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Reproduction of *ab Initio* Electrostatic Potential with Classical Fractional Point Charges

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New additional charge sets, which were added to correct the electrostatic potential (ESP) values on the van der Waals molecular surface using Mulliken net atomic charges, were found to reproduce *ab initio* ESP with the STO-3G basis set. The charge sets were obtained for fundamental small molecules (water, ammonia, methane, ethane, and benzene). Lone pair regions of oxygen and nitrogen atoms show remarkable improvement, while pi electrons do not contribute much to ESP. The present work opens a way for treating large molecules of biological interest.

Keywords——electrostatic potential; van der Waals molecular surface; Mulliken net atomic charge; *ab initio*; water; ammonia; methane; ethane; benzene

Introduction

Drug-receptor interaction is one of the most important subjects to be fully examined when new drugs are developed. The interaction energies are categorized into electrostatic interaction, dipole dipole interaction, polarization interaction, dispersion interaction, charge transfer interaction, hydrogen bonding, hydrophobic interaction, and so on. Among them, electrostatic interaction energy is considered to play a particularly important role in biological systems, as the electrostatic potential (ESP) of the ligand molecule is considered to complement that of the enzyme.[1,2]

In order to comprehend the electrostatic interaction, and to describe it quantitatively, accurate expression of ESP of the molecule is necessary. Accurate ESP can be calculated by applying *ab initio* molecular orbital theory.[3] The theory allows us to obtain ESP at any desired level of approximation based on a strict theoretical foundation, but there is a serious drawback with it, *i.e.*, an extremely long computation time is required.

In biological and pharmaceutical fields, interest is directed toward rather larger and more complex molecules, to which the *ab initio* approach is not easily applied. On this account, a more convenient and simpler, but precise, means of ESP evaluation is needed. The most popular one is to describe the electron distribution by using a set of Mulliken net atomic charges[4] (Mulliken charges, for short). In this method, charge distribution is very easy to handle, because the fractional point charges are located on the atomic nuclei. The charges, however, are not able to represent an inhomogeneous electron cloud, because the continuous

charge distribution is arbitrarily compressed and assigned to atomic nuclei to give the Mulliken charges. Therefore, it is hardly likely that they reflect the real situation to a satisfactory approximation.

In order to overcome this defect of Mulliken charges, several authors have tried setting up new sets of fractional point charges other than Mulliken charges to get a better fit with the *ab initio* ESP. Cox and Williams,[5] and Momany[6] evaluated ESP at grid points in the surrounding region of van der Waals molecular surface, and obtained optimized fractional point charges to get the best least-squares fit with the *ab initio* ESP. Singh and Kollman[7] used the Connolly surface and optimized fractional point charges at points near the surface to reproduce *ab initio* ESP.

Here we tried to develop a new method for reproducing *ab initio* ESP's on the van der Waals molecular surface of some fundamental molecules in an economical and quantitative way. The reason why we adopted the van der Waals molecular surface is that if two molecules interact with each other, they need to be located very close to or in contact with each other, and in such a situation the molecular surface is very important.

The strategy adopted here was that we introduced new fractional point charges into a set of STO-3G[8] Mulliken charges so as to make a new set of charges which well reproduce the *ab initio* STO-3G ESP. Rationalization for this choice of the STO-3G basis set is as follows. According to Cox and Williams[5] and Weiner *et al.*,[9] fractional point charges which reproduce STO-3G ESP around a molecule (ESP-derived charges) can attain best fit to those for 6-31G**[10] which is quite a reliable basis set, just with a scale factor of 1.12. On the other hand, dipole moment calculated with 6-31G** is usually larger than the experimental value and needs adjustment by a factor of 0.91. Therefore STO-3G ESP-derived charges are expected to reproduce true ESP approximately (1.12 times 0.91 makes about 1.0).

The new additional fractional point charges were also applicable to similar portions of compounds other than those to which the optimization procedure was applied. For instance, the fractional point charges determined for the lone pairs of the oxygen atom in a water molecule can also be applied to the oxygen atom in alcoholic or phenolic hydroxyl groups.

In the present paper, we deal with ESP's of a series of fundamental molecules; water, ammonia, methane, ethane, and benzene.

Method

The molecular surface was represented by an assembly of atom spheres. Van der Waals radii of the spheres used were 1.2 Å for hydrogens, 1.7 Å for carbons, 1.6 Å for nitrogens and 1.5 Å for oxygens.[11] The sphere surfaces were divided into small patches by using latitudes and longitudes.[1,2] The centers of the patches were used as points to evaluate ESP's. In the case of water molecule, the numbers of latitudes and longitudes were set at fifteen and thirty, respectively. Consequently, the average distance between points was about 0.05 Å, and the total number of points used per molecule was a thousand or more, in general. This way of picking points did not make their distribution homogeneous, but weight factors were considered for all these points, which not only surmounted the disadvantage of inhomogeneity but also allowed a quite accurate evaluation of ESP's. Moreover, the arrangement of points on the van der Waals surface is convenient for graphical presentation, as can be seen in figures in the present paper, from which one can get three-dimensional images easily without using stereoscopic views.

Electrostatic potential of a molecule at a given point M is formulated in terms of *ab initio* molecular orbital theory as;

$$\mathrm{ESP}^{\mathrm{M}}_{\mathrm{ABIN}}=\sum_{\mathrm{J}}\frac{Z_{\mathrm{J}}}{r_{\mathrm{JM}}}-\int_{r'=r}\frac{1}{r_{\mathrm{M}}}\gamma(r,r')\mathrm{d}r$$

where Z_J: nuclear charge, r_{JM}: distance between nucleus J and point M, r_M: distance between volume element and point M, $\gamma(r, r')$: first-order density matrix.

Electrostatic potential due to Mulliken charges can be simply written as;

$$ESP^{M}_{MLKN} = \sum_{A} \frac{q_A^{MLKN}}{r_{AM}}$$

where q_A^{MLKN}: Mulliken charge of atom A, r_{AM}: distance between nucleus A and point M.

If new fractional point charges are introduced into the Mulliken charge set, the ESP is:

$$ESP^{M}_{NEWCH} = \sum_{A} \frac{q_A^{MLKN}}{r_{AM}} + \sum_{B} \frac{q_B^{NEWCH}}{r_{BM}}$$

where r_{BM}: distance between newly added point charge B and point M. Point B does not need to coincide with any atomic nucleus.

The set of fractional point charges, $\{q_B^{NEWCH}\}$, was determined by minimizing the value:

$$S.D. = \sqrt{\sum_{i} \Delta E_i^2 s_i / \sum_{i} s_i}$$

$$\Delta E_i = ESP^{i}_{NEWCH} - ESP^{i}_{ABIN}$$

where s_i is a weight factor of the area of the i-th patch determined by a pair of latitudes and longitudes.

It was checked that changing the position of poles of "terrestrial globes" did not affect ESP evaluation (to the order of two decimal places in kcal/mol unit). The effect of changing the radii of spheres determining the molecular surface upon STO-3G ESP evaluation was also checked, and it was found that the qualitative trend of ESP was not altered, aside from the magnitudes of the values. Because the behavior of the function defined by point charge potentials would not be so complicated outside the van der Waals surface, this result would apply to more distant regions from the surface. This reasoning is also reinforced by the report by Singh and Kollman[7] in which they studied the effect of different shells around a molecule on derived point charges and elucidated that the point charge model is rather insensitive to the location of the points for evaluating ESP.

The new fractional point charges were added according to three patterns as shown in Fig. 1. Type 1: Three points (marked with Z) are given and they define a plane and an angle. Two new points are added on the bisecting plane of the angle and they are distant from the first plane by the same distance. An example of this case is the two lone pairs of a water molecule. Type 2: Four points are given and they are arranged like a pyramid. One new point is added to the tip of the pyramid resulting in an sp^3 orbital-like structure. An example of this is the lone pair of ammonia. Type 3: Two points are given and a new point is added on the straight line defined by the two points. These three types being used, new charges were optimized by changing parameters (charges, angles, and lengths).

On optimization, attention was paid to the next three points. 1) Original Mulliken charges were preserved, and the total charge of the additional fractional charges should be neutral. 2) New point charges were added at positions of chemical significance, for instance, on a chemical bond, in a lone pair, and so forth. 3) New point charges were located inside the van der Waals molecular surface.

Optimization was done iteratively by the original program DRAG-PMM, which estimated deviation and searched for the steepest descent path numerically. The procedure was continued until satisfactory agreement was obtained or until the gradient sufficiently approached zero.

We did not pay much attention here to multipole moments such as dipole or quadrupole moments, because, if STO-3G ESP is reproduced satisfactorily, the new charges are suitable enough for the purpose of proper treatment of electrostatic intermolecular interactions.

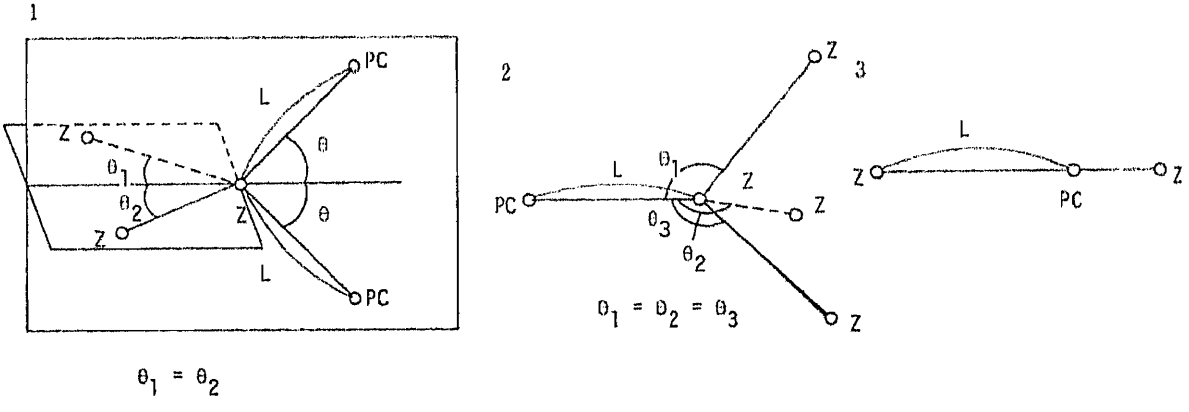


Fig. 1. Three Approaches for Putting Point Charges on a Molecule

Z, atomic nucleus; PC, point charge; θ, angle; L, length.

Ab initio calculations to obtain the molecular orbital, two electron integrals, and Mulliken charges were done with the GAUSSIAN GENERAL program.[12] *Ab initio* ESP's were calculated with our original GAUESP program. The electron density map of benzene was calculated with a modified version of the GAUSSIAN GENERAL program. The optimization procedures of new fractional point charges were performed with the DRAG-PMM program. The above calculations were carried out on a HITAC M-280H at the computer center of the University of Tokyo.

As for water dimer, its stable conformation was determined by a simple random walk procedure. The procedure was done with one water molecule fixed at certain coordinates in three-dimensional space. The other water molecule was allowed to rotate randomly around its oxygen atom by an angle of 1.0° in each step. Constraints on the random rotation were that if the electrostatic energy between the two water molecules, which were represented by two sets of fractional point charges, went up or down by over a certain amount of energy, 0.00001 kcal/mol, then in the next step the molecule was forced to rotate backwards or forwards, respectively. The calculation was done with a program written by us on a PC-9801 microcomputer (Nippon Electric Corporation).

Results

Water

New fractional point charges are presented as "new charge" in Fig. 2, with Mulliken charges based on the STO-3G *ab initio* calculation. In the optimization procedure, in order to represent lone pair electrons, two negative fractional point charges were placed using type 1 structure (see Method). Another two points bearing the same amount of positive charge were added using type 1 too, so that the total charge should be zero. Variables were changed step by step with initial increments, *e.g.*, 0.005 *e* for charge, 0.5° for angle and 0.01 Å for length, which were redetermined automatically in the program.

Electrostatic potential difference ($ESP_{MLKM} - ESP_{ABIN}$) on the molecular surface is depicted in Fig. 3. The region around the two lone pairs is positive, which indicates that negative charge distribution in that region is not expressed in terms of Mulliken charges. On the other hand, the region around the two hydrogen atoms is negative, because the electron distribution along the O–H bond is not described satisfactorily with Mulliken charges.

The "new charge" set gives a better agreement with STO-3G ESP. The difference between the two is presented in Fig. 4. The strongly positive and negative regions in Fig. 3 are improved remarkably. Numerically, the deviation (S.D.) is reduced from 11.7 to 2.6 kcal/mol (Table I).

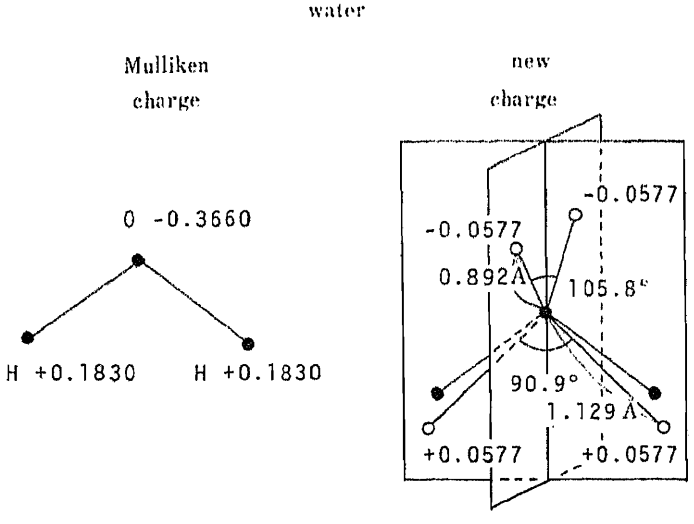


Fig. 2. Mulliken Charges (STO-3G) and Additional New Charges for Water

The STO-3G *ab initio* electrostatic potential is well reproduced by the new charge set (Mulliken charges plus additional new charges).

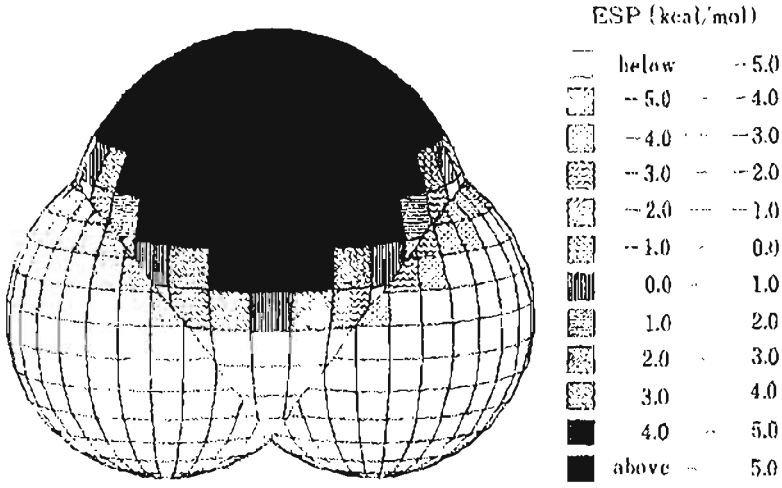


Fig. 3. Electrostatic Potential Difference ($ESP_{MI.KN} - ESP_{AHN}$) for Water

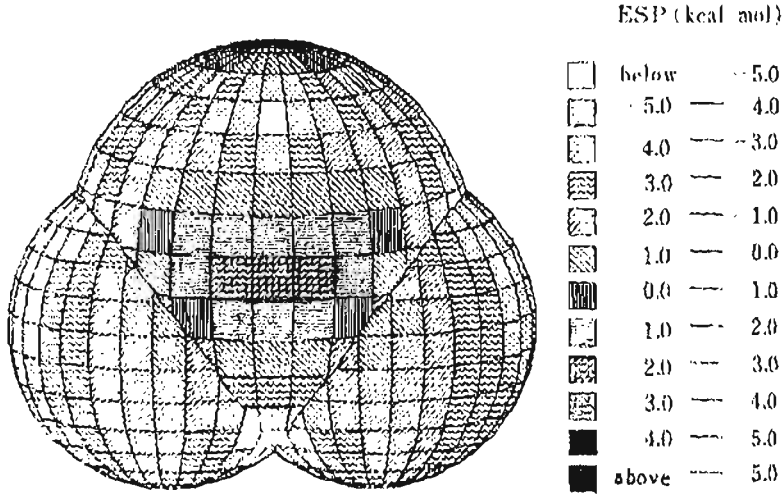


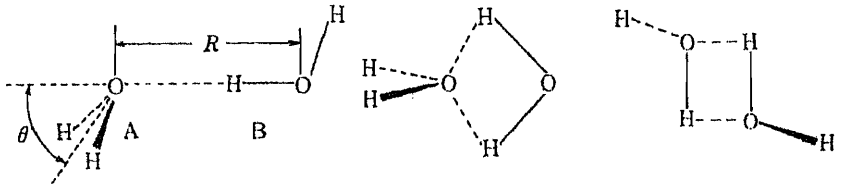
Fig. 4. Electrostatic Potential Difference ($ESP_{NEWCH} - ESP_{AHN}$) for Water

TABLE I. Optimization of S.D.

	Mulliken charge (kcal/mol)	New charge (kcal/mol)
Water	11.7	2.6
Ammonia	13.5	2.8
Methane	3.5	3.4
Ethane	4.3	3.4
Benzene	4.8	3.4

The new fractional point charges thus obtained should give an appropriate picture of the electrostatic relationship of a system in question for our purpose. Nevertheless we made a further check to confirm the validity of the method in conformational analyses, employing a water dimer as an example.

There are numerous studies on the dimer of water molecules.[13-18] Three distinct models are known; linear model, bifurcated model, and cyclic model.



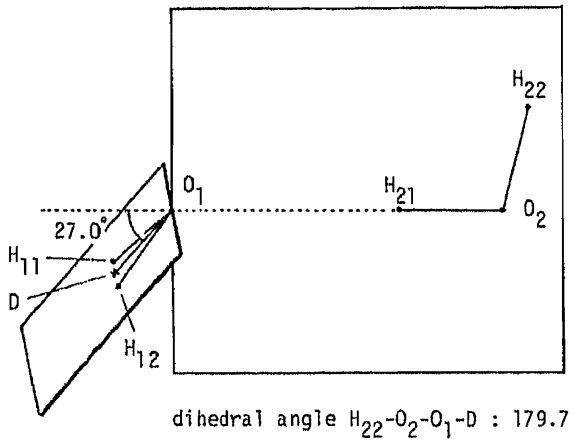


Fig. 5. Result of Random Walk Rotation of the Proton Acceptor Molecule (Left) of a Water Dimer Represented by Two Sets of STO-3G Mulliken Charges

Rotation was started with theta = 0 (see the text) and eventually theta became 27°.

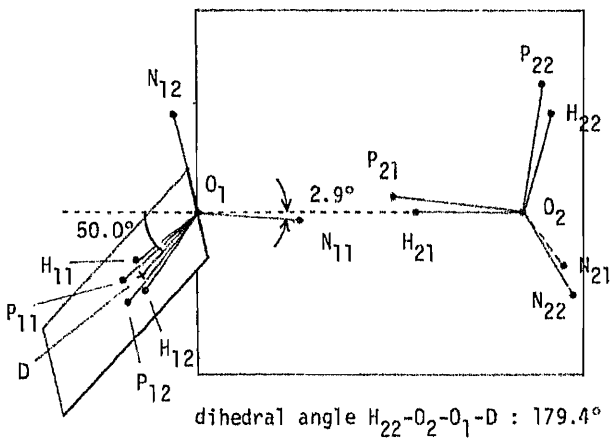


Fig. 6. Result of Random Walk Rotation of the Proton Acceptor Molecule (Left) of a Water Dimer Represented by Two Sets of STO-3G Mulliken Charges and Additional New Charges

Rotation was started with theta = 0° (see the text) and eventually theta became 50°, which is in good agreement with experiments.

Since the study by Morokuma and Pedersen,[13] the linear model has been supported by many *ab initio* calculations. Umeyama and Morokuma[14] carried out detailed *ab initio* calculations using the 4-31G basis set[19] on linear water dimer as well as the other two types of dimers. According to their result, the angle, theta (*vide supra*), is 45°. Experimental studies determined the angle to be 60°.[17,18]

In order to check the predictability of conformations by the new charges, first, we placed STO-3G Mulliken charges on each atomic nucleus, and set the angle, theta, and oxygen oxygen distance, R, in the linear water dimer, to be 0° and 2.96 Å, respectively. The molecule A in the dimer was rotated randomly in the direction of x, y or z. After about one hundred steps of the random walk, the geometry and energy settled around a certain point. The final conformation is shown in Fig. 5. The C_s symmetry of the linear model was almost maintained and theta became 27°.

In the next place, we included the new fractional point charges in the molecular charge set. In the end, the random walk resulted in the linear model with theta of 50° (Fig. 6) and an energy of −3.8 kcal/mol. This value of the angle is quite near the experimental value of 60°. Since the new charge set contains seven fractional point charges, ESP is considered to have been given a significant improvement compared with the use of atom-centered point charges such as Mulliken charges.

Energies were evaluated to be −3.1 and −2.8 kcal/mol for bifurcated and cyclic types of water dimers, respectively, using the new charge set. The geometries for them were taken from the paper by Umeyama and Morokuma.[14] The linear form was proved to be the most

stable among the three.

Ammonia

Figure 7 shows the new charge set for ammonia. In this case, the lone pair electrons of the nitrogen atom were represented by an additional negative charge located according to type 2 structure. Electroneutrality was preserved by three positive charges positioned along the N–H bonds, each having a third of the charge of the first one.

Electrostatic potential difference, $ESP_{MLKN} - ESP_{ABIN}$, on the molecular surface is shown in Fig. 8. The lone pair region is extremely positive, whereas the regions around hydrogen atoms are negative. This implies that the lone pair and electron distribution along chemical bonds can not be represented by Mulliken charges. The situation is quite similar to that in the case of water.

If the new additional charge set "new charge" in Fig. 7 is introduced into the Mulliken charge set, then good agreement is obtained as shown in Fig. 9. Numerically, S.D. dropped from 13.5 to 2.8 kcal/mol (Table I).

As is seen above, it is clear that a "special" distribution of electrons such as a lone pair should be considered explicitly in discussions of electrostatic interaction.

Methane and Ethane

As nonpolar typical aliphatic hydrocarbons, methane and ethane molecules were examined. Electrostatic potential differences ($ESP_{MLKN} - ESP_{ABIN}$) are presented in Fig. 10 for methane and in Fig. 11 for ethane. As can be seen from the figures, although the overall deviations are rather smaller than those for water and ammonia, electron distributions along C–H bondings are not presented satisfactorily. This ESP difference directionality on the molecular surface is so characteristic that it clearly indicates the disadvantage of Mulliken charges. In order to take this effect into account, positive fractional charges were located on extension lines of the C–H bonds, and negative charges were put on carbon atoms for the sake of electroneutrality (Figs. 12 and 13).

Numerical deviations (S.D.'s) are reduced from 5.5 to 3.4 kcal/mol for methane, and from 4.3 to 3.4 kcal/mol for ethane (Table I).

Benzene

As a nonpolar typical aromatic hydrocarbon, benzene was selected. The result of new charge set optimization is depicted in Fig. 14. Standard deviation S.D. for "new charge" in Fig. 14 is 3.4 kcal/mol, while that for Mulliken charges is 4.8 kcal/mol (Table I).

It is a little strange to set positive charges out of the molecular plane where *p* orbital lobes are supposed to be. However, ESP is not determined only by pi electrons but by all the

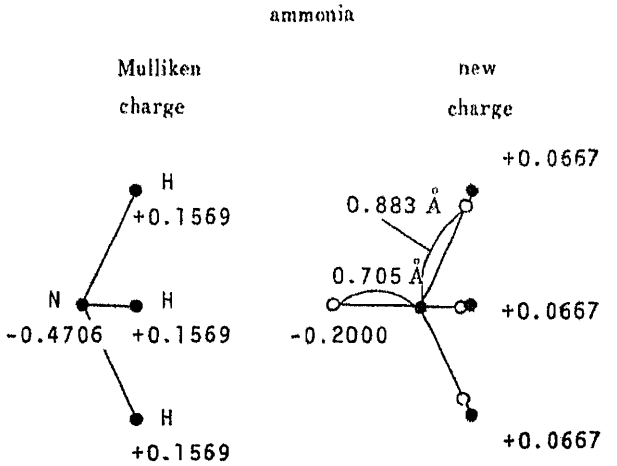


Fig. 7. Mulliken Charges (STO-3G) and Additional New Charges for Ammonia

The STO-3G *ab initio* electrostatic potential is well reproduced by the new charge set (Mulliken charges plus additional new charges).

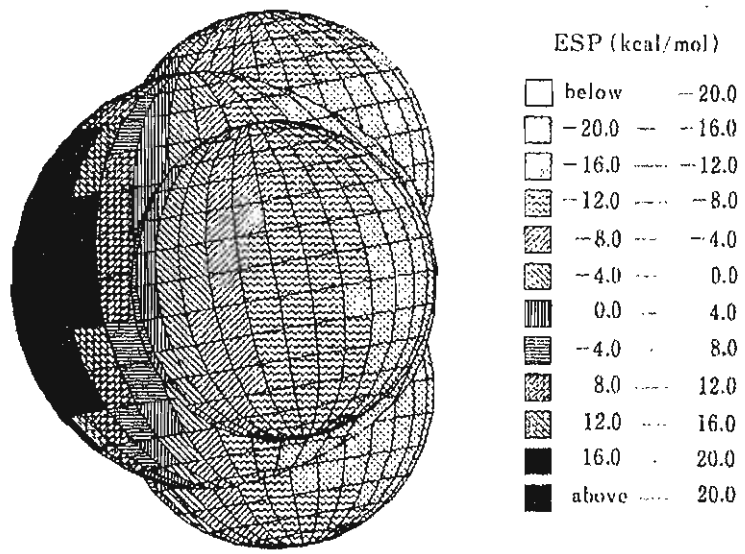


Fig. 8. Electrostatic Potential Difference ($ESP_{MI.KN} - ESP_{ABIN}$) for Ammonia

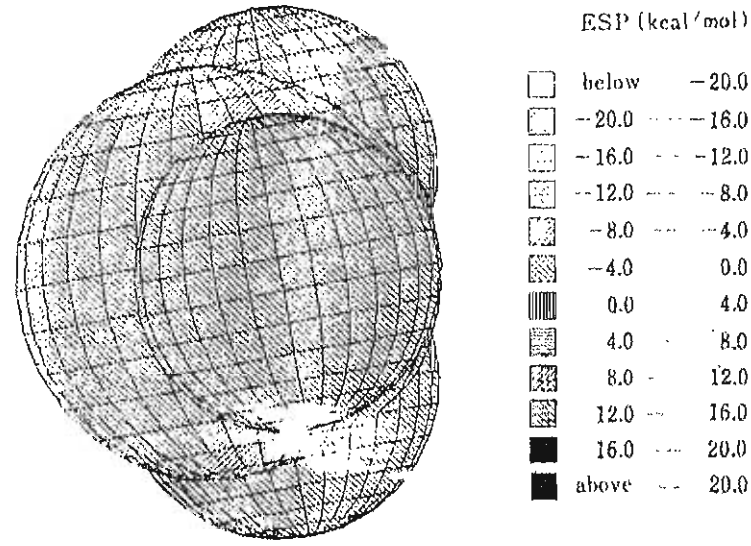


Fig. 9. Electrostatic Potential Difference ($ESP_{NO.WCH} - ESP_{ABIN}$) for Ammonia

electrons the molecule has. Figure 15 shows the total electron density in the plane which contains carbon atoms, C1 and C4, and is perpendicular to the molecular plane. On the other hand, values corresponding to one of two degenerate HOMO's are obtained in the same plane, as in Fig. 16. From Figs. 15 and 16, the extent of pi electron distribution appears not to be broader than the total electron density. Furthermore, the pi orbitals contain only six electrons out of the total of 42 electrons. The question that we should find the answer to is how accurately the "total" electron distribution is reflected by the electrostatic potential.

Discussion

In the present paper, for a series of fundamental molecules, water, ammonia, methane, ethane and benzene, we present fractional point charge sets which overcome the defects of the STO-3G Mulliken charges.

The ESP of water was reproduced by seven fractional point charges (three STO-3G

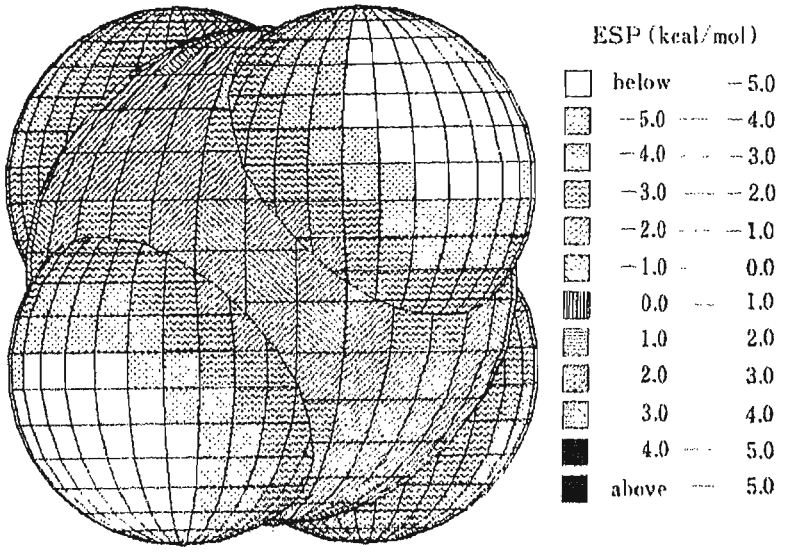


Fig. 10. Electrostatic Potential Difference ($ESP_{ML.KN} - ESP_{ABIN}$) for Methane

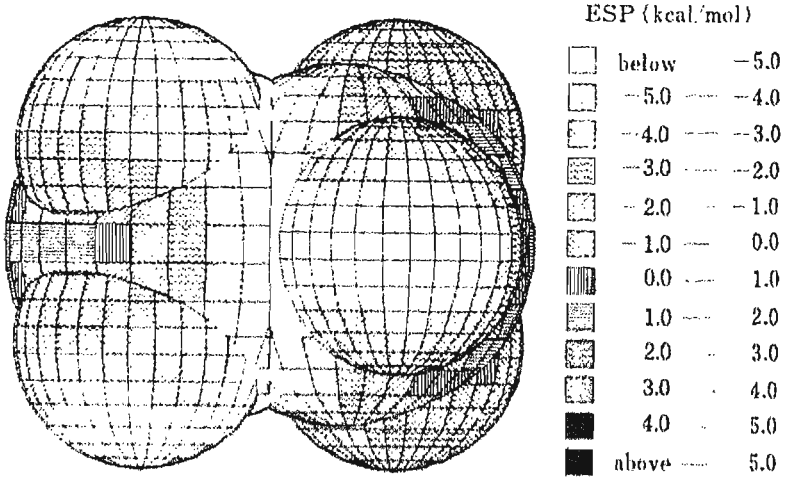


Fig. 11. Electrostatic Potential Difference ($ESP_{ML.KN} - ESP_{ABIN}$) for Ethane

Mulliken charges plus four extra fractional point charges). If the ESP's of molecules are described precisely, the intermolecular electrostatic interactions can be comprehended properly. This means not only that the electrostatic energy is evaluated accurately, but also that the conformation of the interacting system is predicted appropriately. This point was checked on the linear model of water dimer.

Usual atom-centered Mulliken charges lead the conformational change in a random orientational walk to the linear model with the angle, theta, of 27°. Although the overall conformation of the dimer was consistent with theory[13-16] and experiment,[17,18] the magnitude of the angle, theta, was much smaller than those results. The accidental consistency of conformations is considered to be due to the fact that one hydrogen atom of the proton donor molecule, which is located off the line of hydrogen bonding, and two hydrogens of the proton acceptor repelled each other.

On the other hand, the new fractional point charge set obtained in the present work brought the angle, theta, to 50°, which is just in the middle of the theoretical angle of 45°[14] and the experimental value of 60°.[17,18] This result is very encouraging for people who aim at

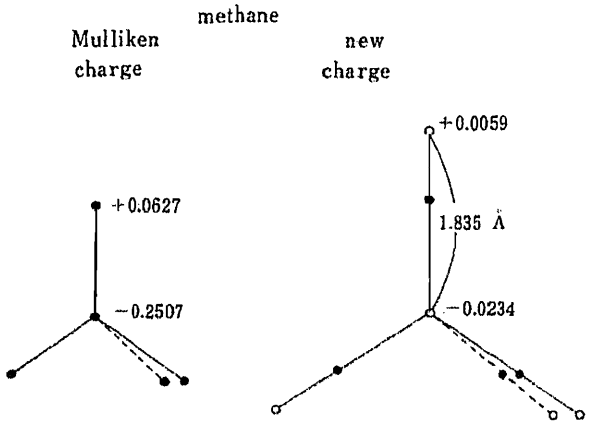


Fig. 12. Mulliken Charges (STO-3G) and Additional New Charges for Methane

The STO-3G *ab initio* electrostatic potential is well reproduced by the new charge set (Mulliken charges plus additional new charges).

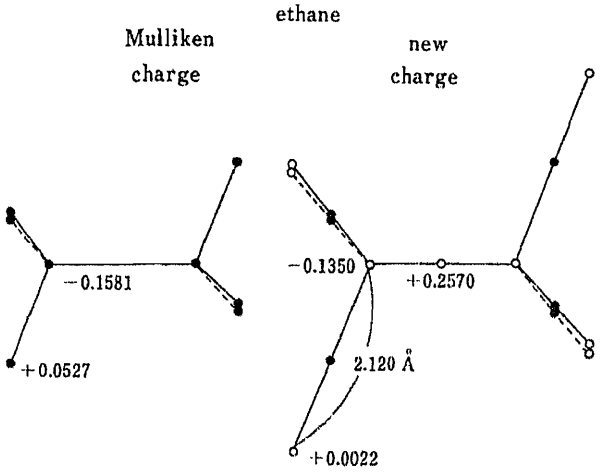


Fig. 13. Mulliken Charges (STO-3G) and Additional New Charges for Ethane

The STO-3G *ab initio* electrostatic potential is well reproduced by the new charge set (Mulliken charges plus additional new charges).

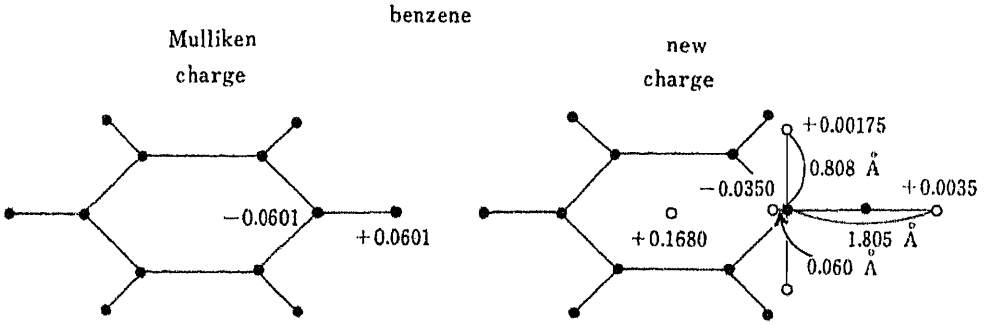


Fig. 14. Mulliken Charges (STO-3G) and Additional New Charges for Benzene

The STO-3G *ab initio* electrostatic potential is well reproduced by the new charge set (Mulliken charges plus additional new charges).

conformational simulation by classical approaches such as molecular mechanics.

According to an *ab initio* energy decomposition analysis by Umeyama and Morokuma[14] based on the 4-31G basis set, electrostatic interaction contributes predominantly (−10.5 kcal/mol) to the total interaction energy (−7.8 kcal/mol) of the linear dimer. The theta dependence of total interaction energy and that of electrostatic energy are quite similar and change almost in parallel upon change of theta. Therefore, the conformation of water dimer should be well reproduced, if estimation of electrostatic energy can be done precisely. In the present study, the conformation of the linear dimer was reproduced as stated above, which is a good confirmation that the result of this work is valid.

In the cases of water and ammonia, it became manifest that explicit treatment of lone pairs is essential. When this localized distribution of electrons is taken into consideration, the overall ESP is improved remarkably. A possible drawback may be that the locations of the charges are off the nuclei of atoms, and that hence handling of the charges may become complex in applications to molecular mechanics or other methods. However, the choice between accuracy of calculation and convenience of handling charges depends on the case. If an appropriate computer program is available, we should choose the former.

In the cases of methane and ethane, the numerical deviation (S.D.) was not large, but the

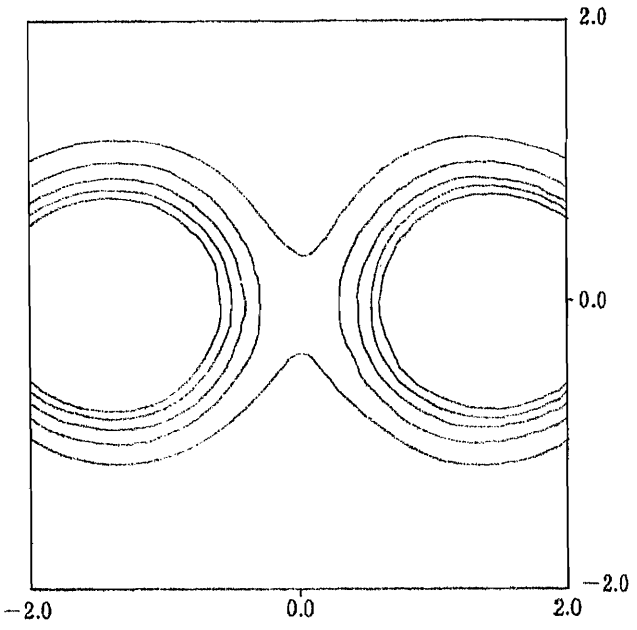


Fig. 15. Total Electron Density of Benzene

The density was drawn on a plane perpendicular to the molecular plane. The picture shows a region of 2.0 Å × 2.0 Å. The contours stand for 0.02, 0.04, 0.06, 0.08, 0.1 $Bohr^{-3}$ from the outermost region. Contours for higher values than 0.1 $Bohr^{-3}$ are omitted. The thickest part corresponds to the C1 and C4 atoms.

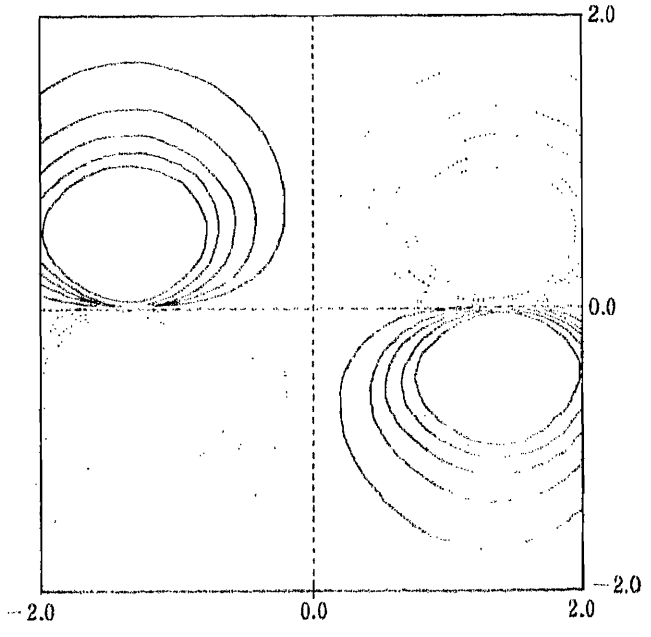


Fig. 16. One of the Two Degenerate Highest Occupied Molecular Orbitals (HOMO's) of Benzene Drawn on a Plane Perpendicular to the Molecular Plane (the Same as in Fig. 15)

The picture shows a region of 2.0 Å × 2.0 Å. Solid and dotted line indicate positive and negative values, respectively. Broken lines stand for nodal planes. The contours stand for 2.0×10^{-7}, 4.0×10^{-7}, 6.0×10^{-7}, 8.0×10^{-7}, 10.0×10^{-7} $Bohr^{-3/2}$ from the outermost region. Contours for values larger in magnitude than 10.0×10^{-7} $Bohr^{-3/2}$ are omitted. Because this figure depicts the molecular orbital itself, the unit is different from that in Fig. 15. The contour (not the value itself), however, is not changed if the corresponding "density" for the "molecular orbital" is plotted.

defect of the Mulliken charge method was clear. It could not describe the directionality of ESP on the van der Waals surface. In particular, the difference was large on the surface near hydrogen atoms. This situation was corrected by adding new positive charges on the extension lines of C–H chemical bonds.

In the case of benzene, the pi electron distribution did not contribute to ESP as significantly as would be expected from the usual oversimplified schematic picture of pi orbitals of benzene. The negative charge is not so dense in that orbital. In the evaluation of ESP, the total cloud of electrons should be taken into account, and one should not stick too firmly to a particular orbital aside from lone pairs.

In previous reports,[5–7] the distance between points used to evaluate ESP was around 0.5 or 1.0 Å, and hence 100 or 300 points were used per molecule, which seems a little too rough. Since no weighting factor was considered for each point, the points needed to be dispersed

spatially in a homogeneous fashion. If one wishes to evaluate graphically the agreement between the new charge ESP and *ab initio* ESP in these respects, one must elaborate a stereoscopic view. Furthermore, the obtained new charges were valid only for the molecules to which the optimization procedure was applied.

In our present work, as is stated in the Method section, the molecular surface was represented by an assembly of atom spheres. The sphere surfaces were divided into small patches by using latitudes and longitudes.[1,2] The average distance between points was typically about 0.05 Å, and the total number of points used per molecule was a thousand or more, in general. Weight factors were considered for all these points, which allowed a quite accurate evaluation of ESP's. Furthermore, the arrangement of points on the van der Waals surface is convenient for graphical use.

New fractional point charges thus obtained for the fundamental molecules are also applicable to other compounds of the same type. For instance, fractional point charges obtained for lone pairs of water can be used for other alcoholic or phenolic hydroxyl groups. Accordingly, if net atomic charges are somehow obtained for molecules larger than those considered in this paper, the new additional charges obtained here can be applied as supplementary charges to those molecules. Actually we have obtained a satisfactory result by applying the charges to some neurotransmitters, such as γ-aminobutyric acid or acetylcholine. Their ESP's were reproduced well and the fit with *ab initio* ESP's was good. A detailed description will be presented elsewhere.

Conclusion

New additional fractional point charges were obtained to correct the electrostatic potentials which Mulliken charges give. The significance of taking lone pair electrons into consideration became clear, while pi electrons did not contribute much to the electrostatic potential. The validity of the new charge sets was supported by successful reproduction of the water dimer conformation reported previously.

In the present paper, we focused on the electrostatic properties on the van der Waals surface, and the approach adopted here allowed accurate numerical evaluation and easy graphical comprehension of the property.

In the field of biological and pharmaceutical sciences, interest is directed toward large-sized molecules in general. To cope with the electrostatic potentials of such molecules, a method to construct economically the potentials of those molecules is requisite. One way of treating this problem is to consider those large molecules as being composed of a number of small parts, and to apply the charge sets reported here as building materials to those molecules. The concrete procedure applied to some neurotransmitters will be reported elsewhere.

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Asymmetric Hydrogenation of 3,4-Methylenedioxy-α-acetamidocinnamic Acid Using Newly Developed Silica Gel-Supported Chiral Rhodium(I)–Phosphine Complexes

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The asymmetric hydrogenation of 3,4-methylenedioxy-α-acetamidocinnamic acid was carried out in the presence of a chiral Rh(I)-complex of (2*S*,4*S*)-*N*-butoxycarbonyl-4-diphenylphosphino-2-diphenylphosphinomethylpyrrolidine (BPPM) adsorbed on hydrophobic silica gel in MeOH–H_2O solvents. Excellent conversions (100%) and a high optical yield (85%) were attained with [Rh(COD)BPPM]$^+ClO_4^-$ on methylated silica gel. The adsorption of the complex was improved by introducing a stearoyl group instead of the butoxycarbonyl group of BPPM. When the recovered catalysts were reused three times, the optical yields decreased gradually from 85 to 69%.

Keywords——asymmetric hydrogenation; rhodium complex; 3,4-methylenedioxy-α-acetamidocinnamic acid; L-DOPA; solid catalyst; silica gel

L-β-(3,4-Dihydroxyphenyl)alanine(L-DOPA), a specific drug for Parkinson's disease, is produced by the optical resolution of the cinchonine salts from racemic DOPA.[1] Since chiral Rh(I)-complexes were first found, the asymmetric hydrogenation of 3,4-methylenedioxy-α-acetamidocinnamic acid (MACA), a precursor of L-DOPA, has been studied extensively.[2] In the asymmetric hydrogenation, reuse of the complex catalyst is essential; these catalysts are composed of a rare metal, Rh, and chiral phosphines obtained by complex synthetic procedures. Therefore, in the preparation of the solid phosphine catalysts, two types of supports for the Rh(I)-complexes have been proposed, organopolymers such as methyl methacrylate polymers,[3] and inorganic powders such as graphite[4] or mineral clays.[5] Charcoal has also been used as a support, and the asymmetric hydrogenation of acetamidocinnamic acid was carried out repeatedly.[6]

Recently, 3,4-diphenylphosphinopyrrolidine has been found to be bound chemically on silica gel by the method of Merrifield and the complex has been used as a solid catalyst.[7] We have conveniently prepared a solid catalyst of chiral Rh(I)-complexes adsorbed on hydrophobic silica gel and used it for the asymmetric hydrogenation of MACA.

Results and Discussion

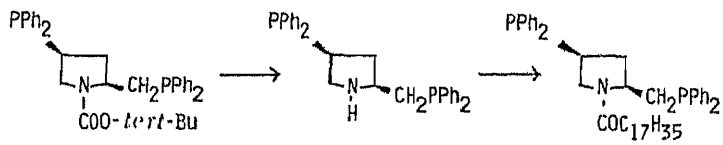
Asymmetric Hydrogenation of MACA with Rh–(−)-2,3-Dihydroxy-1,4-bis(diphenylphosphino)butane (DIOP) and Rh–(2*S*,4*S*)-*N*-Butoxycarbonyl-4-diphenylphosphino-2-diphenylphosphinomethylpyrrolidine (BPPM)

The asymmetric hydrogenation of MACA was carried out in the presence of Rh(I)-complexes adsorbed on a highly methylated silica gel. As chiral phosphine compounds, we used DIOP, BPPM, and (2*S*,4*S*)-*N*-stearoyl-4-diphenylphosphino-2-diphenylphosphinomethylpyrrolidine (SPPM), which was prepared from BPPM in two steps. First, the reaction

TABLE I. Asymmetric Hydrogenation of α-Acetamido-3,4-methylenedioxycinnamic Acid with Rh–BPPM on Methylated Silica Gel

Entry	Catalyst	Substrate (g)	Temp. (°C)	H_2 (kg/cm²)	Conv. (%)	O.y.[d] (%)
1	Rh–BPPM[a]	1.0	50	20	100	84
2	Rh–DIOP[b]	1.0	50	20	100	61
3	Rh–BPPM–$mSiO_2$	1.0	50	20	100	89
4	Rh–DIOP–$mSiO_2$	0.5	50	20	100	51
5	Rh–BPPM–$mSiO_2$	0.5	80	20	100	84
6	Rh–BPPM–$mSiO_2$	0.5	100	20	100	79
7	Rh–BPPM–$mSiO_2$	0.5	50	40	100	84
8	Rh–BPPM–$mSiO_2$	0.5	50	20	100	88
9	Recovered cata. of entry 8	0.5	50	20	68	52
10	Recovered cata. of entry 9	0.5	50	20	49	30
11	Rh–BPPM$^+ClO_4^-$–$mSiO_2$[c]	0.5	50	20	100	85
12	Recovered cata. of entry 11	0.5	50	20	100	77
13	Recovered cata. of entry 12	0.5	50	20	100	69

A molar ratio of the substrate to the Rh(I)-complexes of 1600, MeOH–H_2O (1 : 1, v/v), (30 ml/1 g-substrate), and methylated silica gel ($mSiO_2$ C; 3%) (0.2 g/1 g-substrate) were used. Ethanol was used as the solvent for the hydrogenation in entries 1 and 2. Reaction time, 12 h. *a*) RhCl(COD)BPPM. *b*) RhCl(COD)DIOP. *c*) [Rh(COD)BPPM]$^+ClO_4^-$ on $mSiO_2$. *d*) Optical yield (configuration, *R*).



BPPM PPM SPPM

Chart I

conditions giving good conversion and optical yield were studied by comparing the activities of homogeneous and heterogeneous catalyst systems in the presence of Rh–DIOP or BPPM. The results are shown in Table I. It has been reported by Achiwa[8] that an optical yield of 83% was achieved under homogeneous reaction conditions with Rh–BPPM. Under our reaction conditions, the optical yield of the *R*-isomer reached 84% for Rh–BPPM and 61% for Rh–DIOP at 50 °C. To heterogenize the catalyst, several types of supports were examined, and methylated silica gel ($mSiO_2$) was found to be suitable; Rh(I)-complexes were adsorbed tightly on the support in an MeOH–H_2O (1 : 1, v/v) solvent as described later. With this pale-yellow solid catalyst, the hydrogenation was carried out at 50 °C for 12 h. In the MeOH–H_2O solvent, almost the same optical yield as that in the homogeneous Rh(I)-complex system in ethanol was obtained. When the reaction temperature was increased from 50 to 100 °C, the optical yield decreased from 89 to 79%. However, at lower temperature, the hydrogenation was retarded considerably and a part of the substrate remained insoluble.

On our solid catalyst, the hydrogen pressure hardly affected the optical yield up to 40 kg/cm². Therefore, the hydrogenation was carried out under a hydrogen pressure of 20 kg/cm² as the standard procedure. The recovered catalyst was very sensitive to air; on exposure to air for 5 h, it lost almost all activity. Thus, such procedures as separation of the catalyst and charging of substrates were carried out under an N_2 stream. In the hydrogenation, a cation-type Rh-catalyst [Rh(COD)BPPM]$^+ClO_4^-$ gave higher optical yields than a neutral-type complex

(RhCl(COD)BPPM). A sufficient activity was obtained by using one molecule of the complex per 1600 molecules of the substrate. After the reaction, the catalyst was recovered by centrifugation and used again. However, the color of the solid catalyst gradually changed from yellow to gray; the Rh(I) ion seemed to be reduced to Rh metal during the hydrogenation. Therefore, it was considered that milder reaction conditions might allow better retention of the activity.

Adsorption of Chiral Rh(I) Complexes on $mSiO_2$

The adsorption of Rh–BPPM on $mSiO_2$ was studied in various compositions of MeOH–H_2O solvent (Fig. 1). Finely powdered silica gel (Aerosil R 972) adsorbed Rh–BPPM (a cation type) in a water-rich solvent. However, methylated silica gel (Nipsil ss-10), which is hydrophobic in nature, adsorbed the Rh(I)-complex even in the MeOH–H_2O (1 : 1, v/v) solvent. Then, the effect of the composition of MeOH and H_2O on the adsorption of Rh–BPPM on methylated silica gel (C; 3%) was examined. With increasing ratio of water to MeOH, the amount of Rh(I)-complex adsorbed was increased and reached saturation at MeOH–H_2O (2:3, v/v). At this point, 1.25×10^{-3} mmol of $[Rh(COD)BPPM]^+ClO_4^-$ was adsorbed on 0.1 g of silica gel. The color of the silica gel became pale yellow after the adsorption of the Rh(I)-complexes. Such high adsorption was not observed with unmodified silica gel. When an excess amount (2.5×10^{-3} mmol) of $[Rh(COD)BPPM]^+ClO_4^-$ was added to $mSiO_2$ (0.1 g), a constant amount (1.25×10^{-3} mmol) was adsorbed at 30 °C. The Rh(I)-compelx once adsorbed was not desorbed even by washing three times with MeOH–H_2O (1 : 1, v/v), as checked by ultraviolet (UV) spectroscopy.

The surface area occupied by one molecule of Rh(I)-complex was calculated from the specific surface area of $mSiO_2$ (87 m^2/g) and the adsorbed amount of Rh–BPPM (1.25×10^{-3} mmol/0.1 g-$mSiO_2$). This value of 1.16×10^3 A^2/molecule was close to that estimated from a molecular model[9] (*ca.* 3×10^2 A^2). These results suggest that the Rh(I)-complexes were

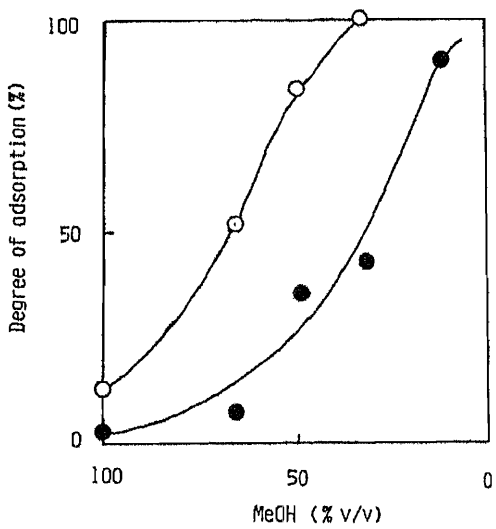


Fig. 1. Effect of the Composition of MeOH–H_2O upon the Adsorption of $[Rh(COD)BPPM]^+ClO_4^-$ on Silica Gel

$[Rh(COD)BPPM]^+ClO_4^-$ (1.25×10^{-3} mmol), silica gel (0.1 g), solvent (15 ml). The complex was adsorbed at 30 °C for 1 h.
○, $mSiO_2$ (Nipsil ss-10, C; 3%); ●, Aerosil (R 972).

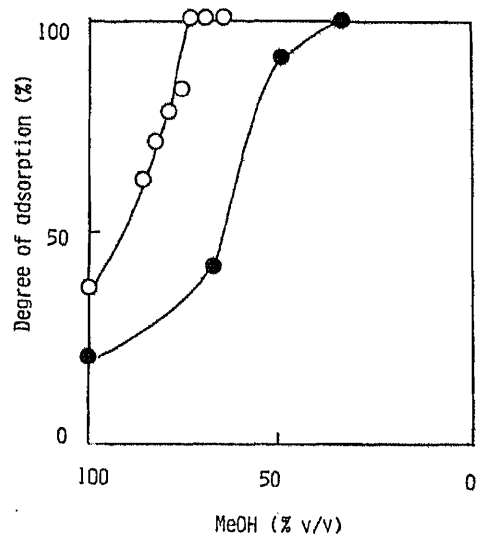


Fig. 2. Effect of the Composition of MeOH–H_2O upon the Adsorption of RhCl(COD)-BPPM and $[Rh(COD)SPPM]^+ClO_4^-$ on Silica Gel

Rh(I)-complexes (1.25×10^{-3} mmol), silica gel (0.1 g), solvent (15 ml). The complexes were adsorbed at 30 °C for 1 h.
○, $[Rh(COD)SPPM]^+ClO_4^-$ on $mSiO_2$ (Nipsil ss-115, C; 4.5%); ●, RhCl(COD)BPPM on $mSiO_2$ (Nipsil ss-10, C; 3%).

TABLE II. Asymmetric Hydrogenation of α-Acetamido-3,4-methylenedioxycinnamic Acid with Rh–SPPM on a Highly Methylated Silica Gel

Entry		$MeOH–H_2O$ (v/v)	Temp. (°C)	H_2 (kg/cm²)	Conv. (%)	O.y.[a] (%)
1	Rh–BPPM	(1:1)	50	20	97	76
2	Rh–SPPM$^+ClO_4^-$–$mSiO_2$	(2:1)	25	20	9	
3	Rh–SPPM$^+ClO_4^-$–$mSiO_2$	(2:1)	35	5	92	74

Substrate (1.5 g), solvent (22.5 ml), and $mSiO_4$ (C; 4.5%) (0.1 g) were used. The molar ratio of the substrate to Rh(I)-complex was 4800. Reaction time, 36 h. *a*) Optical yield (configuration *R*).

adsorbed on $mSiO_2$ as a monomolecular layer.

Activity of Rh–SPPM on a More Methylated Silica Gel

From the results in Table I, it was considered that mild reaction conditions, such as a lower reaction temperature, were important to obtain high optical yields and to increase the life of the catalyst. Thus, we modified (2*S*,4*S*)-4-diphenylphosphino-2-diphenylphosphinomethylpyrrolidine (PPM) with stearoyl chloride to give SPPM, and Rh–SPPM was adsorbed on $mSiO_2$ in methanol-rich solvents. As shown in Fig. 2, Rh–SPPM was completely adsorbed on $mSiO_2$ (C; 4.5%) in the $MeOH–H_2O$ (2:1, v/v) solvent. As a result, the solubility of the substrate was increased and the hydrogenation could be carried out at a lower reaction temperature (35 °C). On this catalyst, about 4500 molecules of the substrate were hydrogenated by one molecule of Rh–SPPM to give an optical yield of 74% (Table II).

In conclusion, the Rh(I)-complexes were heterogenized to make solid catalysts by adsorption of the complexes on methylated silica gel, and these catalysts were used for the asymmetric hydrogenation under mild reaction conditions. Further studies along these lines are in progress.

Experimental

Infrared (IR) spectra were taken on a Hitachi IR-260-30 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL FX-90Q spectrometer using tetramethylsilane as an internal standard. Chemical shifts are given in ppm and the following abbreviation is used: m=multiplet. The optical yield was determined with respect to the value of $[\alpha]_D^{19}$ +53.4° (EtOH)[11] for (*R*)-*N*-acetyl-3-(3,4-methylenedioxyphenyl)alanine by means of a digital polarimeter (Union Giken Co., PM-101).

Reagents——MACA was synthesized according to the method of Yamada *et al.*[1] Rh(I)-cyclooctadienyl complex $[RhCl(COD)]_2$ was prepared from RhCl and cyclooctadiene in MeOH. BPPM was synthesized according to the method of Achiwa.[8] DIOP was obtained from Aldrich Chemical Co. Other reagents were of GR grade. Methylated silica gels, Nipsil ss-10 (C; 3%) and ss-115 (C; 4.5%), were kindly provided by Nippon Silica Co.

Preparation of SPPM——In a two-necked flask, BPPM (3.5 g) and trifluoroacetic acid (10 ml) were stirred at 5 °C for 5 h under an argon atmosphere. After the reaction, the solution was poured into ice-water (300 ml), neutralized with sodium carbonate, and extracted three times with ethyl acetate (80 ml). The ethyl acetate layer was washed with saturated aqueous sodium bicarbonate (50 ml) and dried on magnesium sulfate. By evaporation of the solvent under reduced pressure, an oily product, PPM (3.3 g), was obtained.

A benzene solution of stearoyl chloride (2.1 g, 7.0 mmol) was added to a benzene solution (30 ml) of PPM (3.3 g) and triethylamine (1.3 g, 12.5 mmol) at −5 °C under an argon atmosphere. The solution was stirred for 1.5 h at 5—10 °C. The reaction product was chromatographed on a column packed with Florisil (100—200 mesh, Wako Pure Chemical Co.). Elution with hexane–ethyl acetate (5:1, v/v) gave SPPM (3.5 g) (yield 75.8%) as an oil. *Anal.* Calcd for $C_{47}H_{63}NOP_2$: C, 78.41; H, 8.82; N, 1.95. Found: C, 78.62; H, 8.86; N, 1.9. IR ν_{max}^{neat} cm^{-1}: 3050, 2920, 2850, 1640, 1430, 1410, 740, 700. NMR ($CDCl_3$): 0.7—2.5 (35H, m), 2.5—4.3 (8H, m), 7.0—7.8 (20H, m). $[\alpha]_D^{26}$ −12.3° (*c*=1.02, benzene), −14.4° (*c*=0.92, $CHCl_3$).

Pretreatment of $mSiO_2$ and Adsorption of Rh(I)-Complexes——Methylated silica gel (C; 3 or 4.5%) (0.1 g) was dispersed in $MeOH–H_2O$ (1:1, v/v) (7.5 ml), then acetic acid (0.05 ml) was added, and the mixture was stirred for

30 min at room temperature. The silica gel was washed by centrifugation three times with the same solvent (7.5 ml each). A freshly prepared ethanol solution (1 ml) of Rh(I)-complex containing $[Rh(COD)]^+ClO_4^-$ (0.39 mg, 1.25×10^{-3} mmol) and SPPM (0.89 mg, 1.25×10^{-3} mmol) was added to the pretreated support and the mixture was stirred for 1 h. Adsorption of the complex was carried out in the solvent for half an hour under magnetic stirring at room temperature. After centrifugation, the supernatant was decanted, leaving a pale yellow catalyst. The catalyst was washed three times with the same solvent (7.5 ml each).

Procedures for Asymmetric Hydrogenation——MACA and the catalyst were added to a glass tube (25 × 100 mm) with the solvent (15 ml). The tube was put into a stainless autoclave (100 ml), and hydrogenation was carried out under a hydrogen pressure of 20 kg/cm^2. After the reaction, the catalyst was separated by centrifugation. The products were obtained by evaporation of the solvent. The conversion was determined from the UV absorption at 320 nm. The products were treated with 0.5 N NaOH followed by 1 N HCl, and extracted with ether according to the method of Kagan and Dang.[10] The separation of the catalyst and charging of the substrate were carried out in an N_2 (99.99%) stream, and the recovered catalyst was reused.

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Crystal Structure of (Uracil-1-ylethyl)(adenin-9-ylethyl)tryptophan Dipeptide: An Interaction Model between Nucleic Acid Base and Aromatic Amino Acid[1)]

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As a model compound for the interaction of tryptophan with nucleic acid bases, uracil and adenine, (uracil-1-ylethyl)(adenin-9-ylethyl)tryptophan dipeptide has been synthesized, and its molecular and crystal structure has been analyzed by the X-ray diffraction method. The crystal data are $a=4.989(2)$, $b=30.056(5)$, $c=9.939(1)$ Å, $\beta=94.61(3)°$, $Z=2$, $D_m=1.345(1)$ g·cm^{-3}, space group $P2_1$. The molecule takes a W-shaped extended conformation, and is linked with the neighboring molecule in a head-to-tail fashion through a reverse Watson–Crick base pairing. Consequently the molecules form an infinite chain along the *b*-axis. A weak stacking interaction is observed between the indole ring and the uracil base: the dihedral angle between them is 28.6(3)°, and the average interplanar spacing is 3.95 Å. This stacking mode is formed by the dipole–dipole coupling between the respective aromatic rings. The result may indicate a possible access mode of a tryptophan residue to the uracil base of a single-stranded nucleic acid.

Keywords——(uracil-1-ylethyl)(adenin-9-ylethyl)tryptophan dipeptide; X-ray analysis; indole–uracil stacking interaction; reverse Watson–Crick base pairing

Specific interaction between the chemical groups of amino acid and nucleic acid bases is important for the selective recognition of nucleic acid base sequences by proteins. Several types of specific interactions are possible, and tryptophan, which has the best π-electron donating character among aromatic amino acids,[2)] has been shown to associate with nucleic acid bases by π–π stacking interaction.[3)] Since the stacking, in general, arises from the interaction between the π-orbitals of two aromatic rings, particularly from the coupling between the highest occupied molecular orbital (HOMO) of the indole ring (as a donor) and the lowest unoccupied one (LUMO) of the nucleic acid base (as an acceptor),[4-6)] the interaction could be an important mode of selective recognition of nucleic acid bases by aromatic amino acids. At present, structural knowledge on the tryptophan–nucleic acid base stacking interaction has been mainly derived from solution studies using various spectroscopic methods, and few crystallographic studies have been done because of the difficulty of

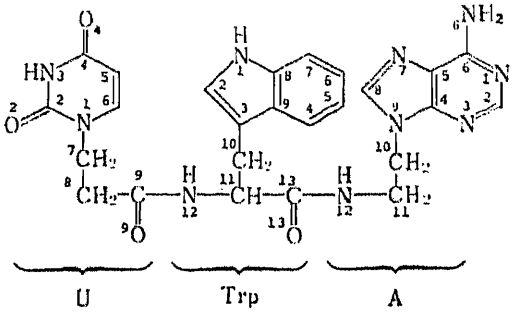


Fig. 1. Chemical Structure of U–Trp–A, and Atomic Numbering

preparing suitable model crystals. However, information at the atomic level is absolutely necessary to establish the details of the interaction.

As a part of our structural studies on the interaction mode between indole ring and nucleic acid base, we have synthesized (uracil-1-ylethyl)(adenin-9-ylethyl)tryptophan dipeptide (U-Trp-A) (see Fig. 1 for its chemical structure), and analyzed the crystal structure by the X-ray diffraction method, in the expectation that this compound would provide information as to the binding geometry of tryptophan residue with adenine(A), uracil(U), or A···U base pair.

Experimental

Synthesis of U-Trp-A——The synthetic scheme is presented in Fig. 2. Uracil-1-ylpropionic acid (**1**) and adenin-9-ylethylamine (**2**) were synthesized from uracil and adenine, respectively, by methods similar to those described by Ueda and Fox,[7] and Leonard and Lambert.[8] U-Trp-A was then synthesized by the normal procedure of peptide synthesis. The crude products were purified by gel filtration on a Sephadex LH-20 column with methanol–water (7 : 3, v/v): *Anal.* Calcd for $C_{25}H_{26}N_{10}O_4 \cdot H_2O$: C, 54.74; H, 5.14; N, 25.53. Found: C, 54.98; H, 5.64; N, 25.90.

X-Ray Data——Single crystals suitable for X-ray study were obtained from 50% aqueous methanol by the vapor diffusion technique. A single crystal, with dimensions of approx. $0.1 \times 0.3 \times 0.5$ mm^3, was used for the X-ray diffraction data collection. The crystallographic data are listed in Table 1. The intensity data were collected with a Rigaku AFC-5 diffractometer using graphite-monochromated Cu K_α radiation at room temperature. A total of 2897 independent reflections within $\sin\theta/\lambda = 0.591$ Å^{-1} was collected in the $\omega - 2\theta$ scan mode and corrected for Lorentz and polarization factors, but not for absorption effects. The scan speed was 6°/min and the background was taken for 3 s at both sides of every reflection.

Structure Determination and Refinement——The structure was solved by the direct method using the MULTAN78 program.[9] An E-map computed with the phase set of the highest figure of merit revealed the positions

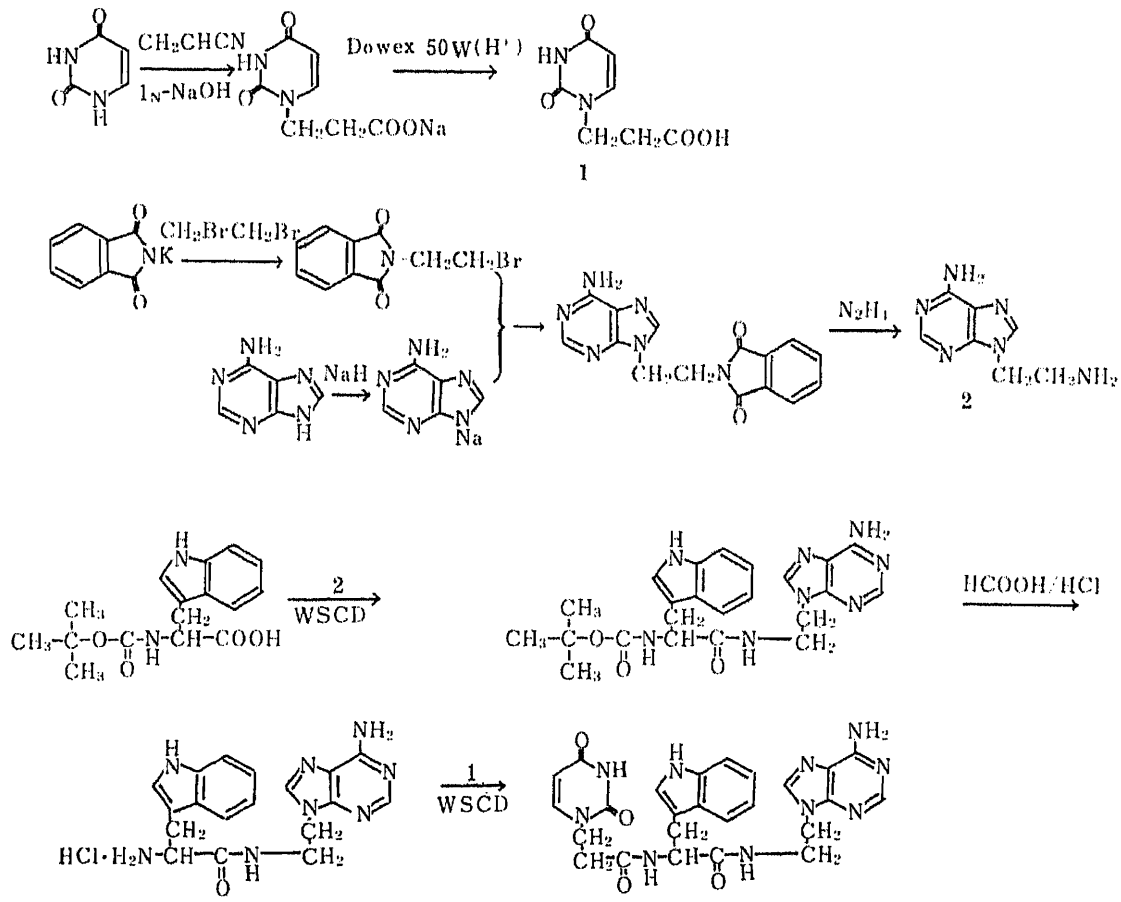


Fig. 2. Synthetic Scheme for U-Trp-A

TABLE I. Crystal Data for U–Trp–A

Molecular formula	$C_{25}H_{26}N_{10}O_4 \cdot 4H_2O$
Molecular weight	602.61
Crystal system	Monoclinic
Space group	$P2_1$
Cell constants	
a (Å)	4.989 (2)
b (Å)	30.056 (5)
c (Å)	9.939 (1)
β (°)	94.61 (3)
Volume (Å^3)	1485.6 (6)
Z	2
ρ (obsd.) (g·cm^{-3})	1.345 (1)
ρ (calcd.)	1.347
F (0 0 0)	636
μ (Cu K_α) (cm^{-1})	8.25

of the A and U bases. The remaining atoms were positioned by successive Fourier syntheses. The structure was refined by the full-matrix least-squares method wih isotropic thermal parameters and then by the block-diagonal least-squares method with anisotropic ones. All the hydrogen atoms, except those of some molecules of water of crystallization, were revealed on a difference Fourier mpa (peak height: 0.3—0.5 e Å^{-3}). The final least-squares refinement including the hydrogen atoms with isotropic thermal parameters was computed to minimize $\Sigma w(|F_o|-|F_c|)^2$ with the following weighting scheme: $w=1.0/[\sigma(F_o)^2-0.02314|F_o|+0.00622|F_o|^2]$, where $\sigma(F_o)$ is the standard deviation based on counting statistics. During the last cycle of refinement, none of the positional parameters shifted by more than one fourth of the estimated standard deviation. The final R and Rw values using 2265 reflections having $F \geqq 3\sigma(F_o)$ are 0.07 and 0.09, respectively. The residual fluctuations in the final difference Fourier map were within the range of −0.3—0.3 eÅ^{-3}. Final atomic coordinates of nonhydrogen atoms are given in Table II.[10]

The structural refinements and other numerical calculations were carried out using the UNICS program[11] and the published scattering factors.[12] All calculations were carried out on an ACOS 900 computer of the Computer Center of Osaka University.

Results and Discussion

Molecular Dimension and Conformation

The bond lengths and angles for nonhydrogen atoms are given in Table III; the average bond distances involving hydrogen atoms are C–H = 1.08 and N–H = 0.87 Å with estimated standard deviations of 0.09 Å. A stereoscopic view of the U–Trp–A molecule is shown in Fig. 3, and the conformational torsion angles are listed in Table IV.

Average bond lengths and angles for neutral A and U bases have been reported by Voet and Rich,[13] and are in good agreement with those of U–Trp–A within the standard errors. The molecular dimensions of the indole ring and the peptide moieties are also normal as judged from those of related indole[14] and peptide compounds.[15] The planarities of U, indole, A, and two peptides were examined in terms of the equations of least-squares planes. The results are summarized in Table V. The rings of U, indole and A are almost planar; maximum deviations are −0.005 Å for the C(2) atom of U, −0.035 Å for the C(5) atom of indole and 0.026 Å for the N(7) atom of A, and the atoms directly attached to the respective rings lay approximately on the planes. The two peptide linkages also form planes with *trans* conformations. The conformation of U–Trp–A, as a whole, is depictable as a W-sharped, extended form. It is characteristic that the side rings of U, indole and A all protrude to the same side of the peptide chain. In order to avoid close atomic contacts, C(8)U and two H(7)U atoms are in a staggered orientation with respect to the C(2)U and C(6)U atoms of the uracil base, and consequently the torsion angle χ_2 is close to 90°. Similar conformations are also

TABLE II. Final Atomic Coordinates of U–Trp–A

Atom	x	y	z	B_{eq} [a]
N(1)A[b]	0.527 (1)	0.2934 (2)	0.8768 (5)	3.7 (2)
C(2)A	0.539 (1)	0.3040 (2)	0.7473 (6)	3.6 (2)
N(3)A	0.7037 (9)	0.3325 (2)	0.6949 (4)	3.3 (2)
C(4)A	0.877 (1)	0.3513 (2)	0.7904 (5)	2.9 (2)
C(5)A	0.881 (1)	0.3437 (2)	0.9276 (6)	3.3 (2)
C(6)A	0.699 (1)	0.3126 (2)	0.9712 (6)	3.2 (2)
N(6)A	0.692 (1)	0.2999 (2)	1.1012 (5)	4.6 (3)
N(7)A	1.081 (1)	0.3693 (2)	0.9952 (5)	3.8 (2)
C(8)A	1.192 (1)	0.3901 (2)	0.8984 (6)	3.7 (2)
N(9)A	1.0763 (9)	0.3800 (2)	0.7716 (5)	3.1 (2)
C(10)A	1.148 (1)	0.3998 (2)	0.6450 (6)	3.4 (2)
C(11)A	0.980 (1)	0.4412 (2)	0.6070 (6)	3.4 (2)
N(12)A	1.0118 (9)	0.4744 (2)	0.7146 (5)	3.5 (2)
N(1)T	0.893 (2)	0.5931 (3)	1.3014 (7)	6.4 (3)
C(2)T	0.742 (2)	0.5771 (3)	1.1868 (7)	5.5 (4)
C(3)T	0.845 (1)	0.5384 (3)	1.1493 (7)	4.3 (3)
C(4)T	1.240 (1)	0.4912 (3)	1.2633 (7)	5.6 (4)
C(5)T	1.430 (2)	0.4901 (4)	1.3707 (9)	7.3 (5)
C(6)T	1.452 (2)	0.5264 (4)	1.460 (1)	8.0 (5)
C(7)T	1.289 (2)	0.5619 (4)	1.4496 (8)	7.2 (5)
C(8)T	1.087 (2)	0.5632 (3)	1.3385 (7)	5.4 (4)
C(9)T	1.067 (1)	0.5278 (3)	1.2466 (7)	4.5 (3)
C(10)T	0.745 (1)	0.5095 (2)	1.0338 (7)	4.2 (3)
C(11)T	0.895 (1)	0.5177 (2)	0.9061 (6)	3.1 (2)
N(12)T	0.8298 (8)	0.5613 (2)	0.8487 (5)	3.1 (2)
C(13)T	0.823 (1)	0.4832 (2)	0.7967 (6)	3.1 (2)
O(13)T	0.5979 (8)	0.4664 (2)	0.7871 (5)	4.4 (2)
N(1)U	1.067 (1)	0.6862 (2)	0.9081 (5)	3.7 (2)
C(2)U	0.868 (1)	0.7155 (2)	0.9351 (6)	3.6 (3)
O(2)U	0.700 (1)	0.7292 (2)	0.8495 (5)	5.2 (2)
N(3)U	0.869 (1)	0.7287 (2)	1.0670 (6)	4.5 (3)
C(4)U	1.043 (1)	0.7152 (3)	1.1707 (7)	4.7 (3)
O(4)U	1.016 (1)	0.7301 (2)	1.2861 (6)	6.4 (3)
C(5)U	1.247 (2)	0.6841 (3)	1.1363 (8)	5.3 (3)
C(6)U	1.249 (1)	0.6713 (2)	1.0094 (7)	4.4 (3)
C(7)U	1.076 (1)	0.6718 (2)	0.7665 (6)	3.8 (3)
C(8)U	0.908 (1)	0.6306 (2)	0.7356 (6)	3.6 (2)
C(9)U	1.014 (1)	0.5890 (2)	0.8040 (5)	2.8 (2)
O(9)U	1.2557 (8)	0.5816 (1)	0.8204 (5)	4.1 (2)
O(1)W	0.104 (1)	0.3577 (3)	0.2837 (5)	7.7 (3)
O(2)W	0.377 (1)	0.8428 (5)	0.5896 (6)	12.2 (6)
O(3)W	0.299 (2)	0.1736 (3)	0.5647 (8)	11.0 (5)
O(4)W	1.365 (3)	0.7309 (6)	0.532 (1)	18.0 (1)

The estimated standard deviations are shown in parentheses.
a) $B_{eq}=4/3(B_{11}a^2+B_{22}b^2+B_{33}c^2+acB_{13}\cos\beta)$. *b*) The suffixes A, T, U and W represent the adenine, tryptophan and uracil moieties of U–Trp–A, and water of crystallization, respectively.

seen in the indole and adenine moieties. The tryptophan moiety takes a conformation that is commonly observed in related compounds[16]: the carbonyl and amino groups are in the *trans* and *gauche*$^-$ positions with respect to the indole ring (C(13)T–C(11)T–C(10)–C(3)T = 170.9(5)° and $\chi_1 = -69.1(7)$°).

Crystal Packing and Hydrogen Bonding Mode

Figure 4 shows the crystal packings viewed along the *a* and *c* axes; molecules of water of

TABLE III. Bond Lengths and Angles of U–Trp–A

1) Bond lengths (Å)

Bond	Distance	Bond	Distance
N(1)A–C(2)A	1.332 (8)	C(5)T–C(6)T	1.41 (2)
N(1)A–C(6)A	1.348 (8)	C(6)T–C(7)T	1.34 (2)
C(2)A–N(3)A	1.323 (8)	C(7)T–C(8)T	1.43 (2)
N(3)A–C(4)A	1.355 (8)	C(8)T–C(9)T	1.40 (1)
C(4)A–C(5)A	1.381 (8)	C(10)T–C(11)T	1.547 (9)
C(4)A–N(9)A	1.342 (7)	C(11)T–N(12)T	1.457 (8)
C(5)A–C(6)A	1.399 (8)	C(11)T–C(13)T	1.524 (8)
C(5)A–N(7)A	1.390 (8)	N(12)T–C(9)U	1.344 (7)
C(6)A–N(6)A	1.349 (8)	C(13)T–O(13)T	1.229 (8)
N(7)A–C(8)A	1.308 (8)	N(1)U–C(2)U	1.367 (8)
C(8)A–N(9)A	1.379 (8)	N(1)U–C(6)U	1.377 (9)
N(9)A–C(10)A	1.462 (8)	N(1)U–C(7)U	1.477 (8)
C(10)A–C(11)A	1.534 (9)	C(2)U–O(2)U	1.217 (8)
C(11)A–N(12)A	1.462 (8)	C(2)U–N(3)U	1.369 (9)
N(12)A–C(13)T	1.320 (8)	N(3)U–C(4)U	1.36 (1)
N(1)T–C(2)T	1.40 (1)	C(4)U–O(4)U	1.25 (1)
N(1)T–C(8)T	1.35 (1)	C(4)U–C(5)U	1.44 (1)
C(2)T–C(3)T	1.34 (1)	C(5)U–C(6)U	1.32 (1)
C(3)T–C(9)T	1.45 (1)	C(7)U–C(8)U	1.515 (9)
C(3)T–C(10)T	1.50 (1)	C(8)U–C(9)U	1.498 (8)
C(4)T–C(5)T	1.37 (2)	C(9)U–O(9)U	1.223 (7)
C(4)T–C(9)T	1.40 (1)		

2) Bond angles (°)

Bond	Angle	Bond	Angle
C(2)A–N(1)A–C(6)A	119.7 (5)	N(1)T–C(8)T–C(9)T	108.4 (7)
N(1)A–C(2)A–N(3)A	127.8 (6)	C(7)T–C(8)T–C(9)T	119.5 (8)
C(2)A–N(3)A–C(4)A	112.1 (5)	C(3)T–C(9)T–C(4)T	133.3 (8)
N(3)A–C(4)A–C(5)A	125.6 (5)	C(3)T–C(9)T–C(8)T	106.2 (7)
N(3)A–C(4)A–N(9)A	127.5 (5)	C(4)T–C(9)T–C(8)T	120.5 (8)
C(5)A–C(4)A–N(9)A	106.9 (5)	C(3)T–C(10)T–C(11)T	112.5 (6)
C(4)A–C(5)A–C(6)A	117.2 (5)	C(10)T–C(11)T–N(12)T	110.8 (5)
C(4)A–C(5)A–N(7)A	109.9 (5)	C(10)T–C(11)T–C(13)T	112.0 (5)
C(6)A–C(5)A–N(7)A	132.9 (5)	N(12)T–C(11)T–C(13)T	107.5 (5)
N(1)A–C(6)A–C(5)A	117.6 (5)	C(11)T–N(12)T–C(9)U	123.3 (5)
N(1)A–C(6)A–N(6)A	118.8 (5)	N(12)A–C(13)T–C(11)T	115.9 (5)
C(5)A–C(6)A–N(6)A	123.6 (5)	N(12)A–C(13)T–O(13)T	124.3 (6)
C(5)A–N(7)A–C(8)A	103.9 (5)	C(11)T–C(13)T–O(13)T	119.8 (5)
N(7)A–C(8)A–N(9)A	113.3 (6)	C(2)U–N(1)U–C(6)U	120.9 (5)
C(4)A–N(9)A–C(8)A	106.0 (5)	C(2)U–N(1)U–C(7)U	117.0 (5)
C(4)A–N(9)A–C(10)A	128.2 (5)	C(6)U–N(1)U–C(7)U	122.1 (5)
C(8)A–N(9)A–C(10)A	125.6 (5)	N(1)U–C(2)U–O(2)U	123.4 (6)
N(9)A–C(10)A–C(11)A	112.0 (5)	N(1)U–C(2)U–N(3)U	115.3 (6)
C(10)A–C(11)A–N(12)A	110.4 (5)	O(2)U–C(2)U–N(3)U	121.3 (6)
C(11)A–N(12)A–C(13)T	123.1 (5)	C(2)U–N(3)U–C(4)U	126.4 (6)
C(2)T–N(1)T–C(8)T	108.7 (7)	N(3)U–C(4)U–O(4)U	118.9 (7)
N(1)T–C(2)T–C(3)T	109.3 (7)	N(3)U–C(4)U–C(5)U	115.8 (7)
C(2)T–C(3)T–C(9)T	107.3 (7)	O(4)U–C(4)U–C(5)U	125.4 (7)
C(2)T–C(3)T–C(10)T	127.4 (7)	C(4)U–C(5)U–C(6)U	118.3 (7)
C(9)T–C(3)T–C(10)T	125.2 (7)	N(1)U–C(6)U–C(5)U	123.2 (7)

2) Bond angles (°)

Bond	Angle	Bond	Angle
C(5)T–C(4)T–C(9)T	119.4 (9)	N(1)U–C(7)U–C(8)U	112.0 (5)
C(4)T–C(5)T–C(6)T	119 (1)	C(7)U–C(8)U–C(9)U	114.8 (5)
C(5)T–C(6)T–C(7)T	124 (1)	N(12)T–C(9)U–C(8)U	116.1 (5)
C(6)T–C(7)T–C(8)T	118 (1)	N(12)T–C(9)U–O(9)U	122.4 (5)
N(1)T–C(8)T–C(7)T	132.1 (9)	C(8)U–C(9)U–O(9)U	121.5 (5)

The estimated standard deviations are shown in parentheses.

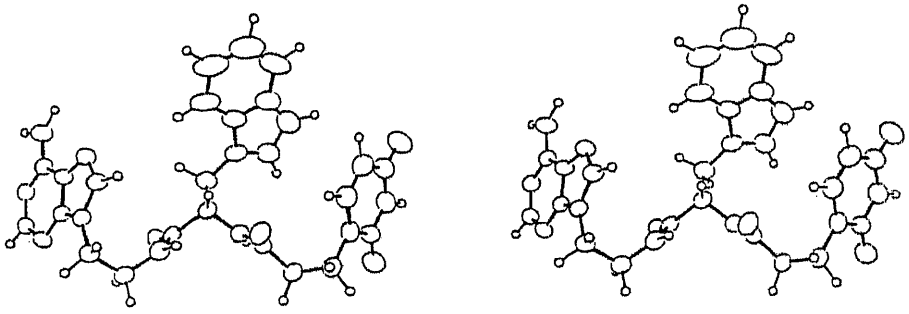


Fig. 3. A Stereoscopic View of U–Trp–A

TABLE IV. Selected Torsion Angles (°) of U–Trp–A

U	ψ	[C(7)U–C(8)U–C(9)U–N(12)T]	141.7 (5)
	ω	[C(8)U–C(9)U–N(12)T–C(11)T]	174.1 (5)
	χ_1	[C(9)U–C(8)U–C(7)U–N(1)U]	−68.5 (7)
	χ_2	[C(8)U–C(7)U–N(1)U–C(2)U]	−89.8 (6)
Trp	ϕ	[C(9)U–N(12)T–C(11)T–C(13)T]	−100.8 (6)
	ψ	[N(12)T–C(11)T–C(13)T–N(12)A]	87.0 (6)
	ω	[C(11)T–C(13)T–N(12)A–C(11)A]	177.9 (5)
	χ_1	[N(12)T–C(11)T–C(10)T–C(3)T]	−69.1 (7)
	χ_2	[C(11)T–C(10)T–C(3)T–C(2)T]	96.3 (9)
A	ϕ	[C(13)T–N(12)A–C(11)A–C(10)A]	−106.5 (6)
	χ_1	[N(12)A–C(11)A–C(10)A–N(9)A]	57.9 (6)
	χ_2	[C(11)A–C(10)A–N(9)A–C(8)A]	−90.7 (7)

The estimated standard deviations are shown in parentheses.

crystallization are indicated by filled circles, and the dotted lines represent possible hydrogen bonds. The possible hydrogen bonds and short contacts observed in the crystal are summarized in Table VI. The polar atoms of the U–Trp–A molecule all participate in hydrogen bonds. The N(12)T—O(9)U and N(12)A—O(13)T atomic pairs of two peptide groups are hydrogen-bonded to those of the neighboring molecules related by *a*-axis translation, consequently forming an infinite layer along this axis (see Fig. 4(A)). On the other hand, U–Trp–A molecules related by a diad screw symmetry are linked in a head-to-tail fashion by two hydrogen bonds between the A and U bases (see Fig. 4(B)): two hydrogen bonds, N(1)A···N(3)U(=2.858(8) Å) and N(6)A···O(2)U(=2.955(8) Å) atomic pairs, form a reversed Watson–Crick base pairing. Similar base pairing has also been observed in the 8-bromo-9-ethyladenine: 1-methyl-5-bromouracil complex.[17]

Four molecules of water of crystallization, which exist near the base pairing parts,

TABLE V. Optimal Planes and Deviations (Å) of Individual Atoms

1) Equations of the best planes expressed as $m_1x+m_2y+m_3z=d$ in the orthogonal space

	Plane	m_1	m_2	m_3	d
I	Uracil base	0.62161	0.76293	−0.17759	16.99311
II	Indole ring	0.68194	0.46386	−0.56551	3.30663
III	Adenine base	−0.66000	0.74617	0.08734	6.08210
IV	C(9)U, O(9)U, N(12)T	0.01127	0.44994	0.89299	15.16247
V	C(13)T, O(13)T, N(12)A	−0.30603	0.72162	−0.62098	4.53965

2) Deviations (Å) from the best plane

I Uracil base		II Indole ring		III Adenine ring		IV C(9)UO(9)UN(12)T		V C(13)TO(13)TN(12)A	
N(1)U[a]	0.003 (7)	N(1)T[a]	0.001 (9)	N(1)A[a]	−0.017 (6)	C(9)U[a]	−0.034 (7)	C(13)T[a]	−0.023 (8)
C(2)U[a]	−0.005 (8)	C(2)T[a]	−0.032 (10)	C(2)A[a]	0.003 (7)	O(9)U[a]	0.023 (6)	O(13)T[a]	0.014 (6)
N(3)U[a]	0.003 (7)	C(3)T[a]	0.010 (9)	N(3)A[a]	0.025 (6)	N(12)T[a]	−0.025 (6)	N(12)A[a]	−0.016 (7)
C(4)U[a]	−0.001 (9)	C(4)T[a]	−0.005 (12)	C(4)A[a]	0.011 (6)	H(12)T[a]	0.030 (20)	H(12)A[a]	0.016 (22)
C(5)U[a]	0.000 (9)	C(5)T[a]	−0.035 (14)	C(5)A[a]	0.016 (6)	C(8)U	−0.083 (18)	C(11)T	−0.032 (20)
C(6)U[a]	−0.002 (9)	C(6)T[a]	−0.003 (15)	C(6)A[a]	−0.021 (7)	C(11)T	−0.104 (18)	C(11)A	−0.052 (20)
O(2)U	−0.016 (9)	C(7)T[a]	0.001 (14)	N(7)A[a]	0.026 (6)				
O(4)U	−0.003 (10)	C(8)T[a]	0.016 (10)	C(8)A[a]	−0.007 (7)				
C(7)U	0.020 (10)	C(9)T[a]	0.019 (9)	N(9)A[a]	−0.030 (6)				
		C(10)T	−0.026 (12)	N(6)A	−0.102 (8)				
				C(10)A	0.000 (8)				

3) Dihedral angles (°) between the best planes

	II	III	IV	V
I	28.6 (3)	81.7 (3)	78.9 (6)	61.9 (6)
II		98.8 (3)	106.8 (6)	61.5 (6)
III			66.0 (6)	46.7 (6)
IV				103.5 (8)

a) Atoms define the plane in each case.

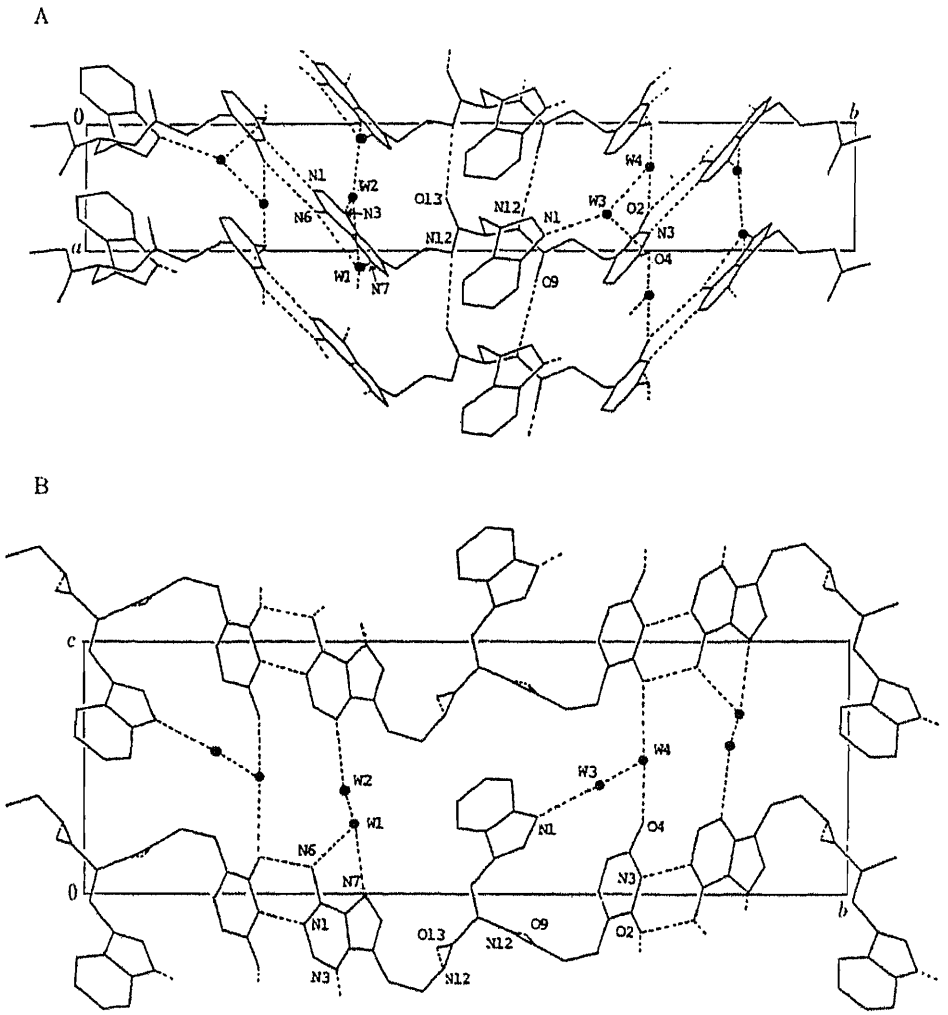


Fig. 4. Crystal Packing of U–Trp–A, Viewed along the *c*-Axis (A) and the *a*-Axis (B)

stabilize the molecular packing in the *c* direction through hydrogen bonds with polar atoms of the aromatic rings.

Stacking Interactions between the Aromatic Rings

One of the structural characteristics observed in this crystal is the presence of four kinds of stacking interactions between the aromatic rings.

1) Stacking Interactions between Adenine Rings and between Indole Rings——Stacking interactions between the adenine rings and between the indole rings are formed in the infinite layers of U–Trp–A arranged along the *a*-axis. The stacking modes are shown in Fig. 5, (A) and (B). The stacking rings are parallel to each other, and the average interplanar spacings are 3.25 Å for the adenine rings and 3.43 Å for the indole rings. These partial overlaps would be stabilized by electrostatic interactions in addition to the crystal packing forces. A comparison of the atomic charges (in atomic units), calculated by the CNDO/2 method,[18] indicates the electrostatic attraction between atomic pairs showing the short contacts (Table VI): −0.283—0.165 for N(1)A—C(8)A, 0.214——0.122 for C(2)A—N(9)A, −0.257—0.086 for N(3)A—C(10)A, 0.024——0.158 for C(6)T—N(1)T and 0.005——0.035 for C(5)T—C(3)T atomic pairs.

2) Stacking Interaction between Adenine and Uracil Bases and between Uracil Base and Indole Ring——Although we expected this compound to be an interaction model of

TABLE VI. Hydrogen Bonds and Short Contacts

1) Hydrogen bonds

Donor (D) at x,y,z	Acceptor (A) at	symmetry operation	Distance (Å) D···A	Angle (°) D-H···A
N(3)U	N(1)A	$1-x, y+0.5, 2-z$	2.858 (8)	175 (4)
N(1)T	O(3)W	$1-x, y+0.5, 2-z$	2.957 (13)	165 (6)
N(12)T	O(9)U	$x-1, y, z$	2.919 (7)	172 (5)
N(6)A	O(2)U	$1-x, y-0.5, 2-z$	2.955 (8)	172 (4)
N(6)A	O(1)W	$1+x, y, 1+z$	3.152 (11)	156 (6)
N(12)A	O(13)T	$1+x, y, z$	2.965 (7)	130 (4)
O(1)W	N(7)A	$x-1, y, z-1$	2.882 (11)	178 (6)
O(1)W	O(2)W	$1-x, y-0.5, 1-z$	2.825 (17)	—
O(2)W	N(3)A	$1-x, y+0.5, 1-z$	2.841 (15)	178 (7)
O(2)W	O(1)W	$-x, y+0.5, 1-z$	2.831 (17)	—
O(3)W	O(4)U	$1-x, y-0.5, 2-z$	2.813 (13)	177 (6)
O(3)W	O(4)W	$2-x, y-0.5, 1-z$	2.639 (21)	—
O(4)W	O(4)U	$x, y, z-1$	2.881 (19)	180 (7)
O(4)W	O(2)U	$x+1, y, z$	3.454 (19)	178 (7)

2) Short contacts (less than 3.5 Å)

Atom at x,y,z	Atom at	symmetry operation	Distance (Å)	Atom at x,y,z	Atom at	symmetry operation	Distance (Å)
C(8)U	O(3)W	$1-x, y+0.5, 1-z$	3.339 (12)	N(1)A	N(7)A	$x-1, y, z$	3.458 (7)
N(1)A	C(8)A	$x-1, y, z$	3.369 (8)	C(2)A	N(9)A	$x-1, y, z$	3.268 (8)
N(3)A	C(10)A	$x-1, y, z$	3.434 (8)	C(6)A	C(8)A	$x-1, y, z$	3.471 (9)
N(1)T	C(6)T	$x-1, y, z$	3.451 (15)	C(3)T	C(5)T	$x-1, y, z$	3.461 (15)
O(13)T	C(8)A	$x-1, y, z$	3.307 (8)	O(13)T	C(10)A	$x-1, y, z$	3.245 (8)
C(2)U	C(6)U	$x-1, y, z$	3.492 (10)	O(2)U	C(6)U	$x-1, y, z$	3.346 (9)
N(3)U	C(5)U	$x-1, y, z$	3.495 (10)	O(2)W	O(4)W	$x-1, y, z$	3.413 (23)
C(4)U	O(3)W	$1-x, y+0.5, 2-z$	3.478 (13)	N(6)A	O(2)W	$1-x, y-0.5, 2-z$	3.376 (15)
N(1)A	C(4)U	$2-x, y-0.5, 2-z$	3.244 (9)	N(1)A	O(4)U	$2-x, y-0.5, 2-z$	3.469 (8)
N(1)A	C(5)U	$2-x, y-0.5, 2-z$	3.477 (10)	C(2)A	C(4)U	$2-x, y-0.5, 2-z$	3.448 (10)
C(2)A	O(4)U	$2-x, y-0.5, 2-z$	3.179 (9)	N(3)A	O(4)U	$2-x, y-0.5, 2-z$	3.379 (8)
C(6)A	N(3)U	$2-x, y-0.5, 2-z$	3.360 (8)	N(6)A	C(2)U	$2-x, y-0.5, 2-z$	3.394 (9)

tryptophan with the A—U Watson-Crick base pairing, the crystal structure showed an extended molecular packing different from that of a self-complementary dinucleotide such as ApU[19]; the adenine base is not parallel to the uracil base intramolecularly, and the dihedral angle is 81.7(3)°. However, an intermolecular stacking interaction is observed between these bases. The adenine and uracil bases translated by a diad screw axis form a parallel alignment, as shown in Fig. 5(C): their dihedral angle is 5.7(3)° and the average interplanar spacing is 3.40 Å. As is clear from this figure, this interaction is stabilized by a strong dipole-dipole coupling interaction, because the directions of the respective dipole moments of the uracil and adenine bases, which were calculated by the CNDO/2 method, are almost antiparallel to each other. Furthermore, electrostatic attractions play a role in the stabilization: −0.283—0.368 for N(1)A—C(4)U, 0.214——0.352 for C(2)A—O(4)U, 0.269——0.236 for C(6)A—N(3)U and −0.244—0.452 for N(6)A—C(2)U atomic pairs.

On the other hand, a weak intramolecular stacking interaction is observed between the uracil base and indole ring, as can be seen in Fig. 5(D). The dihedral angle between them is 28.6(3)°, and the average interplanar spacing is 3.95 Å. Since this spacing value is outside the

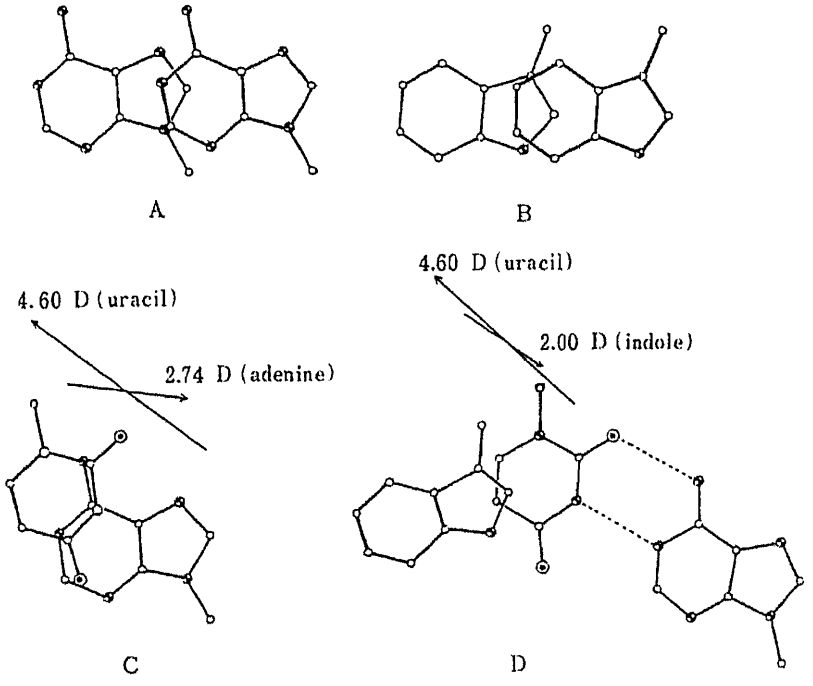


Fig. 5. Stacking Modes between Adenine–Adenine (A), Indole–Indole (B), Adenine–Uracil (C) and Uracil–Indole (D) Rings

Fig. 5(A) or (B) shows the projection perpendicular to the upper adenine or indole ring at x,y,z, and the lower adenine or indole ring is related by the symmetry operation of $x-1,y,z$, respectively. Fig. 5(C) shows the stacking mode of adenine (at x,y,z) and uracil (at $2-x,y-0.5,2-z$) bases, viewed perpendicular to the adenine ring. Fig. 5(D) is a projection perpendicular to the central uracil base (at x,y,z), showing the stacking interaction with the indole ring (at x,y,z) and the hydrogen bonding with the adenine base (at $1-x,y+0.5,2-z$). In Fig. 5(C) and (D), the vectors of permanent dipole moments are represented by arrows. The unit of dipole moment is debye (D).

normal van der Waals separation distance (=3.4 Å), the stacking interaction would be stabilized by the van der Waals contacts between the two aromatic rings. Figure 5(D) shows that the dipole–dipole interaction is important in determining the mutual orientation between the indole ring and the uracil base. A consideration of space-filling models suggests that the observed stacking mode is reasonable, without any steric hindrance, as an interaction mode of tryptophan residue with the uracil base of a single-stranded nucleic acid. Therefore this result means that the dipole–dipole interaction may be an important driving force in placing these two molecules close to each other.

Do the present X-ray results mean that tryptophan interacts with uracil base in preference to adenine base? Several solution studies[20,21] have suggested a preference for the adenine base, and a theoretical calculations[5] has also suggested the existence of indole–adenine stacking interaction. One reason why there is no interaction between the indole ring and the adenine base in this compound, may be that there is a difference between the connecting environments of the uracil and adenine bases to tryptophan, *i.e.*, U–CO–NH– and –CO–NH–A. Therefore further studies using various model compounds are necessary to examine the stacking mode between the indole ring and nucleic acid base at the atomic level.

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Voltammetric and Spectroscopic Studies on the Carcinogen 4-(Hydroxyamino)quinoline *N*-Oxide and Its Analogues

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In connection with the carcinogenic activity of 4-(hydroxyamino)quinoline *N*-oxide (4HAQO), voltammetric studies of the oxidation processes of 4HAQO and related substances were carried out in aqueous solutions. It was verified that 4HAQO forms a reversible redox couple with 4-nitrosoquinoline *N*-oxide (4NOQO) through an intermediate free radical. The reversible reactions in these redox couples were evaluated quantitatively, and the formation constant of the intermediate free radical was calculated. The pH dependence of the above standard redox potential gave the pK_a values, which agreed quite well with the data measured by ultraviolet spectral methods. The molecular structures of species formed by proton addition or dissociation were investigated by analyses of the pK_a values and the electronic spectra, and by their molecular orbital calculations. The *N*-oxide type structure was considered to be a main species in aqueous solutions of 4HAQO and its analogues.

Keywords——carcinogenic activity; 4-(hydroxyamino)quinoline *N*-oxide; 4-nitrosoquinoline *N*-oxide; cyclic voltammetry; electronic spectra; PPP-SCFMO-CI calculation; CNDO/S calculation; pK_a value; free radical formation constant

The fact that the 4-nitroquinoline *N*-oxide (4NQO) is a potent carcinogen is well known.[2,3)] Many chemical and biological studies of the carcinogenicity of 4NQO have indicated that the carcinogenic activity of 4NQO may not be attributed to the molecular interaction ability of 4NQO itself in biological systems,[4)] but should be due to 4-(hydroxyamino)quinoline *N*-oxide (4HAQO),[5)] which is formed from 4NQO by metabolic reduction in biological systems. Thus, much work on 4HAQO and related substances has been carried out by using physicochemical, chemical, and biological techniques from the viewpoint of the carcinogenic activity.[3,5,6)] However, no voltammetric study of the oxidation processes of 4HAQO and its analogues has, to our knowledge, yet been reported, even though the oxidation–reduction processes in hydrophilic media might have a very important bearing on the carcinogenic mechanism. In this paper we present the results of detailed voltammetric investigations on the oxidation processes of 4HAQO and related substances. In addition, the molecular structures and acid dissociation constants of these *N*-oxides in aqueous solutions have been examined by analyses of the ultraviolet (UV) spectra by means of molecular orbital (MO) calculations, because several tautomeric equilibria are possible for 4HAQO.

Experimental

Voltammetric and Spectral Measurements——The oxidation processes of 4HAQO and related substances were studied by recording cyclic voltammograms (CV) on a Yanagimoto P-1000 voltammetric analyzer, equipped with a Watanabe WX-4401 X-Y recorder, an NF-circuit function generator (model FG-121B), an Iwatsu digital memory

(model DM-7100) with a resolution efficiency of 12 bits and 5 μs word^{-1}, and an Iwatsu synchroscope (model SS-5702).[7] The CV curves were recorded at various sweep rates. Positive feedback circuits were added to the above instruments for so-called IR-drop compensation, though the instruments consist of a three-electrode system. The working electrode was a platinum rod, 1 mm in diameter, sealed in a soft glass tube with a diameter of *ca.* 6.5 mm.[7b,8] Just before use, we polished the plane head of the platinum electrode with a commercially available polishing powder, "Royal Diamond Compound." In the case of CV measurements on a sample adsorbed on a working electrode, we employed a cylindrical rod of a pyrolytic graphite carbon (Tomoe Co., Osaka), which was sealed onto a glass tube by heat-shrinkable Teflon tubing. The pyrolytic graphite electrode was cleaved with a razor-blade to produce a basal-plane surface just before use. The electrode surface area was 0.126 cm^2. Our foregoing papers include details pertinent to these working electrodes.[7a] Reference and auxiliary electrodes were a Yanagimoto MR-P2 saturated calomel electrode (SCE) and a coiled platinum wire, respectively. All the measurements were performed at 25$\pm$0.1 °C in suitable buffer solutions (*vide infra*), the oxygen gas dissolved in the test solution being removed by bubbling pure nitrogen gas. The sample concentration was about 3×10^{-4} mol dm^{-3}. Our technique reported in the foregoing papers was applied in order to adsorb 4HAQO and related substances on the surface of a basal-plane pyrolytic graphite electrode (BPGE).[7a,9] For example, in the case of direct adsorption of 4HAQO on the electrode surface under the CV recording conditions, the electrode was exposed to 5.9×10^{-6} mol dm^{-3} buffer sample solution, containing 0.05% methanol (*vide infra*), for *ca.* 3 min under a nitrogen atmosphere at the set potential (0.3 V in pH 1.10 buffer solution), where no change of 4HAQO occurs, and then the CV curve was recorded in the positive direction. The adsorption on the electrode surface under these conditions seems to be in the form of a monomolecular layer with a surface coverage of less than unity on the basis of electricity measurements.

Absorption spectra were recorded in the usual manner with a Hitachi spectrophotometer, model 323, and a Shimadzu spectrophotometer, UV-240, at 25$\pm$0.2 °C, which was maintained during the measurements by circulating constant-temperature water throughout the cell compartment by the use of a Taiyo thermoleader, model EZL-80. Usually the cells used were 1 cm matched quartz cells equipped with stoppers.

All the samples employed here are quite sensitive to (are oxidized by) the oxygen in air, so that all the samples and their solutions were manipulated under a nitrogen atmosphere in a flexible polyethylene bag. Further, we always employed solvents or buffer solutions from which dissolved air had been removed as thoroughly as possible by bubbling nitrogen gas just before use. In the case of the preparation of sample solutions for voltammetric and UV measurements, the stock solution (10^{-3}—10^{-4} mol dm^{-3}) in methanol (for 6-(hydroxyamino)quinoline *N*-oxide alone) or methanol–buffer 1 : 1 mixed solvent was first prepared because of the low solubility of the samples in water, then it was usually diluted 20 times and 10 times with an appropriate buffer solution for spectral and voltammetric measurements, respectively, to get the final measuring solutions, the apparent pH values of which were used to evaluate the physical constants.

Samples and Solvents——The substances employed here were quinoline *N*-oxides substituted with a 4-nitroso group (4NOQO), a 4-N(CH_3)OH group (4NMHAQO), or an NHOH group at the 4-, 5-, or 6-position (4-, 5-, and 6HAQO's). Samples of 4-(hydroxyamino)quinoline (4HAQ), 4-(hydroxyamino)pyridine *N*-oxide (4HAPO), 3-methyl-4-(hydroxyamino)pyridine *N*-oxide (3Me4HAPO), (hydroxyamino)benzene (HAB), and 1-(hydroxyamino)naphthalene (1HAN) were also used for comparison with the above quinoline *N*-oxides. These samples were the same as those used by Kawazoe and his colleagues in order to study the carcinogenic activity of various kinds of aromatic *N*-oxides and related substances.[10] The purity was carefully checked by thin-layer chromatography (TLC) and spectroscopic methods (mass (MS), nuclear magnetic resonance (NMR), infared (IR), and UV). 4NMHAQO, 4HAPO, and 4HAQ were directly used as their HCl salts, the solvents in this case being buffer solutions. The samples mentioned above are autooxidizable and unstable to air and light, so they were put in sample tubes, filled with nitrogen or argon gas, and stored in a refrigerator. The buffer solutions employed were the systems of 0.1 or 0.2 mol dm^{-3} CH_3COONa + HCl, 0.1 mol dm^{-3} Na_2HPO_4 + HCl, and 0.1 mol dm^{-3} Na_2CO_3 + HCl for the pH regions of 3—6, 6—8, and 8—12, respectively. HCl solution alone was used in the pH region smaller than 3. The ionic strength of all the buffer solutions was adjusted to 0.5 by adding NaCl.

Calculation of UV Spectra——In order to interpret the UV spectra of (hydroxyamino)quinoline *N*-oxides, Pariser–Paar–Pople (PPP) and CNDO/S calculations have been performed. In the case of PPP and CNDO/S

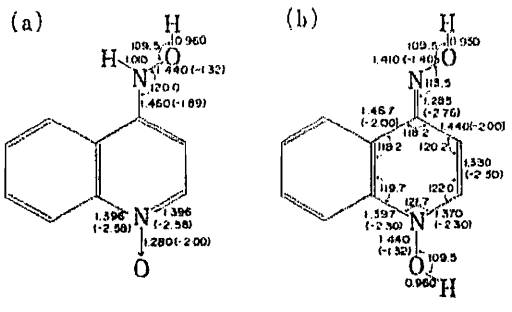


Fig. 1. Molecular Geometries (Å and Degree) and Core-Resonance Energies (β_{core}) Employed in This Study for 4HAQO (a) and Its Hydroxyimino-Type Tautomer (b)

The latter values (eV unit) are shown in parentheses. The ring skeleton of (a) and the benzene ring in (b) are considered to be regular hexagons with a distance of 1.396 Å and $\beta_{core}=-2.37$ eV. See the text for details.

calculations, the valence state ionization potential (I_p) and electron affinity (E_A) of each atom in the molecules were the same as those reported in our foregoing papers.[11] Since two reasonable tautomers can be considered for 4HAQO, as shown in Fig. 1a, b, the molecular dimensions and the core resonance integrals (β_{core}) were separately estimated for these two isomers. The above two variables (I_p and E_A) of the quinoline *N*-oxide part and the $\gtrless$C–NHOH bond distance in 4HAQO were put equal to those employed in our previous studies.[12] The additivity of a single bond atom radius[12b] was applied to estimate the dimensions of the NHOH group, then the β_{core}^{C-N} and β_{core}^{N-O} values of the $\gtrless$C–NHOH group (see Fig. 1a) were evaluated by checking the mutual relations of bond length *vs.* β_{core}^{CN} and bond length *vs.* β_{core}^{NO}, and assigned as -1.89 and -1.32 eV, respectively.[11-13] The ring and the $\gtrless$C=NOH structures of the hydroxyimine-type isomer of 4HAQO were decided by referring to those of *p*-quinones[14] and X-ray crystallographic analyses of similar substances,[15] the details being given in Fig. 1b. The β_{core} values corresponding to the hydroxyimine-type molecular dimensions are given in parentheses in Fig. 1b and were estimated as follows: (i) $\beta_{core}^{C=N}$ for a hydroxyimino group, $\beta_{core}^{C=N}$ in a ring, and β_{core}^{NO} were estimated by using the bond length *vs.* β_{core} relationship (*vide supra*), and (ii) $\beta_{core}^{C=C}$ and β_{core}^{C-C} are taken from the data used for the calculation of *p*-quinones.[14] The bond length of all C–H groups was assumed to be 1.084 Å. The values of Slater exponent, bonding parameter, ($I_p^n + E_A^n$) and ($I\beta + E_A^n$) necessary for the CNDO/S calculation are the same as employed in our previous studies.[11] One and two center repulsion integrals were calculated by using the Pariser-Paar[16] and Nishimoto-Mataga methods,[17] respectively. PPP calculation was carried out with an NEC PC-9801 personal computer in our laboratory, while CNDO/S calculation was done using a FACOM M-382 computer at the Nagoya University Computation Center.

Results and Discussion

Oxidation–Reduction Behavior, Electronic Spectra, and Their pH Dependence of 4HAQO and Related Substances

In the course of the present studies we have clearly found that, as can be seen in Fig. 2, 4HAQO shows a reversible or quasi-reversible CV curve; this voltammogram is especially stable in lower pH regions and consists of a clear redox couple. With increasing pH, however, an effect due to the succeeding chemical reactions appears on the CV curve, so that the measurement of a reversible or quasi-reversible CV curve becomes somewhat difficult. This difficulty could be overcome by recording the CV curve at an increasing sweep rate, in the present case to a maximum 1 V s^{-1}. Under these conditions we could record the CV curves of 4HAQO and the related substances in various pH buffer solutions, and the CV curve and its oxidation and reduction peak potentials (E_p^O and E_p^R) agreed well with those of 4NOQO. If the final oxidation product of 4HAQO is not 4NOQO, the E_p^O and E_p^R values of the CV curve starting from 4HAQO should be different from those obtained from 4NOQO. However, this is not the case. Thus it is clear that 4HAQO and the related substances are as a whole in a reversible two-electron oxidation–reduction couple with 4NOQO and the related nitroso substances, as indicated in Chart 1. This conclusion was supported by peak-height analysis of the CV curves. It was found that the analysis of the anodic peak-height by means of

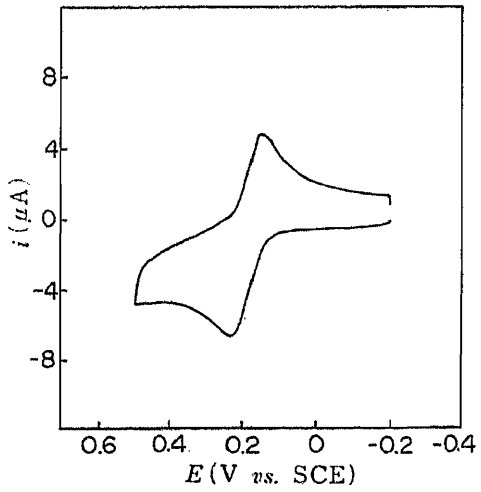


Fig. 2. Cyclic Voltammogram of 4HAQO Using a Platinum Electrode
pH 4.95, $v = 500$ mV s^{-1}.

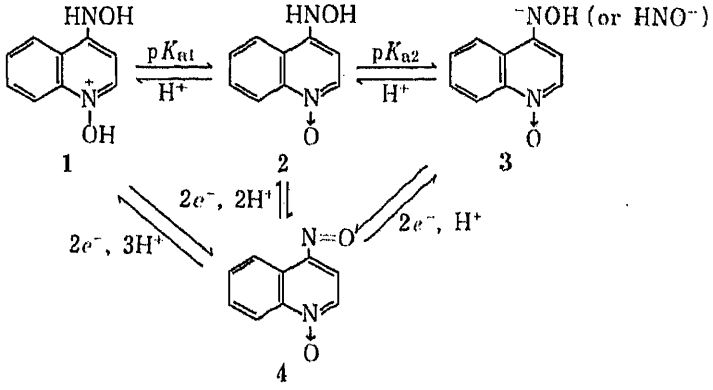


Chart 1. Oxidation–Reduction Mechanisms and Proton Addition and Dissociation Processes in Aqueous Solution are Given for 4HAQO as a Typical Example

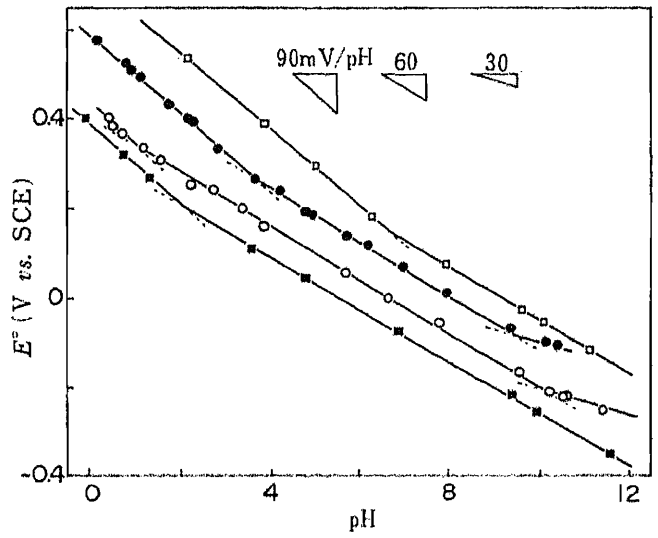
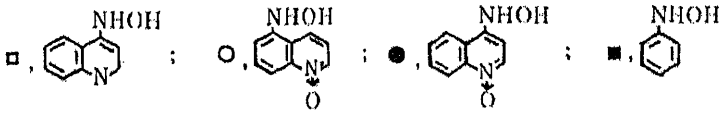


Fig. 3. The pH Dependence of Standard Oxidation–Reduction Potential (E_0) for Several Aromatic Hydroxyamine Compounds



Nicholson's equation[18a] always leads to an electron number (*ca.* 1.7) less than 2 but near it, though some error is inevitable in the measurement of the platinum electrode surface area, because the electrode surface is not completely planar. It appears likely that two electrons participate in this redox couple as a whole. This finding is compatible with reports on the CV study of some related substances.[18b] Here it should be noted that the peak potential difference $|\Delta E_p| = |E_p^O - E_p^R|$ evaluated in a lower pH region than 5, where 4HAQO is relatively stable, is larger (40—60 mV) than the value of *ca.* 30 mV ($2.22RT/2F$)[18a] at room temperature, even if the potential scanning rate is decreased as far as possible, showing that this two electron redox couple may consist of two processes, each of which is in a one-electron redox system.[7,9] Details of this treatment will be discussed in a later section in connection with the stability of the radical intermediate.

The standard oxidation–reduction potential (E_0) of 4HAQO *etc.* is thus determined by

$(E_p^O + E_p^R)/2$, and the pH dependence of E_0 is depicted in Fig. 3 for some typical hydroxyamine substances studied here. In the case of the aqueous solutions the present reversible systems can be written as $Ox + ne^- + mH^+ \rightleftarrows Red$, where Ox and Red are, respectively, for $-N=O$ and $-NHOH$ substituents, and therefore the E_0 value pertinent to this system is given by Eq. 1:

$$E_0 = E_0' - (2.3026RT/F)(m/n)pH \quad (1)$$

Since $2.3026RT/F$ equals 59.2 mV at 25 °C, the coefficient of pH in Eq. 1 is 59.2 mV for $m=n=2$, 29.6 mV for $m=1$ and $n=2$, and 88.8 mV for $m=3$ and $n=2$. Keeping in mind this result, the examination of Fig. 3 indicates that the E_0 *vs.* pH linear relation is divided into three straight lines with slopes of -90, -60, and -30 mV pH^{-1}. These values are very reasonable in comparison with the theoretical values mentioned above. We can now propose the reaction processes given in Chart 1. The present studies indicated that proton addition to the *N*-oxide group oxygen atom of 4NOQO may not occur in the pH region employed here (*i.e.* its pK_a value would be negative) because of the strongly electron-withdrawing nature of the conjugated nitroso group, so that this process is ruled out from Chart 1. The crossing point of the two straight lines of each plot in Fig. 3 gives the corresponding pK_a value. In Table I these pK_a and E_0 (at pH 7) values are listed. The pK_a values were also confirmed by the UV spectral method (*vide infra*).[19] In general, the electronic spectra of *N*-heterocyclic

TABLE I. pK_a Values Obtained by Cyclic Voltammetry and UV Spectroscopy, Maximum Wavelengths (λ) and Maximum Molecular Absorption Coefficients (ε) at the Longest Wavelength, and the Standard Redox Potentials (E_0) at 25 °C and pH 7[a] for Various Aromatic Hydroxyamine Compounds

Substances[b]	pK_{a1}		pK_{a2}		λ_{max}/nm [$\varepsilon_{max}/10^4$]			E_0
	CV	UV	CV	UV	Cation	Neutral	Anion	V *vs.* SCE
4HAQO	3.85	3.92	9.53	9.52	343.5 [1.42]	356.0 [1.02]	419.2 [1.18]	0.063
4NMHAQO	—[c]	3.04	—[c]	9.87	353.0 [1.57]	350.3 [1.09]	420.8 [1.47]	—[c]
5HAQO	1.15	1.15	(10.1)[d]	—	379.1 [0.36]	354.0 [0.73]	—	−0.020
6HAQO	1.55	1.30	—	—	348.6 [0.40]	353.0 [0.41][e]	—	−0.020
4HAQ	6.70	6.73	—	—	328.3 [2.02]	361.9 [1.44]	—	0.130
4HAPO	3.25	3.39	(10.5)[d]	—	279.2 [1.70]	280.6 [2.03]	—	0.062
3M4HAPO	3.35	3.40	(10.0)[d]	—	281.6 [1.63]	280.0 [1.69]	—	0.060
HAB	2.17	2.06	—	—	261.3 [0.10]	277.1 [0.32]	—	−0.084
HAN	—	—	(9.33)[d]	—	—	—	—	−0.094

a) All the substances studied exist in neutral form at this pH. *b*) See the text for abbreviation. *c*) The CV curve of 4NMHAQO is irreversible. *d*) Values in parentheses are not accurate because of the inaccuracy of CV measurements in the region of large pH values. *e*) Shoulder band.

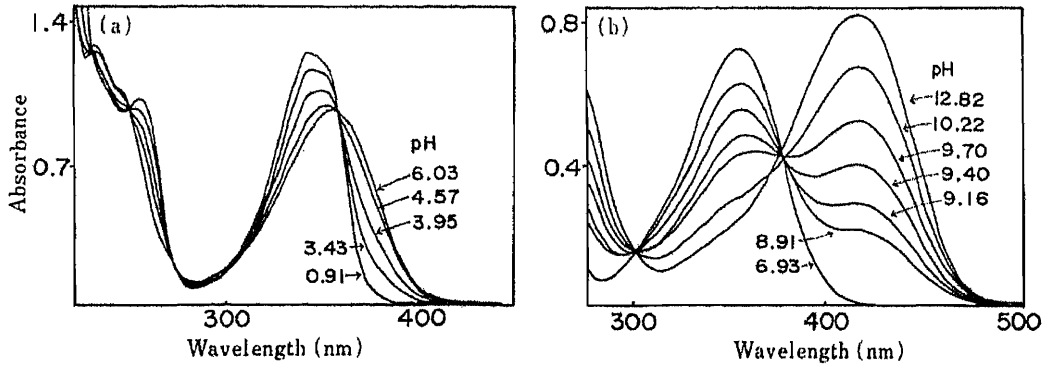


Fig. 4. The Spectral Change of 4HAQO with pH's in an Acidic Region (a) and an Alkaline Region (b)

compounds investigated here are very sensitive to pH, and there are clear isosbestic points that allow easy determination of pK_a values by the application of a usual equation, for example, $pK_a = pH + \log[(E_B - E)/(E - E_{BH^+})]$ for the system $BH^+ \rightleftarrows B + H^+$, where E_B and E_{BH^+} are the optical densities corresponding to B and BH^+ species alone, respectively, E being that at the intermediate pH range. Typical examples are illustrated in Fig. 4 for 4HAQO. When we could not record the spectrum of one of the B, BH^+, or B^- species, such as the BH^+ species of 6HAQO, our revised method was applied to determine the optical density of the species in question.[20] The pK_a values thus obtained by the UV method, and the spectral data, are included in Table I. Good agreement between the UV and electrochemical techniques is apparent.

Interpretation of pK_a's and Electronic Spectra of Hydroxyamino Substituted *N*-Heterocycles

In Table I we can see that the pK_{a1} (6.7) of 4HAQ is much larger than those (3.9—3.0) of 4HAQO, 4NMHAQO, and 4HAPO, and this fact may be ascribed to the proton affinity difference of the ring nitrogen and the *N*-oxide group oxygen atom. It should be noted[2f,21] that the pK_a's of quinoline (4.85—4.94) and quinoline *N*-oxide (0.70) are conspicuously smaller than those of 4HAQ (6.7) and 4HAQO (3.9), respectively. This fact is easily understood by considering the large resonance energies of the cations,[22] as indicated in Chart 2, compared with those of quinoline and quinoline *N*-oxide, where there is no cationic resonance contribution from the NHOH group. Keeping in mind this fact, we can see in Table

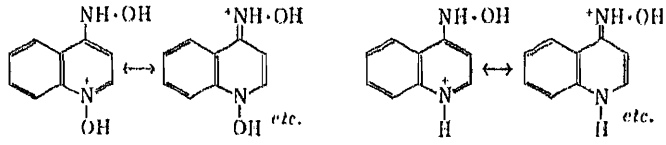


Chart 2. Ion Type Resonance Structures of the Proton Addition Cations of 4HAQO and 4HAQ

I that 5HAQO and 6HAQO have dissociation constants smaller than that of 4HAQO but larger than that of quinoline *N*-oxide, showing the lower contribution from the cation-type resonance structures arising from the NHOH group. This is reasonable, because the NHOH group is bound to a different ring from the *N*-oxide ring. Turning to Table I, let us consider the pK_a difference between 4HAQO and 4NMHAQO, and also the pK_a difference between 4HAPO and the calculated pK_a (2.14—2.06) of 4HAPO, the latter being obtained by the application of the Hammett type equation to the substituent effect on the pK_a's of 4-substituted pyridine *N*-oxides.[21d] It is found that the pK_a's of 4HAQO and 4HAPO are about 0.9 and 1.3 unit larger than those of 4NMHAQO and the above calculated value of 4HAPO, respectively. This finding may be in part ascribed to the possibility that 4HAQO (or 4HAPO) is in tautomeric equilibria as shown in Chart 3.[6,23] Next, we should say that the dissociation constants in the alkaline region of 4HAQO and 4NMHAQO are almost the same. Since the main tautomeric structures seen in Chart 3 are not considered for 4NMHAQO, it is not decided in the present stage whether the first acid dissociation of 4HAQO involves the hydrogen atom bound to the NHOH group nitrogen atom or that to the NHOH group oxygen. Finally, that the pK_{a1} of HAB is 2.1 is quite interesting in comparison with that (4.63) of aniline, because the substituent NHOH seems to have weaker basicity than the NH_2 group. In the case of the NHOH-substituted *N*-heterocycles studied here the resonance interaction between the NHOH group π-electrons and ring nitrogens makes it possible to change the basicity center of pK_{a1} to the ring functional group from the substituent (*vide infra*).

We will next focus our attention on the character of the electronic spectra of 4HAQO, *etc.* Figure 5 (A and B) shows the spectra of proton addition cations and neutral species of

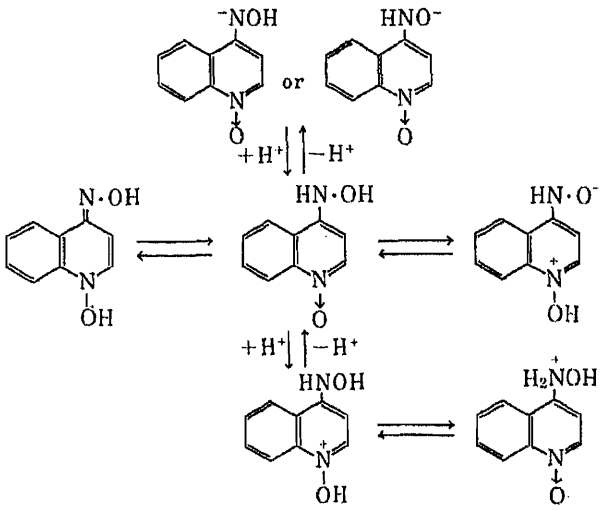


Chart 3. Tautomer Equilibria of 4HAQO and Its Proton Addition and Dissociation Species

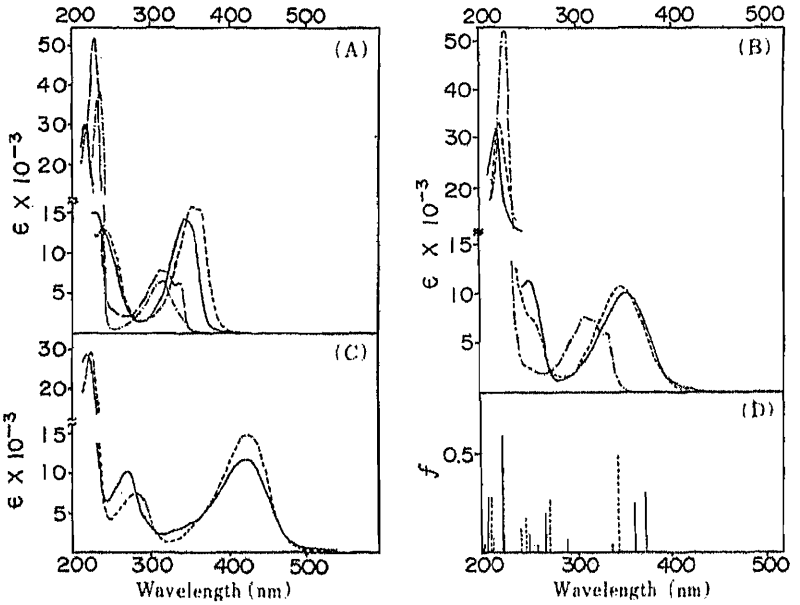
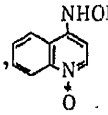
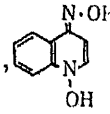


Fig. 5. Electronic Spectra in Aqueous Solutions of Proton Addition Cations (A), Neutral Species (B), and Proton Dissociation Anions (C) of Some Typical Compounds

The calculated results on the UV spectra of 4HAQO and its hydroxyimine-type tautomer are compared in figure (D), where f is the oscillator strength.

(A) Cation: ——, 4HAQO; ------, 4NMHAQO; ·-·-·-, QNO; ——; QNO (neutral).
(B) Neutral: ——, 4HAQO; ------, 4NMHAQO; ·-·-·-, QNO.
(C) Anion: ——, 4HAQO; ------, 4NMHAQO.
(D) PPP-CI calculation: ——,  ; ----, 

4HAQO, 4NMHAQO, and quinoline *N*-oxide (QNO), respectively. The longer wavelength spectra of the proton addition species of 4HAQO and 4NMHAQO are considerably intensified compared with those of the neutral species and also largely red-shifted from those of QNO and its $\gtrless N^+$–OH cation. These results suggest that the proton addition center of these *N*-oxides is the *N*-oxide group oxygen. If it were the case that the proton addition occurs

at the NHOH group nitrogen, the cation spectra should be blue-shifted and decreased in intensity, possibly resembling that of QNO, but this is not the case. The electronic spectra of the anions of 4HAQO and 4NMHAQO are quite similar to each other (see Fig. 5C), so that identification of the first acid dissociation center of 4HAQO is not as clear-cut as has been discussed in the foregoing section (*vide supra*). In Fig. 5D PPP-SCFMO-CI calculation results are depicted for 4HAQO and its hydroxyimine-type tautomer. Generally speaking, the alternant hydrocarbons and their *N*-heterocycles exhibit three clear bands in the near-UV region, *i.e.* ^1L_b, ^1L_a, and ^1B_b bands, and the ^1B_b band intensity is much the strongest. It is well known that these results are well described by the use of semiempirical SCFMO-CI methods such as PPP and CNDO/S.[24] Figure 5D gives the calculation results with the model of 4HAQO; they seem to well reproduce the observed spectrum (see Fig. 5B), especially the strong ^1B_b band at 219.1 nm, and also show that the longer-wavelength band consists of two overlapping bands of ^1L_a and ^1L_b. The same conclusion was also reached from the CNDO/S calculation, although the f (oscillator strength) values obtained by PPP calculation are over- and under-estimated for the ^1L_b and ^1B_b bands of 4HAQO, respectively, in comparison with those calculated by the CNDO/S method (see Table II). On the other hand, the spectra computed using the hydroxyimine-type tautomer of 4HAQO are quite different from those of the 4HAQO type structure: the longest wavelength band has the strongest intensity and the bands in the region of 250—220 nm are not so strong. These results suggest that the main component of the tautomers in an aqueous solution of 4HAQO is the 4-(hydroxyamino)quinoline *N*-oxide.[25,26] This result is in agreement with (i) the discussion in the section on pK_a's and (ii) the fact that the spectra of 4HAQO and 4NMHAQO are both similar. All the above mentioned calculations and experimental results as well as those for other quinoline *N*-oxides are included in Table II. In addition, it should be noted that the spectra in the aqueous phase of 5HAQO and 6HAQO are well reproduced by the *N*-oxide type structure (see Table II).

We next carried out theoretical calculation of the electronic spectra due to the cation or the anion formed by proton addition to or proton dissociation from 4HAQO. The method proposed by Morita *et al.* was used to evaluate the Fock matrix elements under the PPP approximation in the environment of the cation or anion.[27] The effect of the ions is taken into account only for the one-center atom where the proton addition or dissociation occurs. Effective nuclear charge δZ_x at the atom X (oxygen or nitrogen) is best set at $+0.7$ or -0.7 in order to reproduce the observed spectra of the cation or anion in the aqueous phase. The experimental and calculated results are included in Table II; it is clear that the UV spectra of these ions are quite well interpreted on the basis of π-electron structures of the quinoline ring type, but we can not decide whether the first dissociation center of the NHOH group in 4HAQO is the hydrogen atom bound to the nitrogen or that to the oxygen atom.

Stability of the Radical Intermediate Appearing in the Reversible Redox Reaction of 4HAQO and 4NOQO

Many electron spin resonance (ESR) studies have been reported by Nagata, Kawazoe and their colleagues on the free radical as an intermediate of the oxidation process of 4HAQO, and they discussed the structure of the free radical and its significance in carcinogenesis.[6a,c-e] Our present voltammetric study verified that the reversible oxidation–reduction process of 4HAQO can be written as $2 \rightleftarrows 4 + 2e^- + 2H^+$ (see Chart 1) in the neutral pH region, where proton addition or dissociation of all the reaction species does not occur. In the strict sense, however, we believe that this equilibrium is divided into two processes due to a one-electron–one-proton reaction as shown in Chart 4. This viewpoint is strongly supported by the fact that the peak-to-peak separation of the reversible voltammogram depends on the solvent polarity, and increases with increasing quantity of dimethylformamide (DMF) in water–DMF mixed

TABLE II. Observed and Calculated Spectral Data of Neutral, Cation, and Anion Species of 4-(Hydroxyamino)quinoline *N*-Oxide and Related Substances

Substance	E_{obs}[a] (eV)	ε[b]	E_{calcd}[a,e] (eV)	Band character[c]	f_{calcd}[d,e]
4-(Hydroxyamino)quinoline *N*-oxide (neutral)	3.483	10200	3.364 (3.503)	^1L_a	0.311 (0.351)
			3.473 (3.746)	^1L_b	0.256 (0.019)
			4.287 (5.987)		0.076 (0.008)
	4.851	11400	4.632 (4.846)		0.217 (0.041)
			4.788		0.033
			4.929 (4.638)		0.099 (0.009)
	5.659	31300	5.503 (5.011)	^1B_b	0.627 (0.882)
4-(Hydroxyamino)quinoline *N*-oxide (cation)[f]	3.609	14200	3.593	^1L_a	0.435
			3.956	^1L_b	0.020
			4.500		0.054
	5.344	14900	4.730		0.118
			5.233		0.007
			5.405		0.067
	5.740	30200	5.693	^1B_b	1.302
4-(Hydroxyamino)quinoline *N*-oxide (anion)[g]	2.958	11800	2.763	^1L_a	0.353
			3.152	^1L_b	0.067
			3.640		0.050
			4.098		0.021
	4.592	10300	4.304		0.300
			4.765		0.086
			5.039		0.152
			5.202		0.060
			5.344		0.037
	5.575	28700	5.752		0.165
			5.892	^1B_b	0.861
4-(*N*-Methylhydroxyamino)quinoline *N*-oxide (neutral)	3.539	10900	3.352	^1L_a	0.334
			3.462	^1L_b	0.236
			4.271		0.073
	4.758[i]	7500	4.626		0.220
			4.783		0.032
			4.929		0.098
	5.498	32900	5.499	^1B_b	0.613
4-(*N*-Methylhydroxyamino)quinoline *N*-oxide (cation)[f]	3.513	15700			
	5.128	13000			
	5.667	27900			
4-(*N*-Methylhydroxyamino)quinoline *N*-oxide (anion)[h]	2.945	14700	2.437	^1L_a	0.329
			2.777	^1L_b	0.062
			3.320		0.030
			3.814		0.067
	4.376	7600	4.015		0.343
			4.744		0.117
			4.913		0.027
			4.963		0.028
			5.004		0.182
	5.510	28300	5.561		0.207
			5.838	^1B_b	0.627
5-(Hydroxyamino)quinoline *N*-oxide (neutral)	3.502	7250	3.284 (3.461)	^1L_a	0.319 (0.304)
			3.574 (3.733)	^1L_b	0.127 (0.010)
	4.366[i]	5210	4.158 (4.320)		0.190 (0.024)
			4.476		0.019
			4.736 (4.949)		0.123 (0.095)
			5.050		0.184
	5.421	46900	5.134 (5.041)	^1B_b	0.539 (1.000)

TABLE II. (continued)

Substance	E_{obs}[a] (eV)	ε[b]	E_{calcd}[a,c] (eV)	Band character[c]	f_{calcd}[d,e]
6-(Hydroxyamino)quinoline	3.512[i]	4090	3.324		0.069
N-oxide (neutral)	3.861	9040	3.812	$^1L_b+^1L_a$	0.200
	3.979	8970	4.057		0.445
	4.479	11200	4.536		0.290
			4.812		0.221
			5.131		0.021
	5.170	22000	5.222	^1B_b	0.555

a) Observed (E_{obs} at maxima in aqueous solvents[25]) and calculated (E_{calcd}) energies in eV unit. *b*) Molecular absorption coefficient ($dm^3\,mol^{-1}\,cm^{-1}$). *c*) Band classification by Platt's notation[24] is given. *d*) Oscillator strength. Calculated data with *f* values less than 0.005 are omitted from the table for the case of PPP calculation. *e*) Values calculated by the CNDO/S method and having the same character as the PPP calculation results are given in parentheses. *f*) Calculation was carried out for the cation species, in which the *N*-oxide group oxygen atom is the active center for proton addition. *g*) Calculation was made for the anion species, in which the proton bound to the nitrogen atom in NHOH group is the proton dissociation center. *h*) The anion species employed for the calculation is that in which the proton bound to the oxygen atom in the $N(CH_3)OH$ group is the proton dissociation center. *i*) Shoulder band.

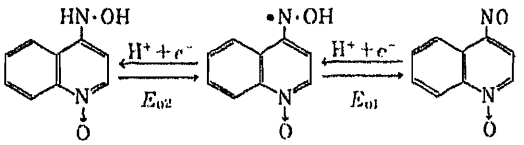


Chart 4. Oxidation-Reduction Mechanism of 4HAQO, Consisting of Two Processes Due to a One-Electron-One-Proton Reaction through an Intermediate Free Radical

solvents, showing that the intermediate free radical **5** is stabilized in less polar solvents (*vide infra*).[28] Note here that this kind of behavior is quite similar to the contribution of semiquinone free radicals in the reversible redox couple of adriamycin in the aqueous phase reported previously by us.[7a,9] Taking into consideration the reaction shown in Chart 4 we can easily write Eq. 2 for the difference of standard potentials ($E_{o1}-E_{o2}$), since E_{o1} and E_{o2} are expressed by $E_{o1}=(RT/F)\ln([5]/[4][H^+])$ and $E_{o2}=(RT/F)\ln([2]/[5][H^+])$, respectively.

$$(E_{o1}-E_{o2})=(RT/F)\ln K, \qquad (2)$$

where $K=[5]^2/[2][4]$ for the equilibrium $2+4\rightleftarrows 25$, the comproportionation reaction of free radicals.[29] If we are able to estimate the $(E_{o1}-E_{o2})$ value, then the K value in Eq. 2 is determined; the larger K is, the greater the stability of the intermediate free radical **5**. The working curve reported by Taube and Richardson for connecting $(E_{o1}-E_{o2})$ to the cathodic and anodic peak separation, ΔE_p, of a reversible CV curve is employed to convert the ΔE_p to the standard potential difference,[30] then the K value was calculated using Eq. 2. The experiment was done in mixed solvents consisting of a protic solvent (0.2 mol dm^{-3} acetate buffer, pH = 5, μ = 0.5 with NaCl[31]) and an aprotic solvent (DMF containing 0.1 mol dm^{-3} tetra-*n*-propylammonium perchlorate). The value of ΔE_p was determined for two cases; one is that evaluated by extrapolating the peak separations, recorded at various sweep rates (v), to $v\rightarrow 0$ in order to exclude the kinetic effect on the CV curves, and the other is the ΔE_p obtained at $v=200\,mV\,s^{-1}$. Table III summarizes the experimental and analyzed results in various solvent systems. It is reasonable that the K value increases with increasing DMF concentration, *i.e.* the intermediate radical is stabilized in such solvent systems. This supports the reaction shown in Chart 4 and the ESR studies carried out by other workers (*vide supra*). Figure 6 shows the CV curve recorded in electrolyte solutions at pH = 1.1 using the BPGE, the surface of which was covered with a monomolecular layer of 4HAQO just before use (see the experimental section). In this CV curve, anodic and cathodic potentials are the same and both

TABLE III. Effect of Solvent Polarity[a] on the Peak-Separation (ΔE_p) of CV Curves, Standard Potential Difference ($E_{o1}-E_{o2}$), and Comproportionation Constant (K)

DMF conc.[b] v/v %	Extrapolated to $v\to 0$			At $v=200\,mV\,s^{-1}$		
	ΔE_p / mV	$E_{o1}-E_{o2}$ / mV	K	ΔE_p / mV	$E_{o1}-E_{o2}$ / mV	K
0	—[c]	—[c]	—[c]	63	41.2	4.9
20	37.3	−24.2	0.39	68	46.4	6.1
40	39.0	−15.0	0.56	70	49.0	6.7
60	43.4	4.8	1.21	100	72.0	16.5
80	53.0	28.0	2.97	128	92.0	35.9
100[d]	181.6	134.2	185.0	191	142.7	258.0

a) This was adjusted by mixing protic and aprotic solvents in the indicated v/v ratio. The former is 0.2 mol dm^{-3} acetate buffer of pH 5 and μ (ionic strength) 0.5 adjusted with NaCl, and the latter is dimethylformamide containing 0.1 mol dm^{-3} tetra-*n*-propylammoniumperchlorate. *b*) Concentration of dimethylformamide to acetate buffer solution in v/v percent. *c*) In the present pH region (pH 5) the reversible CV curve was not recorded at a slow scanning rate because of the succeeding chemical reaction, so that we could not extrapolate ΔE_p to $v\to 0$. *d*) In DMF solvent alone the proton in Chart 4 may not participate in the reaction, or may make a different contribution from that in Chart 4, so that the reaction mechanism seems to be different from that in protic and aprotic mixed solvents. This may be reasonably inferred from the fact that the value of K is by far the largest in DMF solvent.

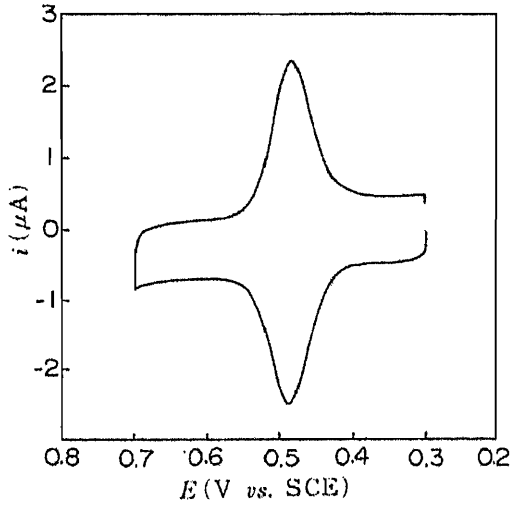


Fig. 6. Cyclic Voltammogram of 4HAQO Adsorbed on the BPGE as in Monomolecular Layer at pH 1.10

the curves are almost symmetrical, as we have reported in detail in the case of the CV curve of adsorbed adriamycin.[7a,9] Such CV curves were especially obtained in the acidic pH region, where 4HAQO is protonated at the *N*-oxide group oxygen atom (*vide supra*) and is very stable. This makes it possible to apply the analysis method[7a,9] pertinent to the adsorbed reversible CV curve due to a two-step one-electron reaction to the experimental result in Fig. 6. Using the half-peak width ($\Delta E_{p/2}$) of 58—60 mV, we obtain values of 0.43—0.56 for the radical formation constant K (see Eq. 2). These outcomes seem quite reasonable in comparison with the results given in Table III. Nevertheless, the present data are for the protonated and adsorbed molecules, so direct comparison with the ones in Table III may not be strictly valid. Finally, we believe that such experimental and theoretical results on 4HAQO and related substances should be very valuable in clarifying their carcinogenic activity.[19]

References and Notes

1) Present address: *Pharmaceutical University of China, Nanjing Jiangsu, The People's Republic of China.*

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25) Blue shift phenomena at the longest wavelength band of 4HAQO were observed on increasing the solvent polarity, *e.g.*, ethanol (372.0 nm), methanol (368.5 nm), and water (355.5 nm). This supports the *N*-oxide structure, since the blue shift phenomenon is one of the general properties of aromatic amine *N*-oxides.[26] However, in such solvents as dichloromethane and ether some irregular spectral behavior was observed, perhaps because of the larger contribution from the hydroxyimine-type tautomer of 4HAQO (see Chart 3) in such nonpolar solvents.
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31) The stability of 4HAQO decreased with increasing pH, but in the region of pH $\simeq$ 5 it is still relatively stable, and the molecule is in a neutral form, so this experiment was carried out using pH 5 buffer solution. In a pH 7 buffer solution, which is close to *in vivo* biological conditions, the succeeding chemical reaction affects the CV curve. Since CV measurement at a slow sweep rate was desired, the experiment at pH 7 was not carried out.

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Phase Separation in Mixed Monolayers of Poly(γ-methyl L-glutamate) and Triolein

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Mixtures of triolein and poly(γ-methyl L-glutamate) (PMLG) were spread on a water–air interface from a helix-favoring solvent and surface pressure–mean area curves were obtained with a surface balance of horizontal float type. The mixtures separated into two or three surface phases at almost all compositions. At relatively low surface pressures, two surface phases, one rich in PMLG and the other rich in triolein, co-existed and at 7 mN/m both changed to a unique duplex film consisting of equi-area PMLG and triolein phases. This duplex film was broken up at 23.5 mN/m. In this system several other phase equilibria between three surface phases were shown.

Keywords——surface pressure; insoluble monolayer; poly(γ-methyl L-glutamate); triolein; phase separation; phase transition; mixed monolayer

Interactions between lipids and proteins have been studied by various technics. Proteins change their conformation or unfold at interfaces such as air–water and solid–water interfaces, and also penetrate into lipid bilayers with a change of conformation.[1-4] The use of spread monolayers is an effective means to investigate lipid–protein interactions. However, it is difficult to spread most proteins as insoluble monolayers on a water surface. Even when proteins were spread as insoluble monolayers on a water surface, surface conformational analysis of the proteins has given ambiguous results.[5] Poly(γ-methyl L-glutamate) (PMLG) can be spread easily as an insoluble monolayer on a water surface. The surface conformation of PMLG has been studied by several methods,[6-9] and has been shown to remain almost the same as in the spreading solvent.[9]

PMLG is a rigid rod shaped polymer when it is in α-helix conformation and a lyotropic liquid crystalline state can be formed by orientation of PMLG molecules.[10] Solutions of rod-like particles of large axial ratio are liable to separate into two phases.[11] This biphasic property is attributed to a loss of mixing entropy resulting from steric hindrance in the isotropic phase.[12] It was expected that mixed monolayers of rod-like molecules and low-molecular-weight molecules would show phase separation.

Phase separations in monolayers of low-molecular-weight compounds have been studied on the bases of variation of transition pressures, such as collapse pressure and surface vapor pressure, with composition.[13,14] Interactions between, and miscibilities of, poly(amino acids) and lipids in mixed monolayers have been studied. Shibata *et al.* used poly(amino acids) which show monolayer–bilayer transitions.[15] The monolayer–bilayer transitions of poly(amino acids) in mixed monolayers were not as clear as the collapses of lipid monolayers. Thus, it is desirable to use lipids having clear collapse pressures in order to study the miscibilities of poly(amino acids) and lipids. Monolayers of PMLG in α-helix conformation have a clear monolayer–bilayer transition point, while triolein also shows a sharp collapse pressure of the monolayer. In this study, the mixtures of PMLG and triolein in the two-dimensional state were investigated. The miscibility and multiphase equilibria are discussed.

Experimental

Materials——PMLG was kindly supplied by Ajinomoto Co., Ltd. PMLG was dissolved in a mixture of dichloroacetic acid and chloroform (1/9, v/v), and precipitated into four fractions by successive addition of ethanol at 25 °C. The number-average molecular weight (M_n) of each fraction was estimated from the intrinsic viscosity[16] [η] in dichloroacetic acid at 25 °C by using the following equaiton: $[\eta]=2.9\times10^{-4}\ M_n^{0.74}$. The axial ratio ($x$) of the helix was calculated by means of the equation[17] $x=1.5P/12.0$, where P is the degree of polymerization. Triolein was purchased from Sigma Co., Ltd. Dichloroacetic acid and chloroform were distilled twice. Water was distilled twice in all glass apparatus.

Surface Pressure Measurement——Surface pressure (F)–mean area (A) isotherms were measured with a horizontal float film balance (Cenco Co., Ltd.). PMLG was dissolved in dichloroacetic acid (about 5 mg/ml) and triolein was dissolved in chloroform (about 1 mg/ml). Both solutions were stored in a freezer at −25 °C. The solutions of PMLG and triolein were mixed just before spreading. The final volume ratio of chloroform to dichloroacetic acid in the spreading solvent was kept at 19/1 by adding chloroform or dichloroacetic acid to the mixed solution. The required amount of the solution was delivered to the air–water interface with an Agla micrometer syringe. After 10 min, compression of the film was started. Surface pressure was measured at 2 min after each stage of compression. The temperature of the subwater was controlled by circulating water from a water bath through a glass tube immersed in the trough.

Results

PMLG Films on Water Surface

The surface pressure–residual area (F–A) curves of PMLG with different molecular weight at 25 °C are shown in Fig. 1. The numbers on the abscissa indicate area per amino acid residue. There was little difference among the F–A curves of PMLG with different molecular weights. In this experiment PMLG of $M_n=1.0\times10^5$, $P=700$ and $x=90$ was used.

The F–A curves of PMLG were very steep at about 18×10^{-20} m^2/residue and showed horizontal portions at 20 mN/m between 9×10^{-20} and 16×10^{-20} m^2/residue. These F–A curves were in good agreement with the results of Malcolm[7] and Loeb and Baier[9] for α-helix conformation, but the area was somewhat smaller than that given by Gouple and Goodrich.[18] Monomolecular films of PMLG at an air–water interface may exist in α-helix, β-pleated sheet and random coil conformations.[8,9] The conformation which is predominant at the air–water interface can be altered by a suitable choice of spreading solvent,[9] because it is known that the same conformation as in the spreading solvent is usually retained in the monolayer. Monomolecular films of PMLG in α-helix conformation have been obtained by Loeb and Baier by using a mixture of chloroform and dichloroacetic acid (9/1, v/v) as the spreading solvent.[9] PMLG exists in α-helix conformation in the spreading solvent used in this experiment.[19] Furthermore, the plateau region in the F–A curve of PMLG appears only in the

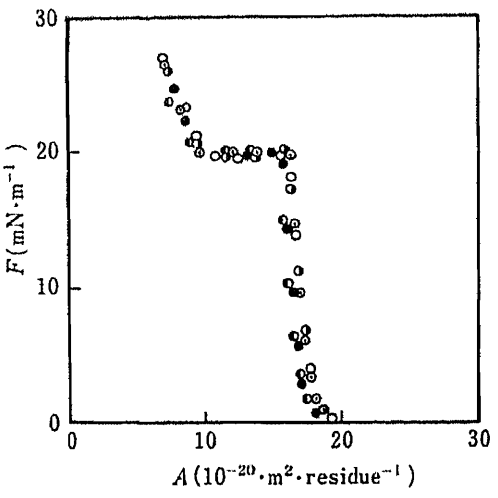


Fig. 1. Surface Pressure (F)–Residual Area (A) Curves for PMLG of Various Molecular Weights at an Air–Water Interface at 25 °C

Molecular weight/10^4: ⊙, 10; ○, 7.3; ◑, 5.9; ◐, 3.6; ●, unfractionated.

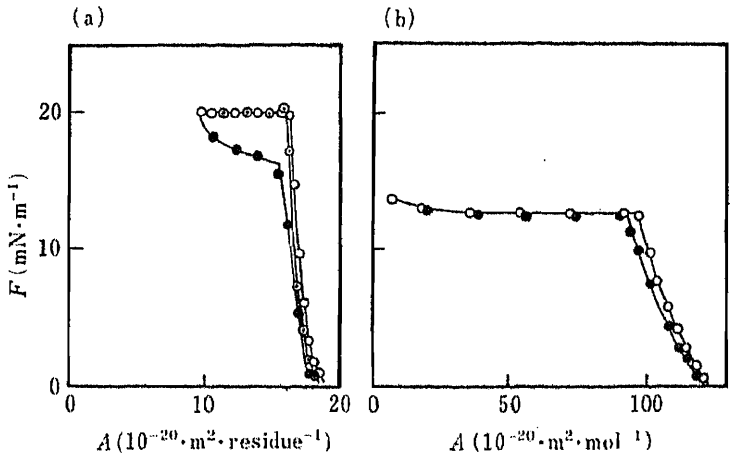


Fig. 2. Compression–Expansion Cycles of the Surface Pressure (F)–Mean Area (A) Curves for PMLG (a) and for Triolein (b)

○, first compression; ●, expansion; ⊖, second compression.

case of α-helix conformation and has been attributed to the overlapping of α-helices in the monolayer to form a bimolecular film.[7,8] In this experiment, therefore, PMLG was probably in α-helix conformation.

The surface pressure increased gently again after completion of bilayer.

Surface pressure in the steep portion of the F–A curve reached a stable value within half a minute after each compression. In the plateau region, the surface pressure increased immediately after compression but then fell rapidly to approach a constant value, 20 mN/m, and did not decrease further for at least one hour. A PMLG film was compressed to an area of 9×10^{-20} m^2/residue and extended to 40×10^{-20} m^2/residue, then recompressed. The resulting F–A curves are shown in Fig. 2(a). The compression–expansion isotherms showed hysteresis. When the film in the plateau region was extended, the surface pressure decreased to approximately zero at first and then increased to the measured value within one minute, but the surface pressure did not recover to 20 mN/m and the plateau was not seen in the F–A curve in expansion. The plateau region appeared again on recompression. This means that molecules in the bilayer easily return to monolayer form when films are left over a relatively large area, while the rearrangement of PMLG molecules to a monolayer from a bilayer may need a long time at near the transition pressure. Malcolm[8] has suggested that the slow relaxation is due to slow irreversible filling of gaps between micelles of ordered PMLG molecules in the monomolecular film.

Triolein Film

The F–A curve of triolein film on the water surface is shown in Fig. 2(b). The F–A curve of triolein showed a break point at 12.5 mN/m due to collapse of the triolein monolayer. As triolein is chemically unstable, the collapse pressure of aged triolein was a little higher than that of fresh triolein. Therefore triolein was used within 3 d after being dissolved in the spreading solvent. The equilibrium spreading pressure, which is the surface pressure obtained by placing droplets of triolein on a clear water surface, was 12.7 mN/m and coincided with the collapse pressure of triolein monolayer. The equilibrium between the collapse phase and the monolayer was established quickly on compression or expansion, as shown in Fig. 2(b). Once the collapse phase had been formed, the surface pressure was constant as the film was compressed or expanded.

Mixed Films

The F–A curves for mixed films of PMLG and triolein are shown in Figs. 3 and 4.

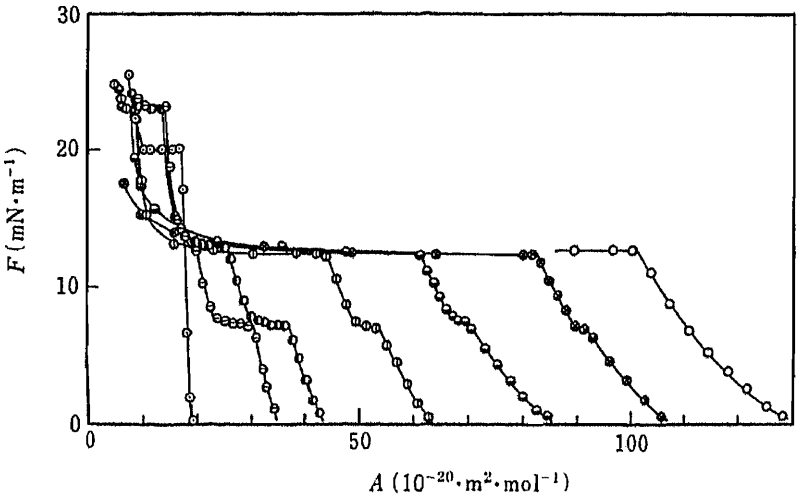


Fig. 3. Surface Pressure (F)–Mean Area (A) Curves for Mixed Films of PMLG and Triolein at an Air–Water Interface at 25 °C

Triolein: PMLG ratio were as follows. ○, 100:0; ⊗, 81:19; ◒, 59:41; ◑, 42:58; ◐, 22:78; ⊖, 16:84; ⊙, 0:100.

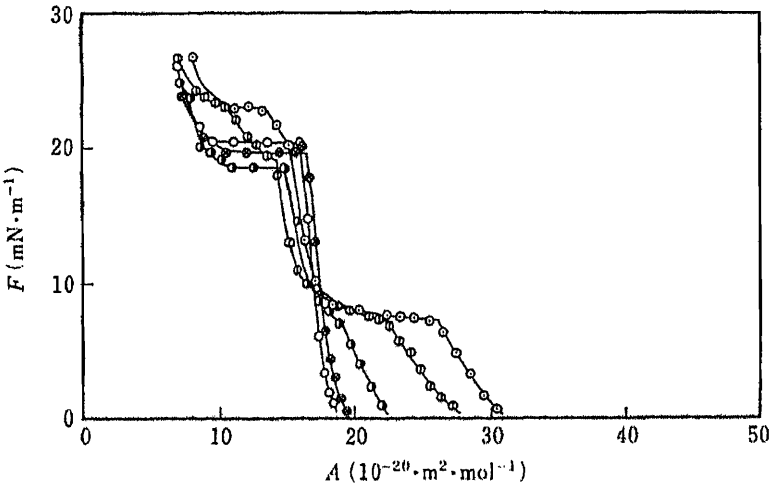


Fig. 4. Surface Pressure (F)–Mean Area (A) Curves for Mixed Films of PMLG and Triolein at an Air–Water Interface at 25 °C

Triolein: PMLG ratio were as follows. ⊙, 11:89; ◑, 8:92; ◑, 4:96; ⊗, 2:98; ○, 0:100.

The mean area (A) is

$$A = A_t/(N_1 + N_2) \tag{1}$$

and residue-mole fractions of PMLG (x_1) and triolein (x_2) are

$$x_1 = N_1/(N_1 + N_2) \tag{2}$$

$$x_2 = N_2/(N_1 + N_2) \tag{3}$$

where A_t is the total area of the surface covered with the film, N_1 is the number of amino acid residues of PMLG and N_2 is that of triolein molecules in the film.

Each F–A curve has one or three break points at surface pressures of 7.0, 12.5, 20.0, and 23.5 mN/m. The relations between the residue-mole fraction and surface pressure or mean area at break points are shown in Fig. 5. The break points at 7.0 and 23.5 mN/m appeared

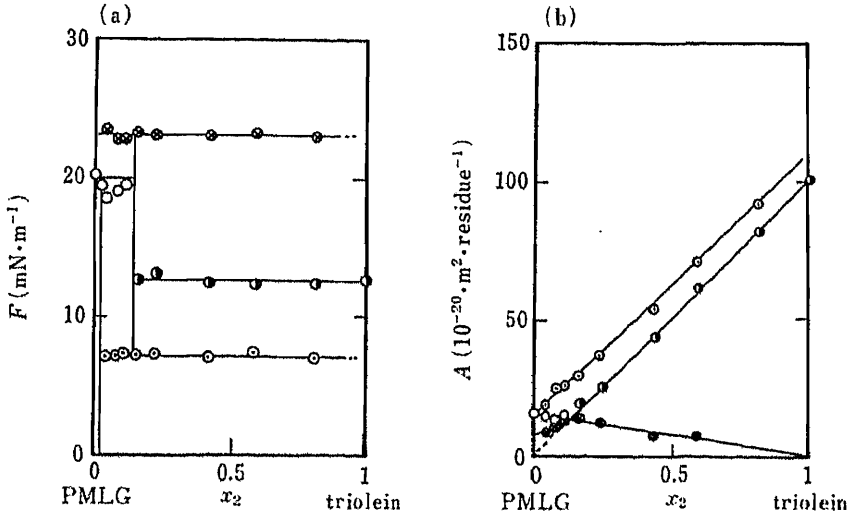


Fig. 5. Relation between the Surface Pressure (F) and Residue-Mole Fraction of PMLG (x_2), (a), or the Mean Area (A) at the Transition Points on the F–A Curves (b)

⊙, formation of the duplex film; ◑, collapse of triolein monolayer; ○, formation of bilayer of PMLG; ⊗, collapse of the duplex film.

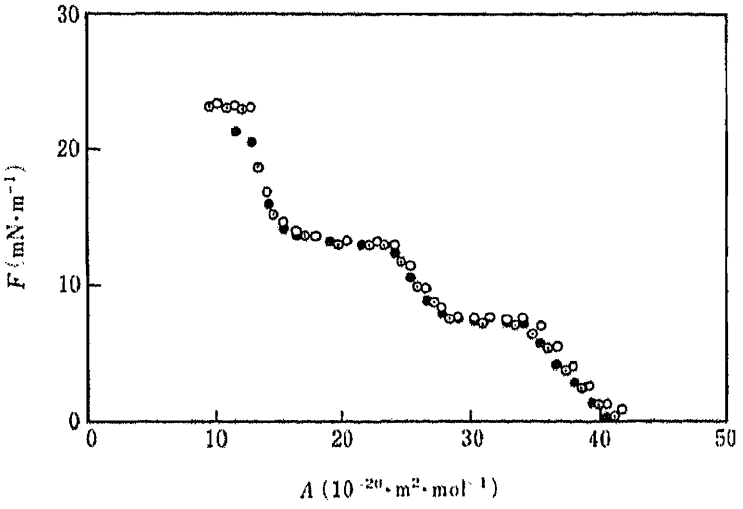


Fig. 6. Compression–Expansion Cycles of the Surface Pressure (F)–Mean Area (A) Curves for the Mixed Film of $x_2 = 0.2$

○, first compression; ●, expansion; ⊙, second compression.

when x_2 was more than 0.04 and the break point at 12.5 mN/m appeared when x_2 was more than 0.15.

The compression–expansion–recompression isotherm of the film with a residue-mole fraction of $x_2 = 0.2$ is shown in Fig. 6. The horizontal portion at 7 mN/m can be clearly seen, though they were not found in the F–A curves of the pure components. The horizontal portions in the F–A curve at 7.0 and 12.5 mN/m appeared both on compression and on expansion, suggesting that the transitions at those surface pressures were reversible. However, the horizontal portion at 23.5 mN/m appeared only as the film was compressed. This behavior at the transition is similar to the monolayer–bilayer transition of PMLG.

Relation Between Mean Area and Composition

The relations between mean area and residue-mole fraction at various surface pressures

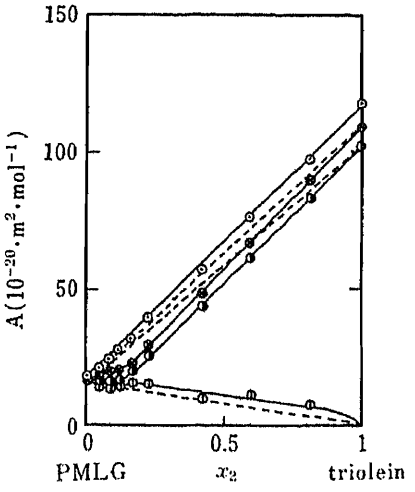


Fig. 7. Change of Mean Area (A) with Residue-Mole Fraction of Triolein (x_1) at Various Surface Pressures

Surface pressure/mN m^{-1}: ⊙, 4; ⊗, 8; ◑, 12; ◐, 16.

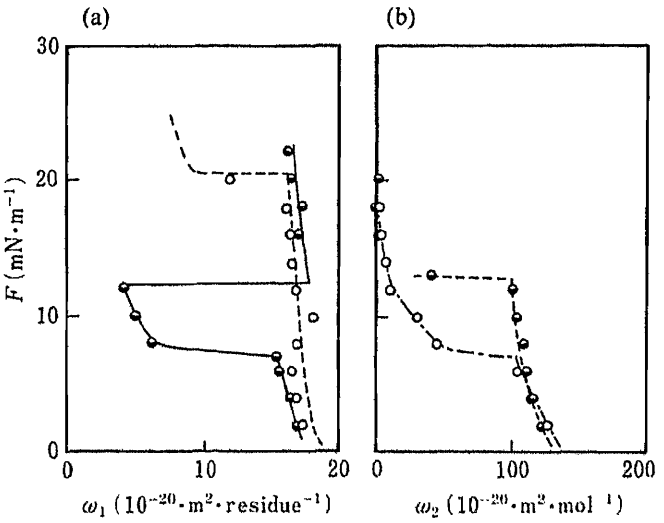


Fig. 8. Partial Residual Area of PMLG (ω_1), (a), and Partial Molecular Area of Triolein (ω_2), (b) at Various Surface Pressures (F)

Dotted curves are the surface pressure-mean area curves for the pure components at an air-water interface. ◒, $x_2 > 0.15$; ○, $x_2 < 0.15$.

are shown in Fig. 7. The dotted lines were calculated by using the following equation

$$A_{\text{calc}} = A_1 x_1 + A_2 x_2 \tag{4}$$

where A_1 and A_2 are the residual area of PMLG and the molecular area of triolein at the air-water interface, respectively. Mean areas below 7 mN/m were represented well by Eq. 4. The mean areas at 4 mN/m are shown in Fig. 7 as an example. Between 7 and 12.5 mN/m the mean areas were less than the areas calculated by using Eq. 4. Maximum decrease of area took place at about $x_2 = 0.15$. Above 12.5 mN/m Eq. 4 again expressed the mean area well.

The partial residual area of PMLG (ω_1) and partial molecular area of triolein (ω_2) changed at about $x_2 = 0.15$. The partial residual areas of PMLG and partial molecular areas of triolein were obtained from the intercepts of the straight lines for $x_2 = 0$ and 1, estimated by the least-squares method. The results are shown in Fig. 8. Below $x_2 = 0.15$, the partial residual areas of PMLG were very similar to the areas of the pure component on water, which are shown by the dotted curve. Above $x_2 = 0.15$, they decreased abruptly at 7 mN/m but recovered to the areas on water again at higher surface pressures. On the other hand, the partial molecular areas of triolein were the same as the areas of triolein on water at any surface pressure above $x_2 = 0.15$, but they decreased at 7 mN/m below $x_2 = 0.15$. The decrease of area at 7 mN/m was attributed to the decrease of ω_2 below $x_2 = 0.15$ and ω_1 above $x_2 = 0.15$.

Discussion

Phase Separation into Lipid Phase and Polymer Phase at a Surface, and Formation of a Duplex Film

Four kinds of transitions were seen in the mixed films of triolein and PMLG at an air-water interface. They were the collapse of triolein monolayer, the monolayer-bilayer transition of PMLG film and two other transitions which were seen only in the mixed system. The surface pressures of these four transitions, as shown in Fig. 5, did not change with composition. Furthermore, as shown in Figs. 3, 4 and 6, plateau regions appeared at those surface pressures. According to the surface phase rule,[20] these results indicate that three

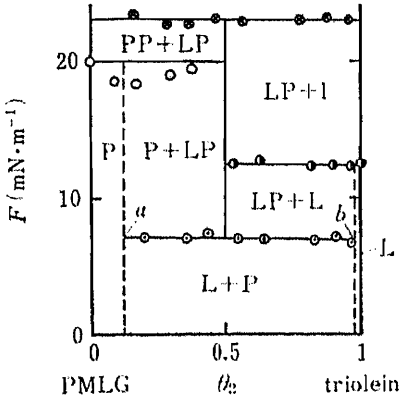


Fig. 9. Relation between Transition Pressure and Apparent Area Fraction of Triolein (θ_2) for Mixed Films

⊙, formation of the duplex film; ◑, collapse of triolein monolayer; ○, formation of bilayer of PMLG; ⊗, collapse of the duplex film.

surface phases were in a state of equilibrium at 7, 12.5, 20 and 23.5 mN/m.

When the transition at 7 mN/m was observed, three surface phases were found in the mixed film of triolein and PMLG. Therefore, below this surface pressure it is inferred that two surface phases co-exist. One is rich in PMLG (P phase) and the other is rich in triolein (L phase).

The nature of the transition at 7 mN/m will be considered next. Volume fractions are usually used for polymer solutions or for solutions of molecules of different sizes in place of mole fractions. In the case of surface phase, area fractions are used. The area fractions of triolein (θ_2) were estimated by using the following equation,

$$\theta_2 = a_2 x_2/(a_1 x_1 + a_2 x_2) \tag{5}$$

where a_1 and a_2 are the partial residual area of PMLG and partial molecular area of triolein, respectively. The apparent area fractions were calculated by putting A_1 and A_2 into a_1 and a_2 in Eq. 5, respectively. The molecular area of triolein above its collapse pressure can be estimated from the following equation of state,[21]

$$F = -174.7 \ln A + 1.079A + 710.0 \tag{6}$$

and a value of 17×10^{-20} m^2/residue was used for the residual area of PMLG above the bilayer transition pressure.

The surface phase diagram of surface pressure *versus* area fraction coordinates is shown in Fig. 9. A new phase appears between 7 and 23.5 mN/m at $\theta_2 = 0.5$. This new phase is composed of equal areas of triolein and PMLG. Although formation of this new phase is accompanied with a decrease of surface area, the partial residual area of PMLG is the same as that of α-helix comformation at higher surface pressure. This indicates that there is little possibility of conformational change of PMLG from α-helix to β-sheet or random coil at that pressure. It was concluded that this new phase is a duplex film (LP phase) formed by overlapping of P phase and L phase. 7 mN/m is the formation pressure and 23.5 mN/m is the collapse pressure of LP phase.

Malcolm[7,8] estimated the surface tension of PMLG film against air (γ_p) as 45.5 mN/m. The value for a typical hydrophobic polymer, polyethylene, is $\gamma_p = 10.2$ mN/m and that of polymethylmethacrylate is $\gamma_p = 13.0$ mN/m.[22] Therefore the surface of PMLG film is not as hydrophobic as that of polyethylene. It is possible that the PMLG layer lies between the triolein film and water. The structure of the duplex film will be discussed in a subsequent paper.

As LP phase co-exists with both P and L phase at the LP formation pressure, the upper and lower limits of composition at which LP formation occurs are the compositions of co-existing L and P phases at that surface pressure. The critical composition of P phase was $x_2 =$

0.04, but the composition of L phase, which was higher than $x_2=0.8$, was not determined, because the decrease of area at the transition was very small as the content of PMLG was low. The area fraction of triolein in P and L phases are shown as points (*a*) and (*b*) in Fig. 9. Triolein dissolved in the P phase up to about $\theta_2=0.1$—0.2, while PMLG was dissolved in triolein up to no more than $\theta_1=0.04$.

The mixed monolayers of triolein and PMLG are two-dimensional solutions composed of two types of molecules that differ very much in their size and shape. It is known that solutions of rod-like particles are liable to separate out ordered phases. Flory[12] has proposed phase diagrams theoretically. Nakajima *et al.*[23] showed that the phase diagram of the poly(γ-benzyl L-glutamate) system was in good accordance with Flory's theory. PMLG in α-helix conformation has been shown to form a lyotropic liquid crystal in which the PMLG molecules are oriented. Therefore PMLG molecules in the P phase may be oriented to some extent.

With mixed monolayers of poly(amino acids) and lipids, both miscible and immiscible cases have been reported.[24] Differences of side chains of poly(amino acids) and polar groups of lipids affect the miscibilities. Poly(*N*-benzyloxycarbonyl L-lysine) is miscible with methyl stearate but immiscible with stearamide. However, poly(γ-benzyl L-glutamate) is miscible with stearamide. Nakajima *et al.*[23] have shown that a slight positive interaction parameter between rod-like polymer and solvent results in large concentration differences between the two phases in equilibrium and that even if the interaction parameter is zero, a phase separation occurs in a narrow range. It may be difficult to detect this phase separation by means of spread monolayer technique. Slight differences in the structures of rod-like poly(amino acid) and lipids would affect the miscibilities.

The Course of L, P and LP Phase

The L phase collapses at almost the same surface pressure as triolein monolayer, as shown in Fig. 9, and triolein molecules are squeezed out from the monolayer to form a collapsed phase (l phase) at the surface. The film will be compressed to a very small area at the same surface pressure, since the area occupied by the l phase is negligibly small.

PMLG molecules in the P phase change to bilayer form at almost the same surface pressure as that of the monolayer–bilayer transition of pure PMLG monolayer, as shown in Fig. 9.

The behavior of a mixed film at the collapse pressure of the LP phase in the compression–expansion cycle was similar to the monolayer–bilayer transition of PMLG film on water. In addition, this transition occurred only when the LP phase appeared. It was concluded that PMLG molecules change from monolayer to bilayer form in the duplex film at the transition pressure.

Triolein in the LP phase does not collapse at the collapse pressure of triolein monolayer, and PMLG in the LP phase does not change to a bilayer at the monolayer–bilayer transition pressure of PMLG films. Triolein and PMLG in the LP phase thus have different properties from those of their individual films. The formation of the duplex film means that PMLG molecules overlapping the triolein monolayer are more stable than those lying on water above 7 mN/m.

Whether the LP phase can co-exist with the L or P phase depends on the areas covered with L and P phases. If the area of the L phase is larger than that of the P phase, an equilibrium between LP and L phases appears. Therefore the total area is practically the same as the area covered with total triolein molecules. PMLG does not take part in the observed area. However, after collapse of the L phase, the surface is covered with LP and l phases, and the area depends practically on PMLG, because the area of the l phase is very small. This is the reason why the partial residual area above $x_2=0.15$ shown in Fig. 8 decreases at the

formation pressure of the LP phase and recovers after collapse of the L phase.

Conclusion

Mixed films of PMLG and triolein separate into two or three surface phases at almost all compositions. A duplex film consisting of equi-area P phase and L phase is formed at 7 mN/m and is broken at 23.5 mN/m.

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Etude Structurale d'un Analogue de L'Ellipticine a Visée Antineoplasique le Dihydro-1, 4 oxo-4 Trimethyl-2,5,11-6*H*-pyrido[3,2-*b*]carbazole par Diffraction de Rayons. X

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The synthesis of 1,4-dihydro-4-oxo-2,5,11-trimethyl-6*H*-pyrido[3,2-*b*]carbazole was described and the lactam–lactim tauromerism was discussed.

$C_{18}H_{16}N_2O$ crystallizes in space group $P2_1/c$ with $D_m = 1.30(2)$ Mg·m^{-3} in acetonitrile with $a = 16.179(4)$, $b = 14.900(3)$, $c = 12.702(3)$ Å, $\beta = 112.28(2)°$, $V = 2833.6$ Å^3, $Z = 8$. The crystal structure was investigated by X-ray diffraction method with final $R = 0.04$ for 2355 independent reflections. In the two molecules, the lactam form was observed while the lactim for was found with many derivatives as well in solid state as in solution. The carbazole ring is planar while the 1,4-dihydro-4-oxo-pyridine deviates from planarity. The molecules have rather small overlapping and the molecular packing involves many hydrogen bonds.

Keywords——crystal structure analysis; pyrido[3,2-*b*]carbazole; lactam–lactim tautomerism

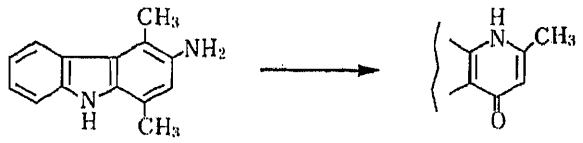
Parmi les dérivés inspirés du modèle de l'ellipticine et à visée antinéoplasique, nous avons étudié antérieurement les structures de deux variétés du dihydro-5,6 pyrimidino[5,4-*c*]-carbazole (DPC en abrégé),[1,2] compose qui, par ailleurs joue un double rôle de ligand et de solvate dans le complexe Co(II)–4DPC dont nous avons effectué la synthèse et établi la structure.[3] En outre, la présence dans le DPC du cycle carbazolique qui est potentiellement électrodonneur, a permis de réaliser un complexe par transfert de charge avec le TCNQ, composé accepteur d'électrons.[4]

Dans le prolongement de ce programme de recherche, nous avons pu élaborer de nouvelles structures dérivées du pyridocarbazole et isomères de l'ellipticine.[5] Plusieurs représentants de cette nouvelle famille présentent une activité cytotoxique démontrée *in vitro* sur des cultures de leucémie L 1210.[6] Ce mémoire décrit la structure cristalline et moléculaire du dihydro-1,4 oxo-4 triméthyl-2,5,11-6*H*-pyrido[3,2-*b*]carbazole (**1**). Les monocristaux utilisés ont été obtenus par évaporation lente d'une solution dans l'acétonitrile.

Au cours de l'investigation d'une nouvelle série d'isomères de l'ellipticine à visée antinéoplasique, nous avons synthétisé **1** par mise en œuvre de la méthode de Conrad–Limpach et en cyclisant l'amino-3 diméthyl-1,4 carbazole[5] (**2**) avec l'acétoacétate d'éthyle.

La pyridocarbazolone **1** présentant la tautomérie lactame–lactime (**1**⇄**1a**) nous avons réalisé une étude en infrarouge (IR) et en résonance magnetique nucléaire ^{1}H (RMN ^{1}H).

L'examen du spectre infrarouge à l'état solide est en faveur de la forme lactamique **1**. On



2 1



1 1a : R = H
3 : R = COCH$_3$

n'y relève pas de bande OH alors qu'on observe une bande carbonyle intense à 1625 cm^{-1} avec un épaulement à 1610 cm^{-1}.

Le spectre de RMN ^{1}H effectué en solution dans le dimethyl sulfoxide-d_6 (DMSO-d_6) présente, entre autres éléments, un blindage important ($\Delta\delta = +1{,}30$ ppm) du proton H3 le plus influençable lorsque l'on compare les δ de la pyridocarbazolone **1** et de l'acétoxy-4 triméthyl-2,5,11 6*H*-pyrido[3,2-*b*]carbazole (**3**). Cet argument unique n'autorise pas toutefois à affirmer qu'il y a prépondérance en solution dans le DMSO de la forme tautomère lactamique **1**.

Par ailleurs, lorsque le spectre IR est déterminé en solution dans l'acétonitrile, il apparaît une très forte bande OH à 3640 cm^{-1} avec un épaulement à 3540 cm^{-1}. Comme il subsiste toutefois encore une bande CO peu intense à 1640 cm^{-1}, on peut estimer qu'en solution dans l'acétonitrile il existe un équilibre tautomère qui est en faveur de la forme lactime **1a**.

Determination de la Structure Cristalline

Le cristal choisi a pour dimensions $150 \times 200 \times 250\,\mu$m.

Les paramètres de la maille déterminés à partir de 25 réflexions avec le rayonnement Mo$K\alpha$ ($5{,}4° < \theta < 13{,}2°$) ont pour valeurs: $a = 16{,}179(4)$, $b = 14{,}900(3)$, $c = 12{,}702(3)$ Å, $\beta = 112{,}28(2)°$, $V = 2833{,}6$ Å^3, $Z = 8$, $D_x = 1{,}29$, $D_{obs} = 1{,}30$ Mg·m^{-3}, $\mu = 0{,}088$ mm^{-1} (MoK_α), $M_r = 276$, groupe spatial $P2_1/c$, $F(000) = 1168$.

Les intensités ont été mesurées sur un diffractomètre automatique CAD-4 ENRAF-NONIUS, avec un balayage $\omega - 2\theta$ d'amplitude $s = 0{,}65 + 0{,}35 \tan\theta$ ($1{,}5° < \theta < 24°$, $-18 \leqslant h \leqslant 18$; $0 \leqslant k \leqslant 17$; $0 \leqslant l \leqslant 14$). Les réflexions $\bar{3}26{,}040$ et $32\bar{6}$ ont été choisies pour contrôler les intensités. L'écart-type relatif moyen sur les intensités est de 0,005; 4585 réflexions indépendantes ont été mesurées; 2355 d'entre elles répondant au critère $I > 3\sigma(I)$ ont été retenues pour l'affinement. Aucune correction d'absorption n'a été effectuée.

La structure a été résolue par les méthodes directes à l'aide de MULTAN 80.[7] L'affinement basé sur F a été effectué par la méthode des moindres carrés à l'aide d'un programme à matrice complète[8]; les facteurs de diffusion corrigés des f' et f'' sont issue de International Tables of Crystallography[9]; au cours de l'affinment, il a été tenu compte des corrections réelles de diffusion anomale pour C, N et O. Les positions des atomes non-hydrogène ainsi que les facteurs d'agitation thermique anisotrope ont été affinés. Les positions des atomes d'hydrogène ont été localisées avec la série de Fourier des différences $F_o - F_c$ puis affinées. Le facteur d'agitation thermique a été laissé fixe et correspond à celui de l'atome porteur. Les valeurs obtenues à l'issue du dernier affinement sont $R = 0{,}04$ et $R_w = 0.03$ (le schéma de pondération correspond à $w = 1/\sigma^2(F)$); la valeur maximale du rapport de la variation Δ (sur les paramètres variables) sur l'écart-type correspond à 0,0009; la valeur maximale de la densité électronique dans la série de Fourier finale des ΔF vaut $\pm 0{,}10\ e \cdot$ Å^{-3}. Les distances, les angles valentiels et de torsion ont été calculés à l'aide du programme ORFFE3,[10] les distances aux plans moyens par le programme BP70C.[11] Le dessin de la molécule (Fig. 1) et la vue stéréoscopique de la maille (Fig. 2) ont été obtenus par le programme ORTEP.[12]

Resultats et Discussion

L'unité asymétrique contient deux molécules cristallographiquement indépendantes. La notation de la première molécule (I) est indiquée sur la Fig. 1 (de 1 à 21). Les atomes de la

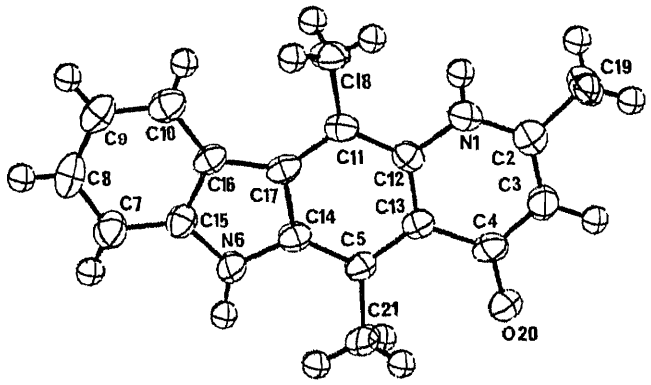


Fig. 1. Conformation de la Molécule I et Numérotation des Atomes

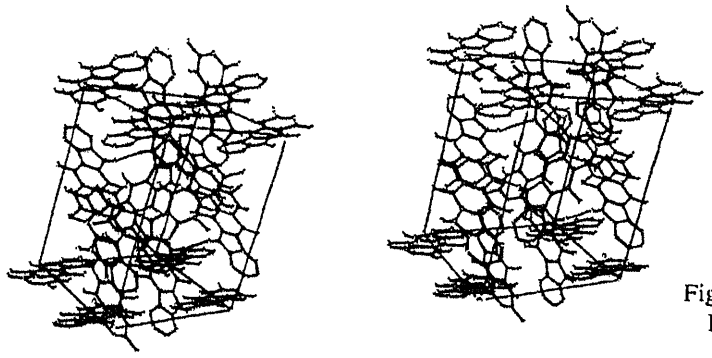


Fig. 2. Vue Stéréoscopique du Contenu de la Maille

TABLEAU 1. Coordonnées Atomiques ($\times 10^4$) et Facteurs de Température Équivalents (les Écarts-Type Sont Mis entre Parenthèses)

	x	y	z	B_{eq} (Å^2)	B_{iso} (Å^2)
N(1)	4657 (2)	6951 (2)	10662 (3)	3,5	
C(2)	4934 (3)	7777 (3)	10511 (4)	3,6	
C(3)	4740 (3)	8104 (3)	9439 (4)	3,7	
C(4)	4209 (3)	7604 (3)	8453 (3)	3,4	
C(5)	3487 (2)	6108 (3)	7721 (3)	2,9	
N(6)	2826 (2)	4579 (2)	7278 (2)	3,7	
C(7)	2339 (3)	3034 (3)	7510 (3)	4,1	
C(8)	2353 (3)	2399 (3)	8312 (4)	4,6	
C(9)	2779 (3)	2573 (3)	9479 (4)	4,3	
C(10)	3192 (3)	3382 (3)	9866 (4)	4,0	
C(11)	3998 (2)	5515 (3)	10048 (3)	2,9	
C(12)	4199 (2)	6374 (3)	9776 (3)	2,9	
C(13)	3957 (2)	6690 (3)	8634 (3)	2,9	
C(14)	3277 (2)	5260 (3)	8004 (3)	3,0	
C(15)	2755 (3)	3852 (3)	7899 (3)	3,3	
C(16)	3185 (2)	4044 (3)	9073 (3)	3,1	
C(17)	3519 (2)	4960 (3)	9153 (3)	2,9	
C(18)	4311 (3)	5179 (3)	11264 (3)	4,0	
C(19)	5452 (3)	8289 (3)	11579 (4)	5,1	
O(20)	3972 (2)	7960 (2)	7479 (2)	4,2	
C(21)	3221 (3)	6322 (3)	6471 (3)	4,4	
N(51)	−0356 (2)	4775 (2)	2730 (3)	3,5	
C(52)	−0164 (3)	4265 (3)	3681 (4)	3,9	
C(53)	0692 (3)	4155 (3)	4401 (3)	3,6	

TABLEAU I. (suite)
(les Écarts-Type Sont Mis entre Parenthèses)

	x	y	z	B_{eq} (Å^2)	B_{iso} (Å^2)
C(54)	1415 (3)	4545 (3)	4188 (3)	3,2	
C(55)	1881 (3)	5372 (3)	2768 (3)	2,9	
N(56)	2119 (2)	6237 (2)	1227 (3)	3,3	
C(57)	1808 (3)	7128 (3)	−0541 (4)	4,1	
C(58)	1128 (3)	7482 (3)	−1477 (4)	4,6	
C(59)	0223 (3)	7355 (3)	−1651 (3)	4,6	
C(60)	−0012 (3)	6855 (3)	−0891 (3)	3,9	
C(61)	0017 (2)	5631 (2)	1379 (3)	2,9	
C(62)	0298 (3)	5148 (2)	2399 (3)	2,9	
C(63)	1208 (3)	5020 (2)	3101 (3)	2,8	
C(64)	1587 (3)	5838 (3)	1747 (3)	3,0	
C(65)	1563 (3)	6645 (3)	0230 (3)	3,4	
C(66)	0662 (3)	6500 (2)	0072 (3)	3,1	
C(67)	0673 (2)	5976 (3)	1044 (3)	2,7	
C(68)	−0971 (3)	5785 (3)	0676 (4)	4,3	
C(69)	−0954 (3)	3877 (3)	3867 (4)	4,6	
O(70)	2206 (2)	4478 (2)	4909 (2)	3,9	
C(71)	2873 (3)	5280 (3)	3423 (4)	4,2	
H(1)	4747	6795	11393		4,7
H(3)	5021	8738	9309		5,1
H(6)	2626	4594	6551		5,0
H(7)	1998	2915	6679		4,2
H(8)	2105	1784	8086		5,9
H(9)	2824	2098	10056		5,7
H(10)	3439	3488	10658		5,1
H(18A)	4604	4692	11379		5,3
H(18B)	4815	5521	11856		5,3
H(18C)	3783	5079	11455		5,3
H(19A)	6124	8140	11906		6,2
H(19B)	5243	8226	12240		6,2
H(19C)	5454	8946	11421		6,2
H(21A)	3703	6680	6348		5,6
H(21B)	2944	5881	5958		5,6
H(21C)	2753	6710	6287		5,6
H(51)	−0963	4835	2222		4,7
H(53)	0865	3787	5155		4,9
H(56)	2706	6341	1560		4,7
H(57)	2437	7134	−0444		5,4
H(58)	1249	7853	−1977		5,9
H(59)	−0209	7656	−2287		5,8
H(60)	−0644	6731	−0996		5,1
H(68A)	−1090	6112	0040		5,5
H(68B)	−1363	5154	0406		5,5
H(68C)	−1248	6187	1083		5,5
H(69A)	−1387	4273	3771		6,0
H(69B)	−0819	3662	4649		6,0
H(69C)	−1117	3345	3612		6,0
H(71A)	3138	5684	4113		5,5
H(71B)	3038	4711	3740		5,5
H(71C)	3238	5404	3006		5,5

$B_{eq} = \frac{4}{3} \sum_i \sum_j \beta_{ij} \mathbf{a}_i \mathbf{a}_j$.

TABLEAU II. Distances Interatomiques (Å), Angles de Liaisons (°) et Écarts-Type entre Parenthèses

1ère molécule		2ème molécule	
Distances (Å)			
N(1)–C(2)	1,348 (5)	N(51)–C(52)	1,358 (5)
N(1)–C(12)	1,386 (4)	N(51)–C(62)	1,394 (4)
C(2)–C(3)	1,366 (5)	C(52)–C(53)	1,349 (5)
C(2)–C(19)	1,504 (5)	C(52)–C(69)	1,501 (5)
C(3)–C(4)	1,430 (5)	C(53)–C(54)	1,421 (5)
C(4)–O(20)	1,264 (4)	C(54)–O(70)	1,263 (4)
C(4)–C(13)	1,464 (5)	C(54)–C(63)	1,473 (5)
C(5)–C(13)	1,416 (5)	C(55)–C(63)	1,409 (5)
C(5)–C(14)	1,390 (5)	C(55)–C(64)	1,386 (5)
C(5)–C(21)	1,514 (5)	C(55)–C(71)	1,509 (5)
N(6)–C(14)	1,380 (5)	N(56)–C(64)	1,400 (4)
N(6)–C(15)	1,370 (4)	N(56)–C(65)	1,385 (5)
C(7)–C(8)	1,383 (5)	C(57)–C(58)	1,382 (6)
C(7)–C(15)	1,390 (5)	C(57)–C(65)	1,388 (5)
C(8)–C(9)	1,401 (5)	C(58)–C(59)	1,408 (5)
C(9)–C(10)	1,376 (5)	C(59)–C(60)	1,382 (5)
C(10)–C(16)	1,406 (5)	C(60)–C(66)	1,398 (5)
C(11)–C(12)	1,397 (5)	C(61)–C(62)	1,400 (5)
C(11)–C(17)	1,381 (5)	C(61)–C(67)	1,382 (5)
C(11)–C(18)	1,517 (5)	C(61)–C(68)	1,524 (5)
C(12)–C(13)	1,432 (5)	C(62)–C(63)	1,416 (5)
C(14)–C(17)	1,431 (5)	C(64)–C(67)	1,424 (5)
C(15)–C(16)	1,415 (5)	C(65)–C(66)	1,410 (5)
C(16)–C(17)	1,458 (5)	C(66)–C(67)	1,455 (5)
Angles (°)			
C(2)–N(1)–C(12)	123,7 (4)	C(52)–N(51)–C(62)	123,1 (3)
N(1)–C(2)–C(3)	120,3 (4)	N(51)–C(52)–C(53)	120,1 (4)
N(1)–C(2)–C(19)	115,9 (4)	N(51)–C(52)–C(69)	115,7 (4)
C(3)–C(2)–C(19)	123,9 (4)	C(53)–C(52)–C(69)	124,2 (4)
C(2)–C(3)–C(4)	121,4 (4)	C(52)–C(53)–C(54)	121,9 (4)
O(20)–C(4)–C(3)	119,8 (4)	O(70)–C(54)–C(53)	120,7 (4)
O(20)–C(4)–C(13)	123,0 (4)	O(70)–C(54)–C(63)	121,7 (4)
C(3)–C(4)–C(13)	117,2 (4)	C(53)–C(54)–C(63)	117,6 (4)
C(14)–C(5)–C(13)	117,0 (3)	C(64)–C(55)–C(63)	115,9 (3)
C(14)–C(5)–C(21)	117,5 (4)	C(64)–C(55)–C(71)	118,5 (3)
C(13)–C(5)–C(21)	125,5 (4)	C(63)–C(55)–C(71)	125,6 (3)
C(15)–N(6)–C(14)	109,6 (3)	C(65)–N(56)–C(64)	108,4 (3)
C(8)–C(7)–C(15)	117,9 (4)	C(58)–C(57)–C(65)	117,2 (4)
C(7)–C(8)–C(9)	121,1 (4)	C(57)–C(58)–C(59)	121,7 (4)
C(10)–C(9)–C(8)	121,1 (4)	C(60)–C(59)–C(58)	120,5 (4)
C(9)–C(10)–C(16)	119,2 (4)	C(59)–C(60)–C(66)	119,1 (4)
C(17)–C(11)–C(12)	117,1 (3)	C(67)–C(61)–C(62)	117,3 (4)
C(17)–C(11)–C(18)	120,5 (4)	C(67)–C(61)–C(68)	121,4 (4)
C(12)–C(11)–C(18)	122,3 (4)	C(62)–C(61)–C(68)	121,3 (4)
N(1)–C(12)–C(11)	118,1 (4)	N(51)–C(62)–C(61)	117,9 (3)
N(1)–C(12)–C(13)	118,4 (4)	N(51)–C(62)–C(63)	119,0 (3)
C(11)–C(12)–C(13)	123,5 (4)	C(61)–C(62)–C(63)	123,2 (4)
C(5)–C(13)–C(12)	118,9 (3)	C(55)–C(63)–C(62)	119,9 (3)
C(5)–C(13)–C(4)	122,4 (4)	C(55)–C(63)–C(54)	122,2 (4)
C(12)–C(13)–C(4)	118,7 (4)	C(62)–C(63)–C(54)	117,9 (3)
N(6)–C(14)–C(5)	127,9 (3)	N(56)–C(64)–C(55)	126,9 (4)
N(6)–C(14)–C(17)	108,9 (3)	N(56)–C(64)–C(67)	108,7 (3)

TABLEAU II. (suite)

1ère molécule		2ème molécule	
Angles (°)			
C(5)–C(14)–C(17)	123,2 (4)	C(55)–C(64)–C(67)	124,4 (4)
N(6)–C(15)–C(7)	128,6 (4)	N(56)–C(65)–C(57)	127,7 (4)
N(6)–C(15)–C(16)	109,4 (3)	N(56)–C(65)–C(66)	109,9 (3)
C(7)–C(15)–C(16)	122,0 (4)	C(57)–C(65)–C(66)	122,4 (4)
C(10)–C(16)–C(15)	118,7 (4)	C(60)–C(66)–C(65)	119,2 (4)
C(10)–C(16)–C(17)	134,8 (4)	C(60)–C(66)–C(67)	134,4 (4)
C(15)–C(16)–C(17)	106,5 (3)	C(65)–C(66)–C(67)	106,4 (3)
C(11)–C(17)–C(14)	120,3 (4)	C(61)–C(67)–C(64)	119,3 (4)
C(11)–C(17)–C(16)	134,0 (4)	C(61)–C(67)–C(66)	134,2 (4)
C(14)–C(17)–C(16)	105,7 (4)	C(64)–C(67)–C(66)	106,5 (3)

Distances interatomiques (Å)					
N(1)–H(1)	0,91	C(19)–H(19B)	0,90	C(60)–H(50)	1,00
C(3)–H(3)	1,09	C(19)–H(19C)	1,00	C(68)–H(68A)	0,90
N(6)–H(6)	0,85	C(21)–H(21A)	1,01	C(68)–H(68B)	1,11
C(7)–H(7)	1,00	C(21)–H(21B)	0,91	C(68)–H(68C)	1,00
C(8)–H(8)	1,00	C(21)–H(21C)	0,91	C(69)–H(69A)	0,89
C(9)–H(9)	1,00	N(51)–H(51)	0,95	C(69)–H(69B)	0,99
C(10)–H(10)	0,94	C(53)–H(53)	1,05	C(69)–H(69C)	0,86
C(18)–H(18A)	0,85	N(56)–H(56)	0,90	C(71)–H(71A)	1,01
C(18)–H(18B)	1,01	C(57)–H(57)	0,98	C(71)–H(71B)	0,93
C(18)–H(18C)	0,98	C(58)–H(58)	0,92	C(71)–H(71C)	0,95
C(19)–H(19A)	1,03	C(59)–H(59)	0,96		

deuxième molécule (II) sont numérotés de 51 à 71. Les coordonnées des atomes et leurs facteurs d'agitation thermique isotrope équivalents sont rassemblés dans le Tableau I. Les distances et angles de liaisons sont indiqués dans le Tableau II.

Chaque molécule est constituée d'un cycle dihydro-1,4 oxo-4 pyridine (A) accolé au noyau carbazole tricyclique (B, C, D). Les équations des plans moyens relatifs aux noyaux A, B, C et D ainsi que celle du plan moyen relatif au noyau carbazole (noté E) sont décrites dans le Tableau III. Le test du χ^2 montre que dans les deux molécules, le cycle A s'écarte légèrement de la planéité à l'inverse des autres cycles B, C et D. Les distances des atomes du cycle A au plan moyen sont compris entre $-0{,}031$ et $+0{,}03$ Å pour la première molécule et entre $-0{,}034$ et $+0{,}042$ Å pour la deuxième molécule. Des groupements méthyles situés sur chacun des cycles A, C(19) reste dans le plan moyen correspondant tandis que C(69) s'en écarte très significativement. Quant aux atomes d'oxygène O(20) et O(70) situés en position méta par rapport aux groupements méthyles, ils sont situés l'un et l'autre nettement en dehors des plans moyens correspondants. Les autres groupements méthyles situés en para sur le cycle B de chaque noyau carbazole restent proches des plans moyens correspondants. Les angles dièdres entre les différents cycles constitutifs du carbazole montrent que ceux-ci sont coplanaires pour les deux molécules; le cycle A de la deuxième molécule s'écarte très légèrement (2,8(1)°) du plan du noyau carbazole correspondant. Par ailleurs, les plans moyens E des noyaux carbazoles homologues font entre eux des angles de 60,77(7)°.

Les longueurs de liaison observées dans les trois cycles B, C et D du carbazole ne présentent pas de différences significatives avec celles décrites dans le carbazole[13] ou dans le diméthyl-5,6 6*H*-pyrido[4,3-*b*]carbazole[14] ainsi que dans le triméthyl-5,6,11-pyrido[4,3-*b*]carbazole[15]; l'écart maximum reste inférieur à 0,05 Å. Au niveau des angles, les écarts ne

TABLEAU III. Equations des Différents Plans Moyens (Pondérés à Partir des Écarts-Type sur des Positions Atomiques), Distances de ces Atomes à ces Plans (Å) et Angles Dièdres entre les Plans

a) Paramètres rapportés à un système de coordonnées cartésiennes en Å

	l	m	n	p	χ_2
Molécule 1					
P1 cycle A pyridinique	−0,9284 (6)	0,371 (2)	0,365 (2)	1,77 (3)	379,1
P2 cycle B benzénique	−0,9369 (6)	0,350 (2)	0,365 (2)	1,48 (2)	41,4
P3 cycle C pyrrolique	−0,9365 (8)	0,351 (2)	0,360 (2)	1,44 (2)	16,0
P4 cycle D benzénique	−0,9304 (7)	0,366 (2)	0,366 (2)	1,62 (2)	2,3
P5 cycle E carbazole	−0,9347 (2)	0,3553 (5)	0,362 (1)	1,52 (1)	137,8
Molécule 2					
P1 cycle A′ pyridinique	−0,244 (2)	0,838 (1)	0,544 (1)	7,968 (3)	529,5
P2 cycle B′ benzénique	−0,204 (2)	0,8473 (9)	0,531 (1)	8,027 (5)	15,6
P3 cycle C′ pyrrolique	−0,194 (2)	0,846 (1)	0,534 (2)	8,03 (1)	36,0
P4 cycle D′ benzénique	−0,198 (2)	0,837 (1)	0,547 (2)	7,94 (1)	25,7
P5 cycle E′ carbazole	−0,197 (1)	0,8439 (3)	0,5365 (5)	8,021 (3)	203,2

Plans moyens: $lx+my+nz=p$.

b) Distances (en Å) des atomes aux plans moyens (les écarts-type sont mis entre parenthèses)

Molécule 1				Molécule 2			
Cycle pyridinique (A)		Cycle benzénique (B)		Cycle pyridinique (A′)		Cycle benzénique (B′)	
N(1)	0,021 (2)	C(5)	−0,007 (3)	N(51)	0,022 (2)	C(55)	0,001 (4)
C(2)	−0,008 (3)	C(11)	−0,012 (3)	C(52)	−0,034 (4)	C(61)	0,006 (3)
C(3)	−0,031 (3)	C(12)	0,005 (3)	C(53)	−0,012 (3)	C(62)	−0,008 (3)
C(4)	0,033 (3)	C(13)	0,006 (3)	C(54)	−0,042 (3)	C(63)	0,004 (3)
C(12)	−0,020 (3)	C(14)	0,000 (3)	C(62)	0,000 (3)	C(64)	−0,003 (3)
C(13)	−0,011 (3)	C(17)	0,009 (3)	C(63)	−0,034 (3)	C(67)	−0,001 (3)
C(19)[a]	−0,008 (7)	C(18)[a]	−0,098 (7)	C(69)[a]	−0,077 (7)	C(68)[a]	−0,052 (7)
O(20)[a]	0,134 (6)	C(21)[a]	−0,073 (7)	C(70)[a]	0,144 (6)	C(71)[a]	−0,000 (7)
Cycle pyrrolique (C)		Cycle benzénique (D)		Cycle pyrrolique (C′)		Cycle benzénique (D′)	
N(6)	−0,004 (2)	C(7)	−0,001 (3)	N(56)	0,007 (2)	C(57)	−0,007 (3)
C(14)	0,001 (3)	C(8)	0,004 (3)	C(64)	−0,010 (3)	C(58)	0,000 (4)
C(15)	0,008 (3)	C(9)	−0,002 (4)	C(65)	−0,007 (3)	C(59)	0,011 (4)
C(16)	−0,006 (3)	C(10)	−0,001 (3)	C(66)	−0,000 (3)	C(60)	−0,009 (3)
C(17)	0,002 (2)	C(15)	−0,001 (3)	C(67)	−0,006 (2)	C(65)	0,005 (3)
		C(16)	0,002 (3)			C(66)	0,003 (3)
Cycle carbazole (E)				Cycle carbazole (E′)			
C(5)	0,002 (3)	C(12)	0,011 (3)	C(55)	0,020 (3)	C(61)	−0,006 (3)
N(6)	−0,014 (3)	C(13)	0,017 (4)	N(56)	−0,018 (3)	C(62)	−0,008 (3)
C(7)	0,012 (4)	C(14)	−0,001 (4)	C(57)	−0,003 (4)	C(63)	0,019 (4)
C(8)	0,024 (4)	C(15)	−0,006 (4)	C(58)	0,020 (5)	C(64)	0,004 (4)
C(9)	0,008 (4)	C(16)	−0,013 (4)	C(59)	0,031 (4)	C(65)	−0,007 (4)
C(10)	−0,008 (4)	C(17)	0,003 (4)	C(60)	−0,005 (4)	C(66)	−0,009 (4)
C(11)	−0,014 (3)					C(67)	−0,011 (4)

a) Atomes exclus du calcul du plan moyen.

c) Angles dièdres en degrés

Molécule 1		Molécule 2	
(A,B) 1,3 (1)	(B,C) 0,3 (1)	(A′,B′) 2,4 (1)	(B′,C′) 0,7 (1)
(A,C) 1,4 (2)	(B,D) 1,0 (1)	(A′,C′) 3,0 (1)	(B′,D′) 1,3 (1)
(A,D) 0,3 (1)	(C,D) 1,1 (2)	(A′,D′) 3,0 (1)	(C′,D′) 0,9 (2)

TABLEAU IV. Liaisons Intermoléculaires

a) Liaisons hydrogène

	Distance (Å)	Angle (°)
N(1) –H(1) ···O(20^{i})	2,918 (4)	133
N(6) –H(6) ···O(70)	2,791 (4)	173
N(56)–H(56)···O(20^{ii})	3,056 (4)	160

b) Liaisons de van der Waals (d<3,70 Å)

N(1)–C(16^{iii})	3,690 (5)	C(10)–C(2^{iii})	3,675 (5)	C(52)–C(59^{vi})	3,506 (6)
C(2)–O(20^{i})	3,582 (5)	C(11)–C(11^{iii})	3,632 (7)	C(53)–C(69^{iv})	3,594 (6)
C(2)–C(10^{ii})	3,675 (5)	C(15)–O(70)	3,677 (5)	C(54)–C(69^{iv})	3,686 (5)
C(2)–C(21^{i})	3,679 (5)	C(18)–O(20^{i})	3,319 (5)	C(55)–C(58^{i})	3,677 (5)
C(3)–C(71^{i})	3,691 (6)	C(19)–O(20^{i})	3,547 (5)	C(58)–C(69^{vi})	3,569 (6)
N(6)–C(69^{iv})	3,639 (5)	C(19)–C(21^{i})	3,608 (6)	C(58)–C(64^{ii})	3,631 (6)
C(8)–O(70^{v})	3,516 (5)	O(20)–C(71^{i})	3,618 (5)	C(60)–C(62^{vi})	3,481 (5)
C(8)–C(54^{v})	3,635 (5)	C(21)–C(69^{iv})	3,540 (6)	C(61)–C(66^{vi})	3,627 (5)
C(8)–C(19^{iii})	3,649 (6)	C(21)–C(57^{i})	3,558 (6)	C(66)–C(68^{vi})	3,620 (6)
C(9)–O(70^{v})	3,298 (5)	N(51)–C(59^{vi})	3,496 (5)	C(67)–C(68^{vi})	3,561 (5)
C(10)–C(68^{iv})	3,613 (6)	N(51)–C(60^{vi})	3,572 (5)		

Code de symétrie: i; x, 3/2−y, 1/2+z. ii; x, 3/2−y, −1/2+z. iii; 1−x, 1−y, 2−z. iv; −x, 1−y, 1−z. v; x, 1/2−y, 1/2+z. vi; −x, 1−y, −z.

dépassent pas 5°.

Quant au cycle pyridinique A, il faut noter le raccourcissement des longueurs de liaisons C(2)–C(3) et C(52)–C(53) dont les valeurs correspondent à celle d'une double liaison Csp^2–Csp^2 confirmant ainsi le caractère partiellement non aromatique du cycle A. Par ailleurs, les longueurs des liaisons N–C, plus courtes que les valeurs des liaisons Nsp^2–Csp^2 (1,426 Å dans $C_6H_5NHCOCH_3$), sont en revanche plus longues que celles décrites dans les dérivés de l'ellipticine,[14,15] ce qui infirme à nouveau le caractère aromatique du cycle A.

Ces observations indiquent l'existence d'une orbitale faiblement délocalisée sur l'ensemble du cycle à laquelle participent vraisemblablement le doublet non partagé de l'azote et les électrons de la liaison CO.

Ainsi, la présence de la forme tautomère lactime **1a** qui a été détectée par l'étude IR sur une solution dans l'acétonitrile n'est pas retrouvée à l'état solide.

La vue stéréoscopique de la structure (Fig. 2) montre que les molécules de types I et II ne donnent lieu à aucun recouvrement, en particulier au niveau des noyaux carbazole, contrairement à ce que l'on avait observé dans les deux formes monocliniques du dihydro-5,6 pyrimidino[5,4-c]carbazole ou dans ses combinaisons moléculaires avec le TCNQ ou le nitrate de cobalt(II). Les molécules du type I sont liées entre elles par la liaison hydrogène N(1)–H(1)···O(20^{i}): 2,918(4) Å; 133°; (code de symétrie i: x, 3/2−y, 1/2+z) formant ainsi des chaînes en zigzag: d'autre part, chaque molécule de type II joue un rôle de pont entre deux molécules voisines du type I par les deux liaisons H:

N(6)–H(6)···O(70): 2,791(4) Å et 173°

et N(56)–H(50)···O(20^{ii}): 3,056(4) Å et 160°

(ii : x, 3/2−y, −1/2+z).

De nombreux contacts de van der Waals (Tableau IV) dont le plus proche vaut

TABLEAU V. Facteurs d'Agitation Thermique Anisotrope ($\times 10^4$)

	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
N(1)	0040 (2)	0043 (2)	0053 (3)	−0002 (2)	0019 (2)	−0003 (2)
C(2)	0032 (2)	0047 (3)	0072 (4)	−0001 (2)	0020 (3)	−0010 (3)
C(3)	0043 (3)	0039 (3)	0070 (4)	−0004 (2)	0024 (3)	−0005 (3)
C(4)	0040 (2)	0041 (3)	0065 (4)	0004 (2)	0030 (3)	0005 (3)
C(5)	0032 (2)	0036 (2)	0046 (4)	0002 (2)	0014 (2)	0007 (2)
N(6)	0055 (2)	0035 (2)	0046 (3)	−0006 (2)	0010 (2)	0005 (2)
C(7)	0048 (3)	0037 (3)	0075 (5)	−0002 (2)	0015 (3)	0004 (3)
C(8)	0047 (3)	0032 (2)	0119 (6)	0000 (2)	0032 (3)	0011 (3)
C(9)	0052 (3)	0038 (3)	0092 (5)	0004 (2)	0030 (3)	0020 (3)
C(10)	0048 (3)	0044 (3)	0072 (4)	0011 (2)	0027 (3)	0015 (3)
C(11)	0035 (2)	0035 (2)	0045 (3)	0007 (2)	0016 (2)	0004 (2)
C(12)	0029 (2)	0039 (2)	0054 (4)	0003 (2)	0018 (2)	−0006 (2)
C(13)	0030 (2)	0034 (2)	0056 (4)	0004 (2)	0018 (2)	0004 (2)
C(14)	0034 (2)	0037 (2)	0047 (4)	0005 (2)	0014 (2)	0008 (2)
C(15)	0041 (2)	0035 (2)	0061 (4)	0004 (2)	0018 (3)	0010 (3)
C(16)	0034 (2)	0036 (2)	0058 (4)	0007 (2)	0020 (2)	0013 (2)
C(17)	0032 (2)	0036 (2)	0050 (4)	0007 (2)	0017 (2)	0012 (2)
C(18)	0063 (3)	0044 (3)	0048 (4)	0010 (2)	0022 (3)	0011 (3)
C(19)	0057 (3)	0062 (3)	0078 (5)	−0015 (3)	0018 (3)	−0022 (3)
O(20)	0066 (2)	0038 (2)	0068 (3)	−0002 (2)	0035 (2)	0006 (2)
C(21)	0063 (3)	0051 (3)	0047 (4)	−0016 (2)	0015 (3)	0008 (3)
N(51)	0035 (2)	0042 (2)	0071 (3)	0001 (2)	0022 (2)	0005 (2)
C(52)	0052 (3)	0038 (2)	0078 (5)	−0001 (2)	0037 (3)	−0006 (3)
C(53)	0051 (3)	0038 (2)	0057 (4)	0002 (2)	0025 (3)	0006 (2)
C(54)	0043 (3)	0029 (2)	0056 (4)	0002 (2)	0022 (3)	−0008 (2)
C(55)	0035 (2)	0031 (2)	0054 (4)	0003 (2)	0019 (2)	0000 (2)
N(56)	0037 (2)	0042 (2)	0058 (3)	0000 (2)	0020 (2)	0004 (2)
C(57)	0057 (3)	0041 (3)	0065 (4)	−0008 (2)	0029 (3)	−0004 (3)
C(58)	0077 (3)	0039 (3)	0063 (4)	−0009 (3)	0029 (3)	0002 (3)
C(59)	0070 (3)	0043 (3)	0064 (4)	0002 (3)	0019 (3)	0003 (3)
C(60)	0051 (3)	0039 (3)	0059 (4)	0001 (2)	0017 (3)	0002 (3)
C(61)	0032 (2)	0029 (2)	0057 (4)	0003 (2)	0016 (2)	−0004 (2)
C(62)	0035 (2)	0030 (2)	0057 (4)	−0002 (2)	0022 (3)	−0002 (2)
C(63)	0039 (2)	0029 (2)	0045 (3)	0003 (2)	0017 (2)	−0001 (2)
C(64)	0037 (2)	0032 (2)	0053 (4)	0001 (2)	0020 (2)	−0006 (2)
C(65)	0052 (3)	0029 (2)	0055 (4)	−0002 (2)	0022 (3)	−0004 (2)
C(66)	0038 (2)	0030 (2)	0052 (4)	−0001 (2)	0012 (2)	−0001 (2)
C(67)	0031 (2)	0030 (2)	0049 (4)	0002 (2)	0013 (2)	−0005 (2)
C(68)	0034 (2)	0058 (3)	0082 (4)	0004 (2)	0018 (3)	0005 (3)
C(69)	0055 (3)	0050 (3)	0115 (5)	−0008 (2)	0052 (3)	0007 (3)
O(70)	0049 (2)	0049 (2)	0052 (3)	0003 (1)	0014 (2)	0005 (2)
C(71)	0028 (2)	0064 (3)	0078 (4)	0006 (2)	0012 (3)	0016 (3)

$T = \exp - \{\beta_{11}h^2 + \beta_{22}k^2 + \beta_{33}l^2 + 2\beta_{12}hk + 2\beta_{13}hl + 2\beta_{23}kl\}$.

3,298(5) Å entre les atomes C(9) et O(70^v) (v: x, $1/2-y$, $1/2+z$) contribuent à la bonne cohésion du cristal.

Ainsi, la planéïté de la molécule, confirmée par l'étude structurale, laisse présager la possibilité de formation d'un complexe par transfert de charge avec le TCNQ: ce taux de transfert devrait être plus important que celui décrit dans le complexe TCNQ–DPC (dont le noyau dihydrocarbazole présente une conformation non plane) et devrait se rapprocher de celui observé par Kobayashi[16] dans le complexe TCNQ–carbazole. Les facteurs d'agitation thermique anisotrope sont indiqués dans le Tableau V.

Conclusion

L'étude cristallographique du dérivé (**1**) a confirmé la présence exclusive de la forme lactame à l'état solide déjà revélée par l'examen du spectre IR. Cependant, l'étude du spectre IR en solution montre la présence d'un équilibre tautomère avec prédominance d'une forme lactime **1a**. Il est envisageable qu'elle puisse jouer un rôle important dans les propriétés biologiques, ce que l'on se propose de vérifier sur d'autres exemples.

Partie Experimentale

Dihydro-1,4 oxo-4 trimethyl-2,5,11-6*H*-pyrido[3,2-*b*]carbazole (1)——Un mélange de 13 g (0,061 mol) d'amino-3 diméthyl-1,4 carbazole (**2**) et de 8,73 g (0,067 mol) d'acétoacétate d'éthyle dans 700 cm^3 de toluène est porté au reflux 8 h. La solution toluénique est évaporée sous pression réduite. L'huile résiduelle obtenue est ajoutée lentement à 50 cm^3 de diphényl éther à 160 °C. On porte ensuite la solution à 230 °C pendant 15 min. Après refroidissement, le mélange est versé dans 500 cm^3 d'éther éthylique. Le précipité jaune est essoré, lavé à l'éther éthylique, séché et recristallisé dans l'acétonitrile. Cristaux marrons. F = 265 °C (déc.), p = 11 g, 65%. IR ν(KBr) cm^{-1}: 3320, 3170 (NH), 1625 (CO). IR (CH_3CN) cm^{-1}: 3650, 3550 (OH), 1630 (CO). RMN (DMSO-d_6) δ: 2,38, 2,98, 3,11 (s, s, s), CH_3 (2), CH_3 (5), CH_3 (11), 9,95, 11,01 (NH), 5,73 (H_3), 8,25 (H10), 7,41 (H8,9), 7,06 (H7). *Anal.* Calc. pour: $C_{18}H_{16}N_2O$: C, 78,23; H, 5,84; N, 10,14. Trouvé: C, 78,14; H, 5,83; N, 10,03.

Les spectres IR sont enregistrés dans le KBr sur Perkin Elmer 257 G et dans l'acétonitrile. Les spectres de RMN ont été mesurés en solution dans le DMSO-d_6 à 90 MHz sur un spectromètre VARIAN EM 390. Les déplacements chimiques (δ) sont exprimés en ppm par rapport au tétraméthylsilane utilisé comme référence interne.

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Thiocarbonyl Ylides. VI.[1] New Generation of Thiocarbonyl Ylides from Organosilicon Compounds Containing Sulfur and Their 1,3-Cycloadditions

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Thermolysis of bromo(trimethylsilyl)methyl trimethylsilylmethyl sulfide was found to give a thiocarbonyl ylide, the 1,3-cycloaddition of which proved a new method for the synthesis of tetrahydrothiophenes. The effect of the silyl group of the ylide on the regio- and stereoselectivity in these 1,3-dipolar cycloadditions is discussed.

Keywords——thiocarbonyl ylide; 1,3-cycloaddition; organosilicon compound; thermolysis; tetrahydrothiophene; regioselectivity

Thiocarbonyl ylides are useful intermediates not only in heterocyclic synthesis but also in carbon–carbon bond formation reactions. Although several methods for the generation of thiocarbonyl ylides have been reported,[2] they are generally not convenient due to the difficulties of synthesizing the starting materials or limited applicability.

As mentioned in the preliminary communication,[3] we have found a convenient method for generating thiocarbonyl ylides and for carrying out their 1,3-dipolar cycloaddition leading to tetrahydrothiophene derivatives by using organosilicon compounds. In this paper we present the details of this work.

Our strategy for the generation of thiocarbonyl ylides involved silicon–carbon bond cleavage,[4] which would be accelerated by stabilization of the carbanion arising from the formation of a thiocarbonyl ylide. We designed an organosilicon compound containing sulfur, bromo(trimethylsilyl)methyl trimethylsilylmethyl sulfide (**2a**), which was easily prepared by bromination of bis(trimethylsilylmethyl) sulfide (**1a**) with *N*-bromosuccinimide (NBS). After several preliminary experiments, thermolysis of **2a** in *N*,*N*-dimethylformamide

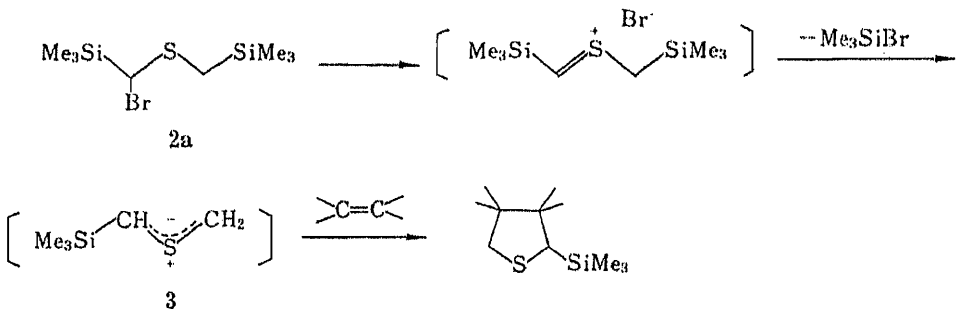
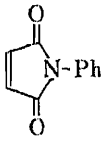
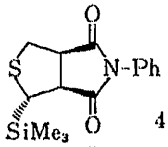
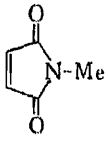
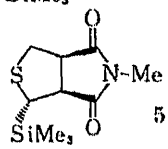
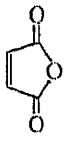
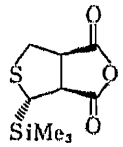
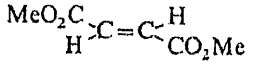
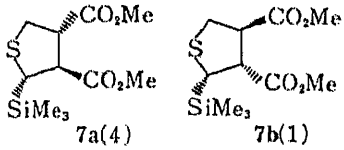
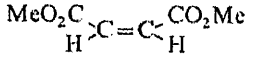
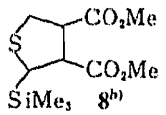
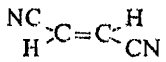
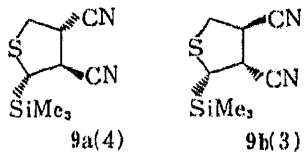


Chart 1

(DMF) has been found to afford trimethylsilylthioaldehyde *S*-methylide (**3**). The results of initial experiments on the 1,3-cycloaddition of **3** to several symmetrical dipolarophiles are shown in Table I.

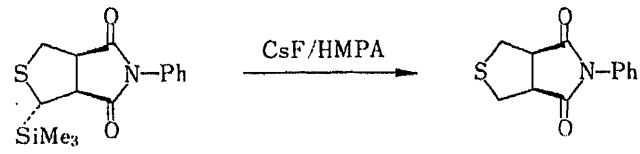
TABLE I. 1,3-Cycloaddition *via* Trimethylsilylthioaldehyde *S*-Methylide (3)

Entry	Dipolarophile	Product[a]	Yield (%)
1		 4	95
2		 5	96
3		 6	91
4		 7a(4) 7b(1)	90
5		 8[b]	94
6		 9a(4) 9b(3)	98

a) The ratio of the isomers is given in parentheses. *b*) A mixture of 3,4-*cis* and 3,4-*trans* isomers.

These reactions were carried out by heating **2a** at 110 °C with dipolarophiles in DMF to give the corresponding 2-trimethylsilyltetrahydrothiophenes (**4**—**9**) in excellent yields. It is noteworthy that the reaction with cyclic dipolarophiles produced a single stereoisomer (entries 1—3). However, dimethyl fumarate or fumaronitrile gave a mixture of two possible stereoisomers, the major one of which was assumed to be the 2,3-*trans* isomer (entries 4 and 6), and dimethyl maleate gave a mixture of four possible isomers which may be formed by epimerization of the 3,4-*cis* isomers initially formed (entry 5). The possibility of cyclopropane ring formation by 1,1-cycloaddition *via* a carbene-like intermediate was definitely excluded on the basis of the proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of the products (Table IV); no signal was observed at the higher magnetic field characteristic of cyclopropane rings. This conclusion was supported by the finding that treatment of **4** with cesium fluoride in hexamethylphosphoramide (HMPA) containing a little water gave the corresponding desilylated tetrahydrothiophenes (**10**) in 73% yield.

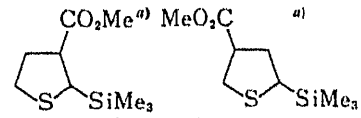
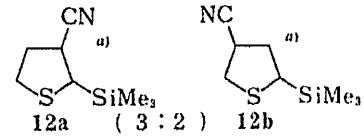
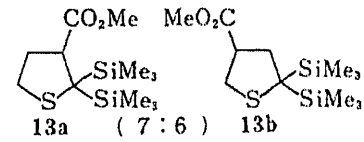
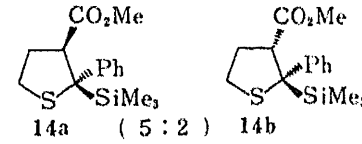
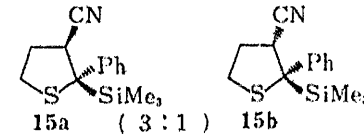
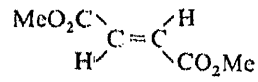
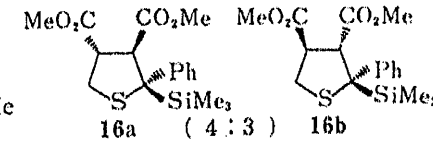
From the mechanistic point of view, the stabilization of the β-cation by silicon[5] promotes the formation of the sulfonium salt in the initial step, and the stabilization of the carbanion due to the formation of thiocarbonyl ylide aids the carbon–silicon bond cleavage by nucleophilic attack of the bromide anion on the silicon atom in the second step (Chart 1). Since we failed to obtain any cycloadduct by the reaction of α-bromo-α-trimethylsilylbenzyl



4 10

Chart 2

TABLE II. Effect of the Silyl Group on Regio- and Stereoselectivity in 1,3-Dipolar Cycloaddition

Entry	Reagent	Dipolarophile	Product (Ratio of isomers)	Total yield (%)
7	$Me_3SiCH(Br)SCH_2SiMe_3$ **2a**	$H_2C=CHCO_2Me$	 **11a** (3 : 1) **11b**	52
8	**2a**	$H_2C=CHCN$	 **12a** (3 : 2) **12b**	56
9	$Me_3Si-C(SiMe_3)(Br)-SCH_2SiMe_3$ **2b**	$H_2C=CHCO_2Me$	 **13a** (7 : 6) **13b**	46
10	$Me_3Si-C(Ph)(Br)-SCH_2SiMe_3$ **2c**	$H_2C=CHCO_2Me$	 **14a** (5 : 2) **14b**	72
11	**2c**	$H_2C=CHCN$	 **15a** (3 : 1) **15b**	87
12	**2c**		 **16a** (4 : 3) **16b**	95

a) A mixture of two possible stereoisomers.

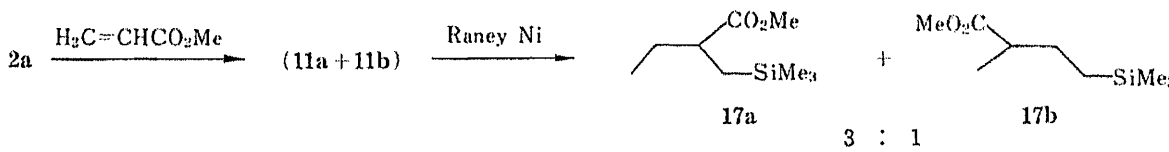


Chart 3

phenyl sulfide with a dipolarophile under similar conditions, the silyl group at the carbon without bromine seems to play a role as a leaving group.

One of the novel features in this reaction is that the generated thiocarbonyl ylide has a silyl group at the terminus. Therefore, in order to reveal the effects[6] of the silyl group on the regio-

and stereoselectivity in 1,3-dipolar cycloadditions, we examined the 1,3-cycloadditions of **2a**—**c** to several unsymmetrical dipolarophiles, and the results are summarized in Table II. Entry 7 (Table II) shows a clear regioselective effect due to the trimethylsilyl group. For the structure elucidation, **11a** and **11b** were desulfurized quantitatively with Raney Ni to 2-(methoxycarbonyl)butyltrimethylsilane (**17a**) and its 3-isomer (**17b**), respectively. The ^{1}H-NMR spectrum of **17a** shows a triplet at 0.87 ppm (J=6.8 Hz) due to the terminal methyl. On the other hand, that of **17b** shows a doublet at 1.15 ppm (J=7.1 Hz) due to the corresponding terminal methyl. The ratio of **11a** to **11b** was determined by gas chromatography (GC) of the mixture of **17a** and **17b** obtained from the crude cycloadduct. The structures and ratio of the isomers in entries 8 and 9 were determined similarly. The regioselectivity observed in entry 9 is not negligible in view of the fact that the 2,2,3-trisubstituted cycloadduct was produced in a slight excess in spite of the large steric hindrance of the two trimethylsilyl groups. Compound **2c** reacted with methyl acrylate to give the corresponding tetrahydrothiophenes (**14a** and **14b**); the product was isolated as a single regio isomer but a mixture of two stereoisomers. The ^{1}H-NMR spectra show two characteristic signals: **14a**, 3.63 ppm (1H, dd, J=4.4, 5.6 Hz, ≥CH), 3.75 ppm (3H, s, OCH_3): **14b**, 3.52 ppm (1H, t, J=6.4 Hz, ≥CH), 3.49 ppm (3H, s, OCH_3). Shift of the signal due to the ester methyl of **14b** to higher magnetic field indicates[7] that the methoxycarbonyl group at the 3-position is *cis* to the phenyl group at the 2-position. The structures of **15a** and **15b** were established by comparison of their ^{1}H-NMR spectra with those of **14a** and **14b**, respectively.

We reported[8] previously that the phenyl group at the dipole terminus affected the stereoselectivity as well as the regioselectivity in the 1,3-dipolar cycloaddition of azomethine ylides. Therefore, the high regioselectivity in entries 10 and 11 seems to be mostly owing to the effect of the phenyl group. Entries 10—12 suggest that the stereoselectivity may be attributed to the steric effect of the silyl group on the conformation of the neighboring phenyl group.

An examination of these effects in terms of molecular orbital theory will be reported in the near future.

Experimental

All melting points and boiling points are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were taken at 90 MHz with a JEOL FX-90Q spectrometer. Chemical shifts are expressed in δ (ppm) values. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were obtained on a Hitachi RMS-4 mass spectrometer.

Bis(trimethylsilylmethyl) Sulfide (1a)——Chloromethyltrimethylsilane (12.3 g, 0.1 mol) and tetrabutylammonium bromide (200 mg) were added to an aqueous solution of sodium sulfide pentahydrate (12 g, 0.05 mol) in 20 ml of water. The mixture was stirred at refluxing temperature for 5 h, then allowed to cool. The organic layer was separated and the aqueous layer was extracted with hexane. The combined hexane solution was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to distillation to give a colorless oil, bp 83—84 °C (15 mmHg), 8.8 g (86%). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (18H, s, $2\times Si(CH_3)_3$), 1.80 (4H, s, $2\times -CH_2-$). *Anal.* Calcd for $C_8H_{22}SSi_2$: C, 46.53; H, 10.74. Found: C, 46.69; H, 10.83.

Bis(trimethylsilyl)methyl Trimethylsilylmethyl Sulfide (1b)——A hexane solution of *sec*-butyllithium (23 ml of 1.3 M solution) was added to a solution of bis(trimethylsilylmethyl) sulfide (**1a**) (4.1 g, 20 mmol) and tetramethylethylenediamine (TMEDA) (2.3 g, 20 mmol) in tetrahydrofuran (THF) (50 ml) with stirring at 0 °C. After the whole had been stirred for 30 min, a solution of chlorotrimethylsilane (2.2 g, 20 mmol) in THF (10 ml) was added dropwise. The solution was stirred for 1 h at 0 °C and for an additional 1 h at room temperature, and then poured into cold water. The organic product was extracted with hexane. The extract was dried over $MgSO_4$ and evaporated under reduced pressure. The residual liquid was subjected to column chromatography on silica gel with hexane to give a colorless oil, 4.0 g (72%), bp 118—119 °C (18 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (9H, s, $Si(CH_3)_3$), 0.09 (18H, s, $2\times Si(CH_3)_3$), 0.52 (1H, s, ≥CH), 1.80 (2H, s, $-CH_2-$). *Anal.* Calcd for $C_{11}H_{30}SSi_3$: C, 47.41; H, 10.85. Found: C, 47.54; H, 10.92.

α-(Trimethylsilyl)benzyl Trimethylsilylmethyl Sulfide (1c)——A solution of chloromethyltrimethylsilane (3.1 g, 25 mmol) in THF (5 ml) was added dropwise to a mixture of sodium α-(trimethylsilyl)benzylthiolate [prepared from α-(trimethylsilyl)benzylthiol[9] (4.9 g, 25 mmol), sodium hydride (35 mmol eq), and THF (30 ml)] with stirring in an ice-bath. After being stirred overnight at room temperature, the reaction mixture was poured into cold water and

TABLE III. Analytical Data for 2-Trimethylsilyltetrahydrothiophenes (4—7, 9)

Compound No.	mp °C (Recryst. solv.[a]) or bp °C (mmHg)	MS *m/z* (M^+)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
4	108—109 (IPE)	305	$C_{15}H_{19}NO_2SSi$	58.98	6.27	4.59
				(58.99	6.24	4.57)
5	98—99 (1)	243	$C_{10}H_{17}NO_2SSi$	49.33	7.04	5.76
				(49.78	6.90	5.40)
6	129—130 (IPE)	230	$C_9H_{14}O_3SSi$	46.93	6.13	
				(46.79	6.10)	
7a	123—124 (8)	276	$C_{11}H_{20}O_4SSi$	47.80	7.29	
				(47.68	7.21)	
7b	Oil[b]	276				
9a	80—82 (IPE)	210	$C_9H_{14}N_2SSi$	51.39	6.71	13.32
				(51.15	6.71	13.16)
9b	82—83 (IPE)	210	$C_9H_{14}N_2SSi$	51.39	6.71	13.32
				(51.11	6.66	13.20)

a) IPE=isopropyl ether. *b*) The amount of this product was too small for distillation.

TABLE IV. NMR Spectral Data for 2-Trimethylsilyltetrahydrothiophenes (4—7, 9)

Compound No.	^{1}H-NMR ($CDCl_3$) δ (J=Hz)	^{13}C-NMR ($CDCl_3$) δ
4	0.23 (9H, s, $Si(CH_3)_3$), 2.56 (1H, dd, J=1.2, 6.8, 2-CH), 2.88—3.16, 3.43—3.65 (4H, m, –CH–CH–CH_2–), 7.16—7.53 (5H, m, C_6H_5)	−1.2 (q), 38.4 (t), 41.4 (d), 50.2 (d), 51.0 (d), 126.6 (d), 128.8 (d), 129.2 (d), 132.3 (s), 176.6 (s), 177.4 (s)
5	0.21 (9H, s, $Si(CH_3)_3$), 2.46 (1H, dd, J=1.7, 5.3, 2-CH), 2.73—3.14, 3.30—3.59 (4H, –CH–CH–CH_2–), 2.98 (3H, s, NCH_3)	−1.4 (q), 25.0 (q), 37.7 (t), 40.7 (d), 50.1 (d), 51.0 (d), 177.3 (s), 178.1 (s)
6	0.23 (9H, s, $Si(CH_3)_3$), 2.50—3.80 (5H, m, –CH–CH–CH–CH_2–)	−1.6 (q), 38.7 (t), 41.6 (d), 50.8 (d), 51.6 (d), 173.5 (s), 174.3 (s)
7a	0.09 (9H, s, $Si(CH_3)_3$), 2.70—3.59 (5H, m, –CH–CH–CH–CH_2–), 3.70 (3H, s, OCH_3), 3.72 (3H, s, OCH_3)	−2.8 (q), 33.1 (t), 36.4 (d), 52.0 (d), 52.2 (d), 53.6 (q), 54.5 (q), 172.2 (s), 173.0 (s)
7b	0.10 (9H, s, $Si(CH_3)_3$), 2.71—3.60 (5H, m, –CH–CH–CH–CH_2–), 3.70 (3H, s, OCH_3), 3.71 (3H, s, OCH_3)	−1.8 (q), 33.2 (t), 35.7 (d), 50.9 (d), 51.9 (d), 52.2 (q), 52.8 (q), 172.3 (s), 172.9 (s)
9a	0.23 (9H, s, $Si(CH_3)_3$), 2.64—3.39 (5H, m, –CH–CH–CH–CH_2–)	−2.9 (q), 33.6 (t), 36.5 (d), 39.1 (d), 39.8 (d), 117.0 (2×s)
9b	0.27 (9H, s, $Si(CH_3)_3$), 2.85—3.68 (5H, m, –CH–CH–CH–CH_2–)	−2.2 (q), 34.0 (t), 35.0 (d), 39.2 (d), 40.9 (d), 117.3 (s), 118.0 (s)

extracted with benzene. The benzene solution was dried over $MgSO_4$ and concentrated uner reduced pressure. The residual oil was subjected to column chromatography on silica gel with hexane to give a colorless oil, 4.6 g (65%). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (18H, s, 2×$Si(CH_3)_3$), 1.64 (2H, s, –CH_2–), 3.26 (1H, s, ≡CH), 7.26 (5H, br s, C_6H_5). *Anal.* Calcd for $C_{14}H_{26}SSi_2$: C, 59.50; H, 9.27. Found: C, 59.72; H, 9.06.

General Procedure for Bromination of 1a—c——*N*-Bromosuccinimide (3.6 g, 20 mmol) was added in portions to a solution of **1a—c** (20 mmol) in carbon tetrachloride (40 ml) with stirring at room temperature. After the mixture had been stirred overnight, the precipitate was removed by filtration. The filtrate was concentrated under reduced pressure (followed by extraction with petroleum ether, if necessary) to give quantitatively enough pure product to be used in the subsequent reaction without further purification. **2a**: ^{1}H-NMR ($CDCl_3$) δ: 0.11 (9H, s, $Si(CH_3)_3$), 0.23 (9H, s, $Si(CH_3)_3$), 1.83 (1H, d, J=12.7 Hz, –CHH_A–), 2.31 (1H, d, J=12.7 Hz, –CHH_B–), 4.68 (1H, s, –CH). ^{13}C-NMR ($CDCl_3$) δ: −2.3 (q), −1.6 (q), 21.8 (t), 58.3 (d). **2b**: ^{1}H-NMR ($CDCl_3$) δ: 0.11 (9H, s, $Si(CH_3)_3$), 0.26 (18H, s,

2×Si(CH_3)$_3$), 2.01 (2H, s, -CH_2-). **2c**: ^{1}H-NMR ($CDCl_3$) δ: 0.09 (9H, s, Si(CH_3)$_3$), 0.17 (9H, s, Si(CH_3)$_3$), 1.85 (2H, brs, -CH_2-), 7.11—7.74 (5H, m, C_6H_5).

General Procedure for 1,3-Cycloaddition of 2a to Symmetrical Dipolarophiles (Table I)——A solution of **2a** (4.5 mmol) and a dipolarophile (3 mmol) in DMF (2 ml) was stirred for 2 h at 110 °C. The reaction solution was diluted with benzene (50 ml), washed with saturated aqueous sodium chloride, and then dried over $MgSO_4$. After removal of the benzene, the residue was subjected to column chromatography on silica gel with hexane–isopropyl ether (IPE) to give the corresponding cycloadduct. Yields and analytical and spectral data for the products are listed in Tables I, III, and IV.

In entry 5 (Table I), the ^{13}C-NMR spectrum of the product (**8**) revealed that it was a mixture of four isomers, two of which were identified as **7a** and **7b**. The GC-MS of each peak of **8** showed the same M^+ peak at 276 (*m/z*) and similar fragment patterns.

Desilylation of 4——A mixture of **4** (300 mg, 1 mmol) and cesium fluoride (150 mg, 1 mmol) in hexamethylphosphoramide (4 ml) containing a drop of water was stirred at 80 °C for 3 h. The reaction mixture was diluted with benzene and washed with water and then saturated aqueous sodium chloride. The benzene solution was dried over $MgSO_4$ and concentrated under reduced pressure to give a solid, 180 mg (77%). **10**: mp 152—153 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ: 2.88—3.64 (6H, m, ring protons), 7.15—7.56 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 36.5 (2×t), 48.5 (2×d), 126.5 (2×d), 128.7 (d), 129.1 (2×d), 132.5 (s), 177.1 (s). *Anal.* Calcd for $C_{12}H_{11}NO_2S$: C, 61.78; H, 4.75; N, 6.00. Found: C, 61.57; H, 4.71; N, 5.91.

General Procedure for 1,3-Cycloaddition of 2a—c to Unsymmetrical Dipolarophiles (Table II)——The reaction by using **2a**, **b**, or **c** (4.5 mmol), a dipolarophile (3 mmol), and DMF (2 ml) and the subsequent work-up were carried out in the same manner as above. The product was obtained as a mixture of two or three isomers, the structures of which were determined as follows.

In entry 7, regioisomers **11a** and **11b** were isolated in a pure state by careful column chromatography on silica gel with THF–hexane (1 : 20). A mixture of the isomer **11a** (100 mg) and Raney Ni (1 g as alloy) in MeOH (2 ml) was stirred for 1 h at room temperature. After a usual work-up, 2-methoxycarbonylbutyltrimethylsilane (**17a**) (85 mg) was obtained. Compound **17b** was similarly obtained from the isomer **11b** in a quantitative yield. **17a**: ^{1}H-NMR ($CDCl_3$) δ: 0.05 (9H, s, Si(CH_3)$_3$), 0.75 (1H, dd, J=6.0, 14.8 Hz, SiCHH_A-), 0.87 (3H, t, J=6.8 Hz, CH_3), 0.88 (1H, dd, J=8.8, 14.8 Hz, SiCHH_B-), 1.40—1.82 (2H, m, -CH_2-), 2.20—2.53 (1H, m, >CH), 3.66 (3H, s, OCH_3). High-resolution MS *m/z*: 188.1232 Calcd for $C_9H_{20}O_2Si$. Found: 188.1192. **17b**: ^{1}H-NMR ($CDCl_3$) δ: 0.05 (9H, s, Si(CH_3)$_3$), 0.88 (2H, t, J=6.9 Hz, SiCH_2-), 1.15 (3H, d, J=7.1 Hz, CH_3), 1.39—1.89 (2H, m, -CH_2-), 2.37 (1H, *ca.* sex. J=7.1 Hz, -CH), 3.68 (3H, s, OCH_3). High-resolution MS *m/z*: 188.1232 Calcd for $C_9H_{20}O_2Si$. Found: 188.1201. The ratio of **11a** to **11b** was determined by GC of the mixture of **17a** and **17b** obtained from the crude cycloadduct.

In entries 8 and 9, the crude cycloadduct was shown to be a mixture of regio- and stereoisomers by GC-MS and ^{13}C-NMR spectroscopy. The ratio of regioisomers was determined by GC of the product obtained by desulfurization of the cycloadduct in the same manner as above. High-resolution MS (**12a**+**12b**) *m/z*: 185.0695 Calcd for $C_8H_{15}NSSi$. Found: 185.0736. High-resolution MS (**13a**+**13b**) *m/z*: 290.1192 Calcd for $C_{12}H_{26}O_2SSi_2$. Found: 290.1161.

In entries 10 and 11, each stereoisomer was isolated in a pure state. **14a**: oil. ^{1}H-NMR ($CDCl_3$) δ: 0.01 (9H, s, Si(CH_3)$_3$), 1.58—1.89 (1H, complicated ddt, -CHH_A-), 2.16—2.49 (1H, com. ddt, -CHH_B-), 2.71—3.26 (2H, com. dt, ddd, SCH_2), 3.63 (1H, dd, J=4.4, 5.6 Hz, >CH), 3.75 (3H, s, OCH_3), 7.11—7.62 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −1.2 (s), 30.6 (t), 33.9 (t), 51.7 (q), 56.2 (s), 57.7 (d), 125.5 (d), 127.4 (2×d), 127.7 (2×d), 145.1 (s), 173.8 (s). High-resolution MS *m/z*: 294.1109 Calcd for $C_{15}H_{22}O_2SSi$. Found: 294.1142. **14b**: oil. ^{1}H-NMR ($CDCl_3$) δ: 0.09 (9H, s, Si(CH_3)$_3$), 2.12—2.39 (2H, m, -CH_2-), 2.69—3.20 (2H, com. ddd, dt, SCH_2-), 3.49 (1H, s, OCH_3), 3.52 (1H, t, J=6.4 Hz, >CH), 7.09—7.61 (6H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −1.7 (q), 29.7 (t), 33.5 (t), 51.3 (q), 54.0 (s), 55.2 (d), 125.1 (d), 127.5 (2×d), 128.2 (2×d), 142.1 (s), 172.8 (s). **15a**: mp 70—72 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.02 (9H, s, Si(CH_3)$_3$), 1.75—2.1 (1H, ddt, J=5.6, 7.3, 13.0 Hz, -CHH_A-), 2.07—2.42 (1H, ddt, 5.6, 7.3, 13.0 Hz, -CHH_B-), 2.61—2.86 (1H, ddt, J=5.6, 7.3, 10.7 Hz, S-CHH_A-), 2.86—2.98 (1H, dt, J=7.3, 10.7 Hz, S-CHH_B-), 3.56 (1H, t, J=5.6 Hz, >CH), 7.04—7.56 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −1.6 (q), 29.9 (t), 35.2 (t), 43.1 (d), 53.5 (s), 120.9 (s), 126.1 (s), 126.8 (2×s), 128.2 (2×d), 143.5 (s). *Anal.* Calcd for $C_{14}H_{19}NSSi$: C, 64.31; H, 7.32; N, 5.36. Found: C, 64.58; H, 7.21; N, 5.42. **15b**: mp 105—106 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.11 (9H, s, Si(CH_3)$_3$), 1.96—2.63 (2H, m, -CH_2-), 2.71—3.25 (2H, m, S-CH_2-), 3.41—3.56 (1H, dd, J=5.6, 7.8 Hz, >CH), 6.99—7.69 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −2.1 (q), 28.9 (t), 33.3 (t), 40.7 (d), 52.9 (s), 119.7 (s), 126.4 (d), 127.9 (2×d), 128.2 (2×d), 140.4 (s). *Anal.* Calcd for $C_{14}H_{19}NSSi$: C, 64.31; H, 7.32; N, 5.36. Found: C, 64.31; H, 7.27; N, 5.28.

In entry 12, the structures of **16a** and **16b** were determined on the basis of the NMR spectra and the ratio of **16a** to **16b** was determined by gas chromatographic analysis. **16a**: oil. ^{1}H-NMR ($CDCl_3$) δ: 0.19 (9H, s, Si(CH_3)$_3$), 2.70—3.36 (4H, m, -CH-CH-CH_2-), 3.65 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 7.07—7.74 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −1.15 (q), 31.2 (t), 48.8 (d), 52.0 (2×q), 53.7 (s), 59.1 (d), 126.3 (d), 127.9 (2×d), 128.4 (2×d), 140.1 (s), 170.9 (s), 173.3 (s). High-resolution MS *m/z*: 352.1164 Calcd for $C_{17}H_{24}O_4SSi$. Found: 352.1168. **16b**: oil. ^{1}H-NMR ($CDCl_3$) δ: 0.19 (9H, s, Si(CH_3)$_3$), 2.70—3.36 (4H, m, -CH-CH-CH_2-), 3.43 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 7.07—7.74 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: −1.5 (q), 32.3 (t), 51.6 (d), 51.9 (q), 52.2 (q), 53.6 (s), 60.6 (d), 125.8

(d), 127.6 (2 × d), 128.0 (2 × d), 144.4 (s), 171.7 (s), 173. 1 (s).

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Enzymatic Procedure for the Synthesis of 11-Deoxyprostaglandins

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This paper describes the enzymatic hydrolysis of the *trans*, *trans*(*meso*)-2-substituted 1,3-bis(acetoxymethyl)cyclopentanes (**4a**, **b**) to afford the monoacetates (**5a**, **b**) in high enantiomeric excess ((−)-**5a**: 96% ee, (−)-**5b**: >99% ee). The absolute stereochemistry of (−)-**5b** was unequivocally determined to be 1*R*,2*R*,3*S* by converting it to a known compound derived from (−)-limonen-10-ol. Starting with (−)-**5b**, a new route to the key intermediate ((−)-**20**) for the synthesis of 11-deoxyprostaglandins was established.

Keywords——prostaglandin; *meso* compound; enzymatic hydrolysis; 11-deoxyprostaglandin; *cis*-1,3-bis(acetoxymethyl)cyclopentane; 1,4-addition

The ability of enzymes to discriminate between enantiotopic groups of symmetrical substrates such as *meso* compounds is highly attractive for the synthesis of natural products. The hydrolytic enzymes have been widely investigated because many of them are capable of transforming *meso*-diesters into chiral monoesters. One of the synthetically useful hydrolytic enzymes is pig liver esterase (PLE). Hydrolysis of *meso* compounds such as monocyclic (three-, four-, and six-membered) ring derivatives with *cis*-1,2-dimethyl esters by commercially available PLE proceeds with high enantioselectivity.[1] However, *cis*-1,2- and *cis*-1,3-bis(methoxycarbonyl)cyclopentanes are hydrolyzed to the corresponding half esters with only 17%[1a] and 34% ee,[2] respectively. According to Laumen and Schneider,[1c] and Jones *et al.*,[3] enzymatic hydrolysis of *cis*-1,2-bis(acetoxymethyl)cyclopentane[4] with PLE or pig pancreatic lipase (PPL) affords the corresponding hydroxy ester in good yield and with fairly good optical purity (88—89% ee). This result is in marked contrast to that of the hydrolysis of the dimethyl esters.

We now report that the enzymatic hydrolysis of the *trans*, *trans*(*meso*)-2-substituted 1,3-bis(acetoxymethyl)cyclopentanes proceeds in a highly enantioselective manner to afford the chiral monoacetates, which provide the new route to the synthesis of a key intermediate[5] for 11-deoxyprostaglandins (PGs).

The *trans*, *trans*(*meso*)-2-substituted 1,3-bis(acetoxymethyl)cyclopentanes used as substrates were synthesized from 1,3-bis(methoxycarbonyl)-1-cyclopentene (**1**)[6] as follows. The 1,4-addition of **1** with $BuCuBF_3$, prepared from BuLi, CuI, and BF_3 etherate, afforded a mixture of the *trans*, *trans*(*meso*) compound (**2a**) and the *cis*, *trans* compound (**3a**) in 70% yield in the ratio of 2 (**2a**) to 1 (**3a**), and these products could be separated by flash chromatography. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the less polar fraction (**2a**), the signals of the C_1-H and C_3-H protons were observed as a broad singlet ($W_{1/2}$ = 14 Hz) at δ 2.47, and two methyl signals due to C_1- and C_3-$COOCH_3$ appeared as a singlet peak at δ 3.67, whereas those of the more polar fraction (**3a**) were observed as separate

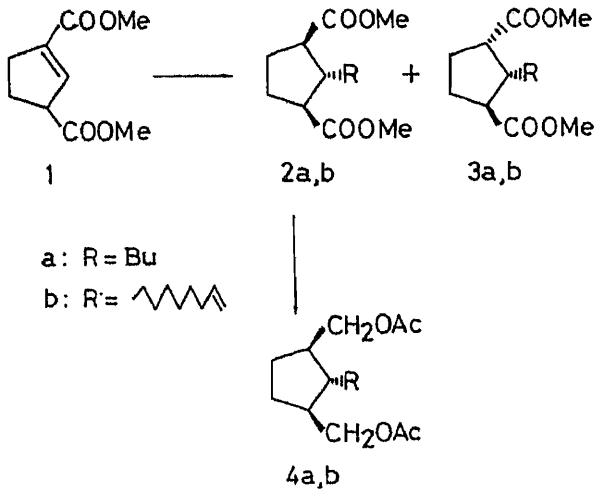
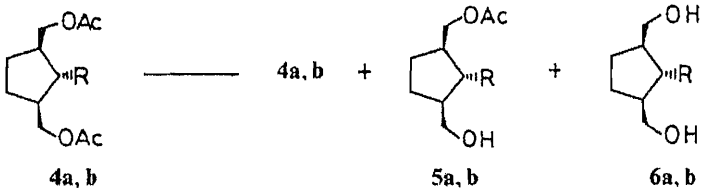


Chart 1

TABLE I. Enzymatic Hydrolysis of **4a, b**



Run	Compd.[a]	Enzyme (mg)	Chem. yield (%)			Reaction time (h)	5	
			4	5	6		% ee[b]	$[\alpha]_D^{20}$ ($CDCl_3$)
1	**4a**	PPL (26)	46	41	5	14	66	−1.39°
	4b	PPL (12)	39	44	4	20	63	−2.13°
2	**4a**	PLE (3.1)	68	4	16	1.5	23	+0.52°
	4b	PLE (1.5)	85	3	1	2.0	59	+2.10°
3	**4a**	RDL (14)	34	62	0	20	96	−2.31°
	4b	RDL (6.2)	18	69	5	68	>99	−3.68°
4	**4a**	—[c]						
	4b	CCL (24.8)	65	14	1	48	21	+1.20°

a) Scale of hydrolysis, **a** = 350, **b** = 200 mg. *b*) Optical purities were determined by examination of the 400 MHz ^{1}H-NMR spectra after conversion of the products to the esters of (+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA).[8] *c*) Not tested.

peaks (see Experimental). These data suggest that compound **2a** is a *meso* form. By assuming approach of the reagent from the less-hindered side of the sterically hindered double bond, the structure of **2a**[7] and **3a** were determined to be 1,2-*trans*-2,3-*trans*(*meso*) and 1,2-*cis*-2,3-*trans*, respectively. In a similar manner, **2b** and **3b** were synthesized from **1** and the cuprate reagent, prepared from 8-bromo-1-octene *via* 8-lithio-1-octene. Reduction of **2** with $LiAlH_4$ followed by treatment with Ac_2O/Py afforded the *trans*, *trans*(*meso*)-2-substituted 1,3-bis(acetoxymethyl)cyclopentanes (**4**).

Enzymatic hydrolyses of **4** were performed in 0.1 M phosphate buffer solution (pH 7) at 30 °C (Table I). Among the four tested enzymes, PLE, PPL, *Rhizopus delemar* lipase (RDL), and *Candida cylindracea* lipase (CCL), RDL afforded the (−)-monoacetate (**5**) in fairly good yield and with high enantiomeric excess ((−)-**5a**: 96% ee, (−)-**5b**: >99% ee). It is noteworthy

that the hydrolysis of **4b** with CCL (or PLE) afforded the (+)-monoacetate (**5b**), whereas PPL (or RDL) gave the (−)-monoacetate (**5b**), although the absolute values of specific rotation are not always identical (see Table I).

The absolute stereochemistry of the hydrolyzed product ((−)-**5b**) was unambiguously determined by converting it to a known compound (**12**)[9] derived from (−)-limonen-10-ol, *via* the removal of the unnecessary hydroxymethyl function. Upon catalytic hydrogenation with H_2/Pt in EtOH and subsequent Swern oxidation ($(COCl)_2$/dimethyl sulfoxide (DMSO)) in the presence of triethylamine, (−)-**5b** afforded the aldehyde (**8**) in 86% yield. The aldehyde function in **8** was smoothly removed by heating in benzonitrile in the presence of Wilkinson complex, and the acetoxy function was reductively transformed into the corresponding

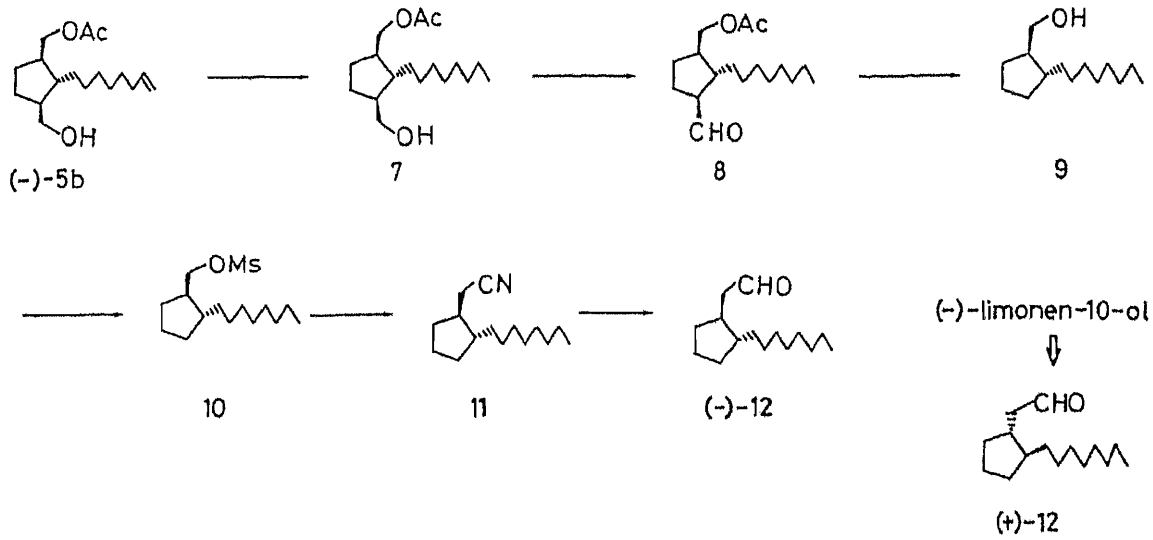


Chart 2

alcohol by treatment with $LiAlH_4$. Mesylation of the alcohol (**9**) with methanesulfonyl chloride/Py followed by substitution with NaCN/DMSO afforded the nitrile (**11**) in 78% yield from **9**. By reduction with diisobutylaluminum hydride (DIBAL-H) and subsequent hydrolysis with 10% H_2SO_4, **11** was converted into the aldehyde ((−)-**12**). The aldehyde (−)-**12** was identical with the standard sample (+)-**12**[9] in terms of the ^{1}H-NMR spectrum, the infrared (IR) spectrum and thin-layer chromatographic (TLC) behavior, except for the sign of specific rotation. Thus, the absolute stereochemistry of (−)-**5b** was unequivocally determined to be 1*R*,2*R*,3*S*.

In the synthesis of 11-deoxy PGs, the absolute stereochemistry of (−)-**5b** suggests that the C_1-CH_2OCOCH_3 group in (−)-**5b** should be converted to the ketone in the five-membered ring. Conversion of (−)-**5b** to the intermediate ((−)-**20**) for 11-deoxy PGs proceeded smoothly with retention of the stereochemistry at C_2 and C_3 in (−)-**5b**. The alcohol function in (−)-**5b** was first protected with 2-methoxyethoxymethyl chloride (MEM chloride)/*N*,*N*-diisopropylethylamine to afford the MEM ether (**13**), and the acetoxy function was converted to the corresponding alcohol with $LiAlH_4$. In order to facilitate the elimination of the alcohol to form the double bond, the alcohol (**14**) was subjected to mesylation with methanesulfonyl chloride/Et_3N and subsequent substitution with NaI in hexamethylphosphoramide (HMPA). Dehydroiodination of the iodide (**16**) with *tert*-BuOK in DMSO at room temperature afforded the diene (**17**) in 68% yield from (−)-**5b**. Concurrent cleavage of the two double bonds in **17** with ozone in CH_2Cl_2, followed by treatment with Zn/AcOH, gave the keto-aldehyde (**18**) in 76% yield. By Jones oxidation of the aldehyde function to the corresponding acid, followed by treatment with CH_2N_2, **18** was converted to

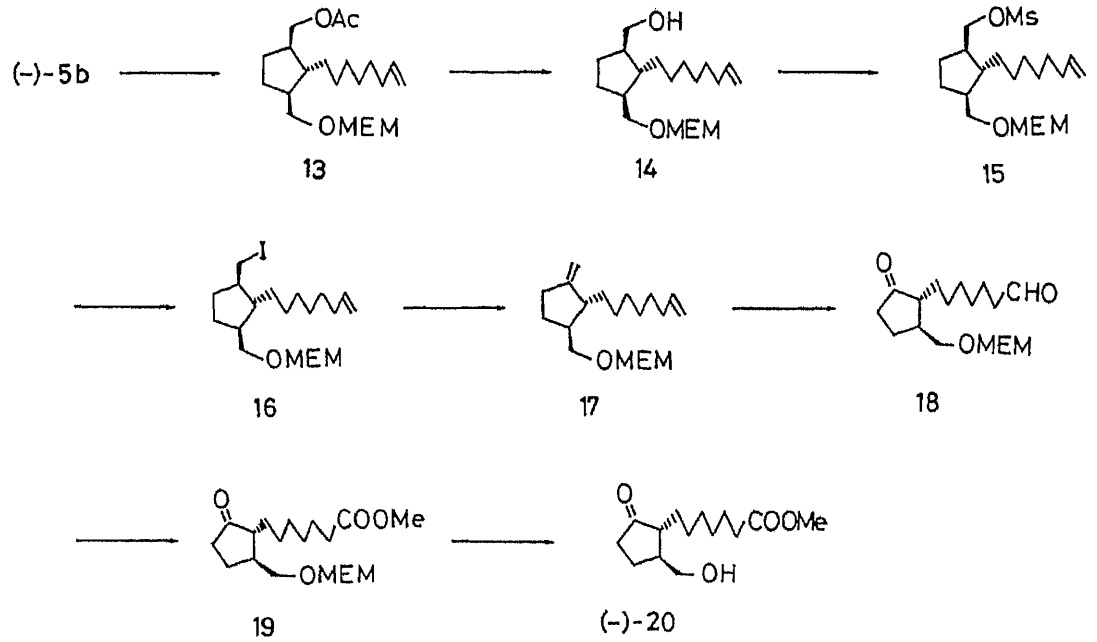


Chart 3

the ester (**19**), and the MEM function was removed by treatment with $TiCl_4$ to afford the intermediate ((−)-**20**),[10] whose ^{1}H-NMR spectrum and IR spectrum were identical with those of a standard sample.[11]

Thus, this synthetic method using enzymatic hydrolysis of *meso* compounds seems to be useful for the synthesis of the optically active target (−)-**20**.

Experimental

IR spectra were measured with a JASCO A-202 spectrometer. ^{1}H-NMR spectra were measured on a JEOL-PS-100 spectrometer. Mass spectra (MS) were taken on a JEOL JMS-D 300 spectrometer. Optical rotations were measured on a JASCO DIP-4 polarimeter. For column chromatography and flash chromatography, silica gel 70–230 mesh (Merck, Kieselgel 60) and 230–400 mesh (Nakarai Chemicals, Silica gel 60) were used, respectively. TLC was performed on Silica gel 60 F_{254} plates (Merck). For enzymatic hydrolysis, PLE (Sigma, type I), PPL (Sigma, type II (pfs)), CCL (Sigma, type VII (pfs)) and RDL (Seikagaku Kogyo, fine grade) were used. All organic solvent extracts were washed with satd. brine and dried over anhydrous sodium sulfate.

(*r*-1,*t*-2,*c*-3)-1,3-Bis(methoxycarbonyl)-2-butylcyclopentane (2a) and (*r*-1,*t*-2,*t*-3)-1,3-Bis(methoxycarbonyl)-2-butylcyclopentane (3a)——BuLi (1.5 M in hexane, 10.9 ml) solution was added dropwise with stirring to a suspension of CuI (3.11 g) in ether (30 ml) at −40 °C under an Ar atmosphere. After 0.5 h, BF_3 etherate (2.12 ml) was added dropwise at −78 °C. The mixture was stirred for 0.5 h, the diester (**1**, 1.003 g) in ether (3 ml) was added dropwise, and the whole was stirred for 1 h at −78 °C, then for 1 h at −10 °C. The reaction mixture was diluted with satd. NH_4Cl, and extracted with ether. The ether extract was washed and dried. The solvent was removed *in vacuo* to afford an oily residue, which was roughly chromatographed on silica gel (10 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded a mixture of **2a** and **3a** (1.10 g), which was subjected to flash chromatography using 2% AcOEt in hexane (v/v). **2a** (702 mg, 53%) and **3a** (344 mg, 26%) were obtained from the less polar fraction and the more polar fraction, respectively. **2a**: IR (neat): 1735, 1430, 1155 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6 Hz, CH_3), 2.47 (2H, br s, CHCO×2), 3.67 (6H, s, $COOCH_3$×2). MS *m/z*: 242 (M^+), 211, 102. **3a**: IR (neat): 1735, 1435, 1160 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, J=6 Hz, CH_3), 2.71 (1H, m, C_3-H),[12] 3.00 (1H, m, C_1-H), 3.64 (3H, s, $COOCH_3$), 3.67 (3H, s, $COOCH_3$). MS *m/z*: 242 (M^+), 210, 182.

(*r*-1,*t*-2,*c*-3)-1,3-Bis(methoxycarbonyl)-2-(7-octenyl)cyclopentane (2b) and (*r*-1,*t*-2,*t*-3)-1,3-Bis(methoxycarbonyl)-2-(7-octenyl)cyclopentane (3b)——8-Bromo-1-octene (5.00 g) in ether (20 ml) was added dropwise with stirring to a suspension of lithium (high sodium, 25% (w/w) dispersion in mineral oil, 2.45 g) in ether (50 ml) in the presence of 1,2-dibromoethane (0.2 ml) at −40 °C under an Ar atmosphere. The resulting 8-lithio-1-octene solution was added dropwise to a stirred solution of CuI (4.29 g) in ether (50 ml) at −40 °C. After 0.5 h, BF_3 etherate (2.93 ml) was added dropwise at −78 °C, and the mixture was stirred for 0.5 h, then the diester (**1**, 1.381 g) in ether (5 ml) was

added dropwise. Stirring was continued for 3 h at −78 °C, and for 1 h at 0 °C, and work-up similar to that described for the synthesis of **2a** and **3a** afforded **2b** (1.40 g, 63%) and **3b** (515 mg, 23%) as colorless oils. **2b**: IR (neat): 1740, 1640, 910 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.46 (2H, br s, CHCO × 2), 3.67 (6H, s, $COOCH_3$ × 2), 4.84—5.15 (2H, m, $=CH_2$), 5.80 (1H, m, CH=). MS *m/z*: 297 ($M^+ + 1$), 296 (M^+), 265, 232. **3b**: IR (neat): 1735, 1640, 910 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.70 (1H, m, C_3-H),[12] 3.02 (1H, m, C_1-H), 3.64 (3H, s, $COOCH_3$), 3.66 (3H, s, $COOCH_3$), 4.82—5.10 (2H, m, $=CH_2$), 5.80 (1H, m, CH=). MS *m/z*: 297 ($M^+ + 1$), 296 (M^+), 264, 232.

(*r*-1,*t*-2,*c*-3)-1,3-Bis(acetoxymethyl)-2-butylcyclopentane (4a) and (*r*-1,*t*-2,*c*-3)-1,3-Bis(acetoxymethyl)-2-(7-octenyl)cyclopentane (4b)——The diester **2a** (484 mg) in ether (5 ml) was added dropwise with stirring to a suspension of $LiAlH_4$ (114 mg) in ether (5 ml) at 0 °C. After 2 h, the reaction mixture was subjected to usual work-up to afford the crude diol (310 mg), which was acetylated in a usual manner. The crude diacetate (450 mg) was purified by column chromatography on silica gel (10 g), and the fraction eluted with 20% AcOEt in hexane (v/v) afforded **4a** (352 mg, 92%) as a colorless oil. Similarly, **4b** was obtained in 94% yield from **2b**. **4a**: IR (neat): 1740, 1240, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, t, J=6 Hz), 2.06 (6H, s, $COCH_3$ × 2). MS *m/z*: 270 (M^+), 223, 211. **4b**: IR (neat): 1735, 1635, 1230 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (6H, s, $COCH_3$ × 2), 4.82—5.12 (2H, m, $=CH_2$), 5.78 (1H, m, CH=). MS *m/z*: 325 ($M^+ + 1$), 264, 222.

General Method for the Enzymatic Hydrolysis. A Typical Example: Hydrolysis of 4b to (1*R*,2*R*,3*S*)-1-Acetoxymethyl-2-(7-octenyl)-3-hydroxymethylcyclopentane (5b) with RDL——Compound **4b** (200 mg) in acetone (1 ml) and RDL (6.2 mg) were successively added with stirring to 0.1 M phosphate buffer (10 ml, pH 7). The whole was stirred at 30 °C, and hydrolysis was terminated by extracting the mixture with AcOEt when a spot of the diol (**6b**) appeared on TLC. The AcOEt extract was washed and dried, then concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel (6 g). The fraction eluted with 3% AcOEt in hexane (v/v) afforded (−)-**5b** (120 mg, 69%) as a colorless oil. $[\alpha]_D^{20}$ −3.68° (*c*=2.18, $CHCl_3$). IR (neat): 3400, 1735, 1635, 1240 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s, $COCH_3$), 3.42 (1H, dd, J=11, 7 Hz, CH_AH_B-OH), 3.82 (1H, dd, J=11, 5 Hz, CH_AH_B-OH), 3.88 (1H, dd, J=10.5, 6.5 Hz, CH_AH_B-OAc), 4.04 (1H, dd, J=10.5, 5.5 Hz, CH_AH_B-OAc). MS *m/z*: 283 ($M^+ + 1$), 252, 234. *Anal.* Calcd for $C_{17}H_{30}O_3$: C, 72.30; H, 10.71. Found: C, 72.43; H, 10.77.

(1*R*,2*R*,3*S*)-1-Acetoxymethyl-3-hydroxymethyl-2-octylcyclopentane (7)——A solution of (−)-**5b** (638 mg) in EtOH (50 ml) was hydrogenated in the presence of PtO_2 (100 mg) under an H_2 atmosphere for 5 h. The catalyst was filtered off, and the filtrate was concentrated *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (13 g). The fraction eluted with 3% AcOEt in hexane (v/v) afforded **7** (613 mg, 95%) as a colorless oil. $[\alpha]_D^{17}$ −2.73° (*c*=1.21, $CHCl_3$). IR (neat): 3400, 1735, 1235 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=6 Hz, CH_3), 2.05 (3H, s, $COCH_3$), 3.28—3.72 (2H, m, CH_2OH), 3.76—4.24 (2H, m, CH_2OAc). MS *m/z*: 285 ($M^+ + 1$), 254, 241.

(1*R*,2*S*,3*S*)-1-Acetoxymethyl-3-formyl-2-octylcyclopentane (8)——DMSO (375 mg) in CH_2Cl_2 (1 ml) was added to a stirred solution of $(COCl)_2$ (305 mg) in CH_2Cl_2 (3 ml) at −60 °C under an Ar atmosphere. After 5 min, **7** (620 mg) in CH_2Cl_2 (2 ml) was added, and the whole was stirred for 15 min, then triethylamine (1.11 g) in CH_2Cl_2 (5 ml) was added at −60 °C. After 20 min, the reaction mixture was warmed to room temperature, diluted with brine (20 ml), and then extracted with AcOEt. The AcOEt extract was successively washed with 3% aq. HCl, 5% aq. $NaHCO_3$, and brine, then dried. Removal of the solvent *in vacuo* gave an oily residue, which was purified by column chromatography on silica gel (15 g). The fraction eluted with 2% AcOEt in hexane (v/v) afforded **8** (559 mg, 91%) as a colorless oil. $[\alpha]_D^{17}$ −19.6° (*c*=1.04, $CHCl_3$). IR (neat): 2700, 1735, 1720, 1235 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s, $COCH_3$), 2.48 (1H, m, CH-CHO), 9.61 (1H, d, J=2 Hz, CHO). MS *m/z*: 281 ($M^+ - 1$), 253, 237, 210.

(1*R*,2*R*)-1-Hydroxymethyl-2-octylcyclopentane (9)——A mixture of **8** (577 mg) and Wilkinson complex (2.08 g) in benzonitrile (25 ml) was heated with stirring for 5 h at 160 °C. The solvent was removed *in vacuo*, and EtOH (20 ml) was added to yield a precipitate, which was filtered off. The filtrate was concentrated *in vacuo* to afford an oily residue which was roughly chromatographed on silica gel (20 g). The fraction eluted with 20% AcOEt in hexane (v/v) afforded the monoacetate contaminated with triphenylphosphine. The crude acetate (250 mg) in ther (5 ml) was added dropwise with stirring to a suspension of $LiAlH_4$ (56 mg) in ether (5 ml) at room temperature, and the whole was stirred for 1 h. The usual work-up afforded a crude oil (183 mg), which was purified by column chromatography on silica gel (20 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded **9** (185 mg) as a colorless oil. $[\alpha]_{546}^{17}$ −55.7°. (*c*=1.12, $CHCl_3$). IR (neat): 3310, 1020 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6 Hz, CH_3), 3.30—3.76 (2H, m, CH_2OH). MS *m/z*: 211 ($M^+ - 1$), 194, 179.

(1*R*,2*R*)-1-Mesyloxymethyl-2-octylcyclopentane (10)——Methanesulfonyl chloride (142 mg) in benzene (2 ml) was added dropwise to a stirred solution of **9** (175 mg) in benzene (5 ml) in the presence of Et_3N (167 mg) at room temperature. After 2 h, the reaction mixture was diluted with 5% aq. HCl (10 ml), and extracted with AcOEt. The AcOEt extract was successively washed with 5% aq. $NaHCO_3$, 5% aq. HCl, and brine, then dried. Removal of the solvent *in vacuo* afforded an oily residue, which was subjected to column chromatography on silica gel (10 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded **10** (207 mg, 87%) as a colorless oil. $[\alpha]_D^{22}$ −39.1° (*c*=1.03, $CHCl_3$). IR (neat): 1355, 1170, 945 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=6 Hz, CH_3), 3.00 (3H, s, SO_2CH_3), 3.93—4.32 (2H, m, CH_2O).

(1*R*,2*R*)-1-Cyanomethyl-2-octylcyclopentane (11)——A mixture of **10** (83 mg) and NaCN (20 mg) in DMSO (5 ml)

was heated with stirring at 100 °C. After 4 h, the reaction mixture was diluted with H_2O (10 ml), and extracted with AcOEt. The AcOEt extract was washed, and dried. The solvent was removed *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (3 g). The fraction eluted with 5% AcOEt in hexane (v/v) gave **11** (57 mg) as a colorless oil. $[\alpha]_D^{25}$ −62.7° (*c*=0.81, $CHCl_3$). IR (neat): 2240, 1460 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6 Hz, CH_3), 2.10—2.64 (2H, m, CH_2CN). MS *m/z*: 220 (M^+-1), 206, 192.

(1*R*,2*R*)-1-Formylmethyl-2-octylcyclopentane (12)——DIBAL-H (tetrahydrofuran solution) (0.4 ml) was added dropwise to a stirred solution of **11** (45 mg) in hexane (5 ml) at −70 °C. The whole was stirred for 0.5 h at −60 °C, and for 5 h at 0 °C, then diluted with 10% H_2SO_4 (10 ml), and extracted with AcOEt. The AcOEt extract was washed, and dried, then concentrated *in vacuo* to leave an oily residue, which was subjected to column chromatography on silica gel (3 g). The fraction eluted with 10% ether in hexane (v/v) afforded (−)-**12** (19 mg) as a colorless oil. $[\alpha]_D^{20}$ −47.6° (*c*=0.84, $CHCl_3$) (the reported value: +43.6°).[8] IR (neat): 2710, 1730, 1030 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=6 Hz, CH_3), 2.25 (1H, ddd, *J*=16, 8, 2.5 Hz, CH_AH_B–CHO), 2.59 (1H, ddd, *J*=16, 6.5, 2.5 Hz, CH_AH_B–CHO), 9.77 (1H, t, *J*=2.5 Hz, CHO). MS *m/z*: 225 (M^++1), 224 (M^+), 206, 193. *Anal.* Calcd for $C_{15}H_{28}O$: C, 80.29; H, 12.58. Found: C, 80.10; H, 12.71.

(1*R*,2*R*,3*S*)-1-Acetoxymethyl-3-(2-methoxyethoxymethoxymethyl)-2-(7-octenyl)cyclopentane (13)——MEM chloride (618 mg) in CH_2Cl_2 (3 ml) was added dropwise to a stirred solution of (−)-**5b** (350 mg) in the presence of *N*,*N*-diisopropylethylamine (650 mg). After 3 h, the reaction mixture was diluted with brine (15 ml), and extracted with AcOEt. The AcOEt extract was washed, and dried, then removal of the solvent *in vacuo* afforded an oily residue, which was chromatographed on silica gel (30 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded **13** (410 mg, 89%) as a colorless oil. $[\alpha]_D^{27}$ +5.56° (*c*=2.16, $CHCl_3$). IR (neat): 1735, 1635, 1105 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s, $COCH_3$), 3.39 (3H, s, OCH_3), 4.70 (2H, s, OCH_2O). MS *m/z*: 371 (M^++1), 370 (M^+), 339, 281.

(1*R*,2*S*,3*S*)-1-Hydroxymethyl-3-(2-methoxyethoxymethoxymethyl)-2-(7-octenyl)cyclopentane (14)——Compound **13** was added dropwise with stirring to a suspension of $LiAlH_4$ (65 mg) in ether (20 ml) at 0 °C, and stirred for 4 h at room temperature. The work-up afforded a crude oil (449 mg), which was subjected to column chromatography on silica gel (15 g). The fraction eluted with 30% AcOEt in hexane (v/v) afforded **14** (347 mg, 93%) as a colorless oil. $[\alpha]_D^{28}$ +7.50° (*c*=2.10, $CHCl_3$). IR (neat): 3425, 1640, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.64 (1H, s, OH), 3.38 (3H, s, OCH_3), 4.84—5.12 (2H, m, $=CH_2$), 5.82 (1H, m, CH=). MS *m/z*: 329 (M^++1), 252, 239, 221.

(1*R*,2*R*,3*S*)-1-Mesyloxymethyl-3-(2-methoxyethoxymethoxymethyl)-2-(7-octenyl)cyclopentane (15)——In a manner similar to that described for the preparation of **10**, **14** (215 mg) afforded **15** (261 mg, 98%) as a colorless oil. $[\alpha]_D^{28}$ +1.26° (*c*=2.11, $CHCl_3$). IR (neat): 1635, 1355, 1175, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.00 (3H, s, SO_2CH_3), 3.39 (3H, s, OCH_3), 4.84—5.10 (2H, m, $=CH_2$), 5.82 (1H, m, CH=). MS *m/z*: 407 (M^++1), 376, 361, 299.

(1*R*,2*R*,3*S*)-1-Iodomethyl-3-(2-methoxyethoxymethoxymethyl)-2-(7-octenyl)cyclopentane (16)——A mixture of **15** (721 mg) and NaI (532 mg) in HMPA (3 ml) was heated under reflux for 6 h, diluted with brine (10 ml), and extracted with AcOEt. The AcOEt extract was washed, and dried, then concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel (15 g). The fraction eluted with 20% AcOEt in hexane (v/v) gave **16** (692 mg, 89%) as a yellow oil. $[\alpha]_D^{25}$ −12.12° (*c*=2.00, $CHCl_3$). IR (neat): 1635, 1110, 905 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.38 (3H, s, OCH_3), 4.82—5.12 (2H, m, $=CH_2$), 5.80 (1H, m, CH=). MS *m/z*: 439 (M^++1), 407, 362, 331.

(2*R*,3*S*)-1-Methylene-3-(2-methoxyethoxymethoxymethyl)-2-(7-octenyl)cyclopentane (17)——A mixture of **16** (240 mg) and *tert*-BuOK (123 mg) in DMSO (5 ml) was stirred for 10 min at room temperature, diluted with 3% aq. HCl (10 ml), and extracted with ether. The ether extract was washed, dried, and then concentrated *in vacuo* to afford an oily residue, which was chromatographed on silica gel (5 g). The fraction eluted with 20% ether in hexane (v/v) afforded **17** (160 mg, 94%) as a colorless oil. $[\alpha]_D^{25}$ −1.54° (*c*=2.26, $CHCl_3$). IR (neat): 1650, 1640, 1110, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.68—5.12 (4H, m, $=CH_2\times2$), 4.70 (2H, s, OCH_2O), 5.82 (1H, m, CH=). MS *m/z*: 311 (M^++1), 234, 220. *Anal.* Calcd for $C_{19}H_{34}O_3$: C, 73.50; H, 11.04. Found: C, 73.38; H, 11.19.

(2*R*,3*S*)-2-(6-Formylhexyl)-3-(2-methoxyethoxymethoxymethyl)-1-cyclopentanone (18)——Ozone gas was bubbled into a solution of **17** (155 mg) in CH_2Cl_2 (15 ml) at −78 °C, and the reaction was monitored by TLC. The resulting ozonide was decomposed with Zn powder (460 mg) and AcOH (3 ml) at 10 to 20 °C. The Zn powder was filtered off, and the filtrate was concentrated *in vacuo*, diluted with brine (20 ml), and then extracted with AcOEt. The AcOEt extract was washed, and dried. The solvent was removed *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (2 g). The fraction eluted with 30% AcOEt in hexane (v/v) afforded **18** (108 mg, 68%) as a colorless oil. $[\alpha]_D^{26}$ −20.87° (*c*=2.18, $CHCl_3$). IR (neat): 2720, 1735, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.40 (3H, s, OCH_3), 4.72 (2H, s, OCH_2O), 9.76 (1H, t, *J*=2 Hz, CHO). MS *m/z*: 315 (M^++1), 314 (M^+), 299, 269.

(2*R*,3*S*)-2-(6-Methoxycarbonylhexyl)-3-(2-methoxyethoxymethoxymethyl)-1-cyclopentanone (19)——Jones reagent (0.2 ml) was added dropwise to a stirred solution of **18** (92 mg) in acetone (10 ml) at −10 °C. After 0.5 h, the excess reagent was decomposed with isopropanol, and the reaction mixture was diluted with brine (5 ml), then extracted with ether. The ether extract was dried, and esterified with CH_2N_2 in the usual manner. Removal of the solvent *in vacuo* afforded an oily residue, which was subjected to column chromatography on silica gel (2 g). The fraction eluted with 20% AcOEt in hexane (v/v) gave **19** (86 mg, 85%) as a colorless oil. $[\alpha]_D^{24}$ −18.98° (*c*=2.06, $CHCl_3$). IR (neat): 1735, 1170, 1110, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.38 (3H, s, OCH_3), 3.70 (3H, s, $COOCH_3$),

4.72 (2H, s, OCH_2O). MS *m/z*: 345 ($M^+ + 1$), 313 ($M^+ - OCH_3$), 299, 269.

(2*R*,3*S*)-3-Hydroxymethyl-2-(6-methoxycarbonylhexyl)-1-cyclopentanone (20)——$TiCl_4$ (142 mg) in CH_2Cl_2 (1 ml) was added dropwise to a stirred solution of **19** (86 mg) in CH_2Cl_2 (2 ml) at 0 °C, and the whole was stirred for 0.5 h. The reaction mixture was diluted with brine (10 ml), and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed, and dried, then concentrated *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (3 g). The fraction eluted with 25% AcOEt in hexane (v/v) afforded (−)-**20** (39 mg, 61%) as a colorless oil. Compound (−)-**20** was identical with the (±)-standard sample[11] in terms of the ^{1}H-NMR and the IR spectra. $[\alpha]_D^{23}$ −39.57° (*c* = 1.87, $CHCl_3$). IR (neat): 3470, 1740, 1170, 1045 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.10—2.64 (18H, m, $CH_2 \times 8$, $CH \times 2$), 1.76 (1H, s, OH), 3.36—3.92 (2H, m, CH_2O), 3.65 (3H, s, $COOCH_3$). MS *m/z*: 257 ($M^+ + 1$), 256 (M^+), 238, 225. *Anal.* Calcd for $C_{14}H_{24}O_4$: C, 65.59; H, 9.44. Found: C, 65.65; H, 9.59.

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8) Optical purities of **5a, b** were determined by 400 MHz ^{1}H-NMR ($CDCl_3$) spectroscopy after conversion to the corresponding (+)-MTPA esters. In the spectrum of the (+)-MTPA ester of (±)-**5a**, methylene protons ($-CH_2O$-MTPA) were observed as two pairs of double-doublet signals originating from two diastereomers at δ 4.107 (dd, J = 10.8, 7.9 Hz), 4.182 (dd, J = 10.8, 7.7 Hz), 4.278 (dd, J = 10.8, 5.1 Hz), 4.340 (dd, J = 10.8, 5.1 Hz). The methylene protons in the (+)-MTPA ester of (−)-**5a** (Table I, run 3) were observed as a major pair of double-double signals at δ 4.182 and 4.278, and the other pair of double-doublet signals at δ 4.107 and 4.340 was remarkably reduced. On the basis of the relative intensity of these two pairs, the optical purity of (−)-**5a** was calculated. By using a similar technique, the optical purity of (−)-**5b** was determined. (+)-MTPA ester of (±)-**5b**: δ 4.104 (dd, J = 10.9, 7.9 Hz), 4.179 (dd, J = 10.8, 7.6 Hz), 4.274 (dd, J = 10.8, 5.1 Hz), 4.336 (dd, J = 10.9, 5.1 Hz). (+)-MTPA ester of (−)-**5b**: δ 4.179 and 4.274.
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10) A route to (±)-11-deoxy PGs from the corresponding (±)-aldehyde was established by Greane and Crabbe.[5]
11) The (±)-standard sample was synthesized by an alternative method.[13]
12) The assignment of C_1- and C_3-H in **3a, b** was based on: H. B. Kagan, "Stereochemistry," Vol. 1, George Thieme Publishers, Stuttgart, 1977, p. 92.
13) K. Sakai, J. Ide, O. Oda, and N. Nakamura, *Tetrahedron Lett.*, **1972**, 1287.

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Trachelosperosides, Glycosides of 19α-Hydroxyursane-Type Triterpenoids from *Trachelospermum asiaticum* (Trachelospermum. III)[1]

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From the whole plant of *Trachelospermum asiaticum*, sixteen triterpenoid glycosides were isolated. Eight of them, including two known glucosides, suavissimoside R1 and the 3,28-bis-*O*-glucoside of 19α-hydroxyasiatic acid, were determined to be 28-*O*- or 3,28-bis-*O*-glucosides of 19α-hydroxyursolic acid derivatives, except for one 28-*O*-xylosyl-glucoside. The most major compound was the 28-*O*-glucoside of 2α,3β,19α,23,24-pentahydroxyurs-12-en-28-oic acid.

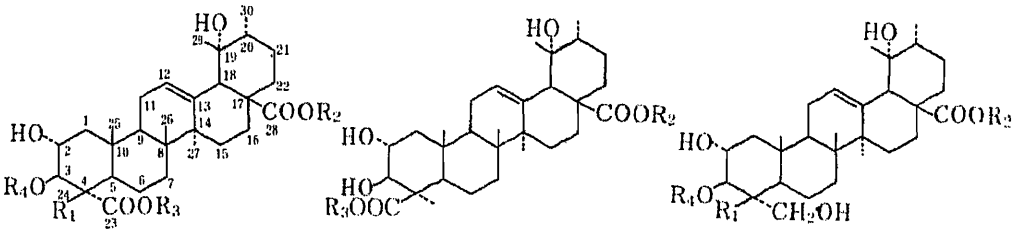
Keywords——*Trachelospermum asiaticum*; Apocynaceae; 19α-hydroxyursolic acid derivative; triterpenoid 28-*O*-β-D-glucopyranosyl ester; trachelosperoside; trachelosperogenin; 2α,3β,19α-trihydroxyurs-12-ene-24,28-dioic acid; 2α,3β,19α,23,24-pentahydroxyurs-12-en-28-oic acid; 2α,3β,19α,24-tetrahydroxyurs-12-ene-23,28-dioic acid; suavissimoside R1

In the course of our studies on the constituents of *Trachelospermum asiaticum* NAKAI (Japanese name: Teikà-kazura) (Apocynaceae), we described the isolation and structure determination of teikaside A, a bisdesmosidic pregnane glycoside,[2] and several lignan glucosides including trachelosiaside, a matairesinol C-glucoside.[1] This paper deals with the isolation and structure determination of polyoxygenated ursolic acid glycosides (I—VIII), as well as the isolation of eight glycosides of oleanane-type triterpenoids (IX—XVI).[3]

The polar fraction of the methanol percolate showed several blue or violet spots on thin layer chromatography (TLC) after spraying dil. H_2SO_4 reagent and heating, as well as the dark spots due to the lignan glucosides. The fraction was subjected to ordinary and reversed-phase column chromatographies and occasionally high-performance liquid chromatography (HPLC) on a preparative scale. Eight glycosides of ursane-type triterpenoids thus isolated are presented in Chart 1.

Compound I, mp 260—265 °C (dec.), $C_{36}H_{56}O_{12}$, afforded a monomethyl ester (I-1) on methylation with CH_2N_2. On enzymic hydrolysis, I-1 gave D-glucose and a monomethyl ester of the aglycone (I-2). From the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum, I-2 was suggested to retain an ursane framework with a 19α-hydroxyl group, two secondary hydroxyl groups which were assignable to 2α and 3β, and one olefinic linkage at C-12. The presence of two carboxyl groups was also suggested on the basis of the molecular formula of I, and two carbonyl carbons at δ 176.9 and 180.1 in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum. In a comparison of the ^{13}C-NMR spectrum of I with that of suavissimoside R1, the 28-*O*-β-D-glucopyranosyl ester of 2α,3β,19α-trihydroxyurs-12-ene-23,28-dioic acid,[4] carbon signals of the two compounds were in good agreement, and finally the structure was confirmed by direct comparison with an authentic sample.

Compound II was isolated as its monomethyl ester (II-1) from the mixture with oleanane glucoside (XVI) after methylation followed by HPLC. Based on the molecular peak at *m/z* 879 ($C_{43}H_{68}O_{17}$+Na) in the fast atom bombardment (FAB) mass spectrum (MS), two



	R_1	R_2	R_3	R_4
I	CH_3	Glc	H	H
I-1	CH_3	Glc	CH_3	H
I-2	CH_3	H	CH_3	H
II	CH_3	Glc	H	Glc
II-1	CH_3	Glc	CH_3	Glc
VI	CH_2OH	Glc	H	H
VI-1	CH_2OH	Glc	CH_3	H
VI-2	CH_2OH	H	H	H
VI-3	CH_2OH	CH_3	CH_3	H
VII	CH_2OH	Glc	H	Glc
VII-1	CH_2OH	Glc	CH_3	Glc
VII-2	CH_2OH	H	H	Glc
VII-3	CH_2OH	CH_3	CH_3	Glc

	R_2	R_3
III	Glc	H
III-1	Glc	CH_3
III-2	H	CH_3

	R_1	R_2	R_4
IV	CH_2OH	Glc	H
IV-1	CH_2OH	H	H
IV-2	CH_2OH	CH_3	H
V	CH_2OH	Glc^2-Xyl	H
VIII	CH_3	Glc	Glc
VIII-1	CH_3	H	Glc

Chart 1

anomeric protons at δ4.88 and 6.29, and the ^{13}C-NMR signals, II was considered to be a bioside. In the ^{13}C-NMR spectrum of II-1, most of the carbon signals coincided with those of I, except for C-3, which showed a downfield shift on glucosylation, and additional carbons of one glucose moiety. Therefore, II was considered to be the 3-*O*-glucopyranoside of suavissimoside R1.

Compound III was obtained as a solid and gave the same molecular formula as I. In the ^{13}C-NMR spectrum of III, the signals due to the sugar moiety and the aglycone showed similar chemical shifts to those of I, except for the carbons of ring A (C-3, C-4, and C-5) and those attached to ring A (C-23, C-24, and C-25). As in the case of I, III afforded a monomethyl ester (III-1) (*m/z* 717, $C_{37}H_{58}O_{12}$+Na) with CH_2N_2, indicating the presence of one free carboxyl group. Enzymic hydrolysis of III-1 provided a monomethyl ester of the aglycone as a solid (III-2) (*m/z* 532.341, $C_{31}H_{48}O_7$). The ^{13}C-NMR spectrum of III showed two carboxyl carbons, suggesting the aglycone of III to be a dioic acid. In the ^{1}H-NMR spectrum of III-2, the signals of two carbinyl protons assignable to 2β-H and 3α-H were shifted downfield (0.55 ppm) and upfield (1.01 ppm), respectively, in comparison with those of the 4α-carboxyl compound (I-2). The characteristic sharp singlet at δ2.92 ascribable to H-18 in III showed a downfield shift to δ3.06 in III-2, as observed in I and I-2, indicating a tertiary hydroxyl group at C-19α and also the glucosyl linkage at the 28-carboxyl group. The electron impact (EI)-MS of III-2 showed the characteristic fragment ion peaks at *m/z* 264, 246 and 201 due to rings D and E, arising from the retro-Diels–Alder cleavage of urs-12-en-28-oic acid with a 19α-hydroxyl group.[5] The peak at *m/z* 268 was assignable to rings A and B with one carboxyl and two hydroxyl groups. On the bases of these spectral data, III was determined to be the 28-*O*-β-D-glucopyranoside of 2α,3β,19α-trihydroxyurs-12-ene-24,28-dioic acid. Compound III and the aglycone were named trachelosperoside A-1 and trachelosperogenin A, respectively.

Compound IV, mp 226—230 °C, $C_{36}H_{58}O_{12}$, was isolated as one of the major glycosides. The ^{1}H- and ^{13}C-NMR spectra indicated that IV was a monoglucoside of an ursane-type triterpenoid. On enzymic hydrolysis, IV furnished an aglycone (IV-1), mp 245—255 °C, *m/z*

TABLE I. ^{1}H Chemical Shifts of Triterpenoid Glycosides and Their Derivatives[a]

	H-2β	H-3α	H-12	H-18	*tert*-Me	H-30	Others
I			5.54 (brs)	2.92 (s)	1.16, 1.21, 1.38, 1.63, 1.72	1.07 (d, 6)	6.29 (H-1′, d, 8)
I-2	4.18 (dt, 4, 10)	4.39 (d, 10)	5.57 (brs)	3.04 (s)	1.05, 1.09, 1.42, 1.59, 1.67	1.12 (d, 6)	3.65 (–COOMe)
II-1			5.51 (brs)	2.92 (s)	1.01, 1.16, 1.38, 1.50, 1.64	1.07 (d, 6)	6.29 (H-1′, d, 8) 4.88 (H-1″, d, 8) 3.87 (–COOMe)
III			5.56 (brs)	2.92 (s)	1.27 (6H), 1.41, 1.70, 1.77	1.07 (d, 6)	6.26 (H-1′, d, 7)
III-1	4.77 (dt, 4, 10)		5.56 (brs)	2.94 (s)	1.01, 1.24, 1.41, 1.65, 1.69	1.08 (d, 6)	6.31 (H-1′, d, 8) 3.63 (–COOMe)
III-2	4.73 (dt, 5, 10)	3.38 (d, 10)	5.59 (brs)	3.06 (s)	0.97, 1.12, 1.44, 1.65, 1.71	1.13 (d, 6)	3.56 (–COOMe)
IV			5.53 (brs)	2.91 (s)	1.17, 1.22, 1.37, 1.61	1.06 (d, 7)	6.28 (H-1′, d, 8) 3.99, 4.62 (H-23, d, 11) 4.26, 4.85 (H-24, d, 11)
IV-1	4.46 (dt, 4, 9)	4.35 (d, 9)	5.58 (brs)	3.04 (s)	1.12, 1.13, 1.42, 1.67	1.11 (d, 6)	3.99, 4.62 (H-23, d, 12) 4.28, 4.88 (H-24, d, 12)
IV-2	4.47 (dt, 4, 10)	4.33 (d, 10)	5.47 (brs)	2.82 (s)	0.89, 1.17, 1.35, 1.63	1.08 (d, 7)	4.03, 4.64 (H-23, d, 12) 4.28, 4.88 (H-24, d, 11) 3.71 (–COOMe)
IV-3[b]	5.19 (dt, 4, 9)	5.15 (d, 9)	5.35 (brs)	2.60 (s)	0.69, 1.10, 1.21, 1.24	0.96 (d, 7)	3.60 (–COOMe), 1.98, 2.02, 2.06, 2.11 (–OAc)
V			5.54 (brs)	2.90 (s)	1.10, 1.12, 1.39, 1.61	1.07 (d, 7)	6.16 (H-1′, d, 8) 5.39 (H-1″, d, 7)
VI			5.51 (brs)	2.92 (s)	1.23, 1.29, 1.38, 1.60	1.07 (d, 7)	6.29 (H-1′, d, 8)
VI-1		4.49 (d, 10)	5.54 (brs)	2.93 (s)	1.23, 1.28, 1.39, 1.64	1.08 (d, 6)	6.30 (H-1′, d, 8) 3.80 (–COOMe)
VI-2	4.41 (dt, 4, 10)	4.69 (d, 10)	5.59 (brs)	3.04 (s)	1.11, 1.25, 1.43, 1.66	1.12 (d, 6)	4.56, 4.98 (H-24, d, 12)
VI-3	4.37 (dt, 4, 10)	4.48 (d, 10)	5.47 (brs)	2.83 (s)	0.88, 1.27, 1.44 1.60	1.09 (d, 7)	3.71, 3.73 (–COOMe) 4.50, 4.94 (H-24, d, 12)
VI-4[b]	5.17 (dt, 4, 11)	5.31 (d, 11)	5.35 (t, 3)	2.60 (s)	0.68, 1.10, 1.21, 1.27	0.94 (d, 7)	3.60, 3.71 (–COOMe) 4.44, 4.77 (H-24, d, 12) 1.97, 1.99, 2.06 (–OAc)
VII-1			5.53 (brs)	2.93 (s)	1.23, 1.25, 1.39, 1.66	1.08 (d, 7)	6.30 (H-1′, d, 8) 4.95 (H-1″, d, 8) 3.89 (–COOMe)
VII-3	4.58 (dt, 4, 9)	4.44 (d, 9)	5.46 (brs)	2.82 (s)	0.86, 1.24, 1.37, 1.63	1.09 (d, 7)	4.93 (H-1″, d, 8) 4.32, 4.87 (H-24, d, 12) 3.70, 3.92 (–COOMe)
VIII			5.53 (brs)	2.92 (s)	1.03, 1.12, 1.21, 1.37, 1.61	1.06 (d, 7)	6.30 (H-1′, d, 8) 5.18 (H-1″, d, 8)

a) δ (ppm) from tetramethylsilane (TMS) in pyridine-d_5, unless otherwise mentioned (*J*/Hz values in parentheses). *b*) Dissolved in $CDCl_3$.

543 ($C_{30}H_{48}O_7$ + Na). In the EI-MS of a methyl ester of IV-1 (IV-2), the peaks at *m/z* 278, 260 and 201 were assigned to the fragments of rings C and D with carboxymethyl at C-17, and the peak at *m/z* 256, to the fragment of rings A and B with four hydroxyl groups. In fact, IV-2 afforded a tetra-*O*-acetyl-monomethyl ester of the aglycone, $C_{39}H_{58}O_{11}$ (IV-3) on usual acetylation. Since almost the same carbon chemical shifts were observed in rings B, C, D and E of IV and I, and two pairs of hydroxyl methylene protons were observed at δ 3.99 and 4.62

TABLE II. ^{13}C Chemical Shifts of Triterpenoid Glycosides and the Methyl Esters, δ (ppm) from TMS in Pyridine-d_5

	I	II-1	III	IV	V	VI	VII-1	VIII
C-1	48.3	47.4	48.5	48.0	48.0	48.6	47.7	47.6
C-2	68.7	66.6	68.7	69.1	69.1	68.6	66.5	67.1
C-3	81.0	92.2	84.4	79.8	79.7	82.2	92.0	88.6
C-4	54.8	54.6	50.0	47.8	47.9	58.6	59.3	44.7
C-5	52.3	52.6	57.2	48.3[a]	48.1	53.0	53.3	47.4
C-6	21.5	20.9	21.2	19.4	19.2	22.7	22.6	18.5
C-7	33.3	33.0	33.9	33.5	33.5	33.9	33.8	33.2
C-8	40.7	40.6	40.6	40.6	40.7	40.8	40.6	40.6
C-9	48.2	47.9	47.3	48.0[a]	48.1	48.4	48.1	47.8
C-10	38.6	37.9	39.0	38.1	38.1	38.7	38.0	37.9
C-11	24.2	24.0	24.5	24.4	24.5	24.4	24.3	24.2
C-12	128.2	128.0	128.5	128.3	128.1	128.1	128.0	128.4
C-13	139.2	139.1	139.3	139.2	139.4	139.2	139.1	139.3
C-14	42.1	42.0	42.3	42.1	42.1	42.0	42.0	42.2
C-15	29.1	29.1	29.2	29.2	29.4	29.2	29.1	29.2
C-16	26.1[a]	26.0[a]	26.2[a]	26.1[b]	25.9[a]	26.1[a]	26.1[a]	26.2[a]
C-17	48.6	48.5	48.7	48.6	48.7	48.6	48.6	48.6
C-18	54.4	54.3	54.5	54.4	54.4	54.4	54.4	54.4
C-19	72.7	72.6	72.7	72.6	72.7	72.7	72.6	72.7
C-20	42.1	42.0	42.1	42.1	42.1	42.0	42.1	42.2
C-21	26.7[a]	26.7[a]	26.7[a]	26.7[b]	26.8[a]	27.0[a]	26.7[a]	26.7[a]
C-22	37.7	37.6	37.6	37.7	37.5	37.7	37.6	37.7
C-23	180.1	176.8[c]	25.3	64.3	64.4	179.1	176.3	64.0
C-24	13.4	13.5	180.1	62.9	62.6	63.0	62.3[b]	14.7
C-25	17.4[b]	17.3[b]	15.5	16.7[c]	16.7	17.2[b]	16.7[c]	17.5[b]
C-26	17.5[b]	17.2[b]	17.4[b]	17.4[c]	17.4[b]	17.5[b]	17.0[c]	17.5[b]
C-27	24.5	24.4	24.7	24.5	24.5	24.4	24.4	24.6
C-28	176.9	177.3[c]	177.0	176.9	176.9	176.9	176.8	176.9
C-29	27.0	26.9	27.0	27.0	27.1	27.0	27.0	27.0
C-30	16.7[b]	16.6[b]	16.7[b]	17.4[c]	17.2[b]	16.8[b]	16.7[c]	16.7[b]
COOMe		52.2					52.1	
C-1′ (28-*O*-Glc)	95.8	95.8	95.8	95.8	93.7	95.9	95.8	95.8
C-2′	74.0	74.0	74.0	74.0	80.7	74.1	74.0	74.1
C-3′	78.9	78.9	78.9	78.9	78.4[c]	78.9	78.9	79.0
C-4′	71.3	71.3	71.3	71.2	71.2	71.2	71.2	71.3
C-5′	79.2	79.1	79.2	79.2	79.0	79.3	79.2	79.2
C-6′	62.4	62.4	62.4	62.3	62.3	62.3	62.4[b]	62.4
		(3-*O*-Glc)			(2′-*O*-Xyl)		(3-*O*-Glc)	(3-*O*-Glc)
C-1″		106.0			106.1		105.7	105.8
C-2″		74.7			76.0		74.8	75.5
C-3″		78.5			78.7[c]		78.7	78.6
C-4″		71.4			71.0		71.4	71.5
C-5″		78.4			67.4		78.3	78.4
C-6″		62.4					62.0[b]	62.4

a—*c*) Signal assignments marked *a*), *b*), or *c*) in each column may be reversed.

(d each, $J=11$ Hz) and at δ4.26 and 4.85 (d each, $J=11$ Hz), two acetylable hydroxyl groups among four were assigned to C-23 and C-24, and the others to 2α and 3β. The presence of 19α-hydroxyl group was also indicated by a sharp singlet at δ2.91 ascribable to H-18 in IV. On reflux with 0.5 N H_2SO_4–50% dioxane, IV was transformed into IV-anhydride as a mixture of Δ^{18}- and Δ^{19}-derivatives. The glucosyl linkage was determined to be at the 28-carboxyl group on the basis of the chemical shift of the anomeric proton at δ6.28, and the downfield shift of

H-18 in IV-1 in comparison with that of IV. Compounds IV and IV-1 were named trachelosperoside B-1 and trachelosperogenin B, respectively.

Compound V was obtained in a small amount as a solid. The presence of two anomeric protons at δ 5.39 and 6.16 suggested V to be a bioside, and D-glucose and D-xylose were detected after acid hydrolysis with 1 N H_2SO_4–50% EtOH. In the ^{13}C-NMR spectrum of V, the signals due to the aglycone coincided with those of IV. Acid hydrolysis, of V with 0.5 N H_2SO_4–50% dioxane afforded IV-anhydride. The xylose was linked to the 2-OH of the glucose on the basis of the downfield shift of C_{glc}-2 in comparison with that of IV. Compound V was named trachelosperoside B-2.

Compound VI (trachelosperoside C-1), mp 235—250 °C (dec.), $C_{36}H_{56}O_{13}$, was also considered to be a monoglucoside of the 19α-hydroxyursane-type triterpenoid on the basis of the ^{1}H- and ^{13}C-NMR spectra. It afforded a monomethyl ester (VI-1), and was hydrolyzed enzymically to an aglycone (trachelosperogenin C, VI-2), which provided a dimethyl ester with CH_2N_2 (VI-3) (*m/z*: 585, $C_{32}H_{50}O_8$ + Na). As in the case of IV-2, VI-3 showed fragment peaks at *m/z* 278, 260 and 201, due to rings C and D with carboxymethyl at C-17. The peak at *m/z* 285 was consistent with the fragment arising from rings A and B with one carboxymethyl and three hydroxyl groups. Upon acetylation, VI-3 gave a triacetate (VI-4). A sharp singlet signal at δ 2.92 due to H-18 in VI was shifted downfield by 0.12 ppm in VI-2, suggesting glucosylation at the 28-carboxyl group as in the cases of I, III, and IV. Since a pair of hydroxyl methylene protons was observed at δ 4.56 and 4.98 (d each, J = 12 Hz) in VI-2, VI was considered to be the 28-*O*-glucoside of 2α,3β,19,24-(or 23)-tetrahydroxyurs-12-ene-23(or 24),28-dioic acid. In the ^{1}H-NMR spectrum of VI-3, the chemical shifts of H-2 (δ 4.37) and H-3 (δ 4.48) were closer to those of the 4α-carboxyl compound (I-2, δ 4.18 and 4.39) than to those of the 4β-carboxyl compound (III-2, δ 4.73 and 3.38). Therefore, the carboxyl carbon was assigned to C-23, and the primary carbinyl group was determined to be C-24. When VI-3 was subjected to reduction with $LiAlH_4$, the product, mp 168—171 °C, was identified as IV-2.

Compound VII was the most polar glycoside in this series, and afforded a monomethyl ester (VII-1) with CH_2N_2. FAB-MS (*m/z*: 895, $C_{43}H_{68}O_{18}$ + Na), as well as the ^{13}C-NMR signals, suggested VII to be a bioside. Compound VII-1 was enzymically hydrolyzed to glucose and a monoglucoside (VII-2), which afforded a dimethyl ester (VII-3) with CH_2N_2. Since the ^{1}H-NMR signal of H-18 in VII-1 was observed at the same chemical shift as that of VI-1, the location of one of the glucose moieties was assigned to the 28-carboxyl group as in the other glucosides. The ^{1}H- and ^{13}C-NMR spectra of VII-1 were similar to those of VI, except for the carbon signals due to one glucose residue, which was considered to be linked at the C-3 hydroxyl group, based on the glycosylation shift at C-3 (+9.8 ppm). Acid hydrolysis of VII-3 with 2 N H_2SO_4 gave D-glucose. Compound VII was therefore the 3-*O*-glucoside of VI, and was named trachelosperoside C-2.

Compound VIII, mp 242—245 °C, afforded the molecular peak at *m/z* 851 ($C_{42}H_{68}O_{16}$ + Na), suggesting it to be a bioside, and the two component sugars were considered to be glucose on the basis of the ^{13}C-NMR signals. On enzymic hydrolysis, one glucose was split off to give a monoglucoside (VIII-1) (*m/z*: 689, $C_{36}H_{58}O_{11}$ + Na). The ^{1}H- and ^{13}C-NMR spectra of VIII suggested that VIII is the 3,28-*O*-bis-β-D-glucoside of 19α-hydroxyasiatic acid, isolated from *Symplocos spicata* by Higuchi *et al.*[5] The ^{13}C-NMR spectra and TLC behavior of the two samples were in good agreement.

In this work, eight glycosides of 19α-hydroxyursane-type triterpenoids including the glycosides of three new triterpenoids, trachelosperogenins A, B, and C, and two known glucosides, suavissimoside R1 and the 3,28-bis-*O*-glucoside of 19α-hydroxyasiatic acid, were identified. It should be noted that a downfield shift of the H-18 signal by 0.12—0.15 ppm was observed in these triterpenoids on removal of the glucose from the 28-carboxyl group, indicating the location of the glucose residue.

19α-Hydroxyursane-type triterpenoids have been isolated from plants of several families, including Rosaceae, Ericaceae, Clethraceae, Staphyleaceae, Symplocaceae, and Rubiaceae. This is, however, the first isolation of 19α-hydroxyursane-type triterpenoid glycosides from Apocynaceae. The structures of the oleanane-type glycosides (IX—XVI) isolated from this plant will be reported shortly.

Experimental

The ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 spectrometer in pyridine-d_5 unless otherwise mentioned. Chemical shifts are given in δ values referred to internal tetramethylsilane, and the following abbreviations are used: s = singlet, d = doublet, t = triplet, dt = doublet of triplets, and brs = broad singlet. EI-MS and FAB-MS were recorded on a JEOL D-300-FD spectrometer. HPLC was run with a Waters model ALC 200, equipped with a radial pack C_{18}, and eluted with MeCN in H_2O (15—25%). For reversed-phase column chromatography, an ODS column (RQ-1, Fuji gel) was used with MeCN in H_2O as a solvent. The following solvent systems were applied for silica gel column chromatography, TLC, and paper chromatography (PC): solv. 1, $CHCl_3$–MeOH–H_2O (bottom layer); solv. 2, EtOAc–MeOH–H_2O (top layer); solv. 3, BuOH–AcOH–H_2O (4 : 1 : 5, top layer). Detection of TLC spots was done by charring the plate after spraying it with 10% H_2SO_4. The aniline hydrogen phthalate reagent was applied for PC.

Extraction and Isolation——*Trachelospermum asiaticum* NAKAI (8 kg) was collected in the suburbs of Fukuoka City in Nov. 1984, and air-dried. The whole plant was powdered and percolated with MeOH (60 l). The MeOH percolate was concentrated *in vacuo* to 4 l, and diluted with 4 l of H_2O. After filtration, the filtrate was extracted with BuOH (ext. 270 g). The BuOH extract was dissolved in H_2O and passed through an MCI gel (CHP-20, Mitsubishi) column, which was eluted stepwise with H_2O containing increasing concentrations of MeOH to give fractions 1—4: fr. 1 (H_2O, ext. 30 g), fr. 2 (30% MeOH, 11 g), fr. 3 (50% MeOH, 60.6 g), fr. 4 (60% MeOH, 16.2 g). Fraction 3 was subjected to chromatographies on silica gel columns with solv. 1 (7 : 3 : 1–7 : 3 : 0.8) and then with solv. 2 (3 : 1 : 2–3 : 1 : 1.5). When two peaks were detected on HPLC in a fraction showing one homogeneous spot on TLC, the reversed-phase chromatography on an ODS column was applied, and in some cases, the mixture was subjected to HPLC on a preparative scale. The following compounds were obtained (yields): I (0.0015%), II (as the monomethyl ester, less than 0.0001%), III (0.00125%), IV (0.011%), V (under 0.0001%), VI (0.0044%), VII (0.0031%), VIII (0.0002%), IX (0.0011%), X (0.0005%), XI (0.0003%), XII (0.0004%), XIII (0.0004%), XIV (0.0005%), XV (0.0002%), and XVI (0.0001%).

Compound I (Suavissimoside R1)——Colorless prisms from MeOH, mp 260—265 °C (dec.), $[\alpha]_D^{18}$ +16.7° (*c* = 0.15, MeOH). FAB-MS *m/z*: 703 ($C_{36}H_{56}O_{12}$ + Na). In a TLC comparison of I with authentic suavissimoside R1 (solv. 1 and 2), the two samples showed the same *Rf* values and all signals in the ^{13}C-NMR spectra were in good agreement. Methylation of I was carried out in the following manner. CH_2N_2 in ether was added dropwise to a methanolic solution of I (50 mg/2 ml). The mixture was allowed to stand at room temperature for 5 h, and the solvent was evaporated off *in vacuo*. The residue was purified by column chromatography to give I-1 as a solid, $[\alpha]_D^{31}$ +11.8° (*c* = 1.25, MeOH). FAB-MS *m/z*: 717 ($C_{37}H_{58}O_{12}$ + Na). Enzymic hydrolysis of I-1 was carried out in the following manner. Cellulase (Sigma Chem. Co., Ltd.) was added to a solution of I-1 (20 mg) in 20% EtOH (2 ml), and the mixture was shaken at 38 °C for 10 h, then extracted with BuOH. The BuOH extract was purified on a silica gel column with solv. 1 (7 : 2 : 2) to give I-2 as a crystalline powder, mp 255—267 °C (dec.), $[\alpha]_D^{30}$ +48.6° (*c* = 0.35, MeOH). FAB-MS *m/z*: 555 ($C_{31}H_{48}O_7$ + Na). The H_2O layer was applied to a charcoal column and eluted with 5% EtOH. The effluent was concentrated *in vacuo* and the residue was examined by PC with solv. 3; *Rf* 0.22 (D-glucose, 0.22).

Compound II (Suavissimoside R1-3-*O*-Glucopyranoside)——A monomethyl ester of II (II-1) was obtained as a solid, $[\alpha]_D^{25}$ −2.6° (*c* = 0.57, MeOH). FAB-MS *m/z*: 879 ($C_{43}H_{68}O_{17}$ + Na).

Compound III (Trachelosperoside A-1)——Homogeneous solid, $[\alpha]_D^{30}$ +12.6° (*c* = 0.35, MeOH). FAB-MS *m/z*: 703.366 (Calcd for $C_{36}H_{56}O_{12}Na$: 703.367). On methylation with CH_2N_2, III afforded a monomethyl ester as a solid (III-1), $[\alpha]_D^{25}$ +20.2° (*c* = 0.52, MeOH). FAB-MS *m/z*: 717 ($C_{37}H_{58}O_{12}$ + Na). The hydrolysis of III-1 (20 mg) with cellulase was carried out in the same manner as that of I-1, and III-2 was obtained as a solid, $[\alpha]_D^{28}$ +37.8° (*c* = 0.80, MeOH). EI-MS *m/z*: 532.341 (Calcd for $C_{31}H_{48}O_7$: 532.340). EI-MS *m/z*: 532, 514, 486, 414, 268, 264, 250, 246, 201. Glucose was detected from the H_2O layer, after treatment in the same manner as described above.

Compound IV (Trachelosperoside B-1)——Colorless prisms from MeOH, mp 226—230 °C, $[\alpha]_D^{18}$ +9.8° (*c* = 0.50, MeOH). FAB-MS *m/z*: 705 ($C_{36}H_{58}O_{12}$ + Na). *Anal.* Calcd for $C_{36}H_{58}O_{12}$: C, 63.32; H, 8.56. Found: C, 63.97; H, 8.66. Acetylation of IV (150 mg) was carried out in the usual manner with Ac_2O and pyridine at room temperature and the acetate was purified on a silica gel column with benzene–acetone (4 : 1) as a solvent. The acetate (190 mg as a solid) was crystallized from MeOH to give prisms, mp 198—200 °C, $[\alpha]_D^{30}$ +12.9° (*c* = 1.20, $CHCl_3$). FAB-MS *m/z*: 1041 ($C_{52}H_{74}O_{20}$ + Na). ^{1}H-NMR ($CDCl_3$): 0.72, 1.11, 1.20, 1.24 (3H each, s), 0.93 (3H, d, *J* = 6 Hz), 2.53 (1H, s, H-18), 2.01, 2.02 (6H, each, s, –OAc), 1.97, 2.05, 2.07, 2.10 (3H each, s, –OAc). Hydrolysis of IV (100 mg) with cellulase

was carried out in the same manner as described for I to give the aglycone (IV-1) as prisms (5 mg) from MeOH, mp 245—255 °C (dec.), $[\alpha]_D^{18}$ +25.2° (c=0.52, MeOH). FAB-MS m/z: 543 ($C_{30}H_{48}O_7$+Na). From the H_2O layer, after BuOH extraction, glucose was detected on PC. The methyl ester of IV-1 (IV-2) was prepared from 10 mg of IV-1 in the same manner as described above and obtained as prisms, mp 171—173 °C, $[\alpha]_D^{18}$ +23.5° (c=0.72, MeOH). FAB-MS m/z: 557 ($C_{31}H_{50}O_7$+Na), EI-MS m/z: 534, 516, 474, 402, 278, 260, 256, 201. Acetylation of IV-2 (20 mg) was carried out in the usual manner and a tetraacetate (IV-3) was obtained as colorless plates (9 mg), mp 130—133 °C from hexane–AcOEt. EI-MS m/z: 702.398 (Calcd for $C_{39}H_{58}O_{11}$: 702.398). Compound IV (20 mg) was dissolved in 1 ml of 0.5 N H_2SO_4–50% dioxane and heated at 100 °C for 30 min. The solution was deacidified with IRA-410 and the dioxane was evaporated off *in vacuo*. The remaining mixture was extracted with BuOH. The BuOH layer was concentrated to dryness *in vacuo* and the residue was purified on a silica gel column to give IV-anhydride as a solid. FAB-MS m/z: 687 (M^++Na). ^{1}H-NMR δ (ppm): (1.77 (br s, 29-H), 0.99 (d, J=5 Hz, 30-H), Δ^{18}-deriv.), (1.60, 1.56 (br s, 29,30-H), 3.51 (br s, 18-H), Δ^{19}-deriv.), *tert*-CH_3 (1.18, 1.17, 1.14, 1.13, 1.02, 0.98), 12-H (5.69, 5.63), 1′-H (6.32, 6.31 (d, J=8 Hz)).

Compound V (Trachelosperoside B-2)——A solid, $[\alpha]_D^{30}$ +3.7° (c=1.32, MeOH). FAB-MS m/z: 837 ($C_{41}H_{66}O_{16}$+Na). Compound V (5 mg) was dissolved in 1.5 ml of 1 N H_2SO_4–50% EtOH and refluxed for 1.5 h. The solution was diluted with H_2O, then deacidified with IRA-410, and the EtOH was evaporated off *in vacuo*. The remaining solution was extracted with BuOH. After BuOH extraction, the H_2O layer was evaporated *in vacuo*. Xylose and glucose were detected from the H_2O layer (PC: solv. 3, 0.29, 0.22 (D-xylose: 0.29, D-glucose: 0.22)). Compound V (2 mg) was dissolved in 0.5 ml of 0.5 N H_2SO_4–50% dioxane, heated at 100 °C for 30 min, and treated in the same manner as described above. The product, obtained from the BuOH layer, was identified as IV-anhydride by ^{1}H-NMR and TLC analyses. FAB-MS m/z: 687.371 (Calcd for $C_{36}H_{56}O_{11}$+Na: 687.372).

Compound VI (Trachelosperoside C-1)——Colorless prisms from MeOH, mp 235—250 °C (dec.), $[\alpha]_D^{18}$ +22.7° (c=0.60, MeOH). FAB-MS m/z: 719 ($C_{36}H_{56}O_{13}$+Na). *Anal.* Calcd for $C_{36}H_{56}O_{13}\cdot 3/2H_2O$: C, 59.73; H, 8.22. Found: C, 59.12; H, 7.83. Methylation of VI with CH_2N_2 afforded a monomethyl ester (VI-1), mp 212—220 °C (from MeOH). FAB-MS m/z: 733 ($C_{37}H_{58}O_{13}$+Na). Hydrolysis of VI (100 mg) with cellulase was carried out in the same manner as described above, and glucose was detected from the H_2O layer. The aglycone, obtained (20 mg) (VI-2), mp 265—280 °C (dec.) from the BuOH layer, was methylated with CH_2N_2 to give a dimethyl ester (VI-3) as fine prisms from MeOH, mp 125—132 °C. FAB-MS m/z: 585 ($C_{32}H_{50}O_8$+Na), EI-MS m/z: 562, 544, 503, 285, 278, 260, 201. Upon acetylation of VI-3 (15 mg), a triacetate of VI-3 (VI-4) was obtained as a crystalline powder, mp 137—140 °C from hexane–AcOEt. EI-MS m/z: 688.386 (Calcd for $C_{38}H_{56}O_{11}$: 688.382). $LiAlH_4$ was added portionwise to a solution of VI-3 (10 mg) in ether, and the mixture was stirred for 2 h. After adding H_2O, the ether layer was further washed with H_2O and then concentrated *in vacuo*. The residue was chromatographed on a silica gel column with solv. 1 (7:3:1.6) to give colorless prisms from MeOH–H_2O, mp 168—171 °C. On admixture with IV-2, no melting point depression was observed and the two samples were identical on TLC. The ^{1}H-NMR spectra of the two samples were superimposable.

Compound VII (Trachelosperoside C-2)——Compound VII was obtained as a monomethyl ester (VII-1), $[\alpha]_D^{18}$ +1.4° (c=1.40, MeOH). FAB-MS m/z: 895 ($C_{43}H_{68}O_{18}$+Na). Hydrolysis of VII-1 with cellulase was carried out in the same manner as described above, and glucose was detected on PC. After hydrolysis, the product (VII-2) from the BuOH layer was again methylated with CH_2N_2 to give a dimethyl derivative (VII-3). FAB-MS m/z: 747.392 ($C_{38}H_{60}O_{13}$+Na). Acid hydrolysis of VII-3 (3 mg) was carried out with 1 ml of 2 N H_2SO_4. After heating at 100 °C for 2 h, the mixture was deacidified with IRA-410. The solution was examined by PC and glucose was detected.

Compound VIII (3,28-Bis-*O*-glucosyl-19α-hydroxyasiatic Acid)——Colorless prisms from MeOH, mp 242—245 °C, $[\alpha]_D^{28}$ −5.9° (c=1.25, MeOH). FAB-MS m/z 851 ($C_{42}H_{68}O_{16}$+Na). Hydrolysis of VIII with cellulase followed by methylation with CH_2N_2 was carried out in the same manner as described above, and a monoglucosyl derivative (VIII-1) was obtained as a solid, FAB-MS m/z: 689 ($C_{36}H_{58}O_{11}$+Na). On TLC (solv. 1, 2) of VIII in parallel with authentic 3,28-bis-*O*-glucosyl-19α-hydroxyasiatic acid, the two samples showed the same Rf values, and the ^{13}C-NMR signals of the two samples were in good agreement.

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An Approach for General Synthesis of Biphenyl Neolignans by the Reaction of 1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione with the Grignard Reagent and Synthesis of Magnaldehyde B

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An approach for general synthesis of biphenyl neolignans by the reaction of 1-oxaspiro[4.5]-deca-6,9-diene-2,8-dione (**3**) (quinol-lactone) with the Grignard reagent **4** was explored. Among the biphenyl derivatives **6**, **8**, and **9** thus obtained, **6** was transformed to magnaldehyde B (**2b**) in three steps.

Keywords——biphenyl neolignan; synthesis; magnaldehyde B; quinol-lactone; Grignard reagent

A number of biphenyl neolignans, *e.g.*, **1a**—**f** and **2a**—**e**, have been isolated from Magnoliaceae and *Rauraceae*.[1] These lignans are of interest because of the medical usage of the parent plants in China and the known pharmacological activities of **1a** and **2a**.[2] In the previous report, we described a synthesis of honokiol (**2a**) and related unsymmetrical biphenyl neolignans utilizing quinol-acetates as reactive intermediates.[3] This paper deals with an approach for general synthesis of biphenyl neolignans by the reaction of 1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (quinol-lactone) (**3**) with the Grignard reagent **4**, and a synthesis of magnaldehyde B (**2b**).

Though the quinol-lactone **3** can be synthesized from *p*-hydroxydihydrocinnamic acid by oxidation with $Pb(OAc)_4$ or H_2O_2, and by anodic oxidation,[4] we carried out by the oxidation of the acid with ferric perchlorate in acetonitrile in 32.8% yield.[5] Reaction of the quinol-lactone **3** with the Grignard reagent **4** was carried out at −78 °C in tetrahydrofuran (THF) for 2 h followed by quenching with NH_4Cl aq. to give **7**, a 1,2-addition product of the ketone group of **3**, in a yield of 73%. Acidic (10% HCl in THF) treatment of the lactone **7** followed by esterification of the acidic portion afforded the biphenyl **8** in 92% yield. Basic (10% KOH in EtOH) treatment of **7** followed by esterification gave another biphenyl **9** in 90% yield. Thus, we could not transform **7** into biphenyls having the skeleton of natural biphenyl neo-lignans such as **6** under the above conditions. On the other hand, the biphenyl **6** was obtained by the reaction of **3** with **4** at −78 °C for 2 h, then at room temperature overnight, followed by quenching with NH_4Cl aq. and esterification of the acidic fraction, in a yield of 75%.

The structures of the biphenyls **6**, **8**, and **9** were confirmed by analyses of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra. The assignments were aided by off-resonance decoupling and by comparison with the spectra of reference compounds, **13**, **14**, **15**, and **16** (Chart 3 and Table I).

In the case of **6**, differences of the carbon signals between the biphenyl ester **6** and the reference compounds **13** and **14** were observed only at C-1′ and C-1. That is, the C-1′ and C-1 signals (at 127.8 and 130.2 ppm) of **6** were observed at lower field than the corresponding signals, 115.5 and 120.8 ppm, of **13** and **14**. This result is in reasonable agreement with the previous report,[6] which indicated that the signals of C-1′ and C-1 may be shifted *ca.* 12—

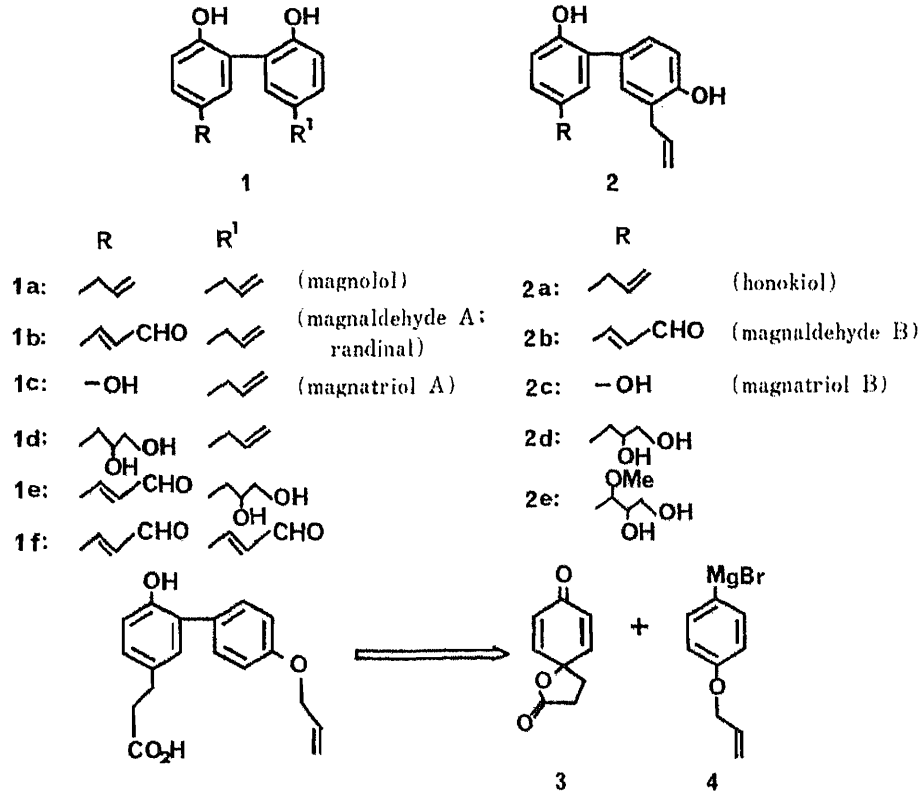


Chart 1

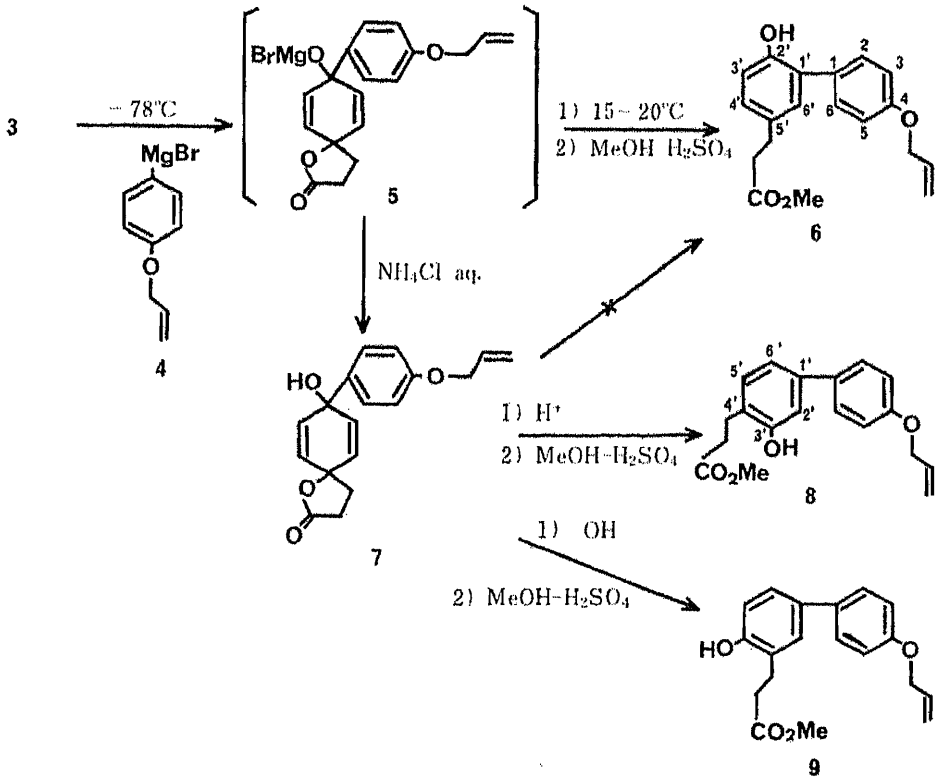


Chart 2

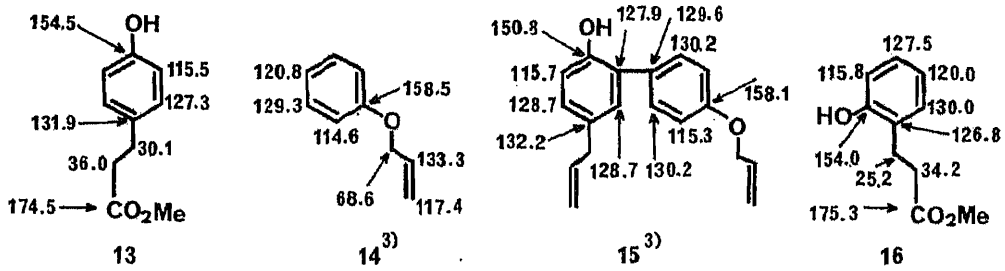


Chart 3. ^{13}C-NMR Spectra of **13**, **14**, **15** and **16** were Measured in $CDCl_3$ at 25 °C (25 MHz)

TABLE I. ^{13}C-NMR Spectral Data for **6**, **8** and **9**[a]

Carbon No.	6	8	9	Carbon No.	6	8	9
1′	127.8 s	140.9 s	133.6 s (or C-1)	1	130.0 s	133.6 s	133.7 s (or C-1′)
2′	151.0 s	115.2 d	128.8 d	2	130.2 d	127.9 d	127.6 d
3′	115.8 d	154.6 s	127.5 s	3	115.4 d	115.2 d	115.0 d
4′	128.4 d	125.8 s	153.4 s	4	158.3 s	158.3 s	157.7 s
5′	132.6 s	130.8 d	117.2 d	5	115.4 d	115.2 d	115.0 d
6′	129.5 d	119.3 d	126.2 d	6	130.2 d	127.9 d	127.6 d
7′	30.2 t	24.7 t	25.1 t	7	68.9 t	69.0 t	68.9 t
8′	36.0 t	34.9 t	34.9 t	8	133.1 d	133.5 d	133.4 d
9′	173.4 s	175.7 s	175.8 s	9	117.7 t	117.6 t	117.6 t
CO_2Me	51.9 q	52.1 q	52.2 q				

a) δ in $CDCl_3$; ^{13}C, 25 MHz at 25 °C; s, singlet; d, doublet; t, triplet; q, quartet.

9 ppm to lower field than those of C-2 of **13** and C-4 of **14**. Thus, the structure of **6** was confirmed as the C–C bond formation product at the C-2 and C-4 positions of **13** and **14**, respectively.

Similarly, the structure of **8** was assigned by comparison of the carbon signals of the biphenyl **8** and the reference compounds **16** and **14**. The C-1′ and C-1 signals (at 140.9 and 133.6 ppm) of **8** were observed at lower field than the corresponding signals, 127.5 and 120.8 ppm, of **16** and **14**. This result shows that **8** is the C–C bond formation product at the C-5 and C-4 positions of **16** and **14**. The structure of **9** was elucidated similarly.

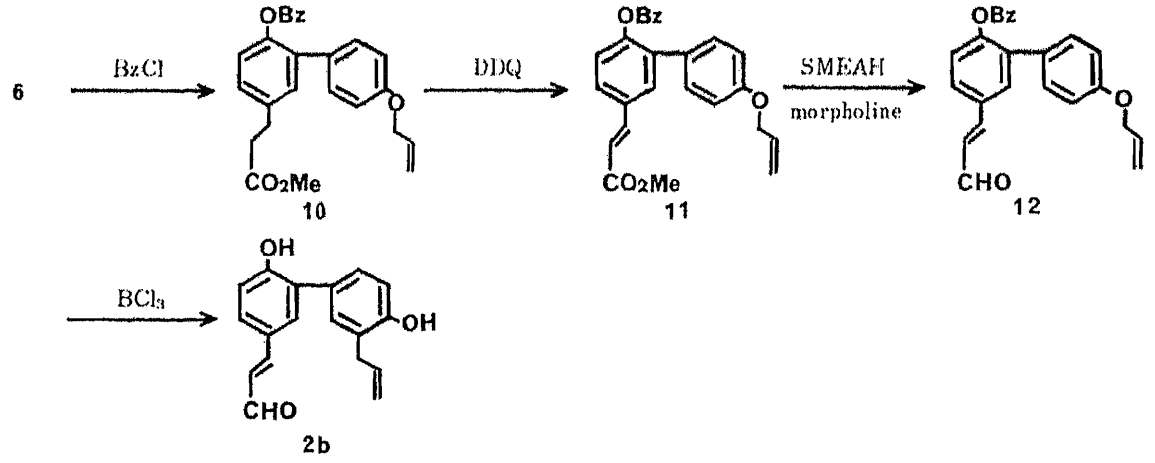


Chart 4

The biphenyl **6** was transformed to magnaldehyde B (**2b**) by means of the following reaction sequence. The O-benzyl derivative **10** was oxidized with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in benzene to give the unsaturated (*E*)-ester **11** in 54% yield. Reduction of **11** with sodium bis(2-methoxyethoxy)aluminum hydride (SMEAH)–morpholine[7] gave the unsaturated (*E*)-aldehyde **12** in 75% yield. The Claisen rearrangement and debenzylation reaction of the aldehyde **12** to give magnaldehyde B (**2b**) were effected by the treatment of **12** with BCl_3 in chlorobenzene at -15 °C in a yield of 63%. All physical data of synthetic magnaldehyde B (**2b**) were identical with those of the natural product.[8]

The results presented here provide the basis for a general approach to the synthesis of biphenyl neo-lignans, because it may be possible to synthesize other natural biphenyl neo-lignans such as **1a** and **1b** by the reaction of the quinol-lactone **3** with suitable Grignard reagents followed by chemical transformations as described above.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded with a Hitachi 260-10 spectrometer, ^{1}H- and ^{13}C-NMR spectra with a JEOL JNM-FX 100 or JEOL JNM-GX 270 spectrometer (with tetramethylsilane as an internal standard; $CDCl_3$ and CD_3COCD_3 sol.) and mass spectra (MS) with a JEOL JMS-D 300 spectrometer. Elementary analyses were done by Ms. A. Sakamoto and Ms. M. Takeda, Kissei Pharmaceutical Company, Matsumoto, Japan. Wako Silica Gel C-200 (200 mesh) and Merck Kieselgel 60 F_{254} were used for column chromatography and thin-layer chromatography (TLC), respectively.

1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione (3)——A solution of *p*-hydroxydihydrocinnamic acid (21.5 g, 0.13 mol) in dry MeCN (200 ml) was added to a solution of $Fe(ClO_4)_3 \cdot 9H_2O$ (145.5 g, 0.31 mol) in dry MeCN (250 ml), and the whole was stirred at room temperature for 2 min. The reaction mixture was poured into ice-water and extracted with $CHCl_3$. The organic layer was washed with H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with $CHCl_3$ gave 7 g (32.8%) of **3** as colorless crystals (EtOH–ether), mp 105—106 °C.[4]

4-Allyloxy-2′-hydroxy-5′-(2-methoxycarbonylethyl)biphenyl (6)——A solution of a small amount of 4-bromoallyloxybenzene (a part of 10.6 g, 50 mmol) and methyl iodide in dry THF (50 ml) was added to a stirred mixture of Mg turnings (1.22 g, 50 mmol) in dry THF under a nitrogen atmosphere, and the mixture was refluxed for *ca.* 2 h. Then, a solution of the remainder of the halide in dry THF (150 ml) was added dropwise and the mixture was refluxed for 2 h until the magnesium turnings disappeared. A solution of **3** (4.1 g, 25 mmol) in dry THF (50 ml) was added dropwise to the Grignard reagent thus prepared under a nitrogen atmosphere at -78 °C, and the whole was stirred under the same conditions for 2 h, then at room temperature overnight. The resultant solution was poured into 5% HCl in ice-water and extracted with AcOEt. The organic layer was extracted with sat. $NaHCO_3$. The $NaHCO_3$ layer was acidified with 10% HCl, and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried and concentrated. A 1% solution of H_2SO_4 in MeOH (5 ml) was added to the residue, and the whole was refluxed for 1 h, poured into ice-water, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with sat. $NaHCO_3$ and H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with 5% AcOEt in $CHCl_3$ gave 5.44 g (75%) of **6** as colorless crystals (MeOH), mp 51—52 °C. IR (KBr) cm^{-1}: 3350, 1700. MS *m/z*: Calcd for $C_{18}H_{18}O_4$ (M^+): 298.1359. Found: 298.1344. *Anal.* Calcd for $C_{18}H_{18}O_4$: C, 72.47; H, 6.08. Found: C, 72.71; H, 6.14.

8-(4′-Allyloxyphenyl)-8-hydroxy-1-oxaspiro[4.5]deca-6,9-diene-2-one (7)——The Grignard reagent was prepared from 4-bromoallyloxybenzene by a procedure similar to that used for **6**, and a solution of **3** (4.1 g, 25 mmol) in dry THF (50 ml) was added dropwise under a nitrogen atmosphere at -78 °C. The whole was stirred under the same conditions for 2 h, then poured into sat. NH_4Cl in ice-water, and extracted with $CHCl_3$. The organic layer was washed with H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with 5% AcOEt in $CHCl_3$ gave 5.85 g (75%) of **7** as colorless crystals (MeOH), mp 136—138 °C. IR (KBr) cm^{-1}: 3400, 1760, and 1610. ^{1}H-NMR ($CDCl_3$) δ: 2.25 [2H, t, J=6.3 Hz, C(4)-H], 2.71 [2H, t, J=6.3 Hz, C(3)-H], 4.52 [2H, d, J=5.4 Hz, C(7′)-H], 5.25 [1H, dd, J=10.0, 1.7 Hz, C(9′)-H], 5.40 [1H, dd, J=17.1, 1.7 Hz, C(9′)-H], 5.85—6.15 [1H, m, C(8′)-H, 5.99, 2H, d, J=10.0 Hz, C(7)- and C(9)-H], 6.12 [2H, d, J=10.0 Hz, C(6)- and C(10)-H], 6.88 [2H, d, J=8.5 Hz, C(2′)- and C(6′)-H], and 7.30 [2H, d, J=8.5 Hz, C(3′)- and C(5′)-H]. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1359. Found: 312.1344. *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.96; H, 6.55.

4-Allyloxy-2′-benzyloxy-5′-(2-methoxycarbonylethyl)biphenyl (10)——A mixture of **6** (1.54 g, 4.9 mmol), benzyl chloride (748 mg, 4.9 mmol) and anhydrous K_2CO_3 (1.2 g, 4.9 mmol) in dry dimethylformamide (DMF) (20 ml) was stirred at 80 °C for 3 h, then poured into ice-water, and extracted with ether. The organic layer was washed with H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with 40% $CHCl_3$ in hexane gave 1.89 g (95%) of **10** as an oil. IR (KBr) cm^{-1}: 1720, 1600. MS *m/z*: Calcd for $C_{26}H_{26}O_4$ (M^+): 402.1831. Found: 402.1856.

TABLE II. ^{1}H-NMR Spectral Data for 6, 8—12 and 2b[a]

Proton	6	8	9	10	11	12	2b[b]
2							7.62
	7.37	7.47	7.44	7.50	7.50	7.49	(d, J=2.2)
6	(d, J=8.8)	(d, J=9.1)	(d, J=9.0)	(d, J=8.8)	(d, J=9.0)	(d, J=9.0)	7.55
							(dd, J=8.3, 2.2)
3	7.02	6.94	6.96	6.95	6.97	6.97	
5	(d, J=8.8)	(d, J=9.1)	(d, J=9.0)	(d, J=8.8)	(d, J=9.0)	(d, J=9.0)	7.05
							(d, J=8.3)
2′		7.07	7.28				
		(br s)	(d, J=2.4)				
3′	6.88			6.93	6.99	7.01	6.91
	(d, J=8.8)			(d, J=8.3)	(d, J=8.3)	(d, J=8.5)	(d, J=8.3)
4′	7.05			7.07	7.41	7.46	7.33
	(dd, J=8.8, 2.2)			(dd, J=8.3, 2.2)	(dd, J=8.3, 2.0)	(dd, J=8.5, 2.2)	(dd, J=8.3, 2.2)
5′		7.11	6.93				
		(d, J=7.6)	(d, J=8.1)				
6′	7.04	7.05	7.30	7.16	7.51	7.53	7.37
	(br s)	(dd, J=7.6, 1.7)	(dd, J=8.1, 2.4)	(d, J=2.2)	(d, J=2.0)	(d, J=2.2)	(d, J=2.2)
7′	2.91	2.93	2.96	2.93	7.67	7.42	7.63
	(t, J=8.3)	(t, J=6.6)	(t, J=6.6)	(d, J=8.1)	(d, J=16.1)	(d, J=15.9)	(d, J=15.9)
8′	2.62	2.73	2.77	2.63	6.35	6.62	6.66
	(t, J=8.3)	(t, J=6.6)	(t, J=6.6)	(d, J=8.1)	(d, J=16.1)	(dd, J=15.9, 7.8)	(dd, J=15.9, 8.0)
7	4.58	4.55	4.57	4.55	4.58	4.57	3.45
	(br d, J=5.1)	(br d, J=6.6)	(br d, J=5.1)	(br d, J=5.1)	(br d, J=5.1)	(br d, J=6.4)	(br d, J=6.6)
8	6.02—6.18	6.02—6.14	6.01—6.17	6.01—6.19	6.02—6.18	6.01—6.18	5.98—6.13
	(m)	(m)	(m)	(m)	(m)	(m)	(m)
9	5.44	5.42	5.44	5.45	5.45	5.44	5.12
	(dd, J=17.3, 1.5)	(dd, J=17.3, 1.7)	(dd, J=17.1, 1.5)	(dd, J=17.1, 1.5)	(dd, J=17.1, 1.5)	(dd, J=17.1, 1.5)	(dd, J=17.1, 2.0)
	5.32	5.29	5.31	5.31	5.31	5.30	5.08
	(dd, J=10.5, 1.5)	(dd, J=10.5, 1.7)	(dd, J=10.5, 1.5)	(dd, J=10.5, 1.5)	(dd, J=10.5, 1.5)	(dd, J=10.5, 1.5)	(dd, J=10.1, 2.0)
OCH_2Ph				7.30	7.32	7.32	
				(s)	(s)	(s)	
OCH_2Ph				5.03	5.12	5.13	
				(s)	(s)	(s)	
CO_2Me	3.67	3.69	3.70	3.66	3.79		
	(s)	(s)	(s)	(s)	(s)		
CHO						9.64	9.65
						(d, J=7.6)	(d, J=8.0)

a) δ in $CDCl_3$; ^{1}H-NMR at 270 MHz (J=Hz). b) Measured in CD_3COCD_3.

4-Allyloxy-3′-hydroxy-4′-(2-methoxycarbonylethyl)biphenyl (8)——A 10% HCl solution (2 ml) was added to a solution of **7** (100 mg, 0.34 mmol) in THF (10 ml), and the whole was stirred at room temperature overnight, poured into ice-water, and extracted with AcOEt. The organic layer was extracted with sat. $NaHCO_3$. The $NaHCO_3$ layer was acidified with 10% HCl, and extracted with AcOEt. The AcOEt layer was washed with H_2O, dried and concentrated. Then 1% H_2SO_4 in MeOH (5 ml) was added to the residue, and the whole was refluxed for 1 h, poured into ice-water, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with sat. $NaHCO_3$ and H_2O, then dried and concentrated. The residue was recrystallized from MeOH to yield 96 mg (92%) of **8** as colorless crystals, mp 134—136 °C. IR (KBr) cm^{-1}: 3400, 1610. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1359. Found: 312.1341. *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.79; H, 6.52.

4-Allyloxy-4′-hydroxy-3′-(2-methoxycarbonylethyl)biphenyl (9)——A 10% solution of KOH in EtOH was added to a solution of **7** (100 mg, 0.34 mmol) in EtOH, and the whole was stirred at room temperature for 2 h, then poured into ice-water, and extracted with AcOEt. The reaction mixture was worked up and esterified by a procedure similar to that used for **8**. The resultant residue was recrystallized from MeOH to yield 94 mg (90%) of **9** as colorless crystals, mp 97—98 °C. IR (KBr) cm^{-1}: 3350, 1610. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1359. Found: 312.1352. *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 73.21; H, 6.52.

4-Allyloxy-2′-benzyloxy-5′-(*E*)-(2-methoxycarbonylethenyl)biphenyl (11)——DDQ (2.09 g, 9.2 mmol) was added to a solution of **10** (1.64 g, 5.3 mmol) in dry benzene, and the whole was refluxed for 24 h. The reaction mixture was cooled, and the precipitates were separated from the solution by filtration. The filtrate was concentrated, and the residue was subjected to silica gel chromatography. The eluate with 50% $CHCl_3$ in hexane gave 881 mg (54%) of **11** as colorless crystals (MeOH), mp 79—80 °C. IR (KBr) cm^{-1}: 1710, 1630, 1600. MS *m/z*: Calcd for $C_{26}H_{24}O_4$ (M^+): 400.1677. Found: 400.1650. *Anal.* Calcd for $C_{26}H_{24}O_4$: C, 77.98; H, 6.04. Found: C, 77.94; H, 6.00.

4-Allyloxy-2′-benzyloxy-5′-(*E*)-(2-formylethenyl)biphenyl (12)——Morpholine (242 mg, 2.77 mmol) in dry toluene (1 ml) was added at 0 °C under a nitrogen atmosphere to a stirred solution of SMEAH [0.3 M in toluene (Aldrich); 8.4 ml, 2.52 mmol] in dry toluene (6 ml) and the whole was stirred under the same conditions for 30 min. Then, a solution of **11** (336 mg, 0.84 mmol) in dry toluene (2 ml) was added at −15 °C under a nitrogen atmosphere to the above solution, and the whole was stirred under the same conditions for 1 h. The reaction mixture was poured into ice-water and extracted with ether. The organic layer was washed with 10% HCl and H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with $CHCl_3$ gave 234 mg (75%) of **12** as colorless crystals (ether–hexane), mp 62—63 °C. IR (KBr) cm^{-1}: 1640, 1600, 1580. MS *m/z*: Calcd for $C_{25}H_{22}O_3$ (M^+): 370.1568. Found: 370.1583. *Anal.* Calcd for $C_{25}H_{22}O_3$: C, 81.05; H, 5.99. Found: C, 81.00; H, 6.00.

3-Allyl-2′,4-dihydroxy-5′-(*E*)-(2-formylethenyl)biphenyl (2b)——A solution of **12** (50 mg, 0.14 mmol) in chlorobenzene (2 ml) was added slowly to a solution of BCl_3 (47.5 mg, 0.41 mmol) in chlorobenzene (4 ml) under a nitrogen atmosphere at −15 °C, and the whole was stirred under the same conditions for 1 h. The reaction mixture was poured into ice-water, and extracted with ether. The organic layer was washed with sat. $NaHCO_3$ and H_2O, then dried and concentrated. The residue was subjected to silica gel chromatography. The eluate with $CHCl_3$ gave 23.8 mg (63%) of magnaldehyde B (**2b**) as colorless crystals (benzene–ether), mp 161—162 °C. IR (KBr) cm^{-1}: 3420, 3250, 1650, 1610. MS *m/z*: Calcd for $C_{18}H_{16}O_3$ (M^+): 282.1098. Found: 280.1088. *Anal.* Calcd for $C_{18}H_{16}O_3$: C, 77.12; H, 5.75. Found: C, 76.75; H, 5.73.

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Synthesis of Biphenyls by Acid-Catalyzed Condensation Reactions of 1-Oxaspiro[4.5]deca-6,9-diene-2,8-dione with Phenols or Phenol-ethers

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Acid catalyzed condensation reactions of 1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (**1**) (quinol-lactone) with phenols and phenol-ethers **2a**—**i** to yield biphenyls **3a**—**i** and **4a**—**f** were found to proceed by an elimination–addition mechanism.

Keywords——condensation; acid catalysis; biphenyl; quinol-lactone; phenol; phenol-ether; elimination–addition mechanism

In the preceding paper, we reported an approach for general synthesis of biphenyl neolignans by the reaction of 1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (quinol-lactone) with the Grignard reagent, as well as a synthesis of magnaldehyde B.[1] This paper describes a synthesis of biphenyls by the acid catalyzed condensation reaction of the quinol-lactone **1** with phenols and phenol-ethers in connection with synthesis of biphenyl lignans, following the synthetic plan shown in Chart 1. This approach is attractive because of the simplicity of the reaction and its possible involvement in biosynthesis of biphenyl neolignans and related natural products.

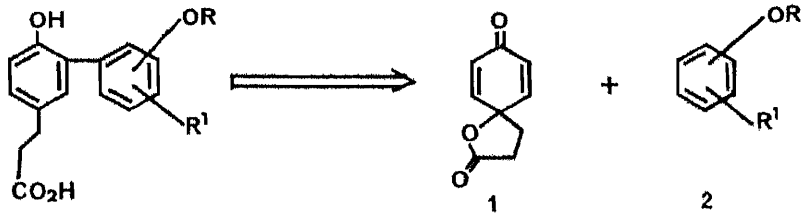
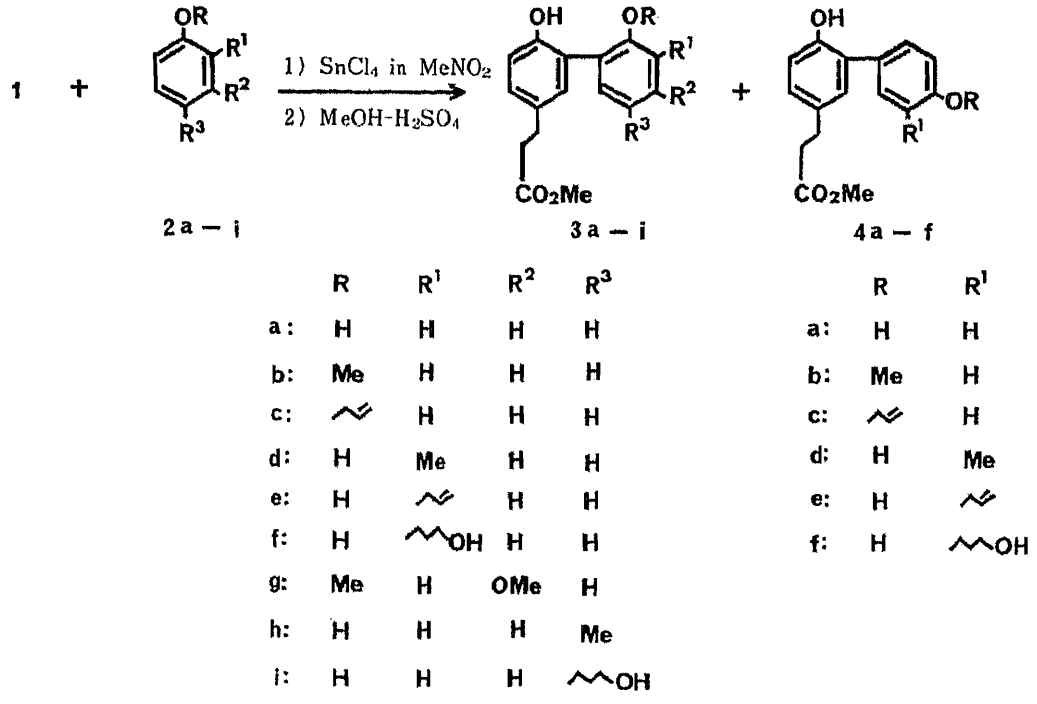


Chart 1

Preliminary experiments on the acid-catalized condensation reaction of the quinol-lactone were done with phenol and anisole as nucleophiles. Though several Lewis acids and solvents, namely, $SnCl_4$, BF_3, $AlCl_3$, *etc.*, and CH_3NO_2, CH_3CN, CH_2Cl_2, *etc.*, were investigated, the best results were obtained with $SnCl_4$ in CH_3NO_2. That is, reactions of the quinol-lactone **1** with phenol or anisole in the presence of equimolar $SnCl_4$ in CH_3NO_2 at room temperature for 2 min afforded the biphenyl acids, which were subsequently transformed to the esters **3a** and **4a** or **3b** and **4b** in yields of 60% and 5% or 61% and 6%, respectively. Similar reactions were explored using the phenols or the phenol-ethers **2c**—**i** and the results are shown in Table I.

Although these reactions may be considered to proceed by an elimination–addition mechanism (elimination of the lactone oxygen followed by addition of nucleophiles), as in the reactions of quinols with phenols or phenol-ethers in the presence of Lewis acids,[2,3] the



	R	R^1	R^2	R^3
a:	H	H	H	H
b:	Me	H	H	H
c:	~	H	H	H
d:	H	Me	H	H
e:	H	~	H	H
f:	H	~OH	H	H
g:	Me	H	OMe	H
h:	H	H	H	Me
i:	H	H	H	~OH

	R	R^1
a:	H	H
b:	Me	H
c:	~	H
d:	H	Me
e:	H	~
f:	H	~OH

Chart 2

TABLE I. Biphenyls **3a**—**i** and **4a**—**f** Formed from the Quinol-lactone **1** and Aromatic Compounds **2a**—**i**

Entry	Substrate	Product	Yield (%)[a]	mp (°C)
1	2a	3a	60	91—92
		4a	5	112—114
2	2b	3b	61	72—73
		4b	6	95—96
3	2c	3c	10	51—52
		4c	10	62—64
4	2d	3d	64	86—87
		4d	6	Oil
5	2e	3e	11	55—56
		4e	8	Oil
6	2f	3f	34	Oil
		4f	9	Oil
7	2g	3g	63	83—84
8	2h	3h	35	86—87
9	2i	3i	51	Oil

a) Yields of the biphenyl esters obtained by esterification of the biphenyl acids.

following results seem noteworthy. In the reaction of **1** with **2a**—**f**, the coupling products at the *ortho* position of the phenols and phenol-ethers predominate over the *para* coupling products, though the reason for this is not clear. In the reaction with **2c** and **2e**, the yields of the biphenyl esters **3c**, **4c** and **3e**, **4e** are very poor, but the yields of **3f** and **4f** in reaction with **2f** are reasonable. This may be due to the occurrence of a Diels–Alder type reaction between the dienone of **1** and the olefinic double bond of **2c** and **2e**.[4]

The structures of **3a**—**i** and **4a**—**f** were confirmed by analyses of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra, as was done in the preceding report.[1] The

TABLE II. ^{13}C-NMR Spectral Data for **3a—i** and **4a—f**[a]

Carbon No.	**3a**	**3b**	**3c**	**3d**	**3e**	**3f**	**4a**	**4b**
1′	125.4 s	126.2 s (or C-1)	126.3 s (or C-1)	124.4 s (or C-1)	124.0 (or C-1)	126.6 s (or C-1)	127.9 s	127.8 s
2′	151.1 s	152.2 s	152.2 s	151.2 s (or C-2)	151.1 s (or C-2)	151.7 s	151.0 s	151.1 s
3′	116.9 d (or C-3)	117.4 d	117.5 d	116.4 d	116.5 d	116.8 d	115.8 d	115.8 d
4′	129.2 d (or C-4)	128.9 d (or C-4)	128.9 d (or C-4)	128.8 d (or C-4)	129.4 d	128.7 d (or C-4)	128.4 d	128.4 d
5′	133.1 s	132.9 s	132.7 s	133.2 s	133.3 s	132.6 s	132.6 s	132.6 s
6′	130.0 d (or C-6)	131.0 d (or C-6)	131.0 d (or C-6)	130.9 d (or C-6)	130.2 d	129.7 d (or C-6)	130.0 d	130.6 d
7′	30.1 t	30.2 t	30.2 t	30.0 t	30.1 t	30.1 t	30.1 t	30.2 t
8′	35.9 t	35.9 t	36.0 t	35.9 t	35.9 t	35.9 t	35.9 t	36.1 t
9′	174.4 s	173.3 s	173.4 s	174.1 s	173.8 s	173.3 s	173.9 s	173.5 s
CO_2Me	51.8 q	51.4 q	51.6 q	51.8 q	51.8 q	51.5 q	51.9 q	51.6 q
1	125.4 s	127.1 s (or C-1′)	127.8 s (or C-1′)	124.6 s (or C-1′)	124.1 s (or C-1′)	126.7 s (or C-1′)	129.2 s	129.4 s
2	152.6 s	155.6 s	154.6 s	151.4 s (or C-2′)	151.3 s (or C-2′)	151.7 s	130.4 d	130.2 d
3	116.8 d (or C-3′)	111.6 d	113.6 d	126.0 s	127.6 s	129.8 s	116.1 d	114.5 d
4	131.3 d (or C-4′)	129.2 d (or C-4′)	129.1 d (or C-4′)	129.3 d (or C-4′)	129.4 d	129.3 d (or C-4′)	155.6 s	159.2 s
5	124.4 d	121.9 d	122.4 d	121.0 d	121.2 d	120.9 d	116.1 d	114.5 d
6	131.5 d (or C-6′)	132.3 d (or C-6′)	132.5 d (or C-6′)	131.4 d (or C-6′)	130.2 d	131.5 d (or C-6′)	130.4 d	130.2 d
7			70.1 t		34.9 t	26.1 t		
8			132.3 d		136.6 d	32.6 t		
9			118.2 t		116.1 t	61.0 t		
Ar-OMe		56.0 q						55.3 q
Ar-Me				16.3 q				

Carbon No.	**4c**	**4d**	**4e**	**4f**	**3g**	**3h**	**3i**
1′	127.8 s	128.0 s	128.0 s	128.3 s	139.3 s	124.4 s (or C-1)	125.1 s (or C-1)
2′	151.0 s	150.9 s	151.1 s	151.0 s	154.1 s	151.4 s (or C-2)	151.3 s (or C-2)
3′	115.8 d	115.7 d (or C-5)	116.4 d	117.0 d	117.8 d	116.4 d	117.0 d (or C-3)
4′	128.4 d	129.0 d	128.4 d	129.5 d	129.3 d	129.3 d (or C-4)	129.2 d (or C-4)
5′	132.6 s	132.4 s	132.6 s	132.6 s	131.3 s	133.2 s	133.1 s (or C-5)
6′	129.5 d	129.7 d	130.0 d	130.0 d	131.1 d	131.2 d (or C-6)	131.2 d
7′	30.2 t	30.1 t	30.2 t	29.7 t	27.8 t	30.1 t	30.1 t
8′	36.0 t	35.9 t	36.1 t	36.1 t	35.3 t	35.9 t	35.9 t
9′	173.4 s	174.1 s	173.8 s	173.6 s	174.4 s	173.8 s	173.8 s
CO_2Me	51.6 q	51.7 q	51.7 q	51.7 q	51.6 q	51.7 q	51.7 q
1	130.0 s	130.0 s	129.4 s	129.3 s	122.9 s	124.1 s (or C-1′)	125.4 s (or C-1′)
2	130.2 s	131.5 d	131.0 d	131.3 d	157.3 s	151.2 s (or C-2′)	150.9 s (or C-2′)

TABLE II. (continued)

Carbon No.	4c	4d	4e	4f	3g	3h	3i
3	115.4 d	127.6 s	126.7 s	128.0 s	98.6 d	120.9 d	116.7 d (or C-3′)
4	158.3 s	153.9 s	154.0 s	154.6 s	160.3 s	128.6 d (or C-4′)	129.0 d (or C-4′)
5	115.4 d	115.5 d (or C-3′)	115.8 d	115.7 d	104.4 d	125.9 s	134.4 s (or C-5′)
6	130.2 d	128.2 d	128.4 d	128.2 d	114.5 d	130.9 d (or C-6′)	131.2 d
7	68.9 t		34.9 t	25.4 t			31.1 t
8	133.1 d		136.1 d	32.1 t			34.0 t
9	117.7 t		116.6 t	60.9 t			62.1 t
Ar-OMe					55.3 q (×2)		
Ar-Me		15.9 q				16.3 q	

a) Spectra were measured at 25 MHz at 25 °C; δ in $CDCl_3$; s, singlet; d, doublet; t, triplet; q, quartet.

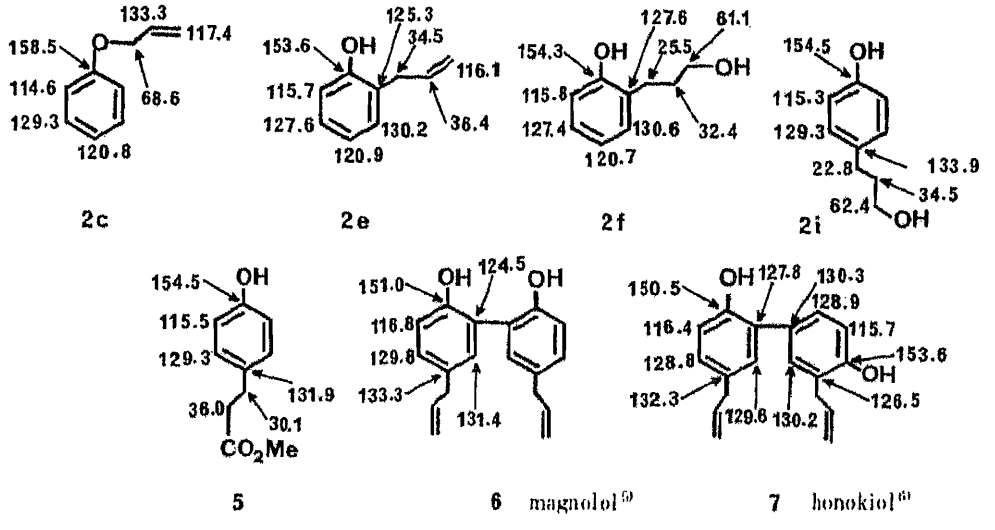


Chart 3. ^{13}C-NMR Spectra of **2c**, **2e**, **2f**, **2i**, **5** and **7** were Measured in $CDCl_3$ at 25 °C (25 MHz)

assignments of the *ortho-ortho* coupling biphenyls **3a—i** were aided by off-resonance decoupling and by comparison with the spectra of the reference compounds **2c**, **2e**, **2i**, **2f**, **5** and **6**[5] (Chart 3 and Table II). In the case of **3c**, differences of the carbon signals between the biphenyl esters **3c** and the reference compounds **5** and **2c** were observed only at the C-1′ and C-1 carbons. That is, the C-1′ and C-1 signals (at 126.3 and 127.8 ppm) of **3c** were observed at lower field than the corresponding signals, 115.5 and 114.6 ppm, of **5** and **2c**, respectively. This result is in reasonable agreement with the previous report,[6] which indicated that the signals of C-1′ and C-1 of **3c** may be shifted about 11—13 ppm to lower field than those of C-2 of **5** and **2c**. Thus, **3c** was confirmed to be the coupling product at the C-2 positions of **5** and **2c**. The structures of **3a** and **3b** were elucidated in the same manner as that of **3c**. The structures of **3d**, **3e**, and **3f** were determined by comparison of the spectra with those of the reference compounds **2e**, **2f** and **5**. The structures of **3g**, **3h** and **3i** were also confirmed by comparison of the spectra with those of the reference compounds **2i**, **5** and **6**.

TABLE III. ^{1}H-NMR Spectral Data for **3a—i** and **4a—f**[a)]

Proton	**3a**	**3b**	**3c**	**3d**	**3e**
3′	6.85 (d, J=8.3)	6.93 (d, J=8.1)	6.95 (d, J=8.1)	6.80 (d, J=8.3)	6.94 (d, J=7.8)
4′	6.92—7.09 (m)	6.97—7.12 (m)	7.08—7.14 (m)	6.99 (dd, J=8.3, 2.0) 7.05 (d, J=2.0)	7.15 (dd, J=7.8, 1.7) 7.12 (br s)
6′					
4	7.18—7.26 (m)	7.29—7.40 (m)	7.31—7.39 (m)	7.04 (br s, J=7.6)	7.22 (dd, J=7.6, 1.7)
2					
6	7.18—7.26 (m)	7.29—7.40 (m)	7.31—7.39 (m)	7.12 (br d, J=7.6)	7.14 (d, J=1.7)
3	6.94 (br d, J=7.8)	7.01 (br d, J=8.3)	7.03 (br d, J=8.1)		
5	6.92—7.09 (m)	6.97—7.12 (m)	7.08—7.14 (m)	6.88 (t, J=7.6)	7.00 (t, J=7.6)
7′	2.90 (t, J=8.1)	2.91 (t, J=8.1)	2.93 (t, J=8.3)	2.85 (t, J=8.1)	2.94 (t, J=7.6)
8′	2.60 (t, J=8.1)	2.61 (t, J=8.1)	2.63 (t, J=8.3)	2.56 (t, J=8.1)	2.65 (t, J=7.6)
7			4.60 (d, J=5.1)		3.52 (d, J=6.8)
8			5.90—6.04 (m)		6.01—6.18 (m)
9			5.22—5.36 (m)		5.12—5.22 (m)
CO_2Me	3.64 (s)	3.64 (s)	3.67 (s)	3.62 (s)	3.69 (s)
Ar-OMe		3.84 (s)			
Ar-Me					
Ar-OH		6.24 (s)	6.33 (s)	6.52 (br s)	5.98 (br s)

Proton	**3f**	**4a**	**4b**	**4c**	**4d**
3′	6.88 (d, J=8.1)	6.86 (d, J=8.8)	6.87 (d, J=8.8)	6.88 (d, J=8.8)	6.84 (d, J=9.1)
4′	7.06 (dd, J=8.1, 2.2)	7.03 (dd, J=8.8, 2.2)	7.04 (dd, J=8.8, 2.2)	7.05 (dd, J=8.8, 2.2)	7.03 (dd, J=9.1, 2.2)
6′	7.10—7.17 (m)	7.01 (br s)	7.04 (d, J=2.2)	7.04 (br s)	7.02 (br s)
4					
2		7.29	7.38	7.37	7.19 (d, J=2.0)
6	7.10—7.17 (m)	(d, J=8.8)	(d, J=8.8)	(d, J=8.8)	7.12 (dd, J=9.1, 2.0)
3		6.91	6.99	7.02	
5	6.95 (t, J=7.8)	(d, J=8.8)	(d, J=8.8)	(d, J=8.8)	6.87 (d, J=9.1)
7′	2.91 (t, J=8.1)	2.90 (t, J=8.3)	2.90 (t, J=8.1)	2.91 (t, J=8.3)	2.90 (t, J=8.1)
8′	2.61 (t, J=8.1)	2.62 (t, J=8.3)	2.62 (t, J=8.1)	2.62 (t, J=8.3)	2.63 (t, J=8.1)

TABLE III. (continued)

Proton	3f	4a	4b	4c	4d
7	2.81 (t, J=6.8)			4.58 (d, J=5.1)	
8	1.82—1.92 (m)			6.01—6.18 (m)	
9	3.58 (t, J=6.1)			5.30—5.45 (m)	
CO_2Me	3.66 (s)	3.67 (s)	3.66 (s)	3.67 (s)	3.68 (s)
Ar-OMe			3.84 (s)		
Ar-Me					2.28 (s)
Ar-OH	4.80 (s) 3.30, (s)	5.14 (s) 5.59 (s)	5.32 (s)	5.20 (s)	5.70 (s) 5.35 (s)

Proton	4e	4f	3g	3h	3i
3′	6.87 (d, J=9.1)	6.88 (d, J=8.8)	7.07 (d, J=8.3)	6.92 (d, J=8.1)	6.84 (d, J=8.1)
4′	7.04 (dd, J=9.0, 2.2)	7.05 (dd, J=8.8, 2.4)	6.72 (dd, J=8.3, 2.7)	7.11 (dd, J=8.1, 2.2)	6.99—7.09 (m)
6′	7.02 (br s)	7.03 (br s)	6.62 (d, J=2.7)	7.04—7.09 (m)	7.05 (br s)
4				7.15—7.21 (m)	6.99—7.09 (m)
2	7.19 (br s)	7.19 (br s)			
6	7.17 (dd, J=9.0, 1.7)	7.17 (dd, J=8.8, 2.2)	7.02 (d, J=9.0)	7.04—7.09 (m)	7.08 (br s)
3			6.54 (d, J=2.2)	6.94 (d, J=7.6)	6.88 (d, J=8.3)
5	6.88 (d, J=9.0)	6.93 (d, J=8.8)	6.52 (dd, J=9.9, 2.2)		
7′	2.90 (t, J=8.1)	2.90 (t, J=8.1)	2.69 (t, J=8.8)	2.91 (t, J=7.8)	2.90 (t, J=7.8)
8′	2.63 (t, J=8.1)	2.62 (t, J=8.1)	2.37 (t, J=8.8)	2.61 (t, J=7.8)	2.58—2.65 (m)
7	3.44 (d, J=6.6)	2.81 (t, J=7.1)			
8	5.95—6.10 (m)	1.85—1.95 (m)			1.78—1.92 (m)
9	5.15—5.22 (m)	3.65 (t, J=5.7)			3.63 (t, J=6.4)
CO_2Me	3.67 (s)	3.67 (s)	3.70 (s)	3.66 (s)	3.65 (s)
Ar-OMe			3.58 (s) 3.83 (s)		
Ar-Me				2.31 (s)	
Ar-OH		5.30 (s)	5.80 (s)	5.72 (br s)	5.30 (s)

a) Spectra were measured at 270 MHz (J=Hz); δ in $CDCl_3$.

Similarly, the structures of *ortho–para* coupling biphenyls **4a**, **4f** were assigned by comparison with the ^{13}C-NMR spectra of the reference compounds **5**, **2c**, **2e**, **2f**, **2i** and **7** in the same way as described in the preceding paper.[1]

These results provide a facile synthetic method for biphenyls which have a propionic acid residue, and some of the synthesized biphenyls, for example **3i**, may also be transformable to natural biphenyl neolignans in the same way as described for the synthesis of magnaldehyde B.[1]

Experimental

All melting point are uncorrected. Infrared (IR) spectra were recorded with a Hitachi 260-10 spectrometer, ^{1}H- and ^{13}C-NMR spectra with a JEOL JNM-FX 100 or JEOL JNM-GX 270 spectrometer (with tetramethylsilane as an internal standard; $CDCl_3$ solvent) and mass spectra (MS) with a JEOL JMS-D 300 spectrometer. Elementary analyses were done by Ms. A. Sakamoto and Ms. M. Takeda, Kissei Pharmaceutical Company, Matsumoto, Japan. Wako Silica Gel C-200 (200 mesh), Florisil (100 mesh), and Merck Kieselgel 60 F_{254} were used for column chromatography and thin-layer chromatography (TLC), respectively.

General Procedure for the Preparation of 3a—i and 4a—f——$SnCl_4$ (207 mg, 0.8 mmol) was added to a solution of 1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (**1**) (100 mg, 0.61 mmol) and an aromatic compound **2a—i** (2.4 mmol) in $MeNO_2$ (6 ml), and the whole was stirred for 2 min, then poured into ice-water, and the solvent was removed under reduced pressure. The residue was extracted with AcOEt. The organic layer was extracted with sat. $NaHCO_3$. The $NaHCO_3$ layer was acidified with 10% HCl, and then extracted with AcOEt. The AcOEt layer was washed with H_2O, dried and concentrated. A 1% solution of H_2SO_4 in MeOH (5 ml) was added to the residue, and the whole was refluxed for 1 h, poured into ice-water, and then extracted with $CHCl_3$. The $CHCl_3$ layer was washed with sat. $NaHCO_3$ and H_2O, dried and concentrated. The residue was subjected to silica gel chromatography using ether–petroleum ether (3 : 1; for **3a** and **4a**), $CHCl_3$ (for **3b** and **4b**, **3c** and **4c**), $CHCl_3$–AcOEt (8 : 1; for **3d** and **4d**, **3e** and **4e**), $CHCl_3$–AcOEt (49 : 1; for **3g** and **3h**), $CHCl_3$–AcOEt (2 : 1; for **3f** and **4f**), and $CHCl_3$–AcOEt (4 : 1; for **3i**). Yields and physical data of **3a—i** and **4a—f** are listed in Tables I, II, and III.

2,2′-Dihydroxy-5′-(2-methoxycarbonylethyl)biphenyl (3a) and 2′,4-Dihydroxy-5′-(2-methoxycarbonylethyl) biphenyl (4a)——These compounds were prepared from **1** and **2a**. **3a** (ether–hexane): IR (KBr) cm^{-1}: 3150, 1725. MS *m/z*: Calcd for $C_{16}H_{16}O_4$ (M^+): 272.1045. Found: 272.1038. *Anal.* Calcd for $C_{16}H_{16}O_4$: C, 70.58; H, 5.92. Found: C, 70.48; H, 6.04. **4a** (ether–hexane): IR (KBr) cm^{-1}: 3250, 1710. MS *m/z*: Calcd for $C_{16}H_{16}O_4$ (M^+): 272.1045. Found: 272.1037. *Anal.* Calcd for $C_{16}H_{16}O_4$: C, 70.58; H, 5.92. Found: C, 70.48; H, 6.04.

2′-Hydroxy-2-methoxy-5′-(2-methoxycarbonylethyl)biphenyl (3b) and 2′-Hydroxy-4-methoxy-5′-(2-methoxy-carbonylethyl)biphenyl (4b)——These were prepared from **1** and **2b**. **3b** (ether–hexane): IR (KBr) cm^{-1}: 3440, 1700. MS *m/z*: Calcd for $C_{17}H_{18}O_4$ (M^+): 286.1206. Found: 286.1213. *Anal.* Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.24; H, 6.46. **4b** (ether–hexane): IR (KBr) cm^{-1}: 3425, 1700. MS *m/z*: Calcd for $C_{17}H_{18}O_4$ (M^+): 286.1206. Found: 286.1187. *Anal.* Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.02; H, 6.41.

2-Allyloxy-2′-hydroxy-5′-(2-methoxycarbonylethyl)biphenyl (3c) and 4-Allyloxy-2′-hydroxy-5′-(2-methoxy-carbonylethyl)biphenyl (4c)——These were prepared from **1** and **2c**. **3c** (ether–hexane): IR (KBr) cm^{-1}: 3380, 1700. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1359. Found: 312.1317. **6c** (ether–hexane): IR (KBr) cm^{-1}: 3350, 1700. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1359. Found: 312.1344. *Anal.* Calcd for $C_{19}H_{20}O_4$: C, 73.06; H, 6.45. Found: C, 72.96; H, 6.55.

2,2′-Dihydroxy-3-methyl-5′-(2-methoxycarbonylethyl)biphenyl (3d) and 2′,4-Dihydroxy-3-methyl-5′-(2-methoxycarbonylethyl)biphenyl (4d)——These were prepared from **1** and **2d**. **3d** (ether–hexane): IR (KBr) cm^{-1}: 3150, 1730. MS *m/z*: Calcd for $C_{17}H_{18}O_4$ (M^+): 286.1206. Found: 286.1218. *Anal.* Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.23; H, 6.40. **4d**: liq. IR (film) cm^{-1}: 3400, 1710. MS *m/z*: Calcd for $C_{17}H_{18}O_4$ (M^+): 286.1196. Found: 286.1196.

3-Allyl-2,2′-dihydroxy-5′-(2-methoxycarbonylethyl)biphenyl (3e) and 3-Allyl-2′,4-dihydroxy-5′-(2-methoxy-carbonylethyl)biphenyl (4e)——These were prepared from **1** and **2e**. **3e** (benzene–hexane): IR (KBr) cm^{-1}: 3100, 1730. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1362. Found: 312.1394. **4e**: liq. IR (film) cm^{-1}: 3350, 1710. MS *m/z*: Calcd for $C_{19}H_{20}O_4$ (M^+): 312.1362. Found: 312.1396.

2,2′-Dihydroxy-3-(3-hydroxypropyl)-5′-(2-methoxycarbonylethyl)biphenyl (3f) and 2′,4-Dihydroxy-3-(3-hydroxypropyl)-5′-(2-methoxycarbonylethyl)biphenyl (4f)——These were prepared from **1** and **2f**. **3f**: liq. IR (film) cm^{-1}: 3250, 1720. MS *m/z*: Calcd for $C_{19}H_{22}O_5$ (M^+): 330.1465. Found: 330.1453. **4f**: liq. IR (film) cm^{-1}: 3350, 1710. MS *m/z*: Calcd for $C_{19}H_{22}O_5$ (M^+): 330.1465. Found: 330.1496.

2′-Hydroxy-3,4-dimethoxy-5′-(2-methoxycarbonylethyl)biphenyl (3g)——This was prepared from **1** and **2g**. **3g** (benzene–hexane): IR (KBr) cm^{-1}: 3400, 1700. MS *m/z*: Calcd for $C_{19}H_{20}O_5$ (M^+): 316.1355. Found: 316.1310.

2,2′-Dihydroxy-5-methyl-5′-(2-methoxycarbonylethyl)biphenyl (3h)——This was prepared from **1** and **2h**. **3h**

(ether–hexane): IR (KBr) cm^{-1}: 3150, 1730. MS *m/z*: Calcd for $C_{17}H_{18}O_4$ (M^+): 286.1205. Found: 286.1238. *Anal.* Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.34; H, 6.36.

2,2′-Dihydroxy-5-(3-hydroxypropyl)-5′-(2-methoxycarbonylethyl)biphenyl (3i)——This was prepared from **1** and **2i**. **3i**: liq. IR (film) cm^{-1}: 3300, 1720. MS *m/z*: Calcd for $C_{19}H_{22}O_5$ (M^+): 330.1465. Found: 330.1469.

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Reactions of Sodium Borohydride. IV.[1] Reduction of Aromatic Sulfonyl Chlorides with Sodium Borohydride

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Aromatic sulfonyl chlorides were reduced with sodium borohydride in tetrahydrofuran at 0 °C to the corresponding sulfinic acids in good yields. Further reduction proceeded when the reaction was carried out under reflux in tetrahydrofuran to give disulfide and thiophenol derivatives *via* sulfinic acid. Furthermore, sulfonamides were reduced with sodium borohydride by heating directly to give sulfide, disulfide and thiophenol derivatives, and diphenyl sulfone was reduced under similar conditions to give thiophenol and biphenyl.

Keywords——reduction; sodium borohydride; aromatic sulfonyl chloride; sulfonamide; sulfone; aromatic sulfinic acid; disulfide; sulfide; thiophenol

Sodium borohydride ($NaBH_4$) itself is a relatively mild reducing agent which is extensively used for the selective reduction of the carbonyl group of ketone, aldehyde and (carboxylic) acid halide derivatives. In the previous papers, we reported that $NaBH_4$ can reduce some functional groups, such as carboxylic anhydrides,[2] carboxylic acids[3] and nitro compounds.[1] As a continuation of these studies, in the present paper, we wish to report the reduction of aromatic sulfonyl chlorides, sulfinic acids and sulfonamides with $NaBH_4$.

Many methods have been reported for the reduction of sulfonyl chlorides to sulfinic acids, such as the use of sodium sulfite,[4a-d] zinc,[5] electrolytic reduction,[6] catalytic reduction,[7] magnesium,[8] sodium amalgam,[9] sodium hydrogen sulfite,[10] stannous chlo-

TABLE I. Reduction of Aromatic Sulfonyl Chlorides with $NaBH_4$ in THF (0 °C)

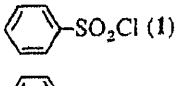
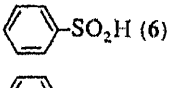
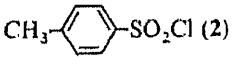
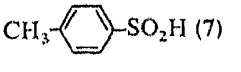
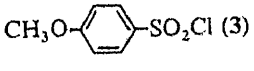
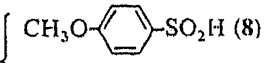
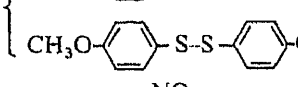
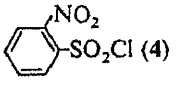
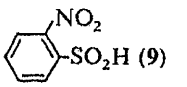
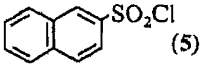
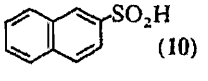
Compound (No.)	$NaBH_4$ (mol eq)	Temp. (°C)	Time (h)	Product (No.)	Yield (%)
 (1)	8.0	0	1	 (6)	78.6
 (2)	5.0	0	1	 (7)	84.5
 (3)	4.0	0	1	 (8)  (11)	79.0 5.2
 (4)	5.0	0	1	 (9)	78.2
 (5)	7.0	0	1	 (10)	70.4

TABLE II. Reduction of Aromatic Sulfonyl Chlorides with $NaBH_4$ in THF (under Reflux)

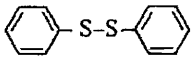
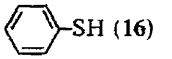
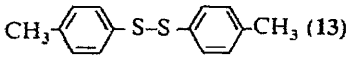
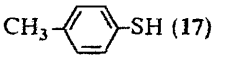
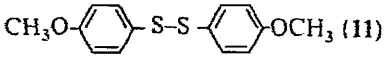
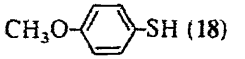
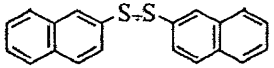
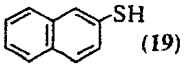
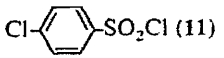
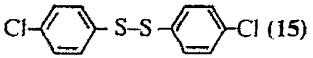
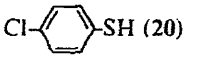
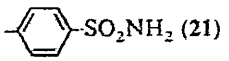
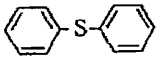
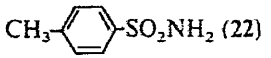
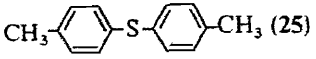
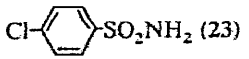
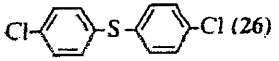
Compound (No.)	$NaBH_4$ (mol eq)	Temp.	Time (h)	Product			
				Disulfide (No.)	Yield (%)	Thiophenol (No.)	Yield (%)
1	8.0	Reflux	4	 (12)	52.0	 (16)	11.2
2	5.0	Reflux	2	 (13)	30.9	 (17)	27.0
2	10.0	Reflux	4	13	60.2	17	29.7
3	6.0	Reflux	2	 (11)	34.6	 (18)	27.1
5	10.0	Reflux	4	 (14)	16.5	 (19)	68.9
 (11)	12.0	Reflux	10	 (15)	39.8	 (20)	41.9

TABLE III. Reduction of Sulfonamides (21—23) with $NaBH_4$ in the Absence of a Solvent

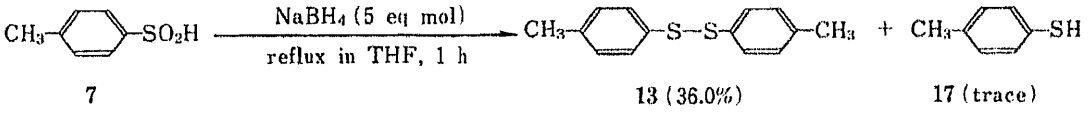
Compound (No.)	$NaBH_4$ (mol eq)	Temp. (°C)	Time (h)	Product					
				Sulfide (No.)	Yield (%)	Disulfide No.	Yield (%)	Thiophenol No.	Yield (%)
 (21)	4.0	300—320	5	 (24)	43.4	12	14.4	16	Trace
 (22)	4.0	260—280	2	 (25)	14.7	13	15.6	17	15.3
 (23)	4.0	260—300	5	 (26)	25.5	15	Trace	20	Trace

ride,[11] and sodium sulfide.[12] Lithium aluminum hydride reduces some sulfonyl chlorides under controlled conditions to sulfinic acids,[13a,b] but if an excess of this hydride is used, thiols are obtained. Brown and Subba Rao[14] reported that sulfonyl chlorides were reduced to thiols with $NaBH_4$–aluminum trichloride. However, the reduction of sulfonyl chloride to sulfinic acid with $NaBH_4$ alone has not been reported previously.

As shown in Table I, aromatic sulfonyl chlorides (**1**—**5**) were reduced with $NaBH_4$ alone in tetrahydrofuran (THF) at 0 °C to give the corresponding sulfinic acids (**6**—**10**) in good yields. In these reductions, small amounts of the corresponding disulfide and thiophenol derivatives were obtained as by-products.

Under more severe conditions (refluxing in THF), the reduction proceeded further to give the corresponding disulfides (**11**—**15**) and thiophenols (**16**—**20**), as shown in Table II.

p-Toluenesulfinic acid (**7**) was reduced with $NaBH_4$ under reflux in THF to give di-*p*-tolyl disulfide (**13**) in 36% yield and a trace of *p*-methylthiophenol (**17**). From the results described above, it is assumed that sulfonyl chlorides were reduced to disulfides and thiophenols *via* sulfinic acids. Therefore, the sequence of reduction of sulfonyl chlorides to thiophenols is considered to be as shown in Chart 1.



7 13 (36.0%) 17 (trace)

reduction pathway

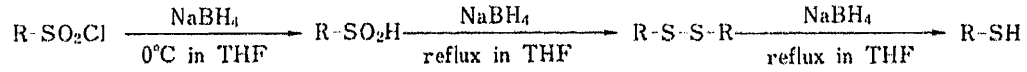


Chart 1

As described above, the reductions of sulfonyl chloride and sulfinic acid proceeded with $NaBH_4$ alone under moderately mild conditions. However, aromatic sulfonamide and sulfone derivatives did not react with $NaBH_4$ under similar conditions. Thus, we examined the reaction of these derivatives on heating directly at 200—320 °C with $NaBH_4$.

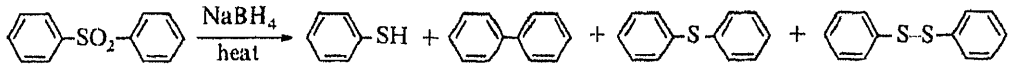
As shown in Table III, aromatic sulfonamide derivatives (**21**—**23**) were reduced by $NaBH_4$ on heating directly at 260—320 °C to give the corresponding sulfides (**24**—**26**), disulfides and thiophenols.

Furthermore, diphenyl sulfone (**27**) was reduced similarly by $NaBH_4$ on heating directly at 200—280 °C to give thiophenol **16** and biphenyl (**28**) with traces of the disulfide **12** and the sulfide **24**. As shown in Table IV, when the reaction temperature was raised gradually from 200 °C to 280 °C, thiophenol **16** was formed in a good yield, but the yield of **16** decreased at temperatures above 250 °C. Taking into account the formation of biphenyl **28** from the sulfone **27**, it is assumed that this reaction proceeded by a radical mechanism to give sulfone and phenyl radicals.

Sulfones are known to undergo loss of sulfur dioxide upon thermolysis with formation of a carbon–carbon bond between the atoms originally attached to sulfur.[15a–c] Da Silva Correa and Waters[16] reported that aromatic sulfonyl radicals afforded aryl thiosulfonates, and Koch *et al.*[17] suggested that aryl thiosulfonates decompose into aryl-SO_2· and aryl-S·, and aryl-S· to give aryl disulfides. Generally, $NaBH_4$ reduces disulfides in excellent yield.[18a–c]

It is assumed that the reactions of sulfonamides and sulfone, as described above, proceeded by a free radical mechanism since the reaction temperature was high and the products were accompanied with coupling products of radicals, such as disulfides and biphenyl. On the other hand, the sulfonamide **21** or the sulfonyl chloride **1** was heated directly at 300 °C to give only traces of biphenyl **28**, the sulfide **24** and the disulfide **12**, respectively.

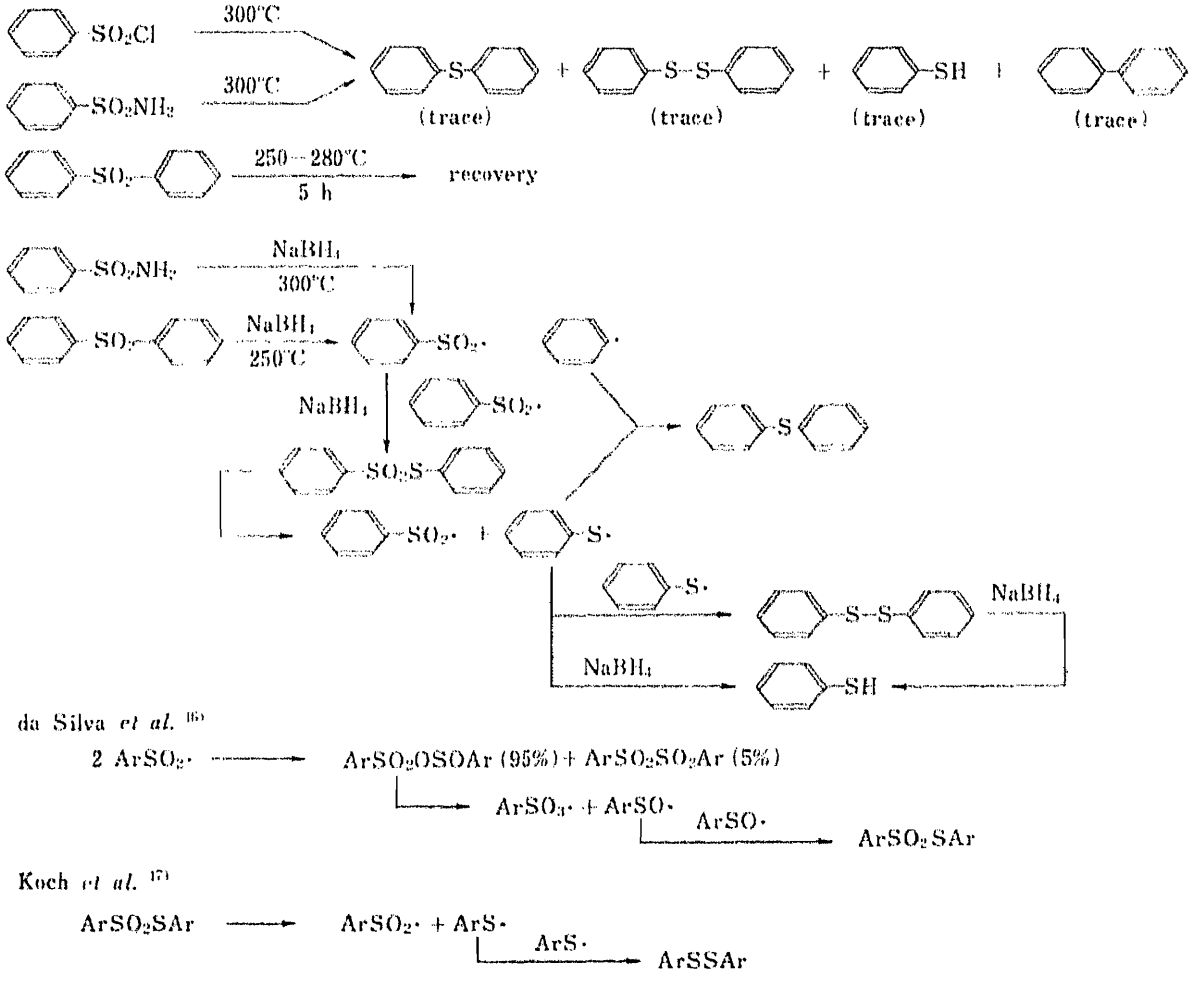
TABLE IV. Reduction of Diphenyl Sulfone (27) with $NaBH_4$ in the Absence of a Solvent



27 16 28 24 12

$NaBH_4$ (mol eq)	Temp. (°C)	Time (h)	Solvent	Yield (%) 16	28	24	12
6.0	200—280	5	None	79.4	19.2	Trace	Trace
6.0	250—280	3	None	33.8	20.8	Trace	Trace

Therefore, it is assumed that $NaBH_4$ accelerated these radical reactions. The sulfur–nitrogen bond of a sulfonamide would be easily cleaved by $NaBH_4$ at temperatures above 200 °C to afford a sulfone radical, and this radical would be reduced with $NaBH_4$ to give disulfide, sulfide and thiophenol derivatives. However, presumably, thiophenols would be obtained *via* disulfides since the similar reaction of sulfides did not yield thiophenols. Since the starting sulfone **27** was recovered unchanged after heating alone at 250—280 °C for 5 h, the carbon sulfur bond of the sulfone **27** seems to be cleaved by $NaBH_4$ at temperatures above 250 °C to give sulfone and phenyl radicals, and the phenyl radicals would afford biphenyl. As an example of a radical reaction in the presence of $NaBH_4$, we reported that alcohols reacted



da Silva *et al.* [16]

Koch *et al.* [17]

Chart 2

with $NaBH_4$ at 300 °C to give the coupling olefins, and these olefins were reduced under similar conditions to afford the corresponding alkanes.[19]

It is noteworthy that aromatic sulfonyl chlorides were reduced with $NaBH_4$ to the corresponding aromatic sulfinic acids under mild conditions (in THF at 0 °C) in good yields, and aromatic sulfonamides and diphenyl sulfone were reduced to the corresponding sulfides, disulfides and thiophenols on heating directly with $NaBH_4$.

Experimental

Commercial $NaBH_4$ was used throughout this work, and THF was distilled from lithium aluminum hydride (a small excess over that required to react with active hydrogen impurities). Melting points were determined on a Yanagimoto micro-melting point apparatus, model MP-S3, and are uncorrected. Infrared spectra (IR) were measured in Nujol mulls or as liquid films with a JASCO A-100 (Nihon Bunko) infrared spectrometer, and ultraviolet spectra (UV) were recorded on a JASCO Uvidec-505 ultraviolet spectrometer. Gas chromatography was done on a JEOL JGC-20K gas chromatograph.

Reduction of Aromatic Sulfonyl Chlorides (1—5)——A) The procedure for the reduction of benzenesulfonyl chloride (**1**) with $NaBH_4$ is described in detail as a typical example. Compound **1** (0.88 g, 5 mmol) was dissolved in THF (30 ml), and $NaBH_4$ (1.51 g, 40 mmol) was added in portions with stirring at 0 °C. After removal of THF, 10 ml of water was added and the mixture was acidified by the addition of 10% hydrochloric acid. The acidic solution was extracted with chloroform, and the chloroform solution was dried over anhydrous magnesium sulfate. After removal of chloroform, the residue was recrystallized from water to give 0.56 g (78.6%) of benzenesulfinic acid (**6**) as colorless prisms, mp 83—84 °C (lit.[20] mp 83—84 °C). This product was identical with an authentic sample on the basis of mixed melting point determination and comparisons of IR and UV spectra.

The following products were similarly obtained, and their yields are listed in Table I; *p*-toluenesulfinic acid (**7**) as colorless needles (from water), mp 84—85 °C (lit.[21] mp 84 °C), *p*-methoxybenzenesulfinic acid (**8**) as colorless needles (from water), mp 96 °C (lit.[22] mp 94—96 °C), bis(*p*-methoxyphenyl) disulfide (**11**) as colorless needles (from ethanol), mp 44—45 °C (lit.[23] mp 44 °C), *o*-nitrobenzenesulfinic acid (**9**) as colorless needles (from ethanol–water), mp 128—129.5 °C (lit.[24] mp 130 °C), and 2-naphthalenesulfinic acid (**10**) as colorless needles (from water), mp 102—104 °C (lit.[25] mp 104 °C). Products **7**—**10** were identical with authentic samples prepared from the corresponding sulfonyl chlorides according to a published procedure[26] on the basis of mixed melting point determination and comparisons of IR and UV spectra.

B) Compound **1** (0.88 g, 5 mmol) was dissolved in THF (30 ml), and $NaBH_4$ (1.51 g, 40 mmol) was added in portions with stirring at room temperature for 10 min, then the reaction mixture was refluxed for 4 h. After removal of THF, 20 ml of water was added to the residue, and the aqueous layer was extracted with ether. The extract was dried over anhydrous magnesium sulfate, and the ether was evaporated off. The residue was recrystallized from ethanol to give 284 mg (52.0%) of diphenyl disulfide (**12**) as colorless needles, mp 61 °C (lit.[27] mp 60 °C). This product was identical with an authentic sample on the basis of mixed melting point determination and comparisons of IR and UV spectra. The aqueous layer was acidified with 10% hydrochloric acid, and extracted with ether. The extract was dried over anhydrous magnesium sulfate. After removal of the ether, the residue was distilled under reduced pressure to give 62 mg (11.2%) of thiophenol (**16**), bp 70—71 °C (15 mmHg) (lit.[28] bp 68 °C (20 mmHg)). All spectral data of **16** were identical with those of an authentic sample.

Sulfonyl chlorides (**2**—**5**) and **11** were reduced similarly to give the following products, respectively (the reaction conditions and results are listed in Table II); bis(*p*-tolyl) disulfide (**13**) as colorless needles (from ethanol), mp 47—47.5 °C (lit.[29] mp 47—48 °C), bis(*p*-methoxyphenyl) disulfide (**11**) as colorless needles (from ethanol), mp 42—43 °C (lit.[23] mp 42—44 °C), bis(2-naphthyl)disulfide (**14**) as light yellow needles (from ethyl acetate), mp 137—138 °C (lit.[30] mp 137—138.5 °C), bis(*p*-chlorophenyl) disulfide (**15**) as colorless needles (from ethanol), mp 70—72 °C (lit.[30] mp 70—72 °C). Products **11**—**15** were identical with the corresponding authentic samples on the basis of mixed melting point determination and comparison of IR and UV spectra. The authentic samples were prepared from the corresponding thiophenol derivatives according to a published procedure.[31] *p*-Methylthiophenol (**17**) bp 90—92 °C (15 mmHg) (lit.[32] bp 89—91 °C (13 mmHg)), mp 42—43 °C (lit.[32] mp 43 °C) (from 70% ethanol), *p*-methoxythiophenol (**18**) bp 98—100 °C (7 mmHg) (lit.[33] bp 89—90 °C (5 mmHg)), 2-naphthalenethiol (**19**) mp 81—82 °C (lit.[34] mp 81.8—82.4 °C) (from ethanol), *p*-chlorothiophenol (**20**) mp 52—53 °C (lit.[35] mp 53 °C) (from 70% ethanol). All spectral data of products **16**—**20** were identical with those of the corresponding authentic samples.

Reduction of *p*-Toluenesulfinic Acid (7)——$NaBH_4$ (0.95 g, 25 mmol) was added to a solution of **7** (0.78 g, 5 mmol) in 30 ml of THF in portions with stirring at room temperature for 20 min, then the reaction mixture was refluxed for 1 h, and treated according to procedure B to give 222 mg (36.0%) of bis(*p*-tolyl) disulfide (**13**), mp 47—48 °C (lit.[29] mp 47—48 °C) (from ethanol) and a trace amount of *p*-methylthiophenol (**17**).

Reduction of Sulfonamides (21—23)——A mixture of benzenesulfonamide (**21**) (1.26 g, 8 mmol) and $NaBH_4$

(1.21 g, 32 mmol) was heated directly at 300—320 °C for 5 h. After the reaction mixture had been brought to room temperature, 20 ml of water was added, and the mixture was extracted with ether. The ether layer was dried over anhydrous magnesium sulfate. After removal of the ether, the residue was gas chromatographed to give diphenyl sulfide (**24**) (43.4%), the disulfide **12** (14.4%) and thiophenol **16** (trace amounts). For characterization of the products, 0.5 g of the residue was chromatographed over a silica gel column to give diphenyl sulfide (**24**) (eluted with pet. ether) bp 147—148 °C (12 mmHg) (lit.[36] bp 145 °C (8 mmHg)), the disulfide **12** (eluted with pet. ether–benzene) and thiophenol (eluted with benzene).

p-Toluenesulfonamide (**22**) and *p*-chlorobenzenesulfonamide (**23**) were reduced similarly to give the following products, respectively (the reaction conditions and results are listed in Table III); di-*p*-tolyl sulfide (**25**) as colorless needles (from ethanol), mp 55.5—57 °C (lit.[37] mp 56—57 °C), di-*p*-chlorophenyl sulfide (**26**) as colorless needles (from pet. ether), mp 94—94.5 °C (lit.[38] mp 94.2—94.5 °C). Sulfides **24**—**26** were identical with the corresponding authentic samples which were prepared from thiophenol derivatives according to a published procedure[39] on the basis of mixed melting point determination and comparisons of IR and UV spectra.

Reduction of Diphenyl Sulfone (27)——A mixture of compound **27** (1.09 g, 5 mmol) and $NaBH_4$ (1.14 g, 30 mmol) was heated directly at 200—280 °C for 5 h, and treated as described above to give 437 mg (79.4%) of thiophenol (**16**), 74 mg (19.2%) of biphenyl (**28**), diphenyl sulfide (**24**) (trace) and diphenyl disulfide (**12**) (trace). Biphenyl (**28**) mp 70.5—71 °C (lit.[40] mp 70.5 °C) (from ethanol). All spectral data of products were identical with those of the corresponding authentic samples.

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Reaction of 8,8-Dibromobicyclo[5.1.0]octanes with Higher-Order Organocuprates

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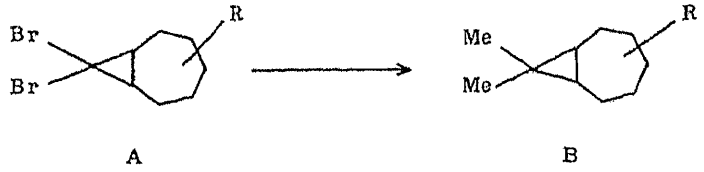
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Treatment of 8,8-dibromobicyclo[5.1.0]octanes (A) with a higher order organocuprate prepared from cuprous thiocyanate (method A) or cuprous cyanide (method B) and methyllithium, followed by the addition of methyl iodide *in situ*, readily afforded 8,8-dimethylbicyclo[5.1.0]octanes (B) in good yields. The results are summarized in Table I. Interestingly, reaction of the *cis*-dibromides (**4** and **19**) under the present conditions gave tricyclic compounds (**15** and **16**, and **20**, respectively).

Keywords——higher order organocuprate; dibromocarbene; dimethyl cyclopropane ring; dibromocyclopropane ring; substitution reaction; stereochemistry; X-ray analysis

Our recent interest in the total synthesis of natural products has been focused on compounds that contain the *gem*-dimethylcyclopropane ring system, especially the 8,8-dimethylbicyclo[5.1.0]octane ring system (B).[1] In connection with the preparation of *gem*-dimethylcyclopropane derivatives, several methods have been developed, *e.g.* substitution of *gem*-dihalocyclopropanes with dimethylcopper lithium (Gilman reagent),[2] addition of dimethylcarbene to olefins,[3] reaction of diphenyl sulfonium isopropylide with conjugated carbonyl compounds,[4] and thermolysis of pyrazolidine prepared by cycloaddition of dimethyldiazomethane to an olefin.[5] Dimethylcopper lithium has usually been used for this purpose. This substitution reaction, however, is occasionally sluggish, requiring a long reaction time.[6-8] Recently, Lipshutz *et al.* have reported that the higher-order organocuprates react readily and efficiently with inactive alkyl iodides and bromides, affording substitution products.[9-11] An attempt was made to apply the Lipshutz method to 8,8-dibromobicyclo[5.1.0]octanes (A) in order to develop an efficient method for preparing 8,8-dimethylbicyclo[5.1.0]octanes (B). We found the reaction of the higher order organocuprate with (A), followed by the addition of methyl iodide *in situ*, suitable for this purpose. We describe here the results of this substitution reaction.



A B

Chart 1

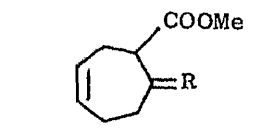
Preparation of 8,8-Dihalobicyclo[5.1.0]octanes (3—8)

Acetalization of methyl (*RS*)-7-oxo-3-cyclohepten-1-carboxylate (**1**)[12] with ethylene

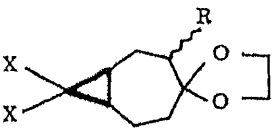
glycol yielded the acetal (**2**) quantitatively. Reaction of **2** with dibromocarbene gave the *trans*-dibromide (**3**) and *cis*-dibromide (**4**) in 64.0 and 35.9% yields, respectively; their stereo-structures were determined by X-ray analysis of **4** (see Fig. 1). 8,8-Dichlorobicyclo-[5.1.0]octanes (**5** and **6**) were prepared by treatment of **2** with dichlorocarbene in 59.7 and 33.3% yields, respectively, and their stereo-structures were elucidated by comparison of their proton nuclear magnetic resonance (^{1}H-NMR) data with those of **3** and **4**. 4-Acetoxy-8,8-dibromobicyclo[5.1.0]octanes (**7** and **8**) were prepared according to the literature.[6]

Results of Substitution Reaction with Higher-Order Organocuprates

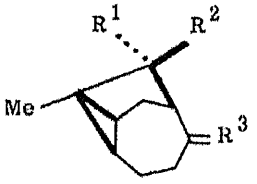
As an attempt at the substitution reaction of **3** with dimethylcopper lithium at −20 °C for 6 d failed, reaction of **3** with higher-order organocuprate was examined. Reaction of **3** with 4 eq of $Me_2Cu(SCN)Li_2$ at −20 °C for 1 h, followed by quenching with aqueous ammonium chloride, gave the dimethylated compound (**10**) together with two compounds (**9**) having a secondary methyl group. Formation of the monomethylated compounds (**9**) suggests that the substitution reaction proceeds *via* a metalated intermediate (C) or its equivalent. Therefore, we envisioned that addition of methyl iodide at the end of the reaction would cause further methylation of the intermediate to afford the desired dimethylated compound (**10**) exclusively. In fact, addition of methyl iodide gave **10** and **11** in 63 and 20 yields, respectively. Then, in order to examine not only the efficiency of the higher-order organocuprate-MeI method but also the difference in reactivity between $Me_2Cu(SCN)Li_2$ (method A) and $Me_2Cu(CN)Li_2$ (method B), reactions of the *trans*-dibromide (**3**) and *cis*-dibromide (**4**) with the two organocuprates were carried out. These results are listed in Table I; $Me_2Cu(CN)Li_2$ is more reactive and nucleophilic, as reported in the literature,[11] yielding over-alkylated by-products. Moreover, it should be noted that the yield is very variable and the ratio of products is dependent on the reaction temperature in the reaction of **4**. On the other hand, reaction of the dichlorides (**5** and **6**) with the organocuprates resulted in recovery of the starting materials.



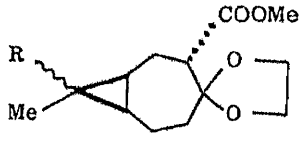
1 : R = O
2 : R = $O(CH_2)_2O$



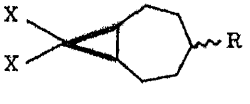
3 : X = Br, R = α-COOMe
4 : X = Br, R = β-COOMe
5 : X = Cl, R = α-COOMe
6 : X = Cl, R = β-COOMe
10 : X = Me, R = α-COOMe
12 : X = Me, R = α-CMe_2OH
13 : X = Me, R = α-COMe
14 : X = Me, R = β-COOMe
18 : X = Br, R = β-CH_2OH
19 : X = Br, R = β-CH_2Cl
21 : X = Me, R = β-CH_2Cl



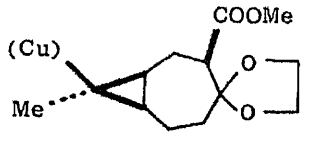
15 : R^1 = Me, R^2 = OH, R^3 = $O(CH_2)_2O$
16 : R^1 = R^2 = O, R^3 = $O(CH_2)_2O$
17 : R^1 = Me, R^2 = OH, R^3 = O
20 : R^1 = R^2 = H, R^3 = $O(CH_2)_2O$



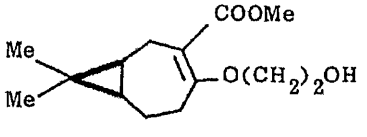
C : R = (Cu)
9 : R = H



7 : X = Br, R = α-OAc
8 : X = Br, R = β-OAc
22 : X = Me, R = α-OAc
23 : X = Me, R = α-OH
24 : X = Me, R = β-OAc



D



11

Chart 2

TABLE I. Results of Substitution Reaction of the Dibromides with Higher-Order Organocuprate-MeI

	Reaction conditions		Products Yield (%)			
	Method	Temperature (°C)				
			10	**11**	**12**	**13**
3	A	−20—−15	63	20	Trace	Trace
	B	−20—−15	62	18	5	3
			14	**15**		**16**
4[a)]	A	−15—−10	15	10		50
	A	−40—−20	57	2		20
	B	−15—−10	9	53		9
	B	−40—−20	38	22		7
			22		**23**	
7	A	−20—−15	75		16	
				24		
8	A	−20—−15		67		
			20		**21**	
19	A	−20—−15	52		34	

a) As yields were very variable, average yields of three experiments are given in this table.

TABLE II. Atomic Parameters for Non-hydrogen Atoms in the *cis*-Dibromide (**4**)

Atom	*x*	*y*	*z*	B_{eq}
Br(1)	0.8132 (1)	0.2970 (1)	0.3334 (0)	6.49 (3)
Br(2)	0.6923 (1)	0.5191 (1)	0.2556 (0)	7.21 (3)
C(1)	0.5454 (4)	0.3832 (5)	0.3202 (2)	4.43 (22)
C(2)	0.5121 (5)	0.2571 (5)	0.3413 (2)	4.44 (21)
C(3)	0.4211 (4)	0.2748 (4)	0.3788 (2)	3.94 (21)
C(4)	0.4921 (5)	0.3071 (5)	0.4355 (2)	4.14 (21)
C(5)	0.5573 (5)	0.4374 (5)	0.4422 (2)	4.88 (24)
C(6)	0.6685 (5)	0.4521 (5)	0.4147 (2)	4.80 (22)
C(7)	0.6240 (5)	0.4810 (5)	0.3572 (2)	4.29 (21)
C(8)	0.6767 (5)	0.4175 (6)	0.3148 (2)	4.70 (22)
C(9)	0.3408 (4)	0.1576 (5)	0.3784 (2)	4.18 (21)
C(10)	0.4395 (9)	0.2206 (7)	0.5096 (3)	7.82 (36)
C(11)	0.5683 (8)	0.1781 (9)	0.5067 (3)	8.48 (37)
C(12)	0.1300 (7)	0.0798 (8)	0.3631 (4)	8.28 (37)
O(1)	0.4007 (4)	0.3051 (4)	0.4677 (2)	5.46 (22)
O(2)	0.5808 (4)	0.2105 (4)	0.4556 (2)	5.60 (21)
O(3)	0.2180 (3)	0.1848 (4)	0.3665 (2)	6.04 (18)
O(4)	0.3805 (4)	0.0516 (4)	0.3866 (2)	6.01 (18)

The structures of products were elucidated on the basis of their spectral data. The stereostructure of **15** was elucidated from the facts that treatment of **16** with methyllithium afforded **15**, which was produced by attack of methyllithium from the less-hindered side, and reaction of **15** with 5% HCl afforded the hydroxy-ketone (**17**). Formation of **16** seems to suggest that this reaction proceeds *via* the metalated intermediate (D). Therefore, it was anticipated that treatment of the chloride (**19**) with organocuprate would give the tricyclic compound (**20**). Application of method A to **19** derived from **4** *via* the alcohol (**18**) produced

TABLE III. Fractional Coordinates and Isotropic Thermal Parameters (Å^2) for Hydrogen Atoms in the *cis*-Dibromide (4)

Atom	x	y	z	B_{iso}
H(1)	0.481 (6)	0.417 (6)	0.294 (2)	7.3 (16)
H(2A)	0.469 (6)	0.198 (5)	0.313 (2)	6.1 (14)
H(2B)	0.590 (5)	0.214 (5)	0.359 (2)	4.9 (12)
H(3)	0.372 (4)	0.342 (4)	0.365 (2)	3.0 (8)
H(5A)	0.591 (5)	0.451 (4)	0.479 (2)	4.2 (11)
H(5B)	0.490 (5)	0.493 (5)	0.429 (2)	4.9 (12)
H(6A)	0.720 (4)	0.370 (4)	0.419 (2)	3.3 (9)
H(6B)	0.712 (8)	0.530 (6)	0.431 (3)	8.7 (20)
H(7)	0.610 (4)	0.572 (4)	0.351 (2)	3.0 (9)
H(10A)	0.442 (8)	0.254 (7)	0.543 (3)	8.5 (17)
H(10B)	0.378 (7)	0.144 (7)	0.506 (3)	7.6 (16)
H(11A)	0.582 (11)	0.093 (10)	0.496 (4)	15.2 (37)
H(11B)	0.623 (10)	0.231 (9)	0.533 (4)	13.2 (30)
H(12A)	0.143 (10)	0.010 (9)	0.340 (4)	12.3 (30)
H(12B)	0.159 (11)	0.029 (9)	0.399 (4)	12.4 (29)
H(12C)	0.049 (7)	0.114 (7)	0.359 (3)	9.2 (20)

TABLE IV. Anisotropic Thermal Parameters (10^4) for Non-H Atoms in the *cis*-Dibromide (4)

Atom	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
Br(1)	98 (1)	188 (1)	28 (0)	18 (1)	17 (0)	−14 (0)
Br(2)	132 (1)	252 (1)	18 (0)	−68 (1)	8 (0)	9 (0)
C(1)	70 (4)	134 (6)	16 (1)	−14 (4)	3 (1)	−1 (2)
C(2)	96 (5)	114 (5)	16 (1)	−11 (4)	9 (2)	−10 (2)
C(3)	76 (4)	93 (5)	17 (1)	6 (3)	7 (1)	0 (2)
C(4)	92 (5)	104 (5)	15 (1)	5 (4)	9 (2)	−5 (2)
C(5)	114 (6)	126 (6)	17 (1)	−17 (5)	15 (2)	−8 (2)
C(6)	89 (5)	128 (6)	19 (1)	−26 (4)	9 (2)	15 (2)
C(7)	84 (5)	107 (5)	18 (1)	−9 (4)	11 (2)	−3 (2)
C(8)	77 (4)	144 (7)	17 (1)	−17 (4)	7 (2)	−4 (2)
C(9)	87 (5)	107 (5)	16 (1)	−8 (4)	10 (2)	−2 (2)
C(10)	282 (14)	133 (8)	24 (1)	4 (8)	38 (4)	5 (2)
C(11)	202 (11)	203 (11)	25 (1)	−72 (8)	−8 (3)	17 (3)
C(12)	112 (7)	169 (9)	51 (2)	−55 (6)	30 (3)	−22 (4)
O(1)	141 (5)	134 (5)	19 (1)	−14 (3)	23 (1)	−4 (1)
O(2)	122 (4)	136 (5)	19 (1)	23 (3)	−1 (1)	−1 (1)
O(3)	73 (3)	119 (4)	37 (1)	−12 (3)	9 (1)	−4 (2)
O(4)	126 (4)	102 (4)	31 (1)	−9 (3)	13 (2)	1 (2)

Estimated standard deviations are given in parentheses. The temperature factor is expressed by the formula, $T=\exp[-(B_{11}h^2+B_{22}k^2+B_{33}l^2+2B_{12}hk+2B_{13}hl+2B_{33}kl)]$.

20 and the dimethyl compound (**21**), as indicated in Table I.

The usefulness of this substitution method was supported by the following experiments. Smith III *et al.* have reported that dimethylation of a mixture of the dibromo-acetates (**7** and **8**) with Gilman reagent (Me_2CuLi) required a long reaction time (9 d) and produced the hydrolyzed dimethyl products.[6] Dimethylation of **7** and **8** by method A, however, yielded the dimethyl acetates (**22** and **24**) in a much shorter reaction time (1—2 h) as well as in comparable yield (see Table I and Experimental).

TABLE V. Bond Length (Å) and Valence Angles (°) in the *cis*-Dibromide (**4**)

Bond length (Å)			
Br(1)–C(8)	1.914 (6)	Br(2)–C(8)	1.908 (6)
C(1)–C(2)	1.503 (8)	C(1)–C(7)	1.522 (7)
C(1)–C(8)	1.497 (7)	C(2)–C(3)	1.543 (8)
C(3)–C(4)	1.534 (7)	C(3)–C(9)	1.500 (7)
C(4)–C(5)	1.526 (7)	C(4)–O(1)	1.430 (8)
C(4)–O(2)	1.407 (7)	C(5)–C(6)	1.536 (8)
C(6)–C(7)	1.491 (7)	C(7)–C(8)	1.504 (8)
C(9)–O(3)	1.320 (5)	C(9)–O(4)	1.190 (7)
C(10)–C(11)	1.476 (13)	C(10)–O(1)	1.390 (9)
C(11)–O(2)	1.405 (10)	C(12)–O(3)	1.441 (9)
Bond angle (°)			
C(2)–C(1)–C(7)	120.3 (4)	C(2)–C(1)–C(8)	123.6 (4)
C(7)–C(1)–C(8)	59.7 (4)	C(1)–C(2)–C(3)	111.3 (4)
C(2)–C(3)–C(4)	112.5 (4)	C(2)–C(3)–C(9)	110.3 (4)
C(4)–C(3)–C(9)	110.3 (4)	C(3)–C(4)–C(5)	115.0 (4)
C(3)–C(4)–O(1)	107.4 (4)	C(3)–C(4)–O(2)	110.2 (4)
C(5)–C(4)–O(1)	107.9 (4)	C(5)–C(4)–O(2)	110.1 (4)
O(1)–C(4)–O(2)	105.8 (4)	C(4)–C(5)–C(6)	115.1 (5)
C(5)–C(6)–C(7)	112.2 (4)	C(1)–C(7)–C(6)	120.1 (5)
C(1)–C(7)–C(8)	59.3 (3)	C(6)–C(7)–C(8)	123.7 (5)
Br(1)–C(8)–Br(2)	111.5 (3)	Br(1)–C(8)–C(1)	120.5 (4)
Br(1)–C(8)–C(7)	120.2 (3)	Br(2)–C(8)–C(1)	117.9 (3)
Br(2)–C(8)–C(7)	117.5 (4)	C(1)–C(8)–C(7)	61.0 (4)
C(3)–C(9)–O(3)	111.8 (4)	C(3)–C(9)–O(4)	125.3 (4)
O(3)–C(9)–O(4)	122.9 (5)	C(11)–C(10)–O(1)	105.7 (7)
C(10)–C(11)–O(2)	105.6 (6)	C(4)–O(1)–C(10)	110.0 (5)
C(4)–O(2)–C(11)	108.7 (5)	C(9)–O(3)–C(12)	117.4 (5)

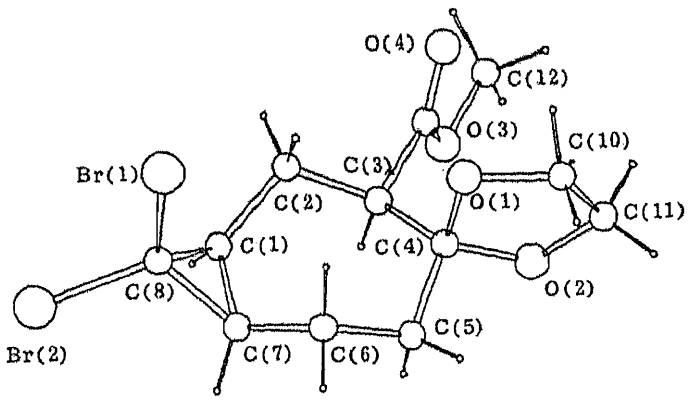


Fig. 1. Molecular Structure of the *cis*-Dibromide (**4**)

In conclusion, the present methods (methods A and B) are efficient for preparing the dimethyl compound (B) from the dibromo compound (A), although method B produced a small amount of over-alkylated products. We are now attempting the synthesis of natural products by applying these results.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus, and are uncorrected. The infrared spectra (IR ν_{max}) were determined on a Shimadzu IR-400 spectrometer in chloroform. The ^{1}H-NMR spectra were obtained in chloroform-*d* at 200 MHz on a JEOL FX 200 instrument with chemical shifts being reported in parts per million downfield from a tetramethylsilane internal standard (δ 0.0), and coupling constants in hertz. Mass spectra (MS) were taken on a JEOL JMS 01SG-2 instrument (direct inlet) at 70 eV. All reactions were carried out under an atmosphere of argon. The reaction mixture was diluted with water and extracted with ether unless otherwise noted. The organic layer was washed with brine, dried over $MgSO_4$, and filtered, then the filtrate was concentrated to dryness *in vacuo*, unless otherwise stated. Column chromatography was carried out on Silica gel 60 (Merck, 70—230 mesh) or Aluminium oxide 90 (Merck, activity II—III). Preparative thin layer chromatography (pTLC) was run on 20 × 20 cm plates coated with a 0.25 mm layer of Merck Silica gel GF_{254}.

Methyl (*RS*)-4-Cycloheptenespiro-2′-(1,3-dioxolane)-2-carboxylate (2)——A mixture of **1**[12] (2.18 g, 13.2 mmol), ethylene glycol (3.33 g, 53.4 mmol), and *p*-toluenesulfonic acid (50 mg) in dry benzene (60 ml) was refluxed for 3 h, while water was removed in a Dean–Stark apparatus. After cooling, the mixture was made basic with 5% aqueous sodium hydrogen carbonate and extracted. The residue in hexane was chromatographed on aluminum oxide (100 g). Elution with hexane–ether (5 : 1) gave **2** (2.71 g, quant.) as an oil. IR: 1725, 1200—1050 cm^{-1}. ^{1}H-NMR: 2.87 (1H, dd, J=9.0, 2.4 Hz, CH), 3.68 (3H, s, COOMe), 3.96 (4H, m, OCH_2CH_2O), 5.65—5.93 (2H, m, olefinic protons). MS Calcd for $C_{11}H_{16}O_4$: 212.1048 (M^+). Found: 212.1050.

Methyl (1*RS*,3*RS*,7*SR*)-8,8-Dibromobicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate (3) and Methyl (1*RS*,3*SR*,7*SR*)-8,8-Dibromobicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate (4)——Bromoform (4.28 g, 17.0 mmol), tributylamine (0.2 ml), and 40% aqueous sodium hydroxide (8 ml) were added successively to a solution of **2** (600 mg, 2.83 mmol) in methylene dichloride (4 ml). The mixture was vigorously stirred under reflux for 4 h, cooled, and poured into ice-water. The residue in hexane–benzene was chromatographed on aluminum oxide (40 g). The fraction eluted with hexane–ether (4 : 1) was recrystallized from hexane to give **3** (695 mg, 64% yield), mp 93—94 °C, as colorless prisms. IR: 1735 cm^{-1}. ^{1}H-NMR: 2.87 (1H, ddd, J=5.9, 2.4, 2.4 Hz, CH), 3.73 (3H, s, COOMe), 3.94 (4H, m, OCH_2CH_2O). *Anal.* Calcd for $C_{12}H_{16}Br_2O_4$: C, 37.52; H, 4.20; Br, 41.61. Found: C, 37.52; H, 4.15; Br, 41.42. The fraction eluted with hexane–ether (3 : 1) was recrystallized from hexane to give **4** (390 mg, 35.9% yield), mp 109—112 °C, as colorless plates. IR: 1730 cm^{-1}. ^{1}H-NMR: 2.88 (1H, dd, J=10.3, 1.0 Hz, CH), 3.69 (3H, s, COOMe), 3.86—3.97 (4H, m, OCH_2CH_2O). *Anal.* Calcd for $C_{12}H_{16}Br_2O_4$: C, 37.52; H, 4.20; Br, 41.61. Found: C, 37.40; H, 4.08; Br, 41.53.

Methyl (1*RS*,3*RS*,7*SR*)-8,8-Dichlorobicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate (5) and Methyl (1*RS*,3*SR*,7*SR*)-8,8-Dichlorobicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate(6)——A solution of **2** (200 mg, 0.94 mmol) in chloroform (1 ml) and tributylamine (0.1 ml) was treated with 50% aqueous sodium hydroxide (2.5 ml). The mixture was vigorously stirred under reflux for 5 h and poured into ice-water. The residue in benzene–hexane was chromatographed on aluminum oxide (30 g). Elution with hexane–ether (4 : 1) gave **5** (165 mg, 59.7% yield), mp 80—81 °C (colorless needles from hexane). IR: 1735 cm^{-1}. ^{1}H-NMR: 2.86 (1H, ddd, J= 5.6, 2.0, 2.0 Hz, CH), 3.73 (3H, s, COOMe), 3.90—3.98 (4H, m, OCH_2CH_2O). MS Calcd for $C_{12}H_{16}Cl_2O_4$: 294.0426 (M^+). Found: 294.0424. Successive elution with the same solvent gave **6** (92 mg, 33.3% yield), mp 87—89 °C (colorless prisms from hexane). IR: 1735 cm^{-1}. ^{1}H-NMR: 2.86 (1H, dd, J=11.2, 1.3 Hz, CH), 3.68 (3H, s, COOMe), 3.85—3.99 (4H, m, OCH_2CH_2O). MS Calcd for $C_{12}H_{16}Cl_2O_4$: 294.0426 (M^+). Found: 294.0419.

(1*R*,4*s*,7*S*)-8,8-Dibromobicyclo[5.1.0]oct-4-yl Acetate (7) and (1*R*,4*r*,7*S*)-8,8-Dibromobicyclo[5.1.0]oct-4-yl Acetate (8)——The title compounds were prepared by the literature method.[6]

Reaction of the *trans*-Dibromide (3) with $Me_2Cu(SCN)Li_2$——The reaction was carried out at −20—−15 °C under the reaction conditions of the general procedure (method A) except methyl iodide. The residue was subjected to column chromatography on silica gel. Elution with chloroform afforded a mixture of methyl (1*RS*,3*RS*,7*SR*)-8-methylbicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylates (**9**) and methyl (1*RS*,3*RS*,7*SR*)-8,8-dimethylbicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate (**10**). The mixture was separated by preparative gas chromatography (15% OV 101 on Chromosorb G; 1 m; column temperature, 160 °C) to afford **9** (10% yield) and **10** (50% yield). **9**: an oil. IR: 1735, 1140—1090 cm^{-1}. ^{1}H-NMR: 0.34 (1H, m, CH), 0.61 (1H, m, CH), 0.98 and 0.99 (total 3H, each d, J=5.8 Hz, CH–Me), 3.72 and 3.73 (total 3H, each s, COOMe), 3.91 (4H, m, OCH_2CH_2O). MS Calcd for $C_{13}H_{20}O_4$: 240.1371 (M^+). Found: 240.1372. **10**: mp 57—59 °C (colorless prisms from pentane). IR: 1730 cm^{-1}. ^{1}H-NMR: 0.35 (1H, ddd, J=11.8, 9.0, 5.8 Hz, CH), 0.68 (1H, ddd, J=11.2, 9.0, 5.6 Hz, CH), 0.99 (3H, s, Me), 1.01 (3H, s, Me), 2.83 (1H, ddd, J=5.9, 2.0, 2.0 Hz, CH), 3.72 (3H, s, COOMe), 3.84—3.96 (4H, m, OCH_2CH_2O). MS Calcd for $C_{14}H_{22}O_4$: 254.1518 (M^+). Found: 254.1510.

General Procedure for Dimethylation of Dibromide with Higher-Order Organocuprate-Methyl Iodide——Cuprous thiocyanate (345 mg, 2.84 mmol) (method A) or cuprous cyanide (253 mg, 2.84 mmol) (method B) dried under vacuum at 50 °C overnight was placed in a flame-dried 30 ml two-necked flask, and dry ether (1 ml) was added. Methyllithium (5.7 ml of 0.9 M ether solution, 5.13 mmol) was added to the suspension at −78 °C and the mixture was gradually warmed to 0 °C over 30 min. Then, the mixture was cooled to the temperature indicated in Tabel I and a

solution of a substance (0.25 mmol) in dry ether (2.2 ml) and hexamethylphosphoric triamide (0.11 ml) was added to the mixture at the same temperature. The reaction mixture was stirred for 1—2 h and methyl iodide (1 ml) was added at −50 °C. After 10 min, the reaction mixture was quenched with saturated aqueous ammonium chloride at −50 °C and precipitates were filtered off on a celite bed. The filtrate was extracted with ether and the organic layer was washed with 5% aqueous ammonia. Yields are given in Table I.

a) Reaction of **3** with Organocuprate-MeI: The residue was subjected to pTLC (hexane : AcOEt = 4 : 1). The highest zone afforded **9**. The middle zone afforded a mixture of (1*RS*,3*SR*,7*SR*)-3-(1-hydroxy-1-methylethyl)-8,8-dimethylbicyclo[5.1.0]octan-4-one ethylene acetal (**12**) and (1*RS*,3*SR*,7*SR*)-8,8-dimethylbicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-yl methyl ketone (**13**), which was further separated by pTLC ($CHCl_3$). The less polar zone gave **12**, mp 96—98 °C (colorless plates from hexane). IR: 3450 cm^{-1}. ^{1}H-NMR: 0.45—0.70 (2H, m, 2 × CH), 1.00 (3H, s, Me), 1.02 (3H, s, Me), 1.29 (3H, s, Me), 1.51 (3H, s, Me), 3.89—4.01 (4H, m, OCH_2CH_2O), 4.57 (1H, s, OH). MS Calcd for $C_{15}H_{26}O_3$: 254.1882 (M^+). Found: 254.1875. The more polar zone gave **13** as an oil. IR: 1710 cm^{-1}. ^{1}H-NMR: 0.27 (1H, ddd, J = 12.0, 9.0, 5.8 Hz, CH), 0.66 (1H, ddd, J = 11.4, 9.0, 5.6 Hz, CH), 0.97 (3H, s, Me), 1.01 (3H, s, Me), 2.20 (3H, s, COMe), 2.92 (1H, ddd, J = 5.6, 2.3, 2.3 Hz, CH), 3.90 (4H, m, OCH_2CH_2O). MS Calcd for $C_{14}H_{22}O_3$: 238.1569 (M^+). Found: 238.1563. The lowest zone afforded **11** as an oil. IR: 3360, 1680, 1620 cm^{-1}. ^{1}H-NMR: 0.61 (1H, ddd, J = 10.0, 10.0, 5.5 Hz, CH), 1.00 (3H, s, Me), 1.07 (3H, s, Me), 3.72 (3H, s, COOMe), 3.65—3.97 (4H, m, OCH_2CH_2O). MS Calcd for $C_{14}H_{22}O_4$: 254.1518 (M^+). Found: 254.1524.

b) Reaction of **4** with Organocuprate-MeI: The residue was subjected to pTLC (hexane : AcOEt = 4 : 1). The highest zone gave methyl (1*RS*,3*SR*,7*SR*)-8,8-dimethylbicyclo[5.1.0]octane-4-spiro-2′-(1,3-dioxolane)-3-carboxylate (**14**), mp 57—58 °C (colorless plates from pentane). IR: 1725 cm^{-1}. ^{1}H-NMR: 0.63 (2H, m, 2 × CH), 1.00 (3H, s, Me), 1.03 (3H, s, Me), 2.86 (1H, dd, J = 11.0, 1.2 Hz, CH), 3.68 (3H, s, COOMe), 3.85—3.97 (4H, m, OCH_2CH_2O). MS Calcd for $C_{14}H_{22}O_4$: 254.1518 (M^+). Found: 254.1517. The middle zone gave (1*RS*,2*RS*,6*SR*,8*SR*,9*SR*)-9-hydroxy-1,9-dimethyltricyclo[4.2.1.$0^{2,8}$]nonan-5-one ethylene acetal (**15**) as an oil. IR: 3450 cm^{-1}. ^{1}H-NMR: 0.80—0.99 (2H, m, 2 × CH), 1.16 (3H, s, Me), 1.34 (3H, s, Me), 3.80—4.05 (4H, m, OCH_2CH_2O), 4.21 (1H, s, OH). MS Calcd for $C_{13}H_{20}O_3$: 224.1412 (M^+). Found: 224.1410. The lowest zone gave (1*RS*,2*SR*,6*RS*,8*SR*)-8-methyltricyclo[4.2.1.$0^{2,8}$]-nonane-5-spiro-2′-(1,3-dioxolane)-7-one (**16**) as an oil. IR: 1700 cm^{-1}. ^{1}H-NMR: 1.24 (3H, s, Me), 3.84—4.00 (4H, m, OCH_2CH_2O). MS Calcd for $C_{12}H_{16}O_3$: 208.1100 (M^+). Found: 208.1103.

c) Reaction of **7** with $Me_2Cu(SCN)Li_2$-MeI: The residue in hexane was chromatographed on silica gel (6 g). Elution with hexane afforded (1*R*,4*s*,7*S*)-8,8-dimethylbicyclo[5.1.0]oct-4-yl acetate (**22**), mp 56—59 °C (colorless plates from pentane). IR: 1720, 1250 cm^{-1}. ^{1}H-NMR: 0.60 (1H, m, CH), 0.97 (3H, s, Me), 1.00 (3H, s, Me), 2.01 (3H, s, COMe), 4.57 (1H, tt, J = 11.0, 3.6 Hz, CH-OAc). MS Calcd for $C_{12}H_{20}O_2$: 196.1463 (M^+). Found: 196.1451. Elution with 30% ether in hexane afforded (1*R*,4*s*,7*S*)-8,8-dimethylbicyclo[5.1.0]octan-4-ol (**23**), mp 63—66 °C (colorless prisms from pentane). IR: 3450 cm^{-1}. ^{1}H-NMR: 0.60 (1H, m, CH), 0.97 (3H, s, Me), 1.00 (3H, s, Me), 3.46 (1H, tt, J = 11.0, 3.6 Hz, CH-OC). MS Calcd for $C_{10}H_{18}O$: 154.1358 (M^+). Found: 154.1357.

d) Reaction of **8** with $Me_2Cu(SCN)Li_2$-MeI: The residue was purified by pTLC (hexane : AcOEt = 19 : 1) to give (1*R*,4*r*,7*S*)-8,8-dimethylbicyclo[5.1.0]oct-4-yl acetate (**24**) as an oil. IR: 1720, 1250 cm^{-1}. ^{1}H-NMR: 0.60 (2H, m, 2 × CH), 1.01 (3H, s, Me), 1.05 (3H, s, Me), 2.03 (3H, s, COMe), 5.21 (1H, m, CH-OAc). MS Calcd for $C_{12}H_{20}O_2$: 196.1463 (M^+). Found: 196.1462.

e) Reaction of **19** with $Me_2Cu(SCN)Li_2$-MeI: The residue was subjected to pTLC (hexane : AcOEt = 19 : 1). The upper zone gave (1*RS*,3*SR*,7*SR*)-3-chloromethyl-8,8-dimethylbicyclo[5.1.0]octan-4-one ethylene acetal (**21**) as an oil. IR: 1160—1050 cm^{-1}. ^{1}H-NMR: 0.62 (2H, m, 2 × CH), 1.01 (3H, s, Me), 1.02 (3H, s, Me), 3.19 (1H, dd, J = 10.2, 10.2 Hz, CH_AH_BCl), 3.80—4.05 (5H, m, OCH_2CH_2O and CH_AH_BCl). MS Calcd for $C_{13}H_{21}ClO_2$: 244.1230 (M^+). Found: 244.1231. The lower zone gave (1*RS*,2*SR*,6*RS*,8*SR*)-8-methyltricyclo[4.2.1.$0^{2,8}$]nonan-5-one ethylene acetal (**20**) as an oil. IR: 1130—1070 cm^{-1}. ^{1}H-NMR: 0.73 (1H, m, CH), 0.91 (1H, m, CH), 1.16 (3H, s, Me), 3.85 (4H, s, OCH_2CH_2O). MS Calcd for $C_{12}H_{18}O_2$: 194.1307 (M^+). Found: 194.1315.

Reaction of 16 with Methyllithium——Methyllithium (0.5 ml of 1.2 M solution in ether, 0.6 mmol) was added to a solution of **16** (26 mg, 0.13 mmol) in dry ether (1 ml) and the reaction mixture was stirred for 1 h under ice cooling. Purification of the residue by pTLC gave **15** (27 mg, 96% yield). The compound was identified by IR and ^{1}H-NMR spectral comparison with authentic **15**.

Deacetalization of 15——Ten drops of 5% aqueous hydrochloric acid were added to a solution of **15** (50 mg, 0.22 mmol) in acetone (2.2 ml). The mixture was stirred at room temperature for 1 h and poured into 5% aqueous sodium hydrogen carbonate. Purification of the residue by pTLC (hexane : AcOEt = 3 : 1) afforded (1*RS*,2*SR*,6*RS*,-7*RS*,8*SR*)-7-hydroxy-7,8-dimethyltricyclo[4.2.1.$0^{2,8}$]nonan-5-one (**17**) (30 mg, 75% yield), mp 57—58 °C (colorless prisms from hexane) as an oil. IR: 3550, 3400, 1695 cm^{-1}. ^{1}H-NMR: 0.95 (1H, m, CH), 1.20 (3H, s, Me), 1.43 (3H, s, Me). MS Calcd for $C_{11}H_{16}O_2$: 180.1150 (M^+). Found: 180.1146.

(1*RS*,3*RS*,7*SR*)-8,8-Dibromo-3-(hydroxymethyl)bicyclo[5.1.0]octan-4-one Ethylene Acetal (18)——Diisobutylaluminum hydride (2 ml of 1.0 M hexane solution, 2 mmol) was added to a solution of **4** (192 mg, 0.5 mmol) in dry toluene (3 ml) at −78 °C. The reaction mixture was stirred for 1 h at −78 °C. Water (1 ml) was added and stirring was continued for an additional 30 min. The precipitate was filtered off and the filtrate was extracted with chloroform. The residue was recrystallized from hexane–ether to afford **18** (150 mg, 84% yield), mp 90—94 °C, as colorless flakes.

IR: 3450, 1150—1030 cm^{-1}. ^{1}H-NMR: 3.5—3.8 (2H, m, CH_2–OH), 4.0 (4H, m, OCH_2CH_2O). *Anal.* Calcd for $C_{11}H_{16}Br_2O_3$: C, 37.11; H, 4.53. Found: C, 37.30; H, 4.56.

(1*RS*,3*SR*,7*SR*)-8,8-Dibromo-3-(chloromethyl)bicyclo[5.1.0]octan-4-one Ethylene Acetal (19)——A mixture of **18** (178 mg, 0.5 mmol) and triphenylphosphine (210 mg, 0.8 mmol) in dry carbon tetrachloride (3 ml) was stirred for 12 h under reflux. After cooling, dry pentane (5 ml) was added and stirring was continued for an additional 15 min. The precipitate was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue in hexane–benzene was chromatographed on silica gel (10 g). Elution with hexane–ether (9:1) gave **19** (176 mg, 94% yield), mp 78—80 °C (colorless prisms from pentane). IR: 1150—1080 cm^{-1}. ^{1}H-NMR: 3.20 (1H, dd, J=10.5, 10.5 Hz, CH_AH_B–Cl), 3.80—4.10 (5H, m, OCH_2CH_2O and CH_AH_B–Cl). *Anal.* Calcd for $C_{11}H_{15}Br_2ClO_2$: C, 35.27; H, 4.04. Found: C, 35.00; H, 4.01.

X-Ray Analysis of 3——Single crystals of **3** were prepared by slow crystallization from hexane. The crystals were monoclinic, space group $A2/a$, with unit cell dimensions of a=10.779(1), b=10.464(1), c=25.964(3) Å, U=2851.4 Å^3, β=103.18(1)°, and D_c=1.789 gcm^{-3} for Z=8 ($C_{12}H_{16}Br_2O_4$, M_r 383.94). A crystal with dimensions of 0.10×0.10×0.20 mm was used for data collection. The intensity data were collected on a Rigaku AFC-5RU diffractometer with monochromated CuK_α radiation (λ=1.54178 Å) using the ω–2θ scan method at an ω scan speed of 16° min^{-1}. Three standard reflections were measured every 56 reflections to monitor intensity fluctuations. Absorption corrections were not applied. A total of 1925 reflection data collected for $0<\theta<60°$ were considered to be usable ($F_o>3\sigma(F_o)$). The structure was solved by the direct method using the MULTAN program[13] and was refined by the full-matrix least-squares method, minimizing the function $\Sigma_w(|F_o|-|F_c|)^2$ with $w=1/\sigma^2$. The last cycles of refinement, which included anisotropic thermal parameters for non-hydrogen atoms and isotropic thermal parameters for hydrogen atoms, converged the discrepancy factors R and R_w to 0.055 and 0.082, respectively. The hydrogen atoms were located from the D-map and refined with isotropic thermal parameters. All computations were performed on a FACOM M382 computer in the Data Processing Center of Kyoto University, using the KPPXRAY program.[14]

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The Constituents of *Eucommia ulmoides* OLIV. V. Isolation of Dihydroxydehydrodiconiferyl Alcohol Isomers and Phenolic Compounds

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Two new lignans, *erythro*-dihydroxydehydrodiconiferyl alcohol (**1**) and its *threo* isomer (**2**), were isolated from the bark of *Eucommia ulmoides* OLIV. (Eucommiaceae), together with six known lignans and three known phenolic compounds. Their structures were elucidated on the basis of spectroscopic analyses and chemical evidence.

Keywords——*Eucommia ulmoides*; lignan; dihydroxydehydrodiconiferyl alcohol; dihydrobenzofuran; phenolic compound; ^{13}C-NMR; HPLC

The bark of *Eucommia ulmoides* OLIV. (Eucommiaceae) is the origin of the crude drug, Eucommiae Cortex which has been used as a staminal tonic and hypotensor in China and Japan. In previous papers, we have reported the isolation of lignan glucosides[1–4] and iridoids.[4] We now wish to report the isolation of non-glucosidic phenolic compounds. Two new lignans, *erythro*-dihydroxydehydrodiconiferyl alcohol (**1**) and its *threo* isomer (**2**), were isolated from this crude drug, together with six known lignans, dihydrodehydrodiconiferyl alcohol (**3**),[5] (+)-epipinoresinol (**4**),[6] (+)-pinoresinol (**5**),[1,7] (+)-medioresinol (**6**),[1,7] (+)-syringaresinol (**7**)[1,7] and (+)-1-hydroxypinoresinol (**8**),[2,6] and three known phenolic compounds, *erythro*-and *threo*-guaiacylglycerol-*β*-coniferyl aldehyde ether (**9**),[8] caffeic acid (**10**)[9] and methyl chlorogenate (**11**),[10] and genipin (**12**).[11]

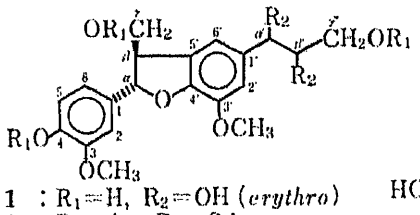
The extraction and separation were carried out as described in the experimental section.

Compound **3** was isolated as a colorless syrup, $[\alpha]_D^{25}$ +5.5° (MeOH). Acetylation of **3** gave a triacetate (**3a**) as a colorless syrup, which gave peaks at *m/z* 486 (M^+), 426 ($M^+ - CH_3COOH$) and 348 in the mass spectrum (MS). Compounds **3** and **3a** were identified as dihydrodehydrodiconiferyl alcohol[5] and its triacetate,[5] respectively, by comparison with authentic samples.

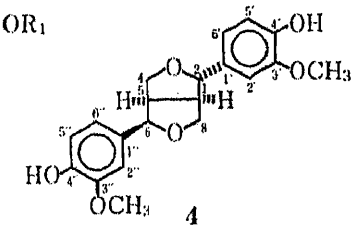
Compound **9** was obtained as a colorless syrup, $[\alpha]_D^{25}$ +20.0° (MeOH), which gave peaks at *m/z* 374 (M^+) and 356 ($M^+ - H_2O$) in the MS. The signals in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **9** were assigned as shown in Table I, by comparison with those of (±)-*erythro*- and *threo*-guaiacylglycerol (**13**),[3] hedyotol C[4,12] and coniferyl alcohol.[13] Acetylation of **9** gave a triacetate (**9a**) as a colorless syrup. Compounds **9** and **9a** were identified as *erythro*- and *threo*-guaiacylglycerol-*β*-coniferyl aldehyde ether[8] and its triacetate[8] by comparison with authentic samples.

Fraction A was obtained as a colorless syrup, $[\alpha]_D^{26}$ 0° (MeOH), which showed a spot at *Rf* 0.56 on thin layer chromatography (TLC) with $CHCl_3$–MeOH–water (70:30:5) as a developing solvent. The MS of fraction A gave peaks at *m/z* 392 (M^+), 374 ($M^+ - H_2O$), 356

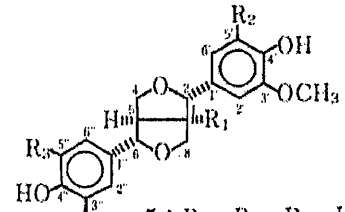
($M^+ - 2 \times H_2O$) and 344. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed the signals due to two methoxyl groups (δ 3.82 and 3.88), and five aromatic protons (δ 6.79 and 6.93). The ^{13}C-NMR chemical shifts were assigned as shown in Table I, by comparison with those of **13** and dehydrodiconiferyl alcohol[5b] (**14**). The shifts of the aromatic and α-, β- and γ-carbons of fraction A were in good agreement with those of **14**. The signals of the C-β' and C-γ atoms of fraction A appeared as doublets at δ 75.5 and 76.1, and at δ 64.3 and 64.6, due to the *erythro* and *threo* isomers, respectively. From these findings, fraction A was concluded to be a mixture of *erythro*- and *threo*-dihydroxydehydrodiconiferyl alcohol. Fraction A was separated into two peaks (**1** and **2**) by high-performance liquid chromatography (HPLC). Peaks **1** and **2** were each isolated and purified by preparative HPLC, yielding **1** and **2**. Compound **1** was obtained as a colorless syrup, $[\alpha]_D^{20}$ 0° (water). The infrared (IR) spectrum showed the presence of hydroxyl groups (3432 cm^{-1}) and aromatic rings (1610 and 1522 cm^{-1}). The ultraviolet (UV) spectrum of **1** showed absorption maxima at 228, 283 and 288 nm. The bathochromic shift of the absorption maxima in the presence of base was very similar to that of **3**. Compound **2** was obtained as a colorless syrup, $[\alpha]_D^{20}$ 0° (water). The IR and UV spectra of **2** were very similar to those of **1**. Acetylation of **1** and **2** gave pentaacetates, **1a** and **2a**, respectively. The MS of **1a** and **2a** were almost identical with each other, showing peaks at *m/z* 602 (M^+) and 542 ($M^+ - CH_3COOH$). The ^{1}H-NMR spectrum of **1a** showed signals due to four alcoholic acetyl groups (δ 2.00, 2.05 and 2.13), a phenolic acetyl group (δ 2.30), two aromatic methoxyl groups (δ 3.82 and 3.91), and five aromatic protons (δ 6.83 and 6.99). That of **2a** showed similar signals. It was reported that *erythro*-guaiacylglycerol tetraacetate and its *threo* isomer could be distinguished on the basis of the chemical shifts and the coupling constants of the benzylic protons.[3,14] The benzylic proton signals of **1a** and **2a** appeared at δ 5.93 (d, J = 5.72 Hz) and δ 5.89 (d, J = 7.48 Hz), which showed **1a** and **2a** to be the *erythro* and *threo* isomers, respectively. From the above results, compounds **1** and **2** were established to be *erythro*-dihydroxydehydrodiconiferyl alcohol and its *threo* isomer, respectively.



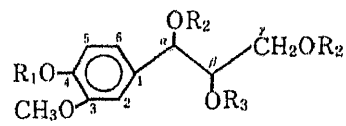
1 : R_1=H, R_2=OH (*erythro*)
1a : R_1=Ac, R_2=OAc
2 : R_1=H, R_2=OH (*threo*)
2a : R_1=Ac, R_2=OAc
3 : R_1=R_2=H
3a : R_1=Ac, R_2=H



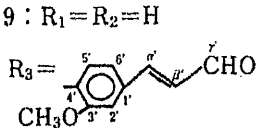
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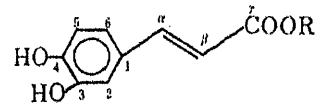
5 : R_1=R_2=R_3=H
6 : R_1=R_3=H, R_2=OCH_3
7 : R_1=H, R_2=R_3=OCH_3
8 : R_1=OH, R_2=R_3=H



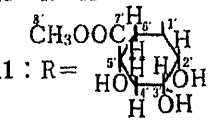
9 : R_1=R_2=H



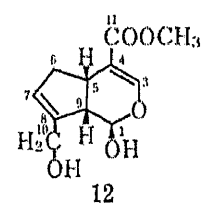
13 : R_1=R_2=R_3=H



10 : R=H



11 : R=



12

Chart I

TABLE I. ^{13}C-NMR Chemical Shifts

	Fraction A[a] (1 and 2)	13[a]	14[b]	9[c]	Guaiacylglycerol moiety of hedyotol C[c]
C-1	134.7	134.7	134.3	132.7, 132.9	133.2
C-1′	136.8[d]		130.4	126.7	
C-2	110.7	111.8, 112.0	110.7	111.2	111.2
C-2′	112.9		111.8	111.6	
C-3	148.9	148.8	148.6	146.9	147.0
C-3′	145.2		145.1	151.3,[d] 151.5[d]	
C-4	147.6	147.1	147.5	145.5	145.4
C-4′	149.1		148.9	149.6[d]	
C-5	116.1	115.8, 115.9	115.8	114.6	114.7
C-5′	129.8[d]		132.0	123.2	
C-6	119.7	120.7, 121.0	119.5	119.0, 119.6	119.4
C-6′	116.6		116.2	114.6	
OCH_3	56.4	56.5		55.4	55.9
OCH_3	56.8			55.7	
α	89.1	76.6,[d] 77.5	88.3	71.1, 71.6	72.2
α′	77.4		130.9	153.3	
β	55.2	76.1,[d] 75.5	54.6	83.4, 83.9	86.1
β′	75.5, 76.1		128.0	126.3	
γ	64.8	64.3, 64.5	64.5	60.2	59.9
γ′	64.3, 64.6		63.3	193.8	

a) In MeOH-d_4. *b*) Data from ref. 5*b*, in Me_2CO-d_6–D_2O (9 : 1). *c*) In DMSO-d_6. *d*) The assignments may be interchanged in each column.

Compounds **4**—**8**, **10**, **11** and **12** were identified as (+)-epipinoresinol,[6] (+)-pinoresinol,[1,7] (+)-medioresinol,[1,7] (+)-syringaresinol,[1,7] (+)-1-hydroxypinoresinol,[2,6] caffeic acid,[9] methyl chlorogenate[10] and genipin[11] by comparison with authentic samples (see Experimental).

Experimental

All melting points are uncorrected. The following instruments were used; melting point, Mettler FP-61; optical rotation value, JASCO DIP-4; HPLC, Hitachi 638 with a UV detector; UV spectra, Hitachi 200-20; IR spectra, Hitachi 270-30; MS, Hitachi RMU-7L; ^{1}H-NMR spectra, JEOL JNM-FX-90; ^{13}C-NMR spectra, JEOL JNM-FX-60, with tetramethylsilane ($\delta=0$) as an internal reference. The abbreviations used for NMR data are as follows: s, singlet; d, doublet; dt, doublet-triplet; t, triplet; m, multiplet.

Precoated TLC plates (Silica gel 60 F_{254}, Merck) were used for TLC. The spots were detected by spraying the plates with 20% H_2SO_4 and by heating. Silica gel (Wako gel C-300, Wako Pure Chemical Co.) and Diaion HP-20 (Nippon Rensui Co.) were used for column chromatography. TSK gel HW-40 (Toyo Soda Co.) was used for gel filtration.

Isolation——The air-dried bark of *Eucommia ulmoides* OLIV. (10 kg, commercial crude drug produced in China) was chopped and extracted with 50% MeOH (20 l × 2) under reflux. The extract was filtered and the filtrate was evaporated under reduced pressure to a small volume, which was suspended in water. This suspension was extracted with EtOAc (3 l × 2) and *n*-BuOH (3 l × 3), successively. The EtOAc extract was evaporated under reduced pressure to afford the residue, which showed spots at *Rf* 0.08 [(+)-cyclo-olivil], 0.10 [(−)-olivil], 0.25 (**3**), 0.33 (**9**), 0.35 (**12**), 0.39 (**8**), 0.53 (**5**), 0.54 (**4**), 0.55 (**6**) and 0.56 (**7**) on TLC with $CHCl_3$–MeOH (10 : 1) as a developing solvent. This residue was subjected to silica gel chromatography with $CHCl_3$–MeOH (40 : 1, 10 : 1), and the eluate was separated into three fractions (frs. 1—3). Fraction 1 was applied to a silica gel column, and eluted successively with $CHCl_3$–MeOH (20 : 1), benzene–EtOAc (5 : 1, 1 : 1) and *n*-hexane–acetone (1 : 1). The eluates showing TLC spots at *Rf* 0.53 (**5**), 0.54 (**4**), 0.55 (**6**) and 0.56 (**7**) were each collected and purified by repeated silica gel chromatography and then gel filtration on TSK gel HW-40 with MeOH, yielding **4** (0.5 g), **5** (3.5 g), **6** (0.6 g) and **7** (0.4 g). After repeated chromatography and gel filtration of fr. 2 and fr. 3, under the same conditions, pure **8** (1.0 g), **9** (0.3 g), **12** (2 g) and **3** (0.1 g) were isolated. The

n-BuOH extract was evaporated under reduced pressure to give the residue, which was subjected to silica gel chromatography with $CHCl_3$–MeOH–water (100 : 10 : 1, 80 : 20 : 3). The eluates were monitored by TLC with $CHCl_3$–MeOH–water (70 : 30 : 5) as a developing solvent. The fractions showing a TLC spot at *Rf* 0.59 (fraction A), 0.38 (**10**) and 0.11 (**11**) were each collected and purified by repeated silica gel chromatography and gel filtration on TSK gel HW-40 with MeOH, yielding fraction A (0.5 g), **10** (0.1 g) and **11** (0.1 g), respectively. Fraction A was separated into two peaks at t_R (min) 6.33 (**1**) and 7.14 (**2**) by HPLC. Conditions: column, Hitachi gel 3056, 4 mm × 25 cm; eluent, CH_3CN–water (15 : 85); flow rate, 1.0 ml/min; detector, UV detector (270 nm). Peaks **1** and **2** were each collected by repeated HPLC under the above conditions, yielding **1** (1.8 mg) and **2** (2.0 mg).

Fraction A(1 and 2)——A colorless syrup, $[\alpha]_D^{20}$ 0° (*c* = 0.11, MeOH). UV λ_{max}^{MeOH} nm: 235, 285. IR ν_{max}^{KBr} cm^{-1}: 3680 (OH), 1608, 1518 (aromatic ring). MS *m/z*: 392 (M^+), 374 ($M^+ - H_2O$), 356 ($M^+ - 2 \times H_2O$), 344. ^{1}H-NMR (in CD_3OD) δ: 3.82, 3.88 (3H, 3H, each s, 2 × OCH_3), 5.54 (1H, d, *J* = 5.71 Hz, C_α-H), 6.79, 6.93 (2H, 3H, each s, arom. H).

***erythro*-Dihydroxydehydrodiconiferyl Alcohol (1)**——A colorless syrup, $[\alpha]_D^{20}$ 0° (*c* = 0.1, water). UV λ_{max}^{EtOH} nm: 228, 283, 288. UV $\lambda_{max}^{EtOH+NaOH}$ nm: 247, 290. IR ν_{max}^{KBr} cm^{-1}: 3432 (OH), 1610, 1522 (aromatic ring). MS *m/z*: 392 (M^+, 1), 374 ($M^+ - H_2O$, 32), 356 ($M^+ - 2 \times H_2O$, 56), 344 (100), 326 (56), 314 (90), 312 (60), 297 (44), 285 (78). ^{1}H-NMR (in CD_3OD) δ: 3.81, 3.88 (3H, 3H, each s, 2 × OCH_3), 5.53 (1H, d, *J* = 5.71 Hz, C_α-H), 6.78, 6.93 (2H, 3H, each s, arom. H).

***threo*-Dihydroxydehydrodiconiferyl Alcohol (2)**——A colorless syrup, $[\alpha]_D^{20}$ 0° (*c* = 0.15, water). UV λ_{max}^{EtOH} nm: 229, 282, 288. UV $\lambda_{max}^{EtOH+NaOH}$ nm: 247.5, 290. IR ν_{max}^{KBr} cm^{-1}: 3424 (OH), 1608, 1520 (aromatic ring). MS *m/z*: 392 (M^+, 1), 374 ($M^+ - H_2O$, 14), 356 ($M^+ - 2 \times H_2O$, 38), 345 (60), 344 (56), 326 (44), 314 (92), 312 (100), 297 (40), 285 (58). ^{1}H-NMR (in CD_3OD) δ: 3.81, 3.87 (3H, 3H, each s, 2 × OCH_3), 5.53 (1H, d, *J* = 6.15 Hz, C_α-H), 6.75—7.00 (5H, m, arom. H). Compounds **1** and **2** were acetylated with acetic anhydride–pyridine, yielding the pentaacetates **1a** and **2a**, respectively.

***erythro*-Dihydroxydehydrodiconiferyl Alcohol Pentaacetate (1a)**——A colorless syrup. IR ν_{max}^{KBr} cm^{-1}: 1764, 1224 ($OCOCH_3$), 1610, 1506 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 2.00, 2.05, 2.13 (3H, 6H, 3H, each s, 4 × alcoholic $OCOCH_3$), 2.30 (3H, s, a phenolic $OCOCH_3$), 3.82, 3.91 (3H, 3H, each s, 2 × OCH_3), 5.53 (1H, d, *J* = 7.03 Hz, C_α-H), 5.93 (1H, d, *J* = 5.72 Hz, $C_{\alpha'}$-H, *erythro*), 6.83, 6.99 (2H, 3H, each s, arom. H). MS *m/z*: 602 (M^+, 64), 542 ($M^+ - CH_3COOH$, 60), 500 ($M^+ - CH_3COOH - CH_2CO$, 100), 482 ($M^+ - 2 \times CH_2COOH$, 17), 440 (20).

***threo*-Dihydroxydehydrodiconiferyl Alcohol Pentaacetate (2a)**——A colorless syrup. IR ν_{max}^{KBr} cm^{-1}: 1764, 1228 ($OCOCH_3$), 1610, 1506 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 2.03, 2.07, 2.08 (12H, each s, 4 × alcoholic $OCOCH_3$), 2.31 (3H, s, a phenolic $OCOCH_3$), 3.82, 3.91 (3H, 3H, each s, 2 × OCH_3), 5.54 (1H, d, *J* = 7.03 Hz, C_α-H), 5.89 (1H, d, *J* = 7.48 Hz, $C_{\alpha'}$-H, *threo*), 6.83, 6.99 (2H, 3H, each s, arom. H). MS *m/z*: 602 (M^+, 52), 542 ($M^+ - CH_3COOH$, 46), 500 ($M^+ - CH_3COOH - CH_2CO$, 100), 440 (12).

Dihydrodehydrodiconiferyl Alcohol (3)——A colorless syrup, $[\alpha]_D^{25}$ +5.5° (*c* = 0.18, MeOH). UV λ_{max}^{EtOH} nm: 228, 283, 288. UV $\lambda_{max}^{EtOH+NaOH}$ nm: 248, 293. IR ν_{max}^{KBr} cm^{-1}: 3408 (OH), 1608, 1520, 1502 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 1.60—2.10 (3H, m, C_β-H, $C_{\beta'}$-H), 2.50—2.75 (2H, m, $C_{\alpha'}$-H), 3.40—4.00 (2H, m, $C_{\gamma'}$-H), 3.66 (2H, t, *J* = 6.6 Hz, $C_{\gamma'}$-H), 3.83, 3.86 (3H, 3H, each s, 2 × OCH_3), 5.51 (1H, d, *J* = 7.47 Hz, C_α-H), 6.65, 6.70—6.80 (2H, s, 3H, m, arom. H). Acetylation of **3** with acetic anhydride–pyridine gave a triacetate (**3a**).

Dihydrodehydrodiconiferyl Alcohol Triacetate (3a)——A colorless syrup. IR ν_{max}^{KBr} cm^{-1}: 1768, 1740, 1248 ($OCOCH_3$), 1608, 1506 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 1.80—2.00 (2H, m, $C_{\beta'}$-H), 2.05, 2.07 (3H, 3H, each s, 2 × alcoholic $OCOCH_3$), 2.31 (3H, s, a phenolic $OCOCH_3$), 2.50—2.75 (2H, m, $C_{\alpha'}$-H), 3.82, 3.90 (3H, 3H, each s, 2 × OCH_3), 4.10 (2H, t, *J* = 6.6 Hz, $C_{\gamma'}$-H), 4.25—4.60 (2H, m, $C_{\gamma'}$-H), 5.52 (1H, d, *J* = 6.6 Hz, C_α-H), 6.65, 6.90—7.10 (2H, s, 3H, m, arom. H).

***erythro*- and *threo*-Guaiacylglycerol-β-coniferyl Aldehyde Ether (9)**——A colorless syrup. $[\alpha]_D^{25}$ +20.0° (*c* = 0.1, MeOH). UV λ_{max}^{MeOH} nm: 229, 287, 332. IR ν_{max}^{KBr} cm^{-1}: 3404 (OH), 1670 (CHO), 1662, 1598, 1514 (aromatic ring). MS *m/z*: 374 (M^+), 356 ($M^+ - H_2O$), 326, 204, 178. ^{1}H-NMR (in $CDCl_3$) δ: 3.88, 3.91 (3H, 3H, each s, 2 × OCH_3), 4.10—4.40 (1H, m, C_β-H), 4.98 (1H, d, *J* = 6.16 Hz, C_α-H), 6.61, 6.62 (1H 2 × dd, *J* = 7.48, 15.39 Hz, $C_{\beta'}$-H), 6.80—7.50 (7H, m, $C_{\alpha'}$-H, arom. H), 9.66, 9.68 (1H, 2 × d, *J* = 7.48 Hz, CHO). ^{13}C-NMR: Table I. Acetylation of **9** gave a triacetate (**9a**).

***erythro*- and *threo*-Guaiacylglycerol-β-coniferyl Aldehyde Ether Triacetate (9a)**——A colorless syrup. IR ν_{max}^{KBr} cm^{-1}: 1746, 1222 ($OCOCH_3$), 1678 (CHO), 1626, 1600, 1514 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 1.99, 2.03, 2.06, 2.09 (6H, 2 × alcoholic $OCOCH_3$), 2.30 (3H, s, a phenolic $OCOCH_3$), 3.83, 3.88 (3H, 3H, each s, 2 × OCH_3), 3.95—4.45 (2H, m, $C_{\gamma'}$-H), 4.60—4.90 (1H, m, $C_{\beta'}$-H), 6.06, 6.11 (1H, 2 × d, *J* = 5.0 Hz; *erythro*, *J* = 6.16 Hz; *threo* C_α-H), 6.61 (1H, dd, *J* = 7.92, 15.83 Hz, $C_{\beta'}$-H), 6.90—7.20 (6H, m, arom. H), 7.40 (1H, d, *J* = 15.83 Hz, $C_{\alpha'}$-H), 9.67 (1H, d, *J* = 7.92 Hz, CHO).

(+)-Epipinoresinol (4)——A white powder (from EtOH), mp 121°C, $[\alpha]_D^{25}$ +70.0° (*c* = 0.1, MeOH). UV λ_{max}^{MeOH} nm: 230, 281. IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 1608, 1516 (aromatic ring). ^{1}H-NMR (in dimethylsulfoxide-d_6 (DMSO-d_6)) δ: 3.76 (6H, s, 2 × OCH_3), 4.28 (1H, d, *J* = 7 Hz, C_6-H), 4.74 (1H, d, *J* = 5 Hz, C_2-H), 6.77—6.88 (6H, m, arom. H). ^{13}C-NMR (in DMSO-d_6) δ: 49.5 (5), 53.8 (1), 55.8 (OCH_3), 68.4 (4), 70.4 (8), 81.4 (6), 86.9 (2), 110.1, 110.5 (2′, 2″), 115.2 (5′, 5″), 118.0, 118.6 (6′, 6″), 129.7 (1″), 132.5 (1′), 145.4, 146.0 (4′, 4″), 147.3, 147.5 (3′, 3″). MS *m/z*:

358 (M^+, 88), 327 (27), 205 (43), 196 (26), 163 (56), 151 (100), 137 (69), 131 (48), 124 (37).

(+)-Pinoresinol (5)——An amorphous powder, $[\alpha]_D^{25}$ +51° ($c=0.1$, $CHCl_3$). UV λ_{max}^{MeOH} nm: 232, 281. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1610, 1510 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 3.0—3.2 (2H, m, $C_{1,5}$-H), 3.87 (6H, s, $2\times OCH_3$), 3.64—4.36 (4H, m, $C_{4,8}$-H), 4.73 (2H, d, $J=5$ Hz, $C_{2,6}$-H), 6.80—6.92 (6H, m, arom. H). ^{13}C-NMR (in DMSO-d_6) δ: 53.7 (1,5), 55.8 (OCH_3), 71.0 (4, 8), 85.2 (2, 6), 110.6 (2′, 2″), 115.2 (5′, 5″), 118.6 (6′, 6″), 132.4 (1′, 1″), 146.0 (4′, 4″), 147.6 (3′, 3″).

(+)-Medioresinol (6)——An amorphous powder, $[\alpha]_D^{25}$ +44.7° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm: 231.5, 280. IR ν_{max}^{KBr} cm^{-1}: 3464 (OH), 1614, 1518 (aromatic ring). ^{1}H-NMR (in DMSO-d_6) δ: 2.92—3.16 (2H, m, $C_{1,5}$-H), 3.76 (9H, s, $3\times OCH_3$), 3.80—4.40 (4H, m, $C_{4,8}$-H), 4.52—4.68 (2H, m, $C_{2,6}$-H), 6.52—6.92 (5H, m, arom. H).

(+)-Syringaresinol (7)——Colorless needles (from EtOH), mp 183.5°C. $[\alpha]_D^{22}$ +44° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm: 237, 273. IR ν_{max}^{KBr} cm^{-1}: 3428 (OH), 1614, 1520 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 2.92—3.18 (2H, m, $C_{1,5}$-H), 3.64—4.27 (4H, m, $C_{4,8}$-H), 3.87 (12H, s, $4\times OCH_3$), 4.61 (2H, d, $J=4$ Hz, $C_{2,6}$-H), 6.60 (4H, s, arom. H).

(+)-1-Hydroxypinoresinol (8)——An amorphous powder, $[\alpha]_D^{25}$ +27° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm: 232, 281. IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 1616, 1518 (aromatic ring). ^{1}H-NMR (in DMSO-d_6) δ: 2.80—3.04 (1H, m, C_5-H), 3.46 4.08 (3H, m, C_{4a}-H, C_8-H), 3.66 (6H, s, $2\times OCH_3$), 4.20—4.48 (1H, m, C_{4c}-H), 4.52 (1H, s, C_2-H), 4.76 (2H, d, $J=5$ Hz, C_6-H), 6.60—7.04 (6H, m, arom. H).

Caffeic Acid (10)——A white powder (from EtOH), mp 163.6°C. IR ν_{max}^{KBr} cm^{-1}: 3440 (OH), 3000—2000, 1646 (COOH), 1620, 1526 (aromatic ring). ^{1}H-NMR (in MeOH-d_4) δ: 6.20 (1H, d, $J=16$ Hz, C_α-H), 6.60—7.10 (3H, m, arom. H), 7.52 (1H, d, $J=16$ Hz, C_β-H). ^{13}C-NMR (in MeOH-d_4) δ: 115.2, 115.7 (2, β), 116.5 (5), 122.7 (6), 127.9 (1), 146.6, 146.9 (3, α), 149.2 (4), 171.0 (γ).

Methyl Chlorogenate (11)——A white powder (from EtOH), mp 100°C. $[\alpha]_D^{25}$ −26.4° ($c=0.1$, MeOH). UV λ_{max}^{MeOH} nm: 220, 242, 298, 330. IR ν_{max}^{KBr} cm^{-1}: 3416 (OH), 1734, 1694 (COO), 1604, 1520 (aromatic ring). ^{1}H-NMR (in MeOH-d_4) δ: 3.60 (3H, s, $COOCH_3$), 5.20 (1H, d, $J=6.4$ Hz, anomeric H), 6.10 (1H, d, $J=16$ Hz, C_α-H), 6.60—7.00 (3H, m, arom. H), 7.42 (1H, d, $J=16$ Hz, C_β-H). ^{13}C-NMR (in MeOH-d_4) δ: 38.2 (4′, 6′), 53.0 (8′), 70.7 (3′), 72.0, 73.0 (1′, 2′), 76.0 (5′), 115.2 (2, β), 116.5 (5), 122.9 (6), 127.7 (1), 146.6, 147.0 (3, α), 149.4 (4), 168.3 (γ), 175.3 (7′). Acetylation of **11** gave a pentaacetate. A white powder, mp 167.9°C. ^{1}H-NMR (in $CDCl_3$) δ: 2.01, 2.10, 2.16 (9H, each s, $3\times$ alcoholic $OCOCH_3$), 2.32 (6H, s, $2\times$ phenolic $OCOCH_3$), 3.74 (3H, s, $COOCH_3$), 6.32 (1H, d, $J=16$ Hz, C_α-H), 7.12—7.48 (3H, m, arom. H), 7.60 (1H, d, $J=16$ Hz, C_β-H).

Genipin (12)——An amorphous powder, $[\alpha]_D^{24}$ +95.4° ($c=1.0$, MeOH). UV λ_{max}^{MeOH} nm: 239. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1692 (C=O). High-resolution MS *m/z*: 226.0849 (M^+, $C_{11}H_{14}O_5$), 208.0747 (M^+-H_2O, $C_{11}H_{12}O_4$). ^{1}H-NMR (in MeOH-d_4) δ; 3.70 (3H, s, $COOCH_3$), 4.22 (2H, s, C_{10}-H), 5.64—5.92 (1H, m, C_7-H), 7.48 (1H, s, C_3-H).

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Aromatic Annelation with α-Phenylsulfinyl-γ-butyrolactones. A Novel Route to 4-(2-Hydroxyalkyl)-1,3-benzenediols

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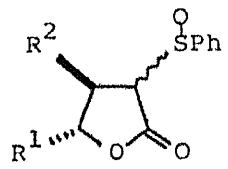
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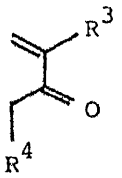
A number of 4-(2-hydroxyalkyl)-1,3-benzenediols (**4**) were synthesized by thermolysis of 1,3-cyclohexadiones (**3**), which were obtained by the reaction of α-phenylsulfinyl-γ-butyrolactones (**1**) with α,β-unsaturated ketones (**2**), providing a new aromatic annelation. One of the compounds thus obtained, 4-(3,4-dihydro-4-hydroxy-7-methoxy-2*H*-1-benzopyran-3-yl)-1,3-benzenediol (**4n**), was converted to isomedicarpin (**5b**) and homopterocarpin (**5c**).

Keywords——annelation; aromatic synthesis; 1,3-benzenediol; α-phenylsulfinyl-γ-butyrolactone; α,β-unsaturated ketone; pterocarpan; homopterocarpin; isomedicarpin

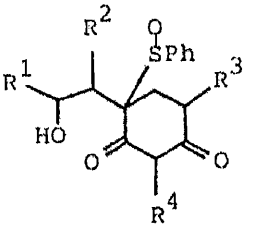
The annelation of aliphatic compounds by means of Michael-type reactions has been utilized for the synthesis of aromatic systems such as phenols,[1a-e] hydroquinones,[1a,e] resorcinols,[1a,f-h] naphthalenes,[1a,i-m] *etc.*,[1n] including natural products. The advantage of forming aromatic rings from aliphatic compounds lies in the ability to control the position of substituents on the aromatic rings according to the starting materials employed. It is effective not only for the regiospecific preparation of aromatic compounds but also for the preparation of synthetic analogues or closely related natural products by means of simple modification of the starting materials.



1a: $R^1=H$, $R^2=H$
1b: $R^1=Me$, $R^2=H$
1c: $R^1=Ph$, $R^2=H$
1d: $R^1=R^2=-(CH_2)_4-$



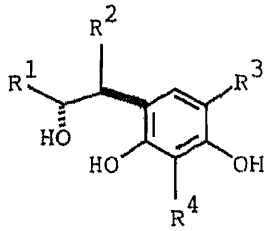
2a: $R^3=R^4=H$
2b: $R^3=Me$, $R^4=H$
2c: $R^3=R^4=Me$

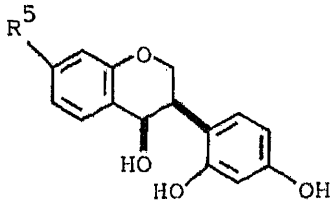


3

Chart 1

As part of our research to develop methods to construct various aromatic compounds from aliphatic compounds, we have developed a new route to 1,3-benzenediols possessing a 2-hydroxyalkyl side chain at the C-4 position of the aromatic ring. This arrangement of the substituents on the aromatic ring is found in naturally occurring isoflavonoids such as the pterocarpans,[2] some of which are well-known as phytoalexins. Here, we describe in detail a new preparation of highly substituted 1,3-benzenediols by annelation with α-phenylsulfinyl-γ-butyrolactones.[3]



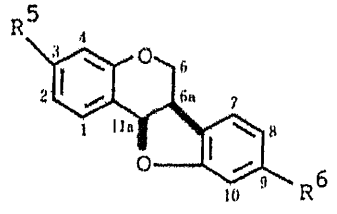


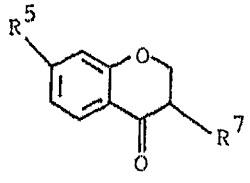
4a:	$R^1=H$,	$R^2=H$,	$R^3=H$	$R^4=H$	
4b:	$R^1=H$,	$R^2=H$,	$R^3=Me$,	$R^4=H$	
4c:	$R^1=H$,	$R^2=H$,	$R^3=Me$,	$R^4=Me$	
4d:	$R^1=Me$,	$R^2=H$,	$R^3=H$,	$R^4=H$	
4e:	$R^1=Me$,	$R^2=H$,	$R^3=Me$,	$R^4=H$	
4f:	$R^1=Me$,	$R^2=H$,	$R^3=Me$,	$R^4=Me$	
4g:	$R^1=Ph$,	$R^2=H$,	$R^3=H$,	$R^4=H$	
4h:	$R^1=Ph$,	$R^2=H$,	$R^3=Me$,	$R^4=H$	
4i:	$R^1=Ph$,	$R^2=H$,	$R^3=Me$,	$R^4=Me$	
4j:	$R^1=R^2=-(CH_2)_4-$,		$R^3=H$,	$R^4=H$	
4k:	$R^1=R^2=-(CH_2)_4-$,		$R^3=Me$,	$R^4=H$	
4l:	$R^1=R^2=-(CH_2)_4-$,		$R^3=Me$,	$R^4=Me$	

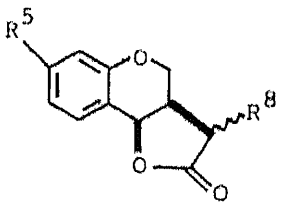
4m: $R^5=H$
4n: $R^5=OMe$

Chart 2

Treatment of the α-phenylsulfinyl-γ-butyrolactones (**1a**—**f**) with the α,β-unsaturated ketones (**2a**—**c**) at room temperature in absolute methanol in the presence of magnesium methoxide afforded unstable intermediates **3**. From their infrared (IR) spectra (1720 cm^{-1}) and the results of the following reactions, it can be presumed that the 1,3-cyclohexadione systems are formed by the Michael-induced ring closure sequence. Thermolysis of these intermediates **3** in refluxing benzene gave the 1,3-benzenediols (**4a**—**n**) with the alcoholic side chain at the 4 position of the aromatic rings in moderate yields. The moiety [-O-$C_5(R^1)$-$C_4(R^2)$-] of the lactones turned into the side chain unit of the produced aromatic rings, which carried the substituents R^3 and R^4 from the α,β-unsaturated ketones. Their structures were confirmed by the spectral data and by chemical transformation. Compound **4n** was transformed to homopterocarpin[4] (**5c**) *via* isomedicarpin[5] (**5b**) in the reported manner[4a] to confirm the alcoholic structure. The pterocarpan framework **5a** was similarly obtained from compound **4m**.







5a:	$R^5=H$,	$R^6=OH$	**6a**:	$R^5=H$,	$R^7=H$	**1e**:	$R^5=H$,	$R^8=S(O)Ph$
5b:	$R^5=OMe$,	$R^6=OH$	**6b**:	$R^5=H$,	$R^7=Br$	**1f**:	$R^5=OMe$,	$R^8=S(O)Ph$
5c:	$R^5=R^6=OMe$		**6c**:	$R^5=H$,	$R^7=CH(CO_2Et)_2$	**7a**:	$R^5=H$,	$R^8=H$
			6d:	$R^5=H$,	$R^7=CH_2CO_2H$	**7b**:	$R^5=H$,	$R^8=SPh$
			6e:	$R^5=OMe$,	$R^7=H$	**7c**:	$R^5=OMe$,	$R^8=H$
			6f:	$R^5=OMe$,	$R^7=Br$	**7d**:	$R^5=OMe$,	$R^8=SPh$
			6g:	$R^5=OMe$	$R^7=CH(CO_2Et)_2$			
			6h:	$R^5=OMe$,	$R^7=CH_2CO_2H$			

Chart 3

The starting lactones employed were obtained as follows. The simple lactones (**1a—d**) were obtained according to the reported method[6] and the three-ring-system lactone, *cis*-3a,9b-dihydro-3-(phenylsulfinyl)-4*H*-furo[3,2-*c*] [1]benzopyran-2(3*H*)-one (**1e**), was synthesized from 2,3-dihydro-4*H*-1-benzopyran-4-one (**6a**). Bromination of **6a** followed by treatment with sodium diethyl malonate gave diethyl (3,4-dihydro-4-oxo-2*H*-1-benzopyran-3-yl)-malonate (**6c**), which was hydrolyzed under acidic conditions to give 3,4-dihydro-4-oxo-2*H*-1-benzopyran-3-acetic acid (**6d**). Reduction of **6d** with aluminum *sec*-butoxide gave the lactone (**7a**). The α-phenylthio-γ-butyrolactone (**7b**) was obtained from **7a** on treatment with lithium diisopropylamide (LDA) followed by the addition of diphenyldisulfide. Oxidation of **7b** with *m*-chloroperbenzoic acid (MCPBA) gave **1e**. Another lactone **1f** was formed from 2,3-dihydro-7-methoxy-4*H*-1-benzopyran-4-one[7] (**6e**) in the same way.

In conclusion, various 4-(2-hydroxyalkyl)-1,3-benzenediols (**4a—n**) were obtained by the annelation reaction of substituted α-phenylsulfinyl-γ-butyrolactones with α,β-unsaturated ketones, and this method was successfully applied to construct the pterocarpan framework by using three-ring-system lactones such as **1e** and **1f**.

Experimental

Melting points were determined on a Yanaco model MP micro melting point apparatus and are uncorrected. IR spectra were obtained on a Hitachi 285 IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-PMX 60si spectrometer or a JEOL JNM-GX270 FT spectrometer using tetramethylsilane (TMS) as an internal standard, and a carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum on a JNM-GX270 FT spectrometer at 67.8 MHz with TMS as an internal standard. High-resolution mass spectra (MS) were obtained on a JEOL JMS-01SG spectrometer. An ultraviolet (UV) spectrum was recorded on a Hitachi model 200-10 spectrophotometer. All extracts were dried over anhydrous $MgSO_4$. Column chromatography was performed with Kieselgel 60 (70—230 mesh) and Kiselgel 60 PF_{254} (Merck), respectively.

Diethyl (3,4-Dihydro-4-oxo-2*H*-1-benzopyran-3-yl)malonate (6c)——A mixture of 2,3-dihydro-4*H*-1-benzopyran-4-one (**6a**)[7] (14.8 g) and $CuBr_2$ (22.3 g) in AcOEt (150 ml) was stirred under reflux for 2 h. The mixture was filtered and the filtrate was washed with sat.aq. $NaHCO_3$, water and brine, and then dried. Evaporation of the solvent *in vacuo* gave 3-bromo-2,3-dihydro-4*H*-1-benzopyran-4-one[8] (**6b**). A solution of diethyl malonate (17 g) in tetrahydrofuran (THF) (50 ml) was added to a suspension of NaH (60%, 4.0 g) in THF (150 ml) at 5 °C, and the mixture was stirred at room temperature for 15 min. Next, a solution of **6b** in THF (80 ml) was added at room temperature and the stirring was continued for 10 h. The reaction mixture was poured into water and acidified with 10% HCl. The product was extracted with Et_2O and the extract was washed with sat.aq. $NaHCO_3$, water, and brine, and then dried. After evaporation of the solvent, the crude product was chromatographed on silica gel with $CHCl_3$ to give pure **6c** (12.5 g, 40.8%) as a pale yellow oil. High-resolution MS: Calcd for $C_{16}H_{18}O_6$ (306.1102). Found: 306.1075 (M^+). IR ($CHCl_3$): 1730, 1685, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t), 1.35 (3H, t), 3.45 (1H, m), 4.05 (1H, m), 4.20 (2H, q), 4.25 (2H, q), 4.60 (2H, m), 6.8—7.5 (3H, m), 7.80 (1H, dd, J=3, 8 Hz).

3,4-Dihydro-4-oxo-2*H*-1-benzopyran-3-acetic Acid (6d)——A mixture of **6c** (20 g), AcOH (10 ml), 90% HCOOH (40 ml), and conc. H_2SO_4 (3 drops) was refluxed for 10 h. After evaporation of the solvent *in vacuo*, the residue was dissolved in Et_2O and the acid was extracted with sat.aq. $NaHCO_3$. The aqueous solution was washed with Et_2O and acidified with 10% HCl. The product was extracted with Et_2O and the extract was washed with brine and dried. After evaporation of the solvent, the residue was recrystallized from benzene to give colorless crystals (9.9 g, 73.5%). mp 109—110 °C. *Anal.* Calcd for $C_{11}H_{10}O_4$: C, 64.07; H, 4.89. Found: C, 63.77; H, 4.84. IR (KBr): 1720, 1690, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.70 (1H, dd, J=8, 17 Hz), 3.05 (1H, dd, J=5, 17 Hz), 3.30 (1H, m), 4.20 (1H, d, J=10 Hz), 4.60 (1H, dd, J=6, 10 Hz), 6.8—7.7 (3H, m), 7.90 (1H, dd, J=3, 8 Hz).

***cis*-3a,9b-Dihydro-4*H*-furo[3,2-*c*][1]benzopyran-2(3*H*)-one (7a)**——A mixture of Al (1.35 g) and $HgCl_2$ (trace) in *sec*-BuOH (100 ml) was refluxed until the solid was dissolved. A suspension of **6d** (5.0 g) in *sec*-BuOH (15 ml) was added to this mixture and the solution was refluxed for 14 h. After evaporation of the solvent *in vacuo*, 10% HCl was added to the residue at 0 °C and the mixture was stirred for 8 h. The resulting precipitates were collected and dried. Recrystallization from EtOH gave colorless needles (4.0 g, 86.7%), mp 102—104 °C. *Anal.* Calcd for $C_{11}H_{10}O_3$: C, 69.46; H, 5.30. Found: C, 69.31; H, 5.21. IR ($CHCl_3$): 1775 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.3—3.2 (3H, m, C_3-H_2, C_{3a}-H), 3.85 (1H, dd, J=8, 10 Hz, C_4-H_{ax}), 4.25 (1H, dd, J=4, 10 Hz, C_4-H_{eq}), 5.50 (1H, d, J=5.5 Hz, C_{9b}-H), 6.8—7.6 (4H, m, aromatic H).

***cis*-3a,9b-Dihydro-3-(phenylthio)-4*H*-furo[3,2-*c*][1]benzopyran-2(3*H*)-one (7b)**——Butyllithium (28 ml of 1.5 M solution in hexane) was added to a solution of diisopropylamine (6.8 ml) in dry THF (40 ml) at −78 °C and the

mixture was stirred at −78 °C for 15 min. Next, a solution of **7a** (3.6 g) in dry THF (25 ml) was added, and the stirring was continued for 1 h at −78 °C. A solution of diphenyldisulfide (4.5 g) and hexamethylphosphoric triamide (3.6 ml) in dry THF (25 ml) was added to the reaction mixture at −78 °C. The stirring was continued for 1 h at −70 °C, for 1 h at −30 °C, and for 1 h at 0 °C. The reaction was quenched by addition of sat.aq. NH_4Cl and the product was extracted with Et_2O. The extract was washed with 10% HCl, sat.aq. $NaHCO_3$, water, and brine, and then dried. After evaporation of the solvent, the residue was chromatographed on silica gel with benzene to give colorless crystals (3.4 g, 60.2%), mp 94—95 °C (from EtOH). *Anal.* Calcd for $C_{17}H_{14}O_3S$: C, 68.43; H, 4.73. Found: C, 68.25; H, 4.76. IR ($CHCl_3$): 1770 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.90 (1H, m, C_{3a}-H), 3.81 (1H, d, J=7 Hz, C_3-H), 4.15 (2H, m, C_4-H_2), 5.40 (1H, d, J=7 Hz, C_{9b}-H), 6.8—7.7 (9H, m, aromatic H).

Diethyl (3,4-Dihydro-7-methoxy-4-oxo-2*H*-1-benzopyran-3-yl)malonate (6g)——This compound (**6g**) was obtained from **6e** *via* 3-bromo-2,3-dihydro-7-methoxy-4*H*-1-benzopyran-4-one (**6f**)[8b] in 45.5% yield as colorless crystals, mp 64—66 °C (from cyclohexane). *Anal.* Calcd for $C_{17}H_{20}O_7$: C, 60.71; H, 5.99. Found: C, 60.47; H, 5.89. IR ($CHCl_3$): 1735, 1680, 1615, 1575 cm^{-1}.

3,4-Dihydro-7-methoxy-4-oxo-2*H*-1-benzopyran-3-acetic Acid (6h)——This compound (**6h**) was obtained from **6g** in 58.7% yield as colorless crystals, mp 131—133 °C (from benzene). *Anal.* Calcd for $C_{12}H_{12}O_5$: C, 61.01; H, 5.12. Found: C, 60.74; H, 4.84. IR (KBr): 1715, 1675, 1610, 1580 cm^{-1}. (lit.[9] mp 137—138 °C).

***cis*-3a,9b-Dihydro-7-methoxy-4*H*-furo[3,2-*c*][1]benzopyran-2(3*H*)-one (7c)**——This compound (**7c**) was obtained from **6h** in 72.9% yield as colorless crystals, mp 88—90 °C (from EtOH). *Anal.* Calcd for $C_{12}H_{12}O_4$: C, 65.44; H, 5.49. Found: C, 65.13; H, 5.30. IR ($CHCl_3$): 1780, 1620, 1580 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.42 (1H, dd, J=3.7, 17.6 Hz, C_3-H), 2.86 (1H, dd, J=8.3, 17.6 Hz, C_3-H), 3.00 (1H, m, C_{3a}-H), 3.79 (3H, s, OMe), 3.78 (1H, m, C_4-H_{ax}), 4.19 (1H, m, C_4-H_{eq}), 5.44 (1H, d, J=5.9 Hz, C_{9b}-H), 6.43 (1H, d, J=3 Hz, C_6-H), 6.61 (1H, dd, J=3, 8 Hz, C_8-H), 7.29 (1H, d, J=8 Hz, C_9-H). (lit.[10] mp 88—88.5 °C).

***cis*-3a,9b-Dihydro-7-methoxy-3-(phenylthio)-4*H*-furo[3,2-*c*][1]benzopyran-2(3*H*)-one (7d)**——This compound (**7d**) was obtained from **7c** in 78.8% yield as colorless crystals, mp 95—97 °C (from Et_2O). *Anal.* Calcd for $C_{18}H_{16}O_4S$: C, 65.83; H, 4.91. Found: C, 65.83; H, 4.79. IR (KBr): 1770, 1625, 1585 cm^{-1}.

Preparation of 1,3-Benzenediols (4a—n)——All of these compounds were prepared by the following general method which is exemplified by the preparation of **4a**.

General Method: 4-(2-Hydroxyethyl)-1,3-benzenediol (4a)——3-Buten-2-one (**2a**) (194 mg, 2.3 mmol) was added to a mixture of dihydro-3-(phenylsulfinyl)-2(3*H*)-furanone (**1a**)[6] (315 mg, 1.5 mmol) and magnesium methoxide (from Mg, 255 mg) in absolute MeOH (5 ml) at room temperature. The resulting solution was stirred for 16 h. After evaporation of the solvent *in vacuo*, 10% HCl was added to the residue at 0 °C and the product was extracted with CH_2Cl_2. The extract was washed with water and dried. Evaporation of the solvent *in vacuo* gave colorless crystals **3** (R^1=R^2=R^3=R^4=H). IR (Nujol): 1720 cm^{-1}. A suspension of these crystals in benzene (3 ml) was refluxed for 3 h. After removal of the solvent, the residue was chromatographed on silica gel with 3% MeOH/$CHCl_3$ (v/v) to give pure **4a** as an oil (130 mg, 56.3%). IR (film): 3300, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 5:1) δ: 2.76 (2H, t, J=6 Hz), 3.80 (2H, t, J=6 Hz), 6.25 (1H, dd, J=3, 8 Hz), 6.36 (1H, br s), 6.88 (1H, d, J=8 Hz). Methylation of this compound with Me_2SO_4/K_2CO_3 in acetone gave 2,4-dimethoxybenzeneethanol, mp 66—67 °C. (lit.[11] mp 67 °C).

4-(2-Hydroxyethyl)-6-methyl-1,3-benzenediol (4b)——This compound (**4b**) was obtained from **1a** and 3-methyl-3-buten-2-one (**2b**)[12] in 45% yield as colorless crystals, mp 101—102 °C (from $CHCl_3$). *Anal.* Calcd for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found: C, 63.96; H, 6.98. IR (KBr): 3400, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.12 (3H, s), 2.76 (2H, t), 3.81 (2H, t), 6.32 (1H, s), 6.76 (1H, s). High-resolution MS: Calcd for $C_9H_{12}O_3$ (168.0785). Found: 168.0779 (M^+).

4-(2-Hydroxyethyl)-2,6-dimethyl-1,3-benzenediol (4c)——This compound (**4c**) was obtained from **1a** and 2-methyl-1-penten-3-one (**2c**)[13] in 30.4% yield as an oil. High-resolution MS: Calcd for $C_{10}H_{14}O_3$ (182.0942). Found: 182.0902 (M^+). IR (film): 3400, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.17 (6H, s), 2.78 (2H, t), 3.89 (2H, t), 6.69 (1H, s). *Anal.* Calcd for $C_{31}H_{20}N_6O_{18}$ (tris-3,5-dinitrobenzoate, mp 128—131 °C from EtOH): C, 48.70; H, 2.64; N, 10.99. Found: C, 48.79; H, 2.70; N, 10.78.

4-(2-Hydroxypropyl)-1,3-benzenediol (4d)——This compound (**4d**) was obtained from dihydro-5-methyl-3-(phenylsulfinyl)-2(3*H*)-furanone (**1b**)[6] and **2a** in 40.7% yield as an oil. High-resolution MS: Calcd for $C_9H_{12}O_3$ (168.0785). Found: 168.0789 (M^+). IR (film): 3300, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 4:1) δ: 1.19 (3H, d, J=6.5 Hz), 2.68 (2H, m), 4.08 (1H, m), 6.30 (1H, dd, J=3, 8 Hz), 6.36 (1H, br s), 6.84 (1H, d, J=8 Hz). *Anal.* Calcd for $C_{11}H_{16}O_3$ (dimethylether, bp 150 °C/1 mmHg, mp 49—50 °C): C, 67.32; H, 8.22. Found: C, 67.05; H, 8.23.

4-(2-Hydroxypropyl)-6-methyl-1,3-benzenediol (4e)——This compound (**4e**) was obtained from **1b** and **2b** in 42.0% yield as an oil. High-resolution MS: Calcd for $C_{10}H_{14}O_3$ (182.0942). Found: 182.0926. IR (film): 3350, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 5:1) δ: 1.19 (3H, d, J=6 Hz), 2.11 (3H, s), 2.65 (2H, m), 4.06 (1H, m), 6.34 (1H, s), 6.72 (1H, s). *Anal.* Calcd for $C_{31}H_{20}N_6O_{18}$ (tris-3,5-dinitrobenzoate, mp 117—120 °C from EtOH): C, 48.70; H, 2.64; N, 10.99. Found: C, 48.72; H, 2.61; N, 10.90.

4-(2-Hydroxypropyl)-2,6-dimethyl-1,3-benzenediol (4f)——This compound (**4f**) was obtained from **1b** and **2c** in 19.9% yield as colorless crystals, mp 103—105 °C (from $CHCl_3$). High-resolution MS: Calcd for $C_{11}H_{16}O_3$ (196.1099). Found: 196.1098 (M^+). IR (KBr): 3400, 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 5:1) δ: 1.20 (3H, d, J=6 Hz), 2.12 (6H, s), 2.72 (2H, m), 4.10 (1H, m), 6.64 (1H, s). *Anal.* Calcd for $C_{32}H_{22}N_6O_{18}$ (tris-3,5-

dinitrobenzoate, mp 239—241 °C from AcOEt–EtOH): C, 49.36; H, 2.84; N, 10.80. Found: C, 49.65; H, 2.82; N, 10.59.

4-(2-Hydroxy-2-phenylethyl)-1,3-benzenediol (4g)——This compound (**4g**) was obtained from dihydro-5-phenyl-3-(phenylsulfinyl)-2(3*H*)-furanone (**1c**)[6] and **2a** in 42.6% yield as colorless crystals, mp 136—138 °C (from benzene). High-resolution MS: Calcd for $C_{14}H_{14}O_3$ (230.0942). Found: 230.0956 (M^+). *Anal.* Calcd for $C_{14}H_{14}O_3$: C, 73.02; H, 6.13. Found: C, 73.22; H, 6.10. IR (KBr): 3400, 1635, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 5:1) δ: 2.87 (1H, dd, J=4, 16 Hz), 3.02 (1H, dd, J=8, 16 Hz), 4.90 (1H, dd, J=4, 8 Hz), 6.29 (1H, dd, J=4, 8 Hz), 6.41 (1H, d, J=4 Hz), 6.75 (1H, d, J=8 Hz), 7.35 (5H, brs).

4-(2-Hydroxy-2-phenylethyl)-6-methyl-1,3-benzenediol (4h)——This compound (**4h**) was obtained from **1c** and **2b** in 44.4% yield as an oil. High-resolution MS: Calcd for $C_{15}H_{16}O_3$ (244.1098). Found: 244.1062 (M^+). IR (film): 3400, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.00 (3H, s), 2.70 (1H, dd, J=3.5, 11 Hz), 2.99 (1H, dd, J=9, 11 Hz), 4.79 (1H, dd, J=3.5, 9 Hz), 6.35 (1H, s), 6.61 (1H, s), 7.24 (5H, s-like). *Anal.* Calcd for $C_{36}H_{28}O_6$ (tribenzoate, mp 161—163 °C from AcOEt): C, 77.68; H, 5.07. Found: C, 77.50; H, 5.04.

4-(2-Hydroxy-2-phenylethyl)-2,6-dimethyl-1,3-benzenediol (4i)——This compound (**4i**) was obtained from **1c** and **2c** in 27.7% yield as an oil. High-resolution MS: Calcd for $C_{16}H_{18}O_3$ (258.1255). Found: 258.1236 (M^+). IR (film): 3400, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (3H, s), 2.08 (3H, s), 2.70 (1H, dd, J=2.5, 14.5 Hz), 3.04 (1H, dd, J=9, 14.5 Hz), 4.86 (1H, dd, J=2.5, 9 Hz), 6.52 (1H, s), 7.34 (5H, s-like). *Anal.* Calcd for $C_{22}H_{24}O_6$ (triacetate, mp 131—132 °C from EtOH): C, 68.73; H, 6.29. Found: C, 68.49; H, 6.36.

4-(*trans*-2-Hydroxycyclohexyl)-1,3-benzenediol (4j)——This compound (**4j**) was obtained from hexahydro-3-(phenylsulfinyl)-2(3*H*)-benzofuranone (**1d**)[6] and **2a** in 21.3% yield as colorless crystals, mp 153—155 °C (from benzene). High-resolution MS: Calcd for $C_{12}H_{16}O_3$ (208.1099). Found: 208.1085 (M^+). *Anal.* Calcd for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74. Found: C, 68.95; H, 7.46. IR (KBr): 3450, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.2—2.2 (9H, m), 2.6—2.9 (1H, t-like), 3.4—3.8 (1H, br), 6.29 (1H, d, J=3 Hz), 6.39 (1H, dd, J=3, 8 Hz), 6.96 (1H, d, J=8 Hz).

4-(*trans*-2-Hydroxycyclohexyl)-6-methyl-1,3-benzenediol (4k)——This compound (**4k**) was obtained from **1d** and **2b** in 16.4% yield as colorless crystals, mp 164—165 °C (from benzene). High-resolution MS: Calcd for $C_{13}H_{18}O_3$ (222.1255). Found: 222.1249 (M^+). *Anal.* Calcd for $C_{13}H_{18}O_3$: C, 70.24; H, 8.16. Found: C, 70.49; H, 8.35. IR ($CHCl_3$): 3300, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 5:1) δ: 1.2—2.1 (9H, m), 2.12 (3H, s), 2.55—2.90 (1H, t-like), 3.30—3.75 (1H, br), 6.33 (1H, s), 6.86 (1H, s).

4-(*trans*-2-Hydroxycyclohexyl)-2,6-dimethyl-1,3-benzenediol (4l)——This compound (**4l**) was obtained from **1d** and **2c** in 10.7% yield as an oil. High-resolution MS: Calcd for $C_{14}H_{20}O_3$ (236.1411). Found: 236.1436 (M^+). IR (film): 3450, 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.2—2.1 (9H, m), 2.15 (3H, s), 2.17 (3H, s), 2.40—2.90 (1H, t-like), 3.20—3.75 (1H, br), 6.73 (1H, s). *Anal.* Calcd for $C_{20}H_{26}O_6$ (triacetate, mp 121—123 °C from EtOH): C, 66.28; H, 7.23. Found: C, 66.07; H, 7.44.

4-(3,4-Dihydro-*cis*-4-hydroxy-2*H*-1-benzopyran-3-yl)-1,3-benzenediol (4m)——A solution of MCPBA (70%, 2.7 g) in CH_2Cl_2 (50 ml) was added to a solution of **7b** (3.0 g) in CH_2Cl_2 (30 ml) with stirring at 0 °C, and the stirring was continued for 2 h. The solution was washed with sat.aq. $NaHCO_3$ and water, and then dried. After evaporation of the solvent *in vacuo* at 20 °C, the residue was chromatographed on silica gel with $CHCl_3$ to give **1e** as colorless crystals (2.6 g, 82.3%), mp 140—145 °C. IR ($CHCl_3$): 1770 cm^{-1}. Compound **4m** was obtained from **1e** and **2a** in 29.4% yield as colorless crystals, mp 182—185 °C. High-resolution MS: Calcd for $C_{15}H_{14}O_4$ (258.0891). Found: 258.0906 (M^+). IR (KBr): 3410, 1620 cm^{-1}. *Anal.* Calcd for $C_{21}H_{20}O_7$ (triacetate, mp 119—124 °C from EtOH): C, 65.61; H, 5.24. Found: C, 65.49; H, 5.37.

4-(3,4-Dihydro-*cis*-4-hydroxy-7-methoxy-2*H*-1-benzopyran-3-yl)-1,3-benzenediol (4n)——Oxidation of **7d** gave **1f** in 78.5% yield as colorless crystals (mp 147—150 °C) in the same manner as noted for **1e**. IR (Nujol): 1760, 1620, 1580 cm^{-1}. Compound **4n** was obtained from **1f** and **2a** in 15.5% yield as colorless crystals, mp 184—187 °C (from EtOH–H_2O). High-resolution MS: Calcd for $C_{16}H_{16}O_5$ (288.0997). Found: 288.0986 (M^+). IR (KBr): 1620, 1580, 1500 cm^{-1}. ^{1}H-NMR ($CDCl_3$–CD_3OD, 20:1) δ: 3.49 (1H, m, C_3-H), 3.79 (3H, s, OMe), 4.20 (1H m, C_2-H_{ax}), 4.64 (1H, dd, J=10.5, 12.5 Hz, C_2-H_{eq}), 4.84 (1H, brs, width, 5.5 Hz, C_4-H), 6.35 (2H, m, C_2-H, C_6-H), 6.42 (1H, d, J=2.2 Hz, C_8-H), 6.51 (1H, dd, J=2.2, 8.1 Hz, C_6-H), 6.90 (1H, d, J=8.1 Hz, C_5-H), 7.18 (1H, d, J=8.8 Hz, C_5-H). *Anal.* Calcd for $C_{22}H_{22}O_8$ (triacetate, mp 152—155 °C from EtOH): C, 63.76; H, 5.35. Found: C, 63.47; H, 5.34.

***cis*-6a,11a-Dihydro-6*H*-benzofuro[3,2-*c*][1]benzopyran-9-ol (5a)**——A mixture of **4m** (100 mg) and AcOH (3 ml) was refluxed for 1 h and the solvent was evaporated off *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$ to afford **5a** as an oil (41 mg, 44.1%). IR ($CHCl_3$): 3400, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.59 (1H, m, C_{6a}-H), 3.63 (1H, m, C_6-H_{ax}), 4.27 (1H, m, C_6-H_{eq}), 5.54 (1H, d, J=6.6 Hz, C_{11a}-H), 6.37 (2H, m, C_8-H, C_{10}-H), 6.95 (1H, d, J=8.1 Hz, C_4-H), 7.03—7.30 (3H, m, C_2-H, C_3-H, C_7-H), 7.53 (1H, d, J=8.0 Hz, C_1-H). *Anal.* Calcd for $C_{22}H_{16}O_4$ (tribenzoate, mp 131—134 °C from EtOH): C, 76.73; H, 4.68. Found: C, 76.68; H, 4.87.

***cis*-6a,11a-Dihydro-3-methoxy-6*H*-benzofuro[3,2-*c*][1]benzopyran-9-ol (*dl*-Isomedicarpin)[5] (5b)**——This compound (**5b**) was obtained in 61.3% yield from **4n** in the same way as noted for **5a**; colorless crystals, mp 92—95 °C (from benzene). High-resolution MS: Calcd for $C_{16}H_{14}O_4$ (270.0891). Found: 270.0910 (M^+). *Anal.* Calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found: C, 71.00; H, 5.06. IR (KBr): 3380, 3270, 1620, 1600, 1580 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.52 (1H, m, C_{6a}-H), 3.63 (1H, m, C_6-H_{ax}), 3.79 (3H, s, OMe), 4.24 (1H, dd-like, J=5, 11 Hz, C_6-H_{eq}), 4.97 (1H, s, OH), 5.51 (1H, d, J=6.6 Hz, C_{11a}-H), 6.32—6.36 (2H, m, C_8-H, C_{10}-H), 6.47 (1H, d, J=2.7 Hz, C_4-H),

6.64 (1H, dd, J=2.7, 8.1 Hz, C_2-H), 7.08 (1H, d, J=8.8 Hz, C_7-H), 7.42 (1H, d, J=8.1 Hz, C_1-H).[14] (lit.[5a] mp 63—64 °C).

***cis*-6a,11a-Dihydro-3,9-dimethoxy-6*H*-benzofuro[3,2-*c*][1]benzopyran (*dl*-Homopterocarpin)[4] (5c)**——A mixture of **5b** (30 mg), K_2CO_3 (40 mg), and Me_2SO_4 (30 mg) in acetone (4 ml) was refluxed for 2 h. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was subjected to preparative thin-layer chromatography (AcOEt–hexane, 4 : 6) to afford **5c** as colorless crystals (27 mg, 85.7%), mp 129—130 °C (from EtOH). *Anal.* Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.67. Found: C, 71.70; H, 5.69. IR ($CHCl_3$): 1620, 1595sh, 1495 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.52 (1H, m, C_{6a}-H), 3.63 (1H, m, C_6-H_{ax}), 3.76 (3H, s, OMe), 3.78 (3H, s, OMe), 4.24 (1H, dd-like, J=5, 11 Hz, C_6-H_{eq}), 5.50 (1H, d, J=6.7 Hz, C_{11a}-H), 6.42—6.49 (3H, m, C_4-H, C_8-H, C_{10}-H), 6.64 (1H, dd, J=2.5, 8.5 Hz, C_2-H), 7.12 (1H, d, J=8.9 Hz, C_7-H), 7.42 (1H, d, J=8.5 Hz, C_1-H).[14] ^{13}C-NMR ($CDCl_3$) δ: 39.5 (d, C_{6a}), 55.4 (q, OMe), 55.5 (q, OMe), 66.6 (t, C_6), 78.6 (d, C_{11a}), 96.9 (d), 101.6 (d), 106.4 (d), 109.2 (d), 112.4 (s), 119.1 (s), 124.7 (d), 131.9 (d), 156.7 (s), 160.8 (s), 161.0 (s), 161.2 (s). UV λ_{max}^{EtOH} nm (log ε): 281sh (3.83), 286 (3.88). (lit.[4a] mp 123—125 °C).

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Synthesis of Coumarins by Nucleophilic Denitrocyclization Reaction

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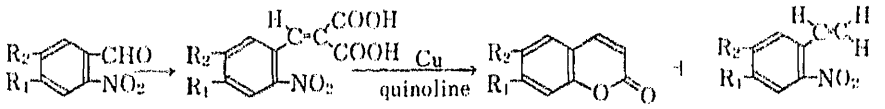
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2-[(2-Nitrophenyl)methylene]propanedioic acids (**2a—c**) were denitrocyclized to coumarins (**3a—c**) by heating in quinoline in the presence of copper powder. 2-(4-Methoxyphenyl)-3-(2-nitrophenyl)-(*Z*)-2-propenoic acids (**6a, b, d, e**) were also similarly denitrocyclized to 3-substituted coumarins (**7a, b, d, e**).

Keywords——coumarin; nucleophilic substitution; denitration; intramolecular cyclization; quinoline

An aromatic nitro group can be selectively replaced with a variety of nucleophiles.[1] In the previous paper[2] we reported a new, facile synthesis of coumarins by nucleophilic intramolecular denitrocyclization reaction, and several naturally occurring coumarins having electron-releasing substituents were synthesized. Now we have extended this reaction to some coumarin analogues having electron-withdrawing groups.

The reaction of electron-withdrawing group (halogeno or nitro)-sustituted *o*-nitrobenzaldehydes (**1a—c**) with malonic acid gave 2-[(2-nitrophenyl)methylene]propanedioic acids (**2a—c**) in almost quantitative yields, and denitrocyclization of **2a—c** in quinoline in the presence of copper powder[3] gave the cyclized products, coumarins (**3a—c**), in 78—91% yields, together with a small amount of by-products (**4a—c**). The yields of the coumarins (**3a—c**) were better than those of the previously reported coumarins[2] having electron-releasing substituents. This suggests that the electron-withdrawing group facilitates nucleophilic attack of the oxygen atom on the aromatic carbon.



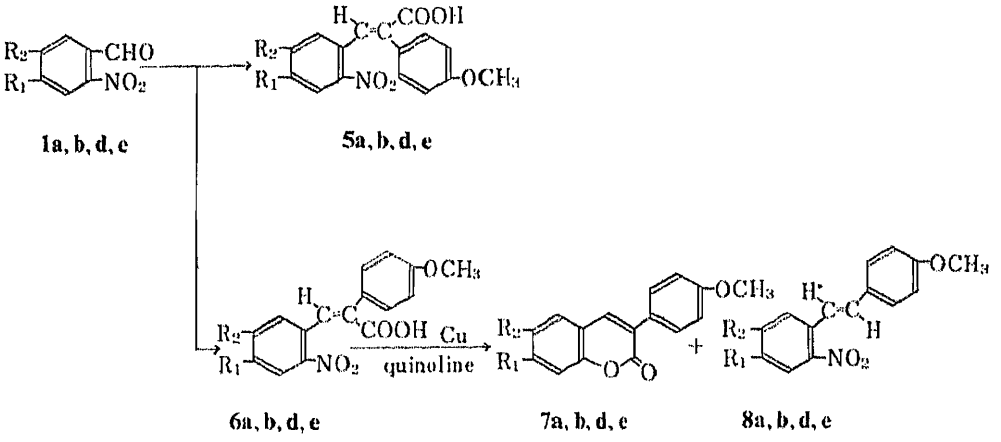
1a—c **2a—c** **3a—c** **4a—c**

	R_1	R_2
a:	H	Br
b:	H	Cl
c:	NO_2	H

Chart 1

3-Substituted coumarin derivatives (**7a,b,d,e**) were also synthesized by this denitrocyclization reaction. For this purpose, *o*-nitrobenzaldehyde derivatives (**1a,b,d,e**) and *p*-methoxyphenylacetic acid were reacted in acetic anhydride in the presence of triethylamine to give 2-(4-methoxyphenyl)-3-(2-nitrophenyl)-2-propenoic acids (**5,6**). The geometrical isomers of these propenoic acids (**5,6**) were separated by fractional precipitation using acetic acid and

hydrochloric acid. The denitrocyclization of the (*Z*)-isomers (**6a,b,d,e**) was carried out in quinoline in the same manner as described for the propanedioic acids (**2a—e**), and the products were separated by silica gel chromatography. The first eluate gave the decarboxylated 1-methoxy-4-[(*E*)-2-nitrophenyl)ethenyl]benzenes (**8a,b,d,e**) (7—21%) and the second eluate gave the desired denitrocyclized coumarins (**7a,b,d,e**) in moderate yields (55—82%).



	R_1	R_2
a:	H	Br
b:	H	Cl
d:	H	H
e:	O-CH_2-O	

Chart 2

In order to examine the application of this denitrocyclization reaction for the formation of heterocyclic rings, we attempted the denitrocyclization of some thiophene derivatives. However, in this case the cyclized compound was not obtained. The failure of cyclization of the thiophene ring is probably due to the electron-rich property of the heterocyclic ring.

Consequently our new method of synthesizing coumarins by nucleophilic intramolecular denitrocyclization is facile and convenient, provided that electron-withdrawing groups are present.

Experimental

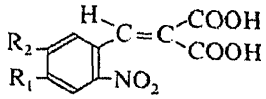
Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Mass spectra (MS) were measured with a JEOL DX-300 mass spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured with a JEOL JNR-100 spectrometer using tetramethylsilane as an internal standard. Infrared (IR) spectra were measured with a JASCO IR-810 spectrophotometer.

2-[(2-Nitrophenyl)methylene]propanedioic Acids (2a—e)——General Procedure: A mixture of 1 mmol of a substituted 2-nitrobenzaldehyde (**1a—e**) and 2 mmol of malonic acid in 5 ml of acetic acid was heated at 60 °C for 48 h. Acetic acid was removed *in vacuo* and the residue was crystallized from MeOH or MeOH-H_2O to give the corresponding propanedioic acid (**2a—e**). Physicochemical data of these compounds are shown in Table I.

Cyclization of 2a—e——General Procedure: A mixture of 1 mmol of **2a—e** and 50 mg of copper powder[3] in 10 ml of quinoline was heated to reflux on a mantle heater for 10 min. After cooling, the mixture was diluted with 50 ml of AcOEt and filtered. The filtrate was washed with 50 ml of 10% HCl to remove quinoline, then washed with water and dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the residue was chromatographed on silica gel[4] with $CHCl_3$. The first eluate gave the corresponding 1-ethenyl-2-nitrobenzene (**4a—e**). The second eluate gave the corresponding 2*H*-1-benzopyran-2-one (**3a—e**). Physicochemical data for these compounds are shown in Tables II and III.

2-(4-Methoxyphenyl-3-(nitrophenyl)-(*E*)-2-propenoic Acids (5a,b,d,e) and 2-(4-Methoxyphenyl)-3-(2-nitrophenyl)-(*Z*)-2-propenoic Acids (6a,b,d,e)——General Procedure: A mixture of 2 mmol of a substituted 2-nitrobenzaldehyde

TABLE I. 2-[(2-Nitrophenyl)methylene]propanedioic Acids (**2a—c**)



Compd. No.	R_1	R_2	mp (°C) (Recry. solv.)	Yield (%) Appearance	Formula	Analysis (%) Calcd (Found)		
						C	H	N
2a	H	Br	185—187 ($MeOH-H_2O$)	99 Colorless prisms	$C_{10}H_6BrNO_6$	38.00 (37.96	1.92 2.05	4.43 4.25)
2b	H	Cl	184—185 ($MeOH-H_2O$)	97 Colorless prisms	$C_{10}H_6ClNO_6$	44.22 (44.49	2.23 2.28	5.16 5.17)
2c	NO_2	H	171—172 (MeOH)	99 Colorless prisms	$C_{10}H_6N_2O_8$	42.59 (42.84	2.14 2.17	9.93 9.67)

Compd. No.	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ (ppm), J (Hz)
2a	3500—2400 (COOH), 1740 (CO), 1700 (CO), 1515 (NO_2), 1340 (NO_2)	7.68 (1H, d, J=2, phenyl 6-H), 7.78 (1H, dd, J=8, 2, phenyl 4-H), 7.88 (1H, s, -CH=C<), 8.08 (1H, d, J=8, phenyl 3-H)
2b	3400—2800 (COOH), 1740 (CO), 1705 (CO), 1515 (NO_2), 1340 (NO_2)	7.52—7.62 (2H, m, phenyl 4,6-H), 8.01 (1H, s, -CH=C<), 8.16 (1H, d, J=8, phenyl 3-H)
2c	3300—2400 (COOH), 1710 (CO), 1540 (NO_2), 1520 (NO_2), 1340 (NO_2)	7.78 (1H, d, J=8, phenyl 6-H), 8.02 (1H, s, -CH=C<), 8.49 (1H, dd, J=8, 2, phenyl 5-H), 8.90 (1H, d, J=2, phenyl 3-H)

TABLE II. 2*H*-1-Benzopyran-2-ones (**3a—c**)

Compd. No.	R_1	R_2	mp (°C)	Yield (%)	Recry. solv.
3a	H	Br	161—163[a]	84	MeOH
3b	H	Cl	162[b]	78	MeOH
3c	NO_2	H	199—202[c]	91	MeOH–H_2O

a) Lit.[5] mp 160 °C. *b*) Lit.[6] mp 161—162 °C. *c*) Lit.[7] mp 198—200 °C.

(**1a,b,d,e**), 2 mmol of 4-methoxyphenylacetic acid and 1 ml of Et_3N in 4 ml of acetic anhydride was heated at 130 °C for 13 h under stirring. Then 50 ml of water was added, the mixture was heated on a water bath for 1 h under stirring, 50 ml of 10% K_2CO_3 was added, and the whole was filtered. The filtrate was washed with ether, and the water layer was acidified with 20% HCl to give a precipitate. The crude yellow mixed acids were collected. For the separation of the (*E*)- and (*Z*)-propenoic acids, the mixed acids were dissolved in 50 ml of 10% K_2CO_3 and then acidified with AcOH. The (*E*)-propenoic acid that precipitated was collected. The mother liquor was strongly acidified with conc. HCl to give the (*Z*)-propenoic acid. The crude (*E*)-propenoic acid was again dissolved in 50 ml of 10% K_2CO_3 and acidified with AcOH to precipitate the (*E*)-propenoic acid. The filtrate was acidified with conc. HCl to precipitate the (*Z*)-propenoic acid. This AcOH–HCl separation was continued until no precipitate was observed upon the addition of conc. HCl. These acids were crystallized from suitable solvents. Physicochemical data for these acids are shown in Tables IV and V.

Denitrocyclization of 6a,b,d,e——General Procedure: A mixture of 1 mmol of a propenoic acid (**6a,b,d,e**) and 50 mg of copper powder[3] in 15 ml of quinoline was heated to reflux for 5 min on a mantle heater. After cooling, the mixture was diluted with 50 ml of AcOEt and filtered. The filtrate was washed with 50 ml of 10% HCl to remove quinoline, then washed with water and dried over $MgSO_4$. The solvent was evaporated off *in vacuo* and the residue was chromatographed on silica gel[4] with $CHCl_3$. The first eluate gave the corresponding 1-methoxy-4-[(*E*)-2-(2-nitrophenyl)ethynyl]benzene (**8a,b,d,e**). From the second eluate, the corresponding 3-(4-methoxyphenyl)-2*H*-1-

TABLE III. 1-Ethenyl-2-nitrobenzenes (**4a—c**)

Compd. No.	R_1	R_2	mp (°C) (Recry. solv.)	Yield (%) Appearance	Formula	Analysis (%) Calcd (Found) C	H	N
4a	H	Br	50—51 (*n*-Hexane)	4 Colorless needles	$C_8H_6BrNO_2$	42.11 (41.96	2.63 2.49	6.14 6.14)
4b	H	Cl	33—34 (*n*-Hexane)	8 Yellow needles	$C_8H_6ClNO_2$	52.34 (52.23	3.29 3.17	7.63 7.50)
4c	NO_2	H	58—59[a] (MeOH)	Trace Yellow needles				

Compd. No.	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ (ppm), J (Hz)	MS (*m/z*)
4a	1525 (NO_2), 1340 (NO_2)	5.52 (1H, d, J=10, $\underline{H}$), 5.73 (1H, d, J=17, $\underline{H}$), 7.17 (1H, dd, J=17, 10 $\underline{H}$)	227, 229 (M^+), 210, 212 (M^+ −OH)
4b	1520 (NO_2), 1340 (NO_2)	5.51 (1H, d, J=10, $\underline{H}$), 5.73 (1H, d, J=17, $\underline{H}$), 7.15 (1H, dd, J=17, 10 $\underline{H}$)	185 (M^+), 168 (M^+ −OH)

a) Lit.[8] mp 58—59 °C.

TABLE IV. 2-(4-Methoxyphenyl)-3-(2-nitrophenyl)-(*E*) and (*Z*)-2-Propenoic Acids (**5a, b, d, e, 6a, b, d, e**)

Compd. No.	R_1	R_2	mp (°C) (Recry. solv.)	Appearance	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N
5a	H	Br	168—170 (MeOH H_2O)	Yellow needles	36	$C_{16}H_{12}BrNO_5$	50.82 (50.88	3.20 3.18	3.70 3.66)
5b	H	Cl	156—157 (MeOH H_2O)	Yellow needles	33	$C_{16}H_{12}ClNO_5$	57.58 (57.41	3.63 3.34	4.20 4.01)
5d	H	H	180—181[a] (Benzene)	Yellow prisms	31	$C_{16}H_{13}NO_5$	64.20 (64.07	4.39 4.34	4.68 4.66)
5e			208—209 (MeOH H_2O)	Yellow prisms	31	$C_{17}H_{13}NO_7$	59.47 (59.37	3.82 3.62	4.08 4.19)
6a	H	Br	176—177 (MeOH–H_2O)	Yellow needles	52	$C_{16}H_{12}BrNO_5$	50.82 (50.84	3.20 3.19	3.70 3.89)
6b	H	Cl	173—174 (MeOH–H_2O)	Yellow needles	47	$C_{16}H_{12}ClNO_5$	57.58 (57.52	3.63 3.34	4.20 4.11)
6d	H	H	164—165 (Benzene)	Yellow prisms	51	$C_{16}H_{13}NO_5$	64.20 (64.17	4.39 4.12	4.68 4.58)
6e			160—161 (MeOH–H_2O)	Yellow needles	49	$C_{17}H_{13}NO_7$	59.47 (59.29	3.82 3.71	4.08 3.99)

a) Lit.[9] mp 177 °C.

TABLE V. Spectral Data for 2-(4-Methoxyphenyl)-3-(2-nitrophenyl)-(*E*) and (*Z*)-2-Propenoic Acids (**5a, b, d, e, 6a, b, d, e**)

Compd. No.	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ (ppm), J (Hz)	MS (*m/z*)
5a	3450—2500 (COOH), 1680 (CO), 1505 (NO_2), 1335 (NO_2)	3.79 (3H, s, OCH_3), 6.78 (2H, d, J=8, 2-phenyl 3,5-H), 7.06 (2H, d, J=8, 2-phenyl 2,6-H), 7.12 (1H, s, –CH=C<), 7.48 (1H, dd, J=8, 2, 3-phenyl 4-H), 7.94 (1H, d, J=8, 3-phenyl 3-H), 8.10 (1H, s, 3-phenyl 6-H), 9.62 (1H, br s, –COOH)	377, 379 (M^+), 332, 334 (M^+ – COOH)
5b	3450—2610 (COOH), 1685 (CO), 1505 (NO_2), 1334 (NO_2)	3.78 (3H, s, OCH_3), 7.00—8.45 (7H, m, phenyl-H), 7.21 (1H, s, –CH=C<)	333 (M^+), 288 (M^+ – COOH)
5d	3300—2400 (COOH), 1680 (CO), 1520 (NO_2), 1340 (NO_2)	3.71 (3H, s, OCH_3), 6.67 (2H, d, J=8, 2-phenyl 3,5-H), 7.08 (2H, d, J=8, 2-phenyl 2,6-H), 7.30—7.40 (3H, m, 3-phenyl 4,5,6-H), 7.84 (2H, s×2, –CH= and 3-phenyl 3-H)	299 (M^+), 254 (M^+ – COOH)
5e	3210—2840 (COOH), 1720 (CO), 1510 (NO_2), 1325 (NO_2)	3.74 (3H, s, OCH_3), 6.10 (2H, s, OCH_2O), 6.30 (1H, s, 3-phenyl 6-H), 6.78 (2H, d, J=8, 2-phenyl 3,5-H), 7.02 (2H, d, J=8, 2-phenyl 2,6-H), 7.58 (1H, s, –CH=C<), 7.84 (1H, s, 3-phenyl 3-H)	343 (M^+), 298 (M^+ – COOH)
6a	3600—3100 (COOH), 1685 (CO), 1505 (NO_2), 1340 (NO_2)	3.84 (4H, s, OCH_3), 6.93 (2H, d, J=8, 2-phenyl 3,5-H), 7.20 (1H, s, –CH=C<), 7.48 (2H, d, J=8, 2-phenyl 2,6-H), 7.68 (1H, s, 3-phenyl 6-H), 7.68 (1H, d, J=8, 3-phenyl 4-H), 8.00 (1H, d, J=8, 3-phenyl 3-H)	377, 379 (M^+), 332, 334 (M^+ – COOH)
6b	3600—2700 (COOH), 1685 (CO), 1510 (NO_2), 1335 (NO_2)	3.88 (3H, s, OCH_3), 6.98 (2H, d, J=8, 2-phenyl 3,5-H), 7.23 (1H, s, –CH=C<), 7.49 (2H, d, J=8, 2-phenyl 2,6-H), 7.52 (1H, s, 3-phenyl 6-H), 7.59 (1H, d, J=8, 3-phenyl 4-H), 8.09 (1H, d, J=8, 3-phenyl 3-H)	333 (M^+)
6d	3300—2400 (COOH), 1685 (CO), 1520 (NO_2), 1340 (NO_2)	3.80 (3H, s, OCH_3), 6.64 (2H, d, J=8, 2-phenyl 3,5-H), 6.97 (2H, d, J=8 Hz, 2-phenyl 2,6-H), 7.17 7.60 (3H, m, 3-phenyl 4,5,6-H), 7.88 (1H, s, –CH=C<), 8.00 (1H, d, J=8, 3-phenyl 3-H)	299 (M^+), 254 (M^+ – COOH)
6e	3400—2800 (COOH), 1715 (CO), 1515 (NO_2), 1340 (NO_2)	3.82 (3H, s, OCH_3), 6.16 (2H, s, OCH_2O), 6.80—7.01 (4H, m, 2-phenyl H), 7.20 (1H, s, 3-phenyl 6-H), 7.54 (1H, s, –CH=C<), 7.61 (1H, s, 3-phenyl 3-H)	343 (M^+), 298 (M^+ – COOH)

benzopyran-2-one (**7a,b,d,e**) was obtained. Physicochemical data for these compounds are given in Tables VI and VII.

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TABLE VI. 3-(4-Methoxyphenyl)-2*H*-1-benzopyran-2-ones (**7a, b, d, e**)

Compd. No.	R_1	R_2	mp (°C) (Recry. solv.)	Yield (%) Appearance	Formula	Analysis (%) Calcd (Found) C	H	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ (ppm), J (Hz)	MS (m/z)
7a	H	Br	208—209 (AcOEt)	79 Colorless plates	$C_{16}H_{11}BrO_3$	57.83 (58.08	3.31 3.33)	1720 (CO)	3.81 (3H, s, OCH_3), 6.94 (2H, d, J=8, phenyl 3′,5′-H), 7.25 (1H, d, J=8, 8-H), 7.64 (2H, d, J=8, phenyl 2′,6′-H), 7.70 (1H, s, 4-H), 7.89 (1H, s, 5-H), 8.04 (1H, d, J=8, 7-H)	332, 330 (M^+), 304, 302 (M^+-CO)
7b	H	Cl	197 (AcOEt)	82 Colorless plates	$C_{16}H_{11}ClO_3$	67.02 (67.08	3.87 3.78)	1715 (CO)	3.83 (3H, s, OCH_3), 6.94 (2H, d, J=8, phenyl 3′,5′-H), 7.24 (1H, d, J=8, 8-H), 7.4 (2H, d, J=8, phenyl 2′,6′-H), 7.45 (1H, s, 5-H), 7.60 (1H, s, 4-H), 7.61 (1H, d, J=8, 7-H)	286 (M^+), 258 (M^+-CO)
7d	H	H	141[a] (AcOEt)	64 Colorless plates	$C_{16}H_{12}O_3$	76.17 (76.22	4.80 4.79)	1735 (CO)	3.80 (3H, s, OCH_3), 6.94 (2H, d, J=8, phenyl 3′,5′-H), 7.21—7.69 (4H, m, 5,6,7,8-H), 7.39 (2H, d, J=8, phenyl 2′,6′-H), 7.70 (1H, s, 4-H)	252 (M^+), 224 (M^+-CO)
7e			243 (AcOEt)	55 Colorless plates	$C_{17}H_{12}O_5$	68.92 (68.88	4.05 3.97)	1690 (CO)	3.83 (3H, s, OCH_3), 6.20 (2H, s, OCH_2O), 6.94 (2H, d, J=8, phenyl 3′,5′-H), 7.18 (1H, s, 8-H), 7.36 (1H, s, 5-H), 7.48 (2H, d, J=8, phenyl 2′,6′-H), 7.50 (1H, s, 4-H)	296 (M^+), 268 (M^+-CO)

a) Lit.[10] mp 136.5—138.5 °C.

TABLE VII. (*E*)-4-Methoxyphenylethenyl-nitrobenzenes (**8a, b, d, e**)

Compd. No.	R_1	R_2	mp (°C) (Recry. solv.)	Yield (%) Appearance	Formula	Analysis (%) Calcd (Found) C	H	N	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$) δ (ppm), J (Hz)	MS (m/z)
8a	H	Br	69—70 (*n*-Hexane)	19 Yellow needles	$C_{15}H_{12}BrNO_3$	53.73 (53.96	3.58 3.37	4.18 4.16)	1505 (NO_2) 1340 (NO_2)	3.82 (3H, s, OCH_3), 6.87 (2H, d, J=8, 2-phenyl 3,5-H), 6.74 (1H, d, J=17, ethylene 2-H), 7.38 (2H, d, J=8, 2-phenyl 2,6-H), 7.41 (1H, d, J=17, ethylene 1-H), 7.64 (1H, s, 1-phenyl 2-H), 7.66 (1H, d, J=8, 1-phenyl 6-H), 7.79 (1H, d, J=8, 1-phenyl 5-H)	333, 335 (M^+), 316, 318 (M^+-OH)
8b	H	Cl	74—76 (*n*-Hexane)	7 Yellow needles	$C_{15}H_{12}ClNO_3$	62.18 (61.95	4.18 4.04	4.84 4.86)	1510 (NO_2) 1345 (NO_2)	3.86 (3H, s, OCH_3), 6.79 (1H, d, J=17, ethylene 2-H), 6.83 (2H, d, J=8, 2-phenyl 3,5-H), 7.04 (1H, d, J=17, ethylene 1-H), 7.17 (2H, d, J=8, 2-phenyl 2,6-H), 7.49 (1H, d, J=8, 1-phenyl 6-H), 7.72 (1H, s, 1-phenyl 2-H), 7.90 (1H, d, J=8, 1-phenyl 5-H)	291 (M^+), 274 (M^+-OH)
8d	H	H	85—87[a] (*n*-Hexane)	11 Yellow prisms	$C_{15}H_{13}NO_3$	70.57 (70.66	5.14 5.20	5.49 5.43)	1505 (NO_2) 1340 (NO_2)	3.81 (3H, s, OCH_3), 6.89 (2H, d, J=8, 1-phenyl 3,5-H), 7.04 (1H, d, J=18, ethylene 1-H), 7.49 (2H, d, J=8, 1-phenyl 2,6-H), 7.51 (1H, d, J=18, ethylene 2-H), 7.59—7.80 (3H, m, 2-phenyl 4,5,6-H), 7.92 (1H, d, J=8, 1-phenyl 3-H)	255 (M^+), 238 (M^+-OH)
8e			121—122 (MeOH–H_2O)	21 Yellow prisms	$C_{16}H_{13}NO_5$	64.21 (64.28	4.35 4.29	4.68 4.65)	1510 (NO_2) 1320 (NO_2)	3.83 (3H, s, OCH_3), 6.05 (2H, s, OCH_2O), 6.84 (1H, d, J=17, ethylene 2-H), 6.85 (2H, d, J=8, 2-phenyl 3,5-H), 7.05 (1H, s, 1-phenyl 6-H), 7.40 (2H, d, J=8, 2-phenyl 2,6-H), 7.45 (1H, s, 1-phenyl 3-H), 7.50 (1H, d, J=17, ethylene 1-H)	299 (M^+), 282 (M^+-OH),

a) Lit.[11] mp 88—90 °C.

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The Constituents of *Eucommia ulmoides* OLIV. VI. Isolation of a New Sesquilignan and Neolignan Glycosides

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A new sesquilignan glycoside, syringylglycerol-β-syringaresinol ether 4'',4'''-di-*O*-β-D-glucopyranoside (**1**), and a new neolignan glycoside, dehydrodiconiferyl alcohol 4,γ'-di-*O*-β-D-glucopyranoside (**2**), were isolated from the bark of *Eucommia ulmoides* OLIV. (Eucommiaceae), together with a known neolignan glycoside, citrusin B (**3**). Their structures were determined on the basis of chemical evidence and spectroscopic analyses.

Keywords——*Eucommia ulmoides*; lignan; sesquilignan; neolignan; dihydrobenzofuran; syringylglycerol-β-syringaresinol ether 4'',4'''-di-*O*-β-D-glucopyranoside; dehydrodiconiferyl alcohol 4,γ'-di-*O*-β-D-glucopyranoside; ^{13}C-NMR

In previous papers, we reported the isolation of a new sesquilignan glycoside, hedyotol C 4',4''-di-*O*-β-D-glucopyranoside (**4**),[1] and new lignans having a β-*O*-4 linkage, *erythro*-dihydroxydehydrodiconiferyl alcohol[2] and its *threo*-isomer,[2] from the bark of *Eucommia ulmoides* OLIV. (Eucommiaceae). As a continuation of this study, we now wish to report the isolation and structures of a new sesquilignan glycoside, syringylglycerol-β-syringaresinol ether 4'',4'''-di-*O*-β-D-glucopyranoside (**1**), and a new neolignan glycoside, dehydrodiconiferyl alcohol 4,γ'-di-*O*-β-D-glucopyranoside (**2**), together with a known neolignan glycoside, citrusin B (**3**).[3] The extraction and separation were carried out as described in the experimental section.

Compound **1** was obtained as an amorphous powder, $[\alpha]_D^{26}$ −11.11° (MeOH), which gave a spot a little higher than that of **4**[1] on thin layer chromatography (TLC) with $CHCl_3$–MeOH–water (70 : 30 : 5) as a developing solvent. The infrared (IR) spectrum of **1** showed the presence of hydroxyl groups (3432 cm^{-1}) and aromatic rings (1598 and 1506 cm^{-1}). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed signals at δ 3.74 due to six aromatic methoxyl groups, and at δ 6.64 due to six aromatic protons on three syringyl rings. The field desorption mass spectrum (FD-MS) gave peaks at *m/z* 1007 (M^++K), 991 (M^++Na), 829 ($M^+ - C_6H_{10}O_5$+Na), 667 ($M^+ - 2\times C_6H_{10}O_5$+Na) and 418. These peaks are 60 mass units larger than the corresponding peaks of **4**.[1] These findings suggest that compound **1** is syringylglycerol-β-syringaresinol ether glycoside. Acetylation of **1** with acetic anhydride–pyridine gave a decaacetate (**1a**) as an amorphous powder, whose ^{1}H-NMR showed signals at δ 1.99, 2.03, 2.15 and 2.18 due to ten alcoholic acetyl groups, at δ 3.76, 3.80 and 3.83 due to six aromatic methoxyl groups, at δ 5.04 (2H, d, J=6.0 Hz) due to two anomeric protons of glucose, and at δ 6.53, 6.56 and 6.60 due to six aromatic protons in three syringyl rings. Except for the glucose moieties, the ^{1}H-NMR spectrum of **1a** was similar to that of syringylglycerol-β-syringaresinol ether tetraacetate.[4] Furthermore, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) signals of **1** and **1a** were assigned as shown in Table I, in

comparison with those of **4**, the decaacetate of **4**[1] and liriodendrin[(+)-syringaresinol di-*O*-*β*-D-glucopyranoside].[5] From the above results, compound **1** was determined to be syringylglycerol-*β*-syringaresinol ether 4″,4‴-di-*O*-*β*-D-glucopyranoside.

Compound **2** was isolated as a white powder, mp 134.7 °C, $[\alpha]_D^{28}$ −62.31° (MeOH), whose FD-MS gave peaks at *m/z* 683 ($M^+ + 1$), 521 ($M^+ - C_6H_{10}O_5 + 1$) and 359 ($M^+ - 2 \times C_6H_{10}O_5 + 1$). The IR spectrum showed the presence of hydroxyl groups (3488 cm^{-1}) and aromatic rings (1614, 1522 and 1502 cm^{-1}). The ^{1}H-NMR spectrum showed signals at δ 3.74 and 3.80 due to two aromatic methoxyl groups, at δ 5.52 (1H, d, J=6.15 Hz) due to a benzylic proton, at δ 6.19 (1H, dt, J=15.83, 6.59 Hz) and δ 6.59 (1H, d, J=15.83 Hz) due to two olefinic protons, and at δ 6.80—7.20 due to five aromatic protons. Acetylation of **2** gave a nonaacetate (**2a**) as an amorphous powder. The ^{1}H-NMR spectrum showed signals at δ 1.96 and 1.98, due to nine alcoholic acetyl groups. The enzymatic hydrolysis of **2** with *β*-glucosidase gave an aglycone (**5**), $[\alpha]_D^{28}$ −20.7° (MeOH), as an amorphous powder, and glucose. Acetylation of **5** afforded a triacetate (**5a**) as an amorphous powder, whose MS gave peaks at *m/z* 484 (M^+), 424 ($M^+ - CH_3COOH$) and 382. The ^{1}H-NMR spectrum showed signals at δ 2.05 and 2.10 due to two alcoholic acetyl groups, and at δ 2.30 due to a phenolic acetyl group. Compounds **5** and **5a** were identified as dehydrodiconiferyl alcohol[6] and its triacetate,[6] respectively, by comparison (IR, ^{1}H-, ^{13}C-NMR and MS) with authentic samples. From the above results, compound **2** was determined to be dehydrodiconiferyl alcohol diglucoside in which one glucose moiety is attached to the phenolic group. This conclusion was supported by the ^{13}C-NMR glucosylation shifts of C-1 (+3.0) and C-3 (+1.6 ppm), in

Table I. ^{13}C-NMR Chemical Shifts (in DMSO-d_6)

Carbon No.	1	4	7	1a[a]	Carbon No.	1	4	7	1a[a]
C-1,5	53.5	53.7	53.6	54.6	Glc-1	102.7	100.4	102.8	101.2
C-4,8	71.1	71.2	71.4	72.4		102.9	101.9		
C-2	84.9	84.8	85.0	86.0	Glc-2	74.0	73.3	74.2	69.9
C-6		85.1					73.5		
C-1′	134.8	134.8	134.0	134.1	Glc-3	76.4	76.8	76.5	72.2
C-1″	133.6	135.3			Glc-4	69.8	69.8	70.0	72.2
C-1‴	133.8	136.2		134.4	Glc-5	76.9	76.8	77.1	73.4
C-2′	103.4	103.5	104.4	103.4	Glc-6	60.9	60.7	61.0	62.6
C-2″	104.3	110.9		103.8			61.1		
C-2‴	105.3	111.6		105.5					
C-3′	152.5	152.6	152.6	153.1	$OCO\underline{C}H_3$				20.7
C-3″		149.0		153.6					
C-3‴	151.9	148.5			$O\underline{C}OCH_3$				169.3
C-4′	137.0	136.9	137.1	137.5					169.5
C-4″		145.9		138.2					169.6
C-4‴	138.1	145.5							170.4
C-5′	152.5	152.6	152.6	153.1					170.5
C-5″		115.6		153.6					170.6
C-5‴	151.9	115.0							
C-6′	103.4	103.5	104.4	103.4					
C-6″	104.3	118.1		103.8					
C-6‴	105.3	119.3		105.5					
C-α	72.1	72.0		72.2					
C-β	85.7	85.9		80.8					
C-γ	59.7	59.8		62.6					
OCH_3	55.9	55.9	56.5	56.3					
	56.3	56.0		56.7					

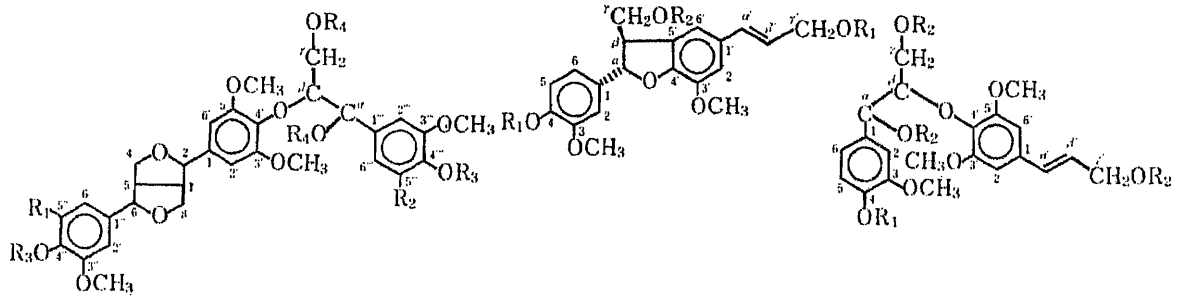
a) In $CDCl_3$. 7: Liriodendrin [(+)-Syringaresinol di-*O*-*β*-D-glucopyranoside].[5]

Table II. ^{13}C-NMR Chemical Shifts (in DMSO-d_6)

Carbon No.	2	5	$\Delta\delta$ (2−5)	3	6	$\Delta\delta$ (3−6)
C-1	135.3	132.3	+3.0	136.2	133.1	+3.1
C-1′	130.3	129.5		132.3	132.2	
C-2	110.6	110.5		111.6	111.2	
C-2′				103.7	103.8	
C-3	149.1	147.5	+1.6	148.4	146.8	+1.6
C-3′	143.7	143.6		152.7	152.6	
C-4	146.3	146.3		145.4	145.2	
C-4′	147.4	147.1		134.9	134.9	
C-5	115.6	115.3		115.0	114.5	
C-5′	131.8	130.5		152.7	152.6	
C-6	118.1	118.4		119.1	119.2	
C-6′	115.2	114.9		103.7	103.8	
C-α	86.9	87.1		71.9	72.0	
C-α′	129.3	128.9	+0.4	130.0	129.9	
C-β	53.1	52.9		85.9	85.9	
C-β′	123.6	127.9	−4.3	128.4	128.4	
C-γ	63.0	62.9		59.6	59.6	
C-γ′	68.7	61.5	+7.2	61.3	61.1	
OCH_3	55.8	55.6		55.7	55.5	
				55.9	55.8	
					56.0	
Glc-1	100.2			100.3		
	101.9					
Glc-2	73.5			73.2		
Glc-3	76.8			76.7		
Glc-4	69.7			69.7		
	70.2					
Glc-5	76.8			76.7		
Glc-6	60.8			60.6		
	61.1					

going to **2** from **5**; they are identical with the 4-*O*-β-D-glucopyranosylation shifts of the guaiacyl group.[7] The position of the other glucose linkage was determined from the ^{13}C-NMR glucosylation shifts of the C-β′ and C-γ′ atoms. As shown in Table II, the signals of **2** at δ 123.6 and 68.7 were assigned to C-β′ and C-γ′, respectively. The shifts of the corresponding carbons in going to **2** from **5** were −4.3 and +7.2 ppm, respectively, which indicated that one glucosyl group in **2** was linked to the C-γ′ atom. Thus, compound **2** was established as a new neolignan glycoside, dehydrodiconiferyl alcohol 4,γ′-di-*O*-β-D-glucopyranoside.

Compound **3** was isolated as an amorphous powder, $[\alpha]_D^{26}$ −16.32° (MeOH). The ultraviolet (UV) (223 and 273 nm) and IR spectra (3412, 1588 and 1510 cm^{-1}) suggested the lignan structure. Acetylation of **3** gave a heptaacetate (**3a**), whose ^{1}H-NMR spectra showed the presence of seven alcoholic acetyl groups (δ 1.97, 2.03, 2.10 and 2.13). The enzymatic hydrolysis of **3** with β-glucosidase afforded an aglycone (**6**) as a colorless syrup, $[\alpha]_D^{27}$ +12.5° (MeOH), and glucose. The MS of **6** gave peaks at *m/z* 406 (M^+), 358 ($M^+ - CH_2OH - OH$) and 210. The ^{1}H-NMR spectrum showed signals at δ 3.74 and 3.77 due to three aromatic methoxyl groups, at δ 6.15 (dt, J=15.83, 5.71 Hz) and δ 6.58 (d, J=15.83 Hz) due to two *trans* olefinic protons, and at δ 6.57 and 6.75—7.20 due to five aromatic protons. The ^{13}C-NMR signals of **3** and **6** were assigned as shown in Table II, by comparison with those of **4** and syringin. From the above results, compound **6** was determined to be guaiacylglycerol-β-*O*-4′-synapyl ether. The ^{13}C-NMR chemical shift differences of the corresponding carbons in going



1: $R_1=R_2=OCH_3$, $R_3=glc$, $R_4=H$
1a: $R_1=R_2=OCH_3$, $R_3=glc(Ac)_4$, $R_4=Ac$
2: $R_1=glc$, $R_2=H$
2a: $R_1=glc(Ac)_4$, $R_2=Ac$
3: $R_1=glc$, $R_2=H$
3a: $R_1=glc(Ac)_4$, $R_2=Ac$
4: $R_1=R_2=R_4=H$, $R_3=glc$
5: $R_1=R_2=H$
5a: $R_1=R_2=Ac$
6: $R_1=R_2=H$

Chart

to **3** from **6** were +3.1 (C-1) and +1.6 ppm (C-3), respectively, which indicated that a glucose moiety in **3** was attached to the phenolic group. The ^1H- and ^{13}C-NMR chemical shifts of **3** were identical with those of citrusin B.[3] Thus, compound **3** was identified as citrusin B.

Experimental

All melting points are uncorrected. The following instruments were used; melting point, Mettler FP-61; optical rotation value, JASCO DIP-4; UV spectra, Hitachi 200-20; IR spectra, Hitachi 270-30; GC, Hitachi 063 with a hydrogen flame ionization detector; electron impact mass spectrum (EI-MS), Hitachi RMU-7L; FD-MS, JEOL 01-SG2; ^1H-NMR spectra, JEOL LMN-FX-90; ^{13}C-NMR spectra, JEOL JMN-FX-60, with tetramethylsilane ($\delta=0$) as an internal reference. The abbreviations used for NMR data are as follows; s, singlet; brs, broad singlet, d, doublet; dt, doublet triplet; t, triplet; m, multiplet.

Precoated TLC plates (Silica gel 60_{F254}, Merck) were used for TLC. The spots were detected by spraying the plates with 20% H_2SO_4 and by heating. Silica gel (Wako gel C-300, Wako Pure Chemical Co.), polyamide C-200 (Wako Pure Chemical Co.) and Diaion HP-20 (Nippon Rensui Co.) were used for chromatography. TSK gel HW-40 (Toyo Soda Co.) was used for gel filtration.

Isolation——The air-dried bark of *Eucommia ulmoides* OLIV. (10 kg, commercial crude drug produced in China) was chopped and extracted with hot water (20 l × 3). The extract was filtered and the filtrate was evaporated under reduced pressure to a small volume, then suspended in water. This suspension was extracted with EtOAc (3 l × 2) and *n*-BuOH (3 l × 2), successively. The aqueous layer was evaporated under reduced pressure to remove *n*-BuOH, and subjected to column chromatography (Diaion HP-20), eluting with water (5 l), 30% MeOH (5 l), 50% MeOH (10 l) and 100% MeOH (15 l), successively. The 50% MeOH eluate was evaporated to dryness under reduced pressure, and then subjected to silica gel chromatography, eluting with $CHCl_3$–MeOH–water (100 : 10 : 1, 80 : 20 : 3, 70 : 30 : 5). The fractions were monitored by TLC using $CHCl_3$–MeOH–water (70 : 30 : 5) as a developing solvent. The fractions showing spots at *Rf* 0.32 (**2**) and 0.63 (**3**) were each collected and purified by repeated silica gel chromatography and gel filtration on TSK gel HW-40 with 50% MeOH, yielding **2** (0.2 g) and **3** (50 mg). The 100% MeOH eluate was evaporated to dryness, and dissolved in water (0.2 l), then, subjected to polyamide C-200 chromatography, eluting with water (2 l) and MeOH (3 l), successively. The MeOH eluate was evaporated under reduced pressure, and subjected to silica gel chromatography; the fractions were monitored under the above conditions. The fractions showing spots at *Rf* 0.50 (**4**) and 0.60 (**1**) were each collected and purified in the same way, yielding **1** (50 mg) and **4** (0.1 g).

Syringylglycerol-β-syringaresinol Ether 4″,4‴-Di-*O*-β-D-glucopyranoside (1)——An amorphous powder, $[\alpha]_D^{26}$ −11.11° ($c=0.14$, MeOH). UV λ_{max}^{MeOH} nm: 228, 270, 279. IR ν_{max}^{KBr} cm^{-1}: 3432 (OH), 1596, 1506 (aromatic ring). FD-MS *m/z*: 1007 (M^++K), 991 (M^++Na), 829 ($M^+-C_6H_{10}O_5$+Na), 667 ($M^+-2\times C_6H_{10}O_5$), 603, 418. ^1H-NMR (in DMSO-d_6) δ: 3.74 (18H, brs, 6 × OCH_3), 6.64 (6H, brs, atom. H). ^{13}C-NMR: Table I.

Acetylation of 1——Compound **1** (25 mg) was acetylated with acetic anhydride–pyridine. The crude acetate was subjected to silica gel chromatography with EtOAc, yielding **1a** (20 mg).

Syringylglycerol-β-syringaresinol Ether 4″,4‴-Di-*O*-β-D-glucopyranoside Decaacetate (1a)——An amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1758, 1228 ($OCOCH_3$), 1598, 1506 (aromatic ring). ^1H-NMR (in $CDCl_3$) δ: 1.99, 2.03, 2.15, 2.18 (30H, each s, 10 × alcoholic $OCOCH_3$), 2.96–3.15 (2H, m, $C_{1,5}$-H), 3.76, 3.80, 3.88 (18H, each s, 6 × OCH_3), 5.04 (2H, d, $J=6.0$ Hz, anomeric H of glucose), 6.00 (1H, d, $J=4.39$ Hz, C_α-H), 6.53, 6.56, 6.60 (each 2H s, arom. H). ^{13}C-NMR: Table I.

Dehydrodiconiferyl Alcohol 4,γ'-Di-*O*-β-D-glucopyranoside (2)——A white powder (from EtOH), mp 134.7 °C, $[\alpha]_D^{28}$ −62.31° (c=0.14, MeOH). UV λ_{max}^{MeOH} nm: 220, 278. IR ν_{max}^{KBr} cm^{-1}: 3396 (OH), 1608, 1504 (aromatic ring). FD-MS m/z: 683 (M^++1), 521 ($M^+ - C_6H_{10}O_5$+1), 359 ($M^+ - 2\times C_6H_{10}O_5$+1). ^{1}H-NMR (in DMSO-$d_6$) δ: 3.74, 3.80 (each 3H s, 2×OCH_3), 5.52 (1H, d, J=6.15 Hz, C_α-H), 6.19 (1H, dt, J=15.83, 6.59 Hz, $C_{\beta'}$-H), 6.59 (1H, d, J=15.83 Hz, $C_{\alpha'}$-H), 6.80—7.20 (5H, m, arom. H). ^{13}C-NMR: Table II. Acetylation of **2** (20 mg) gave a nonaacetate (**2a**, 20 mg).

Dehydrodiconiferyl Alcohol 4,γ'-Di-*O*-β-D-glucopyranoside Nonaacetate (2a)——An amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1760, 1228 ($OCOCH_3$), 1606, 1508 (aromatic ring). ^{1}H-NMR (in DMSO-d_6) δ: 1.96, 1.98 (27H, each s, 9× alcoholic $OCOCH_3$), 3.72, 3.78 (each 3H s, 2×OCH_3), 6.82—7.08 (5H, m, arom. H).

Enzymatic Hydrolysis of 2——Compound **2** (100 mg) was hydrolyzed with β-glucosidase (50 mg, Sigma Chemical Co.) in acetate buffer (0.1 N HOAc–0.1 M NaOAc=1:2, 30 ml, pH 5.0) for 1 d at 37 °C. The reaction mixture was extracted with Et_2O (50 ml×2) and the residue obtained from the Et_2O layer was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (20:1, 10:1) gave a pure aglycone (**5**, 30 mg). The aqueous layer was concentrated under reduced pressure to obtain the residue, which was trimethylsilylated with TMS-PZ (Tokyo Kasei Co.) for 10 min. The reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water and concentrated. The presence of trimethylsilylated α-glucose [t_R (min) 4.8] and β-glucose [t_R (min) 6.9] in this residue was detected by gas chromatography (GC). Conditions: column, 1.5% OV-17, 3 mm×1.5 m; column temperature, 200 °C; carrier gas, N_2, 30 ml/min; injection temperature, 220 °C.

Dehydrodiconiferyl Alcohol (5)——An amorphous powder, $[\alpha]_D^{28}$ −20.7° (c=0.17, MeOH). UV λ_{max}^{MeOH} nm: 219, 278. IR ν_{max}^{KBr} cm^{-1}: 3396 (OH), 1606, 1518, 1500 (aromatic ring). ^{1}H-NMR (in DMSO-d_6) δ: 3.69, 3.74 (each 3H s, 2× OCH_3), 6.43 (1H, d, J=15.83 Hz, C_α-H), 6.71, 6.86 (2H, 3H, each s, arom. H), 8.86 (1H, br s, a phenolic OH). ^{13}C-NMR: Table II.

Acetylation of 5——Compound **5** was acetylated in the usual way, yielding a triacetate (**5a**). An amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1768, 1742, 1232 ($OCOCH_3$), 1608, 1504 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 2.05, 2.10 (each 3H s, 2× alcoholic $OCOCH_3$), 2.30 (3H, s, a phenolic $OCOCH_3$), 3.81, 3.92 (each 3H s, 2×OCH_3), 4.30—4.45 (2H, m, C_γ-H), 4.60—4.85 (2H, m, $C_{\gamma'}$-H), 5.54 (1H, d, J=6.59, C_α-H), 6.13 (1H, dt, J=15.83, 6.15 Hz, $C_{\beta'}$-H), 6.61 (1H, d, J=15.83 Hz, $C_{\alpha'}$-H), 6.88, 6.98 (2H, 3H, each s, arom. H). MS m/z: 484 (M^+, 66), 424 ($M^+ - CH_3COOH$, 25), 382 ($M^+ - CH_3COOH - COCH_2$, 100).

Citrusin B(3)——An amorphous powder, $[\alpha]_D^{26}$ −16.32° (c=0.1, MeOH). UV λ_{max}^{MeOH} nm: 223, 273. IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 1588, 1510 (aromatic ring), ^{1}H-NMR (in DMSO-d_6) δ: 3.76 (9H, s, 3×OCH_3), 6.32—6.53 (1H, m, $C_{\beta'}$-H), 6.67—7.20 (6H, m, $C_{\alpha'}$-H, arom. H). ^{13}C-NMR: Table II.

Acetylation of 3——Compound **3** was acetylated in the usual way, yielding a heptaacetate (**3a**).

Citrusin B Heptaacetate (3a)——An amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1758, 1228, ($OCOCH_3$), 1588, 1514 (aromatic ring). ^{1}H-NMR (in $CDCl_3$) δ: 1.97, 2.03, 2.07, 2.10, 2.13 (3H, 6H, 6H, 3H, 3H, each s, 7× alcoholic $OCOCH_3$), 3.76, 3.80, 3.85 (9H, each s, 3×OCH_3), 4.71 (2H, d, J=5.71 Hz, $C_{\gamma'}$-H), 6.15 (1H, dt, J=15.83, 5.71 Hz, $C_{\beta'}$-H), 6.57 (2H, s, arom. H on syringyl ring), 6.58 (1H, d, J=15.83 Hz, $C_{\alpha'}$-H), 6.75—7.20 (3H, m, arom. H).

Enzymatic Hydrolysis of 3——Compound **3** (30 mg) was hydrolyzed in the same way as **5**, yielding an aglycone (**6**, 10 mg) and glucose. The presence of trimethylsilylated glucose was detected by GC.

Guaiacylglycerol-β-*O*-4'-synapyl Ether (6)——A colorless syrup, $[\alpha]_D^{27}$ +12.5° (c=0.1, MeOH). UV λ_{max}^{MeOH} nm: 227, 278. IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 1588, 1510 (aromatic ring). MS m/z: 406 (M^+, 4), 358 ($M^+ - CH_2OH - OH$, 8), 236 (40), 210 (100), 167 (42), 137 (44). ^{1}H-NMR (in DMSO-d_6) δ: 3.74, 3.77 (9H, each s, 3×OCH_3), 6.20—7.20 (7H, m, $C_{\alpha'}$-H, $C_{\beta'}$-H, arom. H). ^{13}C-NMR: Table II.

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Synthesis of 6,5′-Cyclo-5′-deoxy-5′(*R* and *S*)-(2-hydroxyethyl)-uridines (Nucleosides and Nucleotides. LXXIII[1])

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Oxidation of 6,5′-cyclo-5′-deoxy-2′,3′-*O*-isopropylideneuridine with selenium dioxide gave the 5′-oxo derivative, which was converted to the 5′-ethoxycarbonylmethylidene derivative (**3**). Reduction of **3** afforded, after deprotection, the title compounds (**6**, *R* and *S*). The base-catalyzed epimerization of **6** at the 5′-position was observed, and the equilibrium was in favor of the (*R*)-epimer.

Keywords——*C*-cyclonucleoside; *C*-cyclouridine; 6,5′-cyclo-5′-deoxy-5′-(*RS*)-(2-hydroxyethyl)uridine; 6,5′-cyclo-5′-oxouridine; Wittig reaction; epimerization; NMR; CD

We have reported the synthesis of a number of carbon-bridged purine[2] and pyrimidine[1,3] cyclonucleosides for the study of nucleoside conformations involved in the interactions with enzymes which require nucleosides and nucleotides. As a model of uridine fixed in an *anti* conformation, we have synthesized 6,5′-cyclo-5′-deoxyuridine by the use of a radical cyclization of a 5′-halogeno-5′-deoxyuridine as the key step.[3c] However, this compound lacks the hydroxyl function at the 5′-position which is essential if the nucleoside is to be phosphorylated to the 5′-nucleotide. Fox and co-workers have reported a synthesis of 6,5′(*R* and *S*)-cyclouridines, by an intramolecular aldol reaction of a 5-hydroxyuridine 5′-aldehyde and successive derivatization.[4] Although these compounds have the hydroxyl group at the 5′-position, it is a secondary hydroxyl group. The 5′-hydroxymethyl derivative of 6,5′-cyclo-5′-deoxyuridine has been reported[5]; in that form, the primary hydroxyl group is retained as in uridine. The present report describes a synthesis of *anti*-fixed uridine having the 2-hydroxyethyl group at the 5′-position, thus providing a set of cyclouridines possessing the primary hydroxyl group at the 5′-position.

In the synthesis of 5′-hydroxymethyl-6,5′-cyclouridine by Sasson and Otter,[5] the key step was the reaction of dimethylsulfonium methylide with the 6,5′-cyclo-5′-oxouridine to give the spiro-oxirane, followed by hydrogenolysis. It seems that chain elongation at the 5′-position could be achieved by employing the Wittig reaction of the 5′-oxo nucleosides. In fact, 8,5′-cyclo-5′-deoxy-5′-(2-hydroxyethyl)adenosines were prepared from the 8,5′-cyclo-5′-oxoadenosine.[2d] Therefore, we applied this procedure. Treatment of 6,5′-cyclo-5′-deoxy-2′,3′-*O*-isopropylideneuridine (**1**)[3c] with selenium dioxide in pyridine gave the 6,5′-cyclo-2′,3′-*O*-isopropylidene-5′-oxouridine (**2**) in high yield. The physical properties of **2** were essentially identical with those of the product previously synthesized by sulfur trioxide–pyridine oxidation of the 6,5′(*R*)-cyclouridine.[6] The Wittig reaction of **2** with ethoxycarbonylmethylenetriphenylphosphorane in dichloromethane under reflux afforded 6,5′-cyclo-5′-deoxy-5′-ethoxycarbonylmethylidene-2′,3′-*O*-isopropylideneuridine (**3**) in a crystalline form. Although the *EZ*-geometry at the 6′-position of **3** was not determined, the product seemed to be one of the *EZ*-isomers as judged from its physical data (see Experimental).

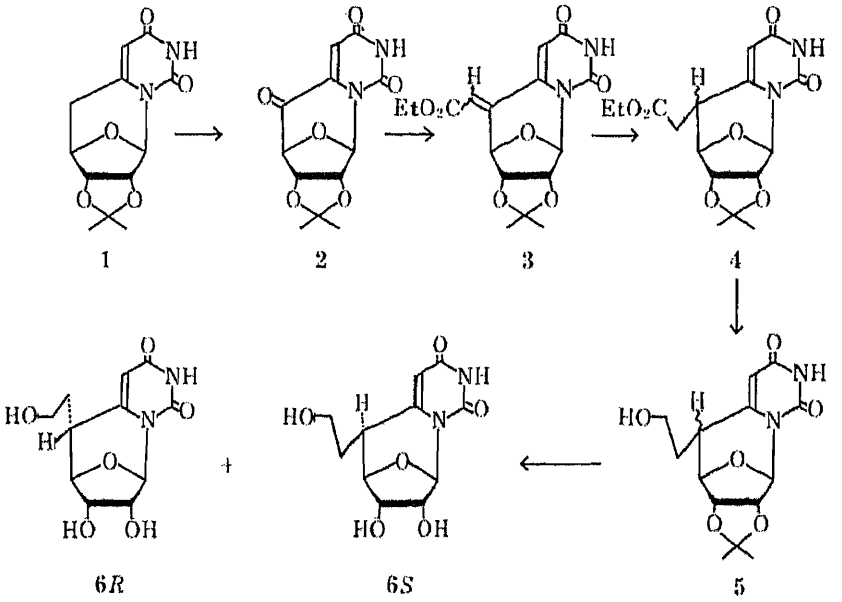


Chart 1

Several attempts at the reduction of the ester group and/or exomethylidene functions of **3** by using metal hydrides were unsuccessful. However, the reduction of **3** with sodium borohydride in the presence of nickel(II) chloride[7] afforded the 5′-ethoxycarbonylmethyl derivative (**4**) in a crystalline form as the 5′-*RS* mixture (1 : 2), based on analysis of the nuclear magnetic resonance spectra (NMR). It seemed that the borohydride reduction preferentially took place from the α-side where the lactol ring oxygen was situated. The reduction of the ester function of **4** was achieved by treatment with an excess of sodium borohydride in aqueous dioxane overnight to give 6,5′-cyclo-5′-deoxy-5′(*RS*)-(2-hydroxyethyl)-2′,3′-*O*-isopropylideneuridine (**5**). The product was obtained in a crystalline form despite being an *RS* mixture. Deprotection of **5** by treatment with 1 M hydrochloric acid in aqueous methanol gave crystalline 6,5′-cyclo-5′-deoxy-5′(*RS*)-(2-hydroxyethyl)uridine (**6**).

A portion of **6** was separated by high performance liquid chromatography (HPLC) into the *R*- and *S*-epimers and their structures were confirmed by NMR spectral analysis at 270 MHz. The 5′(*S*)-epimer showed a doublet due to the 4′-proton coupled to the 5′-proton whereas the 4′-proton signal of the 5′(*R*)-epimer was a singlet. Thus, the assignment of *RS*-configuration at the 5′-position of **6** was made by investigation of molecular models. Similar results had been reported in the cases of the 6,5′-cyclo-5′-deoxy-5′-hydroxymethyluridines[5] and 8,5′-cyclo-5′-deoxy-5′-hydroxyethyladenosines.[2d] In the case of 6,5′-cyclo-5′-deoxyuridine, the signals of both of the 5′-protons were split by the 4′-proton and the coupling constants were 1.7 and 6.4 Hz.[3e] Therefore, the substitution of one of the 5′-protons of **1** with a 2-hydroxyethyl group would have caused the dihedral angle between $H^{4'}$–$C^{4'}$–$C^{5'}$ and $H^{5'}$–$C^{5'}$–$C^{4'}$ to be near 90° in the *R*-epimer of **6**.

As described above, hydride attack on the 5′-methylidene portion of **3** occurred preferentially from the α-side giving the 5′(*R*)- and (*S*)-epimers of **4** in a 1 : 2 ratio. This ratio was apparently maintained during the subsequent borohydride treatment and acidic deprotection. However, the ratio was variable when this reaction was repeated, and the base treatment appeared to be affecting the *RS* ratio. Therefore, the change of the *RS* ratio of **6** (1 : 2 at the beginning) was followed by NMR measurements, based on the peak height of H-5 in sodium deuteroxide solution; the signals of H-5 of the *R*- and *S*-epimers appear at 5.62 and 5.71 ppm, respectively. The *RS* ratio changed with time and after 24 h, it reached equilibrium,

the *RS* ratio being 6:1. During this treatment proton–deuterium exchange at the 5′-position was very fast and the 5′-protons disappeared within 1 h. Therefore, the 5′-epimerization is explained by the dissociation of the 5′-proton in alkaline medium to give a resonant anion, causing the sterically more stable *R*-epimer to become the major component. It should be noted that the rate of proton–deuterium exchange was much faster than that of epimerization at the 5′-position. This epimerization was also monitored by HPLC analysis. Similar 5′-epimerization was reported in the case of 6,5′-cyclo-5′-deoxy-5′-hydroxymethyluridine[5] and 5′-*O*-acetyl-6,5′-cyclo-2′,3′-*O*-isopropylideneuridine.[4b] However, such proton–deuterium exchange by base was not readily observed when 5′-deoxy-6,5′-cyclouridine[3c] was treated under similar conditions. Therefore, the 5′-substituents may contribute to the dissociation of the remaining 5′-proton, the driving force probably being the release of the steric hindrance at the 5′-position.

The circular dichroism spectra (CD) of the 6,5′-cyclouridines exhibited strong positive Cotton bands at the major absorption regions.[3c] The relation of the sign of CD spectra and glycosyl torsion angles has already been discussed.[3g] Compounds **6***R* and **6***S* showed positive Cotton bands, and the molar ellipticity of **6***R* was a little stronger than that of **6***S*. A similar relation had been found in our laboratory for the 5′-substituted 8,5′-cycloadenosines.[2d]

Experimental

Melting points were determined on a Yanagimoto MP-3 micromelting point apparatus and are uncorrected. The ^{1}H-NMR spectra were recorded on a JEOL FX-100FT or FX-270FT spectrometer in an appropriate solvent with tetramethylsilane as an internal standard. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). All exchangeable protons were confirmed by addition of D_2O. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-260 spectrophotometer. Mass spectra (MS) were measured on a JEOL D-300 spectrometer. CD spectra were recorded on a JASCO J-500A spectropolarimeter at room temperature. Silica gel used for column chromatography was Merck Kieselgel 60 (70—230 mesh). Thin layer chromatography (TLC) was performed on Merck Kieselgel F_{254} precoated plates.

6,5′-Cyclo-5′-deoxy-2′,3′-*O*-isopropylidene-5′-oxouridine (2)——A mixture of **1**[3c] (2.66 g, 10 mmol) and SeO_2 (1.67 g, 15 mmol) in pyridine (30 ml) was heated overnight under reflux. The solvent was removed *in vacuo* and the residue was dissolved in MeOH. The insoluble material was filtered off through a celite bed and the filtrate was absorbed to silica gel. The gel was dried and placed on top of a column of silica gel (3.5 × 40 cm). The column was eluted with $CHCl_3$–EtOAc (4:1). The eluate was concentrated and the residue was crystallized from EtOAc to give 2.18 g (78%) of **2**, mp 230—231 °C (lit. 225—226 °C).[6] UV $\lambda_{max}^{H_2O}$: 315 and 269 nm. MS *m/z*: 280 (M^+). NMR ($CDCl_3$): 9.26 (1H, br s, HN^3), 6.42 (1H, br s, H-5), 6.27 (1H, s, H-1′), 4.87 (3H, br, H-2′,3′,4′), 1.68, 1.33 (3H each, s, Me_2C). *Anal.* $C_{12}H_{12}N_2O_6$ requires: C, 51.43; H, 4.32; N, 10.00. Found: C, 51.35; H, 4.44; N, 9.90.

6,5′-Cyclo-5′-deoxy-5′-ethoxycarbonylmethylidene-2′,3′-*O*-isopropylideneuridine (3)——A mixture of **2** (560 mg, 2 mmol) and ethoxycarbonylmethylenetriphenylphosphorane (835 mg, 2.4 mmol) in CH_2Cl_2 (10 ml) was heated for 9 h under reflux. The solvent was removed, the residue was taken up in EtOAc, and *n*-hexane was added to precipitate Ph_3PO. The precipitate was filtered off, then the filtrate was concentrated, and applied to a column of silica gel (3.5 × 45 cm). The eluate with $CHCl_3$–EtOAc (4:1) was concentrated and the residue was crystallized from EtOAc to give 581 mg (83%) of **3**, mp 236 °C (dec.). UV λ_{max}^{MeOH}: 328.2 nm. MS *m/z*: 350 (M^+), 335 ($M^+ - Me$). NMR ($CDCl_3$): 9.47 (1H, br s, HN^3), 6.51 (1H, d, H-1′, J = 1.5 Hz), 6.25 (1H, br s, H-6′), 6.17 (1H, br s, H-5), 6.02 (1H, br s, H-4′), 4.70 (2H, s, H-2′,3′), 4.30 (2H, q, CH_2CH_3), 1.55, 1.35 (3H each, s, Me_2C), 1.35 (3H, t, CH_2CH_3). *Anal.* Calcd for $C_{16}H_{18}N_2O_7$: C, 54.86; H, 5.18; N, 8.00. Found: C, 54.62; H, 5.06; N, 7.91.

6,5′-Cyclo-5′-deoxy-5′(*R* and *S*)-ethoxycarbonylmethyl-2′,3′-*O*-isopropylideneuridine (4*R*,4*S*)——Compound **3** (700 mg, 2 mmol) was dissolved in tetrahydrofuran (20 ml), $NiCl_2 \cdot 6H_2O$ (160 mg, 0.2 mmol) was added, and the solution was stirred for 5 min, then placed in an ice-bath. $NaBH_4$ (152 mg, 4 mmol) was added, and the whole was stirred at room temperature for 30 min, then neutralized by addition of AcOH. The solvent was removed, and the residue was taken up in MeOH, and absorbed on silica gel. The gel was dried and placed on top of a column of silica gel (5 × 10 cm). The eluate with 10% MeOH–$CHCl_3$ was concentrated and the residue was crystallized from MeOH to give 648 mg (92%) of **4** (*R*:*S* = 1:2), mp 175—176 °C. UV λ_{max}^{MeOH} (ε): 267.6 nm (12100). MS *m/z*: 352 (M^+), 337 ($M^+ - Me$). NMR ($CDCl_3$): 8.76 (1H, br s, HN^3), 6.20 (1H, s, H-1′), 5.57, 5.52 (1H, br s, H-5), 4.56 (2.66H, m, H-2′,3′,4′), 4.41 (0.34H, s, H-4′ for *R*), 4.25 (2H, two q, CH_2CH_3), 3.20—2.50 (3H, m, H-5′,6′), 1.50, 1.32 (3H each, s, Me_2C), 1.29 (3H, t, CH_2CH_3). *Anal.* Calcd for $C_{16}H_{20}N_2O_7$: C, 54.54; H, 5.72; N, 7.95. Found: C, 54.28; H, 5.66; N, 7.92.

6,5′-Cyclo-5′-deoxy-5′(*R* and *S*)-(2-hydroxyethyl)-2′,3′-*O*-isopropylideneuridine (5)——Compound **4** (*RS* mixture 176 mg, 0.5 mmol) was dissolved in 80% dioxane (50 ml) and cooled in an ice-bath. $NaBH_4$ (283.5 mg, 7.5 mmol) was added and the solution was stirred overnight at room temperature, then neutralized by addition of AcOH. The solvent was removed *in vacuo*, and the residue was taken up in MeOH, and absorbed on silica gel. The gel was dried and placed on top of a silica gel column (3 × 10 cm). The eluate with 10% MeOH–$CHCl_3$ was concentrated and the residue was crystallized from MeOH to give 132 mg (85%) of **5**, mp 279 °C (dec.). UV λ_{max}^{MeOH} (ε): 270 nm (9400). CD (MeOH)$[\theta]^{25}$(nm): +13800 (270). NMR (DMSO-d_6): 11.30 (1H, brs, HN^3), 5.90 (1H, s, H-1′), 5.49 (1H, brs, H-5), 4.70 (2H, s, H-2′,3′), 4.65 d, 4.42 s (1H, H-4′), 3.55 (2H, m, H-7′), 2.80 (1H, brt, H-5′), 2.1—1.6 (2H, m, H-6′), 1.39, 1.25 (3H each, s, Me_2C). *Anal.* Calcd for $C_{14}H_{18}N_2O_6$: C, 54.19; H, 5.84; N, 9.03. Found: C, 54.18; H, 5.78; N, 8.92.

6,5′-Cyclo-5′-deoxy-5′(*R* and *S*)-(2-hydroxyethyl)uridine (6)——Compound **5** (800 mg, 2.6 mmol) was dissolved in 1 M HCl in 50% MeOH (40 ml) and the solution was heated under reflux for 5 h. The solvent was removed and the residue was crystallized from EtOH to give 522 mg (75%) of **6** (*R*: *S* = 1 : 2), mp 243 °C (dec.). UV $\lambda_{max}^{H_2O}$ (ε): 280 nm (9000). CD (H_2O) $[\theta]$(nm) = +17800 (265). NMR (D_2O): 5.91 (1H, s, H-1′), 5.71 (0.67H, s, H-5 for **6***S*), 5.62 (0.33H, s, H-5 for **6***R*), 4.25 (3H, m, H-2′,3′,4′), 3.76 (2H, t, H-7′), 3.40 (1H, m, H-5′), 2.2—1.6 (2H, m, H-6′). MS *m/z*: 270 (M^+), 226 ($M^+ - CH_2CH_2OH$). *Anal.* Calcd for $C_{11}H_{14}N_2O_6$: C, 48.49; H, 5.22; N, 10.37. Found: C, 48.69; H, 5.20; N, 10.19.

A portion of **6** in H_2O was subjected to HPLC on an MS PACK C-18 column (10 mm × 300 mm) equipped with a JASCO TRI ROTAR-V pump and a UVIDEC-100-V UV spectrophotometer. The flow rate was 2 ml/min with 5% MeOH in H_2O and the retention times of the *S*- and *R*-epimers were 32 and 47 min, respectively. The respective peak fractions were collected and the solvent was removed *in vacuo* to leave **6***S* and **6***R*.

NMR (270 MHz, D_2O) for **6***S*: 6.03 (1H, s, H-1′), 5.81 (1H, s, H-5), 4.55 (1H, d, H-4′, $J_{4',5'}$ = 5.5 Hz), 4.46 (1H, d, H-2′ or 3′, $J_{2',3'}$ = 5.9 Hz), 4.30 (1H, d, H-3′ or 2′), 3.78 (2H, m, H-7′), 3.49 (1H, m, H-5′), 2.2—1.7 (2H, m, H-6′). CD (H_2O)$[\theta]$(nm): +13500 (267).

NMR (270 MHz, D_2O) for **6***R*: 6.04 (1H, s, H-1′), 5.72 (1H, s, H-5), 4.44 (1H, s, H-4′), 4.32 (1H, d, H-2′ or 3′, $J_{2',3'}$ = 5.87 Hz), 4.29 (1H, d, H-3′ or 2′), 3.76 (2H, m, H-7′), 2.99 (1H, dd, H-5′, $J_{5',6'a}$ = 5.9 Hz, $J_{5',6'b}$ = 7.3 Hz), 2.15—1.90 (2H, m, H-6′). CD (H_2O)$[\theta]$(nm): +19900 (268).

Epimerization of 6*R* and 6*S* in Alkaline Solution as Monitored by NMR (100 MHz)——Compound **6** (*R*: *S* = 1 : 2, *ca.* 10 mg) was dissolved in D_2O (0.5 ml) in an NMR tube and 1 N NaOD solution (0.05 ml) was added in the tube with sodium 3-trimethylsilylpropanesulfonate as an internal standard. The peak height of the H-5 signals at 5.72 (**6***R*) and 5.81 (**6***S*) were measured over a period of 24 h at 60 °C. The ratio reached 2.25 : 1 after 2 h, 3 : 1 after 4 h, 4 : 1 after 7 h, and 6 : 1 after 24 h. The H-5′ signals of **6***R* (2.99, d) and **6***S* (3.49, m) disappeared completely within 1 h after addition of 1 N NaOD. The HPLC analysis of this epimerization profile was also similar.

The NMR spectra of 6,5′-cyclo-5′-deoxyuridine[3c] when measured in NaOD–D_2O under similar conditions did not show H–D exchange at H-5′a or b (2.72 and 3.27 ppm) after 1 h. After 24 h at 60 °C, the signals of the 5′-protons had disappeared completely.

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Conversion of (+)-Limonen-10-ol to Bicyclo[3.3.0]octane Skeleton. Synthesis of a Key Intermediate for Carbacyclin

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The conversion of (+)-limonen-10-ol (**7**) into the key intermediate for the synthesis of carbacyclin (**1**), (+)-(1*S*,2*R*,3*R*,5*R*)-3-acetoxy-2-methoxycarbonyl-7-oxobicyclo[3.3.0]octane (**2**), is described. The *cis*-3,4-disubstituted cyclopentanone prepared from **7** *via* a sequence of reactions involving Rh(I)-catalyzed cyclization could be converted to the 3-acetylbicyclo[3.3.0]oct-2-ene skeleton, which was subjected to 1,4-addition reaction with CN^-. The resulting cyano compound was transformed into the key intermediate **2** through appropriate modification of the substituents on the five-membered ring. This synthetic method provides a new route to carbacyclin.

Keywords——prostaglandin; (+)-limonen-10-ol; bicyclo[3.3.0]octene; aldol condensation; carbacyclin; *cis*-3,4-disubstituted cyclopentanol

Prostacyclin (PGI_2) is known to be a potent inhibitor of platelet aggregation as well as a powerful vasodilator. However, the inherent instability due to the labile enol ether linkage limits the clinical use of this compound. Thus, carbacyclin (**1**), which was stabilized by replacing the enol ether oxygen atom with a methylene group, has been synthesized by many groups.[1] It is now known that the biological profile of this compound is similar to that of prostacyclin.

As a part of our studies on the conversion of chiral natural sources to biologically active compounds containing a five-membered ring, such as prostaglandins (PGs), we have previously succeeded in the conversion of methylenomycin A to PG analogs,[2] (+)-limonen-10-ol to brefeldin A[3] and 8-isoprostanoic acid,[4] and (−)-limonen-10-ol to prostanoic acid [4] and 11-deoxy PGs.[5] Limonene and related compounds[6] have several advantages as starting materials. For example, both (*R*)- and (*S*)-limonene are commercially available. Therefore, it is possible to synthesize the enantiomer, if desired, as well as the target compound. Furthermore, the cyclohexene ring in limonene can readily be transformed into the *cis*-3,4-disubstituted five-membered ring ketone with retention of the chirality.[7]

In this paper, we describe the synthesis of an optically active intermediate (**2**) in the synthesis of (+)-carbacyclin (**1**) starting from a natural product, (+)-limonen-10-ol (**7**). The retro synthesis of **2** is shown in Chart 1. The β-methoxycarbonyl and α-acetoxy functions in **2** may be derived from the enone (**3**) *via* 1,4-addition reaction of CN^- from the less-hindered side and subsequent modification of substituents on the five-membered ring. The α,β-unsaturated ketone moiety in **3** may be built up *via* the intramolecular aldol condensation. The aldehyde and ketone functions as required for the aldol condensation (compound **4**) may be formed by oxidation of the corresponding primary and secondary alcohols (compound **5**). The diol **5** should be obtainable from the 1-al-4-ene (**6**) in a stereocontrolled fashion by means of Rh(I)-catalyzed cyclization.[7] Compound **6** with the desired configuration for the synthesis of carbacyclin can be prepared by fission[4] of the cyclohexene ring in naturally abundant (+)-limonen-10-ol (**7**).

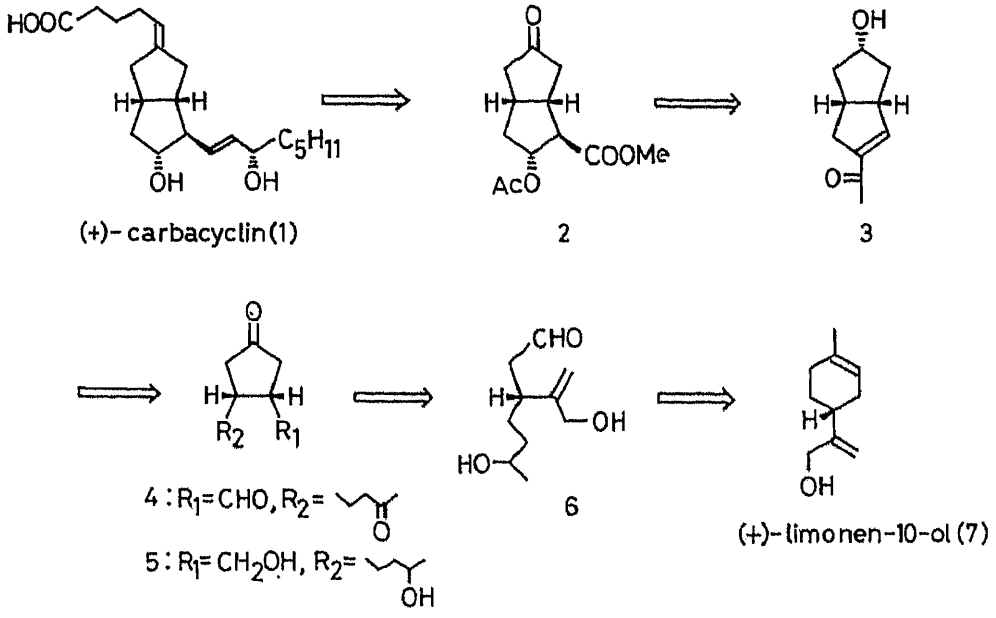


Chart 1

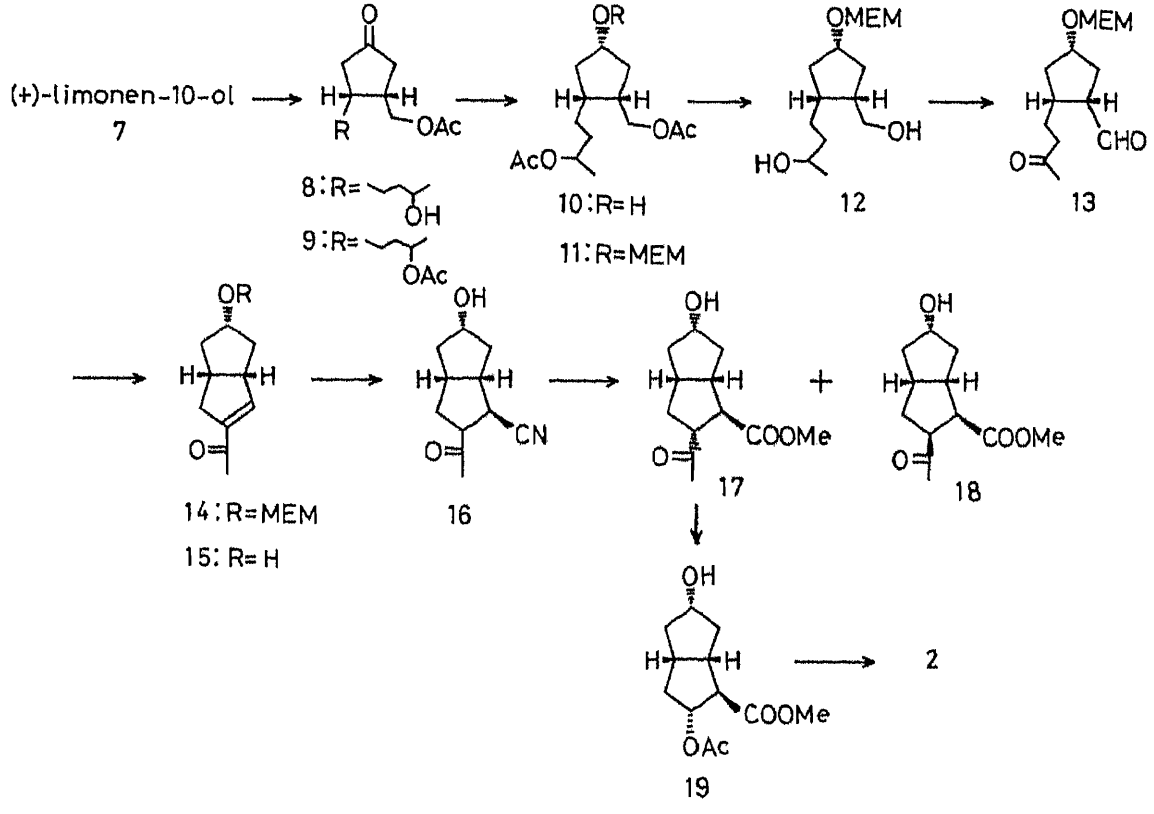


Chart 2

Thus, the designed sequence (Chart 2) starts with reduction of the carbonyl function in the diacetate (**9**), which was obtained by the usual acetylation (Ac_2O/pyridine) of the monoacetate (**8**).[8] The α-side of the carbonyl function in **9** may be shielded by the *cis*-3,4-disubstituents. This shielding effect should lead to stereospecific reduction to afford the C_1α-alcohol (**10**), similarly to our previous case.[3] In accord with our expectation, reduction of the

carbonyl function in **9** with $NaBH_4$/MeOH afforded exclusively the alcohol **10**, and the epimer was not detected on thin layer chromatography (TLC). Therefore, the configuration of C_1-OH in **10** was concluded to be *cis* relative to the *cis*-3,4-disubstituents. The alcohol function in **10** was protected as the 2-methoxyethoxymethyl (MEM) ether by treatment with MEM chloride/*N*,*N*-diisopropylethylamine, and subsequent methanolysis with K_2CO_3/MeOH yielded the diol (**12**) in 83% yield from the diacetate (**9**). Concurrent oxidation of primary and secondary alcohols in **12** with pyridinium chlorochromate (PCC)/CH_2Cl_2 afforded the keto-aldehyde (**13**), which was subjected to intramolecular aldol condensation by treatment with K_2CO_3/MeOH for 9 h at 50 °C. Thus, the chiral building block for carbacyclin, the bicyclo[3.3.0]octene (**14**) was synthesized in 65% yield from **12**. The structure of **14** was supported by the signals of C_2-H (β-hydrogen of α,β-unsaturated ketone) at δ 6.60 (1H, d, J=3 Hz) and C_3-$COCH_3$ at δ 2.32 (3H, s) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, in addition to the absorption bands at 1660 (carbonyl) and 1610 (olefin) cm^{-1} in the infrared (IR) spectrum. Cleavage of the MEM ether in **14** with $TiCl_4/CH_2Cl_2$ at 0 °C afforded the hydroxy compound (**15**) in 80% yield. 1,4-Addition of **15** with KCN in dimethylformamide (DMF) afforded an inseparable mixture (**16**) of stereoisomers in 91% yield. On the assumption that the configuration of the C_2-CN in **16** should be β, based on the approach of CN^- from the convex side, **16** was concluded to be an epimeric mixture of $C_3\alpha$- and $C_3\beta$-$COCH_3$. By treatment with MeOH/HCl, the cyano compound **16** was converted in 76% yield to the corresponding methyl ester, which could be separated into the $C_3\alpha$- and $C_3\beta$-$COCH_3$ forms by careful column chromatography on silica gel. Thus, the α-methyl ketone (**17**) and β-methyl ketone (**18**) were obtained from the less polar fraction and the more polar fraction, respectively. Facile isomerization of **18** to the more stable isomer **17** by treatment with K_2CO_3/MeOH suggests that the C_3-$COCH_3$ in **17** should be assigned as *trans* relative to the $C_2\beta$-$COOCH_3$. By Baeyer–Villiger oxidation with CF_3COOOH/CH_2Cl_2 and subsequent PCC oxidation, **17** was converted to the target compound **2**, which was identical with an authentic sample[9] in terms of TLC behavior, $[\alpha]_D$, and ^{1}H-NMR and IR spectra.

Experimental

IR spectra were measured with a JASCO A-202 spectrometer. ^{1}H-NMR spectra were measured on a JEOL LNP-PS-100 spectrometer. Mass spectra (MS) were taken on a JEOL JMS-D 300 spectrometer. Optical rotations were measured on a JASCO DIP-4 polarimeter. For column chromatography, silica gel (Merck, Kieselgel 60, 70–230 mesh) was used. TLC was performed on Silica gel 60 F_{254} plates (Merck). All organic solvent extracts were washed with satd. brine and dried over anhydrous sodium sulfate.

(3*S*,4*R*)-4-[(3ξ)-3-Acetoxybutyl]-3-acetoxymethylcyclopentanone (9)——The diacetate **9** was obtained from the monoacetate (**8**)[8] by the usual acetylation with Ac_2O/pyridine. IR (neat): 1740, 1730, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, d, J=7 Hz, CH_3), 2.05 (6H, s, $CH_3CO\times2$), 4.12 (2H, m, CH_2O), 4.90 (1H, m, CH-O). MS *m/z*: 272 (M^+), 213, 170.

(1*S*,3*S*,4*R*)-4-[(3ξ)-3-Acetoxybutyl]-3-acetoxymethylcyclopentanol (10)——$NaBH_4$ (140 mg) was added portionwise to a stirred solution of **9** (1.60 g) in MeOH (20 ml) at −15 to 0 °C. After 1 h, the reaction mixture was diluted with brine, and extracted with AcOEt. The AcOEt extract was washed, and dried, then concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel (20 g). The fraction eluted with 50% AcOEt in hexane (v/v) afforded **10** (1.45 g, 94%) as a colorless oil. IR (neat): 3450, 1740, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J=7 Hz, CH_3), 2.03, 2.05 (3H each, s, $CH_3CO\times2$), 4.13 (2H, d, J=7 Hz, CH_2O), 4.34 (1H, m, C_1-H), 4.90 (1H, m, CHOAc). MS *m/z*: 274 (M^+), 256, 194.

(1*S*,3*S*,4*R*)-4-[(3ξ)-3-Acetoxybutyl]-3-acetoxymethyl-1-(2-methoxyethoxymethoxy)cyclopentane (11)——MEM chloride (1.29 g) was added dropwise to a stirred solution of **10** (0.93 g) and *N*,*N*-diisopropylethylamine (1.33 g) in CH_2Cl_2 (5 ml) at room temperature. After being stirred for 4 h, the reaction mixture was diluted with brine, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed, and dried. Removal of the solvent *in vacuo* gave an oily residue, which was subjected to column chromatography on silica gel (25 g). The fraction eluted with 15% AcOEt in hexane (v/v) afforded **11** (1.15 g, 93%) as a colorless oil. IR (neat): 1740, 1450, 1245 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J=7 Hz, CH_3), 2.03 (6H, s, $CH_3CO\times2$), 3.38 (3H, s, OCH_3), 3.50—3.90 (4H, m, OCH_2CH_2O), 3.90—4.35 (3H, m, CH_2O, C_1-H), 4.70—4.95 (3H, m, OCH_2O, CHOAc). MS *m/z*: 362 (M^+), 302, 287, 272.

(1*S*,3*S*,4*R*)-4-[(3ξ)-3-Hydroxybutyl]-3-hydroxymethyl-1-(2-methoxyethoxymethoxy)cyclopentane (12)——K_2CO_3 (840 mg) was added portionwise to a stirred solution of **11** (880 mg) in MeOH (10 ml) at room temperature. After 4 h, the reaction mixture was diluted with brine, and extracted with AcOEt. The AcOEt extract was washed, and dried, then concentrated *in vacuo* to afford an oily residue, which was chromatographed on silica gel (10 g). The fraction eluted with 40% AcOEt in hexane (v/v) gave **12** (640 mg, 95%) as a colorless oil. IR (neat): 3430, 1120, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J=7 Hz, CH_3), 3.38 (3H, s, OCH_3), 3.50—4.00 (7H, m, OCH_2CH_2O, CH_2O, CH-O), 4.13 (1H, m, C_1-H), 4.76 (2H, s, OCH_2O).

(1*S*,3*S*,4*R*)-3-Formyl-4-(3-oxobutyl)-1-(2-methoxyethoxymethoxy)cyclopentane (13)——Compound **12** (300 mg) in CH_2Cl_2 (10 ml) was added dropwise with stirring to a suspension of PCC (950 mg) and NaOAc (80 mg) in CH_2Cl_2 (7 ml) at room temperature. After 3 h, the reaction mixture was diluted with ether, and the resulting precipitate was filtered off. The filtrate was concentrated *in vacuo* to afford an oily residue, which was subjected to column chromatography on silica gel (3 g). The fraction eluted with 5% AcOEt in hexane afforded **13** (282 mg, 90%) as a colorless oil. IR (neat): 2725, 1720, 1120, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.13 (3H, s, CH_3CO), 3.40 (3H, s, OCH_3), 3.47—3.80 (4H, m, OCH_2CH_2O), 4.20 (1H, m, C_1-H), 4.76 (2H, s, OCH_2O), 9.86 (1H, d, J=4 Hz, CHO). MS *m/z*: 272 (M^+), 257, 226.

(1*S*,5*R*,7*S*)-3-Acetyl-7-(2-methoxyethoxymethoxy)bicyclo[3.3.0]oct-2-ene (14)——A mixture of K_2CO_3 (40 mg) and **13** (150 mg) in MeOH (5 ml) was stirred at 50 °C for 9 h. The reaction mixture was diluted with brine, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed, and dried, then concentrated *in vacuo* to leave an oily residue, which was chromatographed on silica gel (2 g). The fraction eluted with 20% AcOEt in hexane (v/v) afforded **14** (101 mg, 71%) as a colorless oil. $[\alpha]_D^{25}$ −83.2° (c=1.31, $CHCl_3$). IR (neat): 3050, 1660, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$). δ: 2.32 (3H, s, CH_3CO), 3.28 (1H, m, C_1-H), 3.40 (3H, s, OCH_3), 3.47—3.79 (4H, m, OCH_2CH_2O), 4.06 (1H, m, C_7-H), 4.68 (2H, s, OCH_2O), 6.60 (1H, d, J=3 Hz, C_2-H). MS *m/z*: 254 (M^+), 211, 178, 148. High-MS for $C_{14}H_{22}O_4$ (M^+): Caled *m/z* 254.15168; Found 254.15118.

(1*S*,5*R*,7*S*)-3-Acetyl-7-hydroxybicyclo[3.3.0]oct-2-ene (15)——Compound **14** (88 mg) in CH_2Cl_2 (3 ml) was added dropwise to a stirred solution of $TiCl_4$ (0.5 ml) in CH_2Cl_2 (2 ml) at 0 °C. After 1 h, the reaction mixture was diluted with brine, and extracted with AcOEt. The AcOEt extract was washed, and dried. Removal of the solvent *in vacuo* afforded an oily residue, which was purified by preparative TLC, and the alcohol **15** was obtained as a colorless oil (48 mg, 80%). $[\alpha]_D^{25}$ −90.5° (c=0.39, $CHCl_3$). IR (neat): 3400, 1655, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.32 (3H, s, CH_3CO), 3.35 (1H, m, C_1-H), 4.24 (1H, tt, J=5, 5 Hz, C_7-H), 6.63 (1H, d, J=3 Hz, C_2-H). MS *m/z*: 166 (M^+), 148, 105.

(1*S*,2*R*,3ξ,5*R*,7*S*)-3-Acetyl-2-cyano-7-hydroxybicyclo[3.3.0]octane (16)——Compound **15** (1.41 g) in DMF (20 ml) was added to a stirred solution of KCN (2.18 g) and NH_4Cl (0.51 g) in H_2O (3 ml). After being stirred for 24 h at room temperature, the reaction mixture was diluted with brine, and extracted with benzene. The benzene extract was washed, and dried, then concentrated *in vacuo* to leave an oily residue, which was subjected to column chromatography on silica gel (30 g). The fraction eluted with 20% AcOEt in hexane (v/v) afforded **16** (1.28 g, 91%) as a yellow oil. IR (neat): 3450, 2230, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.24 (3H, s, $COCH_3$), 3.00—3.56 (2H, m, C_2-, C_3-H), 4.20—4.52 (1H, m, C_7-H). MS *m/z*: 193 (M^+), 175, 132.

(1*S*,2*R*,3*R*,5*R*,7*S*)-3-Acetyl-2-methoxycarbonyl-7-hydroxybicyclo[3.3.0]octane (17) and (1*S*,2*R*,3*S*,5*R*,7*S*)-3-Acetyl-2-methoxycarbonyl-7-hydroxybicyclo[3.3.0]octane (18)——Compound **16** (757 mg) was added to 20% HCl (gas) in MeOH (w/v) (30 ml) at room temperature. After being stirred for 2 h, the reaction mixture was poured into ice water, and extracted with AcOEt. The AcOEt extract was successively washed with brine, 5% $NaHCO_3$, and brine, and then dried. Removal of the solvent *in vacuo* afforded an oily residue, which was chromatographed on silica gel (15 g). The fraction eluted with 15% AcOEt in hexane (v/v) gave **17** (430 mg) as a colorless oil, and 17% AcOEt in hexane (v/v) afforded **18** (173 mg) (**17** + **18** 76%) as a colorless oil. **17**: $[\alpha]_D^{25}$ +12.4° (c=0.50, $CHCl_3$). IR (neat): 3450, 1730, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, s, $COCH_3$), 2.63 (1H, m, C_2-H), 3.20 (1H, m, C_3-H), 3.67 (3H, s, $COOCH_3$), 4.36 (1H, tt, J=5, 5 Hz, C_7-H). MS *m/z*: 226 (M^+), 208, 176, 148. High-MS for $C_{12}H_{18}O_4$ (M^+): Caled *m/z* 226.12040; Found 226.12158. **18**: $[\alpha]_D^{25}$ +3.0° (c=0.80, $CHCl_3$). IR (neat): 3450, 1730, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.18 (3H, s, $COCH_3$), 2.96 (1H, dd, J=5, 11 Hz, C_2-H), 3.43 (1H, dd, J=7, 11 Hz, C_3-H), 3.64 (3H, s, $COOCH_3$), 4.26 (1H, tt, J=5, 5 Hz, C_7-H). MS *m/z*: 226 (M^+), 208, 176, 148. High-MS for $C_{12}H_{18}O_4$ (M^+): Caled *m/z* 226.12040; Found 226.12003.

(1*S*,2*R*,3*R*,5*R*,7*S*)-3-Acetoxy-2-methoxycarbonyl-7-hydroxybicyclo[3.3.0]octane (19)——CF_3COOOH [freshly prepared from $(CF_3CO)_2O$ (6.74 ml) and 60% H_2O_2 (1.5 ml) in CH_2Cl_2 (5 ml)] was added dropwise to a well-stirred solution of **17** (140 mg) in CH_2Cl_2 (4 ml) at room temperature. After 2 h, the reaction mixture was poured into 5% aq. $NaHCO_3$, and extracted with AcOEt. The AcOEt extract was successively washed with 2% aq. KI, 5% aq. $Na_2S_2O_3$, and brine, then dried. Removal of the solvent *in vacuo* afforded an oily residue, which was subjected to column chromatography on silica gel (4 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded **19** (140 mg, 77%) as a colorless oil. $[\alpha]_D^{25}$ −7.2° (c=0.28, $CHCl_3$). IR (neat): 3440, 1735, 1370 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s, $OCOCH_3$), 2.76 (1H, m, C_2-H), 3.68 (3H, s, $COOCH_3$), 4.46 (1H, tt, J=5, 5 Hz, C_7-H), 5.24 (1H, m, C_3-H). MS *m/z*: 242 (M^+), 224, 193.

(1*S*,2*R*,3*R*,5*R*)-3-Acetoxy-2-methoxycarbonyl-7-oxobicyclo[3.3.0]octane (2)——Compound **19** (105 mg) in

CH_2Cl_2 (5 ml) was added dropwise with stirring to a suspension of PCC (200 mg) in CH_2Cl_2 (5 ml) at room temperature. After 2 h, the reaction mixture was diluted with ether, and the resulting precipitate was filtered off. The filtrate was concentrated *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (1 g). The fraction eluted with 10% AcOEt in hexane (v/v) gave **2** (91 mg, 95%) as a colorless oil. $[\alpha]_D^{25}$ −17.1° (c=0.50, $CHCl_3$) (reported value: −16.4°).[9] IR (neat): 1740, 1730, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (3H, s, $OCOCH_3$), 2.96 (1H, m, C_2-H), 3.72 (3H, s, $COOCH_3$), 5.41 (1H, m, C_3-H). MS m/z: 240 (M^+), 209, 180. High-MS for $C_{12}H_{16}O_5$ (M^+): Calcd m/z 240.09967; Found 240.09901.

Isomerization of 18 to 17——A mixture of **18** (85 mg) and K_2CO_3 (200 mg) in MeOH (3 ml) was stirred for 5 h at room temperature, and diluted with brine, then extracted with AcOEt. The AcOEt extract was washed with brine, and dried, then concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel (2 g). The fraction eluted with 15% AcOEt in hexane (v/v) gave **17** (69 mg).

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Tannins and Related Polyphenols of Rosaceous Medicinal Plants. IV.[1] Roxbins A and B from *Rosa roxburghii* Fruits

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From the unripe fruits of *Rosa roxburghii* TRATT., two new hydrolyzable tannins, roxbin A (**1**) (a dimeric ellagitannin) and roxbin B (**9**), were isolated along with rugosin F (**2**), pedunculagin (**3**), casuarictin (**4**), alnusiin (**5**), stachyurin (**6**), tellimagrandin II (**7**), 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose and (+)-catechin. The structures of new tannins were elucidated on the basis of chemical and spectral evidence.

Keywords—*Rosa roxburghii*; Rosaceae; tannin; ellagitannin; roxbin A; roxbin B; centrifugal partition chromatography

Many Rosaceous plants have been traditionally used as folk medicines for a variety of diseases in diverse areas of the world.[2] Among them, the plants used as antidiahrreics, astringents and hemostatics are generally rich in tannins, which are believed to be the active principles. These medicinal plants include *Agrimonia japonica* (MIQ.) KOIDZ., *Geum japonicum* THUNB. and *Rosa rugosa* THUNB., from which we isolated oligomeric ellagitannins and related monomers, such as agrimoniin,[3] gemins A-F[1,4] and rugosins A-G.[5] In a continuation of the investigation on the Rosaceous medicinal plants, we have examined the polyphenolic constituents of *Rosa roxburghii* TRATT., the fruit of which is a Chinese folk medicine used as a stomachic and a tonic. The fruit is also widely used to prepare a healthful drink in China, as it is a rich source of vitamin C. The present paper deals with the isolation and characterization of new tannins, named roxbin A (**1**) and roxbin B (**9**), along with eight known polyphenols, from the fruit extract of this plant.

An aqueous acetone homogenate of the fruits was successively extracted with ether, ethyl acetate and *n*-butanol. The *n*-butanol soluble portion was subjected to centrifugal partition chromatography (CPC)[6] and column chromatography over Toyopearl HW-40 and Sephadex LH-20 to afford a new tannin, roxbin A (**1**), along with rugosin F (**2**),[5b] pedunculagin (**3**),[7] stachyurin (**6**)[7] and 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose.[8] An additional new tannin, roxbin B (**9**), was isolated together with known tannins, casuarictin (**4**),[7] alnusiin (**5**),[9] tellimagrandin II (**7**),[7,10] and (+)-catechin from the EtOAc-soluble portion by a combination of chromatographies over columns of Toyopearl HW-40 and Sephadex LH-20.

Roxbin A (**1**), $C_{75}H_{50}O_{48}\cdot 4H_2O$, was shown to be a dimeric ellagitannin by its similarities to rugosin F (**2**)[5b] in the retention time on high-performance liquid chromatography (HPLC) (normal phase) and in the color reaction with the $NaNO_2$–AcOH reagent. Acid hydrolysis of **1** gave ellagic acid, valoneic acid dilactone and glucose. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited double signals for most of the protons, indicating that **1** exists as a mixture of α- and β-anomers in a ratio of *ca* 4 : 3. The signals of the

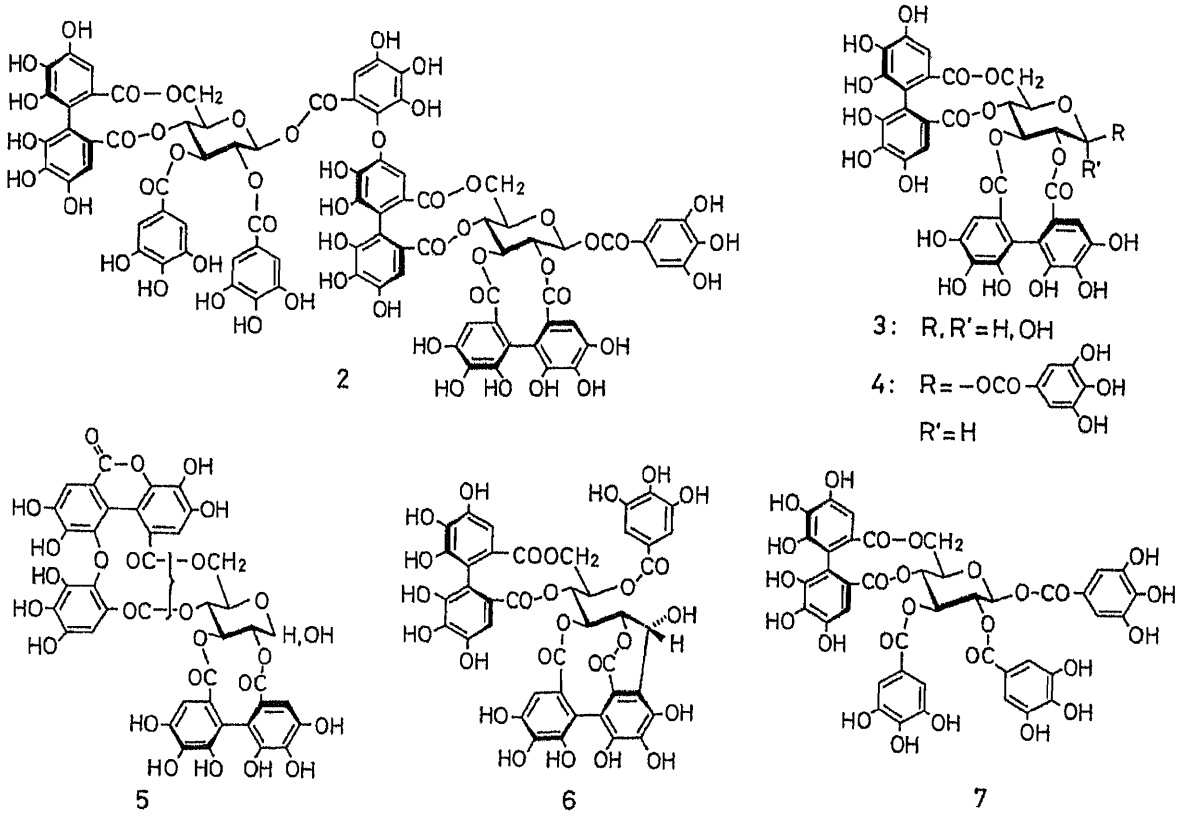
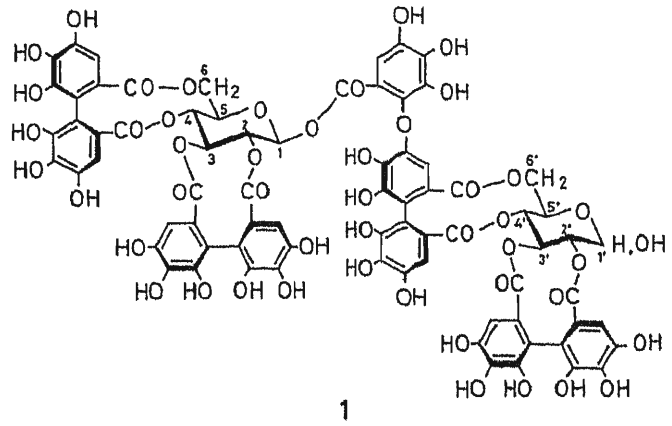


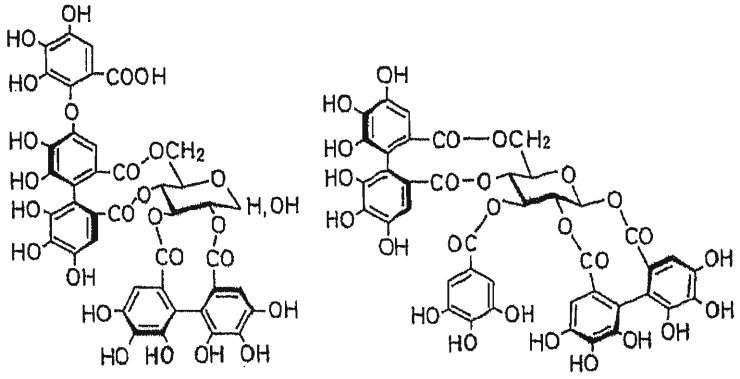
Chart 1

anomeric protons were observed at δ 6.17 and 6.16 (each d, J=9 Hz, 1H in total), and δ 5.43 (d, J=3.5 Hz) and 5.13 (d, J=9 Hz) (1H, in total). The coupling pattern of the other pairs of glucose proton signals indicates that both glucose cores in **1** adopt the $^4C_1$ conformation. Thus the acyloxy group at the anomeric center of one of the glucose cores has the β-configuration. The presence of a valoneoyl and three hexahydroxydiphenoyl (HHDP) groups in **1** was indicated in the aromatic region of the ^{1}H-NMR spectrum by the singlets at δ 7.21, 7.20 (1H in total), 6.69 (1H), 6.62, 6.61 (1H in total), 6.55 (1H), 6.52, 6.46 (1H, in total), 6.39 (3H), and 6.25, 6.24 (1H, in total). Upon treatment with hot water, **1** gave two partial hydrolysates, which were identified as pedunculagin (**3**) and praecoxin A (**8**).[5a] Therefore, roxbin A is a dimer possessing a valoneoyl group, which is biogenetically[11] regarded as a product of intermolecular C–O oxidative coupling between the HHDP group at O-4—O-6 of **3**, and the galloyl group at O-1 of **4**. The proposed structure (**1**) is also consistent with the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum: the glucose carbon resonances are in good agreement with those of **3** and **4**, and hence were assigned as shown in Table I on the basis of the assignments of the peaks of the latter two compounds.[11] Based on the data presented above, the structure **1** was assigned to roxbin A.

Roxbin B (**9**), an off-white amorphous powder, $C_{41}H_{28}O_{26}\cdot 2H_2O$, is a monomeric hydrolyzable tannin as revealed by six sugar carbon signals (Table I) and five ester carbonyl carbon resonances in the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum showed a singlet due to a galloyl group at δ 7.27, and four 1H singlets at δ 7.14, 6.80, 6.73 and 6.60 which are ascribable to two HHDP groups. The presence of these groups was confirmed by acid hydrolysis of **9**, which afforded gallic acid and ellagic acid. The sugar obtained on the hydrolysis was identified as glucose by gas liquid chromatography (GLC) after trimethylsilylation. The conformation of the glucose moiety in **9** was shown to be $^4C_1$ on the basis



1



8 9

Chart 2

TABLE I. ^{13}C Resonances of Glucose Moieties of Roxbin A (1), Roxbin B (9), Pedunculagin (3) and Casuarictin (4) in Acetone-d_6

Carbon	1	3	4[11]	9
C-1	92.10		92.4	92.51
C-2	75.88		76.0	77.24
C-3	77.12		77.3	78.22
C-4	69.11		69.3	69.62
C-5	73.51		73.5	73.24
C-6	63.11		63.1	63.03
α-Anomer				
C-1′	91.75	91.77		
C-2′	75.57	75.55		
C-3′	75.67	75.78		
C-4′	69.63	69.59		
C-5′	67.40	67.40		
C-6′	63.61	63.60		
β-Anomer				
C-1′	95.32	95.40		
C-2′	78.29	78.31		
C-3′	77.44	77.55		
C-4′	69.93	69.89		
C-5′	72.42	72.45		
C-6′	63.61	63.60		

of the large coupling constants (8.5—10 Hz) of the adjacent protons in the ^{1}H-NMR spectrum. The β-configuration of an acyloxy group at C-1 was also demonstrated by the coupling constant ($J_{1,2}$=8.5 Hz) of the anomeric proton signal at δ6.08 (d). The circular dichroism (CD) spectrum of **9** exhibited a positive Cotton effect at 225 nm and a negative one at 255 nm which are indicative[12] of the *S*-configurations of both HHDP groups in **9**. These structural characteristics are similar to those of casuarictin (**4**) (1-*O*-galloyl-2,3;4,6-bis-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose), and thus roxbin B should be an isomer of **4** concerning the positions of the aroyl groups. One of the C_6 methylene protons of **9** resonates at lower field (δ5.30) than the other (δ3.80). Such a large difference in chemical shift between these protons is characteristic of ellagitannins in which the HHDP group is attached to O-6 and O-4 in the $^4C_1$ glucose moiety.[13] The presence of the other HHDP group at O-1—O-3 of glucose core is excluded since it requires a boat conformation, as in brevilagins.[14] Therefore this HHDP group in **9** must be at O-1—O-2 as the $^4C_1$ glucose conformation permits the attachment of an HHDP group only at O-1—O-2 or O-2—O-3 besides O-4—O-6.[13] Upon degalloylation of **9** with tannase, the reversed-phase HPLC of the reaction mixture showed a new single peak and a gallic acid peak. This result is also consistent with the assumption that the galloyl group in **9** is not located at O-1, as degalloylation at C-1 of glucose should result in the formation of an α- and β-anomer mixture which will show two peaks upon reversed-phase HPLC analysis, as found for pedunculagin (**3**),[15] and roxbin A (**1**).

Based on these observations, roxbin B was characterized as 3-*O*-galloyl-1,2;4,6-bis-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (**9**) which is the first example of an ellagitannin possessing the HHDP group at O-1—O-2.

It seems reasonable to presume that ascorbic acid in the fruits of *R. roxburghii*, is stabilized by the abundantly co-existing tannins which were found in the present study, as tannins and related polyphenols are effective inhibitors of the autoxidation of ascorbic acid.[16]

Experimental

The ^{1}H and ^{13}C-NMR spectra were measured on a JEOL FX-270 spectrometer (270 MHz for ^{1}H and 67.8 MHz for ^{13}C) and on a Hitachi R-22FTS (90 MHz for ^{1}H and 22.6 MHz for ^{13}C), with tetramethylsilane as an internal standard. The chemical shifts are given in δ values. The normal phase HPLC was conducted on a Develosil 60-5 column (4×150 mm) using a solvent system of *n*-hexane–MeOH–THF–HCOOH (55:33:11:1) containing oxalic acid (450 mg/l), and the reversed-phase HPLC on a YMC A312 (ODS) column (6×150 mm) with 0.05 M H_3PO_4–0.05 M KH_2PO_4–EtOH–EtOAc (17:17:4:2). Thin layer chromatography (TLC) was carried out on microcrystalline cellulose (Avicel SF, Funakoshi) plates with 7% HOAc, and the spots were visualized by spraying the $FeCl_3$ or $NaNO_2$–HOAc reagents. Column chromatography was performed using Toyopearl HW-40 (coarse and fine grades; Toyo Soda Mfg.) and Sephadex LH-20 (100 μm, Pharmacia Fine Chemicals). CPC was conducted on a CPC model L-90 (Sanki Engineering Ltd.) comprising a centrifuge equipped with twelve cartridges (the internal volume of each cartridge is *ca.* 15 ml).[6]

Isolation of Tannins——The dried unripe fruits (500 g) of *R. roxburghii*, collected in May in Chendgu, China, were homogenized in 70% aqueous acetone. After removal of the acetone, the aqueous solution was extracted with ether, EtOAc and water-saturated *n*-BuOH, successively. A part (5 g) of the EtOAc extract (20.95 g) was fractionated by chromatography on a Toyopearl HW-40C column using aqueous MeOH as the eluant. The 40% MeOH eluate afforded (+)-catechin (0.16 g). The 70% MeOH eluate was rechromatographed over a Sephadex LH-20 with 70% MeOH, followed by purification on Toyopearl HW-40F with 60% MeOH to give roxbin B (**9**) (25 mg). The 80% MeOH fraction was further purified by chromatography on Sephadex LH-20 column using 70% EtOH to give alnusiin (**5**) (154 mg) and casuarictin (**4**) (307 mg). Tellimagrandin II (**7**) (68 mg) was obtained from the MeOH eluate.

A part (3.1 g) of the BuOH extract (19 g) was subjected to CPC (1000 rpm, flow rate 1 ml/min), by the ascending method using the solvent system of *n*-BuOH–*n*-PrOH–H_2O (4:1:5), 10 ml per fraction being collected. This CPC was repeated three times. Fractions 38—80 of each development were combined and further purified by passage through a Toyopearl HW-40F column using the solvent system of MeOH–H_2O–acetone to afford 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (46 mg), pedunculagin (**3**) (29 mg), stachyurin (**6**) (33 mg) and roxbin A (**1**) (147 mg). In a separate experiment, a part (6 g) of the BuOH extract was subjected to chromatography on Toyopearl

HW-40C using the solvent system of 70% EtOH→MeOH. Fractions containing dimers (MeOH eluate) were collected and further purified by chromatography on Sephadex LH-20 and Toyopearl HW-40C to afford roxbin A (**1**) (18 mg) and rugosin F (**4**) (10 mg). Yields of tannin components from dried fruits were as follows: roxbin A (**1**), 0.06%; roxbin B (**9**), 0.02%; (+)-catechin, 0.13%; alnusiin (**5**), 0.13%; casuarictin (**4**), 0.25%; tellimagrandin II (**7**), 0.06%; 2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose, 0.014%; pedunculagin (**3**), 0.007%; stachyurin (**6**), 0.08%; rugosin F (**2**), 0.002%.

Roxbin A (1)——An off-white amorphous powder, TLC, *Rf* 0.32, HPLC (normal phase) t_R 2.33 min, (reversed-phase) t_R 5.29 and 5.91 min. $[\alpha]_D$ +46.5° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{75}H_{50}O_{48}\cdot 4H_2O$: C, 50.28; H, 3.26. Found: C, 50.17; H, 3.58. ^{1}H-NMR (acetone-d_6) δ: 6.17 (d, *J*=9 Hz), 6.16 (d, *J*=9 Hz), 5.43 (d, *J*=3.5 Hz), 5.13 (d, *J*=9 Hz, H-1), 5.11 (dd, *J*=9, 10 Hz), 5.05 (dd, *J*=3.5, 9 Hz), 4.83 (dd, *J*=9, 10 Hz, H-2), 5.22 (t, *J*=10 Hz), 5.45 (t, *J*=10 Hz), 5.42 (t, *J*=10 Hz, H-3), 4.99 (t, *J*=10 Hz), 4.98 (t, *J*=10 Hz), 5.14 (t, *J*=10 Hz), 5.10 (t, *J*=10 Hz, H-4), 4.16 (dd, *J*=6, 10 Hz), 4.56 (dd, *J*=6, 10 Hz), 4.47 (dd, *J*=6, 10 Hz, H-5), 5.33 (dd, *J*=6, 13 Hz), 5.15 (dd, *J*=6, 13 Hz, H-6), 3.86 (d, *J*=13 Hz), 3.73 (d, *J*=13 Hz), 3.67 (d, *J*=13 Hz, H-6'), aromatic protons, see text. ^{13}C-NMR (acetone-d_6) δ: 169.27, 169.21 (1C in total), 169.18, 169.12 (2C in total), 168.90, 168.79 (1C in total), 169.76 (1C'), 167.77, 167.71, 167.61 (3C in total), 163.26, 163.16 (1C in total) (ester CO), Table II. CD (*c*=0.007, MeOH): $[\theta]_{232}$ $+24.6\times10^4$, $[\theta]_{256}$ -10.2×10^4.

Roxbin B (9)——An off-white amorphous powder, TLC, *Rf* 0.32, HPLC (normal phase), t_R 2.94 min, (reversed-phase) t_R 7.73 min. $[\alpha]_D$ −22.8° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{41}H_{28}O_{26}\cdot 2H_2O$: C, 50.62; H, 3.32. Found: C, 50.94; H, 3.65. ^{1}H-NMR (acetone-d_6) δ: 6.08 (d, *J*=8.5 Hz, H-1), 4.90 (dd, *J*=8.5, 9.7 Hz, H-2), 5.21 (t, *J*=9.7 Hz, H-3), 4.89 (dd, *J*=9.7, 10 Hz, H-4), 4.40 (ddd, *J*=1.5, 6.5, 10 Hz, H-5), 5.30 (dd, *J*=6.5, 13 Hz), 3.80 (dd, *J*=1.5, 13 Hz, H-6'), aromatic protons, see text. ^{13}C-NMR (acetone-d_6) δ: 168.26, 167.98, 167.81, 167.16, 164.85 (ester CO), 120.05 (galloyl, C-1), 110.51 (2C, galloyl, C-2, 6), 146.17 (2C, galloyl, C-3, 5), 139.84 (galloyl, C-4), 117.64, 117.44, 115.86, 115.70 (HHDP, C-1, 1'), 126.37, 126.11, 122.23 (2C, HHDP, C-2, 2'), 110.37, 110.16, 108.37, 108.03 (HHDP, C-3, 3'), 145.57, 145.33, 145.23, 145.08, 145.00, 144.86, 144.45, 144.29 (HHDP, C-4, 4', 6, 6'), 138.58, 137.24, 136.46, 136.44 (HHDP, C-5, 5'), Table I. CD (*c*=0.008, MeOH): $[\theta]_{230}$ $+2.2\times10^4$, $[\theta]_{255}$ -0.4×10^4, $[\theta]_{270}$ $+1.5\times10^4$, $[\theta]_{197}$ -1.98×10^4.

Acid Hydrolysis of Roxbin A (1)——A solution of roxbin A (**1**) (2 mg) in 5% H_2SO_4 was kept at 90 °C for 6 h, then extracted with EtOAc. Analysis of the EtOAc extract by reversed-phase HPLC showed the presence of ellagic acid and valoneic acid dilactone. The aqueous layer was neutralized with Amberlite IRA-410 (OH form) and evaporated, and glucose was detected as the trimethylsilyl ether by GLC (3% OV-1, column temperature, 170 °C, N_2 50 ml/min).

Partial Hydrolysis of Roxbin A (1)——A suspension of **1** (70 mg) in H_2O (9 ml) was heated at 82—83 °C for 45 min. The reaction mixture was concentrated and subjected to chromatography over Toyopearl HW-40F using 70% EtOH as the eluant to give pedunculagin (**3**) (9 mg), $[\alpha]_D$ +85° (*c*=0.8, MeOH), and praecoxin A (**8**) (6 mg), $[\alpha]_D$ +51° (*c*=0.6, MeOH). The identities of these products were confirmed by direct comparisons of the spectral data and chromatographic behavior with those of authentic samples.

Acid Hydrolysis of Roxbin B (9)——Roxbin B (**9**) (2 mg) was hydrolyzed as described for **1**, and gallic acid and ellagic acid were detected by HPLC and glucose by GLC after trimethylsilylation.

Degalloylation of 9 with Tannase——Roxbin B (**9**) (1 mg) was treated with tannase at 37 °C for 2 h. The reaction mixture showed upon HPLC (reversed-phase) two peaks of t_R 2.3 and 3.1 min, and the former peak was identical with that of gallic acid.

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Condensed Heteroaromatic Ring Systems. XII.[1] Synthesis of Indole Derivatives from Ethyl 2-Bromocarbanilates

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The palladium-catalyzed reaction of ethyl 2-bromocarbanilate with trimethylsilylacetylene yielded ethyl 2-(trimethylsilylethynyl)carbanilate, which was treated with sodium ethoxide to give indole. The carbanilates having a methyl or a bromo substituent were similarly transformed to corresponding indole derivatives. Furthermore, pyrrolo[3,2-*b*]- and pyrrolo[3,2-*c*]pyridines were synthesized by this method.

Keywords——palladium-catalyzed reaction; trimethylsilylacetylene; ethyl 2-halocarbanilate; ethyl 2-halopyridinecarbamate; ethyl 2-(trimethylsilylethynyl)carbanilate; ethyl *o*-(trimethylsilylethynyl)pyridinecarbamate; indole; pyrrolopyridine

When ethyl 2-nitrophenylpyruvate is hydrogenated in the presence of platinum oxide, the resulting ethyl 2-aminophenylpyruvate undergoes spontaneous dehydro-cyclization to give ethyl 2-indolecarboxylate.[2] The reaction is named as the Reissert indole synthesis. We have previously reported[3] a Reissert-type indole synthesis from 2-(2,2-diethoxyethyl)nitrobenzene, which was obtained by the palladium-catalyzed reaction of 2-bromonitrobenzene and trimethylsilylacetylene (TMSA), followed by the reaction with sodium ethoxide.

On the other hand, there are some reports of the cyclization of 2-ethynylanilines to indoles in the presence of cuprous halides through intramolecular addition of the amino group to the ethynyl group.[4] We describe in this paper another synthetic method to obtain indoles from 2-ethynylaniline derivatives, which were prepared from 2-haloacylanilines by the palladium-catalyzed reaction with TMSA.

The reaction of 2-bromoaniline itself (**1**) with TMSA in the presence of dichlorobis(triphenylphosphine)palladium and cuprous iodide in triethylamine[5] resulted in the recovery of **1**, whereas 2-bromoacetanilide (**2b**) reacted with TMSA under the same conditions to give 2-(trimethylsilylethynyl)acetanilide (**3b**) in moderate yield. When **3b** was heated with sodium ethoxide in ethanol under reflux, indole (**4**) was obtained in 34% yield with a concomitant formation of 2-ethynylaniline (**5**) in 39% yield.

Based on the above results, several acyl derivatives of **1** were examined in order to estimate the efficiency of protecting groups for the cross-coupling reaction with TMSA and the indole cyclization. As a result, ethyl 2-bromocarbanilate (**2e**) was ascertained to be the most satisfactory substrate for both reactions. That is, the carbanilate (**2e**) smoothly reacted with TMSA to give ethyl 2-(trimethylsilylethynyl)carbanilate (**3e**), which was convertible to indole (**4**) by treatment with sodium ethoxide in almost quantitative yield.

Additionally, 2-ethynylaniline (**5**) was heated with sodium ethoxide in ethanol under reflux for 24 h, but no indole formation was observed, and **5** was recovered. Accordingly, it is likely that **3a**—**e** were changed to indole (**4**) by way of the 1-acylindoles (**6**).

According to the procedure described above, all the indole derivatives (**10a**—**d**)

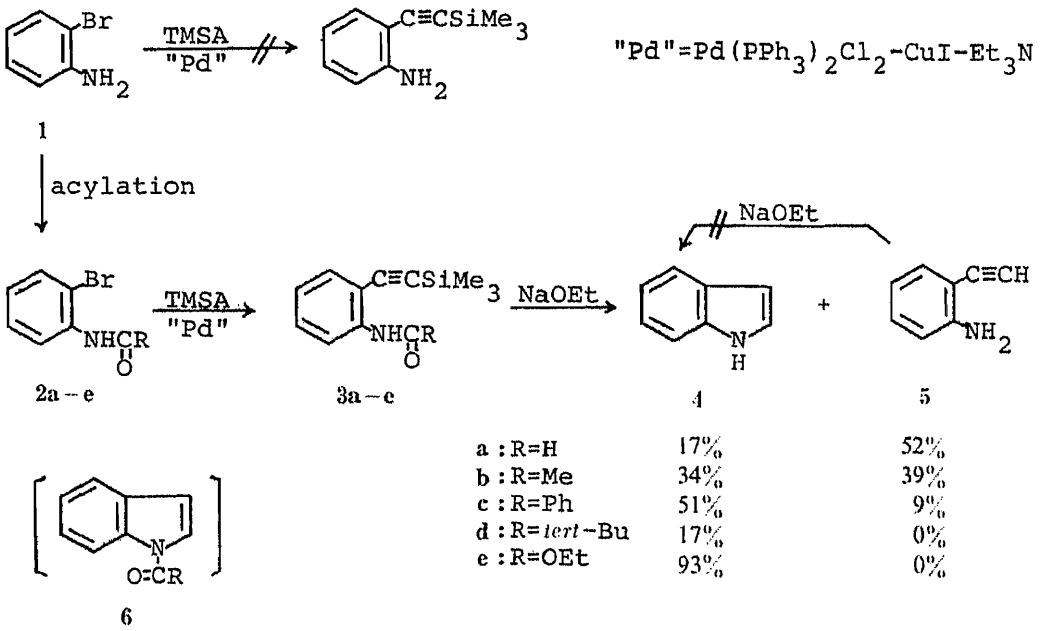


Chart 1

TABLE I. 2-(Trimethylsilylethynyl)acetylenes (**3a**—**e**)

No.	Yield (%)	mp (°C) [bp/mmHg]	IR ($CHCl_3$) cm^{-1}	^{1}H-NMR ($CDCl_3$) δ (ppm)
3a	45	78—80	2120 1700	0.30 (9H, s), 7.0—7.6 (3H, m), 7.90 (1H, brs), 8.3—8.6 (2H, m)
3b	61	96—97	2120 1685	0.30 (9H, s), 2.17 (3H, s), 6.9—7.6 (3H, m), 8.00 (1H, brs), 8.2—8.5 (1H, m)
3c	50	84—85.5	2120 1680	0.30 (9H, s), 6.9—7.7 (6H, m), 7.8—8.1 (2H, m), 8.5—8.8 (1H, m), 8.95 (1H, brs)
3d	51	[130—135/3]	2120 1680	0.29 (9H, s), 1.90 (9H, s), 6.8—7.5 (3H, m), 8.25 (1H, brs), 8.3—8.6 (1H, m)
3e	86	[133/4]	2120 1725	0.30 (9H, s), 1.30 (3H, t, J=7.0 Hz), 4.20 (2H, q, J=7.0 Hz), 6.8—7.5 (4H, m), 8.1—8.3 (1H, m)

containing a methyl group on the fused benzene ring were easily synthesized from the corresponding bromotoluidines (**7a**—**d**) *via* the carbanilates (**8a**—**d**). For example, 2-bromo-5-methylaniline (**7c**) was converted to ethyl 2-bromo-5-methylcarbanilate (**8c**) by treatment with ethyl chloroformate in pyridine. When ethyl 5-methyl-2-(trimethylsilylethnyl)carbanilate (**9c**), obtained from the reaction of **8c** with TMSA, was treated with sodium ethoxide, 6-methylindole (**10c**) was obtained; this is a simple and practical reaction. Further results are listed in Table II.

It is known that in the palladium-catalyzed cross-coupling reaction with terminal acetylenes, aryl iodides commonly show higher reactivity than aryl bromides. Thus, the synthesis of 5-bromo- (**14a**) and 6-bromoindole (**14b**) was achieved by the cross-coupling reaction of ethyl 4-bromo-2-iodocarbanilate (**12a**) and 5-bromo-2-iodocarbanilate (**12b**) with TMSA, followed by treatment with sodium ethoxide.

The above method was successfully applied to the synthesis of pyrrolo[3,2-*b*]pyridine (**18**) and pyrrolo[3,2-*c*]pyridine (**25**), as shown in Chart 3. As reported previously, **18** can be synthesized from 2-bromo-3-nitropyridine (**19**) by using the cross-coupling reaction with

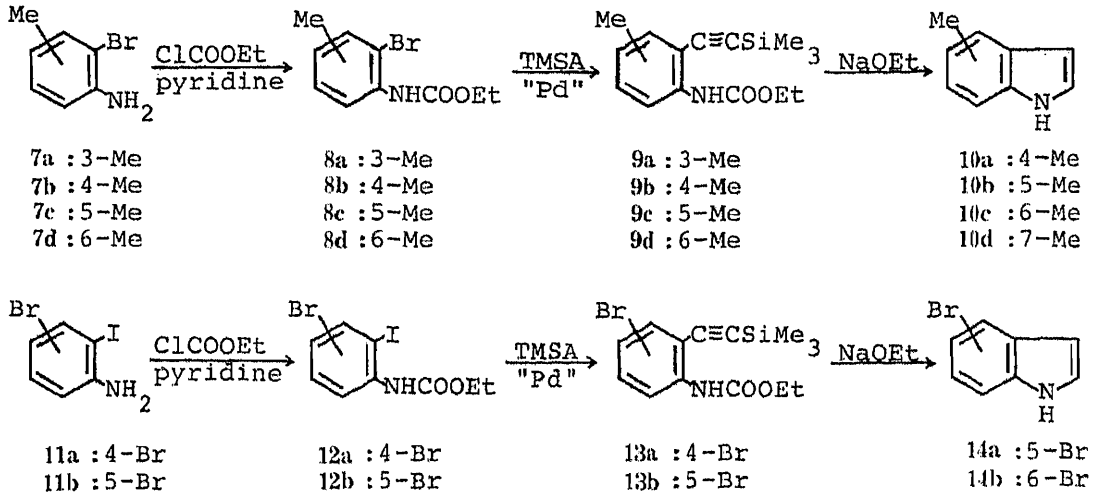


Chart 2

TABLE II. Methyl- (**10a—d**) and Bromoindoles (**14a, b**)

No.	Yield (%)	bp/mmHg [mp] (°C)	Lit. bp/mmHg [mp] (°C)	IR ($CHCl_3$) cm^{-1}	^{1}H-NMR (CCl_4) δ (ppm)
10a	18	120—130/20	90/15[6]	3440	2.50 (3H, s), 6.3—6.5 (1H, m), 6.6—7.1 (4H, m), 7.70 (1H, brs)
10b	78	100—105/3	267[7]	3490	2.41 (3H, s), 5.9—7.6 (6H, m)
10c	47	100—103/3	112/5[8]	3490	2.40 (3H, s), 6.0—7.6 (6H, m)
10d	59	110—115/3	226[7]	3490	2.45 (3H, s), 6.4—7.7 (5H, m), 7.93 (1H, brs)
14a	46	[87—89]	[85.5—86][9]	3480	6.4—6.6 (1H, m), 7.1—7.4 (3H, m), 7.80 (2H, s)
14b	34	[93.5—94]	[93.5—94][10]	3480	6.3—6.5 (1H, m), 6.9—7.5 (4H, m), 7.82 (1H, brs)

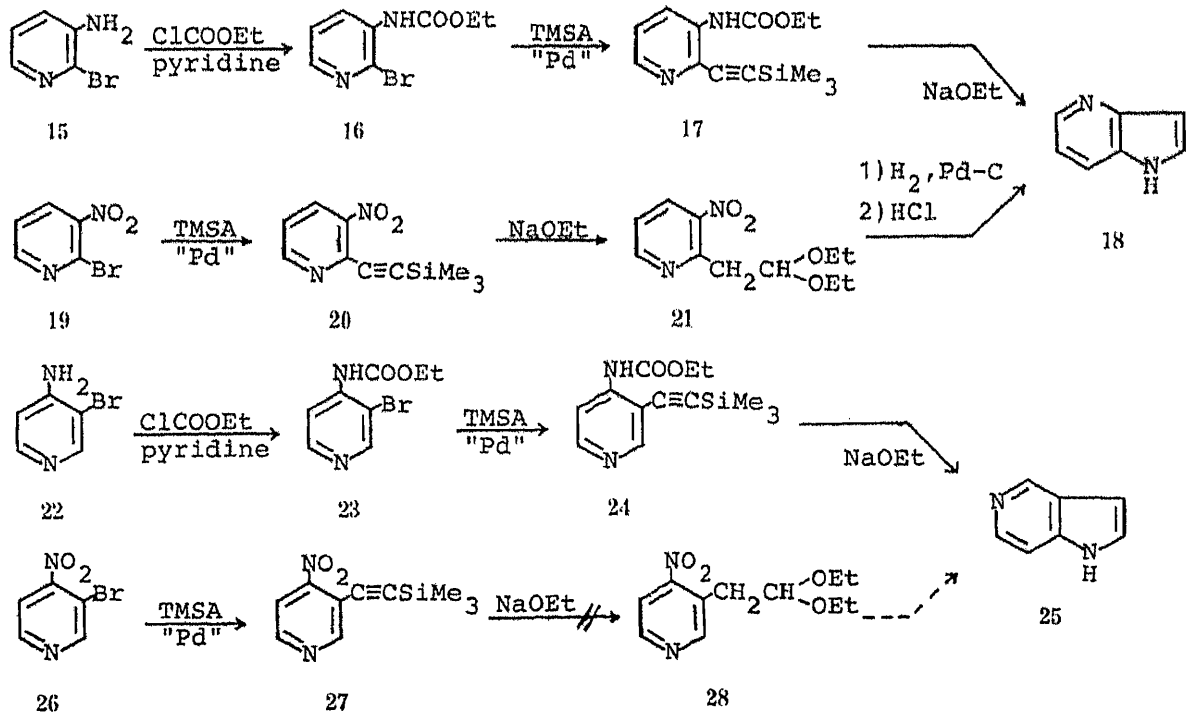


Chart 3

TMSA as a key reaction. The same compound (**18**) was obtained alternatively from **15**, which is more readily available than **19**. An attempted synthesis of **25** from 3-bromo-4-nitropyridine (**26**) failed, because the reaction of 3-(trimethylsilylethynyl)-4-nitropyridine (**27**) with sodium ethoxide gave resinous products instead of the desired compound (**28**). However, **25** was successfully prepared from 4-amino-3-bromopyridine (**22**).

Experimental

All melting points and boiling points are uncorrected. Infrared (IR) spectra were measured with a JASCO IRA-1 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz with a JEOL JMN-PMX 60 spectrometer. Chemical shifts are expressed in δ (ppm) values. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad.

The following starting materials were prepared by the reported procedures: 2-bromo-3-methyl-,[11] 2-bromo-4-methyl-,[12] 2-bromo-5-methyl-,[13] 2-bromo-6-methyl-,[14] and 4-bromo-2-iodoaniline.[15]

General Procedure for the Preparation of 2-(Trimethylsilylethynyl)-*N*-acylanilines (3a—e)——A mixture of a 2-bromo-*N*-acylaniline (5 mmol), TMSA (7.5 mmol), $Pd(PPh_3)_2Cl_2$ (160 mg), CuI (80 mg), and Et_3N (2 ml) was heated in a sealed tube at 100 °C for 5 h. The reaction mixture was diluted with H_2O and extracted with ether. The ethereal extract was purified by SiO_2 column chromatography using C_6H_6 for **3a**, **b**, **d**, hexane–acetone (20 : 1) for **3c**, or hexane–C_6H_6 (3 : 1) for **3e** as an eluent. The crude product was purified by recrystallization or by distillation under reduced pressure.

Reaction of 3a—e with Sodium Ethoxide——A mixture of **3** (2 mmol) and ethanolic sodium ethoxide [prepared from Na (10 mmol) and dry EtOH (20 ml)] was refluxed until **3** was no longer detectable by SiO_2 thin-layer chromatography. After removal of the EtOH, the residue was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ extract was purified by SiO_2 column chromatography using C_6H_6 as an eluent.

5-Bromo-2-iodonitrobenzene——4-Bromoacetanilide (10 g, 47 mmol) was gradually added to fum. HNO_3 (20 ml) below 0 °C. After being stirred for 8 min below 0 °C and for 8 min at room temperature, the mixture was poured into ice-water. A suspension of the resulting precipitate in 3 N KOH (70 ml) was refluxed for 24 h, neutralized with 3 N HCl, and extracted with CH_2Cl_2. Sodium nitrite (1.5 g, 22 mmol) was added to a suspension of the crude product obtained from the CH_2Cl_2 extract in 5 N HCl (12 ml) at 0—10 °C, then an aqueous solution (10 ml) of KI (4.1 g, 25 mmol) was added as a batch. The reaction mixture was stirred at room temperature for 30 min, then heated on a water-bath for 45 min, and made alkaline with K_2CO_3. The resulting precipitate was filtered off and purified by SiO_2 column chromatography using hexane as an eluent. The product, obtained from the hexane eluate, was recrystallized from hexane–ether to give yellow needles, mp 87.5—88 °C. Yield 2.77 g (18%). ^{1}H-NMR ($CDCl_3$): 7.35 (1H, dd, J = 2.0 and 8.0 Hz), 7.86 (1H, d, J = 8.0 Hz), 7.95 (1H, d, J = 2.0 Hz). *Anal.* Calcd for $C_6H_5BrN_2O_2$: C, 21.98; H, 0.92; N, 4.27. Found: C, 22.01; H, 0.85; N, 4.26.

5-Bromo-2-iodoaniline (11b)——A solution of 95% $SnCl_2$ (5.93 g, 30 mmol) in conc. HCl (12 ml) was added to a solution of 5-bromo-2-iodonitrobenzene (2 g, 6.1 mmol) in EtOH (16 ml) at 15—18 °C under stirring. The mixture was heated at 50—60 °C for 15 min, made alkaline with solid KOH, and extracted with $CHCl_3$. The crude product, obtained from the $CHCl_3$ extract, was recrystallized from hexane–ether to give colorless needles, mp 55 °C. Yield 1.25 g (69%). IR ($CHCl_3$) cm^{-1}: 3500, 3390. ^{1}H-NMR (CCl_4): 4.04 (2H, br s), 6.53 (1H, dd, J = 2.0 and 8.0 Hz), 6.75 (1H, d, J = 2.0 Hz), 7.37 (1H, d, J = 8.0 Hz). *Anal.* Calcd for C_6H_5BrIN: C, 24.19; H, 1.69; N, 4.70. Found: C, 23.98; H, 1.65; N, 4.62.

General Procedure for the Preparation of Ethyl 2-Halocarbanilates (8a—d and 12a, b)——Ethyl chloroformate (43 mmol) was added to a solution of a 2-haloaniline (30 mmol) in pyridine (40 ml) at 0—10 °C with stirring, and the

Table III. Analytical Data for 2-(Trimethylsilylethynyl)acylanilines (3a—e)

No.	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
3a	$C_{12}H_{15}NOSi$	66.31	6.96	6.45	66.06	7.19	6.41
3b	$C_{13}H_{17}NOSi$	67.48	7.41	6.05	67.27	7.62	6.07
3c	$C_{18}H_{19}NOSi$	73.68	6.53	4.77	73.72	6.52	4.86
3d	$C_{16}H_{23}NOSi$	70.28	8.48	5.12	70.12	8.78	5.07
3e	$C_{14}H_{19}NO_2Si$	64.33	7.33	5.36	64.18	7.51	5.05

TABLE IV. Ethyl 2-Halocarbanilates (**8a—d** and **12a, b**)

No.	Yield (%)	bp/mmHg [mp] (°C)	IR ($CHCl_3$) cm^{-1}	^1H-NMR (CCl_4) δ (ppm)
8a	99	120/3	3410 1740	1.30 (3H, t, J=7.0 Hz), 2.43 (3H, s), 4.20 (2H, q, J=7.0 Hz), 6.7—7.2 (3H, m), 8.02 (1H, dd, J=2.0 and 8.0 Hz)
8b	89	119—122/4	3420 1735	1.28 (3H, t, J=7.0 Hz), 2.20 (3H, s), 4.16 (2H, q, J=7.0 Hz), 6.7—7.5 (3H, m), 8.04 (1H, d, J=8.0 Hz)
8c	87	115—116/3	3410 1735	1.25 (3H, t, J=7.0 Hz), 2.22 (3H, s), 4.16 (2H, q, J=7.0 Hz), 7.03 (1H, br s), 7.31 (1H, d, J=8.0 Hz), 8.03 (1H, d, J=2.0 Hz)
8d	44	[55—56] (hexane)	3400 1725	1.26 (3H, t, J=7.0 Hz), 2.29 (3H, s), 4.19 (2H, q, J=7.0 Hz), 6.53 (1H, br s), 6.7—7.6 (3H, m)
12a	95	[105—108] (hexane)	3370 1740	1.32 (3H, t, J=7.0 Hz), 4.24 (2H, q, J=7.0 Hz), 6.91 (1H, br s), 7.43 (1H, dd, J=2.0 and 9.0 Hz), 7.88 (1H, d, J=2.0 Hz), 7.98 (1H, d, J=9.0 Hz)
12b	72	[80—81] (hexane)	3385 1735	1.34 (3H, t, J=7.0 Hz), 4.22 (2H, q, J=7.0 Hz), 6.87 (1H, dd, J=2.0 and 8.0 Hz), 7.53 (1H, d, J=8.0 Hz), 8.33 (1H, d, J=2.0 Hz)

TABLE V. Analytical Data for Ethyl 2-Halocarbanilates (**8a—d** and **12a, b**)

No.	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
8a	$C_{10}H_{12}BrNO_2$	46.53	4.70	5.43	46.49	4.61	5.37
8b	$C_{10}H_{12}BrNO_2$	46.53	4.70	5.43	46.61	4.62	5.40
8c	$C_{10}H_{12}BrNO_2$	46.53	4.70	5.43	46.68	4.69	5.31
8d	$C_{10}H_{12}BrNO_2$	46.53	4.70	5.43	46.49	4.91	5.16
12a	$C_9H_9BrINO_2$	29.22	2.45	3.79	29.33	2.45	3.73
12b	$C_9H_9BrINO_2$	29.22	2.45	3.79	29.18	2.34	3.72

mixture was stirred at this temperature for 3 h. After removal of the pyridine, the residue was diluted with H_2O and extracted with ether. The ethereal extract was washed with 3 N HCl and 1 N $NaHCO_3$. The residue, obtained from the ethereal extract was purified by SiO_2 column chromatography, followed by distillation under reduced pressure, or recrystallization.

General Procedure for the Preparation of Methyl- (10a - d) and Bromoindoles (14a, b)——A mixture of **8** or **12** (5 mmol), TMSA (8 mmol), $Pd(PPh_3)_2Cl_2$ (0.25 mmol), CuI (0.4 mmol), and Et_3N (8 mmol) was heated in a sealed tube for 2—3 h at 100—110 °C for **8** or 60 °C for **12**. The mixture was diluted with H_2O and extracted with ether. The ethereal extract was purified by SiO_2 column chromatography. The crude product was added to ethanolic sodium ethoxide (prepared from Na (20 mmol) and dry EtOH (50 ml)), and the mixture was refluxed for 12—24 h. After removal of the EtOH, the residue was diluted with H_2O, and extracted with $CHCl_3$. The $CHCl_3$ extract was purified by SiO_2 column chromatography, distillation under reduced pressure, or recrystallization.

Ethyl 2-Bromo-3-pyridinecarbamate (16)——According to the general procedure for the preparation of ethyl 2-halocarbanilate, 3-amino-2-bromopyridine (**15**) (10 mmol) was treated with ethyl chloroformate (15 mmol) in pyridine (20 ml) to give colorless needles, mp 52—53 °C, which were recrystallized from hexane. Yield 58%. IR ($CHCl_3$) cm^{-1}: 3390, 1730. ^1H-NMR ($CDCl_3$): 1.36 (3H, t, J=7.0 Hz), 4.30 (2H, q, J=7.0 Hz), 7.40 (1H, br s), 8.16 (1H, d, J=6.0 Hz), 8.45 (1H, d, J=6.0 Hz), 8.65 (1H, s). *Anal.* Calcd for $C_8H_9BrN_2O_2$: 39.21; H, 3.70; N, 11.42. Found: C, 39.20; H, 3.59; N, 11.33.

Ethyl 3-Bromo-4-pyridinecarbamate (23)——According to the general procedure for the preparation of ethyl 2-halocarbanilate, 4-amino-3-bromopyridine (10 mmol) was treated with ethyl chloroformate (15 mmol) in pyridine (20 ml) to give colorless needles, mp 60—61.5 °C, which were recrystallized from hexane. Yield 52%. IR ($CHCl_3$) cm^{-1}: 3390, 1730. ^1H-NMR ($CDCl_3$): 1.35 (3H, t, J=7.0 Hz), 4.32 (2H, q, J=7.0 Hz), 7.0—7.3 (1H, m), 7.5—7.9 (2H, m), 8.4—8.5 (1H, m). *Anal.* Calcd for $C_8H_9BrN_2O_2$: 39.21; H, 3.70; N, 11.42. Found: C, 39.16; H, 3.63; N, 11.35.

Pyrrolo[3,2-*b*]pyridine (18)——According to the general procedure for the preparation of indoles, treatment of **16** (5 mmol) with TMSA (7.5 mmol) followed by reaction with NaOEt gave colorless needles, mp 126—127 °C, which

were recrystallized from cyclohexane–acetone. Lit.[16] mp 127—128 °C. IR ($CHCl_3$) cm^{-1}: 3480. ^{1}H-NMR ($CDCl_3$): 7.0—7.3 (1H, m), 7.4—7.9 (2H, m), 8.4—8.6 (1H, m), 10.85 (1H, br s).

Pyrrolo[3,2-*c*]pyridine (25)——According to the general procedure for the preparation of indoles, treatment of **23** (5 mmol) with TMSA (7.5 mmol) followed by reaction with NaOEt gave colorless needles, mp 110—111 °C, which were recrystallized from cyclohexane. Lit.[17] mp 111.5—112.5 °C. Yield 58%. IR ($CHCl_3$) cm^{-1}: 3470. ^{1}H-NMR ($CDCl_3$): 6.6—6.9 (1H, m), 7.2—7.4 (2H, m), 8.35 (1H, d, J=5.6 Hz), 9.20 (1H, s), 10.60 (1H, br s).

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Structure of Acrosome Reaction-Inducing Steroidal Saponins from the Egg Jelly of the Starfish, *Asterias amurensis*

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Three steroidal saponins (designated as Co-ARIS I, II, III) which are essential for inducing the acrosome reaction, were isolated from the egg jelly of the starfish, *Asterias amurensis*, and their structures were elucidated by chemical and spectral means. The structure of Co-ARIS II was determined to be 6α-*O*-{*O*-β-D-fucopyranosyl-(1→2)-*O*-β-D-fucopyranosyl-(1→4)-[*O*-β-D-quinovopyranosyl-(1→2)]-*O*-β-D-quinovopyranosyl-(1→3)-6-deoxy-β-D-*xylo*-hexos-4-ulopyranos-1-yl}-3β-hydroxysulfonyloxy-5α-cholesta-9(11),17(20),24-trien-23-one (**2**), whereas Co-ARIS I (**1**) and Co-ARIS III (**3**) were identical with ovarian asterosaponin-1 (OA-1) and OA-4 previously isolated from the same species of starfish.

Keywords —— starfish; steroid saponin; *Asterias amurensis*; Co-ARIS I; Co-ARIS II; Co-ARIS III; egg jelly; acrosome reaction; ovarian asterosaponin

Introduction

In the starfish, *Asterias amurensis*, the acrosome reaction-inducing activity of the egg jelly has been attributed to two factors: a sulfated glycoprotein (ARIS; acrosome reaction-inducing substance) and an unidentified substance (Co-ARIS). Neither ARIS nor Co-ARIS induces the acrosome reaction on its own, whereas the acrosome reaction is fully induced when they are simultaneously added to the sperm.[1] We have found that Co-ARIS is not a single chemical entity, and have isolated the major Co-ARISs (Co-ARIS I, II and III) by means of a four-step separation: 1) dialysis of the egg jelly against water, 2) elution of the diffusible fraction through a Sep-Pak C_{18} cartridge with 80% methanol, 3) reversed-phase high performance liquid chromatography (HPLC) (ODS column), and 4) preparative thin-layer chromatography (TLC) or column chromatography using silic gel.[2] Chemical and spectroscopic analyses of these compounds revealed that they contain sugars, sulfate and steroid. These characteristics resemble those of the ovarian asterosaponins (OAs) isolated by Okano and Ikegami from the ovaries of the same species of starfish.[3] In this paper we describe the structure elucidation of Co-ARIS I, II and III.

Results and Discussion

Physico-chemical properties of Co-ARIS I (**1**) are as follows; mp 188—199 °C (methanol), ultraviolet (UV): transparent above 220 nm, negative ion fast atom bombardment mass spectrum (FAB-MS) *m/z*: 1239 $(M-H^-)$[4] and 1331 $(M-H+\text{glycerin}^-)$ suggesting $C_{57}H_{92}O_{27}S$ as the molecular formula in the proton form ($C_{57}H_{94}O_{28}S$ for the hydrate form).

The sugars constituting **1** were fucose and quinovose in a *ca.* 1 : 1 ratio as determined by gas liquid chromatography (GLC) after acidic methanolysis followed by trimethylsilyl (TMS) ether derivatization. The lipid fraction obtained by acidic treatment of **1** afforded 3β,6α-dihydroxy-5α-pregn-9(11)-en-20-one (asterone)[5] as a major product, which was characterized in comparison with an authentic sample by TLC and GLC/MS. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (pyridine-d_5–D_2O, 5 : 1) of **1** showed five anomeric carbon signals at δ 102.4, 103.6, 104.4, 105.9 and 106.4 (δ 102.3, 103.6, 103.9, 105.6 and 105.6 in CD_3OD–D_2O (1 : 1), CD_3OD δ 49.0 as a reference), a characteristic signal corresponding to the hydrated ketone carbon of a 6-deoxy-*xylo*-hexos-4-ulopyranosyl residue[5] at δ 92.8, olefinic carbon signals ascribable to the 9(11)-double bond at δ 116.8 and 145.5, and a carbonyl signal at δ 212.9 (C-23). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** in CD_3OD–D_2O (1 : 1) exhibited two singlet methyl signals at δ 0.76 (18-H_3) and 0.99 (19-H_3), two doublet methyl signals at δ 0.90 (J=6 Hz, 26-H_3, 27-H_3), a methine proton signal (m, characteristic of sulfated 3α-H) at δ 4.20 and an olefinic proton signal at δ 5.35 (11-H). These NMR data are quite similar to those repoted for OA-1.[6] Since an authentic sample was not available to us, we isolated OA-1 from the ovary of *A. amurensis* ourselves. Comparison of the chromatographic mobilities, ^{1}H- and ^{13}C-NMR spectra, and negative ion FAB-MS of the two specimens firmly established the identity of Co-ARIS I and OA-1.

Co-ARIS II showed the following properties: mp 192—200 °C, UV λ_{max}: 244 nm (ε 11000 in MeOH), negative ion FAB-MS *m/z*: 1219 ($M-H^-$) and 1311 ($M-H+$glycerin$^-$), $C_{57}H_{88}O_{26}S$ molecular formula in the proton form ($C_{57}H_{90}O_{27}S$ for the hydrate form). Sugar analysis gave the same results as in the case of **1**. The ^{13}C-NMR spectrum (CD_3OD–D_2O, 1 : 1; chemical shifts due to steroidal carbons are given in the experimental section) of **2** showed five anomeric carbon signals and a hydrated ketone carbon signal at the same chemical shifts as observed for **1**. Also the signals of carbons bearing a hydroxy group (δ 72—93), including the steroidal C-3 (δ 80.2) and C-6 (δ 81.0), are superimposable on those of **1** except for the lack of the signal at δ 74.0, attributable to C-20 of **1**, indicating the identity of the sugar portions of **1** and **2**. Other characteristic signals observed are six olefinic carbons at δ 120.7 (C-20), 148.4 (C-17), 123.5 (C-24), 158.8 (C-25), 117.8 (C-11) and 146.5 (C-9), and a

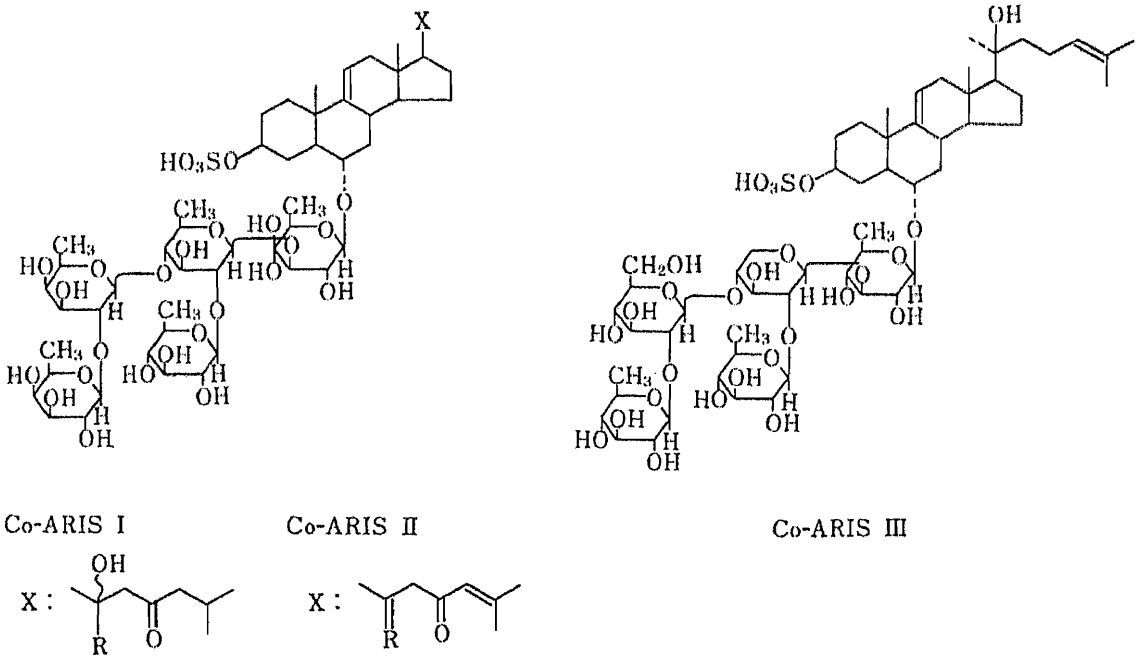


Fig. 1

carbonyl carbon at δ203.9 (C-23). The chemical shifts of C-26 (δ21.1) and C-27 (δ27.9) as well as C-23, C-24 and C-25 are in good agreement with those reported for the known steroid saponin having a 24-en-23-one functionality.[7] The UV absorption supported the presence of this functionality. The ^{1}H-NMR spectrum (400 MHz, CD_3OD) of **2** showed, among others, five singlet methyl signals assignable to 18-H_3 (δ0.84), 19-H_3 (δ1.00), 21-H_3 (δ1.70), 26-H_3 (δ1.90) and 27-H_3 (δ2.11), five doublet methyl signals at δ1.19, 1.26, 1.27, 1.39 and 1.48, and three 1H signals at lower field: δ4.20 (m, 3α-C<u>H</u>OSO_3^-), δ5.40 (br s, 11-H) and δ6.16 (s, 24-H). The location of the remaining double bond was assigned on the basis of the ^{1}H-NMR data. The chemical shift of 21-H_3 and the absence of any additional olefinic protons led us to conclude that the last double bond should be located at the C-17 and C-20 position. Therefore, the structure of Co-ARIS II was formulated as 6α-*O*-{*O*-β-D-fucopyranosyl-(1$\rightarrow$2)-*O*-β-D-fucopyranosyl-(1$\rightarrow$4)-[*O*-β-D-quinovopyranosyl-(1$\rightarrow$2)]-*O*-β-D-quinovopyranosyl-(1$\rightarrow$3)-6-deoxy-β-D-*xylo*-hexos-4-ulopyranos-1-yl}-3β-hydroxysulfonyloxy-5α-cholesta-9(11),17(20),24-trien-23-one (**2**). To our knowledge, a saponin having a 17(20),24-dien-23-one side chain is unprecedented,[8] although a saponin with a 20-hydroxy-24-en-23-one chain was previously isolated from *Acanthaster planci*.[9]

Co-ARIS III showed the following properties: mp 189—201 °C, UV: transparent above 220 nm, negative ion FAB-MS *m/z*: 1227 ($M-H^-$), $C_{56}H_{92}O_{27}S$ molecular formula in the proton form. Sugar analysis showed the presence of quinovose, glucose and xylose in a 3 : 1 : 1 ratio. The ^{13}C-NMR spectrum (CD_3OD) of **3** exhibited olefinic carbon signals at δ117.8 (C-11), 146.4 (C-9), 125.8 (C-24) and 131.8 (C-25), five anomeric carbon signals at δ102.2, 104.7, 104.8, 106.0 and 106.5, characteristic signals of sugar carbon bearing a hydroxyl group at δ62.5 (C-6 of glucose) and δ64.8 (C-5 of xylose), and the steroidal C-3 (δ79.4) and C-6 (δ79.9) signals. The ^{1}H-NMR spectrum (400 MHz, CD_3OD) of **3** showed five singlet methyl signals at δ0.79 (18-H_3), 0.99 (19-H_3), 1.26 (21-H_3), 1.62 (26-H_3) and 1.67 (27-H_3), and lower field 1H-unit signals at δ4.2 (m, characteristic of 3α-C<u>H</u>OSO_3^-), 5.10 (t, J=7 Hz, 24-H) and 5.34 (d, J=5.5 Hz, 11-H). Five anomeric proton signals at δ4.39 (d, J=7.7 Hz), 4.47 (d, J=7.7 Hz), 4.52 (J=7.6 Hz), 4.53 (d, J=7.5 Hz), 4.56 (d, J=7.2 Hz) indicated β-glycoside linkages. Three doublet methyl signals due to the three quinovoses were observed at δ1.27 (d, J=6 Hz), 1.30 (d, J=6.2 Hz) and 1.36 (d, J=6.2 Hz). In addition, the ^{13}C chemical shifts of the steroid moiety are in good agreement with those reported for OA-4 prosapogenins. Based on these data, Co-ARIS III is considered to be identical with OA-4,[10] which was isolated and recently characterized from the ovaries of the same starfish.

Attempted enzymatic hydrolysis of Co-ARISs could not afford useful information until now due to the shortage of the material (*ca.* 10 mg available), but the spectroscopic data, in particular the ^{1}H- and ^{13}C-NMR spectra of the intact saponin molecules described above, provided reasonable bases for the structure elucidation of aglycone steroids. Details of the acrosome reaction-inducing activities will be reported elsewhere.[11]

Experimental

Co-ARISs were isolated as reported previously.[2]

The 200 MHz (unless otherwise stated) and 400 MHz ^{1}H-NMR spectra were recorded on JEOL FX-200 and JEOL GX-400 spectrometers, respectively, in the indicated solvent with tetramethylsilane as an internal reference. ^{13}C-NMR spectra were recorded similarly on the former spectrometer. Negative ion FAB-MS were obtained with a JMS-DX 300 spectrometer. UV spectra were recorded on a Shimadzu UV 200 spectrometer as a methanol solution.

Sugar Analysis——Each Co-ARIS was heated in a sealed tube with dry 1.5 N methanolic HCl for 24 h at 90 °C to obtain the methylglycosides, and they were trimethylsilylated by the method of Sweeley *et al.*[12] The trimethylsilylated derivatives were then analyzed by GLC (Shimadzu GC-7A) using a glass column (3 mm i.d. × 2 m, 2% OV-1 on Chromosorb W, 80—100 mesh).

Chemical Degradation——A solution of Co-ARIS (2 mg) in 2 N HCl (2 ml) was heated at 100 °C for 2 h and the

mixture was extracted with ethyl acetate. The organic layer was washed with brine and concentrated to dryness *in vacuo*. Thin layer chromatographic analysis of the residue (Merck silica gel 60 F_{254} precoated plate, developed with either ethyl acetate or benzene–acetone, 1 : 1) showed that the major spot had the same *Rf* value as authentic asterone. A portion of the hydrolysate was trimethylsilylated (TMS imidazole) and analyzed by GC-MS (Shimadzu DF 9020 spectrometer (70 eV) using a glass column (3 mm i.d. × 1 m, 3% OV-17 on Chromosorb W, 80—100 mesh) at 270 °C). The MS of the major peak showed *m/z*: 476 (M^+), 461, 386, 381, 345, 308, 302, 281, 73 (base peak) and 43, being essentially identical with that of asterone TMS derivative.

The ^{13}C-NMR Data (CD_3OD–D_2O, 1 : 1, CD_3OD δ 49.0 as a Reference) of the Steroid Part of Co-ARIS II——δ 16.8, 19.0, 19.6, 21.1, 22.9, 26.1, 28.0, 29.0, 30.6, 36.1, 36.4, 39.1, 40.7, 41.1, 43.7, *ca.* 50 (the C-5 signal was buried under the CD_3OD signal), 52.8, 54.8, 80.2, 81.0, 117.8, 120.7, 123.5, 146.5, 148.4, 158.8, 203.9.

Acknowledgements We thank Mr. T. Eguchi, Taisho Pharmaceutical Co., Ltd., for the 400 MHz ^{1}H-NMR spectra, and Miss S. Akashi, Ajinomoto Co., Ltd., for the negative ion FAB-MS. Thanks are also due to Prof. Ikegami, Hiroshima University, for providing an authentic sample of asterone and a copy of the ^{13}C-NMR spectrum of OA-1.

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Glycosides of 19α-Hydroxyoleanane-Type Triterpenoids from *Trachelospermum asiaticum* (Trachelospermum. IV)[1]

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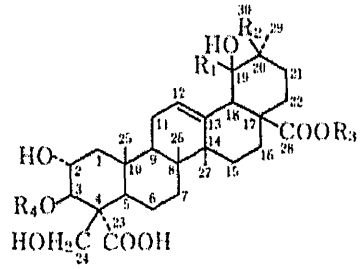
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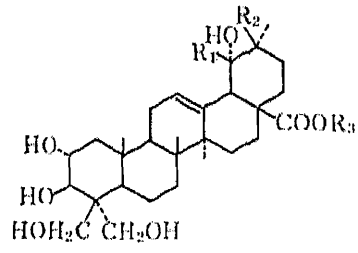
Eight glycosides of 2α,19α-dihydroxyoleanolic acid derivatives, including trachelosperosides D-1, D-2, E-1, F-2, and arjungenin-23,28-bis-*O*-glucoside and -28-*O*-xylopyranosyl-(1→2)-glucopyranoside, were isolated from the whole plant of *Trachelospermum asiaticum*, along with the known glucosides arjunglucoside I and arjungenin-3,28-bis-*O*-glucoside, and their structures were determined.

Keywords——*Trachelospermum asiaticum*; Apocynaceae; oleanane-type triterpenoid; 2α,19α-dihydroxyoleanolic acid derivative; trachelosperoside; trachelosperogenin; 2α,3β,19α,23,24-pentahydroxyolean-12-en-28-oic acid; 2α,3β,19α,24-tetrahydroxyolean-12-ene-23,28-dioic acid; 2α,3β,19α-trihydroxyolean-12-ene-23,28-dioic acid; arjungenin

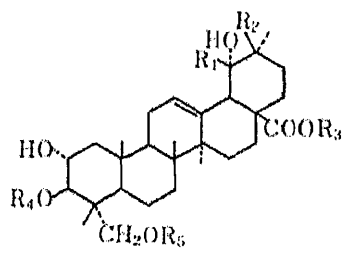
In the preceding paper, we described the isolation from *Trachelospermum asiaticum* of glycosides of 2α,19α-dihydroxyursolic acid derivatives which have additional oxygen functions at C-23 and/or C-24 (trachelosperogenins A, B, and C), along with the known glucosides suavissimoside R1[2] and 19α-hydroxyasiatic acid-3,28-bis-*O*-glucoside[3] (compounds I—VIII).[1] We also mentioned the isolation of the corresponding oleanane-type triterpenoid glycosides. This paper deals with the structures of eight 19α-hydroxyoleanane-type triterpenoid glycosides which were designated as compounds IX—XVI.[1]



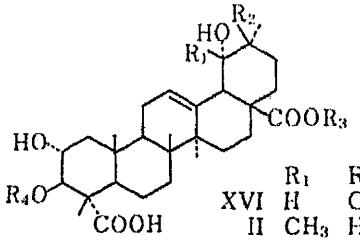
	R_1	R_2	R_3	R_4
IX	H	CH_3	Glc	H
X	H	CH_3	Glc	Glc
VI	CH_3	H	Glc	H



	R_1	R_2	R_3
XI	H	CH_3	Glc
IV	CH_3	H	Glc



	R_1	R_2	R_3	R_4	R_5
XII	H	CH_3	Glc	Glc	H
XIII	H	CH_3	Glc	H	H
XIV	H	CH_3	Glc	H	Glc
XV	H	CH_3	Glc←(2)Xyl	H	H
VII	CH_3	H	Glc	Glc	H



	R_1	R_2	R_3	R_4
XVI	H	CH_3	Glc	Glc
II	CH_3	H	Glc	Glc

Chart 1

TABLE I. ^{1}H Chemical Shifts of Triterpenoid Glycosides and Their Derivatives, δ (ppm) from Tetramethylsilane (TMS) in Pyridine-d_5 (J/Hz Values)

	H-2β	H-3α	H-12	H-18	H-19β	*tert*-Me	Others
IX-1			5.50 (brs)	3.53 (brs)	3.56[a] (brd, 6)	0.99, 1.15, 1.19, 1.27, 1.57	6.38 (H-1′, d, 8), 6.04 (19-OH, d, 6), 3.72 (–COOMe)
IX-3	4.36 (dt, 5, 10)	4.47 (d, 10)	5.47 (brs)	3.41 (brs)	3.53 (brt, 5)	0.88, 1.04, 1.16, 1.27, 1.52	6.03 (19-OH, brs), 4.48, 4.93 (H-24, d, 12), 3.71, 3.75 (–COOMe)
IX-4	5.58 (dt, 4, 10)	5.70 (d, 10)	5.49 (brs)	3.43 (brs)	3.56 (brt, 5)	0.86, 1.04, 1.16, 1.20, 1.62	6.18 (19-OH, d, 6), 4.83, 5.25 (H-24, d, 12), 3.74, 3.80 (–COOMe), 2.00, 2.03, 2.14 (–OAc)
IX-5	5.54 (dt, 4, 10)	5.69 (d, 10)	5.43 (brs)	4.03 (s)		0.83, 1.14 (×2), 1.18, 1.23	4.81, 5.22 (H-24, d, 12), 3.75, 3.77 (–COOMe), 1.99, 2.00, 2.11 (–OAc)
IX-6	4.35 (dt, 5, 10)	4.47 (d, 10)	5.43 (brs)			0.90, 1.04, 1.12, 1.15, 1.20	4.42, 4.90 (H-24, d, 10), 3.66, 3.76 (–COOMe)
X-1			5.49 (brs)	3.53 (brs)	3.58 (brd, 6)	0.99, 1.15, 1.18, 1.23, 1.60	6.37 (H-1′, d, 8), 6.09 (19-OH, d, 6), 4.95 (H-1″, d, 8), 3.91 (–COOMe)
XI			5.50 (brs)	3.52 (brs)	3.56 (brt, 5)	0.98, 1.14, 1.18, 1.20, 1.56	6.39 (H-1′, d, 8), 6.00 (19-OH, d, 5), 4.01, 4.64 (H-23, d, 11), 4.27, 4.89 (H-24, d, 11)
XII			5.49 (brs)	3.53 (brs)	3.56 (brt, 5)	0.98, 1.03, 1.07, 1.14, 1.18, 1.55	6.38 (H-1′, d, 8), 6.00 (19-OH, d, 6), 5.19 (H-1″, d, 7)
XIII			5.50 (brs)	3.53 (brs)	3.55 (brd, 6)	0.98, 1.09, 1.21, 1.14 (×2), 1.55	6.40 (H-1′, d, 8), 5.98 (19-OH, d, 6)
XIV			5.48 (brs)	3.51 (brs)	3.52 (brd, 6)	0.96, 1.02, 1.08, 1.11, 1.15, 1.50	6.36 (H-1′, d, 8), 5.83 (19-OH, d, 6), 4.89 (H-1″, d, 8)
XV			5.50 (brs)	3.48 (brs)	3.54 (brt, 6)	0.97, 1.01, 1.09, 1.13, 1.14, 1.55	6.26 (H-1′, d, 8), 5.83 (19-OH, d, 6), 5.47 (H-1″, d, 8)
XVI-1			5.48 (brs)	3.52 (brs)	3.57 (brt, 6)	0.98, 1.00, 1.12, 1.15, 1.50, 1.58	6.36 (H-1′, d, 8), 6.04 (19-OH, d, 6), 4.88 (H-1″, d, 8), 3.88 (–COOMe)

a) This signal was transformed into brs by D_2O exchange.

Compound IX showed the same *Rf* value as trachelosperoside C-1 (2α,3β,19α,24-tetrahydroxyurs-12-ene-23,28-dioic acid-28-*O*-β-D-glucopyranoside) (VI) on thin layer chromatography (TLC), and was isolated from VI as a methyl ester (IX-1) by reversed-phase chromatographies and high-performance liquid chromatography (HPLC). The molecular formula was the same as that of VI, as determined from the fast atom bombardment (FAB) mass spectrum (MS) of IX-1, and the presence of the ester glycoside linkage was indicated by the anomeric proton peak at δ 6.38 in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum. Since six quaternary carbons were observed in the range from 0 to 60 ppm in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum, along with the peaks ascribable to glucose, IX was considered to be a glucoside of an oleanane-type triterpenoid having a structure similar to that of VI. In fact, the ^{13}C-NMR peaks due to rings A, B, C, and D in VI

TABLE II. ^{13}C Chemical Shifts of Triterpenoid Glycosides and Their Derivatives, δ (ppm) from TMS in Pyridine-d_5

	IX-1	IX-5[f]	X-1	XI	XII	XIII	XIV	XV	XVI-1
C-1	47.9	44.2	47.3	47.6	47.2	47.6	47.1	47.5	47.0
C-2	68.3	69.0	66.4	69.0	67.1	68.9	68.7	68.9	66.6
C-3	81.8	76.8	92.0	79.8	88.6	78.4	77.7	78.2	92.3
C-4	59.1	56.0	59.2	47.9	44.6	43.7	43.5	43.6	54.6
C-5	53.1	51.2	53.3	48.6[a]	47.5	48.5	48.3[a]	48.5[a]	52.6
C-6	22.6	22.7	22.7	19.4	18.5	18.8	18.7	18.7	20.9
C-7	33.4[a]	33.8	33.5[a]	33.3[b]	33.1[a]	33.1[a]	33.0[b]	32.9	33.0[a]
C-8	40.3	39.5	40.3	40.3	40.3	40.4	40.3	40.3	40.2
C-9	48.9	48.0	48.6	48.4[a]	48.5	48.2	48.5[a]	48.0[a]	48.5
C-10	38.3	37.8	38.2	38.3	38.1	38.6	38.5	38.5	38.1
C-11	24.4	23.9	24.4	24.5	24.3	24.3	24.2	24.5	24.1
C-12	123.4	127.6	123.3	123.5	123.5	123.5	123.5	123.5	123.3
C-13	144.3	134.5	144.3	144.3	144.3	144.4	144.5	144.4	144.3
C-14	41.9	42.5	42.0	42.1	42.2	42.2	42.1	42.2	42.1
C-15	29.0[c]	31.6	29.0[b]	29.0[c]	29.1[b]	29.1[b]	28.9	29.5[b]	28.9
C-16	27.9	27.5[a]	27.9	28.0	28.0	28.0	27.9	27.7	27.9
C-17	46.4	44.3	46.4	46.4	46.5	46.5	46.4	46.6	46.4
C-18	44.5	56.1	44.6	44.6	44.7	44.6	44.5	44.7	44.5
C-19	80.9	213.8	80.9	81.0	81.0	81.1	81.0	81.1	80.9
C-20	35.5	51.3	35.5	35.5	35.5	35.5	35.5	35.5	35.5
C-21	28.9[c]	27.8[a]	28.9[b]	28.9[c]	29.0[b]	29.0[b]	28.9	29.0[b]	28.9
C-22	33.0[a]	34.7	33.0[a]	33.0[b]	32.8[a]	32.9[a]	32.9[b]	32.9	32.7[a]
C-23	176.6	173.0	176.3	64.3	64.0	66.7	74.0	67.3	177.1
C-24	62.1[c]	63.1	62.0[c]	62.9	14.6	14.2	14.3	14.2	13.5
C-25	17.3[b]	16.8[c]	16.5	17.5[c]	17.7[d]	17.8[c]	17.7[d]	17.7[d]	17.4[b]
C-26	17.2[b]	16.2[c]	17.1	17.2[c]	17.4[d]	17.4[c]	17.4[d]	17.4[d]	17.2[b]
C-27	24.7[d]	23.7	24.7[d]	24.8[d]	24.9[c]	24.9[d]	25.0[c]	24.8[c]	24.8[c]
C-28	177.1	176.1	177.1	177.1	177.3	177.3	177.2	177.2	177.3
C-29	28.7	26.2[b]	28.7	28.7	28.7	28.7	28.7	28.7	28.7
C-30	24.6[d]	25.9[b]	24.6[d]	24.6[d]	24.7[c]	24.7[d]	24.6[c]	24.7[c]	24.6[c]
COOMe	51.8	52.1 52.7	52.1						52.2
(28-*O*-Glc)									
C-1′	95.8		95.8	95.8	95.9	95.9	95.8	93.7	95.8
C-2′	74.1		74.1	74.1	74.2	74.2	74.1	80.1	74.1
C-3′	78.9		78.9	78.9	79.0	79.0	78.9	78.4[d]	78.9
C-4′	71.0		71.1	71.1	71.2	71.2	71.1	71.1	71.1
C-5′	79.3		79.3	79.2	79.3	79.3	79.2	79.0	79.2
C-6′	62.3[c]		62.2[c]	62.2	62.2	62.3	62.2	62.0	62.2
(Glc or Xyl)			3-*O*-Glc		3-*O*-Glc		23-*O*-Glc	2′-*O*-Xyl	3-*O*-Glc
C-1″			105.8		105.8		105.2	105.8	106.1
C-2″			74.8		75.5		75.1	75.9	74.7
C-3″			78.7		78.0		78.6	78.8[d]	78.5
C-4″			71.5		71.5		71.8	70.7	71.4
C-5″			78.3		78.4		78.3	67.3	78.4
C-6″			62.5[c]		62.5		63.0		62.5

a—*e*) Signal assignments marked *a*), *b*), *c*), *d*) or *e*) in each column may be reversed. *f*) Chemical shifts of acetyl groups: δ 170.2, 170.1, 169.6, 20.8 (×2), 20.3.

were observed in the spectrum of IX, suggesting that two carboxyl groups are present at C-4α and C-17β, and three hydroxyl groups at C-2α, C-3β, and C-24. Enzymic hydrolysis of IX-1 afforded glucose and a monomethyl ester of the aglycone (IX-2). In the ^1H-NMR spectrum of a dimethyl ester of IX-aglycone (IX-3), the peaks of C-2β, C-3α, and C-24 carbinyl protons

were observed. One hydroxyl proton at δ 6.04 which coupled with a broad doublet at δ 3.56, disappeared on D_2O exchange, indicating the presence of an additional secondary hydroxyl group, probably in ring E. In two-dimensional (2D) ^1H–^{13}C chemical shift correlation spectroscopy (COSY), the carbon signal corresponding to this carbinyl proton was observed at δ 80.9 (rather low field), and the hydroxyl group was suggested to be located at C-19.

In order to confirm the 19-hydroxyl group, a dimethyl triacetate of the aglycone (IX-4) was subjected to pyridinium chlorochromate oxidation. In the ^1H-NMR spectrum of the resulting keto compound (IX-5), the signal of H-18 was transformed into a singlet and observed at lower field, and the C-18 (+11.6) and C-20 (+15.8) signals were shifted downfield. The large downfield shifts of C-18 and C-20 can be explained by the introduction of a 19-oxo function. When IX-5 was treated with 1 N KOH, followed by remethylation, the migration of the double bond to form a 13(18)-en-19-one moiety in the product (IX-6) was indicated by the ultraviolet (UV) absorption at 253 nm. The location of the 19-hydroxyl group in IX was thus established, and the orientation was assigned to be α, since the hydroxyl group of IX-3 was hardly reactive in the usual acetylation with Ac_2O and pyridine at room temperature, and the H-19 signal was observed as a broad singlet in IX-1. The electron impact (EI)-MS of IX-2 showed fragment ion peaks at *m/z* 285 (rings A and B) and 264 (rings D and E) besides M^+ peak (*m/z* 548), suggesting the presence of 4α-$COOCH_3$ and 17-COOH. Therefore, the glucose was determined to be attached to the carboxyl group at C-17. Compound IX and the aglycone were named trachelosperoside D-1 and trachelosperogenin D, respectively.

Compound X was also isolated from the ursane-type triterpenoid bis-glucoside, trachelosperoside C-2 (VII), after methylation. On the basis of the similar behavior of X and VII on TLC and HPLC, and the fact that X-monomethyl ester (X-1) was hydrolyzed to IX-2, X was considered to be a glucoside of IX. Based on a ^{13}C-NMR comparison of X-1 with IX-1 and VII-monomethyl ester, X was assigned as the 3-*O*-glucopyranoside of IX, and named trachelosperoside D-2.

Compound XI showed the same *Rf* value and the same molecular formula as trachelosperoside B-1 (2α,3β,19α,23,24-pentahydroxyurs-12-en-28-oic acid-28-*O*-glucopyranoside) (IV) as judged from the FAB-MS peak at *m/z* 705.383 ($C_{36}H_{58}O_{12}Na$). The ^1H-NMR spectrum of XI suggested the presence of two primary hydroxyl groups at C-23 and C-24 (two pairs of doublets at δ 4.01 and 4.64, and δ 4.27 and 4.89, both with coupling constants of 11 Hz, as observed in IV). In the ^{13}C-NMR spectrum, XI showed the same carbon signals of rings A and B as in the case of IV, and of rings C, D, and E as in the case of IX. Compound XI was therefore assigned as the 28-*O*-glucopyranoside of 2α,3β,19α,23,24-pentahydroxyolean-12-en-28-oic acid. Compound XI and the aglycone were named trachelosperoside E-1, and trachelosperogenin E, respectively.

Compound XII, mp 240—246 °C, seemed to be the oleanane-type triterpenoid bis-glucoside corresponding to 19α-hydroxyasiatic acid-3,28-bis-*O*-glucopyranoside (VII), since XII and VII showed similar behavior on TLC and HPLC, and the FAB-MS (*m/z* 851, $C_{42}H_{68}O_{16}Na$) and ^{13}C-NMR spectrum of XII were consistent with the structure of the 3,28-bis-*O*-glucopyranoside of 2α,3β,19α,23-tetrahydroxyolean-12-en-28-oic acid, already known as arjungenin-3,28-bis-*O*-glucopyranoside. Enzymic hydrolysis of XII afforded the 3-*O*-glucoside (XII-1), as in the case of VII. The structure was finally confirmed by direct comparison with an authentic sample, isolated from *Simplocos spicata* by Higuchi *et al.*[3]

Compound XIII, mp 238—242 °C, was hydrolyzed enzymically to the aglycone, arjungenin (XIII-1), and was considered to be arjungenin monoglucoside. The glucosyl linkage seemed to be at the C-17-carboxyl residue, on the basis of ^1H- and ^{13}C-NMR considerations. Compound XIII is therefore arjunglucoside I, formerly isolated from *Terminalia arjuna* by Honda *et al.*[4]

FAB-MS (*m/z* 851, $C_{42}H_{68}O_{16}Na$), the ^{13}C-NMR spectrum and the presence of two anomeric proton signals (δ 4.89 and 6.36) suggested XIV to be a diglucoside of XIII-1, and enzymic hydrolysis of XIV provided XIII-1, as in the case of XIII. One of the glucoside linkages was assigned to the 23-hydroxyl group, based on the glycosylation shift at C-23 (+7.3 ppm) in comparison with that of XIII, and the other one was located at C-28 on the basis of the anomeric proton signal at δ 6.36. The structure of XIV was thus determined to be arjungenin-23,28-bis-*O*-glucopyranoside.

Acid hydrolysis of XV, mp 245—253 °C, afforded XIII-1, glucose, and xylose. Since FAB-MS showed the molecular peak at *m/z* 821 ($C_{41}H_{66}O_{15}Na$), the sugar moiety is composed of one mol each of glucose and xylose. The ^{13}C-NMR signals due to the sugar moiety were superimposable on those of trachelosperoside B-2 (V), which has a β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl moiety, and XV was determined to be arjungenin-28-*O*-xylopyranosyl-(1→2)-glucopyranoside.

Compound XVI was isolated from II after methylation and was suggested to be a bioside of a 19α-hydroxyoleanane-type triterpenoid on the basis of the FAB-MS and ^{13}C-NMR spectrum of XVI-monomethylate (XVI-1). In the ^1H-NMR spectrum of XVI-1, six tertiary methyl groups were observed, and the carbon chemical shifts of rings A and B, and of rings C, D, and E were in good agreement with those of suavissimoside R1-3-*O*-glucoside (II) monomethylate and of IX-1, respectively. The peaks due to the sugar moieties were assigned as those of glucose. The structure of XVI was therefore determined to be the 3,28-bis-*O*-glucopyranoside of 2α,3β,19α-trihydroxyolean-12-ene-23,28-dioic acid. Compound XVI and the aglycone were named trachelosperoside F-2 and trachelosperogenin F, respectively.

In conclusion, eight oleanane-type triterpenoid glycosides, including glycosides of three new aglycones, trachelosperogenins D, E, and F, and four glycosides of arjungenin, were isolated and their structures were elucidated. As well as the ursane-type triterpenoids described in the preceding paper, all oleanane-type triterpenoids isolated from this plant retain the 2α,3β,19α-hydroxyl groups, along with hydroxyl groups at C-23 and/or C-24, and a 4α-carboxyl moiety in some cases.

Experimental

NMR spectra, FAB-MS and EI-MS were measured with the same machines and in the same manner as described in the preceding paper.[1] Column chromatography, HPLC, paper chromatography (PC) and TLC were also carried out in the same manner with the following solvent systems: solv. 1, $CHCl_3$–MeOH–H_2O (7:3:1, bottom layer); solv. 2, EtOAc–MeOH–H_2O (4:1:0.5); solv. 3, CH_3CN–H_2O (for HPLC); solv. 4, benzene–acetone; solv. 5, BuOH–AcOH–H_2O (4:1:5, upper layer). Isolation of the glycosides was described in the preceding paper.[1]

Compound IX (Trachelosperoside D-1)——A methyl ester of IX (IX-1) was obtained as a solid, $[\alpha]_D^{25}$ +27.5 (*c*=1.7, MeOH). FAB-MS *m/z*: 733.379 (Calcd for $C_{37}H_{58}O_{13}Na$: 733.378). The enzymic hydrolysis of IX-1 (20 mg) was carried out with cellulase (Sigma Chem. Co.) (40 mg) at 38 °C for 8 h in 20% ethanolic solution (2 ml). The mixture was extracted with BuOH and the BuOH extract was purified on a silica gel column with solv. 4 (4:1) to give trachelosperogenin D monomethylate (IX-2), as a solid, $[\alpha]_D^{29}$ +49.1° (*c*=1.6, MeOH). EI-MS *m/z*: 548 (M$^+$, $C_{31}H_{48}O_8$), 502, 486, 430, 285, 264, 246. The H_2O layer was concentrated, applied to a charcoal column, and eluted with 5% EtOH. The effluent was concentrated *in vacuo* and the residue was examined by PC with solv. 5 (*Rf* 0.22; D-glucose, 0.22). On methylation with CH_2N_2 in ether, IX-2 afforded trachelosperogenin D dimethylate (IX-3) as a crystalline powder from MeOH, mp 147—151 °C, $[\alpha]_D^{29}$ +47.9° (*c*=0.33, MeOH). EI-MS *m/z*: 562.350 (Calcd for $C_{32}H_{50}O_8$: 562.350). On acetylation of IX-3 with pyridine and Ac_2O at room temperature, trachelosperogenin D dimethyl triacetate (IX-4) was obtained as a solid, $[\alpha]_D^{28}$ +15.6° (*c*=1.4, $CHCl_3$). FAB-MS *m/z*:711 ($C_{38}H_{56}O_{11}Na$). Pyridinium chlorochromate (100 mg) was added portionwise to a solution of IX-4 (50 mg) in CH_2Cl_2 (5 ml), and the mixture was stirred overnight, diluted with $CHCl_3$ and then washed with H_2O. The $CHCl_3$ extract was purified on a silica gel column with solv. 4 (8:1). 19-Dehydrotrachelosperogenin D dimethyl triacetate (IX-5) was obtained as a crystalline powder (MeOH), mp 119—122 °C, $[\alpha]_D^{28}$ +50.6° (*c*=0.75, MeOH). EI-MS *m/z*: 686.365 (Calcd for $C_{38}H_{54}O_{11}$: 686.366). Compound IX-5 (10 mg) was dissolved in 2 ml of 1 N KOH in MeOH and refluxed for 1 h. The mixture was diluted with H_2O and extracted with BuOH. The BuOH layer was concentrated *in vacuo* and the residue was crystallized from MeOH to give colorless prisms, mp 265—275 °C (dec.). This product was subjected to

methylation with CH_2N_2 in ether to give IX-6 as a solid. EI-MS *m/z*: 560.335 (Calcd for $C_{32}H_{48}O_8$; 560.335). UV λ_{max}^{MeOH} nm (ε): 253 (7100).

Compound X (Trachelosperoside D-2)——A monomethyl ester (X-1) was obtained as a solid, $[\alpha]_D^{31}$ +3.6° (*c*=0.95, MeOH). FAB-MS *m/z*: 895 ($C_{43}H_{68}O_{18}Na$). Compound X-1 (2 mg) was heated with 2 N H_2SO_4 (0.5 ml) at 100 °C for 2 h, and the mixture was deacidified with IRA-410. The mixture was then concentrated *in vacuo*, and IX-2 and glucose were detected on TLC (solvs. 1, 2, and 5).

Compound XI (Trachelosperoside E-1)——Obtained as a solid, $[\alpha]_D^{31}$ +15.5° (*c*=0.60, MeOH). FAB-MS *m/z*: 705.383 (Calcd for $C_{36}H_{58}O_{12}Na$; 705.383).

Compound XII (Arjungenin-3,28-bis-*O*-glucopyranoside)——Obtained as colorless prisms from MeOH, mp 240—246 °C, $[\alpha]_D^{25}$ +1.1° (*c*=0.35, MeOH). FAB-MS *m/z*: 851 ($C_{42}H_{68}O_{16}Na$). Hydrolysis of XII (20 mg) with cellulase afforded XII-1 as fine prisms (MeOH), mp 238—248 °C (dec.). FAB-MS *m/z*: 689 ($C_{36}H_{58}O_{11}Na$). On admixture of XII with an authentic sample isolated from *Symplocos spicata*,[3] no melting point depression was observed. The two samples showed the same *Rf* values on TLC (solvs. 1 and 2), and the ^{13}C-NMR spectra were superimposable.

Compound XIII (Arjunglucoside I)—— Obtained as prisms from MeOH, mp 238—242 °C, $[\alpha]_D^{31}$ +4.6° (*c*=0.75, MeOH). FAB-MS *m/z*: 689.388 (Calcd for $C_{36}H_{58}O_{11}Na$; 689.388). Enzymic hydrolysis of XIII (20 mg) with cellulase was carried out in the same manner as described for IX, and arjungenin (XIII-1) was obtained as prisms by crystallization from MeOH, mp 285—295 °C (dec.), $[\alpha]_D^{30}$ +26.2° (*c*=0.70, MeOH) [ref. arjungenin mp 293—294 °C, $[\alpha]_D$ +29°].[4] EI-MS *m/z*: 504.344 (Calcd for $C_{30}H_{48}O_6$; 504.345).

Compound XIV (Arjungenin-23,28-bis-*O*-glucopyranoside)——Obtained as a homogeneous solid, $[\alpha]_D^{30}$ +2.2° (*c*=0.95, MeOH). FAB-MS *m/z*: 851 ($C_{42}H_{68}O_{16}Na$). On enzymic hydrolysis of 50 mg of XIV with cellulase, XIII-1 (18 mg) was obtained and crystallized from MeOH to give prisms, mp 290—295 °C.

Compound XV (Arjungenin-28-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside)——Obtained as colorless prisms from MeOH, mp 245—253 °C, $[\alpha]_D^{30}$ +3.9° (*c*=0.65, MeOH). FAB-MS *m/z*: 821 ($C_{41}H_{66}O_{15}Na$). After shaking of XV in 25% EtOH with cellulase for 8 h at 38 °C, no hydrolysate was obtained and XV was recovered. On reflux of XV (5 mg) with 1 N H_2SO_4–50% EtOH (1 ml) for 1 h, the mixture was diluted with MeOH, and deacidified with IRA-410. The mixture was concentrated *in vacuo*, diluted with H_2O, and extracted with BuOH. On TLC of the BuOH extract, arjungenin (XIII-1) was detected (solvs. 1 and 2). The H_2O layer, after BuOH extraction, was concentrated *in vacuo* and subjected to PC (solv. 5) (*Rf* 0.22, 0.29; glucose 0.22, xylose 0.29). On refluxing of XV (5 mg) with 0.5 N H_2SO_4–50% dioxane (1 ml) for 30 min, followed by deacidification with IRA-410 and extraction with BuOH, XIII was detected on TLC (solvs. 1 and 2).

Compound XVI (Trachelosperoside F-2)——The monomethyl ester of XVI (XVI-1) was obtained as a solid, $[\alpha]_D^{25}$ +0.8° (*c*=0.52, MeOH). FAB-MS *m/z*: 879 ($C_{43}H_{68}O_{17}Na$).

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Studies on Cardiac Ingredients of Plants. III.[1] Structural Confirmation and Biological Activity of Reduced Proscillaridins

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Catalytic hydrogenation of proscillaridin (I) gave five compounds (II—VI) which were successfully separated by reversed-phase high performance liquid chromatography. The structures of II—VI were determined on the basis of the proton, carbon-13 and two dimensional nuclear magnetic resonance (^{1}H-, ^{13}C- and 2D-NMR) spectra. The configurations of IV and V were determined by circular dichroism spectral analyses.

Relationships between the chemical structures and biological activities of I—VI were studied by the use of isolated guinea-pig papillary muscle and an Na^+, K^+-adenosine triphosphatase preparation from dog kidney. The biological activity of each compound was evaluated in terms of pD_2 and pIC_{50} values, while the inotropic speed of each compound was evaluated in terms of T_{50} value. The activities of the reduced compounds (II—VI) were weaker than that of proscillaridin (I), but the inotropic speeds were faster.

A significant correlation was obtained between the ^{13}C-NMR chemical shifts of C-24 and the pD_2 values of I—VI ($r=0.95$, $p<0.01$). The T_{50} values and ^{13}C-NMR chemical shifts of C-18 also showed a remarkable correlation ($r=0.96$, $p<0.01$).

Keywords—proscillaridin; proscillaridin catalytic hydrogenation; 20*R*-, 20*S*-tetrahydroproscillaridin; reversed-phase HPLC; ^{13}C-NMR; 2D-NMR; CD; Na^+, K^+-ATPase; positive inotropic effect; guinea-pig papillary muscle

The cardiac glycosides digitoxin, digoxin and ouabain have been widely used in the therapy of congestive heart failure in spite of their toxicity and the narrow safety margin.[2] In 1957, Vick *et al.*[3] indicated that hydrogenation of the lactone rings of digitoxin, digoxin and ouabain increased the safety margin of the parent glycosides without a corresponding loss of positive inotropic effect (PIE) on the dog heart–lung preparation. Since then, a number of studies[4] have been undertaken to examine the difference in action between the natural glycosides and their hydrogenated derivatives. The reduction of the unsaturated lactone rings of cardiac glycosides introduces a center of asymmetry at C-20.[5] However, most of the pharmacological investigations[6] on dihydroglycosides have been carried out with mixtures of the 20*R*- and 20*S*-epimers because of the lack of methodology for effective separation of the two epimers. We have, therefore, been interested in separation of the individual 20*R*- and 20*S*-dihydroepimers and subsequent evaluation of their pharmacological differences.

In a previous paper,[1] we reported the synthesis and isolation of 20*R*- and 20*S*-dihydroouabain, and clarified the differences in PIE between the two epimers. As an extension of our program directed towards the development of new cardiac glycosides with a lower risk of toxicity due to an improved ratio of therapeutic and toxic doses, we wish to report in this paper our results concerning the hydrogenation of proscillaridin (I), one of the most potent

cardiac glycosides, and the positive inotropic activities of the resulting reduced proscillaridins (II—VI). In addition, we will describe the relationship between the PIE and chemical shifts in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra.

Chemistry

Catalytic hydrogenation of proscillaridin (I) over 5% Pd–C gave five compounds (II—VI), which were separated and purified by high performance liquid chromatography (HPLC) with a reversed-phase column (Chart 1).

Compound II was obtained as colorless needles, mp 216—221 °C, $[\alpha]_D^{22}$ −60.7°, $C_{30}H_{44}O_8$ (*m/z* 532, M^+) and confirmed to be 3*β*-[(6-deoxy-α-L-mannopyranosyl)oxy]-14*β*-hydroxybufa-4,20(22)-dienolide by a comparison of the mass spectrum (MS), and infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra with those of an authentic sample prepared according to Repke and Berlin[7] by Al–Hg reduction of I.

Compound III was obtained as a crystalline powder, mp 187—188 °C, $[\alpha]_D^{22}$ −23.2°, $C_{30}H_{44}O_8$ (*m/z* 532, M^+). The IR spectrum showed a carbonyl group absorption at 1745 cm^{-1}. In the two-dimensional ^{1}H shift-correlated (2D-COSY) spectrum of III (Fig. 1), the C-21 olefinic proton signal was observed at *δ* 6.54 as a singlet and the signals of the 22-H_2 and 23-H_2 protons of the lactone ring at *δ* 2.46 (1H, m), 2.93 (1H, m) and 2.50—2.64 (2H, m), respectively. The ^{13}C-NMR spectrum showed the signals of five carbons of the lactone ring at *δ* 21.0 (t), 29.0 (t), 121.6 (s), 138.4 (d) and 169.4 (s). From these results, compound III was confirmed to be 3*β*-[(6-deoxy-α-L-mannopyranosyl)oxy]-14*β*-hydroxybufa-4,20-dienolide.

Compound IV (colorless needles, mp 245—249 °C, $[\alpha]_D^{22}$ −41.1°) and compound V (colorless needles, mp 236—238 °C, $[\alpha]_D^{22}$ −56.8°) were found to have the same molecular formula, $C_{30}H_{46}O_8$, by elementary analyses. The MS of IV and V showed the same fragment ion at *m/z* 388 ($M^+ - C_6H_{10}O_4$). The IR spectra of IV and V showed carbonyl absorptions due to a saturated *δ*-lactone at 1720 and 1725 cm^{-1}, respectively. The ^{1}H- and ^{13}C-NMR spectra showed no signals in the olefinic lactone region. In the ^{1}H-NMR spectrum (Table I), the signals of protons of the lacton ring in IV were observed at *δ* 1.95 (1H, m, 22-H), 2.15 (1H,

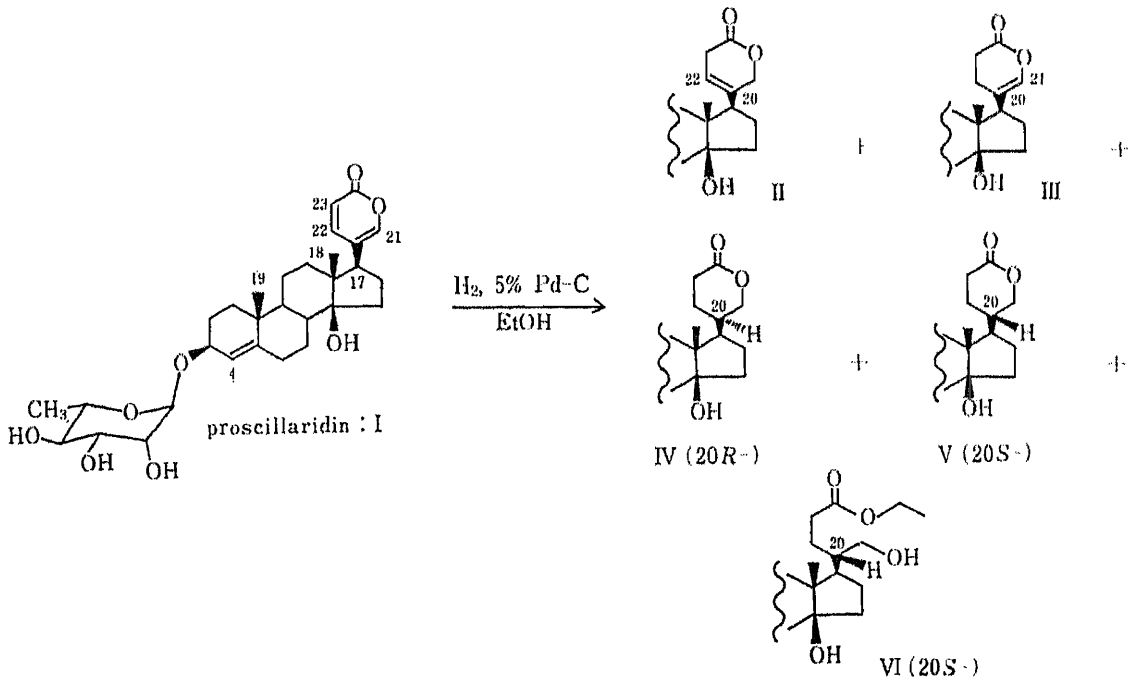


Chart 1

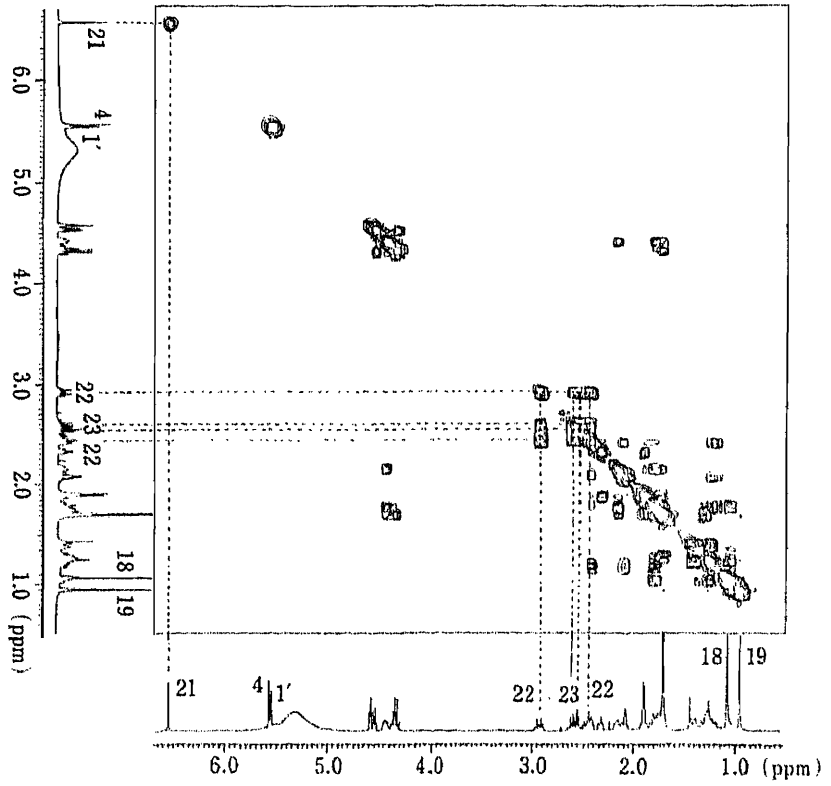


Fig. 1. Contour Plot of the COSY Map of Compound III

m, 22-H), 2.20—2.24 (1H, m, 20-H), 2.45 (1H, ddd, 23-H), 2.56 (1H, ddd, 23-H), 3.98 (1H, dd, 21-H) and 4.26 (1H, dd, 21-H). The signals of V were similarly observed at δ 1.58 (1H, m, 22-H), 1.82 (1H, m, 22-H), 2.16—2.25 (1H, m, 20-H), 2.54 (1H, ddd, 23-H), 2.64 (1H, ddd, 23-H), 4.38 (1H, dd, 21-H) and 4.69 (1H, dd, 21-H). The above signal assignments of IV and V were supported by 2D-COSY. These observations suggested that compounds IV and V are 3β-[(6-deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4-enolides, and they are clearly the epimers of the chiral center at C-20.

The C-20 configuration of each epimer was deduced by circular dichroism (CD) spectroscopy. Beecham[8] reported that the sign of CD curve for δ-lactones is associated with the location of the β-carbon relative to the plane formed by the lactone moiety and the α-carbon (C_α–CO–O). The CD curve is positive when the conformation of the δ-lactone has the β-carbon above the plane and negative when the β-carbon is below the plane (Fig. 2). The CD spectra of compounds IV and V revealed that IV had a positive CD curve and V had a negative CD curve (Fig. 3). These results indicate that the β-carbon of the lactone is located as in Fig. 2A or 2B for compound IV and as in Fig. 2C or 2D for compound V. Molecular model examination of IV and V suggested that the energetically preferred position for the steroid substituent on C-20 is quasi-equatorial as shown in Fig. 2. We concluded that compound IV has the *R*-configuration at C-20 and compound V has the *S*-configuration.

Compound VI was obtained as a crystalline powder, mp 189—191 °C, $[\alpha]_D^{22}$ −29.4°, $C_{32}H_{52}O_9$. The IR spectrum showed a carbonyl group absorption at 1725 cm^{-1}. The ^{1}H-NMR spectrum showed the appearance of new signals attributed to an ethoxy group at δ 1.20 (3H, t) and 4.18 (2H, q). In the ^{13}C-NMR spectrum, ethoxy carbons were also observed at δ 14.4 (q) and 60.2 (t), respectively, and no signals of olefinic lactone ring carbons were observed. The signal of C-21 was observed at δ 63.5 (t), whereas the corresponding signals of IV and V were observed at δ 73.5 (t) and 71.6 (t) Such a high-field shift of C-21 suggests

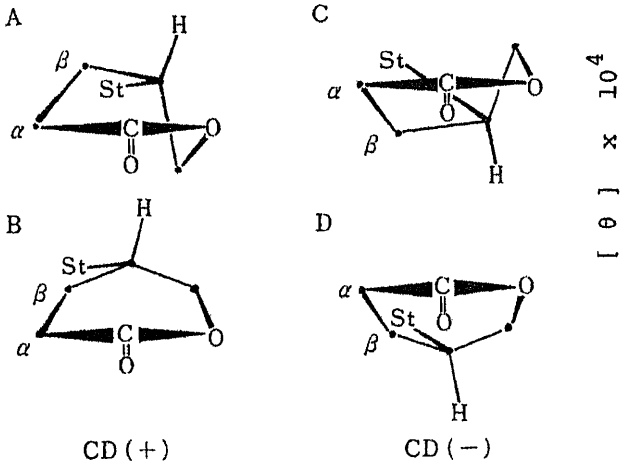


Fig. 2. Perspective Representation of the Four Stable Conformations of the δ-Lactone

A positive CD spectrum is associated with conformation A or B, and a negative CD spectrum with C or D. St represents the steroid nucleus.

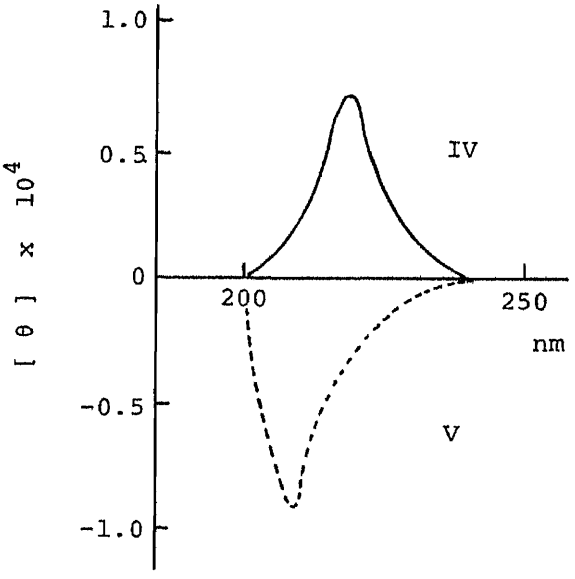


Fig. 3. CD Spectra of IV and V

——, IV (20*R*-tetrahydroproscillaridin); ------, V (20*S*-tetrahydroproscillaridin).

the cleavage of the lactone ring followed by the formation of the ester. Moreover, when IV and V were allowed to stand in EtOH under the same reductive conditions, V was readily converted to VI while IV remained unchanged. On the basis of these results, compound VI was concluded to be 20*S*-ethyl-3β-[(6-deoxy-α-L-mannopyranosyl)oxy]-14β,21-dihydroxychola-4-enoate.

The spatial structures of I—VI were examined by one-dimensional nuclear Overhauser effect (NOE) and two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY). NOE enhancements common in I—VI were observed among (18-H and 19-H), (19-H and 6-H), (4′-H and 5′-H), (5′-H and 6′-H) and (6′-H and 19-H). Further NOE examinations were undertaken in order to reveal the stereochemical features around the lactone ring, which is well known to be closely related to PIE development. Irradiation of the C-18 methyl signal in I caused 2.9% and 4.4% enhancements of the integral intensities of H-21 and H-22 signals, 4.1% and 1.4% in II, and 2.0% and 4.3% in III, respectively. NOE enhancements between the C-18 methyl and H-21 or H-22 signals were not observed in other compounds (IV—VI). These NOE examinations suggest that the rotation of the C-17 and C-20 linkage in compounds having a double bond in the lactone ring is restricted on account of the steric interaction between the C-18 methyl group and the C-21 or C-22 ring proton, while the corresponding rotation in IV—VI appears to be relatively free due to absence of the steric interaction.

Biological Results and Discussion

The biological activities (pD_2, pIC_{50} and T_{50} values) of I—VI were examined by means of measurements of PIE in isolated guinea-pig papillary muscle and enzyme activity of an Na^+, K^+-adenosine triphosphatase (ATPase) preparation from dog kidney. The results are summarized in Table III. Although the biological activities (pD_2 and pIC_{50}) of II—VI were less potent than that of the parent compound (I), compound III showed the most expanded concentration range in PIE development using guinea-pig papillary muscles, and reduced occurrence of the arrhythmia as judged from recordings of irregular contraction in guinea-pig

TABLE I. ^{1}H-NMR Chemical Shifts of I—VI[a]

Compd.	4-H	18-H	19-H	20-H	21-H	22-H	23-H	1'-H
I	5.58 (1H, s)	0.93 (3H, s)	0.94 (3H, s)		7.49 (1H, d, J=2.7)	8.24 (1H, dd, J=2.7, 9.8)	6.37 (1H, d, J=9.8)	5.55 (1H, s)
II	5.57 (1H, s)	1.07 (3H, s)	0.94 (3H, s)		5.12 (1H, d, J=15.1) 5.42 (1H, d, J=15.1)	5.68 (1H, t, J=3.9)	3.14 (1H, dd, J=3.9, 15.5) 3.19 (1H, dd, J=3.9, 15.5)	5.55 (1H, s)
III	5.56 (1H, s)	1.07 (3H, s)	0.95 (3H, s)		6.54 (1H, s)	2.46 (1H, m) 2.93 (1H, m)	2.50—2.64 (2H, m)	5.54 (1H, s)
IV	5.58 (1H, s)	1.16 (3H, s)	0.95 (3H, s)	2.20—2.24 (1H, m)	3.98 (1H, dd, J=10.6, 10.9) 4.26 (1H, dd, J=5.3, 10.9)	1.95 (1H, m) 2.15 (1H, m)	2.45 (1H, ddd, J=6.8, 7.2, 16.9) 2.56 (1H, ddd, J=7.2, 7.2, 16.9)	5.55 (1H, s)
V	5.57 (1H, s)	1.18 (3H, s)	0.95 (3H, s)	2.16—2.25 (1H, m)	4.38 (1H, dd, J=9.5, 11.5) 4.69 (1H, dd, J=4.8, 11.5)	1.58 (1H, m) 1.82 (1H, m)	2.54 (1H, ddd, J=8.2, 8.3, 16.6) 2.64 (1H, ddd, J=6.4, 8.2, 16.6)	5.54 (1H, s)
VI[b]	5.57 (1H, s)	1.36 (3H, s)	0.96 (3H, s)	1.75—1.84 (1H, m)	3.88 (1H, dd, J=6.8, 10.8) 4.25 (1H, dd, J=5.6, 10.8)	2.06—2.17 (2H, m)	2.59—2.63 (2H, m)	5.55 (1H, s)

a) Measured in C_5D_5N and coupling constants in Hz. b) Ethoxy signal: 1.20 (3H, t, J=7.1, OCH_2CH_3), 4.18 (2H, q, J=7.1, OCH_2CH_3).

heart-lung preparation (data not shown). The speed (T_{50}) of PIE with II—VI was clearly faster than that of the parent compound (I), and IV was found to show the fastest speed of PIE, being superior to ouabain (8.8 min).[9] More detailed biological studies on I—VI will be reported elsewhere.

An excellent correlation was obtained between the ^{13}C-NMR (C_5D_5N) chemical shifts of C-24 and the pD_2 values of I—VI, as shown in Fig. 4. The regression line was expressed by the following equation (r, correlation coefficient); $\delta_{C\text{-}24} = -4.0\ pD_2 + 193.4$ ($r = -0.95$, $p < 0.01$). Similarly, Fig. 5 shows a significant correlation between the ^{13}C-NMR (C_5D_5N) chemical shifts of C-18 and the T_{50} values of compounds (I—VI); $\delta_{C\text{-}18} = 0.03\ T_{50} + 16.09$ ($r = 0.96$, $p < 0.01$).

Na^+, K^+-ATPase is generally recognized to be a receptor of cardiac glycosides,[10] and this has prompted many molecular-level studies on the relationship between chemical structure and biological activity of cardiac glycosides. Fullerton *et al.*[11] synthesized nine digitoxigenin analogues in order to examine the relationship between genin structure and Na^+, K^+-ATPase inhibitory activity. With the use of a multidisciplinary approach including X-ray crystallography and conformational energy calculations, they found a striking correlation between the position of the C-17 side group carbonyl oxygen and biological activity.

Table II. ^{13}C-NMR Chemical Shifts of I—VI

Carbon	I	II	III	IV	V	VI
C-4	121.6 (d)	121.6 (d)	121.6 (d)	121.6 (d)	121.6 (d)	121.5 (d)
C-5	146.8 (s)	146.9 (s)	146.9 (s)	146.9 (s)	147.0 (s)	147.1 (s)
C-13	48.7 (s)	49.1 (s)	48.9 (s)	47.5 (s)	47.4 (s)	47.4 (s)
C-14	84.2 (s)	84.0 (s)	83.8 (s)	83.9 (s)	84.1 (s)	84.7 (s)
C-15	32.8 (t)	32.8 (t)	32.9 (t)	32.4 (t)	32.3 (t)	32.7 (t)
C-16	29.4 (t)	26.0 (t)	26.0 (t)	29.9 (t)	29.7 (t)	29.7 (t)
C-17	51.3 (d)	56.5 (d)	53.6 (d)	51.3 (d)	53.6 (d)	52.9 (d)
C-18	17.2 (q)	16.4 (q)	16.3 (q)	16.2 (q)	16.4 (q)	16.7 (q)
C-19	19.0 (q)	19.0 (q)	19.0 (q)	19.0 (q)	19.0 (q)	19.0 (q)
C-20	123.4 (s)	140.6 (s)	121.6 (s)	34.9 (d)	35.3 (d)	43.1 (d)
C-21	149.4 (d)	69.9 (t)	138.4 (d)	73.5 (t)	71.6 (t)	63.5 (t)
C-22	147.5 (d)	120.1 (d)	21.0 (t)	21.5 (t)	24.3 (t)	23.2 (t)
C-23	115.3 (d)	31.8 (t)	29.0 (t)	24.1 (t)	26.6 (t)	33.3 (t)
C-24	162.2 (s)	171.2 (s)	169.4 (s)	172.4 (s)	172.6 (s)	173.8 (s)
$OCH_2\underline{CH_3}$						14.4 (q)
$O\underline{CH_2}CH_3$						60.2 (t)

Measured in C_5D_5N.

Table III. Biological Activities of I—VI

Compd.	pD_2[a]	pIC_{50}[b]	T_{50}[c]
I	7.61±0.14	7.44±0.02	32.99±2.19
II	6.18±0.04	7.02±0.04	15.21±2.08
III	5.81±0.12	7.26±0.04	7.13±1.51
IV	5.34±0.07	6.96±0.04	2.31±0.19
V	4.91±0.05	6.47±0.02	6.69±0.38
VI	5.02±0.06	5.54±0.03	22.42±1.96

a) pD_2 is the concentration of test compounds required for 50% of the maximum PIE in guinea-pig papillary muscles ($n=5$—6, mean±S.E.). *b*) pIC_{50} is the concentration of test compounds required for 50% of the maximum inhibition of the activity of an Na^+, K^+-ATPase preparation from dog kidney ($n=3$, mean±S.E.). *c*) T_{50} is the time (min) to the point of half-maximum PIE after addition of test compounds at the pD_2 concentration in guinea-pig papillary muscles ($n=5$, mean±S.E.).

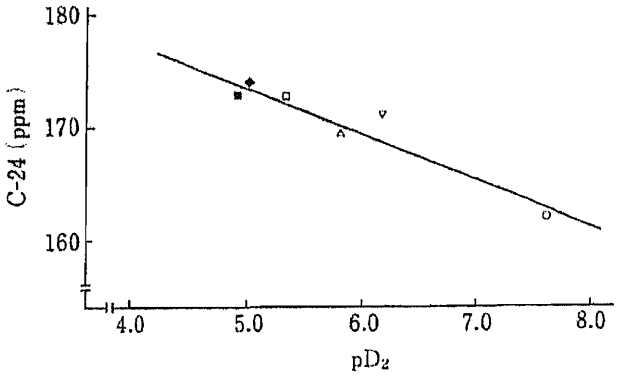


Fig. 4. Correlation between pD_2 Values and Chemical Shifts of Carbon-24 (C-24) in I—VI

○, I; ▽, II; △, III; □, IV; ■, V; ◆, VI. The pD_2 value of each compound is the mean value. The chemical shifts (C-24) of I—VI are those in the ^{13}C-NMR (C_5D_5N) spectra.

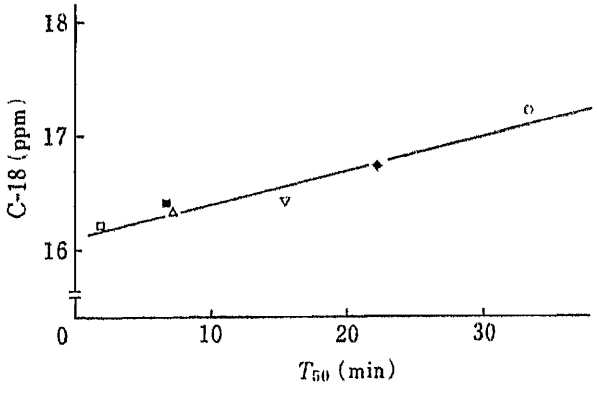


Fig. 5. Correlation between T_{50} Values and Chemical Shifts of Carbon-18 (C-18) in I—VI

○, I; ▽, II; △, III; □, IV; ■, V; ◆, VI. The T_{50} value of each compound is the mean value. The chemical shifts (C-18) of I—VI are those in the ^{13}C-NMR (C_5D_5N) spectra.

On the other hand, we employed the readily obtainable ^{13}C-NMR chemical shift value as a novel parameter for the elucidation of structure–activity relationships, since this method is applicable to non-crystalline compounds such as I—VI, which are not suitable for X-ray analysis, and further, the *in vivo* molecular conformation of the compound is more likely to be duplicated in the liquid medium than in the solid state.

First of all, we investigated the relationship between the cardiotonic activity (pD_2 value) on isolated myocardium in the guinea pig and the ^{13}C-NMR chemical shifts of carbonyl carbons in the lactone. There was an excellent correlation between PIE and the C-24 chemical shifts. This method could therefore be a convenient and useful means to predict the PIE potency of new compounds. Further application to other analogues of cardiac glycosides is under investigation.

A significant correlation was also obtained between the T_{50} values and the C-18 methyl chemical shifts. The steric interactions between the C-18 methyl group and lactone protons are presumed to be closely related to the differences in C-18 chemical shift and T_{50} value, but the chemical shift differences and NOE enhancements of the compounds are too small for us to conclude that the observed significant correlation resulted almost exclusively from the steric interactions.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. The MS were measured with a JEOL JMS DX-300 mass spectrometer. The ultraviolet (UV) spectra were recorded with a Shimadzu UV-210A spectrometer, the IR spectra with a JASCO IRA-2 spectrometer, and the CD spectra with a JASCO J-40S spectrometer. The optical rotations were taken with a JASCO DIP-140 automatic polarimeter. The ^{1}H- and 2D-NMR spectra were recorded with JEOL JNM-MH-100 and JEOL GX-400 spectrometers and the ^{13}C-NMR spectra with a JEOL JNM-FX 100 spectrometer in C_5D_5N using tetramethylsilane as an internal standard. The following abbreviations are used; s, singlet; d, doublet; dd, double doublet; ddd, double double-doublet; t, triplet; q, quartet; m, multiplet. The apparatus used for HPLC was a JASCO TWINCL high-performance liquid chromatograph equipped with a JASCO UVIDEC 100-III detector, and the eluate was monitored at 210 nm. HPLC was carried out on a Develosil ODS-10/20 column (0.76 cm i.d. × 25 cm, Nomura Chem. Co., Seto) at a flow rate of 2.0 ml/min.

Hydrogenation of Proscillaridin (I) — Proscillaridin (1.0 g) was added to a hydrogenation bottle containing 50 ml of EtOH and 1.5 g of 5% Pd–C catalyst. Proscillaridin was hydrogenated for 1.5 h at room temperature under atmospheric pressure. The catalyst was removed and the EtOH was evaporated off *in vacuo* to afford a residue (1.1 g).

Separation by HPLC — The above residue was dissolved in EtOH and subjected to HPLC. Elution with MeCN–H_2O (2:3) gave three fractions, 1 (675 mg), 2 (194 mg) and 3 (compound III, 86 mg). Fraction 1 was rechromatographed with MeCN–MeOH–H_2O (3:2:6) to give compound IV (277 mg) and compound V (331 mg). Similarly, fraction 2 was rechromatographed with MeCN–H_2O (1:2) to give compound II (97 mg) and compound VI (68 mg).

3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4,20,22-trienolide; Proscillaridin (I) — Colorless needles, mp 221—223 °C (MeOH) (lit.[12] mp 220—222 °C). $[\alpha]_D^{22}$ −65.6° (c=0.1, EtOH). UV λ_{max}^{EtOH} nm (log ε): 211 (3.88), 299 (3.75). IR ν_{max}^{KBr} cm^{-1}: 3480 (OH), 1725 (C=O). *Anal.* Calcd for $C_{30}H_{42}O_8$: C, 67.90; H, 7.98. Found: C, 67.78; H, 8.08. ^{1}H-NMR: Table I. ^{13}C-NMR Table II.

3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4,20(22)-dienolide (II)——Colorless needles, mp 216—221 °C (Me_2CO–Et_2O (1:1)). $[\alpha]_D^{22}$ −60.7° (c=0.1, EtOH). UV λ_{max}^{EtOH} nm (log ε): 212 (3.54), 300 (1.51). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1725 (C=O). MS *m/z*: 532 (M^+), 386 ($M^+ - C_6H_{10}O_4$), 368 ($M^+ - C_6H_{10}O_4 - H_2O$). *Anal.* Calcd for $C_{30}H_{44}O_8 \cdot 1/2H_2O$: C, 66.52; H, 8.37. Found: C, 66.42; H, 8.21. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4,20-dienolide (III)——Crystalline powder, mp 187—188 °C (Me_2CO–isopropyl ether (1:1)). $[\alpha]_D^{22}$ −23.2° (c=0.1, EtOH). UV λ_{max}^{EtOH} nm (log ε): 224 (3.72), 293 (2.42). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1745 (C=O). MS *m/z*: 532 (M^+), 386 ($M^+ - C_6H_{10}O_4$), 368 ($M^+ - C_6H_{10}O_4 - H_2O$). *Anal.* Calcd for $C_{30}H_{44}O_8 \cdot H_2O$: C, 65.43; H, 8.42. Found: C, 65.16; H, 8.47. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

20*R*-3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4-enolide (IV)——Colorless needles, mp 245—249 °C (Me_2CO–Et_2O (1:1)). $[\alpha]_D^{22}$ −41.1° (c=0.1, EtOH). CD (c=0.02, EtOH) $[\theta]^{20}$ (nm): $+1.9 \times 10^3$ (221) (positive maximum). UV λ_{max}^{EtOH} nm (log ε): 213 (2.94), 298 (1.84). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1720 (C=O). MS *m/z*: 388 ($M^+ - C_6H_{10}O_4$), 370 ($M^+ - C_6H_{10}O_4 - H_2O$). *Anal.* Calcd for $C_{30}H_{46}O_8 \cdot 1/2H_2O$: C, 66.27; H, 8.71. Found: C, 66.22; H, 8.44. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

20*S*-3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-14β-hydroxybufa-4-enolide (V)——Colorless needles, (mp 236—238 °C (Me_2CO). $[\alpha]_D^{22}$ −56.8° (c=0.1, EtOH). CD (c=0.02, EtOH) $[\theta]^{20}$ (nm): -1.0×10^4 (208) (negative maximum). UV λ_{max}^{EtOH} nm (log ε): 214 (2.96), 297 (2.46). IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 1725 (C=O). MS m/z: 388 ($M^+-C_6H_{10}O_4$), 370 ($M^+-C_6H_{10}O_4-H_2O$). *Anal.* Calcd for $C_{30}H_{46}O_8\cdot1/2H_2O$: C, 66.27; H, 8.71. Found: C, 66.04; H, 8.51. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

20*S*-Ethyl-3β-[(6-deoxy-α-L-mannopyranosyl)oxy]-14β,21-dihydroxychola-4-enoate (VI)——Crystalline powder, mp 189—191 °C (Me_2CO–isopropyl ether (1 : 1)). $[\alpha]_D^{22}$ −29.4° (c=0.1, EtOH). UV λ_{max}^{EtOH} nm (log ε): 210 (3.41), 295 (1.51). IR ν_{max}^{KBr} cm^{-1}: 3360 (OH), 1725 (C=O). MS m/z: 388 ($M^+-C_6H_{10}O_4-C_2H_5OH$), 370 ($M^+-C_6H_{10}O_4-C_2H_5OH-H_2O$). *Anal.* Calcd for $C_{32}H_{52}O_9\cdot1/2H_2O$: C, 65.17; H, 9.06. Found: C, 65.26; H, 8.75. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Biological Activity——PIE (pD_2, T_{50}, and pIC_{50} values) of test compounds were examined by the use of isolated guinea-pig papillary muscle preparations and Na^+, K^+-ATPase preparation from dog kidney, respectively. The measurements were performed according to the methods described in out previous paper.[1)]

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Stereoselectivity in Reduction of Steroidal 7-Ketones

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Several 7-ketones of lanostane, 4,4-dimethylcholestane and cholestane derivatives were subjected to catalytic hydrogenation on platinum, reduction with complex hydrides and reduction with sodium in *tert*-butanol, and the product ratios (7α-ol/7β-ol) were determined by gas chromatography or high-performance liquid chromatography. Catalytic hydrogenation of 3β-hydroxylanostan-7-one and 3β-hydroxy-4,4-dimethylcholestan-7-one yielded the 7β-alcohols as the major products, whereas their 3-acetates gave principally the 7α-alcohols. Reduction of various 7-ketones with sodium in *tert*-butanol gave mainly the 7β-equatorial alcohols, while the epimeric 7α-ols were the major products on reduction with lithium tri-*sec*-butylborohydride. The stereoselectivity of reduction with sodium borohydride and lithium aluminum hydride was highly dependent on the neighboring double bond and 4,4-dimethyl and/or 14α-methyl substituent(s).

Keywords— reduction; 7-oxygenated sterol; stereoselectivity; lithium tri-*sec*-butylborohydride; 3β-hydroxy-4,4-dimethylcholestan-7-one

In connection with another project concerning oxygenated sterols as inhibitors of cholesterol biosynthesis,[1] we have prepared various 7-oxygenated sterols. During these experiments, it was found that hydrogenation of 3β-hydroxylanostan-7-one (**1a**) on platinum in ethyl acetate containing acetic acid gave the 7β-ol (**3a**) as the major product, whereas under similar conditions the corresponding 3β-acetate (**1b**) was reported[2] to yield principally the 7α-ol (**2b**). These observations prompted us to investigate systematically the stereochemistry of steroidal 7-ketone reductions. Thus, several 7-ketones of cholestane, 4,4-dimethylcholestane and lanostane derivatives were subjected to catalytic hydrogenation, reduction with complex hydrides and reduction with sodium in *tert*-butanol, and the product ratios (7α-ol to 7β-ol) were determined by gas chromatography (GC) and/or high-performance liquid chromatography (HPLC) (Table I).

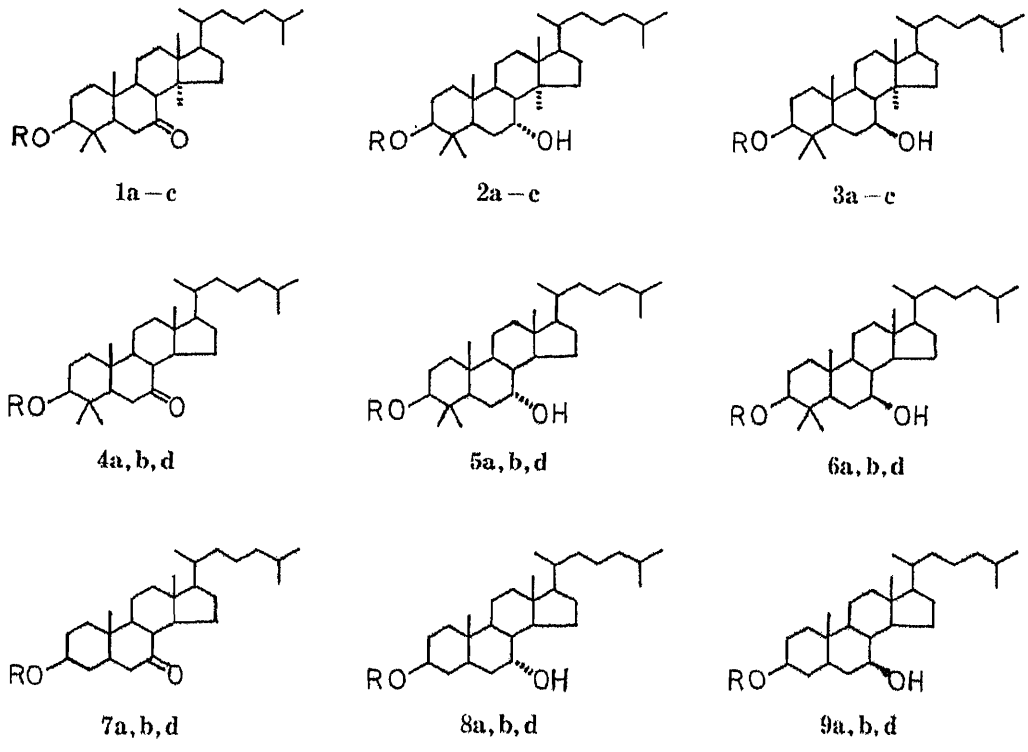
Preparation of the Substrate 7-Ketones

The cited methods were used for the preparation of 3β-hydroxylanotan-7-one (**1a**) and its acetate (**1b**),[2] 3β-hydroxylanost-8-en-7-one (**1c**),[3] 3β-hydroxycholestan-7-one (**7a**) and its acetate (**7b**),[4] and 3β-acetoxycholest-5-en-7-one (**7d**).[5] 3β-Acetoxy-4,4-dimethylcholest-5-en-7-one (**4d**) was prepared by Collins oxidation of 3β-acetoxy-4,4-dimethylcholest-5-ene.[6] The enone (**4d**) was hydrogenated on platinum oxide, and then treated with pyridinium chlorochromate to reoxidize the partially produced 7-hydroxyl moiety. The resulting ketoacetate (**4b**) was saponified to yield 3β-hydroxy-4,4-dimethylcholestan-7-one (**4a**).[7]

Catalytic Hydrogenation

First we repeated the reported hydrogenation[2] of 3β-acetoxylanostan-7-one (**1b**). The crude product was saponified and treated with trimethylsilylimidazole to give the trimethylsilyl (TMS) ethers. Gas chromatography–mass spectrometry (GC-MS) of this sample revealed it to be a mixture of the 7α-ol (**2a**) and 7β-ol (**3a**) in a ratio of 73 : 27, together with an

unidentified compound appearing at a shorter retention time on GC. In similar catalytic hydrogenation, the 3β-alcohol (**1a**) yielded the 7α-ol and the 7β-ol in a ratio of 21:79. Identification of the two alcohols (**2a** and **3a**) was based on a comparison of the GC-MS data with those of authentic samples, which were prepared by the reported methods.[2] Other 7-epimeric alcohols described below were also identified in the same manner. It was thus established that the stereoselectivity of the 7-ketone hydrogenation is reversed depending on the nature of the 3β-substituent, although the reason for this remains obscure. This reversal of stereoselectivity was also observed with 4,4-dimethylcholestane derivatives: the 7α-ol (**5b**) or the 7β-ol (**6a**) was the major hydrogenation product from the 3β-acetoxy-7-ketone (**4b**) or the 3β-hydroxy-7-ketone (**4a**), respectively. In contrast, both 7-oxocholestan-3β-ol (**7a**) and its acetate (**7b**) gave the 7α-ol and 7β-ol in an almost 1:1 ratio, although these results appear to be somewhat different from the data reported a few decades ago.[4,8] Thus, the 4,4-dimethyl group seems to be an essential factor for the reversal of stereoselectivity in the 7-ketone reduction. Analogous catalytic hydrogenation of the $\Delta^{8(9)}$-lanostan-7-one derivative (**1c**) produced the $\Delta^{7,9(11)}$-diene and $\Delta^{8(9)}$-olefin, in accord with the earlier results.[9]



1a–c **2a–c** **3a–c**

4a,b,d **5a,b,d** **6a,b,d**

7a,b,d **8a,b,d** **9a,b,d**

a: R=H **b**: R=Ac **c**: R=Ac, $\Delta^{8(9)}$ **d**: R=Ac, $\Delta^{5(6)}$

Chart

Reduction with Sodium in *tert*-Butanol

It is accepted[10,11] that reduction of steroidal ketones by alkali metals gives the thermodynamically more stable equatorial alcohol. Indeed, the results presented in Table I together with the literature data[11] indicate that the 7β-equatorial alcohols, *e.g.* **3a**, **6a** and **9a**, were the sole or the major products on reduction with sodium in *tert*-butanol. The predominance of 7β-ol over 7α-ol appears to increase with methyl substitution(s) at the 14 and 4 positions, which would sterically interfere with the 7α-hydroxyl moiety and destabilize the 7α-axial alcohols.

TABLE I. Stereoselectivity in Reductions of Steroidal 7-Ketones

Substrate	Alcohols produced on reaction with				
	H_2/Pt 7α:7β	Na/*tert*-BuOH 7α:7β	$NaBH_4$ 7α:7β	$LiAlH_4$ 7α:7β	L-Selectride 7α:7β
1a	21:79	0:100	47:53	38:62	100:0
1b	73:27	—	36:64	48:52	—
1c	*a*)	—	62:38	71:29	*b*)
4a	21:79	14:86	67:33	47:53	99:1
4b	61:39	—	—	—	—
4d	—	—	8:92	13:87	57:43
7a	51:49	27:73	61:39	51:49	99:1
7b	43:57	—	—	—	—
7d	—	—	18:82	37:63	76:24

a) This reaction gave no 7-hydroxyl compound but yielded the $\Delta^{7,9(11)}$-diene and the $\Delta^{8(9)}$-olefin. However, see Added in Proof. *b*) The starting ketone was recovered.

Reduction with Complex Hydride

As indicated in Table I, the stereochemistry of reduction of steroidal 7-ketones with complex hydrides is highly dependent on the reagent used (sodium borohydride, lithium aluminum hydride and lithium tri-*sec*-butylborohydride) and the neighboring groups (double bond and 4- or 14-methyl group) of the substrate 7-ketones.[7,10,12] Approach of the hydride reagent from the β-side of the 7-ketones appears to be hindered by the 10- and 13-methyl groups. Accordingly, the 7β-alcohol, which is also the thermodynamically more stable epimer (*vide supra*), will be preferentially produced. Nevertheless, sodium borohydride reduction of 3β-hydroxycolestan-7-one (**7a**) gave the 7α-ol (**8a**) and 7β-ol (**9a**) in a ratio of 61 : 39. Similar reduction with lithium aluminum hydride increased the amount of the thermodynamically controlled product, *i.e.*, the 7β-ol (**9a**), as expected from the established rationalization.[10] Introduction of the $\Delta^{5(6)}$-double bond into the 7-ketone removes the 5α-axial hydrogen, and accordingly the approach of the reagent from the α-side of the Δ^5-7-ketone (**7d**) may be facilitated to yield a higher amount of the 7β-ol (**9d**). In contrast, introduction of the 4,4-dimethyl group into the 7-ketone did not appreciably affect the stereoselectivity of 7-ketone reduction, as shown by the fact that the product ratio of the 7α-ol (**5a**) to the 7β-ol (**6a**) was almost the same as in the above-mentioned reduction of the desmethyl analog (**7a**). Introduction of the Δ^5-double bond again increased the 7α-ol/7β-ol ratio. The influence of the 14α-methyl group on complex hydride reduction appears to be mainly thermodynamic, because compared with the 14α-H analogs (**4a** and **7a**), 3β-hydroxylanostan-7-one (**1a**) gave a lower 7α-ol/7β-ol ratio despite the α-side attack of the reagent being highly hindered by the 14α-methyl group. Introduction of the $\Delta^{8(9)}$-double bond into the lanostan-7-one nucleus also has a pronounced effect on its reduction. Thus, the 7-oxo-8(9)-ene (**1c**) was quite resistant to reduction with sodium borohydride, and HPLC analysis of the 7-epimeric alcohols produced in low yield revealed the presence of the 7α-ol (**2c**) and the 7β-ol (**2d**) in a ratio of 62 : 38.[9] Analogous reduction of **1c** with lithium aluminum hydride gave **2c** and **2d** in a ratio of 71 : 29. Flattening of the molecule caused by introduction of the $\Delta^{8(9)}$-double bond would partially relieve the steric interaction between the 7α-hydroxyl group and the 14α-methyl group, and the β-side approach of the hydride reagent would be facilitated by the absence of the 8β-axial hydrogen.

Lithium tri-*sec*-butylborohydride (L-Selectride, Aldrich) is a hydride reagent that selectively yields the axial alcohol.[13] In the present experiments, the selectivity for the 7α-axial alcohol was found to be almost complete in the reduction of the three saturated 7-ketones (**1a**,

4a and **7a**). The axial nature of the 7α-hydroxyl group of the unsaturated compounds (**5d** and **8d**) is decreased, especially in the 4,4-dimethylated compound (**5d**), leading to the formation of a significant amount of the 7β-ol (**6d**). L-Selectride failed to reduce the highly unreactive (*vide supra*) unsaturated ketone (**1c**). It should finally be pointed out that reduction of 3β-acetoxycholest-5-en-7-one (**7d**) with L-Selectride followed by saponification afforded 7α-hydroxycholesterol in 73% yield, and this method seems to be most convenient to prepare this biologically important sterol.[14]

Experimental

Melting points were measured on a micro melting point apparatus (Yanako) and are uncorrected. Ultraviolet (UV) absorption spectra were taken with a Hitachi EPS-3T spectrometer for solutions in methanol. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL JNM-GX270 (270 MHz) spectrometer for solutions in deuterochloroform. Chemical shifts (δ) are given in ppm from internal tetramethylsilane. GC were performed on a Shimadzu GC-8A gas chromatograph using a 1.5% OV-17 column (1m) at 260 °C or a 1.5% OV-1 column (2 m) at 270 °C. HPLC were performed on a Shimadzu LC-8A liquid chromatograph using a Zorbax ODS column (4.6 mm i.d. × 15 cm; detector setting, UV 220 nm) with methanol as the solvent at a flow rate of 1.0 ml/min. Mass spectra (MS) were taken with a JEOL JMS D-100 mass spectrometer using a direct inlet system or a Shimadzu 9020-DF spectrometer using a GC-MS system. Column chromatography was performed with Kieselgel 60 (Merck, 70—230 mesh). Thin layer chromatography (TLC) was carried out with precoated Kieselgel 60 F_{254} plates (Merck, 0.25 mm thick). The usual work-up refers to dilution with brine, extraction and evaporation under vacuum. The samples (*ca.* 0.5 mg) to be analyzed by GC were derivatized to TMS ethers by heating with trimethylsilylimidazole (50 μl) in a sealed tube at 80 °C for 1 min, and a 1-μl portion of the resultant solution was injected into the GC column. Relative retention times (rt_R) on 1.5% OV-1 of the TMS ethers of standard samples: cholesterol, 1.00; 7-one **1a**, 2.35; 7α-ol **2a**, 2.23; 7β-ol **3a**, 2.02; 7-one **4a**, 2.19; 7α-ol **5a**, 1.48; 7β-ol **6a**, 1.78; 7-one **7a**, 1.61; 7α-ol **8a**, 1.04; 7β-ol **9a**, 1.41; 7-one **7d**, 1.69; 7α-ol **8d**, 0.95; 7β-ol **9d**, 1.05.

3β-Acetoxy-4,4-dimethylcholest-5-en-7-one (4d)——4,4-Dimethylcholest-5-en-3β-ol (1.5 g)[6] was treated with pyridine (4 ml)–acetic anhydride (2 ml) to give the acetate (1.6 g), mp 152—154 °C (from methanol). ^{1}H-NMR δ: 0.67 (3H, s, 13-Me), 0.86 (6H, d, J=6.6 Hz, 25-Me_2), 0.91 (3H, d, J=6.6 Hz, 20-Me), 1.07, 1.08, 1.14 (each 3H, s, 4-, 4-, 10-Me), 3.25 (1H, m, 3-H), 5.56 (1H, m, 6-H). A solution of this acetate (1.5 g) in dry dichloromethane (16 ml) was added to a stirred mixture of CrO_3 (8 g), pyridine (6.4 g), P_2O_5 (2.4 g) and dichloromethane (180 ml). This mixture was refluxed for 2 h, and then filtered through a Florisil column with dichloromethane–ethyl acetate (1 : 1) to give the crude 7-ketone (1.5 g). Recrystallization from methanol afforded **4d**, mp 142—143 °C. ^{1}H-NMR δ: 0.67 (3H, s, 13-Me), 0.86, 0.87 (each 3H, d, J=6.2 Hz, 25-Me_2), 0.92 (3H, d, J=6.5 Hz, 20-Me), 1.11, 1.21, 1.27 (each 3H, s, 4-, 4-, 10-Me), 2.08 (3H, s, 3-OAc), 4.60 (1H, m, 3-H), 5.92 (1H, s, 6-H).

3β-Acetoxy-4,4-dimethylcholestan-7-one (4b)——The enone (**4d**, 570 mg) was stirred with platinum oxide (50 mg) in acetic acid (40 ml) under hydrogen at 30 °C for 18 h. The catalyst was filtered off and the filtrate was evaporated to dryness. TLC of the residue indicated the presence of three components. A solution of this crude product (510 mg) in dry dichloromethane (7 ml) was added in one portion to a suspension of pyridinium chlorochromate (1.0 g) in dry dichloromethane (40 ml). The mixture was stirred for 1 h. The two polar components (7α- and 7β-hydroxyl compounds) were no longer detectable on TLC. The mixture was filtered through a Florisil column with dichloromethane–ethyl acetate (1 : 1) to give an amorphous material (0.50 g), and recrystallization from methanol–dichloromethane gave **4b**, mp 176—178 °C. ^{1}H-NMR δ: 0.64 (3H, s, 13-Me), 0.84, 0.92, 1.14 (each 3H, s, 4-, 4-, 10-Me), 2.06 (3H, s, 3-OAc), 4.48 (1H, m, 3-H).

3β-Hydroxy-4,4-dimethylcholestan-7-one (4a)——The ketone (**4b**, 450 mg) in 5% KOH–methanol (5 ml) was refluxed for 1 h. The usual work-up followed by column chromatography on silica gel with benzene–ethyl acetate (10 : 1) gave **4a** (147 mg), mp 198—199 °C (from methanol). ^{1}H-NMR δ: 0.64 (3H, s, 13-Me), 0.85, 0.96, 1.12 (each 3H, s, 4-, 4-, 10-Me), 3.25 (1H, m, 3-H).

Catalytic Hydrogenation——3β-Hydroxylanostan-7-one (**1a**, 20 mg)[2a] in 1 : 1 ethyl acetate–acetic acid (2 ml) was stirred with platinum oxide (3 mg) under hydrogen for 30 h. The catalyst was filtered off and the usual work-up using ethyl acetate for extraction gave a crude material (22 mg). After trimethylsilylation, three peaks of TMS ethers were found on GC (270 °C) at retention times of 6.9, 8.0 and 12.1 min. The retention times and MS coincided with those of authentic samples[2a] of lanostan-3β,7β-diol bis TMS ether (*m/z*: 590, 575, 500, 485, 450, 411, 395, 371, 366), lanostan-3β,7α-diol bis TMS ether (*m/z*: 590, 500, 485, 450, 411, 395, 371, 366) and 3β-hydroxylanostan-7-one TMS ether (*m/z*: 516, 501, 426, 387, 294, 129), respectively.

3β-Acetoxylanostan-7-one (**1b**, 20 mg)[2] was similarly hydrogenated and the product was refluxed with 5% KOH–methanol (4 ml) for 1 h. After TMS derivatization of the saponified product, four peaks were seen on GC and GC-MS at retention times of 6.2 min (*m/z*: 588, 573, 498, 483, 417, unidentified compound), 6.9 min (3β,7β-diol bis TMS ether), 8.0 min (3β,7α-diol bis TMS ether) and 12.1 min (7-oxo-3β-ol TMS ether).

3β-Acetoxylanost-8-en-7-one (**1c**, 20 mg)[3] was similarly hydrogenated. The products were less polar than **1c** on TLC and showed ^{1}H-NMR δ: 0.56 (3H, s, 13-Me), 5.32, 5.46 (each 1H, 7- and 11-H), 4.50 (1H, m, 3-H) together with 0.69 (3H, s, 13-Me of 3β-acetoxylanost-8-ene). UV λ_{max}: 237, 245, 253 nm. MS *m/z*: 470, 468, 455 and 453.

Other 7-ketones (**4a**, **4b**, **7a** and **7b**) were similarly hydrogenated (and then saponified in the cases of **4b** and **7b**) and the product TMS ethers were analyzed by GC.

Reduction with Sodium in *tert*-Butanol—— Sodium (50 mg) was added to a solution of 3β-hydroxylanostan-7-one (**1a**, 10 mg) in dry *tert*-butanol (2 ml) at 80 °C. The mixture was refluxed for 2 h, and methanol (*ca.* 1 ml) was added to decompose excess sodium. The usual work-up using dichloromethane for extraction gave a crude product (11 mg), which after TMS derivatization, showed two peaks on GC at rt_R 2.02 (3β,7β-diol bis TMS ether) and 2.35 (7-oxo-3β-ol TMS ether). The crude reaction product was recrystallized from methanol to give the 3β,7β-diol (**3a**), mp 209—211 °C (lit.[2a] 204—206 °C). ^{1}H-NMR δ: 0.78 (3H, s, 13-Me), 0.81, 0.88, 0.92, 0.98 (each 3H, s, 4-, 4-, 10-, 14-Me), 3.22 (1H, m, 3-H), 3.61 (1H, m, 7-H).

3β-Hydroxy-4,4-dimethylcholestan-7-one (**4a**) was similarly treated with sodium in *tert*-butanol and the product, after TMS derivatization, was analyzed by GC. The crude reduction product was chromatographed on silica gel to give the ketone (**4a**) and the 3β,7β-diol (**6a**), mp 182—185 °C (from methanol), ^{1}H-NMR δ: 0.67 (3H, s, 13-Me), 0.81, 0.89, 0.98 (each 3H, s, 4-, 4-, 10-Me), 3.23 (1H, m, 3-H), 3.33 (1H, m, 7-H).

3β-Hydroxycholestan-7-one (**7a**) was similarly treated with sodium in *tert*-butanol and the product, after TMS derivatization was analyzed by GC. The crude reduction product showed ^{1}H-NMR δ: 0.68 (3H, s, 13-Me), 0.83 (3H, s, 10-Me), 3.37 (1H, m, 7-H), 3.60 (1H, m, 3-H).

Reduction with Sodium Borohydride——3β-Hydroxylanostan-7-one (**1a**, 22 mg) in methanol (7 ml) was stirred with sodium borohydride (30 mg) for 1 h. The usual work-up using ethyl acetate for extraction gave a crude product, which after TMS derivatization, showed two peaks on GC at rt_R 2.02 (3β,7β-diol bis TMS ether) and 2.23 (3β,7α-diol TMS ether).

Similarly, 3β-acetoxylanostan-7-one (**1b**, 4 mg) in a mixture of methanol (0.5 ml) and tetrahydrofuran (0.3 ml) was stirred with sodium borohydride (8 mg) for 20 h, and then the product was heated in 5% KOH–methanol (0.8 ml) at 65 °C for 30 min. After TMS derivatization of the saponified product, two peaks were seen on GC at rt_R 2.02 (3β,7β-diol bis TMS ether) and 2.23 (3β,7α-diol bis TMS ether).

Similarly, 3β-acetoxylanost-8-en-7-one (**1c**, 100 mg) in a mixture of methanol (10 ml) and tetrahydrofuran (4 ml) was treated with sodium borohydride (350 mg) for 19 h. The product showed four peaks on HPLC at 5.7 min (**3c**, 7β-OH), 6.8 min (**1c**, 7-oxo), 7.3 min (**2c**, 7α-OH) and 23 min (3β-acetoxylanosta-7,9(11)-diene). The column-chromatographically isolated **2c** and **3c** showed the following ^{1}H-NMR signals: **2c**, 0.61 (3H, s, 13-Me), 0.90, 0.92, 0.98, 1.03 (each 3H, s, 4-, 4-, 10-, 14-Me), 4.20 (1H, m, 7-H), 4.56 (1H, m, 3-H); **3c**, 0.77 (3H, s, 13-Me), 0.88, 0.90, 0.92, 1.10 (each 3H, 4-, 4-, 10-, 14-Me), 4.36 (1H, m, 7-H), 4.47 (1H, m, 3-H).

Other 7-ketones (**4a**, **4d**, **7a** and **7d**) were similarly reduced with sodium borohydride (and then saponified in the cases of **4d** and **7d**), and the TMS ethers were analyzed by GC.

Reduction with Lithium Aluminum Hydride——3β-Hydroxylanostan-7-one (**1a**, 22 mg) in tetrahydrofuran (1.5 ml) was stirred with lithium aluminum hydride (10 mg) for 1 h. The usual work-up gave a crude product, which after TMS derivatization, showed two peaks on GC at rt_R 2.02 (3β,7β-diol bis TMS ether) and 2.23 (3β,7α-diol bis TMS ether).

3β-Acetoxylanost-8-en-7-one (**1c**, 24 mg) was similarly treated with lithium aluminum hydride (50 mg). The product showed three peaks on HPLC at retention times of 8.4 min (3β,7β-diol), 10.2 min (3β,7α-diol) and 25.8 min (lanosta-7,9(11)-dien-3β-ol).

Other 7-ketones (**1b**, **4a**, **4d**, **7a** and **7d**) were similarly reduced with lithium aluminum hydride and the TMS ethers were analyzed by GC.

Reduction with L-Selectride——3β-Hydroxylanostan-7-one (**1a**, 22 mg) in dry tetrahydrofuran (2 ml) was stirred with L-Selectride (1 M tetrahydrofuran solution, Aldrich, 150 μl) at 0 °C for 4 h under nitrogen. The usual work-up using dichloromethane for extraction gave a crude product, which after TMS derivatization, showed two peaks on GC at rt_R 2.23 (3β,7α-diol bis TMS ether) and 2.35 (3β-hydroxy-7-one TMS ether). The reduction product was chromatographed on silica gel with *n*-hexane–ethyl acetate (20 : 1) to give **2a** (15 mg, 68%), mp 157—158 °C (from methanol, lit.[2a] 163—165 C). ^{1}H-NMR δ: 0.74 (3H, s, 13-Me), 0.81, 0.93, 0.97, 1.08 (each 3H, s, 4-, 4-, 10-, 14-Me), 4.06 (1H, m, 7-H), 3.27 (1H, m, 3-H).

Other 7-ketones (**4a**, **4d**, **7a** and **7d**) were similarly reduced with L-Selectride (and then saponified in the cases of **4d** and **7d**) and the products TMS ethers were analyzed by GC.

Similar treatment of 3β-acetoxylanost-8-en-7-one (**1c**, 9.7 mg) with L-Selectride (100 μl) in tetrahydrofuran (0.5 ml) at room temperature for 17 h gave the recovered ketone (**1c**).

Preparation of 7α-Hydroxycholesterol——L-Selectride solution (1 M tetrahydrofuran, Aldrich, 1.8 ml) was added to an ice-cooled solution of the 3β-acetoxycholest-5-en-7-one (**7d**, 200 mg) in dry tetrahydrofuran (6 ml) under nitrogen. The mixture was stirred for 1.5 h, then 5% KOH–methanol (5 ml) was added and the whole was refluxed for 0.5 h. Work-up as usual and crystallization from methanol–CH_2Cl_2 gave 3β,7α-dihydroxycholest-5-ene (77 mg, mp 176—178 °C, ref.15, 176—178 °C). Column chromatography of the mother liquor afforded a further crop of 7α-

hydroxycholesterol (54 mg). Total yield of 7α-hydroxycholesterol: 132 mg (73%).

Added in Proof (February 20, 1987) Very recently, Professor Y. Sato and his coworkers obtained, on similar reduction of **1c**, 3β-acetoxylanostan-7α-ol (**2c**) in 26% yield in addition to the olefinic products. Y. Sonoda, Y. Tanoue, M. Yamaguchi and Y. Sato, *Chem. Pharm. Bull.*, **35**, 394 (1987).

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Chemical Studies on the Constituents of the Thymelaeaceous Plants. III.[1] Structure of a Novel Spiro Biflavonoid, Daphnodorin C, from *Daphne odora* THUNB.

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The structure of a novel spiro biflavonoid, daphnodorin C (3), previously isolated from the root and bark of *Daphne odora* THUNB., was established on the basis of chemical and spectral studies, and confirmed by an X-ray diffraction analysis.

Keywords——*Daphne odora*; Thymelaeaceae; daphnodorin C; flavan; biflavonoid; ruixianggen; biflavonoid X-ray analysis

In the course of our studies of the phenolic components of Thymelaeaceous plants, we investigated the constituents of the root (ruixianggen) and the bark of *Daphne odora* THUNB. In previous papers, we had reported the isolation of three new flavans, daphnodorin A (1), daphnodorin B (2) and daphnodorin C (3), together with four known coumarins,[2] and elucidated the structures of 1 and 2.[1,3] This paper deals with the structure of compound 3.[4]

Daphnodorin C (3), $C_{30}H_{22}O_9$, pale yellow crystals, mp 233—235 °C (dec.), gave a reddish brown coloration with Gibb's reagent and a dark brown coloration with $FeCl_3$ reagent. The optical rotatory dispersion (ORD) spectrum of 3 (dioxane) showed a negative Cotton effect [−7211.3° (342) (trough), 0° (332), +5558.7° (316) (peak)]. The circular dichroism (CD) spectrum of 3 showed negative Cotton effects at 333 (Δ_ε −11.3) and 280 (Δ_ε −14.9), and positive Cotton effects at 303 (Δ_ε +4.1) and 243 (Δ_ε +1.9). The ultraviolet (UV) spectrum of 3 (dioxane) showed absorption maxima at 222, 283 and 322 nm, and the infrared (IR) spectrum of 3 showed absorption bands at 3450, 3250, 1680, 1640, 1620 and 1530 cm^{-1}. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of 3 (δ, dimethylsulfoxide-d_6 (DMSO-d_6)) (Table I) exhibited two pairs of A_2X_2 signals, suggesting the presence of two 4-oxyphenyl groups, at 7.04 and 6.68 (each 2H, br d, J_{AX} = 8.8 Hz) and 6.85 and 6.59 (each 2H, br d, J_{AX} = 8.8 Hz), signals due to two aromatic protons situated in meta positions at 5.83 and 5.52 (each 1H, d, J = 1.7 Hz), a signal due to a benzylmethine proton at 5.47 (1H, s), and signals due to five phenolic hydroxyl groups at 9.25—10.63. Further, the signals due to protons at the C-6 (or C-8), C-2, C-3 and C-4 positions of a 2,8(or 6)-disubstituted 5,7-dioxy-3,4-dihydrobenzopyran ring were observed at 6.10 (1H, s), 4.78 (1H, br d, J = 10.4 Hz), 2.27 (1H, m), 1.55 (1H, m) and 2.55 (2H, m), respectively. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of 3 (δ, DMSO-d_6) (Table I) showed signals arising from twenty five sp^2 carbons [>C=O, =C–O ×8, =C– ×5, =CH– ×11] and five sp^3 carbons [–C–, –CH– ×2, $-CH_2-$ ×2].

From the above spectral data, it was considered that 3 is a flavan derivative similar to 1 and has the partial structures shown in Fig. 1. The methylation of 3 with diazomethane afforded a pentamethyl ether (4) and the acetylation of 3 with acetic anhydride and pyridine afforded a pentaacetate (5). Compound 4 was decomposed upon heating in AcOH with

TABLE I. NMR Data for Daphnodorin C (in DMSO-d_6)

	Chemical shifts (δ, ppm)			
	Carbon	J_{CH} (Hz)	Proton	J (Hz)
2	75.89	145.8	4.78 d	10.4
3	19.29	130.1	1.55 m, 2.27 m	
4	28.25	128.7	2.55 m	
4a	102.66[a]	6.0		
5	160.80	3.0		
6	89.42	162.6	6.10 s	
7	172.42	2.9		
8	102.57	6.0		
8a	152.40	6.0		
		3.0		
1′	131.14	7.3		
		3.7		
2′,6′	126.06	159.1	6.85 m	8.8 (J_{AX})
		7.3		
		2.2		
3′,5′	114.84	157.7	6.59 m	8.8 (J_{AX})
4′	157.65[b]	6.0		
		3.0		
2″	90.87	150.8	5.47 s	
		5.1		
3″	93.91	1.5		
4″	193.30	0.18 (half width)		
4a″	102.78[a]	6.0		
5″	159.10[c]	3.0		
		3.0		
6″	96.10	160.4	5.83 d	1.7
7″	157.71[c]	2.4		
		2.4		
8″	89.37	162.6	5.52 d	1.7
8a″	168.00	2.9		
		2.9		
9″	123.42	7.3		
		3.7		
10″,14″	129.00	159.4	7.04 m	8.8 (J_{AX})
		7.3		
		4.4		
11″,13″	114.84	157.7	6.68 m	8.8 (J_{AX})
12″	156.26[b]	6.0		
		3.0		
OH			10.63	
			10.42	
			9.88	
			9.50	
			9.25	

a—*c*) Assignments may be reversed. Assignments based on C/H two-dimensional correlations and an LSPD experiment.

H_2SO_4 to give four degradation products (**7**—**10**), which were identical with 4-methoxybenzoic acid (**7**), 3,5-dimethoxyphenol (**8**), 2-hydroxy-4,6-dimethoxybenzoic acid (**9**), and a furanoflavan derivative (**10**) prepared by the cleavage of daphnodorin A pentamethyl ether (**6**) in the same way. Upon alkaline hydrolysis, **4** afforded **7**, **8**, **9** and a carboxylic acid (**11**). The

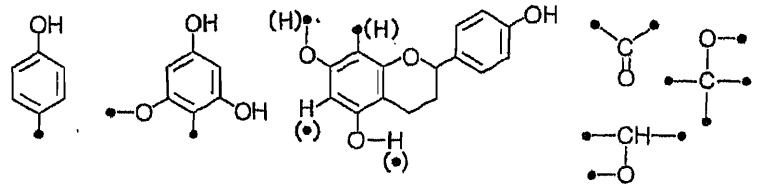


Fig. 1

^{1}H-NMR spectrum of **11** (δ, DMSO-d_6, ppm) showed signals due to two methoxyl groups at 3.75 and 3.67 (each 3H, s), signals due to a carboxyl group and a phenolic hydroxyl group at 8.96 (1H, br) and 5.66 (1H, s) and signals due to an adjacent methylene to carbonyl group at 3.40 and 3.33 (each 1H, d, J=16.0 Hz), in addition to signals assignable to an 8-monosubstituted 5,7,4′-trioxyflavan skeleton. From the above spectral data, it appeared that **11** is an 8-carboxymethyl-7-hydroxy-5,4′-dimethoxyflavan. Upon heating in EtOH with a small quantity of H_2SO_4, **3** and **4** gave daphnodorin A (**1**) and daphnodorin A pentamethyl ether (**6**), respectively.

The reduction of **4** with sodium borohydride in ethanol–tetrahydrofuran gave an alcohol (**12**), $C_{35}H_{34}O_9$, M^+ 598.2219, mp 127—129 °C, whose ^{1}H-NMR spectrum (δ, $CDCl_3+D_2O$) showed a sharp singlet signal due to a methine proton formed during the reduction of the carbonyl group (C-4″) at 5.37 ppm. From this result, it was concluded that there is no methine group adjacent to the position of the carbonyl group. A ^{13}C-NMR long-range

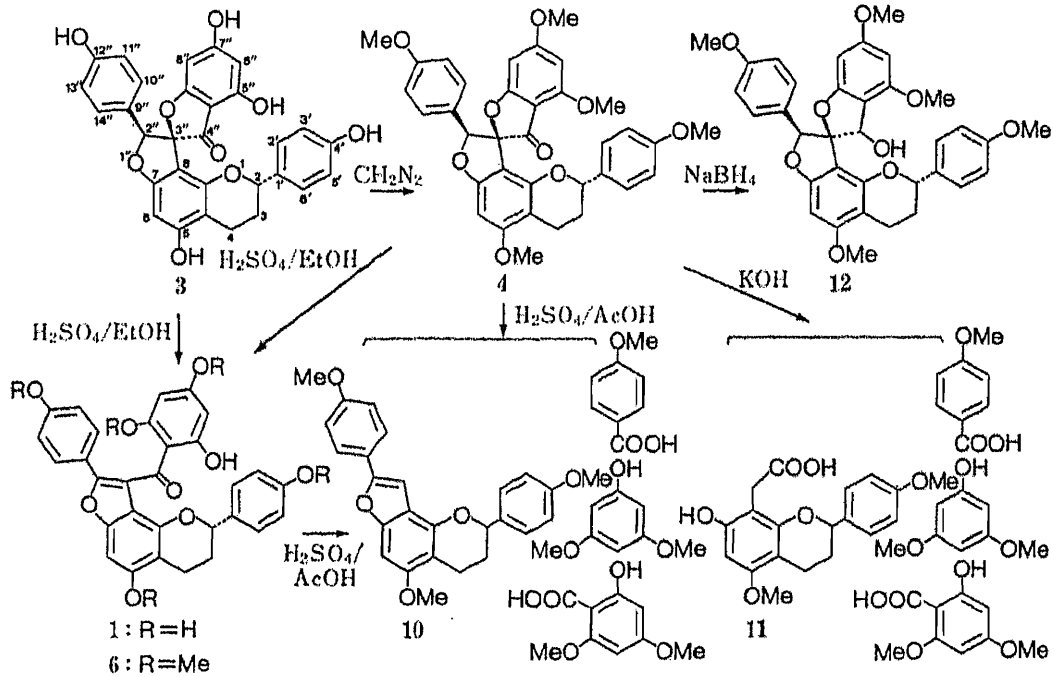


Chart 1

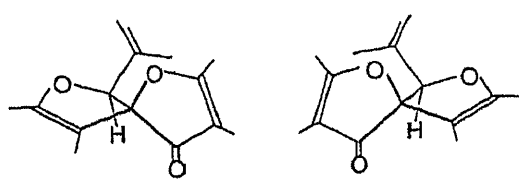


Fig. 2

TABLE II. Atomic Parameters of Nonhydrogen Atoms for Daphnodorin C with Anisotropic Temperature Factors

Atom	x	y	z	B_{eq}
O1	0.8430 (2)	0.5917 (5)	0.1137 (2)	2.6 (1)
C2	0.8747 (2)	0.5361 (6)	0.0619 (2)	2.3 (1)
C3	0.8295 (2)	0.6227 (6)	−0.0305 (3)	2.6 (1)
C4	0.7412 (2)	0.5709 (7)	−0.0885 (2)	2.8 (1)
C5	0.6266 (2)	0.5740 (6)	−0.0717 (2)	2.5 (1)
C6	0.5947 (2)	0.6007 (6)	−0.0236 (3)	2.6 (1)
C7	0.6493 (2)	0.6220 (6)	0.0706 (3)	2.4 (1)
C8	0.7317 (2)	0.6115 (6)	0.1161 (3)	2.3 (1)
C9	0.7628 (2)	0.5905 (6)	0.0655 (2)	2.2 (1)
C10	0.7093 (2)	0.5755 (6)	−0.0310 (2)	2.3 (1)
O11	0.5725 (2)	0.5505 (6)	−0.1651 (2)	3.7 (1)
C1′	0.9621 (2)	0.5726 (6)	0.1244 (3)	2.5 (1)
C2′	1.0113 (3)	0.4908 (7)	0.2104 (3)	3.0 (1)
C3′	1.0938 (3)	0.5241 (7)	0.2703 (3)	3.1 (1)
C4′	1.1278 (2)	0.6399 (7)	0.2439 (3)	2.9 (1)
C5′	1.0828 (3)	0.7180 (7)	0.1613 (3)	2.9 (1)
C6′	1.0006 (2)	0.6885 (7)	0.1007 (3)	2.6 (1)
O7′	1.2089 (2)	0.6683 (6)	0.3045 (2)	3.7 (1)
O1″	0.6301 (2)	0.6496 (5)	0.1311 (2)	2.48 (9)
C2″	0.7047 (2)	0.6943 (6)	0.2197 (3)	2.2 (1)
C3″	0.7717 (2)	0.6087 (6)	0.2188 (2)	2.1 (1)
C4″	0.8546 (2)	0.6943 (6)	0.2794 (2)	2.2 (1)
C5″	0.9097 (2)	0.5579 (6)	0.3341 (2)	2.2 (1)
C6″	0.9931 (2)	0.5573 (6)	0.3952 (2)	2.4 (1)
C7″	1.0325 (2)	0.4002 (6)	0.4294 (3)	2.6 (1)
C8″	0.9868 (2)	0.2459 (6)	0.4035 (3)	2.3 (1)
C9″	0.9032 (2)	0.2453 (6)	0.3457 (3)	2.3 (1)
C10″	0.8673 (2)	0.4020 (6)	0.3127 (2)	2.1 (1)
C11″	0.7010 (2)	0.6501 (6)	0.2973 (3)	2.5 (1)
C12″	0.7655 (2)	0.6878 (7)	0.3876 (3)	2.8 (1)
C13″	0.7605 (2)	0.6627 (7)	0.4613 (2)	2.8 (1)
C14″	0.6906 (2)	0.6004 (7)	0.4453 (2)	2.5 (1)
C15″	0.6254 (2)	0.5565 (7)	0.3536 (3)	3.0 (1)
C16″	0.6317 (2)	0.5834 (7)	0.2829 (3)	2.8 (1)
O17″	0.8684 (2)	0.8477 (5)	0.2775 (2)	3.0 (1)
O18″	1.0357 (2)	0.7053 (5)	0.4199 (2)	3.4 (1)
O19″	1.0219 (2)	0.0897 (5)	0.4346 (2)	2.9 (1)
O21″	0.7854 (1)	0.4286 (4)	0.2499 (2)	2.13 (9)
O23″	0.6807 (2)	0.5791 (6)	0.5141 (2)	3.3 (1)

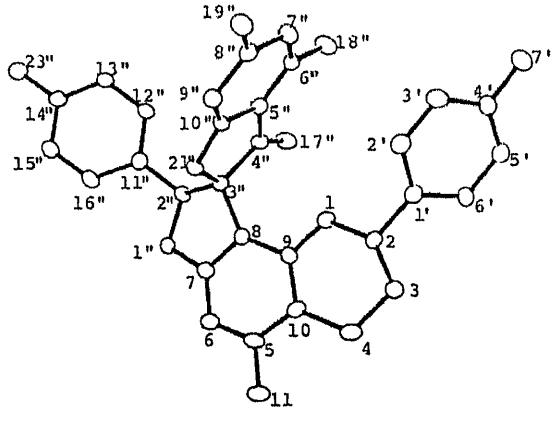


Fig. 3. ORTEP Drawing of Daphnodorin C

selective proton decoupling (LSPD) experiment was carried out on the proton signals of **3** at 5.47 and 4.78 ppm. The irradiation of the signal at 5.47 ppm reduced the doublet signal at 172.42 ppm to a singlet, the doublet signal at 93.91 ppm to a singlet, the broad singlet signal at 193.30 ppm (carbonyl carbon) to a singlet (half-width 0.18→0.09 Hz), the triplet signal at 168.00 ppm to a doublet, the triplet doublet signal at 123.42 ppm to a triplet and the doublet doublet doublet (ddd) signal at 129.00 ppm to doublet doublet (dd). The irradiation of the signal at 4.78 ppm reduced the ddd signal at 131.14 ppm to dd, and the dd signal at 126.06 ppm to a doublet.

From the above results, **3** was considered to have the structure **3**, possessing a spiro ring.

In a nuclear Overhauser effect (NOE) experiment on **12**, irradiation of the H-2'' signal at 5.69 resulted in a 3.8% NOE enhancement of the H-4'' signal at 5.86. Accordingly, it was decided that the relative configuration of C-2'' and C-3'' of **3** is as shown in Fig. 2. The absolute configuration at the C-2 position of **3** has been determined to be *S* from the facts that **3** and **4** were converted into **1** and **6**, respectively. In order to confirm the absolute configurations at the C-2'' and C-3'' positions, and to investigate further the stereostructure of **3**, X-ray diffraction analysis of **3** was carried out. The X-ray diffraction intensities from a pale yellow crystal (0.3 × 0.4 × 0.6 mm) of daphnodorin C (**3**) were measured on a Rigaku AFC-5 diffractometer equipped with a graphite monochromator. The atomic parameters of nonhydrogen atoms and an ORTEP drawing of **3** are shown in Table II and Fig. 3. The crystal data are presented in the experimental section.

Since the absolute configuration at the C-2 position of **3** was decided to be *S* as described above, from this X-ray analysis the absolute configurations of C-2'' and C-3'' were concluded to be both *S*.

Experimental

All melting points were measured on a Büchi melting point apparatus and are uncorrected. The UV spectra were recorded with a Shimadzu UV-200s spectrometer, the IR spectra with a Hitachi EPI-G2 spectrometer, the ORD spectra with a JASCO ORD/UV-5 spectrometer and the CD spectra with a JASCO J-500 spectropolarimeter. The mass spectrum (MS) were taken with Hitachi M-80 and Nihondenshi JEOL JMS-HX 100 spectrometers. The ^{1}H-NMR spectra were taken with Varian XL-300 and Nihondenshi JEOL FX-200 spectrometers with tetramethylsilane (TMS) as an internal standard, and the ^{13}C-NMR spectra with Varian XL-300 and Nihondenshi JEOL FX-100 spectrometers with TMS as an internal standard. Column chromatography was carried out on Merck Silica gel 60 (70—230 mesh) and Sephadex LH-20. For thin layer chromatography (TLC) and preparative TLC, Merck Silica gel 60 F_{254} plates (0.25 mm) and Merck Silica gel 60 F_{254} plates (concentrating zone, 2 mm) were employed and the developed spots were detected under a UV lamp (253.6 and 365 nm).

Daphnodorin C (3)——Recrystallized from $CHCl_3$–MeOH to give pale yellow crystals, $C_{30}H_{22}O_9$, mp 233—235 °C (dec.), M$^+$ 526, Gibb's test: reddish brown, $FeCl_3$ test: dark brown, Mg–HCl test: negative. UV $\lambda_{max}^{dioxane}$ nm (log ε): 222 (4.84), 283 (4.45), 322 (3.72). UV λ_{max}^{EtOH} nm (log ε): 217 (4.81), 287 (4.35), 323 (3.71). IR ν_{max}^{Nujol} cm^{-1}: 3450, 3250, 1680, 1640, 1620, 1530. ORD (c=0.21, dioxane) $[\alpha]^{22}$ (nm): −262.9° (589), −338.0° (550), −469.5° (500), −657.3° (450), −1164.3° (400), −4507.0° (350), −7211.3° (342) (trough), 0° (332), +5558.7° (316) (peak), 0° (306). CD ($c = 2.28 \times 10^{-5}$, dioxane) $\Delta\varepsilon^{23}$ (nm): 0 (400), −11.3 (333), 0 (315), +4.1 (303), 0 (292), −14.9 (280), 0 (248), +1.9 (243). *Anal.* Calcd for $C_{30}H_{22}O_9$: C, 68.44; H, 4.21. Found: C, 68.53; H, 4.24. The NMR data are summarized in Table I.

Conversion of 3 to 1——**3** (100 mg) was dissolved in EtOH (3 ml) and 3 drops of H_2SO_4 were added. The mixture was heated on a boiling water bath for 2 min, then diluted with ice water, and extracted with EtOAc. The EtOAc solution was treated with 5% $NaHCO_3$, washed with water, dried and concentrated *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$: MeOH = 5 : 1) to afford **1** (25 mg). This product was identical with an authentic sample of daphnodorin A, based on comparisons of the spectral data.

Methylation of 3 with CH_2N_2——A solution of **3** (1 g) in MeOH (10 ml) was methylated with CH_2N_2–Et_2O in the usual way. The product was purified by column chromatography on silica gel with hexane–EtOAc (3 : 2) to afford the 3-pentamethyl ether (**4**) (0.6 g).

4: White fine crystals, $C_{35}H_{32}O_9$, mp 125—127 °C. MS *m/z* (rel. int.): 596 (13, M$^+$), 462 (3), 416 (23), 282 (41), 181 (41), 134 (100). UV $\lambda_{max}^{dioxane}$ nm (log ε): 220 (4.55), 282 (4.06). IR ν_{max}^{Nujol} cm^{-1}: 1710, 1615, 1590, 1515, 1460. ORD (c=0.035, dioxane) $[\alpha]^{20}$ (nm): −228.57° (589), −342.86° (550), −457.14° (500), −571.43° (450), −914.29°

(400), −4914.29° (342) (trough), 0° (330), +4800.00° (324) (peak), 0° (313). CD ($c=2.01\times10^{-5}$, dioxane) $\Delta\varepsilon^{23}$ (nm): 0 (400), −8.7 (340), −12.3 (328), 0 (320), +1.5 (317), + 4.8 (307), +1.2 (298), 0 (294), −21.1 (278), 0 (251), +3.9 (245). ^{1}H-NMR ($CDCl_3$) δ: 7.27, 6.80 (each 2H, m, $J_{AX}=8.8$ Hz), 6.99, 6.72 (each 2H, m, $J_{AX}=8.8$ Hz), 6.20 (1H, s), 5.81 (1H, s), 5.79, 5.76 (each 1H, d, $J=1.8$ Hz), 4.80 (1H, dd, $J=10.7$, 1.8 Hz), 2.74 (1H, ddd, $J=16.9$, 5.3, 2.3 Hz), 2.56 (1H, ddd, $J=16.9$, 11.7, 5.8 Hz), 2.16 (1H, m), 1.81 (1H, m), 3.84, 3.83, 3.77, 3.77, 3.69 (each 3H, s). ^{13}C-NMR ($CDCl_3$) δ: 194.63 (s), 173.77 (s), 169.75 (s), 161.86 (s), 161.41 (s), 159.66 (s), 158.59 (s), 158.45 (s), 152.21 (s), 133.31 (s), 128.78 (d)×2, 126.62 (d)×2, 125.47 (s), 113.42 (d)×2, 113.27 (d)×2, 104.87 (s), 104.02 (s), 103.69 (s), 95.21 (s), 92.51 (d), 91.07 (d), 88.17 (d), 86.40 (d), 77.02 (d), 28.85 (t), 19.64 (t), 55.89 (q), 55.79 (q)×2, 55.20 (q)×2.

Conversion of 4 to 6——**4** (500 mg) was dissolved in EtOH (6 ml) and 5 drops of H_2SO_4 were added. The mixture was heated on a boiling water bath for 30 min, diluted with ice water, and extracted with EtOAc. The EtOAc solution was treated with 5% $NaHCO_3$, washed with water, dried and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with $CHCl_3$ to afford **6** (116 mg). This product was identical with an authentic sample of daphnodorin A pentamethyl ether, based on comparisons of the spectral data.

Acetylation of 3 with Ac_2O and Pyridine——A solution of **3** (100 mg) in a mixture of Ac_2O (6 ml) and pyridine (6 ml) was allowed to stand at room temperature overnight. The reaction mixture was treated in the usual way, and the products were purified by column chromatography on silica gel with $CHCl_3$: MeOH (10:1) to afford **5** (92.8 mg).

5: White powder, $C_{40}H_{32}O_{14}$, M^+ 736. UV $\lambda_{max}^{dioxane}$ nm (log ε): 217 (4.74), 265 (4.13), 327 sh (3.72). IR ν_{max}^{Nujol} cm^{-1}: 1770, 1730, 1620, 1460. ORD ($c=0.285$, dioxane) $[\alpha]^{23}$ (nm): −147.37° (589), −178.95° (550), −231.58° (500), −347.37° (450), −694.74° (400), −3045.61° (363), −3733.33° (357), −3045.61° (354). ^{1}H-NMR ($CDCl_3$) δ: 7.28, 7.03 (each 2H, m, $J_{AX}=8.6$ Hz), 7.04, 6.98 (each 2H, m, $J_{AX}=8.6$ Hz), 6.43 (1H, s), 6.45, 6.40 (each 1H, d, $J=2.2$ Hz), 5.82 (1H, s), 4.89 (1H, dd, $J=2.0$, 10.5 Hz), 2.58 (2H, m), 2.22, 1.83 (each 1H, m), 2.39, 2.31, 2.29, 2.25, 2.20 (each 3H, s).

Cleavage of 4 with H_2SO_4/AcOH (Formation of 7, 8, 9 and 10)——**4** (500 mg) was dissolved in AcOH (5 ml) and 4 drops of H_2SO_4 were added. The mixture was heated on a boiling water bath for 5 min, diluted with ice water (100 ml), and extracted with EtOAc. The EtOAc solution was treated with 5% $NaHCO_3$, washed with water, dried and concentrated *in vacuo*. The residue was purified by preparative TLC (hexane–EtOAc=2:1) to afford **7** (65 mg), **8** (30 mg), **9** (28 mg) and **10** (54 mg). The ^{1}H-NMR spectra of **7**, **8**, **9** and **10** coincided with those of authentic samples of 4-methoxybenzoic acid, 3,5-dimethoxyphenol, 2-hydroxy-4,6-dimethoxybenzoic acid and the furanoflavan derivative obtained from daphnodorin A, respectively.

Hydrolysis of 4 with 40% KOH (Formation of 7, 8, 9 and 11)——**4** (700 mg) was dissolved in 40% KOH (2 ml) and heated on a sand bath for 1.5 h at 220 °C. Then the reaction mixture was diluted with ice water (100 ml), acidified with dil. HCl and extracted with EtOAc. The EtOAc solution was washed with H_2O, dried and concentrated *in vacuo*. The residue (876 mg) was purified by column chromatography on silica gel with $CHCl_3$: MeOH (30:1) to afford **7** (85 mg), **8** (45 mg), **9** (37 mg) and **11** (65 mg). The products, **7**, **8** and **9**, were identical with authentic samples, based on comparisons of the spectral data.

11: Pale yellow powder, $C_{19}H_{20}O_6$, MS *m/z*: 326 (18, M−H_2O), 192 (9, M−134), 134 (100, M−192). Optically inactive. IR ν_{max}^{Nujol} cm^{-1}: 3450, 3200–2500, 1680, 1620, 1510. ^{1}H-NMR (DMSO-d_6) δ: 8.96 (1H, br), 7.32, 6.92 (each 2H, d, $J=8.6$ Hz), 6.05 (1H, s), 5.66 (1H, s), 4.95 (1H, dd, $J=10.3$, 2.0 Hz), 2.50 (2H, m), 2.12, 1.80 (each 1H, m), 3.75, 3.67 (each 3H, s), 3.40, 3.33 (each 1H, d, $J=16.0$ Hz).

Reduction of 4 with $NaBH_4$ (Formation of 12)——A mixture of **4** (250 mg) in tetrahydrofuran (12 ml) and $NaBH_4$ (350 mg) in EtOH (10 ml) was stirred for 2 h and then allowed to stand at room temperature overnight. The reaction mixture was diluted with water (100 ml), acidified with dil. HCl and extracted with Et_2O. The Et_2O solution was washed with H_2O, then dried and concentrated *in vacuo*. The residue was recrystallized from EtOH to give an alcohol **12** (200 mg).

12: White crystalline powders, $C_{35}H_{34}O_9$, mp 127–129 °C, M^+ 598.2219. UV $\lambda_{max}^{dioxane}$ nm (log ε): 220 (4.78), 280 (3.71). IR ν_{max}^{Nujol} cm^{-1}: 3475, 1615, 1505, 1460. CD ($c=2.51\times10^{-5}$, dioxane) $\Delta\varepsilon^{23}$ (nm): 0 (400), +6.3 (275), +8.7 (248), 0 (243). ^{1}H-NMR ($CDCl_3$) δ: 7.31, 6.80 (each 2H, d, $J_{AX}=9.0$ Hz), 6.94, 6.74 (each 2H, d, $J_{AX}=8.5$ Hz), 6.24 (1H, s), 5.51 (1H, s), 5.86, 5.69 (each 1H, d, $J=2.0$ Hz), 5.37 (1H, d, $J=12.0$ Hz), 4.85 (1H, dd, $J=10.1$, 1.6 Hz), 3.91 (1H, d, $J=12.0$ Hz), 2.83 (1H, ddd, $J=16.9$, 5.9, 2.4 Hz), 2.62 (1H, ddd, $J=16.9$, 11.4, 6.1 Hz), 2.32 (1H, m), 2.05 (1H, m), 3.84, 3.77, 3.76, 3.68, 3.65 (each 3H, s). ^{13}C-NMR ($CDCl_3$) δ: 162.98 (s), 161.10 (s), 160.92 (s), 160.39 (s), 159.77 (s), 159.19 (s), 157.48 (s), 152.21 (s), 131.82 (s), 129.51 (d)×2, 127.43 (d)×2, 125.47 (s), 113.78 (d)×2, 113.33 (d)×2, 107.34 (s), 104.65 (s), 104.36 (s), 99.75 (s), 92.98 (d), 91.47 (d), 87.59 (d), 87.25 (d), 77.70 (d)×2, 26.68 (t), 19.58 (t), 55.78 (q), 55.42 (q), 55.28 (q), 55.19 (q), 55.08 (q).

Crystal Data of 3——$C_{30}H_{22}O_9$, monoclinic, $C2$, $a=20.622$ (7), $b=7.723$ (1), $c=17.927$ (7), $\beta=126.13$ (4), $z=4$, $D_m=1.496$ (1) g/cm^3. The crystal structure was solved by a direct method. All heavy atoms were refined with anisotropic temperature factors. The *R* factor was reduced to 0.067, including hydrogen atoms. Calculations for structure determinations and refinements were carried out using mostly the UNICS and AUTO library of crystallographic programs (Crystallographic Research Center, Institute for Protein Research of Osaka University).

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1,3-Oxazines and Related Compounds. XIII.[1] Reaction of Acyl Meldrum's Acids with Schiff Bases Giving 2,3-Disubstituted 5-Acyl-3,4,5,6-tetrahydro-2*H*-1,3-oxazine-4,6-diones and 2,3,6-Trisubstituted 2,3-Dihydro-1,3-oxazin-4-ones

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The reactions of Schiff bases (**2**) with various acyl Meldrum's acids (**1**) were investigated. Refluxing of **1** and **2** in benzene caused an exchange reaction of the acetone moiety of **1** with the Schiff base moiety through the intermediate acylketenes **11** formed *in situ* from **1** to give 2,3-disubstituted 5-acyl-3,4,5,6-tetrahydro-2*H*-1,3-oxazine-4,6-diones (**3**). The oxazindiones **3** underwent thermal conversion to afford 2,3,6-trisubstituted 2,3-dihydro-1,3-oxazin-4-ones (**4**) in good yield. The intermediates **11** were found to be formed by thermal cleavage of both **1** and **3**.

Keywords——acyl Meldrum's acid; Schiff base; 1,3-oxazine-4,6-dione; 1,3-oxazin-4-one; 1,3-oxazine-5-carboxylic acid; acylketene; cycloaddition

In recent years, acyl Meldrum's acids (**1**, 5-acyl-2,2-dimethyl-1,3-dioxane-4,6-diones) have been widely utilized as acylacetylating agents,[2] because of the characteristic that **1** loses acetone and carbon dioxide with ease to generate acylketenes (**12**). An additional unique reactivity of **1** was found by Kato *et al.*, though only one example was described[3a]; when a solution of acetyl Meldrum's acid (**1a**) and *N*-benzylidenepropylamine (**2a**) in toluene was refluxed, exchange reaction between a C–O moiety of the dioxane ring in **1a** and C=N moiety of the Schiff base took place with loss of acetone to produce the 1,3-oxazinedione derivative **3a**, without involvement of an acetylketene intermediate, along with a small amount of the oxazin-4-one **4a**.



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

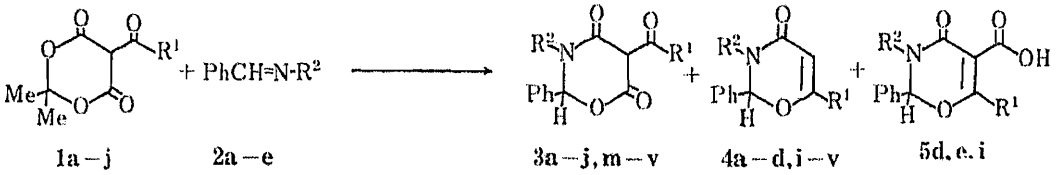
Chart 1

In this series of studies, our interest has been focused on an exploration of such reactivities of acyl Meldrum's acids **1**. This paper describes an investigation of the reaction of various acyl Meldrum's acids **1** with Schiff bases **2**, including the reaction modes of **1** and the transformation of the products **3** into **4**, which would open a way for further synthesis of 1,3-oxazine derivatives.

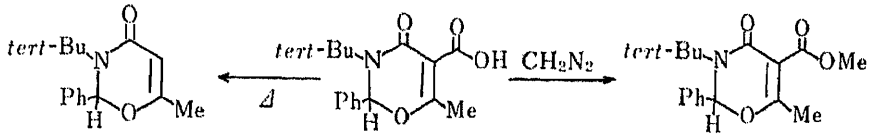
According to the reported method[3] except for the use of benzene as a solvent instead of toluene, a solution of acetyl Meldrum's acid **1a** and **2a** in benzene was refluxed for 25 min to give the oxazine-4,6-dione **3a** as a sole product in good yield. Similarly, the oxazine-4,6-diones **3b**—**i**[7] were obtained from **1a**—**e** (R^1: aliphatic) and **2b**—**e** (Table I). In contrast, similar

treatment of acyl Meldrum's acids **1f—i** (R^1: aromatic) with **2d, e** gave rise to 1,3-oxazin-4-one derivatives **4m—t**[7] instead of the expected **3**. Reaction between **2d, e** and **1j** afforded both **3** and **4**.

In the reactions of **1a, b** with the Schiff bases **2d, e**, very small amounts of 1,3-oxazine-5-carboxylic acids **5d, e, i** were found to be formed as by-products; they are isomers of the corresponding oxazine-4,6-diones **3d, e, i**. The structural assignment of **5** was accomplished on the basis of microanalysis and the spectroscopic data, such as infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR) and mass spectra (MS) (summarized in Table VI). The following reaction provides further evidence for the structure of **5**; refluxing a solution of **5d** in toluene caused decarboxylation to give **4d**, and treatment of **5d** with diazomethane gave the corresponding methyl ester (**6**), the IR spectrum of which showed the characteristic C=O band due to the ester group at 1715 cm^{-1}.



1a—j **2a—e** **3a—j, m—v** **4a—d, i—v** **5d, e, i**



4d **5d** **6**

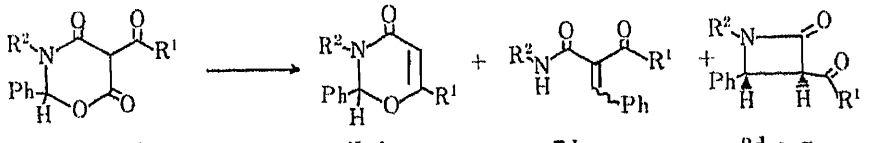
1a : R^1 = Me
1b : R^1 = Et
1c : R^1 = styryl
1d : R^1 = $PhCH_2$
1e : R^1 = cyclohexyl

1f : R^1 = Ph
1g : R^1 = *p*-Me-C_6H_4
1h : R^1 = *p*-MeO-C_6H_4
1i : R^1 = *p*-Cl-C_6H_4
1j : R^1 = *p*-NO_2-C_6H_4

2a : R^2 = Pr
2b : R^2 = iso-Pr
2c : R^2 = $PhCH_2$
2d : R^2 = *tert*-Bu
2e : R^2 = Ph

Chart 2

It was found that the product alternative of **3** or **4** in the reactions depends on the reaction temperature; thus, treatment of **1f—j** with **2d, e** in benzene at 50 °C (bath temperature) for 4 h gave exclusively the expected diones **3** in satisfactory yields (Table I). Therefore, in order to examine the thermal stability of **3**, a solution of **3** in benzene was refluxed. The results obtained are shown in Table II. The diones **3a—c**, possessing an aliphatic acyl group, were relatively stable and were mostly recovered unchanged, whereas **3h, i, m**, were transformed into **4h, i, m**[8] with the loss of carbon dioxide, and **3d, e, g** were converted into the amide derivatives **7d, e, g**[8] together with a small amount of the *β*-lactams **8**.[9] The oxazines **4** were also obtained by refluxing a solution of **3a—i** in xylene, or by treatment of **1** and **2** in refluxing xylene (Table I).



3a—e, g—i, m **4h, i, m** **7d, e, g** **8d, e, g**

Chart 3

Mechanisms for the formation of **4**, **7**, and **8** have been proposed by Kato *et al.*[3]; the oxazines **4** are formed by 1,4-cycloaddition of acetylketene (**12a**) across the C=N double bond of the Schiff bases **2**, whereas 1,2-cycloaddition of **12a** to the C=N bond yields the β-lactams **8**, and compounds **7** are produced *via* pyrimidine derivatives intermediately formed from 1 eq of **12a** and 2 eq of the Schiff bases. It is strongly suggested that the transformations of **3** into **4**, **7**, and **8** each involve generation and addition of acylketenes **12** in the reaction systems, that is, retro Diels–Alder type cleavage of **3** into **2** and **12**, followed by decarboxylation of **11** leading to **12**.

Generation of the important intermediates **11** from **1** by the retro Diels–Alder reaction

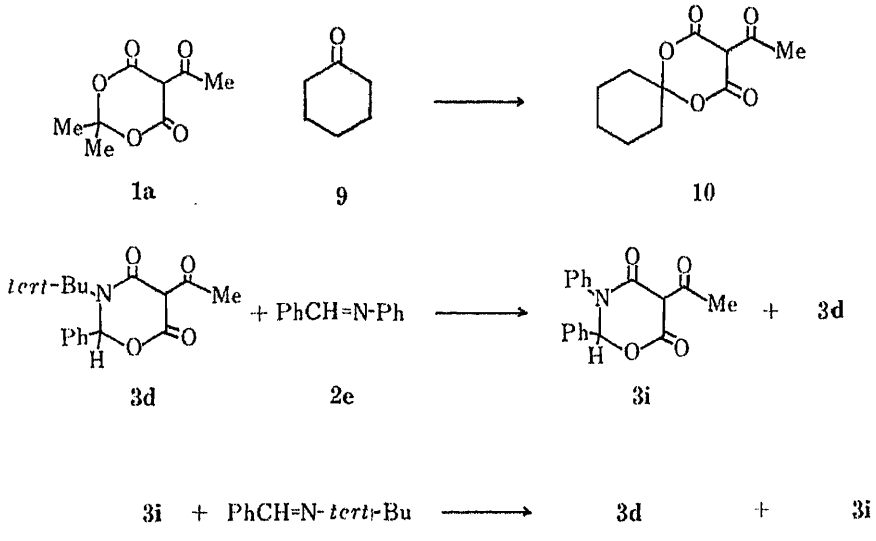


Chart 4

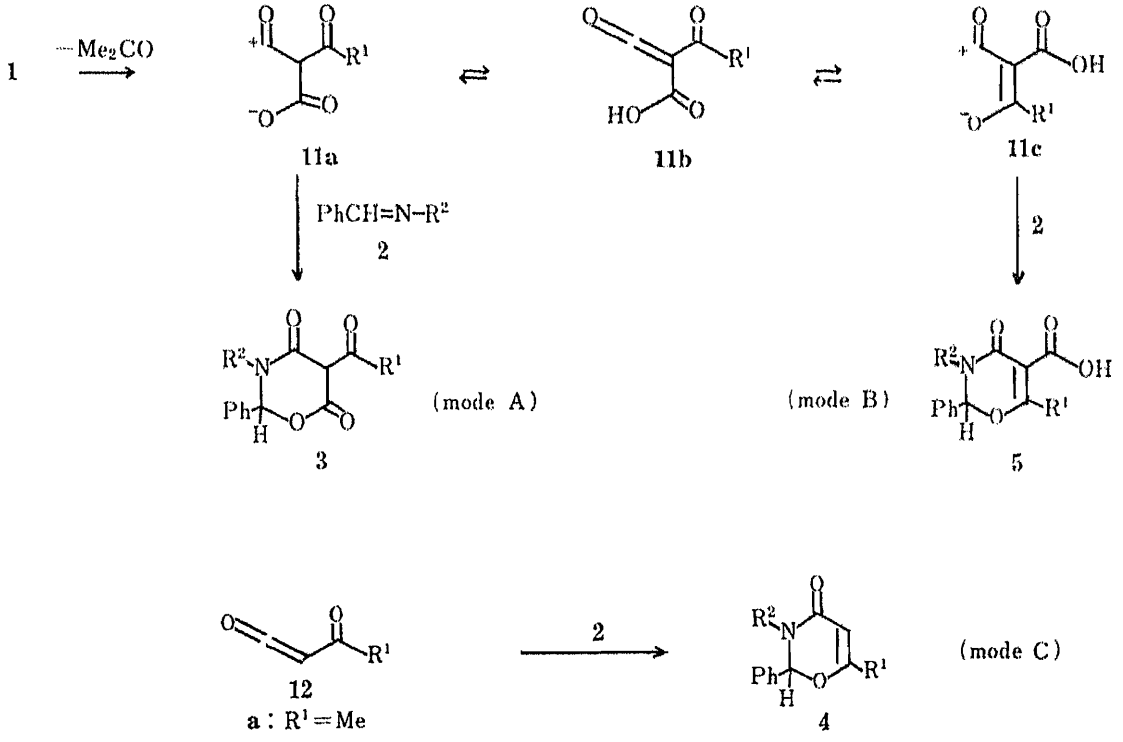


Chart 5

TABLE I. Reaction of Acyl Meldrum's Acids **1a**—**j** with Schiff Bases **2a**—**e**

Substituent		Product No.	Yield (%)	Method[a]	mp (°C) (Solvent) (Lit. mp)	Product No.	Yield (%)	Method[a]	mp (°C) (Solvent) (Lit. mp)
R^1	R^2								
Me	Pr	**3a**	83.5	I	74—75 (P.E.)[b] (73—74)[c]	**4a**	43	III	Oil
Me	iso-Pr	**3b**	72	I	92—93 (Et_2O)	**4b**	57	III	100—102 (Et_2O–P.E.)[b] (100—102)[c]
Me	$PhCH_2$	**3c**	80	I	113—114 (AcOEt)	**4c**	54	III	77—78 (Et_2O–P.E.)[b] (77—79)[c,e]
Me	*tert*-Bu	**3d**	65	I	107—109 (dec.) (Et_2O)	**4d**	24	III	89—91 (Et_2O–P.E.)[b] (99—100)[c]
Et	*tert*-Bu	**3e**	63	I	94—95 (dec.) (Et_2O)				
Styryl	*tert*-Bu	**3f**	79	I	141—142 (dec.) (AcOEt)				
$PhCH_2$	*tert*-Bu	**3g**	68	I	105—107 (dec.) (Et_2O)				
Cyclohexyl	*tert*-Bu	**3h**	61.5	I	112—114 (dec.) (Et_2O)				
Me	Ph	**3i**	68.5	I	102—104 (dec.) (Et_2O)	**4i**	68	III	100—101 (Et_2O–P.E.)[b] (94—94.5)[c,e]
Et	Ph					**4j**	64	III	101—102 (Et_2O–P.E.)[b]
$PhCH_2$	Ph					**4k**	61.5	III	135—136 (AcOEt)
Cyclohexyl	Ph					**4l**	75	III	156—157 (AcOEt)
Ph	*tert*-Bu	**3m**	54	II	131—132 (dec.) (AcOEt)	**4m**	50	I	157—158 (AcOEt) (159—161)[d]
p-Me C_6H_4	*tert*-Bu	**3n**	74	II	107—108 (dec.) (Et_2O)	**4n**	65	I	137—139 (Et_2O)
p-MeO–C_6H_4	*tert*-Bu	**3o**	62	II	117—118 (dec.) (Et_2O)	**4o**	52	I	115—117 (Et_2O)
p-Cl–C_6H_4	*tert*-Bu	**3p**	75	II	110—111 (dec.) (Et_2O)	**4p**	66	I	123—124 (Et_2O)
Ph	Ph	**3q**	73	II	121—122 (dec.) (AcOEt)	**4q**	74	I	153—154 (AcOEt) (146—147)[d]
p-Me–C_6H_4	Ph	**3r**	80	II	124—125 (dec.) (AcOEt)	**4r**	55	I	158—160 (AcOEt)

TABLE I. (continued)

Substituent		Product No.	Yield (%)	Method[a]	mp (°C) (Solvent) (Lit. mp)	Product No.	Yield (%)	Method[a]	mp (°C) (Solvent) (Lit. mp)
R^1	R^2								
p-MeO-C_6H_4	Ph	**3s**	68	II	128—130 (dec.) (AcOEt)	**4s**	68	I	151—153 (AcOEt)
p-Cl-C_6H_4	Ph	**3t**	80	II	133—134 (dec.) (AcOEt)	**4t**	51	I	154—157 (AcOEt)
p-NO_2-C_6H_4	*tert*-Bu	**3u**	29	I	122—124 (dec.) (Et_2O)	**4u**	32	I	127—129 (Et_2O)
			76	II					
p-NO_2-C_6H_4	Ph	**3v**	10	I	121—122 (dec.) (Et_2O)	**4v**	38	I	192—194 (AcOEt)
			86	II					

a) I=refluxed in benzene for 25 min. II=heated at 50° in benzene for 4 h. III=refluxed in xylene. *b*) P.E.=petroleum ether. *c*) Ref. 3. *d*) Ref. 4. *e*) Ref. 6.

TABLE II. Thermal Decomposition of 1,3-Oxazine-4,6-diones **3a—e, g—i, m**

Compd. No.	R^1	R^2	Time	Product					
				4		7		8	
				Yield (%)	mp (°C) (Solvent) (Lit. mp)	Yield (%)	mp (°C) (Solvent) (Lit. mp)	Yield (%)	mp (°C) (Solvent) (Lit. mp)
3a	Me	Pr	24 h	Recovery (87%)					
3b	Me	iso-Pr	24 h	Recovery (85%)					
3c	Me	$PhCH_2$	24 h	Recovery (86%)					
3d	Me	*tert*-Bu	24 h	—		56	110—111 (Et_2O) (112—113)[a,d]	3	89—90 (Et_2O) (90—92)[a]
3e	Et	*tert*-Bu	29 h	—		42	120—122 (Et_2O-P.E.)[c]	2	78—79 (Et_2O-P.E.)[c]
3g	$PhCH_2$	*tert*-Bu	24 h	—		45	150—152 (Et_2O)	4	89—90 (Et_2O-P.E.)[c]
3h	Cyclohexyl	*tert*-Bu	32 h	54	115—116 (P.E.)[c]	—		—	
3i	Me	Ph	37 h	37	100—101 (Et_2O-P.E.)[c] (94—94.5)[a,c]	—		—	
3m	Ph	*tert*-Bu	25 min	72	157—158 (AcOEt) (159—161)[b]	—		—	

a) Ref. 3. *b*) Ref. 4. *c*) P.E.=petroleum ether. *d*) Ref. 5. *e*) Ref. 6.

was proven by means of an interchange reaction of the acetone moiety in **1a** with a cyclohexanone moiety on refluxing **1a** and cyclohexanone (**9**) in benzene (Chart 4). In addition, the intermediates **11** were found to be formed from **3**; when a solution of equimolar

amounts of **3d** and **2e** in benzene was refluxed for 1 h, exchange reaction between the Schiff bases **2d** and **2e** took place giving **3i** and **3d** in 52% and 21% yields, respectively. Meanwhile, **3d** and **3i** were obtained from **3i** and **2d** by the same procedure in 32% and 50% yields, respectively.

TABLE III. Acyl Meldrum's Acids **1a—j**

Compd. No.	R^1	mp (°C) (Lit. mp)	Recrystn. solvent[a]	Yield (%)
1a	Me	83—84 (dec.) (82—85)[b]	A	79
1b	Et	52—53 (55)[c]	B	83
1c	Styryl	114—115 (dec.)	A	58
1d	$PhCH_2$	95—96 (dec.) (96—97)[c]	A	76
1e	Cyclohexyl	81—82	B	63
1f	Ph	103—104 (dec.)	C	80
1g	*p*-Me-C_6H_4	105—106 (dec.)	C	76
1h	*p*-MeO-C_6H_4	111—112 (dec.)	C	79
1i	*p*-Cl-C_6H_4	106 (dec.)	C	71
1j	*p*-NO_2-C_6H_4	113—114 (dec.)	C	75.5

a) A=ether, B=petroleum ether, C=acetone. *b*) Ref. 3*a*. *c*) Ref. 2*a*.

TABLE IV. Spectral and Analytical Data for Acyl Meldrum's Acids **1a—j**

Compd. No.	IR ν_{max}^{KBr} cm^{-1}	NMR ($CDCl_3$) δ	Formula	Analysis (%) Calcd (Found)	
				C	H
1a	1735 1660	1.73 (6H, s), 2.66 (3H, s), 15.00 (1H, br)	—	—	—
1b	1735 1650	1.26 (3H, t, J=7 Hz), 1.73 (6H, s), 3.13 (2H, q, J=7 Hz), 15.40 (1H, br)	—	—	—
1c	1720 1655	1.77 (6H, s), 7.23—7.86 (5H, m), 8.00 (1H, d, J=16 Hz), 8.33 (1H, d, J=16 Hz), 14.90 (1H, br)	$C_{15}H_{14}O_5$	65.69 (65.39	5.14 5.18)
1d	1740 1650	1.70 (6H, s), 4.43 (2H, s), 7.20—7.53 (5H, s), 15.33 (1H, br)	—	—	—
1e	1730 1660	1.07—2.07 (10H, m), 1.73 (6H, s), 3.50—4.07 (1H, m), 15.53 (1H, br)	$C_{13}H_{18}O_5$	61.41 (61.29	7.13 6.91)
1f	1735 1655	1.80 (6H, s), 7.20—7.83 (5H, m), 15.47 (1H, br)	$C_{13}H_{12}O_5$	62.90 (62.62	4.87 4.87)
1g	1725 1655	1.80 (6H, s), 2.40 (3H, s), 7.20 (2H, d, J=9 Hz), 7.56 (2H, d, J=9 Hz), 15.23 (1H, br)	$C_{14}H_{14}O_5$	64.12 (64.04	5.38 5.42)
1h	1740 1650	1.80 (6H, s), 3.83 (3H, s), 6.90 (2H, d, J=9 Hz), 7.70 (2H, d, J=9 Hz), 15.47 (1H, br)	$C_{14}H_{14}O_6$	60.43 (60.30	5.07 5.04)
1i	1730 1660	1.83 (6H, s), 7.40 (2H, d, J=9 Hz), 7.63 (2H, d, J=9 Hz), 15.17 (1H, br)	$C_{13}H_{11}ClO_5$	55.24 (55.23	3.92 3.66)
1j	1740 1665	1.83 (6H, s), 7.73 (2H, d, J=9 Hz), 8.26 (2H, d, J=9 Hz), 15.53 (1H, br)	$C_{13}H_{11}NO_7$	53.25 (53.34	3.78 3.60)

In conclusion, the reaction of **1** with **2** proceeds through the three reaction modes A, B, and C as illustrated in Chart 5; acyl Meldrum's acids **1** lose the acetone moiety, generating the intermediates **11**, which can be represented by formulae **11a**, **11b**, and **11c** as illustrated in Chart 5. At a relative low temperature, 1,4-cycloadditions of **11a** and **11c** across the C=N bond of **2** proceed leading to the corresponding **3** (mode A) and **5** (mode B) with considerable predomination of mode A over B, whereas at a relative high temperature **11a** or **11c** undergoes decarboxylation, giving the acylketenes **12** and 1,4-addition of **12** to the C=N bond occurs to yield **4** (mode C).

Experimental

Melting points were determined on a Mel-Temp melting point apparatus with an open capillary tube, and are

TABLE V. Spectral Data for 5-Acyl-tetrahydro-1,3-oxazine-4,6-diones **3a—i, m—v**

Compd. No.	IR ν_{max}^{KBr} cm^{-1} (C=O)	NMR ($CDCl_3$) δ	Formula MS (*m/z*, M^+)
3a	1710	0.90 (3H, t, *J*=7 Hz), 1.30—2.00 (2H, m), 2.43 (3H, s), 2.56—3.16 (1H, m), 3.53—4.20 (1H, m), 6.13 (1H, s), 7.37 (5H, s), 16.93 (1H, br)	—
3b	1725	1.10 (3H, d, *J*=7 Hz), 1.33 (3H, d, *J*=7 Hz), 2.43 (3H, s), 4.07—4.93 (1H, m), 6.23 (1H, s), 7.33 (5H, s), 16.80 (1H, br)	$C_{15}H_{17}NO_4$ (275)
3c	1710	2.53 (3H, s), 3.90 (1H, d, *J*=15 Hz), 5.37 (1H, d, *J*=15 Hz), 6.07 (1H, s), 7.03—7.50 (10H, m), 16.76 (1H, br)	$C_{19}H_{17}NO_4$ (327)
3d	1730	1.56 (9H, s), 2.40 (3H, s), 6.50 (1H, s), 7.37 (5H, s), 17.03 (1H, br)	$C_{16}H_{19}NO_4$ (289)
3e	1720	1.03 (3H, t, *J*=7 Hz), 1.56 (9H, s), 2.80 (2H, q, *J*=7 Hz), 6.47 (1H, s), 7.30 (5H, s), 17.36 (1H, br)	$C_{17}H_{21}NO_4$ (303)
3f	1725	1.56 (9H, s), 6.56 (1H, s), 7.16—7.63 (11H, m), 7.90 (1H, d, *J*=16 Hz), 16.93 (1H, br)	$C_{23}H_{23}NO_4$ (377)
3g	1735	1.53 (9H, s), 4.13 (2H, dd, *J*=10, 24 Hz), 6.47 (1H, s), 7.03—7.20 (5H, m), 7.30 (5H, s), 17.00 (1H, br)	$C_{22}H_{23}NO_4$ (365)
3h	1720	1.00—2.03 (10H, m), 1.56 (9H, s), 3.21—3.70 (1H, m), 6.46 (1H, s), 7.30 (5H, s), 17.36 (1H, br)	$C_{21}H_{27}NO_4$ (357)
3i	1730	2.53 (3H, s), 6.50 (1H, s), 7.20—7.50 (10H, m), 16.67 (1H, br)	$C_{18}H_{15}NO_4$ (309)
3m	1735	1.63 (9H, s), 6.60 (1H, s), 7.10—7.63 (10H, m), 17.00 (1H, m)	$C_{21}H_{21}NO_4$ (351)
3n	1730	1.63 (9H, s), 2.30 (3H, s), 6.50 (1H, s), 7.07 (4H, s), 7.26—7.63 (5H, m), 16.07 (1H, br)	$C_{22}H_{23}NO_4$ (365)
3o	1735	1.63 (9H, s), 3.77 (3H, s), 6.60 (1H, s), 6.77 (2H, d, *J*=9 Hz), 7.23 (2H, d, *J*=9 Hz), 7.30—7.67 (5H, m), 16.83 (1H, br)	$C_{22}H_{23}NO_4$ (381)
3p	1730	1.60 (9H, s), 6.56 (1H, s), 6.93—7.63 (9H, m), 17.00 (1H, br)	$C_{21}H_{20}ClNO_4$ (386)
3q	1730	6.60 (1H, s), 7.07—7.67 (15H, m), 16.56 (1H, br)	$C_{23}H_{17}NO_4$ (371)
3r	1730	2.33 (3H, s), 6.60 (1H, s), 6.96—7.70 (14H, m), 16.56 (1H, br)	$C_{24}H_{19}NO_4$ (385)
3s	1730	3.83 (3H, s), 6.67 (1H, s), 6.83 (2H, d, *J*=9 Hz), 7.23—7.80 (12H, m), 16.67 (1H, br)	$C_{24}H_{19}NO_5$ (401)
3t	1730	6.60 (1H, s), 7.13—7.73 (14H, m), 16.73 (1H, br)	$C_{23}H_{16}ClNO_4$ (406)
3u	1725	1.63 (9H, s), 6.63 (1H, s), 7.30 (2H, d, *J*=9 Hz), 7.47 (5H, s), 8.13 (2H, d, *J*=9 Hz), 16.83 (1H, br)	$C_{21}H_{20}N_2O_6$ (396)
3v	1725	6.63 (1H, s), 7.13—7.70 (12H, m), 8.13 (2H, d, *J*=9 Hz), 16.03 (1H, br)	$C_{23}H_{16}N_2O_6$ (416)

TABLE VI. 1,3-Oxazine-5-carboxylic Acids **5d, e, i**

Compd. No.	R^1	R^2	mp (°C) (Solvent)	IR ν_{max}^{KBr} cm^{-1}	NMR ($CDCl_3$) δ	Formula MS (*m/z*, M^+)	Yield (%)
5d	Me	*tert*-Bu	130—131 (dec.) (Et_2O)	2800—2100 1725	1.56 (9H, s), 2.33 (3H, s), 6.66 (1H, s), 7.37 (5H, s), 14.33 (1H, s)	$C_{16}H_{19}NO_4$ (289)	Trace
5e	Et	*tert*-Bu	119—121 (dec.) (Et_2O)	2800—2100 1725	0.93 (3H, t, J=7 Hz), 1.53 (9H, s), 2.70 (2H, q, J=7 Hz), 6.70 (1H, s), 7.40 (5H, s), 14.43 (1H, s)	$C_{17}H_{21}NO_4$ (303)	Trace
5i	Me	Ph	159—160 (dec.) (AcOEt)	2800—2100 1730	2.53 (3H, s), 6.63 (1H, s), 7.07—7.56 (10H, m), 13.73 (1H, s)	$C_{21}H_{21}NO_4$ (309)	Trace

TABLE VII. Spectral Data for 2,3-Dihydro-1,3-oxazin-4-ones **4a—d, i—v**

Compd. No.	IR $\nu^{a)}$ cm^{-1} (C=O)	NMR ($CDCl_3$) δ	Formula MS (*m/z*, M^+)
4a	1650	0.86 (3H, t, J=7 Hz), 1.23—1.77 (2H, m), 1.83 (3H, s), 2.53—3.07 (1H, m), 3.53—4.10 (1H, m), 5.20 (1H, s), 6.13 (1H, s), 7.33 (5H, s)	—
4b	1655	1.07 (3H, d, J=7 Hz), 1.30 (3H, d, J=7 Hz), 1.76 (3H, s), 4.60—5.10 (1H, m), 5.23 (1H, s), 6.33 (1H, s), 7.40 (5H, s)	—
4c	1670	1.83 (3H, s), 3.80 (1H, d, J=15 Hz), 5.30 (1H, s), 5.33 (1H, d, J=15 Hz), 6.03 (1H, s), 7.00—7.50 (15H, m)	—
4d	1660	1.50 (9H, s), 1.73 (3H, s), 5.10 (1H, s), 6.53 (1H, s), 7.30 (5H, m)	—
4i	1660	1.86 (3H, s), 5.37 (1H, s), 6.60 (1H, s), 7.03—7.60 (10H, m)	
4j	1665	1.00 (3H, t, J=7 Hz), 2.17 (2H, q, J=7 Hz), 5.33 (1H, s), 6.56 (1H, s), 7.00—7.60 (10H, m)	$C_{18}H_{17}NO_2$ (279)
4k	1660	3.43 (2H, s), 5.33 (1H, s), 6.56 (1H, s), 6.83—7.50 (15H, m)	$C_{23}H_{19}NO_2$ (341)
4l	1660	0.60 (11H, m), 5.30 (1H, s), 6.50 (1H, s), 7.00—7.60 (10H, m)	$C_{22}H_{23}NO_2$ (333)
4m	1640	1.57 (9H, s), 5.80 (1H, s), 6.80 (1H, s), 7.26—7.70 (10H, m)	—
4n	1640	1.56 (9H, s), 2.33 (3H, s), 5.73 (1H, s), 6.77 (1H, s), 7.03—7.60 (9H, m)	$C_{21}H_{23}NO_2$ (321)
4o	1645	1.56 (9H, s), 3.80 (3H, s), 5.70 (1H, s), 6.76 (1H, s), 6.83 (2H, d, J=11 Hz), 7.20—7.66 (5H, m), 7.56 (2H, d, J=11 Hz)	$C_{21}H_{23}NO_3$ (337)
4p	1640	1.56 (9H, s), 5.73 (1H, s), 6.77 (1H, s), 7.13—7.63 (9H, m)	$C_{20}H_{20}ClNO_2$ (342)
4q	1660	6.03 (1H, s), 6.83 (1H, s), 7.20—7.86 (15H, m)	—
4r	1645	2.33 (3H, s), 5.96 (1H, s), 6.77 (1H, s), 7.03—7.73 (14H, m)	$C_{23}H_{19}NO_2$ (341)
4s	1650	3.77 (3H, s), 5.90 (1H, s), 6.77 (1H, s), 6.90 (2H, d, J=9 Hz), 7.17—7.80 (10H, m), 7.63 (2H, d, J=9 Hz)	$C_{23}H_{19}NO_3$ (357)
4t	1655	5.96 (1H, s), 6.80 (1H, s), 7.10—7.73 (14H, m)	$C_{22}H_{16}ClNO_2$ (362)
4u	1660	1.60 (9H, s), 5.93 (1H, s), 7.23—7.60 (5H, m), 7.70 (2H, d, J=9 Hz), 8.17 (2H, d, J=9 Hz)	$C_{20}H_{20}N_2O_4$ (352)
4v	1675	6.13 (1H, s), 6.83 (1H, s), 7.20—7.66 (10H, m), 7.80 (2H, d, J=9 Hz), 8.20 (2H, d, J=9 Hz)	$C_{22}H_{16}N_2O_4$ (372)

a) Spectra of **4b—d, i—v** were taken in KBr. The spectrum of **4a** was taken neat.

TABLE VIII. Spectral Data for Amide Derivatives **7d, e, g** and β-Lactams **8d, e, g**

Compd. No.	IR ν_{max}^{KBr} cm^{-1} (C=O)	NMR ($CDCl_3$) δ	Formula MS (*m/z*, M^+)
7d	1695 1625	1.37 (9H, s), 2.40 (3H, s), 5.47—5.86 (1H, br), 7.23—7.70 (6H, m)	—
7e	1695 1630	1.10 (3H, t, J=7 Hz), 1.37 (9H, s), 2.77 (2H, q, J=7 Hz), 5.63 (1H, br)	$C_{16}H_{21}NO_2$ (259)
7g	1690 1655	1.33 (9H, s), 4.07 (2H, s), 6.50 (1H, br), 7.03—7.67 (11H, m)	$C_{21}H_{23}NO_2$ (321)
8d	1745 1710	1.23 (9H, s), 2.26 (3H, s), 3.90 (1H, d, J=2 Hz), 5.00 (1H, d, J=2 Hz), 7.30—7.50 (5H, m)	—
8e	1745 1710	1.07 (3H, t, J=7 Hz), 1.60 (9H, s), 2.53 (2H, q, J=7 Hz), 3.90 (1H, d, J=2 Hz), 5.00 (1H, d, J=2 Hz), 7.33 (5H, s)	$C_{16}H_{21}NO_2$ (259)
8g	1745 1710	1.23 (9H, s), 3.86 (2H, s), 3.93 (1H, d, J=2 Hz), 4.96 (1H, d, J=2 Hz), 7.03—7.46 (10H, m)	$C_{21}H_{23}NO_2$ (321)

uncorrected. IR spectra were taken on a Shimadzu IR-430 spectrometer. MS were recorded with a Hitachi RMU-6MG spectrometer. ^{1}H-NMR spectra were measured on a JEOL JNM-PMX 60 instrument. Chemical shifts are reported in δ values downfield relative to internal tetramethylsilane. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad, and dd=double doublet.

Preparation of Acyl Meldrum's Acids (1). General Procedure——A solution of an acid chloride (60 mmol) in $CHCl_3$ (50 ml) was added dropwise with stirring over a period of 1 h to a solution of Meldrum's acid (7.20 g, 50 mmol) and pyridine (7.9 g, 100 mmol) in $CHCl_3$ (150 ml), in an ice-salt bath. The temperature was maintained for an additional 1 h, then the reaction mixture was allowed to stand at room temperature for 1 h. The resulting mixture was washed with 10% HCl (3×30 ml) and then water (50 ml). The organic layer was separated, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting crude product was purified by recrystallization from the solvent indicated in Table III. When aroyl chlorides were used as the acid chloride, the use of 4-dimethylaminopyridine instead of pyridine resulted in satisfactory yields. The results and physical data (mp, IR and ^{1}H-NMR spectra) are summarized in Tables III and IV.

Preparation of 3 from 1 and 2. General Procedure——A solution of **1** (10 mmol) and **2** (10 mmol) in benzene (50 ml) was refluxed for 25 min, or kept at 50 °C (bath temperature) for 4 h. After removal of the solvent under reduced pressure, the remaining crude product (**3**) was purified by recrystallization from the solvent indicated in Table I. The results obtained are summarized in Table I. Reactions of **1j** with **2d** and **2e** gave **4u** and **4v**, respectively, besides **3u** and **3v** (see Table I).

Reaction of 1a, b with 2d, e to Give 3d, e, i and 5d, e, i——Benzene solutions of **1a** and **2d**, **1b** and **2d**, and **1a** and **2e** were each refluxed for 25 min. After work-up as described above, a mixture of **3** and **5** was obtained in each case. The products were separated by fractional recrystallization from ether and purified by recrystallization from the solvent shown in Table VI. Table VI also summarizes the results.

Decarboxylation of 5d to 4d——A solution of **5d** (1.19 g, 4.1 mmol) in toluene (30 ml) was refluxed for 10 min, and then washed successively with 10% Na_2CO_3 aqueous solution (3×10 ml) and water (10 ml). The organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated to give crude **4d**. Recrystallization of the crude product from ether–petroleum ether gave 0.1 g (10%) of **4d**, mp 89—91 °C. The IR spectrum of this product was identical with that of the sample obtained above. The starting **5d** was recovered in 65% yield by acidification of the aqueous layer. Prolonged reflux caused decomposition of **4d**.

Esterification of 5d to 6——A solution of excess diazomethane (prepared by the general method from *p*-toluenesulfonyl-*N*-methylnitrosoamide and KOH aqueous solution) in ether was added dropwise with stirring to a solution of **5d** (0.45 g, 1.55 mmol) in ether, with cooling in an ice bath. After completion of the addition, stirring was continued for 30 min. After evaporation of the solvent under reduced pressure, the residue was recrystallized from ether–petroleum ether to give **6**, 0.4 g (85%), mp 94—96 °C. *Anal.* Calcd for $C_{17}H_{21}NO_4$: C, 67.31; H, 6.98; N, 4.61. Found: C, 67.50; H, 7.02; N, 4.51. IR (KBr): 1715, 1650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53 (9H, s), 2.03 (3H, s), 3.73 (3H, s), 6.56 (1H, s), 7.37 (5H, s). MS *m/z*: 303 (M^+).

Synthesis of 4. General Procedure——i) Reaction of **1f—i** with **2d, e**: A solution of **1f—i** (10 mmol) and **2d, e**, in benzene (50 ml) was refluxed for 25 min. After work-up as described above, the crude **4m—t** obtained was purified by recrystallization from the solvent indicated in Table I. The results are summarized in Table I.

ii) Thermal Decomposition of **3h, i, m** to **4h, i, m**: A solution of **3h, i, m** (5 mmol) in benzene (50 ml) was refluxed for the period shown in Table II, and then the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography using hexane–ether (1 : 4) as eluent to give **4h, i, m**. The results are summarized

TABLE IX. Elemental Analyses of Compounds 3—5, 7 and 8

Compd. No.	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
3b	$C_{15}H_{17}NO_4$	65.44	6.22	5.09	65.43	6.12	4.93
3c	$C_{19}H_{17}NO_4$	70.58	5.30	4.33	70.68	5.24	4.56
3d	$C_{16}H_{19}NO_4$	66.42	6.62	4.84	66.21	6.48	4.74
3e	$C_{17}H_{21}NO_4$	67.31	6.98	4.62	67.07	7.02	4.37
3f	$C_{23}H_{23}NO_4$	73.19	6.14	3.71	73.24	6.13	3.56
3g	$C_{22}H_{23}NO_4$	72.31	6.34	3.83	72.24	6.28	3.63
3h	$C_{21}H_{27}NO_4$	70.56	7.61	3.92	70.78	7.67	3.78
3i	$C_{18}H_{15}NO_4$	69.88	4.91	4.53	69.83	4.88	4.44
3m	$C_{21}H_{21}NO_4$	71.78	6.02	3.99	71.80	5.93	3.92
3n	$C_{22}H_{23}NO_4$	72.31	6.34	3.83	72.31	6.39	3.82
3o	$C_{22}H_{23}NO_5$	69.28	6.08	3.67	69.21	6.00	3.51
3p	$C_{21}H_{20}ClNO_4$	65.37	5.22	3.63	65.35	5.02	3.48
3q	$C_{23}H_{17}NO_4$	74.38	4.61	3.77	74.12	4.72	3.77
3r	$C_{24}H_{19}NO_4$	74.79	4.97	3.63	74.90	4.86	3.55
3s	$C_{24}H_{19}NO_5$	71.81	4.77	3.49	71.57	4.67	3.29
3t	$C_{23}H_{16}ClNO_4$	68.07	3.97	3.45	67.93	4.01	3.51
3u	$C_{21}H_{20}N_2O_6$	63.63	5.08	7.07	63.59	5.23	6.91
3v	$C_{23}H_{16}N_2O_6$	66.34	3.87	6.73	66.44	4.03	6.63
4j	$C_{18}H_{17}NO_2$	77.40	6.13	5.10	77.14	6.05	4.72
4k	$C_{23}H_{19}NO_2$	80.92	5.61	4.10	81.06	5.81	3.93
4l	$C_{22}H_{23}NO_2$	79.25	6.95	4.20	79.30	7.02	4.00
4n	$C_{21}H_{23}NO_2$	78.47	7.21	4.36	78.53	7.10	4.19
4o	$C_{21}H_{23}NO_3$	74.75	6.87	4.15	74.99	7.00	4.22
4p	$C_{20}H_{20}ClNO_2$	70.27	5.90	4.10	70.34	5.85	4.04
4r	$C_{23}H_{19}NO_2$	80.92	5.61	4.10	80.84	5.58	4.11
4s	$C_{23}H_{19}NO_3$	77.29	5.36	3.92	77.31	5.35	3.87
4t	$C_{22}H_{16}ClNO_2$	73.03	4.46	3.87	73.16	4.47	3.81
4u	$C_{20}H_{20}N_2O_4$	68.19	5.72	7.95	68.12	5.77	7.71
4v	$C_{22}H_{16}N_2O_4$	70.96	4.33	7.52	70.90	4.40	7.37
5d	$C_{16}H_{19}NO_4$	66.42	6.62	4.84	66.18	6.55	4.68
5e	$C_{17}H_{21}NO_4$	67.31	6.98	4.64	67.22	6.88	4.50
5i	$C_{21}H_{21}NO_4$	69.88	4.91	4.53	69.93	4.72	4.23
7e	$C_{16}H_{21}NO_2$	74.10	8.16	5.40	73.97	8.35	5.21
7g	$C_{21}H_{23}NO_2$	78.47	7.21	4.36	78.48	7.30	4.08
8e	$C_{16}H_{21}NO_2$	74.10	8.16	5.40	73.96	8.28	5.19
8g	$C_{21}H_{23}NO_2$	78.47	7.21	4.36	78.22	7.28	4.13

in Table II.

Interchange Reaction of 3d and 3i with 2d and 2e——A solution of **3d** (1.45 g, 5 mmol) and **2e** (0.90 g, 5 mmol) in benzene (50 ml) was refluxed for 1 h and the solvent was removed under reduced pressure. The remaining mixture of **3d** and **3i** was purified by fractional recrystallization from ether to afford **3i** (0.8 g, 52%) and **3d** (0.3 g, 21%). Similar treatment of **3i** (1.55 g, 5 mmol) and **2d** (0.81 g, 5 mmol) in benzene (50 ml) gave **3d** (0.46 g, 32%) and **3i** (0.77 g, 50%).

Reaction of 1a with Cyclohexanone (9) Giving 10——A solution of **1a** (0.93 g, 5 mmol) and **9** (4.9 g, 50 mmol) was refluxed for 1 h, allowed to stand to room temperature, and then extracted with 10% Na_2CO_3 aqueous solution (3 × 20 ml). The aqueous layer was acidified with 10% HCl aqueous solution and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over $MgSO_4$ and concentrated under reduced pressure. The precipitate formed was collected by filtration and purified by recrystallization from petroleum ether to give **10** (0.8 g, 80%) as yellow prisms, mp 75—76.6 °C.

Thermal Transformation of 3d, e, g to 7d, e, g and 8d, e, g. General Procedure——A solution of **3d, e, g** (5 mmol) in benzene (50 ml) was refluxed for the time shown in Table II. After removal of the solvent, the residue was purified by silica gel column chromatography using hexane–ether (1 : 4) as the eluent to give the corresponding **7d, e, g** and **8d,**

e, g. The results are shown in Table II. The IR spectra of **7d** and **8d** were identical with those of corresponding authentic samples prepared in the reported manner.[3,5] The structural assignments of **7e, g** and **8e, g** were achieved on the basis of comparison of their IR and NMR spectra with those of authentic **7d** and **8d**.

Acknowledgment The authors are grateful to Dr. S. Suzuki of this college for mass spectral measurements.

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7) IR spectra of the known compounds were identical with those of corresponding authentic samples prepared by the reported methods.
8) The reasons for the differences of thermal stability of **3** and the alternative decompositions of **3** to **4** or **7** are not clear.
9) Chemoselective synthesis of the 1,3-oxazine-5-carboxylic acids **5** and the β-lactams **8** from acyl Meldrum's acids **1** and Schiff bases **2** will be described in the accompanying paper: Y. Yamamoto and Y. Watanabe, *Chem. Pharm. Bull.*, **35**, 1871 (1987).

[Chem. Pharm. Bull. 35(5)1871—1879(1987)]

1,3-Oxazines and Related Compounds. XIV.[1] Facile Synthesis of 2,3,6-Trisubstituted 2,3-Dihydro-1,3-oxazine-5-carboxylic Acids and 1,4-Disubstituted 3-Acyl-β-lactams from Acyl Meldrum's Acids and Schiff Bases

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Simple preparations of 2,3,6-trisubstituted 2,3-dihydro-1,3-oxazine-5-carboxylic acids (**4**) and 1,4-disubstituted 3-acyl-β-lactams (**5**) from acyl Meldrum's acids and Schiff bases were carried out. 2,3-Disubstituted 5-acyl-3,4,5,6-tetrahydro-2*H*-1,3-oxazine-4,6-diones (**3**) were isomerized to **4** in a chlorotrimethylsilane–triethylamine system and to **5** by hydrogen chloride.

Keywords——acyl Meldrum's acid; Schiff base; 1,3-oxazine-4,6-dione; 1,3-oxazine-5-carboxylic acid; β-lactam; acylketene; chlorotrimethylsilane; triethylamine

In the preceding paper,[1] we reported that the reaction of acyl Meldrum's acids (**1**, 5-acyl-2,2-dimethyl-1,3-dioxane-4,6-diones) and Schiff bases (**2**) afforded 5-acyl-1,3-oxazine-4,6-dione derivatives (**3**) as the major product together with a trace of 2,3,6-trisubstituted 2,3-dihydro-1,3-oxazin-4-one-5-carboxylic acids (**4**), which are isomers of **3**. In addition, the products (**3**) undergo thermal decomposition to give the β-lactams (**5**), though the yield was very low (Chart 1). We considered that improvement of the reaction conditions to give chemoselective formation of **4** and **5** would increase the yields and might also be useful for synthesis of the unique carboxylic acids (**4**) and the β-lactams (**5**). This paper describes facile and novel methods for the syntheses of **4** and **5**.

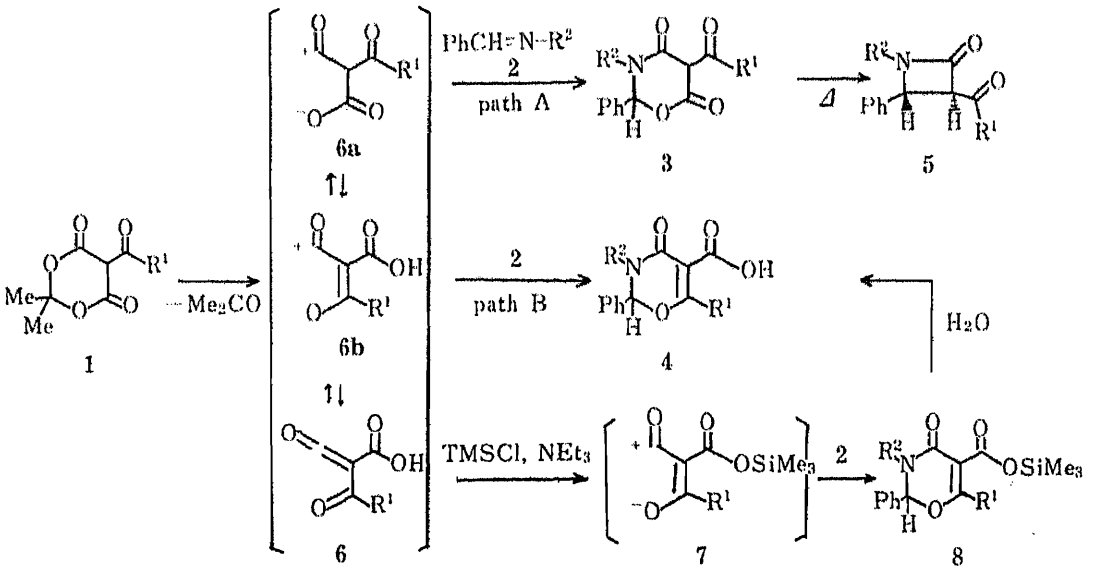
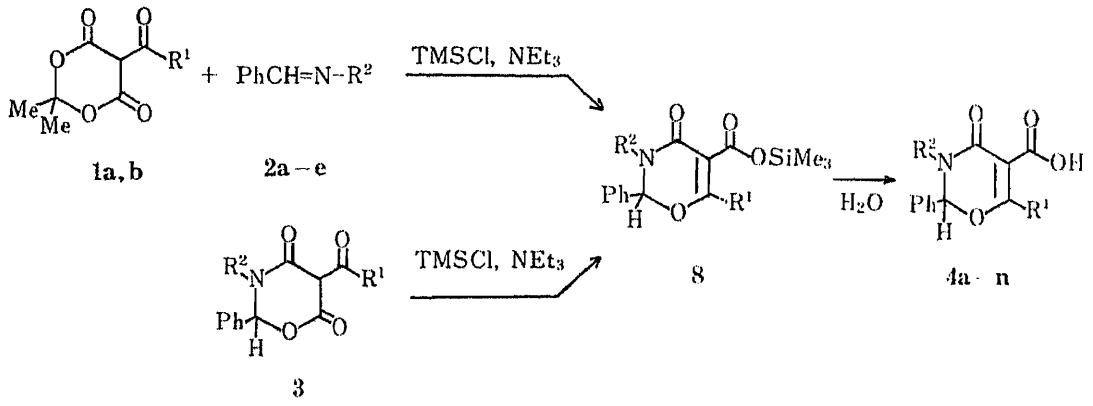


Chart 1

Synthesis of 1,3-Oxazine-5-carboxylic Acids (4)

In the preceding paper,[1] we showed that the carboxylic acids (**4**) are formed *via* the intermediates (**6**) generated *in situ* from **1** in the reaction of **1** with **2**, as illustrated in Chart 1. If the proportion of **6b** could be increased in the equilibrium of **6**, the yield of **4** would exceed that of **3**. It was expected that silylation of **6** would proceed to form the silyl esters (**7**) of the intermediates (**6**), and then 1,4-cycloaddition across the C=N bond through path B would take preference over that through path A, leading to the corresponding silyl esters (**8**) of **4**. In fact, it was found that reaction between **1** and **2** in the presence of chlorotrimethylsilane (TMSCl) and triethylamine (Et_3N) resulted in the effective formation of the desired **4**. Thus, a solution of 1 eq each of **1** and **2**, and 1.2 eq each of TMSCl and Et_3N in benzene was allowed to stand overnight with stirring at room temperature. The resulting mixture was washed with water, and usual work-up gave the carboxylic acids (**4**) in good yield. The results are summarized in Table I. If the aqueous treatment described above was omitted, the corresponding trimethylsilyl esters (**8**) could be isolated as distillable liquids. However, some difficulty in the isolation was experienced, since these silyl esters are highly sensitive to moisture.



1a : R^2 = Me
1b : R^1 = Ph
2a : R^2 = Pr
2b : R^2 = iso-Pr
2c : R^2 = $PhCH_2$
2d : R^2 = *tert*-Bu
2e : R^2 = Ph

Chart 2

In the preceding paper[1] we also showed that the diones (**3**) readily undergo retro Diels-Alder type reaction to give the acylketene intermediates (**6**) *in situ*. Therefore, isomerization of **3** into **4** in this fashion was achieved successfully; a benzene solution of **3** in the presence of 1.2-fold molar excess of both TMSCl and Et_3N was allowed to stand overnight at room temperature, followed by work-up as described above, to give the carboxylic acids (**4**) (Table I).

In addition, interchange reaction of the Schiff base moiety in the oxazine ring of **3** and simultaneous isomerization to **4** under the same conditions were found to take place; analogous treatment of **3d** in benzene with **2e** in the presence of TMSCl and Et_3N afforded the exchanged product (**4i**) in good yield along with **4d**. Similar treatment of **3i** with **2d** gave **4i** as the major product together with a small amount of **4d**.

On the basis of these observations, the sequential steps formulated in Chart 1 are suggested as the reaction pathway for the formation of **4**, involving generation of the silylated acylketene intermediates (**7**) *in situ* from **1** or **3**.

Synthesis of the β-Lactams (5)

A noteworthy observation concerning the reactivity of **3** was its transformation into the

TABLE I. Preparation of 1,3-Oxazine-5-carboxylic Acids (**4a—n**)

Compd. No.	R^1	R^2	Yield (%)	Method[a]
4a	Me	Pr	88	I
			75	II
4b	Me	iso-Pr	87	I
4c	Me	$PhCH_2$	84	I
4d	Me	*tert*-Bu	83	I
			75.5	II
4e	Et	*tert*-Bu	71	II
4f	Styryl	*tert*-Bu	90	II
4g	$PhCH_2$	*tert*-Bu	70	II
4h	Cyclohexyl	*tert*-Bu	77.5	II
4i	Me	Ph	89	I
			84	II
4j	Ph	Pr	90	I
4k	Ph	iso-Pr	89	I
4l	Ph	$PhCH_2$	88	I
4m	Ph	*tert*-Bu	90	I
			72	II
4n	Ph	Ph	75	I

a) I, from acyl Meldrum's acids (1) and Schiff bases (2); II, by the isomerization of 3.

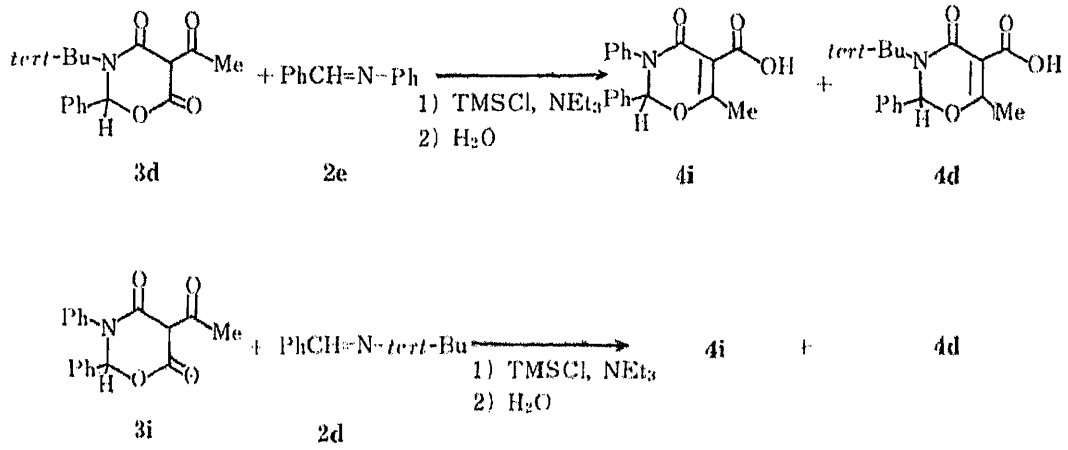
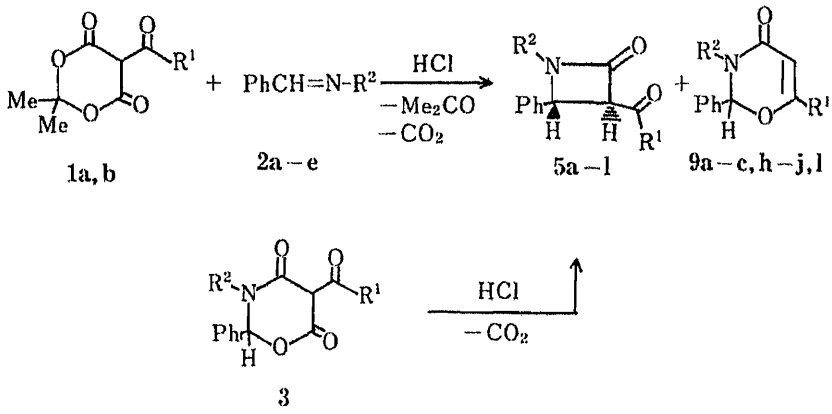


Chart 3

β-lactams (**5**); thus, it was found that the use of hydrogen chloride instead of the TMSCl-Et_3N system in the reaction of **1** with **2** described above resulted in formation of the β-lactams (**5**) exclusively. This provides an additional synthetic methodology for β-lactams and extends the practical utility of acyl Meldrum's acids in synthetic organic chemistry.

An ice-cooled solution of **1a** and **2a** in benzene was saturated with hydrogen chloride followed by heating under reflux to give the β-lactam (**5a**) along with the 1,3-oxazin-4-one derivative (**9**) as a by-product. Thus, the β-lactams (**5b—d, h—l**) were prepared. In the case of **1b**, use of xylene instead of benzene as a solvent gave a better result. The β-lactams (**5**) were also obtained from the oxazines (**3**) by analogous treatment (Table II). Thus, a solution of **3** in benzene or xylene was heated under reflux while hydrogen chloride was bubbled into the solution; usual work-up gave **5** as a sole product. Attempts to use various protic or Lewis acids were unsuccessful.[6] Furthermore, it was found that a small amount of hydrochloride of **2** was also effective, but a prolonged reaction period was required. Taking these findings into

consideration, formation of the β-lactams (**5**) could be explained in terms of [2+2] cycloaddition between the Schiff bases (**2**) and acylketenes (**10**) generated *in situ*, so hydrogen chloride would presumably play a role in decreasing the basicity of the N atom in **2** to cause preferential C–C bond formation between the carbon atom of the C=N group and the α-carbon of **10**.[7]



1a, b **2a–e** **5a–l** **9a–c, h–j, l** **3**

1a : R^1=Me
1b : R^1=Ph

2a : R^2=Pr **2b** : R^2=iso-Pr **2c** : R^2=$PhCH_2$ **2d** : R^2=*tert*-Bu **2e** : R^2=Ph

Chart 4

TABLE II. Preparation of the β-Lactams (**5a—l**)

5	Substituent		Solvent[a]	Product		Method[b]
	R^1	R^2		**5** Yield (%)	**9** Yield (%)	
a	Me	Pr	A	52	21	I
			A	Recovery (87%)		II
			B	12	—	II
b	Me	iso-Pr	A	68	9	I
			A	Recovery (80%)		II
c	Me	$PhCH_2$	A	42	30	I
			A	Recovery (86%)		II
d	Me	*tert*-Bu	A	82	—	I
			A	82	—	II
e	Et	*tert*-Bu	A	77	—	II
f	$PhCH_2$	*tert*-Bu	A	81	—	II
g	Cyclohexyl	*tert*-Bu	A	64	—	II
h	Ph	Pr	B	43	34	I
i	Ph	iso-Pr	B	61	14	I
j	Ph	$PhCH_2$	B	35	6	I
k	Ph	*tert*-Bu	B	85	—	I
			A	46	—	II
l	Ph	Ph	B	54	12	I

a) A, benzene; B, xylene. *b*) I, from acyl Meldrum's acids (**1**) and Schiff bases (**2**); II, by the transformation of 1,3-oxazine-4,6-diones (**3**).

Therefore, reactions of 2,2,6-trimethyl-1,3-dioxin-4-one (**11**) and diketene (**12**), both well-known precursors of acetylketene, with the Schiff bases (**2**) in the presence of hydrogen chloride were carried out. In both cases, the expected β-lactams (**5**) were obtained in satisfactory yields.

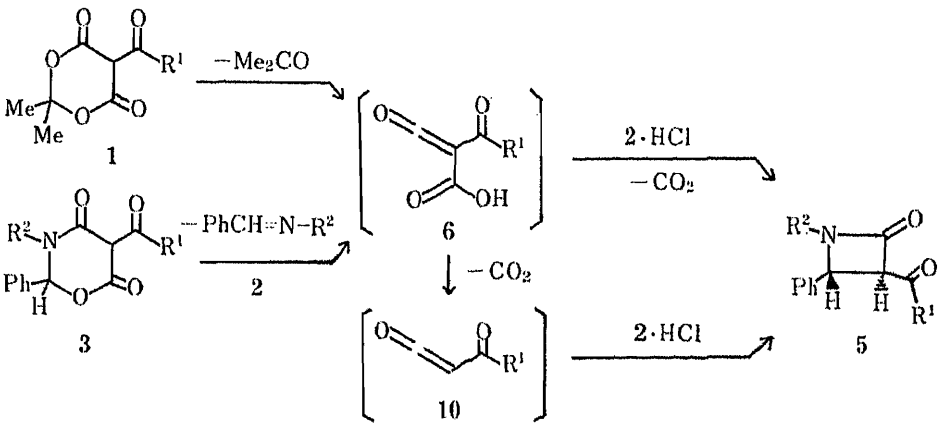


Chart 5

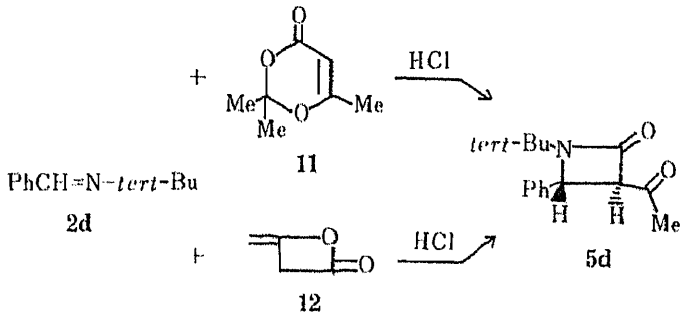


Chart 6

Experimental

Melting points were determined on a Mel-Temp melting point apparatus with an open capillary tube, and are uncorrected. Infrared (IR) spectra were taken on a Shimadzu IR-430 spectrometer. Mass spectra (MS) were recorded with a Hitachi RMU-6MG spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a JEOL JNM-PMX 60 instrument. Chemical shifts are reproted in δ values downfield relative to internal tetramethylsilane. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, and dd = double doublet.

Preparation of 3,4-Dihydro-2*H*-1,3-oxazin-4-one-5-carboxylic Acids (4). General Procedure——i) From Acyl Meldrum's Acids (1) and Schiff Bases (2): A solution of TMSCl (0.65 g, 6 mmol) in dry benzene (10 ml) was added with stirring at room temperature to a solution of an acyl Meldrum's acid (**1**, 5 mmol), a Schiff base (**2a**—**e**, 5 mmol) and Et_3N (0.61 g, 6 mmol) in dry benzene (30 ml). The reaction mixture was allowed to stand overnight with stirring at room temperature. The precipitate formed was removed by filtration and the filtrate was washed successively with 10% HCl (2 × 20 ml) and water (10 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the crude product (**4a**—**d**, **i**—**n**), which was purified by recrystallization from the solvent shown in Table III. The results are summarized in Table I.

ii) Isomerization of **3** to **4**. Typical Procedure: A solution of TMSCl (0.65 g, 6 mmol) in dry benzene (10 ml) was added dropwise with stirring to a solution of **3d** (1.45 g, 5 mmol) and Et_3N (0.61 g, 6 mmol) in dry benzene (30 ml) at room temperature. The reaction mixture was allowed to stand overnight with stirring at room temperature, followed by the same procedure as described above in i). Recrystallization of the crude product gave colorless prisms of **4d**, 1.2 g (83%). The IR spectrum of this product was identical with that of the sample obtained in i).

Reaction of the Oxazines (3) with Schiff Bases (2) in the Presence of TMSCl and Et_3N——i) A solution of TMSCl (0.65 g, 6 mmol) in dry benzene was added dropwise with stirring at room temperature to a solution of **3d** (1.45 g, 5 mmol), **2e** (0.90 g, 5 mmol) and Et_3N (0.61 g, 6 mmol) in dry benzene (30 ml). The reaction mixture was stirred overnight at room temperature. The precipitate formed was removed by filtration and the filtrate was washed successively with 10% HCl (2 × 20 ml) and water (10 ml). The resulting organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Fractional recrystallization of the residual solid from ethyl acetate gave **4i** (1.1 g, 71%) along with **4d** (0.1 g, 7%). The IR spectra of these products were identical with those of the respective samples obtained in the above run.

TABLE III. Physical and Analytical Data for **4a**—**n**

Compd. No.	mp (°C)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found)		
				C	H	N
4a	165—166 (dec.)	B	$C_{15}H_{17}NO_4$	65.44 (65.60	6.22 6.16	5.09 5.08)
4b	155—156 (dec.)	B	$C_{15}H_{17}NO_4$	65.44 (65.38	6.22 6.18	5.09 4.80)
4c	115—116	B	$C_{19}H_{17}NO_4$	70.58 (70.62	5.30 5.20	4.33 4.12)
4d	130—131 (dec.)	A	$C_{16}H_{19}NO_4$	66.42 (66.18	6.62 6.55	4.84 4.68)
4e	119—121 (dec.)	A	$C_{17}H_{21}NO_4$	67.31 (67.22	6.98 6.88	4.62 4.50)
4f	156—157 (dec.)	B	$C_{23}H_{23}NO_4$	73.19 (73.15	6.14 6.32	3.71 3.49)
4g	142—143 (dec.)	B	$C_{22}H_{23}NO_4$	72.31 (72.25	6.34 6.19	3.83 3.53)
4h	159—160 (dec.)	A	$C_{21}H_{27}NO_4$	70.56 (70.31	7.61 7.77	3.92 3.79)
4i	159—160 (dec.)	B	$C_{18}H_{15}NO_4$	69.88 (69.93	4.91 4.72	4.53 4.23)
4j	135—136 (dec.)	B	$C_{20}H_{19}NO_4$	71.20 (71.05	5.68 5.69	4.15 3.94)
4k	143—144 (dec.)	B	$C_{20}H_{19}NO_4$	71.20 (70.92	5.68 5.82	4.15 4.04)
4l	149—150 (dec.)	C	$C_{24}H_{19}NO_4$	74.79 (74.85	4.97 4.97	3.63 3.45)
4m	137—138 (dec.)	B	$C_{21}H_{21}NO_4$	71.78 (71.77	6.02 6.00	3.99 3.71)
4n	130 (dec.)	B	$C_{23}H_{17}NO_4$	74.38 (74.34	4.61 4.53	3.77 3.68)

a) A, ether; B, ethyl acetate; C, acetone.

ii) The reaction between **3i** and **2d** under the same conditions as described in i) afforded **4i** (1 g, 64.5%) and **4d** (0.15 g, 7%).

The IR spectra were identical with those of the respective samples obtained in the above run.

Synthesis of the Trimethylsilyl Ester (8) of 3a——A solution of TMSCl (0.65 g, 6 mmol) in benzene (10 ml) was added dropwise with stirring to a solution of acetyl Meldrum's acid (**1a**, 0.93 g, 5 mmol), the Schiff base (**2a**, 0.74 g, 5 mmol), and Et_3N (0.61 g, 6 mmol) in benzene (30 ml) at room temperature. The reaction solution was allowed to stand overnight with stirring. After removal of the precipitates formed by filtration, the filtrate was distilled under an atomosphere of nitrogen to give 1.9 g, (55%) of **8** as a pale yellow liquid. IR (CCl_4): 1660 cm^{-1}, ^{1}H-NMR ($CDCl_3$) δ: 0.33 (9H, s), 0.63—1.10 (3H, m), 1.17—1.86 (2H, m), 2.17 (3H, s), 2.63—3.17 (1H, m), 3.40—4.00 (1H, m), 6.17 (1H, s), 7.43 (5H, s).

Preparation of the β-Lactams (5)——i) From Acyl Meldrum's Acids (**1**) and Schiff Bases (**2**). General Procedure: Hydrogen chloride was passed into an ice-cooled solution of **1** (5 mmol) and **2** (5 mmol) in dry benzene (50 ml) until saturation. The resulting mixture was refluxed for 1 h and then washed with water (2 × 30 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using hexane–ether (1 : 1) as an eluent to give **5**. Subsequent elution with ether gave **9**. In the reaction of **1b**, xylene was used as a solvent instead of benzene. The results obtained are summarized in Table II.

ii) Transformation of **3** into **5**. General Procedure: A solution of **3** (5 mmol) in benzene (50 ml) was refluxed for 3 h while hydrogen chloride was passed into the reaction solution. The resulting solution was washed with water (2 × 30 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residual solid was purified by recrystallization from the solvent shown in Table IV.

iii) Reaction of **1a** and **2d** in the Presence of the Hydrochloride of **2d**: A solution of **1a** (0.93 g, 5 mmol), the Schiff

TABLE IV. Physical and Analytical Data for the β-Lactams (**5a**—**l**)

Compd. No.	mp (°C) or bp (°C/Torr) (Lit. mp)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found)		
				C	H	N
5a	101/0.15[b]		—		—	
5b	101—103/0.2[b]		—		—	
5c	129—130/0.15[b]		—		—	
5d	89—91 (90—91)[b]	E	—		—	
5e	78—79	D	$C_{16}H_{21}NO_2$	74.10 (73.96	8.16 8.28	5.40 5.19)
5f	89—90	D	$C_{21}H_{23}NO_2$	78.47 (78.22	7.21 7.28	4.36 4.13)
5g	38—39	C	$C_{20}H_{27}NO_2$	76.64 (76.35	8.68 8.98	4.47 4.34)
5h	83—84	D	$C_{19}H_{19}NO_2$	77.79 (77.95	6.53 6.61	4.77 4.51)
5i	93—94	E	$C_{19}H_{19}NO_2$	77.79 (77.79	6.53 6.47	4.77 4.59)
5j	76—77	D	$C_{23}H_{19}NO_2$	80.92 (81.01	5.61 5.38	4.10 4.14)
5k	133—134	E	$C_{20}H_{21}NO_2$	78.15 (78.35	6.89 7.16	4.56 4.37)
5l	42—44	C	$C_{22}H_{17}NO_2$	80.71 (80.76	5.23 5.11	4.28 4.20)

a) C, petroleum ether; D, ether–petroleum ether; E, ether; F, ethyl acetate. *b*) Ref. 2.

TABLE V. Physical and Analytical Data for **9a**—**c**, **h**—**j**, **l**

Compd. No.	mp (°C) or bp (°C/Torr) (Lit. mp)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found)		
				C	H	N
9a	100—101/0.2[b]		—		—	
9b	100—102 (100—102)[b]	A	—		—	
9c	73—75 (77—79)[b,c]	A	—		—	
9h	134—135	C	$C_{19}H_{19}NO_2$	77.79 (77.76	6.53 6.53	4.77 4.59)
9i	143—144	B	$C_{19}H_{19}NO_2$	77.79 (77.88	6.53 6.28	4.77 4.56)
9j	123—124	C	$C_{23}H_{19}NO_2$	80.92 (81.12	5.61 5.74	4.10 3.99)
9l	153—155 (146—148)[d]	C	—		—	

a) A, ether–petroleum ether; B, ether; C, ethyl acetate. *b*) Ref. 2. *c*) Ref. 5. *d*) Ref. 3.

base (**2d**, 0.81 g, 5 mmol), and a trace of the hydrochloride[8] of **2d** in benzene (50 ml) was refluxed for 6 h with stirring. The resulting solution was washed with water (2 × 30 ml), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was recrystallized from ether–petroleum ether to give **5d**, 1.0 g (81%).

iv) Reaction of 2,2,6-Trimethyl-1,3-dioxin-4-one (**11**) with the Schiff Base (**2d**): Hydrogen chloride was passed into an ice-cooled solution of **11** (0.71 g, 5 mmol) and **2d** (0.81 g, 5 mmol) in dry xylene (50 ml). The reaction mixture was refluxed for 1 h and then washed with water (2 × 30 ml). The organic layer was dried over anhydrous Na_2SO_4 and

TABLE VI. Spectral Data for 1,3-Oxazine-5-carboxylic Acids (**4a—n**)

Compd. No.	IR ν_{max}^{KBr} cm^{-1}	NMR ($CDCl_3$) δ	Formula MS (m/z, M^+)
4a	2800—2100 1735	0.86 (3H, t, J=7 Hz), 1.26—1.96 (2H, m), 2.43 (3H, s), 2.67—3.23 (1H, m), 3.56—4.10 (1H, m), 6.26 (1H, s), 7.43 (5H, s), 14.10 (1H, s)	$C_{15}H_{17}NO_4$ (275)
4b	2800—2100 1730	1.10 (3H, d, J=7 Hz), 1.33 (3H, d, J=7 Hz), 2.37 (3H, s), 4.40—5.13 (1H, m), 6.46 (1H, s), 7.40 (5H, s), 14.20 (1H, s)	$C_{15}H_{17}NO_4$ (275)
4c	2800—2100 1730	2.46 (3H, s), 3.96 (1H, d, J=15 Hz), 5.30 (1H, d, J=15 Hz), 6.20 (1H, s), 6.96—7.60 (10H, m), 14.07 (1H, s)	$C_{19}H_{17}NO_4$ (323)
4d	2800—2100 1725	1.56 (9H, s), 2.33 (3H, s), 6.66 (1H, s), 7.37 (5H, s), 14.33 (1H, s)	$C_{16}H_{19}NO_4$ (289)
4e	2800—2200 1725	0.93 (3H, t, J=7 Hz), 1.53 (1H, s), 2.70 (2H, q, J=7 Hz), 6.70 (1H, s), 7.40 (5H, s), 14.43 (1H, s)	$C_{17}H_{21}NO_4$ (303)
4f	2800—2200 1720	1.60 (9H, s), 6.83 (1H, s), 7.20—7.70 (11H, m), 7.96 (1H, d, J=16 Hz), 14.63 (1H, s)	$C_{23}H_{23}NO_4$ (377)
4g	2800—2200 1730	1.53 (9H, s), 3.67 (1H, d, J=14 Hz), 4.60 (1H, d, J=14 Hz), 6.60 (1H, s), 6.86—7.33 (10H, m), 14.53 (1H, s)	$C_{22}H_{23}NO_4$ (365)
4h	2800—2200 1730	0.60—1.90 (10H, m), 1.53 (9H, s), 3.20—3.80 (1H, m), 6.67 (1H, s), 7.33 (5H, m), 14.56 (1H, s)	$C_{21}H_{27}NO_4$ (357)
4i	2800—2100 1730	2.50 (3H, s), 6.63 (1H, s), 7.07—7.56 (10H, m), 13.73 (1H, s)	$C_{18}H_{15}NO_4$ (309)
4j	2800—2100 1730	0.86 (3H, t, J=7 Hz), 1.26—1.93 (2H, m), 2.80—3.33 (1H, m), 3.53—4.03 (1H, m), 7.23—7.63 (10H, m), 14.03 (1H, s)	$C_{20}H_{19}NO_4$ (337)
4k	2800—2100 1730	1.16 (3H, d, J=7 Hz), 1.37 (3H, d, J=7 Hz), 4.50—5.13 (1H, m), 6.60 (1H, s), 7.10—7.80 (10H, m), 14.00 (1H, s)	$C_{20}H_{19}NO_4$ (337)
4l	2800—2100 1720	4.07 (1H, d, J=15 Hz), 5.23 (1H, d, J=15 Hz), 6.33 (1H, s), 6.93—7.67 (15H, m), 13.90 (1H, s)	$C_{24}H_{19}NO_4$ (385)
4m	2800—2200 1730	1.60 (9H, s), 6.86 (1H, s), 7.26—7.67 (10H, m), 14.17 (1H, s)	$C_{21}H_{21}NO_4$ (351)
4n	2800—2100 1730	6.80 (1H, s), 7.20—7.77 (15H, m), 13.63 (1H, s)	$C_{23}H_{17}NO_4$ (371)

concentrated under reduced pressure. The residual solid was recrystallized from ether–petroleum ether to give **5d**, 0.84 g (68%).

v) Reaction of Diketene (**12**) with the Schiff Base (**2d**): Hydrogen chloride was introduced into a ice-cooled solution of **2d** (0.81 g, 5 mmol), and then diketene (**12**, 0.42 g, 5 mmol) was added. The reaction mixture was refluxed for 6 h and then washed with water (2 × 30 ml). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was recrystallized from ether–petroleum ether to give **5d**, 0.7 g (57%).

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References and Notes

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6) As protic or Lewis acids, sulfuric acid, trifluoroacetic acid, *p*-toluenesulfonic acid, hydrogen bromide, boron trifluoride etherate, and titanium trichloride were examined. Only hydrogen bromide (30% HBr in acetic acid was used) was slightly effective.
7) T. Kato *et al.* have investigated in detail the reaction of Schiff bases and acylketenes and have demonstrated that the reaction under basic or neutral conditions gives the [2+4] cycloaddition products (1,3-oxazin-4-one derivatives (**8**)) as the major products. See ref. 2*b*.
8) A few drops of a benzene solution of the hydrochloride, which was prepared just before use by passing hydrogen chloride into a solution of **2d**, were employed.

TABLE VII. Spectral Data for the β-Lactams (**5a—l**)

Compd. No.	IR $\nu^{a)}$ cm^{-1} (C=O)	NMR ($CDCl_3$) δ	Formula MS (m/z, M^+)
5a	1740 1710	0.86 (3H, t, J=7 Hz), 1.20—1.86 (2H, m), 2.26 (3H, s), 2.56—3.70 (2H, m), 4.03 (1H, d, J=2 Hz), 4.96 (1H, d, J=2 Hz), 7.26 (5H, s)	—
5b	1740 1710	1.07 (3H, d, J=7 Hz), 1.30 (3H, d, J=7 Hz), 2.30 (3H, s), 3.46—3.96 (1H, m), 4.00 (1H, d, J=2 Hz), 5.00 (1H, d, J=2 Hz), 7.37 (5H, s)	—
5c	1745 1710	2.30 (3H, s), 3.80 (1H, d, J=15 Hz), 4.07 (1H, d, J=2 Hz), 4.76 (1H, d, J=15 Hz), 4.83 (1H, d, J=2 Hz), 6.90—7.46 (10H, m)	—
5d	1745 1710	1.23 (9H, s), 2.26 (3H, s), 3.90 (1H, d, J=2 Hz), 5.00 (1H, d, J=2 Hz), 7.30 (5H, s)	—
5e	1745 1710	1.07 (3H, t, J=7 Hz), 1.60 (9H, s), 2.53 (2H, q, J=7 Hz), 3.90 (1H, d, J=2 Hz), 5.00 (1H, d, J=2 Hz), 7.33 (5H, s)	$C_{16}H_{21}NO_2$ (259)
5f	1745 1710	1.23 (9H, s), 3.86 (2H, s), 3.93 (1H, d, J=2 Hz), 4.96 (1H, d, J=2 Hz), 7.03 (1H, d, J=2 Hz)	$C_{21}H_{23}NO_2$ (321)
5g	1745 1700	0.67—2.86 (11H, m), 1.23 (9H, s), 3.96 (1H, d, J=2 Hz), 4.90 (1H, d, J=2 Hz)	$C_{20}H_{27}NO_2$ (313)
5h	1745 1670	0.93 (3H, t, J=7 Hz), 1.23—1.90 (2H, m), 2.60—3.82 (2H, m), 4.73 (1H, d, J=2 Hz), 5.62 (1H, d, J=2 Hz), 7.10—7.63 (8H, m), 7.96—8.20 (2H, m)	$C_{19}H_{19}NO_2$ (293)
5i	1740 1670	1.10 (3H, d, J=7 Hz), 1.33 (3H, d, J=7 Hz), 3.50—4.03 (1H, m), 4.63 (1H, d, J=2 Hz), 5.26 (1H, d, J=2 Hz), 7.20—7.63 (8H, m), 7.90—8.20 (2H, m)	$C_{19}H_{19}NO_2$ (293)
5j	1750 1670	3.86 (1H, d, J=15 Hz), 4.77 (1H, d, J=2 Hz), 4.83 (1H, d, J=15 Hz), 5.13 (1H, d, J=2 Hz), 6.93—7.56 (13H, m), 7.90—8.16 (2H, m)	$C_{23}H_{19}NO_2$ (341)
5k	1750 1670	1.26 (9H, s), 4.56 (1H, d, J=2 Hz), 5.30 (1H, d, J=2 Hz), 7.20—7.60 (8H, m), 7.86 (2H, m)	$C_{20}H_{21}NO_2$ (307)
5l	1750 1675	4.80 (1H, d, J=2 Hz), 5.73 (1H, d, J=2 Hz), 6.73—8.30 (15H, m)	$C_{22}H_{17}NO_2$ (327)

a) The spectra of **5d—l** were taken in KBr. Those of **5a—c** were taken neat.

TABLE VIII. Spectral Data for 2,3-Dihydro-1,3-oxazin-4-ones (**9a - c, h—j, l**)

Compd. No.	IR $\nu^{a)}$ cm^{-1} (C=O)	NMR ($CDCl_3$) δ	Formula MS (m/z, M^+)
9a	1650	0.86 (3H, t, J=7 Hz), 1.23—1.77 (2H, m), 1.83 (3H, s), 2.53—3.07 (1H, m), 3.53—4.10 (1H, m), 5.20 (1H, s), 6.13 (1H, s), 7.33 (5H, s)	—
9b	1655	1.07 (3H, d, J=7 Hz), 1.30 (3H, d, J=7 Hz), 1.76 (3H, s), 4.60—5.10 (1H, m), 5.23 (1H, s), 6.33 (1H, s), 7.40 (5H, s)	—
9c	1670	1.83 (3H, s), 3.80 (1H, d, J=15 Hz), 5.30 (1H, s), 5.33 (1H, d, J=15 Hz), 6.03 (1H, s), 7.00—7.50 (15H, m)	—
9h	1650	0.90 (3H, t, J=7 Hz), 1.30—1.86 (2H, m), 2.63—3.20 (1H, m), 3.63—4.20 (1H, m), 5.90 (1H, s), 6.37 (1H, s), 7.20—7.73 (10H, m)	$C_{19}H_{19}NO_2$ (293)
9i	1640	1.13 (3H, d, J=7 Hz), 1.37 (3H, d, J=7 Hz), 4.56—5.20 (1H, m), 5.86 (1H, s), 6.50 (1H, s), 7.20—7.73 (10H, m)	$C_{19}H_{19}NO_2$ (293)
9j	1645	3.90 (1H, d, J=15 Hz), 5.43 (1H, d, J=15 Hz), 5.96 (1H, s), 6.26 (1H, s), 7.03—7.73 (15H, m)	$C_{23}H_{19}NO_2$ (341)
9l	1660	6.03 (1H, s), 6.83 (1H, s), 7.20—7.86 (15H, m)	—

a) The spectra of **8b, c, h—j, l** were taken in KBr. The spectrum of **8a** was taken neat.

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Electrostatic Interaction Energy and Solvent Accessibility in the Methotrexate–Reduced Nicotinamide Adenine Dinucleotide Phosphate–Dihydrofolate Reductase Ternary Complex

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The electrostatic interaction energies among dihydrofolate reductase (DHFR), coenzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH) and methotrexate (MTX) were calculated by means of the Debye–Hückel equation taking the solvent accessibility into account; the results obtained were comparable with the experimental values. The atoms of guest molecules exposed on the molecular surface in NADPH and MTX may contribute to the electrostatic stabilization on the molecular complex formed with the host molecules. The electrostatic effects due to the replacement of charged amino acid residues with other ones or uncharged ones were visualized as the difference of two electrostatic correlation potentials by the use of computer graphics. It is shown that Arg43, Arg44, Arg57, and Asp26 of DHFR play important roles from the view point of electrostatic potential. A distance graphics method was used to express the distance from a patch on the van der Waals' surface of the guest molecule to the nearest host-molecular surface in order to visualize the buried ligand, and host-atom contacts with the guest atom are given as the contact ratio of the guest-atoms with the proper host atoms.

Keywords——electrostatic complementarity; solvent accessibility; Debye–Hückel equation; electrostatic correlation potential; distance graphics; buried effect; dihydrofolate reductase; methotrexate; electrostatic interaction energy; NADPH

Dihydrofolate reductase (DHFR) is an enzyme which catalyzes the conversion from dihydrofolate to tetrahydrofolate with the coenzyme reduced nicotinamide adenine dinucleotide phosphate (NADPH). Methotrexate (MTX), a competitive substrate inhibitor of DHFR, is used as an anticancer drug. The structure of the ternary complex, MTX–NADPH–DHFR, has been determined by X-ray diffraction experiments.[1] Many amino acid residues in DHFR directly interact through van der Waals' contacts with NADPH and MTX.[1] Side chains of Arg43 and Arg44 interact with NADPH through adenine–ribose phosphate and pyrophosphate groups, respectively. In the DHFR family, amino acid residues at position 43 are basic amino acid residues such as Arg or Lys,[2] and also those at position 44 are similar residues such as Arg, Lys or His.[2] These facts indicate that the positive charges of basic amino acids are needed to stabilize the negative charges of NADPH at those positions. MTX binds to DHFR at the pteridine ring (pK_a more than 10) due to ion-pair formation of the pteridine ring with Asp26 anion.[3] The pK_a shift from 5.7 to 10 means that the system gains free energy of more than 6.1 kcal/mol at 37 °C through an increase in electrostatic interaction energy. Since the moieties of MTX other than the pteridine ring are the same as those of dihydrofolate, the substrate, the interaction modes of the C-terminal and side chain carboxyl groups of the glutamic acid moiety in MTX should reflect those in the enzymatic reaction. The side chain of Arg57 forms an ion-pair with the C-terminal carboxyl group of MTX.[1] Arg57 is completely conserved in the DHFR family,[2] and is thought to be

electrostatically important. The His28 side chain interacts with the side chain carboxyl group of the glutamic acid moiety in MTX,[1] but is not necessarily conserved as a positively charged amino acid residue in the DHFR family.[2] This indicates that the stabilization by the electrostatic interaction energy is not required or is small in the enzymatic reaction.

If the various types of electrostatic interaction energies mentioned above are estimated quantitatively, elucidation of the enzymatic reaction mechanism of the substrate and the inhibition mechanism of inhibitors may become much easier. In this paper, we introduce first the equations of electrostatic interaction energy including apparent dielectric constant as a function of the solvent accessibility, and then we estimate the electrostatic potentials, which can be visualized by a computer graphics method. The roles of Arg43, Arg44, Asp26, Arg57, and His28 of DHFR in the electrostatic interaction were examined by graphical simulation. Computer graphics studies on the inhibitor binding to DHFR, trypsin, and prealbumin *etc.* by Kollman *et al.*,[4] Hansch *et al.*,[5] and Langridge *et al.*,[4a,5a-c,6] dealt with solvent-accessible surface and electrostatic potential. We have also performed a computer graphics study. The elctrostatic-potential complementarity between the guest and host molecules was examined by using two models for dielectric constant (ε), *i.e.*, assuming a constant value ($\varepsilon=4$)[7] and assuming a linear distance dependence.[8] Moreover, the use of the normalized electrostatic correlation potential was proposed to visualize the electrostatic-potential complementarity of the guest and host potentials.[9] In this paper, the difference electrostatic correlation potential and the contact of the guest and host molecules are visualized on the molecular surface of the guest molecule.

Method

Atomic coordinates of DHFR, NADPH and MTX (except hydrogens) were obtained from the Protein Data Bank[10] (code number 3DFR). Positions of the hydrogen atoms except $-CH_3$, $-NH_3^+$, $-CONH_2$, $-OH$, $-NHC(NH)NH_2$, and $-NHC(NH_2)_2^+$ of DHFR were determined geometrically. The positions of the hydrogen atoms in NADPH were determined as follows: the coordinates of HNO3* and HAO3* (see Fig. 1a for atom designation) were set so that they formed hydrogen bonds to O of His28 and intramolecular APO3, respectively. HNO2* was positioned so as to point towards O of Gly17. Coordinates of other hydrogen atoms in NADPH and MTX were determined geometrically, using the values of bond length, bond angle and dihedral angle of methane, benzene, pyridine, aniline, and adenine.

The electrostatic energy was calculated according to the following equation[11]

$$E=\sum_{i>j}\sum\{q_i\cdot q_j/(\varepsilon_{ij}\cdot r_{ij})\}\cdot e^{-K\cdot r_{ij}},$$

where r_{ij} is the distance between atoms i and j, ε_{ij} is an apparent dielectric constant, and $e^{-K\cdot r_{ij}}$ is the term obtained from the Debye–Hückel equation. ε_{ij} was determined so as to include solvent effects such as hydration, considering the solvent accessibility according to the following equation;

$$\varepsilon_{ij}=2/(1/\varepsilon_i+1/\varepsilon_j),$$

$$\varepsilon_i=(78.5-4.0)\cdot A_i+4.0$$

The maximum solvent accessibility of all the atoms of each side chain including the C_α carbon and that of all the C, O and N atoms of the main-peptide chain were used as the above solvent accessibility, A_i. The solvent accessibility of each atom of monomer or complex was determined on the solvent-accessible surface of the heavy atoms excluding hydrogens. The dielectric constants in the protein and the aqueous solution were assumed to be 4.0 and 78.5, respectively. The Debye–Hückel parameter, K, was determined to be 0.127 $Å^{-1}$ considering the dielectric constant of water and the ionic strength, 0.308 M·dm^{-3}, in the case of biological 0.154 M NaCl solution at 37 °C.

Matthew and Richards reported the solvent-accessibility-modified, Tanford–Kirkwood, discrete charge model for electrostatic effects of ribonuclease.[12] The apparent dielectric constant was estimated by using the equation

$$\varepsilon^{ap}=\varepsilon_{H_2O}/(1-A_{ij})\cdot e^{-K\cdot r_{ij}},$$

$$A_{ij}=(A_i+A_j)/2$$

where A_{ij} is the average solvent accessibility of charged sites i and j on ribonuclease, K is the usual Debye–Hückel

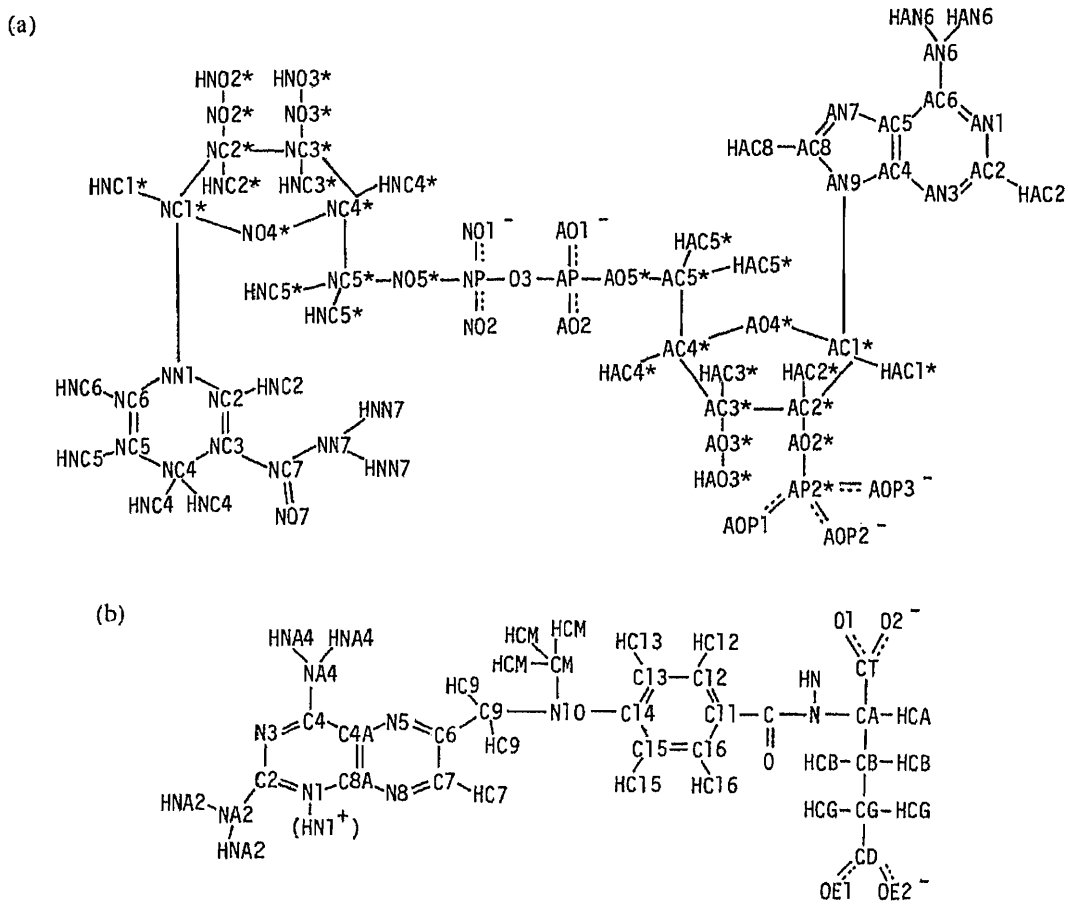


Fig. 1. Structures and Atom Numbering of NADPH (a) and MTX (b)

parameter proportional to the square root of the ionic strength, and ε_{H_2O} is the dielectric constant of water. They used 78.5 and 4.0 as the bulk dielectric constants of the external medium and the interior of the sphere, respectively, which are the same as in our report. We determined the apparent dielectric constant essentially according to their theoretical structure, since they could predict the behavior of individual titratable sites and the pH-dependent free energy of denaturation. The maximum solvent accessibility was used to include the adsorption phenomena of water due to thermal motion on the protein surface, and it was constant overall to preserve local dipole moments. Moreover Matthew and Richards used a similar approach to solvent accessibility to attain the maximum value in a model tripeptide.[12] Thomas *et al.* concluded from the pH dependence of enzyme catalysis that electrostatic effects should be highly masked over a distance of 14—15 Å at ionic strength 1.0.[13] They quoted the Debye-Hückel theory to explain the decreased effect of the ionic charge of the enzyme. Therefore our use of the Debye-Hückel parameter for the electrostatic interaction seems to be justified.

Mulliken net charges were used for the net charges due to the heterogeneous electron distribution in the molecule. Those of DHFR were obtained from the charge table of amino acid monomers presented by Sheridan and Allen.[14] Lys, Arg, Glu, Asp, N-terminal and C-terminal residues are ionized, and His18, His28, His89 and His153 were assumed to be ionized, considering their environment. Other His residues were regarded as neutral. Since the coordinates of the side chain atoms beyond C_β of Lys51 are ambiguous, +1 charge was added to C_β of Lys51. Mulliken net charges were calculated with the STO-3G basis set [15] using our Gaussian General Program revised from IMSPACK.[16] Mulliken net charges of arginine of which the side chain is neutral were determined as follows: *ab initio* calculation for the guanidine moiety was performed, and those of arginine which is cationic were replaced by them in part. The Mulliken net charges are listed in Table Ia. Since NADPH and MTX have too many molecular orbitals for direct *ab initio* calculation, they were first separated into four portions (adenine, 5-dehydroxymethylribose-2-phosphate dianion, methyl 5-(1-dehydroxyribose) diphosphate dianion, and 1-methyldihydronicotinamide) and three portions (1-hydro-2,4-diamino-6-methylpteridine cation, *p*-dimethylaminobenzoylamide, and glutamic acid dianion), respectively. $NADP^+$, NADH, NAD^+, and (1-neut)MTX, in which the pteridine N1 ring position of MTX is neutral, were similarly treated. The net charges of overlapping atoms in these moieties were distributed on the average to all atoms of both the neighbor segments. The structures and atom numbering for NADPH and MTX are shown in Fig. 1. All the Mulliken net charges obtained for four co-factors, NADPH, $NADP^+$, NADH and NAD^+, and MTX in

TABLE Ia. Mulliken Net Atomic Charges of Arginine Residue of Which the Side Chain is not Ionized

Atom	Net charge (q)
N	−0.3780
HN	0.2010
C	0.3100
O	−0.3030
CA	0.0910
HCA	0.0940
CB	−0.0980
HCB	0.0680
CG	−0.1030
HCG	0.0740
CD	0.0040
HCD	0.0850
NE	−0.2830
CZ	0.3700
NH1	−0.1230
NH2	−0.1230

TABLE Ib. Mulliken Net Atomic Charges of NADPH, $NADP^+$, NADH, and NAD^+

Atom	Mulliken net atomic charge (q)			
	NADPH	$NADP^+$	NADH	NAD^+
NN1	−0.2744	−0.1751	−0.2744	−0.1751
NC2	0.0828	0.1154	0.0828	0.1154
HNC2	0.0633	0.1313	0.0633	0.1313
NC3	−0.0378	0.0130	−0.0378	0.0130
NC4	−0.0999	0.0799	−0.0999	0.0799
HNC4	0.0729	0.1674	0.0729	0.1674
HNC4	0.0730		0.0730	
NC5	−0.0680	−0.0516	−0.0680	−0.0516
HNC5	0.0651	0.1382	0.0651	0.1382
NC6	0.0399	0.1346	0.0399	0.1346
HNC6	0.0772	0.1611	0.0772	0.1611
NC7	0.2540	0.2709	0.2540	0.2709
NO7	−0.2908	−0.2103	−0.2908	−0.2103
NN7	−0.4325	−0.4189	−0.4325	−0.4189
HNN7	0.2191	0.2584	0.2191	0.2584
HNN7	0.2046	0.2234	0.2046	0.2234
NC1*	−0.0676	−0.0696	−0.0676	−0.0696
HNC1*	0.0752	0.1212	0.0752	0.1212
NC2*	0.0543	0.0569	0.0558	0.0584
HNC2*	0.0710	0.0736	0.0725	0.0751
NO2*	−0.3293	−0.3268	−0.3279	−0.3253
HNO2*	0.1852	0.1878	0.1867	0.1892
NC3*	0.0506	0.0532	0.0521	0.0547
HNC3*	0.0569	0.0595	0.0584	0.0610
NO3*	−0.3319	−0.3293	−0.3304	−0.3278
HNO3*	0.1912	0.1938	0.1927	0.1953
NC4*	0.0753	0.0779	0.0768	0.0794
HNC4*	0.0857	0.0882	0.0871	0.0897
NO4*	−0.2478	−0.2453	−0.2463	−0.2438

TABLE Ib. (continued)

Atom	Mulliken net atomic charge (q)			
	NADPH	$NADP^+$	NADH	NAD^+
NC5*	−0.0039	−0.0013	−0.0024	0.0002
HNC5*	0.0509	0.0535	0.0524	0.0549
HNC5*	0.0552	0.0578	0.0568	0.0592
NO5*	−0.4700	−0.4675	−0.4686	−0.4660
NP	1.3395	1.3420	1.3410	1.3435
NO1	−0.7341	−0.7315	−0.7326	−0.7300
NO2	−0.7419	−0.7393	−0.7404	−0.7378
O3	−0.6909	−0.6883	−0.6894	−0.6868
AP	1.3358	1.3384	1.3373	1.3398
AO1	−0.7393	−0.7367	−0.7378	−0.7352
AO2	−0.7621	−0.7595	−0.7606	−0.7580
AO5*	−0.4495	−0.4469	−0.4480	−0.4454
AC5*	−0.0908	−0.0882	−0.0893	−0.0867
HAC5*	0.0414	0.0440	0.0429	0.0455
HAC5*	0.0428	0.0454	0.0443	0.0469
AC4*	0.0398	0.0398	0.0406	0.0406
HAC4*	0.0731	0.0731	0.0827	0.0827
AO4*	−0.3094	−0.3094	−0.2660	−0.2660
AC3*	0.0472	0.0472	0.0596	0.0596
HAC3*	0.0081	0.0081	0.0822	0.0822
AO3*	−0.3857	−0.3857	−0.2882	−0.2882
HAO3*	0.2603	0.2603	0.2066	0.2066
AC2*	0.0530	0.0530	0.0904	0.0904
HAC2*	0.0371	0.0371	0.0629	0.0629
AO2*	−0.4865	−0.4865	−0.3527	−0.3527
HAO2*			0.2323	0.2323
AP2*	1.1191	1.1191		
AOP1	−0.7777	−0.7777		
AOP2	−0.7962	−0.7962		
AOP3	−0.8019	−0.8019		
AC1*	−0.0059	−0.0059	0.0052	0.0052
HAC1*	0.0565	0.0565	0.0928	0.0928
AN9	−0.3249	−0.3249	−0.3217	−0.3217
AC8	0.1659	0.1659	0.1691	0.1691
HAC8	0.0841	0.0841	0.0872	0.0872
AN7	−0.2705	−0.2705	−0.2674	−0.2674
AC6	0.2928	0.2928	0.2960	0.2960
AN6	−0.4081	−0.4081	−0.4049	−0.4049
HAN6	0.2360	0.2360	0.2391	0.2391
HAN6	0.2442	0.2442	0.2474	0.2474
AC5	0.0086	0.0086	0.0117	0.0117
AC4	0.1762	0.1762	0.1793	0.1793
AN3	−0.2483	−0.2483	−0.2451	−0.2451
AC2	0.1582	0.1582	0.1614	0.1614
HAC2	0.0849	0.0849	0.0881	0.0881
AN1	−0.3308	−0.3308	−0.3276	−0.3276

two charge states, MTX and (1-neut)MTX, are listed in Table Ib and c, respectively. The total net charges were -4, -3, -2, -1, -1, and -2, respectively.

Electrostatic potential at a "patch" k, that is, one of polyhedrons into which the van der Waals' surface of the guest molecule is divided by some meridians and parallels, was calculated by means of the following equation by a similar method to the electrostatic energy calculation.[11]

TABLE Ic. Mulliken Net Atomic Charges of MTX

Atom	Net charge (q)		Atom	Net charge (q)	
	MTX[a]	(1-neut)MTX[b]		MTX	(1-neut)MTX
N1	−0.3077	−0.2980	C12	−0.0136	−0.0140
HN1	0.2940		HC12	0.0854	0.0850
C2	0.4523	0.3482	C13	−0.0996	−0.1000
NA2	−0.3950	−0.4181	HC13	0.0768	0.0764
HNA2	0.2625	0.2334	C14	0.1822	0.1818
HNA2	0.2801	0.2207	C15	−0.1033	−0.1037
N3	−0.3209	−0.3501	HC15	0.0615	0.0611
C4	0.3564	0.2961	C16	−0.0265	−0.0269
NA4	−0.3796	−0.4154	HC16	0.0835	0.0830
HNA4	0.2872	0.2405	C	0.2786	0.2782
HNA4	0.2954	0.2526	O	−0.2992	−0.2996
CA4	0.0566	0.0246	N	−0.4263	−0.4267
N5	−0.2071	−0.2339	HN	0.1398	0.1394
C6	0.1237	0.0845	CA	0.0255	0.0255
C7	0.0756	0.0522	HCA	0.0576	0.0576
HC7	0.1202	0.0770	CB	−0.0989	−0.0989
N8	−0.2322	−0.2406	HCB	0.0632	0.0632
CA8	0.2490	0.1868	HCB	0.0111	0.0111
C9	−0.1652	−0.1658	CG	−0.1259	−0.1259
HC9	0.0987	0.0765	HCG	−0.0330	−0.0330
HC9	0.1021	0.0825	HCG	−0.0122	−0.0122
N10	−0.2721	−0.2725	CD	0.1440	0.1440
CM	−0.0666	−0.0670	OE1	−0.4961	−0.4961
HCM	0.0792	0.0788	OE2	−0.4535	−0.4535
HCM	0.0885	0.0881	CT	0.1746	0.1746
HCM	0.0790	0.0786	O1	−0.4944	−0.4944
C11	−0.0847	−0.0851	O2	−0.5707	−0.5707

a) N1 cationic state. *b*) N1 neutral state.

$$\psi_k = \Sigma\{q_j/(\varepsilon_j \cdot r_{jk})\} \cdot e^{-K \cdot r_{jk}}$$

Guest (G) and host (H) potentials were calculated as ψ_k^{G} and ψ_k^{H}, respectively, at the patch k.[7] Electrostatic correlation potential ψ_k^{GH} was calculated by using ψ_k^{G} and ψ_k^{H}.

$$\psi_k^{\mathrm{GH}} = \psi_k^{\mathrm{G}} \cdot \psi_k^{\mathrm{H}}$$

The change of electrostatic correlation potentials was described as C_k^{GH}, when some substitutions of guest or host molecule were made.

$$C_k^{\mathrm{GH}} = \psi_k^{\mathrm{GH}}(\text{changed}) - \psi_k^{\mathrm{GH}}(\text{original})$$

Molecular orbital calculations were performed at the Computer Center of the Institute for Molecular Science. Electrostatic calculations and computer graphics were performed at the Computer Center of the University of Tokyo, on a HITAC M-280H computer with a color graphic video terminal (HT-5425-E82).

Steric stick models of NADPH and MTX bound on DHFR are shown in Figs. 2 and 3. CPK models corresponding to Figs. 2 and 3 are shown in Figs. 4 and 5. Electrostatic calculation and computer graphics were carried out by the DRAG-ES programs developed from TERAS,[17] written in Fortran, using the 'CGDM' software graphics package[18] for the latter. If the electrostatic correlation potential (ECP) is averaged for the total molecular surface area, the ECP coefficient is calculated as follows.

$$\bar{\psi}^{\mathrm{GH}} = \Sigma\psi_k^{\mathrm{GH}} \cdot S_k / \Sigma S_k ,$$

where S_k is the area of patch k. Similarly the averaged C_k^{GH} becomes

$$\bar{C}^{\mathrm{GH}} = \Sigma C_k^{\mathrm{GH}} \cdot S_k / \Sigma S_k ,$$

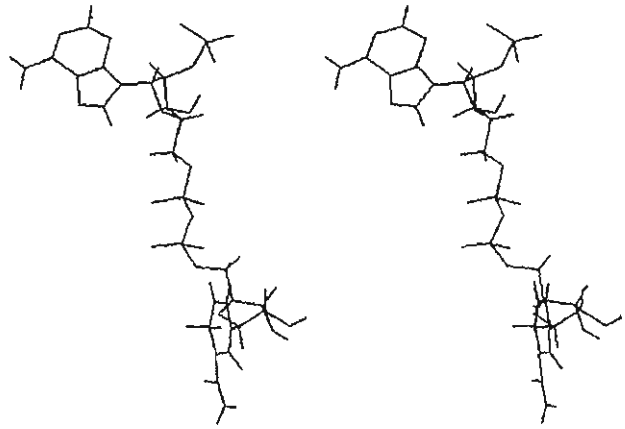


Fig. 2. Steric Stick Model of NADPH in the DHFR–NADPH–MTX Ternary Complex

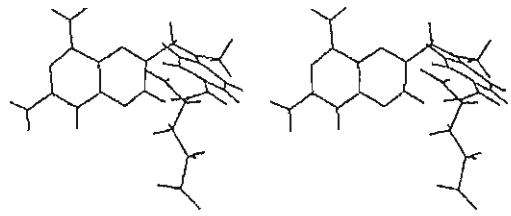


Fig. 3. Steric Stick Model of MTX in the DHFR–NADPH–MTX Ternary Complex

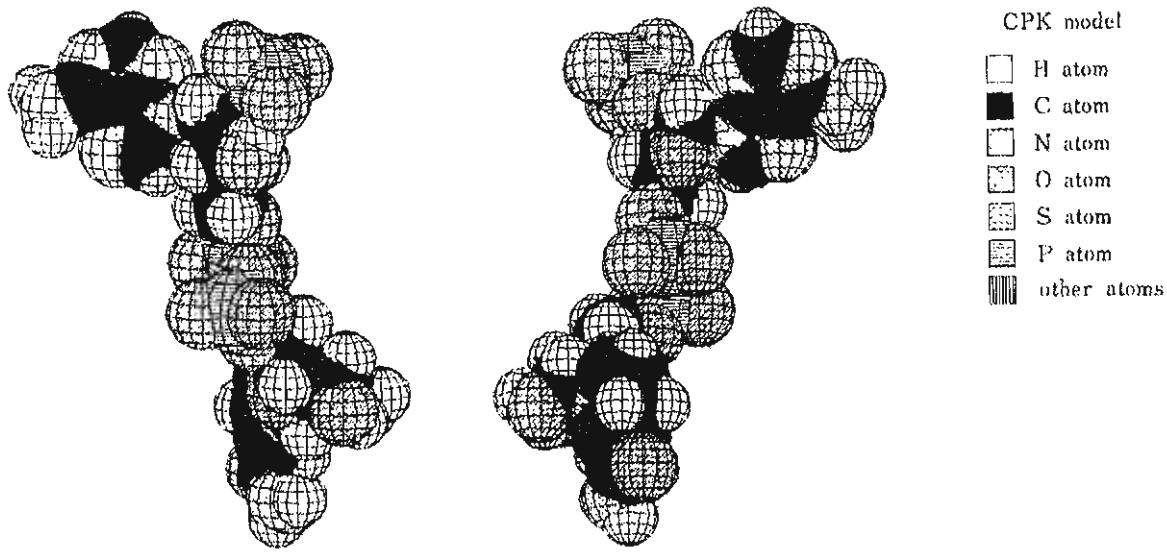


Fig. 4. CPK Model of NADPH Corresponding to Fig. 2 and the Reverse Side

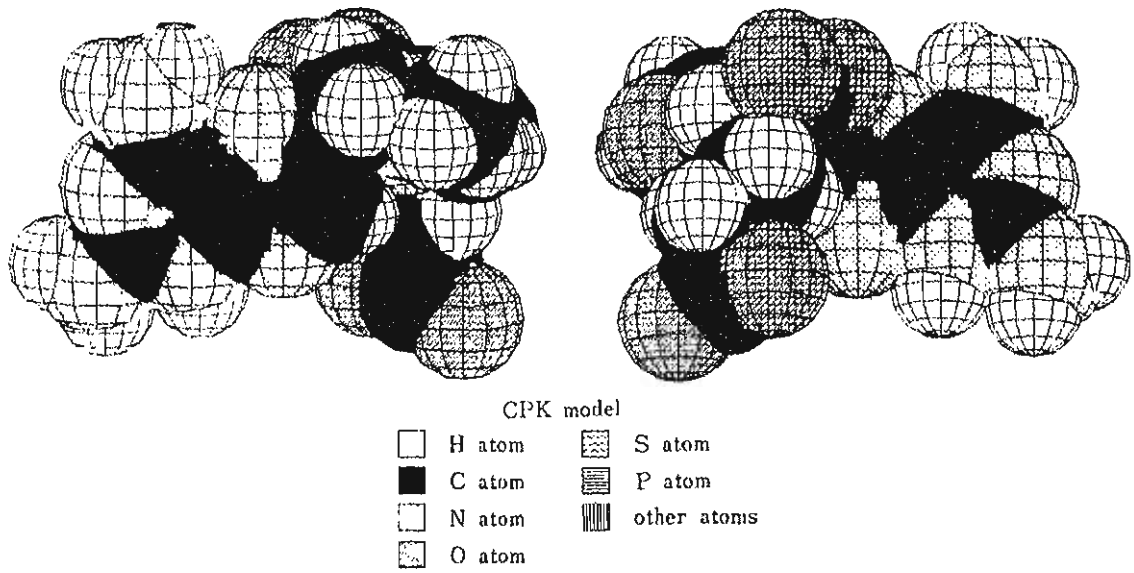


Fig. 5. CPK Model of MTX Corresponding to Fig. 3 and the Reverse Side

and the $\bar{C}^{GH}$ value is useful to estimate quantitatively the change of the electrostatic complementarity.

The distance-graphics method has been proposed to describe the contact state between guest and host molecules. Information on the distance from a guest-atom patch to the nearest host-atom surface is reflected in the value given to the patch, and the color spectrum for all the patches of the guest-molecular surface is visualized on a computer graphics terminal. The areas of the patches selecting the proper host-atom on one guest-atom are divided by the total areas of all the patches on the same guest-atom, and the value multiplied by 100 is called the "contact ratio" of the guest-atom with the proper host-atom.

Results and Discussion

Electrostatic Interaction Energies

Fersht *et al.* examined the role of complementary hydrogen bonding by protein engineering[19]; deletion of an uncharged hydrogen bond and charge deletion from an ionic hydrogen bond weakened the binding energies by 0.5—1.5 and 3.5—4.5 kcal/mol, respectively. Electrostatic interaction energies (E^{GH}) were calculated for various combination types of guest and host molecules, and the results are shown in Table II. The peptide NH groups of Gly99 and Gln101 in DHFR form two hydrogen bonds separately with pyrophosphate of NADPH. The net charge of hydrogen of the NH group was added to that of nitrogen at the position of the nitrogen to delete the hydrogen bond effect of the NH group. The effects

Table II. Electrostatic Interaction Energy between Guest and Host Molecules

Guest	Molecule	Host	E^{GH} (kcal/mol)
NADPH	——	DHFR	−11.0
$NADP^+$	——	DHFR	−12.4
NADH	——	DHFR	−7.1
NAD^+	——	DHFR	−8.5
NADPH	——	DHFR (Arg43(neut))[a]	−5.2
NADPH	——	DHFR (Arg44(neut))	−6.6
NADPH	——	DHFR (Gly99(>N(H)))[b]	−9.7
NADPH	——	DHFR (Ala100(>N(H)))	−10.7
NADPH	——	DHFR (Gln101(>N(H)))	−9.8
(1-neut)MTX[c]	——	DHFR–NADPH	−8.7
MTX	——	DHFR–NADPH	−22.0
MTX	——	DHFR–$NADP^+$	−17.0
MTX	——	DHFR	−18.0
MTX	——	DHFR–NADPH (Asn26)[d]	−13.2
MTX	——	DHFR–NADPH (His28(neut))	−19.8
MTX	——	DHFR–NADPH (Lys51(neut))	−20.8
MTX	——	DHFR–NADPH (Arg57(neut))	−15.1

a) The side chain was assumed to be in the neutral state. *b*) The net charge of hydrogen of the NH group was added to that of nitrogen at the position of the nitrogen. *c*) The pteridine ring of MTX was assumed to be in the neutral state at the N1 ring position. *d*) Aspartic acid was replaced with asparagine at residue 26 of DHFR.

of the two hydrogen bonds were estimated to be 1.3{$=-9.7-(-11.0)$} and 1.2 {$=-9.8-(-11.0)$} kcal/mol for the former and the latter, respectively. Arg43, Arg44 and His28 in DHFR are amino acid residues forming ion-pairs separately with NADPH and MTX, and they can be replaced by other basic amino acid residues in the DHFR family.[2] Electrostatic interaction energies were calculated by changing the net charge of the side chain from cation type to neutral, and the decreased energy values were estimated to be 5.8{$=-5.2-(-11.0)$}, 4.4{$=-6.6-(-11.0)$} and 2.2{$=-19.8-(-22.0)$} kcal/mol for Arg43, Arg44 and His28, respectively (Table II). Our results are in good agreement with the experiments by Fersht *et al.*,[19] and this confirms our electrostatic calculation method based on the solvent accessibility and the classical Debye–Hückel theory, which is only approximate at high ionic strength.

The one negative charge of Asp26, buried in the MTX–DHFR complex, is completely conserved in the DHFR family,[2] and forms an ion-pair with the pteridine ring of MTX. The amino acid substitution of Asp→Asn27 in DHFR from *Escherichia coli* markedly decreases the enzymatic activity.[20] The Asp27 corresponds to Asp26 of DHFR from *Lactobacillus casei*.[1a,2] The amino acid residue Asp26 was modified to Asn, and the electrostatic interaction energy was calculated. The decrease of electrostatic energies produced by changing the charged state from -1 to 0 was estimated to be 8.8{$=-13.2-(-22.0)$} kcal/mol. The value of 8.8 kcal/mol explains the experimental fact that the free energy needed to cause the pK_a-shift of the MTX pteridine ring in the complex formation with DHFR is more than 6.1 kcal/mol at 37 °C. Moreover, this confirms that the charge state of the amino acid residue in the position corresponding to Asp26 should be one negative charge (as in Asp or Glu).

Arg57, which is buried in MTX–DHFR complex, is completely conserved,[2] and interacts with the C-terminal carboxyl group of MTX. The decrease of electrostatic energy on changing the charge state from $+1$ to 0 was estimated to be 6.9{$=-15.1-(-22.0)$} kcal/mol, which is larger than those for Arg43 and Arg44. This result shows that Arg57 plays a significant role in the inhibition mechanism and the enzymatic process from an electrostatic point of view. Arg57 is also thought to be sterically important, since substitution by Lys or His is not found in the DHFR family in the position corresponding to Arg57.

When MTX binds to DHFR with NADPH, the pteridine ring of MTX is positively charged in the N1 ring position.[3] The electrostatic interaction energy is more stabilized in the cationic state than in the neutral state by 13.3{$=-8.7-(-22.0)$} kcal/mol. Thus, the ion-pair between the pteridine ring of MTX and Asp26 of DHFR is of great importance.

NADPH is converted into $NADP^+$ in the enzymatic reaction by hydride-ion transfer from the nicotinamide ring of NADPH to the substrate. From X-ray diffraction experiments, it is known that NADPH forms a stable ternary complex with DHFR and MTX.[1a] We calculated the electrostatic interaction energy of $NADP^+$ with the DHFR–MTX complex, and its energy was destabilized by 5.0{$=-17.0-(-22.0)$} kcal/mol in comparison with the stable ternary complex of NADPH, MTX and DHFR. This is thought to be because the positive charges of the pteridine ring of MTX and the nicotinamide ring of $NADP^+$ repel each other strongly.

Subramanian and Kaufman reported calorimetric experiments on the complex formation of MTX with DHFR of chicken liver, showing that the enthalpy (ΔH°) is -13.3 kcal/mol, which contributes much more than the entropy term to the reaction.[21] Our electrostatic energy of -18.0 kcal/mol may cast light on the nature of the interaction of ligand and enzyme, although of course the electrostatic interaction energy is only one part of the total interaction energy.

Electrostatic Potentials

MTX and NADPH bind to DHFR with good potential-complementarity between guest

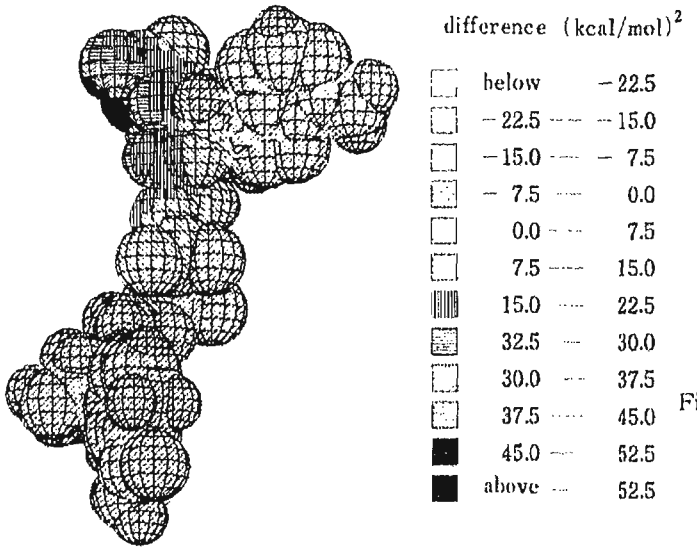


Fig. 6. Difference Electrostatic Correlation Potentials on the Molecular Surface of NADPH on Changing the Positive Charge of Side Chain of Arg43 to Neutral

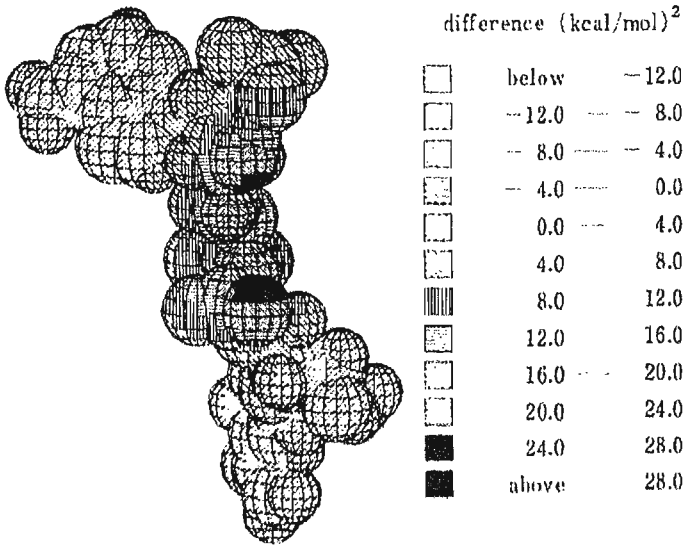


Fig. 7. Difference Electrostatic Correlation Potentials on the Molecular Surface of NADPH on Changing the Positive Charge of Side Chain of Arg44 to Neutral

and host molecules.[7,9] In this paper, the difference of two electrostatic correlation potentials (C_k^{GH}) at each position on van der Waals' molecular surface is visualized by means of computer graphics. When NADPH binds as a guest molecule to the DHFR host molecule, the guest molecule interacts directly with Arg43 and Arg44 of the host molecule to stabilize the complex. Figure 6 shows C_k^{GH} on the molecular surface of NADPH on changing the positive charge of the side chain of Arg43 to neutral. The C_k^{GH} of ribose-phosphate group takes a positive large value, showing that the electrostatic complementarity is very good between the ribose-phosphate group and the Arg43 side chain. Figure 7 shows C_k^{GH} on the same surface of NADPH on changing the positive charge of the side chain of Arg44 to neutral. The C_k^{GH} of the pyrophosphate group takes a large positive value, again showing that very good complementarity exists between the pyrophosphate group of NADPH and the Arg44 side chain.

When MTX comes into the DHFR–NADPH complex, the former and the latter are the guest and host molecules, respectively. Good electrostatic complementarity was already reported in a crude electrostatic model.[7] The host molecule was modified at Asp26, since modification of Asp→Asn26 is predicted to decrease greatly the enzymatic activity.[20] Figure 8 shows C_k^{GH} on the MTX molecular surface of the guest molecule in the case of the Asp→Asn26. The two hydrogens covalently bound to the N1 and NA2 nitrogens of the

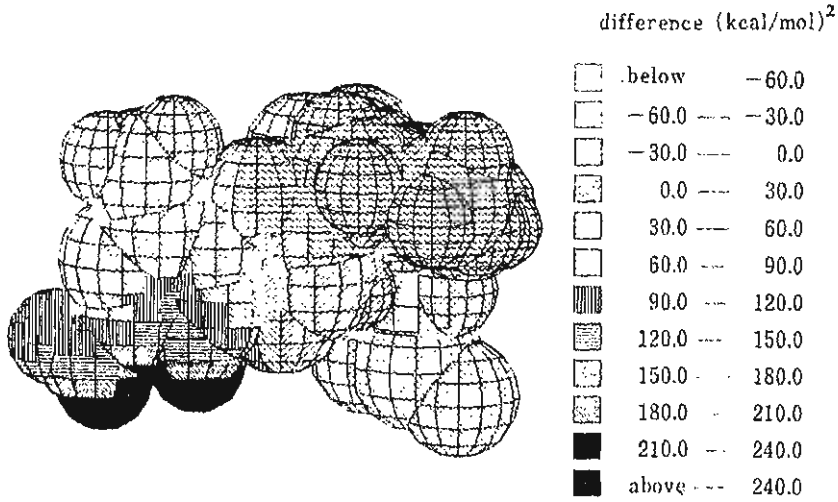


Fig. 8. Difference Electrostatic Correlation Potentials on the MTX Molecular Surface on Replacing Asp26 with Asn

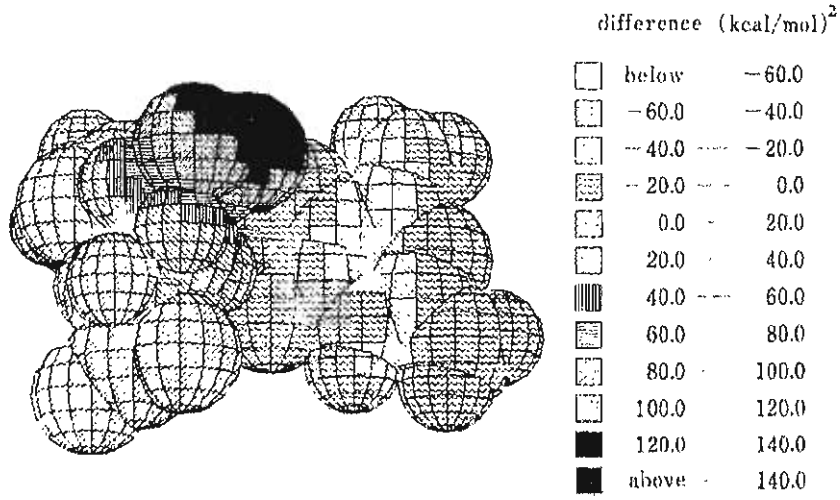


Fig. 9. Difference Electrostatic Correlation Potentials at the MTX Molecular Surface on Changing the Charge State of the Arg57 Side Chain from +1 to 0

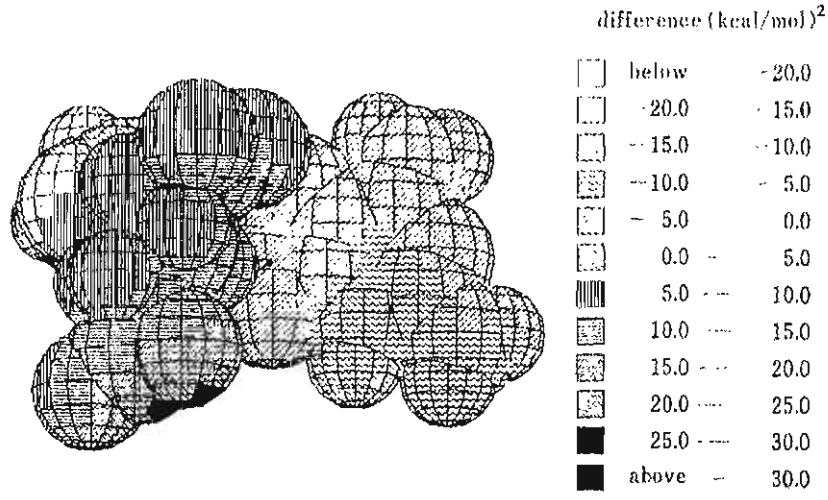


Fig. 10. Difference Electrostatic Correlation Potentials at the MTX Molecular Surface on Changing the Charge State of the His28 Side Chain from Cationic to Neutral

pteridine ring have large positive values of C_k^{GH} (over 210 kcal^2/mol^2). This shows that Asp26 contributes to the good guest–host complementarity. Arg57 is completely conserved in the DHFR family,[2] and, since the ion-pair of the Arg57 side chain and C-terminal carboxyl group of MTX is buried in the complex formation of MTX and DHFR–NADPH, the electrostatic complementarity is of great interest. The effect of changing the charge state of the Arg57 side chain from +1 to 0 on C_k^{GH} is shown in Fig. 9. The two C-terminal carboxyl oxygens of MTX have values of over 140 kcal^2/mol^2, although these are smaller than the positive values discussed in Fig. 8. It is clear that the electrostatic complementarity of the C-terminal carboxyl group of MTX and the Arg57 side chain is very large. His28 of DHFR interacts with the carboxyl group of the glutamic acid side chain of MTX in a position that is exposed to solvent. Figure 10 shows C_k^{GH} on changing the charge state of the His28 side chain from cationic to neutral. The carboxyl oxygens of the glutamic acid side chain of MTX have positive values of over 30.0 kcal^2/mol^2, which is very much smaller than those discussed in Figs. 8 and 9. This shows that, although electrostatic complementarity between the His28 side chain and the side chain carboxyl group of the MTX glutamic acid exists to some degree, it does not greatly stabilize the guest–host complex.

In Figs. 6—9, NADPH and MTX form complexes with the host molecules to show high complementarity. It is clear that Arg43 and Arg44 are significant for NADPH binding and that Asp26 and Arg57 are significant for MTX binding.

TABLE III. Electrostatic Correlation Potential Coefficient for Various Guest and Host Molecules

Guest Molecule		Host	$\bar{\psi}^{GH}$ (kcal/mol)^2
NADPH	———	DHFR	1.7
$NADP^+$	———	DHFR	−6.9
NADH	———	DHFR	1.8
NAD^+	———	DHFR	−7.6
NADPH	- -	DHFR (Arg43(neut))	9.4
NADPH	- - -	DHFR (Arg44(neut))	6.5
NADPH	·	DHFR (Gly99(>N(H)))	4.5
NADPH		DHFR (Ala100(>N(H)))	2.7
NADPH	- - - -	DHFR (Gln101(>N(H)))	4.2
(1-neut)MTX	- -	DHFR–NADPH	13.8
MTX	———	DHFR–NADPH	−74.2
MTX	———	DHFR–$NADP^+$	−42.2
MTX	———	DHFR	−35.9
MTX	———	DHFR–NADPH (Asn26)	−39.5
MTX	———	DHFR–NADPH (His28(neut))	−72.1
MTX	———	DHFR–NADPH (Lys51(neut))	−73.2
MTX	———	DHFR–NADPH (Arg57(neut))	−63.2

Change of Electrostatic-Potential Complementarity

The $\bar{\psi}^{GH}$ in Table III was calculated to estimate quantitatively the electrostatic-potential complementarity. From this table, the $\bar{C}^{GH}$ value is estimated as the difference of two $\bar{\psi}^{GH}$ values. The $\bar{C}^{GH}$ values for changing the charge state of Arg43, Arg44 and Arg57 are 7.7(=9.4−1.7), 4.8(=6.5−1.7), and 11.0{=−63.2−(−74.2)}, respectively. The largest contribution of Arg57 to the electrostatic-potential complementarity is of great interest in relation to the complete conservation of Arg57 in the DHFR family. The $\bar{C}^{GH}$ value for Asp→Asn26 is estimated to be 34.7{=−39.5−(−74.2)}, and that for the cationic state →the neutral state of the pteridine ring of MTX is 87.9{=13.8−(−74.2)}. These values show quantitatively that the ion-pair of Asp26 and the cationic pteridine ring of MTX greatly contribute to the electrostatic-potential complementarity. The $\bar{C}^{GH}$ value for His28 (cation)→His28 (neutral) is estimated to be 2.0{=−72.1−(−74.2)}. Although His28 forms an ion-pair with the carboxyl ion of the glutamic acid side chain in MTX, the electrostatic-potential complementarity is not as large as that of Arg57, which forms an ion-pair with the C-terminal carboxyl ion of the glutamic acid moiety in MTX.

The $\bar{\psi}^{GH}$ value for various converted states (Table III) is plotted against the electrostatic interaction energy (Table II) in Fig. 11. The result shows that the former is correlated very well to the latter with a correlation coefficient of 0.922. In the MTX–NADPH–DHFR complex system, the electrostatic-potential complementarity is proportional to the electrostatic interaction energy, and, hence, the electrostatic interaction on the molecular surface is thought to be significant. Figure 12 shows a histogram of electrostatic energy for each atom

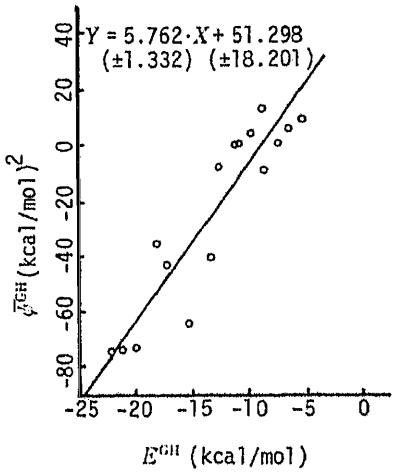


Fig. 11. Relation of Electrostatic Correlation Potential Coefficient for Various Guest and Host Molecules to the Electrostatic Interaction Energy

In the regression equation, the figures in parentheses are the 95% confidence intervals, n is the number of data points used in the regression analysis, r the correlation coefficient, and s the standard error of the estimate.

$n=17$, $r=0.922$ ($p<0.05$), $s=13.081$.

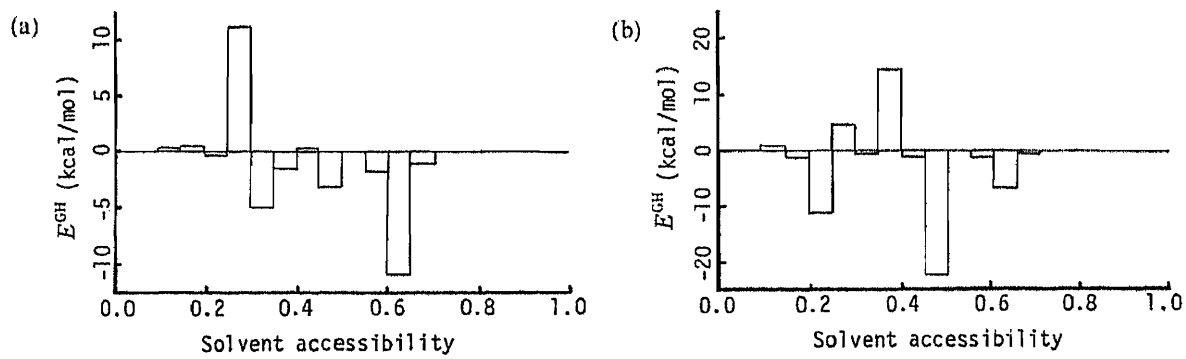


Fig. 12. Histogram of the Solvent Accessibility of Each Guest-Atom (Abscissa) and the Electrostatic Interaction Energy for the Atom of the Guest Molecule (Ordinate) (a) NADPH, (b) MTX

of the guest molecule against the solvent accessibility of the guest-atoms. The guest-atoms exposed on the molecular surface have more stabilized electrostatic energies in both cases (NADPH and MTX). This shows that the electrostatic interaction energy of guest atoms on the molecular surface contributes to the stabilization of the complex formation of NADPH or MTX.

Distance Graphics

The distance between the patch k of the guest molecule and the nearest molecular surface of the host molecule is illustrated in Figs. 13 and 14 for NADPH and MTX, respectively. The buried moieties are easily distinguishable from the moieties exposed to the solvent. The

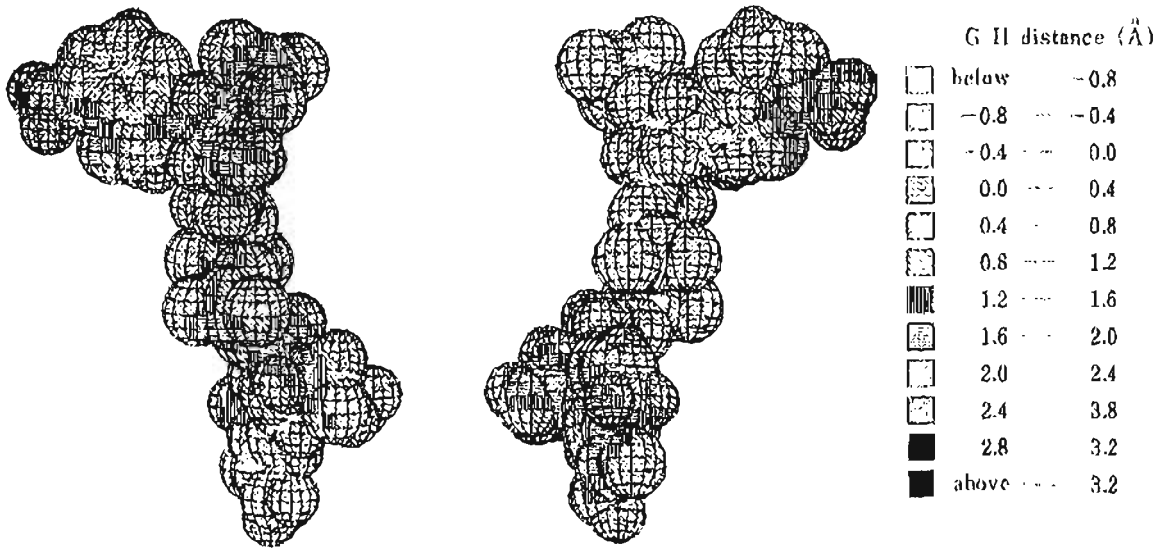


Fig. 13. Distance Graphics of NADPH

The distance from each guest-atom patch to the nearest host-atom surface is visualized on the molecular surface.

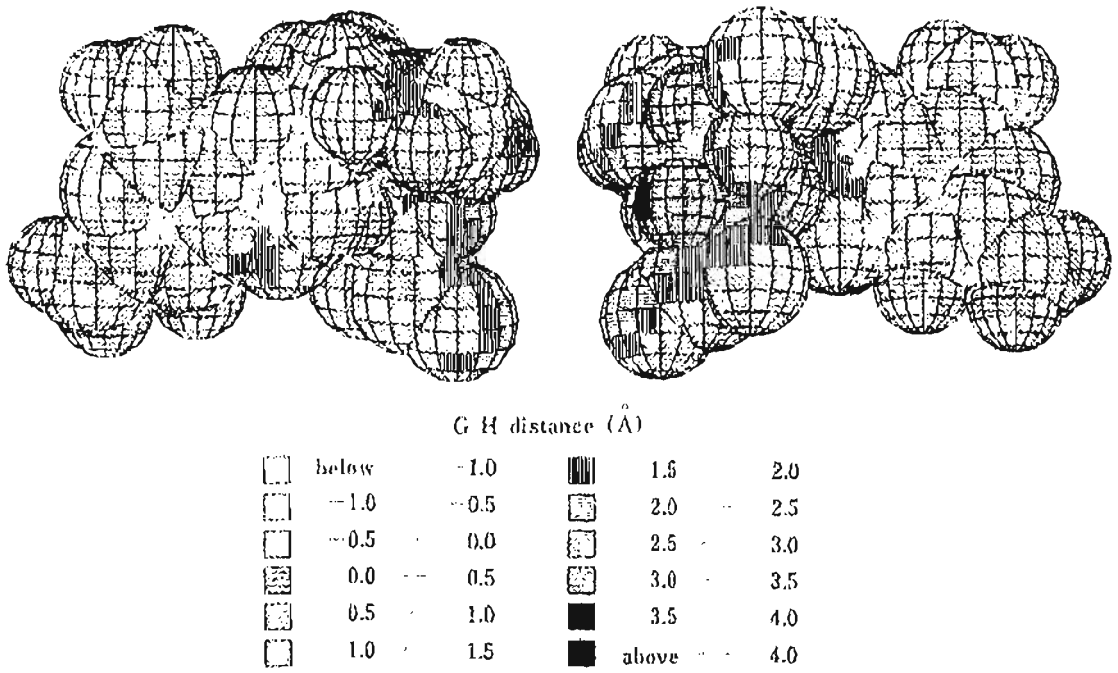


Fig. 14. Distance Graphics of MTX

information on host-atom contact with the guest-atom is given as the contact ratio of the guest-atom with the proper host-atom. AOP1 and AOP3 oxygens of ribose-phosphate of NADPH interact with Arg43 NE and NH2 nitrogens at distances of 2.69 and 2.96 Å with contact ratios of 28 and 82%, respectively. The existence of the two hydrogen bonds may explain why Arg43 is almost wholly conserved in the DHFR family. The NO2 oxygen of the NADPH pyrophosphate interacts with the Arg44 NH1 nitrogen at 3.08 Å distance with a contact ratio of 43%. However, three oxygens NO1, AO1, and AO2 of the pyrophosphate do not contact the Arg44 atoms, and they interact with the Gln101 main-chain hydrogen, the Gly99–Gln–101–Ile102 main chain hydrogens, and the Gly99 main chain hydrogen-Thr45 main chain hydrogen-Thr45 OG1 oxygen with contact ratios of 37, 56 and 83%, respectively. That the Arg44 side chain forms only one hydrogen bond with the pyrophosphate group may explain the replacement of Arg→Lys44 in the DHFR family. The OE2 carboxyl oxygen of MTX contacts the His28 HND1 hydrogen at a distance of 1.70 Å with a contact ratio of 60%. The O1 and O2 carboxyl oxygens of MTX interact with the Arg57 NH1 and NH2 nitrogens at distances of 2.68 and 3.04 Å, with contact ratios of 36 and 43%, respectively. The existence of the two hydrogen bonds between the carboxyl and guanidium groups is consistent with the fact that Arg57 is completely conserved in the DHFR family. The hydrogen covalently bonded to the N1 nitrogen and the hydrogen covalently bonded to the NA2 nitrogen of the pteridine ring contact the OD1 and OD2 oxygens of Asp26 at distances of 1.64 and 1.88 Å with contact ratios of 100 and 83%, respectively. The existence of such parallel hydrogen bonds explains the importance of the carboxyl group of Asp26. As mentioned above, the contact ratio is a good index of the importance of various moieties in ligands and enzymes.

Buried Ligands in DHFR

When NADPH interacts with DHFR, NADPH is buried as shown in the above distance graphics. The electrostatic interaction energy was calculated for the buried state in Table II. If NADPH is far distant from DHFR and is exposed completely to the solvent, the moieties of the contact region in the complex are occupied by solvent molecules in place of the host or guest molecule. The electrostatic interaction energy of the experimental complex structure was calculated in the state in which both NADPH and DHFR are exposed to solvent molecules, and it was −1.8 kcal/mol. The increased stabilization energy arising from removal of solvent molecules in the process of the complex formation is estimated to be 9.2{= −1.8−(−11.0)} kcal/mol. Similarly, the electrostatic interaction energy of the experimental complex structure between two solvent-exposed molecules of MTX and NADPH–DHFR was calculated to be −8.8 kcal/mol. The increased stabilization energy for MTX due to the removal of solvents in the MTX–NADPH–DHFR complex formation is estimated to be 13.2{= −8.8−(−22.0)} kcal/mol. Accordingly it is clear that the decreased solvent accessibility of ligands such as NADPH and MTX plays an important role in stabilizing the electrostatic interaction energy due to complex formation with the DHFR enzyme. Matthew and Richards reported similar facilitation of complex formation between flavodoxin and cytochrome c.[12]

Conclusion

A method of calculating the electrostatic interaction energy is proposed by using the solvent accessibility and the Debye–Hückel equation, and it gave results comparable with the experimental values. A computer graphics approach was employed to visualize the electrostatic effects of replacing or charging amino acid residues as the difference electrostatic correlation potential. It was shown that Arg43, Arg44, Arg57 and Asp26 of DHFR are important from an electrostatic point of view. Further, the electrostatic interaction energy of the guest-atoms on the molecular surface of NADPH or MTX contributes to the electrostatic

stabilization of the complex formation with the host molecule. A distance graphics method was used to visualize the buried ligand, and the contact ratio was estimated quantitatively.

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Synthesis and Antibacterial Activities of Optically Active Ofloxacin and Its Fluoromethyl Derivative

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Two optically active (100% enantiomeric excess) isomers (**13a** and **13b**) of ofloxacin (**1**) [(±)-ofloxacin; DL-8280; (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid] and their fluoromethyl derivatives (**14a** and **14b**) were prepared *via* their optically active intermediates resolved by use of high-performance liquid chromatography (HPLC). The isomers (**13a** and **13b**) were also obtained efficiently by an alternative route *via* separation of the diastereoisomers (**18**, **19**) prepared in the reaction of benzoxazine (**17**) with L-prolinyl chloride.

The (−)-isomers of **1** and its fluoromethyl derivative (**2**) were approximately twice as active as the corresponding racemates, while the (+)-isomers were considerably less active than the racemates.

The absolute configuration at the C_3 position in the oxazine ring in a series of (−)-compounds (**b**) was confirmed by X-ray analysis of the hydrochloride of the (−)-benzoxazine derivative (**15b**) to be *S*.

Keywords——ofloxacin; DL-8280; optically active ofloxacin; 7,8-difluoro-2,3-dihydro-3-methyl-4*H*-[1,4]benzoxazine; *N*-*p*-toluenesulfonyl-L-proline; X-ray analysis; absolute configuration; antibacterial activity; DNA gyrase

Considerable progress in the activity of quinolone carboxylic acid antibacterial agents (quinolones) has recently been achieved by the development of new quinolones having both a fluorine atom and a piperazinyl group in their parent compounds. The active center of this class of compounds is considered to be the N-1 substituted 1,4-dihydro-4-oxopyridine-3-carboxylic acid moiety, and a variety of N-1 substituents having no asymmetric center have been reported. The roles of these substituents have also been discussed.[1] However, the new quinolone ofloxacin (**1**),[2] which is characterized by a tricyclic structure, possesses a methyl group at the C_3 position in the oxazine ring, and so has an asymmetric center at this position.

In this paper, we describe the synthesis of optically active (+)- and (−)-ofloxacin (**13a** and **13b**)[3] and their 3-fluoromethyl derivatives (**14a** and **14b**) through optical resolution of racemic intermediates of **1** both by high-performance liquid chromatography (HPLC) and by an alternative route consisting of derivatization from the diastereomers using an optically active proline analogue.

The Riker Laboratories group[4] has recently disclosed that the *S*-(−)-isomer of 6,7-dihydro-5,8-dimethyl-9-fluoro-1-oxo-1*H*,5*H*-benzo[*ij*]quinolizine-2-carboxylic acid (S-25930) had potent antibacterial activity *in vitro* while the *R*-(+)-isomer showed much weaker activity.

Chemistry

The (±)-3,5-dinitrobenzoyl derivative (**4**) of the racemic alcohol (**3**)[5] was resolved

completely by HPLC to yield optically pure benzoyl esters (**5a** and **5b**). The benzoyl ester (**5b**) was hydrolyzed with an ethanolic aqueous $NaHCO_3$ solution to yield an alcohol (**6b**), which was converted to an iodide (**7b**) with triphenylphosphite methiodide[6] in *N*,*N*-dimethylformamide (DMF). Optically active carboxylic acid (**9b**) was obtained by reduction of **7b** with tri-*n*-butyltin hydride in EtOH, followed by hydrolysis of the resulting ester (**8b**) in concentrated HCl–AcOH. Reaction of **9b** with boron trifluoride etherate in Et_2O[7] gave a chelate compound (**12b**), which was reacted with 1-methylpiperazine in dimethyl sulfoxide (DMSO), and then with triethylamine in 95% MeOH to give (−)-ofloxacin (**13b**) in good yield. Similarly, the benzoyl ester (**5a**)was converted through **6a**, **7a**, **8a**, **9a** and **12a** to (+)-ofloxacin (**13a**).

Optically active fluoromethyl derivatives (**14a** and **14b**) were synthesized as follows: fluorination of **6a** and **6b** with diethylaminosulfur trifluoride (DAST)[8] in dry CH_2Cl_2 afforded **10a** and **10b**, which were hydrolyzed in concentrated HCl–AcOH to give the carboxylic acids (**11a** and **11b**). The acids (**11a** and **11b**) were reacted with 1-methylpiperazine by heating in DMSO to afford the desired compounds (**14a** and **14b**) in good yield.

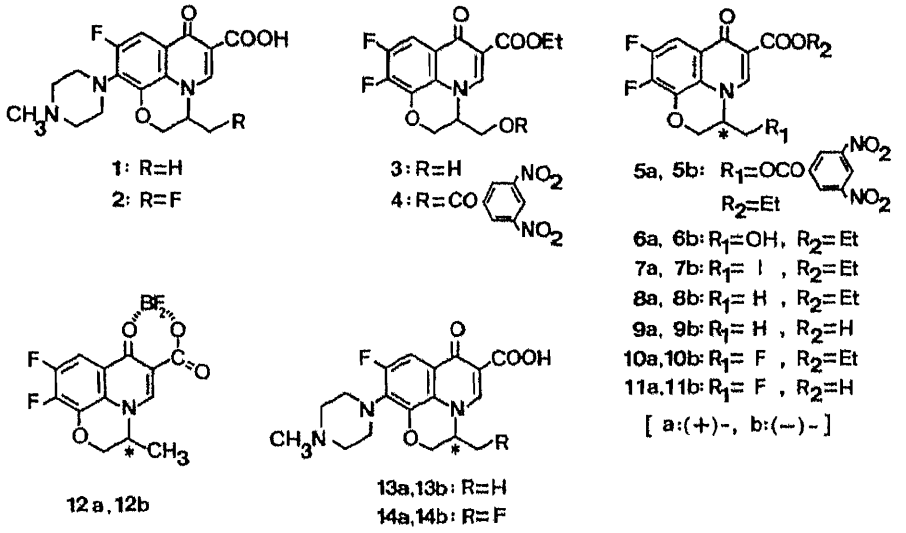


Chart I

In order to obtain a large quantity of optically active ofloxacin, we investigated effective methods of preparing optical isomers of (±)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-[1,4]benzoxazine (**17**),[2] a key intermediate of **1**. We have already reported[9] an asymmetric hydrolysis of (±)-7,8-difluoro-2,3-dihydro-3-acetoxymethyl-4*H*-[1,4]benzoxazine with enzymes to give (+)- and (−)-7,8-difluoro-2,3-dihydro-3-methyl-4*H*-[1,4]benzoxazine, (**15a** and **15b**), from which optically active esters (**8a** and **8b**) were prepared in a manner similar to that reported in the previous paper.[2]

An alternative method of obtaining **15a** and **15b** that was similar to the technique reported by Gerster and his co-workers[4] was employed. Reaction of **17** with *N*-*p*-toluenesulfonyl-L-prolinyl chloride[10] in the presence of pyridine in dry CH_2Cl_2 afforded amides (**18** and **19**). Most of **19** was removed by fractional crystallization of the mixture of **18** and **19** from AcOEt–hexane. Then, the desired pure **18** was easily obtained from the filtrate by silica gel column chromatography. Hydrolysis of the first eluate (**18**) was carried out by heating in 1 N NaOH–EtOH solution to give **15b** in excellent yield. The physical data of the optically active compounds are shown in Table I.

The hydrochloride of **15b** was crystallized from EtOH–AcOEt to give colorless prisms

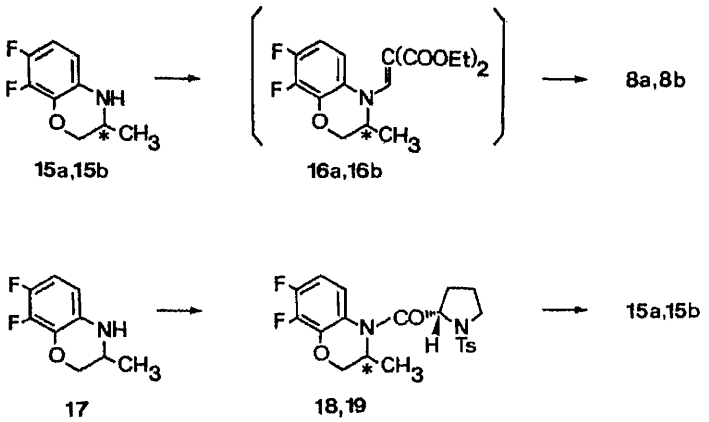


Chart 2

TABLE I. Physical Data for Optically Active Compounds

Compds. No.	mp (°C)	$[\alpha]_D^{23}$ (c, solvent)	Compds. No.	mp (°C)	$[\alpha]_D^{23}$ (c, solvent)
5a	235—240	+90.8° (0.852, DMF)	**11a**	>300	+92.4° (0.675, DMSO)
5b	244—249	−92.5° (0.889, DMF)	**11b**	>300	−92.7° (0.550, DMSO)
6a	231—234	+125.9° (0.715, DMF)	**12a**	>300	+9.6° (0.540, DMSO)
6b	235—240	−125.9° (0.918, DMF)	**12b**	>300	−9.4° (0.490, DMSO)
8a	259—260	+68.9° (0.700, AcOH)	**13a**	222—225 (dec.)	+76.7° (0.350, 0.05 N NaOH)
8b	258—259	−68.1° (0.520, AcOH)	**13b**	225—227 (dec.)	−76.9° (0.385, 0.05 N NaOH)
9a	>300	+65.6° (0.903, DMSO)	**14a**	225—235 (dec.)	+109.4° (0.435, DMSO)
9b	>300	−65.6° (0.950, DMSO)	**14b**	228—239 (dec.)	−109.6° (0.500, DMSO)
10a	275—278	+102.5° (0.640, AcOH)	**18**	107—108	+70.7 (0.953, $CHCl_3$)
10b	277—279	−102.1° (0.425, AcOH)	**19**	194—196	−277.2° (2.04, $CHCl_3$)

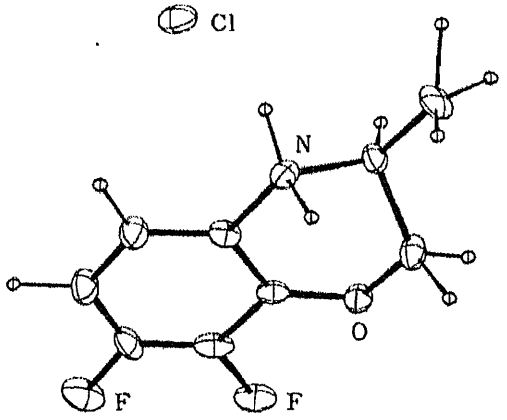


Fig. 1. The Molecular Structure of the Hydrochloride of **15b**

[mp 90—105 °C; $[\alpha]_D^{23}$ −33.2° (c=0.68, EtOH)], which belong to the orthorhombic system and have unit cell dimensions of a=10.8282(3), b=14.2060(6) and c=6.6090(4) Å. The space group was $P2_12_12_1$ and Z=4. The lattice parameters and intensities were measured on a Philips four-circle diffractometer with monochromated CuK_α radiation. A total of 764 independent reflections were obtained by the θ–2θ scan method. The crystal was sealed in a glass capillary, since it easily decomposed. The size of the crystal was approximately 0.3×0.2×0.2 mm.

The structure was solved by the direct method with the program MULTAN. Refinement

TABLE II. *In Vitro* Antibacterial Activities

Organism	MIC (μg/ml)					
	13b	**13a**	**1**	**14b**	**14a**	**2**
Staphylococcus aureus 209P	0.20	25.0	0.39	0.10	12.5	0.20
S. aureus SMITH	0.10	12.5	0.20	0.10	6.25	0.20
S. epidermidis 56500	0.78	>100	1.56	0.39	50.0	0.78
S. epidermidis 56556	0.39	25.0	0.39	0.39	50.0	0.39
Streptococcus pyogenes G-36	1.56	>100	3.13	1.56	100	1.56
S. faecalis ATCC 19433	1.56	>100	3.13	1.56	>100	1.56
Escherichia coli NIHJ	≦0.05	0.39	≦0.05	≦0.05	1.56	≦0.05
E. coli 05136	≦0.05	0.78	≦0.05	≦0.05	3.13	≦0.05
Salmonella enteritidis IID 604	≦0.05	1.56	0.10	≦0.05	3.13	≦0.05
Proteus vulgaris 08601	≦0.05	0.39	≦0.05	≦0.05	3.13	≦0.05
P. morganii IID 602	≦0.05	1.56	0.10	≦0.05	6.25	0.10
Klebsiella pneumoniae type 2	≦0.05	1.56	0.10	≦0.05	6.25	0.10
Enterobacter cloacae 03400	≦0.05	0.78	≦0.05	≦0.05	3.13	≦0.05
Serratia marcescens 10100	≦0.05	1.56	0.10	≦0.05	6.25	0.10
Pseudomonas aeruginosa 32104	0.39	12.5	0.78	0.78	100	0.78
P. aeruginosa 32121	0.10	6.25	0.20	0.10	12.5	0.10
P. maltophilia IID 1275	0.39	12.5	0.78	0.39	50.0	0.78

was carried out by the block-diagonal least-squares method using anisotropic temperature factors for non-hydrogen atoms and isotropic ones for hydrogen atoms. The final R value was 0.096. The absolute configuration was determined by the use of the anomalous scattering effect of the chlorine atom for CuK_α radiation.

The structure of the resulting hydrochloride of **15b** is shown in Fig. 1, and confirms that the absolute configuration of the chiral center is *S*. Consequently, the C_3 position in the oxazine ring in a series of the (−)-compounds (**b**) derived from **15b** was of the *S* configuration.

Biological Activities

Antibacterial activities of the optically active ofloxacins (**13a** and **13b**) and the corresponding fluoromethyl derivatives (**14a** and **14b**), together with their racemates (**1** and **2**),[11] were tested *in vitro*. The results are summarized in Table II in terms of the minimum inhibitory concentration (MIC, μg/ml) of the compounds, as determined by the serial dilution method.[12] *S*-(−)-Ofloxacin (**13b**) was 8 to 128 times more active than *R*-(+)-ofloxacin (**13a**), and about twice as active as the racemate (**1**) against both the gram-positive and the gram-negative bacteria tested. In the case of fluoromethyl derivatives, the *S*-(−)-isomer, **14b**, also showed an activity 32 to 128 times higher than the *R*-(+)-isomer, **14a**. These results indicate that the configuration of the methyl or fluoromethyl group at the C_3 position of **1** or **2** has a significant effect on the antibacterial activity.

Quinolone antibacterial agents are generally called desoxyribonucleic acid (DNA) gyrase inhibitors because of their mode of action.[13] In relation to this, a study on the activities of **13a** and **13b** toward bacterial DNA gyrase has recently been reported.[14]

Experimental

Melting points were taken in a Yanagimoto micromelting point apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a JEOL FX-90Q (90 MHz) or a Varian XL-200 (200 MHz) spectrometer. The optical rotations were recorded with a Horiba SEPA-200 polarimeter. The description in this experimental section refers to both of the optically active compounds.

(±)-Ethyl 9,10-Difluoro-2,3-dihydro-3-(3,5-dinitrobenzoyloxy)methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benz-

oxazine-6-carboxylate (4)——A mixture of **3** (1.0 g), 3,5-dinitrobenzoyl chloride (1.6 g) and pyridine (0.5 g) in dry tetrahydrofuran (THF) (100 ml) was heated under reflux for 1.5 h. Precipitate was formed around the end of the reaction. Removal of the solvent gave a solid, which was washed with MeOH and Et_2O to give **4** (1.2 g, 75%) as a colorless solid, mp 240—242 °C. ^{1}H-NMR ($CDCl_3$–5%DMSO-d_6) δ: 1.30 (3H, t, J=7 Hz, $CH_2\underline{CH_3}$), 4.26 (2H, q, J=7 Hz, $\underline{CH_2}CH_3$), 4.4—5.4 (5H, m, 2×CH_2 and CH), 7.76 (1H, dd, J=11, 7 Hz, C_8-H), 8.8 (1H, s, C_5-H), 9.0 (2H, d, J=3 Hz, 2×ArH), 9.2 (1H, t, J=3 Hz, ArH). *Anal.* Calcd for $C_{22}H_{15}F_2N_3O_{10}$: C, 50.88; H, 2.91; N, 8.09. Found: C, 50.75; H, 2.95; N, 8.30.

Optical Resolution of the (±)-Benzoate (4)——The (±)-benzoate (**4**) (6 mg) was dissolved in DMF (0.6 ml), and the solution was subjected to HPLC to give the optically pure isomers (**5a**) (2.5 mg), and (**5b**) (2.5 mg). Each isomer (250 mg) was obtained by repeating this operation. HPLC: SUMIPAX OA-4200 column (20×250 mm) (Sumitomo Chemical Co., Ltd.). Solvent: hexane–1,2-dichloroethane–EtOH=6:3:1. Flow rate: 8 ml/min. Retention times: **5a**, 56—76 min; **5b**, 78—98 min.

(−)-Ethyl 9,10-Difluoro-2,3-dihydro-3-hydroxymethyl-7-oxo-7*H*-pyrido[1,2,3,-*de*][1,4]benzoxazine-6-carboxylate (6b)——A suspension of **5b** (120 mg), EtOH (10 ml) and satd. $NaHCO_3$ solution (4 ml) was stirred with heating at 50—60 °C for 2 h. The mixture was concentrated to give the residue, which was washed with water, 95% EtOH and Et_2O to afford **6b** (68 mg, 91%) as colorless crystals. *Anal.* Calcd for $C_{15}H_{13}F_2NO_5$: C, 55.39; H, 4.03; N, 4.31. Found: C, 55.44; H, 4.01; N, 4.49. The NMR spectrum and thin-layer chromatography (TLC) behavior were identical with those of the racemate (**3**).[5] In the same manner as described above, the (+)-compound (**6a**) was synthesized from **5a**. *Anal.* Calcd for $C_{15}H_{13}F_2NO_5$: C, 55.39; H, 4.03; N, 4.31. Found: C, 55.48; H, 4.11; N, 4.20.

(−)-Ethyl 9,10-Difluoro-2,3-dihydro-3-iodomethyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylate (7b)——A suspension of **6b** (63 mg) and triphenylphosphite methiodide (341 mg) in DMF (12 ml) was stirred at room temperature for 1.5 h. After evaporation of the solvent, the residue was dissolved in $CHCl_3$, washed with aqueous $Na_2S_2O_3$ solution and brine, then dried over $MgSO_4$. Evaporation of the solvent gave a semi-solid, which was triturated with Et_2O to afford **7b** (78 mg, 93%) as a colorless powder, mp 214—217 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (3H, t, J=7 Hz, $CH_2\underline{CH_3}$), 3.38—3.70 (2H, m, CH_2), 4.36—4.58 (2H, m, 2×CH), 4.42 (2H, q, J=7 Hz, $\underline{CH_2}CH_3$), 5.04 (1H, dd, J=13, 2 Hz, CH), 7.86 (1H, dd, J=11, 7 Hz, C_8-H), 8.44 (1H, s, C_5-H). *Anal.* Calcd for $C_{15}H_{12}F_2INO_4$: C, 41.40; H, 2.78; N, 3.22. Found: C, 41.16; H, 2.58; N, 2.99. The (+)-compound (**7a**) was obtained in the same manner as described above.

(−)-Ethyl 9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylate (8b)——A mixture of **7b** (78 mg) and tri-*n*-butyltin hydride (0.2 ml) in EtOH (30 ml) was stirred at 50—60 °C for 1 h. Evaporation of the solvent gave a pale yellow powder, which was purified by preparative TLC on silica gel (developing solvent: $CHCl_3$–MeOH=20:1) and recrystallized from EtOH to afford **8b** (40 mg, 73%) as colorless needles. The NMR spectrum and TLC behavior were identical with those of the racemate.[2] *Anal.* Calcd for $C_{15}H_{13}F_2NO_4$: C, 58.26; H, 4.24; N, 4.53. Found: C, 58.49; H, 4.40; N, 4.49. The (+)-compound (**8a**) was obtained in the same manner as described above. *Anal.* Calcd for $C_{15}H_{13}F_2NO_4$: C, 58.26; H, 4.24; N, 4.53. Found: C, 58.25; H, 4.23; N, 4.58.

(−)-9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic Acid (9b)——A solution of **8b** (40 mg) in concd. HCl–AcOH (2:1) (6 ml) was refluxed for 40 min. Concentration of the solution to 2 ml gave crystals, which were filtered, washed with water, EtOH and Et_2O, then dried *in vacuo* to afford **9b** (22 mg, 61%) as colorless needles. *Anal.* Calcd for $C_{13}H_9F_2NO_4$: C, 55.52; H, 3.23; N, 4.98. Found: C, 55.79; H, 3.20; N, 4.91. The NMR spectrum and TLC behavior were identical with those of the racemate.[2] The (+)-compound (**9a**) was obtained in the same manner as described above. *Anal.* Calcd for $C_{13}H_9F_2NO_4$: C, 55.52; H, 3.23; N, 4.98. Found: C, 55.32; H, 3.25; N, 4.94.

(−)-9,10-Difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic Acid BF_2-chelate (12b)——Boron trifluoride etherate (0.3 ml) was added to a suspension of **9b** (21 mg) in Et_2O (5 ml) and the resulting mixture was stirred at room temperature for 1 h. The precipitate was filtered, washed with Et_2O and dried *in vacuo* to afford **12b** (24 mg, 98%) as pale yellow prisms. ^{1}H-NMR (DMSO-d_6) δ: 1.57 (3H, d, J=7 Hz, CH_3), 4.61 (1H, dd, J=12, 2 Hz, 1H of OCH_2), 4.87 (1H, d, J=12 Hz, 1H of OCH_2), 5.37 (1H, m, C_3-H), 8.21 (1H, dd, J=12, 8 Hz, C_8-H), 9.72 (1H, s, C_5-H). *Anal.* Calcd for $C_{13}H_8BF_4NO_4$: C, 47.45; H, 2.45; N, 4.26. Found: C, 47.43; H, 2.48; N, 4.33. The (+)-compound (**12a**) was obtained in the same manner as described above. *Anal.* Calcd for $C_{13}H_8BF_4NO_4$: C, 47.45; H, 2.45; N, 4.26. Found: C, 47.11; H, 2.55; N, 4.25.

(−)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic Acid (13b)——A solution of **12b** (24 mg) and 1-methylpiperazine (50 mg) in DMSO (3 ml) was stirred at room temperature for 17 h. After evaporation of the solvent, the residual oil was triturated with Et_2O to give a solid, which was dissolved in 95% MeOH (10 ml) and triethylamine (0.2 ml), and the solution was refluxed for 6 h. Evaporation of the solvent gave a residue, which was purified by preparative TLC on silica gel (developing solvent: lower layer solution of $CHCl_3$–MeOH–H_2O=15:3:1) to afford **13b** (14 mg, 52%) as a pale yellow powder. Crystallization from EtOH–Et_2O gave needles. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, d, J=7 Hz, C_3-CH_3), 2.38 (3H, s, N-CH_3), 2.54—2.60 (4H, m, 2×CH_2N), 3.40—3.44 (4H, m, 2×CH_2N), 4.35—4.52 (3H, m, CH and CH_2), 7.76 (1H, d, aromatic ring C_8-H), 8.64 (1H, s, C_5-H). *Anal.* Calcd for $C_{18}H_{20}FN_3O_4 \cdot 1/2H_2O$: C, 58.37 H, 5.72; N, 11.35. Found:

C, 58.17; H, 5.58; N, 11.27. (+)-Ofloxacin (**13a**) was obtained in the same manner as described above. *Anal.* Calcd for $C_{18}H_{20}FN_3O_4 \cdot 1/2H_2O$: C, 58.37; H, 5.72; N, 11.35. Found: C, 58.50; H, 5.64; N, 11.44.

(−)-Ethyl 9,10-Difluoro-3-fluoromethyl-2,3-dihydro-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylate (10b)——An excess of diethylaminosulfur trifluoride was added to a suspension of **6b** (70 mg) in dry CH_2Cl_2 (60 ml) and the solution was stirred at room temperature for 2 h. After addition of EtOH (2 ml), the reaction mixture was stirred at room temperature for 30 min, and washed with brine, then dried over Na_2SO_4. Evaporation of the solvent gave a pale yellow oil, which was chromatographed on silica gel using $CHCl_3$–MeOH (50 : 1 v/v) to yield **10b** (59 mg) as a powder. The (+)-compound (**10a**) was obtained in the same manner as described above.

(−)-9,10-Difluoro-3-fluoromethyl-2,3-dihydro-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic Acid (11b)——A solution of **10b** (57 mg) in concd. HCl–AcOH (2 : 1) (9 ml) was refluxed for 1 h. Concentration of the solvent to 1 ml gave a residue, which was filtered, washed with water, EtOH and Et_2O, and dried *in vacuo* to afford **11b** (43 mg, 83%) as a colorless powder. The (+)-compound (**11a**) was obtained in the same manner as described above.

(−)-9-Fluoro-3-fluoromethyl-2,3-dihydro-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic Acid (14b)——A solution of **11b** (41 mg) and 1-methylpiperazine (50 mg) in DMSO (5 ml) was stirred at 110—120 °C for 40 min. The solvent was evaporated *in vacuo*, and the residue was triturated with Et_2O to give a powder, which was purified by preparative TLC on silica gel (developing solvent: lower layer solution of $CHCl_3$–MeOH–H_2O = 7 : 3 : 1) to yield a yellow oil. The oil was dissolved in water, and the solution was lyophilized to yield **14b** (40 mg, 77%) as an amorphous solid. ^{1}H-NMR ($CDCl_3$) δ: 2.35 (3H, s, N-CH_3), 2.45—2.7 (4H, m, 2 × CH_2N), 3.3—3.5 (4H, m, 2 × CH_2N), 4.40, 4.75 (each 1H, brd, J = 12 Hz, 2 × C_2-H), 4.46, 4.85 (each 1H, dm, J = 36 Hz, CH_2F), 4.9—5.1 (1H, m, C_3-H), 7.70 (1H, d, J = 12 Hz, C_8-H), 8.63 (1H, s, C_5-H). *Anal.* Calcd for $C_{18}H_{19}F_2N_3O_4$: C, 56.99; H, 5.05; N, 11.08. Found: C, 56.67; H, 5.19; N, 10.82. The (+)-compound (**14a**) was obtained in the same manner as described above. *Anal.* Calcd for $C_{18}H_{19}F_2N_3O_4$: C, 56.99; H, 5.05; N, 11.08. Found: C, 56.76; H, 5.23; N, 10.80.

(+)- and (−)-7,8-Difluoro-2,3-dihydro-3-methyl-4-[(*S*)-*N*-*p*-toluenesulfonyl]prolinyl-4*H*-[1,4]benzoxazine (18) and (19)——(*S*)-*N*-*p*-Toluenesulfonylprolinyl chloride, prepared from (*S*)-*N*-*p*-toluenesulfonylproline[10] (61.9 g) and thionyl chloride (50 ml) in benzene (350 ml), was added to a solution of **17** (32.8 g) and pyridine (28 ml) in CH_2Cl_2 (300 ml) at room temperature, and the solution was stirred for an additional 2 h. The reaction mixture was washed with 10% HCl, satd. $NaHCO_3$ and brine, then dried over $MgSO_4$. Evaporation of the solvent gave an oil, which was crystallized from AcOEt–hexane to afford the (−)-amide (**19**). Concentration of the mother liquor gave an oil, which was chromatographed on silica gel using benzene–AcOEt (25 : 1, v/v) as an eluent to afford an oil. Crystallization from EtOH–Et_2O yielded the (+)-amide (**18**) (33.4 g, 86%) as colorless crystals.

18: ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, d, J = 7.2 Hz, CH_3), 2.42 (3H, s, ArMe), 6.78 (1H, dt, J = 7.2, 11 Hz, C_9-H), 7.32 (2H, d, J = 9 Hz, 2 × ArH), 7.42—7.66 (1H, m, C_6-H), 7.78 (2H, d, J = 9 Hz, 2 × ArH). *Anal.* Calcd for $C_{21}H_{22}F_2N_2O_4S$: C, 57.79; H, 5.08; N, 6.42. Found: C, 58.05; H, 5.14; N, 6.47.

19: ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J = 7 Hz, CH_3), 2.40 (3H, s, ArMe), 6.5—6.9 (2H, m, C_9-H, C_6-H), 7.0—7.4 (4H, m, 4 × ArH). *Anal.* Calcd for $C_{21}H_{22}F_2N_2O_4S$: C, 57.79; H, 5.08; N, 6.42. Found: C, 57.90; H, 5.03; N, 6.40.

(−)-7,8-Difluoro-2,3-dihydro-3-methyl-4*H*-[1,4]benzoxazine (15b)[9]——A solution of **18** (32.8 g) in EtOH (1000 ml) and 1 N NaOH (500 ml) was refluxed for 2 h. The solvent was evaporated and the residue was dissolved in benzene; this solution was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave an oil, which was chromatographed on silica gel using benzene as an eluent to afford the (−)-benzoxazine (**15b**) (12.2 g, 88%) as a colorless oil. $[\alpha]_D^{23}$ −7.8 (c = 6.80, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J = 6 Hz, CH_3), 3.53 (1H, m, C_3-H), 3.81 (1H, dd, J = 8, 10 Hz, C_2-H), 4.31 (1H, dd, J = 3, 10 Hz, C_2-H), 6.30 (1H, m, ArH), 6.60 (1H, m, ArH).

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Semisynthetic β-Lactam Antibiotics. IV. Synthesis and Antibacterial Activity of New Ureidocephalosporin and Ureidocephamycin Derivatives Containing a Catechol Moiety or Its Acetate

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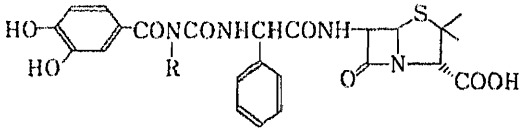
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New ureidocephalosporin and ureidocephamycin derivatives containing a catechol moiety or its acetate were prepared and their minimum inhibitory concentration values against various microorganisms were determined. Among these compounds, the ureidocephalosporins (**2**, **3Aa**, **3Ba**) and ureidocephamycins (**4**, **5**) carrying a methyl group on the nitrogen atom of the ureido bond showed strong activities against *Pseudomonas aeruginosa*. 7β-[(*R*)-2-[3-(3,4-Dihydroxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic acid (**5**) had the most potent activity *in vitro* against gram-negative bacteria, its activity being 8- to 32-fold and 4-fold greater than those of cefoperazone and ceftazidime, respectively, against two strains of *P. aeruginosa*. The structure–activity relationship is discussed.

Keywords—ceftazidime; anti-*Pseudomonas* activity; ureidocephalosporin; ureidocephamycin; catechol moiety; catechol acetate moiety; minimum inhibitory concentration; structure–activity relationship

In recent years, new cephalosporin and cephamycin derivatives with an expanded antibacterial spectrum and greater β-lactamase stability have been reported,[1~10] and ceftazidime[4] was proved to have potent activity against *Pseudomonas aeruginosa* strains, which are generally less sensitive to β-lactam antibiotics. Aiming at a further improvement, we have been continuing the research effort.

In our previous papers,[11,12] we reported the synthesis, antibacterial activity and structure–activity relationship of new ureidopenicillins having a catechol moiety, which we had synthesized in the expectation that penicillins carrying an iron-sequestering moiety would be readily incorporated into bacterial cells. In the course of our studies, we found that 6-[(*R*)-2-[3-(3,4-dihydroxybenzoyl)-3-methylureido]-2-phenylacetamido]penicillanic acid (**1a**) had the highest *in vitro* activity against gram-negative bacteria, including *P. aeruginosa*, and its derivative (**1b**) showed remarkable activity against *P. aeruginosa in vivo* as well as *in vitro*.



1a : R=Me
1b : R=$(CH_2)_3OH$

Chart 1

These findings encouraged us to synthesize ureidocephalosporins and ureidocephamycins having a catechol moiety to investigate whether these compounds also have strong anti-

bacterial activity and a broad spectrum. In the course of synthesizing the ureidocephalosporins (**3**) with a catechol moiety, various substituents (R_2), *e.g.*, methyl, 1-hydroxypropyl, cyanoethyl, methoxyethyl, or ethoxycarbonylmethyl were introduced on the nitrogen atom of the ureido bond in combination with an acetoxymethyl or 1-methyl-1*H*-tetrazole-5-thiomethyl group at the C-3 position. Encouraged by the result that cephalosporins (**2**, **3Aa**, **3Ba**; R_2=Me) showed high *in vitro* activity, we introduced a 3-(3,4-diacetoxybenzoyl)-3-methyl-1-ureido group or a 3-(3,4-dihydroxybenzoyl)-3-methyl-1-ureido group into the C-7 side chain of 7α-methoxy-3-cephem derivatives carrying an acetoxymethyl or a 1-methyl-1*H*-

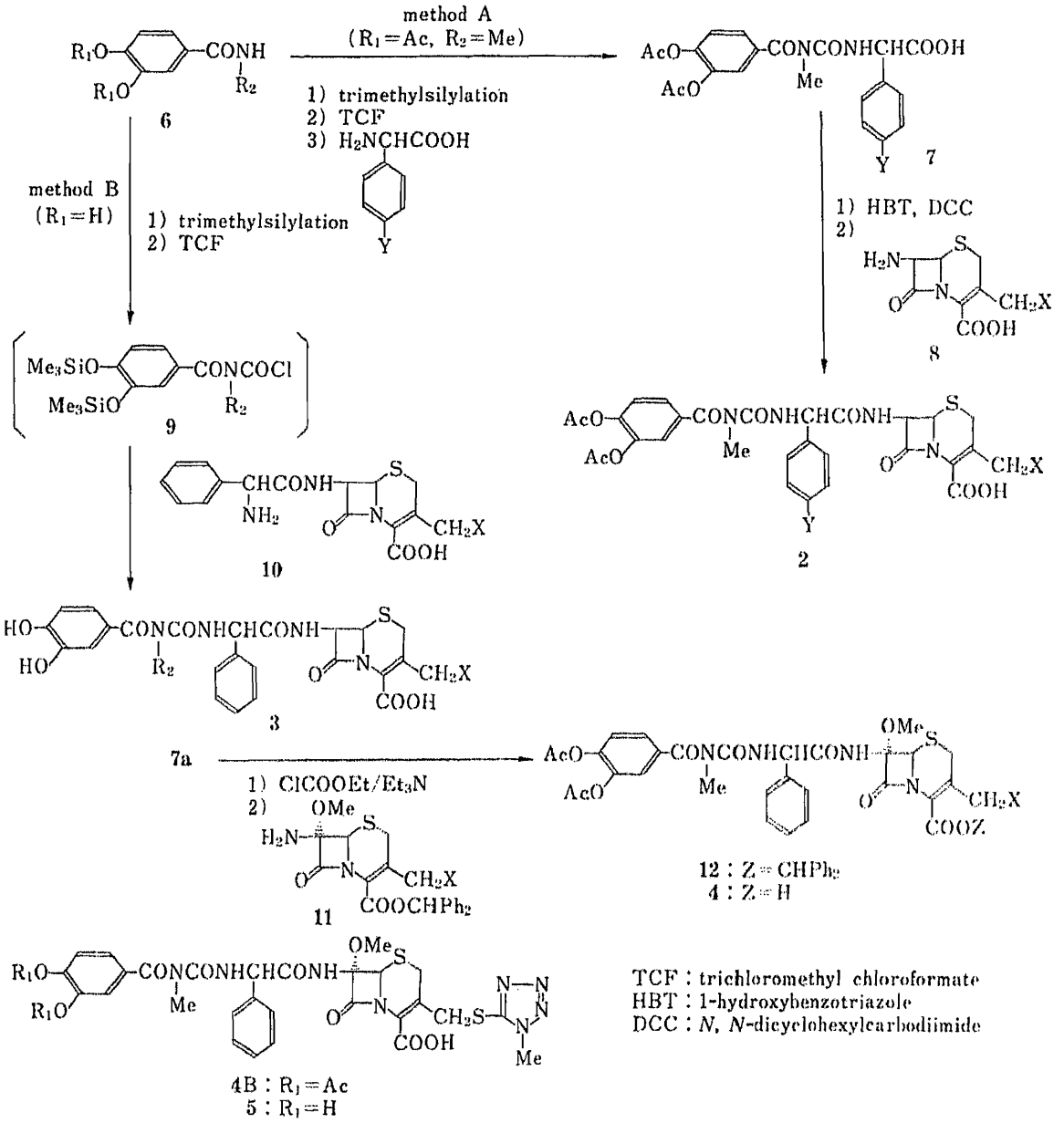
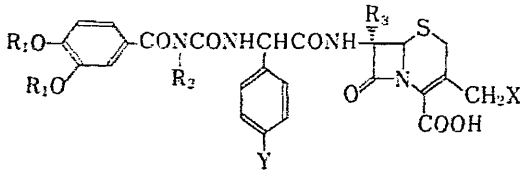


Chart 2

TABLE I. Antibacterial Activities of Cephalosporins (2, 3) and Cephamycins (4, 5)



Compd. No.	R_1	R_2	R_3	X	Y	MIC (μg/ml)								
						S. aureus		*E. coli* NIHJ	*K. Pneumoniae*	*P. morganii*	*S. marcescens*	*E. cloacae*	*P. aeruginosa*	
						209P JC-1	JU-5[a]	JC-2	JU-90	JU-241	FU-104	FU-250	Ps-6	Ps-33
2Aa	Ac	CH_3	H	OAc	H	0.78	3.13	0.20 (1.56)[b]	0.10	3.13	1.56 (100)[b]	0.39	1.56	1.56
2Ab	Ac	CH_3	H	OAc	OH	1.56	3.13	0.20 (1.56)	0.05	3.13	1.56 (100)	0.39	1.56	1.56
2Ba	Ac	CH_3	H	MTT[c]	H	0.78	1.56	0.20 (1.56)	0.10	3.13	1.56 (100)	0.39	1.56	1.56
2Bb	Ac	CH_3	H	MTT	OH	1.56	3.13	0.20 (1.56)	0.05	1.56	0.78 (50)	0.20	0.78	3.13
3Aa	H	CH_3	H	OAc	H	0.78	3.13	0.20 (12.5)	0.05	3.13	1.56 (50)	0.39	0.78	0.78
3Ab	H	$(CH_2)_3OH$	H	OAc	H	0.39	1.56	3.13 (12.5)	0.10	12.5	25 (400)	12.5	6.25	6.25
3Ac	H	$(CH_2)_2CN$	H	OAc	H	0.78	1.56	3.13 (25)	0.10	12.5	6.25 (400)	6.25	25	25
3Ad	H	$(CH_2)_2OMe$	H	OAc	H	0.78	1.56	6.25 (25)	0.10	12.5	12.5 (400)	12.5	25	50
3Ae	H	CH_2COOEt	H	OAc	H	1.56	3.13	6.25 (50)	0.20	25	100 (400)	50	12.5	12.5
3Ba	H	CH_3	H	MTT	H	0.78	3.13	0.20 (6.25)	0.05	0.78	0.78 (200)	0.78	0.78	0.78
3Bb	H	$(CH_2)_3OH$	H	MTT	H	0.78	0.78	3.13 (12.5)	0.10	6.25	6.25 (100)	1.56	12.5	6.25
4A	Ac	CH_3	OMe	OAc	H	6.25	6.25	0.39 (1.56)	0.10	6.25	3.13 (50)	0.78	6.25	6.25
4B	Ac	CH_3	OMe	MTT	H	1.56	3.13	0.20 (0.78)	0.05	1.56	0.78 (25)	0.20	1.56	1.56
5	H	CH_3	OMe	MTT	H	1.56	3.13	0.10 (0.78)	0.05	0.78	0.39 (6.25)	0.20	0.78	0.78
Cefoperazone						0.78	1.56	0.20 (0.78)	0.05	1.56	3.13 (25)	0.10	6.25	25
Ceftazidime						6.25	3.13	0.39 (0.39)	0.20	0.10	0.39 (0.78)	0.39	3.13	3.13

a) Penicillinase producer. *b*) One loopful of 1×10^9/ml. *c*) MTT: 1-methyl-1*H*-tetrazole-5-thiol.

tetrazole-5-thiomethyl group at C-3.

This paper deals with the synthesis and structure–activity relationships of ureidocephalosporins (**2**, **3**) and ureidocephamycins (**4**, **5**) containing a catechol moiety or its acetate.

Chemistry

As outlined in Chart 2, we prepared the cephalosporins listed in Table I by two general methods. The cephalosporins (**2**) having two acetoxy groups on the benzene ring were prepared by acylation of the corresponding 7-aminocephalosporins (**8**) with active esters derived from 1-hydroxybenzotriazole (HBT) and the ureidocarboxylic acids (**7**)[11] (method A). An alternative method (method B) involving reaction of the carbamoyl chloride (**9**)[11,12] and silylated phenylglycylcephalosporins (**10**), was developed for the preparation of the cephalosporins (**3**) having a catechol moiety. The cephamycins (**4**) were synthesized by acylation of the diphenylmethyl 7β-amino-7α-methoxy-3-cephem-4-carboxylates (**11**)[8,9] with the mixed anhydride derived from (*R*)-2-[3-(3,4-diacetoxybenzoyl)-1-ureido]phenylacetic acid (**7a**) and ethyl chloroformate, followed by cleavage of the diphenylmethyl protecting group using trifluoroacetic acid (TFA)/anisole. Ureidocephamycin (**5**) having a catechol group was obtained by removal of the acetyl groups from cephamycin (4B) using *N*,*N*-diethylethanolamine in *N*,*N*-dimethylformamide (DMF).[11]

Antibacterial Activity

The minimum inhibitory concentration (MIC) values of the cephalosporins (**2**, **3**) and the cephamycins (**4**, **5**) were determined by the serial two-fold agar dilution method against two species of gram-positive and seven species of gram-negative bacteria. The *in vitro* activities of these compounds (**2**—**5**) compared to those of cefoperazone and ceftazidime are listed in Table I. The substituent (R_2) on the ureido bond influenced the antibacterial activity against gram-negative bacteria. The methyl analogs (**3Aa**, **3Ba**) showed the highest activity. The compound (**3Ab**) having a hydroxypropyl group as the substituent (R_2) was less potent against gram-negative bacteria including *P. aeruginosa* than **3Aa**, contrary to our expectation based on the case of penicillins. Enhanced antibacterial activity against gram-negative bacteria is often observed as a result of replacing the acetoxy group at C-3 with heteroaromatic thiols, such as 1-methyl-1*H*-tetrazole-5-thiol.[13,14] Again, contrary to our expectation, no significant improvement was observed when 1-methyl-1*H*-tetrazole-5-thiol was introduced into cephalosporins (**2Aa**, **2Ab**, **3Aa**). In the case of cephamycin (**4B** *vs.* **4A**), however, some improvement was attained. The cephamycin (**5**) bearing 1-methyl-1*H*-tetrazole-5-thiol as the substituent (X) was more active than the corresponding cephalosporin (**3Ba**) against *E. coli* NIHJ JC-2 and *S. marcescens* FU-104 when the inoculum size of the two strains was increased from 10^6 to 10^9 cfu/ml. Furthermore, this compound proved to be strongly active *in vitro* against *P. aeruginosa* with an 8- to 32-fold increase in activity compared with cefoperazone, and 4-fold compared with ceftazidime.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi EPI-G3 spectrometer. The nuclear magnetic resonance (NMR) spectra were measured on a Hitachi R-20A spectrometer using tetramethylsilane (TMS) as an internal standard. All chemical shifts are reported in δ ppm.

7-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]cephalosporanic Acid (2Aa)——*N*,*N*-Dicyclohexylcarbodiimide (DCC) (2.1 g) in tetrahydrofuran (THF) (5 ml) was added to a stirred solution of (*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-phenylacetic acid (**7a**, 5.2 g) and 1-hydroxybenzotriazole (1.4 g) in THF (50 ml) under cooling. The mixture was stirred at 0—5 °C for 1 h, and used for the following reaction.

N,*O*-Bis(trimethylsilyl) acetamide (BSA) (6.5 ml) was added to a stirred suspension of 7-aminocephalosporanic acid (7-ACA) (3.6 g) in CH_2Cl_2 (100 ml) at room temperature. Stirring was continued for 5 min, then trimethylchlorosilane (0.5 ml) was added to the mixture under ice-cooling. Stirring was continued for 1 h at 5 °C. To the resulting solution was added the reaction mixture containing activated ester described above at 5—10 °C, and the

whole was stirred at the same temperature for 3 h. After the organic solvents had been evaporated off, the residue was taken up in THF (150 ml). The insoluble materials were removed by filtration. Then, the filtrate was concentrated *in vacuo*, and the residue was purified by chromatography on a silica gel column using $CHCl_3$–MeOH (30 : 1) to give **2Aa** (2.0 g, 24%) as a white powder. IR (KBr): 1780, 1745—1680, 1510 cm^{-1}. NMR (acetone-d_6): 2.00 (3H, s), 2.28 (6H, s), 3.15 (3H, s), 3.5 (2H, br s), 4.78 and 5.09 (2H, AB q, J=13 Hz), 5.03 (1H, d, J=5 Hz), 5.5—6.0 (2H, m), 7.2—7.7 (8H, m), 8.33 (1H, d, J=8 Hz), 9.93 (1H, d, J=7 Hz).

7-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-(4-hydroxyphenyl)acetamido]cephalosporanic Acid (2Ab)——Compound **2Ab** was prepared from (*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-(4-hydroxyphenyl)-acetic acid (**7b**) and 7-ACA by the method described for **2Aa**. Yield 20%. IR (KBr): 1775, 1740—1670, 1510 cm^{-1}. NMR (acetone-d_6): 2.00 (3H, s), 2.28 (6H, s), 3.16 (3H, s), 3.5 (2H, br s), 4.78 and 5.11 (2H, AB q, J=13 Hz), 5.03 (1H, d, J=5 Hz), 5.5—6.1 (2H, m), 6.7—7.7 (7H, m), 8.29 (1H, d, J=8 Hz), 9.87 (1H, d, J=7 Hz).

7-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic Acid (2Ba)——Compound **2Ba** was prepared from (*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-phenylacetic acid (**7a**) and 7-amino-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic acid (**8B**) by the method described for **2Aa**. Yield 22%. IR (KBr): 1775, 1740—1680, 1510 cm^{-1}. NMR (acetone-d_6): 2.27 (6H, s), 3.13 (3H, s), 3.75 (2H, br s), 3.93 (3H, s), 4.37 (2H, br s), 5.11 (1H, d, J=5 Hz), 5.6—5.9 (2H, m), 7.2—7.7 (8H, m), 8.40 (1H, d, J=8 Hz), 9.95 (1H, d, J=7 Hz).

7-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-(4-hydroxyphenyl)acetamido]-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic Acid (2Bb)——Compound **2Bb** was prepared from (*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-(4-hydroxyphenyl) acetic acid (**7b**) and **8B** by the method described for **2Aa**. Yield 20%. IR (KBr): 1775, 1720—1680, 1510 cm^{-1}. NMR (acetone-d_6): 2.29 (6H, s), 3.17 (3H, s), 3.8 (2H, br s), 3.98 (3H, s), 4.40 (2H, br s), 5.09 (1H, d, J=5 Hz), 5.4—6.0 (2H, m), 6.7—7.7 (7H, m), 8.37 (1H, d, J=8 Hz), 9.78 (1H, d, J=7 Hz).

7-[(*R*)-2-[3-(3,4-Dihydroxybenzoyl)-3-methylureido]-2-phenylacetamido]cephalosporanic Acid (3Aa)——Triethylamine (10.1 g) was added dropwise to a stirred suspension of *N*-methyl-3,4-dihydroxybenzamide (**6a**, 5.0 g) and trimethylchlorosilane (10.9 g) in CH_2Cl_2 (70 ml) under ice-cooling. The mixture was refluxed for 1 h. After cooling of the reaction mixture, trichloromethyl chloroformate (TCF) (3.6 g) was added at 0 °C. Stirring was continued for 3 h at 20—30 °C and then for 1 h at the same temperature under reduced pressure to remove excess phosgene. The residue was used for the following reaction.

Silylated cephaloglycin (**10A**) [prepared from **10A** (15.8 g), trimethylchlorosilane (12.5 g) and triethylamine (8.9 g)] in CH_2Cl_2 (200 ml) was added to the residue described above at 0—5 °C. Stirring was continued for 1.5 h at 5—10 °C. After the organic solvents had been evaporated off, the residue was taken up in mixed solvents (EtOAc : THF = 1 : 1, 300 ml) and 1 N HCl (100 ml) under ice-cooling. The organic layer was separated, washed with brine, dried over $MgSO_4$ and evaporated to dryness to give a crude product, which was dissolved in 5% $NaHCO_3$ solution (10 ml) and chromatographed over Diaion HP-20 with elution by H_2O and 30% aqueous acetone in order. The fractions containing the product were combined and adjusted to pH 2.5 with 2 N HCl after the addition of EtOAc (200 ml). The EtOAc layer was separated, washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was triturated with *n*-hexane to give **3Aa** (1.4 g, 8%) as a white powder.

Compounds **3Ab**—**3Ae**, **3Ba**, **3Bb** were prepared in a manner similar to that described for **3Aa**. In the cases of **3Ba** and **3Bb**, 7-[(*R*)-2-amino-2-phenylacetamido]-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic acid was utilized in place of **10A**. NMR and IR spectral data of these compounds and **3Aa** are given in Table II.

Diphenylmethyl 3-Acetoxymethyl-7β-[(*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-cephem-4-carboxylate (12A)——*N*-Methylmorpholine (0.32 g) in CH_2Cl_2 (5 ml) was added to a stirred solution of **7a** (1.38 g) and ethyl chloroformate (0.35 g) in CH_2Cl_2 (30 ml) at −15—−10 °C. Stirring was continued at the same temperature for 1 h, then diphenylmethyl 7β-amino-7α-methoxycephalosporanate (**11A**, 1.50 g) in CH_2Cl_2 (10 ml) was added dropwise to the reaction mixture below −10 °C. The whole was stirred at −15—−10 °C for 2 h. Furthermore, stirring was continued while allowing the temperature to rise to room temperature over 2 h. Then the solvent was evaporated off and EtOAc (100 ml) was added to the residue. The EtOAc solution was washed successively with saturated $NaHCO_3$ solution (100 ml) and brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by chromatography on a silica gel column. Elution with EtOAc–benzene (1 : 2) gave **12A** (0.45 g, 16%) as a pale yellow powder. IR (KBr): 3300, 1775, 1740—1680, 1510—1490 cm^{-1}. NMR ($CDCl_3$): 2.01 (3H, s), 2.28 (6H, s), 3.19 (3H, s), 3.2 (2H, br s), 3.51 (3H, s), 4.76 and 5.03 (2H, AB q, J=13 Hz), 5.02 (1H, s), 5.62 (1H, d, J=7 Hz), 6.90 (1H, s), 7.0—7.7 (19H, br s), 9.97 (1H, d, J=7 Hz).

Diphenylmethyl 7β-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylate (12B)——Compound **12B** was prepared from **7a** and diphenylmethyl 7β-amino-7α-methoxy-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylate (**11B**) by the method described for **12A**. Yield 25%. IR (KBr): 3400, 1775, 1730—1680, 1510—1490 cm^{-1}. NMR ($CDCl_3$): 2.28 (6H, s), 3.17 (3H, s), 3.50 (3H, s), 3.74 (3H, s), 3.8 (2H, br s), 4.3 (2H, m), 5.01 (1H, s), 5.67 (1H, d, J=7 Hz), 6.89 (1H, s), 7.1—7.7 (19H, br s), 9.99 (1H, d, J=7 Hz).

3-Acetoxymethyl-7β-[(*R*)-2-[3-(3,4-diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-

TABLE II. IR and NMR Spectral Data for Cephalosporins (3)

Compound No.	IR ν_{max}^{KBr} cm^{-1} (C=O)	NMR δ value (solvent)
3Aa	1775, 1730, 1675	(DMSO-d_6): 2.02 (3H, s), 3.12 (3H, s), 3.5 (2H, brs), 4.66 and 5.03 (2H, AB q, J=13 Hz), 5.5—5.9 (2H, m), 6.8—7.6 (8H, m), 9.42 (1H, d, J=8 Hz), 9.74 (1H, d, J=8 Hz)
3Ab	1775, 1715, 1675	(DMSO-d_6): 1.4—2.0 (2H, m), 2.00 (3H, s), 3.1—4.0 (6H, m), 4.65 and 5.01 (2H, AB q, J=13 Hz), 5.01 (1H, d, J=5 Hz), 5.4—5.9 (2H, m), 6.7—7.6 (8H, m), 9.22 (1H, d, J=8 Hz), 9.33 (1H, d, J=8 Hz)
3Ac	1775, 1730, 1690	(DMSO-d_6): 2.01 (3H, s), 2.6—3.0 (2H, m), 3.5 (2H, brs), 3.7—4.2 (2H, m), 4.68 and 5.03 (2H, AB q, J=13 Hz), 5.05 (1H, d, J=5 Hz), 5.4—5.9 (2H, m), 6.8—7.6 (8H, m), 9.18 (1H, d, J=8 Hz), 9.40 (1H, d, J=8 Hz)
3Ad	1775, 1725, 1680	(DMSO-d_6): 2.01 (3H, s), 3.1—4.1 (6H, m), 3.18 (3H, s), 4.67 and 5.02 (2H, AB q, J=13 Hz), 5.04 (1H, d, J=5 Hz), 5.4—5.9 (2H, m), 6.7—7.6 (8H, m), 9.18 (1H, d, J=8 Hz), 9.40 (1H, d, J=8 Hz)
3Ae	1775, 1730, 1690	(DMSO-d_6): 1.0—1.4 (3H, m), 2.00 (3H, s), 3.5 (2H, brs), 3.9—4.3 (2H, m), 4.4 (2H, brs), 4.66 and 5.03 (2H, AB q, J=13 Hz), 5.05 (1H, d, J=5 Hz), 5.5—6.0 (2H, m), 6.8—7.7 (8H, m), 9.44 (1H, d, J=8 Hz), 9.54 (1H, d, J=8 Hz)
3Ba	1775, 1680	(DMSO-d_6): 3.10 (3H, s), 3.6 (2H, brs), 3.93 (3H, s), 4.3 (2H, brs), 5.03 (1H, d, J=5 Hz), 5.4—5.8 (2H, m), 6.8—7.6 (8H, m), 9.43 (1H, d, J=8 Hz), 9.73 (1H, d, J=8 Hz)
3Bb	1775, 1680	(DMSO-d_6): 1.4—2.0 (2H, m), 3.2—4.0 (6H, m), 3.94 (3H, s), 4.3 (2H, brs), 5.02 (1H, d, J=5 Hz), 5.4—5.9 (2H, m), 6.7—7.6 (8H, m), 9.28 (1H, d, J=8 Hz), 9.41 (1H, d, J=8 Hz)

cephem-4-carboxylic Acid (4A)——Compound **12A**, (0.40 g) was dissolved in anisole (1.5 ml) and CH_2Cl_2 (3 ml), and trifluoroacetic acid (3 ml) was added to the solution under ice-cooling. The mixture was stirred for 30 min at 5°C, then the excess TFA and the organic solvents were removed *in vacuo*. The residual oil was treated with Et_2O to give **4A** (0.25 g, 78%) as a pale yellow powder. IR (KBr): 1775, 1740—1680, 1505 cm^{-1}. NMR (acetone-d_6): 2.01 (3H, s), 2.28 (6H, s), 3.17 (3H, s), 3.2 (2H, brs), 3.50 (3H, s), 4.26 and 5.07 (2H, AB q, J=13 Hz), 5.09 (1H, s), 5.72 (1H, d, J=7 Hz), 7.2—7.7 (8H, m), 8.6 (1H, brs), 9.85 (1H, d, J=7 Hz).

7β-[(*R*)-2-[3-(3,4-Diacetoxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic Acid (4B)——Compound **4B** was prepared in a manner similar to that described for **4A**. Yield 80%. IR (KBr): 1775, 1720—1670, 1510 cm^{-1}. NMR (acetone-d_6): 2.28 (6H, s), 3.16 (3H, s), 3.48 (3H, s), 3.8 (2H, brs), 3.94 (3H, s), 4.22 and 4.49 (2H, AB q, J=13 Hz), 5.03 (1H, s), 5.70 (1H, d, J=7 Hz), 7.2—7.6 (8H, m), 8.6 (1H, brs), 9.82 (1H, d, J=7 Hz).

7β-[(*R*)-2-[3-(3,4-Dihydroxybenzoyl)-3-methylureido]-2-phenylacetamido]-7α-methoxy-3-[(1-methyl-1*H*-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylic Acid (5)——2-Diethylaminoethanol (1 ml) was added to a solution of **4B** (0.35 g) in DMF (1 ml) under ice-cooling, and stirring was continued at room temperature for 5 h. When the reaction was over, the solution was poured into 1 N HCl (50 ml), EtOAc (50 ml) and acetone (10 ml). The aqueous layer was separated and further extracted with EtOAc (30 ml) and acetone (6 ml). The combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was treated with Et_2O to afford **5** (0.25 g, 81%) as a pale yellow powder. IR (KBr): 1775, 1680, 1515 cm^{-1}. NMR (acetone-d_6): 3.18 (3H, s), 3.49 (3H, s), 3.5 (2H, brs), 3.94 (3H, s), 4.23 and 4.51 (2H, AB q, J=13 Hz), 5.03 (1H, s), 5.70 (1H, d, J=7 Hz), 6.9—7.7 (8H, m), 8.6 (1H, brs), 9.90 (1H, d, J=7 Hz).

Determination of *in Vitro* Antibacterial Activity——All the *in vitro* antibacterial activities are given as MIC (in μg/ml) required to prevent growth of the bacterial culture. MIC's were determined by the agar dilution method using Antibiotic Medium No. 3 agar after incubation at 37°C for 20 h, with an inoculum size of about 10^6 cfu/ml.

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Chemical Modification of Glycyrrhetinic Acid in Relation to the Biological Activities[1)]

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18β-Olean-12-ene-3β,30-diol (deoxoglycyrrhetol) (**4a**) was prepared with a view to eliminating pseudoaldosteronism, a side-effect of glycyrrhetinic acid (**1b**), which is the sapogenin of Licorice saponin, glycyrrhizin (**1a**), while maintaining or enhancing the therapeutic activities.

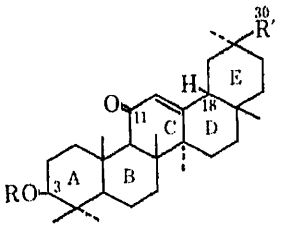
On reduction of the 11-keto and 30-carboxyl groups of **1b** with $NaAlH_2$-$(OCH_2CH_2OCH_3)_2$, olean-12-ene-3β,11β,30-triol (**9**) and olean-12-ene-3β,11α,30-triol (**10**) were obtained. On catalytic hydrogenation of **9** and **10** with Pd–C as a catalyst, **4a** was formed in an overall yield of 80%. On treatment with conc. HCl, **9** yielded 18β-olean-9(11),12-diene-3β,30-diol (**11a**), while **10** yielded olean-11,13(18)-diene-3β,30-diol (**12a**). Mono- and di-β-carboxy-propionyl and mono- and di-*o*-phthaloyl esters of **4a**, **11a** and **12a** were prepared to increase the hydrophilic character.

The competitive inhibition of 5β,Δ^4-reductase of corticosteroids in the liver which is caused by **1b** to induce pseudoaldosteronism was not observed in the case of **4a**. Compounds **4a**, **11a** and **12a** and their β-carboxypropionyl and *o*-phthaloyl esters were studied pharmacologically, and showed antiulcerogenic, antiallergic and antiinflammatory activities.

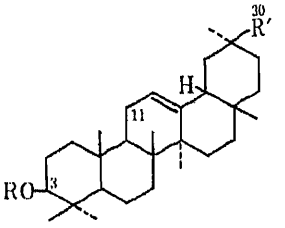
Keywords—glycyrrhizin; glycyrrhetinic acid; 18β-olean-12-ene-3β,30-diol; deoxoglycyrrhetol; 18β-olean-9(11),12-diene-3β,30-diol; 18β-olean-11,13(18)-diene-3β,30-diol

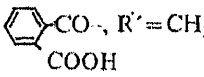
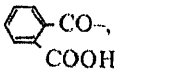
Glycyrrhizin (**1a**) and glycyrrhetinic acid (**1b**), a major saponin of licorice root (*Glycyrrhiza* spp. (Leguminosae)) and its aglycone, are known to be effective as antiallergic,[3)] antiinflammatory[4)] and antiulcer[5)] agents. Disodium 3-*O*-β-carboxypropionyl-glycyrrhetinate (carbenoxolone sodium)[6)] (**1c**) is used orally as a remedy for stomach ulcer, and a preparation of ammonium glycyrrhizinate combined with L-cysteine and glycine (strong neominophagen-C) (SNMC) is clinically applied by intravenous injection as an antiallergic agent. Recently SNMC was shown to be effective against chronic hepatitis in a clinical double blind trial.[7)] Antiviral activity of ammonium glycyrrhizinate *in vitro* was also reported.[8)]

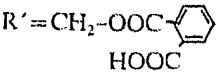
Thus glycyrrhizin and glycyrrhetinic acid are noteworthy drugs among phytotherapeutics, while the licorice root itself has long been used in traditional or folkloric medicine of both the East and the West. However, a side effect (induction of edema and hypertension) has been observed in patients given high doses of glycyrrhizin and glycyrrhetinic acid for prolonged periods. This side effect, a mineral corticoid-like action noted as pseudoaldosteronism, is due to the inhibition of the metabolic clearance of endogenous corticoid with

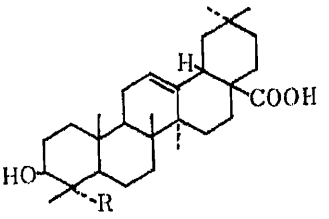


1a: R = GlcAβ(1→2)GlcA, R′ = COOH (glycyrrhizin)
1b: R = H, R′ = COOH (glycyrrhetinic acid)
1c: R = NaOOC(CH$_2$)$_2$–CO–, R′ = COOH
3: R = H, R′ = CH$_2$OH (glycyrrhetol)

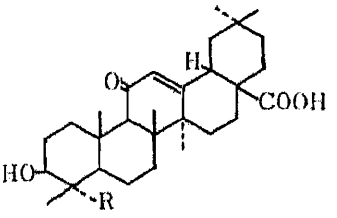


2: R = H, R′ = COOH (deoxoglycyrrhetinic acid)[17]
4a: R = H, R′ = CH$_2$OH (deoxyoglycyrrhetol)
4b: R = HOOC(CH$_2$)$_2$–CO–, R′ = CH$_2$OH
4c: R = HOOC(CH$_2$)$_2$–CO–, R′ = CH$_2$OOC(CH$_2$)$_2$–COOH
4d: R =  , R′ = CH$_2$OH
4e: R =  ,





5: R = CH$_2$OH (hederagenin)
7: R = CH$_3$ (oleanolic acid)



6: R = CH$_2$OH (11-oxohederagenin)
8: R = CH$_3$ (11-oxo-oleanolic acid)[18]

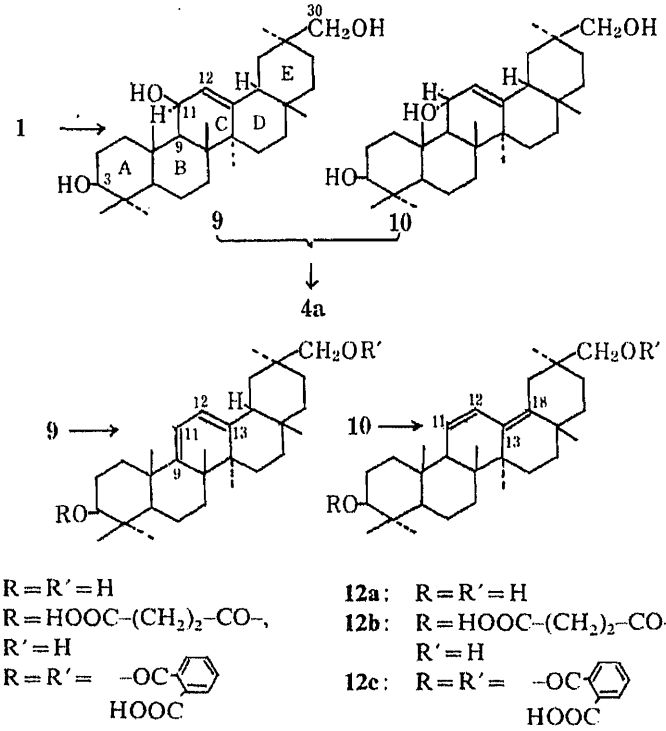
Chart 1

retention of Na^+ and water and excretion of K^+.

Kumagai *et al.*[9] and Atherden[10] found significant inhibition of the reduction of the Δ^4-3-keto system of cortical steroid by glycyrrhizin and glycyrrhetinic acid in rat liver, which contains soluble Δ^4-5β-reductase and microsomal Δ^4-5α-reductase (the former is the major enzyme in human liver). Atherden[10] showed that 11-deoxo-glycyrrhetinic acid (**2**) does not inhibit rat liver Δ^4-reductase and suggested that the presence of the 11-keto-$\Delta^{12(13)}$ system in ring C of glycyrrhetinic acid is essential for the inhibitory activity against the reductase. Baran *et al.*[11] prepared several derivatives of glycyrrhetinic acid to examine the reductase-inhibiting activities, and reached the same conclusion as above, while they observed loss of the antiulcer activity of the parent compound on modification of the structure. It seems plausible that the 11-oxo-$\Delta^{12(13)}$ system in ring C of glycyrrhetinic acid is competitive with the 3-oxo-$\Delta^{4(5)}$ system in ring A of cortical steroids at the active site of the reducing enzyme.

The present study has been designed to prepare modified compounds of glycyrrhetinic acid with a view to eliminating the pseudoaldosteronism while retaining or enhancing the therapeutic activities.

For the exclusion of pseudoaldosteronism, destruction of the 11-oxo-$\Delta^{12(13)}$ system in ring C of glycyrrhetinic acid was attempted, and for the enhancement of the therapeutic activities, a modification of the terminal carboxyl attached to ring E of the parent compound into hydroxymethyl was examined. The significance of the presence of a hydroxymethyl group attached to the terminal ring of oleanane-type triterpenoids for enhancing pharmacological activities was shown in the cases of saikosaponins a and d,[12] platycodins A and D,[13] *Aesculus hippocastanum* seed saponins, "aescin,"[14] and *Sapindus mukurossi* saponins and their aglycone, hederagenin.[15] It is remarkable to note that saikosaponin c is pharmacologically ineffective,[16] presumably because it possesses no hydroxymethyl group in ring A while the other part of the structure is almost the same as in its homologues a and d.



11a: R=R'=H
11b: R=HOOC-$(CH_2)_2$-CO-, R'=H
11c: R=R'= -OC(HOOC)C$_6$H$_4$

12a: R=R'=H
12b: R=HOOC-$(CH_2)_2$-CO-, R'=H
12c: R=R'= -OC(HOOC)C$_6$H$_4$

Chart 2

Chemistry

The target compound of the present study is 18β-olean-12-ene-3β-30-diol (18β-deoxoglycyrrhetol) (**4a**), which satisfies the above requirements. Some other oleanane derivatives (**2**, **3**, **5**, **6**, **7** and **8**) were also prepared as the reference compounds to compare their biological activities with those of deoxoglycyrrhetol (**4a**).

Almost all the above compounds except 11-oxohederagenin (**6**) are known in the literature, though the reported preparation procedures have been modified to some extent in the present study. Introduction of a keto group at the 11-position in ring C of oleanane-type triterpenes was performed by the following procedure: Acetylation of hederagenin (**5**) and oleanolic acid (**7**) at the hydroxyl group followed by oxidation with *tert*-butylchromate and deacetylation with alkali in dioxane afforded **6** and **8**, respectively.

Compound **4a** was synthesized by Ryabinin and Konovalova[19] starting from methyl glycyrrhetinate by catalytic reduction of the 11-keto group followed by reduction of the 30-carbomethoxyl to hydroxymethyl with $LiAlH_4$. Compound **4a** was also prepared by Canonica *et al.*[20] from naturally occurring olean-12-en-11-oxo-3β,30-diol (glycyrrhetol, a minor sapogenin of Licorice root) (**3**) by hydrogenation using PtO_2 as the catalyst.

During preliminary studies of the foregoing procedures, the following process was found to be the most efficient in giving a high yield of **4a**. Glycyrrhetinic acid (**1a**) was reduced with sodium bis(2-methoxyethoxy)aluminum hydride (vitride)[21] in tetrahydrofuran (THF) to afford olean-12-ene-3β,11β,30-triol (**9**), mp 252 °C $[\alpha]_D$ +89.6° (dioxane) and olean-12-ene-3β,11α-30-triol (**10**), mp 224 °C $[\alpha]_D$ +5.75° (dioxane), whose stereochemistry at the 11-position was established by the magnitude of the coupling constants of the proton nuclear magnetic resonance (^{1}H-NMR) signals due to 9α-H, 11ξ-H and 12-H. The 11-α-H of **9a** bisects the dihedral angle between 9α-H and 12-H (*ca.* 90°) to give a triplet ^{1}H-NMR signal (J=6 Hz) at δ 4.4. The double doublets (J_1=4 Hz and J_2=8 Hz) at δ 4.4 in the NMR spectrum of **10** agree well with the result given by Finucane and Thomson[24] for 3β-

acetoxyolean-12-en-11α-ol.

The major product possesses 11-β-hydroxyl (**9**) and the minor one 11α-hydroxyl (**10**), both of which are convertible to an equilibrium mixture on standing in chloroform at room temperature. The determination of the stereochemistry at C-11 and the assignment of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) chemical shifts of C-11 and C-12 of the triol (**9**) were performed by means of a deuteration experiment using $NaAlD_4$ as the reducing agent for methyl glycyrrhetinate (30-methyl ester of **1b**) a deuterated triol, $C_{30}H_{47}O_3D_3$, which gave the same *Rf* value on thin layer chromatography (TLC) as the major triol (**9**), was afforded as the major product. $LiAlD_4$ should attack the C-11 carbonyl of **1b** from the less-hindered α-direction to produce α-D, β-OH at this position. On acetylation, **9** and **10** gave the same triacetate, mp 96 °C, $[\alpha]_D$ +220°, having β-OAc at the 11-position. On acid treatment, **9** afforded only a homoannular diene (**11a**), while **10** yielded a heteroannular diene (**12a**).

On acid treatment the major deuterated triol having α-D, β-OH at C-11 was converted into a homoannular diene which retained the deuterium atom at C-11. The ^{13}C-NMR spectrum of this compound showed, in comparison with that of non-deuterated **11a**, a specific corruption of a doublet signal at δ 115.6, assignable to C-11. Thus the doublet signal at δ 121.5 was assigned to C-12, and those at δ 154.5(s) and 146.3(s) to C-9 and C-13 or *vice versa*.

On catalytic reduction of either **9** or **10** with Pd–C as the catalyst, **4a**, mp 250—251 °C, $[\alpha]_D$ +87.5° (dioxane), was produced in a high overall yield. The ^{1}H-NMR spectrum of **4a** showed that 12-H forms a dihedral angle of *ca.* 40° with 11-H_2, giving a triplet (1H) signal (J= 4 Hz) at δ 5.18.

Oxidation of **9** and **10** with manganese dioxide[23] in chloroform yielded a dienone compound, 11-oxo-18β-olean-12-ene-3β,30-diol(glycyrrhetol) (**3**), mp 282 °C, $[\alpha]_D$ +97.6° (dioxane). Compound **3** was also prepared from **4a** diacetate by the oxidation of its allylic methylene at C-11 with *tert*-butyl chromate[24] followed by deacetylation under the same conditions as used for the preparation of **6** and **8** from **5** and **7**, respectively. To obtain a water-soluble derivative of deoxoglycyrrhetol, the 3-*O*-β-carboxypropionate (**4b**) was prepared by the following procedure.

For the purpose of temporarily protecting the primary alcohol, **4a** was tritylated with trityl pyridinium fluoroborate[25] or with trityl chloride and 4-dimethylaminopyridine in dichloromethane.[26]

The 30-tritylated **4a** was reacted with succinic anhydride, 4-dimethylaminopyridine, triethylamine and pyridine in dichloromethane to afford the 3-*O*-β-carboxypropionate of the 30-trityl ether of deoxoglycyrrhetol, from which the trityl group was removed by heating with 80% acetic acid[27] or by the action of 10% Pd–C and 10% formic acid[28] to yield **4b** of deoxoglycyrrhetol. Compound **4b** was also prepared by partial hydrolysis of the 3β,30-di-β-carboxypropionate of **4a** (**4c**) with alkali. The 3β-*O*-hemiphthalate (**4d**) and 3β,30-di-*O*-hemiphthalate (**4e**) of **4a** were also prepared by the reaction of phthalic anhydride with **4a** in the presence of pyridine or triethylamine, and their sodium salts were employed for pharmacological experiments.

Biochemistry

To evaluate pseudoaldosteronism *in vitro*, the activities of **4a** and some related oleanane derivatives against the liver reductase of 3-keto-Δ^4-steroid were assayed using aldosterone as the substrate. The assay was carried out by the procedure developed by Kumagai *et al.*[29,30]

The experimental results were reported preliminarily.[1] Compound **4a** showed no inhibition of 5α or 5β-Δ^4-reductase (-7.1 ± 2.2% and 0 ± 1.1%, respectively), whereas glycyrrhetinic acid (**1b**) inhibited 9.2 ± 2.2% of the activity of 5α-reductase and 87.7 ± 2.2% of that of 5β-reductase. Thus the destruction of the αβ-unsaturated C=O system in ring C of

glycyrrhetinic acid appears to eliminate pseudoaldosteronism. The details of this experiment will be reported elsewhere.

Using our samples, Inoue *et al.*[31] reported that the disodium salt of **4e** showed a strong inhibition of 5- and 12-lipoxygenases. The disodium salt of the hemiphthalate of the homoannular diene (**11a**) and heteroannular diene (**12a**) compounds corresponding to **4e** also inhibited lipoxygenases. Since 5-lipoxygenase is involved in the biosynthesis of several leukotrienes,[32] the above triterpenoid compounds related to **4e** may be effective as antiinflammatory and antiallergic agents.

Pharmacology

The antiulcerogenic, antiallergic and antiinflammatory activities of deoxoglycyrrhetol (**4a**) and related compounds were reported preliminarily,[1] and further experiments have been performed using the hemisuccinates and hemiphthalates of this series of triterpenoid compounds. The results will be reported in detail elsewhere.

Experimental

Optical rotations were determined in dioxane with a Jasco J20 ORD/CD polarimeter. Ultraviolet (UV) spectra were measured with dioxane as a solvent. Melting points were taken on a Yanaco micro melting points apparatus and are uncorrected. NMR spectra were recorded on a JEOL FX-60 NMR spectrometer for all ^{13}C-NMR spectra using 10% solutions in 10 mm (o.d.) tubes at 60 MHz or on a JEOL PS 100 for all ^{1}H-NMR spectra in 20—30 mg/0.3 ml $CDCl_3$ or d_5-pyridine as a solvent, with Me_4Si as an internal standard. Chemical shifts are reported in parts per million upfield from this standard. Mass spectral analyses were performed with a JEOL G-300 instrument coupled with a JEOL data analysis system (model JMA 2000 disk system).

Liquid chromatography was performed using three different types of apparatus.

(A) The liquid chromatograph consisted of a Nihon Seimitsu NSP-800-12DX solvent pumping system with a Bellows Damper (NBD-III), Shodex SE-11 (RI-detector) and a Rheodyne model 7125 injection valve. Chromatograms were recorded on a Rikadenki model R 201 recorder. The experiments were performed in a column (30 cm × 8 mm i.d.) (Nihon Seimitsu, Tokyo) packed with Nucleosil 50-5 (5 μm) (Macherey, Nagel Co., Duren, G.F.R.) or Whatman Partisil 5 (5 μm) (Whatman Ltd., Springfield Mill, England) fitted with a guard column system, holder 140-200 and column SI-GU (Brownlee Labs. Inc., Santa Clara, U.S.A.).

(B) Du Pont model 830 liquid chromatograph. The experiments were performed on a column (50 cm × 8 mm i.d.) (Kyowa Seimitsu, Tokyo) packed with Lichrosorb SI-60 (5 μm) (E. Merck, Darmstadt, Dr. Theodor Schachardt Co.).

(C) Waters system 500A (Waters Associates, Inc., Milford, Mass, U.S.A.) Column; Prepak-500/silica.

The term "treated in the usual way" refers to the treatment of extracts, and indicates that they were washed with water till neutral, dried over Na_2SO_4, and evaporated to dryness.

High-performance liquid chromatography (HPLC) was applied for the separation and isolation of glycyrrhetinic acid derivatives. By using the Waters system 500A liquid chromatograph, olean-12-ene-3β,30-diol (**4a**) could be purified in large quantities within 10 min. Semipreparative HPLC was also efficient for the isolation of compounds for analysis.

18β-Glycyrrhetinic Acid (3β-Hydroxy-11-oxo-18β-olean-12-en-30-oic Acid) (1b)——Glycyrrhizin, a diglucuronide saponin of licorice root (*Glycyrrhiza* spp.), colorless needles, mp 335 °C, was hydrolyzed to prepare **1b**, which was used as a starting compound for chemical modification. ^{13}C-NMR (d_5-pyridine) δ ppm: 199.4 (s, C-11), 178.9 (s, C-30), 169 (s, C-13), 128.5 (d, C-12), 77.8 (d, C-3), 62.1 (d, C-9), 55.2 (d, C-5).

Methyl Ester: mp 235.5—237 °C. $[\alpha]_D$ (dioxane): +158.3°.

11-Deoxo-18β-glycyrrhetinic Acid (3β-Hydroxy-18β-olean-12-en-30-oic Acid) (2)——A solution of 4.0 g of **1b** dissolved in 170 ml of AcOH was added to prereduced Pt (prepared from 2.4 g of PtO_2) in 20 ml of AcOH, under an atmosphere of H_2. The mixture was stirred, and absorbed 420 ml of H_2 over 8 h at room temperature, when the reaction product separated out. Then 100 ml of AcOH was added, and the reaction mixture was heated on a boiling water bath and filtered. On evaporation of the solvent, crude **2** was obtained. Recrystallization from AcOH gave colorless needles, mp >330 °C, in 80% yield. ^{13}C-NMR (d_5-pyridine) δ ppm: 179.4 (s, C-30), 145.0 (s, C-13), 122.8 (d, C-12), 78.0 (d, C-3), 55.7 (d, C-5). MS *m/z* Calcd for $C_{30}H_{48}O_3$: M^+ 456.3603. Found: M^+ 456.3602.

18β-Olean-12-ene-3β,11α and β,30-triol——Glycyrrhetinic acid (**1b**) (0.94 g, 2 mM) dissolved in dry THF (20 ml) was added to a solution of $NaAlH_2(OCH_2CH_2OCH_3)_2$ (70% in toluene) (2.9 ml, 10 mM) in dry THF (20 ml) under vigorous stirring at 60 °C in an N_2 atmosphere. The reaction was continued for 1 h. After cooling, the reaction mixture was decomposed with 10% HCl to adjust the pH to 3—4. The filtrate was extracted with $CHCl_3$, and the

organic layer was washed with water 3 times then dried over anhydr. Na_2SO_4 for 6 h. The solvent was evaporated off to leave a residue, which was separated by HPLC (Waters system 500A flow rate 150 ml/min) with $CHCl_3$–EtOH MeOH (94 : 5 : 1) as a solvent system to afford 18β-olean-12-ene-3β,11β,30-triol (yield 660 mg) at the retention time of 11 min, and olean-12-ene-3β,11α,30-triol (yield 165 mg) at the retention time of 14 min. The yield ratio of isomeric products at the 11-hydroxyl group was 4 : 1.

18β-Olean-12-ene-3β,11β,30-triol (**9**), colorless needles, mp 248—252 °C. ^{13}C-NMR (d_5-pyridine) δ ppm: 146.3 (s, C-13), 128.1 (d, C-12), 78.0 (d, C-3), 66.9 (d, C-11), 65.6 (t, C-30). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.3 (d, 1H, J=5 Hz, C-12-H), 4.4 (t, 1H, J=6 Hz, C-11-H), 3.5 (s, 2H, C-30-H_2), 3.2 (t, 1H, J=8 Hz, C-3-H). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3728.

18β-Olean-12-ene-3β,11α,30-triol (**10**), colorless needles, mp 222—224 °C. ^{1}H-NMR (d_5-pyridine) δ ppm: 5.8 (d, 1H, J=4 Hz, C-12-H), 4.4 (dd, 1H, J=4 Hz, J_2=8 Hz, C-11-H), 3.7 (dd, 2H, J_1=9 Hz, J_2=20 Hz, C-30-H_2), 3.5 (t, 1H, J=8 Hz, C-3-H). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3706. Calcd for $C_{30}H_{48}O_2$: M$^+$ $-H_2O$ 440.3654. Found: M$^+$ 440.3736.

18β-Olean-12-ene-3β,11β,30-triol Triacetate——On acetylation, **9a** and **9b** gave the same triacetate, mp 96 °C $[\alpha]_D$ +220°. ^{13}C-NMR (d_5-pyridine) δ ppm: 170.6, 170.2, 169.9 (s, acetyl, C=O), 147.9 (s, C-13), 122.4 (d, C-12), 80.7 (d, C-3), 68.5 (d, C-11), 67.8 (t, C-30). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.44 (t, 1H, J=6 Hz, C-11-H), 5.12 (d, 1H, J=6 Hz, C-12-H), 4.50 (t, 1H, J=9 Hz, C-5-H), 3.94 (br s, 2H, C-30-H_2), 2.00 (s, 9H, acetyl CH_3). MS *m/z* Calcd for $C_{36}H_{56}O_6$: M$^+$ 584.4077. Found: M$^+$ 584.4199.

18β-Olean-9(11),12-diene-3β,30-diol (11a)——A solution of 92 mg of **9** in $CHCl_3$ was acidified with 0.5 ml of conc. HCl at room temperature. After standing for 10 min the reaction mixture was evaporated at 60 °C to afford colorless needles, mp 230.5—232 °C. $[\alpha]_D$ +271° (dioxane). UV $\lambda_{max}^{dioxane}$ nm (log ε): 282 (3.79) (homoannular diene). Yield: 79 mg (90%). ^{13}C-NMR ($CDCl_3$) δ: 154.5 (s), 146.3 (s, C-9 or C-13 or *vice versa*), 121.1 (d, C-12), 115.7 (d, C-11). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.60 (dd, 2H, J_1=4 Hz, J_2=8 Hz, C-11-H, C-12-H), 3.30 (t, 1H, J=7 Hz, C-3-H), 3.60 (dd, 2H, J_1=9 Hz, J_2=14 Hz, C-30-H_2). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found : M$^+$ 440.3663.

18β-Olean-11,13(18)-diene-3β,30-diol (12a)——A solution of 92 mg of **10** in $CHCl_3$ was acidified with 0.5 ml of conc. HCl at room temperature. After standing for 10 min, the reaction mixture was evaporated at 60 °C to afford colorless needles, mp 230—232 °C. $[\alpha]_D$ (dioxane) −61.5°. Yield: 79 mg (90%). UV $\lambda_{max}^{dioxane}$ nm (log ε): 254 (4.23), 260 (4.21) (heteroannular diene). ^{13}C-NMR (d_5-pyridine) δ ppm: 137.8 (s), 134.2 (s), 126.0 (d), 125.9 (d), 78.1 (d, C-3), 73.4 (t, C-30), 55.4, 54.6. ^{1}H-NMR ($CDCl_3$) δ ppm: 6.42 (dd, 1H, J_1=4 Hz, J_2=10 Hz, C-11-H), 5.60 (br d, 1H, J=10 Hz, C-12-H), 3.30 (m, 1H, C-3-H). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found: M$^+$ 440.3658 (bp).

18β-Olean-12-ene-3β,30-diol (Deoxoglycyrrhetol) (4a)——Olean-12-ene-3β,11α or β-30-triol (**10** or **9**) (920 mg, 2 mM) dissolved in EtOH (100 ml, pretreated with $NaHCO_3$) was hydrogenated over 10% Pd–C (3.7 g). After uptake of H_2 (20 mM), the reaction mixture was worked up in the usual way. The product was purified by HPLC (Du Pont solvent system, $CHCl_3$ MeOH (96 : 4)) to obtain **4a**, mp 250—251 °C. $[\alpha]_D$ +87.5° (dioxane). Yield: 500 mg. ^{13}C-NMR (d_5-pyridine) δ ppm: 145.0 (s, C-13), 122.5 (d, C-12), 78.2 (d, C-3), 66.0 (t, C-30). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.18 (1H, t, J=9 Hz, C-12-H), 3.50 (2H, br s, C-30-H_2), 3.18 (1H, t, J=8 Hz, C-3-H). MS *m/z* Calcd for $C_{30}H_{50}O_2$: M$^+$ 442.3811. Found: M$^+$ 442.3842.

30-*O*-Tritylate——(1) A mixture of **4a** (88.5 mg, 0.2 mM), tritylchloride (279 mg, 1.0 mM), triethylamine (0.02 ml) and 4-dimethylaminopyridine (24.5 mg, 0.2 mM) in CH_2Cl_2 was stirred under reflux for 20 h. Chloroform (100 ml) and water (100 ml) were added to the reaction mixture, and the organic layer was separated. After removal of the solvent, the yellowish residue was purified by column chromatography to afford colorless needles, mp 131—133 °C. $[\alpha]_D$ (dioxane) +103.2°. Yield: 123 mg (90%). ^{13}C-NMR (d_5-pyridine) δ ppm: 144.9 (s, C-13), 122.5 (d, C-12), 128.2, 127.3, 125.1 (d, phenyl C of trityl group), 86.4 (s, trityl C), 78.1 (d, C-3), 66.1 (t, C-30). ^{1}H-NMR ($CDCl_3$) δ ppm: 7.55—7.2 (m, trityl H), 4.9—5.2 (1H, m, C_{12}-H), 3.12 (1H, t, J=8 Hz, C_3-H), 2.85 (2H, br s, C-30-H_2).

(2) A solution of **4a** (3.3 g) in dry dimethylformamide was treated with tritylpyridinium fluoroborate (3.5 g), and the solution was stirred at 60 °C for 48 h. After cooling, the solution was diluted with water (400 ml) and extracted with chloroform 3 times (400 ml each). The combined chloroform solution was washed with water 3 times (500 ml each) and dried. The residue obtained on evaporation of the organic solvent was treated as above to give colorless needles, mp 131—133 °C (Yield: 67.7%), identical with the sample prepared by method (1).

3β-*O*-β-Carboxypropionyl-30-*O*-trityl 18β-Olean-12-ene-3β,30-diol (3-*O*-β-Carboxypropionyl-30-*O*-trityl-deoxoglycyrrhetol)——In a 200 ml round-bottomed flask, fitted with a magnetic stirrer and a condenser protected from moisture, 30 ml of dry pyridine and 30 ml of dry triethylamine were placed. Under vigorous stirring, 5 g of succinic anhydride was added to the above mixture and a solution of 30-*O*-trityl 18β-olean-12-ene-3β,30-diol (2.5 g) in 30 ml of triethylamine was subsequently added. The reaction mixture was maintained at 60 °C for 24 h in an oil bath, and then poured into 100 ml of ice-water and acidified with HCl. The solution was extracted with 150 ml of $CHCl_3$ 3 times, and the combined extracts were separated by HPLC to afford 3β-*O*-β-carboxypropionyl-30-*O*-trityl 18β-olean-12-ene, colorless needles, mp 126 °C, $[\alpha]_D$ +87.7°. ^{13}C-NMR (d_5-pyridine) δ ppm: 173.2 (s, succinyl C=O), 144.9 (s, C-13), 129.3, 128.1, 127.2 (phenyl C of trityl group), 86.3 (s, trityl C), 80.7 (d, C-3), 66.2 (t, C-30), 55.6 (d, C-5).

The conditions of HPLC were as follows: Whatman silica gel column, 8 mm i.d. × 30 cm; solvent: 1% MeOH/$CHCl_3$; flow rate: 3 ml/min; t_R: 9.0 min.

3β-*O*-β-Carboxypropionyl 18β-Olean-12-ene-3β,30-diol (3-*O*-β-Carboxypropionyldeoxoglycyrrhetol) (4b)——(1) The 30-*O*-tritylate of **4a** (1 g) was dissolved in MeOH (50 ml) containing 10% HCOOH. The mixture was added to a suspension of 10% Pd–C (2.5 g) in MeOH under an atmosphere of N_2. After a few hours, the catalyst was filtered off and washed successively with CH_3OH and $CHCl_3$, and the combined filtrate was evaporated. The product was purified by HPLC (Du Pont column, 50 cm × 8 mm, packed with Lichrosorb SI-60 (5 μm) using $CHCl_3$–MeOH–AcOH (95 : 5 : 0.05) as the solvent system (3 ml/min) retention time, 12 min) to obtain colorless needles, mp 172—174 °C. $[\alpha]_D$ (dioxan) +79.3°. Yield: 0.59 g.

(2) The 30-*O*-tritylate of **4a** (2 g) was dissolved in 80% CH_3COOH (200 ml) and heated at 110 °C for 1 h. The reaction mixture was extracted with $CHCl_3$. The extracts were separated over a silica gel column using $CHCl_3$–CH_3OH as the developing solvent to obtain colorless needles, mp 172—174 °C. Yield: 1.25 g.

Methyl Ester of **4b**: MS *m/z* Calcd for $C_{35}H_{56}O_5$: M^+ 556.4128. Found: M^+ 556.4116.

3β,30-*O*-Di-β-carboxypropionyl 18β-Olean-12-ene-3β,30-diol 3β,30-Di-*O*-β-carboxypropionyldeoxoglycyrrhetol) (4c)——Compound **4a** (6.5 g) was dissolved in dried pyridine (400 ml). Next, succinic anhydride (144.1 g) and triethylamine (67 ml) were successively added, and the mixture was heated under stirring in an oil bath at 90—100 °C for 7 h. After cooling, the reaction mixture was poured into ice and dil. HCl to obtain dark-colored precipitates which were separated and washed with water. The precipitates were extracted 3 times with $CHCl_3$ (each 700 ml), and the $CHCl_3$ layer was washed 3 times with water (each 500 ml). The $CHCl_3$ was distilled off from the dried extracts to obtain the crude product (3.7 g), which was recrystallized from CH_3OH to obtain pure **4c**, mp 226—228 °C.

Dimethyl Ester of **4c**: MS *m/z* Calcd for $C_{40}H_{62}O_8$: M^+ 670.4445. Found: M^+ 670.4482.

4b: Compound **4c** (6.4 g) was dissolved in boiling ethanol (225 ml). A solution of KOH (3.2 g) in water (70 ml) was added to an ethanolic solution of **4c**, and refluxed for 40 min on a boiling water bath.

Then 10% HCl was added to separate crystals, which were extracted 3 times with $CHCl_3$ (each 150 ml). The combined $CHCl_3$ layer was washed with water (each 200 ml) and dried over anhydr. Na_2SO_4. The solvent was distilled off to obtain a mixture of products (5 g) which was chromatographed over silica gel using $CHCl_3$–CH_3OH as a developing solvent to separate **4b** (2.9 g), mp 172—174 °C, and **4a**, needles (2 g), mp 250—251 °C. **4a** was submitted to re-esterification.

3β,30-*O*-Di-*o*-phthaloyl 18β-Olean-12-ene-3β,30-diol (Deoxoglycyrrhetol 3β,30-Dihemiphthalate) (4e)——Phthalic anhydride (110 g) and triethylamine (35 ml) were added to a solution of **4a** (13.2 g) in dried pyridine (200 ml). The mixture was heated at 90—100 °C in an oil bath for 7 h under stirring. After cooling, the reaction mixture was poured into an excess amount of dil. HCl mixed with ice. The precipitates formed were filtered off, washed with water, and then transferred to a 1 l beaker containing water (700 ml) and heated on a water bath under stirring. The precipitates were filtered off while hot and washed with hot water 3 times. The product was recrystallized from MeOH to obtain a colorless powder, mp 162.4—164 °C. $[\alpha]_D^{20}$ +65.8° (*c* = 0.41, THF). Yield: 20.5 g. ^{13}C-NMR (C_5D_5N) δ: 169.8, 169.6, 168.9, 168.3 (s, phthaloyl C=O), 144.4 (s, C-13), 122.7 (s, C-12), 82.4 (d, C-3), 69.4 (t, C-30). *Anal.* Calcd for $C_{40}H_{58}O_8 \cdot 2H_2O$: C, 71.29; H, 8.06. Found: C, 71.13; H, 8.01.

Dimethyl Ester: MS *m/z* Calcd for $C_{48}H_{62}O_8$: M^+ 767. Found: M^+ 767.

Di-Na Salt: **4e** (14.8 g) was dissolved in THF (300 ml), and $^1/_{10}$ N NaOH (400 ml) was added. The mixture was refluxed for 30 min on a boiling water bath, and then concentrated to form a paste. THF (400 ml) was added to the paste, and the mixture as refluxed again to obtain colorless crystals, mp 294—295 °C. Yield: 15.2 g.

3β,30-*O*-Di-*o*-diphthaloyl 18β-Olean-9(11),12-diene-3β,30-diol (11c)——Compound **11a** (26.4 g) was treated with phthalic anhydride and pyridine as above to afford the 3β,30-*O*-dihemiphthalate, mp 157—158 °C. $[\alpha]_D^{20}$ +62.9° (*c* = 0.39, pyridine). ^{13}C-NMR (C_5D_5N) δ: 168.7, 168.5, 168.0, 167.1 (s, phthaloyl C=O), 153.0 (s, C-9), 145.0 (s, C-13), 121.5 (d, C-12), 115.0 (d, C-11), 81.1 (d, C-3), 68.2 (t, C-30). UV λ_{max}^{EtOH} nm: 203.5, 226, 282. *Anal.* Calcd for $C_{40}H_{56}O_8 \cdot 2H_2O$: C, 71.48; H, 7.82. Found: C, 71.13; H, 7.69.

Dimethyl Ester: MS *m/z* Calcd for $C_{48}H_{60}O_8$: M^+ 765. Found: M^+ 765.

Di-Na Salt: mp 279.5—281 °C.

3β,30-*O*-Di-*o*-phthaloyl 18β-Olean-11,13(18)diene-3β,30-diol (12c)——Compound **12a** (13.2 g) was treated with phthalic anhydride and pyridine as above to afford the 3β,30-*O*-dihemiphthalate, mp 156 °C. $[\alpha]_D^{20}$ −22.4 (*c* = 0.42, pyridine). Yield: 21 g. ^{13}C-NMR (C_5D_5N) δ: 169.0, 168.9, 167.8, 167.3 (s, phthaloyl C=O), 135.4, 132.7 (s, C-13, C-18 or *vice versa*), 124.9, 124.8 (d, C-11, C-12 or *vice versa*), 81.2 (d, C-3), 74.8 (t, C-30). UV λ_{max}^{EtOH} nm: 204, 236, 242, 250, 260.

Dimethyl Ester: MS *m/z* Calcd for $C_{48}H_{60}O_8$: M^+ 765. Found: M^+ 765.

Di-Na Salt: mp 297—298 °C.

3β-*O*-β-Carboxypropionyl 18β-Olean-9(11),12-diene-3β,30-diol (11b)——Succinic anhydride (12 g) and triethylamine (5.5 ml) were added to a solution of **11a** (2.2 g) in dried pyridine (35 ml), and the mixture was refluxed for 7 h in an oil bath. After cooling, the reaction mixture was poured into ice water, and an excess of dil. HCl was added to obtain a dark-colored precipitate, which was extracted 3 time with $CHCl_3$ (50 ml each). Dark-colored crystals separated from the $CHCl_3$ solution on evaporation. On purification over a silica gel column using $CHCl_3$–MeOH (98 : 2) as the developing solvent, 3β,30-di-*O*-β-carboxypropionyl 18β-olean-9(11)12-diene-3β,30-diol, mp 224 °C (2.7 g), was isolated.

On partial hydrolysis of the 3β,30-di-*O*-β-carboxypropionate by refluxing for 2.5 h with 3.2% KOH (29 ml) in EtOH (100 ml), **11b** (1.4 g) was obtained from the acidified reaction mixture by extraction with $CHCl_3$ and chromatography of the extract on silica gel using $CHCl_3$–MeOH (99 : 1) as the developing solvent. mp 201—215 °C. $[\alpha]_D^{20}$ +254° (*c*=0.50, $CHCl_3$). UV λ_{max}^{EtOH} nm: 203, 208 (EtOH). ^{13}C-NMR (d_5-pyridine) δ ppm: 174.5, 172.3 (s, succinyl C=O), 154.2 (s, C-9), 147.1 (s, C-13), 121.3 (d, C-12), 116 (d, C-11), 80.7 (d, C-3), 65.7 (t, C-30).

Methyl Ester: MS *m/z* Calcd for $C_{35}H_{54}O_5$: M^+ 554.3957. Found: M^+ 554.3953.

3β-*O*-β-Carboxypropionyl 18β-Olean-11,13(18)-diene-3β,30-diol (12b)——**12a** (2.2 g) was treated as described above for **11a** to obtain **12b** (1.3 g). mp 224—226 °C. $[\alpha]_D^{20}$ −55° (*c*=1.00, $CHCl_3$). UV λ_{max}^{EtOH} nm: 243, 251, 260. ^{13}C-NMR ($CDCl_3$) δ ppm: 177.0, 171.8 (s, succinyl C=O), 136.8, 134.2 (s, C-13, C-18 or *vice versa*), 125.8, 125.5 (d, C-11, C-12 or *vice versa*), 81.6, (d, C-3), 74.1 (d, C-30).

Methyl Ester: MS *m/z* Calcd for $C_{35}H_{54}O_5$: M^+ 554.3957. Found: M^+ 554.3980.

3β,30-Di-*O*-β-carboxypropionyl 18β-Olean-11,13(18)diene-3β,30-diol: mp 183 °C.

3β,23-Dihydroxy-11-oxo-18β-olean-12-ene-28-oic Acid (11-Oxohederagenin) (6)——3,23-*O*-Diacetylhederagenin [prepared from hederagenin (**5**) (5.5 g) by treatment with Ac_2O and pyridine at room temperature for 12 h] was dissolved in CCl_4 (44.8 ml) and the solution was stirred with AcOH (11.2 ml) and Ac_2O (2.9 ml) at 55 °C for 45 min. *tert*-Butylchromate, CrO_2 (O-*tert*-Bu)$_2$ (CrO_3: 7.6 g), in CCl_4 (41.1 ml) and a mixture of AcOH (11.2 ml) and Ac_2O (2.9 ml) were added to the above solution, and the reaction was allowed to proceed overnight at 60—65 °C. After cooling of the mixture, 10% oxalic acid solution (207 ml) was added dropwise under stirring for 1 h. The reaction mixture was extracted three times with $CHCl_3$ (500 ml each).

The solution was evaporated *in vacuo* and the residue was purified by silica gel column chromatography using hexane–acetone (9 : 1) as the solvent. The product was deacetylated with 3% KOH in 50% dioxane (300 ml) at room temperature for 2 h. The reaction mixture was treated as usual and purified by chromatography using $CHCl_3$ as the solvent to obtain 11-oxo-hederagenin (**6**), colorless needles, mp 294 °C. Yield: 3.07 g (53.5%, Calcd from hederagenin (**5**)). MS *m/z* Calcd for $C_{30}H_{46}O_5$: M^+ 486.3345. Found: M^+ 486.3334. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1688 (COOH), 1644 ($\alpha\beta$ unsat. 6 membered C=O). ^{13}C-NMR (d_5-pyridine) δ ppm: 199.9 (s, C-11), 179.5 (s, C-28), 169.5 (s, C-13), 128.0 (d, C-12), 72.7 (d, C-3), 67.2 (t, C-24), 62.3 (d, C-9). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.88 (1H, s, C-12H), 4.13 (2H, br s, C-24H), 3.59 (1H, dd, C-18H), 2.53 (1H, s, C-9H).

11-Oxo-18β-olean-12-ene-3β,30-diol (Glycyrrhetol) (3)——AcOH (1.3 ml) and Ac_2O (0.34 ml) were added to a solution of **4a** diacetate (650 mg) in CCl_4 (5.2 ml), and the mixture was kept at 55—60 °C for 45 min under continuous stirring. A solution consisting of AcOH (1.3 ml), Ac_2O and *tert*-butyl chromate, CrO_2 (O-*tert*-Bu)$_2$ (CrO_3: 0.9 g), dissolved in CCl_4 (7.3 ml) was added to the above mixture, and the whole was kept at 60—65 °C for 20 h under stirring. After cooling of the reaction mixture to room temperature, 10% aq. oxalic acid solution (24 ml) was added dropwise. After stirring for additional 1 h, the reaction mixture was extracted with $CHCl_3$. After removal of the solvent, the residue was deacetylated with 5% KOH–50% aq. dioxane under stirring for 30 min at 80 °C. The reaction product was purified by HPLC on a Shodex silica gel column (8 mm i.d. × 30 cm) with hexane–acetone (75 : 25) as the solvent (flow rate 2.5 ml/min) to obtain colorless needles, mp 281—282 °C. ^{13}C-NMR (d_5-pyridine) δ ppm: 199.4 (s, C-11), 169.7 (s, C-13), 128.5 (d, C-12), 77.8 (d, C-3), 65.4 (t, C-30), 62.1 (d, C-9), 55.3 (d, C-5). ^{1}H-NMR ($CDCl_3$) δ ppm: 5.59 (1H, s, C-12H), 3.48 (2H, br s, C-30-H_2), 3.19 (1H, t, J=8 Hz, C-3-H), 2.75 (1H, dt, J=12, 4 Hz), 2.30 (1H, s, C-9-H). MS *m/z* Calcd for $C_{30}H_{48}O_3$: M^+ 456.3603. Found: M^+ 456.3624.

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References and Notes

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1,5-Benzoxathiepin Derivatives. I. Synthesis and Reaction of 1,5-Benzoxathiepin Derivatives

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Methyl 3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**3a**—**f**) were synthesized by regioselective Dieckmann reaction of methyl 2-methoxycarbonylmethylthiophenoxyacetates (**2a**—**f**) [readily prepared from 2-mercaptophenols (**1a**—**f**)] in fairly good yields. Alkylation of the ketoester (**3b**) with alkyl halides gave 4-alkylated derivatives (**7** and **8**). A substituent at the 2-position on the 1,5-benzoxathiepin ring was introduced by Dieckmann reaction of methyl α-substituted 2-methoxycarbonylmethylthiophenoxyacetates (**11**, **12b** and **12f**). Thorpe-Ziegler reaction of 2-cyanomethylthiophenoxyacetonitriles (**18a**—**d**) gave 3-amino-2*H*-1,5-benzoxathiepin-4-carbonitriles (**19a**—**d**). Novel heterocycles, 4-amino-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin derivatives (**24**—**32**), were synthesized by the reaction of enaminonitriles (**3a** and **3b**) with amidines or guanidines.

Keywords——1,5-benzoxathiepin; Dieckmann reaction; enaminonitrile; 11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin derivative; seven-membered heterocyclic compound

Synthesis of seven-membered 1,5-benzoxathiepin derivatives has not been extensively studied.[1-4] Cabiddu *et al.* were the first to describe the introduction of a functional group into the 3-position of the 1,5-benzoxathiepin ring by the reaction of 2-mercaptophenols with epichlorohydrin in an aqueous alkaline solution.[3] It has also been demonstrated that the Dieckmann cyclization of methyl methoxycarbonylmethylthiomethyloxyacetate gave regioselectively methyl 5-oxo-1,3-oxathian-4-carboxylate.[5] In this paper, we report the preparation of 1,5-benzoxathiepins with functional groups at the 2-, 3-, and 4-positions *via* the route involving Dieckmann cyclization of the *O*-, *S*-diacetic acid esters prepared from 2-mercaptophenols.

2-Mercaptophenols (**1a**—**f**) were alkylated with two equivalent amounts of methyl bromoacetate in refluxing acetone in the presence of potassium carbonate to give methyl 2-methoxycarbonylmethylthiophenoxyacetates (**2a**—**f**) (Table V). Examination of the reaction conditions for Dieckmann reaction of **2a** showed that the reaction occurred smoothly when 28% sodium methoxide or potassium *tert*-butoxide was used as a base in *N*,*N*-dimethylformamide (DMF) at ambient temperature to give methyl 3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (**3a**) as the sole cyclized product in 71% yield. Compounds **2b**—**f** were similarly cyclized to give methyl 3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**3b**—**f**) in fairly good yields (Table I). Decarboxylation of the ketoesters (**3a** and **3b**) in boiling acetic acid containing 65% sulfuric acid (1 : 1, v/v) gave 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ones (**4a** and **4b**). Subsequent hydride reduction of the ketones (**4a** and **4b**) with sodium borohydride in ethanol gave 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ols (**5a** and **5b**) the structures of which were confirmed by direct comparison with authentic samples prepared by the reaction of 2-mercaptophenols (**1a** and **1b**) with epichlorohydrin according to the method of Cabiddu *et al.*[3] Regioselectivity of the Dieckmann cyclization was deduced on the basis of the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectral data of the resulting product (**3a**),

which exhibited two doublet peaks (2H) at δ 4.55 and 4.90 ppm (J=17 Hz) assignable to the geminal methylenic protons at the 2-position adjacent to the oxygen atom in the 1,5-benzoxathiepin ring.

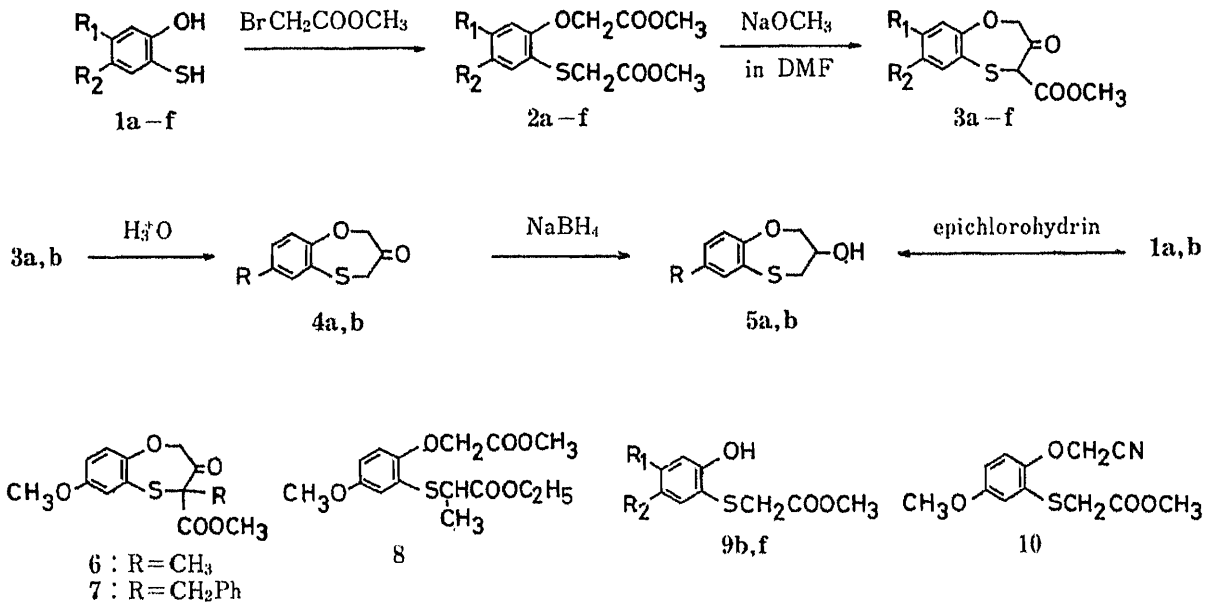


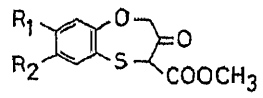
Chart 1

Retro-Dieckmann reaction of **3b** occurred readily in boiling methanol in the presence of a catalytic amount of sodium methoxide to yield the starting diester (**2b**) quantitatively. Michael addition of **3b** to acrylonitrile using 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) in refluxing acetonitrile or potassium fluoride and 18-crown-6 in refluxing toluene[6] did not occur. Mannich reaction of **3b** with dimethylamine hydrochloride and formalin in ethanolic hydrogen chloride resulted in only the recovery of the starting compound (**3b**). However, direct alkylation of **3b** with alkyl halides (methyl iodide, benzyl bromide) proceeded smoothly by the use of potassium carbonate in DMF to give the 4-methyl and 4-benzyl derivatives (**6**, 81% and **7**, 97%).

Neither methyl 2-(1-ethoxycarbonylethyl)thio-4-methoxyphenoxyacetate (**8**) nor methyl 2-cyanomethyloxy-5-methoxyphenylthioacetate (**10**) gave any isolable cyclized product under Dieckmann conditions similar to those described above.

Next, in order to introduce a substituent into the 2-position of the 1,5-benzoxathiepin ring, we examined Dieckmann reaction of 2-methoxycarbonylmethylthiophenoxyacetates bearing a hydroxyethyl or methoxycarbonylethyl group at the α-position of the *O*-acetate group. *O*-Alkylation of methyl 2-hydroxyphenylthioacetates (**9b** and **9f**) with α-bromo-γ-butyrolactone and dimethyl 2-mesyloxy-glutarate gave methyl 5-methoxy-2-(2-oxo-tetrahydrofuryloxy)phenylthioacetate (**11**) and dimethyl 2-(2-methoxycarbonylmethylthiophenoxy)-glutarates (**12d** and **12f**), respectively. Dieckmann reaction of the esters (**11**, **12b**, and **12f**) thus obtained yielded methyl 2-hydroxyethyl-7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (**13**, 80%) and methyl 3-(4-methoxycarbonyl-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionates (**14b**, 62% and **14f**, 68%), respectively. Heating the 2-hydroxyethyl derivative (**13**) in a mixture of acetic acid and concentrated hydrochloric acid (1 : 1, v/v) caused chlorination of the hydroxy group along with decarboxylation to give 2-chloroethyl-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (**15**) in 18% yield. Decarboxylation of **13** with 20% sulfuric acid in dioxane gave the ketal product (**16**, 70%), which

TABLE I. Methyl 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**3a**—**f**)



Compound No.	R_1	R_2	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)	
						C	H
3a	H	H	71	Oil	$C_{11}H_{10}O_4S$	55.45 (55.33	4.23 4.41)
3b	H	OCH_3	77	79—81[a]	$C_{12}H_{12}O_5S$	53.72 (54.04	4.51 4.68)
3c	H	CH_3	73	Oil	$C_{12}H_{12}O_4S$	57.13 (57.21	4.79 4.88)
3d	H	Cl	67	92—94[a]	$C_{11}H_9ClO_4S$	48.45 (48.45	3.33 3.06)
3e	H	OCH_2Ph	68	101—102[a]	$C_{18}H_{16}O_5S$	62.78 (62.71	4.68 4.38)
3f	OCH_3	H	67	Oil	$C_{12}H_{12}O_5S$	53.72 (53.98	4.51 4.66)

a) Crystallized from AcOEt-hexane.

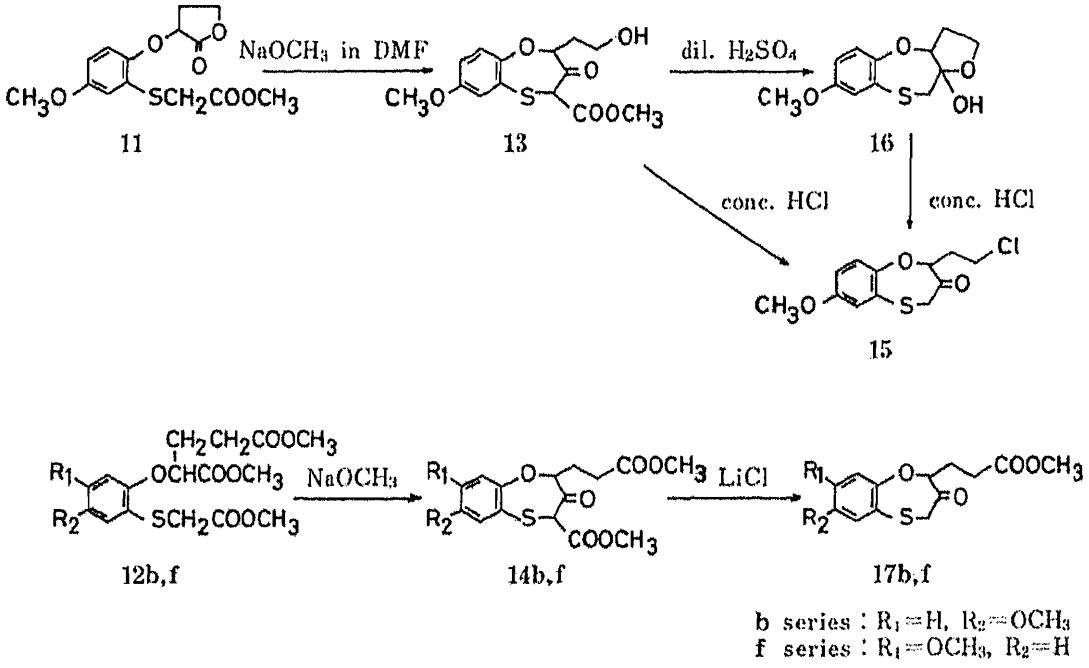


Chart 2

was converted to the 2-chloroethyl derivative (**15**) in 40% yield on heating in a mixture of acetic acid and concentrated hydrochloric acid. Heating the propionates (**14b** and **14f**) in DMF by the use of lithium chloride and water at 100 °C gave methyl 3-(3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionates (**17b**, 62% and **17f**, 61%).

Success with the regioselective Dieckmann reaction in 1,5-benzoxathiepin synthesis prompted us to study Thorpe–Ziegler reaction of 2-cyanomethylthiophenoxyacetonitriles. 2-Mercaptophenols (**1a**—**d**) were reacted with two equivalent amounts of chloroacetonitrile to

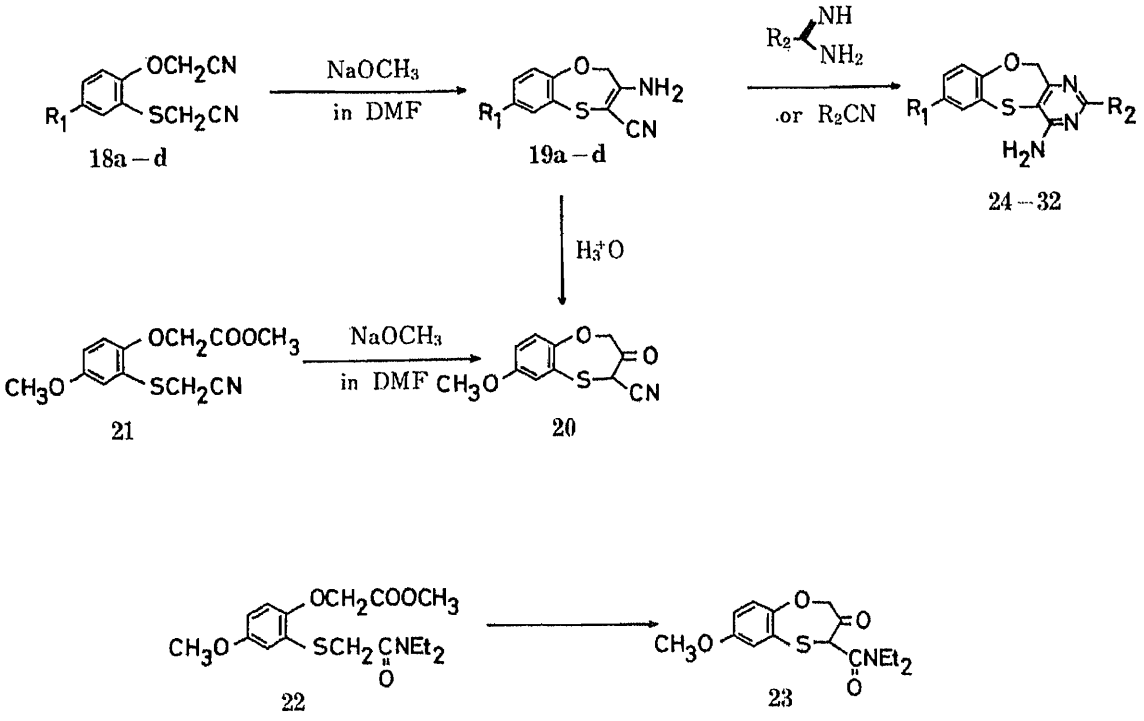
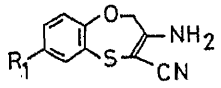


Chart 3

TABLE II. 3-Amino-2*H*-1,5-benzoxathiepin-4-carbonitriles (**19a—d**)



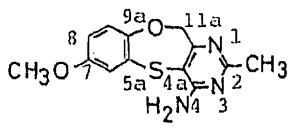
Compound No.	R_1	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
19a	H	65	209—210[a]	$C_{10}H_8N_2OS$	58.81 (58.63	3.95 3.82	13.72 13.46)
19b	OCH_3	65	203—205[b]	$C_{11}H_{10}N_2O_2S$	56.39 (56.41	4.30 4.29	11.89 11.85)
19c	CH_3	62	230—231[a]	$C_{11}H_{10}N_2OS$	60.53 (60.54	4.62 4.52	12.83 12.83)
19d	Cl	60	285—288[a]	$C_{10}H_7ClN_2OS$	50.31 (50.28	2.96 2.88	11.74 11.72)

a) Crystallized from AcOEt. *b*) Crystallized from $CHCl_3$.

give the desired dinitriles (**18a—d**) in good yields. The Thorpe–Ziegler reaction of dinitriles (**18a—d**) gave 3-amino-2*H*-1,5-benzoxathiepin-4-carbonitriles (**19a—d**) in fairly good yields (Table II). Hydrolysis of **19b** in refluxing ethanol containing concentrated hydrochloric acid led to the formation of 7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carbonitrile (**20**, 80%) upon removal of the crystalline deposit of ammonium chloride. The ketonitrile (**20**) was also obtained by the Dieckmann reaction of methyl 2-cyanomethylthio-4-methoxyphenoxyacetate (**21**) in 40% yield.

On the other hand, Dieckmann reaction of methyl 2-*N*,*N*-diethylcarbamoylmethylthio-4-methoxyphenoxyacetate (**22**) afforded only a complicated mixture of products on thin-layer chromatography (TLC). The main product isolated by column chromatography on silica gel

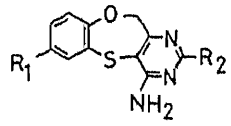
TABLE III. ^{13}C-NMR Spectral Data for 4-Amino-7-methoxy-2-methyl-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin (**26**)



C-2	C-11a	C-4	C-7	C-9a	C-5a	C-9	C-8	C-6	C-4a	C-11	OCH_3	2-CH_3
164.19	161.82	161.12	154.92	152.12	125.91	122.35	115.64	115.59	105.57	73.69	55.53	24.71

ppm (δ) in DMSO-d_6. Assigned by the use of the low-power selective proton decoupling technique.

TABLE IV. 4-Amino-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin Derivatives (**24**—**32**)

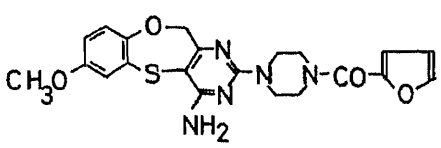


Compd. No.	R_1	R_2	Yield (%)	mp (°C)	^{1}H-NMR (DMSO-d_6) δ (C_{11}-H)	Formula	Analysis (%) Calcd (Found)			MS *m/z* (M^+)
							C	H	N	
24	H	Ph	34	170—172[a]	5.38	$C_{17}H_{13}N_3OS$	66.43 (66.33	4.26 4.11	13.67 13.46)	307
25	OCH_3	H	20	213—215[b]	5.08	$C_{12}H_{11}N_3O_2S$	55.16 (55.05	4.24 4.24	16.08 15.97)	261
26	OCH_3	CH_3	33	193—195[a]	5.02	$C_{13}H_{13}N_3O_2S$	56.71 (56.61	4.76 4.88	15.26 14.96)	275
27	OCH_3	Ph	35	148—150[a]	5.26	$C_{18}H_{15}N_3O_2S$	64.08 (63.89	4.48 4.77	12.45 12.69)	337
28	OCH_3	NH_2	71	194—195[c]	5.02	$C_{12}H_{12}N_4O_2S$	52.17 (52.16	4.38 4.63	20.28 20.38)	276
29	OCH_3		52	193—194[c]	5.08	$C_{16}H_{19}N_5O_2S$	55.63 (55.35	5.54 5.78	20.28 20.54)	345
30	OCH_3		58	158—160[a]	5.10	$C_{17}H_{20}N_4O_2S$	59.28 (59.52	5.85 5.63	16.27 16.58)	344
31	OCH_3		20	169—171[a]	5.10	$C_{16}H_{18}N_4O_3S$	56.71 (56.45	4.76 4.82	15.26 15.02)	346
32	OCH_3	3-Pyridyl	45	219—220[b]	5.18	$C_{17}H_{14}N_4O_2S$	60.34 (60.33	4.17 4.06	16.56 16.36)	338

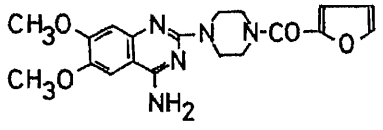
a) Crystallized from AcOEt. *b*) Crystallized from MeOH. *c*) Crystallized from EtOH.

was identified as 7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-*N*,*N*-diethylcarboxamide (**23**, 9%) from its ^{1}H-NMR spectral data.

The formation of the pyrimidine ring by the reaction of enaminonitrile with amidines has been established as one of the more useful methods in heterocyclic synthesis.[7] We tried to synthesize novel 2-amino-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin derivatives from the enaminonitrile (**19**). Heating **19b** and acetamidine hydrochloride in refluxing ethanol with a slight excess of sodium methoxide gave colorless needles having the molecular formula $C_{13}H_{13}N_3O_2S$ as judged from elemental analysis data and mass spectral data (*m/z*: 275, M^+). The absence of a nitrile absorption band in the infrared (IR) spectrum and the presence of two singlet peaks at δ 2.22 (3H) and 5.02 (2H) ppm assignable to methyl protons at the 2-position



33



prazocin

Chart 4

and methylenic protons at the 11-position in the ^{1}H-NMR spectrum indicate the formation of 4-amino-2-methyl-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin (**26**). The structure (**26**) was confirmed on the basis of carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data; all ^{13}C-carbon signals were assigned as shown in Table III. Similar reaction of the enaminonitriles (**19a** and **19b**) with some amidines and guanidines gave the 2-substituted 4-amino-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin derivatives (**24**—**32**) (Table IV). As an application to the synthesis of biologically active compounds, we conducted acylation of **29** with 2-furoic acid in DMF in the presence of diethyl phosphorocyanidate (DEPC)[8] and obtained **33** as a tricyclic analogue of the α_1-blocker, prazocin. However, **33** did not show any significant α_1-blocking action on *in vitro* biological evaluation.

Thus, a convenient method for synthesis of 1,5-benzoxathiepins with substituents at the 2-, 3-, and 4-positions of the heterocyclic skeleton was established. Further extension of this method to the synthesis of biologically active compounds will be described in the following paper.[9]

Experimental

All melting points were determined on a micro melting point apparatus (Yanagimoto) and are uncorrected. The following instruments were used for obtaining the spectral data: IR spectra, Hitachi 215 and 260-10 spectrometers; ^{1}H-NMR spectra, Varian T-60 and EM-390 instruments; ^{13}C-NMR spectra, a JEOL JNM-GX400 FT NMR spectrometer; mass spectra (MS), a JEOL JMS-01SC mass spectrometer. In the ^{1}H-NMR spectra, chemical shifts are given on the δ (ppm) scale with tetramethylsilane (TMS) as an internal standard.

Methyl 2-Methoxycarbonylmethylthiophenoxyacetate (2a)——A mixture of 2-mercaptophenol (**1a**), (20 g, 160 mmol), methyl bromoacetate (53 g, 350 mmol) and anhydrous K_2CO_3 (45 g, 330 mmol) was boiled under reflux for 5 h with stirring under an atmosphere of dry N_2. The inorganic precipitates were filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in AcOEt and the organic layer was washed with H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. Recrystallization of the residue afforded **2a** (35.5 g, 83%) as colorless needles. IR ν_{max}^{KBr} cm^{-1}: 1770, 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ: 3.66 (3H, s, $COOCH_3$), 3.73 (2H, s, SCH_2), 3.78 (3H, s, $COOCH_3$), 4.72 (2H, s, OCH_2).

Methyl 4- or 5-substituted 2-methoxycarbonylmethylthiophenoxyacetates (**2b**—**f**) were similarly prepared from the corresponding 2-mercaptophenols,[10-12] and their physical data are included in Table V.

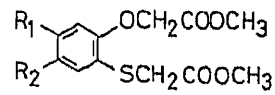
Methyl 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (3a)——A solution of **2a** (10 g, 37 mmol) in DMF (10 ml) was added dropwise to a mixture of 28% $NaOCH_3$ (7.7 g, 40 mmol) and DMF (30 ml) at room temperature with stirring under an atmosphere of dry N_2. After being stirred for 1 h, the reaction mixture was poured into ice-H_2O containing conc. HCl (10 ml), and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (3 : 2) as the eluant to give **3a** (6.3 g, 71%) as a colorless oil. IR ν_{max}^{neat} cm^{-1}: 1765 (ester), 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.78 (3H, s, $COOCH_3$), 4.55 (1H, d, J=17 Hz, C_2-H), 4.85 (1H, s, C_4-H), 4.90 (1H, d, J=17 Hz, C_2-H).

Similarly, Dieckmann reaction of methyl 4- or 5-substituted 2-methoxycarbonylmethylthiophenoxyacetates (**2b**—**f**) gave **3b**—**f** (Table I).

Demethoxycarbonylation of Methyl 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (3a and 3b)——A solution of the ketoester (**3a** or **3b**) (100 mmol), AcOH (60 ml) and 65% H_2SO_4 (60 ml) was boiled under reflux for 1 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (3 : 2) as the eluant to give **4a** or **4b**.

3,4-Dihydro-2*H*-1,5-benzoxathiepin-3-one (4a)——A colorless oil (79% yield) which crystallized upon standing at

TABLE V. Methyl 2-Methoxycarbonylmethylthiophenoxyacetates (**2a**—**f**)



Compound No.	R_1	R_2	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)	
						C	H
2a	H	H	83	65—66[a]	$C_{12}H_{14}O_5S$	53.32 (53.20	5.22 5.29)
2b	H	OCH_3	75	78[a]	$C_{13}H_{16}O_6S$	51.99 (52.18	5.37 5.37)
2c	H	CH_3	72	45—46[b]	$C_{13}H_{16}O_5S$	54.92 (55.10	5.67 5.70)
2d	H	Cl	73	76—77[a]	$C_{12}H_{13}ClO_5S$	47.30 (47.40	4.30 4.29)
2e	H	OCH_2Ph	70	52—53[c]	$C_{19}H_{20}O_6S$	60.63 (60.75	5.36 5.39)
2f	OCH_3	H	76	Oil	$C_{13}H_{16}O_6S$	51.99 (52.06	5.37 5.51)

a) Crystallized from AcOEt–Hexane. *b*) Crystallized from MeOH. *c*) Crystallized from Et_2O–hexane.

room temperature for several days, mp 28—31 °C. *Anal.* Calcd for $C_9H_8O_2S$: C, 59.98; H, 4.47. Found: C, 59.96; H, 4.50. IR ν_{max}^{neat} cm^{-1}: 1725 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.80 (2H, s, SCH_2), 4.65 (2H, s, OCH_2).

7-Methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (4b)——Recrystallization of crude **4a** from Et_2O–petroleum ether gave colorless prisms (72% yield), mp 47—48 °C. *Anal.* Calcd for $C_{10}H_{10}O_3S$: C, 57.13; H, 4.79. Found: C, 57.28; H, 4.64. IR ν_{max}^{KBr} cm^{-1}: 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.75 (2H, s, SCH_2), 4.45 (2H, s, OCH_2).

Hydride Reduction of 3,4-Dihydro-2*H*-1,5-benzoxathiepin-3-ones (4a and 4b)——Sodium borohydride (1.0 g, 26 mmol) was added in small portions to a solution of **4a** or **4b** (30 mmol) and EtOH (30 ml) with stirring at room temperature. Stirring was continued for 2 h, then the reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane AcOEt (1 : 1) as the eluant to give **5a** or **5b**.

3,4-Dihydro-2*H*-1,5-benzoxathiepin-3-ol (5a)——Colorless plates (from aqueous EtOH), mp 93—94 °C. Yield 60%. *Anal.* Calcd for $C_9H_{10}O_2S$: C, 59.32; H, 5.53. Found: C, 59.31; H, 5.47. IR ν_{max}^{KBr} cm^{-1}: 3300 (OH). ^{1}H-NMR ($CDCl_3$) δ: 3.0 (2H, br s, SCH_2), 4.1—4.8 (3H, m). MS *m/z*: 182 (M^+).

7-Methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (5b)——Colorless prisms (from 50% EtOH), mp 62—63 °C. Yield 95%. *Anal.* Calcd for $C_{10}H_{12}O_3S$: C, 56.59; H, 5.70. Found: C, 56.70; H, 5.66. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1600, 1580, 1480, 1430, 1290, 1260, 1235, 1205. ^{1}H-NMR ($CDCl_3$) δ: 2.8—3.1 (2H, m), 3.70 (3H, s, OCH_3), 3.6—4.4 (3H, m). **5a** and **5b** thus obtained were identical with those prepared by the method of Cabiddu *et al.*,[3] based on comparisons of the IR and NMR spectra and melting point.

Retro-Dieckmann Reaction of 3b——A solution of **3b** (2.7 g, 10 mmol) in MeOH (20 ml) containing 50 mg (0.25 mmol) of 28% NaOMe was boiled under reflux for 2 h. Evaporation of the solvent and recrystallization of the residue afforded **2b** (2.88 g, 96%) as colorless prisms.

Alkylation of 3b——An alkyl halide (methyl iodide or benzyl bromide; 3.7 mmol) and anhydrous K_2CO_3 (0.6 g, 4.4 mmol) were added to a solution of **3b** (1.0 g, 3.7 mmol) in DMF (10 ml), and the mixture was stirred at room temperature for 0.5 h. The reaction mixture was poured into cold H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (2 : 1) as the eluant to give 4-alkylated derivatives (**6** and **7**).

Methyl 7-Methoxy-4-methyl-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (6)——Colorless prisms (from hexane–AcOEt), mp 92—93 °C. *Anal.* Calcd for $C_{13}H_{14}O_5S$: C, 55.31; H, 5.00. Found: C, 55.38; H, 4.94. IR ν_{max}^{KBr} cm^{-1}: 1760 (ester), 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.62 (3H, s, 4-CH_3), 3.75 (6H, m), 4.43 (1H, d, J = 18 Hz, C_2-H), 4.77 (1H, d, J = 18 Hz, C_2-H).

Methyl 4-Benzyl-7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (7)——A colorless oil (97% yield). *Anal.* Calcd for $C_{19}H_{18}O_5S$: C, 63.63; H, 5.06. Found: C, 63.41; H, 5.21. IR ν_{max}^{neat} cm^{-1}: 1760 (ester), 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.08 (1H, d, J = 14 Hz, CH_2Ph), 3.40 (3H, s), 3.58 (3H, s), 3.63 (1H, d, J = 14 Hz, CH_2Ph), 4.48

(1H, d, J=17 Hz, C_2-H), 4.72 (1H, d, J=17 Hz, C_2-H). MS m/z: 358 (M^+).

Methyl 2-(1-Ethoxycarbonylethyl)thio-4-methoxyphenoxyacetate (8)——A mixture of **1b** (15.4 g, 0.1 mol) and ethyl 2-chloropropionate (13.6 g, 0.1 mol) in acetone (100 ml) in the presence of anhydrous K_2CO_3 (15 g, 0.11 mol) was stirred at room temperature for 5 h. Then methyl bromoacetate (15.3 g, 0.1 mol) and anhydrous K_2CO_3 (13.8 g, 0.1 mol) were added, and the reaction mixture was heated under reflux with stirring for 5 h. After filtration, the filtrate was concentrated. The residue was chromatographed on silica gel with hexane and AcOEt (1 : 1) to give **6** (17.1 g, 67%) as a colorless oil. *Anal.* Calcd for $C_{15}H_{20}O_6S$: C, 54.86; H, 6.14. Found: C, 55.09; H, 6.16. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (3H, t, J=7 Hz, OCH_2CH_3), 1.50 (3H, d, J=7 Hz, $SCHCH_3$), 3.72 (3H, s), 3.75 (3H, s), 4.62 (2H, s, OCH_2CO).

Methyl 2-Hydroxy-5-methoxyphenylthioacetate (9b)——A mixture of **1b** (1.76 g, 10 mmol), methyl bromoacetate (1.5 g, 10 mmol) and anhydrous K_2CO_3 (1.37 g, 10 mmol) in acetone (15 ml) was stirred at room temperature for 6 h. After usual work-up, chromatography on silica gel with hexane–AcOEt (1 : 1) as the eluant gave **9b** (2.0 g, 88%) as a pale brown oil. *Anal.* Calcd for $C_{10}H_{12}O_4S$: C, 52.60; H, 5.30. Found: C, 52.81; H, 5.44.

Methyl 2-Hydroxy-4-methoxyphenylthioacetate (9f)——Similar alkylation of **1f** with methyl bromoacetate gave **9f** in 85% yield as a pale brown oil. *Anal.* Calcd for $C_{10}H_{12}O_4S$: C, 52.60; H, 5.30. Found: C, 52.41; H, 5.16. IR ν_{max}^{neat} cm^{-1}: 3430 (OH), 1730 (ester).

Methyl 2-Cyanomethyloxy-5-methoxyphenylthioacetate (10)——Anhydrous K_2CO_3 (2.2 g, 16 mmol) and KI (0.4 g, 2.4 mmol) were added to a solution of **9b** (3.0 g, 13 mmol) and chloroacetonitrile (1.2 g, 15.9 mmol) in acetone (20 ml) and the mixture was heated under reflux for 3 h. Filtration and subsequent concentration of the filtrate gave **10** (3.2 g, 91%) as colorless needles, mp 97—99 °C (from MeOH). *Anal.* Calcd for $C_{12}H_{13}NO_4S$: C, 53.92; H, 4.90; N, 5.24. Found: C, 53.81; H, 4.76; N, 5.11. ^{1}H-NMR ($CDCl_3$) δ: 3.62 (2H, s, SCH_2), 3.65 (3H, s), 3.72 (3H, s), 4.75 (2H, s, OCH_2CN). MS m/z: 267 (M^+). Dieckmann reaction of **10** using 28% NaOMe in DMF at 0 °C gave a complicated mixture of products.

Methyl 5-Methoxy-2-(2-oxo-tetrahydrofuryl)oxyphenylthioacetate (11)——Anhydrous K_2CO_3 (37.5 g, 0.27 mol) and KI (9 g, 54 mmol) were added to a solution of **9b** (50 g, 235 mmol) and α-bromo-γ-butyrolactone (42 g, 255 mmol) in acetone (300 ml), and the reaction mixture was boiled under reflux with stirring for 5 h. Usual work-up and chromatography on silica gel with hexane–AcOEt (2 : 1) as the eluant gave **11** (58 g, 85%) as a colorless oil. *Anal.* Calcd for $C_{14}H_{16}O_6S$: C, 53.84; H, 5.16. Found: C, 53.91; H, 5.24. IR ν_{max}^{neat} cm^{-1}: 1780 (lactone), 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ: 2.4—2.8 (2H, m), 3.53 (2H, s, SCH_2), 3.58 (3H, s), 3.65 (3H, s), 4.0—5.0 (3H, m).

Dimethyl 2-(4-Methoxy-2-methoxycarbonylmethylthiophenyloxy)glutarate (12b)——Anhydrous K_2CO_3 (18 g, 131 mmol) and KI (1.0 g, 6 mmol) were added to a solution of **9b** (34 g, 133 mmol) and dimethyl 2-mesyloxyglutarate[13] (34 g, 131 mmol) in acetone (300 ml), and the reaction mixture was boiled under reflux with stirring for 8 h. Usual work-up and chromatography on silica gel with hexane–AcOEt (2 : 1) as the eluant gave **12b** (21 g, 41%) as a colorless oil. *Anal.* Calcd for $C_{17}H_{22}O_8S$: C, 52.84; H, 5.74. Found: C, 52.99; H, 5.76. IR ν_{max}^{neat} cm^{-1}: 1730 (ester), 1485, 1270, 1200. ^{1}H-NMR ($CDCl_3$) δ: 2.2—2.8 (4H, m), 3.70 (3H, s), 3.77 (3H, s), 4.72 (1H, t, J=5 Hz, OCH—).

Dimethyl 2-(5-Methoxy-2-methoxycarbonylmethylthiophenoxy)glutarate (12f)——Similarly, **12f** was prepared by alkylation of **9f** with dimethyl 2-mesyloxyglutarate as described for **12b**. A colorless oil (40% yield). *Anal.* Calcd for $C_{17}H_{22}O_8S$: C, 52.84; H, 5.74. Found: C, 52.98; H, 5.74. ^{1}H-NMR ($CDCl_3$) δ: 2.2—2.8 (4H, m), 3.73 (2H, s, SCH_2), 3.75 (3H, s), 3.78 (3H, s), 4.73 (1H, t, J=5 Hz, OCH—).

Dieckmann Reaction of 11b, 12b and 12f——A solution of methyl α-substituted 2-methoxycarbonylmethylthiophenoxyacetate (**11b**, **12b** and **12f**) (10 mmol) in DMF (5 ml) was added dropwise to an ice-cooled solution of 28% NaOMe (2.5 g, 13 mmol) and DMF (15 ml) under an atmosphere of dry N_2 with stirring, and the reaction mixture was stirred for 2 h. The reaction mixture was poured into ice-H_2O containing conc. HCl (3 ml) and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (2 : 1) as the eluant to give the following 2-substituted 1,5-benzoxathiepin derivatives (**13**, **14b** and **14f**).

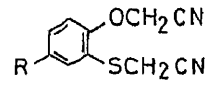
Methyl 2-(2-Hydroxyethyl)-7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (13)——A colorless oil (80% yield). *Anal.* Calcd for $C_{14}H_{16}O_6S$: C, 53.84; H, 5.16. Found: C, 54.12; H, 5.38. IR ν_{max}^{neat} cm^{-1}: 3500—3400 (OH), 1745 (ester), 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.1—2.8 (2H, m), 3.62 (3H, s), 3.68 (3H, s), 3.7—4.7 (4H, m).

Methyl 3-(7-Methoxy-4-methoxycarbonyl-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionate (14b)——A colorless oil (62% yield). *Anal.* Calcd for $C_{16}H_{18}O_7S$: C, 54.23; H, 5.12. Found: C, 54.42; H, 5.23. IR ν_{max}^{neat} cm^{-1}: 1770, 1750 (ester), 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.8 (4H, m), 3.68 (3H, s), 3.75 (3H, s), 3.78 (3H, s), 3.80 (3H, s), 4.5—5.0 (2H, m).

Methyl 3-(8-Methoxy-4-methoxycarbonyl-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionate (14f)——A colorless oil (68% yield). *Anal.* Calcd for $C_{16}H_{18}O_7S$: C, 54.23; H, 5.12. Found: C, 54.28; H, 5.12. IR ν_{max}^{neat} cm^{-1}: 1745 (ester), 1720 (CO), 1485, 1440, 1315. ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.8 (4H, m), 3.70 (3H, s), 3.76 (3H, s), 3.80 and 3.83 (3H), 4.5—5.0 (2H, m).

Demethoxycarbonylation of 13——Method A: A mixture of **13** (1.0 g, 3.2 mmol), 20% H_2SO_4 (3 ml) and dioxane (5 ml) was heated at 100 °C for 10 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel

TABLE VI. 2-Cyanomethylthiophenoxyacetonitriles (**18a—d**)



Compound No.	R	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
18a	H	73	76—77[a]	$C_{10}H_8N_2OS$	58.81 (58.77	3.95 3.70	13.72 13.47)
18b	OCH_3	79	53—54[b]	$C_{11}H_{10}N_2O_2S$	56.39 (56.57	4.30 4.32	11.96 11.78)
18c	CH_3	68	88—90[a]	$C_{11}H_{10}N_2OS$	60.53 (60.47	4.62 4.54	12.83 12.60)
18d	Cl	70	141—143[a]	$C_{10}H_7ClN_2OS$	50.32 (50.41	2.96 2.97	11.74 11.70)

a) Crystallized from MeOH. *b*) Crystallized from EtOH.

with hexane–AcOEt (2:1) to give 7-methoxy-3,3a,10,10a-tetrahydrofuro[3,2-*b*][1,5]benzoxathiepin-10a-ol (**16**) as a colorless oil (0.6 g, 74%). *Anal.* Calcd for $C_{12}H_{14}O_4S$: C, 56.68; H, 5.55. Found: C, 56.69; H, 5.23. IR ν_{max}^{neat} cm^{-1}: 3450—3300 (OH), 1485, 1440, 1215, 1115. ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.7 (2H, m), 2.83 (1H, d, J=14 Hz, C_{10}-H), 4.0—4.5 (2H, m), 4.58 (1H, dd, J=6, 2 Hz, C_{10a}-H). MS *m/z*: 254 (M^+).

Method B: A mixture of **13** (1.0 g, 3.2 mmol), conc. HCl (3 ml) and AcOH (3 ml) was heated at 100 °C for 20 h. The reaction mixture was diluted with H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (3:1) as the eluant to give 2-chloroethyl-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (**15**) (160 mg, 18%) as colorless prisms, mp 70—71 °C (from AcOEt). *Anal.* Calcd for $C_{12}H_{13}ClO_3S$: C, 52.84; H, 4.80. Found: C, 53.18; H, 4.88. IR ν_{max}^{KBr} cm^{-1}: 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.1—2.6 (2H, m), 3.08 (1H, d, J=12 Hz, C_4-H), 3.62 (3H, s, OCH_3), 3.6—4.0 (2H, m), 4.52 (1H, d, J=12 Hz, C_4-H), 4.7—5.0 (1H, m, C_2-H). **15** was also obtained by heating of **16** in conc. HCl and AcOH for 5 h in 40% yield.

Methyl 3-(7-Methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionate (17b)——A mixture of **14b** (3.54 g, 10 mmol), LiCl (0.5 g, 12 mmol), H_2O (0.2 ml) and DMF (12 ml) was heated at 100 °C for 5 h. The reaction mixture was diluted with H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (2:1) as the eluant to give **17b** (1.84 g, 62%) as colorless prisms, mp 118—120 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{14}H_{16}O_5S$: C, 56.74; H, 5.44. Found: C, 56.94; H, 5.52. IR ν_{max}^{KBr} cm^{-1}: 1735 (ester), 1715 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.8 (4H, m), 3.17 (1H, d, J=12 Hz, C_4-H), 3.68 (3H, s), 3.73 (3H, s), 4.63 (1H, d, J=12 Hz, C_4-H), 4.6—4.9 (1H, m, C_2-H).

Methyl 3-(8-Methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionate (17f)——**17f** was prepared by similar demethoxycarbonylation of **14f**. A colorless oil (61% yield). *Anal.* Calcd for $C_{14}H_{16}O_5S$: C, 56.74; H, 5.44. Found: C, 56.89; H, 5.59. IR ν_{max}^{neat} cm^{-1}: 1735, 1730, 1725, 1480, 1390, 1200. ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.7 (4H, m), 3.17 (1H, d, J=9 Hz, C_4-H), 3.67 (3H, s), 3.77 (3H, s), 4.40 (1H, d, J=9 Hz, C_4-H), 4.93 (1H, dd, J=3, 5 Hz, C_2-H).

2-Cyanomethylthiophenoxyacetonitrile (18a)——Anhydrous K_2CO_3 (3.4 g, 25 mmol) and KI (0.1 g, 0.6 mmol) were added to a mixture of **1a** (1.26 g, 10 mmol), chloroacetonitrile (1.9 g, 25 mmol) and acetone (20 ml), and the reaction mixture was boiled under reflux for 5 h. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was recrystallized from MeOH to give **18a** (1.46 g) as colorless plates. IR ν_{max}^{KBr} cm^{-1}: 2230 (CN). ^{1}H-NMR ($CDCl_3$) δ: 3.57 (2H, s, SCH_2), 4.85 (2H, s, OCH_2).

Compounds (**18b—d**) were prepared by similar alkylation of substituted 2-mercaptophenols and are included in Table VI.

3-Amino-2*H*-1,5-benzoxathiepin-4-carbonitrile (19a)——A solution of **18a** (1.46 g, 10 mmol) dissolved in DMF (3 ml) was added dropwise to a mixture of 28% NaOMe (3.0 g, 15 mmol) and DMF (12 ml) under ice-cooling in an atmosphere of dry N_2, and the reaction mixture was stirred for 5 h, then poured into cold H_2O containing AcOH (2 ml). The resulting precipitates were collected and recrystallized from $CHCl_3$ to give **19a** as colorless needles. IR ν_{max}^{KBr} cm^{-1}: 3440, 3350, 3250, 2200 (CN), 1650. ^{1}H-NMR (DMSO-d_6) δ: 3.28 (2H, s, NH_2), 5.26 (2H, s, C_2-H), 6.6—7.6 (4H, m).

Compounds (**19b—d**) were similarly prepared by means of the Thorpe–Ziegler reaction of the corresponding dinitriles (**18b—d**), and their physical data are included in Table II.

3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carbonitrile (20a)——A solution of **19a** (2.92 g, 20 mmol) in EtOH (100 ml) containing conc. HCl (18 ml) was boiled under reflux for 1 h. The resulting precipitates (NH_4Cl) were filtered off and the filtrate was evaporated *in vacuo*. The residue was recrystallized from AcOEt–hexane to yield **20a** (1.37 g, 67%) as colorless prisms, mp 93—95 °C. *Anal.* Calcd for $C_{10}H_7NO_2S$: C, 58.52; H, 3.44; N, 6.82. Found: C, 58.68; H, 3.52; N, 6.66. IR ν_{max}^{KBr} cm^{-1}: 3350 (enol OH), 2250 (CN), 1730 (CO). ^{1}H-NMR ($CDCl_3$) δ: 4.73 (1H, d, J=17 Hz, C_2-H), 5.03 (1H, d, J=17 Hz, C_2-H), 5.25 (1H, s, C_4-H), 7.2—7.4 (4H, m).

7-Methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carbonitrile (20b)——Method A: Hydrolysis of **19b** as described for **20a** gave **20b** in 85% yield. Colorless prisms, mp 132—133 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{11}H_9NO_3S$: C, 56.16; H, 3.86; N, 5.95. Found: C, 56.08; H, 3.79; N, 5.85. IR ν_{max}^{KBr} cm^{-1}: 3380 (enol OH), 2280, 2220 (CN), 1740 (CO). ^{1}H-NMR ($CDCl_3$) δ: 3.73 (3H, s, OCH_3), 4.63 (1H, d, J=18 Hz, C_2-H), 4.93 (1H, d, J=18 Hz, C_2-H), 5.30 (1H, s, C_4-H), 6.6—7.2 (3H, m).

Method B: **20b** was also prepared by Dieckmann reaction of methyl 2-cyanomethyl-4-methoxyphenyloxyacetate (**21**) using 28% NaOMe in DMF. Yield 40%.

Methyl 2-Cyanomethylthio-4-methoxyphenoxyacetate (21)——A mixture of **1b** (2.0 g, 13 mmol), chloroacetonitrile (1.0 g, 13 mmol) and anhydrous K_2CO_3 (1.8 g, 13 mmol) in acetone (20 ml) was boiled under reflux for 3 h. Then methyl bromoacetate (1.0 g, 13 mmol) and K_2CO_3 (1.8 g) were added to the reaction mixture, and the mixture was boiled under reflux for a further 5 h. Usual work-up and chromatography on silica gel with hexane and AcOEt (1 : 1) gave **21** (2.08 g, 60%) as colorless prisms, mp 99—101 °C (from AcOEt). *Anal.* Calcd for $C_{12}H_{13}NO_4S$: C, 53.92; H, 4.90; N, 5.24. Found: C, 53.97; H, 4.81; N, 5.20. IR ν_{max}^{KBr} cm^{-1}: 1735 (ester), 1490, 1435, 1245, 1205. ^{1}H-NMR ($CDCl_3$) δ: 3.63 (2H, s, SCH_2), 3.65 (3H, s), 3.72 (3H, s), 4.75 (2H, s, OCH_2).

Methyl 2-*N*,*N*-Diethylcarbamoylmethylthio-4-methoxyphenoxyacetate (22)——Anhydrous K_2CO_3 (25 g, 0.18 mol) was added to a solution of **1b** (28 g, 0.18 mol) and *N*,*N*-diethyl chloroacetamide (26.9 g, 0.18 mol) in acetone (300 ml), and the mixture was stirred at room temperature under an atmosphere of dry N_2 for 5 h. After filtration of the precipitates, methyl bromoacetate (25 g, 0.16 mol) and K_2CO_3 (25 g) were added to the filtrate, and the mixture was boiled under reflux for 5 h. Usual work-up and chromatography on silica gel with hexane–AcOEt (1 : 1) gave **22** (45 g, 73%) as a colorless oil. *Anal.* Calcd for $C_{16}H_{23}NO_5S$: C, 56.29; H, 6.79; N, 4.10. Found: C, 56.23; H, 6.77; N, 4.18. IR ν_{max}^{neat} cm^{-1}: 1770 (ester), 1660 (amide). ^{1}H-NMR ($CDCl_3$) δ: 1.27 (6H, t, J=7 Hz, $CH_2\underline{CH_3}$), 3.77 (3H, s), 3.87 (3H, s), 4.23 (4H, q, J=7 Hz, $C\underline{H_2}CH_3$), 4.60 (2H, s, OCH_2), 6.6—7.3 (3H, m).

7-Methoxy-3-oxo-3,4-dihydro-*N*,*N*-diethyl-2*H*-1,5-benzoxathiepin-4-carboxamide (23)——A solution of **22** (43 g, 0.126 mol) in DMF (50 ml) was added dropwise to a mixture of 28% NaOMe (30 g, 0.155 mol) and DMF (160 ml) at room temperature under an atmosphere of dry N_2, and the mixture was stirred for 6 h. The reaction mixture was poured into ice-H_2O containing AcOH (15 ml) and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was chromatographed on silica gel with hexane–AcOEt (1 : 1) to give **23** (3.4 g, 9%) as colorless prisms, mp 112—113 °C (from AcOEt). *Anal.* Calcd for $C_{15}H_{19}NO_4S$: C, 58.23; H, 6.19; N, 4.53. Found: C, 58.17; H, 6.06; N, 4.54. IR ν_{max}^{KBr} cm^{-1}: 1730 (CO), 1635 (amide). ^{1}H-NMR ($CDCl_3$) δ: 1.18 (6H, t, J=7 Hz, $CH_2\underline{CH_3}$), 3.0—3.7 (4H, m), 3.73 (3H, s, OCH_3), 4.51 (1H, d, J=17 Hz, C_2-H), 4.95 (1H, d, J=17 Hz, C_2-H) 5.70 (1H, s, C_4-H), 6.3—7.2 (3H, s).

4-Amino-7-methoxy-2-methyl-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin (26)——A solution of 28% NaOMe (2.5 g, 13 mmol) was added to a solution of acetamidine·HCl (1.25 g, 13 mmol) in EtOH (30 ml), and the mixture was boiled for 0.5 h. Then, **19b** (1.6 g, 6.8 mmol) was added, and the mixture was boiled under reflux for 20 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and evaporated *in vacuo*. The residue was recrystallized from AcOEt to give **26** (0.62 g) as colorless plates. IR ν_{max}^{KBr} cm^{-1}: 3440, 3310 (NH_2), 1640, 1560, 1490, 1200, 1040. ^{1}H-NMR (DMSO-d_6) δ: 2.22 (3H, s, 2-CH_3), 3.68 (3H, s, OCH_3), 5.02 (2H, s, C_{11}-H), 6.8—7.0 (3H, m).

Compounds **24**—**25** and **27**—**31** were similarly prepared from enaminonitriles (**19a** and **19b**) and the corresponding amidines or guanidines. **31** was prepared by similar treatment of **19b** with 3-cyanopyridine. Physicochemical properties of **24**—**32** are listed in Table IV.

4-Amino-7-methoxy-2-[4-(2-furoyl)-1-piperazinyl]-11*H*-pyrimido[4,5-*c*][1,5]benzoxathiepin (33)——Diethyl phosphorocyanidate (0.21 g, 1.2 mmol) and Et_3N (0.18 ml) were added to a mixture of **29** (0.3 g, 0.9 mmol), 2-furoic acid (0.11 g, 1.0 mmol) and DMF (4 ml), and the mixture was stirred at room temperature for 2 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. Usual work-up and chromatography on silica gel with hexane–AcOEt (1 : 2) as the eluant gave **33** (0.31 g, 82%) as colorless crystals, mp 170—171 °C (from AcOEt). *Anal.* Calcd for $C_{21}H_{21}N_5O_4S$: C, 57.39; H, 4.82; N, 15.94. Found: C, 57.53; H, 4.94; N, 15.55. IR ν_{max}^{KBr} cm^{-1}: 3480, 3350 (NH_2), 1620, 1600. ^{1}H-NMR ($CDCl_3$) δ: 3.72 (3H, s, OCH_3), 5.18 (2H, s, C_{11}-H).

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1,5-Benzoxathiepin Derivatives. II.[1] Synthesis and Serotonin S_2-Receptor-Blocking Activity of Aminoalkyl-Substituted 3,4-Dihydro-2*H*-1,5-benzoxathiepin-3-ols and Related Compounds

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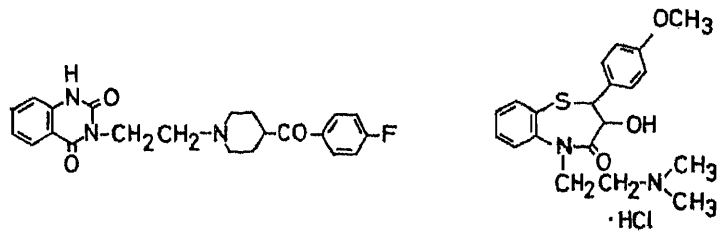
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Novel 1,5-benzoxathiepin derivatives, 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ols with an aminoalkyl group at the 2-, 3- or 4-position, were synthesized and evaluated for serotonin S_2-receptor-blocking activity and adrenergic α_1-receptor-blocking activity. Methyl 4-aminoalkyl-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates showed significant S_2-receptor-blocking activities. Structure–activity relationships (including the results of a conformational study and skeletal modifications) were examined. In the series of 1,5-benzoxathiepin, 1-benzoxepin and 1-benzothiepin derivatives, methyl *cis*-3-hydroxy-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate hydrochloride (CV-5197) showed the most potent and the most selective S_2-receptor-blocking activity in the binding profile, and was chosen as a candidate for further pharmacological evaluation.

Keywords——seven-membered heterocycle; 1,5-benzoxathiepin derivative; aminoalkyl-substituted 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol; serotonin S_2-receptor antagonist; structure–activity relationship; 1,5-benzoxathiepin derivative conformation; CV-5197

Serotonin (5-hydroxytryptamine, 5-HT) is known to act as a chemical mediator in a wide variety of physiological actions. Peroutka and Snyder demonstrated the existence of distinct populations of serotonin receptors, S_1 or 5-HT_1 and S_2 or 5-HT_2 receptors, based on the binding characteristics of radio-labeled serotonin and spiperone in rat brain homogenate, respectively.[2] Peripheral vascular serotonergic receptors have the pharmacological characteristics of receptors such as S_2-receptors in the central nervous systems.[3] Ketanserin, which is a potent and selective S_2-receptor antagonist, is a member of a new class of drugs possessing effective antihypertensive activity.[4] However, recent studies suggest that the antihypertensive effect of ketanserin in animal models is more related to its postsynaptic adrenergic α_1-receptor-blocking activity than to its antagonism of vascular S_2-receptors, and the apparent role of serotonin in hypertension is still uncertain.[5] Nevertheless, platelet aggregation due to serotonin is mediated through S_2-receptors.[6] Furthermore, serotonin has an amplifying effect on vascular and platelet actions, which is also mediated through S_2-receptors.[4,6,7] Thus, the selective antagonism of S_2-receptors might be important in preventing peripheral circulatory disorders in which vasoconstriction and platelet aggregation are suggested to be involved. In a study of the structure–activity relationships of S_2-receptor antagonists, no distinct structural relationships among serotonin and antagonists were found except for the essential amino function in the molecules.[8] Diltiazem, classified as a Ca antagonist having a structure with the seven-membered 1,5-benzothiazepine skeleton, caused reduction of the serotonin-induced contraction in isolated rabbit basilar artery, whereas such a reduction was diminished in the aorta.[9]



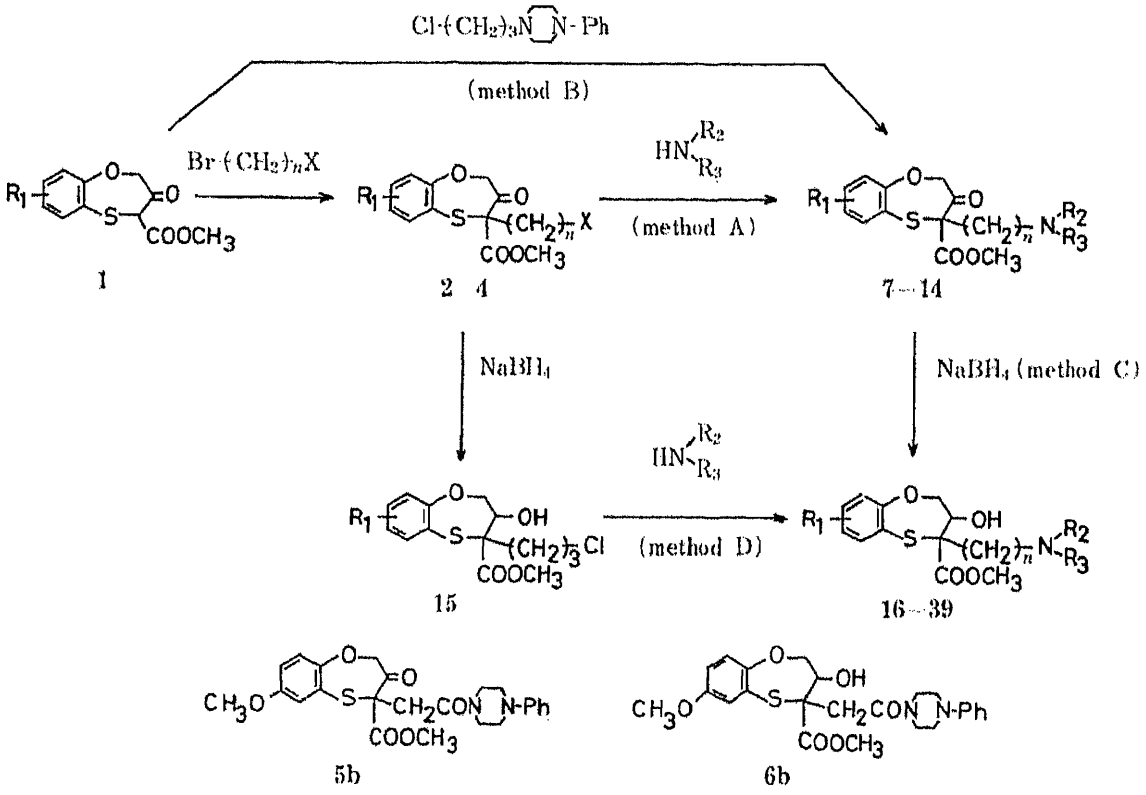
ketanserin diltiazem

Chart 1

With the aim of finding a novel S_2-receptor blocker, we synthesized 1,5-benzoxathiepin derivatives having structures analogous to 1,5-benzothiazepines and found that the 3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ols with an aminoalkyl group at the 4-position showed significant S_2-receptor-blocking activities. In this paper, we report the syntheses of the 1,5-benzoxathiepins with an aminoalkyl group at the 2-, 3-, or 4-position and related compounds, and the structure–activity relationships of S_2-receptor-blocking activity.

Synthesis of Aminoalkyl-Substituted 1,5-Benzoxathiepin Derivatives and Related Compounds

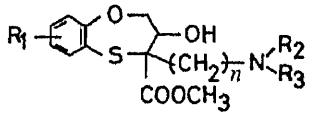
In the previous paper, we reported a novel synthetic route to the 1,5-benzoxathiepin skeleton and some modifications at its 2-, 3-, and 4-positions.[1] We first examined the introduction of an aminoalkyl group into the 4-position of methyl 3-oxo-3,4-dihydro-2*H*-1,5-



a series: R_1 = H, b series: R_1 = 7-OCH_3,
c series: R_1 = 8-OCH_3, d series: R_1 = 7-Cl,
e series: R_1 = 7-CH_3, f series: R_1 = 7-OCH_2Ph,
g series: R_1 = 7-OH

Chart 2

TABLE I. Physicochemical Properties of Methyl 4-Aminoalkyl-Substituted 3-Hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**16**—**39**)



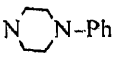
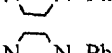
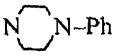
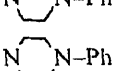
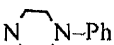
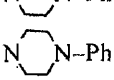
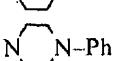
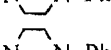
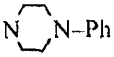
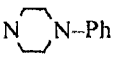
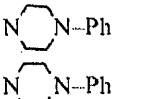
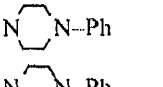
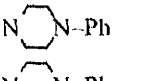
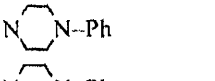
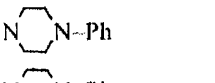
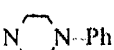
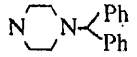
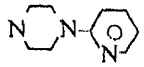
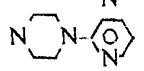
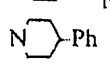
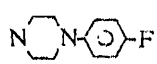
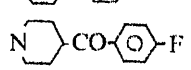
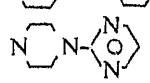
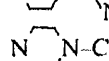
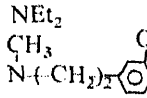
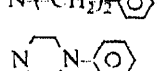
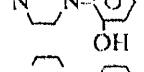
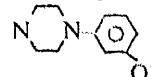
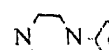
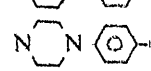
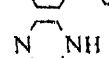
Compd. No.	R_1	$N<^{R_2}_{R_3}$	n	Method[a]	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
cis-**16b**	7-OCH_3		2	F	42	213—216	$C_{24}H_{30}N_2O_5S\cdot 2HCl$	54.24 (54.14)	6.07 (6.08)	5.27 (5.29)
cis-**17a**	H		3	C	52	196—198	$C_{24}H_{30}N_2O_4S\cdot 2HCl$	55.92 (55.73)	6.26 (6.15)	5.45 (5.51)
trans-**17a**	H		3	C	38	165—170	$C_{24}H_{30}N_2O_4S\cdot 2HCl\cdot 1/4H_2O$	55.43 (55.47)	6.30 (6.19)	5.39 (5.40)
cis-**17b**	7-OCH_3		3	C D	47 56	154—155	$C_{25}H_{32}N_2O_5S\cdot HCl\cdot 2H_2O$	55.09 (55.46)	6.84 (6.77)	5.14 (5.09)
trans-**17b**	7-OCH_3		3	C	31	140—145	$C_{25}H_{32}N_2O_5S\cdot HCl\cdot H_2O$	56.97 (56.67)	6.69 (6.70)	5.31 (5.35)
cis-**17c**	8-OCH_3		3	C	46	195—198	$C_{25}H_{32}N_2O_5S\cdot HCl\cdot 1/4H_2O$	58.47 (58.43)	6.57 (6.44)	5.46 (5.62)
trans-**17c**	8-OCH_3		3	C	30	170—175	$C_{25}H_{32}N_2O_5S\cdot 2HCl\cdot 1/2H_2O$	54.12 (54.08)	6.36 (6.02)	5.05 (5.03)
cis-**17d**	7-Cl		3	C	42	205—207	$C_{24}H_{29}ClN_2O_4S\cdot 2HCl\cdot 1/2H_2O$	51.57 (51.77)	5.77 (5.79)	5.01 (4.97)
trans-**17d**	7-Cl		3	C	38	150—160	$C_{24}H_{29}ClN_2O_4S\cdot 2HCl$	52.42 (52.24)	5.68 (5.76)	5.09 (4.97)
cis-**17e**	7-CH_3		3	C	48	170—175	$C_{25}H_{32}N_2O_4S\cdot 2HCl$	56.70 (56.73)	6.47 (6.54)	5.01 (5.04)
trans-**17e**	7-CH_3		3	C	33	145—155	$C_{25}H_{32}N_2O_4S\cdot 2HCl\cdot 1/4H_2O$	56.23 (56.39)	6.51 (6.53)	5.25 (5.24)
cis-**17f**	7-OCH_2Ph		3	D	61	198—201	$C_{31}H_{38}N_2O_5S\cdot HCl$	63.62 (63.32)	6.37 (6.41)	4.79 (4.52)
cis-**17g**	7-OH		3	E	64	207—209	$C_{24}H_{30}N_2O_5S$	62.86 (62.61)	6.59 (6.50)	6.11 (5.88)
cis-**18b**	7-OCH_3		4	C	75	168—171	$C_{26}H_{34}N_2O_5S\cdot 2HCl$	55.81 (55.92)	6.49 (6.54)	5.01 (5.04)
trans-**18b**	7-OCH_3		4	C	11	128—130	$C_{26}H_{34}N_2O_5S$	64.17 (64.32)	7.04 (6.95)	5.76 (5.62)
cis-**19b**	7-OCH_3		5	C	22	125—128	$C_{27}H_{36}N_2O_5S\cdot HCl\cdot 1^1/_2H_2O$	57.48 (57.20)	7.14 (6.98)	4.97 (4.94)
cis-**20b**	7-OCH_3		3	C	55	145—150	$C_{25}H_{31}ClN_2O_5S\cdot 2HCl\cdot 1/2H_2O$	50.98 (50.67)	5.82 (6.12)	4.76 (4.61)
trans-**20b**	7-OCH_3		3	C	33	111—113	$C_{25}H_{31}ClN_2O_5S\cdot$	59.22 (59.28)	6.16 (6.27)	5.52 (5.34)
cis-**21b**	7-OCH_3		3	C	50	140—145	$C_{26}H_{34}N_2O_6S\cdot HCl\cdot 2H_2O$	54.30 (54.57)	6.83 (6.64)	4.87 (4.70)
trans-**21b**	7-OCH_3		3	C	20	170—175	$C_{26}H_{34}N_2O_6S\cdot 2HCl\cdot H_2O$	52.61 (52.99)	6.45 (6.24)	4.72 (4.72)
cis-**22b**	7-OCH_3		3	C	55	185—193	$C_{26}H_{34}N_2O_6S\cdot 2HCl$	54.26 (54.56)	6.30 (6.29)	4.87 (5.07)
trans-**22b**	7-OCH_3		3	C	22	178—182	$C_{26}H_{34}N_2O_6S\cdot 2HCl$	54.26 (54.05)	6.31 (6.36)	4.88 (4.66)
cis-**23b**	7-CH_3		3	C	65	133—135	$C_{32}H_{38}N_2O_5S$	68.30 (68.48)	6.81 (6.73)	4.98 (4.97)

TABLE I. (continued)

Compd. No.	R_1	$N<^{R_2}_{R_3}$	n	Method[a]	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
trans-**23b**	7-OCH_3		3	C	33	173—176	$C_{32}H_{38}N_2O_5S$	68.30 (68.34)	6.81 6.81	4.98 4.82)
cis-**24b**	7-OCH_3		3	C	63	173—176	$C_{24}H_{31}N_3O_5S\cdot 2HCl\cdot 1/2\,H_2O$	51.89 (51.96)	6.17 6.40	7.56 7.32)
trans-**24b**	7-OCH_3		3	C	25	222—225	$C_{24}H_{31}N_3O_5S\cdot 2HCl$	52.74 (52.31)	6.09 6.02	7.69 7.65)
cis-**25b**	7-OCH_3		3	D	51	135—140	$C_{26}H_{33}NO_5S\cdot HCl\cdot 1/2\,H_2O$	60.39 (60.48)	6.82 6.84	2.71 2.70)
cis-**26b**	7-OCH_3		3	D	36	140—150	$C_{25}H_{31}FN_2O_5S\cdot 2HCl\cdot 1/2\,H_2O$	52.44 (52.71)	5.90 5.82	4.89 4.79)
cis-**27b**	7-OCH_3		3	D	43	150—153	$C_{27}H_{32}FNO_6S\cdot HCl\cdot H_2O$	56.68 (56.71)	6.17 6.08	2.45 2.46)
cis-**28b**	7-OCH_3		3	D	24	210—213	$C_{23}H_{30}N_4O_5S\cdot 2HCl\cdot H_2O$	48.85 (48.94)	6.06 5.84	9.91 9.99)
cis-**29b**	7-OCH_3		3	D	35	142—145	$C_{20}H_{30}N_2O_5S\cdot 1/2\,H_2O$	57.29 (57.45)	7.45 7.40	6.68 6.71)
cis-**30b**	7-OCH_3		3	D	47	205—210	$C_{19}H_{20}NO_6S\cdot HCl$	52.59 (52.57)	6.50 6.72	3.23 3.19)
cis-**31b**	7-OCH_3	NEt_2	3	D	34	185—188	$C_{19}H_{29}NO_5S\cdot HCl$	54.34 (54.07)	7.20 7.23	3.34 3.34)
cis-**32b**	7-OCH_3		3	D	41	—[b]	$C_{26}H_{35}NO_7S\cdot HCl\cdot 1/2\,H_2O$	56.67 (56.68)	6.77 6.97	2.54 2.51)
cis-**33b**	7-OCH_3		3	D	50	175—190	$C_{25}H_{32}N_2O_6S\cdot 2HCl\cdot 1/2\,H_2O$	52.63 (52.91)	6.18 5.94	4.91 4.95)
cis-**34b**	7-OCH_3		3	D	65	175—180	$C_{25}H_{32}N_2O_6S\cdot 2HCl\cdot H_2O$	51.81 (52.00)	6.26 6.02	4.83 4.72)
cis-**35b**	7-OCH_3		3	D	50	240—245	$C_{25}H_{32}N_2O_6S\cdot 2HCl$	53.43 (53.20)	6.10 5.97	4.99 5.21)
cis-**36f**	7-OCH_2Ph		3	D	57	247—251	$C_{31}H_{36}N_2O_6S\cdot HCl$	61.93 (61.91)	6.20 6.01	4.66 4.65)
cis-**37f**	7-OCH_2Ph		3	F	64	193—200	$C_{25}H_{32}N_2O_5S\cdot 2HCl\cdot 1/2\,H_2O$	54.15 (54.10)	6.36 6.30	5.05 5.11)
cis-**38g**	7-OH		3	E	72	226—229	$C_{24}H_{30}N_2O_6S$	60.74 (60.47)	6.37 6.39	5.90 5.71)
cis-**39g**	7-OH		3	E	90	233—240	$C_{18}H_{26}N_2O_5S\cdot 2HCl$	47.47 (47.19)	6.20 6.36	6.15 5.86)

a) Method E, catalytic reduction of the corresponding *O*-benzyl ether. Method F, see experimental section. *b*) Amorphous powder.

benzoxathiepin-4-carboxylates (**1a**—**f**) by alkylation of the reactive methine carbon (Chart 2). The ketoesters (**1a**—**f**) were alkylated with ω-halogenoalkyl bromides having a side chain of various lengths ($n=3$—5) in refluxing acetonitrile by using potassium carbonate and a catalytic amount of potassium iodide to yield a mixture of the desired *C*-alkylated compounds (30—40%) and *O*-alkylated enole ethers (10—20%) as a by-product. Chromatographic separation of the reaction mixture gave methyl 4-(ω-halogenoalkyl)-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**2**—**4**). The 4-[2-oxo-2-(4-phenyl-1-piperazinyl)ethyl] derivative (**5b**) was prepared by alkylation of **1b** with 1-chloroacetyl-4-phenylpiperazine in 69% yield. Conversion of the halogeno moiety of **2**—**4** into the amino group by sub-

stitution with some amines was carried out by heating in *N*,*N*-dimethylformamide (DMF) at 70 °C in the presence of potassium carbonate and potassium iodide to give methyl 4-aminoalkyl-substituted 3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**7**—**14**) in fairly good yields (58—76%) (method A). Direct introduction of an *N*-phenylpiperazinylpropyl moiety into the 4-position of **1** by alkylation using 4-phenyl-1-piperazinylpropyl chloride gave *N*-phenylpiperazinylpropyl derivatives (**7a**—**e**) in 18—31% yields (method B). Reduction of **7**—**14** with sodium borohydride ($NaBH_4$) in an ice-cooled solution of methanol and tetrahydrofuran (THF), and subsequent separation of the resulting stereoisomeric products by column chromatography on silica gel gave methyl *cis*- and *trans*-4-aminoalkyl-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**17**—**24**) (method C) (Table I). The configurational nomenclature in this paper refers to the relative configuration between the 3-hydroxy group and the 2- or 4-substituent with higher priority. In each case, $NaBH_4$ reduction produced predominantly *cis*-alcohols over *trans*-isomers in the range of 1 : 1—2 : 1 ratios. Similar $NaBH_4$ reduction of **2b** and **2f** afforded *cis*-4-chloropropyl-3-ols (*cis*-**15b**, 63% and *cis*-**15f**, 50%) and *trans*-alcohols (*trans*-**15b**, 25% and *trans*-**15f**, 30%), respectively. Substitution of *cis*-**15b** and *cis*-**15f** with various kinds of amines gave *cis*-alcohols with a modified amino group (*cis*-**25**—*cis*-**36**) (method D). The *N*-phenylpiperazinylethyl derivative (*cis*-**16b**) was synthesized by $NaBH_4$ reduction of **5b**, followed by selective reduction of the amide moiety with sodium monoacetoxyborohydride[10] in boiling THF. 7-Hydroxy derivatives (*cis*-**17g**, *cis*-**36g**, and *cis*-**39g**) were obtained by catalytic reduction of the corresponding 7-benzyloxy derivatives (*cis*-**17f**, *cis*-**36f**, and *cis*-**37f**) using palladium black as a catalyst in ethanol containing hydrochloric acid (method E). The stereochemistries of the compounds bearing substituents at the 3- and 4-positions were determined on the basis of various data including X-ray crystallographic analysis, proton nuclear magnetic resonance (^{1}H-NMR) spectral data and *Rf* values of each stereoisomer. The physicochemical properties of methyl *cis*- and *trans*-4-aminoalkyl-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**16**—**39**) thus obtained are summarized in Table I.

The *N*-phenylpiperazinylpropyl group was introduced into the 2-position by converting the ester group of methyl 3-(3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionates (**40b** and **40c**)[11] in three steps (Chart 3). Reduction of **40b** with $NaBH_4$ and subsequent chromatographic separation gave *cis*-**41b** (47%) and *trans*-**41b** (36%). On the other hand, similar reduction of **40c** gave *cis*-**41c** (86%) and a minor product (7%) which could not be

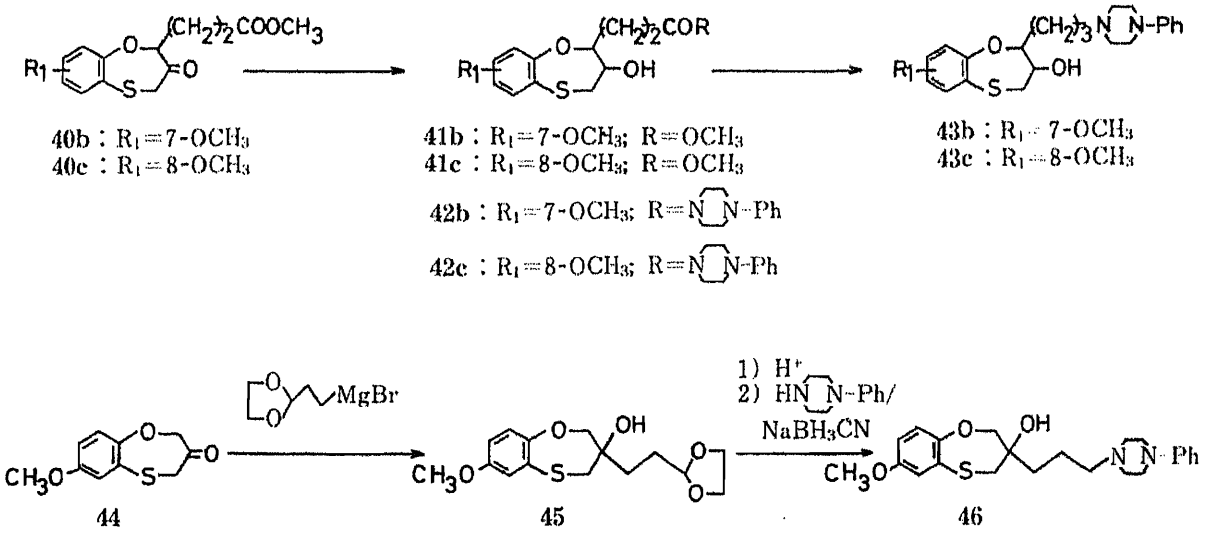


Chart 3

identified as *trans*-**41c**. Heating a mixture of the ester (*cis*-**41b**) and *N*-phenylpiperazine at 90 °C for 5 h afforded the amide (*cis*-**42b**), which was reduced with lithium aluminum hydride ($LiAlH_4$) in THF to yield *cis*-7-methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-**43b**). Similarly, *trans*-**43b** and *cis*-**43c** were prepared *via* the amides (*trans*-**42b** and *cis*-**42c**).

Grignard reaction of 7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (**44**)[11] with 2-(1,3-dioxolan-2-yl)ethylmagnesium bromide[11] in THF gave 3-(1,3-dioxolan-2-yl)ethyl-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (**45**) in 82% yield. Next, deprotection of the acetal group to aldehyde by treatment with dilute hydrochloric acid and subsequent reductive amination with *N*-phenylpiperazine using sodium cyanoborohydride afforded 7-methoxy-3-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (**46**) in 57% yield (Chart 3).

The compound (**50b**) lacking the 4-methoxycarbonyl group in *cis*-**17b**, which showed the most potent antagonistic activity against S_2-receptors, was prepared in several steps from **2b** as illustrated in Chart 4. Heating **2b** in DMF at 100 °C for 6 h in the presence of aqueous lithium chloride afforded 4-(3-chloropropyl)-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (**47b**) in 48% yield. Either $NaBH_4$ reduction of **47b** and subsequent replacement of the chloro group by *N*-phenylpiperazine or initial amination of **47b** and subsequent $NaBH_4$ reduction gave the same single product, the structure of which was confirmed as the *cis*-isomer (*cis*-**50b**) by X-ray crystallographic analysis. In order to prepare the *trans*-isomer, we examined another synthetic route and found that decarboxylation of *cis*-4-(3-chloropropyl)-3-hydroxy-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylic acid (*cis*-**51b**) obtained by alkaline hydrolysis of *cis*-**15b** with heating at 180 °C for 30 min gave *trans*-**48b** with stereochemical retention of the 3-hydroxy and 4-(3-chloropropyl) groups in 16% yield. Subsequent substitution reaction of *trans*-**48b** with *N*-phenylpiperazine gave *trans*-**50b**.

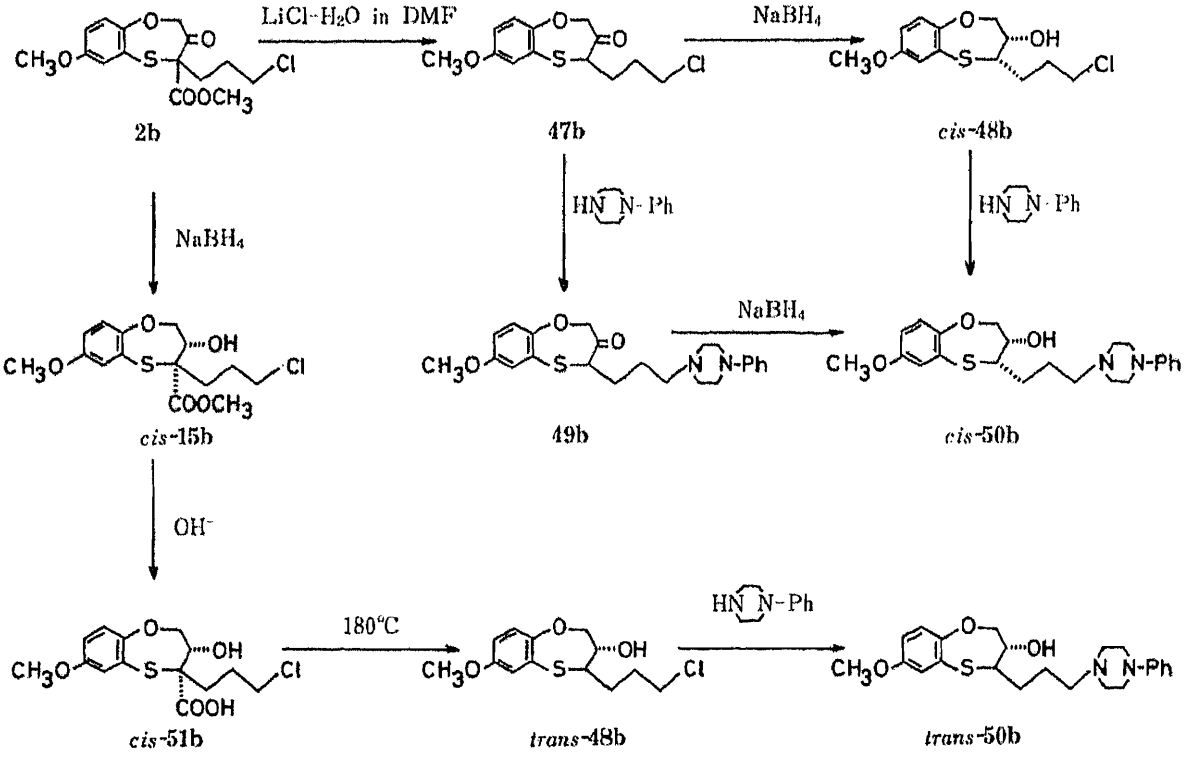


Chart 4

Removal of the 3-hydroxy group from *cis*-**17b** was unsuccessful. Tosylation of *cis*-**17b** with tosyl chloride in pyridine and subsequent catalytic reduction of the crude product using 10% palladium charcoal in ethyl acetate yielded two isomeric products (**52** and **53**) which were also obtained by reduction of *cis*-**15b** with red phosphorus and hydriodic acid in aqueous acetic acid, followed by treatment with *N*-phenylpiperazine. The infrared (IR) spectra of **52** and **53** showed similar absorptions to each other. The 400 MHz NMR spectrum of **52** exhibited two double doublets at δ 4.115 (J=12.0, 5.4 Hz) and 4.263 (J=12.0, 2.1 Hz) due to methylenic protons adjacent to the oxygen atom and a sextet (ddd) at δ 3.612 (J=2.1, 5.4, 9.5 Hz) assignable to the methine proton attached to the carbon bearing the sulfur atom. On irradiation at δ 3.612, the two double doublets were transformed into two doublets with geminal coupling (J=12.0 Hz). The ^{1}H-NMR spectrum of **53** showed similar signals with ABX coupling at δ 4.184 (1H, dd, J=12.2, 2.0 Hz), 4.439 (1H, dd, J=12.2, 4.2 Hz), and 3.599 (1H, ddd, J=2.1, 4.2, 9.8 Hz). These results indicate the presence of the $OCH_2\underset{|}{C}HS$ group and the absence of the OCH_2CH_2 group as partial structures of **52** and **53**. Thus, the structures of **52** and **53** were assigned as isomeric methyl 5-(4-phenyl-1-piperazinyl)-2-(6-methoxy-1,4-benzoxathian-3-yl)pentanoates, which might be produced by rearrangement involving the episulfonium ion intermediates[12] generated by elimination of the hydroxy group at the 3-position. Compound **58**, which lacks the 3-hydroxy group in addition to the 4-ester of *cis*-**17b**, was synthesized as illustrated in Chart 5. Grignard reaction of 3-chloropropanal with 2-(1,3-dioxolan-2-yl)ethylmagnesium bromide in THF gave 1-chloro-5-(1,3-dioxolan-2-yl)pentan-3-ol (**54**) in 73% yield. Then **54** was converted into 5-(1,3-dioxolan-2-yl)-3-mesyloxypentyl benzoate (**55**) by the reaction of **54** with sodium benzoate in DMF, followed by mesylation.

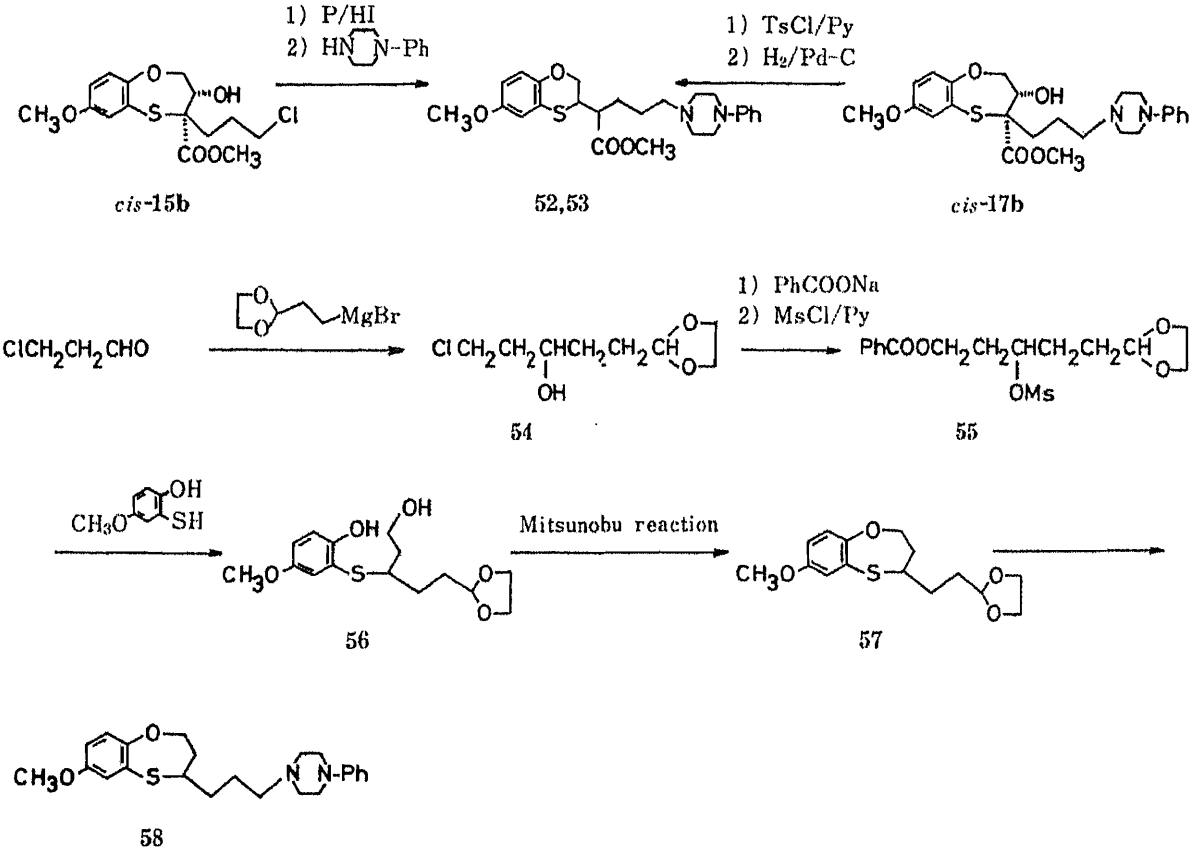
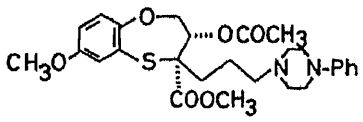


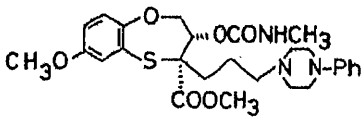
Chart 5

Treatment of **55** with 4-methoxy-2-mercaptophenol and subsequent alkaline hydrolysis gave the precursor (**56**) in 43% yield from **55**. Ring closure of **56** was conducted by means of the Mitsunobu reaction[13] using triphenylphosphine and ethyl azodiformate in toluene to afford 7-methoxy-4-[2-(1,3-dioxolan-2-yl)ethyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin (**57**, 71%), which was converted into the desired 7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin (**58**, 51%) by reductive amination after deprotection of the acetal group of **57**.

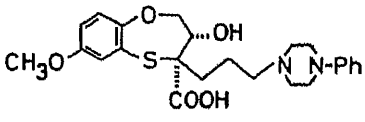
Modifications of the 3-hydroxy and the 4-ester groups of *cis*-**17b** were done by *O*-acetylation, *O*-carbamoylation using methyl isocyanate, alkaline hydrolysis, esterification of the resulting 4-carboxylic acid with diethyl sulfate, and lithium aluminum hydride reduction, to give the *O*-acetate (*cis*-**59b**), the 3-*N*-methylcarbamoyloxy derivative (*cis*-**60b**), 4-carboxylic acid (*cis*-**61b**), the 4-ethyl ester (*cis*-**62b**) and the 4-methanol (*cis*-**63b**), respectively (Chart 6).



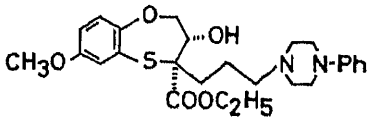
cis-**59b**



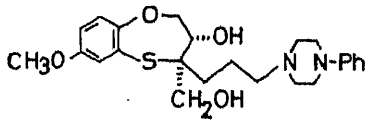
cis-**60b**



cis-**61b**



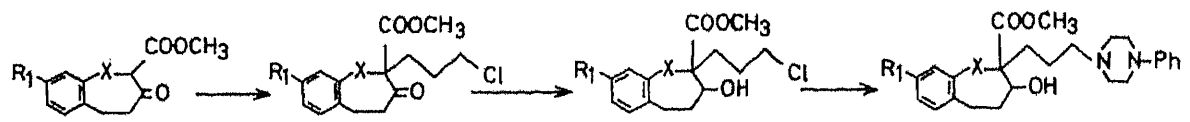
cis-**62b**



cis-**63b**

Chart 6

We also tried to prepare the 1-benzoxepin and 1-benzothiepin analogs of *cis*-**17b** in order to clarify the pharmacological significance of the hetero atoms in the 1,5-benzoxathiepin ring (Chart 7). Methyl 8-methoxy-3-oxo-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (**64**) was obtained by Dieckmann reaction of methyl 3-(4-methoxy-2-methoxycarbonylmethyloxy)phenylpropionate according to the method described by Huckle *et al.*[14] Methyl 3-oxo-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylates (**65** and **66**) were prepared by similar Dieckmann reaction according to the procedure of Huckle *et al.*[14] *via* the Newman's reaction[15] of methyl 2-hydroxyphenylpropionates. The syntheses of *N*-phenylpiperazinylpropyl-substituted 1-benzoxepin and 1-benzothiepin derivatives (**73**, **74**, and **75**) from the ketoesters (**64**, **65**, and **66**) were done by methods similar to those described for 1,5-benzoxathiepin derivatives, involving alkylation with 3-bromo-1-chloropropane, subsequent $NaBH_4$ reduction and finally substitution with *N*-phenylpiperazine (Chart 7) (Table II).

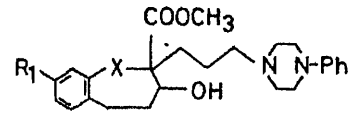


64	**67**	**70**	**73**
65	**68**	**71**	**74**
66	**69**	**72**	**75**

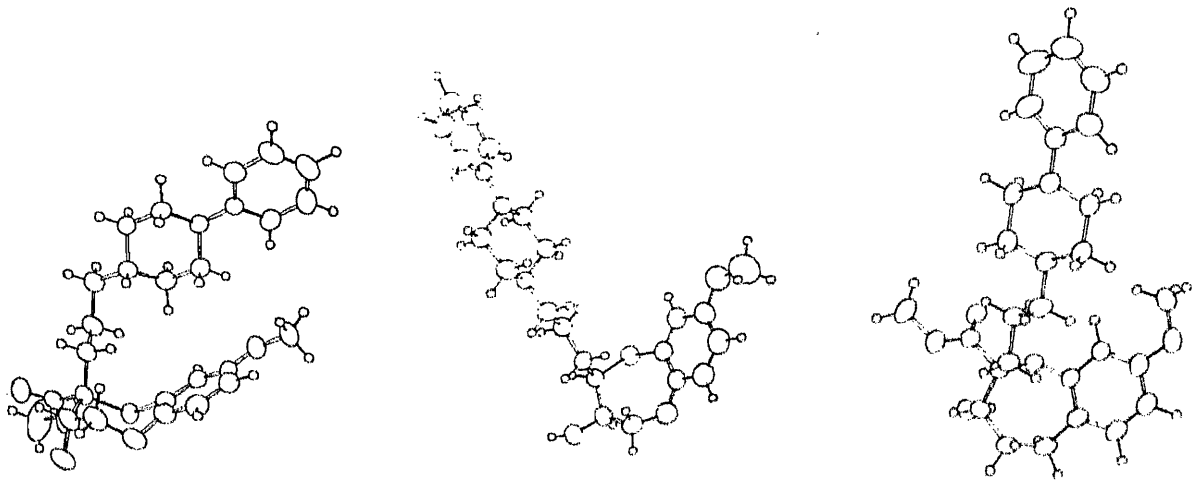
64, **67**, **70**, **73**: $R_1 = OCH_3$; X = O
65, **68**, **71**, **74**: $R_1 = H$; X = S
66, **69**, **72**, **75**: $R_1 = OCH_3$; X = S

Chart 7

TABLE II. Physicochemical Properties of *N*-Phenylpiperazinylpropyl-Substituted 1-Benzoxepin and 1-Benzothiepin Derivatives (73—75)



Compd. No.	R_1	X	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
cis-73	OCH_3	O	47	126—128	$C_{26}H_{34}N_2O_5$	68.70	7.54	6.16
						(68.42	7.62	6.02)
trans-73	OCH_3	O	72	140—155	$C_{26}H_{34}N_2O_5 \cdot 2HCl$	59.20	6.88	5.31
						(59.12	6.96	5.23)
cis-74	H	S	60	152—154	$C_{25}H_{32}N_2O_3S$	68.15	7.32	6.36
						(68.40	7.34	6.36)
cis-75	OCH_3	S	80	145—150	$C_{26}H_{34}N_2O_4S \cdot HCl \cdot 1/2\,H_2O$	60.51	7.03	5.43
						(60.39	7.36	5.49)



cis-**17b**·HCl (CV-5197)[a] *cis*-**50b** *cis*-**73**

Fig. 1. X-Ray Structures of *cis*-**17b**·HCl, *cis*-**50b**, and *cis*-**73**
a) Shown without Cl⁻.

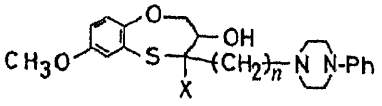
In the case of 1-benzothiepin derivatives, the hydride reduction of the 3-carbonyl moiety gave exclusively *cis*-alcohols (*cis*-**71** and *cis*-**72**) without detectable stereoisomers on thin-layer chromatography (TLC). The configurations of these compounds were determined by X-ray chrystallographic analysis of *cis*-**71** and comparison of the 400 MHz NMR spectral data of the products (**71** and **72**).

Configurations and Conformations of 1,5-Benzoxathiepin Derivatives and Related Compounds

The conformations of seven-membered compounds have been investigated theoretically and experimentally from the viewpoint of interconversion and pseudorotation.[16,17]

We first determined the configurations of *cis*-**17b**, *cis*-**50b**, *cis*-**58b**, *cis*-**71**, and *cis*-**73** by X-ray crystallographic analysis and then examined the conformations in solution based on 400 MHz NMR spectra data. The X-ray results have shown that the chair form of the seven-membered ring is the common conformation for 1,5-benzoxathiepin (*cis*-**17b**, *cis*-**50b**, and *cis*-**58b**), 1-benzoxepin (*cis*-**73**), and 1-benzothiepin (*cis*-**71**) derivatives (Fig. 1). The most striking

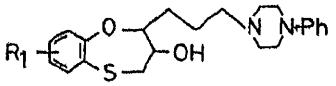
TABLE III. Selected 400 MHz NMR Spectral Data for 4-(4-Phenyl-1-piperazinyl)alkyl-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ols



Compd. No.	X	*n*	Salt	NMR (DMSO-d_6)[a]					
				Chemical shift δ (ppm)			Coupling constant (Hz)		
				C_2-H_a	C_2-H_b	C_3-H	$J_{2a,3}$	$J_{2b,3}$	$J_{3,4}$
cis-**16b**	$COOCH_3$	2	2HCl	4.022	4.168	4.080	2.7	6.1	—
cis-**17b**	$COOCH_3$	3	HCl	3.873	4.155	3.999	0—1.0	4.6	—
cis-**17b**	$COOCH_3$	3	Free base	3.843	4.249	4.078	0—1.0	3.4	—
trans-**17b**	$COOCH_3$	3	HCl	3.794	4.107	4.383	8.4	3.8	—
cis-**18b**	$COOCH_3$	4	2HCl	3.920	4.151	4.017	2.3	5.6	—
trans-**18b**	$COOCH_3$	4	2HCl	3.783	4.063	4.314	7.8	3.7	—
cis-**19b**	$COOCH_3$	5	HCl	3.913	4.149	4.011	2.3	5.5	—
cis-**50b**	H	3	2HCl	3.776	4.017	4.152	8.5	3.8	3.8[b]
trans-**50b**	H	3	2HCl	3.855	4.329	3.785	4.9	2.8	7.7[c]

a) *cis*-**17b** (free base) was determined in $CDCl_3$ solution. *b*) δ 3.206 ppm (C_4-H). *c*) δ 3.031 ppm (C_4-H).

TABLE IV. Selected 400 MHz NMR Spectral Data for 2-[3-(4-Phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ols

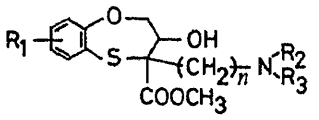


Compd. No.	R_1	NMR ($CDCl_3$)						
		Chemical shift δ (ppm)				Coupling constant (Hz)		
		C_2-H	C_3-H	C_4-H_a	C_4-H_b	$J_{2,3}$	$J_{3,4a}$	$J_{3,4b}$
cis-**43b**	7-OCH_3	3.609	3.975	2.953	3.039	0—1.0	2.1	5.4
trans-**43b**	7-OCH_3	3.96	3.96	2.735	3.534	—[a]	4.8	2.3
cis-**43c**	8-OCH_3	3.668	3.970	2.886	2.966	0—1.0	2.1	5.4

a) Not determined.

characteristic of *cis*-**17b** is the folded conformation of the whole molecule and the quasi-axial orientation of the sterically bulky *N*-phenylpiperazinylpropyl substituent. The 400 MHz NMR spectral data of the 4-[3-(4-phenyl-1-piperazinyl)propyl]-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin derivatives also support the occurrence of solution conformations that are similar to the solid state conformations, except in the case of *trans*-**50b** (Table III). The rather large coupling constant in *trans*-**17b** ($J_{2a,3}$ = 8.4 Hz) suggests the presence of a quasi-equatorial 3-hydroxy group in *trans*-**17b**, while the small values in *cis*-**17b** ($J_{2a,3}$ = 0—1.0 Hz and $J_{2b,3}$ = 4.6 Hz) indicate a major contribution of a conformation similar to the X-ray conformation in solution. The excellent agreement of the correlative *J* values of *cis*-**50b** and *trans*-**17b** indicates that these compounds possess similar conformations except for the structural difference of the absence or presence of the 4-ester moiety. However, the discrepancy between the *J* values in the NMR spectral data of *trans*-**50b** and *cis*-**17b** suggests that the major conformer of *trans*-**50b** differs from the X-ray conformer of *cis*-**17b**. The $J_{3,4}$

TABLE V. Biological Activities of Methyl 4-Aminoalkyl-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates



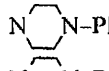
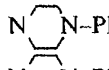
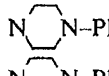
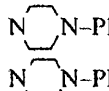
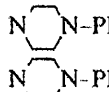
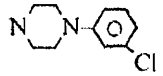
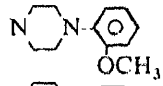
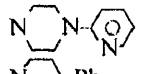
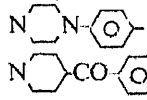
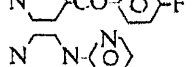
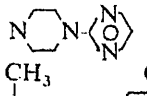
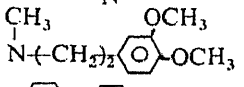
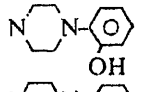
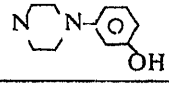
Compd. No.	R_1	$N<^{R_2}_{R_3}$	n	Serotonin S_2-blocking activity[a]			Adrenaline α_1-blocking activity[b]	
				10^{-5}	10^{-6}	10^{-7} M	10^{-5}	10^{-6} M
cis-**16b**	7-OCH_3		2	68			80	
cis-**17a**	H		3	100	77	3	100	30
trans-**17a**	H		3	100	18		100	75
cis-**17b**	7-OCH_3		3		100	77	5	
trans-**17b**	7-OCH_3		3	100	89	15	66	
cis-**17c**	8-OCH_3		3	92	71	3	31	
trans-**17c**	8-OCH_3		3	96	28		53	
cis-**17d**	7-Cl		3	100	85	31	45	
trans-**17d**	7-Cl		3	100	67	12	70	
cis-**17e**	7-CH_3		3	100	77	40	35	
trans-**17e**	7-CH_3		3	100	27	0	58	
cis-**17g**	7-OH		3	79	16		0	
cis-**18b**	7-OCH_3		4	66			95	
trans-**18b**	7-OCH_3		4	66	35		90	
cis-**19b**	7-OCH_3		5	56			90	
cis-**20b**	7-OCH_3		3	94	35		47	
cis-**21b**	7-OCH_3		3	88	12		100	28
cis-**24b**	7-OCH_3		3	100	77	55	100	18
cis-**25b**	7-OCH_3		3	100	88	25	40	
cis-**26b**	7-OCH_3		3	100	99	12	30	
cis-**27b**	7-OCH_3		3	100	88	50	100	
cis-**28b**	7-OCH_3		3	68			0	
cis-**32b**	7-OCH_3		3	83	27		15	
cis-**33b**	7-OCH_3		3	100	12		55	
cis-**34b**	7-OCH_3		3	100	36	16	10	

TABLE V. (continued)

Compd. No.	R_1	$N<^{R_2}_{R_3}$	n	Serotonin S_2-blocking activity[a]			Adrenaline α_1-blocking activity[b]	
				10^{-5}	10^{-6}	10^{-7} M	10^{-5}	10^{-6} M
cis-**35b**	7-OCH_3	N⟨⟩N-⟨O⟩-OH	3	100	100	79	56	
cis-**38g**	7-OH	N⟨⟩N-⟨O⟩-OH	3	100	0		8	

a) % inhibition of 5-HT-induced contraction in pig coronary artery. *b*) % inhibition of norepinephrine-induced contraction in rabbit aorta.

TABLE VI. Biological Activities of 3-(4-Phenyl-1-piperazinyl)propyl-Substituted 3,4-Dihydro-2*H*-1,5-benzoxathiepin Derivatives and Related Analogs

Compd. No.	Serotonin S_2-blocking activity[a]			Adrenaline α_1-blocking activity[b]
	10^{-5}	10^{-6}	10^{-7} M	10^{-5} M
7b	100	80	2	63
cis-**43b**	56	13		50
trans-**43b**	42	0		77
cis-**43c**	100	63		53
46	25	0		99
cis-**50b**	99	35		50
trans-**50b**	72			55
58	58			60
cis-**59b**	100	92	19	40
cis-**60b**	100	47		72
cis-**62b**	100	83	19	0
cis-**63b**	90	17	0	50
cis-**73**	100			44
trans-**73**	100	24		62
cis-**74**	96	60		36
cis-**75**	100	69		31

a) % inhibition of 5-HT-induced contraction in pig coronary artery. *b*) % inhibition of norepinephrine-induced contraction in rabbit aorta.

value (7.7 Hz) of *trans*-**50b** suggests a substantial contribution of quasi-equatorial orientation of the *N*-phenylpiperazinylpropyl group at the 4-position. In the case of the derivatives with a side chain of various lengths (**16**, **18** and **19**), it was deduced that the main conformation of each stereoisomer was similar to that found for the corresponding isomer of **17b** on the basis of the NMR spectral data, supposing the half-chair conformation for the seven-membered ring. On the other hand, the equatorial orientation of the *N*-phenylpiperazinylpropyl moiety at the 2-position was estimated from the NMR spectral data of *cis*-**43b**, **c** and *trans*-**43b** (Table IV). X-ray crystallographic analyses and the NMR spectral data of 1-benzoxepin (**73**) and 1-benzothiepin (**74** and **75**) derivatives indicated that these compounds also had major conformers similar to those observed in **17b**. The main contribution of *trans*-1,2-diaxial orientation of the sterically bulky substituents for *cis*-**17b** in contrast with *trans*-**50b** is readily rationalizable in terms of the stabilizing effect associated with the favorable hydrogen bonding between the 3-hydroxy function and the 4-ester carbonyl in *cis*-**17b** compared with

trans-**50b**, lacking the ester group.

Biological Results and Discussion

The S_2-receptor-blocking activity and selectivity toward S_2-receptors over adrenergic α_1-receptors of the aminoalkyl-substituted 1,5-benzoxathiepin derivatives and related compounds synthesized in this paper were evaluated in terms of ability to antagonize serotonin-induced contraction in the isolated pig coronary artery and to block norepinephrine-induced contraction in the isolated rabbit aorta. The results of *in vitro* evaluation are shown in Tables V and VI.

Methyl 4-aminoalkyl-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates showed significant S_2-receptor-blocking activities. The inhibitory potency for serotonin-induced contraction increased in the order of 3>2, 4>5 of side chain length (*n*). The substituents on the benzene ring of the 1,5-benzoxathiepin skeleton also influenced the biological activities. Introduction of the methoxy group into the 7-position (**17b**) resulted in marked enhancement of the activity, whereas shift of the methoxy group from the 7- to the 8-position diminished the effect. Substituents of the amino group in the side chain also caused changes (**17b**,**20b**—**35b**) in the structure–activity relationships. The nitrogen atom attached to the 4-propyl group might play an important role in the interaction with S_2-receptors since the 4-phenylpiperidyl derivative (*cis*-**25b**) showed activity comparable to that of the *N*-phenylpiperazinyl derivative (*cis*-**17b**). The presence of the aromatic ring within a distance of two or three methylenic chains from the nitrogen atom described above seemed to be preferable for S_2-receptor-blocking activity. Among the stereochemical isomers, the *cis*-isomers, rather than the *trans*-isomers, showed more potent and more selective antagonism toward S_2-receptors over adrenergic α_1-receptors. When the 3-(4-phenyl-1-piperazinyl)propyl moiety was introduced into the 2-position, the presence of the methoxy group at the 8-position (*cis*-**43c**) instead of the 7-position (**43b**) was critical for the manifestation of biological activities. On the other hand, substitution of the 3-(4-phenyl-1-piperazinyl)propyl group at the 3-position (**46**) resulted in a marked reduction of S_2-receptor-blocking activity and considerably increased inhibitory potency for adrenergic α_1-receptors. In the series of methyl aminoalkyl-substituted 3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates, methyl *cis*-3-hydroxy-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate hydrochloride (*cis*-**17b**, CV-5197) showed the most potent S_2-receptor-blocking activity and the highest selectivity over the adrenergic α_1-blocking activity. Some modifications of the 3-hydoxy and 4-ester groups of *cis*-**17b** gave the following results. The activities were unchanged upon modifications of the 3-hydroxy group, *e.g.*, *O*-acetate (*cis*-**59b**) and *N*-methylcarbamoyloxy (*cis*-**60b**) derivatives and the 3-carbonyl compound (**7b**). Removal of the 4-ester moiety gave quite different results in different stereochemical isomers. The *cis*-isomer (*cis*-**50b**) showed activities comparable to those of *trans*-**17b**. However, the S_2-receptor-blocking activity of *trans*-**50b** was 100 times less potent than that of *cis*-**17b** in spite of the configurational retention of the 3-hydroxy and 4-aminoalkyl moieties in both molecules. Removal of the 3-hydroxy and 4-ester groups (**58**) resulted in further reduction of the activity. Ring-contraction of 1,5-benzoxathiepin skeleton (**52** and **53**) resulted in loss of S_2-receptor antagonistic activity. In the series of skeletal modifications, methyl *cis*-3-hydroxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (*cis*-**74**) showed almost equipotent activity to the corresponding 1,5-benzoxathiepin derivative (*cis*-**17a**). However, introduction of the methoxy group (*cis*-**75**) did not produce the potentiation of the activity that was observed in the case of the 1,5-benzoxathiepins (**17a**→**17b**), and the inhibitory potency of the 1-benzoxepin derivative (*cis*-**73**) was 10 times less than that of *cis*-**17b** in spite of the conformational similarity between the two compounds (Fig. 1). These results suggest that the oxygen atom in the 1,5-benzoxathiepin ring of *cis*-**17b**

might also play a significant role in the interaction with S_2-receptors.

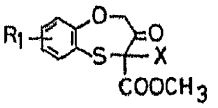
X-Ray crystallographic analyses of known S_2-receptor antagonists have shown that ketanserin,[18] metergoline,[19] pipanperon,[20] and haloperidol[21] have a common extended conformation, but spiperone, with the neuroleptics displaying dopamine-blocking activity and potent S_2-receptor-blocking activity as well, had the folded conformation.[22] A recent investigation by Azibi *et al.* showed the conformational flexibility of spiperone and confirmed the existence of extended and folded conformers in two polymorphs of spiperone by X-ray analysis.[23] The structure–activity relationships in our study indicate that the favorable structure interacting with S_2-receptors approximates the folded conformation proposed as the preferred form of spiperone.[24] A consideration of the folded conformation shown by the X-ray analysis of *cis*-**17b** (CV-5197) suggests that two aromatic rings, a 7-methoxy group, a 3-quasi-axial hydroxy group along with the oxygen atom in the skeleton and the essential nitrogen atom attached to the 4-propyl side chain are significant for the biological activity.

Further evaluation of CV-5197 revealed higher selectivity for the S_2 binding sites than the S_1 ones in the radioligand binding assay in rat brain synaptosomes and practically no inhibitory action on any other agonist, including histamine and acetylcholine.[25] The detailed pharmacological profiles of CV-5197 and its actions on circulatory disorders in experimental animal models will be described elsewhere.[26]

Experimental

All melting points were determined on a micro melting point apparatus (Yanagimoto) and are uncorrected. IR spectra were obtained with Hitachi 215 and 260-10 spectrophotometers. ^{1}H-NMR spectra were measured with Varian T-60, EM-390, and JEOL JNM-GX400 NMR spectrometers and the 60 MHz spectral data are given, unless otherwise mentioned. Mass spectra (MS) were taken on JEOL JMS-01SC and Hitachi M-80A (high-resolution MS)

Table VII. Physicochemical Properties of Methyl 4-Substituted 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (**2**–**5**)



Compd. No.	R_1	X	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
2b	7-OCH_3	$(CH_2)_3Cl$	44	64–65	$C_{15}H_{17}ClO_5S$	52.33 (52.25	5.10 4.97)	
2c	8-OCH_3	$(CH_2)_3Cl$	42	Oil	$C_{15}H_{17}ClO_5S$	52.33 (52.14	5.10 5.23)	
2d	7-Cl	$(CH_2)_3Cl$	32	Oil	$C_{14}H_{14}Cl_2O_4S$	48.15 (48.36	4.04 4.27)	
2e	7-CH_3	$(CH_2)_3Cl$	37	Oil	$C_{15}H_{17}ClO_4S$	54.79 (54.52	5.21 5.48)	
2f	7-OCH_2Ph	$(CH_2)_3Cl$	36	88–89	$C_{21}H_{21}ClO_5S$	59.93 (59.89	5.03 5.02)	
3b	7-OCH_3	$(CH_2)_4Br$	32	Oil	$C_{16}H_{19}BrO_5S$	47.65 (47.39	4.75 4.86)	
4b	7-OCH_3	$(CH_2)_5Br$	31	Oil	$C_{17}H_{21}BrO_5S$	48.92 (48.81	5.07 5.26)	
5b	7-OCH_3	CH_2CON⟨⟩NPh	69	146–148	$C_{24}H_{26}N_2O_6S$	61.26 (61.40	5.57 5.60	5.94 5.90)

mass spectrometers. In the NMR spectra, chemical shifts are given in the δ (ppm) scale with tetramethylsilane as an internal standard.

Reactions were run at room temperature unless otherwise noted, and followed by TLC on Merck F-254 silicagel plates. Standard work-up procedures were as follows. The reaction mixture was partitioned between the indicated solvent and water. The organic extract was washed with water (H_2O). The extract was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Chromatographic separation was done on Merck Silica gel 60 with the indicated eluant.

Methyl 4-(ω-Halogenoalkyl)-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (2—5, Table VII)——A typical example of the experimental procedure used to obtain **2**—**5** is as follows. A mixture of methyl 7-methoxy-3-oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (**1b**) (2.0 g, 7 mmol), 1-bromo-3-chloropropane (2.3 g, 15 mmol), K_2CO_3 (1.5 g, 11 mmol), KI (1.2 g, 7 mmol) and CH_3CN (30 ml) was refluxed with stirring under an N_2 stream for 3 h. After filtration of the reaction mixture, the filtrate was concentrated *in vacuo*. The residue was diluted with H_2O and worked up (AcOEt; H_2O). The residue was subjected to column chromatography on silica gel (hexane : CH_2Cl_2 : AcOEt = 30 : 15 : 1). Compound **2b** (1.12 g, 44%) was obtained from the first fraction as colorless prisms (recrystallized from EtOH). IR ν_{max}^{KBr} cm^{-1}: 1760, 1725, 1600, 1485, 1260, 1240, 1210, 1175, 1040. ^{1}H-NMR ($CDCl_3$) δ: 1.8—2.2 (4H, m), 3.57 (2H, t, J = 6 Hz, CH_2Cl), 3.73 (3H, s), 3.75 (3H, s), 4.47 (1H, d, J = 18 Hz, C_2-H), 4.73 (1H, d, J = 18 Hz, C_2-H), 6.55 (1H, dd, J = 8, 2 Hz, C_8-H), 6.57 (1H, d, J = 2 Hz, C_6-H), 6.88 (1H, d, J = 8 Hz, C_9-H).

The second fraction yielded methyl 3-(3-chloropropyloxy)-7-methoxy-2*H*-1,5-benzoxathiepin-4-carboxylate (0.38 g, 15%) as a colorless oil. MS *m/z*: 344, 346 (M^+). IR ν_{max}^{neat} cm^{-1}: 1720 (ester). ^{1}H-NMR ($CDCl_3$) δ: 2.18 (2H, m), 3.70 (2H, t, J = 6 Hz, CH_2Cl), 3.72 (3H, s), 3.80 (3H, s), 4.10 (2H, t, J = 6 Hz, $O\underline{CH_2}CH_2$), 5.10 (2H, s, C_2-H). Compounds **2**—**5** were prepared by similar alkylation of **1a**—**f** with the corresponding ω-halogenoalkylbromides, and their physical data are listed in Table VII.

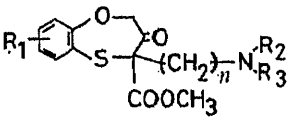
Methyl *cis*- and *trans*-3-Hydroxy-7-methoxy-4-[2-oxo-2-(4-phenyl-1-piperazinyl)ethyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (*cis*- and *trans*-6b)——$NaBH_4$ (0.38 g, 10 mmol) was added in small portions to a solution of **5b** (3.1 g, 6.7 mmol) in MeOH (50 ml). The mixture was stirred for 1 h and then poured into ice-H_2O. The resulting precipitates were collected by filtration and recrystallized from AcOEt to give *cis*-**6b** (1.8 g, 57%) as colorless prisms, mp 213—215 °C. *Anal.* Calcd for $C_{24}H_{28}N_2O_6S$: C, 61.00; H, 5.97; N, 5.93. Found: C, 60.87; H, 5.84; N, 5.86. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1740 (ester), 1650 (amide). ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 3.907 (1H, dd, J = 1.2, 13.2 Hz, C_2-H), 4.128 (1H, dd, J = 1.2, 4.4 Hz, C_3-H), 4.341 (1H, dd, J = 4.4, 13.2 Hz, C_2-H). Chromatographic purification of the mother liquor gave *trans*-**6b** (0.4 g, 13%) as a colorless oil, which was converted into the hydrochloride, colorless crystals, mp 170—180 °C (dec.). *Anal.* Calcd for $C_{24}H_{28}N_2O_6S \cdot HCl \cdot 1/2H_2O$: C, 55.64; H, 5.83; N, 5.40. Found: C, 55.38; H, 5.73; N, 5.44. IR ν_{max}^{KBr} cm^{-1}: 3550, 1740, 1650. ^{1}H-NMR (400 MHz) (DMSO-d_6) δ: 4.082 (1H, dd, J = 5.7, 12.6 Hz, C_2-H), 4.185 (1H, ddd, J = 2.2, 5.7, 7.3 Hz, C_3-H), 4.300 (1H, dd, J = 2.2, 12.6 Hz, C_2-H), 5.546 (1H, d, J = 7.3 Hz, OH).

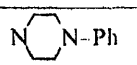
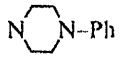
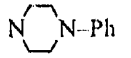
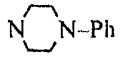
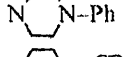
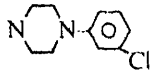
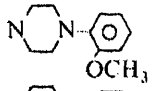
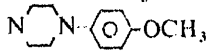
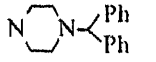
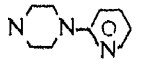
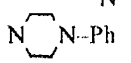
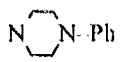
Methyl 4-Aminoalkyl-Substituted 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (7—14, Table VIII)——A typical example of the experimental procedure used to obtain **7**—**14** is as follows. Method A) A mixture of **2b** (56 g, 0.16 mol), *N*-phenylpiperazine (40 g, 0.26 mol), K_2CO_3 (34 g, 0.25 mol), KI (5.5 g, 33 mmol) and DMF (250 ml) was stirred at 70 °C for 7 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was worked up and the residue was recrystallized from MeOH to give **7b** (57 g, 75%) as colorless crystals. IR ν_{max}^{KBr} cm^{-1}: 1755 (ester), 1720 (CO), 1600, 1485, 1265, 1240, 1225, 1205, 1045. ^{1}H-NMR ($CDCl_3$) δ: 1.2—3.3 (14H, m), 3.68 (3H, s), 3.72 (3H, s), 4.38 (1H, d, J = 18 Hz, C_2-H), 4.72 (1H, d, J = 18 Hz, C_2-H), 6.4—7.4 (8H, m). Method B) A mixture of **1b** (5 g, 19 mmol), 3-(4-phenyl-1-piperazinyl)propyl chloride (6.7 g, 28 mmol), K_2CO_3 (4.65 g, 34 mmol), KI (3 g, 18 mmol) and CH_3CN (150 ml) was refluxed with stirring for 4 h. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was diluted with H_2O and extracted with AcOEt. The organic layer was worked up and the residue was purified by column chromatography on silica gel (hexane : AcOEt = 2 : 1) to give **7b** (2.7 g, 31%) as colorless crystals. Compounds **7**—**14** were similarly prepared by the substitution of **2**—**4** with the corresponding amines (method A) or by the alkylation of **1** with 3-(4-phenyl-1-piperazinyl)propyl chloride (method B), and their physical data are listed in Table VIII.

Methyl *cis*- and *trans*-4-(3-Chloropropyl)-3-hydroxy-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (*cis*- and *trans*-15b)——$NaBH_4$ (0.3 g, 8 mmol) was added in small portions to an ice-cooled solution of **2b** (2.0 g, 6 mmol) in MeOH (15 ml) and THF (8 ml) with stirring. The mixture was stirred for 1 h, then poured into ice-H_2O and worked up (AcOEt; H_2O). The residue was subjeced to column chromatography on silica gel (hexane : AcOEt = 2 : 1) to give *trans*-**15b** (0.5 g, 25%) from the first fraction, as colorless needles, mp 108—110 °C (recrystallized from AcOEt–hexane). *Anal.* Calcd for $C_{15}H_{19}ClO_5S$: C, 51.95; H, 5.52. Found: C, 51.63; H, 5.51. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 (ester). ^{1}H-NMR ($CDCl_3$) δ: 1.95 (4H, m, $C\underline{H_2}C\underline{H_2}CH_2Cl$), 3.48 (2H, t, J = 6.5 Hz, $C\underline{H_2}Cl$), 3.58 (3H, s), 3.70 (3H, s), 4.0—4.2 (3H, m).

The second fraction yielded *cis*-**15b** (1.25 g, 63%) as colorless prisms, mp 80—82 °C (recrystallized from EtOH). *Anal.* Calcd for $C_{15}H_{19}ClO_5S$: C, 51.95; H, 5.52. Found: C, 51.61; H, 5.48. IR ν_{max}^{KBr} cm^{-1}: 3540 (OH), 1735 (ester), 1600, 1485, 1440, 1250, 1210. ^{1}H-NMR ($CDCl_3$) δ: 1.4—2.4 (4H, m), 3.40 (2H, t, J = 4 Hz, CH_2Cl), 3.75 (3H, s), 3.80

TABLE VIII. Physicochemical Properties of Methyl 4-Aminoalkyl-Substituted 3-Oxo-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (7—14)



Compd. No.	R_1	n	$N<^{R_2}_{R_3}$	Method	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
7a	H	3		B	18	176—178	$C_{24}H_{28}N_2O_4S\cdot HCl\cdot 1/2\,H_2O$	59.67 (59.49)	6.26 (6.33)	5.83 (5.79)
7b	7-OCH_3	3		A B	75 31	110—112	$C_{25}H_{30}N_2O_5S$	63.81 (63.50)	6.43 (6.37)	5.95 (5.71)
7c	8-OCH_3	3		B	32	140—145	$C_{25}H_{30}N_2O_5S\cdot 2HCl\cdot H_2O$	53.47 (53.55)	6.10 (5.87)	4.99 (5.00)
7d	7-Cl	3		A B	76 27	197—199	$C_{24}H_{27}ClN_2O_4S\cdot 2HCl\cdot 1/2\,H_2O$	51.76 (52.02)	5.43 (5.12)	5.03 (5.08)
7e	7-CH_3	3		A B	78 29	145—150	$C_{25}H_{30}N_2O_4S\cdot 2HCl\cdot 1/2\,H_2O$	55.96 (56.11)	6.20 (6.19)	5.22 (5.11)
8b	7-OCH_3	3		A	73	153—156	$C_{25}H_{29}N_2O_5S\cdot 2HCl$	51.95 (51.85)	5.41 (5.42)	4.85 (4.74)
9b	7-OCH_3	3		A	75	185—190	$C_{26}H_{32}N_2O_6S\cdot 2HCl$	54.45 (54.46)	5.98 (5.94)	4.89 (4.83)
10b	7-OCH_3	3		A	76	133—135	$C_{26}H_{32}N_2O_5S\cdot 1/2\,H_2O$	61.27 (60.95)	6.26 (6.30)	5.50 (5.48)
11b	7-OCH_3	3		A	62	152—155	$C_{32}H_{36}N_2O_5S\cdot 2HCl\cdot 2^1/_2\,H_2O$	56.63 (56.64)	6.39 (6.16)	4.13 (4.15)
12b	7-OCH_3	3		A	58	158—162	$C_{24}H_{29}N_3O_5S\cdot 2HCl\cdot 1^1/_2\,H_2O$	50.44 (50.69)	5.99 (5.81)	7.35 (7.30)
13b	7-OCH_3	4		A B	49 22	155—165	$C_{26}H_{32}N_2O_5S\cdot 2HCl\cdot 1/2\,H_2O$	55.12 (55.30)	6.22 (6.19)	4.95 (4.96)
14b	7-OCH_3	5		A B	44 18	130—150	$C_{27}H_{34}N_2O_5S\cdot 2HCl\cdot 1/2\,H_2O$	55.85 (56.00)	6.42 (6.41)	4.83 (4.81)

(3H, s), 3.7—4.4 (3H, m), 6.80 (1H, dd, $J=2$, 4 Hz, C_7-H), 6.9—7.1 (2H, m). Similar $NaBH_4$ reduction of **2f** gave *cis*- and *trans*-**15f**, respectively.

Methyl *cis*-7-Benzyloxy-4-(3-chloropropyl)-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-15f) Yield 50%. Colorless oil. *Anal.* Calcd for $C_{21}H_{23}ClO_5S$: C, 59.64; H, 5.48. Found: C, 59.77; H, 5.39.

Methyl *trans*-7-Benzyloxy-4-(3-chloropropyl)-3-hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*trans*-15f) Yield 30%. Colorless prisms, mp 97—99 °C (recrystallized from AcOEt–hexane). *Anal.* Calcd for $C_{21}H_{23}ClO_5S$: C, 59.64; H, 5.48. Found: C, 59.80; H, 5.51.

Methyl *cis*-3-Hydroxy-7-methoxy-4-[2-(4-phenyl-1-piperazinyl)ethyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-16b) —— AcOH (0.48 g, 8 mmol), was added to a suspension of $NaBH_4$ (0.30 g, 8 mmol) in THF (20 ml). The mixture was gently boiled for 0.5 h, and then *cis*-**6b** (0.5 g, 7 mmol) was added to the above mixture. The mixture was refluxed for 20 h. The reaction mixture was worked up (AcOEt; H_2O) and the residue was purified by column chromatography on silica gel (hexane: AcOEt = 1 : 1) to give *cis*-**16b** (0.20 g, 42%) as a colorless oil, which was converted into the hydrochloride, *cis*-**16b**·2HCl, colorless crystals (from MeOH). IR ν_{max}^{KBr} cm^{-1}: 3520, 1740 (ester). ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 4.022 (1H, dd, $J=2.7$, 12.7 Hz, C_2-H), 4.080 (1H, dd, $J=2.7$, 6.1 Hz, C_3-H), 4.168 (1H, dd, $J=6.1$, 12.7 Hz, C_2-H).

Methyl 4-Aminoalkyl-Substituted 3-Hydroxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylates (17—36, Table I) ——Method C) $NaBH_4$ (0.51 g, 13.5 mmol) was added in small portions to an ice-cooled solution of **7b** (12.6 g, 27 mmol) in MeOH (100 ml) and THF (30 ml) with stirring. The mixture was stirred for 3 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was worked up. The residue obtained was subjected to column chromatography on silica gel (hexane: AcOEt = 2 : 1—1 : 3). *trans*-**17b** was obtained from the

first fraction as a pale yellow oil, which was isolated as the hydrochloride, *trans*-**17b**·HCl (4.6 g), colorless prisms (recrystallized from 50% EtOH). IR ν_{max}^{KBr} cm^{-1}: 3530, 3500—3200, 2700—2300, 1720, 1600, 1485, 1255, 1235, 1205, 1035. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 3.667 (3H, s), 3.746 (3H, s), 3.794 (1H, dd, J=8.4, 12.7 Hz, C_2-H), 4.107 (1H, dd, J=3.8, 12.7 Hz, C_2-H), 4.383 (1H, dd, J=3.8, 8.4 Hz, C_3-H).

The second fraction yielded *cis*-**17b** as a colorless oil, which was converted into the hydrochloride, *cis*-**17b**·HCl (6.9 g), colorless prisms (recrystallized from 50% EtOH). IR ν_{max}^{KBr} cm^{-1}: 3600—3300, 1735, 1720, 1595, 1480, 1250. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 3.693 (3H, s), 3.758 (3H, s), 3.873 (1H, dd, J=0—1, 13.1 Hz, C_2-H), 3.999 (1H, dd, J=0—1, 4.6 Hz, C_3-H), 4.155 (1H, dd, J=4.6, 13.1 Hz, C_2-H). Method D) A mixture of *cis*-**15b** (95 g, 0.20 mol), *N*-phenylpiperazine (50 g, 0.33 mol), K_2CO_3 (42 g, 0.31 mol) and DMF (400 ml) was stirred at 70 °C for 8 h. The reaction mixture was worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (hexane : AcOEt = 2 : 3) to give *cis*-**17b**, which was isolated as the hydrochloride (83.7 g, 56%).

Compounds **17**—**36** were similarly prepared by method C or method D, and their physicochemical properties are listed in Table I.

Methyl *cis*-3,7-Dihydroxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-17 g, Table I)——Method E) Pd black (1.0 g) was added to a solution of *cis*-**17f** (2.45 g), conc. HCl (1.1 ml), and MeOH (200 ml). The mixture was hydrogenated under atmospheric pressure of H_2 for 20 h. After filtration of the catalyst, the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane : AcOEt : MeOH = 30 : 30 : 1), followed by recrystallization from AcOEt to give *cis*-**17g** (1.31 g, 64%) as colorless crystals. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1730 (ester). ^{1}H-NMR ($CDCl_3$) δ: 1.1—3.4 (14H, m), 3.72 (3H, s, $COOCH_3$), 4.0—4.1 (3H, m), 6.6—7.4 (8H, m). MS *m/z*: 458 (M^+).

Similarly, catalytic hydrogenation of *cis*-**36f** and *cis*-**37f** gave *cis*-**38g** and *cis*-**39g** (Table I), respectively.

Methyl *cis*-7-Benzyloxy-3-hydroxy-4-(3-piperazinylpropyl)-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-37f, Table I)——A mixture of *cis*-**15f** (2.0 g, 4.7 mmol), *N*-*tert*-butyloxycarbonylpiperazine (1.76 g, 9.5 mmol), K_2CO_3 (0.98 g, 7 mmol), KI (0.4 g, 2.4 mmol) and CH_3CN (20 ml) was refluxed for 6 h. The reaction mixture was worked up (AcOEt; H_2O) and the residue was purified by column chromatography on silica gel (hexane : AcOEt = 3 : 2) to give the *tert*-butyloxycarbonyl derivative of *cis*-**37f** (1.8 g, 69%) as a colorless oil. MS *m/z*: 572 (M^+), which was treated with HCl–AcOEt to give *cis*-**37f** (1.6 g) as colorless prisms (recrystallized from MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (ester). ^{1}H-NMR (DMSO-d_6) δ: 1.2—2.4 (6H, m), 3.40—3.60 (8H, m), 3.68 (3H, s, $COOCH_3$), 3.9—4.1 (3H, m), 5.16 (2H, s, CH_2Ph), 6.7—7.5 (8H, m).

Methyl *cis*- and *trans*-3-(3-Hydroxy-7-methoxy-3,4-dihydro)2*H*-1,5-benzoxathiepin-2-yl)propionate (*cis*- and *trans*-41b)——$NaBH_4$ (0.2 g, 5.3 mmol) was added in small portions to a solution of **40b**[1] (2.0 g, 6.7 mmol) in MeOH (20 ml) and THF (20 ml) with stirring. The mixture was stirred for 3 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was worked up. The residue was subjected to column chromatography on silica gel (hexane : CH_2Cl_2 : AcOEt = 3 : 3 : 1) to give *cis*-**41b** (0.94 g, 47%) from the first fraction, as colorless crystals, mp 88—89 °C (recrystallized from Et_2O-petroleum ether). *Anal.* Calcd for $C_{14}H_{18}O_5S$: C, 56.36; H, 6.08. Found: C, 56.50; H, 6.04. IR ν_{max}^{KBr} cm^{-1}: 3490 (OH), 1730 (ester). ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 2.950 (1H, dd, J=2.0, 14.3 Hz, C_4-H), 3.000 (1H, dd, J=5.0, 14.3 Hz, C_4-H), 3.574 (1H, ddd, J=0—1, 3.3, 10.4 Hz, C_2-H), 3.966 (1H, ddd, J=0—1, 2.0, 5.0 Hz, C_3-H).

From the second fraction, *trans*-**41b** (0.72 g, 36%) was obtained as colorless crystals, mp 65—66 °C (recrystallized from Et_2O-petroleum ether). *Anal.* Calcd for $C_{14}H_{18}O_5S$: C, 56.36; H, 6.08. Found: C, 56.48; H, 6.10. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1725 (ester). ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 2.739 (1H, dd, J=4.6, 14.6 Hz, C_4-H), 3.558 (1H, dd, J=2.6, 14.6 Hz, C_4-H), 3.910 (1H, ddd, J=2.4, 6.0, 8.0 Hz, C_2-H), 3.950 (1H, ddd, J=2.6, 4.6, 8.0 Hz, C_3-H).

Methyl *cis*-(3-Hydroxy-8-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-2-yl)propionate (*cis*-41c)——$NaBH_4$ reduction of **40c** as described for **40b** gave *cis*-**41c** (86% yield) as a colorless oil. *Anal.* Calcd for $C_{14}H_{18}O_5S$: C, 56.36; H, 6.08. Found: C, 56.55; H, 6.19. IR ν_{max}^{neat} cm^{-1}: 3480 (OH), 1730 (ester).

***cis*-7-Methoxy-2-[3-oxo-3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-42b)**——A mixture of *cis*-**41b** (300 mg, 1 mmol) and *N*-phenylpiperazine (1 ml) was stirred at 90 °C for 3 h. The reaction mixture was worked up (AcOEt; H_2O). The residue was recrystallized from AcOEt–Et_2O to yield *cis*-**42b** (320 mg, 74%) as colorless crystals, mp 110—111 °C. *Anal.* Calcd for $C_{23}H_{28}N_2O_4S$: C, 64.46; H, 6.59; N, 6.54. Found: C, 64.62; H, 6.51; N, 6.52. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1645 (amide).

Similar amidation of *trans*-**41b** and *cis*-**41c** gave *trans*-**42b** and *cis*-**42c**, respectively.

***trans*-7-Methoxy-2-[3-oxo-3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*trans*-42b)**——Yield 75%. Recrystallization from AcOEt gave colorless crystals, mp 139—140 °C. *Anal.* Calcd for $C_{23}H_{28}N_2O_4S$: C, 64.46; H, 6.59; N, 6.54. Found: C, 64.52; H, 6.31; N, 6.61.

***cis*-8-Methoxy-2-[3-oxo-3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-42c)**——Yield 71%. Colorless prisms, mp 126—127 °C (recrystallized from AcOEt). *Anal.* Calcd for $C_{23}H_{28}N_2O_4S$: C, 64.46; H, 6.31; N, 6.61. Found: C, 64.30; H, 6.60; N, 6.43.

***cis*-7-Methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-43b)**——A solution of *cis*-**42b** (350 mg, 0.8 mmol) in THF (10 ml) was added dropwise to a suspension of $LiAlH_4$ (100 mg, 2.6 mmol)

in dry Et_2O (20 ml) with stirring under an atmosphere of dry N_2. The mixture was refluxed for 3 h. The reaction mixture was diluted with 20% NaOH and filtered off. The filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane : AcOEt : MeOH = 10 : 10 : 1) to give ***cis*-43b** (270 mg, 80%) as colorless crystals, mp 107—109 °C (recrystallized from AcOEt). *Anal.* Calcd for $C_{23}H_{30}N_2O_3S$: C, 66.64; H, 7.29; N, 6.76. Found: C, 66.59; H, 7.22; N, 6.90. IR ν_{max}^{KBr} cm^{-1}: 3450—3300 (OH), 1585, 1475, 1230, 1030, 915. ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 2.953 (1H, dd, J = 2.1, 14.2 Hz, C_4-H), 3.039 (1H, dd, J = 5.4, 14.2 Hz, C_4-H), 3.609 (1H, ddd, J = 0—1, 4.0, 9.6 Hz, C_2-H), 3.975 (1H, ddd, J = 0—1, 2.1, 5.4 Hz, C_3-H).

Similar $LiAlH_4$ reduction of *trans*-**42b** and *cis*-**42c** gave *trans*-**43b** and *cis*-**43c**, respectively.

***trans*-7-Methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*trans*-43b)** Yield 77%. Recrystallization from AcOEt gave colorless crystals, mp 128—130 °C. *Anal.* Calcd for $C_{23}H_{30}N_2O_3S$: C, 66.64; H, 7.29; N, 6.76. Found: C, 66.50; H, 6.94; N, 6.56. IR ν_{max}^{KBr} cm^{-1}: 3450—3000 (OH), 1585, 1490, 1235, 1035. ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 2.735 (1H, dd, J = 4.8, 14.5 Hz, C_4-H), 3.534 (1H, dd, J = 2.3, 14.5 Hz, C_4-H), 3.96 (2H, m, C_2-H, C_3-H).

***cis*-8-Methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-43c)**——Yield 76%. Recrystallization from AcOEt gave colorless prisms, mp 149—150 °C. *Anal.* Calcd for $C_{23}H_{30}N_2O_3S$: C, 66.64; H, 7.29; N, 6.76. Found: C, 66.71; H, 7.26; N, 6.79. IR ν_{max}^{KBr} cm^{-1}: 3400—3200 (OH), 1595, 1475, 1290, 1240, 1160, 1005. ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 2.886 (1H, dd, J = 2.1, 14.2 Hz, C_4-H), 2.966 (1H, dd, J = 5.4, 14.2 Hz, C_4-H), 3.668 (1H, ddd, J = 0—1, 4.0, 9.4 Hz, C_2-H), 3.970 (1H, dd, J = 0—1, 2.1, 5.4 Hz, C_3-H).

3-[2-(1,3-Dioxolan-2-yl)ethyl]-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (45)——A solution of **44**[1] (2.0 g, 9.5 mmol) in THF (10 ml) was added dropwise to a solution of Grignard reagent prepared from Mg (350 mg, 14 mmol), 2-(1,3-dioxolan-2-yl)ethyl bromide (2.6 g, 14 mmol) and THF (30 ml) with stirring. The mixture was stirred for 1 h. The reaction mixture was diluted with 1 N NaOH (20 ml) and worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (CH_2Cl_2 : Et_2O = 5 : 1) to give **45** (2.44 g, 82%) as a colorless oil. *Anal.* Calcd for $C_{15}H_{20}O_5S$: 57.67; H, 6.45. Found: C, 57.41; H, 6.52. IR ν_{max}^{neat} cm^{-1}: 3230 (OH), 1595, 1490, 1475, 1195. ^{1}H-NMR (90 MHz) ($CDCl_3$) δ: 1.6—1.8 (4H, m), 2.83 (2H, s, C_4-H), 3.75 (3H, s, OCH_3), 3.6—4.2 (6H, m), 5.93 (1H, t, J = 3 Hz, $CH<^{O}_{O}$). MS *m/z*: 312 (M^+).

7-Methoxy-3-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (46)——A 50% H_2SO_4 solution (2 ml) was added to a solution of **45** (2.9 g, 9.3 mmol) in acetone (20 ml) and H_2O (10 ml). The mixture was stirred for 3 h. The reaction mixture was concentrated to *ca.* 10 ml *in vacuo* and extracted with CH_2Cl_2. The organic layer was worked up and the residue was dissolved in CH_3CN (20 ml). *N*-Phenylpiperazine (1.6 g, 10 mmol) was added to the above solution, the mixture was stirred for 10 h, and then $NaBH_3CN$ (130 mg, 20 mmol) and MeOH were added. The reaction mixture was stirred for 3 h, then diluted with 3 N HCl (10 ml) and stirred for 2 h. The mixture was washed with AcOEt. The aqueous layer was made alkaline with 3 N NaOH (20 ml) and extracted with AcOEt. The organic layer was worked up and the residue was purified by column chromatography on silica gel (hexane : AcOEt : MeOH = 10 : 10 : 1) to give **46** as a colorless oil, which was converted into the hydrochloride, **46**·HCl (2.4 g, 57%), colorless plates, mp 216—219 °C (recrystallized from 50% EtOH). *Anal.* Calcd for $C_{23}H_{30}N_2O_3S \cdot HCl$: C, 61.25; H, 6.93; N, 6.21. Found: C, 61.43; H, 6.70; N, 6.27. IR ν_{max}^{KBr} cm^{-1}: 3500—3200 (OH), 1595, 1485. ^{1}H-NMR (90 MHz) (DMSO-d_6) δ: 1.6—2.1 (4H, m), 2.90 (2H, s, C_4-H), 3.2—4.0 (10H, m), 3.67 (3H, s, OCH_3), 3.93 (2H, s, C_2-H).

4-(3-Chloropropyl)-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (47b)——A mixture of **2b** (5.0 g, 14.5 mmol), LiCl (1.5 g, 35 mmol), H_2O (0.3 ml) and DMSO (30 ml) was stirred at 100 °C for 5 h. The reaction mixture was poured into ice-H_2O and extracted with AcOEt. The organic layer was washed with H_2O, dried and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 2 : 1) to give **47b** (2.0 g, 48%) as a colorless oil. *Anal.* Calcd for $C_{13}H_{15}ClO_3S$: C, 54.45; H, 5.27. Found: C, 54.90; H, 5.24. IR ν_{max}^{neat} cm^{-1}: 1730, 1595, 1490, 1205. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.4 (4H, m), 3.4—4.0 (3H, m), 3.68 (3H, s, OCH_3), 4.43 (1H, d, J = 17 Hz, C_2-H), 4.75 (1H, d, J = 17 Hz, C_2-H).

***cis*-4-(3-Chloropropyl)-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-48b)**——$NaBH_4$ reduction of **47b** in MeOH and THF (5 : 1) and usual work up gave *cis*-**48b** (84% yield) as a colorless oil. *Anal.* Calcd for $C_{13}H_{17}ClO_3S$: C, 54.07; H, 5.93. Found: C, 54.46; H, 6.11. IR ν_{max}^{neat} cm^{-1}: 3450 (OH), 1580, 1485. ^{1}H-NMR (400 MHz) ($CDCl_3$) δ: 3.044 (1H, ddd, J = 1.2, 5.6, 8.8 Hz, C_4-H), 3.659 (1H, dd, J = 0.7, 12.5 Hz, C_2-H), 3.903 (1H, ddd, J = 0.7, 1.2, 4.0 Hz, C_3-H), 4.367 (1H, dd, J = 4.0, 12.5 Hz, C_2-H).

7-Methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-one (49b)——A mixture of **47b** (1.8 g) and *N*-phenylpiperazine (2.4 g) was heated at 100 °C for 1 h. The reaction mixture was worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (hexane : AcOEt = 1 : 1) to give **49b** (1.68 g, 65%) as a colorless oil. *Anal.* Calcd for $C_{23}H_{28}N_2O_3S$: C, 66.97; H, 6.84; N, 6.79. Found: C, 66.62; H, 6.73; N, 6.68. IR ν_{max}^{neat} cm^{-1}: 1725 (CO), 1595. ^{1}H-NMR ($CDCl_3$) δ: 1.5—2.0 (4H, m), 2.3—2.8 (4H, m), 3.65 (3H, s, OCH_3), 4.42 (1H, d, J = 8 Hz, C_4-H), 4.73 (1H, d, J = 18 Hz, C_2-H), 4.80 (1H, d, J = 18 Hz, C_2-H).

***cis*-7-Methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-50b)**——a) Substitution reaction of *cis*-**48b** with *N*-phenylpiperazine, as described for *cis*-**17b** (method D), gave *cis*-**50b** (42% yield) as

colorless prisms, mp 112—113 °C (recrystallized from AcOEt). *Anal.* Calcd for $C_{23}H_{30}N_2O_3S$: C, 66.64; H, 7.29; N, 6.76. Found: C, 66.95; H, 7.34; N, 6.92. IR ν_{max}^{KBr} cm^{-1}: 3500—3000, 1595, 1490, 1440, 1200, 1035. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 3.206 (1H, ddd, J=3.8, 4.6, 8.1 Hz, C_4-H), 3.776 (1H, dd, J=8.5, 12.2 Hz, C_2-H), 4.017 (1H, dd, J=3.8, 12.2 Hz, C_2-H), 4.152 (1H, dt, J=3.8, 8.5 Hz, C_3-H).

b) $NaBH_4$ reduction of **49b** in a solution of THF and MeOH (10 : 1) gave *cis*-**50b** (93% yield). The structure of *cis*-**50b** was determined by X-ray crystallographic analysis (Fig. 1).

***cis*-4-(3-Chloropropyl)-3-hydroxy-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylic Acid (*cis*-51b)**——A 1 N NaOH solution (8 ml, 8 mmol) was added to a solution of *cis*-**16b** (2.0 g, 5.8 mmol) in MeOH (20 ml). The mixture was stirred for 14 h, then poured into ice-H_2O containing conc. HCl (5 ml). The resulting precipitates were collected by filtration and recrystallized from AcOEt to give *cis*-**51b** (1.25 g, 75%) as colorless prisms, mp 174—176 °C. *Anal.* Calcd for $C_{14}H_{17}ClO_5S$: C, 50.53; H, 5.15. Found: C, 50.60; H, 5.12. IR ν_{max}^{KBr} cm^{-1}: 3430, 1740, 1485, 1205, 1035. ^{1}H-NMR (DMSO-d_6) δ: 1.2—2.3 (4H, m), 3.43 (2H, t, J=4 Hz, CH_2Cl), 3.75 (3H, s, OCH_3), 3.7—4.4 (3H, m).

***trans*-4-(3-Chloropropyl)-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*trans*-48b)**——*cis*-**51b** (700 mg) was heated at 180 °C for 0.5 h under atmosphere of dry N_2. After cooling, the residue was subjected to column chromatography on silica gel (hexane : CH_2Cl_2 : AcOEt = 3 : 3 : 1) to give *trans*-**48b** (101 mg, 16%) as a colorless oil. *Anal.* Calcd for $C_{13}H_{17}ClO_3S$: C, 54.07; H, 5.93. Found: C, 54.33; H, 6.06. IR ν_{max}^{neat} cm^{-1}: 3450 (OH), 1600, 1485, 1200, 1035.

***trans*-7-Methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*trans*-50b)**——A mixture of *trans*-**48b** (100 mg) and *N*-phenylpiperazine (200 mg) was heated at 90 °C for 2 h. The reaction mixture was subjected to column chromatography on silica gel (hexane : AcOEt : MeOH = 10 : 10 : 1) to give *trans*-**50b** as a colorless oil, which was converted into the hydrochloride, *trans*-**50b**·2HCl (50 mg, 29%), amorphous powder. *Anal.* Calcd for $C_{23}H_{28}N_2O_3S \cdot 2HCl \cdot H_2O$: C, 56.42; H, 6.85; N, 5.51. Found: C, 56.32; H, 6.75; N, 5.44. IR ν_{max}^{KBr} cm^{-1}: 3400, 2800—2200, 1595, 1490, 1440, 1200, 1035. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 3.031 (1H, ddd, J=3.4, 7.7, 9.3 Hz, C_4-H), 3.785 (1H, ddd, J=2.7, 4.9 7.7 Hz, C_3-H), 3.855 (1H, dd, J=5.0, 12.6 Hz, C_2-H), 4.329 (1H, dd, J=2.8, 12.6 Hz, C_2-H).

Methyl 5-(4-Phenyl-1-piperazinyl)-2-(6-methoxy-1,4-benzoxathian-3-yl)pentanoates (52 and 53)——a) A mixture of TsCl (3.0 g, 16 mmol) in pyridine (3 ml) was added dropwise to an ice-cooled solution of *cis*-**17b** (5.0 g, 11 mmol) in pyridine (15 ml). The mixture was stirred at 0—5 °C for 6 h and then poured into ice-H_2O. The supernatant was removed by decantation and the residue was worked up (AcOEt; H_2O). The residue was subjected to column chromatography on silica gel (hexane : AcOEt = 1 : 1) to give a gummy residue [2.0 g, MS *m/z*: 454 (M^+)]. Catalytic hydrogenation of the product obtained (2.0 g) in AcOEt (20 ml) was carried out in the presence of 5% Pd–C (100 mg) under atmospheric pressure of H_2. After hydrogen absorption had ceased (100 ml), the catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel (hexane : AcOEt : MeOH = 10 : 10 : 1) to give **52** from the first fraction as a colorless oil, which was converted into the hydrochloride, **52**·HCl (360 mg, 7% from *cis*-**17b**), colorless prisms, mp 121—123 °C (recrystallized from 50% EtOH). *Anal.* Calcd for $C_{25}H_{32}N_2O_4S \cdot HCl \cdot H_2O$: C, 58.75; H, 6.90; N, 5.48. Found: C, 58.39; H, 6.79; N, 5.30. IR ν_{max}^{KBr} cm^{-1}: 3500, 3430, 2700—2300, 1730 (ester), 1595, 1485, 1260, 1205, 1195. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 2.730 (1H, ddd, J=3.4, 9.5, 10.0 Hz, $-CH(COOCH_3)$), 3.612 (1H, ddd, J=2.1, 5.4, 9.5 Hz, C_3-H), 4.115 (1H, dd, J=5.4, 12.0 Hz, C_2-H), 4.263 (1H, dd, J=2.1, 12.0 Hz, C_2-H).

The second fraction gave **53** as a colorless oil, which was isolated as the hydrochloride **53**·HCl (320 mg, 6% from *cis*-**17b**). Recrystallization from 50% EtOH gave colorless prisms, mp 152—154 °C. *Anal.* Calcd for $C_{25}H_{32}N_2O_4S \cdot HCl$: C, 60.90; H, 6.75; N, 5.68. Found: C, 60.53; H, 6.83; N, 5.70. IR ν_{max}^{KBr} cm^{-1}: 3520, 3450, 2700—2300, 1725, 1595, 1490, 1440, 1260, 1245, 1200. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 2.805 (1H, ddd, J=3.4, 9.8, 10.5 Hz, $-CH(COOCH_3)$), 3.599 (1H, ddd, J=2.0, 4.2, 9.8 Hz, C_3-H), 4.184 (1H, dd, J=2.0, 12.2 Hz, C_2-H), 4.439 (1H, dd, J=4.2, 12.2 Hz, C_2-H).

b) A mixture of red P (300 mg, 10 mmol), I_2 (90 mg) and AcOH (6 ml) was stirred for 0.5 h. *cis*-**16b** (3.0 g, 8.7 mmol) and H_2O (0.1 ml) were added to the above mixture and the mixture was refluxed for 1 h. The reaction mixture was worked up (AcOEt; H_2O) and the residue was subjected to column chromatography on silica gel (hexane : AcOEt = 2 : 1) to give a colorless oil [1.38 g, MS *m/z*: 330, 332 (M^+)], which was heated with *N*-phenylpiperazine (4 ml) at 90 °C for 2 h. The mixture obtained was subjected to column chromatography on silica gel (hexane : AcOEt : MeOH = 10 : 10 : 1) to give first **52** (isolated as **52**·HCl; 0.46 g, 11% from *cis*-**15b**) and then **53** (isolated as **53**·HCl; 0.38 g, 9% from *cis*-**15b**).

1-Chloro-5-(1,3-dixolan-2-yl)pentan-3-ol (54)——A solution of 3-chloropropanal (3.3 g, 36 mmol) in THF (10 ml) was added to a solution of 2-(1,3-dioxolan-2-yl)ethylmagnesium bromide prepared from Mg (1.0 g, 41 mmol), 2-(1,3-dioxolan-2-yl)ethyl bromide (7.5 g, 41 mmol) and THF (20 ml). The mixture was stirred for 2 h, then diluted with 1 N NaOH (20 ml) and worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (CH_2Cl_2 : Et_2O = 5 : 1) to give **54** (2.6 g, 73%) as a colorless oil. *Anal.* Calcd for $C_8H_{15}ClO_3$: C, 49.36; H, 7.77. Found: C, 49.55; H, 7.51. IR ν_{max}^{neat} cm^{-1}: 3400 (OH), 1450, 1415, 1345, 1210, 1040. ^{1}H-NMR ($CDCl_3$) δ: 1.2—2.2 (6H, m), 3.4—4.2 (7H, m), 4.91 (1H, t, J=4 Hz, O–CH–O).

5-(1,3-Dioxolan-2-yl)-3-mesyloxypentyl Benzoate (55)——A mixture of **54** (3.0 g, 15 mmol), sodium benzoate (3.0 g, 21 mmol), KI (1.0 g, 6 mmol), CH_3CN (30 ml) and DMF (20 ml) was stirred at 80 °C for 5 h. The reaction mixture was worked up (AcOEt; H_2O) and the residue was purified by column chromatography on silica gel (CH_2Cl_2 : Et_2O = 5 : 1) to give 5-(1,3-dioxolan-2-yl)-3-hydroxypentyl benzoate (1.8 g, 42%) as a colorless oil [MS *m/z*: 280 (M^+)]. MsCl (1.8 g, 6.4 mmol) was added to the alcohol obtained above (1.8 g) in pyridine (10 ml). The mixture was stirred for 2 h, poured into ice-H_2O containing conc. HCl (20 ml) and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (CH_2Cl_2 : Et_2O = 5 : 1) to give **55** (0.9 g, 54%) as a colorless oil. MS *m/z*: 358 (M^+), 357. High-resolution MS Calcd for $C_{10}H_{22}O_7S$: 358.1085. Found: 358.1088. IR ν_{max}^{neat} cm^{-1}: 1725, 1710, 1595, 1445, 1350, 1280, 1270, 1175. ^{1}H-NMR ($CDCl_3$) δ: 1.7—2.4 (6H, m), 3.08(3H, s, OSO_2CH_3), 3.2—4.2 (4H, m), 4.2—4.6 (3H, m), 4.9—5.1 (1H, t, J = 4 Hz, O-CH-O).

2-[1-(1,3-Dioxolan-2-yl)-5-hydroxy-3-pentyl]thio-4-methoxyphenol (56)——A mixture of **55** (1.4 g, 3.9 mmol), 2-mercapto-4-methoxyphenol (0.8 g, 5 mmol), K_2CO_3 (0.6 g, 4.4 mmol) and acetone (20 ml) was stirred for 15 h and then filtered, and the filtrate was concentrated *in vacuo*. A 1 N NaOH solution (10 ml) was added to a solution of the above residue in MeOH (30 ml). The resulting mixture was stirred for 5 h, neutralized with 1 N HCl and worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (hexane : AcOEt = 1 : 1) to give **56** (0.7 g, 43%) as a colorless oil. *Anal.* Calcd for $C_{15}H_{22}O_5S$: C, 57.30; H, 7.05. Found: C, 57.51; H, 7.21. IR ν_{max}^{neat} cm^{-1}: 3450—3200 (OH), 1600, 1480, 1275, 1250, 1220, 1205. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.2 (6H, m), 2.8—3.2 (1H, m), 3.77 (3H, s, OCH_3), 3.6—4.2 (6H, m), 4.90 (1H, t, J = 4 Hz, O-CH-O). MS *m/z*: 314 (M^+), 252, 213, 200, 183.

4-[2-(1,3-Dioxolan-2-yl)ethyl]-7-methoxy-3,4-dihydro-2*H*-1,5-benzoxathiepin (57)——Ph_3P (0.85 g, 3.2 mmol) was added to a solution of **56** (0.9 g, 2.9 mmol) and toluene (10 ml) with stirring. Then, a solution of ethyl azodiformate (0.55 g, 3.2 mmol) in toluene (1 ml) was added dropwise to the above mixture. The whole was stirred for 2 h and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 2 : 1) to give **57** (0.61 g, 71%) as a colorless oil. *Anal.* Calcd for $C_{15}H_{20}O_4S$: C, 60.79; H, 6.80. Found: C, 60.88; H, 6.71. IR ν_{max}^{neat} cm^{-1}: 1595, 1485, 1435, 1280, 1265, 1200. ^{1}H-NMR ($CDCl_3$) δ: 1.5—2.4 (6H, m), 2.9—3.1 (1H, m, C_4-H), 3.75 (3H, s, OCH_3), 3.7—4.6 (6H, m), 4.88 (1H, t, J = 4 Hz, O-CH-O).

7-Methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin (58)——A mixture of **57** (680 mg, 2.3 mmol), 50% H_2SO_4 (0.5 ml), H_2O (2 ml) and acetone (10 ml) was stirred for 2 h, then the reaction mixture was worked up (AcOEt; H_2O). *N*-Phenylpiperazine (0.4 g, 2.5 mmol) was added to a solution of the residual oil in CH_3CN (10 ml) and the mixture was stirred for 4 h. Then, $NaBH_3CN$ (190 mg, 3 mmol) was added. The reaction mixture was stirred for 4 h and then worked up (AcOEt; H_2O). The resulting residue was purified by column chromatography on silica gel (hexane : AcOEt = 1 : 1) to give **58** as a colorless oil, which was converted into the hydrochloride, **58**·2HCl (550 mg, 51%), white crystals, mp 150—153 °C (recrystallized from 50% EtOH). *Anal.* Calcd for $C_{23}H_{30}N_2O_2S$·2HCl: C, 58.59; H, 6.84; N, 5.94. Found: C, 58.48; H, 6.75; N, 5.64. IR ν_{max}^{KBr} cm^{-1}: 3500—3400, 2600—2200, 1595, 1480.

Methyl *cis*-3-Acetoxy-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-59b)——Acetylation of *cis*-**17b** with Ac_2O in pyridine gave *cis*-**59b** as colorless prisms, mp 168—170 °C (recrystallized from AcOEt) in 83% yield. *Anal.* Calcd for $C_{27}H_{34}N_2O_6S$: C, 63.01; H, 6.66; N, 5.44. Found: C, 63.01; H, 6.69; N, 5.40. IR ν_{max}^{KBr} cm^{-1}: 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ: 2.08 (3H, s, $OCOCH_3$), 3.62 (3H, s), 3.63 (3H, s).

Methyl *cis*-7-Methoxy-3-*N*-methylcarbamoyloxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-60b)——*cis*-**60b** was prepared by the reaction of *cis*-**17b** with CH_3NCO in DMF and isolated as the hydrochloride in 89% yield. Recrystallization from EtOH gave *cis*-**60b**·2HCl as colorless prisms, mp 167—172 °C. *Anal.* Calcd for $C_{27}H_{35}N_3O_6S$·2HCl: C, 53.82; H, 6.19; N, 6.97. Found: C, 53.56; H, 6.42; N, 6.71. IR ν_{max}^{KBr} cm^{-1}: 3400, 1720 (ester). ^{1}H-NMR (DMSO-d_6–D_2O) δ: 2.75 (3H, s, $NHCH_3$), 3.73 (6H, s, 7-OCH_3 + $COOCH_3$), 5.25 (1H, m, C_3-H).

***cis*-3-Hydroxy-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylic Acid (*cis*-61b)**——A mixture of *cis*-**17b** (3.0 g, 6.3 mmol), 1 N NaOH (12 ml) and MeOH (40 ml) was stirred at 60 °C for 5 h. After evaporation of the MeOH, the residual mixture was acidified with 1 N HCl. The resulting precipitates were collected by filtration and recrystallized from EtOH to give *cis*-**61b** (2.4 g, 95%) as colorless crystals, mp 250—260 °C (dec.). *Anal.* Calcd for $C_{24}H_{30}N_2O_5S \cdot H_2O$: C, 60.48; 6.77; N, 5.88. Found: C, 60.27; H, 6.73; N, 5.66. IR ν_{max}^{KBr} cm^{-1}: 3600, 3510—3300, 2600—2200, 1640—1590, 1485, 1370, 1210.

Ethyl *cis*-3-Hydroxy-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-4-carboxylate (*cis*-62b)——A mixture of *cis*-**61b** (1.2 g, 2.5 mmol), Et_2SO_4 (0.5 g, 3.2 mmol), $NaHCO_3$ (1.0 g, 12 mmol) and EtOH (25 ml) was refluxed for 3 h, then worked up (AcOEt; H_2O). The residue was purified by column chromatography on silica gel (hexane : AcOEt = 1 : 1) to give *cis*-**62b** as colorless oil, which was isolated as the hydrochloride *cis*-**62b**·2HCl (0.5 g, 42%), colorless prisms, mp 186—188 °C (from EtOH). *Anal.* Calcd for $C_{26}H_{34}N_2O_5S$·2HCl: C, 55.81; H, 6.49; N, 5.01. Found: C, 55.74; H, 6.56; N, 5.03. IR ν_{max}^{KBr} cm^{-1}: 3540 (OH), 1740 (ester). ^{1}H-NMR (DMSO-d_6–D_2O) δ: 1.30 (3H, t, J = 7 Hz, $OCH_2\underline{CH_3}$), 3.72 (3H, s, 7-OCH_3), 4.1—4.2 (3H, m, C_2-H + C_3-H), 4.28 (2H, t, J = 7 Hz, $OC\underline{H_2}CH_3$).

***cis*-4-Hydroxymethyl-7-methoxy-4-[3-(4-phenyl-1-piperazinyl)propyl]-3,4-dihydro-2*H*-1,5-benzoxathiepin-3-ol (*cis*-63b)**——A solution of *cis*-**17b** (400 mg, 0.8 mmol) in dry Et_2O (10 ml) was added dropwise to a suspension of $LiAlH_4$ (90 mg, 2.4 mmol) and dry Et_2O (20 ml) with stirring. The mixture was refluxed for 0.5 h. Excess $LiAlH_4$ was decomposed by adding H_2O and 15% NaOH. The inorganic deposit was filtered and the filtrate was concentrated *in vacuo*. The residue was recrystallized from AcOEt to give *cis*-**62** (300 mg, 80%) as colorless needles, mp 163—165 °C. *Anal.* Calcd for $C_{24}H_{32}N_2O_4S$: C, 64.84; H, 7.25; N, 6.30. Found: C, 64.76; H, 7.31; N, 6.39. IR ν_{max}^{KBr} cm^{-1}: 3540 (OH). ^{1}H-NMR (400 MHz) ($CDCl_3$–D_2O) δ: 3.923 (1H, dd, J=1.0, 4.9 Hz, C_3-H), 3.995 (1H, dd, J=1.0, 12.8 Hz, C_2-H), 4.138 (1H, dd, J=4.9, 12.8 Hz, C_2-H).

Methyl 8-Methoxy-3-oxo-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (64)——A solution of methyl 3-(4-methoxy-2-methoxycarbonylmethyloxyphenyl)propionate (15 g, 53 mmol) in toluene (200 ml) was added dropwise to a gently boiling suspension of 60% NaH (5.6 g, 140 mmol), *tert*-BuOH (0.4 ml) and toluene (200 ml) (8 h). After refluxing for 0.5 h, the reaction mixture was allowed to stand overnight and then poured into ice-H_2O containing AcOH (10 ml). The organic layer was separated, washed with H_2O, dried and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (hexane : AcOEt = 4 : 1) to give **64** (9.5 g, 71%) as a colorless oil. *Anal.* Calcd for $C_{13}H_{14}O_5S$: C, 62.39; H, 5.64. Found: C, 62.18; H, 5.86. IR ν_{max}^{neat} cm^{-1}: 1760, 1720. ^{1}H-NMR ($CDCl_3$) δ: 2.9—3.1 (4H, m), 3.72 (3H, s), 3.80 (3H, s), 5.00 (1H, s, C_2-H), 6.5—7.2 (3H, m).

Methyl 3-Oxo-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (65)——**65** was prepared in 81% yield by Dieckmann reaction of methyl 3-(2-methoxycarbonylmethylthiophenyl)propionate, as described for **64**. The starting methyl 3-(2-methoxycarbonylmethylthiophenyl)propionate was prepared in 5 steps from methyl 3-(2-hydroxyphenyl)propionate *via* the route involving thiocarbamoylation with dimethylthiocarbamoyl chloride (74% yield), thermal rearrangement at 260—270 °C (70% yield), alkaline hydrolysis, *S*-alkylation with methyl bromoacetate and esterification with dimethyl sulfate (55% yield). Chromatographic purification of the crude product gave **65** as a colorless oil. *Anal.* Calcd for $C_{12}H_{12}O_3S$: C, 61.00; H, 5.12. Found: C, 61.23; H, 5.28. MS *m/z*: 236 (M^+). IR ν_{max}^{neat} cm^{-1}: 1740, (ester), 1700 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.6—3.4 (4H, m), 3.62 (3H, s, $COOCH_3$), 4.20 (1H, s, C_2-H), 6.9—7.7 (4H, m).

Methyl 8-Methoxy-3-oxo-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (66)——**66** was similarly prepared by Dieckmann reaction of methyl 3-(4-methoxy-2-methoxycarbonylmethylthiophenyl)propionate in 80% yield. Recrystallization from AcOEt–hexane gave **66** as colorless prisms, mp 76—78 °C. *Anal.* Calcd for $C_{13}H_{14}O_4S$: C, 58.68; H, 5.30. Found: C, 58.59; H, 5.26. IR ν_{max}^{KBr} cm^{-1}: 1740 (ester), 1710 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.9—3.1 (4H, m), 3.68 (3H, s), 3.78 (3H, s), 4.22 (1H, s, C_2-H), 6.7—7.4 (3H, m).

Methyl 2-(3-Chloropropyl)-8-methoxy-3-oxo-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (67)——**67** was prepared by alkylation of **64** with 3-bromo-1-chloropropane, as described for **2**. Chromatographic purification gave a colorless oil. MS *m/z*: 326, 328 (M^+). High-resolution MS Calcd for $C_{16}H_{19}ClO_5$: 326.0919. Found: 326.0925. IR ν_{max}^{neat} cm^{-1}: 1750 (ester), 1720 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.0—2.2 (4H, m), 2.92 (4H, s, C_4-H + C_5-H), 3.52 (2H, t, J=6 Hz, CH_2Cl), 3.64 (3H, s), 3.72 (3H, s), 6.4—7.1 (3H, m). Compound **67** thus obtained was found to contain a small amount of enol ether (2—3%) as a by-product, but was used for the following step without further purification. **68** and **69** were similarly prepared by alkylation of **65** and **66**, respectively.

Methyl 2-(3-Chloropropyl)-3-oxo-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (68)——A colorless oil (41% yield). MS *m/z*: 312, 314 (M^+). High-resolution MS Calcd for $C_{15}H_{17}ClO_3S$: 312.0586. Found: 312.0582. IR ν_{max}^{neat} cm^{-1}: 1720 (ester, CO). ^{1}H-NMR ($CDCl_3$) δ: 1.7—3.6 (10H, m), 3.65 (3H, s, $COOCH_3$), 7.1—7.8 (4H, m).

Methyl 2-(3-Chloropropyl)-8-methoxy-3-oxo-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (69)——A colorless oil (50% yield). MS *m/z*: 342, 344 (M^+). High-resolution MS Calcd for $C_{16}H_{19}ClO_4S$: 342.0692. Found: 342.0693. IR ν_{max}^{neat} cm^{-1}: 1740 (ester), 1700 (CO). ^{1}H-NMR ($CDCl_3$) δ: 1.8—2.0 (4H, m), 2.8—3.0 (4H, m), 3.42 (2H, t, J=7 Hz, CH_2Cl), 3.62 (3H, s), 3.72 (3H, s), 6.7—7.4 (3H, m).

Methyl *cis*- and *trans*-2-(3-Chloropropyl)-3-hydroxy-8-methoxy-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (*cis*- and *trans*-70)——$NaBH_4$ reduction of **67** in an ice-cooled solution of THF and MeOH (1 : 4) and subsequent column chromatography on silica gel (hexane : AcOEt = 3 : 1) gave *cis*-**70** (from the first fraction) and *trans*-**70** (from the second fraction).

cis-**70**: A colorless oil (46% yield). *Anal.* Calcd for $C_{16}H_{21}ClO_5$: C, 58.45; H, 6.44. Found: C, 58.66; H, 6.59. IR ν_{max}^{neat} cm^{-1}: 3500 (OH), 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ: 1.8—2.0 (4H, m), 2.4—3.5 (6H, m), 3.72 (3H, s), 3.75 (3H, s), 6.5—7.2 (3H, m).

trans-**70**: A colorless oil (44% yield). *Anal.* Calcd for $C_{16}H_{21}ClO_5$: C, 58.45; H, 6.44. Found: C, 58.78; H, 6.21. IR ν_{max}^{neat} cm^{-1}: 3500 (OH), 1740 (ester). ^{1}H-NMR ($CDCl_3$) δ: 1.9—2.0 (4H, m), 2.4—3.2 (4H, m), 3.48 (2H, t, J=7 Hz, CH_2Cl), 3.66 (3H, s), 3.72 (3H, s), 4.0—4.2 (1H, m), 6.46—7.02 (3H, m).

Methyl *cis*-2-(3-Chloropropyl)-3-hydroxy-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (*cis*-71)——$NaBH_4$ reduction of **68** gave *cis*-**71** (80% yield) as colorless prisms, mp 108—110 °C (recrystallized from AcOEt). *Anal.* Calcd for $C_{15}H_{19}ClO_3S$: C, 57.23; H, 6.08. Found: C, 57.27; H, 6.11. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1730 (ester). ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 4.051 (1H, dd, J=2.7, 5.4 Hz, C_3-H).

Methyl *cis*-2-(3-Chloropropyl)-3-hydroxy-8-methoxy-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (*cis*-72)——$NaBH_4$ reduction of **69** gave *cis*-**72** (74% yield) as a colorless oil. *Anal.* Calcd for $C_{16}H_{21}ClO_4S$: C, 55.73; H, 6.14.

Found: C, 55.98; H, 6.00. IR ν_{max}^{neat} cm^{-1}: 3530 (OH), 1740. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 4.056 (1H, dd, J=2.7, 5.4 Hz, C_3-H).

Methyl *cis*-3-Hydroxy-8-methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (*cis*-73, Table II)——A mixture of *cis*-**70** (1.0 g, 3.1 mmol), *N*-phenylpiperazine (1.1 g, 6.8 mmol), and KI (0.25 g, 1.5 mmol) was stirred at 90 °C for 3 h. The reaction mixture was subjected to column chromatography on silica gel (hexane : AcOEt : MeOH = 30 : 20 : 1) to give *cis*-**73** (0.65 g, 47%) as colorless prisms (recrystallized from AcOEt). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1760, 1730. ^{1}H-NMR (400 MHz) of *cis*-**73**·2HCl (DMSO-d_6–D_2O) δ: 4.064 (1H, dd, J=3.4, 3.9 Hz, C_3-H).

Similar treatment of *trans*-**70**, *cis*-**71** and *cis*-**72** gave *trans*-**73**, *cis*-**74** and *cis*-**75**, respectively (Table II).

Methyl *trans*-3-Hydroxy-8-methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-2,3,4,5-tetrahydro-1-benzoxepin-2-carboxylate (*trans*-73)——Recrystallization of the hydrochloride from MeOH–Et_2O gave *trans*-**73**·2HCl as colorless crystals. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 4.070 (1H, dd, J=2.7, 7.3 Hz, C_3-H).

Methyl *cis*-3-Hydroxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (*cis*-74)——Recrystallization from AcOEt gave *cis*-**74** as colorless prisms. IR ν_{max}^{KBr} cm^{-1}: 3450—3100 (OH), 1720 (ester).

Methyl *cis*-3-Hydroxy-8-methoxy-2-[3-(4-phenyl-1-piperazinyl)propyl]-2,3,4,5-tetrahydro-1-benzothiepin-2-carboxylate (*cis*-75)——Recrystallization of the hydrochloride from 30% EtOH gave *cis*-**75**·HCl as colorless crystals. ^{1}H-NMR (400 MHz) (DMSO-d_6–D_2O) δ: 4.056 (1H, dd, J=2.2, 5.1 Hz, C_3-H).

X-Ray Analyses of *cis*-17b, *cis*-50b, *cis*-58b, *cis*-71 and *cis*-73——All single-crystal measurements were made with a Rigaku AFC-5 automatic diffractometer. The structures were solved by the direct method[27] and refined by a block-diagonal least-squares method[28] using unit weight. In the final refinement, non-hydrogen and hydrogen atoms were refined with anisotropic and isotropic temperature factors, respectively. Details of the X-ray analyses will be published elsewhere.

Serotonin S_2-Receptor-Blocking Activity and Adrenergic α_1-Receptor-Blocking Activity *in Vitro*——Pig hearts were obtained from a slaughterhouse under ice-cooling and the left circumflex or anterior descending coronary artery was dissected out within 3 h after death. The coronary artery was cut into a ring preparation of 3 mm in width. On the other hand, the thoracic aorta was dissected out from albino rabbits (2—3 kg body weight, male) after exsanguination. The rabbit aorta was cut into a spiral preparation of about 2 mm in width and about 2 cm in length. These blood vessel preparations were suspended in organ baths containing 20 ml of Krebs–Henseleit solution with a pair of suspending hooks. One of the hooks was fixed to the bottom of the organ bath, while the other was connected to a strain-gauge transducer, and the tension developed by these preparations was isometrically measured. The organ bath was maintained at 37 °C, and the Krebs–Henseleit solution was saturated with a gas mixture of 97% O_2 + 3% CO_2. As agonists, serotonin (10^{-6} M) and norepinephrine (10^{-7} M) were used in the porcine coronary and rabbit aortic preparations, respectively.

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Synthesis of 2-Phenylthiazolidine Derivatives as Cardiotonic Agents. I. 2-Phenylthiazolidine-3-thiocarboxamides

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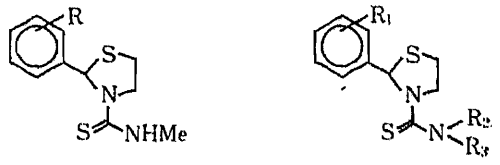
(Received October 9, 1986)

A series of novel 2-phenylthiazolidine-3-thiocarboxamides (II) was synthesized and tested for positive inotropic activity in the isolated guinea pig heart and in anesthetized dogs. Reaction of the benzaldehydes (VI, XI, XIV and XV) with cysteamine followed by treatment with isothiocyanates readily gave II. Structure-activity relationships were investigated by varying the structural parameters. *N*-Methyl-2-phenylthiazolidine-3-thiocarboxamides having an *ortho* substituent such as a Me or OMe group exhibited significant positive inotropic action, which was not blocked by propranolol. Among the various *ortho*-alkoxyphenyl derivatives synthesized, the 2-(2-(3-(4-phenylpiperazino)propoxy)phenyl) derivative (I_{67}) was found to exhibit more potent and longer-lasting activity than amrinone without any significant effect on heart rate or blood pressure.

Keywords——2-phenylthiazolidine-3-thiocarboxamide; positive inotropic activity; structure–activity relationship; cardiotonic agent

Cardiac glycosides and sympathomimetic agents have been used for the treatment of congestive heart failure (CHF) for many years. Although cardiac glycosides such as digitoxin and digoxin have been the principal agents used for CHF, their use is limited by arrhythmogenic liability and very narrow therapeutic index. The use of sympathomimetic agents such as dobutamine and dopamine is also limited because of their chronotropic liability and oral ineffectiveness.[1,2] Thus, the absence of safe, orally effective, positive inotropic agents for the treatment of CHF has stimulated the development of several new nonglycoside, nonsympathomimetic, cardiotonic agents such as amrinone, milrinone, sulmazole, and fenoximone.[3]

In the course of our studies on new antiulcer agents, 2-phenylthiazolidine-3-thiocarboxamides (I), conformationally restricted analogues[4a] of metiamide, have been synthesized. Unexpectedly, in the isolated guinea pig heart, these compounds were found to display positive inotropic activity which was not blocked by propranolol. In view of the structural novelty of this class of compounds as cardiotonic agents, we have synthesized a large number of derivatives by varying the structural parameters. In this paper, we report the synthesis and cardiotonic activity of a series of new 2-phenylthiazolidines represented by the general formula II and related compounds.

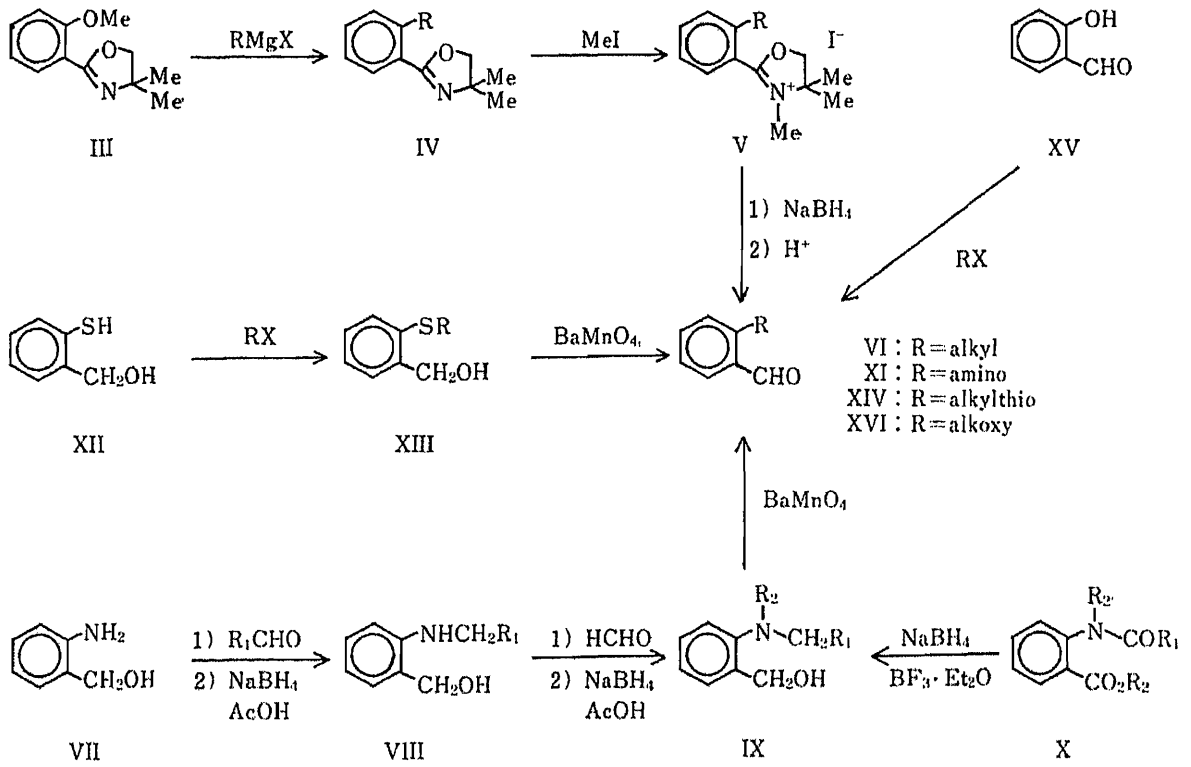


I II

Chart 1

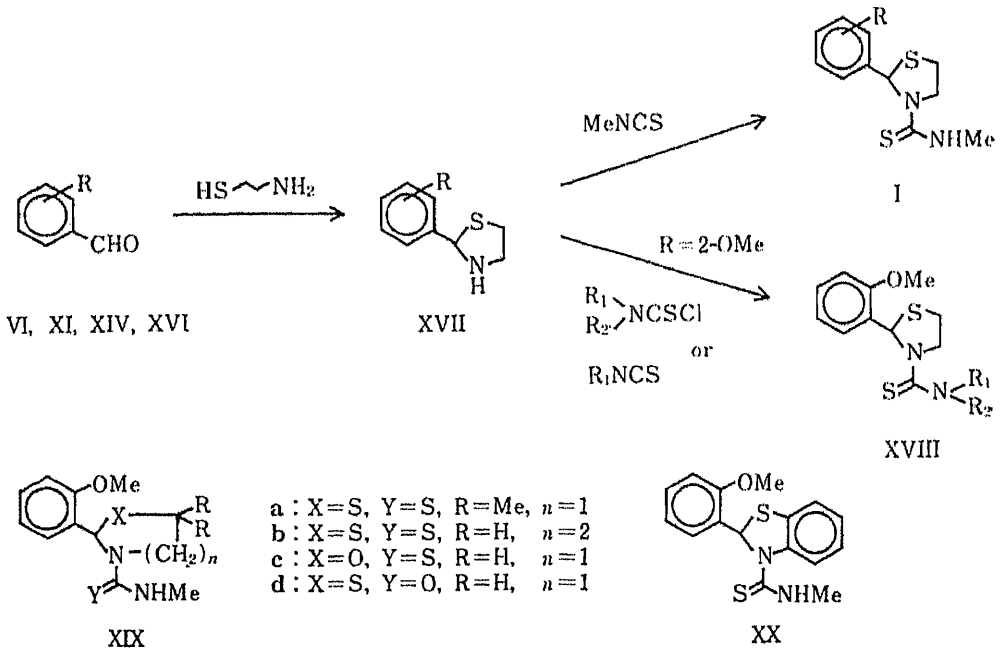
Chemistry

Our chemical approach was to examine the effects of varying three structural parameters on the activity of I; (1) the position and nature of substituents on the phenyl ring, (2) the effect of substituents on nitrogen, and (3) modification of the thiazolidine ring. These thiazolidine derivatives could be readily obtained from substituted benzaldehydes. The requisite benzaldehydes were synthesized by diverse routes depending on the nature of the substituents (Chart 2) or by known procedures.



III IV V XV XII XIII VII VIII IX X

Chart 2



VI, XI, XIV, XVI XVII I XVIII

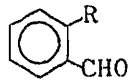
a : X=S, Y=S, R=Me, n=1
b : X=S, Y=S, R=H, n=2
c : X=O, Y=S, R=H, n=1
d : X=S, Y=O, R=H, n=1

XIX XX

Chart 3

Reaction of the oxazoline (III)[5a] with alkylmagnesium halides according to Meyers and Mihelich[5b] effected the conversion of the methoxy group of III to various alkyl groups, giving the 2-(2-alkylphenyl)oxazolines (IV). Quaternization of IV with methyl iodide followed by reduction with $NaBH_4$[6] gave, after acidic hydrolysis, various 2-alkylbenzaldehydes (VI) listed in Table I.

TABLE I. 2-Substituted Benzaldehydes (VI, XI, XIV, XVI)



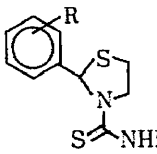
Compd. No.	R	Yield (%)	^{1}H-NMR ($CDCl_3$) δ ppm
VIa	C_2H_5	63[a]	1.25 (3H, t, J=7.5 Hz), 3.06 (2H, q, J=7.5 Hz), 10.27 (1H, s)
VIb	C_3H_7	61[a]	0.98 (3H, t, J=7.0 Hz), 1.51—1.78 (2H, m), 10.28 (1H, s)
VIc	iso-C_3H_7	61[a]	[b]
VId	C_4H_9	57[a]	0.93 (3H, t, J=7.0 Hz), 1.20—1.75 (4H, m), 10.28 (1H, s)
VIe	C_6H_{13}	69[a]	0.88 (3H, t, J=6.0 Hz), 1.10—1.70 (8H, m), 10.29 (1H, s)
VIf[c]	$CH_2N(CH_3)_2$	64	2.24 (6H, s), 3.75 (2H, s), 10.44 (1H, s)
VIg	$(CH_2)_3OCH_3$	28[a]	1.76—2.11 (2H, m), 3.38 (3H, s), 10.25 (1H, s)
XI[d]a	$N(CH_3)(CH_2)_2OCH_3$	55[e]	[b]
XIb	$N(CH_3)(CH_2)_2N(CH_3)_2$	21[e]	2.34 (6H, s), 3.04 (3H, s), 10.39 (1H, s)
XIc	$N(CH_3)(CH_2)_2SCH_3$	57[e]	2.07 (3H, s), 2.91 (3H, s), 10.29 (1H, s)
XId	$N(C_2H_5)_2$	66[f]	1.05 (6H, t, J=7 Hz), 3.18 (4H, q, J=7 Hz), 10.33 (1H, s)
XIe	$N(C_3H_7)_2$	62[f]	0.84 (6H, t, J=7 Hz), 1.20—1.90 (4H, m), 10.37 (1H, s)
XIf	$N(C_4H_9)_2$	55[f]	0.70—1.05 (6H, m), 1.10—1.90 (8H, m), 10.35 (1H, s)
XIV[g]a	$S(CH_2)_2N$ 	79[h]	1.62—2.00 (4H, m), 2.45—3.26 (8H, m), 10.47 (1H, s)
XIVb	$S(CH_2)_2N$ 	34[h]	1.30—1.75 (6H, m), 2.33—3.23 (8H, m), 10.42 (1H, s)
XIVc	$S(CH_2)_2N$  $N-CH_3$	34[h]	[b]
XIVd	$S(CH_2)_3N$ 	84[h]	1.30—2.08 (8H, m), 2.31—2.98 (8H, m), 10.40 (1H, s)
XVI[i]a	$O(CH_2)_2OCH_3$	82	[b]
XVIb	$O(CH_2)_2SCH_3$	93	2.19 (3H, s), 2.91 (2H, t, J=6.5 Hz), 10.44 (1H, s)
XVIc	$O(CH_2)_2SC_6H_5$	84	3.32 (2H, t, J=6.5 Hz), 4.25 (2H, t, J=6.5 Hz), 10.32 (1H, s)

a) Overall yield from III. *b*) See Experimental. *c*) This compound was prepared by reduction of 2-(dimethylaminocarbonyl)benzoic acid with B_2H_6 and subsequent oxidation with $BaMnO_4$. *d*) 2-Dimethylamino and 2-acetaminobenzaldehyde were prepared by the reported procedures. See reference 9. *e*) Overall yield from VII. *f*) Overall yield from X. *g*) Other known 2-alkylthiobenzaldehydes were prepared by the reported procedures. See reference 10. *h*) Overall yield from XII. *i*) Other known 2-alkoxybenzaldehydes were prepared by the reported procedures. See reference 11.

2-Aminobenzaldehydes (XI) were prepared from 2-aminobenzylalcohol (VII). Two successive reductive alkylations of VII gave IX, which could also be obtained by diborane reduction of the *N*-acyl anthranilates (X). Oxidation of IX with $BaMnO_4$[7] in refluxing $CHCl_3$ readily gave various 2-aminobenzaldehydes (XI) (Table I). 2-Alkylthiobenzaldehydes (XIV, Table I) were prepared by alkylation of 2-mercaptobenzyl alcohol (XII)[8] followed by $BaMnO_4$ oxidation. Alkylation of salicylaldehyde (XV) with alkyl halides gave various 2-alkoxybenzaldehydes (XVI), listed in Table I.

Reaction of substituted benzaldehydes (VI, XI, XIV, and XVI) with cysteamine at room temperature gave the 2-phenylthiazolidines (XVII). Without isolation, the products (XVII) were allowed to react with methyl isothiocyanate (MeNCS) to give the *N*-methylthiocarboxamides (I) (Table II). Since the 2-methoxyphenyl derivative (I_{37}) exhibited sig-

TABLE II. 2-Aryl-*N*-methylthiazolidine-3-thiocarboxamides (I)



Compd. No.	R	Yield (%)	Salt[a]	mp (°C)	Recrystn.[b] solvent	Analysis (%) Calcd (Found)	Myocardial contractility: Anesthetized dog[c] Dose mg/kg i.v.	LV$dP/dt_{max}$ Δ%	Duration min	Isolated guinea pig heart[d] MED[e] μg/heart
						C H N				
1	H	91	—	168—169	A	$C_{11}H_{14}N_2S$ 55.45 5.92 11.76 (55.52 5.92 11.74)	1	7	<10	3
2	2-Cl	90	—	204—205	B	$C_{11}H_{13}ClN_2S_2$ 48.43 4.80 10.27 (48.29 4.83 10.29)	1	9.5	<10	10
3	3-Cl	57	—	128—130	C	$C_{11}H_{13}ClN_2S_2$ 48.43 4.80 10.27 (48.42 4.85 10.15)	1	8.5	3	30
4	4-Cl	57	—	141.5—144	C	$C_{11}H_{13}ClN_2S_2$ 48.43 4.80 10.27 (48.61 4.91 10.02)	1	12	6	30
5	2-CH_3	58	—	183—184	C	$C_{12}H_{16}N_2S_2$ 57.10 6.39 11.10 (57.04 6.39 11.12)	1	40	10	3
6	3-CH_3	60	—	131—133	A	$C_{12}H_{16}N_2S_2$ 57.10 6.39 11.10 (57.21 6.46 11.15)	1	4.5	3	30
7	4-CH_3	52	—	134—135	A	$C_{12}H_{16}N_2S_2$ 57.10 6.39 11.10 (57.07 6.40 11.11)	1	6.8	3	30
8	2-C_2H_5	68	—	157—159	A	$C_{13}H_{18}N_2S_2$ 58.61 6.81 10.52 (58.49 6.89 10.56)	1	23	15	10

9	2-C_3H_7	88	—	156—159	A	$C_{14}H_{20}N_2S_2$ 59.96 7.19 9.99 (60.26 7.29 9.96)	1	20	19	*f)*
10	2-iso-C_3H_7	58	—	184—186	A	$C_{14}H_{20}N_2S_2$ 59.96 7.19 9.99 (59.69 7.13 9.89)	1	14	11	100
11	2-C_4H_9	81	—	137—139	A	$C_{15}H_{22}N_2S_2$ 61.18 7.53 9.51 (61.38 7.59 9.50)	1	34	20	10
12	2-C_6H_{13}	73	—	106—108	A	$C_{17}H_{26}N_2S_2$ 63.33 8.09 8.69 (63.06 8.19 8.87)	1	48	39	100
13	2-$CH_2N(CH_3)_2$	88	HCl	193—197 (dec.)	D	$C_{14}H_{21}N_3S_2 \cdot HCl$ 50.66 6.68 12.66 (50.48 6.71 12.56)	1	6	1	100
14	2-$(CH_2)_3OCH_3$	71	—	116—119	A	$C_{15}H_{22}N_2OS_2$ 58.03 7.14 9.02 (58.24 7.20 8.98)	1	33	20	10
15	2-CO_2H	51	—	202—203	E	$C_{12}H_{14}N_2O_2S_2$ 51.04 5.00 9.92 (50.94 4.98 9.80)	1	0	0	*f)*
16	2-CO_2CH_3	78	—	172—175	C	$C_{13}H_{16}N_2O_2S_2$ 52.68 5.44 9.45 (52.94 5.58 9.20	1	33	10	3
17	2-NH_2[g]	27	—	159—161	A	$C_{11}H_{15}N_3S_2$ 52.14 5.97 16.58 (52.15 6.14 16.29)	1	0	0	100
18	2-$NHCOCH_3$	19	—	191—193	A	$C_{13}H_{17}N_3OS_2$ 52.85 5.80 14.22 (52.89 5.82 14.18)	1	0	0	*f)*
19	2-$N(CH_3)_2$	70	H_2SO_4	141—142.5 (dec.)	F	$C_{13}H_{19}N_3S_2 \cdot H_2SO_4$ 41.13 5.58 11.07 (41.26 5.70 10.95)	0.3	46	10	3
20	2-$N(C_2H_5)_2$	84	H_2SO_4	114—118 (dec.)	F	$C_{15}H_{23}N_3S_2 \cdot H_2SO_4 \cdot 1/2\,H_2O$ 43.25 6.29 10.08 (42.96 6.51 9.84)	0.3	20	8.5	10
21	2-$N(C_3H_7)_2$	32	H_2SO_4	143—146 (dec.)	F	$C_{17}H_{27}N_3S_2 \cdot H_2SO_4$ 46.87 6.71 9.65 (46.98 6.57 9.90)	0.3	16	10	30

TABLE II. (continued)

Compd. No.	R	Yield (%)	Salt[a]	mp (°C)	Recrystn.[b] solvent	Analysis (%) Calcd (Found)	Myocardial contractility: Anesthetized dog[c] Dose mg/kg i.v.	LVdP/dt_{max} Δ%	Duration min	Isolated guinea pig heart[d] MED[e] μg/heart
						C H N				
22	2-N(C_4H_9)$_2$	55	H_2SO_4	163—164 (dec.)	F	$C_{19}H_{31}N_3S_2 \cdot H_2SO_4$ 49.22 7.17 9.06 (49.41 7.29 9.03)	1	60	25	100
23	2-N(CH_3)(CH_2)$_2$N(CH_3)$_2$	43	2HBr	105—110 (dec.)	F	$C_{16}H_{26}N_4S_2 \cdot 2HBr$ 38.41 5.64 11.20 (38.63 5.72 11.40)	1	2	5	*f*)
24	2-N(CH_3)(CH_2)$_2$SCH_3	81	HCl	143—145 (dec.)	F	$C_{15}H_{23}N_3S_3 \cdot HCl$ 47.66 6.40 11.12 (47.47 6.56 10.95)	1	213	22	1
25	2-N(CH_3)(CH_2)$_2$OCH_3	59	HCl	122—128	F	$C_{15}H_{23}N_3S_2 \cdot HCl \cdot H_2O$ 47.41 6.89 11.06 (47.50 6.82 10.80)	1	43	6	1
26	2-SCH_3	81	—	180—182	C	$C_{12}H_{16}N_2S_3$ 50.69 5.67 9.85 (50.65 5.70 9.68)	1	30	10	10
27	2-SO_2CH_3	62	—	237—238.5	G	$C_{12}H_{16}N_2O_2S_3$ 45.55 5.10 8.85 (45.67 5.19 8.84)	1	3	1	*f*)
28	2-SC_2H_5	76	—	161.5—163.5	A	$C_{13}H_{18}N_2S_3$ 52.31 6.08 9.39 (52.35 6.11 9.32)	1	16	8.5	100
29	2-SC_3H_7	75	—	143.5—145	A	$C_{14}H_{20}N_2S_3$ 53.81 6.45 8.96 (54.00 6.55 8.98)	1	26	17.5	100
30	2-SC_4H_9	81	—	162—164	A	$C_{15}H_{22}N_2S_3$ 55.17 6.79 8.58 (55.06 6.81 8.32)	1	11	11.5	*f*)
31	2-S(CH_2)$_2$N⟨ ⟩	77	HCl	193—195 (dec.)	D	$C_{17}H_{25}N_3S_2 \cdot HCl$ 50.53 6.49 10.40 (50.68 6.70 10.19)	1	19	27	30

32	2-S(CH$_2$)$_2$N⟨⟩	30	HCl	186—189 (dec.)	H	$C_{18}H_{27}N_3S_3 \cdot HCl$ 51.71 6.75 10.05 (51.66 6.86 9.84)	1	45	40	100
33	2-S(CH$_2$)$_2$N⟨O⟩	84	HCl	204—206 (dec.)	I	$C_{17}H_{25}N_3OS_3 \cdot HCl$ 48.61 6.24 10.00 (48.70 6.48 9.69)	1	37	10	3
34	2-S(CH$_2$)$_2$N⟨N–CH$_3$⟩	79	2HCl	209—211 (dec.)	F	$C_{18}H_{28}N_4S_3 \cdot 2HCl \cdot 1/2\,H_2O$ 45.18 6.53 11.71 (45.37 6.60 11.44)	1	12	17.5	*f*)
35	2-S(CH$_2$)$_3$N⟨⟩	69	HCl	216—218 (dec.)	F	$C_{19}H_{29}N_3S_3 \cdot HCl$ 52.81 7.00 9.72 (52.67 7.04 9.59)	1	15	37	*h*)
36	2-OH	37	—	177—178	A	$C_{11}H_{14}N_2OS_2$ 51.94 5.55 11.01 (51.87 5.50 11.18)	1	2	1	*f*)
37	2-OCH$_3$	80	—	153—154	A	$C_{12}H_{16}N_2OS_2$ 53.70 6.01 10.44 (53.69 6.03 10.41)	1	30	10	3
38	3-OCH$_3$	86	—	81—82	D	$C_{12}H_{16}N_2OS_2$ 53.70 6.01 10.44 (53.55 5.99 10.28)		NT		*f*)
39	4-OCH$_3$	90	—	104—105	D	$C_{12}H_{16}N_2OS_2$ 53.70 6.01 10.44 (53.75 6.14 10.41)		NT		*f*)
40	2.4-(OCH$_3$)$_2$	54	—	119.5—121	J	$C_{13}H_{18}N_2O_2S_2$ 52.32 6.08 9.39 (52.23 6.13 9.34)	1	4.5	< 10	3
41	2.5-(OCH$_3$)$_2$	84	—	178—180	K	$C_{13}H_{18}N_2O_2S_2$ 52.32 6.08 9.39 (52.21 6.04 9.35)		NT		*f*)
42	2-OC$_2$H$_5$	78	—	169.5—171.5	K	$C_{13}H_{18}N_2OS_2$ 55.29 6.42 9.92 (55.21 6.38 9.76)	1	28	10	10
43	2-OC$_3$H$_7$	89	—	149—152	A	$C_{14}H_{20}N_2OS_2$ 56.72 6.80 9.45 (56.68 6.83 9.39)	1	21	20	30
44	2-OC$_4$H$_9$	60	—	138—140	L	$C_{15}H_{22}N_2OS_2$ 58.03 7.14 9.02 (58.09 7.21 9.01)	1	39	30	30

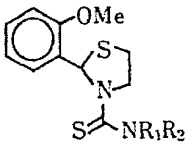
TABLE II. (continued)

Compd. No.	R	Yield (%)	Salt[a]	mp (°C)	Recrystn.[b] solvent	Analysis (%) Calcd (Found) C	H	N	Myocardial contractility: Anesthetized dog[c] Dose mg/kg i.v.	LV$dP/dt_{max}$ Δ%	Duration min	Isolated guinea pig heart[d] MED[e] μg/heart
						$C_{16}H_{24}N_2OS_2$						
45	2-OC_5H_{11}	60	—	117—119	C	59.22	7.45	8.63	1	6	8	100
						(59.32	7.51	8.60)				
						$C_{17}H_{26}N_2OS_2$						
46	2-OC_6H_{13}	62	—	115—118	C	60.30	7.74	8.28	1	29	9	*f*)
						(60.01	7.51	8.20)				
						$C_{23}H_{38}N_2OS_2$						
47	2-$OC_{12}H_{25}$	37	—	109—112	C	67.35	9.06	6.63	1	0	0	*f*)
						(67.08	9.10	6.78)				
						$C_{17}H_{18}N_2OS_2$						
48	2-OC_6H_5	80	—	160—162	C	61.79	5.49	8.48	1	32.5	26.5	100
						(61.82	5.47	8.45)				
						$C_{18}H_{20}N_2OS_2$						
49	2-$OCH_2C_6H_5$	68	—	177.5—181	K	62.76	5.85	8.13		NT		30
						(62.59	5.95	8.10)				
						$C_{14}H_{18}N_2O_3S_2$						
50	2-$OCH_2CO_2CH_3$	83	—	139.5—140.5	A	51.52	5.55	8.58	1	8	4	*f*)
						(51.47	5.62	8.44)				
						$C_{13}H_{16}N_2O_3S_2$						
51	2-OCH_2CO_2H[f]	61	—	184—185	B	49.98	5.16	8.97	1	2	2	*f*)
						(49.96	5.21	8.88)				
						$C_{14}H_{20}N_2O_2S_2$						
52	2-$O(CH_2)_2OCH_3$	75	—	142—144	A	53.80	6.45	9.00	1	7	3	100
						(53.79	6.44	9.02)				
						$C_{14}H_{20}N_2OS_3$						
53	2-$O(CH_2)_2SCH_3$	80	—	153—155	M	51.19	6.14	8.53	1	15	12	100
						(51.40	6.20	8.49)				
						$C_{19}H_{22}N_2OS_3$						
54	2-$O(CH_2)_2SC_6H_5$	86	—	142—145	K	58.43	5.68	7.17	1	25	5	*f*)
						(58.73	5.75	7.15)				
						$C_{15}H_{23}N_3OS_2 \cdot HCl$						
55	2-$O(CH_2)_2N(CH_3)_2$	52	HCl	209—212 (dec.)	F	49.78	6.68	11.61	1	−5	—	*f*)
						(49.77	6.68	11.66)				

56	2-O(CH_2)$_2$N(C_2H_5)$_2$	61	HCl	210—211 (dec.)	F	$C_{17}H_{27}N_3OS_2 \cdot HCl$ 52.36 7.24 10.77 (52.12 7.26 10.72)	1	−1	—	*f*)
57	2-O(CH_2)$_2$N⟨ ⟩	81	2HCl	140—145	D	$C_{17}H_{25}N_3OS_2 \cdot 2HCl \cdot 1/2\,H_2O$ 47.10 6.51 9.69 (47.39 6.59 9.51)	1	5	—	*h*)
58	2-O(CH_2)$_2$N⟨ ⟩	41	HCl	209—211 (dec.)	F	$C_{18}H_{27}N_3OS_2 \cdot HCl$ 53.78 7.02 10.45 (53.65 7.02 10.49)	0.3	20	16	*f*)
59	2-O(CH_2)$_2$N⟨ O ⟩	91	HCl	207—208	D	$C_{17}H_{25}N_3O_2S_2 \cdot HCl$ 50.34 6.49 10.40 (50.52 6.48 10.42)	1	6	12.5	100
60	2-O(CH_2)$_3$N(CH_3)$_2$	73	HCl	213—215 (dec.)	F	$C_{16}H_{25}N_3OS_2 \cdot HCl$ 51.11 6.97 11.18 (50.96 6.96 11.09)	1	−25	5	*f*)
61	2-O(CH_2)$_3$N(C_2H_5)$_2$	54	HCl	195—196 (dec.)	F	$C_{18}H_{29}N_3OS_2 \cdot HCl$ 53.51 7.48 10.40 (53.57 7.53 10.35)	1	−23	—	*f*)
62	2-O(CH_2)$_3$N(C_3H_7)$_2$	87	HCl	192—194 (dec.)	F	$C_{20}H_{33}N_3OS_2 \cdot HCl$ 55.60 7.93 9.73 (55.73 7.94 9.76)	1	−12	—	*h*)
63	2-O(CH_2)$_3$N⟨ ⟩	71	2HCl	122—125	D	$C_{18}H_{27}N_3OS_2 \cdot 2HCl \cdot H_2O$ 47.36 6.85 9.21 (47.64 7.04 9.27)	1	2	1	*h*)
64	2-O(CH_2)$_3$N⟨ ⟩	55	2HCl	129—133	D	$C_{19}H_{29}N_3OS_2 \cdot 2HCl \cdot H_2O$ 48.50 7.07 8.93 (48.48 7.37 8.91)	1	−4	—	*h*)
65	2-O(CH_2)$_3$N⟨ O ⟩	72	2HCl	160—165	D	$C_{18}H_{27}N_3O_2S_2 \cdot 2HCl$ 47.57 6.43 9.25 (47.29 6.51 9.08)	1	19	15	*f*)
66	2-O(CH_2)$_3$N⟨ N–CH_3 ⟩	86	2HCl	195—198 (dec.)	D	$C_{19}H_{30}N_4OS_2 \cdot 2HCl \cdot H_2O$ 47.00 7.06 11.54 (47.18 6.95 11.57)	1	2	1	*f*)
67	2-O(CH_2)$_3$N⟨ N–C_6H_5 ⟩	63	Fum	125—128	D	$C_{24}H_{32}N_4OS_2 \cdot C_4H_4O_4$ 58.72 6.34 9.78 (58.28 6.31 9.68)	0.3	68	60	10
	Amrinone						0.3	25	25	10

a) Fum=fumarate. *b*) A=EtOH, B=MeOH–H_2O, C=AcOEt–hexane, D=EtOH–Et_2O, E=MeOH–THF, F=MeOH–Et_2O, G=DMF–iso-Pr_2O, H=iso-PrOH–Et_2O, I=MeOH–iso-Pr_2O, J=EtOH–iso-Pr_2O, K=AcOEt, L=iso-PrOH, M=THF. *c*) For methodology, see reference 14. *d*) For methodology, see reference 2*a*. *e*) Minimum effective dose (minimum dose required to cause an increase in contractile force of more than 0.3 g). *f*) Ineffective at 100 μg/heart. *g*) Prepared from the *N*-acetate (I_{18}) by alkaline hydrolysis. *h*) Caused a decrease in contractile force. *i*) This compound was prepared by alkaline hydrolysis of the ester (I_{50}). NT=not tested.

TABLE III. *N*-Substituted-2-(2-methoxyphenyl)thiazolidine-3-thiocarboxamides (XVIII)



Compd. No.	R_1	R_2	Yield (%)	Salt	mp (°C)	Recrystn.[a] solvent	Analysis (%) Calcd (Found)			Myocardial contractility			
										Anesthetized dog[b]			Isolated guinea pig heart[c] MED[d] μg/heart
							C	H	N	Dose mg/kg i.v.	LV$dP/dt_{max}$ Δ%	Duration min	
							$C_{11}H_{14}N_2OS_2$						
1	H	H	52[e]	—	182.5—184.5	N	51.94 (51.96)	5.55 (5.57)	11.01 (10.96)	1	−3	—	*f*
							$C_{13}H_{18}N_2OS_2$						
2	H	C_2H_5	64	—	131.5—134	D	55.29 (55.49)	6.42 (6.47)	9.92 (9.91)	1	−7	—	*f*
							$C_{15}H_{22}N_2OS_2$						
3	H	iso-C_4H_9	55	—	86—91	H	58.03 (58.14)	7.14 (7.20)	9.02 (9.21)	1	2.3	1	*f*
							$C_{17}H_{18}N_2OS_2$						
4	H	C_6H_5	64	—	142—144	C	61.79 (61.63)	5.49 (5.52)	8.48 (8.46)	1	3.5	1	*f*

							$C_{15}H_{23}N_3OS_2 \cdot HCl$					
5	H	$(CH_2)_2N(CH_3)_2$	82	HCl	182—185 (dec.)	A	49.78 6.68 11.61 (49.50 6.83 11.56)	1	−6	—	*f*)
							$C_{15}H_{20}N_2O_3S_2$					
6	H	$CH_2CO_2C_2H_5$	89	—	79—83	J	52.92 5.92 8.23 (52.81 5.75 8.11)	1	0	0	*f*)
							$C_{13}H_{16}N_2O_3S_2$					
7	H	CH_2CO_2H	80[g]	—	145—146 (dec.)	O	49.98 5.16 8.97 (50.15 5.18 8.95)	1	0	0	*f*)
							$C_{14}H_{18}N_2O_3S_2$					
8	H	$CO_2C_2H_5$	89	—	139—141.5	N	51.51 5.56 8.58 (51.44 5.54 8.58)	1	1.4	1	*f*)
							$C_{13}H_{16}N_2O_2S_2$					
9	H	$COCH_3$	28	—	143—146	K	52.68 5.44 9.45 (52.48 5.51 9.32)	1	0	0	*f*)
							$C_{18}H_{18}N_2O_2S_2$					
10	H	COC_6H_5	78	—	142—144.5	C	60.31 5.06 7.81 (60.25 4.92 7.74)	1	0	0	*f*)
							$C_{13}H_{18}N_2OS_2$					
11	CH_3	CH_3	2	—	170—173	C	55.29 6.42 9.92 (55.49 6.55 9.81)	1	−2	—	*f*)

a) N=MeOH, O=AcOEt–benzene. See also footnote *b* in Table II. *b*—*d*) See footnotes *c*—*e* in Table II, respectively. *e*) Prepared by alkaline hydrolysis of the *N*-acetate ($XVIII_9$). *f*) See footnote *f* in Table II. *g*) Prepared by alkaline hydrolysis of the ester ($XVIII_6$).

nificant cardiotonic activity (see below), the effect of varying the substituents on the thiocarboxamide group was examined with this compound. Reaction of XVII (R=2-OMe) with various isothiocyanates or dimethylthiocarbamoyl chloride gave the *N*-substituted thiocarboxamides (XVIII) listed in Table III.

With regard to modification of the thiazolidine ring, reaction of 2-methoxybenzaldehyde with 2-aminomethyl-2-propanethiol[12] and 3-amino-1-propanethiol followed by treatment with MeNCS gave the *gem*-dimethyl derivative (XIXa) and the 1,3-thiazine derivative (XIXb), respectively (Chart 3). The oxazolidine analogue (XIXc) was prepared by reaction of 2-methoxybenzaldehyde with ethanolamine[13] followed by treatment with MeNCS. The benzothiazoline derivative (XX) was prepared in a similar manner by the use of 2-aminothiophenol. Physical properties of XIX and XX are summarized in Table IV.

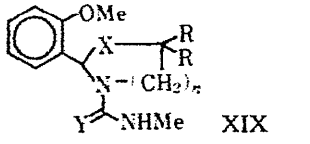
Pharmacology and Structure–Activity Relationships

The positive inotropic activities of the novel 2-phenylthiazolidine derivatives were determined in the isolated guinea pig heart by measuring the increase in cardiac contractile force[2a] and also in anesthetized dogs by measuring percent increase in maximum dP/dt of left ventricular pressure[14] after i.v. administration by the method reported previously. The results are listed in Tables II—IV together with comparative data for amrinone.

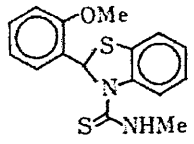
Effects of the substituents on the benzene ring of *N*-methyl-2-phenylthiazolidine-3-thiocarboxamides (I) were examined first (Table II). The presence of substituents such as Me (I_5) and OMe (I_{37}) at the *ortho* position to the thiazolidine group conferred potent positive inotropic activity, which was not blocked by propranolol. Substitution at any other position was ineffective, as exemplified by the corresponding *meta* and *para* isomers ($I_{6,7}$ and $I_{38,39}$). The 2-dimethylamino (I_{19}), 2-methoxycarbonyl (I_{16}), and 2-methylthio (I_{26}) derivatives also exhibited potent activity. On the other hand, introduction of hydrophilic substituents such as OH (I_{36}), NH_2 (I_{17}), NHAc (I_{18}), and CO_2H (I_{15}) at the *ortho* position resulted in disappearance of the activity. In a series of *ortho*-alkoxyphenyl derivatives (I_{37}, I_{42-47}), the duration of action in anesthetized dogs tends to increase with increasing length of the alkylene chain, the butoxy derivative (I_{44}) exhibiting the most prolonged activity. However, lengthening of the alkylene chain in this series caused a major decrease in *in vitro* activity. Similar results were generally observed in the 2-alkyl and 2-alkylthio series. The activity of 2-dialkylaminophenyl derivatives decreased with increasing bulkiness of the *N*-alkyl group. Thus, a change from methyl to butyl (I_{19}—I_{22}) resulted in gradual decrease in both *in vitro* and *in vivo* activities. The positive inotropic activity of these 2-aminophenyl derivatives was invariably accompanied by significant elevation of blood pressure. This was also the case for the 2-((2-methylthio)ethylamino)phenyl derivative (I_{24}), one of the most potent compounds in this series. After examination of a number of *ortho*-alkoxy and -alkylthio derivatives having a hetero atom in the alkylene chain, the phenylpiperazinopropoxy derivative (I_{67}) was found to exhibit more potent and longer-lasting positive inotropic activity than amrinone with only small effects on both heart rate and blood pressure.

The effects of *N*-substituents on the thiocarboxamide group on the activity were examined in a series of 2-methoxyphenyl derivatives (XVIII), listed in Table III. Replacement of the methyl group of I_{37} by hydrogen, a larger alkyl group, or an acyl group all resulted in disappearance of the activity. The *N*-dimethyl derivative ($XVIII_{11}$) was also ineffective. The effect of modifying the thiazolidine moiety was also examined in a series of 2-methoxyphenyl derivatives (Table IV). Introduction of a geminal dimethyl group at C_5 of the thiazolidine ring (XIXa) and conversion of thiazolidine to the 1,3-thiazine (XIXb), oxazolidine (XIXc), or benzothiazoline (XX) all resulted in a marked fall in activity. Finally, conversion of the *N*-methylthiocarboxamide (I_{37}) to the corresponding carboxamide (XIXd) also conferred reduced activity.

TABLE IV. Modified Thiazolizine Analogues (XIX and XX)



XIX



XX

Compd. No.	X	Y	R	n	Yield (%)	mp (°C)	Recrystn.[a] solvent	Analysis (%) Calcd (Found) C	H	N	Myocardial contractility: Anesthetized dog[b] Dose mg/kg i.v.	LV$dP/dt_{max}$ Δ%	Duration min	Isolated guinea pig heart[c] MED[d] μg/heart
XIXa	S	S	CH_3	1	68	139—140	C	$C_{14}H_{20}N_2OS_2$ 56.72 (56.78)	6.80 (6.86)	9.45 (9.39)	1	0	0	100
XIXb	S	S	H	2	75	141—143	A	$C_{13}H_{18}N_2OS_2$ 55.29 (55.41)	6.42 (6.49)	9.92 (9.95)	1	−6.5		e)
XIXc	O	S	H	1	63	142—147	A	$C_{12}H_{16}N_2O_2S$ 57.12 (57.10)	6.39 (6.38)	11.10 (11.03)		NT		30
XIXd	S	O	H	1	75	161—164	D	$C_{12}H_{16}N_2O_2S$ 57.12 (57.39)	6.39 (6.31)	11.10 (11.09)		NT		30
XX					14	185—186	C	$C_{16}H_{16}N_2OS_2$ 60.73 (66.77)	5.10 (5.04)	8.85 (8.78)	1	0	0	e)

a) See footnote *b* in Table II. *b*—*e*) See footnotes *c*—*f* in Table II, respectively. NT = not tested.

Thus, examination of the positive inotropic activities of a large number of novel 2-phenylthiazolidine-3-thiocarboxamides (II) and related compounds clarified the structure–activity relationship of this series and led to the discovery of the phenylpiperazino derivative (I_{67}) with potent and long-lasting activity. Further modification of this new lead as a cardiotonic agent is in progress and will be the subject of a forthcoming 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 at 60 MHz on a JEOL PMX-60 spectrometer with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were measured with Hitachi RMU-6M instrument.

2-Isopropylbenzaldehyde (VIc)——An ethereal solution of isopropylmagnesium bromide (prepared from isopropyl bromide (9.0 g) and Mg (1.96 g)) was added to a stirred solution of III[5a] (5.0 g, 24.4 mmol) in tetrahydrofuran (THF) (20 ml) at room temperature. The mixture was stirred for 3.5 h, then aq. NH_4Cl solution was added dropwise under ice-cooling, and the aq. layer was extracted with Et_2O. The combined organic layer was washed with water, dried over Na_2SO_4, and evaporated to give IV (R = isopropyl, 5.23 g, 99%) as an oil. IR ν_{max}^{neat} cm^{-1}: 1645 (C=N). ^{1}H-NMR ($CDCl_3$) δ: 1.23 (6H, d, J = 7 Hz, $CH(CH_3)_2$), 1.37 (6H, s, 2 × CH_3), 3.69 (1H, m, $CH(CH_3)_2$), 4.05 (2H, s, OCH_2–), 6.99—7.67 (4H, m, Ar-H). MS m/z: 217 (M^+). This oil (1.0 g, 4.6 mmol) was heated with methyl iodide (2.25 ml) in nitromethane (2 ml) at 70 °C overnight. The cooled reaction mixture was diluted with Et_2O. The precipitate was collected, washed with Et_2O, and dried to give V (R = isopropyl, 1.54 g, 96%), mp 153—156 °C. $NaBH_4$ (0.17 g, 4.5 mmol) was added portionwise to a solution of V (R = isopropyl, 1.5 g, 4.3 mmol) in EtOH (10 ml) at room temperature, and the mixture was stirred for 4 h. After removal of the solvent, the residual oil was heated with 5% aq. HCl (10 ml) at 65 °C for 20 min and extracted with Et_2O. The extracts were washed with water, dried, and concentrated. The residue was chromatographed on silica gel with benzene to give VIc (0.41 g, 64%; 61% overall yield from III) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (6H, d, J = 7 Hz, $CH(CH_3)_2$), 3.95 (1H, m, $CH(CH_3)_2$), 7.15—7.90 (4H, m, Ar-H), 10.43 (1H, s, CHO). MS m/z: 148 (M^+). Compounds VIa, b, d, e, g were prepared in a similar manner and are listed in Table I.

2-(*N*-(2-Methoxyethyl)-*N*-methylamino)benzyl Alcohol (IX, $R_1 = CH_2OCH_3$, $R_2 = CH_3$)——A mixture of VII (2.0 g, 16.3 mmol) and 2-methoxyacetaldehyde (1.80 g, 24.5 mmol) in MeOH (30 ml) was stirred at room temperature overnight. The MeOH was evaporated off, and the resulting oil was dissolved in THF (10 ml). This solution was added dropwise to a mixture of $NaBH_4$ (1.0 g, 26.4 mmol) in AcOH (8 ml) under ice-cooling, and the mixture was stirred at room temperature for 5 h. The reaction mixture was made alkaline with aq. K_2CO_3 and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was chromatographed on silica gel with AcOEt–benzene (1 : 4) to give VIII ($R_1 = CH_2OCH_3$, 2.27 g, 77%) as an oil. IR ν_{max}^{neat} cm^{-1}: 3350 (OH), 1600. ^{1}H-NMR ($CDCl_3$) δ: 3.2—4.0 (5H, m, 2 × CH_2 and NH), 3.31 (3H, s, OCH_3), 4.54 (2H, s, CH_2O), 4.5—4.8 (1H, br, OH), 6.5—7.4 (4H, m, Ar-H). Treatment of this oil (2.2 g, 12.3 mmol) with 37% HCHO (3 ml) and $NaBH_4$ (1.0 g, 26.4 mmol) in the same manner as described above gave IX ($R_1 = CH_2OCH_3$, $R_2 = CH_3$, 2.0 g, 83.4%; 64.2% overall yield from VII) as an oil. IR ν_{max}^{neat} cm^{-1}: 3400 (OH). ^{1}H-NMR ($CDCl_3$) δ: 2.69 (3H, s, N–CH_3), 3.29 (3H, s, OCH_3), 3.05—3.65 (4H, m, 2 × CH_2), 4.66 (2H, s, CH_2O), 5.20 (1H, br s, OH), 7.1—7.4 (4H, m, Ar-H). Compounds IXb, c were prepared in a similar manner.

***N*,*N*-Dibutylaminobenzyl Alcohol (IX: $R_1 = C_3H_7$, $R_2 = C_4H_9$)**——BF_3-etherate (27 g) was added dropwise to a mixture of X ($R_1 = C_3H_7$, $R_2 = C_4H_9$, 12.4 g, 38.9 mmol) and $NaBH_4$ (9.0 g, 238 mmol) in THF (200 ml) with stirring. The mixture was refluxed overnight, then decomposed with water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated *in vacuo*. The residual oil was chromatographed on silica gel with AcOEt–benzene (1 : 6) to give IX ($R_1 = C_3H_7$, $R_2 = C_4H_9$, 6.29 g, 69%) as an oil. ^{1}H-NMR ($CDCl_3$) δ: 0.65—1.10 (6H, m, 2 × CH_2CH_3), 1.10—1.80 (8H, m, 2 × CH_2CH_2), 2.80—3.05 (4H, m, 2 × CH_2N), 4.80 (2H, s, CH_2O), 6.08 (1H, s, OH), 7.05—7.30 (4H, m, Ar-H).

2-(*N*-(2-Methoxyethyl)-*N*-methylamino)benzaldehyde (XIa)——A mixture of IX ($R_1 = CH_2OCH_3$, $R_2 = CH_3$, 2.00 g, 10.3 mmol) and $BaMnO_4$ (5.23 g) in $CHCl_3$ (40 ml) was refluxed for 6 h with stirring. The inorganic material was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel with AcOEt–hexane (1 : 4) to give XIa (1.7 g, 85.5%; 55% overall yield from VII) as an oil. IR ν_{max}^{neat} cm^{-1}: 1680 (CHO). ^{1}H-NMR ($CDCl_3$) δ: 2.94 (3H, s, NCH_3), 3.31 (3H, s, OCH_3), 3.31—3.80 (4H, m, 2 × CH_2), 7.00—7.85 (4H, m, Ar-H), 10.31 (1H, s, CHO). Compounds XIb—f were prepared in a similar manner and are listed in Table I.

2-(2-(4-Methylpiperazino)ethylthio)benzaldehyde (XIVc)——A mixture of 2-mercaptobenzyl alcohol (XII, 3.6 g, 25.7 mmol), 1-chloroethyl-4-methylpiperazine dihydrochloride (6.0 g, 25.7 mmol), and K_2CO_3 (10.5 g, 3 × 25.7 mmol) in dimethyl formamide (DMF, 25 ml) was stirred overnight under argon, then poured into water. Extraction with AcOEt followed by evaporation of the solvent gave XIII (R = CH_2CH_2N⟨⟩N–CH_3, 4.9 g) as an oil, which was

oxidized with $BaMnO_4$ (15 g) in CH_2Cl_2 (150 ml) under reflux for 4 h. The inorganic material was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel with $CHCl_3$–MeOH (10:1) to give XIVc (2.3 g, 34.2% overall yield from XII) as an oil. IR ν_{max}^{neat} cm^{-1}: 1680 (CHO). ^{1}H-NMR ($CDCl_3$) δ: 2.27 (3H, s, NCH_3), 2.47—2.77 (10H, m, 5 × CH_2), 2.99—3.24 (2H, m, SCH_2), 7.17—7.92 (4H, m, Ar-H), 10.48 (1H, s, CHO). MS *m/z*: 264 (M^+). Compounds XIVa, b, d were prepared in a similar manner and are listed in Table I.

2-(2-Methoxyethoxy)benzaldehyde (XVIa)——A mixture of XV (2.63 g, 21.6 mmol), 1-bromo-2-methoxyethane (4.00 g, 28.8 mmol), and K_2CO_3 (3.0 g, 21.7 mmol) in DMF (30 ml) was stirred for 16 h at room temperature, then heated at 50 °C for 16 h. The mixture was cooled, diluted with water, and extracted with AcOEt. The extracts were washed with water, dried, and evaporated. The residue was chromatographed on silica gel and eluted with AcOEt–hexane (1:9) to give 3.20 g of XVIa as an oil. IR ν_{max}^{neat} cm^{-1}: 1680 (CHO). ^{1}H-NMR ($CDCl_3$) δ: 3.43 (3H, s, OCH_3), 3.70—3.90 (2H, m, CH_2–C), 4.10—4.30 (2H, m, C–CH_2), 6.80—7.90 (4H, m, Ar-H), 10.49 (1H, s, CHO). MS *m/z*: 180 (M^+). Compounds XVIb, c were prepared in a similar manner (Table I).

Preparation of 2-Aryl-*N*-methylthiazolidine-3-thiocarboxamides (I)——The following prodedure is representative. A solution of 2-methoxybenzaldehyde (10.0 g, 73.4 mmol) in EtOH (20 ml) was added to a stirred suspension of cysteamine hydrochloride (8.34 g, 73.4 mmol) and NaOH (3.00 g, 75 mmol) in EtOH (160 ml) under argon. The mixture was refluxed for 1 h, and a solution of methyl isothiocyanate (5.90 g, 80.7 mmol) in EtOH (20 ml) was added, then the whole was refluxed for 3 h. After removal of the solvent, the residue was chromatographed on silica gel with $CHCl_3$–EtOH (30:1) to give 14.6 g of 2-(2-methoxyphenyl)-*N*-methylthiazolidine-3-thiocarboxamide (I_{37}), mp 154—155 °C (from EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3365, 3280 (NH). ^{1}H-NMR ($CDCl_3$) δ: 2.99 (3H, d, J=4.5 Hz, $NHCH_3$), 2.90—3.25 (2H, m, SCH_2), 3.93 (3H, s, OCH_3), 4.09—4.80 (2H, m, NCH_2), 5.50 (1H, br, NH), 6.31 (1H, s, S–CH–N), 6.30—7.40 (4H, m, Ar-H). MS *m/z*: 268 (M^+).

2-(2-Aminophenyl)-*N*-methylthiazolidine-3-thiocarboxamide (I_{17})——A mixture of I_{18} (0.95 g, 3.2 mmol) and 20% KOH (5 ml) in MeOH (30 ml) was refluxed for 30 h. The reaction mixture was evaporated *in vacuo* and diluted with water. The precipitate was collected and recrystallized from EtOH to give 0.22 g of I_{17}, mp 159—161 °C. IR ν_{max}^{neat} cm^{-1}: 3445, 3360, 3330 (NH_2). ^{1}H-NMR ($CDCl_3$) δ: 3.00 (3H, d, J=4.5 Hz, $NHCH_3$), 3.00—3.20 (2H, m, SCH_2), 3.8 (2H, br s, NH_2), 4.10—4.70 (2H, m, NCH_2), 5.55 (1H, br s, NH), 6.12 (1H, s, S–CH–N), 6.68—7.27 (4H, m, Ar-H). MS *m/z*: 253 (M^+). Compound $XVIII_1$ was prepared from $XVIII_0$ in a similar manner.

2-(2-Carboxymethoxyphenyl)-*N*-methylthiazolidine-3-thiocarboxamide (I_{51})——A mixture of the ester (I_{50}, 7.00 g, 21.5 mmol) and NaOH (0.945 g, 23.7 mmol) in 50% aq. MeOH (100 ml) was stirred at room temperature overnight. The mixture was acidified with 10% HCl and extracted with AcOEt. The extracts were washed with water, dried over Na_2SO_4, and evaporated. The residue was recrystallized from aq. MeOH to give 4.1 g of I_{51}, mp 184—185 °C. IR ν_{max}^{Nujol} cm^{-1}: 3380 (NH), 1720 (COOH). ^{1}H-NMR (DMSO-d_6) δ: 2.86 (3H, d, J=4 Hz, N–CH_3), 3.00—3.21 (2H, m, –SCH_2), 3.80—4.30 (2H, m, N–CH_2), 4.72 (2H, s, O–CH_2), 6.80—7.30 (5H, m, S–CH–N and Ar-H), 7.50 (1H, br s, NH). MS *m/z*: 312 (M^+). Compound $XVIII_7$ was prepared from $XVIII_6$ in a similar manner.

2-(2-Methoxyphenyl)-*N*,*N*-dimethylthiazolidine-3-thiocarboxamide ($XVIII_{11}$)——A solution of dimethylthiocarbamoyl chloride (2.96 g, 24 mmol) in DMF (10 ml) was added to a solution of 2-(2-methoxyphenyl)thiazolidine (XVII, R=2–OCH_3, 3.90 g, 20 mmol) and 4-dimethylaminopyridine (2.93 g, 24 mmol) in DMF (40 ml) under ice-cooling, and the mixture was stirred at room temprature for 20 h, then poured into water and extracted with AcOEt. The extracts were washed with 5% HCl, sat. $NaHCO_3$, and water, successively and dried over Na_2SO_4. After removal of the solvent, the residual oil was chromatographed on silica gel with benzene–AcOEt (100:1) to give 0.10 g of $XVIII_{11}$, mp 170—173 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 1600 (Ar). ^{1}H-NMR ($CDCl_3$) δ: 3.30 (6H, s, $N(CH_3)_2$), 2.93—3.75 (3H, m, S–CH_2, NCH–H), 3.88 (3H, s, OCH_3), 4.50—4.85 (1H, m, NCH–H), 6.59 (1H, s, S–CH–N), 6.76—7.35 (4H, m, Ar-H). MS *m/z*: 282 (M^+).

2-(2-Methoxyphenyl)-*N*-methyloxazolidine-3-thiocarboxamide (XIXc)——A solution of MeNCS (1.40 g, 18.4 mmol) was added to a solution of 2-(2-methoxyphenyl)oxazolidine[13] (3.00 g, 16.7 mmol) in EtOH (30 ml), and the mixture was refluxed for 4 h. The mixture was concentrated *in vacuo*, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with water, dried, and evaporated. The crystalline residue was collected by filtration, washed with Et_2O, and recrystallized from EtOH to give 2.66 g of XIXc, mp 142—147 °C. IR ν_{max}^{Nujol} cm^{-1}: 3400, 3250 (NH). ^{1}H-NMR ($CDCl_3$) δ: 3.00 (3H, d, J=4.5 Hz, $NHCH_3$), 3.92 (3H, s, OCH_3), 3.81—4.50 (4H, m, –OCH_2CH_2–N), 5.50 (1H, br, NH), 6.46 (1H, s, O–CH–N), 6.87—7.50 (4H, m, Ar-H). MS *m/z*: 252 (M^+).

2-(2-Methoxyphenyl)-*N*-methylthiazolidine-3-carboxamide (XIXd)——A solution of methyl isocyanate (3.80 g, 66 mmol) in THF (10 ml) was added to a solution of 2-(2-methoxyphenyl)thiazolidine (XVII, R=2–OCH_3, 10.7 g, 55 mmol) in THF. The mixture was refluxed for 4 h and concentrated *in vacuo*, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with water, dried, and evaporated. The crystalline residue was collected by filtration, washed with Et_2O, and recrystallized from EtOH–Et_2O to give 10.4 g of XIXd, mp 161—164 °C. IR ν_{max}^{Nujol} cm^{-1}: 3310 (NH), 1635 (CO). ^{1}H-NMR ($CDCl_3$) δ: 2.73 (3H, d, J=4.5 Hz, $NHCH_3$), 2.88—3.08 (2H, m, S–CH_2), 3.60—4.50 (3H, m, N–CH_2 and NH), 3.89 (3H, s, OCH_3), 6.16 (1H, s, S–CH–N), 6.78—7.41 (4H, m, Ar-H).

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Studies on Sesquiterpene Glycosides from *Prenanthes acerifolia* BENTH.

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A new guaiane-type sesquiterpene, prenanthelide A (1), and three new guaiane-type sesquiterpene glycosides, prenanthesides A (3), B (4) and C (7), have been isolated from *Prenanthes acerifolia* BENTH., together with three known guaiane-type sesquiterpene glycosides, ixerin D (2), 8-epidesacylcynaropicrin glucoside (5) and crepiside E (6). The structures of the new compounds were established on the basis of chemical and spectral data.

Keywords——*Prenanthes acerifolia*; Compositae; sesquiterpene glycoside; guaianolide; prenanthelide; prenantheside

In connection with a study on the sesquiterpene glycosides of some plants in Compositae, we have also investigated *Prenanthes acerifolia* BENTH., which is distributed widely in mountainous regions of Japan. From the polar fraction of the water extract of the whole plants, a new guaiane-type sesquiterpene lactone, prenanthelide A (**1**), and three new guaiane-type sesquiterpene glycosides, prenanthesides A (**3**), B (**4**) and C (**7**), have been isolated together with three known guaiane-type sesquiterpene glycosides, ixerin D (**2**), 8-epidesacylcynaropicrin glucoside (**5**) and crepiside E (**6**). The structures of the new compounds were determined on the basis of some chemical transformations and spectroscopic studies.

The three known sesquiterpene glycosides, ixerin D (**2**), 8-epidesacylcynaropicrin glucoside (**5**) and crepiside E (**6**), were identified by direct comparison [proton nuclear magnetic resonance (^{1}H-NMR), carbon-13 nuclear magnetic resonance (^{13}C-NMR) and infrared (IR) spectra] with authentic samples.[1~3]

Prenanthelide A (**1**), $C_{15}H_{20}O_4$, $[\alpha]_D$ +3.5°, mp 200—202 °C, was obtained as colorless plates. The IR spectrum showed hydroxyl (3500 cm^{-1}), γ-lactone ring (1760 cm^{-1}) and five-membered ring ketone (1730 cm^{-1}) absorptions. The ^{1}H-NMR spectrum exhibited a singlet methyl signal at δ 1.22, a doublet methyl signal at δ 1.24 (J=7 Hz), a lactonic proton signal at δ 4.12 (t, J=10 Hz) and two doublet signals at δ 5.54 (J=3.1 Hz), 6.25 (J=3.5 Hz), which are characteristic of an exocyclic α-methylene-γ-lactone. The ^{13}C-NMR spectrum exhibited a tertiary carbon signal at δ 73.1 and this was shifted downfield to δ 82.8 in the acetate (**1a**) which was obtained on acetylation with acetic anhydride under reflux. Furthermore, in the ^{1}H-NMR spectrum of **1a** a proton signal at δ 2.96 (m), which was assigned to H-7 by means of a decoupling experiment, was shifted upfield by 0.14 ppm compared with that of **1**. From these data, **1** was assumed to be a guaianolide-type sesquiterpene lactone having an α-oriented tertiary hydroxyl group at C-10.[1] The circular dichroism (CD) spectrum of **1** showed positive Cotton effects $[\theta]_{303}$ +3757, $[\theta]_{295}$ +3960 and a negative Cotton effect $[\theta]_{253}$ −2310, as in the case of eregoyazin.[4] Therefore, the orientation of the methyl group at C-4 was assumed to be α. Dehydration of **1** with phosphorus oxychloride and pyridine afforded **1b** as a colorless oil. The mass spectrum (MS) of **1b** showed a molecular ion peak at m/z: 246 in agreement with a molecular formula of $C_{15}H_{18}O_3$. The ^{1}H-NMR spectrum exhibited exomethylene proton signals at δ 4.69 (s) and 5.02 (s), which are characteristic of an exomethylene at C-14 of a 3-

keto guaianolide.[1] Thus, **1b** was assumed to be estafiatone, and this was confirmed by comparison of the ^{1}H-NMR spectrum with that of authentic estafiatone.[5] From these data, the structure of prenanthelide A was decided to be **1**.

Prenantheside A (**3**), $C_{21}H_{30}O_9 \cdot H_2O$, $[\alpha]_D$ +10.3°, was obtained as an amorphous powder. The ^{1}H-NMR spectrum exhibited a singlet methyl signal at δ 1.37, a vinyl methyl signal at δ 1.98 (br s) and two olefinic proton signals at δ 5.37 (d, J=3.1 Hz), 6.22 (d, J=3.4 Hz), which were assigned to H_2-13. Acid hydrolysis afforded glucose as the sugar moiety and enzymatic hydrolysis afforded **1** as the aglycone. Therefore, the structure of prenantheside A was decided to be **3**, which is an enol-type glycoside like ixerin S and ixerin T.[2]

Prenantheside B (**4**), $C_{29}H_{36}O_{11} \cdot 1/2H_2O$, $[\alpha]_D$ −7.4°, was obtained as an amorphous powder. The ultraviolet (UV) spectrum showed absorption maxima at 277 and 284 nm. The ^{1}H-NMR spectrum was similar to that of **2** except for the appearance of signals due to *p*-hydroxyphenylacetate [δ 3.73 (2H, s), 7.10 (2H, d, J=8 Hz), 7.31 (2H, d, J=8 Hz)].[1-3] Saponification afforded **2** and *p*-hydroxyphenylacetic acid. In the ^{13}C-NMR spectrum, the carbon signals due to the aglycone moiety showed almost the same chemical shifts, but among the carbon signals due to the glucopyranosyl moiety, C-6′ (δ 64.8) was shifted downfield by 1.7 ppm and C-5′ (δ 74.9) was shifted upfield by 3.5 ppm compared with those of **2**. Therefore, the structure of prenantheside B was decided to be **4**, which has a *p*-hydroxyphenylacetyl group at C-6′ of the glucose moiety.

Prenantheside C (**7**), $C_{30}H_{34}O_{12}$, $[\alpha]_D$ +61.9°, was obtained as an amorphous powder. The IR spectrum showed hydroxyl (3450 cm^{-1}), γ-lactone ring (1760 cm^{-1}) and α,β-unsaturated ester (1715 cm^{-1}) absorptions. The UV spectrum showed absorption maxima at 249, 300 and 331 nm. The ^{1}H-NMR spectrum exhibited a doublet methyl signal at δ 1.55 (J=7 Hz), a proton signal at δ 4.81 (br t, J=6 Hz), two pair of exomethylene proton signals at δ 5.02, 5.21 (each s), 5.45, 5.51 (each br s) and a pair of olefinic proton signals at δ 6.56 and 7.93 (each d, J=16 Hz). Acid hydrolysis afforded glucose as the sugar moiety and saponification afforded caffeic acid and **7a** as the desacyl compound. Comparison with the ^{1}H- and

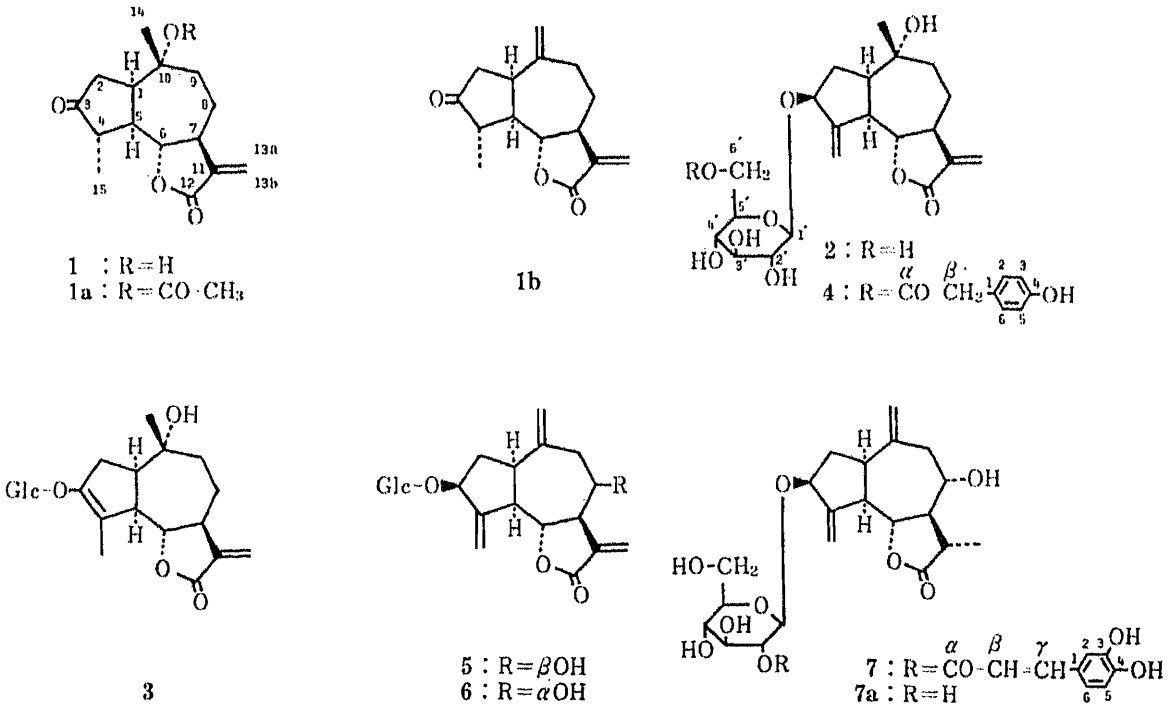


Chart 1

TABLE I. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	1[a]	1a[a]	1b[a]
Aglycone moiety			
6	4.12 (1H, t, J=10 Hz)	4.09 (1H, dd, J=10, 11 Hz)	4.00 (1H, t, J=9 Hz)
7	3.10 (1H, m)	2.96 (1H, m)	
13a	5.54 (1H, d, J=3.1 Hz)	5.52 (1H, d, J=3.1 Hz)	5.58 (1H, d, J=3.1 Hz)
13b	6.25 (1H, d, J=3.5 Hz)	6.23 (1H, d, J=3.6 Hz)	6.30 (1H, d, J=3.5 Hz)
14	1.22 (3H, s)	1.49 (3H, s)	4.69 (1H, s) 5.02 (1H, s)
15	1.24 (3H, d, J=7 Hz)	1.24 (3H, d, J=7 Hz)	1.26 (3H, d, J=7 Hz)
OAc	2.06 (3H, s)		

Proton No.	3[b]	4[b]	7[b]
Aglycone moiety			
3			4.81 (1H, br t, J=6 Hz)
6		4.46 (1H, t, J=10 Hz)	
13a	5.37 (1H, d, J=3.1 Hz)	5.32 (1H, d, J=3.1 Hz)	1.55 (3H, d, J=7 Hz)
13b	6.22 (1H, d, J=3.4 Hz)	6.18 (1H, d, J=3.6 Hz)	
14	1.37 (3H, s)	1.34 (3H, s)	5.02 (1H, s) 5.21 (1H, s)
15	1.98 (3H, br s)	5.46 (1H, br s) 5.66 (1H, br s)	5.45 (1H, br s) 5.51 (1H, br s)
Sugar moiety			
1		5.07 (1H, d, J=8 Hz)	5.07 (1H, d, J=7 Hz)
2			5.76 (1H, t, J=9 Hz)
Ester moiety			
β		3.73 (2H, s)	6.56 (1H, d, J=16 Hz)
γ			7.93 (1H, d, J=16 Hz)
2,6		7.31 (2H, d, J=8 Hz)	
3,5		7.10 (2H, d, J=8 Hz)	

Run at 89.55 MHz in *a*) $CDCl_3$ solution and *b*) pyridine-d_5 solution.

^{13}C-NMR spectral data of **6** indicated that **7a** in the 11,13-dihydro derivative of **6**. The structure of **7a** was finally established by direct comparison (IR, ^{1}H-NMR, ^{13}C-NMR) with the $NaBH_4$ reduction product of **6**.[6] In the ^{13}C-NMR spectrum of **7**, the C-1′ (δ 99.2) and C-3′ (δ 74.9) signals were shifted upfield by 4.8 and 3.6 ppm, respectively, and the C-2′ (δ 76.1) signal was shifted downfield by 0.8 ppm compared with those of **7a**. Thus, the structure of prenantheside C was decided to be **7**, which has a caffeoyl group at C-2 of the glucose moiety. The stereochemistry of the anomeric center was decided to be β from the coupling constant $J_{C_1-H_1}$ (160 Hz).[7]

Experimental

Melting points were taken on a Yanaco MP-500 micromelting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. IR spectra were run on a JASCO A-201 IR spectrometer and UV spectra on a Shimadzu UV-360 recording spectrometer. MS were measured on a JEOL LMS-D 100 mass 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 Hitachi K53 gas chromatograph. High-performance liquid chromatography (HPLC) was conducted on a Kyowa Seimitsu model K880 instrument.

TABLE II. ^{13}C-NMR Chemical Shifts and Coupling Constant

Carbon No.	1[a]	1a[a]	1b[a]	3[b]	4[b]	7[b]
Aglycone moiety						
1	46.4[c]	43.7	39.9	50.4[d]	50.3[g]	45.2
2	40.3	39.0	43.9	32.1[e]	34.8[h]	37.3
3	218.9	217.5	218.8	151.4	79.9	79.7[i]
4	48.2[c]	47.5	47.0	113.6	150.3	150.1
5	50.6	50.7	50.8	52.6[d]	50.8[g]	51.7
6	85.8	85.5	88.7	85.5	81.7	79.3[i]
7	44.5	44.3	44.1	44.0	44.5	56.6
8	25.8	25.5	31.8	25.5	24.8	74.9
9	40.3	31.8	38.5	37.2[e]	35.6[h]	46.3
10	73.1	82.8	148.7	73.2	73.4	144.1
11	141.3	140.2	138.9	142.0	142.7	42.5
12	169.7	169.9	169.7	170.1	170.1	179.0
13	119.3	120.2	121.0	118.5	118.3	16.3
14	27.2	24.6	113.0	28.9	30.6	115.9[j]
15	15.8	16.2	14.3	12.1	113.5	114.3
OAc		22.3, 169.1				
Sugar moiety						
1				101.8 (160 Hz)	103.3	99.2
2				75.0	74.9	76.1
3				78.7[f]	78.0	74.9
4				71.5	71.5	71.8
5				78.5[f]	74.9	78.1
6				62.6	64.8	62.5
Ester moiety						
α					171.9	166.4
β					40.6	115.5[j]
γ						145.8
1					125.2	126.6
2					130.8*	115.0[j]
3					116.2	148.7[k]
4					157.7	147.4[k]
5					116.2	116.5[j]
6					130.8	121.9

Run at 22.5 MHz in *a*) $CDCl_3$ solution and *b*) pyridine-d_5 solution. *c*—*k*) Assignments may be interchanged in each column.

Isolation——Fresh whole plants of *P. acerifolia* (9.5 kg) collected in April 1985, in Shizuoka, Japan, were extracted twice with hot water. The extract was passed through an Amberlite XAD-2 column and the absorbed material was eluted with methanol. The methanol eluate (20 g) was chromatographed on a polyamide column and eluted with water–methanol (75:25). After repeated chromatography of the eluate (13 g) on silica gel with a chloroform–methanol system, seven sesquiterpene lactones were isolated.

Prenanthelide A (1)——Recrystallized from methanol as colorless plates (330 mg), mp 200—202 °C, $[\alpha]_D^{25}$ +3.5° (c=0.85, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.03; H, 7.65. IR ν_{max}^{KBr} cm^{-1}: 3500, 1760, 1730, 1410, 1315, 1260, 1170, 1105, 985, 960, 940, 825. MS *m/z*: 264 (M^+, 5), 246 ($M^+ - H_2O$, 32), 175 (25), 109 (45), 81 (100). CD (c=0.26, $CHCl_3$) [θ] (nm): +3757 (303), +3960 (295), −2310 (253). ^{1}H- and ^{13}C-NMR: Tables I and II.

Ixerin D (2)——Recrystallized from water–methanol as colorless needles (1.0 g), mp 221—222 °C. *Anal.* Calcd for $C_{21}H_{30}O_9 \cdot 1/2H_2O$: C, 57.92; H, 7.18. Found: C, 57.96; H, 7.06. IR ν_{max}^{KBr} cm^{-1}: 3480, 1780, 1670, 1255, 1090, 990. ^{1}H-NMR (pyridine-d_5) δ: 1.28 (3H, s, H_3-14), 5.05 (1H, d, J=7 Hz, H-1′), 5.32 (1H, d, J=3.1 Hz, H-13a), 5.44, 5.70 (each 1H, br s, H_2-15), 6.18 (1H, d, J=3.6 Hz, H-13b). ^{13}C-NMR (pyridine-d_5) δ: 25.1 (C-8), 30.2 (C-14), 35.7 (C-2, C-9), 44.8 (C-7), 50.3, 50.7 (C-1/C-5), 63.1 (C-6′), 72.0 (C-4′), 73.7 (C-10), 75.4 (C-2′), 78.4, 78.6 (C-3′/C-5′), 79.9 (C-3), 82.2 (C-6), 103.9 (C-1′), 112.9 (C-15), 118.7 (C-13), 142.8 (C-11), 150.9 (C-4), 170.5 (C-12).

Prenantheside A (3)——Amorphous powder (0.8 g), $[\alpha]_D^{25}$ +10.3° (c=1.12, pyridine). *Anal.* Calcd for

$C_{21}H_{30}O_9 \cdot H_2O$: C, 56.75; H, 7.26. Found: C, 56.71; H, 7.20. IR ν_{max}^{KBr} cm^{-1}: 3450, 1765, 1640, 1220, 1080, 1040, CD (c=0.14, MeOH) [θ] (nm): −1765 (263). ^{1}H- and ^{13}C-NMR: Tables I and II.

Prenantheside B (4)——Amorphous powder (120 mg), $[\alpha]_D^{25}$ −7.4° (c=1.12, MeOH). *Anal.* Calcd for $C_{29}H_{36}O_{11} \cdot 1/2H_2O$: C, 61.15; H, 6.55. Found: C, 61.41; H, 6.50. IR ν_{max}^{KBr} cm^{-1}: 3450, 1745, 1670, 1620, 1600, 1520, 1450, 1270, 1230, 1155, 1080, 1060, 1015. UV λ_{max}^{MeOH} nm (log ε): 277 (3.37), 284 (sh, 3.33). ^{1}H- and ^{13}H-NMR: Table I and II.

8-Epidesacylcynaropicrin Glucoside (5)——Amorphous powder (20 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1760, 1665, 1645, 1075. ^{1}H-NMR (pyridine-d_5) δ: 4.84 (1H, br t, J=8 Hz, H-3), 4.99, 5.18 (each 1H, s, H_2-14), 5.59, 5.89 (each 1H, br s, H_2-15), 5.69 (1H, d, J=3.1 Hz, H-13a), 6.49 (1H, d, J=3.5 Hz, H-13b). ^{13}C-NMR (pyridine-d_5) δ: 38.5 (C-2), 43.7 (C-9), 45.1 (C-1), 50.0, 50.7 (C-5/C-7), 63.0 (C-6′), 66.0 (C-8), 71.8 (C-4′), 75.3 (C-2′), 78.2, 78.5 (C-6/C-3′/C-5′), 80.8 (C-3), 104.6 (C-1′), 111.9 (C-15), 116.1 (C-14), 121.0 (C-13), 137.7 (C-11), 144.9 (C-10), 150.8 (C-4), 170.1 (C-12).

Crepiside E (6)——Amorphous powder (30 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1760, 1645. ^{1}H-NMR (pyridine-d_5) δ: 5.58, 5.77 (each 1H, br s, H_2-15), 6.41 (2H, m, H_2-13). ^{13}C-NMR (pyridine-d_5) δ: 38.4 (C-2), 43.0 (C-9), 46.0 (C-1), 51.2, 52.0 (C-5/C-7), 63.0 (C-6′), 71.9 (C-4′), 72.2 (C-8), 75.3 (C-2′), 78.2, 78.5 (C-3′/C-5′), 79.0 (C-6), 80.5 (C-3), 103.4 (C-1′), 114.3 (C-15), 116.2 (C-14), 121.6 (C-13), 140.5 (C-11), 144.4 (C-10), 150.0 (C-4), 170.0 (C-12).

Prenantheside C (7)——Amorphous powder (125 mg), $[\alpha]_D^{25}$ +61.9° (c=1.09, MeOH). *Anal.* Calcd for $C_{30}H_{34}O_{12}$: C, 61.43; H, 5.84. Found: C, 61.57; H, 6.09. IR ν_{max}^{KBr} cm^{-1}: 3450, 1760, 1715, 1640, 1605, 1525, 1450, 1270, 1180, 1075, 1030. UV λ_{max}^{MeOH} nm (log ε): 249 (4.03), 300 (sh, 4.06), 331 (4.20). ^{1}H- and ^{13}C-NMR: Tables I and II.

Acetylation of Prenanthelide A (1)——Prenanthelide A (**1**) (27 mg) was refluxed with acetic anhydride (1.5 ml) for 2 h. Excess acetic anhydride was evaporated off *in vacuo*. The residue was chromatographed on silica gel using hexane–acetone (1:1) as the solvent to give the acetate (**1a**) (11 mg) as an amorphous powder. MS m/z: 264 ($M^+ - CH_2=C=O$, 3), 246 ($M^+ - AcOH$, 100). ^{1}H- and ^{13}C-NMR: Tables I and II.

Dehydration of Prenanthelide A (1)——Prenanthelide A (**1**) (20 mg) was treated with pyridine (1.5 ml) and $POCl_3$ (10 drops) for 3.5 h at room temperature. The reaction mixture was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was chromatographed on silica gel using hexane–acetone (93:7) as the eluent to give estafiatone (**1b**) (7.5 mg) as a colorless oil. MS m/z: 246 (M^+, 19), 218 ($M^+ - C=O$, 10), 190 (16), 175 (13), 121 (22), 105 (29), 91 (43), 79 (46), 69 (57), 55 (59), 53 (65), 42 (100).

Enzymatic Hydrolysis of Prenantheside A (3)——A solution of prenantheside A (**3**) (26 mg) in water (3 ml) was treated with crude hesperidinase (30 mg) at 38 °C for 5 h. The reaction mixture was diluted with water and then passed through an Amberlite XAD-2 column, which was washed with water. The methanol eluate was purified on a silica gel column using benzene–acetone (93:7) as the eluent and recrystallized from methanol to give prenanthelide A (**1**) (7 mg), colorless plates, mp 199—200 °C (mixed mp 199—200 °C), as an aglycone. IR and ^{1}H-NMR spectra were identical to those of the natural product.

Saponification of Prenantheside B (4) and C (7)——A solution of a glycoside (*ca.* 0.1 mg) in 2% NaOH aq. (2 drops) was stirred for 1 h at room temperature under a nitrogen atmosphere. The solution was acidified with 2 N HCl and extracted with *n*-butanol. **2** and **7a** were detected by HPLC in the *n*-butanol extract from **4** and **7**, respectively. Conditions: column, YMC Pack AM-212 C_8, 6 mm × 15 cm; flow rate, 1.3 ml/min; detector, UV 205 nm; solvent, water–acetonitrile (82:18); t_R 4.8 min (**2**), 5.2 min (**7a**). The remaining *n*-butanol extract was concentrated to dryness and the residue was refluxed with acetyl chloride–methanol (1:20) for 20 min. The reaction mixture was concentrated to give methyl *p*-hydroxyphenylacetate and methyl caffeate, which were detected by HPLC, from **4** and **7**, respectively. Conditions: column, TSK GEL LS-410AK, 4.6 mm × 30 cm; flow rate, 1.3 ml/min; detector, UV 245 nm; solvent, water–methanol (1:1); t_R 4.7 min (methyl *p*-hydroxyphenylacetate), 6.2 min (methyl caffeate).

Saponification of Prenantheside C (7)——A solution of prenantheside C (**7**) (45 mg) in 2% NaOH aq. (2 ml) was stirred for 1 h at room temperature under a nitrogen atmosphere. The solution was acidified with dil. HCl and passed through an Amberlite XAD-2 column, which was washed with water. The methanol eluate was concentrated and the residue was chromatographed on a polyamide column. From the water eluate, **7a** was obtained as an amorphous powder (30 mg). IR ν_{max}^{KBr} cm^{-1}: 3450, 1765, 1665, 1650, 1080, 1040. ^{1}H-NMR (pyridine-d_5) δ: 1.65 (3H, d, J=7 Hz, H_3-13), 4.84 (1H, br t, J=7 Hz, H-3), 5.01, 5.14 (each 1H, br s, H_2-14), 5.06 (1H, d, J=7 Hz, H-1′), 5.54, 5.87 (each 1H, br s, H_2-15). ^{13}C-NMR (pyridine-d_5) δ: 16.5 (C-13), 37.9 (C-2), 42.3 (C-11), 44.4 (C-1), 46.6 (C-9), 50.8 (C-5), 56.3 (C-7), 62.9 (C-6′), 71.8 (C-4′), 75.1, 75.3 (C-8/C-2′), 78.2, 78.5 (C-3′/C-5′), 79.4 (C-6), 80.5 (C-3), 104.0 (C-1′), 112.3 (C-15), 115.1 (C-14), 145.1 (C-10), 150.8 (C-4), 179.0 (C-12).

Sodium Borohydride Reduction of Crepiside E (6)——A solution of crepiside E (**6**) (25 mg) in methanol (3 ml) was treated at 0 °C with sodium borohydride (18 mg), and the mixture was stirred for 15 min. The reaction mixture was acidified with acetic acid, diluted with water, and then passed through an Amberlite XAD-2 column, which was washed with water. The reaction product was eluted with methanol to give **7a** (20 mg) as an amorphous powder. The IR, ^{1}H- and ^{13}C-NMR spectra were identical with those of the saponification product of prenantheside C.

Acid Hydrolysis of Prenanthesides A (3), B (4) and C (7)——A solution of a glycoside (*ca.* 0.1 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 IRA-45 column and the eluate was concentrated to give a residue, which was reduced with sodium borohydride (*ca.* 2 mg) for

1 h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and the eluate was concentrated to dryness. Boric acid was removed by co-distillation with methanol and the residue was acetylated with acetic anhydride and pyridine (1 drop each) at 100 °C for 1 h. The reagents were evaporated off *in vacuo*. From each glycoside, glucitol acetate was detected by GC. Conditions: column, 1.5% OV-17, 3 mm × 1 m; column temperature, 215 °C; carrier gas, N_2; t_R 6.0 min.

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Studies on the Sesquiterpenoids of *Panax ginseng* C. A. MEYER. Isolation and Structure Determination of Sesquiterpene Alcohols, Panasinsanols A and B

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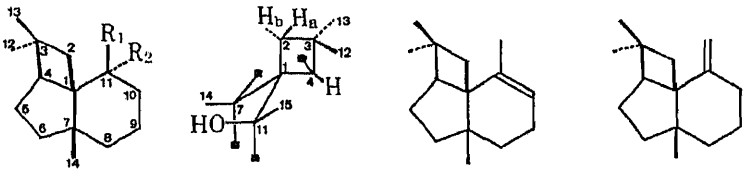
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Two sesquiterpene alcohols, panasinsanol A (**1**) and panasinsanol B (**2**), were isolated from the rootlets of *Panax ginseng* C. A. MEYER (Araliaceae) together with known sesquiterpene hydrocarbons, α-panasinsene (**3**), β-panasinsene (**4**), α-neoclovene (**5**), and β-neoclovene (**6**). The structures of **1** and **2** were established by spectral evidence, chemical correlations to congener hydrocarbons (**3**, **4**, **5**, and **6**), and finally by their syntheses from (−)-β-caryophyllene. This is the first isolation of **1** from a natural source, and **2** is a novel panasinsane-type sesquiterpene.

Keywords—*Panax ginseng*; Araliaceae; *Panax* spp.; sesquiterpenoid; panasinsanol A; panasinsanol B

The roots and rootlets of *Panax ginseng* C. A. MEYER (Araliaceae) are a well-known and very important crude drug in the prescriptions of traditional oriental medicine. In the previous paper,[1] we reported methoxy- and alkylpyrazines from the basic fraction of the ethereal extract of *P. ginseng*. Many reports on saponins of this crude drug have been published,[2a,b] but only a few chemical studies have been done on the constituents other than these compounds.[2b-d,3a] During our study of the constituents of this crude drug,[1] the results of gas chromatography-mass spectrometry (GC-MS) indicated that many sesquiterpenoids were contained in the neutral fraction of the ethereal extract. Thus far, several sesquiterpenoids have been reported,[2d,3a] but no investigation of sesquiterpenoids other than these compounds has yet been made in detail. We therefore investigated the oxygen-containing sesquiterpenoids and have now isolated two sesquiterpene alcohols, named panasinsanols A and B, together with the related known sesquiterpene hydrocarbons, α-panasinsene, β-panasinsene, α-neoclovene, and β-neoclovene.[3a] In this paper, we wish to report the structural elucidation of panasinsanols A and B, as well as some findings obtained by the comparative analyses of the neutral volatile oils of *Panax* spp., *P. ginseng*, *P. japonicum*, *P. quinquefolium*, and *P. notoginseng*.

The neutral fractions[1] were chromatographed on a silica-gel column using hexane, hexane–ether, ether and acetone as eluants. From the hexane–ether (4:1) fractions, panasinsanols A and B were isolated by further silica-gel column chromatography and preparative high-performance liquid chromatography (HPLC). The least polar fractions eluted with hexane were further chromatographed on a silver nitrate ($AgNO_3$)-impregnated silica-gel column, followed by preparative HPLC at low temperature, yielding four analytically pure sesquiterpene hydrocarbons. These compounds were identified as α-panasinsene (**3**), β-panasinsene (**4**), α-neoclovene (**5**), and β-neoclovene (**6**) by comparisons of the spectral data with reported values.[3a-d]

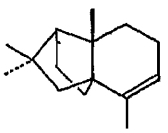


1 : R_1=Me[15], R_2=OH
2 : R_1=OH, R_2=Me[15]

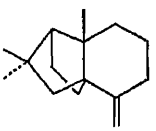
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3

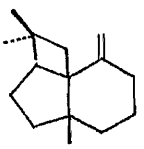
4



5



6



7

Chart 1

Panasinsanol A (**1**) was isolated as a minor compound, colorless oil, $[\alpha]_D^{25}$ −51.9° ($CHCl_3$). The molecular formula $C_{15}H_{26}O$ of **1** was determined from the high-resolution mass spectrum (HR-MS) together with the carbon-13 nuclear magnetic resonance (^{13}C-NMR) data, which indicated the presence of three degrees of unsaturation. The infrared (IR) absorption at 3630 cm^{-1} and a dehydration ion at *m/z* 204 ($M^+ - 18$, H_2O) in the MS of **1** indicated the presence of a hydroxy group, and a quaternary carbon signal at δ 72.6 in the ^{13}C-NMR spectrum indicated the hydroxy group to be tertiary. The IR absorptions at 1375 and 1380 cm^{-1} and fragment ion at *m/z* 166 ($M^+ - 56$, C_4H_8) suggested that two of four methyl groups were assignable to a geminal dimethyl group.[4] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed signals due to four tertiary methyl groups at δ 0.91 (6H, s), 1.12, 1.20 (each 3H, s), and a methine proton at δ 2.26 (1H, m), and its ^{13}C-NMR spectrum showed no sp^2 carbon. These observations suggest that panasinsanol A is a saturated tricarbocyclic sesquiterpenoid possessing a tertiary hydroxy group and a geminal dimethyl group.

Dehydration of **1** with thionyl chloride ($SOCl_2$) in pyridine gave two olefins which were identified as **3** and **4** by comparisons of the MS and retention times on GC with those of authentic samples.[3a] From the above results, two formulas (**1** and **2**) were suggested for the structure of panasinsanol A. One of them, **1**, has been previously synthesized as a key intermediate in the total syntheses of sesquiterpene hydrocarbons (**4**, **5**),[3c,d] and the chemical shift values of methyl signals in the ^{1}H-NMR spectrum and optical rotation of our **1** were similar to those of the reported **1**.[3c,d] McKillop and his collaborators have reported that this alcohol was converted to **5** by treatment with concentrated sulfuric acid (H_2SO_4) in anhydrous ether.[3c] Indeed, on similar treatment of our **1**, two different olefins were formed. These compounds were directly identified as **5** and **6** by comparisons of the MS and retention times on GC with those of the authentic samples.[3a] All these results indicate that the structure of panasinsanol A is represented by formula **1**, whose relative configuration at the carbinol center is as shown by Johnson and Meanwell.[3d] To our knowledge, this is the first isolation of **1** from a natural source.

Panasinsanol B (**2**) was obtained as a colorless oil, $[\alpha]_D^{25}$ −44.3° ($CHCl_3$). The molecular formula $C_{15}H_{26}O$ of **2** was determined from the HR-MS together with the ^{13}C-NMR data, which indicated the presence of three degrees of unsaturation, and its MS showed a similar fragmentation to that of **1**. By analogy with **1**, the IR absorption at 3630 cm^{-1} and dehydration ion at *m/z* 204 ($M^+ - 18$, H_2O) in the MS of **2** indicated the presence of a hydroxy group, and a quaternary carbon signal at δ 73.2 (s) in the ^{13}C-NMR spectrum indicated the hydroxy group to be tertiary. Also the IR absorptions at 1370 and 1380 cm^{-1} and a fragment

ion at m/z 166 ($M^+ - 56$, C_4H_8) suggested that two of four methyl groups were assignable to a geminal dimethyl group.[4] The ^{1}H-NMR spectrum of **2** showed signals due to four tertiary methyl groups at δ 0.95, 1.04, 1.16, 1.41 (each 3H, s), and a methine proton at δ 2.08 (1H, m), and the ^{13}C-NMR spectrum showed no sp^2 carbon. Furthermore, in the ^{1}H-NMR spectrum, signals were observed as A and B parts of an ABX system at δ 1.62 (1H, br d, J=12.9 Hz) and 1.92 (1H, dd, J=2.1 and 12.9 Hz). These observations are similar to those in the case of **1**. Hence, it is suggested that panasinsanol B is a similar saturated tricarbocyclic sesquiterpenoid to **1**.

Dehydration of **2** with $SOCl_2$ in pyridine gave two olefins and these compounds were identified as **3** and **4** by comparisons of the spectral data, optical rotations and retention times on GC with those of authentic samples.[3a-d] On treatment of **2** with concentrated H_2SO_4 in anhydrous ether as described for **1**,[3c] three compounds were obtained. The major product was identified as **5** by comparisons of the spectral data, optical rotation and retention time on GC with those of an authentic sample.[3a-c,5] The second product was directly identified as **6** by comparisons of the MS and retention time on GC with those of an authentic sample.[3a] The remaining product appeared to be a new alcoholic compound. From the chemical evidence, the formula **2** was suggested for the structure of panasinsanol B.

To confirm the structure of **2**, ^{1}H-NMR studies on **2**, including proton spin decoupling, proton spin decoupling difference, and nuclear Overhauser effect (NOE) difference experiments, were undertaken. In the ^{1}H-NMR spectrum of **2**, a methyl signal at δ 1.41 was assignable to the equatorial carbinol methyl group (15-H_3) from its chemical shift value. In the spin decoupling experiments, irradiation at the frequency of a double doublet at δ 1.92 (2-H_a) collapsed a broad doublet at δ 1.62 (2-H_b) to a broad singlet and a multiplet at δ 2.08 (4-H) to a broad doublet. Irradiation at the frequency of the multiplet at δ 1.62 (2-H_b) collapsed the double doublet at δ 1.92 (2-H_a) to a doublet and slightly influenced the multiplet at δ 2.08 (4-H). Also, irradiation with the frequency of the multiplet at δ 2.08 (4-H) collapsed the signals at δ 1.62 (2-H_b) and 1.92 (2-H_a) to AB-type signals. In the proton spin decoupling difference experiments, long-range coupling between two methyl signals at δ 0.95 (13-H_3) and 1.16 (12-H_3) was found, supporting the presence of a geminal dimethyl group. In the NOE experiments on the geminal dimethyl group, irradiation at the frequency of the methyl signal at δ 0.95 (13-H_3) resulted in NOE enhancement of the signal at δ 1.62 (2-H_b), and irradiation at the frequency of the methyl signal at δ 1.16 (12-H_3) resulted in NOE enhancements of the signals at δ 1.41 (15-H_3), 1.92 (2-H_a), and 2.08 (4-H). Also, irradiation at the frequency of the methyl signal at δ 1.41 (15-H_3) resulted in NOE enhancements of the signals at δ 1.16 (12-H_3), 1.92 (2-H_a), and 2.08 (4-H). Irradiation at the frequency of the methyl signal at δ 1.04 (14-H_3) resulted in NOE enhancement of the signal at δ 1.62 (2-H_b). These NOE findings are indicated by the double-headed arrows in Fig. 1. These observations are consistent with the partial structure **2a**. Hence, all these results indicate that the structure of panasinsanol B is represented by formula **2**. Furthermore, this result indirectly supports the relative con-

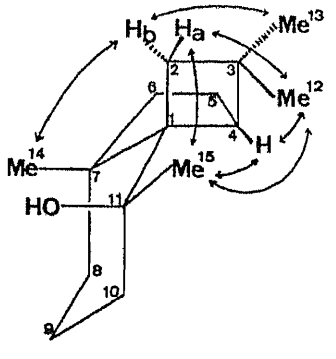


Fig. 1. NOE Findings for **2** (400 MHz)

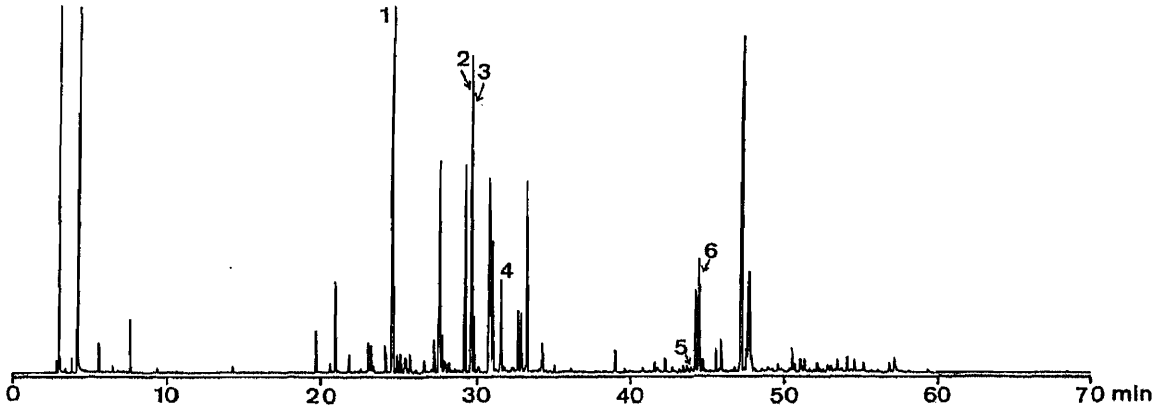


Fig. 2. Gas Chromatogram of the Neutral Volatile Oil of *P. ginseng* C. A. MEYER

Peak 1, β-panasinsene (4); peak 2, α-panasinsene (3); peak 3, α-neoclovene (5); peak 4, β-neoclovene (6); peak 5, panasinsanol A (1); peak 6, panasinsanol B (2).

figuration at the carbinol methyl group in **1** as axial, a choice so far based on the result of a ^{1}H-NMR experiment on this alcohol using a shift reagent, and the ^{13}C-NMR and IR spectra of its related compounds.[3d]

Finally, the stereostructures of **1** and **2** were confirmed by total syntheses from the tricarbocyclic ketone (**7**), which was obtained from (−)-β-caryophyllene of known absolute configuration[3e] by a method similar to that reported by McKillop and his collaborators.[3c] Treatment of **7** with methyl lithium produced a 11 : 1 mixture of the desired two tertiary alcohols (**1** and **2**) in good yield.[3f] The spectral data, optical rotations and retention times on GC of these synthetic alcohols were in good agreement with those of naturally occurring **1** and **2**. Thus, the structures of panasinsanols A and B were established as (4*S*,7*R*,11*R*)-(**1**) and (4*S*,7*R*,11*S*)-3,3,7,11-tetramethyltricyclo[5,4,0,$0^{1,4}$]undecan-11-ol (**2**) respectively.

It has been reported by Yoshihara and Hirose that there was little difference between the gas chromatograms of the volatile oils obtained from dried rootlets of *P. ginseng* collected in Japan and in Korea.[3a] Indeed, it was proved by detailed analyses by GC and GC-MS that these materials all contained not only **3**—**6** but also **1** and **2**. Furthermore, it was also proved that white and red ginsengs both contained **1**—**6**. A typical gas chromatogram of the neutral volatile oil of the rootlets collected in Japan is shown in Fig. 2. On the other hand, the GC analyses clearly showed that the constituents of the neutral volatile oils obtained from dried roots of other *Panax* spp., *P. japonicum* C. A. MEYER (chikusetsu-ninjin), *P. quinquefolium* L. (American ginseng) and *P. notoginseng* (BURK) F. H. CHEN (sanchi-ginseng), were different from those of *P. ginseng*. Detailed GC-MS analyses showed that trace amounts of **3**, **4** and **5** were contained only in *P. quinquefolium*. Also, **1**, **2** and **6** could not be found in any *Panax* spp. other than *P. ginseng*.

P. ginseng contains other sesquiterpenoids which are under investigation in our laboratories.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured with tetramethylsilane (TMS) as an internal reference. Chemical shifts are expressed in ppm downfield from TMS and coupling constants (J) in Hz. Abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. ^{1}H-Gated decoupling measurements without NOEs were employed to confirm the number of carbon atoms. Intensive nuclei enhanced by polarization transfer and single frequency off-resonance decoupling measurements were also employed to delineate the ^{13}C signals. The following instruments were used: IR spectra, Shimadzu IR-410 IR spectrometer; ^{1}H-NMR spectra, JEOL FX-100, FX-200, and GX-400 FT NMR spectrometers; ^{13}C-NMR spectra, JEOL FX-200 spectrometer; GC-MS, Hitachi M-80 gas chromatograph-

mass spectrometer and Hewlett Packard 5970B mass selective detector combined with a Hewlett Packard 5890A gas chromatograph; HR-MS, Hitachi M-80 gas chromatograph-mass spectrometer; GC, Hewlett Packard 5890A equipped with a Hewlett Packard 3392A integrated recorder; circular dichroism (CD) spectrum, JASCO J-500C spectropolarimeter; optical rotation, Optical Activity AA-10 digital polarimeter. For GC and GC-MS analyses, DB-WAX fused-silica capillary columns (J & W Scientific, INC., i.d. 0.25 mm × 60 m, i.d. 0.33 mm × 60 m) were used and the column temperature was programmed at 3 °C/min from 60 (held for 5 min) to 210 °C and then held. For preparative HPLC, a Waters 6000A solvent delivery system, U6K injector, R-401 differential refractometer, and stainless steel column packed with Chemcosorb 5 Si (Chemco, Inc., i.d. 10 mm × 30 cm) were used. For column chromatography, Wakogel C-100 and C-200 were used.

Extraction and Isolation——The ethereal extract and the neutral fraction of *P. ginseng* collected in Nagano Prefecture (2 kg, dried rootlets) were obtained as described in the previous paper.[1] The neutral fractions (25.6 g) were chromatographed on a silica-gel column using hexane (1000 ml), hexane–ether (9 : 1, v/v, 600 ml), hexane–ether (4 : 1, v/v, 800 ml), ether (800 ml), and acetone (1000 ml) as eluants. The least polar fractions eluted with hexane (2.30 g) were further chromatographed on a silica-gel column impregnated with 10% $AgNO_3$ using hexane as an eluant. Each eluted fraction was further subjected to preparative HPLC (solvent, hexane; column temperature, −45 °C; flow rate, 1.0 ml/min), yielding analytically pure α-panasinsene (**3**) (35.1 mg), β-panasinsene (**4**) (20.4 mg), α-neoclovene (**5**) (32.8 mg), and β-neoclovene (**6**) (11.9 mg). The fractions eluted with hexane–ether (4 : 1, v/v) (1.20 g) were further chromatographed on a silica-gel column using solvents of increasing polarity from benzene to ether. The fractions eluted with benzene–ether (9 : 1, v/v) were further subjected to preparative HPLC (solvent, benzene–ether (97 : 3, v/v); flow rate, 2.5 ml/min), yielding crude panasinsanol A (**1**) and analytically pure panasinsanol B (**2**) (25 mg). Isolation of **1** was carried out by preparative HPLC (solvent, hexane–ether (9 : 1, v/v); column temperature, −45 °C; flow rate, 1.0 ml/min), yielding analytically pure **1** (4.5 mg).

Panasinsanol A (**1**): Colorless oil. $[\alpha]_D^{25}$ −51.9° (*c* = 0.54, $CHCl_3$). IR (CCl_4): 3630, 1380, 1375 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.91 (6H, s), 1.12, 1.20 (each 3H, s), 2.26 (1H, m). ^{13}C-NMR ($CDCl_3$, 50 MHz) δ: 20.4 (t), 20.7 (q), 23.4 (q), 24.1 (t), 25.0 (q), 28.4 (s), 29.4 (q), 36.5 (t), 37.6 (t), 39.0 (t), 43.4 (t, s), 49.0 (d), 53.8 (s), 72.6 (s). MS *m/z* (% rel. int.): 222 (M^+, 3), 207 (4), 204 (6), 189 (10), 166 (10), 161 (7), 151 (26), 138 (12), 133 (14), 125 (15), 123 (26), 108 (21), 81 (26), 67 (21), 55 (26), 43 (100), 41 (53), 39 (19). HR-MS *m/z*: M^+ Calcd for $C_{15}H_{26}O$ 222.1983. Found: *m/z* 222.1952.

Panasinsanol B (**2**): Colorless oil. $[\alpha]_D^{25}$ −44.3° (*c* = 0.70, $CHCl_3$). IR (CCl_4): 3630, 1370, 1365 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.95 (3H, s, 13-H_3), 1.04 (3H, s, 14-H_3), 1.16 (3H, s, 12-H_3), 1.41 (3H, s, 15-H_3), 1.62 (1H, br d, *J* = 12.9 Hz, 2-H_b), 1.92 (1H, dd, *J* = 2.1, 12.9 Hz, 2-H_a), 2.08 (1H, m, 4-H). ^{13}C-NMR ($CDCl_3$, 50 MHZ) δ: 17.5 (t), 21.4 (q), 24.7 (t), 25.7 (q), 28.4 (s), 29.5 (q), 31.4 (q), 36.4 (t), 37.2 (t), 37.7 (t), 42.9 (t, s), 50.4 (d), 51.9 (s), 73.2 (s). MS *m/z* (% rel. int.): 222 (M^+, 3), 207 (3), 204 (4), 189 (4), 166 (12), 151 (37), 148 (10), 138 (16), 133 (11), 125 (15), 123 (24), 108 (24), 93 (15), 81 (37), 67 (20), 55 (24), 43 (100), 41 (59), 39 (20). HR-MS *m/z*: M^+ Calcd for $C_{15}H_{26}O$ 222.1983. Found: *m/z* 222.1961.

α-Panasinsene (**3**): Colorless oil. $[\alpha]_D^{25}$ −17.0° (*c* = 1.17, $CHCl_3$). The spectral data (^{1}H-NMR, IR, and MS) were identical with those reported for α-panasinsene.[3a]

β-Panasinsene (**4**): Colorless oil. $[\alpha]_D^{25}$ −22.9° (*c* = 0.87, $CHCl_3$). The spectral data (^{1}H-NMR, IR, and MS) were identical with those reported for β-panasinsene.[3a,d]

α-Neoclovene (**5**): Colorless oil. $[\alpha]_D^{25}$ −61.0° (*c* = 1.20, $CHCl_3$). The spectral data (^{1}H-NMR, IR, and MS) were identical with those reported for α-neoclovene.[2a~c]

β-Neoclovene (**6**): Colorless oil. $[\alpha]_D^{25}$ −33.5° (*c* = 0.75, $CHCl_3$). The spectral data (^{1}H-NMR, IR, and MS) were identical with those reported for β-neoclovene.[3a]

Dehydration of 1 with Thionyl Chloride——A solution of **1** (3 mg) in dry pyridine (0.5 ml) at 0 °C was treated with thionyl chloride (0.02 ml). After 2 h, the mixture was poured into ice-water and extracted with ether. The ether solution was washed with saturated brine and dried over anhydrous Na_2SO_4. After removal of the solvent, the residual substance was chromatographed on a silica-gel column. A GC analysis revealed that the least polar fraction (1.2 mg) eluted with hexane consisted of **3** (peak area %, 30%) and **4** (65%), which were directly identified as α-panasinsene and β-panasinsene by comparisons of the MS and retention times on GC with those of authentic samples.[3a]

Dehydration of 2 with Thionyl Chloride——A solution of **2** (15 mg) in dry pyridine (0.7 ml) at 0 °C was treated with thionyl chloride (0.05 ml). After 2 h, the mixture was worked up according to the previously outlined procedure. A GC analysis revealed that the least polar fraction (9 mg) eluted with hexane consisted of **3** (peak area %, 47%) and **4** (53%), which were isolated by preparative HPLC (solvent: hexane column temperature, −45 °C; flow rate, 1.0 ml/min) and identified as α-panasinsene and β-panasinsene by comparisons of the spectral data (^{1}H-NMR, IR, and MS), optical rotations and retention times on GC with those of authentic samples.[3a,d]

Acid-Catalyzed Rearrangement of 1——A solution of **1** (2 mg) in anhydrous ether (1 ml) was added with stirring to a solution (1 ml) prepared from concentrated H_2SO_4 (1 ml) and anhydrous ether (7 ml) at 0 °C.[3b,c] The mixture was stirred for 20 min at 0 °C and then for 30 min at room temperature. The reaction mixture was diluted with ice-water, neutralized with 2 N NaOH solution, and extracted with ether. The ether solution was washed with brine and dried

over anhydrous Na_2SO_4. After removal of the solvent, the residual substance was chromatographed on a silica-gel column. A GC analysis revealed that the least polar fraction (1.3 mg) eluted with hexane consisted of **5** (peak area %, 95%) and **6** (5%), which were directly identified as α-neoclovene and β-neoclovene by comparisons of the MS and retention times on GC with those of authentic samples.[3a]

Acid-Catalyzed Rearrangement of 2——A solution of **2** (7 mg) in anhydrous ether (1 ml) was added with stirring to a solution (1 ml) prepared from concentrated H_2SO_4 (2 ml) and anhydrous ether (7 ml) at 0 °C.[3b,c] The mixture was stirred for 20 min at 0 °C and then for 30 min at room temperature. The reaction mixture was worked up according to the previously outlined procedure. A GC analysis revealed that the least polar fraction (4.2 mg) eluted with hexane consisted of **5** (peak area %, 95%) and **6** (3%). Isolation of **5** was carried out by preparative HPLC (solvent, hexane; column temperature, −45 °C; flow rate, 1.0 ml/min), and **5** was identified as α-neoclovene by comparisons of the spectral data (^{1}H-, ^{13}C-NMR (benzene-d_6), IR, and MS), optical rotations and retention times on GC with those of an authentic sample.[3a-c,5] **6** was directly identified as β-neoclovene by comparisons of the MS and retention times on GC with those of an authentic sample.[3a] Elution with ether gave an unknown alcohol (1.6 mg), but its structure could not be elucidated.

Synthesis of 1 and 2 from (−)-β-Caryophyllene——A solution of commercial grade (−)-β-caryophyllene (Tokyo Kasei) in hexane was washed with diluted 2 N NaOH and then water until the water layer was neutral. The hexane layer was washed with 50% $AgNO_3$ solution and then water. After being dried over anhydrous Na_2SO_4, the hexane extract was concentrated under reduced pressure and the residual oil was carefully chromatographed on a silica-gel column impregnated with 10% $AgNO_3$ and eluted with hexane.[3b,c] Evaporation of the eluate afforded β-caryophyllene, $[\alpha]_D^{25}$ −13.5° (c=3.30, $CHCl_3$).[3g] The GC analysis of β-caryophyllene thus obtained indicated it be 98.5% pure. The synthesis of the tricyclic ketone (**7**) was carried out by the method reported by McKillop *et al.*[3c]

7——Colorless oil. $[\alpha]_D^{25}$ −37.6° (c=1.65, $CHCl_3$). CD ($c=1.21\times10^{-3}$, MeOH) $\Delta\varepsilon_{294}$: +2.7. IR (film): 2950, 1690, 1475, 1425, 1385, 1370, 1325, 1290, 1165, 1140, 935 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 100 MHz) δ: 0.86 (6H, s), 0.98 (3H, s). MS *m/z* (% ref. int.): 206 (M^+, 32), 191 (68), 163 (32), 151 (100), 135 (73), 107 (57).

The spectral data of **7** were in good agreement with those of the known tricyclic ketone.[3a,c] A solution of **7** (50 mg) in anhydrous ether (3 ml) was added to 1.8 N methyl lithium (10 ml) and the mixture was heated to reflux for 1 h under a nitrogen atmosphere.[3f] After this time the solution was cooled and then water was added dropwise. The ether layer was separated, washed with water, and concentrated under reduced pressure, yielding the desired tertiary alcohols (52 mg) (97%), which showed hydroxy but not carbonyl absorption in the IR spectrum, and a GC analysis revealed that the products consisted of two alcohols. Preparative HPLC (solvent, benzene–ether (97 : 3, v/v)) of the mixture gave **1** (44 mg) and **2** (4 mg). The spectral data (^{1}H-NMR, IR) and optical rotation of **1** thus obtained were in good agreement with reported data.[3c,d] Further, the spectral data (^{1}H-, ^{13}C-NMR, IR, and MS), optical rotations, and retention times on GC of these two alcohols thus obtained were in good agreement with those of our naturally occurring **1** and **2**, respectively.

GC and GC-MS Analyses of the Neutral Volatile Oils——The following materials were used: *P. ginseng*, dried rootlets (collected in Nagano and Fukusima Prefectures, each 100 g, and in Korea, 25 g), white ginseng (collected in Nagano Prefecture, cultivated for 5 years, 150 g), red ginseng (collected in Nagano Prefecture, cultivated for 5 years, 75 g); *P. japonicum* (collected in Nagano Prefecture, 150 g); *P. quinquefolium* (obtained from Hong Kong, 30 g); *P. notoginseng* (obtained from China, 100 g). All materials were obtained from Mikuni Co. (Osaka). The neutral volatile oils of these materials were prepared from the neutral fractions of their ethereal extracts by simultaneous steam distillation-extraction using a Nickerson-Likens apparatus.[6] The simultaneous steam distillation-extractions (ether–H_2O (1 : 10, v/v)) were continued for 30 min. The ether solutions were dried over anhydrous Na_2SO_4 and the solvents were removed under reduced pressure at 45 °C, yielding the neutral volatile oils.

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Studies on the Consituents of Leguminous Plants. X.[1] The Structures of New Triterpenoid Saponins from the Fruits of *Gymnocladus chinensis* BAILLON

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Four new triterpenoid saponins, gymnocladus saponins F_2 (**1**), F_1 (**2**), E (**3**) and D_1 (**4**), were isolated from the fruits of *Gymnocladus chinensis* BAILLON. On the basis of chemical and physicochemical evidence, they were characterized structurally as 3,28-bisglycosides of 2β,23-dihydroxyacacic acid having a glycosyl monoterpene carboxylate as an acyl moiety.

Keywords——*Gymnocladus chinensis*; Leguminosae; gymnocladus saponin F_2; gymnocladus saponin F_1; gymnocladus saponin E; gymnocladus saponin D_1; 2β,23-dihydroxyacacic acid; triterpenoid; saponin; glycosyl monoterpene

In the previous paper,[1] we reported the structure elucidation of a triterpenoid saponin, gymnocladus saponin G (**5**), isolated from the fruits of *Gymnocladus chinensis* BAILLON. We have also reported[2] the inhibitory effect of **5** on Epstein–Barr virus (EBV) early antigen induction by 12-*O*-tetradecanoylphorbol-13-*O*-acetate (TPA).[3]

Four new triterpenoid saponins, named gymnocladus saponins F_2 (**1**), F_1 (**2**), E (**3**) and D_1 (**4**), were isolated from the same source, and characterized as 3,28-bisglycosides of 2β,23-dihydroxyacacic acid (**6**) bearing a glycosyl monoterpene carboxylate as an acyl moiety, on the basis of carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrometry and some chemical degradations.

Gymnocladus saponin F_2 (**1**) was obtained as a white powder, $C_{96}H_{154}O_{50}$, $[\alpha]_D$ −3.6°. In the infrared (IR) spectrum (1680 cm^{-1}) and the ultraviolet (UV) spectrum (λ_{max} 217 nm), **1** showed the presence of a conjugated acyl group. In the ^{13}C-NMR spectrum, **1** showed three singlet signals of carbonyl carbons at δ 176.7, 174.7 and 167.7, four olefinic carbons at δ 143.8, 142.1, 128.6 and 115.2, in addition to 123.9 (C-12) and 143.4 (C-13), and nine anomeric carbons at δ 106.3, 106.1, 106.0, 105.6, 103.1, 102.0, 101.3, 99.8 and 95.0.

On acid hydrolysis of **1** with 10% H_2SO_4, 2β,23-dihydroxyacaco-28,21β-lactone (**7**)[4] and four kinds of sugars, glucose, xylose, arabinose and rhamnose were obtained and identified by comparison with authentic samples. On alkaline hydrolysis of **1** with 2% $NaHCO_3$ in ethanol, a partially deacylated compound (**8**) was obtained as a hygroscopic white powder, $C_{91}H_{146}O_{49}\cdot 3H_2O$, $[\alpha]_D$ −2.4°. The IR spectrum (1680 cm^{-1}), the UV spectrum (λ_{max} 217 nm) and ^{13}C-NMR spectrum (signals at δ 174.8 and 167.8 due to carbonyl carbons, at δ 144.0, 142.3, 128.6 and 115.0 due to olefinic carbons, and at δ 106.3, 106.1, 106.0, 105.5, 103.1, 102.0, 101.4, 99.9 and 95.0 due to anomeric carbons) of **8** suggested the presence of a conjugated acyl group, which was not hydrolyzed under the conditions used, and the same oligosaccharide as in **1**. The five prominent signals of **1** at δ 176.7, 41.2, 27.0, 11.5 and 16.5 corresponding to the signals of the 2-methylbutanoyl group disappeared in the ^{13}C-NMR spectrum of **8**. On hydrolysis of **1** with 10% KOH in ethanol, a monoterpene glycoside (**9**) and a prosapogenin

(**10**) were obtained. The monoterpene glycoside (**9**) was found to be identical with the reported compound obtained from the same plant.[5] The prosapogenin (**10**) was also found to be identical with the reported saponin, gymnocladus saponin B,[4] based on thin layer chromatographic (TLC), IR and ^{13}C-NMR comparisons.

On methylation by Hakomori's method,[6] **1** and **8** gave the same permethylate (**11**), showing an absorption maximum at 212 nm in the UV spectrum, and signals due to a terminal olefin at δ 5.13 (dd, J=17.5, 1.5 Hz), 5.21 (dd, J=11.0, 1.5 Hz) and 5.83 (dd, J=17.5, 11.0 Hz), and nine anomeric protons of sugars at δ 4.22 (d, J=8.5 Hz), 4.25 (d, J=7.8 Hz), 4.44 (d, J=8.0 Hz), 4.62 (d, J=7.4 Hz), 4.67 (d, J=7.6 Hz), 4.69 (d, J=7.2 Hz), 4.83 (br s), 5.10 (br s) and 5.59 (d, J=6.0 Hz) in the ^{1}H-NMR spectrum.

On reduction with lithium aluminum hydride ($LiAlH_4$), **11** gave a mixture of a monoterpene glycoside (**12**), an oligosaccharide alcohol (**13**) and a triterpene glycoside (**14**). Compounds **12** and **13** were found to be identical with reported compounds,[1] based on TLC behavior, optical rotation and IR and proton nuclear magnetic resonance (^{1}H-NMR) spectral comparisons. In the ^{1}H-NMR, **14** showed two anomeric protons at δ 4.22 (d, J=7.8 Hz) and 4.68 (d, J=7.7 Hz), ten O-methyl protons, and six C-methyl protons. Compound **14** was methanolyzed with 2 N hydrogen chloride (HCl) in methanol to afford methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside, methyl 3,4,6-tri-*O*-methyl-D-glucopyranoside and a triterpene alcohol (**15**). The triterpene alcohol (**15**) was identical with an authentic sample of olean-12-ene-2β,16β,23-trimethoxy-3β,21β,28-triol,[7] on the basis of TLC behavior, and IR and ^{1}H-NMR spectral comparisons. Therefore, the deacyl saponin (**8**) was concluded to have the structure shown in Chart 1.

As shown in Table I, in the ^{13}C-NMR spectrum of **1**, the signals assigned to the triterpene moiety and the oligosaccharide moieties at C-3 and C-28 were superimposable on those of **8** within $\pm$0.1 ppm, except for the signals of the glycosylated monoterpene moiety. The signals of the glycosylated monoterpene moieties of **1** and **8** were, respectively, superimposable on those of gymnocladus saponin G (**5**) and its deacylated derivative.[1] From all of the results described above, and a consideration of the acylation shift rule,[8] it was concluded that a 2-methylbutanoyl group was located at C-4 of arabinose in the molecule of **1**, as in **5**.

Therefore, the structure of gymnocladus saponin F_2 (**1**) was concluded to be 2β,23-dihydroxy-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-21-*O*-{(6*S*)-2-*trans*-2,6-dimethyl-6-*O*-[3-*O*-(β-D-glucopyranosyl)-4-*O*-(2-methylbutanoyl)-α-L-arabinopyranosyloxy]-2,7-octadienoyl}-acacic acid 28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl ester.

Gymnocladus saponins F_1 (**2**), E (**3**) and D_1 (**4**) were each obtained as a hygroscopic white powder. The IR and UV spectra of these saponins suggested that all of them have conjugated acyl groups.

As shown in Table II, F_1 (**2**), E (**3**) and D_1 (**4**) were each hydrolyzed with 2% $NaHCO_3$ in ethanol to afford partially deacylated saponins (**16**, **17** and **18**, respectively). On hydrolysis of **2**, **3** and **4** with 10% KOH in ethanol, a monoterpene glycoside (**19**) and a triterpene 3-*O*-glycoside (**20**) were obtained from **2**, a monoterpene glycoside (**9**) and a triterpene 3-*O*-glycoside (**21**) from **3**, and **19** and **21** from **4**. When Hakomori's method was applied, **2**, **3** and **4** gave the permethylates (**22**, **23** and **24**, respectively). On reductive cleavage with $LiAlH_4$, an oligosaccharide alcohol (**13**) was obtained from each of **22**, **23** and **24**, and a monoterpene glycoside (**25**) and a triterpene alcohol (**26**) were obtained from **22**, a monoterpene glycoside (**12**) and a triterpene alcohol (**27**) from **23**, and **25** and **27** from **24**. Compounds **25**, **26** and **27** were identical with the corresponding compounds derived from gymnocladus saponin G and gymnocladus saponin D, on the basis of TLC, IR and ^{1}H-NMR comparisons.[1] Therefore, the structures of the deacylated saponins (**16**, **17** and **18**) were concluded to be as shown in Chart 1.

TABLE I. Carbon-13 NMR Chemical Shifts (δ) of Gymnocladus Saponins F_2(**1**), F_1(**2**), E(**3**) and D_1(**4**) and the Deacylated Compounds (**8**, **16**, **17** and **18**) in Pyridine-d_5[a]

Carbon	**1**	**8**	**2**	**16**	**3**	**17**	**4**	**18**
Triterpene								
1	44.1	44.1	44.1	44.1	44.8	44.8	44.8	44.8
2	70.2	70.3	70.4	70.5	71.1	71.1	71.1	71.1
3	83.2	83.2	83.5	83.5	81.5	81.5	81.5	81.5
4	42.6	42.7	42.7	42.7	42.9	42.9	42.9	43.0
5	47.5[b]	47.5[b]	47.5[b]	47.5[b]	47.6[b]	47.6[b]	47.6[b]	47.6[b]
6	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3
7	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2
8	40.2	40.2	40.2	40.2	40.2	40.2	40.2	40.3
9	47.9[b]	47.9[b]	47.8[b]	47.8[b]	47.9[b]	47.9[b]	47.9[b]	48.0[b]
10	37.0	37.0	37.0	37.0	37.1	37.1	37.1	37.2
11	24.0	24.1	24.1	24.1	24.1	24.1	24.1	24.1
12	123.9	123.9	123.9	123.9	123.9	123.9	123.9	123.9
13	143.4	143.4	143.4	143.3	143.4	143.4	143.3	143.4
14	42.2	42.3	42.2	42.2	42.2	42.3	42.3	42.3
15	35.9	35.9	36.0	35.9	36.0	35.9	35.9	36.0
16	73.4	73.5	73.5	73.5	73.3	73.4	73.4	73.5
17	51.7	51.8	51.8	51.8	51.8	51.8	51.8	51.8
18	40.9	40.9	40.9	40.9	40.9	40.9	40.9	40.9
19	48.0	48.0	47.9	47.9	47.9	47.8	47.8	48.0
20	35.3	35.4	35.4	35.4	35.4	35.4	35.4	35.4
21	76.9	77.0	77.0	77.0	77.0	77.0	77.0	77.0
22	36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5
23	65.9	65.9	65.6	65.6	65.2	65.2	65.2	65.2
24	14.9	14.9	15.0	15.0	14.9	14.8	14.8	14.9
25	17.4[c]	17.4[c]	17.3[c]	17.3[c]	17.5[c]	17.6[c]	17.5[c]	17.6[c]
26	17.5[c]	17.5[c]	17.5[c]	17.6[c]	17.7[c]	17.7[c]	17.6[c]	17.7[c]
27	27.1	27.2	27.2	27.2	27.1	27.2	27.1	27.2
28	174.7	174.8	174.7	174.7	174.8	174.8	174.8	174.8
29	29.1	29.2	29.2	29.1	29.2	29.2	29.2	29.2
30	19.2	19.2	19.2	19.2	19.2	19.2	19.2	19.2
C3-*O*-	Glc		Glc		Rham		Rham	
1	103.1	103.1	105.7	105.7	104.1	104.1	104.1	104.2
2	83.1	83.1	75.4	75.4	72.7	72.7	72.7	72.8
3	76.3	76.4	78.1[f]	78.2	72.5[f]	72.5[f]	72.4[f]	72.5[f]
4	70.9	71.0	71.6	71.6	73.9[g]	73.9[g]	73.9[g]	73.9[g]
5	77.7	77.8	76.0	76.0	70.2	70.3	70.3	70.3
6	62.3	62.3	68.4	68.4	18.6	18.6	18.6	18.6
	Glc		Ara					
1	105.6	105.5	101.5	101.5				
2	75.2	75.2	79.3	79.4				
3	78.3	78.3	72.3	72.3				
4	71.1	71.2	67.4	67.4				
5	78.0	78.1	63.6	63.6				
6	62.5	62.5	Xyl					
			105.8	105.9				
			75.0[d]	75.0[d]				
			78.0[g]	78.0				
			70.8[h]	70.8[f]				
			67.0	67.0				
C-28-	Glc		Glc		Glc		Glc	
1	95.0	95.0	95.0	95.1	95.0	95.0	95.0	95.0
2	79.0	79.0	79.0	78.9	78.9	78.9	78.9	78.9
3	77.8	78.0	78.0[g]	77.8	77.9	77.9	77.9	77.9
4	71.4	71.4	71.5	71.5	71.4	71.4	71.5	71.5
5	76.6	76.6	76.6	76.6	76.8	76.8	76.8	76.9
6	66.8	66.8	66.9	66.9	66.9	66.8	66.8	66.9
	Rham		Rham		Rham		Rham	
1	101.3	101.4	101.4	101.4	101.5	101.5	101.5	101.6
2	71.9	71.7	71.7	71.7	71.8	71.6	71.6	71.6

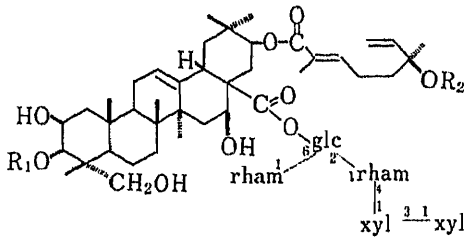
TABLE I. (continued)

Carbon	1	8	2	16	3	17	4	18
3	72.5	72.5	72.5[f]	72.5	72.5[f]	72.5[f]	72.4[f]	72.5[f]
4	84.1	84.2	84.2	84.1	84.3	84.3	84.4	84.4
5	68.3	68.4	68.3	68.3	68.4	68.4	68.4	68.4
6	18.5	18.6	18.7	18.6	18.6	18.7	18.6	18.7
	Xyl		Xyl		Xyl		Xyl	
1	106.3	106.3	106.3	106.3	106.4	106.4	106.4	106.4
2	75.0	75.0	75.0	75.0	75.1	75.1	75.1	75.1
3	87.9	87.9	87.8	87.7	87.7	87.7	87.7	87.8
4	68.8	68.8	68.9	68.9	68.9	68.9	68.9	68.9
5	67.4	67.4	67.1	67.1	67.4	67.4	67.4	67.5
	Xyl		Xyl		Xyl		Xyl	
1	106.0	106.0	106.0	106.0	106.0	106.0	106.0	106.0
2	75.1	75.1	75.1[d]	75.1[d]	75.2	75.2	75.2	75.2
3	78.2[f]	78.2[f]	78.1[f]	78.1	78.2	78.1	78.1	78.2
4	70.7	70.7	70.8[h]	70.8[f]	70.8	70.8	70.8	70.8
5	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.4
	Rham		Rham		Rham		Rham	
1	102.0	102.0	102.0	102.1	102.0	102.0	102.0	102.1
2	72.0	72.0	72.0	72.0	72.0	72.0	72.1[h]	72.1
3	72.6	72.6	72.6	72.6[g]	72.6	72.6	72.6	72.7[h]
4	73.9	73.9	73.9	73.9	73.9[g]	73.9[g]	73.9[g]	73.9[g]
5	69.6	69.6	69.7	69.6[h]	69.6	69.6	69.6	69.7
6	18.6	18.6	18.6	18.6	18.6	18.6	18.6	18.6
C-21-*O*-								
1	167.7	167.8	167.8	167.8	167.8	167.9	167.9	167.9
2	128.6	128.6	128.6	128.6	128.6	128.6	128.6	128.6
3	142.1	142.3	142.2	142.3	142.2	142.4	142.3	142.4
4	23.6	23.6	23.6	23.6	23.6	23.6	23.5	23.7
5	40.3	40.5	40.3	40.5	40.3	40.4	40.5	40.5
6	79.9	79.7	79.9	79.6	79.9	79.7	79.7	79.6
7	143.8	144.0	143.9	144.0	143.8	144.0	143.9	144.1
8	115.2	115.0	115.2	115.0	115.2	115.1	115.2	115.0
9	12.7	12.7	12.7	12.7	12.7	12.7	12.7	12.7
10	23.7	23.7	23.7	23.7	23.7	23.7	23.6	23.7
	Ara		Ara		Ara		Ara	
1	99.8	99.9	100.2	100.1	99.8	99.9	100.0	100.2
2	71.8	71.5[g]	72.5[i]	72.6[g]	71.7[h]	71.5[h]	72.1[h]	72.7[h]
3	81.2	84.0	73.0	74.7	81.2	84.0	77.0	74.7
4	71.6	69.2	72.8	69.6[h]	71.6	69.1	76.3	69.6
5	64.2	66.7	64.4	66.7	64.2	66.7	66.5	66.7
	Glc				Glc			
	106.1	106.1	168.2		106.1	106.1	168.2	
	75.6	75.7	128.0		75.6	75.6	128.0	
	78.2[f]	78.6	143.6		78.3	78.5	143.8	
	71.7	71.5[g]	23.9		71.7[h]	71.5[h]	23.9	
	78.1	78.2[f]	41.3		78.2	78.2	41.3	
	63.0	62.7	72.1		63.0	62.6	72.0	
			146.5				146.4	
	176.7[e]		111.7		176.8[e]		111.7	
	41.2		12.5		41.2		12.5	
	27.0		28.5		27.0		28.5	
	11.5				11.5			
	16.5				16.6		176.6[e]	
							41.3	
							27.1	
							11.7	
							16.7	

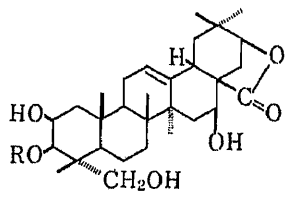
a) Measured with TMS as an internal standard, at 75.0 MHz, on a Varian XL-300. *b*, *c*, *d*) Assignments may be interchanged in each column. *e*) These five signals were assigned to a 2-methylbutyroyl group. *f*—*i*) These signals overlapped in each column.

TABLE II. Compounds Obtained from Each Saponin in Various Reactions

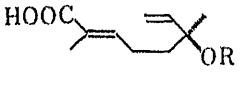
Saponin	10% H_2SO_4	2% $NaHCO_3$	10% KOH	Permethylation	Reductive cleavage of permethylate
F_2 (1)	7	8	9+10	11	12+13+14
F_1 (2)	7	16	19+20	22	25+13+26
E (3)	7	17	9+21	23	12+13+27
D_1 (4)	7	18	19+21	24	25+13+27



8 : R_1=-glc$^{2\text{-}1}$glc, R_2=-ara$^{3\text{-}1}$glc
16 : R_1=-glc$^{6\text{-}1}$ara$^{2\text{-}1}$xyl, R_2=-ara
17 : R_1=-rham, R_2=-ara$^{3\text{-}1}$glc
18 : R_1=-rham, R_2=-ara

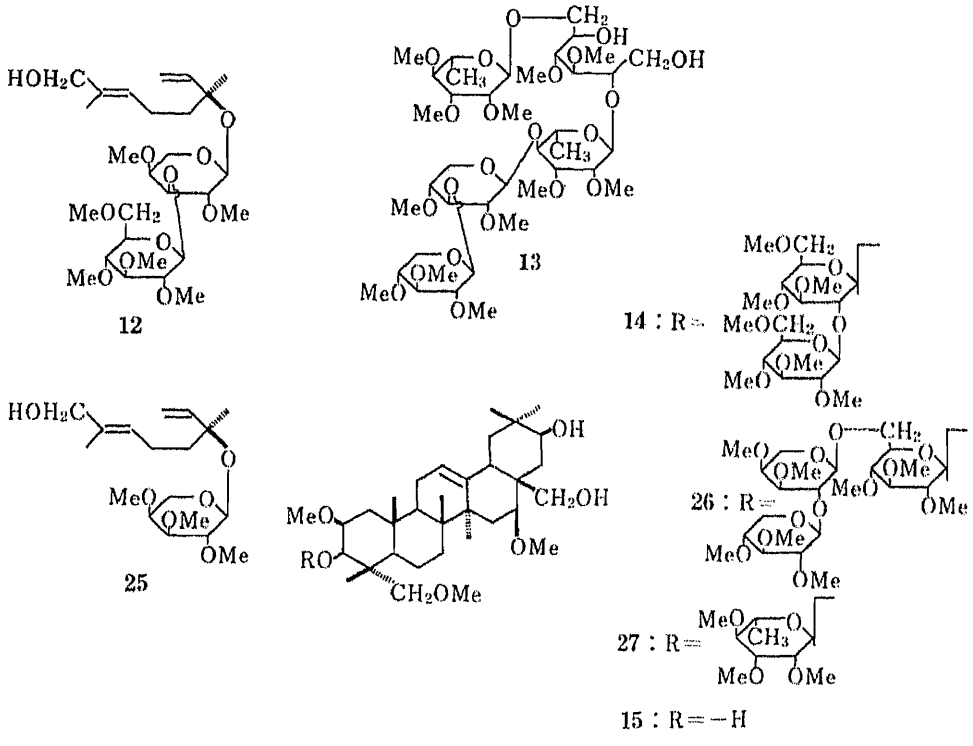


7 : R=-H
10 : R=-glc$^{2\text{-}1}$glc
20 : R=-glc$^{6\text{-}1}$ara$^{2\text{-}1}$xyl
21 : R=-rham



9 : R=-ara$^{3\text{-}1}$glc
19 : R=-ara
28 : R=-H

Chart 1



12

13

14 : R=

26 : R=

27 : R=

15 : R=-H

Chart 2

As shown in Table I, the ^{13}C-NMR signals assigned to the triterpene moieties and the oligosaccharide moieties at C-3 and C-28 of the saponins (**2**, **3** and **4**) were superimposable on those of the deacylated saponins (**16**, **17** and **18**, respectively), except for the signals of arabinose of the glycosylated monoterpene moiety. In the ^{13}C-NMR spectrum of **2**, the five prominent signals at δ 168.2, 146.5, 143.6, 128.0 and 111.7, in addition to signals due to the

arabinosyloxy monoterpene carboxylate, were assigned to the other monoterpene carboxylate. The signals attributable to C-3, C-4 and C-5 of the arabinose of the glycosyl monoterpene moiety at δ 74.7, 69.6 and 66.7, respectively, in **16**, were found to be shifted -1.7, $+3.2$ and -2.3 ppm in the case of the saponin F_1 (**2**). The monoterpene carboxylic acid (**28**) was obtained from **2** by alkaline hydrolysis, and was identical with a sample obtained from **2** by alkaline hydrolysis, and was identical with a sample obtained from gleditsia saponin C.[9]

From these results, and a consideration of the acylation shift rule, the hydroxyl group at C-4 of the arabinopyranosyloxy monoterpene carboxylate was concluded to be acylated by the monoterpene carboxylic acid (**28**) in the molecule of saponin F_1 (**2**). Therefore, gymnocladus saponin F_1 (**2**) was concluded to be 2β,23-dihydroxy-3-*O*-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl]-21-*O*-{(6*S*)-2-*trans*-2,6-dimethyl-6-*O*-[4-*O*-((6*S*)-2-*trans*-2,6-dimethyl-6-hydroxy-2,7-octadienoyl)-α-L-arabinopyranosyloxy]-2,7-octadienoyl}-acacic acid 28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl ester.

In the ^{13}C-NMR spectrum of **3**, the prominent five signals at δ 176.8, 41.2, 27.0, 11.5 and 16.6, and the signals due to the glucopyranosylarabinopyranosyloxy monoterpene moiety were superimposable on those of **1**. The signals of the glycosyl monoterpene moiety of the deacylated compound (**17**) were also superimposable on those of **8**. These results suggested that the acyl group at C-21 of the 2β,23-dihydroxyacacic acid of **3** is (6*S*)-2-*trans*-2,6-dimethyl-6-[3-*O*-(β-D-glucopyranosyl)-4-*O*-(2-methylbutanoyl)-α-L-arabinopyranosyloxy]-2,7-dienoic acid, as in **1**. Therefore, gymnocladus saponin E (**3**) was concluded to be 2β,23-dihydroxy-3-*O*-α-L-rhamnopyranosyl-21-*O*-{(6*S*)-2-*trans*-2,6-dimethyl-6-[3-*O*-(β-D-gluco-

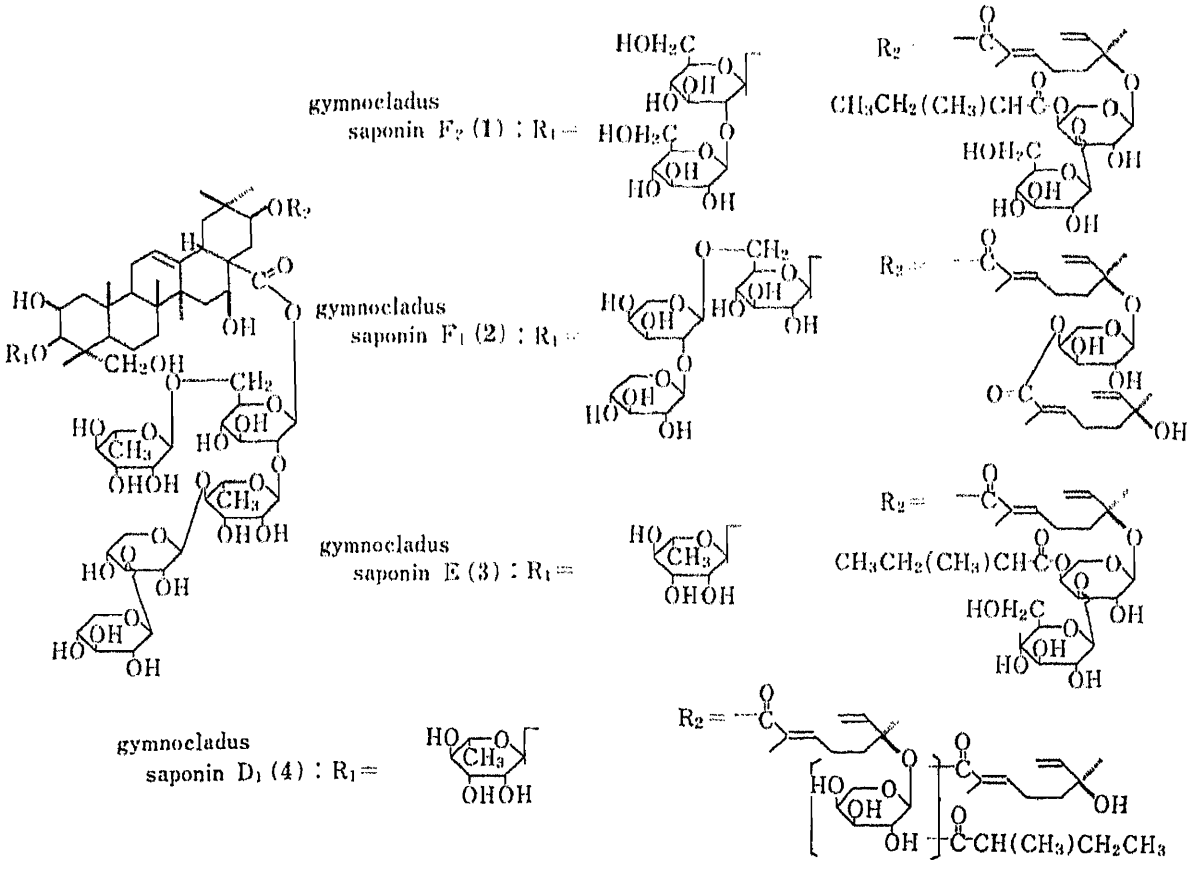


Chart 3

pyranosyl)-4-*O*-(2-methylbutanoyl)-α-L-arabinopyranosyloxy]-2,7-octadienoyl}-acacic acid 28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl ester.

From the ^{13}C-NMR spectrum of gymnocladus saponin D_1 (**4**), it was clear that two acyl groups are located on the arabinosyloxy monoterpene carboxylate. One of the acyl groups is a 2-methylbutanoyl group and the other is a (6*S*)-2-*trans*-2,6-dimethyl-6-hydroxy-2,7-dienoyl group. Studies on the positions of these acyl groups, and on the inhibitory effects of all four saponins and the deacylated saponins on EBV activation induced by TPA and by teleocidin B are in progress.

Experimental

^{1}H-NMR spectra were measured on a Varian XL 300 NMR spectrometer in $CDCl_3$ at 300 MHz with tetramethylsilane (TMS) as an internal standard. UV spectra were measured on a Shimadzu UV 240 spectrometer in 95% EtOH. High performance liquid chromatography (HPLC) was carried out on a μ-Bondapak C-18 (Waters Ltd) column, 7.8 mm × 30 cm, at a solvent flow rate of 1.8 ml/min. Conditions of TLC and gas liquid chromatography (GLC) for identification of methylated monosaccharides were reported in previous papers.[4,10]

Purification and Properties of 1, 2, 3 and 4——The crude saponin fraction[11] was chromatographed repeatedly on a silica gel column with $CHCl_3$: MeOH : H_2O = 65 : 35 : 10 (lower layer) to afford four fractions. These four fractions were subjected to gel filtration on Sephadex LH20 with MeOH several times, and purified by preparative HPLC with MeOH : H_2O = 68 : 32 (in the cases of **1** and **2**) or MeOH : H_2O = 65 : 35 (in the cases of **3** and **4**).

Gymnocladus saponin F_2 (**1**) was obtained as a hygroscopic white powder (yield: 0.005% from the dried fruits). $[\alpha]_D^{23}$ −3.6° (*c* = 0.77, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm (ε): 217 (18000). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{96}H_{154}O_{50}\cdot H_2O$: C, 54.23; H, 7.40. Found: C, 54.19; H, 7.51.

Gymnocladus saponin F_1 (**2**) was obtained as a hygroscopic white powder (yield: 0.002%). $[\alpha]_D^{23}$ −2.0° (*c* = 0.71, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm (ε): 212 (10000), 217 (18000). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{99}H_{156}O_{49}\cdot 7H_2O$: C, 52.70; H, 7.59. Found: C, 52.60; H, 7.59.

Gymnocladus saponin E (**3**) was obtained as a hygroscopic white powder (yield: 0.004%). $[\alpha]_D^{23}$ −5.4° (*c* = 0.76, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm (ε): 217 (18000). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{90}H_{144}O_{44}\cdot 2H_2O$: C, 54.98; H, 7.59. Found: C, 55.11; H, 7.87.

Gymnocladus saponin D_1 (**4**) was obtained as a hygroscopic white powder (yield: 0.002%). $[\alpha]_D^{23}$ +28.0° (c = 1.10, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm (ε): 212 (10000), 217 (18000). ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{94}H_{148}O_{41}\cdot 8H_2O$: C, 54.33; H, 7.95. Found: C, 54.30; H, 7.68.

Hydrolysis of 1, 2, 3 and 4 with 10% H_2SO_4——A solution of **1** (50 mg) in EtOH (10 ml) and 10% H_2SO_4 (10 ml) was refluxed for 2.5 h and then neutralized with Amberlite IRA 450. The reaction mixture was concentrated to half the initial volume *in vacuo*, and extracted with EtOAc successively. The organic layer was evaporated, and the residue was recrystallized from MeOH–H_2O to afford 2β,23-dihydroxyacaco-28,21β-lactone (**7**, 5 mg) as colorless needles, mp 187—188°C, (identical with an authentic sample). From the water layer, glucose, xylose, arabinose and rhamnose were identified by paper partition chromatography (PPC). From each of saponins F_1 (**2**), E (**3**) and D_1 (**4**), compound **7** and the same four sugars were similarly obtained and identified.

Hydrolysis of 1, 2, 3 and 4 with 2% $NaHCO_3$——A solution of **1** (130 mg) in EtOH (20 ml) and 2% $NaHCO_3$ (20 ml) was refluxed for 1.5 h. The reaction mixture was neutralized with Dowex 50 W × 8, and evaporated to dryness *in vacuo*. The residue showed two major spots on TLC (*Rf* 0.23 and 0.17, $CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower layer). The mixture was chromatographed on a silica gel column and purified by HPLC (MeOH : H_2O = 7 : 3) to give 30 mg of **1** (corresponding to *Rf* 0.23), and 60 mg of **8** (corresponding to *Rf* 0.17) as a hygroscopic white powder. $[\alpha]_D^{30}$ −2.7° (c = 0.86, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm: 217. ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{91}H_{140}O_{49}\cdot 3H_2O$: C, 52.59; H, 7.37. Found: C, 52.40; H, 7.38.

From saponin F_1 (**2**, 100 mg), **16** (38 mg) was obtained as a hygroscopic white powder in the same manner. $[\alpha]_D^{30}$ −5.6° (*c* = 0.64, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm: 217. ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{89}H_{142}O_{47}\cdot 3H_2O$: C, 52.97; H, 7.39. Found: C, 52.88; H, 7.40.

From saponin E (**3**, 120 mg), **17** (43 mg) was obtained as a hygroscopic white powder in the same manner. $[\alpha]_D^{30}$ −2.0° (*c* = 0.95, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm: 217. ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{85}H_{136}O_{43}\cdot 2H_2O$: C, 54.25; H, 7.50. Found: C, 54.21; H, 7.57.

From saponin D_1 (**4**, 80 mg), **18** (25 mg) was obtained as a hygroscopic white powder in the same manner. $[\alpha]_D^{30}$ −4.8° (c = 0.91, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3400—3600 (OH), 1680, 1720 (COOR). UV λ_{max}^{EtOH} nm: 217. ^{13}C-NMR: Table I. *Anal.* Calcd for $C_{79}H_{126}O_{38}\cdot 3H_2O$: C, 54.60; H, 7.66. Found: C, 54.52; H, 7.72.

Hydrolysis of 1, 2, 3 and 4 with 10% KOH——A solution of **1** (90 mg) in EtOH (20 ml) and 10% KOH (20 ml) was refluxed for 3 h, and neutralized with Dowex 50 W × 8. The solution was evaporated to half the initial volume,

and extracted with *n*-BuOH saturated with H_2O. The organic layer was evaporated to dryness *in vacuo* to give a brown oily residue (60 mg), which showed two major spots on TLC (*Rf* 0.60 and 0.45, $CHCl_3$: MeOH : H_2O = 65 : 35 : 10, lower layer). The mixture was subjected to chromatography on a silica gel column and the product was purified by HPLC (MeOH : H_2O = 66 : 34) to give 24 mg of **10**, corresponding to *Rf* 0.60. This product was concluded to be identical with a reported sample[4] of gymnocladus saponin B on the basis of TLC, IR and ^{13}C-NMR comparisons. From the second fraction, 10 mg of **9** was obtained and found to be identical with a reported sample[5] of a monoterpene glycoside by TLC, IR and ^{13}C-NMR comparisons.

The saponin F_1 (**2**, 60 mg) was hydrolyzed in the same manner as described above to afford gymnocladus saponin C (**20**, 14 mg), a monoterpene glycoside (**19**, 8 mg) and a monoterpene carboxylic acid (**28**, 3 mg), which were identical with reported samples.[4,5]

The saponin E (**3**, 85 mg) was hydrolyzed in the same manner as described above to afford the 3-*O*-rhamnopyranoside of 7 (**21**) and a monoterpene glycoside (**9**), which were identical with reported samples.[7]

The saponin D_1 (**4**, 45 mg) was hydrolyzed in the same manner to afford **21**, **19** and **28**, which were identical with reported samples.[5,7]

Permethylation of 1, 2, 3 and 4——According to Hakomori's method,[6] the reagent mixture (10 ml) prepared from NaH (2 g) and dimethyl sulfoxide (DMSO) (50 ml) was added to a solution of **1** (150 mg) in DMSO (10 ml). The reaction mixture was stirred for 1 h at room temperature under an N_2 stream. After addition of CH_3I (15 ml), the reaction mixture was stirred for 3 h at room temperature, then poured into ice-water and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried over $MgSO_4$ and evaporated to give a yellow oily product (180 mg). This oily product was purified by chromatography on a silica gel column (benzene : acetone = 8 : 2) to give the permethylate (**11**, 60 mg) as a colorless syrup, $[\alpha]_D^{29}$ −21.4° (c = 0.99, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720 (COOR), 1100 (C–O–C). UV λ_{max}^{EtOH} nm: 212. ^{1}H-NMR δ: 0.76, 0.83, 0.90, 1.00, 1.20 (3H, each s, $CH_3 \times 5$), 1.25, 1.27 (6H, each s, $CH_3 \times 4$), 1.29 (3H, s, CH_3), 4.22 (1H, d, J = 8.5 Hz, anomeric H), 4.25 (1H, d, J = 7.8 Hz, anomeric H), 4.44 (1H, d, J = 8.0 Hz, anomeric H), 4.62 (1H, d, J = 7.4 Hz, anomeric H), 4.67 (1H, d, J = 7.6 Hz, anomeric H), 4.69 (1H, d, J = 7.2 Hz, anomeric H), 4.83 (1H, br s, anomeric H), 5.10 (1H, br s, anomeric H), 5.13 (1H, dd, J = 17.5, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.21 (1H, dd, J = 11.0, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.59 (1H, d, J = 6.0 Hz, anomeric H), 5.83 (1H, dd, J = 17.5, 11.0 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$). *Anal.* Calcd for $C_{119}H_{202}O_{49}$: C, 59.13; H, 8.42. Found: C, 58.96; H, 8.44.

The saponin F_1 (**2**, 115 mg) was permethylated by the same method to afford the permethylate (**22**, 30 mg) as a colorless syrup, $[\alpha]_D^{29}$ −14.3° (c = 1.00, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720 (COOR), 1100 (C–O–C). UV λ_{max}^{EtOH} nm: 212. ^{1}H-NMR δ: 0.77, 0.83, 0.92, 1.00, 1.20 (3H, each s, $CH_3 \times 5$), 1.25 (12H, s, $CH_3 \times 4$), 1.29 (3H, s, CH_3), 4.19 (1H, d, J = 7.3 Hz, anomeric H), 4.28 (1H, d, J = 7.4 Hz, anomeric H), 4.44 (1H, d, J = 7.4 Hz, anomeric H), 4.52 (1H, d, J = 4.2 Hz, anomeric H), 4.61 (1H, d, J = 7.6 Hz, anomeric H), 4.69 (1H, d, J = 7.4 Hz, anomeric H), 4.82 (1H, br s, anomeric H), 5.10 (1H, br s, anomeric H), 5.13 (1H, dd, J = 17.6, 1.4 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.20 (1H, dd, J = 11.0, 1.4 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.57 (1H, d, J = 6.0 Hz, anomeric H), 5.83 (1H, dd, J = 17.6, 11.0 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$). *Anal.* Calcd for $C_{115}H_{194}O_{47}$: C, 59.31; H, 8.40. Found: C, 59.20; H, 8.49.

The saponin E (**3**, 149 mg) was permethylated by the same method to afford the permethylate (**23**, 40 mg) as a colorless syrup, $[\alpha]_D^{29}$ −22.8° (c = 0.96, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720 (COOR), 1100 (C–O–C). UV λ_{max}^{EtOH} nm: 212. ^{1}H-NMR δ: 0.77, 0.83, 0.86, 1.00, 1.19 (3H, each s, $CH_3 \times 5$), 1.25 (15H, s, $CH_3 \times 5$), 1.29 (3H, s, CH_3), 4.25 (1H, d, J = 7.6 Hz, anomeric H), 4.44 (1H, d, J = 7.6 Hz, anomeric H), 4.62 (1H, d, J = 7.6 Hz, anomeric H), 4.69 (1H, d, J = 7.4 Hz, anomeric H), 4.82 (1H, br s, anomeric H), 4.85 (1H, br s, anomeric H), 5.10 (1H, br s, anomeric H), 5.13 (1H, dd, J = 17.5, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.20 (1H, dd, J = 11.0, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.58 (1H, d, J = 6.2 Hz, anomeric H), 5.83 (1H, dd, J = 17.5, 11.0 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$). *Anal.* Calcd for $C_{109}H_{184}O_{43}\cdot H_2O$: C, 59.98; H, 8.50. Found: C, 59.81; H, 8.67.

The saponin D_1 (**4**, 90 mg) was methylated by the same method to afford the permethylate (**24**, 20 mg) as a colorless syrup, $[\alpha]_D^{29}$ −18.9° (c = 0.92, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720 (COOR), 1100 (C–O–C). UV λ_{max}^{EtOH} nm: 212. ^{1}H-NMR δ: 0.77, 0.84, 0.86, 1.00, 1.19 (3H, each s, $CH_3 \times 5$), 1.26 (15H, s, $CH_3 \times 5$), 1.13 (3H, s, CH_3), 4.28 (1H, d, J = 6.9 Hz, anomeric H), 4.62 (1H, d, J = 7.6 Hz, anomeric H), 4.69 (1H, d, J = 7.0 Hz, anomeric H), 4.83 (1H, br s, anomeric H), 4.85 (1H, br s, anomeric H), 5.10 (1H, br s, anomeric H), 5.13 (1H, dd, J = 17.6, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.21 (1H, dd, J = 10.8, 1.5 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$), 5.59 (1H, d, J = 6.0 Hz, anomeric H), 5.83 (1H, dd, J = 17.6, 10.8 Hz, $\overset{H}{R}\!>\!C\!=\!C\!<\!\overset{H}{H}$). *Anal.* Calcd for $C_{100}H_{168}O_{38}$: C, 60.71; H, 8.56. Found: C, 60.59; H, 8.70. From the deacyl saponins (**8**, **16**, **17** and **18**), the permethylates (**11**, **22**, **23** and **24**, respectively) were also obtained in the same manner.

Reductive Cleavage of 11, 22, 23 and 24 with $LiAlH_4$——$LiAlH_4$ (100 mg) was added to a solution of the permethylate (**11**, 120 mg) in anhydrous tetrahydrofuran (THF) (10 ml), and the mixture was refluxed for 3 h. The excess $LiAlH_4$ was decomposed with wet Et_2O and mixture was extracted with Et_2O and EtOAc successively. The Et_2O extract was chromatographed on a silica gel column (benzene : acetone = 5 : 3) to give 15 mg of **12** as a colorless syrup. From the second fraction, a triterpenoid glycoside alcohol (**14**, 25 mg) was obtained as an amorphous powder, $[\alpha]_D^{21}$ +14.8° (c=1.10, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450 (OH), 1100 (C–O–C). ^{1}H-NMR δ: 0.87, 0.91, 0.92, 0.96, 1.21, 1.29 (3H, each s, C$\underline{H}_3$ × 6), 3.29, 3.31, 3.32, 3.35, 3.37, 3.52, 3.53, 3.60, 3.64, 3.65 (3H, each s, OC$\underline{H}_3$ × 10), 4.02 (1H, dd, J=11.2, 5.4 Hz, C-18-H), 4.22 (1H, d, J=7.8 Hz, anomeric H), 4.68 (1H, d, J=7.7 Hz, anomeric H), 5.28 (1H, t like, C-12-H). *Anal.* Calcd for $C_{52}H_{90}O_{16}\cdot H_2O$: C, 63.13; H, 9.37. Found: C, 63.01; H, 9.38. The EtOAc extract was purified by preparative TLC (benzene : acetone = 1 : 1) to give an oligosaccharide alcohol (**13**, 15 mg) as a colorless oil. The monoterpene glycosyl alcohol (**12**) and the oligosaccharide alcohol (**13**) were found to be identical with reported samples derived from gymnocladus saponin G by TLC, IR, ^{1}H-NMR and optical rotation comparisons. From the permethylate (**22**), the oligosaccharide alcohol (**13**), the monoterpene glycosyl alcohol (**25**) and the triterpenoid glycosyl alcohol (**26**) were obtained in the same manner, and identified by comparison with the reported compounds (TLC, IR and ^{1}H-NMR). From the permethylate (**23**), **13**, **12** and **27** were similarly obtained and identified. From the permethylate (**24**), **13**, **25** and **27** were similarly obtained and identified.

Methanolysis of 14, 26 and 27——A solution of **14** (10 mg) in 2 N methanolic HCl (10 ml) was refluxed for 3 h, then neutralized with Ag_2CO_3 and filtered. The filtrate was chromatographed on a silica gel column (benzene : acetone = 5 : 4) to give a triterpene alcohol (**15**, 3 mg), methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside and methyl 3,4,6-tri-*O*-methyl-D-glucopyranoside. **15** was identical with olean-12-ene-2β,16β,23-trimethoxy-3β,21β,28-triol, which was derived from gymnocladus saponin D in the same manner, as judged from TLC, IR and ^{1}H-NMR comparisons. Both **26** and **27** also gave the same triterpene alcohol (**15**) in the same manner. Methyl 2,3,4-tri-*O*-methyl-D-xylopyranoside, methyl 3,4-di-*O*-methyl-L-arabinopyranoside and methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside were also obtained from **26**, and methyl 2,3,4-tri-*O*-methyl-L-rhamnopyranoside from **27**.

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New Heterobifunctional Cross-Linking Reagents for Protein Conjugation, *N*-(Bromoacetamido-*n*-alkanoyloxy)succinimides

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Five heterobifunctional reagents, *N*-(bromoacetamido-*n*-alkanoyloxy)succinimides having a glycine or *ω*-amino acid (*β*-alanine, *γ*-aminobutyric acid, *δ*-aminovaleric acid or *ε*-aminocaproic acid) residue, were synthesized. Bromoacetamido-*n*-alkanoyl groups could be introduced into horseradish peroxidase and hen egg-white lysozyme and its reduced and *S*-3-(trimethylated amino)propylated product by using the reagents. The number of bromoacetamido-*n*-alkanoyl groups introduced into the proteins could be successfully estimated from the amount of glycine or *ω*-amino acid (non-protein amino acid) produced from the introduced groups by acid hydrolysis. *N*-(*β*-Bromoacetamido-*n*-propionoyloxy)succinimide, one of the reagents, was examined as a reagent for the preparation of horseradish peroxidase–insulin conjugate. A single *β*-bromoacetamido-*n*-propionoyl group was first introduced into Gly^{A1}, Phe^{B1}-dicitraconylinsulin through the *ε*-amino group of its $Lysine^{B29}$ residue and the product was subjected to decitraconylation with dilute acetic acid to obtain Lys^{B29}-(*β*-bromoacetamido-*n*-propionoyl)insulin. The insulin was reacted with thiolated horseradish peroxidase to give peroxidase–insulin conjugate (molar ratio of 1 : 1).

Keywords—heterobifunctional cross-linking reagent; protein conjugation; *N*-(bromoacetamido-*n*-alkanoyloxy)succinimide; *ω*-amino acid; amino acid analysis; enzyme label; horseradish peroxidase; insulin

Bifunctional cross-linking reagents are essential for the preparation of protein–protein conjugates, protein–low molecular compound conjugates, ligand-linked solid matrices and immobilized enzymes, which have been used in biological, physiological, pharmacological and analytical researches. Among these reagents, heterobifunctional reagents are especially useful to bind either the same or different proteins with each other without the undesirable linking inherent in the use of homobifunctional reagents.[1,2] A currently available heterobifunctional reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)[3] has been used in the preparation of enzyme-labeled Fab' of immunoglobulin G (IgG),[4,5] enzyme-labeled deoxyribonucleic acid (DNA),[6] calmodulin-linked thiol-Sepharose[7] and antibody-linked liposomes.[8] Combinations of functional groups in other heterobifunctional cross-linking reagents were maleimidyl and succinimidyl groups,[8,9] and chloroformyl and bromoacetyl groups.[10]

This paper describes the synthesis and application of a new series of heterobifunctional cross-linking reagents, *N*-(bromoacetamido-*n*-alkanoyloxy)succinimides (II(*n*), Chart 1). The reagents can react with primary amino group(s) of a protein at the *N*-succinimidyl ester side to yield the protein carrying a bromoacetamido-*n*-alkanoyl group(s), and then with sulfhydryl group(s) of another protein through the introduced bromoacetyl group(s) to give a protein-protein conjugate. These reagents have a glycine residue (II(1)) or one of the *ω*-amino acid (II(2)—II(5); *β*-alanine,[11] *γ*-aminobutyric acid, *δ*-aminovaleric acid and *ε*-aminocaproic acid, respectively) residues (Chart 1). This enabled us to develop a powerful method for the estimation of the number of bromoacetamido-*n*-alkanoyl group(s) introduced into protein,

and the molar ratio of protein to protein in a conjugate prepared with II(n) except for II(1).

To verify the utility of the reagents, we investigated the introduction of bromoacetamido-n-alkanoyl groups of II(n) into horseradish peroxidase (HRP), hen egg-white lysozyme and its reduced and *S*-3-(trimethylated amino)propylated product (RT-lysozyme),[12] and we also prepared an HRP–insulin conjugate using II(2) as an example of protein–protein conjugate preparation.

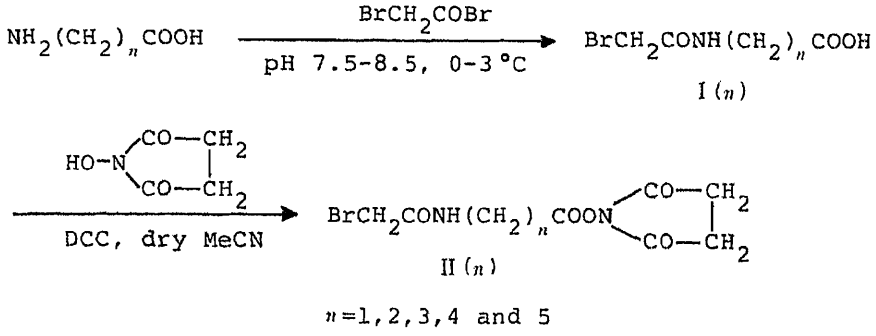


Chart 1. Synthesis of II(n)

II(1), *N*-(bromoacetamidoacetoxy)succinimide; II(2), *N*-(β-bromoacetamido-*n*-propionoyloxy)succinimide; II(3), *N*-(γ-bromoacetamido-*n*-butyryloxy)succinimide; II(4), *N*-(δ-bromoacetamido-*n*-valeroyloxy)succinimide; II(5), *N*-(ε-bromoacetamido-*n*-caproyloxy)-succinimide.

Experimental

Materials and Apparatus——Deionized water was used, which was filtered through a Milli-QII (Japan Millipore Ltd., Tokyo, Japan) before use. Diisopropyl ether (iso-Pr_2O) was passed through a column of neutral alumina AG 7 (100—200 mesh; Bio-Rad, Richmond, U.S.A.) to remove peroxide, which was checked with Peroxide Test (Japan Merck, Tokyo, Japan). Aqueous urea was passed through an Amberlite MB 3 (mixed resin 20—50 mesh; Bio-Rad) column. HRP (285 purpurogallin units/mg, type VI; Sigma, St. Louis, U.S.A.). Hen egg-white lysozyme and RT-lysozyme[12] (both from Seikagaku Kogyo, Tokyo, Japan) were used. Gly^{A1},Phe^{B1}-Dicitraconylinsulin (porcine) was prepared as described previously.[13] All other chemicals were of reagent grade.

Melting points were measured on a Yanagimoto micromelting point apparatus. Absorbances were measured with a Hitachi 150-20 spectrophotometer in semimicro-quartz cells (10-mm optical path length, 1 ml). Electron impact mass spectra were taken with a JEOL JMS-DX300 mass spectrometer. Fluorescence intensities in the microassay of HRP activity[14] were measured with a Hitachi MPF-4 spectrofluorimeter in semimicro-quartz cells (10-mm width parallel to the excitation beam, 3-mm length parallel to the emission beam, 1 ml).

Chromatographic Conditions——Thin layer chromatography (TLC) was performed on Silica gel F-254 precoated aluminum-backed sheets (Japan Merck) with $CHCl_3$–MeOH (19 : 1, v/v; solvent A) and (4 : 1, v/v; solvent B) as developing solvents. Fractions on the plates were detected by fluorescence quenching under ultraviolet light or by the use of ninhydrin (1% in butanol) spray followed by heating at 50 °C for 1 min after successive treatments with ammonia vapor and AcOH vapor. *N*-Hydroxysuccinimide esters were detected by the ferric chloride method.[15]

Adsorption column chromatography was carried out on a Silica gel 60 (Japan Merck) column (400 × 15 mm i.d.) with stepwise elution using 100 ml of $CHCl_3$ and 200 ml each of $CHCl_3$ solutions containing 0.5, 1, 2, and 3% (v/v) MeOH.

Anion-exchange high-performance liquid chromatography (HPLC) was carried out on a TSK gel DEAE-2SW column (300 × 7.8 mm i.d.; Toyo Soda, Tokyo, Japan) with a Hitachi 655 liquid chromatograph equipped with a 655 proportioning valve pump for gradient elution, a 650—60 recording processor, a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Toyo Soda UV-8 model II spectrophotomonitor operated at 278 nm. Elution with an NaCl concentration gradient during 40 min was done at a flow rate of 1.4 ml/min with eluant A (0.05 M Na-K phosphate buffer (pH 7.0) containing 4 M urea) and eluant B (eluant A containing 0.5 M NaCl). The NaCl gradient was as follows: the initial eluant contained 90% of eluant A and 10% of eluant B. The content of eluant B was increased to 30% linearly during 20 min and to 100% linearly during another 20 min. The used column was regenerated by passing eluant B for 20 min and then the initial eluant for 20 min. Chromatogram peaks were identified by the 1-fluoro-2,4-dinitro-benzene method.[13,16]

Gel-permeation HPLC was done on a TSK gel G3000SW column (600 × 7.5 mm i.d.; Toyo Soda) with a Waters

M-45 liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (200-μl loop) and a Waters 440 absorbance detector operated at 254 and 405 nm. The mobile phase was 0.05 M Na phosphate buffer (pH 7.0) containing 0.15 M NaCl, and the flow rate was 0.7 ml/min.

Amino Acid Analysis——Performed on a Hitachi 835 amino acid analyzer after hydrolysis of protein samples in 6 M HCl *in vacuo* at 110 °C for 24 h, using a column (150 × 4 mm i.d.) packed with a Hitachi 2619 custom ion-exchanger resin with the sodium citrate buffer system as the mobile phase. The column temperature was 53 °C. The stepwise elution of amino acids was carried out as described in the handling manual[17] except that the concentrations of benzyl alcohol in eluants III and IV described in the manual (0 and 5% (v/v), respectively) were changed to 5 and 10% (v/v), respectively.

Determination of Bromoacetamido-*n*-alkanoyl Groups Introduced into Proteins——The number of bromoacetamido-*n*-alkanoyl groups ($BrCH_2CONH(CH_2)_nCO$–, $n=1$, 2, 3, 4 or 5) incorporated into proteins was determined from the amount of glycine (calculated by subtracting the amount of glycine in intact protein), β-alanine, γ-aminobutyric acid, δ-aminovaleric acid or ε-aminocaproic acid, respectively; the chromatogram peaks of these ω-amino acids (non-protein amino acids) were observed in that order between the peaks for tyrosine and phenylalanine, phenylalanine and lysine, lysine and ammonia, and histidine and arginine, respectively. When the peak area obtained with glycine was taken as 100, those with β-alanine, γ-aminobutyric acid, δ-aminovaleric acid and ε-aminocaproic acid were 51.2, 98.1, 81.1 and 60.1, respectively.

Synthesis of *N*-(Bromoacetamido-*n*-alkanoyloxy)succinimides (II(*n*)) *via* Bromoacetamido-*n*-alkylcarboxylic Acids (I(*n*))——I(1)—I(3) and I(5) (Chart 1) were synthesized by the previous method.[18] I(4) (Chart 1) was prepared by bromoacetylation of δ-aminovaleric acid with bromoacetyl bromide in the same way as reported previously, with some modifications.[18] The reaction was carried out at pH 7.5—8.5 in an ice-water bath. I(1) was obtained in sufficiently pure form. I(2)—I(5) were purified by adsorption chromatography. Fractions (40 ml each) containing I(2)—I(5) were collected, checked by TLC (Rf values (solvent A, solvent B): I(2) (0.10, 0.49); I(3) (0.11, 0.57); I(4) (0.13, 0.63); I(5) (0.18, 0.66)), concentrated to dryness and recrystallized from tetrahydrofuran (THF)–iso-Pr_2O. The melting points of I(1)—I(3) were in agreement with reported data.[18] That of I(5) was 78—79 °C, though it was reported to be 69—70 °C.[18] I(4) (mp 77—78 °C, colorless powder, yield 20%) was characterized by the following data. *Anal.* Calcd for $C_7H_{12}BrNO_3$: C, 35.31; H, 5.08; N, 5.88. Found: C, 35.03; H, 5.03; N, 5.86. Mass spectrum (MS) m/z: 238 (M^+H), 240 (M^+H+2).

I(*n*) (1.0 mmol) and *N*-hydroxysuccinimide (1.0 mmol) were dissolved in 15 ml of dry MeCN, and then 1.0 mmol of *N*,*N*′-dicyclohexylcarbodiimide (DCC) in 5 ml of dry MeCN was added. The mixture was stirred under protection from moisture at 4 °C overnight. The resulting precipitates were filtered off, the filtrate was concentrated to dryness, and the residue was recrystallized from THF–iso-Pr_2O to give II(*n*) (all colorless needles). The melting points, yields, elemental analyses and MS data are summarized in Table 1.

Introduction of Bromoacetamido-*n*-alkanoyl Groups into HRP——HRP (62.5 nmol, 2.5 mg) was dissolved in 500 μl of 0.1 M Na phosphate buffer (pH 7.5), and a 10 to 100-fold molar excess of II(*n*) in 100 μl of MeCN was added under stirring. The mixture was kept at 23 °C for 1 h, dialyzed against 1 l of water (7 times) at 4 °C and lyophilized. The number of bromoacetamido-*n*-alkanoyl groups introduced into HRP was calculated from the amino acid analysis data.

TABLE 1. Data for II(*n*)

Compound[a]	mp[b] (°C)	Yield (%)	Formula Molecular weight	Elemental analysis Calcd (Found) (%)			MS peak[c] (m/z)		Rf in TLC Solvent[d]	
									A	B
II(1)	119—121	76.1	$C_8H_9BrN_2O_5$ 293.1	32.78 (32.69	3.09 3.14	9.55 9.44)	293 (M^+H)	295 (M^+H+2)	0.21	0.73
II(2)	104—106	50.6	$C_9H_{11}BrN_2O_5$ 307.1	35.19 (35.32	3.61 3.66	9.12 9.32)	307 (M^+H)	309 (M^+H+2)	0.25	0.73
II(3)	98—100	53.3	$C_{10}H_{13}BrN_2O_5$ 321.1	37.40 (37.87	4.08 4.27	8.72 8.70)	321 (M^+H)	323 (M^+H+2)	0.25	0.73
II(4)	78—79	52.4	$C_{11}H_{15}BrN_2O_5$ 335.2	39.42 (39.39	4.51 4.51	8.35 8.42)	334 (M^+) 336 (M^++2)	335 (M^+H) 337 (M^+H+2)	0.27	0.73
II(5)	89—90	69.8	$C_{12}H_{17}BrN_2O_5$ 349.2	41.28 (41.08	4.90 5.03	8.02 7.91)	348 (M^+) 350 (M^++2)	349 (M^+H) 351 (M^+H+2)	0.35	0.76

a) Stable for 3 months when kept dry under protection from light. *b*) Uncorrected. *c*) Assigned by analogy with the peak for M^+H+2 of II(1) (found, m/z 294.9777; calcd for $C_8H_{10}{}^{81}BrN_2O_5$, m/z 294.9753) in the high-resolution MS. *d*) See the experimental section.

Introduction of Bromoacetamido-*n*-alkanoyl Group into Lysozyme and RT-Lysozyme——Lysozyme or RT-lysozyme (22.5 nmol; 320 and 350 μg, respectively) was dissolved in 360 μl of 0.1 M Na phosphate buffer (pH 7.5) and a 10 to 100-fold molar excess of II(*n*) in 20 μl of dry MeCN was added under stirring. The mixture was maintained at 23 °C for 1 h, dialyzed against 1 l of water (5—7 times) at 4 °C and lyophilized. The number of introduced bromoacetamido-*n*-alkanoyl groups was calculated from the amino acid analysis data.

Preparation of Lys^{B29}-(β-Bromoacetamido-*n*-propionoyl)insulin (BAP-insulin)——A solution of II(2) (17 μmol, 5.2 mg) in 100 μl of MeCN was added to a solution of Gly^{A1},Phe^{B1}-dicitraconylinsulin (0.17 μmol, 1 mg) in 1.0 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl at 30 °C under stirring, and stirring was continued for 1 h. The resulting mixture containing Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin was dialyzed against 1 l of 1 M AcOH (pH 2.5) (3 times) at 4 °C. The dialyzate was allowed to stand at 30 °C for 20 h to decitraconylate, dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The resulting powder was dissolved in 200 μl of 0.1 M Na phosphate buffer (pH 7.0) and subjected to anion-exchange HPLC. The BAP-insulin fraction was dialyzed against 1 l of water (4 times) at 4 °C and lyophilized. The colorless powder (0.5 mg) thus obtained was stored at −20 °C. The molar ratio of BAP group to insulin moiety was estimated by the amino acid analysis data.

Preparation of Gly^{A1},Phe^{B1}-Dicitraconyl-BAP-insulin and Phe^{B1}-Citraconyl-BAP-insulin——Gly^{A1},Phe^{B1}-Dicitraconylinsulin (0.85 μmol, 5 mg) was reacted with II (2) (85 μmol, 26 mg) in the same way as described above using 5 volumes of the solvents. The resulting mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The lyophilizate was dissolved in 300 μl of 0.1 M AcOH. This solution was allowed to stand at 4 °C for 10 h, then 3 ml of water was added, and the mixture was neutralized with 2.5 M NaOH. The neutralized mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized again. The resulting powder was dissolved in 400 μl of eluant A. A portion (40 μl) of the solution was subjected to anion-exchange HPLC. The eluates corresponding to the peaks of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin, Phe^{B1}-citraconyl-BAP-insulin and BAP-insulin were collected. This procedure was repeated 10 times and the combined individual eluates were dialyzed against 1 l of water (5 times) at 4 °C and lyophilized. Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin (0.3 mg) and Phe^{B1}-citraconyl-BAP-insulin (1.5 mg) were obtained along with BAP-insulin (0.3 mg).

Preparation of Thiolated HRP (HRP-SH)——A 250 μl aliquot of 0.1 M *S*-acetylmercaptosuccinic anhydride in dimethylformamide was added to 5 mg (125 nmol) of HRP in 2.5 ml of 0.1 M Na phosphate buffer (pH 7.5) containing 0.1 M NaCl under stirring at 30 °C, and the mixture was stirred continuously for 1 h. The resulting mixture was dialyzed against 1 l of 0.1 M Na phosphate buffer (pH 6.0, evacuated) at 4 °C. To the dialyzate (2.7 ml), 540 μl of 0.1 M Tris–HCl buffer (pH 7.0, evacuated), 54 μl of 0.1 M ethylenediaminetetra acetic acid (EDTA)·2Na in the Tris–HCl buffer and 190 μl of 1 M hydroxylamine·HCl in the Tris–HCl buffer were added. Immediately after the addition, the pH of the solution was adjusted to 7.3 with 2.5 M NaOH (evacuated) at 30 °C and the mixture was stirred continuously for 20 min. The resulting mixture was dialyzed against 1 l of 0.1 M Na phosphate buffer (pH 6.0) containing 0.05 M NaCl and 5 mM EDTA·2Na (evacuated) (4 times) at 4 °C. A 3.5 ml aliquot of HRP-SH solution (35.7 nmol/ml) was obtained. The number of sulfhydryl groups in the HRP-SH was determined as 2.15 by the 4,4′-dithiodipyridine method.[19]

Preparation of HRP–Insulin (1 : 1) Conjugate——BAP-insulin (150 nmol, 0.9 mg) was dissolved in 700 μl of the HRP-SH solution (evacuated) and the pH of the mixture was adjusted to 8.0 with 2.5 M NaOH (evacuated) under stirring at 30 °C. The mixture was kept at 30 °C for 1 h. The resulting mixture was dialyzed against 1 l of water (3 times) at 4 °C and lyophilized. The resulting powder was dissolved in 200 μl of 0.1 M Na phosphate buffer (pH 7.0) and subjected to gel-permeation HPLC. The eluate from the peak of HRP–insulin was collected, and dialyzed against 1 l of water (2 times) at 4 °C. The dialyzate was concentrated to 2.0 ml (10.3 nmol/ml HRP–insulin conjugate) under vacuum, and the solution was stored at −20 °C. The molar ratio of HRP to insulin in the conjugate was estimated from the amino acid analysis data. The concentration of the HRP–insulin conjugate was calculated from the absorbance at 403 nm, where the absorption is only due to the HRP moiety. The molar absorptivity of HRP at 403 nm was taken as $1.02 \times 10^5\ M^{-1}\ cm^{-1}$.[20]

Results and Discussion

The *N*-succinimidyl ester moiety in the reagents undergoes nucleophilic attack by a primary amine to form an amide bond.[21] Because HRP and its derivatives have been widely used in biological investigations, the introduction of bromoacetamido-*n*-alkanoyl groups into HRP was examined.

Table II shows the number of bromoacetamido-*n*-alkanoyl groups introduced per HRP under the described conditions. The maximum number was 2.1—3.2. A similar result was observed in the introduction of the 3-(2-pyrydyldithio)propionoyl or ε-maleimidocaproyl group into HRP using SPDP or *N*-(ε-maleimidocaproyloxy)succinimide (EMCS),[22] re-

TABLE II. Number of Bromoacetamido-*n*-alkanoyl Groups Introduced into HRP[a]

Reagent	Reagent/HRP (mol/mol) in the reaction				
	10	15	25	50	100
II(1)	0.5	0.6	1.0	1.6	2.3
II(2)	0.8	1.1	1.5	1.8	2.1
II(3)	1.3	1.6	2.2	2.5	2.6
II(4)	1.2	1.8	2.3	2.9	3.2
II(5)	1.3	1.8	2.1	2.6	2.6

a) Based on 23 residues of alanine in an HRP molecule.

TABLE III. Number of II(*n*) Moieties Introduced into Lysozyme and RT-Lysozyme[a]

Reagent	Lysozyme or RT-lysozyme[b]	Reagent/lysozyme or RT-lysozyme (mol/mol) in the reaction				
		10	15	25	50	100
II(1)	L	0.7	1.2	1.3	4.5	5.8
	RT-L	1.2	1.6	2.7	6.0	7.6
II(2)	L	5.5	6.8	7.7	8.1	10.9
	RT-L	6.1	7.8	9.1	9.7	11.1
II(3)	L	3.0	4.1	5.0	7.1	8.7
	RT-L	4.0	5.0	7.3	8.5	9.6
II(4)	L	3.4	4.5	5.1	6.4	7.9
	RT-L	4.7	6.7	7.5	8.5	9.6
II(5)	L	4.5	5.7	6.5	8.8	10.0
	RT-L	6.4	8.1	8.7	9.9	10.5

a) Based on 12 residues of alanine in a lysozyme molecule. *b*) L, lysozyme; RT-L, RT-lysozyme.

spectively (data not given). There is no evidence of alkylation of lysine or histidine residues of HRP, indicating that the reaction occurred only at the *N*-succinimidyl ester side of II(*n*). These observations suggest that II(*n*) can be used for controlled introduction of bromoacetamido-*n*-alkanoyl groups into protein amino groups.

The reactions of II(*n*) with lysozyme and RT-lysozyme were also investigated (Table III). Lysozyme is useful to examine various reactions because the protein contains all the kinds of amino acid residues that occur in natural proteins. The number of II(*n*) moieties introduced into RT-lysozyme was greater than that for lysozyme. This can be explained on the basis of the unfolded structure of RT-lysozyme. The introduction of more than 7 II(*n*) moieties was observed, though lysozyme and RT-lysozyme both have 7 amino groups. The reason for this result is not known.

In general, haloacyl groups readily react with the thiolate ion, and under certain conditions, they also react with other nucleophilic sites (amino, imidazole and thioether groups) of proteins.[23] Thus, II(*n*) should be usable for cross-linking between a protein without a reactive sulfhydryl group and a protein with a reactive sulfhydryl group.

Because of our interest in the use of enzymelabeled insulin in an assay of insulin receptor, we prepared HRP–insulin conjugate by using II(2) (Chart 2). HRP was selectively linked to the ε-amino group of the lysine^{B29} residue of insulin. In the first step, a single BAP group was introduced into Gly^{A1},Phe^{B1}-dicitraconyl-insulin to obtain Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin. Then, the citraconyl groups of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-

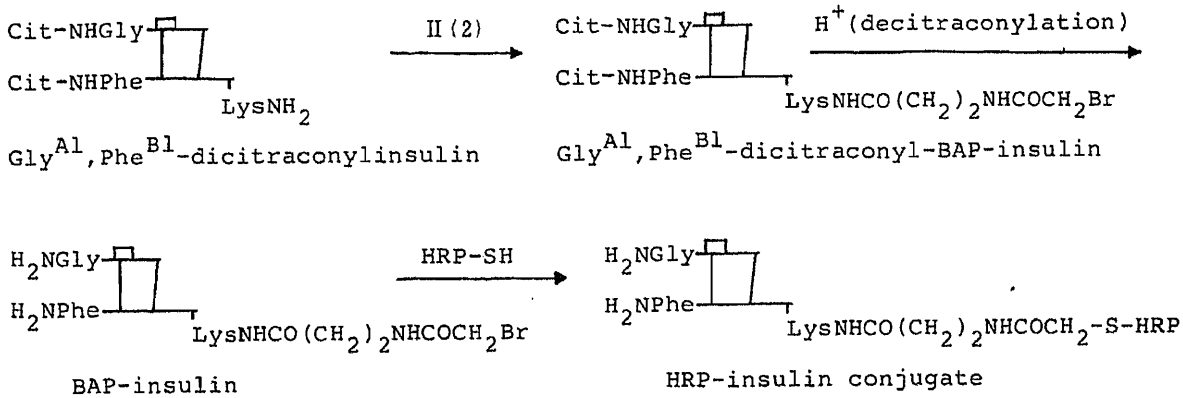


Chart 2. Introduction of a Single BAP Group into Gly^{A1},Phe^{B1}-Dicitraconylinsulin and Preparation of HRP-Insulin Conjugate

Cit: citraconyl.

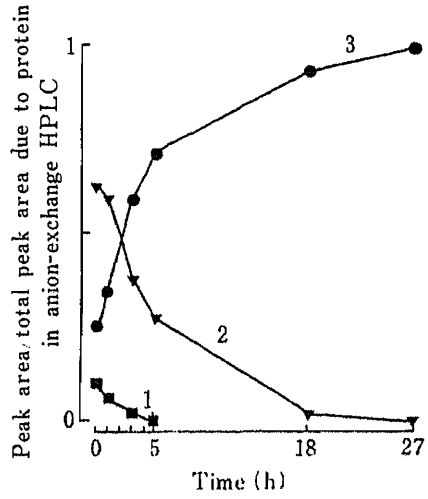


Fig. 1. Time-Course of Decitraconylation of Gly^{A1},Phe^{B1}-Dicitraconyl-BAP-Insulin and Phe^{B1}-Citraconyl-BAP-Insulin

The dialyzate obtained after the reaction of II (2) with Gly^{A1},Phe^{B1}-dicitraconylinsulin in the procedure for the preparation of BAP-insulin was allowed to stand at 30 °C for various periods (1—27 h) in the presence of 1 M acetic acid. Portions (10 μl) of the dialyzate and the mixtures at various reaction periods were subjected to anion-exchange HPLC. Curves: 1, Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin; 2, Phe^{B1}-citraconyl-BAP-insulin; 3, BAP-insulin.

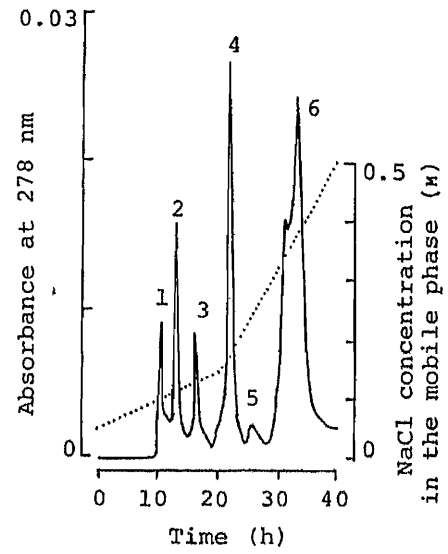


Fig. 2. Chromatogram of Insulin Derivatives

A portion (60 μl) of the neutralized mixture obtained in the preparation of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin and Phe^{B1}-citraconyl-BAP-insulin was subjected to anion-exchange HPLC. Peaks: 1, insulin; 2, BAP-insulin; 3, citraconic acid; 4, Phe^{B1}-citraconyl-BAP-insulin; 5, Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin; 6, unknown (non-protein).

insulin were eliminated with 1 M acetic acid to obtain BAP-insulin. The time-course of the decitraconylation could be followed by anion-exchange HPLC (Fig. 1). A chromatogram of the neutralized mixture obtained in the preparation of Gly^{A1},Phe^{B1}-dicitraconyl-BAP-insulin and Phe^{B1}-citraconyl-BAP-insulin is shown in Fig. 2. The decitraconylation had to be carried out prior to the conjugation with HRP, otherwise a marked deactivation of HRP was observed. The BAP group of the insulin was stable in the acidic medium. On the other hand, when EMCS was used instead of II(2), the ε-maleimidocaproyl group introduced into Gly^{A1},Phe^{B1}-dicitraconylinsulin was destroyed by the acid treatment (data not given). These

observations suggest that the acid-resistant nature of the bromoacetyl group can be effectively employed in the course of preparation of protein conjugates.

The reaction of the HRP-SH with an approximately 6-fold molar excess of BAP-insulin gave HRP–insulin (1 : 1) conjugate. Excess BAP-insulin could be recovered easily by gel-permeation HPLC because the molecular size of BAP-insulin is much smaller than that of HRP–insulin conjugate. The molar ratio of HRP to insulin in the conjugate could be calculated as 1 : 1.01 from the results of amino acid analysis of the conjugate. In the calculation, the molar ratio of β-alanine to alanine in BAP-insulin (1 : 2) was used and the amount of alanine derived only from the HRP moiety of the conjugate could be estimated by using the amount of β-alanine. The principle of the method should be applicable for the estimation of the molar ratio of protein to protein in other protein–protein conjugates prepared with II(n) except for II(1).

The HRP–insulin conjugate retained 66.7% of native HRP activity. The actual use of the conjugate will be described elsewhere.

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Non-enzymatic Reduction of Sennidins and Sennosides by Reduced Flavin

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Reduced flavin-adenine dinucleotide ($FADH_2$), which was produced by flavin-adenine dinucleotide (FAD)-dependent sennidin reductase, reduced methyl orange non-enzymatically in a molar ratio of 2:1 under anaerobic conditions. Similarly, sennidins and sennosides were reduced non-enzymatically to rheinanthrone and 8-glucosylrheinanthrone, respectively, by the reduced forms of cofactors such as FAD, flavin mononucleotide (FMN), riboflavin and benzyl viologen. The reaction was inhibited by oxygen. $FADH_2$ reduced sennidins equimolarly, but sennosides were reduced much less effectively than sennidins (about one-tenth).

The polarographic half-wave potentials of sennidins A and B, and sennosides A and B were −115, −133, −184 and −201 mV, respectively.

Therefore, it was confirmed that the reduction of sennidins and sennosides by FAD-dependent sennidin reductase consists of the following two reactions; (1) the reductase reduces cofactors such as FAD and (2) sennidins and sennosides are reduced non-enzymatically by the reduced forms of cofactors.

On the other hand, oxidation of rheinanthrone to sennidins in a neutral solution occurred with oxygen but not with FAD.

Keywords——sennidin; sennoside; sennidin reductase; non-enzymatic reduction; sennidin reduction; rheinanthrone; $FADH_2$

Introduction

Sennosides A and B, the main laxative constituent of Sennae Folium or Rhei Rhizoma, are prodrugs which are converted to an active principle, rheinanthrone, by intestinal bacteria.[1-6] Our previous investigations[5-7] showed that sennosides were metabolized to rheinanthrone through two different pathways by intestinal bacteria. These involved hydrolysis of sennosides and 8-glucosylrheinanthrone to the respective aglycones by β-glucosidase, and reduction of sennosides and sennidins to 8-glucosylrheinanthrone and rheinanthrone, respectively. Several species of human intestinal bacteria were able to reduce sennidin A to rheinanthrone.

We purified sennidin reductase from *Peptostreptococcus intermedius*, which is predominant in human intestine.[7] The purified enzyme required reduced nicotinamide adenine dinucleotide (NADH) as an electron donor and flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), riboflavin or benzyl viologen as an electron carrier for the reduction of sennidins and sennosides. These reductions were inhibited by molecular oxygen. This enzyme also reduced methyl orange, whose mode of reduction resembles that of sennidin. Gingell and Walker[8] and Mason *et al.*[9] reported non-enzymatic reduction of azo-dyes by

reduced flavins which were generated by the enzyme obtained from *Streptococcus faecalis* and by azo-reductase from rat liver microsomes under anaerobic conditions.

In this paper, we report non-enzymatic reduction of sennidins and sennosides to rheinanthrone and 8-glucosylrheinanthrone, respectively, by the reduced forms of cofactors such as FAD, FMN, riboflavin and benzyl viologen.

Experimental

Bacterium and Culture Conditions——*Peptostreptococcus intermedius* from human feces was kindly provided by Prof. T. Mitsuoka of The University of Tokyo. The bacterium, maintained in General Anaerobic Medium (GAM, Nissui) containing 0.15% agar, was first grown in GAM broth for 24 h at 37 °C in an anaerobic box, transferred to 9 volumes of the medium, and then cultured for 4 h at 37 °C under anaerobic conditions.

Purification of FAD-Dependent Sennidin Reductase——Purification was carried out principally according to the procedures described in our previous paper.[7] The bacterial cell suspension was sonicated and then centrifuged at $11000 \times g$ to obtain the supernatant fraction. The supernatant fraction was concentrated by ultrafiltration, then applied to a Sephadex G-200 column equilibrated with 20 mM K-phosphate buffer (pH 7.2), and eluted with the same buffer. Fractions containing sennidin- and methyl orange-reductase activities were collected, and applied to a diethylaminoethyl (DEAE)-cellulose column equilibrated with 20 mM K-phosphate buffer (pH 7.2). The column was eluted with 50 mM K-phosphate buffer (pH 7.2) and then with a linear gradient from 0 to 0.5 M NaCl in 50 mM K-phosphate buffer (pH 7.2). Fractions containing FAD-dependent sennidin- and methyl orange-reductase activities were collected and concentrated by ultrafiltration.

Non-enzymatic Reduction of Sennidins, Sennosides and Methyl Orange——Non-enzymatic reduction of these compounds was carried out in a Thunberg-type tube under nitrogen. In the main part of the tube, 0.2—20 μmol of a cofactor such as FAD, FMN, riboflavin or benzyl viologen was first reduced with 4—100 μmol of NADH in the presence of the purified sennidin reductase in 2 ml of 0.1 M K-phosphate buffer at 37 °C. Then 0.19 μmol of sennidin A or B, 0.23 μmol of sennoside A or B, or 0.20 μmol of methyl orange containing 2 μmol of *p*-chloromercuriphenylsulfonic acid (*p*CMS) (to inactivate the enzyme) in the side arm was mixed with the reduced cofactor in the main tube at room temperature. Rheinanthrone and 8-glucosylrheinanthrone, the reduction products of sennidins and sennosides, respectively, were determined as the azometin derivatives by adding *p*-nitroso-*N*,*N*′-dimethylaniline as described in the previous paper.[6] Reduced methyl orange was determined by measuring the absorbance at 500 nm as described previously.[7]

Determination of Half-Wave Potentials——Polarographic measurements were carried out at 25 °C for 0.1 mM compounds in 0.1 M K-phosphate buffer (pH 7.0) in an electrolytic microcell. A dropping mercury electrode from Yanagimoto Mfg. Co., Ltd., Kyoto, was used as the working electrode and a mercury pool as the counter electrode. A saturated calomel electrode was used as the reference electrode but all potentials in this paper are described *vs.* the normal hydrogen electrode. The dropping mercury electrode had the following characteristics: $m = 0.93$ mg/s (in pure water at zero applied potential) and $t = 3.0$ s. The head of mercury was 75 cm. Dissolved oxygen was removed from the solution by bubbling oxygen-free nitrogen for 30 min before electrolyzing. Polarograms were recorded with a Hokuto model PS-1000 potentiostat, an LS-1D potential sweeper and a Rikadenki model RW-21T XY recorder. The potential sweep rate was 250 mV/min. Half-wave potentials were determined from polarograms obtained by the ordinary method.

Preparation of Reduced Riboflavin——Riboflavin (20 mg) was dissolved in 200 ml of water and reduced by the addition of an excess of sodium hydrosulfite powder. When kept at room temperature, the reduced riboflavin precipitated because of its insolubility. The precipitate thus obtained was washed sufficiently with water to remove remaining hydrosulfite.

Oxidation of Rheinanthrone——Rheinanthrone (3 mg) was dissolved in 3 ml of 0.1 M K-phosphate buffer, pH 7.2, under anaerobic conditions. The solution was incubated for 5—60 min at 37 °C under air without FAD or under anaerobic conditions with 9 mg of FAD. One volume of 1 N HCl and two volumes of butanol were added to an aliquot of the reaction mixture, and the whole was shaken vigorously and centrifuged at 3000 rpm for 5 min. The upper layer, containing sennidins A and B, and rhein, was chromatographed on a silica gel thin layer plate (Merck, Silica gel 60, layer thickness 0.25 mm) and analyzed quantitatively by densitometry (Shimadzu CS-910) according to the method reported previously.[6] Remaining rheinanthrone was determined as the azometin derivative, as described above.

Chemicals——Sennosides A and B were isolated from a crude sennoside powder (Nippon Funmatsu Yakuhin Co., Ltd.) according to the method reported previously.[5] Sennidins A and B, and rheinanthrone were prepared by the methods of Stoll *et al.*[10,11] NADH was purchased from Oriental Yeast Co., Ltd., and FAD and FMN from Seikagaku Kogyo Co., Ltd.

Results

Non-enzymatic Reduction of Methyl Orange

FAD-dependent sennidin reductase from *Peptostreptococcus intermedius* was able to reduce methyl orange in the presence of FAD.[7] Reduced flavin-adenine dinucleotide ($FADH_2$) generated by the enzyme reduced methyl orange non-enzymatically after inactivation of the enzyme with *p*CMS. When one equivalent amount of $FADH_2$ with respect to methyl orange was used, complete decoloration of the reaction mixture was observed under anaerobic conditions, but the orange color was fully restored by exposure to air. When a double molar amount of $FADH_2$ was used, 62.6% of methyl orange was reduced non-enzymatically even after exposure to air as shown in Table I. Accordingly, it was suggested that 1 mol of azo group of methyl orange was reduced non-enzymatically and irreversibly to 2 mol of amine by 2 mol of $FADH_2$.

When the mixture was heated at 100 °C for 10 min in order to denature the enzyme in place of the addition of *p*CMS, similar findings were also obtained. These results show that the reduction of methyl orange by the enzyme is due to non-enzymatic reduction with $FADH_2$ generated from NADH by the enzyme.

Non-enzymatic Reduction of Sennidins and Sennosides

Sennidins A and B were also reduced non-enzymatically and instantaneously to rheinanthrone by excess amounts of reduced forms of cofactors such as FAD, FMN, riboflavin and benzyl viologen. Sennoside A was also reduced to 8-glucosylrheinanthrone, though the extent of reduction (%) was less than in the case of sennidins (Table II). Using an equivalent amount of reduced forms of cofactors such as FAD, FMN, riboflavin and benzyl viologen against sennidin A, 60—80% of sennidin A was reduced.

Reduction of sennidin A or B was proportional to the amount of $FADH_2$ added (Fig. 1). The slope shows that 1 mol of sennidin A or B was reduced by 1 mol of $FADH_2$. However,

Table I. Non-enzymic Reduction of Methyl Orange by $FADH_2$

$FADH_2$ (μmol)	Methyl orange (μmol)	$FADH_2$/methyl orange	Reduction of[a] methyl orange (%)
0.2	0.2	1	1
0.4	0.2	2	62.6
0.5	0.2	2.5	81.6

a) Measurement after exposure to oxygen by transferring the sample solutions from Thunberg tubes to cuvettes.

Table II. Non-enzymic Reduction of Sennidins and Sennoside A by Excess Amounts of Reduced Cofactors

Cofactor	Reduction (%)		
	Sennidin A	Sennidin B	Sennoside A
None	0	0	0
FAD (2 μmol)	114	76.0	64.5
FMN (2 μmol)	110	61.3	50.1
Benzyl viologen (20 μmol)	88.4	110	71.4
Riboflavin (2 μmol)	95.5	89.2	51.3

The original amounts per tube were 0.19 μmol of sennidins A and B, and 0.23 μmol of sennoside A.

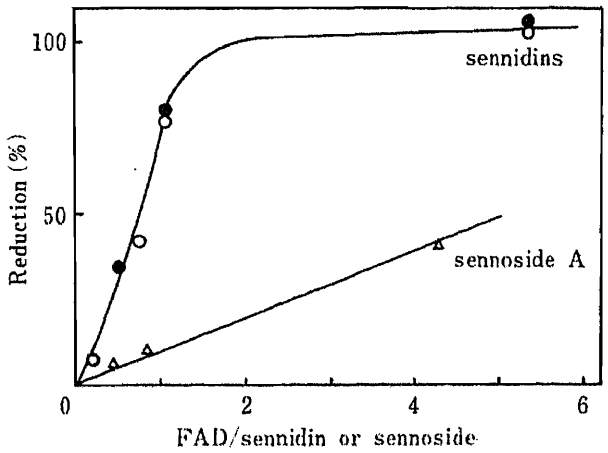


Fig. 1. Non-enzymatic Reduction of Sennidins and Sennoside A by $FADH_2$

Molar ratio of FAD with respect to sennidin or sennoside is represented on the abscissa and reduction (%) of sennidins or sennoside A on the ordinate.

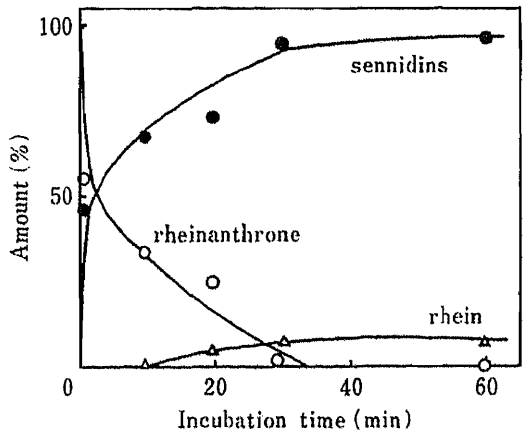


Fig. 2. Aerobic Oxidation of Rheinanthrone to Sennidins

The ordinate represents production (%) of sennidins and rhein and decrease (%) of rheinanthrone on a molar basis.

TABLE III. Polarographic Half-Wave Potentials of Sennidins and Sennosides

	Half-wave potential (mV)
Sennidin A	−115
Sennidin B	−133
Sennoside A	−184
Sennoside B	−201

about ten times more $FADH_2$ was required for the reduction of sennoside A.

Chemically reduced riboflavin also reduced sennidins and sennosides non-enzymatically. It was ascertained that sennidins were reduced non-enzymatically with an equivalent amount of reduced forms of cofactors such as FMN, riboflavin or benzyl viologen, as was the case of FAD.

Polarographic Half-Wave Potentials of Sennidins and Sennosides

Polarographic half-wave potentials, representing oxidation–reduction potentials in general, of sennidins and sennosides were measured. The values of sennidins A and B were −115 and −133 mV, respectively, higher than those of −184 and −201 mV of sennosides A and B (Table III). These results are compatible with the observation that sennidins were reduced easily in comparison with sennosides.

Oxidation of Rheinanthrone to Sennidins

Rheinanthrone was unstable when exposed to air and was readily oxidized to rhein in an acidic solution. During incubation in 0.1 M K-phosphate buffer (pH 7.2) at 37 °C under air, the amount of rheinanthrone decreased and sennidins were produced, as shown in Fig. 2. At neutral pH, a little rhein was produced and the yield of sennidins reached 90% of added rheinanthrone on a molar basis at 60 min. The ratio of sennidin A to B was three to two.

On the other hand, when rheinanthrone was incubated with FAD (oxidized form) under anaerobic conditions, no sennidins were produced, indicating that the reduction of sennidins by $FADH_2$ is irreversible.

Discussion

In the reduction of sennidins, sennosides and methyl orange, the enzyme purified from *P. intermedius* required NADH as an electron donor and FAD, FMN or benzyl viologen as an electron carrier.[7] The present study shows that this enzyme reduced the cofactors used as electron carriers, and methyl orange, sennidins and sennosides were reduced non-enzymatically by the reduçed forms of these cofactors under anaerobic conditions. Therefore, this enzyme is considered to be flavin reductase, and the reduction of sennidins, sennosides and methyl orange is presumed to proceed through the following two steps; (1) the enzyme reduces the cofactor and (2) sennidins, sennosides or methyl orange are reduced non-enzymatically by the reduced cofactor, as was the case in the reduction of azo-dyes by azo-reductase reported by Gingell and Walker[8] or Mason *et al.*[9]

Moreover, Mason *et al.*[9] reported that a radical was generated in the process of reduction of azo-dyes and this radical reacted very easily with oxygen. The presence of the radical of methyl orange was supported by our data. Namely, when methyl orange was mixed with 1 eq amount of $FADH_2$, decoloration of methyl orange was observed, but the original color was restored on exposure to oxygen. Lemli *et al.*[12] reported that anthraquinone dimers such as sennosides and sennidins easily form free radicals at the C10–C10′ bond. Therefore, it is suggested that sennidins or sennosides form a radical by accepting an electron, followed by cleavage to anthraquinones.

Polarographic half-wave potentials for sennidins A and B (−115 and −133 mV) were higher than the oxidation–reduction potentials of cofactors such as FAD, FMN and riboflavin, indicating that non-enzymatic reduction of sennidins by these cofactors can occur. It was also suggested that the less effective reduction of sennosides A and B by reduced FAD and riboflavin was due to the polarographic half-wave potentials of sennosides A and B (−184 and −201 mV) being close to the oxidation–reduction potentials of these cofactors. In accordance with this, cysteine (−140 mV) did not reduce sennidins and sennosides.

Aside from *P. intermedius*, many species of human intestinal bacteria are able to reduce sennidin A to rheinanthrone.[6] *Clostridium* sp. such as *C. sphenoides* and *C. perfringens* isolated from human feces converted sennidin A to rheinanthrone more effectively than other human intestinal bacteria. A large amount of riboflavin was produced by *Clostridium* sp. of intestinal bacteria.[13] Moreover, since the amount of riboflavin excreted into feces is more than that of riboflavin fed to various animals, biosynthesis of riboflavin by intestinal bacteria in gut probably occurs. In humans, 200—600 μg of riboflavin is excreted into feces per day even though the amount of riboflavin ingested is limited to 60—90 μg per day.[14] The amount of riboflavin in feces is enough to reduce sennidins, though the amounts of FAD and FMN in feces are not known. Accordingly, it seems likely that sennidins are converted in the gut to rheinanthrone, a laxative principle, non-enzymatically by the reduced forms of flavin produced by flavin reductases in bacteria.

We have proposed two different pathways for sennoside metabolism to rheinanthrone in the gut[5-7]; sennosides are either reduced or hydrolyzed to 8-glucosylrheinanthrone or sennidins and the respective metabolites are next hydrolyzed or reduced to rheinanthrone, a laxative principle. However, sennidins are reduced more easily by the reduced forms of flavin than sennosides, and the pathway in which sennosides are first hydrolyzed to the aglycone by β-glucosidase and then reduced to rheinanthrone seems to be the major one in the human intestine, as observed in the metabolism of sennosides by rat feces.[6] Namely, it is suggested that sennosides are metabolized in the gut to rheinanthrone by the successive involvement of β-glucosidase-positive strain(s) and flavin reductase-positive one(s), such as *Clostridium* sp. and *P. intermedius*, which do not contain sennoside β-glucosidase.

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Macrophage Activation and Immunostimulating Activity of *Sphaerotilus natans* and Its Slime Fraction

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The macrophage activation and immunostimulating effects of the slime of *Sphaerotilus natans* IAM 12068, which is an aquatic sheathed bacterium, and GF-P-1, which was isolated from the slime fraction by Sepharose 4B gel filtration and has antitumor activity against Ehrlich ascites tumor in mice, were investigated. C57BL/6 mice peritoneal exudate macrophage induced by GF-P-1, which exhibited more effective antitumor activity than slime fraction, had the strongest cytostatic activity against EL-4 leukemic cells *in vitro*. Addition of GF-P-1 to mouse peritoneal macrophages *in vitro* caused marked spreading morphology, but the slime fraction did not. Acid phosphatase activity in the peritoneal cells induced by slime and GF-P-1 was also augmented as compared with that of resident macrophages. *S. natans* fractions, as well as *Salmonella typhimurium* lipopolysaccharide, were able to induce the mitogenic response in cultured spleen cells of C57BL/6 mice. Since spleen cells pretreated with rabbit anti-mouse thymocyte antiserum showed an unaffected mitogenic response to slime, the slime may be a B-lymphocyte mitogen. When slime fraction and GF-P-1 were injected intraperitoneally into ddY mice, they exhibited an enhancing effect on antibody response *in vivo*. These results indicate that slime and GF-P-1 are able to activate macrophages and exhibit immunostimulating effects.

Keywords——*Sphaerotilus natans*; slime fraction; cytostatic activity; macrophage activation; mitogenicity; adjuvanticity

Sphaerotilus natans, which is a gram-negative, aquatic sheathed bacterium, belongs to the order *Chlamydobacteriales*.[1] The organisms of the group are characterized by the presence of slime on the surface of the sheath.

We previously reported that slime fraction of *S. natans* has antitumor activity against Ehrlich carcinoma cells in mice.[2] Furthermore, two fractions designated as GF-P-1 and GF-P-2, which are mainly composed of protein, carbohydrate, and lipid, were isolated from slime fraction by Sepharose 4B gel filtration. GF-P-1, which contains large amounts of fucose and unidentified sugar as neutral sugar, showed marked antitumor activity at half the dose of the slime fraction, whereas the antitumor activity of GF-P-2, which is composed mainly of protein, was weak.

Various microorganisms and their components are capable of suppressing tumor growth and have immunostimulating effects such as macrophage activation, mitogenic activity for lymphocytes or an adjuvant effect on antibody response. It is generally considered that such an immunostimulant effect of microorganisms plays some role in their antitumor activity. In this paper, we describe the *in vitro* cytostatic activity of C57BL/6 mice peritoneal macrophages induced by slime fraction and GF-P-1 against syngeneic EL-4 leukemic cells and the immunostimulating effects, such as mitogenic activity and an adjuvant effect.

Materials and Methods

Strain and Medium——*Sphaerotilus natans* IAM 12068 was provided by the Institute of Applied Microbiology, University of Tokyo, Tokyo. The organism was cultured under aeration at 30 °C for 24 h in modified Stokes' medium.[3] The grown cells were harvested by continuous centrifugation at 4 °C and were washed twice with distilled water.

Preparation of Slime Fraction of *S. natans*——The procedure for the preparation of *S. natans* slime and its fractions, GF-P-1 and GF-P-2, was the same as described previously.[2] In brief, washed cells were homogenized in distilled water with a Waring blender and centrifuged. The supernatant fraction was treated with 2% streptomycin sulfate and the precipitate was removed by centrifugation. The supernatant was lyophilized to provide the slime fraction. Furthermore, the slime fraction was applied to a column of Sepharose 4B (Pharmacia Fine Chemical, Uppsala, Sweden) and separated into two fractions designated as GF-P-1 and GF-P-2, respectively. Their chemical compositions were reported in the previous paper.[2]

Animals——C57BL/6 mice, 5—8 weeks old, were used in the experiments. These mice were supplied by our animal colony. Male ddY mice, weighing 20—25 g, were purchased from the Shizuoka Agricultural Cooperative for Laboratory Animals, Hamamatsu.

Cell Line——Mouse EL-4 leukemic cells were kindly provided by Dr. Kyoko Tanaka, the Aichi Cancer Center, Nagoya. The cells were maintained in tissue culture flask (Falcon, #3013) containing 10 ml of RPMI-1640 medium (Nissui Seiyaku, Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS; GIBCO) at 37 °C in 5% CO_2/air.

Preparation of Peritoneal Macrophages——C57BL/6 mice were injected intraperitoneally (i.p.) with *S. natans* whole cells (1.0 mg), slime (0.5 mg), GF-P-1 (0.2 mg), or GF-P-2 (0.2 mg) on days 1, 3, 5, and 7. Three days later, the peritoneal cells were collected with a syringe and washed twice with Hanks' balanced salt solution (Nissui Seiyaku). After centrifugation, the cells were resuspended in RPMI-1640 medium, poured into a plastic Petri dish (Falcon, #3002) and then incubated at 37 °C for 90 min. The adherent cells were collected as peritoneal macrophages in phosphate buffered-saline containing 0.2% ethylenediaminetetraacetic acid (EDTA) with a rubber policeman.

Assay of Cytostatic Activity of Mouse Peritoneal Macrophages——The *in vitro* cytostatic activity against tumor cells by C57BL/6 mouse peritoneal macrophages, which were induced by i.p. injection of *S. natans* or its fractions, was determined by measuring the inhibition of tumor cell deoxyribonucleic acid (DNA) synthesis using ^{3}H-thymidine (^{3}H-TdR) according to the method of Benacerraf *et al.*[4] EL-4 leukemic cells (100 μl; 2.5×10^4 EL-4 cells/ml) as the target cells, and 100 μl of a suspension at an appropriate number of peritoneal macrophages in RPMI-1640 medium supplemented with 10% FCS were seeded in 96-well microplates (Falcon, #3072), and cultured at 37 °C for 40 h in 5% CO_2/air. Each well was pulsed with 0.1 μCi of ^{3}H-TdR (Radiochemical Centre, England) for the final 16 h of incubation. The cultured EL-4 leukemic cells and macrophages were harvested on a glass fiber filter (Whatman GF/C) with an automatic cell harvester. The radioactivity taken up by these cells was measured with a scintillation counter (Aloka, LSC-661, Aloka Co., Tokyo) in toluene scintillant containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per liter of toluene.

The results were expressed as percentage of ^{3}H-TdR incorporation according to the following formula.

$$\text{incorporation (\%)} = \text{cpm (tumor cells + effector cells)} - \text{cpm(effector cells)/cpm(tumor cells)} \times 100$$

Assay for Acid Phosphatase Activity——Mice were injected i.p. with slime or its fraction. Four days later, peritoneal exudate cells were collected with a syringe and washed three times with saline. The cells were disrupted by freezing and thawing in distilled water, and centrifuged at 4 °C. Acid phosphatase activity of the supernatant was assayed with *para*-nitrophenyl phosphate as the substrate.[5]

Mitogen and Adjuvant——Concanavalin A (Boehringer Mannheim, West Germany) was used as a specific T-lymphocyte mitogen.[6] Lipopolysaccharide (LPS), which was extracted from *Salmonella typhimurium* LT-2 by the hot phenol-water method,[7] was used as an adjuvant and a specific B-lymphocyte mitogen.[8]

Mitogenic Studies——Suspensions of splenic and thymic cells of C57BL/6 mice were prepared as described previously.[9] The cells were suspended in RPMI-1640 medium supplemented with 10% FCS, and 20 μl of a sample solution and 180 μl (5×10^5 cells) of the cell suspension were placed in wells of a 96-well microplate. The plate was incubated at 37 °C in 5% CO_2/air. Each well was pulsed with 0.25 μCi of ^{3}H-TdR for the final 18 h of incubation. After incubation for 48 h, the cultured spleen cells were harvested on a glass fiber filter. The radioactivity taken up by the cells was measured with a scintillation counter.

Rabbit Anti-thymocyte Serum (ATS) Treatment of Spleen Cells——ATS was prepared according to the method of Gray *et al.*[10] The prepared ATS showed cytotoxicity titers of 2^9 and 2^3 for mouse thymocytes and bone marrow cells, respectively, based on the Trypan Blue dye exclusion method.[11]

The spleen cells from C57BL/6 mice were incubated with suitably diluted ATS at 37 °C for 45 min in the presence of complement (guinea pig serum) in order to kill T-lymphocytes in the spleen cell population. As a reference, normal rabbit serum (NRS) was used.

Hemolytic Plaque Assay——Sheep red blood cells (SRBC) were used as the antigen. SRBC (10^8) and/or *S. natans* and its fraction were injected i.p. into ddY mice. Spleen cells were prepared from each mouse and the number

of plaque-forming cells (PFC) in the spleen was determined by the technique of localized hemolysis in agar.[12] The results are expressed as mean PFC per five dishes, with S.D.

Results

In Vitro Cytostatic Activity of Peritoneal Exudate Macrophages from *S. natans*-Injected Mice against EL-4 Leukemic Cells

In vitro cytostatic activity against EL-4 cells by peritoneal exudate macrophages was evaluated by measuring the inhibition of tumor cell DNA synthesis in terms of the incorporation of ^{3}H-TdR. Peritoneal macrophages induced by injection of slime (0.5 mg/d) or GF-P-1 (0.2 mg/d) exhibited a marked cytostatic activity at effector-to-tumor cell (E/T) ratios of 10 and 20 (Fig. 1).

On the other hand, peritoneal macrophages induced by whole cells (0.5 mg/d) of *S. natans* and resident macrophages showed no inhibitory activity at the E/T ratio of 10.

Next, the cytostatic activity of peritoneal exudate macrophages induced by GF-P-1 or GF-P-2 was compared (Fig. 2). GF-P-1 exerted marked cytostatic activity at E/T ratios of 10 and 20, whereas GF-P-2 showed inhibition at the E/T ratio of 20, but exhibited no activity at the E/T ratio of 10.

Morphological Change of Macrophages Treated with Fractions of the Slime

We examined the morphological change of mouse peritoneal macrophages on addition of GF-P-1 or GF-P-2 *in vitro*. As shown in Fig. 3-b, the macrophages incubated with GF-P-1 for 40 h exhibited marked spreading, but macrophages incubated with GF-P-2 (Fig. 3-c) and resident macrophages (Fig. 3-a) did not show any morphological change. The results suggest that GF-P-1 can cause the activation of peritoneal macrophages.

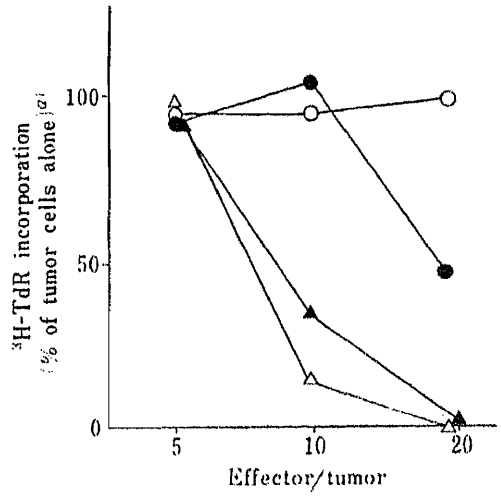


Fig. 1. Cytostatic Activity of Peritoneal Macrophages Induced by Treatment with *S. natans* and Its Fraction, Measured in Terms of ^{3}H-TdR Incorporation of EL-4 Leukemic Cells *in Vitro*

C57BL/6 mice were injected i.p. with *S. natans* whole cells, slime, or GF-P-1 on days 1, 3, 5, and 7. The peritoneal marcophages were harvested on day 10.

—○—, saline (control); —●—, *S. natans* whole cell; —△—, slime; —▲—, GF-P-1.

$$^3\text{H-TdR incorporation} = \frac{\text{cpm (effector+tumor)} - \text{cpm effector alone}}{\text{cpm tumor alone}} \times 100$$

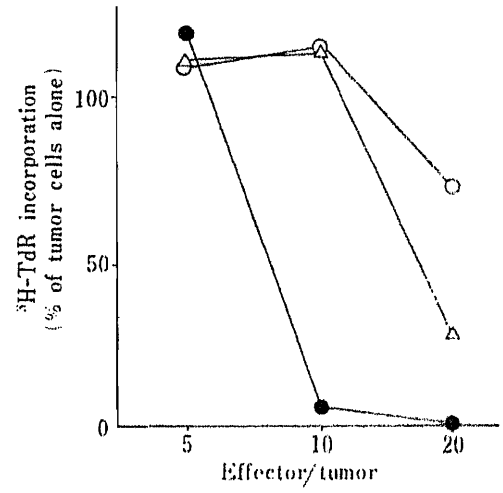


Fig. 2. Cytostatic Activity of Peritoneal Macrophages Induced by Treatment with GF-P-1 or GF-P-2, Measured in Terms of ^{3}H-TdR Incorporation of EL-4 Leukemic Cells *in Vitro*

C57BL/6 mice were injected i.p. with GF-P-1 or GF-P-2 on days 1, 3, 5, and 7. The peritoneal macrophages were harvested on day 10.

—○—, saline (control); —●—, GF-P-1; —△—, GF-P-2.

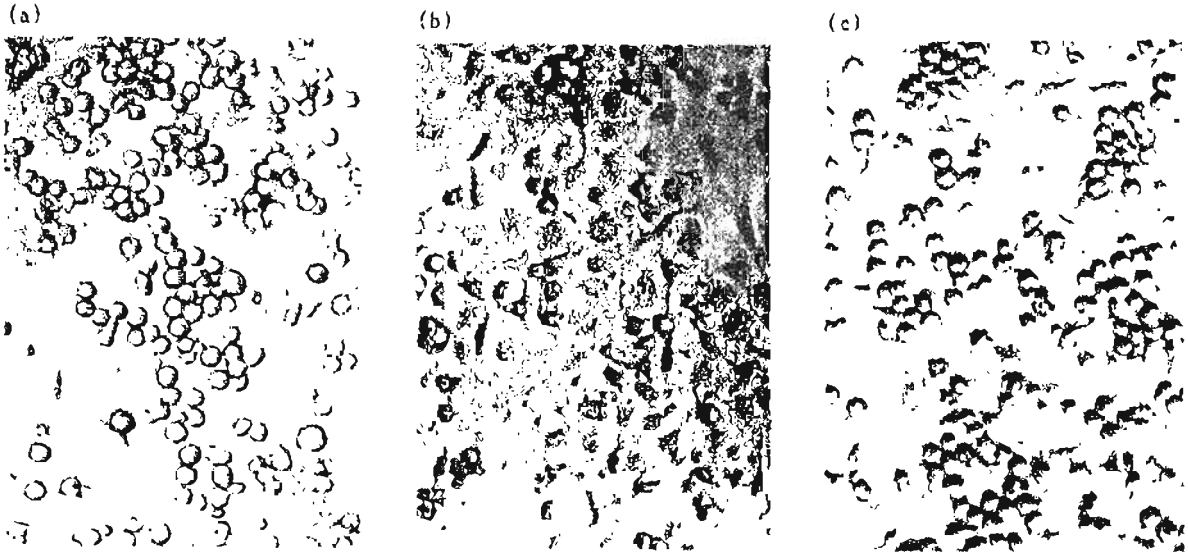


Fig. 3. Effect of GF-P-1 and GF-P-2 on Spreading of Mouse Peritoneal Macrophages

Macrophages (2.5×10^5 per well) were plated and incubated at 37°C for 40 h. (a) Saline (control). (b) GF-P-1 (0.2 mg/ml). (c) GF-P-2 (0.2 mg/ml).

TABLE I. Effect of *S. natans* Fractions on Acid Phosphatase Activity in Mouse Peritoneal Exudate Cells

Treatment	Dose (mg/d)	Acid phosphatase activity (milliunits/mg protein)[a)]	Stimulation index
Saline (control)	0	10.8	1.0
S. natans whole cells	1.0	51.9	4.8
Slime	0.5	40.3	3.7
GF-P-1	0.2	25.7	2.4
GF-P-2	0.2	21.9	2.0

Mice were injected i.p. with each fraction on days 1 and 3. The activity of acid phosphatase was assayed on day 5. *a*) One unit was defined as the enzymatic activity which liberates 1 μmol of *para*-nitrophenol per min at 37 °C.

Acid Phosphatase Activity in Peritoneal Exudate Cells

Acid phosphatase activity in peritoneal exudate cells from mice that had received i.p. *S. natans* whole cells, slime, GF-P-1, or GF-P-2 was measured (Table I). The activity of peritoneal exudate cells as a control (saline) was 10.8 milliunits/mg protein, whereas the group of mice treated with GF-P-1 (0.2 mg) showed a high activity of 25.7 milliunits/mg protein.

However, the acid phosphatase activity induced by GF-P-1 or GF-P-2 (each 0.2 mg) was considerably weak in comparison with that induced by *S. natans* whole cells (1.0 mg) or slime (0.5 mg).

Mitogenic Effect of *S. natans* Fraction on Cultured Spleen Cells

To evaluate the mitogenic effect of *S. natans* fraction, different doses (1—100 μg/ml) of each fraction were added to the cultured spleen cells of C57BL/6 mice and the incorporation of ^{3}H-TdR into these cells *in vitro* was measured (Table II). *S. natans* slime and its fractions exhibited a strong mitogenicity, and the dose-response relationships of *S. natans* slime and GF-P-1 were almost the same. Although slime and GF-P-1 were most effective at a dose of 10 μg/ml, GF-P-2 was most effective at a dose of 100 μg/ml. The mitogenic activities of GF-P-1

TABLE II. Mitogenic Effect of *S. natans* Fractions on Cultured Spleen Cells of C57BL/6 Mice *in Vitro*[a]

Stimulant	Dose (μg/ml)	^{3}H-TdR incorporation (cpm ± S.D.)	Stimulation index[b]
None (control)	0	422 ± 36	1.0
Concanavalin A	2	14420 ± 640	34.1
S. typhimurium LPS	10	4202 ± 100	10.0
S. natans slime	100	2314 ± 223	5.5
	10	3170 ± 124	7.5
	1	1155 ± 82	2.7
GF-P-1	100	3483 ± 181	8.2
	10	5364 ± 258	12.7
	1	2407 ± 223	5.7
GF-P-2	100	5363 ± 434	12.7
	10	1723 ± 275	4.1
	1	538 ± 18	1.3

a) Assays were performed in triplicate with 5×10^5 spleen cells per well. The response was determined after 48 h of culture. *b*) Stimulation index = experimental cpm/control cpm.

TABLE III. Mitogenic Effect of *S. natans* Slime on Cultured Spleen Cells of C57BL/6 Mice *in Vitro*[a]

Stimulant	Dose (μg/ml)	NRS-treated cell stimulation index[b]	ATS-treated cell stimulation index
None (control)	0	1.0[c]	1.0
Concanavalin A	2	18.8	0.9
S. typhimurium LPS	10	3.8	4.1
S. natans slime	100	3.2	3.1
	10	4.5	2.9
	1	1.9	1.4

a) Assays were performed in triplicate with 5×10^5 spleen cells per well. The response was determined after 48 h of culture. *b*) See footnote *b*) in Table II. *c*) The mean incorporation of ^{3}H-TdR in the control cultures was 322 ± 69 cpm in the NRS-treated cells and 833 ± 97 cpm in the ATS-treated cells.

and GF-P-2 were more potent than that of the original slime fraction.

In order to determine whether the slime fraction is a T or B-lymphocyte mitogen, T-lymphocytes in a spleen cell population were killed by ATS in the presence of complement. After the treatment with ATS, the mitogenic response of cultured cells to concanavalin A, a T-lymphocyte mitogen,[6] was markedly reduced, whereas the incorporation of ^{3}H-TdR into ATS-treated cells cultured with *S. typhimurium* LPS, a B-lymphocyte mitogen,[8] was unaffected. The slime fraction was also capable of increasing the incorporation of ^{3}H-TdR into ATS-treated spleen cells (Table III).

On the other hand, ^{3}H-TdR incorporation of thymocytes stimulated by the slime fraction was weak (data not shown). These findings suggest that the slime acts on a population of B-lymphocytes.

Adjuvant Effect of *S. natans* Slime and Its Fraction on Antibody Response

The adjuvant effect of *S. natans* whole cells, slime, and its fractions on the antibody response to xenogeneic erythrocytes in ddY mice was determined (Table IV). The whole cells of *S. natans* and its fraction exhibited an adjuvant effect on day 2 after immunization. Slime fraction (0.5 mg) and GF-P-1 (0.25 mg) showed stimulation index values of 6.7 and 7.6,

TABLE IV. Adjuvant Effect of *S. natans* and Its Fractions on Anti-SRBC Response in the Spleen of ddY Mice

Test material[a]	Dose (mg/mouse)	No. of PFC/spleen[b] (mean ± S.D.)	Stimulation[c] index
None (control)	0	400 ± 190	1.0
S. natans whole cells	1.0	1320 ± 610	3.3
S. natans slime	0.5	2680 ± 380	6.7
GF-P-1	0.25	3040 ± 1337	7.6
GF-P-2	0.25	1232 ± 768	3.1

a) Mice were injected i.p. with 10^8 SRBC and/or a test material on day 0. *b*) Assayed 2 d later. Five mice per group. *c*) Stimulation index = No. of PFC in the spleen of the treated group/No. of PFC in the spleen of the control group.

respectively. In contrast, the activity of *S. natans* whole cells (1.0 mg) and GF-P-2 (0.25 mg) was weak.

Discussion

Previously, we examined the antitumor activity of *S. natans* and found that the slime, which is formed on the outer sheath of the organism, and GF-P-1, which was obtained from the slime fraction by Sepharose 4B gel filtration, have remarkable antitumor activity against Ehrlich ascites tumor cells in mice.[2] Since the antitumor activity was abrogated by treatment of mice with i.p. injection of silica, the activity seemed to be mediated by macrophages activated by *S. natans* fractions.

Various immunomodulators, including LPS or lipid A, can induce spreading morphology of peritoneal macrophages *in vitro*,[13] this being one of the criteria for macrophage activation.[14] Activation of macrophages by GF-P-1 may be necessary for expression of antitumor activity *in vivo* and *in vitro*. In this paper, we have shown that GF-P-1 is able to activate mouse peritoneal macrophages, as judged by the induction of morphological change, enhancement of acid phosphatase activity, and exhibition of cytostatic activity against tumor cells *in vitro*. Peritoneal macrophages incubated with GF-P-1 induced marked spreading morphology (Fig. 3-b). On the other hand, macrophages incubated with GF-P-2 and resident macrophages showed spherical morphology (Fig. 3-a, -c). Moreover, cytostatic activity by *S. natans* whole cells and GF-P-2 was weak. Our results indicate that spreading morphology of macrophages may result in an enhancement of the potency of cytostatic activity.

S. natans fractions exhibited strong mitogenic activity. In particular, 10 μg/ml of GF-P-1 was more effective than the same dose of *S. typhimurium* LPS. However, 100 μg/ml of GF-P-2, which possesses weak antitumor activity, exhibited the most effective mitogenic activity in this experiment, except for concanavalin A (Table II). The potency of the mitogenic activity did not coincide with that of the antitumor activity.

Slime and GF-P-1 had effective adjuvant activity 2 d after SRBC injection. After 4 d, there was no difference in the number of plaque-forming cells among the groups (data not shown). This result suggests that slime and GF-P-1 as well as bacterial LPS[15] are capable of initiating nonspecifically the maturation of antibody-producing cell precursors and converting them to antibody-producing cells at an early stage of antibody production. When various immunomodulators stimulate macrophages, they produce many kinds of cytokine.[16] To elucidate the mechanism of immunological activity of GF-P-1, production of cytokines should be investigated, for instance, macrophage-arming factor, interferon and so on.

It is well known that bacterial LPSs show mitogenicity and adjuvanticity.[17] *S. natans*

belongs to the category of gram-negative bacteria and may possess LPS in the outer membrane. Previously, we carried out isolation of LPS from the organism by the hot phenol–water technique,[7] but the yield and the biological activity were very low in comparison with those of LPS from other gram-negative bacteria. The immunostimulating effect of GF-P-1 does not seem to be due to LPS, since the content of LPS in GF-P-1 detected by the *Limulus* test[18] was only trace (data not shown). To elucidate the mode of action of antitumor activity of GF-P-1, further purification of the substance will be necessary.

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Chemical Modification of Essential Histidine Residues in Mn(III)-Acid Phosphatase by Diethylpyrocarbonate in the Presence of F^- Ion

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Mn(III)-acid phosphatase [EC 3.1.3.2] from sweet potato was inactivated by diethylpyrocarbonate (DEPC) in the presence of the F^- ion. Upon treatment of the inactivated enzyme with NH_2OH, the enzyme activity was significantly restored. The difference absorption spectra of the modified *vs.* native enzyme preparations, both in the presence and absence of the F^- ion, exhibited two prominent peaks at around 242 and 275 nm. The pH-dependence of the inactivation rate suggested that an amino acid residue having a pK value of approximately 7.3 was involved in the inactivation. These results indicate that the inactivation was due to the modification of histidine residues. The correlation between the spectral change at 242 nm and the activity change of the enzyme in the presence and absence of the F^- ion revealed that 1 histidine residue per subunit was essential for the enzyme activity. The inactivation by DEPC occurred only in the presence of the F^- ion, which has been found to interact with the Mn in the active site, suggesting that the histidine residue to be modified is located at the active site of the enzyme.

Keywords——acid phosphatase; histidine modification; active site; diethylpyrocarbonate; sweet potato

A violet-colored acid phosphatase from sweet potato contains 1 atom of Mn(III) ion per enzyme subunit, and this ion coordinates directly with amino acid residues including cysteine sulfhydryl and tyrosine phenolate groups and plays an essential role in the catalytic reaction of the enzyme.[1-5] The nature of the participation of amino acid residues in the enzyme activity, however, is little known. We showed previously that the enzyme contained essential histidine residues, on the basis of the results of chemical modification studies with *N*-ethylmaleimide.[5]

In addition, we have found that the enzyme is significantly inactivated by diethylpyrocarbonate (DEPC), a reagent that is relatively selective for histidine residue, in the presence of the F^- ion. Interestingly, the F^- ion has been found to interact with the Mn center of the enzyme.[4] The present results provide further evidence concerning the existence of an essential histidine residue located at the active site containing the Mn ion.

Experimental

Chemicals——DEPC was obtained from Aldrich Chemical Co. All other chemicals used were of the highest purity available.

Enzyme——Mn(III)-acid phosphatase was purified from sweet potato.[5] Enzyme activity was routinely determined at 37 °C using *p*-nitrophenyl phosphate (2.5 mM) as a substrate in acetate buffer (0.1 M, pH 5.8), and the protein concentration was determined from the absorbance at 280 nm taking $A^{1\%}_{1cm} = 26.8$, as described previously.[5]

Enzyme Inactivation——Chemical modification of the enzyme with DEPC was carried out at 32 °C in a reaction medium containing 3-(*N*-morpholino)propanesulfonic acid (Mops)–NaOH buffer (0.1 M, pH 7.5), enzyme (50 μg/ml),

KF (10 mM) and DEPC (10 mM), unless otherwise stated. The reaction was started by the addition of DEPC dissolved in ethanol and an aliquot of the modified enzyme was assayed for remaining activity at the indicated intervals. The reaction of imidazole groups with DEPC was not affected by the F^- ion, as assessed by treatment of α-*N*-acetyl-L-histidine with DEPC. The number of histidine residues modified by DEPC was calculated from the increase in absorbance at 242 nm using a value of $4.0 \times 10^3\ M^{-1} \cdot cm^{-1}$, which was determined by reaction with *N*-acetylhistidine in Mops-NaOH buffer (0.1 M, pH 7.5).

Results and Discussion

Inactivation of Mn(III)-Acid Phosphatase by DEPC in the Presence of the F^- Ion

Incubation of the enzyme with DEPC (10 mM) at 32 °C in the presence of the F^- ion (10 mM) in Mops-NaOH buffer (0.1 M, pH 7.5) caused a time-dependent decrease of the enzyme activity, though significant activity loss did not occur on treatment with DEPC alone, as shown in Fig. 1. The reaction of the enzyme and DEPC in the presence of the F^- ion followed initially pseudo-first-order kinetics. When the inactivation by DEPC (10 mM) was examined at various concentrations of the F^- ion, the inactivation rate was dependent upon the concentration of the F^- ion and became almost constant at F^- ion concentrations above 5 mM, as shown in Fig. 2. In the presence of the F^- ion (10 mM), the inactivation rate was also found to be proportional to the DEPC concentration (1.0—10 mM) (not shown). Thus, the following experiments were carried out at the concentrations of 10 mM DEPC and 10 mM F^- ion.

Identification of the Amino Acid Residues Modified in Mn(III)-Acid Phosphatase by DEPC in the Presence of the F^- Ion

DEPC has been shown to carbethoxylate preferentially histidine residues in proteins,[6] but reactions with other amino acid residues, including cysteine, tyrosine and lysine, have also been described.[7-9] To identify the amino acid residue(s) modified in the enzyme by DEPC plus the F^- ion, the following experiments were carried out.

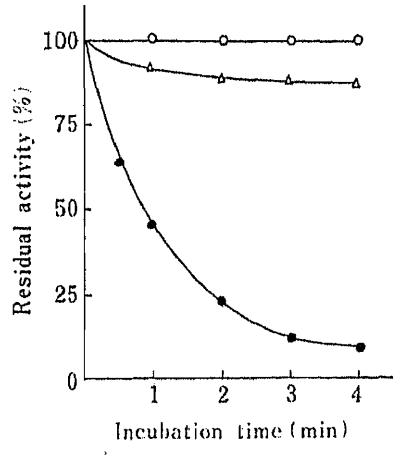


Fig. 1. Inhibitory Effect of DEPC on Acid Phosphatase in the Presence and Absence of the F^- Ion

The enzyme (50 μg/ml) was incubated at 32 °C with DEPC (10 mM) in the presence (●) and absence (△) of the F^- ion (10 mM) in Mops-NaOH buffer (0.1 M, pH 7.5). Aliquots were withdrawn at intervals and the enzyme activity was determined as described in Experimental. A control experiments (○) were run in which DEPC or DEPC and the F^- ion were omitted from the reaction mixture.

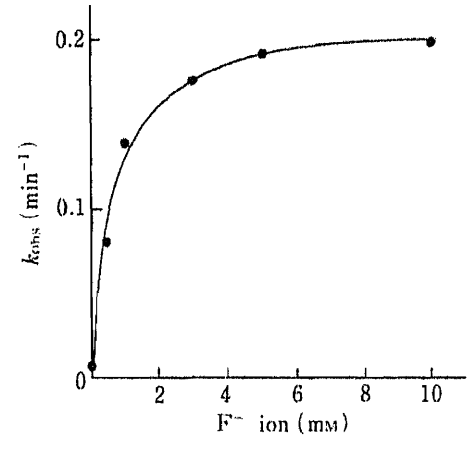


Fig. 2. Effect of the F^- Ion on the Inactivation Rate of Acid Phosphatase by DEPC.

The enzyme (50 μg/ml) was incubated at 32 °C with DEPC (10 mM) in the presence of the indicated concentration of the F^- ion in Mops-NaOH buffer (0.1 M, pH 7.5). The rate of inactivation is presented in terms of the pseudo-first-order rate constant, k_{obs}.

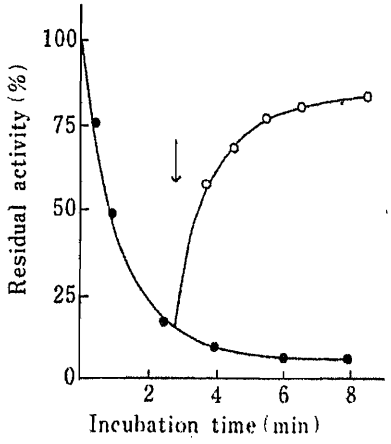


Fig. 3. Reactivation of DEPC-Inactivated Acid Phosphatase with NH_2OH

The enzyme (50 μg/ml) was incubated at 32 °C with DEPC (10 mM) in the presence of the F^- ion (10 mM) in Mops-NaOH buffer (0.1 M, pH 7.5) (●). After 2.5 min, NH_2OH (pH 7.5) was added up to a final concentration of 0.1 M (○). At intervals, aliquots were withdrawn and the enzyme activity was determined as described in Experimental. The arrow indicates the addition of NH_2OH.

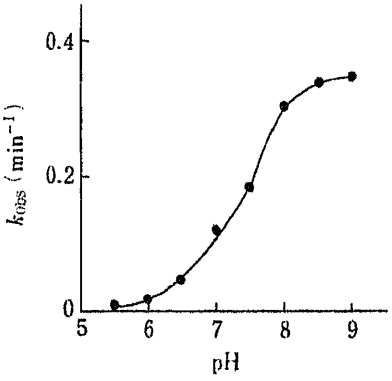


Fig. 4. pH-Dependence of the Inactivation Rate of Acid Phosphatase by DEPC in the Presence of the F^- Ion

The enzyme (50 μg/ml) was incubated at 32 °C with DEPC (10 mM) in the presence of the F^- ion in acetate buffer (0.1 M, pH 5.5—6.0) and Mops-NaOH buffer (0.1 M, pH 6.0—9.0). The rate of inactivation is presented in terms of the pseudo-first-order rate constant, k_{obs}.

i) Reactivation of the Inactivated Enzyme with NH_2OH——NH_2OH has been shown to remove the ethoxyformyl group from modified histidine and tyrosine residues, but does not affect the more stable ethoxyformyl-sulfhydryl and *N*-ethoxyformyllysyl bonds.[10] When the enzyme inactivated by DEPC plus the F^- ion (residual activity, 18%) was treated with NH_2OH (0.1 M) in the same buffer at 32 °C, the enzyme activity was significantly restored (residual activity, 85%) within 5 min, as shown in Fig. 3. This finding indicates that the inactivation of the enzyme was not due to the modification of cysteine or lysine residues.

ii) Effect of pH——Since DEPC is known to react with the unprotonated imidazole group, but not with the protonated group,[11] measurements of activity loss of the enzyme in a series of buffered solutions from pH 5.5 to 9.0 were carried out. The inactivation of the enzyme by DEPC plus the F^- ion was pH-dependent and was found to be slower at acidic pH's, and faster at alkaline pH's, as shown in Fig. 4. The apparent p*K* value was around pH 7.3, suggesting that the inactivation might due to histidine modification.

iii) Absorption Change——Figure 5 shows the difference absorption spectra between the DEPC-treated and native enzyme preparations, both in the presence and absence of the F^- ion. The increase of the absorbance at around 240 nm upon addition of DEPC is a characteristic of histidine modification,[12] and the decrease of the absorbance at around 280 nm indicates the occurrence of tyrosine modification.[9] In the case of the enzyme, absorbance changes were observed at around 242 and 275 nm, indicating that the modification of both residues occurred. However, a similar extent of absorbance change at around 275 nm was found to occur in both enzyme preparations obtained by treatment with DEPC in the presence and absence of the F^- ion, though the residual activities of the enzymes prepared in the presence and absence of the F^- ion were 15 and 85%, respectively. These findings suggest that the modification of tyrosine residue(s) was not responsible for the activity loss. The absorbance of the enzyme in the visible region (λ_{max} = 540 nm) is attributed to the Mn ion directly coordinated with some amino acid residues, including the tyrosine phenolate group.[1,5] No significant decrease of the absorbance of the enzyme at 540 nm was observed following treatment with DEPC. These facts suggest that the tyrosine residue(s) modified by

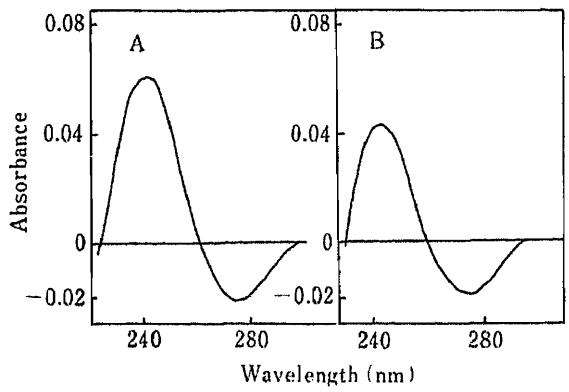


Fig. 5. Difference Absorption Spectra of DEPC-Treated *vs.* Native Acid Phosphatase Preparations in the Presence and Absence of the F^- Ion

A) Both sample and reference cuvettes contained the enzyme (0.3 mg/ml) and the F^- ion (10 mM) in 2.5 ml of Mops–NaOH buffer (0.1 M, pH 7.5).

B) Experimental conditions were the same as described in A, except the reaction mixture did not contain the F^- ion. DEPC (10 mM) dissolved in ethanol was added to the sample cuvette, and an equivalent volume of ethanol to the reference cuvette. The spectra were recorded at 25 °C after 4 min.

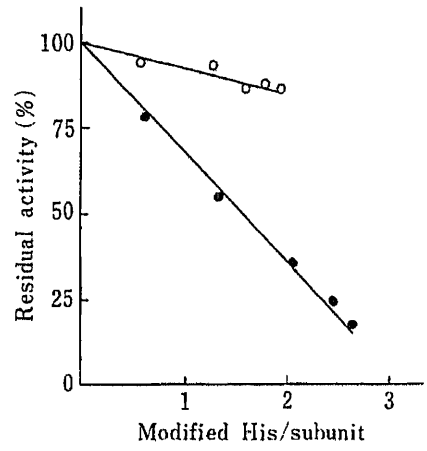


Fig. 6. Correlation between the Enzyme Activity and the Number of Modified Histidine Residues

The enzyme (0.6 mg/ml) was incubated at 25 °C with DEPC (10 mM) in the presence (●) and absence (○) of the F^- ion (10 mM) in Mops–NaOH buffer (0.1 M, pH 7.5). At intervals, aliquots were withdrawn and the enzyme activity was determined. Absorbance at 242 nm was monitored, and the number of modified histidine residues was calculated as described in Experimental.

DEPC may be not involved in the coordination to the Mn ion.

iv) Correlation between the Enzyme Activity and the Number of Modified Residues——The number of histidine residues that reacted in the absence and presence of the F^- ion was also estimated from the change in absorbance at 242 nm. The correlation between the enzyme activity and the number of the modified residues is shown in Fig. 6. In the presence of the F^- ion, modification of approximately 3 histidine residues per subunit resulted in an almost complete inactivation of the enzyme, whereas in the absence of the F^- ion, only a 15% activity loss occurred, despite the modification of 2 histidine residues. These results may indicate that 2 out of 3 residues modified in the presence of the F^- ion are modified without causing inactivation and are not necessary for the enzyme activity, that is, 1 histidine residue per subunit is essential for the enzyme activity. The result is compatible with the finding that the acid phosphatase 96%-inactivated by treatment with *N*-ethylmaleimide has lost about 1 histidine per subunit.[5]

The enzyme is composed of 2 subunits having an identical molecular weight, and contains 2 atoms of Mn(III) ion per mol of the enzyme.[5,13] However, the identity between the 2 subunits in respect of chemical composition, including amino acids and Mn, has not yet been determined, and reversible dissociation of the enzyme to subunits has not been achieved. At the present stage, it is also obscure whether modification of histidine by DEPC occurs on both subunits or only on either one of the 2 subunits.

Concerning the state of the histidine residue modified by DEPC in the presence of the F^- ion, based on the present results and other findings, we speculate that the functional histidine residue may be located in close proximity to the Mn in the enzyme molecule, since significant inactivation of the enzyme by DEPC occurred only in the presence of the F^- ion, which has been found to interact with the Mn in the enzyme,[4] and the enzyme contains 1 atom of Mn per subunit.[5]

Although the mechanism of the accelerating effect of the F^- ion is obscure, we also speculate that the essential histidine residue, which interacts with the Mn in the enzyme, is displaced by the F^- ion added and this results in susceptibility to DEPC, or that a conformational change of the active site caused by the binding of the F^- ion to Mn results in susceptibility of the essential histidine residue to DEPC.

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Synthesis of the Revised Amino Acid Sequence of Thymopoietin II and Examination of Its Immunological Effect on the Impaired T-Lymphocyte Transformation of a Uremic Patient with Pneumonia[1)]

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The nonatetracontapeptide corresponding to the revised amino acid sequence of thymopoietin II was synthesized by assembling ten peptide fragments in solution followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio, 1:1) in trifluoroacetic acid in the presence of dimethylselenide and *m*-cresol. The synthetic nonatetracontapeptide was tested for effect on impaired T-lymphocyte transformation by phytohemagglutinin (PHA) in a uremic patient with pneumonia. The synthetic peptide was found to have restoring activity on the impaired PHA stimulation of T-lymphocytes; the minimum effective concentration was 1 μg/ml.

Keywords——thymopoietin II; trifluoromethanesulfonic acid deprotection; uremic patient; pneumonia; impaired T-lymphocyte transformation; fluorometric blast-formation test

Two T-cell-differentiating hormones of thymus, thymopoietins I and II, were isolated from bovine thymus by Goldstein.[2)] Each peptide contains 49 amino acid residues, and the two peptides are closely related, differing by only two amino acid residues.[3)] Fujino and coworkers[4)] reported the first total synthesis of thymopoietin II using the HONB–DCC procedure in 1977. In 1981, the proposed structures of thymopoietins I and II were revised by Goldstein *et al.*[5)] They also reported the isolation of thymopoietin III from bovine spleen and the complete amino acid sequence of thymopoietin III.[5)] The revised structure of thymopoietin II differs from the previously proposed structure of thymopoietin II in the replacement of five amino acid residues: Ser (position 1), Gln (position 2), Gln (position 38), Thr (position 43) and Val (position 47) in the unrevised thymopoietin II are replaced by Pro, Glu, Glu, Ser

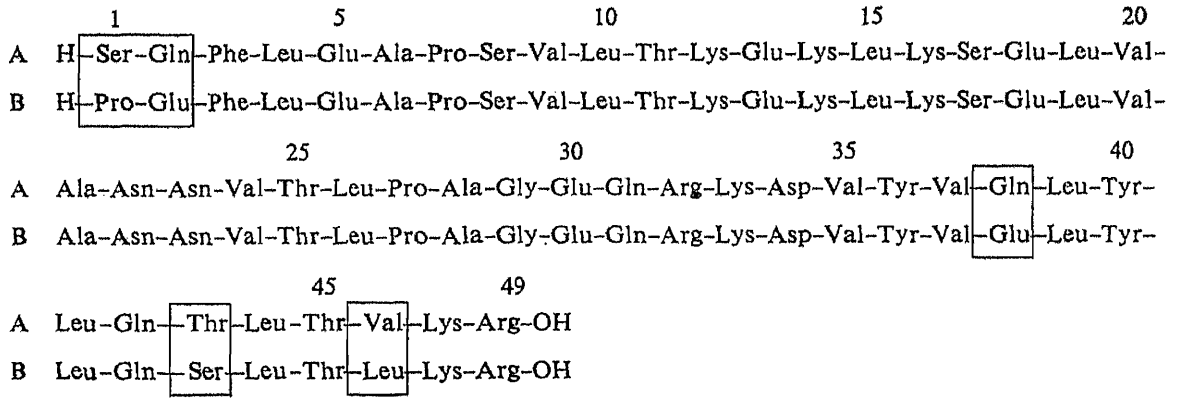


Fig. 1. Comparison of the Formerly Proposed Amino Acid Sequence of the Thymopoietin II (A) and the Revised Sequence of Thymopoietin II (B); Differences in the Sequences are Enclosed in Boxes[5)]

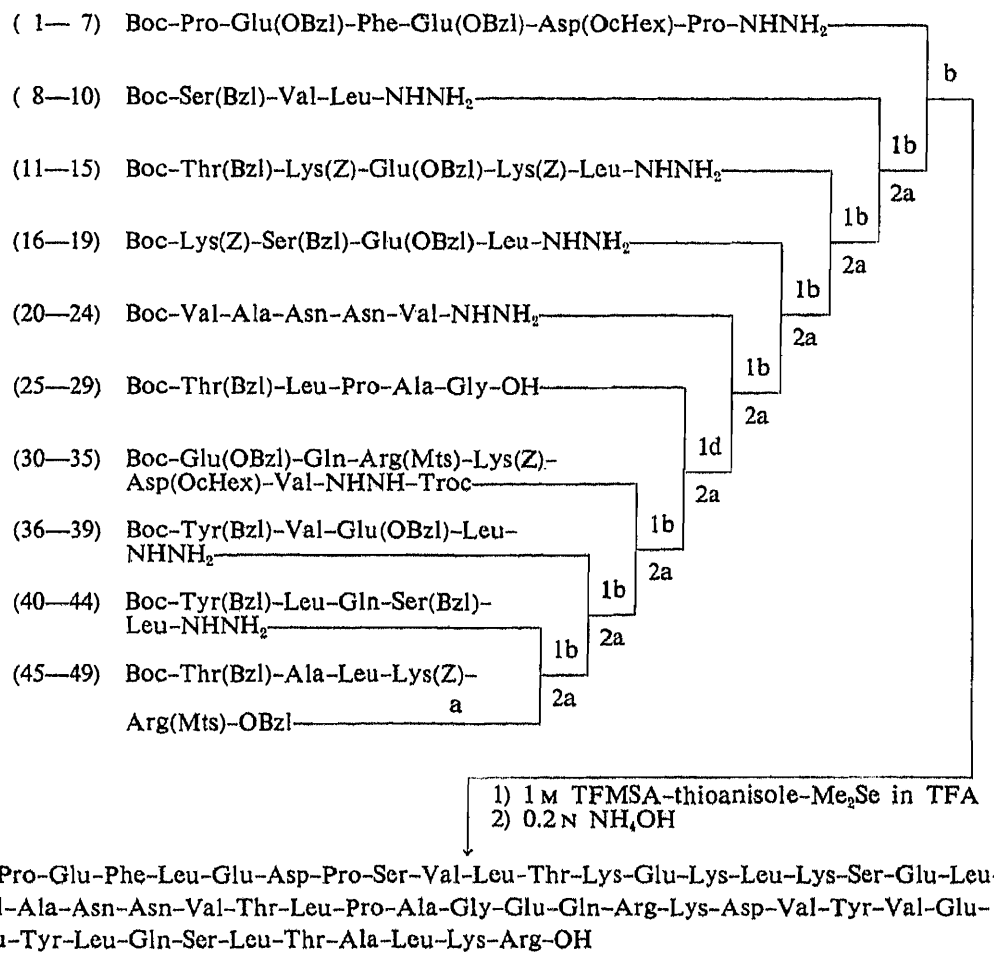


Fig. 2. Synthetic Route to Thymopoietin II

a, TFA-anisole; b, azide; c, Zn-AcOH; d, HOSu-WSCI.

and Leu, respectively.

In 1982, we reported[6] the synthesis of the octadecapeptide (32—49), which corresponds to a part of the revised structure of thymopoietin II, and showed that the biological activity of the revised thymopoietin II fragment (32—49) on low E-rosette-forming cells of an aged patient with chronic renal failure was equal to that of the unrevised thymopoietin II fragment (32—49).[7]

Following the syntheses of thymopoietins I and III[8,9] we wish to report the solution synthesis of the revised structure of thymopoietin II, *via* the route illustrated in Fig. 2 and to describe the *in vitro* effect of this peptide on the impaired PHA stimulation of T-lymphocytes from a uremic patient with pneumonia.

The life of patients with chronic renal failure has been prolonged by recent advances in hemodialysis therapy. However, the complication of infectious diseases in chronic hemodialysis patients still poses a grave problem in term of their prognosis. It is an accepted fact that there is a decrease in immunity, particularly cell-mediated immunity, in uremic patients.[10] This impairement is reflected in both *in vitro* and *in vivo* depressed cell-mediated immune function. Patients with chronic uremia may also have thymic atrophy. The thymus may show a marked reduction in lymphoid elements and extensive replacement with fat. These observations[11] suggested to us that one of the cell-mediated immune abnormalities seen chronic uremia might be attributable to thymic hormone deficiency. Thus, we were interested in the immunological effect of the revised amino acid sequence of thymopoietin II

on the impaired immune function in uremic patients.

The synthetic routes that we employed here are almost the same as those employed for our previous syntheses of thymopoietins I and III.[8,9] As illustrated in Fig. 2, the TFA-labile Boc group was employed for N^{α}-protection and amino acid derivatives bearing protecting groups removable by the thioanisole-mediated TFMSA deprotecting procedure[12,13] were employed, *i.e.*, Lys(Z), Glu(OBzl), Thr(Bzl), Ser(Bzl), Tyr(Bzl), Asp(OcHex)[14] and Arg(Mts).[15] N^{α}-Deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction as usual. Asp(OcHex) was employed to minimize aspartimide formation during the synthesis of Asp-containing peptides.[14] Eight protected peptide hydrazides, one protected pentapeptide and the C-terminal protected pentapeptide ester were selected as building blocks to construct the revised amino acid sequence of thymopoietin II (Fig. 2). Of these, eight fragments, Boc–(1—7)–$NHNH_2$ [10], Boc–(8—10)–$NHNH_2$ [9], Boc–(11—15)–$NHNH_2$ [8], Boc–(16—19)–$NHNH_2$ [7], Boc–(20—24)–$NHNH_2$ [6], Boc–(25—29)–OH [5], Boc–(36—39)–$NHNH_2$ and Boc–(45—49)–OBzl [1], are available from our previous syntheses of thymopoietins I and III.[8,9] The protected pentapeptide ester, Boc–(40—44)–OMe, was prepared stepwise starting from H–Leu–OMe·HCl by the HOBT–WSCI procedure[16] except for the introduction of Gln, which was introduction by the NP active ester procedure.[17] The protected pentapeptide ester thus obtained was smoothly converted to the corresponding hydrazide, Boc–(40—44)–$NHNH_2$ [2], in the usual manner. The hydrazine test on the thin-layer chromatograms and elemental analysis data of this peptide fragment were consistent with homogeneity of the desired product. Next, for the preparation of the protected hexapeptide fragment containing Glu(OBzl) and Asp(OcHex), Boc–(30—35)–NHNH–Troc, we employed a substituted hydrazide, Troc–$NHNH_2$,[18] a protecting group which is known to be removed by Zn[19] in AcOH without affecting side chain protecting groups such as Boc, Mts, Z, OBzl and OcHex. Boc–(30—35)–NHNH–Troc was prepared stepwise starting from Boc–Val–NHNH–Troc by the HOBT–WSCI procedure[16] except for the introduction of the Gln residue, which was introduced by the NP active ester procedure,[17] and the introduction of the Arg(Mts) residue, which was introduced by the MA procedure.[20] The protected hexapeptide, Boc–(30—35)–NHNH–Troc, was treated with Zn[19] in AcOH to remove the Troc group, and the zinc acetate was removed by treatment with EDTA to give the required hydrazide, Boc–(30—35)–$NHNH_2$ [2], in analytically pure form. The hydrazine test on the thin-layer chromatograms and elemental analysis data were consistent with homogeneity of the desired product.

The ten fragments were assembled successively by the azide procedure[21] and the HOSu–WSCI procedure[22] according to the routes illustrated in Fig. 2. The amount of the acyl component in each fragment condensation was increased from 2 to 5 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. Some of the intermediates were purified by repeated precipitation from DMF or DMSO with MeOH and others were purified by gel-filtration on Sephadex LH-60 using DMF or DMSO as the eluent. Throughout this synthesis, Ala or Gly was taken as the diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Ala or Gly with those of newly incorporated amino acids, satisfactory incorporation of each fragment in each condensation reaction was confirmed.

Starting with the side-chain-protected pentapeptide ester corresponding to positions 45 to 49 of thymopoietin II, Boc–(45—49)–OBzl, nine fragments, Boc–(40—44)–$NHNH_2$, Boc–(36—39)–$NHNH_2$, Boc–(30—35)–$NHNH_2$, Boc–(25—29)–OH, Boc–(20—24)–$NHNH_2$, Boc–(16—19)–$NHNH_2$, Boc–(11—15)–$NHNH_2$, Boc–(8—10)–$NHNH_2$ and Boc–(1—7)–$NHNH_2$, were successively condensed by the azide procedure[21] and the HOSu–WSCI procedure[22] as shown in Fig. 2 to give the protected nonatetracontapeptide corresponding to

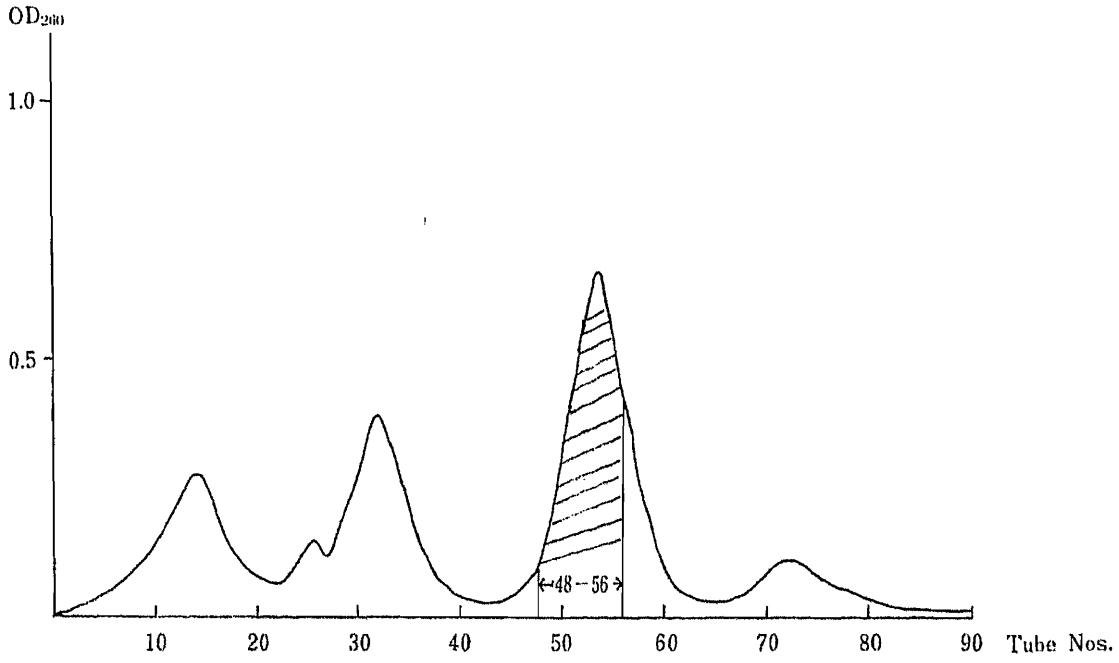


Fig. 3. Purification of Synthetic Thymopoietin II by Ion-Exchange Chromatography on a CM-Biogel A Column

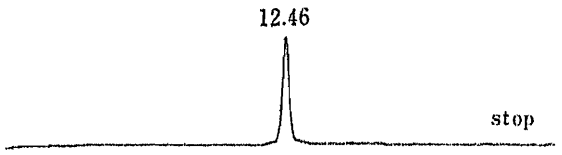


Fig. 4. HPLC of Synthetic Thymopoietin II

the entire amino acid sequence of thymopoietin II. The homogeneities of the peptides were checked by elemental analysis, TLC and amino acid analyses of the acid hydrolysates.

In the final step of the synthesis, the protected nonatetracontapeptide ester was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and Me_2Se. *m*-Cresol was used as an additional cation scavenger to suppress a side reaction, *i.e.*, *O*-sulfonation of Tyr residues.[15] Me_2Se was employed to facilitate acidolytic cleavage of protecting groups.[23] The deprotected peptide was next precipitated with dry ether, converted to the corresponding acetate with Amberlite IR-400 (acetate form) and then treated with 0.2 N NH_4OH to reverse a possible N→O shift at the Ser and Thr residues.[24] The crude peptide was purified by gel-filtration on Sephadex G-50 and then by ion-exchange column chromatography on a CM-Biogel A column with linear gradient elution using pH 6.5 ammonium acetate buffer (0→0.25 M), followed by preparative TLC.

Desalting on Sephadex G-25 gave a fluffy powder, which exhibited a single spot (ninhydrin- and Sakaguchi-positive) on TLC in two different solvent systems and on paper electrophoresis (pH 2.80 acetate buffer). The peptide also exhibited a single peak on HPLC (Fig. 4). Homogeneity of the synthetic thymopoietin II was further ascertained by amino acid analyses, after 6 N HCl hydrolysis and enzymatic digestion.

The immunological effect of the synthetic thymopoietin II was examined by the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[25] In contrast to normal persons, the transformation of the T-lymphocytes into lymphoblasts with mitotic activity after PHA stimulation is depressed in severely uremic patients with pneumonia. The *in vitro* effect of the synthetic peptide on the impaired PHA

TABLE I. Effect of the Synthetic Thymopoietin II on the Impaired PHA Stimulation of T-Lymphocytes of a Uremic Patient with Pneumonia

Peptide	Dose (μg/ml)	$SI^{a,b)}$
—[c]	—	311.4±60.8
—[d]	—	129.7±64.7
Synthetic thymopoietin II[d,e]	0.1	118.6±63.1
	1.0	192.6±66.3
	2.0	283.2±59.4
	3.0	280.3±62.4

a) Each value represents the mean±S.D. of triplicate measurements. *b*) *SI* (stimulation index) was calculated according to the following formula: $SI=\{(I_2-I_0)/(I_1-I_0)\}\times 100$, 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 ethidium bromide. *c*) Normal venous lymphocytes. *d*) Patient's venous lymphocytes. *e*) Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 in air for 12 h.

stimulation of T-lymphocytes from a uremic patient with pneumonia is shown in Table I. When peripheral T-lymphocytes isolated from the patient were incubated with various amounts of the synthetic thymopoietin II from 0.1 to 3 μg/ml, restoration of the impaired PHA stimulation of T-lymphocytes was observed at concentrations of 1 μg/ml and above. In normal subjects, no effects of thymopoietin II were observed (data not shown). The results suggest that the synthetic thymopoietin II is able to restore the impaired T-lymphocyte transformation *in vitro* in severely uremic patients with infectious diseases such as pneumonia.

Experimental

General experimental procedures used in this paper are essentially the same as described in the previous papers.[8,9] An azide was prepared according to Honzl and Rudinger[21] 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—45 °C. Boc groups of protected peptides were removed by TFA–anisole treatment in the presence of anisole. 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 entire amino acid sequence of thymopoietin II was chromatographed on a cellulose plate (Merck). Rf^3 value refers to BuOH–AcOH–H_2O (4:1:1) and Rf^4 value refers to BuOH–pyridine–AcOH–H_2O (30:20:6:24).[27] Troc–$NHNH_2$ was purchased from Kokusan Chemical Works Ltd., Japan. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. Preparations of protected intermediates were repeated several times in order to obtain sufficient quantities for the next step. Papain (No. p-3125) and leucine aminopeptidase (No. L-9876) were purchased from Sigma Chemical Co. Venous blood was obtained from a severely uremic patient with pneumonia. Venous blood from three healthy doners was used as a control. The fluorescence excitation spectra was measured with a UVLOG-FIOUSPEC-11A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus coupled to a μBondapak C_{18} column.

Boc–Ser(Bzl)–Leu–OMe (I)——H–Leu–OMe·HCl (1.8 g) was dissolved in THF (10 ml) containing NMM (1.1 ml). To this ice-chilled solution, Boc–Ser(Bzl)–OH (3 g), HOBT (1.5 g) and WSCI (2.1 g) were successively added. After being stirred at 4 °C for 12 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 petroleum ether: Yield 4 g (95%), mp 71—74 °C, $[\alpha]_D^{21}-6.8°$ (c=1.0, DMF), Rf^1 0.52, Rf^2 0.60, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{34}N_2O_6\cdot H_2O$: C, 59.98; H, 8.24; N, 6.36. Found: C, 59.86; H, 8.32; N, 6.27.

Boc–Gln–Ser(Bzl)–Leu–OMe (II)——I (3.5 g) was treated with TFA–anisole (10 ml–2 ml) in an ice-bath for 40 min, and TFA was then removed by evaporation. The residue was washed with *n*-hexane, and dried over KOH pellets *in vacuo* for 2 h. The dried residue was dissolved in DMF (20 ml) together with NMM (0.93 ml). Boc–Gln–ONp (3.6 g) was added and the solution was stirred at room temperature for 12 h. The reaction mixture was diluted

with 1 N NH_4OH (3 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 stirring, and the precipitate thereby formed was washed successively with 1 N NH_4OH, H_2O, 5% citric acid and H_2O. The product was reprecipitated from DMF with 1 N NH_4OH: Yield 4.1 g (87%), mp 139—144 °C, $[\alpha]_D^{21}$ −16.2° (*c* = 1.0, DMF), Rf^1 0.46, Rf^2 0.47, single ninhydrin-positive spot. *Anal.* Calcd for $C_{27}H_{52}N_4O_8$: C, 57.84; H, 9.35; N, 9.99. Found: C, 57.49; H, 9.40; N, 9.81.

Boc–Leu–Gln–Ser(Bzl)–Leu–OMe (III)——II (2.2 g) was treated with TFA–anisole (10 ml–2 ml) as described above and the resulting powder was dissolved in DMF (15 ml) containing NMM (0.44 ml). To this ice-chilled solution, Boc–Leu–OH (1.1 g), HOBT (594 mg) and WSCI (844 mg) were successively added. After 12 h at 4 °C, 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 EtOH with ether: Yield 1.9 g (73%), mp 136—143 °C, $[\alpha]_D^{21}$ −24.6° (*c* = 1.0, DMF), Rf^1 0.48, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{33}H_{63}N_5O_9 \cdot 2\ H_2O$: C, 55.38; H, 9.51; N, 9.87. Found: C, 55.74; H, 9.46; N, 9.74.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–OMe (IV)——This compound was prepared essentially in the same manner as described for the preparation of III by using III (1.4 g), Boc–Tyr(Bzl)–OH (819 mg), HOBT (298 mg) and WSCI (422 mg). The product was reprecipitated from acetone with ether: Yield 1.3 g (68%), mp 124—132 °C, $[\alpha]_D^{21}$ −11.4° (*c* = 1.0, DMF), Rf^1 0.68, Rf^2 0.69, single ninhydrin-positive spot. *Anal.* Calcd for $C_{49}H_{78}N_6O_{11}$: C, 63.48; H, 8.48; N, 9.06. Found: C, 63.17; H, 8.69; N, 8.85.

Boc–Tyr(Bzl)–Leu–Gln–Ser(Bzl)–Leu–NHNH$_2$ (V)——IV (927 mg) was dissolved in DMF–MeOH (1 : 1, 10 ml). Hydrazine hydrate (0.48 ml) was added, and the solution was kept standing at room temperature for 36 h. After evaporation of the MeOH, the residue was poured into ice-chilled H_2O with vigorous stirring. The precipitate thereby formed was filtered off and washed with H_2O. The dried product was recrystallized from hot MeOH: Yield 742 mg (80%), mp 136—143 °C, $[\alpha]_D^{21}$ −15.6° (*c* = 1.0, DMF), Rf^1 0.44, Rf^2 0.47, single hydrazine-test-positive spot. *Anal.* Calcd for $C_{48}H_{78}N_8O_{10}$: C, 62.18; H, 8.48; N, 12.09. Found: C, 62.03; H, 8.76; N, 11.87.

Boc–Asp(OcHex)–Val–NHNH–Troc (VI)——This compound was prepared essentially in the same manner as described for the preparation of III by using Boc–Val–NHNH–Troc (1 g), Boc–Asp(OcHex)–OH (821 mg), HOBT (327 mg) and WSCI (528 mg). The product was reprecipitated from EtOAc with petroleum ether: Yield 1.1 g (85%), mp 70—73 °C, $[\alpha]_D^{21}$ −8.4° (*c* = 1.0, DMF), Rf^1 0.50, Rf^2 0.51, single ninhydrin-positive spot. *Anal.* Calcd for $C_{23}H_{37}Cl_3N_4O_8$: C, 45.74; H, 6.18; N, 9.28. Found: C, 45.62; H, 6.32; N, 9.45.

Boc–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VII)——This compound was prepared essentially in the same manner as described for the preparation of III by using VI (1 g), Boc–Lys(Z)–OH · DCHA (1.1 g), HOBT (248 mg) and WSCI (352 mg). The product was reprecipitated from EtOAc with *n*-hexane: Yield 1.3 g (93%), mp 78—84 °C, $[\alpha]_D^{21}$ −21.3 (*c* = 1.0, DMF), Rf^1 0.54, Rf^2 0.57, single ninhydrin-positive spot. *Anal.* Calcd for $C_{37}H_{55}Cl_3N_6O_{11} \cdot 2\ H_2O$: C, 49.26; H, 6.59; N, 9.31. Found: C, 49.52; H, 6.73; N, 9.18.

Boc–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (VIII)——VII (1.1 g) was treated with TFA–anisole (10 ml–2 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo*, and dissolved in DMF–THF (1 : 1, 10 ml) containing NMM (0.14 ml). To this ice-chilled solution, a solution of the mixed anhydride [prepared from 741 mg of Boc–Arg(Mts)–OH · DCHA with 0.16 ml of ethylchlorocarbonate at −10 °C] in THF (4 ml) and acetonitrile (4 ml) was added. The mixture was stirred at 4 °C for 2 h and then at room temperature for 6 h. The mixture was evaporated *in vacuo*, the residue 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 1 g (67%), mp 74—77 °C, $[\alpha]_D^{21}$ −15.7 (*c* = 1.0, DMF), Rf^1 0.60, Rf^2 0.54, single ninhydrin-positive spot. *Anal.* Calcd for $C_{52}H_{77}Cl_3N_{10}O_{14}S$: C, 51.85; H, 6.44; N, 11.63. Found: C, 51.73; H, 6.70; N, 11.59.

Boc–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (IX)——This compound was prepared from VIII (803 mg) and Boc–Gln–ONp (313 mg) essentially as described for the preparation of IV. The dried product was reprecipitated from EtOAc with ether: Yield 759 mg (86%), mp 123—129 °C, $[\alpha]_D^{21}$ −30.8° (*c* = 1.0, DMF), Rf^1 0.49, Rf^2 0.52, single ninhydrin-positive spot. *Anal.* Calcd for $C_{57}H_{85}Cl_3N_{12}O_{16}S$: C, 51.37; H, 6.43; N, 12.61. Found: C, 51.23; H, 6.74; N, 12.24.

Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH–Troc (X)——This compound was prepared from IX (666 mg), Boc–Glu(OBzl)–OH (186 mg), HOBT (74 mg) and WSCI (106 mg) essentially as described for the preparation of III. The reaction mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O. The dried product was reprecipitated from MeOH with ether: Yield 592 mg (76%), mp 132—137 °C, $[\alpha]_D^{21}$ −16.2° (*c* = 1.0, DMF), Rf^1 0.56, Rf^2 0.55, single ninhydrin-positive spot. *Anal.* Calcd for $C_{69}H_{98}Cl_3N_{13}O_{19}S \cdot 3\ H_2O$: C, 51.60; H, 6.53; N, 11.34. Found: C, 51.62; H, 6.48; N, 11.73.

Boc–Glu(OBzl)–Gln–Arg(Mts)–Lys(Z)–Asp(OcHex)–Val–NHNH$_2$ (XI)——X (517 mg) in a mixture of AcOH (4 ml) and DMF (4 ml) was treated with Zn dust (218 mg) at 4 °C for 2 h and then at room temperature for 8 h. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with 3% EDTA and then with $NaHCO_3$ to adjust the pH to neutral. The resulting powder was washed with H_2O and reprecipitated from DMF with H_2O: Yield 419 mg (89%), mp 164—173 °C, $[\alpha]_D^{21}$ −16.2° (*c* = 1.0, DMF), Rf^1 0.50, Rf^2 0.54, single hydrazine-

test-positive spot. *Anal.* Calcd for $C_{66}H_{97}N_{13}O_{17}S \cdot 2\,H_2O$: C, 56.12; H, 7.21; N, 12.89. Found: C, 56.09; H, 7.41; N, 12.91.

Synthesis of the Protected Thymopoietin II——1. Successive azide condensations of nine fragments except for Boc-(25—29)-OH [5], which was condensed by the HOSu–WSCI procedure, were carried out according to the Fig. 2. Prior to condensation, the Boc group was removed from the respective amino component (1 ml per 0.1 g of the peptide) in the presence of anisole (10 eq) in an ice-bath for 40 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) containing NMM (1.1 eq). The corresponding azide (the amount was increased from 2 to 5 eq as chain elongation progressed) in DMF or DMF–DMSO (1 : 1) and NMM (1.1 eq) were added to the above ice-chilled solution and the mixture was stirred at −10 °C until the solution became negative to the ninhydrin test. The mixture was poured into ice-chilled 5% citric acid with stirring. The precipitate thereby formed was successively washed with 5% citric acid and H_2O. The dried product was purified by one of the following two procedures. A: Precipitation from DMF or DMSO with MeOH. B: Gel-filtration on Sephadex LH-60 using DMF or DMSO as an eluant. In procedure B, eluates (5 ml fractions) were examined by measuring the ultraviolet (UV) absorption at 260 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 afford a powder.

2. The HOSu–WSCI condensation procedure: Boc-(30—49)-OBzl was treated with TFA–anisole and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF–DMSO (1 : 1) together with NMM (1.1 eq). To this were added Boc-Thr(Bzl)-Leu-Pro-Ala-Gly-OH (3 eq), HOSu (3 eq) and WSCI (3 eq) at 0 °C. After 24 h, the reaction mixture was evaporated *in vacuo* and then 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 reprecipitaion three times from DMSO with MeOH. The purification procedure, yield, physical constants and analytical data of protected thymopoietin II and its intermediates are listed in Tables II and III.

H–Pro–Glu–Phe–Leu–Glu–Asp–Pro–Ser–Val–Leu–Thr–Lys–Glu–Lys–Leu–Lys–Ser–Glu–Leu–Val–Ala–Asn–Asn–Val–Thr–Leu–Pro–Ala–Gly–Glu–Gln–Arg–Lys–Asp–Val–Tyr–Val–Glu–Leu–Tyr–Leu–Gln–Ser–Leu–Thr–Ala–Leu–Lys–Arg–OH (Corresponding to Thymopoietin II)——The protected nonatetracontapeptide (85 mg) was treated with 1M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (70 μl) and Me_2Se (60 μl) in an ice-bath for 110 min, then dry ether was added. The resulting powder was collected by centrifugation, and dried over KOH pellets *in vacuo* for 60 min. The deprotected peptide thus obtained was dissolved in H_2O (5 ml) and treated with Amberlite IR-400 (acetate form, approximately 2 g) for 30 min with stirring. The pH of the filtrate was adjusted to 8.0 with ice-chilled 0.2 N NH_4OH, then after 30 min, to 6.0 with 0.2 N AcOH, and the solution was lyophilized to give a

TABLE II. Characterization of the Protected Thymopoietin II (Revised Amino Acid Sequence) and Its Intermediates

	Puri. proc. (Yield %)	Rf^{1}	mp (°C)	$[\alpha]_D^{21}$ (c = 1.0, DMF)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Boc-(40—49)-OBzl	A (76)	0.72	186—195	−17.4	$C_{104}H_{131}N_{15}O_{21}S \cdot 8H_2O$	59.39	7.04	9.99
						(59.57	7.10	9.87)
Boc-(36—49)-OBzl	A (79)	0.79	207—215	−26.3	$C_{143}H_{189}N_{19}O_{28}S \cdot 10H_2O$	60.60	7.34	9.39
						(60.48	7.52	9.22)
Boc-(30—49)-OBzl	B (73)	0.72	224—234	−25.4	$C_{204}H_{274}N_{30}O_{43}S_2 \cdot 13H_2O$	59.29	7.32	10.17
						(59.31	7.59	9.85)
Boc-(25—49)-OBzl	A (75)	0.61	218—227	−16.3	$C_{231}H_{313}N_{35}O_{49}S_2 \cdot 15H_2O$	59.74	7.31	10.56
						(59.51	7.42	10.29)
Boc-(20—49)-OBzl	A (71)	0.51	241—259	−26.4	$C_{242}H_{348}N_{42}O_{56}S_2 \cdot 11H_2O$	58.09	7.45	11.76
						(58.01	7.67	11.52)
Boc-(16—49)-OBzl	A (80)	0.48	218—229	−14.9	$C_{294}H_{401}N_{47}O_{65}S_2 \cdot 16H_2O$	58.99	7.29	11.00
						(58.82	7.45	10.87)
Boc-(11—49)-OBzl	B (74)	0.52	231—242	−21.6	$C_{351}H_{474}N_{54}O_{77}S_2 \cdot 14H_2O$	60.24	7.23	10.18
						(60.13	7.41	10.56)
Boc-(8—49)-OBzl	B (71)	0.50	211—224	−17.6	$C_{372}H_{505}N_{57}O_{81}S_2 \cdot 16H_2O$	59.90	7.31	10.70
						(59.99	7.52	10.57)
Boc-(1—49)-OBzl	B (52)	0.44	180—197	−31.2	$C_{431}H_{578}N_{64}O_{94}S_2 \cdot 17H_2O$	60.69	7.23	10.51
						(60.47	7.38	10.29)

A, precipitation from DMF or DMSO with MeOH. B, gel-filtration on Sephadex LH-60.

TABLE III. Amino Acid Ratios in 6 N HCl Hydrolysates of the Protected Thymopoietin II (Revised Amino Acid Sequence) and Its Intermediates[a]

	Protected peptides									Residue
	40—49	36—49	30—49	25—49	20—49	16—49	11—49	8—49	1—49	
Leu	3.02	4.03	4.01	5.02	4.99	6.10	7.08	8.04	9.06	9
Val		1.01	1.92	2.03	4.02	4.02	4.00	5.02	5.01	5
Ala	1.00	1.00	2.10	3.04	3.05	2.94	3.01	3.10	3.08	3
Gly				1.00	1.00	1.00	1.00	1.00	1.00	1
Pro				0.91	0.93	0.91	0.86	0.87	2.84	3
Phe									0.92	1
Tyr	0.81	1.94	1.92	1.97	1.96	1.92	1.83	1.90	1.87	2
Ser	0.89	0.87	0.88	0.87	0.86	1.89	1.80	2.80	2.84	3
Thr	0.91	0.89	0.90	1.84	1.88	1.87	2.85	2.88	2.83	3
Glu	0.94	1.95	4.02	4.01	3.92	4.87	5.89	5.86	7.91	8
Asp			0.99	0.92	2.90	2.86	2.94	2.91	3.90	4
Lys	1.01	0.97	2.01	1.98	2.04	3.02	4.92	4.90	4.91	5
Arg	0.89	0.88	1.87	1.85	1.84	1.82	1.85	1.83	1.84	2

a) The results are expressed as ratios to the value for Ala or Gly, which was taken as the diagnostic amino acid in acid hydrolysates.

fluffy powder. The powder was dissolved in 1% AcOH (2 ml), applied to a column of Sephadex G-50 (3.6 × 97 cm) and eluted with 1% AcOH. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. The front peak (tube Nos. 79—86) was collected and the solvent was removed by lyophilization. Next, the Sephadex-purified sample was dissolved in H_2O (2 ml) and the solution was applied to a column of CM-Biogel A (2.3 × 12 cm), which was eluted first with H_2O (100 ml) and then with a linear gradient from H_2O (250 ml) to 0.25 M NH_4OAc buffer (250 ml, pH 6.50). Individual fractions (4 ml each) were collected and the absorbancy at 260 nm was determined. The main peak (tube Nos. 48—56) was collected and the solvent was removed by lyophilization. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf 0.57 (main) and Rf 0.74 (minor). The crude peptide was dissolved in a small amount of H_2O and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) as a developing solvent. The zone corresponding to Rf 0.57 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume, applied to a Sephadex G-25 column (3.6 × 90 cm), and eluted with 2% AcOH. The single main peak fractions were combined and the solvent was removed by lyophization to give a fluffy powder: Yield 6.1 mg (11%), $[\alpha]_D^{21}$ −73.2° (c = 0.3, 1 N AcOH), Rf^3 0.58, Rf^4 0.57, single ninhydrin- and Sakaguchi-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), acetate buffer at pH 2.80, Mobility, 8.0 cm from the origin toward the anode, after running at 1.5 mA, 600 V for 60 min. The synthetic peptide exhibited a single peak on HPLC using a μBondapak C_{18} column (0.39 × 30 cm) at a retention time of 12.46 min, when eluted with a gradient of acetonitrile (30—35% in 20 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate: Leu 9.04, Val 4.92, Ala 3.09, Gly 1.00, Pro 2.82, Phe 0.94, Tyr 1.89, Ser 2.85, Thr 2.90, Glu 7.95, Asp 3.89, Lys 4.92, Arg 1.83 (recovery of Gly 83%). Amino acid ratios in papain plus leucine aminopeptidase digest: Leu 9.01, Val 5.04, Ala 2.98, Gly 1.00, Pro 2.91, Phe 0.99, Tyr 2.03, Ser 2.91, Thr 2.94, Glu 5.92, Asp 1.87, Lys 5.01, Arg 1.93; Asn and Gln were not determined (recovery of Gly 80%).

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 Hypague-Ficoll gradient.[28] 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 peptide (0.1—3 μg/ml) 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; the lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) 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 peptide are those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature: *Eur. J. Biochem.*, **138**, 9 (1984); *Int. J. Peptide Protein Res.*, **24**, No. 1 (1984). Other abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; AcOH, acetic acid; EtOAc, ethyl acetate; NMM, *N*-methylmorpholine; MeOH, methanol; THF, tetrahydrofuran; TFA, trifluoroacetic acid; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; ONp, *p*-nitrophenyl ester; NP, *p*-nitrophenyl; Mts, mesitylene-2-sulfonyl; OBzl, benzyl ester; Troc, *β*,*β*,*β*-trichloroethyloxycarbonyl; Bzl, benzyl; OcHex, cyclohexyl ester; EDTA, ethylenediaminetetraacetic acid; OMe, methyl ester; CM, carboxymethyl; FCS, fetal calf serum; RPMI, Rosewell Park Memorial Institute; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; E-rosette, a rosette with sheep erythrocytes; TFMSA, trifluoromethanesulfonic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DCC, *N*,*N*′-dicyclohexylcarbodiimide; HONB, *N*-hydroxy-5-norbornene-2,3-dicarboximide; PHA, phytohemagglutinin; MA, mixed anhydride; DCHA, dicyclohexylamine.
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Calcium-Binding Protein Isolated from Rat Liver Cytosol Reverses Activation of Pyruvate Kinase by Ca^{2+}

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The effect of a calcium-binding protein (CaBP) isolated from rat liver cytosol on enzyme activation by Ca^{2+} was investigated. Pyruvate kinase (adenosine triphosphate: pyruvate 2-*O*-phosphotransferase; EC 2.7.1.40) isolated from liver cytosol was activated by addition of Ca^{2+} in the range of 1.0—10^3 μM; the concentration giving a half-maximal effect was 10 μM Ca^{2+}. The enzyme activation by 10 μM Ca^{2+} addition was reversed by the presence of CaBP (4—40 μg/ml). With increasing concentrations of Ca^{2+} (1, 10 and 100 μM Ca^{2+}), the pyruvate kinase activity increased progressively. The increase of the enzyme activity by Ca^{2+} was clearly prevented by the presence of CaBP (20 μg/ml). CaBP had no effect on pyruvate kinase activity in the absence of Ca^{2+}. Meanwhile, calmodulin (2.5—10 μg/ml) also reversed the activation of pyruvate kinase by Ca^{2+} (1 and 10 μM). These results indicate that CaBP may regulate the activation of pyruvate kinase by Ca^{2+}. In particular, a novel CaBP may play an important role in the regulation of the Ca^{2+} effect on pyruvate kinase.

Keywords——calcium-binding protein; calcium ion; calmodulin; liver; pyruvate kinase

In recent years, it has been demonstrated that the liver participates in the regulation of calcium metabolism due to the hepatic bile system in rats.[1,2] On the basis of this finding, a calcium-binding protein (CaBP) has been found in the cytosol of rat liver.[3—7] The molecular weight of CaBP isolated from rat liver cytosol was estimated to be 28800, and the Ca^{2+} binding constant was found to be 4.19×10^5 M^{-1} by equilibrium dialysis.[5] Thus, this protein differs from calmodulin, which modulates many biochemical effects of Ca^{2+} in cells.[8,9] Although the cell physiological role of this protein is not fully resolved, it has been demonstrated that the CaBP regulates the activation of fructose 1,6-bisphosphatase in hepatic cytosol by Ca^{2+}-calmodulin.[7] The present investigation was undertaken to elucidate the cell physiological role of CaBP. Pyruvate kinase participates in the rate-controlling process of hepatic gluconeogenesis.[10] The present report describes the effect of Ca^{2+} on liver pyruvate kinase and clarifies the effect of CaBP on the enzyme activation by Ca^{2+}. It was found that CaBP reverses the activation of pyruvate kinase by Ca^{2+}.

Materials and Methods

Animals——Male Wistar rats, weighing 130—150 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.

Chemicals and Enzyme——Calcium chloride and all other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). Pyruvate kinase (30 U/mg protein, adenosine triphosphate: pyruvate 2-*O*-phosphotransferase; EC 2.7.1.40) isolated from rabbit liver and calmodulin (43500 U/mg protein) isolated from bovine brain were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Isolation of CaBP——The livers of rats were perfused with ice-cold 0.25 M sucrose solution, frozen imme-

diately, and then cut into small pieces, which were suspended 1:4 in an ice-cold 0.25 M sucrose solution and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 105000 × *g* in a refrigerated centrifuge for 60 min and the supernatant fluid was collected to obtain the cytosol. 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.[4]

Analytical Methods——Pyruvate kinase assay was carried out under optimal conditions.[11] The usual reaction mixture contained 125 μmol of Tris-HCl buffer (pH 7.5), 25 μmol of $MgCl_2$, 187.5 μmol of KCl, 4.0 μmol of trisodium phosphoenolpyruvate, 4.0 μmol of the sodium salt of adenosine diphosphate (ADP) brought to pH 7.0 with NaOH, and 21 μg of pyruvate kinase, in a final volume of 3.2 ml. In some experiments, the test system contained calcium chloride (10^{-3}—10^3 μM Ca^{2+}), calmodulin (1.0—10 μg/ml) and/or CaBP (4.0—40 μg/ml) in the above assay system. Assays were conducted by incubation at 30 °C, and aliquots of 0.2 ml of assay mixture were removed for pyruvate determination at 1 and 3 min after initiation of the reaction by the addition of ADP. Pyruvate was determined in 0.2-ml aliquots of reaction mixture by a modification of the 2,4-dinitrophenylhydrazine method.[12] The aliquots were added to 0.5 ml of cold 0.0125% 2,4-dinitrophenylhydrazine in 0.2 N HCl, and incubated for 10 min at 37 °C, then 1.2 ml of 2.4 N NaOH containing 2 mM ethylenediaminetetraacetic acid (EDTA) was added. The tubes were allowed to stand for 10 min at room temperature, during which time color developed. The optical density of the solution was measured at 510 nm in a spectrophotometer. The enzyme activity was expressed as μmol of pyruvate released per min per mg protein. The protein concentration was determined by the method of Lowry *et al.*[13]

Statistical Methods——The significance of differences between values was estimated by using Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

The effect of Ca^{2+} addition on pyruvate kinase activity isolated from liver cytosol is shown in Fig. 1. Pyruvate kinase activity was significantly increased by addition of Ca^{2+} in the range of 1.0—10^3 μM. A half-maximal effect was observed at 10 μM Ca^{2+}. With higher levels of Ca^{2+}, the enzyme activity increased, and the maximal effect was observed at 100 μM Ca^{2+}. This enzyme activity was not enhanced by the presence of calmodulin (5 μg/ml).

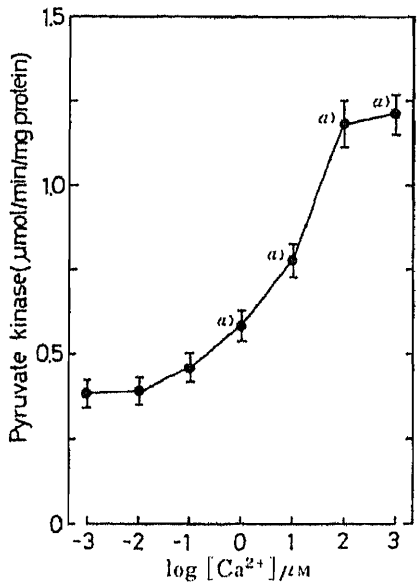


Fig. 1. Effect of Increasing Concentrations of Ca^{2+} on the Activity of Pyruvate Kinase Isolated from Liver Cytosol

Addition of 10^{-3} and 10^{-2} μM Ca^{2+} did not affect the enzyme activity. Each value represents the mean ± SEM of 5 experiments. *a)* $p < 0.01$, as compared with the value without Ca^{2+}

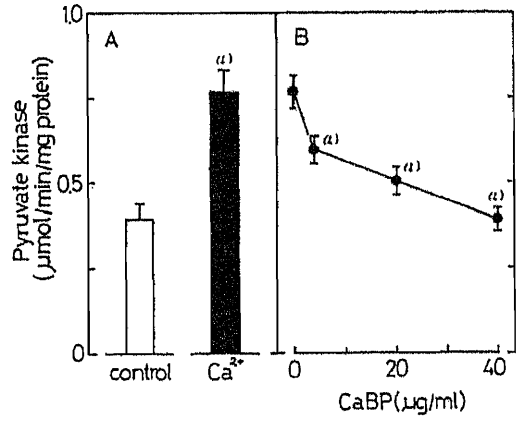


Fig. 2. Effect of CaBP Isolated from Rat Liver Cytosol on the Activation of Pyruvate Kinase by Ca^{2+}

Figure A shows the effect of 10 μM Ca^{2+} on pyruvate kinase activity. Figure B shows the effect of increasing concentrations of CaBP (4, 20 and 40 μg/ml). Each value represents the mean ± SEM of 5 experiments. *a)* $p < 0.01$, as compared with the control value. □, control; ■, 10 μM Ca^{2+} addition.

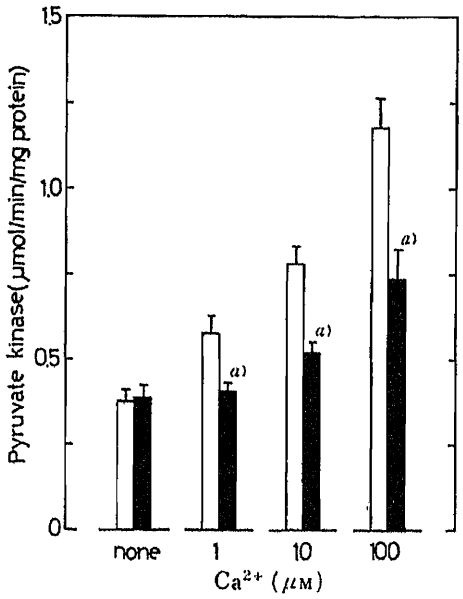


Fig. 3. Effect of Increasing Concentrations of Ca^{2+} on Pyruvate Kinase Activity in the Presence of CaBP

The enzyme reaction mixture contained 20 μg/ml of CaBP. Each value represents the mean±SEM of 5 experiments. *a*) $p<0.01$, as compared with the control value. □, control; ■, CaBP.

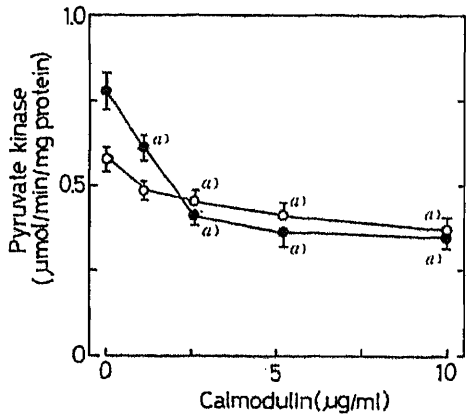


Fig. 4. Effect of Calmodulin on the Activation of Pyruvate Kinase by Ca^{2+}

The enzyme reaction mixture contained 1.1—10.0 μg/ml of calmodulin with 1.0 μM Ca^{2+} or 10 μM Ca^{2+}. The enzyme activity of the control reaction mixture, which did not contain Ca^{2+} or calmodulin was 0.35± 0.029 (μmol/min/mg protein). Each value represents the mean±SEM of 5 experiments. *a*) $p<0.01$, as compared with the enzyme activity without Ca^{2+}. —○—, 1.0 μM Ca^{2+} addition; —●—, 10 μM Ca^{2+} addition.

The effect of CaBP on the activation of pyruvate kinase by addition of Ca^{2+} (10 μM) was examined, and the result is shown in Fig. 2. The increase in the enzyme activity by Ca^{2+} addition was reversed to the control level by CaBP, and the reversal was complete at the concentration of 40 μg/ml. This indicates that the calcium added to the reaction mixture was bound to CaBP, because this protein has 6-7 high-affinity binding sites per molecule of protein.[5]

The reversible effect of CaBP on the activation of pyruvate kinase by addition of Ca^{2+} (1.0—100 μM) was examined (Fig. 3). The activation of pyruvate kinase due to addition of 1.0 μM Ca^{2+} was completely reversed by the presence of CaBP (20 μg/ml). An appreciable activating effect of Ca^{2+} on pyruvate kinase in the presence of CaBP was observed with higher concentrations of Ca^{2+} (10 and 100 μM), although CaBP significantly reversed the enzyme activation by Ca^{2+}. Thus, the Ca^{2+} effect on the enzyme was also weakened by CaBP at higher concentrations of Ca^{2+}.

Calmodulin is a kind of Ca^{2+}-binding protein.[8] Pyruvate kinase isolated from liver cytosol was not activated by calmodulin. Rather, calmodulin reversed the activation of pyruvate kinase by Ca^{2+} (1.0 and 10 μM) (Fig. 4). This effect is consider to be due to the binding of Ca^{2+} to calmodulin, because the protein can bind Ca^{2+} [8,9]

Discussion

It is well known that Ca^{2+} has many biological effects in liver cells.[14] Pyruvate kinase is an enzyme involved in gluconeogenesis in the hepatic cytosol.[10,11] The present result, that

liver pyruvate kinase is activated by Ca^{2+}, is a novel finding. The Ca^{2+} effect on liver cell function is amplified through calmodulin.[8,14] However, the activation of liver pyruvate kinase by Ca^{2+} was not enhanced by calmodulin. This indicates that pyruvate kinase is activated by only Ca^{2+}, independently of calmodulin. Since liver cytosolic Ca^{2+} concentration is raised by hormones,[14] pyruvate kinase may be regulated by the cytosolic Ca^{2+}. The effect of Ca^{2+} on pyruvate kinase may have a cell physiological role. It has been reported, on the other hand, that partially purified muscle pyruvate kinase is inhibited by Ca^{2+} addition[15,16]; appreciable inhibition is observed at Ca^{2+} concentrations greater than 2.0 mM. The present study has shown that isolated liver pyruvate kinase was activated by addition of Ca^{2+} in the range of 1.0—10^3 μM used. Presumably, lower Ca^{2+} concentrations may also activate pyruvate kinase.

The activating effect of Ca^{2+} on pyruvate kinase isolated from liver cytosol was modified by CaBP isolated from rat liver cytosol.[4,5] We found that CaBP could prevent the activation by Ca^{2+}, and the effect of CaBP reached a saturated level on the addition of increasing concentrations of CaBP to a reaction system containing a fixed concentration of Ca^{2+}. Also, CaBP suppressed the elevation of pyruvate kinase activity by increasing concentrations of Ca^{2+}. The results indicate that CaBP does not directly inhibit pyruvate kinase. Although the mechanism by which CaBP modulates the Ca^{2+} effect on pyruvate kinase is unknown, CaBP may affect the binding of Ca^{2+} to the enzyme. Which of CaBP and the enzyme can bind Ca^{2+} may be decided by the relative concentrations of the two proteins and Ca^{2+} in hepatic cytosol. The Ca^{2+}-binding constant of CaBP was found to be 4.19×10^5 M^{-1} by equilibrium dialysis, and there appear to be 6—7 high affinity binding sites for Ca^{2+} per molecule of the protein.[5] Thus, the capacity of Ca^{2+} binding by CaBP is greater than that of calmodulin.[8] Presumably, CaBP may be able to preempt the binding of Ca^{2+} to other proteins in hepatic cytosol.

Interestingly, calmodulin, which can activate various enzyme dependently on Ca^{2+}, reversed the activation of pyruvate kinase by the metal. Calmodulin itself had no effect on pyruvate kinase activity. This calmodulin effect is presumably based on the binding of Ca^{2+} to the protein, because calmodulin is a kind of Ca^{2+}-binding protein.[8]

The previous report[7] demonstrated that CaBP reverses the activation of liver fructose 1,6-bisphosphatase by Ca^{2+}-calmodulin, suggesting that CaBP plays an important role in the regulation of Ca^{2+} action by influencing the interaction of Ca^{2+} with calmodulin. Interestingly, the present investigation has clearly demonstrated that the liver pyruvate kinase is activated by Ca^{2+} independently of calmodulin, and that this activation is reversed by CaBP. This suggests an important direct role of CaBP in regulating the effects of Ca^{2+} on liver cell functions. Further investigation is in progress to clarify the role of this protein in liver cells.

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The Effects of Valproate on Plasma Concentration of Phenytoin in Normal and D-Galactosamine-Treated Rats

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The effect of valproate on the plasma concentration of phenytoin was examined in both normal and D-galactosamine-treated rats. In normal rats the total plasma concentration of phenytoin decreased and the plasma concentration of unbound phenytoin increased after the injection of valproate (300 mg/kg). By contrast, in rats showing low binding as a result of D-galactosamine treatment, the total and unbound concentrations of phenytoin in plasma were almost unchanged after the injection of valproate. Possible mechanisms to explain the above results are discussed in this paper on the basis of the results of plasma protein binding and tissue distribution studies. One such possible mechanism was thought to be that decrease in the total plasma concentration of phenytoin after valproate injection in normal rats may have resulted from an increased volume of distribution which is dependent on increase in the unbound fraction of phenytoin in plasma. On the other hand, one possible reason why valproate produced no significant influence on the total and unbound concentrations of phenytoin in plasma in the rats showing low binding may have been an unchanged volume of distribution of phenytoin preceding and following the injection of valproate which would have been dependent on the lack of change in the unbound fraction. The molar ratio of nonesterified fatty acid (NEFA)/albumin of the plasma protein was increased by D-galactosamine treatment. As NEFA is considered to be an important inhibitor of phenytoin binding to rat plasma, it could be speculated that valproate does not act as an inhibitor of phenytoin binding in rat plasma when the NEFA/albumin molar ratio of plasma protein is increased.

Keywords——phenytoin; valproate; tissue distribution; plasma protein binding; drug-drug interaction; liver injury; pharmacokinetics

Introduction

Many epileptic patients are treated with combinations of several drugs. Valproate, which shows a marked degree of binding to plasma protein in humans[1] and rabbits,[2] is frequently used in combination with phenytoin (PHT) and may displace phenytoin from its binding site on the albumin molecule.[3] Although there are several reports on the interaction between valproate and PHT in man,[4–6] the rhesus monkey,[7] and the rat,[8] the effects of valproate on the tissue distribution of PHT have not been elucidated.

On the other hand, Blaschke *et al.*[9] have reported the influence of acute viral hepatitis on PHT kinetics in humans. They observed that the unbound fraction of PHT increased during the acute phase of viral hepatitis. Accordingly, in view of the wide use of valproate and PHT in the therapy of many epileptic patients, it seems to be important to understand how valproate influences the disposition of PHT in disease states such as liver disease compared with that in the normal state. Keppler *et al.*[10] have reported a method for producing hepatitis similar to human viral hepatitis in the rat through treatment with D-galactosamine (GAL). The points of resemblance to human viral hepatitis are increases in transaminase activity and bilirubin in serum, an intense inflammatory reaction in the periportal areas and the

appearance of uni- and multicellular necrosis and a lack of fatty infiltration. Yamano *et al.*[11] have also reported a decrease in the levels of microsomal drug-metabolizing enzymes in rats treated with GAL. For these reasons, GAL-treated rats were used in this present study as an animal model of liver disease.

The purpose of this study was to examine the effects of valproate on the plasma concentrations of PHT in both normal rats and rats with liver injury produced by GAL treatment. Some of the possible mechanisms for the effects of valproate on the plasma concentrations of PHT in both normal and GAL-treated rats are discussed on the basis of plasma protein binding and tissue distribution studies.

Experimental

Materials——Sodium phenytoin was purchased from Tokyo Kasei Kogyo Co., Ltd. and was also obtained as an injectable solution (Aleviatin, 250 mg/5 ml) from Dainippon Seiyaku Co., Ltd. Sodium valproate was kindly supplied by Kyowa Hakko (Tokyo). All other chemicals were of reagent grade and were used without further purification.

Animals and Pretreatment with GAL——Male Wistar rats (weight 250—300 g) were used for the experiments without prior fasting. GAL dissolved in 0.9% NaCl was injected intraperitoneally at a dose of 750 mg/kg. Subsequent studies on rats with liver injury were performed for 72 h after the injection of GAL.[11]

***In Vivo* Studies**——Rats lightly anesthetized with ether were catheterized *via* the femoral artery with polyethylene tubing (No. 10, Igarashi Ika Kogyo Co., Ltd.). A saline solution of PHT was infused (178 μg/min/kg) through the jugular vein and an intravenous bolus dose of PHT (10 mg/kg) was immediately administered through the femoral vein. Sodium valproate (VPA) (300 mg/kg) was then added 90 min after the start of PHT infusion. The infusion rate of PHT was controlled to produce levels of 10—20 μg/ml of PHT.

Blood (0.3—1.0 ml) samples were obtained at designated times *via* a catheter inserted into the femoral or carotid artery, and centrifuged for 10 min at 3000 rpm to obtain plasma samples for the assay of total and unbound PHT and total VPA.

PHT unbound concentration was determined from the filtrate solution obtained by ultrafiltration of the plasma sample with a disposable Centrifree MPS-3 device (Amicon Corporation, Danver, Mass). In order to obtain the filtrate solution, the plasma samples were preincubated at 37 °C for 20 min. About 0.2 ml of ultrafiltrate was obtained from 0.5 ml of plasma after centrifugation (1000 × *g*) at 37 °C for 5 min. The coefficient of variation for determination of the unbound fraction was less than 2%.

***In Vitro* Serum Protein Binding Studies**——Aliquots of serum containing various concentrations of PHT (4—50 μg/ml) and VPA (0—1000 μg/ml) were preincubated at 37 °C for 20 min. These samples were centrifuged at 37 °C for 5 min (1000 × *g*) with a disposable Centrifree MPS-3 device to obtain the filtrate solution. An adequate concentration of PHT was dissolved in methanol, and added with a microsyringe in serum according to the method of Monks *et al.*[12] The content of methanol in serum was less than 0.6%. Unbound PHT in the ultrafiltrates was assayed by using the method described below.

***In Vivo* Tissue-to-Plasma Concentration Ratio (K_p) and Tissue-to-Plasma Unbound Concentration Ratio ($K_{p,f}$) in Normal Rats**——At 150 min after the start of PHT infusion for determinations of K_p and $K_{p,f}$ of PHT in the absence of VPA, the animals were sacrificed by exsanguination after the collection of blood samples *via* a carotid artery. Determinations of K_p and $K_{p,f}$ of PHT in the presence of VPA were performed at 190 min after the start of PHT infusion (this time corresponds to 30 min after the bolus injection of 300 mg/kg of VPA). Each tissue (brain, heart, lung, liver, kidney, small intestine, muscle, skin and adipose tissue around the kidneys and bladder) was quickly excised and rinsed with cold saline. A sample (0.5—1 g) of each tissue was homogenized with 4 ml of 0.34 M perchloric acid containing ethylenediaminetetraacetic acid (EDTA) (1×10^{-5} M) in a grinder.

Tissue concentrations and total and unbound concentrations of PHT in plasma in the presence and absence of VPA were then determined.

Analytical Method——The total and unbound concentrations of PHT in plasma and serum were determined by using a high-performance liquid chromatographic (HPLC) system (LC-6A, Shimadzu, Kyoto, Japan). To 0.1 ml of each sample, 1 ml of pH 5.0, 0.1 M phosphate buffer and 2 ml of ethyl acetate containing amobarbital (6 μg/ml) as an internal standard were added. The samples were shaken for 15 min, then 1.5 ml of the ethyl acetate layer was removed. The residue remaining after subsequent evaporation of the ethyl acetate sample was reconstituted with 0.1 ml of the mobile phase, and 20 μl was injected onto the HPLC column. The concentration of PHT in tissues was determined by the HPLC method of Stout and LeVaine[13] with the following modification. To 0.5 ml of each tissue homogenate, 0.5 ml of distilled water, 1.0 ml of pH 11.4 phosphate buffer and 0.1 ml of MPPH (5-(*p*-methylphenyl)-phenylhydantoin, 50 μg/ml) solution as an internal standard were added. For the extraction of drugs, 2 ml of ethyl acetate was used.

The mobile phase was a 55:45 (v/v) mixture of methanol and 0.01 M NaH_2PO_4 for plasma and filtrates, and a 40:60 (v/v) mixture of acetonitrile and 4×10^{-4} M KH_2PO_4 for tissues. Detection was at 230 nm for plasma and filtrates and at 254 nm for tissues. A reversed-phase packed column (Shim-Pack CLC-ODS, Shimadzu) was used. The detection limit of these methods was about 1 μg/ml for both plasma and tissue samples. The regression line was linear for PHT concentrations of more than 1 μg/ml ($r > 0.99$).

Fluorescence polarization immunoassay was used for VPA determination according to the procedure recommended by the manufacturer (Dainabot, Japan).

The concentrations of albumin, nonesterified fatty acid (NEFA) and bilirubin in plasma were determined by using commercial kits (albumin B-Test, NEFA C-Test and Bilirubin-Test, Wako Pure Chemical Co., Ltd., Japan).

Statistical Analysis——All means are presented with their standard error (mean ± S.E.). Statistical analysis was performed by using Student's *t*-test.

Results

In Vivo Plasma Levels and Unbound Fraction of PHT in Normal Rats

The effects of VPA on total and unbound levels of PHT in plasma are illustrated in Fig. 1 for normal rats. The total levels of PHT in plasma before VPA treatment were almost at a steady state throughout the experimental period. After the injection of VPA, when the total VPA concentration in plasma was in the range from 700 to 500 μg/ml, the total plasma concentration of PHT decreased dramatically ($p < 0.05$) and the unbound concentration of PHT increased significantly. The unbound fraction of PHT after the injection of VPA was twice as great as that before VPA treatment (see open circles in Fig. 3).

In Vivo Plasma Levels and Unbound Fraction of PHT in GAL-Treated Rats

In GAL-treated rats, the interindividual variability was large. However, in some GAL-treated rats, the total and unbound concentrations of PHT in plasma were almost unchanged preceding and following the injection of VPA (left panel in Fig. 2). In some of the others, the total and unbound levels of PHT showed a similar pattern to that observed in normal rats

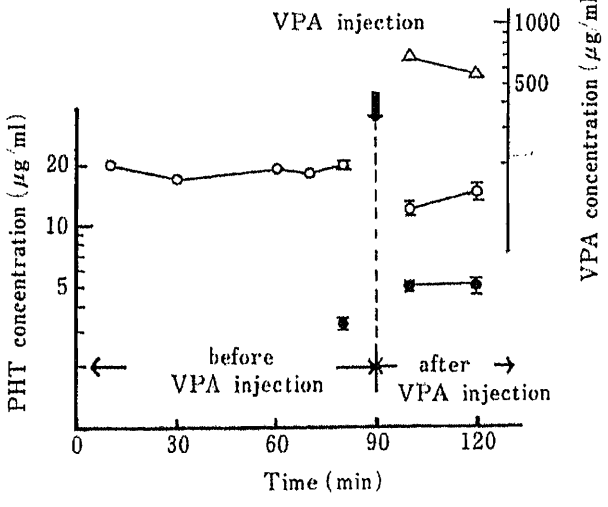


Fig. 1. Total and Unbound PHT Concentrations in Plasma during the Infusion of PHT (178 μg/min/kg) before and after VPA Administration i.v. (300 mg/kg) in Normal Rats

○, plasma total concentration of PHT; ●, plasma unbound concentration of PHT; △, plasma total concentration of VPA ($n = 2$ to 4).

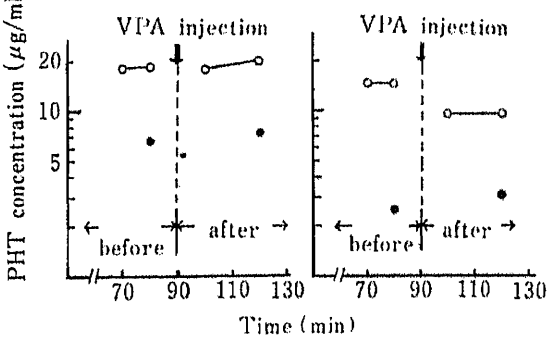


Fig. 2. Typical Plots of Total and Unbound PHT Concentrations in Plasma during the Infusion of PHT (178 μg/min/kg) before and after VPA Administration i.v. (300 mg/kg) in GAL-Treated Rats

The left panel shows an example in which the total and unbound concentration of PHT was almost unchanged. The right panel shows an example of a pattern similar to that in normal rats.

○, plasma total concentration of PHT; ●, plasma unbound concentration of PHT.

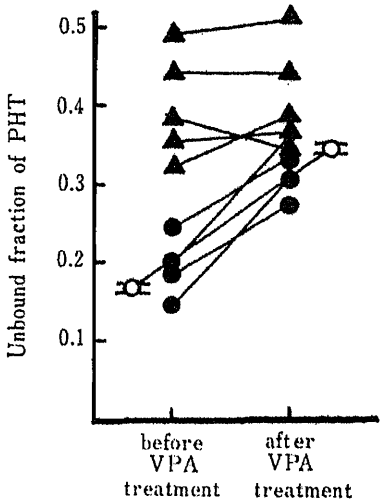


Fig. 3. Effect of VPA on the Plasma-Unbound Fraction of PHT *in Vivo* in Normal and GAL-Treated Rats

○, normal rats; ● and ▲, GAL-treated rats. The solid triangles represent the unbound fraction of PHT in the low-binding rats, which had already shown a tendency toward exhibiting low-binding behavior due to GAL treatment before the injection of VPA. The solid circles represent the data for other GAL-treated rats.

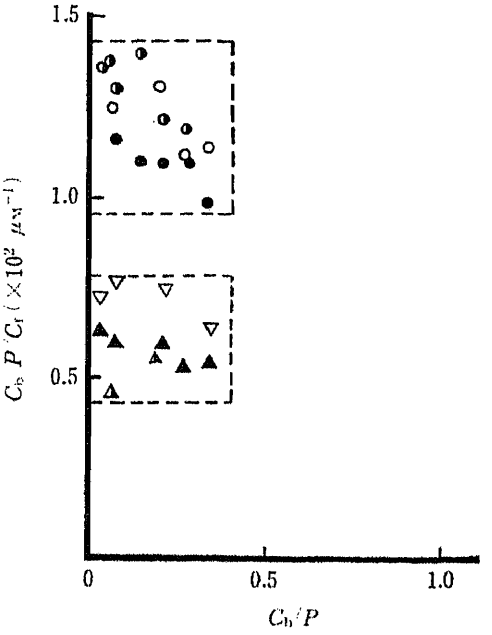


Fig. 4. Scatchard Plots of Data for *in Vitro* Serum Protein Binding of PHT to Normal Rat Serum (○) and GAL-Treated Rat Serum (Other Symbols) at 37 °C

(right panel in Fig. 2). Figure 3 shows the changes in the PHT unbound fraction before and after VPA treatment in normal rats (open circles) and in GAL-treated rats (solid circles solid triangles). The data represented by the solid circles in Fig. 3 showed a similar pattern to that observed in normal rats before and after VPA treatment. On the other hand, the data represented by the solid triangles showed a tendency toward a low level of PHT binding with plasma protein in comparison to that in normal rats before VPA treatment. In such rats (hereinafter referred to as low-binding rats), VPA had no significant influence on the plasma protein binding of PHT.

In Vitro Serum Protein Binding in Normal and GAL-Treated Sera

Figure 4 shows the Scatchard plots for the serum protein binding of PHT *in vitro* with a drug concentration range of between 4 and 50 μg/ml using serum from normal and GAL-treated rats. In some of the GAL-treated rat serum samples (see triangle symbols in Fig. 4), the values of $C_b/P/C_f$ (ordinates) were decreased compared to those in normal samples, although the other sets of GAL-treated rat sera appeared to show similar binding behavior to that of normal rat serum (0.0046—0.0076 μM-1 to 0.0098—0.014 μM-1). C_b and C_f represent the bound and unbound concentration of PHT in serum, and P represents the albumin concentration in serum.

In Vivo Unbound Fraction of PHT and Albumin, Bilirubin and NEFA Concentration in Plasma from Normal and GAL-Treated Rats

The unbound fraction before and after VPA treatment and albumin, bilirubin and NEFA concentrations in plasma from normal and GAL-treated rats are summarized in Table I. The data obtained from GAL-treated rats Nos. 1, 2, 3, 4 and 5 correspond to the data

TABLE I. Unbound Fraction of PHT and Laboratory Values in Plasma of Normal and GAL-Treated Rats

	f_p		Albumin (mM)	Bilirubin (mg/dl)	NEFA (meq/l)	NEFA/Albumin (meq/l/mM)
	Before	After				
Normal rats mean ± S.E. $n=4$	0.167 ± 0.0065	0.345 ± 0.0067	0.539 ± 0.0094	0.132 ± 0.0067	0.434 ± 0.049	0.801 ± 0.079
GAL-treated rats						
1	0.33	0.39	0.320	0.89	1.29	4.031
2	0.49	0.51	0.290	2.07	1.29	4.448
3	0.38	0.35	0.377	0.77	2.08	5.517
4	0.44	0.44	0.325	0.69	0.75	2.308
5	0.35	0.37	—[a]	—	—	—
6	0.20	0.36	0.428	N.D.[b]	0.30	0.701
7	0.25	0.34	0.393	0.09	0.73	1.856
8	0.14	0.31	0.378	0.09	0.53	1.402
9	0.18	0.32	—	—	—	—

a) Not determined. *b*) Not detected.

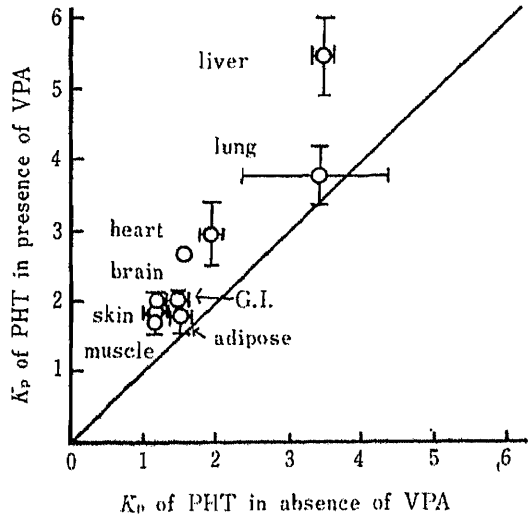


Fig. 5. Relationship between the Tissue-to-Plasma Concentration Ratios (K_p) of PHT in the Absence and Presence of VPA in Normal Rats

The line has a slope of 1.

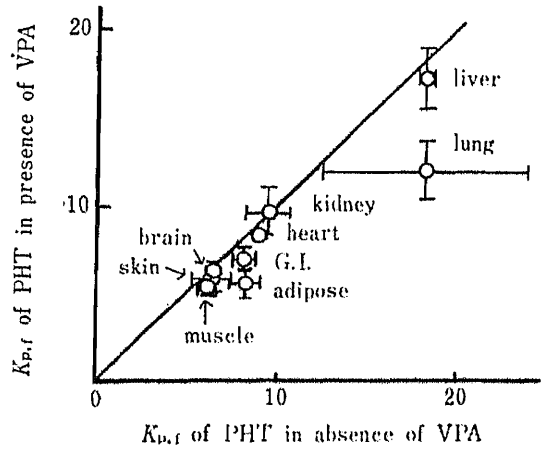


Fig. 6. Relationship between the Tissue-to-Plasma Unbound Concentration Ratios ($K_{p,f}$) of PHT in the Absence and Presence of VPA in Normal Rats

The line has a slope of 1.

represented by the solid triangles in Fig. 3. The mean value ±S.E. of the molar ratio of NEFA/albumin in the plasma of GAL-treated rats Nos. 1, 2, 3 and 4 was 4.08 ± 0.667. This mean value of NEFA/albumin was significantly increased compared with that in normal rat plasma.

Tissue Distribution Study in Normal Rats

The tissue-to-plasma concentration ratio (K_p) and the tissue-to-plasma unbound concentration ratio ($K_{p,f}$ of PHT in the absence of VPA in normal rats were determined using data obtained at 150 min after the start of infusion, K_p and $K_{p,f}$ in the presence of VPA were also determined by using data obtained at 190 min (30 min after the bolus injection of VPA) after

the start of infusion. The values of K_p and $K_{p,f}$ in the absence and presence of VPA were calculated by using the following equations.

$$K_p = K_{p,app} = C_{tissue}/C_{p,a} \quad (1)$$

$$K_{p,f} = K_{p,f,app} = C_{tissue}/C_{p,f} \quad (2)$$

where C_{tissue} is the concentration of PHT in each tissue, and $C_{p,a}$ and $C_{p,f}$ are the total and unbound concentrations of PHT in arterial plasma, respectively. The liver is the disposing organ for PHT, so the proper correction is necessary to estimate K_p and $K_{p,f}$. However, the K_p and $K_{p,f}$ for liver were approximated by using the above equations in the present study, because PHT has a low extraction ratio.

The relationship between K_p and $K_{p,f}$ values in the absence and presence of VPA are shown in Fig. 5 and Fig. 6, respectively. Although the K_p values for many tissues except for adipose, G.I. and lung were significantly increased in the presence of VPA, the $K_{p,f}$ values except for adipose and G.I. exhibited no significant change in the presence of VPA.

Discussion

The effects of VPA on the plasma concentration of PHT was examined in the normal and GAL-treated rats. In the normal rats VPA showed a significant influence on the total and unbound concentrations and the unbound fraction of PHT in plasma (Fig. 1 and open circles in Fig. 3). By contrast, in the rats exhibiting a low binding as a result of GAL treatment, the total and unbound concentrations of PHT in plasma were not influenced by VPA (left panel in Fig. 2).

As PHT has a low extraction ratio, only the unbound drug should be considered to be available in kinetic studies.[14] Although substrate saturation of the metabolic enzyme for PHT is well known to occur in rats,[15] if the linear kinetic theory is assumed for the sake of simplicity, the total plasma concentration of PHT at the steady state (C_{ss}) can be expressed as follows.[16]

$$C_{ss} = \frac{I_0}{CL_{tot}} = \frac{I_0}{f_p CL_{int}} \quad (3)$$

where I_0 is the infusion rate, CL_{tot} is the total body clearance of PHT, CL_{int} is the intrinsic clearance, and f_p is the unbound fraction of PHT in plasma.

The total plasma concentration of PHT in the steady state is also given by[16]:

$$C_{ss} = \frac{I_0 T_{1/2}}{0.693\, V} \quad (4)$$

where $T_{1/2}$ is the elimination half-life, and V is the apparent volume of distribution during the terminal phase of PHT. Since V is approximately equal to the volume of distribution at the steady state (V_{dss}),[16] C_{ss} can be expressed as follows.

$$C_{ss} \simeq \frac{I_0 T_{1/2}}{0.693\, V_{dss}} \quad (5)$$

Based on Eqs. 3 and 5, possible mechanisms that might cause the total plasma concentration of PHT in normal rats to decrease after VPA injection are considered to be as follows: a) the total body clearance increases according to Eq. 3; b) the volume of distribution at the steady state increases according to Eq. 5, provided that the elimination half-life is assumed to be unchanged.

The data in Fig. 3 (open circles) suggest that VPA displaced PHT from rat plasma

protein in normal rats. This result is in agreement with the observation of O'Leary and Sanson.[8] This finding according to Eq. 3, also suggests that the total body clearance increases after VPA injection, and consequently the total plasma concentration of PHT decreases. However, the fact that the plasma concentration of unbound PHT increased after VPA injection cannot be explained according to Eq. 3, because the unbound concentration is independent of change in the unbound fraction, and so would not be expected to change if the sole interaction is the displacement of PHT from plasma proteins by VPA. One of the most probable mechanisms for explaining an increased concentration of unbound PHT after VPA injection is that the intrinsic clearance decreases. However, O'Leary and Sanson[8] showed that no significant change of the intrinsic clearance of PHT occurred in rats injected with PHT–VPA. Therefore, it is unlikely that decrease in the total plasma concentration after VPA injection results solely from an increase in total body clearance dependent on increase in the unbound fraction.

Therefore another possibility, that the volume of distribution increases after VPA injection, was investigated. The volume of distribution at the steady state is related to the unbound fraction in plasma, blood-to-plasma concentration ratio (*RBP*), the blood volume (V_B) and the tissue volume (V_T) as follows[16]:

$$V_{dss} = RBPV_B + \sum K_p V_T = RBPV_B + \sum f_p K_{p,f} V_T \quad (6)$$

According to Eq. 6, the data in Figs. 5 and 6 suggest that the volume of distribution of PHT increased in the presence of VPA and was dependent on the unbound fraction of PHT in plasma. Equation 5 suggests that a possible mechanism for the decrease in the total plasma concentration of PHT in normal rats after the injection of VPA may be an increased volume of distribution, which is dependent on increase in the unbound fraction of PHT. This suggestion is based on the fact that $T_{1/2}$ of PHT did not significantly change after bolus injection of VPA–PHT in rats.[8]

By contrast, one of the possible reasons why VPA had no significant influence on the total and unbound concentrations in plasma in the low-binding rats (left panel in Fig. 2) may have been an unchanged volume of distribution of PHT preceding and following the injection of VPA, which would have been dependent on the absence of change in the unbound fraction of PHT. The data for the low-binding rats in Fig. 3 (solid triangles) suggest that VPA had no significant influence on the plasma protein binding. Perucca[17] has reviewed the factors which may affect the plasma protein binding of PHT in man. These factors include a decrease in plasma albumin concentration, and the presence of endogenous bound ligands such as NEFA and bilirubin. The results shown in Fig. 4 indicate that some change affecting the serum protein binding of PHT such as alteration of the intrinsic binding properties of the serum protein and/or the presence of endogenous ligands took place in some of the sera treated with GAL (triangle and circle symbols). Also, marked differences in the molar ratio of NEFA/albumin of the plasma protein were obtained between normal rats and low-binding rats (GAL-treated rats Nos. 1, 2, 3 and 4 in Table I). These data suggest that NEFA may be one of the important inhibitors of PHT binding to rat plasma. The data in Table I may also be interpreted as demonstrating that VPA did not play an important role as an inhibitor of PHT binding in rat plasma when the molar ratio of the NEFA/albumin of the plasma protein increased. Blaschke *et al.*[9] concluded that a small decline in serum albumin concentration and elevated serum bilirubin level may be responsible for the alterations in plasma protein binding of PHT seen in patients with acute viral hepatitis. In our present results, the plasma bilirubin levels from the low-binding rats were about 10-fold greater than that observed in normal rats. However, the molar ratio of bilirubin/albumin in the low-binding rats was about 0.06. Therefore, bilirubin is unlikely to have played a predominant role in displacing PHT from the plasma protein in low-binding rats, as the bilirubin–albumin complex accounts for less than

10% of albumin.

In this study, the effects of VPA on PHT pharmacokinetics were elucidated in normal rats and in GAL-treated rats (especially in the low-binding rats). In normal rats, decreases in the total plasma concentration of PHT after VPA injection may have resulted from an increased volume of distribution, which is dependent on increase in the unbound fraction. By contrast, one of the possible reasons why VPA had no significant influence on the total and unbound concentrations in plasma in the low-binding rats after VPA injection may have been an unchanged volume of distribution of PHT, which would have been dependent on the lack of change in the unbound fraction. However, it is an extremely important finding in relation to the therapy of epilepsy that after VPA injection the plasma concentration of unbound PHT increases, associated with decrease in the total plasma concentration. This finding is supported by the observation of Riva *et al.*[5] in humans. As it has been reported that the intrinsic clearance of PHT is decreased by VPA in humans,[6] further studies will be necessary to investigate exactly how VPA influences the volume of distribution and the intrinsic clearance of PHT not only in rats, but also in humans.

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Studies on the Factors Affecting Pulmonary Absorption of Xanthine Derivatives in the Rat

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In an attempt to develop a suitable dosage form of systemically acting drugs for pulmonary administration, the absorption of xanthine derivatives from the lung of rats was studied. The pulmonary absorption of xanthine derivatives was rapid, and these drugs were transferred into the circulation. For example, in the case of theophylline (500 μg/rat), the absorption within 1 min was about 90%, and in the cases of aminophylline (500 μg/rat) and caffeine (1000 μg/rat), 96% and 97%, respectively. The maximum plasma levels in all cases were achieved within 30 s. The pulmonary absorption of these drugs was not affected by body weight or wet weight of lung. When the dose of these drugs was raised 10- to 15-fold, the amount of compound absorbed was directly proportional to the dose, the percentage absorption remaining constant. The pulmonary absorption of these drugs was not significantly different at pH between 6.4 and 8.4, but at pH 9.4, the absorption of theophylline and aminophylline was significantly decreased ($p<0.001$), though the absorption of caffeine remained nearly constant. Comparison of the partition coefficients of these drugs indicated that the pulmonary absorption of xanthine derivatives depends on lipid solubility. The pulmonary absorption of theophylline was unaffected by changes of the osmotic pressure of drug solutions. These results suggest that the pulmonary absorption of xanthine derivatives can indeed occur very rapidly, and that the lung may afford a favorable route of administration to obtain a systemic effect of drugs.

Keywords——xanthine derivative; pulmonary absorption; pulmonary administration; pH effect; partition coefficient; theophylline; aminophylline; caffeine; rat

Recent investigations concerning the permeability characteristics of lung mucosa[1] indicated that the lung may provide a useful route of administration for systemically acting drugs such as cyanocobalamin,[2] gentamicin[3] and kanamycin,[4] which are not absorbed or are absorbed very slowly from the gastrointestinal tract. Moreover, Schanker *et al.* suggested that the lung membrane possesses at least two absorption processes: a nonsaturable process[5] and saturable carrier-type transport.[6] According to his data, drug absorption from the lung is rapid, as had been expected from the histological and physiological characteristics of the lung. However, drug administration to the lung is usually done for topical treatment, but not systemic treatment, because dosage forms suitable for pulmonary administration, except for gaseous drugs, have not been developed adequately.

The present study was designed to obtain basic data for developing an effective dosage form for pulmonary administration of systemically acting drugs by investigating the influence of various factors on the absorption of xanthine derivatives from the rat lung.

Experimental

Materials——Three xanthine derivatives: theophylline (Nakarai Chemicals, Ltd.), aminophylline (Eisai Co., Ltd.) and caffeine (Shizuoka Caffeine Co., Ltd.) were selected. They were purchased from commercial sources and used without further purification.

Preparation of Solutions for Administration and for Determination of Partition Coefficient——Test solutions of xanthine derivatives were prepared with Krebs-Ringer phosphate solution (pH 6.4, 7.4 and 8.4)[7] or isotonic sodium borate buffer solution (pH 8.4 and 9.4), and for the study of osmotic pressure effect on the pulmonary absorption, drug solutions having different osmolalities were prepared by adding sodium chloride.

Determination of Partition Coefficient——Isotonic buffered solutions (pH 6.4—9.4) containing xanthine derivatives (200 and 5000 μg/ml) were prepared, and the partition coefficients between the buffer and chloroform, which has been used to evaluate the lipid solubility of substances,[8] were determined. After shaking with an equal volume of the chloroform at 37 °C for 1 h, the drug content in the aqueous phase was determined and the partition coefficient was calculated.

Animal Procedures——Male Wistar rats weighing 170—190 g were used in most experiments. However, for the study of the effect of the body weight or of the lung weight on the pulmonary absorption of theophylline, rats having 170—300 g body weight were used. The animals were fed a standard laboratory diet and given tap water *ad libitum* prior to experiments.

With the rats under sodium pentobarbital (Nembutal, 40 mg/kg i.p.) anesthesia, the trachea was exposed through a ventral midline incision in the neck. A 2.5-cm length of polyethylene tubing, which served as a tight-fitting tracheal cannula, was inserted through an incision between the fourth and fifth tracheal rings caudal to the thyroid cartilage to a depth of 0.6 cm. The incision in the skin was then closed with a wound clip and the body temperature was maintained at 37±1 °C with a 40-W incandescent lamp suspended above the animals.

Procedure of Absorption Experiments——According to the method developed by Enna and Schanker,[9] 0.1 ml of solution was injected into the lungs through polyethylene tubing attached to a calibrated 100-μl syringe (Hamilton Company). The injection tubing was inserted through the tracheal cannula to a point 1 mm above the bifurcation of the trachea, the drug solution was injected within 1 to 2 s, and the tubing was quickly withdrawn. At the end of an absorption period, the blood supply to the lungs was quickly halted with an arterial clamp, and then the lungs and attached trachea were removed. These tissues were assayed for unabsorbed drugs.

Determination of Drug Level in Plasma——The blood was collected with a heparinized syringe from the descending aorta at given times after drug administration. The plasma was separated by centrifuging at 3000 *g* for 20 min, and drug concentration was determined.

Analytical Methods——(1) Theophylline and Aminophylline: Removed lung tissue and attached trachea were weighed, and placed in a glass homogenizer together with sufficient distilled water to make 5 ml. After homogenization, the lung was extracted with 20 : 1 chloroform–isopropanol (30 ml), and determined spectrophotometrically at a wavelength of 275 nm using a Hitachi spectrophotometer, model 124, according to the modified method of Vasiliades and Turner.[10] The recovery of xanthine derivatives was always more than 90%, so that the method appeared to be suitable for this study. Essentially the same procedures were applied for the determination of the drug in plasma.

(2) Caffeine: Tissue caffeine and plasma caffeine were analyzed spectrophotometrically at a wavelength of 460 nm using a Hitachi spectrophotometer (model 124) after extraction with chloroform (20 ml).[11]

Student's *t* test was used to examine the statistical significance of differences. The 0.05 level of probability was regarded as significant.

Results

The results of the pulmonary absorption of xanthine derivatives in rat are shown in Fig. 1. The semilogarithmic plots of percentage of unabsorbed drugs against time were not linear. The pulmonary absorption curves demonstrated that drug absorption from the lung can occur rapidly. The 1-min absorption value of theophylline (500 μg/rat) at pH 7.4 was about 90%, and the percentages absorbed in 1 min at pH 7.4 in the cases of aminophylline (500 μg/rat) and caffeine (1000 μg/rat) were 96 and 97, respectively.

To examine in more detail the rapid disappearance of these drugs, the plasma levels of xanthine derivatives were measured after administration. In Fig. 2, the time courses of plasma level after pulmonary administration are illustrated. The plasma levels were investigated up to 30 min in the cases of theophylline and aminophylline, and 15 min in the case of caffeine. The plasma level of these drugs reached the maximum within 30 s after pulmonary administration, and almost all of these drugs was transferred to the plasma very rapidly.

In order to study the relation between pulmonary absorption and body weight or lung wet weight of animals, the absorption of theophylline from the lung mucosa of rats weighing 170—300 g was investigated. As shown in Figs. 3 and 4, the pulmonary absorption of

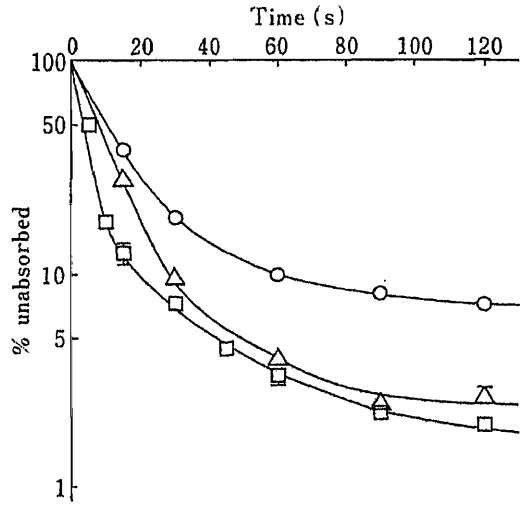


Fig. 1. Time Course of Percent Unabsorbed of Xanthine Derivatives after Pulmonary Administration to Rats

Each point is the mean for five animals. Vertical bars indicate the S.E.; the absence of bars indicates that the S.E. is within the size of the symbol.
○—○, theophylline 500 μg/rat (pH 7.4); △—△, aminophylline 500 μg/rat (pH 7.4); □—□, caffeine 1000 μg/rat (pH 7.4).

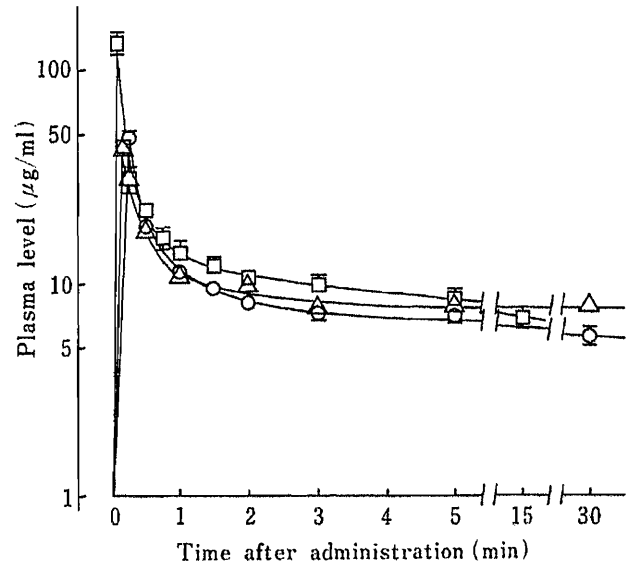


Fig. 2. Plasma Levels of Xanthine Derivatives after Pulmonary Administration to Rats

Each point is the mean for 5—6 animals. Vertical bars indicate the S.E.; the absence of bars indicates that the S.E. is within the size of the symbol.
○—○, theophylline 500 μg/rat (pH 7.4); △—△, aminophylline 500 μg/rat (pH 7.4); □—□, caffeine 1000 μg/rat (pH 7.4).

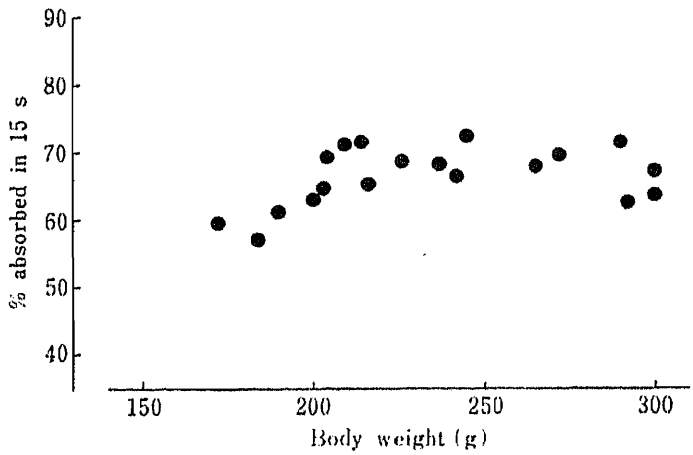


Fig. 3. Relationship between Pulmonary Absorption of Theophylline and Body Weight of Rats

Dose: theophylline 500 μg/rat (pH 7.4). $Y=0.038X+57.6$, $r=0.355$ ($n=17$, not significant). X: body weight of rat. Y: percent absorbed in 15 s after pulmonary administration of theophylline.

theophylline (500 μg/rat) was not significantly affected by the body weight or lung wet weight, and there was no direct relationship between pulmonary absorption of theophylline and body weight of rats ($Y=0.038X+57.6$, $r=0.355$ ($n=17$, not significant)), or lung wet weight of rats ($Y=-5.9X+73.1$, $r=0.16$ ($n=17$, not significant)).

The effect of dose on the pulmonary absorption is shown in Fig. 5. When the dose of theophylline and aminophylline was varied over a 10-fold range (theophylline 50—500 μg/rat, aminophylline 100—1000 μg/rat), and that of caffeine over a 15-fold range (caffeine 100—1500 μg/rat), the amount of drugs absorbed was directly proportional to the dose, and the percentage absorption was almost constant in these studies.

As shown in Fig. 6, the pH value of administered solution was varied between 6.4 and 9.4. The pulmonary absorption of all drugs in 15 s remained constant at pH 6.4, 7.4 and 8.4.

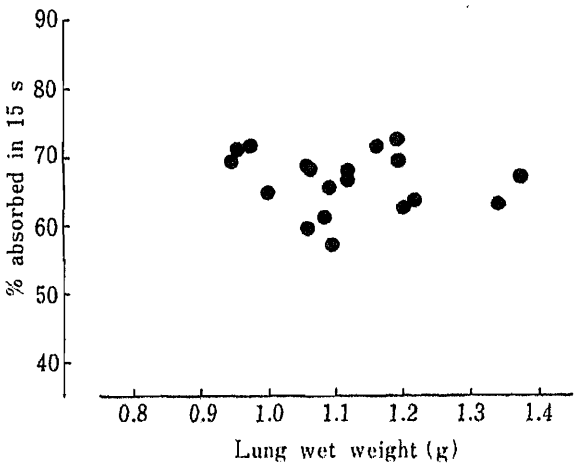


Fig. 4. Relationship between Pulmonary Absorption of Theophylline and Lung Wet Weight of Rats

Dose: theophylline 500 μg/rat (pH 7.4). $Y = -5.9X + 73.1$, $r = 0.16$ ($n = 17$, not significant). X: lung wet weight of rat. Y: percent absorbed in 15 s after pulmonary administration of theophylline.

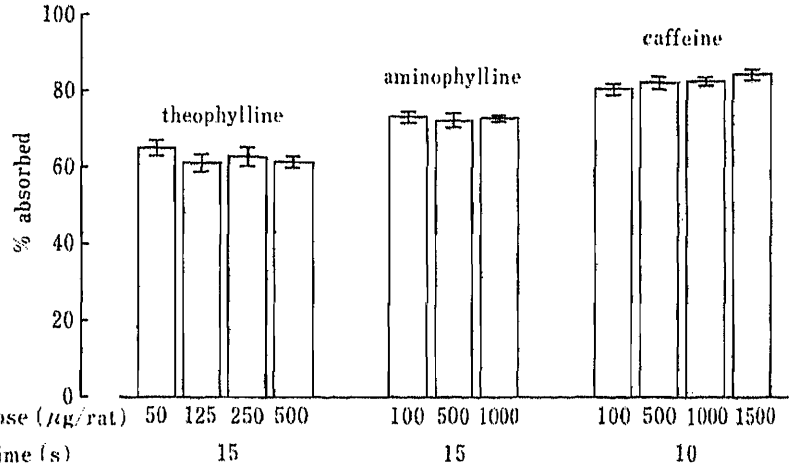


Fig. 5. Effect of Dose on Pulmonary Absorption of Xanthine Derivatives

Each value is the mean for five animals. Vertical bars indicate S.E.

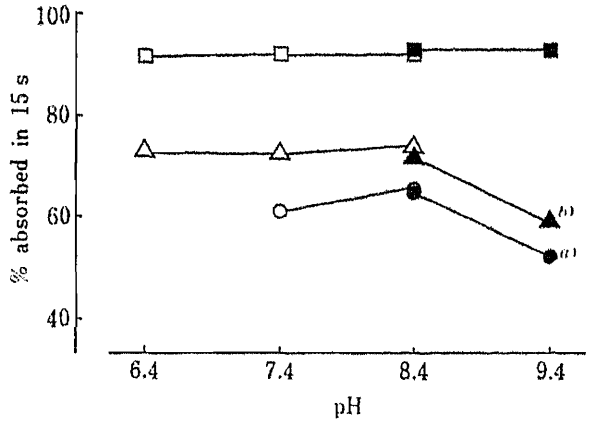


Fig. 6. Effect of pH on Pulmonary Absorption of Xanthine Derivatives

Each point is the mean for 5–11 animals. ○-●, theophylline 500 μg/rat; △-▲, aminophylline 500 μg/rat; □-■, caffeine 1000 μg/rat.

○, △, □, all drugs were dissolved in Krebs-Ringer phosphate buffer solution; ●, ▲, ■, all drugs were dissolved in isotonic sodium borate buffer solution.

a) Significantly different from the other three values ($p < 0.001$). *b*) Significantly different from the other four values ($p < 0.001$).

In contrast to these results, at pH 9.4, the pulmonary absorptions of theophylline and aminophylline in 15 s were significantly decreased ($p < 0.001$), but the absorption of caffeine did not change.

The partition coefficients of xanthine derivatives at various pH values are summarized in Table I. When the partition coefficient values of theophylline and aminophylline at pH 9.4 decreased from 0.233 to 0.119 and from 0.185 to 0.078, respectively, the 15-s absorption

TABLE I. Comparison between Rates of Absorption and Physical Properties of Xanthine Derivatives

Compound	Mol. weight	pH	% absorbed in 15 s (Mean ± S.E.)	Partition coefficient (Chloroform/buffer; at 37 °C)
Theophylline	180.2	6.4	—	0.341
		7.4	61.2 ± 1.4 (5)	0.325
		8.4	65.6 ± 1.4 (6)	0.278
		8.4[a]	64.9 ± 1.0 (6)	0.233
		9.4[a]	52.4 ± 1.2 (9)[b]	0.119
Aminophylline	420.4 + 18.02X	6.4	72.8 ± 1.2 (10)	0.294
		7.4	72.4 ± 1.8 (5)	0.278
		8.4	73.7 ± 0.7 (11)	0.201
		8.4[a]	71.5 ± 1.2 (5)	0.185
		9.4[a]	59.0 ± 0.8 (6)[c]	0.078
Caffeine	212.2	6.4	91.8 ± 0.7 (6)	27.4
		7.4	92.3 ± 0.6 (6)	28.0
		8.4	92.1 ± 0.4 (6)	26.9
		8.4[a]	93.3 ± 0.3 (6)	21.2
		9.4[a]	93.1 ± 0.4 (6)	21.1

a) Test drug was dissolved in isotonic sodium borate buffer solution. Dose levels were theophylline 500 μg/rat, aminophylline 500 μg/rat, caffeine 1000 μg/rat. *b*) Significantly different from the other three values ($p < 0.001$). *c*) Significantly different from the other four values ($p < 0.001$).

TABLE II. Effect of Osmotic Pressure on Pulmonary Absorption of Theophylline

Component of drug solution	No. of rats	Time after administration	% absorbed (Mean ± S.E.)
Distilled water	5	15 s	62.9 ± 2.2
0.9% NaCl	5	15 s	65.6 ± 2.4
2.7% NaCl	5	15 s	65.1 ± 2.1

Dose: theophylline 500 μg/rat.

values of these drugs at pH 9.4 also decreased to 52.4 ± 1.2% ($p < 0.001$) and 59.0 ± 0.8% ($p < 0.001$), respectively. In the case of caffeine, neither the partition coefficient value at pH 9.4 nor the 15-s absorption value at pH 9.4 changed significantly. On the other hand, on comparing the partition coefficients and pulmonary absorption of these three compounds at pH 7.4, caffeine, which has 86- to 101-fold (pH 7.4) greater partition coefficient than the other two drugs, was absorbed more rapidly than the other two drugs. These results suggest that the partition coefficient of the derivatives may affect the pulmonary absorption.

The effect of osmotic pressure of the drug solution administered on the absorption of theophylline is summarized in Table II. The 15-s absorptions from drug solutions in distilled water, 0.9% NaCl soln. and 2.7% NaCl soln. showed no significant difference.

Discussion

It is well known that the lung possesses a membrane for exchange of gas between blood and air; it is located deep inside the chest and is divided into microscopic subsections (alveoli) at the ends of the many bronchi. Moreover, the alveolar membrane is very thin and the alveoli are surrounded by a dense vessel network. Accordingly, it is expected that drugs will be transferred rapidly from the alveoli into the circulation.

In the present study, it was confirmed that xanthine derivatives (theophylline, amin-

ophylline and caffeine) were absorbed from the rat lung very rapidly, suggesting that absorption of these drugs from the lung is fast compared to absorption from the gastrointestinal tract. For example, as reported by Hogben *et al.*,[12] the 30-min absorption of theophylline from the rat small intestine is about 29%, whereas, as shown in the present study, the 1-min absorption of theophylline was about 90% in the case of rat lung (Fig. 1). Since absorption of drugs from the lung is much more rapid than from the intestinal tract, it may be possible to utilize the lungs as a site of drug administration for therapeutic agents that are poorly absorbed from the gastrointestinal tract.

An interesting feature of the present study is the nonlinear, semilogarithmic plots obtained (Fig. 1). In agreement with these findings, Schanker *et al.*[13] found that digoxin and digitoxin showed multiexponential absorption kinetics. They suggested that one or more of the following factors may be responsible for the secondary phase of slower absorption: 1) reversible binding of drugs to macromolecules in lung tissue or in the pulmonary tree; 2) absorption rates might be different depending on the region of pulmonary tissue involved; or 3) restricted diffusion of drugs within the pulmonary tree owing to entrapment in fluids with high viscosity. However, these possibilities were not examined in the present study.

To confirm in more detail the fate of these drugs after administration into the lung, we measured plasma levels of the drugs (Fig. 2). As the plasma level of these drugs reached the maximum within 30 s, it is thought that the lung mucosa may afford a useful route of administration for systemically acting drugs, provided that pathologic and histological damage to the lung mucosa does not occur. In particular, the pulmonary route may be suitable for the administration of endogenous substances which have a short biological half-life and are effective at a small dose, for example, calcitonin[14] and prostaglandin E.[15]

The present investigations demonstrated that there was no direct relationship between pulmonary absorption of theophylline and body weight or lung wet weight of rats, and that the percentage absorption remained nearly constant (Figs. 3 and 4). From these findings, it seems possible that the total absorption from the lung may be independent of both the body weight and the lung wet weight of rats, since theophylline may be absorbed rapidly from a part of the lung before diffusing throughout the whole lung. Drug administration *via* this route might be very favorable in clinical therapy because the absorption rate is independent of factors such as body weight and lung wet weight, because the absorption rates of drugs did not vary from individual to individual, and because effective plasma concentrations of drugs can be maintained easily.

To examine the effect of dose on the rate of absorption, these compounds were administered over a wide range of initial dose (Fig. 5). The percent absorbed was not affected by the dose, suggesting that the amount of drug absorbed would be proportional to the dose.

In order to examine the effect of pH on the pulmonary absorption, the pH value of administered drug solution was varied between 6.4 and 9.4. The absorption rates of theophylline, aminophylline and caffeine were unchanged at pH 6.4, 7.4 and 8.4, but at pH 9.4, the absorption rates of theophylline and aminophylline decreased markedly, though that of caffeine did not (Fig. 6). With respect to theophylline and aminophylline, Trochta *et al.*[16] have reported that both drugs have a pK_a value of about 8.8. Thus, the absorption rates of both drugs at pH 9.4 may have decreased significantly because both drugs exist in almost wholly nonionized form at pH 6.4, 7.4 and 8.4, but almost wholly ionized form at pH 9.4. In contrast, caffeine, having pK_a 0.8,[17] exists in a nonionized form at pH 9.4. Therefore, these results suggest that the lung mucosa may be preferentially permeable to the nonionized form of drugs, according to the pH-partition hypothesis.[18] Thus, the pH value of a dosage form must be carefully adjusted.

To examine the effect of ionic components on the absorption, Krebs–Ringer phosphate solution and isotonic sodium borate solution of pH 8.4 were also studied, and no difference in

absorption was found. From these results, it may be possible to conclude that ionic components did not effect on the absorption of drug through pulmonary mucosa, contrary to our previous findings in the case of intestinal absorption.[19]

On comparing the absorption rates with the lipid/water partition coefficients of these drugs, since both theophylline and aminophylline have a pK_a value of about 8.8,[16] the absorption rates of both drugs at pH 9.4 decreased together with the decrease of the partition coefficients, while, in the case of caffeine, neither the partition coefficient nor the absorption rate decreased at any of the pH values used in the present study (Table I). Moreover, caffeine, having 86- to 101-fold (pH 7.4) greater partition coefficient than theophylline and aminophylline, was absorbed more rapidly than the latter two drugs. These results indicate that the absorption of xanthine derivatives from the lung may depend on their lipid solubilities.

To clarify the effect of osmolality of the solution administered on pulmonary absorption, hypotonic, isotonic and hypertonic theophylline solutions were administered to the rat lung (Table II). The results obtained suggest that the absorption from the lung mucosa is not affected by the tonicity on the drug solution. Kitazawa *et al.*[19] found that the results obtained for intestinal absorption were quite different from those for pulmonary absorption. Namely, the intestinal drug absorption increased with decreasing tonicity of the perfusate. This difference may be related to the unique physiological function of the lung. However, further investigations are required.

In conclusion, the present study has provided some basic data for the development of dosage forms suitable for pulmonary administration.

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Effect of Ionic Interaction on the Entrapping of Drug into Porous Microspheres and Drug Release Characteristics

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Polymer-coated porous microspheres containing salicylic acid as a model drug were prepared by graft copolymerization by a pre-irradiation method using gamma rays. Methyl methacrylate (MMA), dimethylaminoethyl methacrylate (DM), and diethylaminoethyl methacrylate (DE) were used as monomers. Irradiated spheres were brought in contact with monomer solution containing the drug. The amount of salicylic acid entrapped increased linearly with increasing extent of graft polymerization of DM-MMA and DE-MMA. The amount of salicylic acid released was proportional to the square root of time, and diffusion of salicylic acid through solution-filled pores in the matrix was the rate-limiting step. The diffusion rate constant of salicylic acid increased with increasing content of amino ester in the graft copolymer.

Keywords——salicylic acid release; porous microsphere; graft polymerization; pre-irradiation; gamma ray; basic vinyl monomer; methyl methacrylate; dimethylaminoethyl methacrylate; diethylaminoethyl methacrylate

Much research has been done on means to minimize harmful side effects and to maintain drug levels in a therapeutically desirable range by employing porous structures as an inert matrix to provide controlled drug release.[1~3] We have studied the employment of porous microspheres in the preparation of capsules containing a drug, and the drug release profiles from such porous microspheres. In previous papers,[4,5] it was found that the amount of drug entrapped in porous microspheres was increased significantly by graft polymerization of methyl methacrylate into the microspheres, although the original microspheres had no ability to absorb the drug. Furthermore, pretreatment of the sphere surfaces and/or the internal parts of spheres with metal oxide before irradiation led to an increase in the amount of drug entrapped in the spheres and a decrease of the drug release rate. It was considered that interaction between the substrate and the drug was an important factor in entrapment of the drug.

In this report, polymer-coated porous microspheres containing salicylic acid as a model drug were prepared by graft polymerization of methyl methacrylate (MMA) and MMA containing amino esters such as dimethylaminoethyl methacrylate (DM) and diethylaminoethyl methacrylate (DE) by a pre-irradiation method. The effects of adding amino esters on the entrapment of salicylic acid into porous microspheres and on the release profile of salicylic acid are discussed.

Experimental

Materials——Commercial grade cellulofine GC-700m, composed mainly of cellulose (Chisso Corporation;

particle size distribution, 45—105 μm; molecular weight in elimination limit, 70000) was used as porous microspheres. Salicylic acid was of guaranteed reagent grade (Kanto Chemical Co., Inc). The monomers of MMA, DM, and DE were of commercial reagent grade, and were used without further purification.

Preparation of Modified Microspheres by Graft Polymerization——Polymer-coated porous microspheres containing salicylic acid were prepared by graft polymerization by a pre-irradiation method using gamma ray from Co-60 as described in a previous paper,[4] to prevent degradation of salicylic acid.

Microspheres (0.5 g) were pre-irradiated under a pressure of 10^{-4} mmHg at -78 °C. The irradiated spheres were then brought into contact with monomer solution containing 150 mg of salicylic acid (1.89 g of monomer, 0.25 ml of ethanol, 0.5 ml of water) *in vacuo* and kept for a specified time at 40 °C. The concentration of amino ester in monomer solutions was 3 or 10 mol%. The latter concentration was equal to the initial amount of salicylic acid used. The spheres were washed five times with 20 ml of 50% methanol solution to eliminate excess salicylic acid and monomer, then dried to constant weight in a vacuum oven at 25 °C.

The weight increase of spheres (W) was defined as follows:

$$W(\%)=(W_g-W_0)\times 100/W_0 \quad (1)$$

where W_0 and W_g are the weights of dry spheres before and after graft polymerization, respectively. Furthermore, the degree of grafting (G) was defined as follows:

$$G(\%)=(W_g-W_0-W_d)\times 100/W_0 \quad (2)$$

where W_d is the amount of salicylic acid entrapped in the modified spheres.

About 0.2 g of dry spheres was accurately weighed out into a 200-mesh stainless steel basket, and kept in pH 1.2 hydrochloric acid solution for two weeks at 37°C. Then the spheres were wiped with filter paper to remove the excess acid solution from the surface, and weighed quickly. The weight swelling ratio (S) was defined as follows:

$$S=(W_0+W_w)/W_0 \quad (3)$$

where W_w is the weight of hydrochloric acid solution absorbed by the spheres during two weeks.

The dissolution test of salicylic acid from modified spheres was carried out using a Shimazu double-beam spectrophotometer, model UV-240. About 3 mg of dry spheres was put in a basket equipped with a magnetic stirring bar and the basket was placed into an ultraviolet cell containing 3 ml of pH 1.2 hydrochloric acid solution at 37 °C. The amount of salicylic acid released from the spheres was determined by measuring the absorbance at 299.6 nm of the dissolution medium at appropriate time intervals.

The sizes of both dry and wet particles were read from microphotographs, and the mean weight diameter values were calculated from approximately 300 spheres. A wet sphere was one which had been saturated with hydrochloric acid solution for two weeks.

Results and Discussion

Graft Polymerization of MMA Containing Basic Monomer

Monomer solutions were absorbed almost completely into spheres upon contact with irradiated spheres and graft polymerization was initiated immediately. On the other hand, unirradiated spheres absorbed monomer solution only slightly. Graft polymerization only occurred in the presence of 50% ethanol solution. These facts suggested that graft polymerization proceeded inside the spheres.

Figure 1 shows that the weight of graft-polymerized DM–MMA and DE–MMA spheres increased with increasing reaction time. The degree of weight increase decreased with increasing content of DM or DE in the comonomer.

The variations of particle sizes of graft-polymerized DM–MMA and DE–MMA spheres are shown in Fig. 2. In the dry state, the sizes of modified spheres were larger than that of the original spheres, and increased with increasing particle weight. Morever, the particle size increased with increasing content of amino ester in the comonomer. Graft copolymerization may proceed among the cellulose molecular chains in the spheres, causing distortion of the arrangement of cellulose chains and a consequent increment of particle size.

The densities of graft copolymers formed should not be very different, because the densities of poly-MMA, poly-DM, and poly-DE are 1.08, 1.15, and 1.14, respectively.[6] DM–MMA copolymer obtained by copolymerization in the absence of spheres dissolved in water

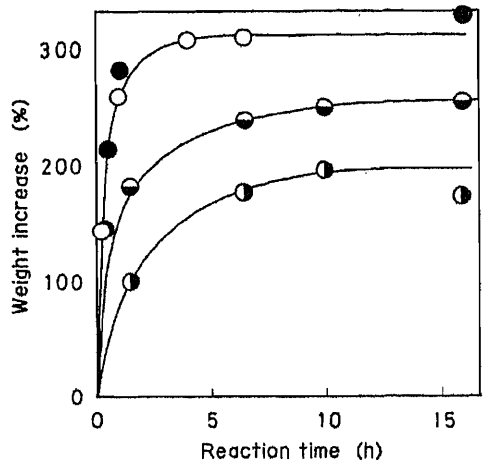


Fig. 1. Weight Increase of Porous Spheres Owing to Graft Polymerization of MMA Containing Basic Monomers

○, MMA; ◑, DM-MMA (molar ratio 1:9); ●, DM-MMA (molar ratio 3:97); ◒, DE-MMA (molar ratio 1:9).

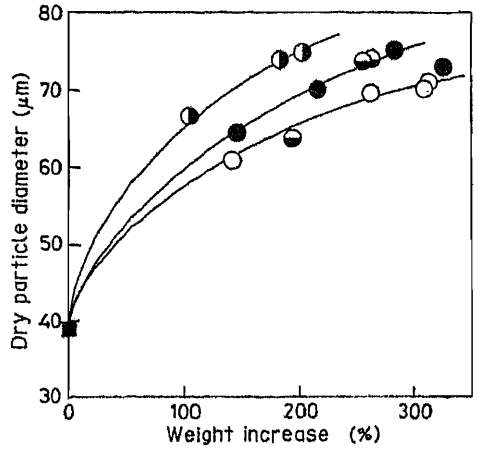


Fig. 2. Variations of Particle Size Owing to Graft Polymerization of MMA Containing Basic Monomers

○, MMA; ◑, DM-MMA (molar ratio 1:9); ●, DM-MMA (molar ratio 3:97); ◒, DE-MMA (molar ratio 1:9); ■, original sphere.

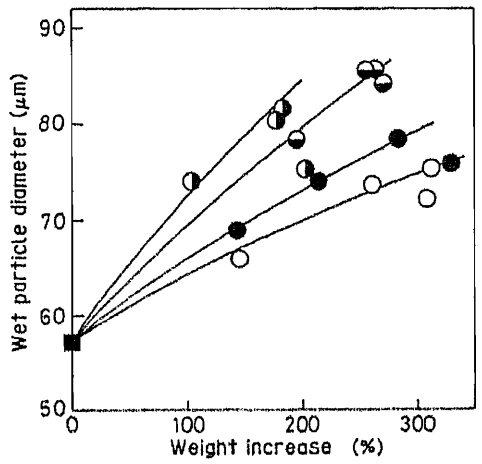


Fig. 3. Relationship between Wet Particle Size and Weight Increase

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

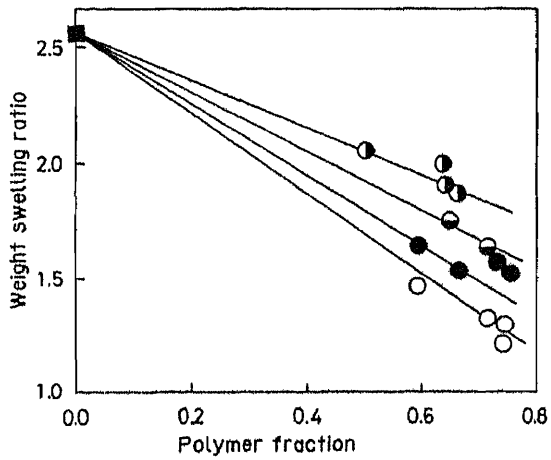


Fig. 4. Effect of the Polymer Fraction on Weight Swelling Ratio of Modified Spheres

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

when the DM content in copolymer exceeded 20%. This suggests that the extension of the graft copolymer chains increased with increasing amount of DM. The differences of particle size at various values of monomer component content and monomer ratio may be due to different degrees of extension of the graft copolymer chains in the spheres in 50% ethanol solution.

The sizes of modified spheres in the wet state are also shown in Fig. 3. In all cases, the particle sizes in the wet state were larger than those in the dry state. Similar results were also obtained as the content of amino ester in the comonomer was increased.

Figure 4 shows the relationship between the weight swelling ratio of modified spheres in pH 1.2 hydrochloric acid solution and polymer fraction, where the polymer fraction represents the fractional weight of graft copolymer in modified spheres. The weight swelling ratio decreased linearly with increasing polymer fraction and the ratio became larger with increasing content of amino ester in the comonomer at a given polymer fraction. Furthermore, the weight swelling ratio of spheres of graft DM-MMA was bigger than that of

DE-MMA at the same polymer fraction. These results clearly indicate that both the wet particle size and the weight swelling ratio of modified spheres are dependent on the extension of graft copolymer chains in the hydrochloric acid solution.

Maximum Amount of Drug Entrapped in Porous Microspheres

Figure 5 shows the effect of dissolution media on the release of salicylic acid from spheres modified with MMA, where the weight increase of the spheres was about 300%. The maximum amount of released salicylic acid in pH 1.2 hydrochloric acid solution was about 70% of that in 50% methanol solution, and the dissolution time became about 30 times that in 50% methanol solution. When methanol was used as the dissolution medium, the maximum amount of salicylic acid released was the same as in 50% methanol solution. The solubility values of salicylic acid in methanol and hydrochloric acid solution were 40 g/100 g and 0.4 g/100 g, respectively. This indicates that salicylic acid entrapped in modified spheres is dissolved completely in the dissolution medium. The maximum amount of salicylic acid entrapped in spheres modified with MMA was determined by measuring the maximum amount of salicylic acid released in 50% methanol solution.

Figure 6 shows the effect of dissolution media on the release of salicylic acid from spheres modified with MMA containing 10 mol% DM, where the weight increase of the spheres is about 200%. The maximum amount of released salicylic acid in pH 1.2 hydrochloric acid solution was greater than that in 50% methanol solution. The dissolution time in the former case was within 4 h. A similar tendency was observed in the dissolution test of spheres modified with DE-MMA. Furthermore, the maximum amount of salicylic acid released from spheres modified with DM-MMA was not affected by the pH of the dissolution medium, because the maximum amount of salicylic acid released in 0.1 N sodium hydroxide solution was the same as that in hydrochloric acid solution. These results suggest that salicylic acid is entrapped in modified spheres as a salt, and can be dissolved in the water. The maximum amount of salicylic acid entrapped in modified spheres was determined by measuring the maximum amount of salicylic acid released in pH 1.2 hydrochloric acid solution.

Figure 7 shows the effect of grafting on the amount of salicylic acid entrapped in spheres. The amount of salicylic acid entrapped increased exponentially in the case of MMA and

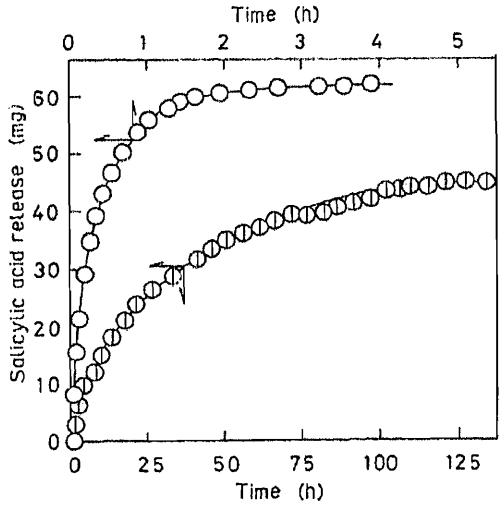


Fig. 5. Effect of Dissolution Media on the Amount of Salicylic Acid Released from Spheres Modified with MMA

○, 50% methanol solution; ①, hydrochloric acid solution at pH 1.2.

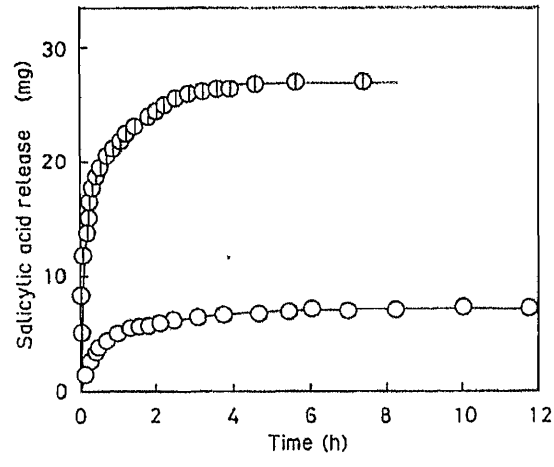


Fig. 6. Effect of Dissolution Media on the Amount of Salicylic Acid Released from Spheres Modified with DM-MMA

○, 50% methanol solution; ①, hydrochloric acid solution at pH 1.2.

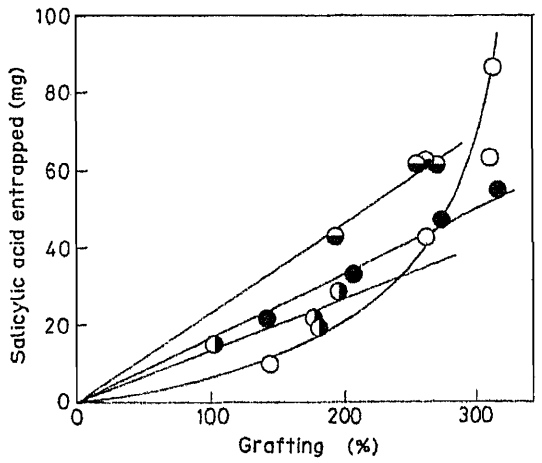


Fig. 7. Effect of Grafting of Basic Monomers on Salicylic Acid Entrapment in Spheres

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

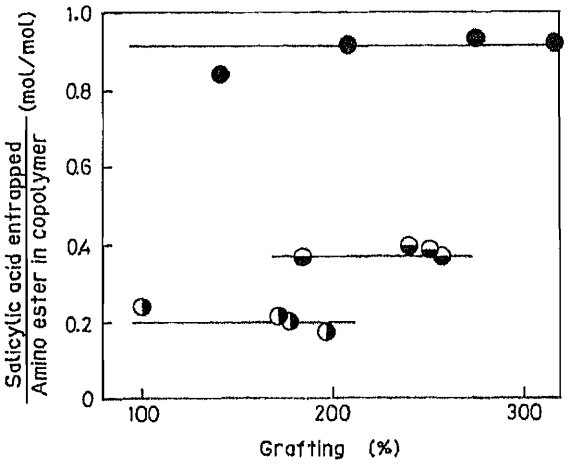


Fig. 8. Amount of Salicylic Acid Entrapped per Unit of Basic Group

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

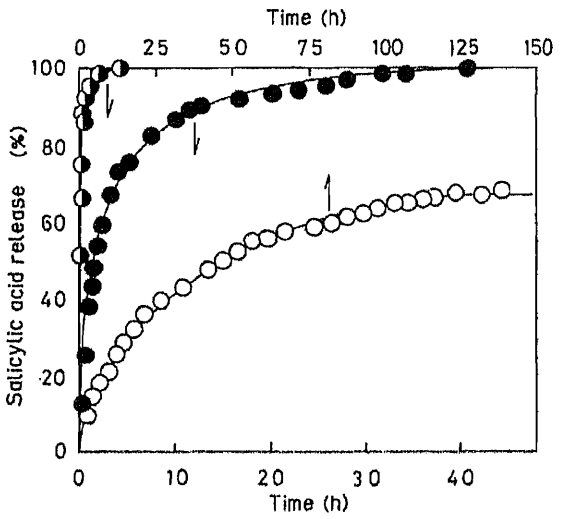


Fig. 9. Release Profiles of Salicylic Acid from Spheres Modified with DM-MMA

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

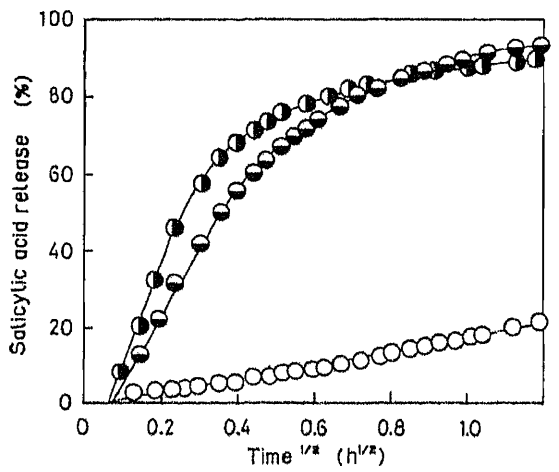


Fig. 10. Relationship between the Amount of Salicylic Acid Released and the Square Root of Dissolution Time

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

linearly for the other monomer components. DM-MMA entrapped less salicylic acid than DE-MMA and the amount decreased with increasing content of amino ester in the comonomer.

The molar ratio of salicylic acid entrapped by amino ester groups in graft copolymers is plotted against degree of grafting in Fig. 8. The variation of copolymer composition with degree of grafting was calculated by the method proposed by Skeist,[7] and developed by Spinner *et al.*[8] and Meyer and Lowry.[9] The component ratio in graft copolymers did not vary significantly with degree of grafting. In each case, the molar ratio was independent of the degree of grafting but dependent upon the monomer components and component ratio. For a given component ratio of 1 : 9, the molar raio of DE-MMA is higher than that of DM-MMA, whereas the molar ratio of DM-MMA decreased with increasing content of DM in the graft copolymer. The fact that the molar ratio was independent of the degree of grafting suggests

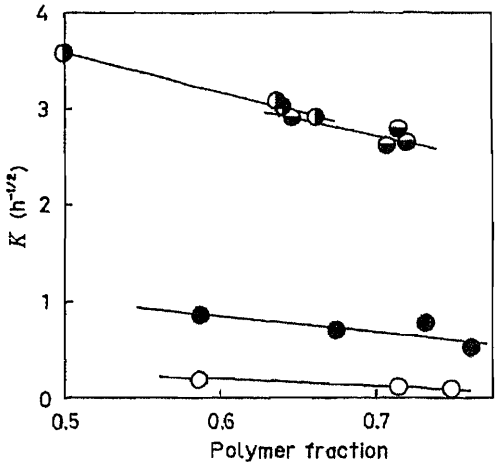


Fig. 11. Dependency of K on the Polymer Fraction in Modified Spheres

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

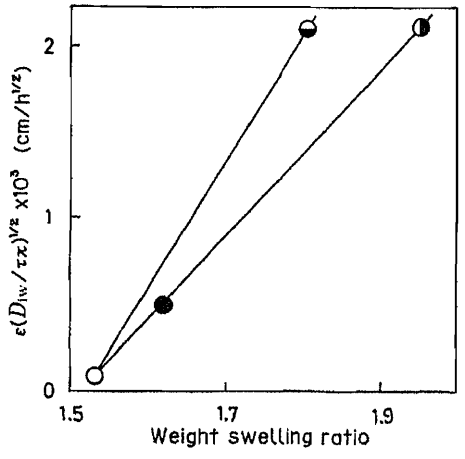


Fig. 12. Relationship between Diffusion Coefficient of Salicylic Acid from Spheres and Weight Swelling Ratio

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

that salicylic acid was entrapped in the modified spheres as a salt. The pK_b values of DM and DE are 6.04 and 5.34, respectively.[6] This means that the degree of dissociation of DM is greater than that of DE. The difference in the amount entrapped in modified spheres between DM and DE is due to the different degrees of dissociation of these components.

The extension of the graft copolymer chains in 50% ethanol solution increased with increasing DM content in the copolymer as described above. It is therefore suggested that the degree of extension of the graft copolymer chain affects the degree of dissociation of salts. Both the extension and the dissociation of graft copolymer chains are important for entrapping the drug.

In Fig. 9, release profiles of salicylic acid in pH 1.2 hydrochloric solution are plotted; the weight increase of the spheres with grafting of MMA is about 300%, that of spheres with MMA containing 3 mol% DM is about 330%, and that of spheres with MMA containing 10 mol% DM is about 200%. The amount released from spheres with grafting of MMA increased with time, and reached a saturated value at 140 h; this was about 60% of the maximum amount of entrapped salicylic acid. On the other hand, the amount released increased with the addition of DM to MMA (3:97), and maximum release occurred within 40 h. Salicylic acid could be released within 4 h by further increasing the content of DM in the graft copolymer. From this figure, it is clear that the dissolution time can be adjusted by controlling the DM content in the comonomer.

Elution of Salicylic Acid from Modified Spheres

The drug was dispersed in the matrix and diffusion occurred through solution-filled pores in the matrix. The amount of salicylic acid released can be written as follows;

$$C/C_0=(6\varepsilon/r)(D_{iw}/\tau\pi)^{1/2}t^{1/2} \quad (4)$$

where C is the amount of drug released at any given time t, C_0 is the initial amount of drug in the spheres, r is the radius of the wet particle, ε is the porosity of the matrix, τ is the tortuosity of the matrix, and D_{iw} is the diffusion coefficient of the drug in the solution.[10,11] The value of C/C_0 is plotted against the square root of time in Fig. 10, where the weight increase of the spheres with grafting of MMA is about 150%, that of the spheres with DM–MMA is about 180%, and that of the spheres with DE–MMA is about 190%. A linear relationship was

obtained within a certain time range and then gradually changed to a saturated curve as shown in Fig. 10.

The K values obtained from the relationship between C/C_0 and the square root of time are plotted as a polymer fraction in Fig. 11; K represents the release rate constant of salicylic acid from the modified spheres. The K values decreased with increasing polymer fraction, and thus the release rate of salicylic acid was suppressed with increasing polymer fraction. The K values increased with increasing amino ester content in the graft copolymer.

As expressed in Eq. 4, the diffusion rate constant depends on the radius of the wet particle, the porosity and the tortuosity. The porosity can be approximately expressed as the weight swelling ratio.

The value of $\varepsilon(D_{iw}/\tau\pi)^{1/2}$ determined by using the K values in Fig. 11 and wet particle sizes in Fig. 3 is plotted as a function of the weight swelling ratio in Fig. 12, where each value is obtained at a polymer fraction of 0.6. Figure 12 shows that the porosity increased with increasing content of amino ester in the graft copolymer, and the diffusion of salicylic acid through the pores filled with solution occurs more easily with increasing porosity.

The diffusion rate constant $\varepsilon(D_{iw}/\tau\pi)^{1/2}$ in spheres modified with DE–MMA was greater than that in spheres modified with DM–MMA taken at the same swelling ratio. The difference between DE and DM may be due to different tortuosity of the pores. This is probably due to the difference in the extension of graft copolymer chains, as described above.

Conclusion

Polymer-coated porous microspheres containing salicylic acid were prepared by graft polymerization by a pre-irradiation method. The amount of salicylic acid entrapped in porous microspheres was significantly increased by such grafting. When DM or DE was added to MMA, the amount of salicylic acid entrapped in modified spheres linearly increased with the degree of grafting. Salicylic acid was entrapped as a salt. Increasing amino ester content led to an increase in particle size and a decrease in the amount of entrapped salicylic acid. The amount of salicylic acid released was proportional to the square root of time, and diffusion of salicylic acid through solution-filled pores in the matrix was rate-limiting. The diffusion rate coefficient increased with increase in the weight swelling ratio of modified spheres. It was concluded that the extension of graft copolymer chains in solution was very important factor affecting the entrapment and the release rate of drug in the case of porous microspheres.

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Antimalarial Agents. III.[1] Mechanism of Action of Artesunate against *Plasmodium berghei* Infection

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The antimalarial agent, artesunate has been observed to suppress the infection growth rate of *Plasmodium berghei* in CF_1 mouse red blood cells. The inhibition of growth was due to a number of biochemical mechanisms. Deoxyribonucleic acid (DNA) synthesis in the *Plasmodium berghei* was inhibited significantly by the drug with marginal inhibition of protein synthesis. The inhibition of DNA synthesis seemed to be due to a block of purine synthesis at the regulatory enzyme site of inosine monophosphate (IMP) dehydrogenase. Whereas there was no evidence to demonstrate that the drug alkylated nucleotide bases or caused cross linking of DNA strands of the plasmodium, the drug did appear to cause non-specific binding to bases that interfered with template activity. There was marked inhibition of messenger ribonucleic acid (mRNA) polymerase activity with moderate inhibition of DNA polymerase activity. The drug caused significant reduction of basal oxygen consumption of *Plasmodium berghei* with a significant inhibition of succinic acid dehydrogenase activity. The drug interacted with reduced nicotinamide adenine dinucleotide (NADH), suggesting the possibility that artesunate may interfere with energy required for cell growth.

Keywords——artesunate; antimalarial agent; *Plasmodium berghei*; DNA synthesis; inhibitor; oxidative phosphorylation suppressor

Introduction

The Chinese herb *Artemisia annua* which is a member of the Compositae family, contains an active principle, Arteannuin (qinghaosu, QHS, artemisinie) which has been shown to have antimalarial activity *in vivo*.[2,3] A number of its derivatives, *e.g.* dihydroqinghaosu (DHQ), artemether, and α-propoxycarboxydihydroartemisinin (SM242) have been synthesized and also investigated for antimalarial activity in the United States and Britain.[4,5] Qinghaosu is relatively insoluble in biological aqueous medium; however, the agent artesunate which has been used in the People's Republic of China to treat malaria has been reported to not only be soluble in an aqueous sodium, but more potent than DHQ.[2,3] Artesunate has been shown to be active against chloroquine-resistant and -sensitive strains of *Plasmodium berghei*,[2] *Plasmodium knowlesi*,[2] and *Plasmodium falciparum*[4] as well as cerebral malaria infections.

Thus we selected artensunate as a proto-type drug of the qinghaosu chemical class in order to study its *in vitro* metabolic effects on *Plasmodium berghei*. Infected red blood cells (RBC) from CF_1 mice were used since this rodent species acted as a suitable host for the plasmodium. These results are reported at this time.

Experimental

Source of Drug——Artesunate was obtained as a white crystalline powder in ampules of 100 and 500 mg quantities from the Pharmaceutical Factory of Guilin, Guangxi, People's Republic of China and the drug is reported to be greater than 99% pure.

Preparation of Drug——The artesunate (1 mM stock solution) was prepared in 1% carboxymethylcellulose–H_2O by homogenization followed by sterilization by passage through Gelman acrodisc (0.45 μm) filter assemblies. The control contained sterile 1% carboxymethylcellulose–H_2O as vehicle for all of the assays.

***In Vitro* Antimalarial Activity**——*Plasmodium berghei* infections which were maintained RBC of CF_1 mice (*ca.* 30 g) were used as a source of the parasite organism. The CF_1 male mice (*ca.* 30 g) were bled from the eye orbital to collect 1 ml of infected RBC inocula. Infected blood was centrifuged, the white blood cells and platelets were removed and the RBC were washed twice in isotonic phosphosaline buffer, pH 7.4. The infected RBC were incubated with isolated uninfected normal RBC from mice and incubated in RMPI 1640, 25 mM HEPES, 32 mM $NaHCO_3$ and 10% heat inactivated (40 min at 60 °C) mouse plasma so that the percentage of infection was 0.8% and the hematocrit was 2%.[6,7] Serial dilutions of the drug at 10^{-4}—10^{-7} M (final concentration) were added to the tubes which were flushed with N_2 gas, capped and placed in a CO_2 (humidified) incubator at 37 °C.

At 24, 48, 72 and 96 h, aliquots were removed and assessed for percent parasitemia with *Plasmodium berghei*. One drop of the aliquots was smeared on a microscopic slide, fixed in 100% methyl alcohol and dried. Giemsa stain solution (1 : 10 KH_2PO_4, pH 7.2) was dropped on the slide and stained for 45—60 min. The slides were washed in water and dried. The percent parasitemia was estimated by counting the number of infected RBC per approximately 1000 RBC under oil immersion.

***Plasmodium berghei* Infected Mouse RBC**——CF_1 male mice (*ca.* 30 g) were injected intraperitoneally with 0.3 ml isotonic saline suspension containing 5×10^6 infected RBC. After 7 d the parasitemia reached 20%. These RBC cells were used for the biochemical *in vitro* studies. An uninfected CF_1 mouse RBC preparation was also prepared and assayed. The incorporation and enzymatic values obtained for this normal RBC preparation were subtracted from the infected RBC preparation to obtain the activity of the *P. berghei*, itself.

Deoxyribonucleic Acid (DNA) Ribonucleic Acid (RNA) and Protein Incorporation Studies——^{3}H-Thymidine (21.5 Ci/mmol), [5,6-^{3}H]-uridine (22.4 Ci/mmol) or [4,5-^{3}H]-leucine (52 Ci/mmol) (1 μCi) was incubated with 10^4 infected RBC, suspended in RMPI 1640 medium, 25 mM HEPES, 32 mM $NaHCO_3$ in a total volume of 1 ml for 4 h in a CO_2 incubator at 37 °C.[8] The reactions were terminated for the nucleic acid synthesis with 10% perchloric acid containing 1% sodium pyrophosphate and the protein synthesis with 10% trichloroacetic acid. Acid insoluble DNA, RNA, and protein were collected on GF/F glass fiber disks, nitrocellulose Millipore filters, and Whatman No. 1 filters, respectively, by vacuum suction. The filters were dried, placed in scintillation vials with 8 ml of scintillation fluid (toluene, Triton-X 100, 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)) and counted in a Packard Beta Scintillation Counter. After corrected for quenching, results are expressed as dpm.

Polymerase Activity——DNA polymerase activity was determined by the method of Sawada *et al.*[9] except that [methyl-^{3}H]-deoxythymidine triphosphate (82.4 Ci/mmol) (1 μCi) was used with 5×10^5 infected cells. The perchloric acid insoluble nucleic acid was collected on Whatman GF/F filters and counted.

Messenger ribonucleic acid (mRNA) polymerase activity was determined by the method of Anderson *et al.*[9] using ^{3}H-uridine triphosphate (23.2 Ci/mmol) (2 μCi) and 5×10^5 cells. The perchloric acid insoluble precipitate was collected on nitrocellulose filter and counted.

DNA Binding Studies——DNA (1 mg/1 ml) was prepared in isotonic saline and incubated with varying concentrations of drug and scanned spectrophotometrically from 190 to 340 nm. Scans of the DNA, DNA + drug and drug alone were compared for hyperchromic shifts of the DNA absorption.[10] Artesunate was also incubated with pure guanosine monophosphate (GMP) for 24 h at room temperature and nuclear magnetic resonance (NMR) scans were compared for chemical interaction.

DNA Strand Scission——Drug (10^{-4} M) was added to 5×10^5 infected RBC cultures in RMPI medium with 10 μCi ^{3}H-thymidine (21.5 Ci/mmol)) and incubated 24 h at 37 °C in a CO_2 incubator. Cells were harvested by centrifugation and washed twice in isotonic phosphosaline buffer, pH 7.4. The cells were resuspended in 1 ml of the same buffer and an aliquot (0.2 ml) was loaded onto 35 ml of 1—10% alkaline sucrose gradients overlaid with 1.0 ml of lysis buffer [0.5 M NaOH, 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.01% Triton X-100 and 2.5% sucrose]. The gradients were centrifuged at 7500 for 16 h at 25 °C in a SW-28 rotor. Fractions (2 ml) were removed from the top of the gradient, acidified with 500 μl of 6 N HCl and counted.[12-14]

DNA Viscosity Studies——Viscosity studies were conducted using a No. 50 Cannon Ubbelohde Viscometer, according to the method of Cohen and Eisenberg.[15] Calf thymus DNA (Sigma) was further purified by treatment with ribonuclease (RNase) (bovine pancreas) and deproteinized in isoamyl alcohol–chloroform by the method of Marmur.[16] The purified DNA was dissolved in saline–citrate buffer adjusted to a concentration of 1 mg/ml and sonicated with an E/MC Ultrasonic Cleaner.[15] For the viscosity determinations, 50 μg of DNA (154 μM) in saline–citrate buffer was incubated with drug at 50, 100 or 200 μM final concentration in a total volume of 10 ml for 24 h at 37 °C. The flow time was recorded from the viscometer and the intrinsic viscosities were reported according to Cohen and Eisenberg.[17]

DNA Thermal Denaturation Studies——The melting point of purified double-stranded calf thymus DNA[14,15] was determined by the method of Britten *et al.*[18] and Mandel and Marmur.[19] The DNA (50 μg/ml) was placed in 10 ml of saline citrate buffer with 50, 100 or 200 μM drug for 60 min at 25 °C. This solution was diluted 1 : 1 with the buffer and placed in the center well of the jacketed cell which had water circulating through it from a Hawk

circulating water bath with a Nesland Programmer TP-2 temperature regulator. The initial temperature in the outer well was 30 °C. Gradually the temperature was increased and aliquots (1 ml) of the medium were analyzed for ultraviolet (UV) absorption at 260 nm. Samples were taken for every 4 °C change in temperature until 100 °C was achieved. The absorption of the DNA was plotted against the temperature to determine the melting point (T_m value)[18] of DNA.

Formate Incorporation into Purines——(^{14}C)-Formate (52.0 Ci/mmol) incorporation into the purine of 5×10^5 *P. berghei* RBC was determined by the method of Spassova *et al.*[20] Purines were separated on silica gel thin layer chromatography (TLC) plates eluted with *n*-butanol–acetic acid–water (4 : 1 : 5). Areas appropriate with adenine and guanine standards were scraped and counted. Formate incorporation into DNA was determined by collecting the perchloric acid insoluble labeled DNA on GF/F filters and counted.

Phosphoribosyl Pyrophosphate (PRPP) Amido Transferase Activity——The spectrophotometric method of Wyngaarden and Ashton[21] was used to measure PRPP amido transferase activity of 5×10^5 *P. berghei* cells by measuring the reduction of nicotinamide adenine dinucleotide (NAD) at 340 nm over a 30 min period using 5×10^5 *P. berghei* cells.

Inosine Monophosphate (IMP) Dehydrogenase Activity——This enzyme activity was determined with ^{14}C-IMP (61 μCi/mmol). IMP was separated from xanthosine monophosphate (XMP) by plating on PEI plates and eluting with 0.5 M $(NH_4)_2SO_4$.[22] By using a XMP standard, the appropriate area was scraped and counted.

Carbamoyl Phosphate Synthetase——The spectrophotometric method of Kelman *et al.*[23] was utilized in the presence of ornithine and ornithine transcarbamoylase. The citrulline in the assay formed was measured spectrophotometrically by the method of Archibald.[24]

Aspartate Transcarbamoylase Activity——The method of Kelman *et al.*[23] was used. The concentration of carbamoyl aspartate generated was determined by the colorimetric method of Koritz and Cohen.[25]

Orotidine Monophosphate (OMP) Decarboxylase Activity——The method of Appel[26] with ^{14}C-OMP (34.9 mCi/mmol) was utilized to assess OMP decarboxylase activity. The $^{14}CO_2$ generated was trapped in the center well of Warburg flasks in Hyamine hydroxide and an aliquot was counted.

Thymidylate Synthetase Activity——Enzyme activity was determined using [5-^{3}H]-deoxyuridine monophosphate (11 μCi/mmol) by the method of Kampf *et al.*[27] The reaction medium was incubated with activated charcoal, and filtered; the ^3H_2O filtrates were counted.

Ribonucleotide Reductase Activity——This enzyme activity was determined by the method of Moore and Hurlbert[28] using ^{3}H-cytidine-5′-diphosphate (25 mCi/mmol). After termination of the reaction, the medium was incubated with alkaline phosphatase enzyme to release inorganic phosphate from the nucleotides. Ribose and deoxyribose nucleotides were separated on PEI plates eluted with 4% boric acid–4 M LiCl (4 : 3) and the area at the *Rf* corresponding to the standard deoxycitidine diphosphate (dCDP) was scraped and counted.

Thymidine Kinase and Thymidine Monophosphate (TMP) Kinase——These activities were measured, respectively with ^{3}H-thymidine and ^{3}H-TMP using the medium described by Maley and Ochoa.[29] PEI TLC was used to separated the thymidine from TMP and TMP from thymidine diphosphate (TDP) by eluting with 0.5 M formic acid and 0.6 M LiCl (1 : 1). Using standards, appropriate areas were scraped and counted.

Type of Protein Synthesis Inhibition——In order to distinguish the type of protein synthesis inhibition by artesunate, a kinetic study for a 14 min period was performed in order to compare the pattern of inhibition with known standards, *i.e.*, pyrocatechol violet for an initiation inhibitor and emetine for an elongation inhibitor.[30]

Time Course Studies——Enzyme studies were performed for DNA, RNA and protein synthesis and IMP dehydrogenase activity by incubating for varying times, 2, 3 and 4 h, at the drug concentration which afforded the ID_{50} for the specific assay.

Concentration Dependent Studies——The individual enzyme assays were conducted at 0.25, 0.5, and 1.00×10^{-4} M concentration of drug.

Protein Content——The protein concentration of the cellular fraction used in each of the enzyme tests was determined by the Lowry *et al.* technique.[31]

Respiration Rates——Oxygen consumption of infected RBC with *Plasmodium berghei* and normal RBC were conducted using a Gilson oxygraph with an oxygen electrode.[32] The reaction vessel contained 55 μM of sucrose, 22 μM of potassium chloride, 22 μM of dibasic potassium phosphate, 90 μM of succinate or 60 μM of α-ketoglutamate, 2 μM of adenosine triphosphate (ATP), 3 μM artesunate and RBC (0.2 ml) in a total volume of 1.8 ml.

Succinic Dehydrogenase Activity——The activitiy was determined by the spectrophotometric method of Cooperstein *et al.*[33] using 0.2 ml of infected RBC.

NAD, Reduced Nicotinamide Adenine Dinucleotide (NADH), Cytochrome c and Cytochrome Oxidase UV Binding Studies with Artesunate——These studies were carried out by incubating 10^{-4} M drug with a 1 mM solution of NAD at 200—290 nm, NADH at 290—380 nm, cytochrome c at 450—650 nm and cytochrome oxidase at 220—280 nm to determine if the drug affects the absorption of these components. The absorption was measured with the Bausch & Lomb spectronic 1001.

Results

In vitro cytotoxic assays demonstrated that artesunate effectively inhibited the percentage infection of *Plasmodium berghei* of mouse RBC *i.e.* the increase number of RBC containing plasmodium (Fig. 1). A concentration of 10^{-4}, 10^{-5} and 10^{-6} M caused cell lysis over a 3-d incubation period, while a concentration of 10^{-7} M markedly reduced the growth of the plasmodium but did not lyse the cells in 3 d. Artesunate inhibited DNA, RNA and protein synthesis of *Plasmodium berghei*. DNA synthesis was preferentially inhibited with an ID_{50} of 1.4×10^{-4} M concentration of artesunate incubated for 1 h (Table I). DNA synthesis was suppressed by artesunate greater than 50% after 2 h incubation; protein synthesis was inhibited approximately 40% and RNA synthesis was suppressed approximately 28% at 10^{-4} M concentration of drug (Fig. 2). Formate incorporation into purine followed a similar pattern with an ID_{50} value of 1.35×10^{-4} M concentration for 1 h. The inhibition in *de novo* purine synthesis did not appear to be at the first regulatory site of the pathway, PRPP amido transferase, but rather the pathway was inhibited at the second regulating site, IMP dehydrogenase (Fig. 3). The ID_{50} for IMP dehydrogenase inhibition for 1 h was 2.1×10^{-5} M concentration of drug. Pyrimidine synthesis did not appear to be inhibited since the activity of rate limiting enzymes carbamoyl-phosphate synthetase, aspartate transcarbamoylase, or OMP decarboxylase were unaffected by artesunate's presence (Table I). The suppression of the ^{14}C-carbamoyl-phosphate incorporation into DNA could be accounted for by the observed inhibition in purines also required for the DNA synthetic process. No significant inhibition by the drug was evident for thymidylate synthetase, thymidine kinase or TMP kinase enzymatic activities (Table I). Ribonucleotide reductase activity was not significantly inhibited in the presence of artesunate suggesting no supression of the conversion of ribonucleotides to deoxyribonucleotides.

DNA polymerase and mRNA polymerase activities were suppressed in *Plasmodium berghei* by 24% reduction of the former and 50% reduction of the latter at 10^{-4} M concentration of drug (Table I). These data suggest that DNA may be a target of the drug. Analysis of UV spectra demonstrated binding of the drug to calf thymus DNA with a hyperchromic shift to a lower wave length. Binding from 10–30 μM concentration reached a maximum at 25 μM concentration of drug and then the magnitude of the binding reversed itself (Fig. 7). NMR studies demonstrated no direct chemical interaction between pure deoxyguanosine monophosphate (dGMP) and the drug which is probably indicative of no

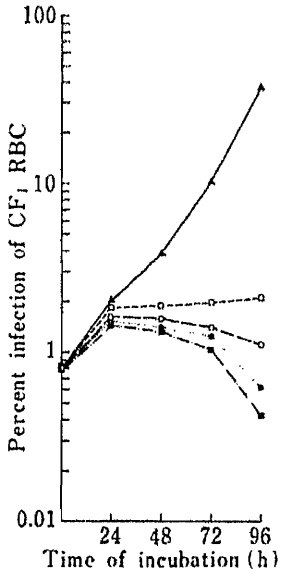


Fig. 1. Effects of Artesunate on *Plasmodium berghei* Infection Rate of CF_1 Mice RBC in Tissue Culture

CF_1 mice RBC with an infection rate of 0.8% with *Plasmodium berghei* were incubated with non-infected mice RBC in RMPI 1640, 25 mM HEPES, 32 mM sodium bicarbonate and 10% heat inactivated mouse plasma in a 5% carbon dioxide humidified incubator at 37°C. Aliquots were removed at 24, 48, 72, and 96 h stained and assessed for the infection rate at 0 (▲——▲), 10^{-4} M (■- - -■), 10^{-5} M (●------●), 10^{-6} M (○——○), 10^{-7} M (□——□) concentration of drug.

TABLE I. The Effects of Artesunate on the Activity of Enzyme Involved in *de novo* Synthesis of Nucleic Acid after 60 min Incubation

	Percent control ($\bar{X}\pm$S.D.)			
	Control	0.25×10^{-4} M	0.5×10^{-4} M	1.0×10^{-4} M
^{3}H-Thymidine incorporation into DNA	$100\pm5^{a)}$	93 ± 5	87 ± 6	$58\pm3^{r)}$
^{3}H-Uridine incorporation into RNA	$100\pm6^{b)}$	101 ± 6	91 ± 4	85 ± 5
^{3}H-Leucine incorporation into protein	$100\pm5^{c)}$	90 ± 3	81 ± 5	$72\pm4^{r)}$
^{14}C-Formate incorporation into DNA	$100\pm4^{d)}$	$72\pm3^{r)}$	65 ± 6	$60\pm3^{r)}$
^{14}C-Carbamoyl phosphate incorporation into DNA	$100\pm4^{e)}$	—	—	$69\pm4^{r)}$
^{14}C-Formate incorporation into purines	$100\pm5^{f)}$	84 ± 4	$74\pm4^{r)}$	$62\pm3^{r)}$
DNA polymerase	$100\pm4^{g)}$	91 ± 4	85 ± 3	$76\pm4^{r)}$
mRNA polymerase	$100\pm3^{h)}$	82 ± 5	$76\pm3^{r)}$	$50\pm1^{r)}$
Thymidine kinase	$100\pm8^{i)}$	—	—	81 ± 7
TMP kinase	$100\pm7^{j)}$	—	—	98 ± 6
Thymidylate synthetase	$100\pm3^{k)}$	103 ± 7	106 ± 8	104 ± 7
Carbamoyl phosphate synthetase	$100\pm4^{l)}$	98 ± 3	103 ± 5	105 ± 5
Aspartate transcarbamoylase	$100\pm6^{m)}$	102 ± 5	109 ± 6	117 ± 7
OMP decarboxylase	$100\pm5^{n)}$	97 ± 3	98 ± 4	96 ± 5
PRPP amido transferase	$100\pm4^{o)}$	—	—	120 ± 5
IMP dehydrogenase	$100\pm4^{p)}$	$48\pm5^{r)}$	$18\pm4^{r)}$	$15\pm2^{r)}$
Ribonucleotide reductase	$100\pm5^{q)}$	—	—	93 ± 6

a) dpm/10^4 infection RBC. *b*) 1001892 dpm/10^4 infected RBC. *c*) 13545 dpm/10^4 infected RBC. *d*) 3380 dpm/5×10^5 infected RBC. *e*) 1192 dpm/5×10^5 infected RBC. *f*) 1716 dpm/5×10^5 infected RBC. *g*) 16518 dpm/5×10^5 infected RBC. *h*) 58306 dpm/5×10^5 infected RBC. *i*) 106560 dpm/5×10^5 infected RBC. *j*) 119100 dpm/5×10^5 infected RBC. *k*) 174507 dpm/5×10^5 infected RBC. *l*) 0.855 optical density unit/5×10^5 RBC. *m*) 0.637 optical density unit/5×10^5 RBC. *n*) 15611 dpm/5×10^5 infected RBC. *o*) 0.102 optical density units/min/5×10^5 infected RBC. *p*) 141052 dpm/5×10^5 infected cells. *q*) 10304 dpm/5×10^5 infected RBC. *r*) $p\leq0.001$.

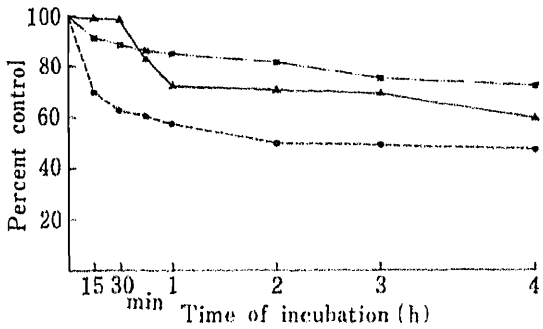


Fig. 2. The Effects of Artesunate on DNA, RNA, and Protein Synthesis of CF_1 Mice RBC Infected with *Plasmodium berghei*

CF_1 mice RBC infected with *Plasmodium berghei* (0.8%) were incubated in RMPI 1640, HEPES and sodium bicarbonate and 1 μCi of radiolabeled isotope for 4 h at 37 C and 10^{-4} M drug concentration to determine the thymidine incorporation (●——●), uridine incorporation (■——■), leucine incorporation (▲——▲), at 0.25, 0.5, 0.75, 1, 2, 3, and 4 h.

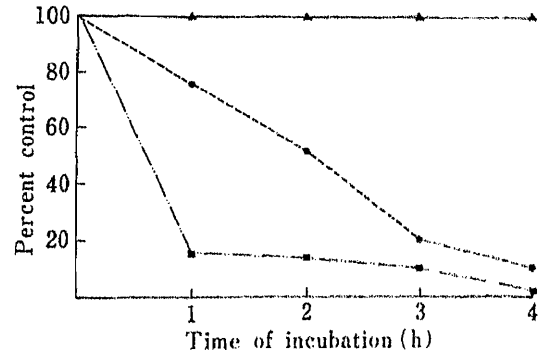


Fig. 3. The Effects of Artesunate on IMP Dehydrogenase Activity of *Plasmodium berghei* Infected CF_1 Mice RBC

Incubations of 5×10^5 infected CF_1 mice RBC with radiolabeled IMP and artesunate at 0, control (▲——▲), 2.1×10^{-5} M (■——■), 1.0×10^{-6} M (●——●), concentration. Aliquots of the reaction medium were plated on PEI plates and eluted with 0.5 M ammonium sulfate. The spot corresponding to the standard XMP was scraped and counted.

direct alkylation of the bases by the drug in the concentration range of 10—30 μM. Studies with *Plasmodium berghei* incubating with drug for 24 h showed that the drug did not cause cross linking of DNA strands of *Plasmodium berghei* but did result in DNA strand scission at 10^{-4} M concentration (Fig. 4). These studies were repeated twice affording similar

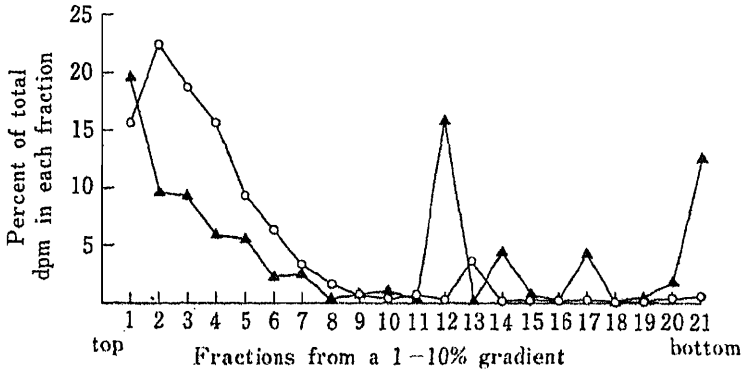


Fig. 4. The Effects of Artesunate on DNA Strand Scission of *Plasmodium berghei* Infected CF_1 Mice RBC ($N=3$)

Incubations with 5×10^5 infected mice RBC, and radiolabeled thymidine in RMPI 1640 medium at 37 C for 24 h with drug at 0, control (▲—▲), 10^{-4} M (○—○) concentration drug. Fractions from the 1—10% alkaline sucrose gradient from top to bottom of the gradient were counted for radioactivity. The presence of artesunate caused a shift of the labeled DNA to the top of the tube to fraction 2 indicating lower molecular weight DNA compared to the control with its major peak at fraction 12.

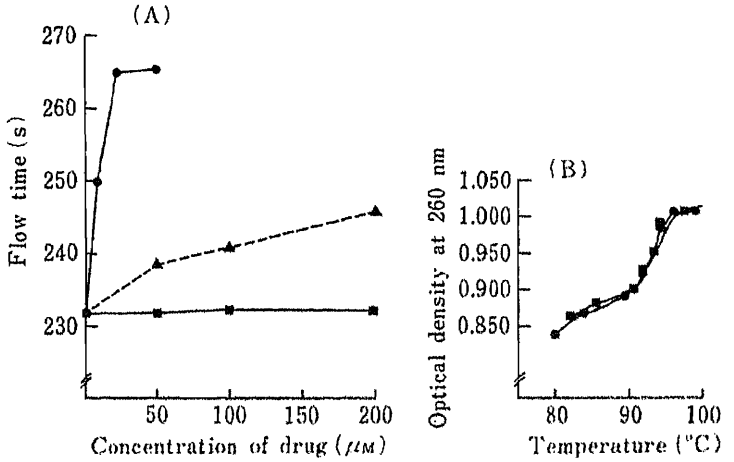


Fig. 5. The Effects of Artesunate on Calf Thymus DNA Viscosity and Thermal Denaturation

(A) Viscosity: After 24 h incubation of purified calf thymus DNA with phosphate saline buffer, (control) (■—■), artesunate from 0–200 μM (▲—▲), or chloroquine at 50 μM (standard) (●—●), the DNA viscosity was determined.

(B) Thermal denaturation: After 60 min incubation of purified calf thymus DNA with phosphate saline buffer (control) (■—■), artesunate at 200 μM (●—●), the thermal denaturation was determined.

results. DNA viscosity and DNA thermal denaturation studies demonstrated only minimal binding of the drug to DNA; however, the binding did not appear to be by cross linking of the two strands (Fig. 5).

Protein synthesis was also suppressed by artesunate; however, this inhibition was not of the same magnitude as the observed DNA synthesis inhibition and probably only accounted for moderate inhibition of plasmodium growth. The effects on protein synthesis appeared to be afforded by the drug at the initiation steps of the protein synthesis process since the pattern of inhibition resembles that of pyrocatechol violet more so than emetine, as reference standards (Fig. 6).

Infected RBC with *P. berghei* demonstrated a much higher rate of basal respiration than non-infected RBC normal cells (results not shown). Artesunate at 3×10^{-6} M concentration

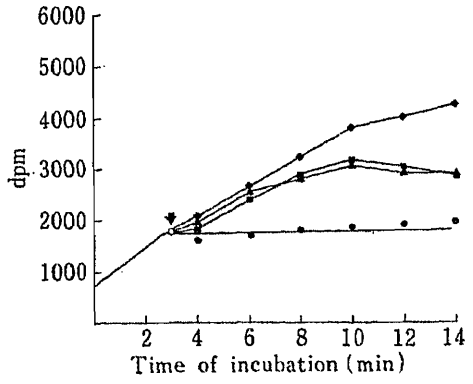


Fig. 6. The Effects of Artesunate on Protein Synthesis of *Plasmodium berghei* Infected CF_1 Mice RBC

The leucine incorporation into protein was measured for 14 min with 5×10^5 infected mice RBC for the control (◆—◆). At 3 min artesunate at 10^{-4} M (▲—▲), pyrocatechol violet 100 μM (■—■), or emetine 100 μM (●—●), concentration was added. Since emetine is an elongation protein synthesis inhibotor, leucine incorporation stops immediately; however, with pyrocatechol violet an initiation protein synthesis inhibitor there is a lag in time before leucine incorporation is retarded because the drug has to wait until the next round of protein synthesis. The pattern of protein synthesis afforded by artesunate mimics that of pyrocatechol violet rather than the pattern of emetine.

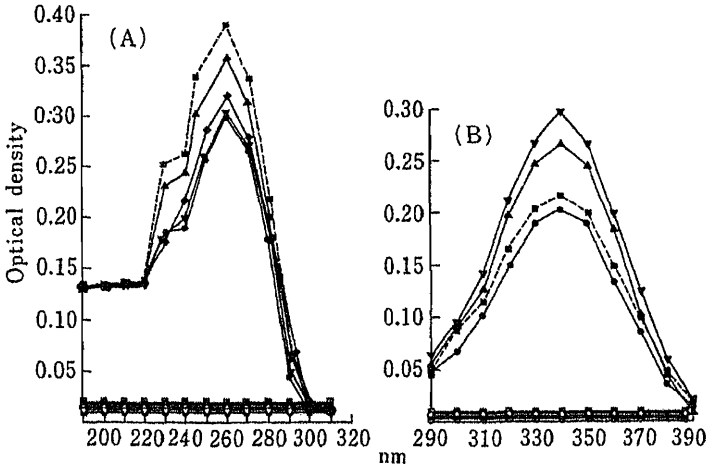


Fig. 7. The UV Binding of Artesunate to Calf Thymus DNA (A) and NADH (B)

(A) Artesunate was incubated with purified calf thymus DNA at 0 (●—●), 10 μM (▼—▼), 20 μM (◆—◆), 25 μM (■---■), 30 μM (▲—▲) concentration of drug for 1 h at 37 °C and the UV absorption (190—310 nm) was measured. The open figures represent the drug's absorption alone at the identical concentration as described above.

(B) Artesunate was also incubated with NADH for 1 h at 37 °C with 0 (●—●), 10 μM (■---■), 20 μM (▲—▲), 30 μM (▼—▼), concentration of drug. The UV absorption was measured from 290—390 nm.

caused a 63% reduction of the basal respiration rate when sodium succinate was used as a substrate and a 67% reduction when α-ketoglutarate was used as a substrate (Table II). Succinic dehydrogenase activity of *Plasmodium berghei* was suppressed 95% at 3×10^{-6} M concentration of drug (Table II). UV spectra studies demonstrated no binding of artesunate to cytochrome oxidase, cytochrome c, NAD or nicotinamide adenine dinucleotide phosphate (NADP); however the drug did preferentially interact to NADH which demonstrated a concentration dependent hyperchronic shift from 10—30 μM (Fig. 7).

Discussion

Artesunate proved to be an effective antimalarial agent against the growth of *Plasmodium berghei in vitro* using CF_1 mouse RBC as the host cells. This observation is consistent with previous observations that artesunate is active against the infection of *P. berghei in vivo* in mice.[34] The drug was observed to have a safe therapeutic index.[34] Artesunate is a sesquiterpene lactone containing a peroxide function, a moiety which

TABLE II. The Effects of Artesunate on the Oxygen Consumption and Succinic Acid Dehydrogenase Activity of *Plasmodium berghei* Infected RBC from CF_1 Mice

($N=8$)	Basal oxygen consumption		Succinic dehydrogenase activity
	Succinate	α-Ketoglutarate	
Control	100[a] ± 4	100[b] ± 3	100[c] ± 6
Artesunate 3×10^{-6} M	37 ± 2[d]	33 ± 3[d]	5 ± 2[d]

a) 8.726 μl O_2 consumed/h/ml infected RBC. *b*) 5.743 μl O_2 consumed/h/μg infected RBC. *c*) 0.102 ΔO.D. changes/3 min/5×10^5 cells. *d*) $p \leq 0.001$.

supposedly is responsible for its antimalarial activity. When the peroxide function is destroyed the compound loses its pharmacological activity.[3,35] Known sesquiterpene lactones, *e.g.* the antitumor agent helenalin, have been reported to suppress DNA and protein synthesis in P-388 lymphocytic leukemia cells *via* suppression of IMP dehydrogenase,[36] DNA polymerase,[36] eIF-3[37] and eIF-2 kinase[38] activities. Furthermore, helenalin in tumor cells has been shown to inhibit the activity of dehydrogenases involved in anaerobic and aerobic glycolysis[39] as well as the basal respiration of these cells.[32] Examination of artesunate effects on *P. berghei* cells has shown that DNA synthesis is preferentially inhibited over RNA and protein synthesis of the plasmodium. The DNA synthesis inhibition apparently is afforded through a number of mechanisms. Firstly, *de novo* purine synthesis is inhibited in a parallel manner as the DNA synthesis. The site in the purine pathway which is sensitive to the drug appears to be IMP dehydrogenase which is inhibited in a concentration dependent manner by the drug. This is at a concentration 10 times lower than inhibition of other enzymes involved in DNA synthesis by artesunate. No suppression of the other regulatory site of purine synthesis, *i.e.* PRPP amido transferase, was evident in the presence of the drug (Table I). The suppression by the drug of purine synthesis would account for the observed lowering of DNA synthesis of the plasmodium and may account for inhibition of RNA synthesis since ribonucleotide levels would be reduced also. Since the ribonucleotide pools are much higher than the deoxyribonucleotide pools in most cells,[40] it is not surprising that the effect of artesunate on RNA synthesis was not as marked, *i.e.* 15% inhibition as its effects on DNA synthesis, *i.e*; 42% inhibition after 1 h. Sencondly, the effects of the drug on DNA polymerase activity would account also for DNA synthesis inhibition; however, this did not appear to be of a magnitude to account for the entire observed 42% inhibition of DNA synthesis *Plasmodium berghei* (Table I).

The fact that both DNA and mRNA polymerase activities were suppressed by artesunate may be more closely related to the fact that the drug binds to the DNA molecule *in vitro* and caused what appears to be DNA strand scission. If the template activity of the DNA was interfered with the drug, then inhibition of the polymerase would be apparent and, in fact, usually mRNA polymerase activity is affected more than DNA polymerase activity. Indeed inhibition in mRNA polymerase activity was observed after incubating with artesunate at 10^{-4} M concentration with *P. berghei* (Table I). The drug did not appear to cross link the nucleotide bases of DNA as do other antimalarial drugs, *e.g.* chloroquine, in that no significant changes in viscosity of DNA were observed with artesunate from 0—200 μM; furthermore the drug did not alter the melting point (T_m) of calf thymus DNA *in vitro* at 0—200 μM concentrations (Fig. 5). The binding of the drug to DNA appears to be either nonspecific in nature or only affects the template function of the DNA polymerases.

The inhibition of protein synthesis by artesunate appears to be due to interfering in the initiation events of the protein synthesis process. Whereas no detailed study was performed

here, this data would be consistent with the previous observations[37] that helenalin which contains an α-methylene-γ-lactone moiety, blocks the initiation steps of protein synthesis. However, the inhibition of protein synthesis again does not appear to be of a magnitude to explain the suppressed growth of the plasmodium organism. Although few studies have been performed with qinghaosu derivatives, these findings in *P. berghei* are consistent with those observed in *P. falciparum* where qinghaosu inhibited ^{3}H-hypoxanthine incorporation into DNA[4] and ^{3}H-isoleucine incorporation into protein[41] over 5 h. In the current study artesunate in *P. berghei* cells achieved a higher level of DNA synthesis inhibition in 1 h than qinghaosu did in *P. falciporum*.

Artesunate had significant effects on basal respiration of the *Plasmodium berghei* lowering the oxygen consumption significantly (Table II). This depression of O_2 consumption by the drug was observed with succinic acid, a non-NAD dependent substrate, as well as α-ketoglutarate which is a NAD dependent substrate in the Krebs cycle. Examination of the succinic dehydrogenase activity demonstrated that the drug inhibited its enzymatic activity markedly, which would explain the inhibitory effects by the drug on basal oxygen consumption of *Plasmodium berghei*. This inhibition was at the low concentration of 3×10^{-6} M of drug which would suggest that the lack of oxygen may be a decided factor in the death of the organism.

Spectrophotometric binding studies demonstrated no interaction between drug and NAD, cytochrome c, or cytochrome oxidase, components which are required in the electron chain of mitochondria (Fig. 7). However, the drug did bind to NADH, the reduced form of the cofactor. If such a binding was non-reversible, then the NADH could not be reoxidized to NAD for the use in the electron transport chain and thus oxygen consumption by the cell would be reduced. From a chemical standpoint, one could speculate that the hydride ion of NADH might cleave the peroxide bridge of the artesunate to form a diol which eliminates the NADH from participating in the electron transport chain. Studies with *P. berghei* have shown that there are important mitochondrial changes in the life cycle with alterations in the dehydrogenase enzyme concentration at different stages.[42] Cytochrome oxidase is present at all stages of the cycle. Furthermore, artesunate has been shown by a histochemical technique to lower cytochrome oxidase activity in the plasmodium.[35] The present studies demonstrated that no chemical spectrophotometric interaction with cytochrome oxidase itself had occurred; however, if the electron transport chain was blocked early by the drug then alteration in cytochrome oxidase activity might be possible since it is the final electron acceptor in the electron transport chain.

These studies have demonstrated a number of effects of artesunate on the metabolism of *Plasmodium berghei in vitro*. The inhibition of DNA synthesis would certainly reduce the growth of the organism, whereas the inhibition of respiration observed by the drug may be related more to the reduction of energy processes of cells, thus reducing ATP necessary for synthetic processes. Oxygen lack may also lead to RBC lysis which is observed after treatment with the drug for 96 h.

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Purification of Two Specific Antibodies against Drug and Carrier Protein Molecules

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Affinty purification procedures for antibodies specific to a drug and to a carrier protein were examined in detail with the use of three enzyme immunoassays (EIAs). Many blasticidin S (BLS) molecules were coupled to an affinity ligand with pig serum albumin as a spacer protein. The influence of the spacer on the affinity purification of an antibody specific to BLS was quantitatively analyzed. The antibody showed higher binding to BLS bound to the solid matrix with a carrier protein than without. The stability of specific antibody in six representative eluents, used for affinity chromatography of specific antibodies, was examined, and 0.1 M potassium chloride–0.008 N hydrochloric acid buffer was selected as the preferred eluent based on the stability of the specific antibody and convenience in handling. The ability of the buffer to elute the specific antibody from an affinity column was studied, and was improved by modifying the concentration of potassium chloride in the buffer to 0.3 M. Complete purification of anti-BLS antibody was performed by affinity chromatography under the chosen conditions. The specific anti-BLS and anti-carrier protein antibodies were purified quantitatively. Formation of denatured specific antibody was hardly detected by a sensitive EIA, under the chosen conditions. The purity of the standard anti-BLS antibody was demonstrated by the affinity chromatographic method with the aid of two EIAs for rabbit immunoglobulin G and for BLS.

Keywords——affinity chromatography; affinity ligand; enzyme immunoassay; blasticidin S; drug–protein conjugate; drug-specific antibody

Introduction

Specific antibodies are key reagents in a variety of studies in the biochemical, immunological and pharmacological fields.[1-9] Affinity chromatography is regarded as the best way to isolate specific antibodies from specific antisera.[10-15] Isolation of highly purified specific antibodies is not easy; the elution of a purified specific antibody from its bound form on the affinity column usually involves drastic conditions which may partially inactivate unstable antibodies. The optimum conditions for elution have not been established so far. This may be the reason why a number of enzyme-linked immunosorbent assay (ELISA)[16,17] procedures for specific antibodies use tentatively defined titers or dilutions of the antibody or antiserum as standards. It is difficult to determine accurately the content of an antibody specific to a drug in a specific antiserum, owing to the difficulty in preparing a standard specific antibody.

Previously, we developed a new method for preparing hapten immunogens,[7,8,18-22] with the use of hetero-bifunctional reagents of maleimide succinimidyl ester type.[23-25] Application of the method allowed us to prepare blasticidin S (BLS) and bovine serum albumin (BSA) conjugate as the hapten immunogen.[20] Separation and purification of the two specific antibodies, anti-BLS and anti-BSA, from the rabbit antiserum to BLS were studied quantitatively, with the use of EIAs for BLS,[20] BSA and rabbit immunoglobulin G (IgG).[27] We report here the results of a detailed examination aimed at preparing standard samples of these two specific antibodies.

Materials and Methods

Reagents——Cyanogen bromide-activated Sepharose 4B was bought from Pharmacia Fine Chemicals (Uppsala). *N*-(Gamma-maleimidebutyryloxy)succinimide (GMBS)[26] (Dojin Chemicals, Kumamoto), BLS (Kaken Kagaku Ind., Tokyo) and Amino-Dylark cylinders (6 mm diameter, 4 mm in height) (Sekisui Chem. Ind., Osaka) were commercial products. Rabbit antiserum to BLS,[20] β-galactosidase-labeled BLS (Gal-BLS)[20] and Gal-labeled goat anti-rabbit IgG antibody[27] were prepared by the cited methods. EIAs for rabbit IgG[27] and BLS[20] were performed by the cited methods. All other chemicals were of reagent grade.

Preparation of the Affinity Ligand for Anti-BLS Antibody——The scheme for a three-step synthesis of BLS–GMBS–pig serum albumin (PSA) conjugate is shown in Fig. 1.

Step 1. GMBS-Acylated BLS: A solution of BLS (10 μmol) in 1 ml of 0.05 M sodium phosphate buffer (pH 7.0) was incubated at 30 °C for 30 min with 2.8 mg of GMBS (10 μmol) dissolved in 0.5 ml of tetrahydrofuran under stirring. Tetrahydrofuran was removed by flushing with nitrogen, then excess GMBS was extracted from the reaction mixture three times with 5 ml of methylene chloride. The aqueous layer was immediately used for step 3.

Step 2. Sodium Borohydride Reduction of Disulfide Bonds in PSA: $NaBH_4$ (total 20 mg) and *n*-butanol (0.2 ml) were alternately added portionwise to 10 mg of PSA (0.15 μmol) dissolved in 2 ml of 6 M urea–0.1 M ethylenediaminetetraacetic acid (EDTA). The mixture was incubated at 30 °C for 30 min and then excess $NaBH_4$ was decomposed by adding 1 ml of 0.1 M sodium phosphate (monobasic) and 0.4 ml of acetone. The solution was immediately used for the next step.

Step 3. Conjugation of GMBS-Acylated BLS to the Reduced PSA: The solution of the reduced PSA (step 2) was incubated at 25 °C for 2 h with the aqueous layer of GMBS-acylated BLS (step 1) and the mixture was then loaded onto a 2.5 × 57 cm column of Sephadex G-100 and eluted with 3 M urea.

BSA and BLS–GMBS–PSA conjugate, in the pooled fraction, were coupled with cyanogen bromide activated Sepharose 4B according to the manual of Pharmacia Fine Chemicals. A 3 M urea solution did not disturb the coupling reaction.

Preparation of BLS–GMBS–PSA-Loaded Amino-Dylark Cylinders——Amino-Dylark cylinders were immersed at 25 °C for 1 h in 1% glutaraldehyde under mechanical shaking. The cylinders were washed with 0.01 M phosphate-buffered saline, pH 7.0 (PBS), and then immersed at 25 °C for 20 min in a 0.01% solution of BLS–GMBS–PSA in PBS, and then at 4 °C for 2 h. After successive washing with PBS and buffer A (0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA and 0.1% NaN_3), Amino-Dylark cylinders loaded with BLS–GMBS–PSA were stored in buffer A at 4 °C. BSA- and BLS-loaded Amino-Dylark cylinders were prepared in a similar way.

Isolation of Rabbit Anti-BLS Antibody——Rabbit antiserum to BLS (10 ml) was fractionated with 50% saturated ammonium sulfate. The precipitate was washed with 33% saturated ammonium sulfate solution and dissolved in Tris–HCl buffer, pH 8.5 (4 ml). The electric conductivity was adjusted to 1 mU, and the solution was then dialyzed overnight against the same buffer. The IgG solution was preincubated overnight at room temperature with 1 ml of buffer B (0.06 M sodium phosphate-buffered saline, pH 7.4, containing 0.1% BSA and 0.01 M EDTA). The solution was loaded onto the affinity column (1.5 × 5 cm) of the BLS–GMBS–PSA-coupled Sepharose 4B previously

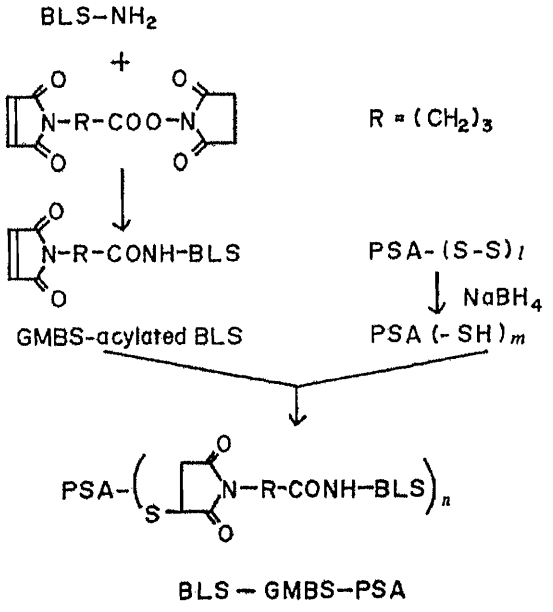


Fig. 1. Scheme for Preparation of BLS–PSA Conjugate as Affinity Ligand for Anti-BLS Antibody

swollen with 0.02 M Tris–HCl buffered saline, pH 8.5. The column was eluted with 100 ml of the same buffer containing 1% BSA, and non-specific IgG and anti-BSA were eluted. BSA was then eluted with 300 ml of 0.05 M sodium phosphate-buffered saline, pH 7.0. The eluent was changed to 0.3 M KCl–0.008 N HCl buffer, pH 2.3. Each fraction was immediately neutralized with 0.1 M glycine–0.1 N NaOH buffer, pH 10.3. IgG content and immune reactivity in each fraction were assayed by means of EIAs for rabbit IgG and BLS.

Results

Preparation of BLS–GMBS–PSA Conjugate

BLS–GMBS–PSA conjugate, with covalent bonds between the amino groups of BLS and the thiol groups of chemically modified PSA, was prepared with the use of the hetero-bifunctional cross-linker GMBS (Fig. 1). BLS–GMBS–PSA conjugate was isolated by chromatography on a Sephadex G-100 column; the elution profiles are shown in Fig. 2.

Effect of a Spacer Protein

The effect of a spacer on the binding of anti-BLS antibody to BLS was studied. The amino group (groups) in free BLS or BLS–GMBS–PSA conjugate was coupled covalently to amino groups of Amino-Dylark cylinders by using glutaraldehyde as the coupling reagent. Binding activities of anti-BLS antiserum in variously diluted solutions to BLS-coupled to Amino-Dylark cylinders with or without the spacer GMBS–PSA were examined. Gal-labeled goat anti-rabbit IgG antibody was used as the tracer, in the presence of an excess amount of BSA to prevent the cross-reactive binding of anti-BSA antibody to PSA (Fig. 3).

Stability Test of Anti-BLS Antibody in Various Eluents

An aliquot of anti-BLS antiserum was incubated at 30 °C for 3 h in 10 solutions, 6 of which are common eluents for affinity chromatography of specific antibodies. Every solution was then diluted 100 times with buffer C. The binding activity of anti-BLS antibody in an aliquot of the diluted solution against BLS–GMBS–PSA-coupled Amino-Dylark cylinders

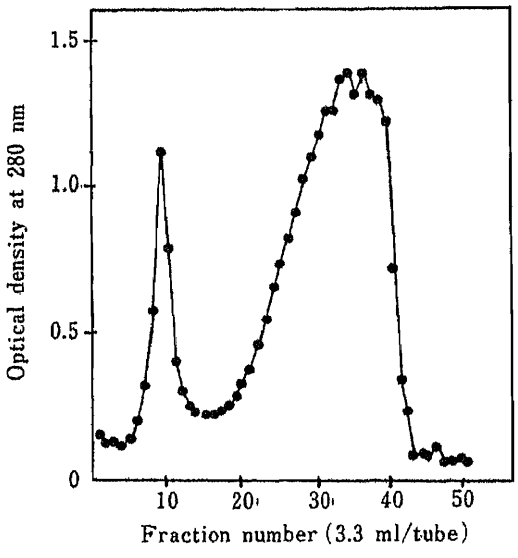


Fig. 2. Elution Profile of BLS–GMBS–PSA Conjugate from a 2.5 × 57 cm Sephadex G-100 Column with 3 M Urea as an Eluent

Closed circles; optical density at 280 nm.

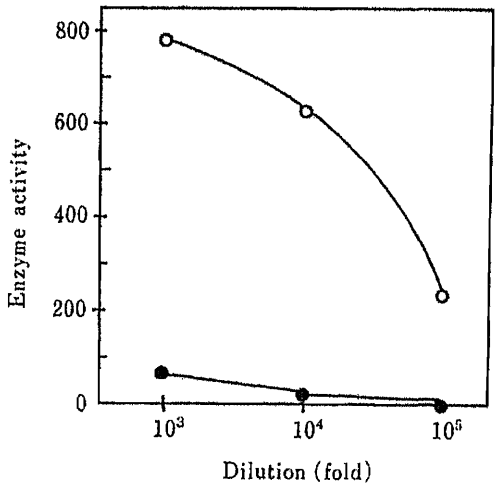


Fig. 3. Effect of Spacer Protein on the Binding of Rabbit Anti-BLS Antiserum to BLS-Linked Amino-Dylark Cylinders

Enzyme activity of the bound Gal-labeled anti-rabbit IgG was plotted against the dilution of rabbit anti-BLS antiserum. BLS–GMBS–PSA- (open circles) and BLS- (closed circles) loaded Amino-Dylark cylinders were used as the solid-phase antigens.

TABLE I. Recovery of Immuno-Reactive Rabbit Anti-BLS Antibody after Incubation in Buffer Solutions at 25 °C for 3 h

Solution	Recovery percent of anti-BLS activity[a)]	Reference No.
Control (buffer C)	100.0± 8.1	
0.2 M Glycine–HCl buffer (pH 2.3) (I)	97.2± 5.4	11
50% Ethylene glycol (II)	91.7±10.6	12
10% Dioxane (III)	89.2± 3.7	12
6 M Gaunidine (IV)	22.4± 3.7	13
4.5 M $MgCl_2$ (V)	99.8±12.3	14
8 M Urea (VI)	87.0± 3.7	29
3.0 M KSCN (VII)	94.7± 8.1	30
0.1 M KCl–0.008 N HCl (pH 2.3) (VIII)	94.0± 8.3	31
10% Dioxane–0.1 M KCl–0.008 N HCl (pH 2.3) (IX)	92.3±12.3	
0.3 M KCl–0.008 N HCl (pH 2.3) (X)	105.9±11.9	

An aliquot of 100-fold diluted anti-BLS antiserum in buffer C (BSA in buffer B was replaced by the corresponding amount of horse serum albumin) was further diluted 100 times with a test solution. After the 3 h incubation the test solution was diluted 100 times with buffer C. Anti-BLS activity in the test solution was measured in terms of binding to BLS–GMBS–PSA coupled Amino-Dylark cylinders using Gal-labeled goat anti-rabbit IgG as the indicator. *a*) Mean±S.D. (number of assays was 5).

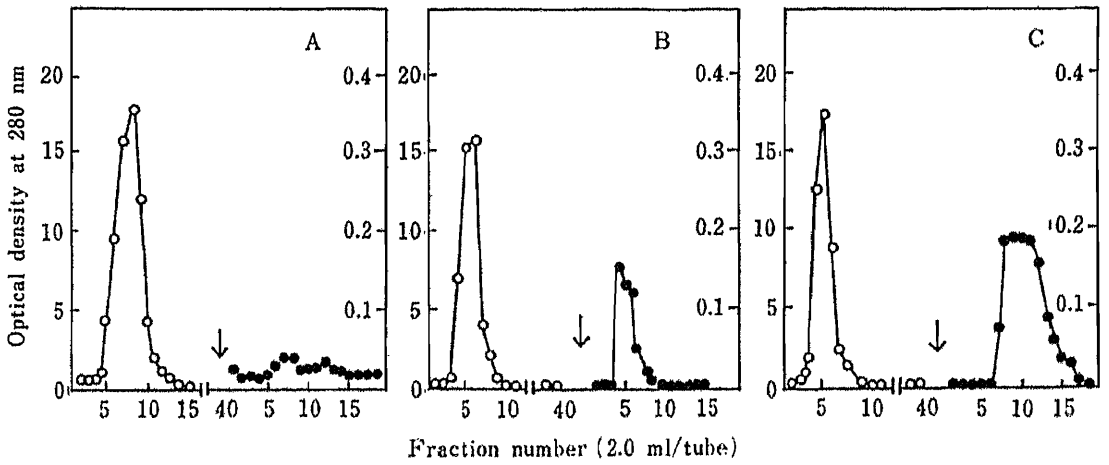


Fig. 4. Elution Profiles of Anti-BSA Antiserum from a 1.5×5 cm BSA-Coupled Sepharose 4B Column

Each column was first eluted with 100 ml of 0.02 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl to remove non-specific IgG. Anti-BSA antibody was then eluted with three different eluents: A, elution profile with 0.2 M glycine–HCl buffer, pH 2.5; B, elution profile with 4.5 M $MgCl_2$, pH 7.0; C, elution profile with 0.1 M KCl–0.008 N HCl buffer, pH 2.3. The OD at 280 nm of each fraction was measured; open circles, the scale for OD values is shown on the left (0—20); closed circles, the scale for OD values is shown on the right (0—0.4).

was measured by using Gal-labeled anti-rabbit IgG antibody as the tracer. The anti-BLS activities remaining after the incubation are summarized in Table I. A marked reduction in the immuno-reactivity of anti-BLS was observed only in 6 M guanidine. Three solutions, 0.2 M glycine–HCl buffer, 4.5 M $MgCl_2$ and 0.1 M KCl–0.008 N HCl buffer, were chosen as candidates for the best eluent, based on the results of the stability test.

Selection of the Eluent for Affinity Chromatography

Anti-BSA antibody contained in anti-BLS–MBS–BSA antiserum was adsorbed on a BSA-coupled Sepharose 4B column. The eluting abilities of the above 3 eluents in affinity

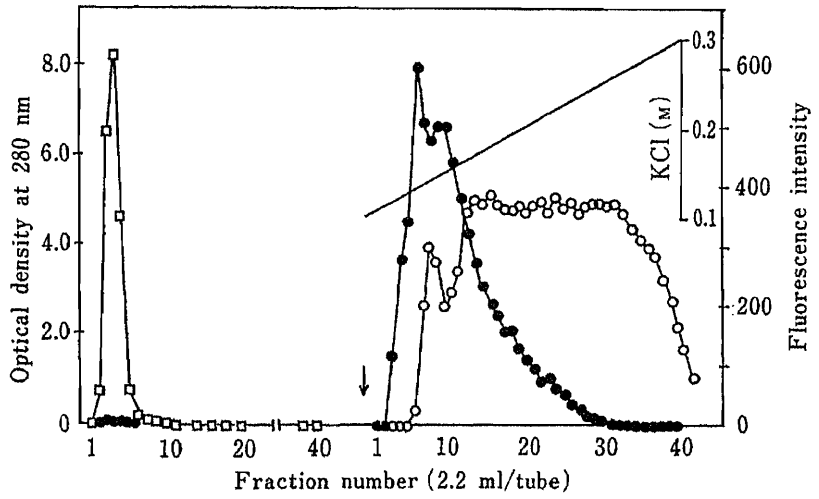


Fig. 5. Elution Profiles of Anti-BSA Antibody from a BSA-Coupled Sepharose 4B Column (1.5 × 5 cm) Using 0.008 N HCl Buffer, pH 2.3, Containing Either 0.1 M KCl (Open Circles) or a Gradient of 0.1 to 0.3 M KCl (Closed Circles) as the Second Eluent

Open squares, optical density at 280 nm; open and closed circles, anti-BSA activities determined by EIA for anti-BSA antibody.

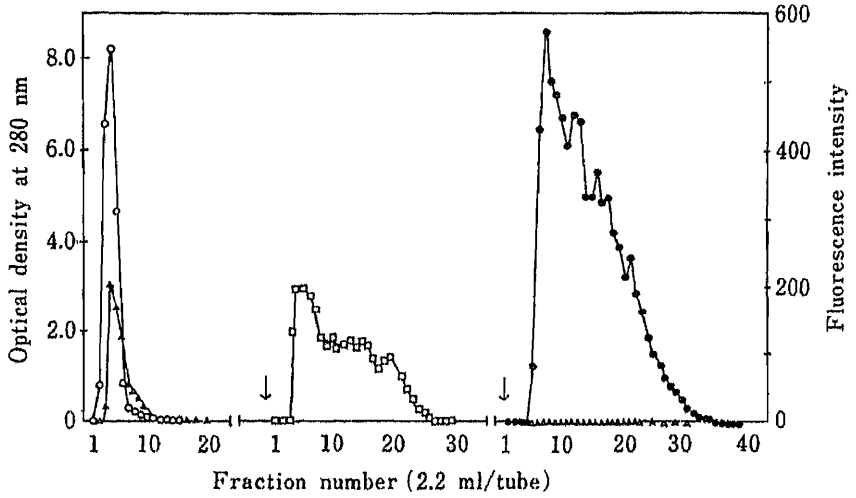


Fig. 6. Elution Profile of Anti-BLS Antiserum from a BLS–GMBS–PSA-Coupled Sepharose 4B Column (1.5 × 5 cm)

The IgG fraction, separated from anti-BLS antiserum by the ammonium sulfate method, was applied to an affinity column. The column was first eluted with 100 ml of 0.02 M Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl and 0.1% BSA to remove non-specific and anti-BSA antibodies. BSA was then eluted with 0.05 M sodium phosphate-buffered saline, pH 7.0 (300 ml). Anti-BLS antibody was eluted with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. The elution profile was measured in terms of OD at 280 nm (open circles), anti-BSA antibody activity (binding activity against the BSA-coupled Amino-Dylark cylinders; open triangles), sandwich EIA for rabbit IgG (open squares) and anti-BLS activity (binding activity against BLS–GMBS–PSA conjugate-coupled Amino-Dylark cylinders; closed circles) using Gal-labeled anti-rabbit IgG antibody as the common indicator.

chromatography of anti-BSA antibody were compared. The elution profiles determined by the optical density measurement at 280 nm are shown in Fig. 4.

Improvement of Eluent for Affinity Chromatography

Anti-BSA antibody, adsorbed on the BSA-coupled Sepharose 4B column, was eluted

TABLE II. Amounts of Rabbit IgG Applied to and Recovered from the BLS–GMBS–PSA-Coupled Sepharose 4B Column at the 1st Affinity Chromatography for Crude IgG Fraction, and at the 2nd One for the Standard Anti-BLS Antibody

	Non-anti-BLS IgG		Anti-BLS antibody	
	Content (mg)	Yield (%)	Content (μg)	Yield (%)
Crude rabbit IgG	108	100		
First purification	100	92.6	408[a]	
Second purification	Not detected[b]		75.0	75.0

Affinity chromatographies were performed by the procedures described in Materials and Methods. Amounts of nonspecific and specific IgGs were determined by sandwich EIA for rabbit IgG. *a*) The content is 0.38% of total IgG. *b*) The detection limit was 0.1 ng/0.1 ml sample solution.

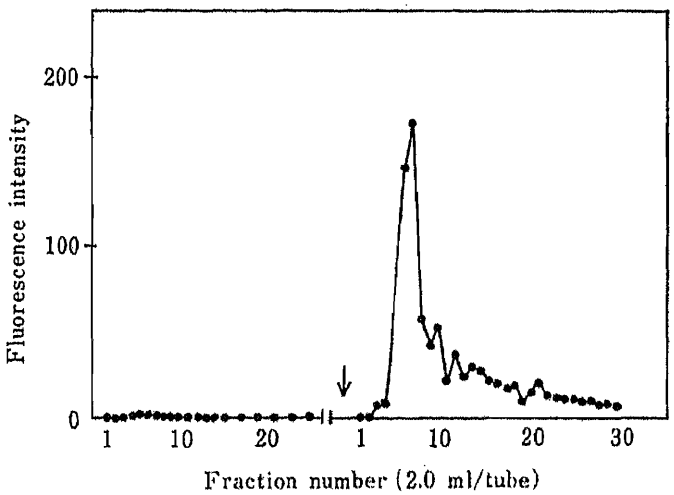


Fig. 7. Elution Profile of the Purified Anti-BLS Antibody on the 2nd Affinity Chromatography Using the Same Affinity Column as in the 1st Purification of the Antibody

The elution conditions were slightly modified since the purified antibody did not contain BSA. The column was first eluted with 100 ml of 0.02 M Tris–HCl-buffered saline, pH 8.5, followed by elution with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. The elution profile was measured by means of sandwich EIA for rabbit IgG.

with a KCl concentration gradient from 0.1 to 0.3 M in 0.008 N HCl buffer, pH 2.3 (Fig. 5). The peak fraction of anti-BSA antibody was at 0.15 M KCl concentration. Consequently, 0.3 M KCl was deduced to be a sufficient concentration for the improved eluent, and a stability test in this solution was carried out (Table I).

Affinity Chromatography of Anti-BLS Antibody

An aliquot of the crude IgG, separated from anti-BLS antiserum by the ammonium sulfate method, was incubated overnight at 25 °C with buffer B which contained a large excess of BSA. The mixture was then applied to affinity column of the BLS–GMBS–PSA-coupled Sepharose 4B. The column was eluted with Tris–HCl buffered saline, pH 8.5, containing 0.1% BSA. Non-specific IgG and anti-BSA antibody bound to BSA were eluted under these conditions. BSA was washed with 0.05 M sodium phosphate-buffered saline, pH 7.0. The bound anti-BLS antibody was eluted with 0.3 M KCl–0.008 N HCl buffer, pH 2.3. Elution profiles in terms of the optical density at 280 nm, and the immune specificities against BSA and BLS-Gal are shown in Fig. 6.

Amounts of non-specific IgG and specific antibody to BLS were determined by sandwich EIA for rabbit IgG (Table II).

Purity of Anti-BLS Antibody

The purity of the isolated anti-BLS antibody was examined by an affinity chromatographic analysis. The purified anti-BLS antibody (100 μg) was chromatographed again on the same affinity column under the same conditions used for purification of the antibody. The elution profiles were followed by means of EIAs for rabbit IgG and BLS. Non-specific IgG was not detected by the sensitive EIA for rabbit IgG (less than 1 ng/ml; Fig. 7, Table II).

Discussion

Affinity purification of antibodies specific to a drug BLS and to the carrier protein BSA was examined in detail with use of a combination of three enzyme immunoassays. The effect of spacer protein on affinity chromatography of an anti-drug antibody was first studied. PSA was chosen as a protein able to bind many BLS molecules.

BLS–GMBS–PSA conjugate was prepared applying the method of Kitagawa *et al.*[18-22] with the use of the heterobifunctional cross-linker, GMBS[26] (Fig. 1). GMBS differs from MBS in the substitution of *n*-butyric acid for benzoic acid, to avoid cross-reaction of anti-BLS antibody with MBS, which was used for preparation of the BLS-immunogen.[20] The BLS–GMBS–PSA conjugate gave a homogeneous band on sodium dodecyl sulfate electrophoresis and the number of BLS molecules coupled per PSA was calculated as 15 based on the molecular weight of the conjugate, determined by the electrophoresis (data not shown; *cf.* references).[20,21]

The amino group of BLS was linked by glutaraldehyde directly or indirectly to amino groups of Amino-Dylark cylinders, with or without the use of the spacer. Gal-babeled anti-rabbit IgG antibody was used as the indicator. There was a large difference between the two preparations (Fig. 3), and BLS–GMBS–PSA conjugate was chosen for further study.

The best eluent for affinity chromatography of anti-BLS antibody was then investigated. The stability of the antibody was examined in 6 representative solutions, commonly used as eluents for affinity chromatography of specific antibodies. A period of 3 h was chosen for the test since most elutions for affinity chromatography would be finished within that time. Anti-BLS antibody was stable in these solutions except for 6 M guanidine (Table I).

The driving force of these solutions in eluting the specific antibody from its bound form would be a reversible change in molecular conformation of IgG. The antibody should revert its active form when the driving force is removed. High salt concentration and acidity of eluents should be effective for conformational change of the specific antibody, as judged from the constituents of the eluents shown in Table I. Eluates containing highly concentrated salts would require dialysis. For acidic eluents, pH adjustment should be enough to recover the native conformation of the eluted antibody.

Three eluents chosen on the base of stability results were then examined for ability to elute anti-BSA antibody bound to the BSA-coupled Sepharose 4B. The 4.5 M $MgCl_2$ and 0.1 M KCl–0.008 N HCl buffers showed good eluting activities (Fig. 4). Improvement of the latter buffer was then attempted. When a KCl concentration gradient from 0.1—0.3 M in 0.008 N HCl solution was applied, the peak fraction of the specific antibody was observed at 0.15 M KCl. Thus, 0.3 M KCl–0.008 N HCl buffer was tested as the eluent and anti-BSA antibody was isolated effectively.

Anti-BLS–MBS–BSA antiserum contains anti-BSA antibody. Complete removal of the specific anti-BSA antibody from anti-BLS antibody is required during the affinity purification of anti-BLS antibody. Anti-BSA antibody in anti-BLS–MBS antiserum was reacted overnight

with a large excess of BSA, so that only anti-BLS antibody would be absorbed on the affinity ligand, BLS–GMBS–PSA-coupled Sepharose 4B. Under these conditions, most anti-BSA antibody was collected in the non-specific IgG fractions and very little anti-BSA antibody was eluted in the fractions obtained soon after those containing the non-specific IgG. After anti-BSA antibody had been eluted completely, the eluent was changed to phosphate buffered saline to wash out BSA completely (Fig. 6).

The bound anti-BLS antibody absorbed on the affinity column was then eluted with 0.3 M KCl–0.008 N HCl solution. No contamination by anti-BSA antibody in the isolated fraction was found in the binding test with the BSA-coupled Amino-Dylark cylinders. Amounts of anti-BLS were determined by sandwich EIA for rabbit IgG.[27]

The purity of the isolated anti-BLS antibody was confirmed by the recovery test in rechromatography on the BLS-loaded affinity column; the antibody contained a negligible amount of non-specific IgG (Fig. 7 and Table II).

Very recently, we have developed a convenient new method for measuring the affinity constants of specific antibodies by the use of EIA.[32] It was found that the binding of anti-BLS to BLS is among the strongest immuno-reactions, judging from its binding constant. The present purification method for specific antibody was successfully applied for several specific antibodies to antigens and haptens (unpublished data). A purified goat anti-rabbit IgG antibody, eluted from an affinity column with 0.3 M–0.008 N HCl buffer and then neutralized with 0.1 N NaOH (the specific IgG content was 1.5 mg/ml), could be stored for more than 1 year at −80 °C. Applications of the purified specific antibodies for enzyme immunoassay and for immunohistochemistry are under way.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Human Urinary Kallikrein. II. Analysis of Asparagine-Linked Oligosaccharides by Using Lectins

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The patterns of micro-heterogeneity in relation to the carbohydrate structures of active- and latent-types of normal human urinary kallikrein (HUK) were analyzed by serial lectin agarose chromatographies (concanavalin A–, erythroagglutinating phytohemagglutinin–, lentil lectin– and wheat germ agglutinin–agarose chromatographies) and crossed affino-immunoelectrophoresis. In the case of active HUK, the species carrying tri- and/or tetra-antennary oligosaccharide(s), core-fucosylated bi-antennary oligosaccharide(s), and bi-antennary oligosaccharides containing outer galactose residues and an *N*-acetylglucosamine residue linked β1,4 to a β-linked mannose residue (bisecting *N*-acetylglucosamine residue) amounted to approximately 36, 33 and 17% of the total active-type HUK, respectively. The micro-heterogeneity of the latent-type HUK found by serial lectin affinity chromatographies was similar to that of the active-type HUK.

Keywords——human urinary kallikrein; active-type; latent-type; micro-heterogeneity; carbohydrate structure; serial lectin agarose chromatography; affino-immunoelectrophoresis

Human urinary kallikrein (HUK) is a glycoenzyme with a molecular weight of approximately 40000[1] and is generally considered to be of renal origin.[2—4] HUK is composed of several micro-heterogeneous forms which can be separated by isoelectricfocusing; these forms show almost identical molecular weights, enzymochemical and immunological properties and so on.[5,6] Therefore, as in the case of micro-heterogeneity of hog pancreatic kallikrein,[7] that of HUK may arise mainly from the sialic acid contents, and the protein structures of the micro-heterogeneous forms of HUK might be identical.

It has recently been reported that changed patterns of micro-heterogeneity and/or structures of sugar chains of glycoproteins such as α-fetoprotein,[8] γ-glutamyltranspeptidase,[9,10] chorionic gonadotrophin[11,12] and thyroglobulin[13,14] were induced with malignant transformation. Therefore, such changes in HUK might also be useful as markers for the diagnosis of renal disease. For example, in the case of Bartter's syndrome, HUK showed the same immunoreactivities as normal HUK against anti-normal HUK antibody, but the electrophoretic mobility of the HUK was slower than that of normal HUK.[6] These preliminary results suggest that in Bartter's syndrome, the transformational change(s) were induced mainly in the carbohydrate moieties of the HUK molecule. For these reasons there has been considerable interest in the study of the carbohydrate structures and/or the micro-heterogeneity of HUK from patients, but it is difficult to deal with multiple and complicated carbohydrate structures. The major impediment is that only very limited amounts of HUK are available, and as a consequence, it is difficult to perform detailed structural studies. To overcome these problems, we have developed a lectin affinity analysis procedure similar to those applied in the analysis of Asn-linked oligosaccharides of glycoprotein by Cummings and Kornfeld,[15] and Cowan *et al.*[16]

This paper deals with the pattern of micro-heterogeneity and the carbohydrate structures of both active- and latent-types of HUKs derived from normal subjects as elucidated by serial lectin affinity chromatographies and crossed affino-immunoelectrophoresis.

Materials and Methods

Materials

Concanavalin A (Con A) and wheat germ agglutinin (WGA) were purified from jack bean meal and wheat germ according to the methods reported by Agrawal and Goldstein,[17] and Vretblad,[18] respectively. Purified Con A and WGA were each coupled to carboxyl-Sepharose 4B by the procedure of Matsumoto *et al.*[19] Erythroagglutinating phytohemagglutinin (E-PHA)-, leukoagglutinating phytohemagglutinin (L-PHA)- and lentil lectin-agarose were purchased from E-Y Laboratories, San Mateo, U.S.A. α-Methyl-D-glucose (α-MeGlc) was from Nakarai Chemicals, Kyoto, Japan. Trypsin (type XII-S) and soybean trypsin inhibitor (type I-S, SBTI) were from Sigma Chemical, St. Louis, U.S.A. H-Prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide (H-Pro-Phe-Arg-MCA) was from the Protein Research Foundation, Osaka, Japan. Anti-HUK antibody was prepared as described previously.[6] Seakem Agarose (ME) was from Marine Colloids, Rockland, U.S.A. Other chemicals were of guaranteed reagent grade.

Methods

Kallikrein Assay——Amidolytic activity of HUK toward H-Pro-Phe-Arg-MCA was determined by a slight modification of the method of Iwanaga *et al.*[20] A mixture of 0.1 ml of sample solution and 1.5 ml of 0.1 mM substrate in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.01 M $CaCl_2$, was incubated at 30 °C for 30 min. Thereafter, the reaction was terminated by addition of 1.4 ml of 0.2 M glycine-HCl buffer, pH 2.7. The generated fluorescence intensity was measured with a spectrofluorometer (380 nm excitation and 460 nm emission). One amidase unit (AU) was defined as the amount of enzyme that could hydrolyze 1 μmol of the substrate per min under the conditions described above.

The amount of active HUK was determined as differential activity in the absence and presence of anti-HUK antibody. After activation with trypsin,[21] differential activity in the absence and presence of the antibody was measured as total (active plus latent) HUK activity, and the latent HUK activity was defined as differential activity between total and active HUK.

Purification of HUKs——Highly purified active HUK was prepared according to our previously described method[22] with minor modifications from crude urinary protein fractions of normal men, kindly supplied by Dr. S. Morichi of Green Cross Corp., Osaka, Japan. The purified enzyme was analyzed by electrophoresis. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate was performed in 10% (w/v) gel at pH 7.0, for 6 h at 2 mA/tube. The gels were stained for protein with Coomassie Brilliant Blue R-250 or for sugar with periodate-Schiff reagent.[23]

Individual samples of normal HUK (HUK-Na, -Nb, -Nc and -Nd) were partially purified from fresh urine (each 5–7 l) of normal subjects (Na, Nb, Nc and Nd, respectively) by the following steps, *i.e.*, DEAE-Sephadex A-50 adsorption and elution (batch method) after dialysis against tap water and ammonium sulfate precipitation (80% saturation precipitate). The precipitate was dissolved in deionized water and dialyzed against TBS (0.01 M Tris, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% (w/v) NaN_3, pH 8.0) for 24 h at 4 °C.

Analysis of Sugars——Contents of fucose, mannose and galactose in kallikrein molecules were analyzed by the gas chromatographic method of Spiro.[24] A sample (1.5 mg) was hydrolyzed in 1.0 ml of 1 N H_2SO_4 for 8 h at 100 °C in an evacuated, sealed tube. Then, the hexoses were converted to the corresponding alditolacetate derivatives, and the amount of each derivative was determined with a Shimadzu GC-6A gas chromatograph. The contents of *N*-acetylglucosamine and *N*-acetylgalactosamine were determined on a Hitachi automatic liquid chromatograph, model 034, with a column of Hitachi custom ion exchange resin #2611, after hydrolysis of the sample (0.6—0.8 mg) with 1.0 ml of 4 N HCl for 8 h at 100 °C. Neuraminic acid was determined as the amount of *N*-acetylneuraminic acid by the periodate-resorcinol method of Jourdian *et al.*[25]

Lectin Affinity Chromatographies——Serial lectin affinity chromatographies were carried out at 4 °C according to the method of Cummings and Kornfeld.[15]

Con A-Sepharose 4B Affinity Chromatography: Partially purified HUKs (equivalent to 400—1750 ml of urine) were applied to a Con A-Sepharose 4B column (0.76 × 32.5 cm) previously equilibrated with TBS. HUK was eluted firstly with TBS (ten fractions of 8 ml each were collected), then with 10 mM α-MeGlc in TBS (ten fractions of 8 ml each were collected) and finally with 500 mM α-MeGlc in TBS (ten fractions of 8 ml each were collected). The flow rate was 10 ml/h.

E-PHA-Agarose Affinity Chromatography: Con A unbound HUKs (eluted with TBS) were dialyzed against deionized water and lyophilized. The lyophilized material was dissolved in 1 ml of PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.02% (w/v) NaN_3, pH 7.4) and applied to an E-PHA-agarose column (0.69 × 20.5 cm). HUK was eluted with PBS (twenty-five fractions of 2.5 ml each were collected). The flow rate was 12 ml/h.

Lentil Lectin–Agarose Affinity Chromatography: HUKs weakly bound to Con A (eluted with 10 mM α-MeGlc in TBS) were dialyzed against deionized water and lyophilized. The lyophilized material was dissolved in 1 ml of TBS and applied to a lentil lectin-agarose column (0.69 × 13.0 cm). HUK was eluted firstly with TBS (ten fractions of 2.5 ml each were collected), then with 10 mM α-MeGlc in TBS (ten fractions of 2.5 ml each were collected) and finally with 500 mM α-MeGlc in TBS (ten fractions of 2.5 ml each were collected). The flow rate was 12 ml/h.

WAG–Sepharose 4B Affinity Chromatography: HUKs strongly bound to Con A (eluted with 500 mM α-MeGlc in TBS) were dialyzed against deionized water and lyophilized. The lyophilized material was dissolved in 1 ml of PBS and applied to a WGA–Sepharose 4B column (0.69 × 13.5 cm). HUK was eluted firstly with PBS (ten fractions of 2.5 ml each were collected) and then with 500 mM GlcNAc in PBS (ten fractions of 2.5 ml each were collected). The flow rate was 10 ml/h.

Crossed Affino-immunoelectrophoresis——Crossed affino-immunoelectrophoresis with free Con A in the first-dimensional gel was carried out according to the method of Bøg-Hansen *et al.*[26] Con A (120 μg/cm²) was imbibed at 56 °C into 1.0% (w/v) agarose melted in Tris–Veronal buffer, pH 8.6 (72 mM Tris, 24 mM Veronal, 0.4 mM calcium lactate and 0.2 mM NaN_3) containing 0.7 mM $CaCl_2$, 0.7 mM $MgCl_2$ and 0.7 mM $MnCl_2$. The first-dimensional electrophoresis was carried out at 2 mA/cm for 1 h. The electrophoresis in the second-dimensional gel containing anti-HUK antibody was carried out at 2 V/cm for 22 h. The gel was stained with Coomassie Brilliant Blue R-250 after removal of surplus proteins.

Results

Carbohydrate Composition of Purified Active HUK

The carbohydrate composition of active HUK is summarized in Table I. The neutral sugars, fucose (Fuc), mannose (Man) and galactose (Gal), aminosugar, *N*-acetylglucosamine (GlcNAc), and *N*-acetylneuraminic acid (NANA) were mainly detected. Furthermore, small amounts of *N*-acetylgalactosamine (GalNAc) were also detected. The sugar contents of HUK were about 3—4 times those of hog pancreatic kallikrein A or B.

Analysis of the Pattern of Micro-heterogeneity of Normal HUK by Using Immobilized Lectins

Each partially purified HUK sample (HUK-Na, -Nb, -Nc and -Nd) containing active- and latent-types of the enzyme derived from individual normal subjects (Na, Nb, Nc and Nd, respectively) was fractionated on a Con A–Sepharose 4B column as shown in Fig. 1A, in the case of HUK-Na. Both active and latent HUKs were mainly detected in unbound (fraction I) and weakly bound (eluted with 10 mM α-MeGlc, fraction III) fractions, and also to a small extent in the retarded (fraction II) and strongly bound (eluted with 500 mM α-MeGlc, fraction IV) fractions. The relative proportions of these fractions of individual normal HUKs are summarized in Table II.

Next, the unbound HUK (fraction I) from the Con A–Sepharose 4B column was applied to a column of E-PHA–agarose (Fig. 1B); 67—68% of active- and latent-types of HUK passed through the column (fraction I-1), while 32—33% of both types of HUK was detected in the retarded fraction (fraction I-2).

Fraction III from the Con A–Sepharose 4B column was next applied to a column of lentil lectin–agarose (Fig. 1C, Table III); 61% of both types of HUK was found in the strongly

TABLE I. Carbohydrate Compositions of HUK and Hog Pancreatic β-Kallikreins A and B (mol/mol of kallikrein)

Kallikrein	Fuc	Man	Gal	GlcNAc	GalNAc	NANA	Total sugar (%, w/w)
HUK	1.88	18.61	9.50	5.48	0.29	7.77	23.51
Hog panc. β-K.A[a]	0.80	2.20	0.76	2.69	ND	0.65	5.41
Hog panc. β-K.B[a]	2.44	3.92	1.78	4.63	ND	1.07	9.59

The values were calculated on the basis that the molecular weights of HUK and hog pancreatic β-kallikreins A and B are 40000, 26800 and 28600, respectively. ND, not detected. *a*) M. Ikekita, H. Moriya, K. Kizuki and S. Ozawa, *Chem. Pharm. Bull.*, **28**, 1948 (1980).

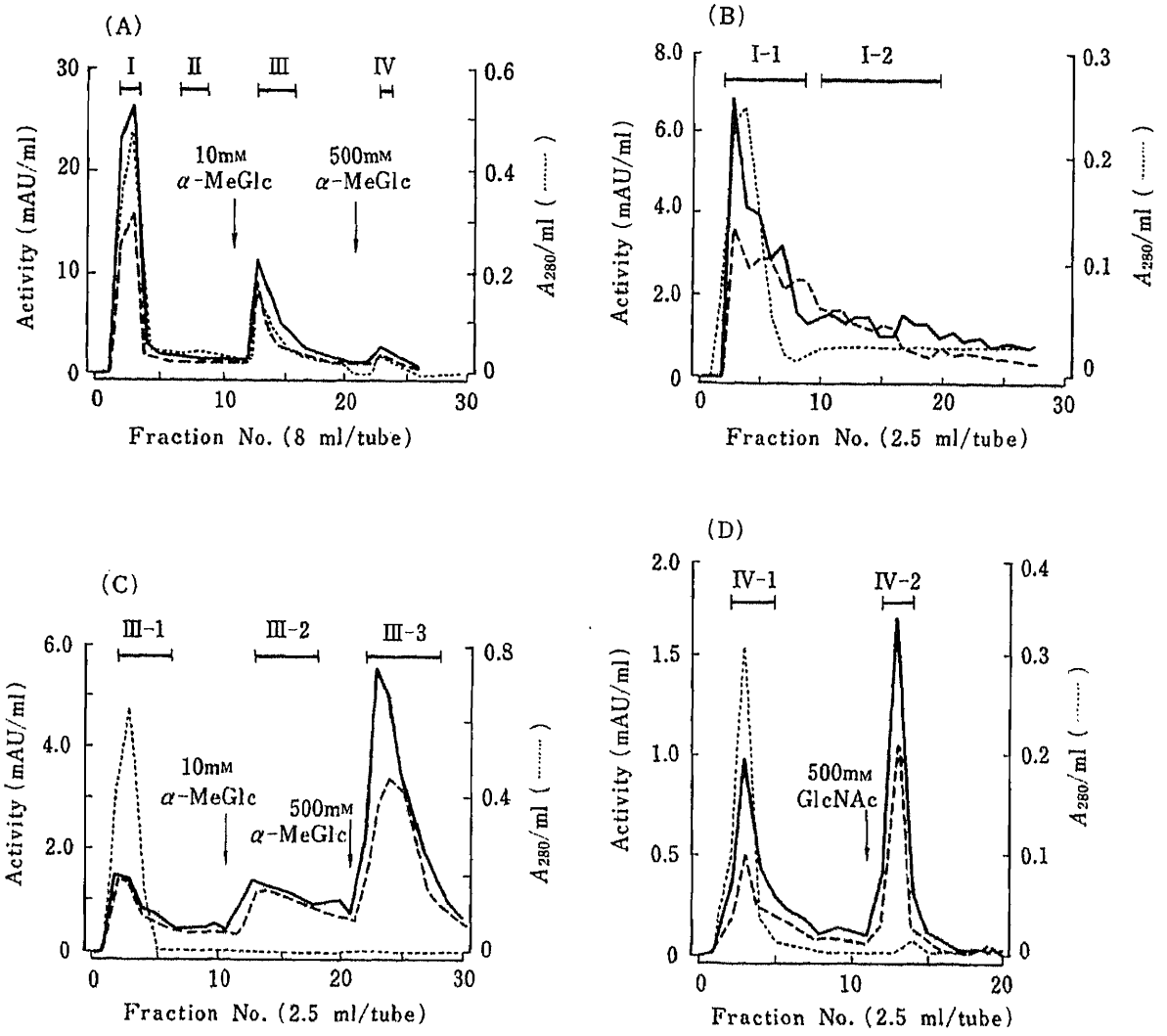


Fig. 1. Lectin–Agarose Affinity Chromatography of HUK from One Normal Individual (HUK-Na)

Partial purified HUK-Na was applied to Con A–Sepharose (A), E-PHA–agarose (B), lentil lectin–agarose (C) and WGA–Sepharose (D) columns as described in Materials and Methods and Results.
Activity: - - -, active HUK; ——, latent HUK.

TABLE II. Fractionation of HUKs from Normal Individuals on a Con A–Sepharose 4B Column

Subject	Fraction			
	Unbound (I) (%)	Retarded (II) (%)	Bound	
			Weakly (III) (%)	Strongly (IV) (%)
Na	51.3/46.9	—	40.6/45.8	8.1/7.3
Nb	55.4/48.4	—	40.6/45.2	4.0/6.4
Nc	56.9/55.6	—	37.6/37.4	5.5/7.0
Nd	48.7/60.5	8.8/7.8	36.1/25.8	6.4/5.9
Mean±S.E.	53.1±1.9/52.9±3.2	8.8/7.8	38.7±1.1/38.6±4.7	6.0±0.9/6.7±0.3

The values in the numerator and denominator indicate the distributions of active and latent HUKs, respectively. —: not detected.

Table III. Fractionation of HUKs Weakly Bound to Con A on a Lentil Lectin–Agarose Column

Subject	Fraction		
	Unbound (III-1) (%)	Bound	
		Weakly (III-2) (%)	Strongly (III-3) (%)
Na	16.2/16.7	19.9/19.8	63.9/63.5
Nb	17.4/13.3	24.7/25.2	57.9/61.5
Nc	8.5/14.2	22.7/23.8	68.8/62.0
Nd	15.8/12.4	29.8/32.1	54.4/55.5
Mean ± S.E.	14.5 ± 2.0/14.2 ± 0.9	24.3 ± 2.1/25.2 ± 2.6	61.3 ± 3.2/60.6 ± 1.8

The values in the numerator and denominator indicate the distributions of active and latent HUKs, respectively.

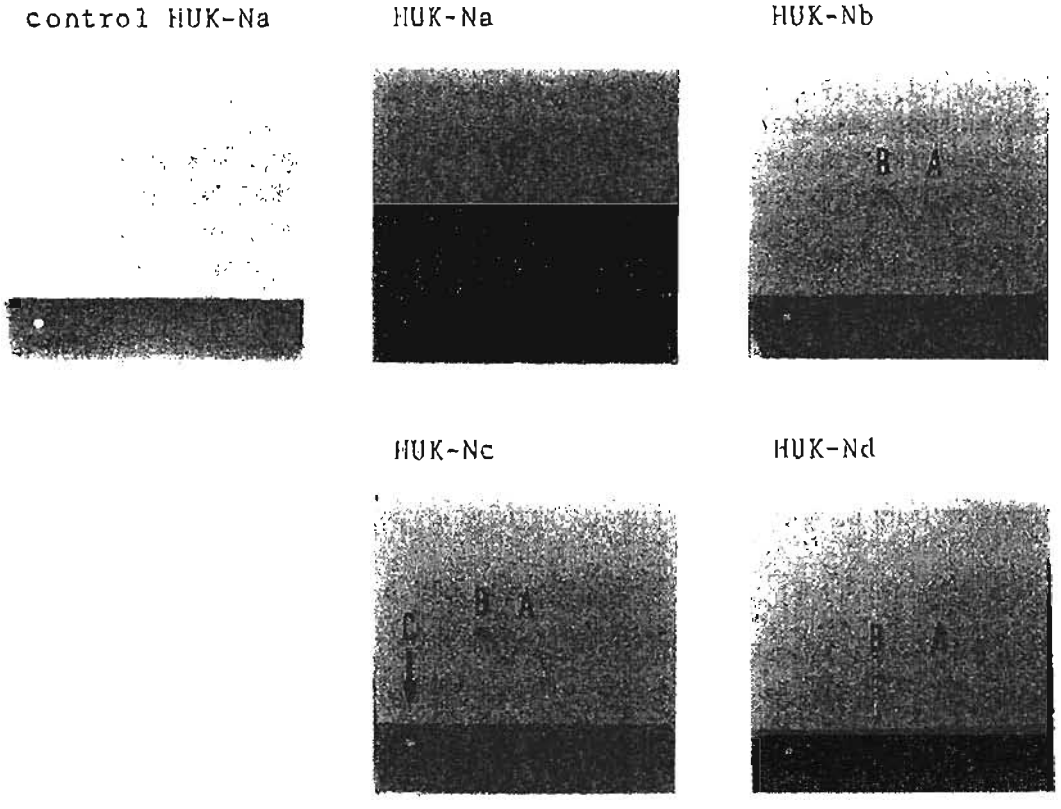


Fig. 2. Crossed Affino-immunoelectrophoresis of HUKs from Normal Individuals

Experimental details are given in the text.
Control HUK-Na was subjected to electrophoresis without free Con A in the first-dimensional gel and other procedures were similar to those applied to the test samples.

bound fraction (fraction III-3), and 24—25% and 14—15% of both HUKs were detected in the weakly bound (fraction III-2) and flow-through (fraction III-1) fractions, respectively.

When fraction IV from the Con A–Sepharose 4B column was applied to a WGA–Sepharose 4B column, 52% and 59% of active and latent HUKs were found in the flow-through fraction (fraction IV-1), respectively, and 49% and 42% of both types of HUKs were

TABLE IV. Fractionation of HUKs Strongly Bound to Con A on a WGA–Sepharose 4B Column

Subject	Fraction	
	Unbound (IV-1) (%)	Bound (IV-2) (%)
Na	51.0/56.2	49.0/43.8
Nb	46.6/45.0	53.4/55.0
Nc	46.6/63.5	53.4/36.5
Nd	61.8/70.7	38.2/29.3
Mean±S.E.	51.5±3.6/58.9±5.5	48.5±3.6/41.2±5.5

The values in the numerator and denominator indicate the distributions of active and latent HUKs, respectively.

TABLE V. Fractionation of HUKs from Normal Individuals by Crossed Affino-immuno-electrophoresis with Con A

Subject	Fraction		
	Faster-migrating (%)	Slower-migrating (%)	No migration (%)
Na	43.0	57.0	—
Nb	45.9	54.1	—
Nc	46.5	53.1	0.4
Nd	51.8	48.2	—
Mean±S.E.	46.8±1.8	53.1±1.8	0.4

—: not detected.

found in the bound fraction (fraction IV-2), respectively (Fig. 1D, Table IV).

Analysis of the Pattern of Micro-heterogeneity of Normal HUK by Crossed Affino-immuno-electrophoresis

Normal HUKs (HUK-Na, -Nb, -Nc and -Nd) were also analyzed by crossed affino-immunoelectrophoresis with Con A. The electrophoregrams are shown in Fig. 2. The ratio of each fraction was estimated by the peak area method, and the results are summarized in Table V. The conventional crossed immunoelectrophoresis of normal HUK (HUK-Na) with a second-dimensional gel impregnated with monospecific anti-HUK antibody gave a single precipitation peak (Fig. 2, control HUK-Na). Including free Con A in the first-dimensional gel leads to the appearance of two main peaks (peaks A and B) which reflect different degrees of interaction and/or different numbers of binding sites between the Con A and HUK molecules (containing active and latent HUKs) (Fig. 2, Na—Nd). It was found that peak A included nonreactive HUK, and that peak B corresponded to weakly and strongly reactive HUKs (data not shown). In individual normal HUKs, fractions A and B, accounted for 36—52% and 48—57% of the total HUK, respectively. In the case of HUK-Nc, a small nonmigrating fraction (peak C) was seen.

Discussion

Active HUK purified from urine of healthy men is a glycoprotein with a carbohydrate content of 23.5% (w/w), and as the main amino sugar is *N*-acetylglucosamine, the oligosaccharides bound to the HUK molecule might be mainly Asn-linked type(s). Furthermore, preliminary observations obtained from analysis of the sugar moieties of active-type HUK showed that the charge heterogeneity of HUK may arise from micro-heterogeneity in the sugar moiety of this enzyme. In order to obtain a better understanding of the nature of micro-heterogeneity in both active- and latent-types of HUK, we have developed procedures of serial lectin affinity chromatographies and lectin affino-immunoelectrophoresis for the detection and fractionation of molecular variants depending on slight differences in the affinities of Asn-linked oligosaccharides bound for lectins. Lectins have been widely used for the study of the structures and functions of glycoproteins. Previous studies from a number of laboratories have established that Asn-linked oligosaccharides can be effectively fractionated on columns of immobilized lectins.[15,16] This approach takes advantage of presently available immobilized lectin gels displaying different affinities for a given glycoprotein.

In this study, we showed that the majority of the Asn-linked oligosaccharides of both

TABLE VI. Patterns of Micro-heterogeneity of Active- and Latent-Types of Normal HUKs Determined by Serial Lectin Affinity Chromatographies

Fraction	Proportion (%) Active (Latent)	Characteristics of oligosaccharides bound to HUKs
I-1	36 (36)	Tri- or tetra-antennary
I-2	17 (17)	Bi-antennary with bisecting GlcNAc
II	9 (8)	
III-1	6 (6)	Bi-antennary without fucose
III-2	9 (10)	Bi-antennary with fucose
III-3	24 (23)	
IV-1	3 (4)	High-mannose type
IV-2	3 (3)	Hybrid type

active- and latent-types of HUK can be fractionated by serial chromatographies on Con A–, E-PHA–, lentil lectin– and WGA–agarose columns, according to the method of Cummings and Kornfeld.[15] This method of fractionation is simple, rapid and sensitive, and allows for quantitative estimates of the component Asn-linked oligosaccharide(s) of active- and latent-types of HUK. For example, in the case of active HUK, the species containing tri- and/or tetra-antennary oligosaccharide(s) account for approximately 36%, the species containing core-fucosylated bi-antennary oligosaccharide(s) account for 33%, and the species containing bi-antennary oligosaccharides with outer galactose residues and an *N*-acetylglucosamine residue linked β1,4 to a β-linked mannose residue (bisecting *N*-acetylglucosamine residue) account for 17% of the total active-type HUK. The profiles of micro-heterogeneity found by serial lectin affinity chromatographies of the latent-type HUK were similar to those of the active-type HUK. These data are summarized in Table VI. Our results suggest that the oligosaccharides of both active- and latent-types of HUK in normal urine might be specially rich in complex type oligosaccharides and furthermore, both types of HUK might be biosynthesized in the same organ(s) and excreted into urine *via* the same route. The analysis of affinity for Con A was carried out by two methods, *i.e.* the enzymochemical method of affinity chromatography and the immunochemical method of crossed affino-immunoelectrophoresis. The results obtained by the two different analytical methods mostly coincided well with each other.

In this paper, we succeeded in fractionating both active- and latent-types of HUK molecules. Because of their simplicity, serial lectin agarose chromatographies and crossed affino-immunoelectrophoresis are expected to provide a powerful general approach for the detection of subtle changes of oligosaccharides of both active and latent HUKs that might occur in various disorders. Studies of carbohydrate micro-heterogeneity in HUKs from various patients with various renal disorders is in progress. This work could help to solve the problem of the physiological and/or pathological significance of the tissue kallikreins.

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Studies on the Temporal Variations in the Pharmacokinetics and Metabolism of Theophylline

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Temporal variations in the pharmacokinetics and metabolism of theophylline were investigated in seven healthy volunteers following single oral dosing of an immediate-release theophylline tablet formulation at 9:00 a.m. and 9:00 p.m. Theophylline concentration in the plasma and theophylline and its metabolites excreted in the urine were measured by a high-performance liquid chromatographic (HPLC) method. The pharmacokinetic and bioavailability parameters obtained in this study showed no difference between morning and evening dosing. Cumulative urinary excretion and urinary recovery of theophylline and its metabolites also showed no difference between morning and evening dosing. The present study shows that there are no temporal variations in the pharmacokinetics and metabolism of theophylline.

Keywords——temporal variation; theophylline pharmacokinetics; theophylline metabolism; immediate-release tablet formulation; healthy volunteer; high-performance liquid chromatographic method

Theophylline is regarded as being useful in the treatment of patients with reversible obstructive airway diseases, and its bronchodilator effect is well established.[1,2] In a number of recent studies, theophylline has been shown to have circadian variations in absorption and disposition.[3-7] On the other hand, it has been reported that there are no diurnal variations in theophylline metabolism or excretion and the diurnal variations in theophylline disposition are due to slower absorption of the evening dose.[8-10] In our earlier studies, we also concluded that there is likely to be diurnal variation in the trough theophylline concentrations in the steady state, following multiple oral dosing of a sustained-release theophylline tablet formulation.[11]

Theophylline has a narrow therapeutic range of 10—20 μg/ml, and since the risk of side effects increases as plasma concentrations exceed this range, the routine monitoring of plasma theophylline concentrations to adjust the dosing schedule in order to maintain this range is recommended. Thus, circadian variations in theophylline pharmacokinetics could be important in relation to therapeutic efficacy. However, the mechanism of circadian variations in theophylline disposition has not yet been established.

The present study was carried out to investigate the influence of time of administration on the disposition and metablism of theophylline following single oral dosing of an immediate-release theophylline tablet formulation in healthy volunteers.

Experimental

Drug——The drug used in this study was an immediate-release theophylline tablet formulation, aminophylline (Neophylline, which contains 80 mg of theophylline per tablet; Eisai Co., Japan).

TABLE I. Characteristics of the Normal Healthy Volunteers

Subject	Sex	Age (years)	Height (cm)	Body weight (kg)	Smoking
A	M	22	176	62	No
B	M	22	168	61	No
C	M	23	173	66	No
D	M	23	180	78	No
E	M	24	170	54	20 cigarettes/d
F	M	26	162	60	20 cigarettes/d
G	M	21	177	63	10 cigarettes/d
Mean		23.0	172.3	63.4	
S.E.M.		0.6	2.3	2.3	
CV%[a]		7.1	3.6	11.7	

a) Coefficient of variation.

Procedure Used with Healthy Volunteers——Characteristics of the volunteers tested are given in Table I. We obtained informed consent from each subject after full explanation of procedures. They were judged to be healthy on the basis of routine laboratory tests. Each subject received four tablets corresponding to 320 mg of theophylline with 150 ml of water at 9:00 a.m. and 9:00 p.m. They were not allowed to take xanthine-containing food or drink for 8 h before and 12 h after each dosing and received no food for 2 h after each dosing. The drug administration trials were carried out in a cross-over fashion with 2-week intervals. Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 8, and 12 h after the dosing. Urine was also collected and the urine collection periods were as follows: 0—2 h, 2—4 h, 4—6 h, and 6—12 h. The volume and pH of each fraction were measured and recorded. Plasma samples obtained by centrifugation and 10 ml of each urine fraction were stored at −40 °C until analyzed. Total theophylline concentration in the plasma was determined by means of the high-performance liquid chromatographic (HPLC) method previously reported.[12] Urine was also assayed for theophylline and its metabolites, 1-methyluric acid (1-MU), 3-methylxanthine (3-MX), and 1,3-dimethyluric acid (1,3-DMU) by an HPLC method.[13]

Pharmacokinetic Analysis——The area under the plasma concentration–time curve (AUC) was calculated by applying the linear trapezoidal rule and the AUC from the last data point to infinity was extrapolated by dividing the last data point by the apparent first-order elimination rate constant (k_{el}). The bioavailability was evaluated from the observed maximum concentration of theophylline (C_{max}), time to C_{max} (T_{max}) and the AUC. Plasma concentration data after single oral dosing of the drug were fitted to a one-compartment model including a first-order absorption process according to Eq. 1.

$$C_t = \frac{F \cdot D \cdot k_a}{Vd(k_a - k_{el})}(e^{-k_{el} \cdot t} - e^{-k_a \cdot t}) \quad (1)$$

where k_a is the apparent absorption rate constant and F is the fraction absorbed. D is the dose (oral administration). Values of C_t were calculated assuming $F = 1.0$. The pharmacokinetic parameters were analyzed by nonlinear least-squares regression analysis (MULTI[14]).

Values in this paper are expressed as the mean ± S.E.M., and the statistical significance of differences was assessed by means of Student's paired t-test.

Results and Discussion

Figure 1 shows the mean plasma theophylline concentration–time data after oral dosing of the drug to seven healthy volunteers in the morning (9:00 a.m.) and in the evening 9:00 p.m.). The overall mean plasma theophylline concentrations in the terminal elimination phase were higher in the evening than in the morning, but the difference was not significant.

Tables II and III summarize the values of the various pharmacokinetic parameters and bioavailability parameters, respectively. The mean plasma theophylline concentration–time curves after dosing at the two dosing times were similar, but the terminal elimination rate constant (k_{el}) in the evening ($0.125\,h^{-1}$) was slower than that in the morning ($0.144\,h^{-1}$), although the difference was not significant. The plasma theophylline clearance, CL, in the

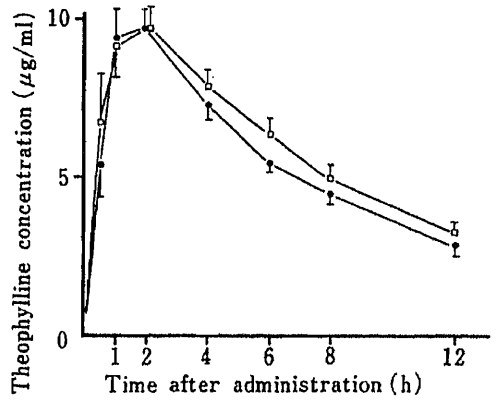


Fig. 1. Mean Plasma Theophylline Concentration–Time Data in Seven Subjects after Oral Dosing of the Immediate-Release Theophylline Tablet Formulation at 9:00 a.m. (●) and 9:00 p.m. (□)

Each value represents the mean ± S.E.M.

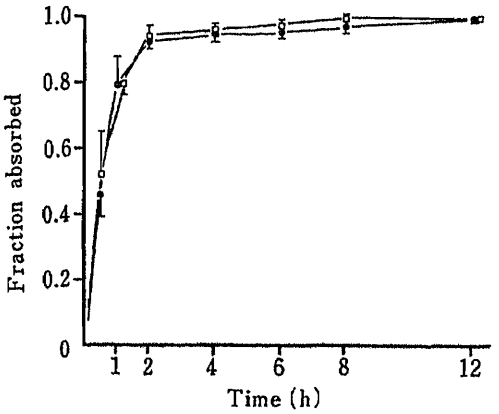


Fig. 2. Wagner–Nelson Analysis of the Mean Plasma Theophylline Concentration–Time Curves Obtained after Oral Dosing of the Immediate-Release Theophylline Tablet Formulation at 9:00 a.m. (●) and 9:00 p.m. (□)

Each value represents the mean ± S.E.M.

TABLE II. Computer Estimates of Pharmacokinetic Parameters Following Administration of Immediate-Release Theophylline Tablets at 9:00 a.m. and 9:00 p.m.

Parameter	9:00 a.m.	9:00 p.m.
k_{el} (h^{-1})	0.144 ± 0.007	0.125 ± 0.005 ($p<0.10$)
Vd (l/kg)	0.420 ± 0.023	0.419 ± 0.025
k_a (h^{-1})	1.665 ± 0.386	2.673 ± 0.830
CL (ml/kg/h)	59.94 ± 3.27	52.09 ± 3.15

Each value represents the mean ± S.E.M. of seven subjects. Plasma theophylline clearance, CL, was calculated as $k_{el} \cdot Vd$.

TABLE III. Bioavailability Parameters Following Administration of Immediate-Release Theophylline Tablets at 9:00 a.m. and 9:00 p.m.

Parameter	9:00 a.m.	9:00 p.m.
C_{max} (μg/ml)	10.03 ± 0.86	10.11 ± 0.88
T_{max} (h)	1.57 ± 0.20	1.43 ± 0.20
AUC (μg·h/ml)	68.97 ± 4.60	74.41 ± 5.41

Each value represents the mean ± S.E.M. of seven subjects. Area under the plasma concentration–time curve (AUC) from time zero to 12 h was calculated by using the trapezoidal rule. Maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were observed values.

morning (59.94 ml/kg/h) was very similar to that in the evening (59.09 ml/kg/h). The bioavailability parameters such as C_{max}, T_{max}, and AUC also showed no significant differences between in the morning and in the evening.

In the present study, we estimated the pharmacokinetic characteristics for theophylline after a single oral dose of the drug based on the assumption of apparent first-order absorption and elimination kinetics. In a number of studies on the elimination kinetics of theophylline, it has been stated that the pathways of theophylline metabolism are both first-order and capacity-limited processes, and that the major metabolite formation followed Michaelis–Menten kinetics and becomes capacity-limited in the therapeutic concentration range of

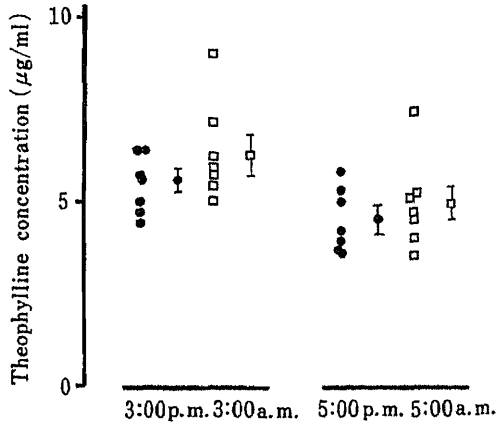


Fig. 3. Individual Plasma Theophylline Concentration Data at 6 and 8 h after Oral Dosing of the Immediate-Release Theophylline Tablet Formulation at 9:00 a.m. (●) and 9:00 p.m. (□)

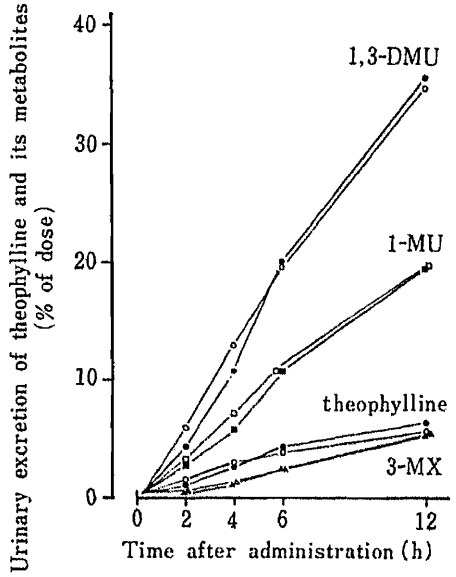


Fig. 4. Mean Cumulative Urinary Excretion of Theophylline and Its Metabolites Obtained after Oral Dosing of the Immediate-Release Theophylline Tablet Formulation at 9:00 a.m. (Closed Symbols) and 9:00 p.m. (Open Symbols)

Values of metabolites are expressed on a molar basis and represent the amount excreted in 12 h as a percentage of the theophylline dose.

theophylline.[15,16] On the other hand, it has also been reported that the elimination kinetics were linear at both low and high doses of theophylline.[17] However, the kinetics of theophylline elimination and the elimination of its major metabolites after a single oral dose of theophylline have not been clarified. Whether or not theophylline kinetics are altered at lower and higher theophylline concentrations cannot be answered on the basis of the present study.

As shown in Fig. 2, the fraction absorbed–time profile calculated from the plasma theophylline concentration after dosing of the drug by use of the Wagner–Nelson method[18] obtained in the morning was very similar to that obtained in the evening. No significant difference was found in the rate or extent of absorption of the drug between in the morning and in the evening.

It is usually recommended that immediate-release theophylline formulations are administered every 6 or 8 h to maintain a therapeutic plasma theophylline concentration. We therefore compared plasma concentrations of theophylline at 6 and 8 h after morning and evening dosing (Fig. 3). As shown, plasma theophylline concentrations at 6 and 8 h after administration in the morning were 5.56 ± 0.27 and 4.50 ± 0.33 μg/ml, and those in the evening were 6.23 ± 0.55 and 4.91 ± 0.47 μg/ml, respectively. The differences at the respective times are not significant.

Lesko *et al.*[3] reported circadian variations in the disposition of theophylline after administration of rapid-acting and sustained-release theophylline formulations. Others[4,5] have also reported similar results for a sustained-release theophylline formulation. On the other hand, Taylor *et al.*[7] reported that there are no significant differences in theophylline disposition, but absorption is slower after evening dosing. In recent years, other investigators have concluded that the most important factor contributing to temporal variation in plasma theophylline concentration is delayed gastrointestinal absorption of theophylline at night.[8,9]

TABLE IV. Mean Urinary Recovery of Theophylline and Its Metabolites

Metabolite	9:00 a.m.	9:00 p.m.
1-MU	19.40±1.75	19.51±1.20
3-MX	5.54±0.55	5.66±0.41
1,3-DMU	34.61±2.55	34.14±1.69
Theophylline	6.45±0.82	6.07±0.49
Total	66.01±4.83	65.38±2.94

Each value represents the mean±S.E.M. of six subjects. Values of metabolites are expressed on a molar basis and represent the amount excreted in 12 h as a percentage of the theophylline dose.

The present study did not find such delayed absorption of theophylline after evening dosing, but showed a tendency for slower elimination after evening dosing. However, the mechanism of temporal variation in theophylline pharmacokinetics is unclear. For example, one possible mechanism proposed to explain a temporal variation in the disposition of phenacetin was variations in hepatic metabolism.[19] Since theophylline is known to be metabolized by microsomal oxidases,[20,21] temporal variation in their activity may account for the changes in theophylline disposition.

In the present study, in order to clarify whether or not changes in hepatic metabolism are important in relation to theophylline, we compared the concentrations of theophylline and its major metabolites excreted in the urine after morning and evening dosing. Table IV shows the mean percentage of the dose recovered in the urine after 12 h. Figure 4 shows the mean cumulative urinary excretion of theophylline and its major metabolites. The percentage of the dose recovered in the urine was not significantly different between in the morning and in the evening (66.0±4.8% and 65.4±2.9%). The mean urine volume excreted from 9:00 a.m. to 9:00 p.m. was 957.0±118.5 ml and that excreted from 9:00 p.m. to 9:00 a.m. was 882.1±58.6 ml. It was found that there are no significant differences in the rate or extent of urinary excretion of theophylline and its major metabolites between in the morning and in the evening. On the basis of the results on total urinary excretion of theophylline and its major metabolites, it is concluded that there was no variation in theophylline biotransformation.

In conclusion, it is unlikely that significant temporal variations in the disposition of theophylline and its metabolites occur in humans.

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Notes

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Synthesis of Proline Derivatives by 1,3-Dipolar Cycloaddition of Azomethine Ylide Generated from an Organosilicon Compound

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The 1,3-dipolar cycloaddition of azomethine ylides generated from methyl *N*-(trimethylsilylmethyl)iminoacetate and benzyl bromide or a catalytic amount of trifluoroacetic acid has been found to give the corresponding proline derivatives.

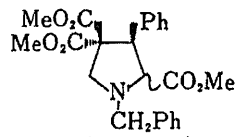
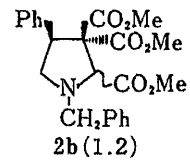
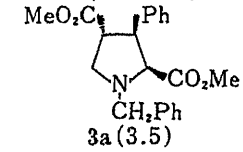
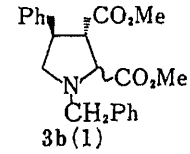
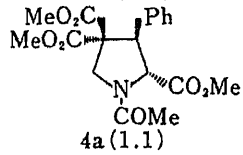
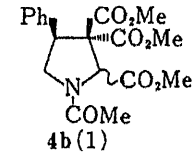
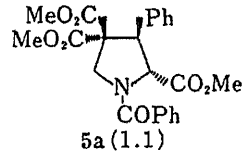
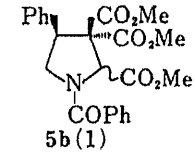
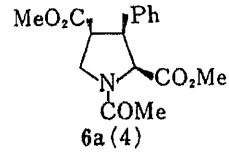
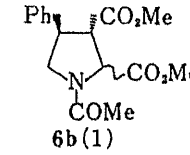
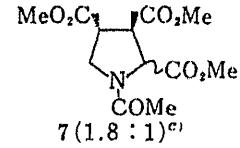
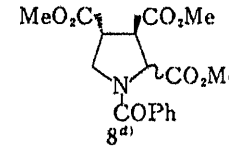
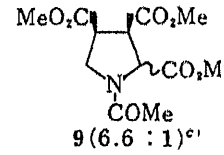
Keywords——1,3-dipolar cycloaddition; methyl *N*-(trimethylsilylmethyl)iminoacetate; Schiff base; azomethine ylide; *N*-acylation; proline derivative; ambivalent

As a part of our studies on 1,3-dipolar cycloaddition of azomethine ylides,[1,2] in the previous communication, we have reported a new synthesis of proline derivatives by 1,3-dipolar cycloaddition of methyl *N*-(trimethylsilylmethyl)iminoacetate (**1**) with olefinic dipolarophiles in the presence of benzyl bromide or a catalytic amount of trifluoroacetic acid (TFAA).[3] The details of this new cycloaddition are described in this paper.

The Schiff base (**1**) easily decomposed in the neat state and also in hexamethylphosphoramide (HMPA), the reaction solvent in the subsequent 1,3-dipolar cycloaddition, but it was relatively stable in ether, so that this starting material (**1**) was freshly prepared from methyl α-hydroxy-α-methoxyacetate and trimethylsilylmethylamine using $MgSO_4$ as a drying agent and ether as a solvent (at room temperature for 30 min). The subsequent 1,3-dipolar cycloaddition of the freshly prepared Schiff base (**1**) with dimethyl benzylidenemalonate or methyl cinnamate in the presence of benzyl bromide in HMPA at 60 °C for 7 h proceeded smoothly to give the corresponding *N*-benzylproline derivatives. The TFAA-catalyzed reaction of **1** with olefinic dipolarophiles (dimethyl benzylidenemalonate, methyl cinnamate, dimethyl fumarate, and dimethyl maleate) also proceeded smoothly in HMPA on ice-cooling for 1.5 h to give the corresponding *N*-unsubstituted proline derivatives, which were purified by silica gel column chromatography (SGCC) after *N*-acylation because of their instability. These data are summarized in Table I. In entries 1—5, the products were obtained as mixtures of regio- and/or stereoisomers. In entries 6—8, they were obtained as mixtures of two stereoisomers. The structures of these products were determined on the basis of infrared (IR), proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) and mass spectral (MS) analyses, and physical data as indicated in Experimental.

The mechanism of the reaction may to be as follows. The Schiff base (**1**) reacts with benzyl bromide to produce the *N*-benzyliminium salt (**10**), and the subsequent heterolysis of the silicon–carbon bond gives the non-stabilized *N*-benzylazomethine ylide (**11**), 1,3-dipolar cycloaddition of which with olefinic dipolarophiles proceeds to give the corresponding *N*-benzylproline derivatives. The TFAA-catalyzed cycloaddition of **1** with olefinic dipolarophiles proceeds *via* the *N*-protonated iminium salt (**12**) and the *N*-protonated azomethine ylide to give the corresponding *N*-unsubstituted proline derivatives (see Chart 1).

TABLE I. Synthesis[a] of Proline Derivatives

Entry	Dipolarophile reagents	Product[b]	Total yield (%)
1	$PhCH=C(CO_2Me)_2$ $PhCH_2Br$	 2a (*cis* : 2.6)  2b (1.2)	51
2	 $PhCH_2Br$	2a' (*trans* : 1)  3a (3.5)  3b (1)	25
3	$PhCH=C(CO_2Me)_2$ CF_3CO_2H, MeCOCl	 4a (1.1)  4b (1)	62
4	$PhCH=C(CO_2Me)_2$ CF_3CO_2H, PhCOCl	 5a (1.1)  5b (1)	48
5	 CF_3CO_2H, MeCOCl	 6a (4)  6b (1)	49
6	 CF_3CO_2H, MeCOCl	 7 (1.8 : 1)[c]	49
7	 CF_3CO_2H, PhCOCl	 8[d]	85
8	 CF_3CO_2H, MeCOCl	 9 (6.6 : 1)[c]	27

a) Reaction conditions are described in Experimental. *b*) Ratio of the isomers is given in parentheses. *c*) Ratio of the stereoisomers at the 2-position was estimated on the basis of the NMR spectra. *d*) The product 8 was estimated to be a mixture of two isomers on the basis of the number of carbonyl carbons (^{13}C-NMR spectra) but the ratio of the two isomers could not be estimated.

This reaction was found to give the proline derivatives with low regioselectivity, possibly due to the ambivalence of the 1,3-dipole termini, and **11a**↔**11b** (or **11c**) may be the structures of the intermediary azomethine ylide in this cycloaddition. This ambivalence was confirmed by a comparison with the base-promoted 1,3-dipolar cycloaddition of *N*-(phenylthiomethyl)-α-amino acid methyl ester (**14**).[1f,m] That is to say, the 1,3-dipolar cycloaddition of the Schiff base (**1**) and benzyl bromide as the synthon **11a** gave almost the same regioselectivity of the

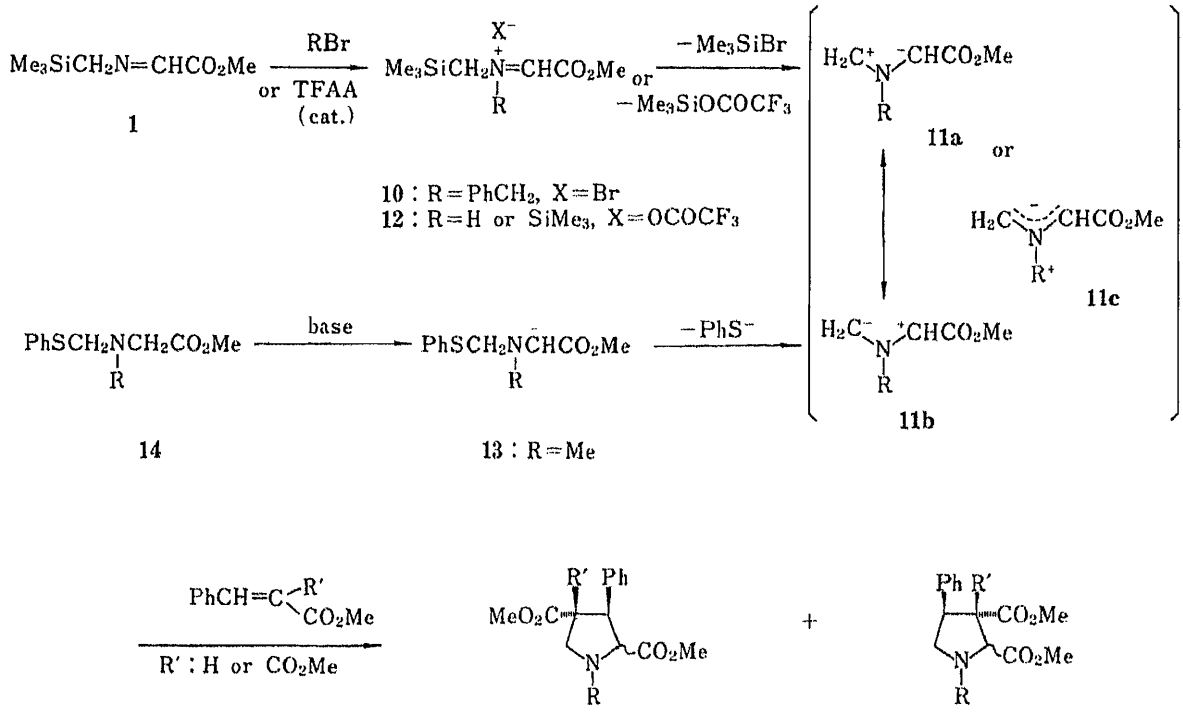


Chart 1

products as the base-promoted reaction of **14** as the synthon **11b**. This result indicates that both of the 1,3-dipolar cycloadditions proceed *via* similar ambivalent azomethine ylide intermediates (**11a**↔**11b** or **11c**).

It should be noted that the 1,3-dipolar cycloaddition of **1** is a convenient method for the formation of proline derivatives, that the present reaction is superior to the base-promoted one in regard to the product's stereoselection because no epimerization of the products is observed (entries 2, 5, and 8), and that intermediary azomethine ylides are ambivalent.

Experimental

All melting and boiling points are uncorrected. Infrared (IR) spectra were measured with a Hitachi EPI-G2 IR spectrometer. NMR spectra were taken on a JEOL JNM90Q spectrometer (90 MHz) and all chemical shifts are given downfield from tetramethylsilane (TMS). MS data were recorded on a Hitachi RMS-4 MS spectrometer.

***N*-Benzylproline Derivatives (2 and 3)**——Trimethylsilylmethylamine (13.2 mmol) was added dropwise to a stirred suspension of methyl α-hydroxy-α-methoxyacetate[4] (12 mmol) and $MgSO_4$ in dried ether (10 ml) under ice-cooling. The mixture was stirred at room temperature for 30 min and filtered. The filtrate was added dropwise to an ice-cooled solution of dimethyl benzylidenemalonate or methyl cinnamate (2 mmol) and benzyl bromide (2.2 mmol) in HMPA (20 ml) with stirring, and the whole was stirred at 60 °C for 7 h under a nitrogen atmosphere. The reaction mixture was diluted with benzene (40 ml), washed with sat. aq. NaCl–10% aq. $KHCO_3$ (60 ml+20 ml) and sat. aq. NaCl (80 ml), and dried over $MgSO_4$. After removal of the benzene, the residual oil was subjected to thin layer chromatography (TLC) on silica gel using benzene–iso-Pr_2O (9 : 1) or benzene in the case of dimethyl benzylidenemalonate or methyl cinnamate, respectively, as an eluent. The reaction with dimethyl benzylidenemalonate gave three isomers **2a**, **2a′**, and **2b** in the ratio of 2.6 : 1 : 1.2 in 51% total yield. Compound **2a** was isolated in pure form. **2a**: bp 250 °C (0.1 mmHg) (by bulb-to-bulb distillation). MS (*m/z*): 411 (M^+, Calcd for $C_{23}H_{25}NO_6$ 411.1682. Found 411.1684). ^{1}H-NMR ($CDCl_3$, J=Hz) δ: 2.65 (1H, d, J=11.1, 5-CH_A), 3.06 (3H, s, 4-CO_2Me oriented *cis* to the 3-phenyl group), 3.26 (3H, s, 2-CO_2Me oriented *cis* to the 3-phenyl group), 3.30 (1H, d, J=12.6, NCH_APh), 3.67 (3H, s, 4-CO_2Me oriented *trans* to the 3-phenyl group), 3.95 (1H, d, J=6.6, 2-CH), 4.05 (1H, d, J=11.1, 5-CH_B), 4.29 (1H, d, J=12.6, NCH_BPh), 4.36 (1H, d, J=6.6, 3-CH), 6.91—7.52 (10H, m, C_6H_5 × 2). ^{13}C-NMR ($CDCl_3$) δ: 51.1 (q, OCH_3), 51.9 (q, OCH_3), 53.0 (q, OCH_3), 53.6 (d, 3-C), 58.0, 58.5 (t, t, 5-C, NCH_2Ph), 63.6 (s, 4-C), 70.3 (d, 2-C), 127.2, 127.4, 127.8, 128.4, 128.8, 129.8, 137.2, 138.4 (d, d, d, d, d, d, s, s, C_6H_5 × 2), 168.2 (s, CO), 170.5 (s, CO), 171.7

(s, CO). Compounds **2a'** and **2b** were obtained as a mixture. In ^{1}H-NMR spectra of the mixture (**2a'** and **2b**), the signals of the methyl protons of the methoxycarbonyl groups appear as singlets at 3.09 and 3.17 ppm (CO_2 Me oriented *cis* to the phenyl groups), 3.59, 3.61, and 3.69 ppm (CO_2Me oriented *trans* to the phenyl groups). **2a'** and **2b** (mixture): ^{13}C-NMR ($CDCl_3$) δ: 49.3 (d, 4-C of **2b**), 51.8 (q, OCH_3 of **2a'** and **2b**), 52.0 (q, OCH_3 of **2b**), 52.2 (q, OCH_3 of **2a'**), 52.6 (q, OCH_3 of **2b**), 52.9 (q, OCH_3 of **2a'**), 53.6 (d, 3-C of **2a'**), 57.2 (t, N$\underline{C}H_2$Ph of **2a'** and **2b**), 58.2 (t, 5-C of **2b**), 58.5 (t, 5-C of **2a'**), 64.5 (s, 4-C of **2a'**), 68.3 (s, 3-C of **2b**), 70.7 (d, 2-C of **2a'**), 71.7 (d, 2-C of **2b**), 127.3, 127.5, 128.0, 128.1, 128.3, 129.0, 137.7, 138.2 (d, d, d, d, d, d, s, s, $C_6H_5 \times 4$), 169.0 (s, CO), 169.1 (s, CO), 169.3 (s, CO), 170.8 (s, CO), 171.5 (s, CO), 172.1 (s, CO). In the reaction with methyl cinnamate, two regioisomers **3a** and **3b** were isolated in 25% total yield. **3a**: bp 250 °C (0.5 mmHg) (by bulb-to-bulb distillation). MS (*m/z*): 353 (M^+, Calcd for $C_{21}H_{23}NO_4$ 353.1627. Found 353.1623). ^{1}H-NMR ($CDCl_3$) δ: 2.80—4.07 (7H, m, 2-CH, 3-CH, 4-CH, 5-CH_2, NC$\underline{H}_2$Ph), 3.20 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 7.05—7.42 (10H, m, $C_6H_5 \times 2$). ^{13}C-NMR ($CDCl_3$) δ: 48.2 (d, 4-C), 50.7 (q, OCH_3), 50.8 (q, OCH_3), 51.9 (d, 3-C), 55.6 (t, N$\underline{C}H_2$Ph), 57.5 (t, 5-C), 70.7 (d, 2-C), 127.2, 128.3, 129.0, 138.2, 139.1 (d, d, d, s, s, $C_6H_5 \times 2$), 171.4 (s, CO), 173.9 (s, CO). **3b**: bp 250 °C (0.5 mmHg) (by bulb-to-bulb distillation). MS (*m/z*): 353 (M^+, Calcd for $C_{21}H_{23}NO_4$ 353.1627. Found 353.1703). ^{1}H-NMR ($CDCl_3$) δ: 2.88—4.14 (7H, m, 2-CH, 3-CH, 4-CH, 5-CH_2, NC$\underline{H}_2$Ph), 3.57 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 7.19—7.34 (10H, m, $C_6H_5 \times 2$). ^{13}C-NMR ($CDCl_3$) δ: 45.7 (d, 3-C), 51.5 (q, OCH_3), 51.9 (q, OCH_3), 54.9 (d, 4-C), 56.9 (t, N$\underline{C}H_2$Ph), 59.7 (t, 5-C), 67.3 (d, 2-C), 126.7, 127.2, 127.4, 128.3, 128.6, 128.8, 138.1, 142.5 (d, d, d, d, d, d, s, s, $C_6H_5 \times 2$), 171.4 (s, CO), 171.6 (s, CO).

***N*-Acylproline Derivatives (4—9)**——General Procedure: The Schiff base **1**, which was prepared freshly from α-hydroxy-α-methoxyacetate (12 mmol) and trimethylsilylmethylamine (13.2 mmol) in a manner similar to that described for the *N*-benzylproline derivatives (**2** and **3**), was added dropwise on ice-cooling to a stirred solution of an olefinic dipolarophile (2 mmol) and a catalytic amount of TFAA (0.2 mmol) in HMPA (20 ml). The whole was stirred at the same temperature for 1.5 h under a nitrogen atmosphere. Work-up of the reaction mixture was carried out by treatment with benzene (40 ml), sat. aq. NaCl–10% aq. $KHCO_3$ (60 ml + 20 ml), and sat. aq. NaCl (80 ml). The benzene layer was dried over $MgSO_4$, filtered, and evaporated to dryness. Acetyl chloride (13.2 mmol) was added dropwise to an ice-cooled solution of the resultant residual oil in pyridine (5 ml). The mixture was stirred at the same temperature for 1.5 h under a nitrogen atmosphere and diluted with dichloromethane (40 ml). The solution was washed with 15% aq. HCl (20 ml), sat. aq. NaCl–10% aq. $KHCO_3$ (60 ml + 20 ml) and sat. aq. NaCl (80 ml), and then dried over $MgSO_4$. After removal of the dichloromethane, the residual oil was submitted to SGCC using hexane–ethyl acetate (1 : 3) as an eluent in the cases of all dipolarophiles.

In entry 3, two regioisomers **4a** and **4b** were isolated. Compound **4a** was purified by bulb-to-bulb distillation for elemental analysis. **4a**: bp 250 °C (0.5 mmHg). IR ν_{max}^{neat} cm^{-1}: 1741 (CO), 1660 (NCO). MS (*m/z*): 363 (M^+). *Anal.* Calcd for $C_{18}H_{21}NO_7$: C, 59.49; H, 5.83; N, 3.86. Found: C, 59.29; H, 5.83; N, 3.96. ^{1}H-NMR ($CDCl_3$) δ: 2.22 (3H, s, $COCH_3$), 3.35 (3H, s, CO_2CH_3), 3.70 (3H, s, CO_2CH_3), 3.74 (2H, s, 5-CH_2), 3.77 (3H, s, CO_2CH_3), 3.95—5.26 (2H, m, 2-CH, 3-CH), 7.16—7.35 (5H, m, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 22.2 (q, CO$\underline{C}H_3$), 52.2 (t, 5-C), 52.5 (q, $CO_2\underline{C}H_3$), 52.6 (q, $CO_2\underline{C}H_3 \times 2$), 53.4 (d, 3-C), 64.4 (d, 2-C), 64.6 (s, 4-C), 128.1, 128.4, 128.6, 137.0 (d, d, d, s, C_6H_5), 167.5 (s, CO), 169.1 (s, CO × 2), 170.8 (s, CO). Compound **4b** was recrystallized from EtOH. **4b**: mp 168—170 °C. IR ν_{max}^{KBr} cm^{-1}: 1739 (CO), 1659 (NCO). MS (*m/z*): 363 (M^+). *Anal.* Calcd for $C_{18}H_{21}NO_7$: C, 59.49; H, 5.83; N, 3.86. Found: C, 59.46; H, 5.84; N, 3.79. ^{1}H-NMR ($CDCl_3$) δ: 2.12 (3H, s, $COCH_3$), 3.41 (3H, s, CO_2CH_3), 3.69 (3H, s, CO_2CH_3), 3.73 (3H, s, CO_2CH_3), 3.62—4.69 (3H, m, 4-CH, 5-CH_2), 5.26 (1H, s, 2-CH), 7.27 (5H, br s, C_6H_5). ^{13}C-NMR ($CDCl_3$) δ: 21.9 (q, CO$\underline{C}H_3$), 49.1 (d, 4-C), 52.2 (t, 5-C), 52.5 (q, $CO_2\underline{C}H_3 \times 2$), 53.0 (q, $CO_2\underline{C}H_3$), 64.5 (d, 2-C), 66.4 (s, 3-C), 128.2, 128.5, 136.3 (d, d, s, C_6H_5), 167.5 (s, CO), 168.0 (s, CO), 169.1 (s, CO), 170.0 (s, CO).

In the cases of entries 4 and 5, the products were obtained as mixtures of two regioisomers (**5a**, **5b** and **6a**, **6b**, respectively). **5a** and **5b** (mixture): ^{1}H-NMR ($CDCl_3$) δ: 3.29 (s, OCH_3), 3.38 (s, OCH_3), 3.71 (s, $OCH_3 \times 2$), 3.74 (s, $OCH_3 \times 2$), 3.53—4.67 (m, 2-CH × 2, 3-CH of **5a**, 4-CH of **5b**, 5-$CH_2 \times 2$), 6.83—7.84 (m, $C_6H_5 \times 4$). **6a** and **6b** (mixture): bp 240 °C (0.5 mmHg) (by bulb-to-bulb distillation). IR ν_{max}^{neat} cm^{-1}: 1740 (CO), 1660 (NCO). MS (*m/z*): 305 (M^+). *Anal.* Calcd for $C_{16}H_{19}NO_5$: C, 62.94; H, 6.27; N, 4.59. Found: C, 62.73; H, 6.29; N, 4.66. ^{1}H-NMR ($CDCl_3$) δ: 1.96 (s, $COCH_3$ of **6b**), 2.10 (s, $COCH_3$ of **6a**), 3.23 (s, CO_2CH_3 of **6a**), 3.59 (s, CO_2CH_3 of **6a** and **6b**), 3.70 (s, CO_2CH_3 of **6b**), 3.53—4.37 (m, 2-CH × 2, 4-CH × 2, 5-$CH_2 \times 2$), 4.37—4.99 (m, 3-CH × 2), 7.16—7.38 (m, $C_6H_5 \times 2$). ^{13}C-NMR ($CDCl_3$) δ: 21.7 (q, CO$\underline{C}H_3 \times 2$), 43.7 (d, 3-C of **6b**), 45.5 (d, 4-C of **6a**), 49.3 (t, 5-C of **6b**), 49.6 (d, 3-C of **6a**), 50.1 (t, 5-C of **6a**), 51.3 (q, $CO_2\underline{C}H_3$ of **6b**), 51.5 (q, $CO_2\underline{C}H_3$ of **6a**), 51.9 (q, $CO_2\underline{C}H_3$ of **6b**), 52.2 (q, $CO_2\underline{C}H_3$ of **6a** and **6b**), 53.8 (d, 4-C of **6b**), 61.0 (d, 2-C of **6b**), 64.0 (d, 2-C of **6a**), 127.3, 127.9, 128.5, 129.0, 134.4, 137.6 (d, d, d, d, s, s, $C_6H_5 \times 2$), 168.8 (s, CO of **6b**), 169.1 (s, CO of **6a**), 170.8 (s, CO of **6a** and **6b**), 171.6 (s, CO of **6a**), 172.0 (s, CO of **6b**).

In entries 6—8, compounds **7**—**9** were obtained as mixtures of two stereoisomers at the 2-position. **7** (mixture): bp 210 °C (1 mmHg) (by bulb-to-bulb distillation). IR ν_{max}^{neat} cm^{-1}: 1740 (CO), 1659 (NCO). MS (*m/z*): 287 (M^+). *Anal.* Calcd for $C_{12}H_{17}NO_7$: C, 50.17; H, 5.97; N, 4.88. Found: C, 49.89; H, 5.97; N, 5.00. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (s, COC′H_3), 2.09 (s, $COCH_3$), 3.56—4.05 (m, 2-CH × 2, 3-CH × 2, 4-CH × 2, 5-$CH_2 \times 2$), 3.69, 3.74, 3.78 (s, s, s, $CO_2CH_3 \times 3$ and $CO_2C'H_3 \times 3$). ^{13}C-NMR ($CDCl_3$) δ: 21.8 (q, CO$\underline{C}H_3$), 22.0 (q, CO$\underline{C}'H_3$), 44.6 (d, 3-C), 45.8 (d, 3-C′), 47.8 (d, 4-C′), 47.9 (d, 4-C), 48.7 (t, 5-C), 49.0 (t, 5-C′), 52.4, 52.6, 52.7, 52.9 (q, q, q, q, $CO_2\underline{C}H_3 \times 3$ and

$CO_2C'H_3 \times 3$), 60.3 (d, 2-C), 61.1 (d, 2-C'), 169.1, 169.3, 169.4, 169.7, 170.7, 171.0, 171.8 (s, s, s, s, s, s, s, CO × 4 and C'O × 4). **8** (mixture): bp 250 °C (0.5 mmHg) (by bulb-to-bulb distillation). IR ν_{max}^{neat} cm^{-1}: 1740 (CO), 1641 (NCO). MS (*m/z*): 349 (M^+). *Anal.* Calcd for $C_{17}H_{19}NO_7$: C, 58.45; H, 5.48; N, 4.01. Found: C, 58.24; H, 5.49; N, 3.98. ^{1}H-NMR ($CDCl_3$) δ: 3.70, 3.72, 3.77 (s, s, s, $OCH_3 \times 6$), 3.12—4.20 (m, 2-CH × 2, 4-CH × 2, 5-$CH_2 \times 2$), 4.89—5.24 (m, 3-CH × 2), 7.44 (br s, $C_6H_5 \times 2$). ^{13}C-NMR ($CDCl_3$) δ: 52.5 (q, $OCH_3 \times 6$), 127.1, 127.3, 128.5, 130.6, 135.2 (d, d, d, d, s, $C_6H_5 \times 2$), 169.4, 169.7, 169.9, 170.6, 171.0, 171.3 (s, s, s, s, s, s, CO × 6). **9** (mixture): mp 100—102 °C (from EtOH). IR ν_{max}^{neat} cm^{-1}: 1759 (CO), 1740 (CO), 1722 (CO), 1659 (NCO). MS (*m/z*): 287 (M^+). *Anal.* Calcd for $C_{12}H_{17}NO_7$: C, 50.17; H, 5.97; N, 4.88. Found: C, 50.30; H, 5.96; N, 4.87. ^{1}H-NMR ($CDCl_3$) δ: 2.01 (s, $COC'H_3$), 2.11 (s, $COCH_3$), 3.34—4.06 (m, 2-CH × 2, 4-CH × 2, 5-$CH_2 \times 2$), 3.72, 3.74, 3.77 (s, s, s, $CO_2CH_3 \times 3$ and $CO_2C'H_3 \times 3$), 4.81 (br s, 3-CH × 2). ^{13}C-NMR ($CDCl_3$) δ: 21.7 (q, $COC'H_3$), 21.9 (q, $COCH_3$), 43.5 (d, 3-C'), 44.6 (d, 3-C), 47.5 (d + t, 4-C', 5-C'), 47.9 (d + t, 4-C, 5-C), 52.3, 52.6, 52.7, (q, q, q, $CO_2CH_3 \times 3$ and $CO_2C'H_3 \times 3$), 61.3 (d, 2-C), 62.1 (d, 2-C'), 169.5, 170.2, 170.5, 170.9 (s, s, s, s, CO × 4 and C'O × 4).

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Studies on Antitumor Agents. VI.[1] Syntheses and Antitumor Activities of Acyl Derivatives of 2′-Deoxy-5-trifluoromethyluridine

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Various *O*-acyl and *N*-acyl derivatives of 2′-deoxy-5-trifluoromethyluridine (F_3Thd) were synthesized; namely 5′-*O*-acyl, 3′,5′-di-*O*-acyl, N^3-acyl, 3′,5′-di-*O*-acetyl-N^3-acyl, 3′,5′-di-*O*-carbamoyl and 3′,5′-di-*O*-ethoxycarbonyl compounds. 5′-*O*-Acyl derivatives of 2′-deoxy-5-trifluoromethylcytidine were also synthesized.

The antitumor activities of these compounds against sarcoma 180 were examined by oral administration to mice. Among the 5′- and 3′,5′-diester compounds with aliphatic acids, the 5′-*O*-hexanoyl compound showed the highest activity. Full protection of the sugar moiety with aroyl or carbamoyl groups considerably decreased the activities, and those of the 3′,5′-di-*O*-*m*-fluorobenzoyl and 3′,5′-di-*O*-butylcarbamoyl compounds were the smallest. N^3-Benzoyl compounds were slightly more effective than F_3Thd but none of them showed higher activity than the effective *O*-acyl compounds. In the case of 5′-*O*-acylates of 2′-deoxy-5-trifluoromethylcytidine, the 5′-*O*-benzoyl compound showed the highest activity.

Keywords——2′-deoxy-5-trifluoromethyluridine; 2′-deoxy-5-trifluoromethylcytidine; acyl derivative; antitumor activity; pyrimidine nucleoside acylation; saponification

2′-Deoxy-5-trifluoromethyluridine (F_3Thd) was first synthesized by Heidelberger and his coworkers in 1962.[2] It has considerable biological activities in a number of systems.[3–7] For example, F_3Thd has been reported to be a potent compound in cancer chemotherapy. F_3Thd effectively inhibits the growth of various mammalian cells in culture.[6] The antitumor activities of F_3Thd against transplanted tumors such as adenocarcinoma 755 and L 1210 leukemia are equal to or higher than that of 2′-deoxy-5-fluorouridine (FUdR).[7]

However, F_3Thd showed unsatisfactory results in clinical cancer chemotherapy.[8] A disadvantage of F_3Thd for practical medicinal use is its short half-life in plasma after injection, mainly because of rapid metabolic degradation by thymidine phosphorylase to biologically inactive 5-trifluoromethyluracil (F_3T).[9] Thus, depot forms of F_3Thd which resist degradation by thymidine phosphorylase would be expected to maintain higher concentrations of F_3Thd in plasma and thus show greater antitumor activity *in vivo*.

If the depot form of F_3Thd is activated slowly after absorption, it is also expected to show low toxicity to the gastro-intestinal tract, minimizing the decrease of body weight,[10] and thus giving a high therapeutic index. Therefore, various acyl compounds of F_3Thd were synthesized and their antitumor activities were evaluated. Although similar attempts to enhance the activity of FUdR by *N*-benzoylation[10] or *O*-acylation[11] have been reported, it should be noted that 5-fluorouracil, the metabolite of FUdR, should itself be active, as well as FUdR. In the present case, F_3Thd is active only in the form of the nucleoside, and F_3T is inactive.[7]

TABLE I. Antitumor Effects of Acyl Derivatives of F_3Thd on Sarcoma 180

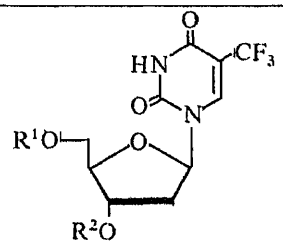
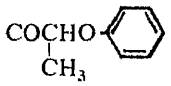
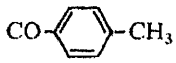
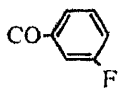
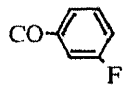
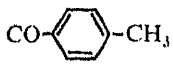
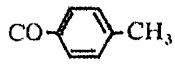
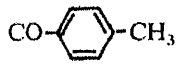
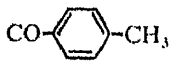
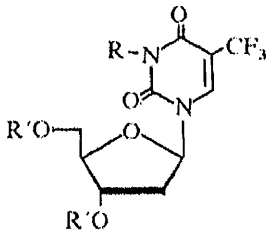
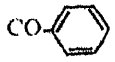
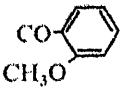
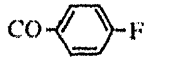
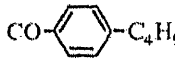
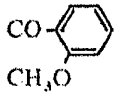
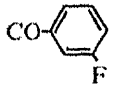
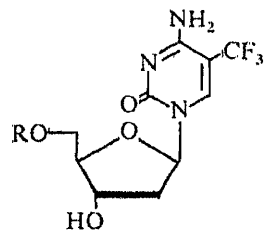
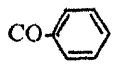
	Compd.	R^1	R^2	ED_{50} (mg/kg/d)
	1	$COCH_3$	H	31
	2	$CO(CH_2)_2CH_3$	H	27
	3	$CO(CH_2)_4CH_3$	H	19
	4	$CO(CH_2)_8CH_3$	H	58
	5	$CO(CH_2)_{14}CH_3$	H	40
	6		H	80
	7		H	52
	8	$COCH_3$	$COCH_3$	37
	9	$CO(CH_2)_2CH_3$	$CO(CH_2)_2CH_3$	31
	10	$CO(CH_2)_4CH_3$	$CO(CH_2)_4CH_3$	25
	11	$CO(CH_2)_8CH_3$	$CO(CH_2)_8CH_3$	38
	12	$CO(CH_2)_{14}CH_3$	$CO(CH_2)_{14}CH_3$	40
	13	$CO(CH_2)_{18}CH_3$	$CO(CH_2)_{18}CH_3$	40
	14			80
	15			80
	16	$CO(CH_2)_4CH_3$		80
	17		$CO(CH_2)_4CH_3$	57
	18	$CONH(CH_2)_3CH_3$	$CONH(CH_2)CH_3$	80
	19	$CO_2C_2H_5$	$CO_2C_2H_5$	29
	20		H	45
	21		H	38
	22		H	43
	23		H	34
	24		$COCH_3$	40
	25		$COCH_3$	32
	26	$CO(CH_2)_4CH_3$		33
	27	$CO(CH_2)_{14}CH_3$		80
	28			24
	F_3Thd			63

TABLE II. Physical Constants and Analytical Data for Acyl Derivatives of F_3Thd

Compound	mp (°C)	Recryst. solvent	Yield (%)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
1	167—168	EtOH	32	$C_{12}H_{13}F_3N_2O_6$	42.61 (42.61	3.87 3.91	8.28 8.35)
2	159—160	EtOH	35	$C_{14}H_{17}F_3N_2O_6$	45.91 (45.89	4.68 4.86	7.65 7.58)
3	158—159	EtOH	38	$C_{16}H_{21}F_3N_2O_6$	48.73 (48.60	5.37 5.71	7.10 7.20)
4	158—160	EtOH	46	$C_{20}H_{29}F_3N_2O_6$	53.33 (53.48	6.49 6.79	6.22 6.18)
5	136— (dec.)	EtOH	52	$C_{26}H_{41}F_3N_2O_6$	58.41 (58.90	7.73 7.92	5.24 4.81)
6	Foam		42	$C_{19}H_{19}F_3N_2O_6$	51.35 (51.09	4.30 4.44	6.30 6.37)
7	201—203	EtOH	62	$C_{18}H_{17}F_3N_2O_6$	52.17 (51.96	4.14 4.20	6.76 6.68)
8	139—140.5	EtOH	92	$C_{14}H_{15}F_3N_2O_6$	44.22 (44.30	3.98 3.98	7.37 7.33)
9	Oil		49	$C_{18}H_{23}F_3N_2O_7$	49.54 (49.39	5.31 5.42	6.42 6.20)
10	Oil		64	$C_{22}H_{31}F_3N_2O_7$	53.65 (53.70	6.34 6.42	5.69 5.72)
11	Oil		59	$C_{30}H_{47}F_3N_2O_7$	59.59 (59.39	7.83 7.98	4.63 4.59)
12	Oil		53	$C_{42}H_{71}F_3N_2O_7$	65.26 (65.67	9.26 9.90	3.62 3.55)
13	55—56	EtOH	80	$C_{50}H_{87}F_3N_2O_7$	68.38 (67.86	9.18 9.54	3.19 3.15)
14	196—197	EtOH	72	$C_{24}H_{17}F_3N_2O_7$	53.24 (53.14	3.16 3.06	5.17 5.18)
15	181—182	EtOH	68	$C_{26}H_{23}F_3N_2O_7$	58.65 (58.51	4.35 4.24	5.26 5.33)
16	Foam		66	$C_{24}H_{27}F_3N_2O_7$	56.25 (56.41	5.31 5.44	5.47 5.55)
17	Oil		57	$C_{24}H_{27}F_3N_2O_7$	56.25 (56.39	5.31 5.34	5.47 5.40)
18	184—185	EtOH–ether	20	$C_{20}H_{29}F_3N_2O_7$	48.58 (48.54	5.91 6.13	11.33 11.45)
19	Foam		36	$C_{16}H_{19}F_3N_2O_6$	43.64 (43.26	4.35 4.44	6.36 6.28)
20	155—156	Ether–pet. ether	56	$C_{17}H_{15}F_3N_2O_6$	51.01 (51.27	3.78 3.85	7.00 7.03)
21	Foam		42	$C_{18}H_{17}F_3N_2O_7$	50.24 (50.25	3.98 4.03	6.51 6.54)
22	Foam		46	$C_{17}H_{14}F_4N_2O_6$	48.81 (49.00	3.37 3.49	7.70 7.64)
23	Oil		62	$C_{21}H_{23}F_3N_2O_6$	55.26 (54.98	5.08 5.27	6.14 6.44)
24	Foam		59	$C_{22}H_{21}F_3N_2O_6$	51.36 (51.69	4.11 4.26	5.45 5.45)
25	Foam		45	$C_{21}H_{18}F_4N_2O_8$	50.21 (50.06	3.61 3.76	5.58 5.62)
26	199—202	EtOH	51	$C_{16}H_{22}F_3N_3O_5$	48.85 (48.79	5.64 5.66	10.68 10.93)
27	198—200	EtOH	32	$C_{26}H_{42}F_3N_3O_5$	58.52 (58.74	7.93 8.21	7.87 7.61)
28	202	EtOH	38	$C_{17}H_{16}F_3N_3O_5$	51.13 (51.58	4.04 4.02	10.52 10.31)

This paper describes the antitumor activities against sarcoma 180 of various acyl derivatives of F_3Thd on oral administration to mice.

Materials and Methods

Chemicals——Melting points were determined with a Yanagimoto MP-3 micro melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a JEOL LMN-FX 100 spectrometer (using tetramethylsilane as an internal standard).

5′-*O*-Acyl compounds (**1**—**7**), 3′,5′-di-*O*-acyl compounds (**8**—**17**) and the 3′,5′-di-*O*-ethoxycarbonyl compound (**19**) were synthesized by treatment of F_3Thd with a small excess of the appropriate acyl chloride in pyridine at suitable temperatures. 5′-*O*-Acyl derivatives of 2′-deoxy-5-trifluoromethylcytidine (**26**—**28**) were similarly prepared. 3′,5′-Di-*O*-Acyl compounds (**8**—**15**) can be obtained more easily by the reaction of F_3Thd and acid anhydride in acetonitrile using dimethylaminopyridine as a catalyst.[12] Reaction of F_3Thd and butyl isocyanate in dimethylformamide (DMF) gave 3′,5′-di-*O*-butylcarbamoyl-F_3Thd (**18**).

Reaction of F_3Thd and 1 eq of acyl chloride in the presence of triethylamine in dimethylacetamide (DMA) gave N^3-acyl compounds (**20**–**23**) selectively. The acylation in solvents other than DMA gave 5′-*O*-acyl compound. The procedure for the preparation of N^3-benzoyl-2′-deoxy-5-trifluoromethyluridine (**20**) is described as a typical example. Benzoyl chloride (11.2 g, 0.08 mol) was added to a solution of F_3Thd (24 g, 0.08 mol) and triethylamine (16 ml) in DMA and the mixture was stirred for 12 h at room temperature. The reaction mixture was filtered and the filtrate was concentrated. The residue was dissolved in $CHCl_3$ (150 ml), then water (15—20 ml) was added with stirring to afford a precipitate of **20**. The precipitate was recrystallized from ether–pet. ether, giving 18 g of **20** in 56% yield.

The structures of these compounds were confirmed by the elemental analyses as well as by ^{1}H-NMR measurements. Physical constants are listed in Table II.

Antitumor Test——Mice of the ICR strain (Japan Clea Inc., Tokyo, Japan) were used. Five-week-old male ICR mice were inoculated sobcutaneously in the axillary region with 5×10^6 sarcoma 180 cells and given text compounds orally once a day for 7 consecutive days beginning 24 h after inoculation of the tumor cells. Groups of seven mice were used for each dose and the test compounds were suspended in 0.5% carboxymethylcellulose (CMC) solution containing 0.1% Tween 80. On day 10, the tumors were excised and weighed. The inhibitory effects of test compounds were calculated from the ratio of the tumor weight in the test group to that in the control group.

Inhibitory effects of F_3Thd derivatives on the growth of solid tumor S 180 are shown in terms of the ED_{50} values in Table I.

Results and Discussion

Among the 5′- and 3′,5′-diester compounds with aliphatic acids, the 5′-*O*-hexanoyl compound (**3**) showed the highest activity and the ED_{50} value was one-third of that of F_3Thd. Although the effect of esterification of F_3Thd by aliphatic acids was similar to that in the case of FUdR,[11] the range of optimal chain length was more restricted.

ED_{50} values of other 5′-*O*-acyl compounds with aryl functions, such as the 2-phenoxypropionyl (**6**) or benzoyl (**7**) compound, were equal to that of F_3Thd. The full protection of the sugar moiety by aroyl or carbamoyl groups considerably decreased the activities and the 3′,5′-di-*O*-*m*-fluorobenzoyl (**14**) and 3′,5′-di-*O*-butylcarbamoyl (**18**) compounds showed no activity at the same dose as that of F_3Thd.

The rates of saponification of these inactive compounds to F_3Thd by mouse intestinal homogenate[13] were significantly slower than those of effective *O*-aliphatic acyl compounds. It is therefore assumed that the F_3Thd-releasing ability is the most important factor affecting the activity of *O*-acylated derivatives of F_3Thd. It has been reported that the enzymatic saponification rate of 5′-*O*-acyl FUdR was greater than that of 3′-*O*-acyl FUdR.[13a] This would mean that, in 3′,5′-di-*O*-acyl compounds of F_3Thd, the cleavage of the ester bond at the 3′-position may be the rate-limiting step in activation. In fact, compounds **14**, **15**, **16** and **18**, in which the 3′-position is modified by a benzoyl or carbamoyl group, showed the lowest activities.

N^3-Benzoyl compounds (**20**—**25**) were slightly more effective than F_3Thd but none of them showed higher activity than the effective *O*-acyl compounds. 2′-Deoxy-5-

trifluoromethylcytidine is assumed to be a prodrug of F_3Thd, since it is deaminated to F_3Thd *in vivo*.[14] However, the antitumor activity of the 5′-*O*-benzoyl compound was higher than that of the 5′-*O*-hexanoyl compound, in contrast to the result with F_3Thd. It should be noted that *O*-acyl compounds of 2′-deoxy-5-trifluoromethylcytidine may have different metabolic pathways from the *O*-acyl compounds of F_3Thd.

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Synthesis and Spasmolytic Activity of Aminoalkyl 2-Substituted-2-(1,2-benzisoxazol-3-yl)acetates

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Several aminoalkyl esters of 2-(1,2-benzisoxazol-3-yl)-2-cyclohexylacetic acid, 1-(1,2-benzisoxazol-3-yl)-1-cyclopentanecarboxylic acid, and 1-(1,2-benzisoxazol-3-yl)-1-cyclohexanecarboxylic acid and their quaternary ammonium salts were synthesized. The anticholinergic (anti-Ach) and musculotropic (anti-KCl) activities of these compounds were examined. Among them, 3-(*N*,*N*-diethylamino)-propyl 2-(1,2-benzisoxazol-3-yl)-2-cyclohexylacetate (**8b**) showed potent anti-Ach and anti-KCl activities.

Keywords——1,2-benzisoxazole derivative; aminoalkyl ester; musculotropic activity; antimuscarinic activity; antispasmodic; structure–activity relationship

Although a number of compounds having antimuscarinic activity are used clinically as antispasmodics, there is an unavoidable problem of their undesirable anticholinergic side effects such as blurred vision, dry mouth, and tachycardia. As one approach to reducing these side effects, Toson *et al.* mentioned that an antispasmodic agent with balanced antimuscarinic and papaverine-like musculotropic activities exerts antispasmodic action with less side effects than a pure antimuscarinic agent and with less cardiovascular effect than a typical smooth muscle relaxant such as papaverine.[1] They claimed that 2-diethylamino-1-methylethyl *cis*-2-cyclohexyl-2-hydroxy-1-cyclohexanecarboxylate (rociverine) is the first antispasmodic agent with balanced antimuscarinic activity (sometimes referred to as anticholinergic or anti-Ach activity) and musculotropic activity (sometimes referred to as papaverine-like or anti-KCl activity).

As a part of our search for new antispasmodic agents, we reported the syntheses and spasmolytic activities of 3-substituted-1,2-benzisoxazole derivatives in previous papers.[2] Since we were interested in agents having both antimuscarinic and musculotropic activities, and considering that rociverine has two cyclohexyl rings, we prepared aminoalkyl esters of 1,2-benzisoxazole-3-acetic acid having a cycloalkyl ring such as cyclohexyl, tetramethylene, and pentamethylene at the α-position in the hope of finding new antispasmodics having dual modes of action. This paper deals with the synthesis of these compounds and the results of their primary pharmacological evaluations.

Chemistry

The target esters (**8**, **9**, and **10**) were synthesized as shown in Chart 1. Methyl 1,2-benzisoxazole-3-acetate (**1**) was converted to **2** by treatment with sodium hydride (NaH) (1 eq) in dimethylformamide, followed by alkylation with cyclohexyl iodide (1.9 eq) in 33% yield. This reaction also afforded a small amount of 2*H*-azirine derivative as a by-product.[3] The carboxylic acid (**5**) was prepared by hydrolysis of **2** with sodium hydroxide (NaOH) (1.5 eq) in aqueous ethanol. The carboxylic acids (**6** and **7**) were also obtained on treatment of **1** with

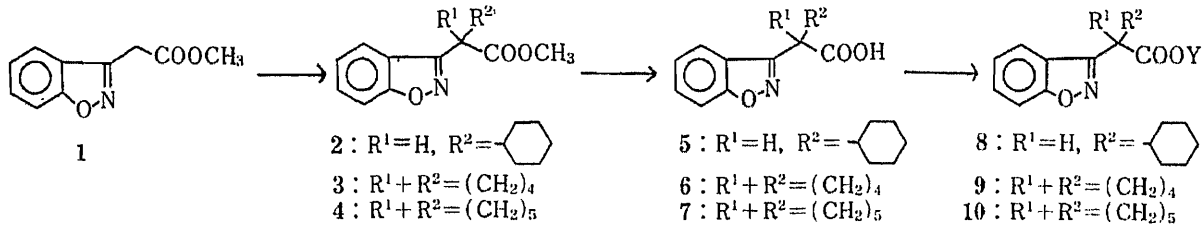
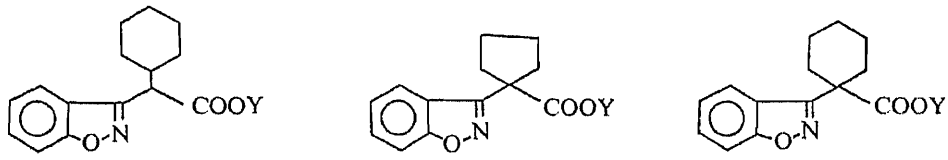


Chart I

TABLE I. Pharmacological Data



8 **9** **10**

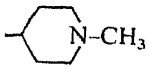
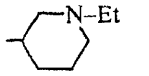
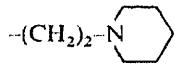
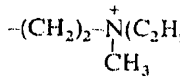
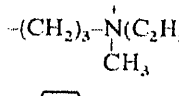
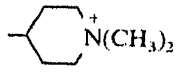
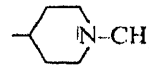
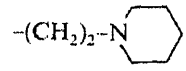
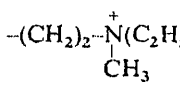
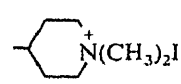
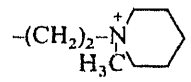
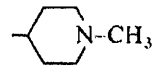
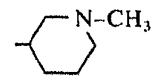
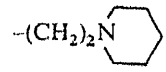
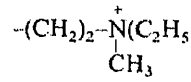
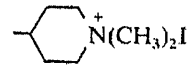
Compound No.	Y	anti-Ach[a] ID_{50} (mM)	anti-KCl[b] ID_{50} (mM)
8a	$-(CH_2)_2-N(C_2H_5)_2$	2.7×10^{-4}	2.3×10^{-3}
8b	$-(CH_2)_3-N(C_2H_5)_2$	3.5×10^{-4}	3.8×10^{-4}
8c	$-(CH_2)_4-N(C_2H_5)_2$	1.0×10^{-3}	NT
8d	$-CH(CH_3)-CH_2-N(C_2H_5)_2$	9.3×10^{-4}	5.5×10^{-3}
8e	$-CH(CH_3)-(CH_2)_3-N(C_2H_5)_2$	1.4×10^{-3}	NT
8f		8.4×10^{-5}	1.0×10^{-2}
8g		1.0×10^{-4}	NT
8h		8.9×10^{-4}	5.9×10^{-3}
8i		5.2×10^{-4}	NT
8j		4.0×10^{-4}	$>5.0\times10^{-2}$
8k		2.3×10^{-5}	$>5.0\times10^{-2}$
9a	$-(CH_2)_2-N(C_2H_5)_2$	8.8×10^{-4}	NT
9b	$-(CH_2)_3-N(C_2H_5)_2$	7.8×10^{-4}	3.8×10^{-2}
9c	$-CH(CH_3)-CH_2-N(C_2H_5)_2$	3.3×10^{-3}	NT
9d		1.1×10^{-4}	$>1.0\times10^{-1}$
9e		5.0×10^{-4}	$>5.0\times10^{-2}$
9f		2.3×10^{-4}	$>1.0\times10^{-1}$
9g		5.3×10^{-5}	NT

TABLE I. (continued)

Compound No.	Y	anti-Ach[a] ID_{50} (mM)	anti-KCl[b] ID_{50} (mM)
9h		6.6×10^{-4}	NT
10a	$-(CH_2)_2-N(C_2H_5)_2$	8.7×10^{-4}	NT
10b	$-(CH_2)_3N(C_2H_5)_2$	5.4×10^{-4}	8.5×10^{-3}
10c	$-CH(CH_3)-CH_2N(C_2H_5)_2$	3.9×10^{-4}	NT
10d		8.7×10^{-5}	$>5.0\times10^{-2}$
10e		2.1×10^{-3}	NT
10f		5.9×10^{-4}	NT
10g		3.7×10^{-4}	$>1.0\times10^{-1}$
10h		2.9×10^{-5}	$>1.0\times10^{-1}$
Scopolamine-*N*-butyl bromide		2.0×10^{-4}	$>5.0\times10^{-1}$
Papaverine hydrochloride		3.5×10^{-2}	3.5×10^{-3}

a) Concentration required to produce 50% inhibition of the response induced by acetylcholine (1.1×10^{-4} mM). *b*) Concentration required to produce 50% inhibition of the response induced by potassium chloride (40 mM). NT: Not tested.

NaH (2.2 eq) and 1,4-dibromobutane or 1,5-dibromopentane (1.2 eq) in dimethyl sulfoxide, followed by hydrolysis of the resulting esters **3** and **4** with an equimolar amount of NaOH in aqueous ethanol, in 76% and 50% yields, respectively. The aminoalkyl esters **8** were obtained by esterification of the aminoalkanols with **5** using *p*-toluenesulfonyl chloride in toluene. The esters **9** and **10** were obtained by reaction of the corresponding acid chloride with aminoalkanols. Quaternization was carried out in acetone using methyl iodide. The yields and physicochemical properties of **8**, **9**, and **10** are summarized in Table II.

Pharmacological Results and Discussion

The anticholinergic (anti-Ach) activity of the esters (**8**, **9**, and **10**) was examined by measuring the inhibitory effect on the response of isolated guinea pig ileum to acetylcholine (Ach) according to the method previously reported.[2a] The musculotropic (anti-KCl) activity of several compounds which showed relatively potent anti-Ach activity was measured in terms of the inhibitory effect on the response induced by potassium chloride using isolated guinea pig taenia coli. Based on the anti-Ach and anti-KCl activities shown in Table I, some structure–activity relationships of these esters may be summarized as follows. (a) When the aminoalkanol part of the esters was fixed, anti-Ach activity increased according to the kind of α-substituent in the order $-(CH_2)_4- < -(CH_2)_5- <$ cyclohexyl. (b) The order of increasing anti-Ach activity for the aminoalkanol part was as follows: linear aminoalkanol < cyclic aminoalkanol < quaternized cyclic aminoalkanol. However, the reverse order was found for the anti-KCl activity. As shown in Table I, several compounds showed remarkable anti-Ach activity. Among these esters, quaternary ammonium salts (**8k** and **10h**) exhibited about ten times more potency than scopolamine-*N*-butyl bromide, but these salts lacked musculotropic activity. The initially designed linear aminoalkanol esters having a cyclohexyl substituent

showed not only a significant antimuscarinic activity, but also a potent musculotropic activity. Of these compounds, the anti-KCl activities of **8a** or **8b** were equal to or ten times stronger than that of papaverine hydrochloride, respectively. Compound **8b** is of interest as a candidate antispasmodic having potent dual effects. An *in vivo* investigation of these compounds is in progress.

Experimental

Chemistry

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken with a Hitachi 260-10 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Varian EM-360 instrument (60 MHz) with tetramethylsilane as an internal standard. Abbreviations used: s, singlet; d, doublet; m, multiplet. Mass spectra (MS) were recorded with a Hitachi RMU-6L spectrometer. For thin layer chromatography (TLC), Merck precoated silica gel $60F_{254}$ plates were used. Organic extracts were dried over anhydrous Na_2SO_4.

Methyl α-Cyclohexyl-1,2-benzisoxazole-3-acetate (2)——NaH (60% dispersion in mineral oil, 0.5 g, 10 mmol) was added portionwise to a solution of **1** (1.9 g, 10 mmol) and cyclohexyl iodide (4 g, 19 mmol) in dry dimethylformamide (15 ml) under ice cooling, and the mixture was stirred at room temperature for 0.5 h. Water (50 ml) was added to the reaction mixture and then the mixture was acidified with dil.HCl (pH 5—6). The solution was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water and dried. After removal of the solvent, the residue was chromatographed on silica gel with hexane–$CHCl_3$ (1 : 1) as an eluent. The fractions were monitored by TLC developed with $CHCl_3$. Concentration of the fractions showing a spot at *Rf* 0.7 gave **2** (0.89 g, 33%) as a colorless oil. MS *m/z*: 273 (M^+). IR cm^{-1}: $\nu_{C=O}$ 1735 (film). ^{1}H-NMR ($CDCl_3$) δ: 0.7—2.6 (11H, m), 3.72 (3H, s), 3.98 (1H, d, J=10 Hz), 7.2—8.2 (4H, m).

The next fractions (*Rf* 0.6) afforded the starting material (0.58 g, 31%) and the following fractions (*Rf* 0.2) afforded methyl 3-(2-hydroxyphenyl)-2-cyclohexyl-2*H*-azirine-2-carboxylate (0.07 g, 2.6%) as a by-product.[3]

α-Cyclohexyl-1,2-benzisoxazole-3-acetic Acid (5)——A solution of **2** (6.3 g, 23 mmol) and NaOH (1.4 g, 35 mmol) in water (8 ml) and EtOH (70 ml) was stirred at 60 °C for 0.5 h. After evaporation of the solvent, dil. HCl (5%, 100 ml) was added to the residue. The mixture was extracted with ethyl acetate. The organic layer was dried and evaporated. Recrystallization of the residue from ethyl acetate–hexane gave **5** as colorless needles (5 g, 84%), mp 135—137 °C. *Anal.* Calcd for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.07; H, 6.56; N, 5.37.

1-(1,2-Benzisoxazol-3-yl)-1-cyclopentanecarboxylic Acid (6)——NaH (6.9 g, 173 mmol) was added portionwise to a solution of **1** (15 g, 79 mmol) in dimethyl sulfoxide (120 ml). The mixture was stirred for 0.5 h at room temperature, then 1,4-dibromobutane (20.4 g, 98 mmol) was added to the mixture, and the solution was stirred for 4 h at room temperature. Water (100 ml) and conc. HCl (8 ml) were added to the solution and the mixture was extracted with toluene. The toluene layer was washed with water and dried. After removal of the solvent, the residue was chromatographed on silica gel. Elution with toluene gave **3** as a colorless oil (18 g). IR cm^{-1}: $\nu_{C=O}$ 1725 (film). ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.2 (4H, m), 2.2—2.9 (4H, m), 3.67 (3H, s), 7.1—7.8 (4H, m). A solution of **3** (18 g, 73 mmol) and NaOH (3 g, 75 mmol) in water (12 ml) and EtOH (150 ml) was stirred for 40 min at 60 °C. After evaporation of the solvent, dil. HCl (5%, 100 ml) was added to the residue. The mixture was extracted with ethyl acetate. The organic layer was dried and evaporated. The residue was recrystallized from ethyl acetate–hexane to give **6** as colorless needles (13.8 g, 76% from **1**), mp 124—126 °C. *Anal.* Calcd for $C_{13}H_{13}NO_3$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.50; H, 5.93; N, 6.17. IR cm^{-1}: $\nu_{C=O}$ 1680—1700 (KBr).

1-(1,2-Benzisoxazol-3-yl)-1-cyclohexanecarboxylic Acid (7)——Compound **7** was prepared from **1** (15 g, 79 mmol) and 1,5-dibromopentane (21.6 g, 94 mmol) by a procedure similar to that described for the preparation of **6**. **4**: A colorless oil (15 g). IR cm^{-1}: $\nu_{C=O}$ 1720 (film). ^{1}H-NMR ($CDCl_3$) δ: 1.3—2.7 (10H, m), 3.67 (3H, s), 7.1—7.9 (4H, m). **7**: Colorless needles from toluene–hexane (9.63 g, 50% from **1**), mp 116—118 °C. *Anal.* Calcd for $C_{14}H_{15}NO_3$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.52; H, 6.36; N, 5.42. IR cm^{-1}: $\nu_{C=O}$ 1690 (KBr).

General Procedure for Preparation of the Esters (8a—h)——*p*-Toluenesulfonyl chloride (0.8 g, 4.2 mmol) was added to a solution of **5** (1 g, 3.9 mmol) in dry toluene (30 ml) and triethylamine (0.6 ml). The mixture was stirred for 1 h at room temperature and an appropriate aminoalkanol (1.2 eq) was added to the solution. The mixture was stirred for 3 h at 70 °C. The reaction mixture was diluted with toluene and washed with an aqueous solution of Na_2CO_3. The toluene layer was extracted with dil. HCl. The aqueous layer was made alkaline with conc. NH_4OH under ice cooling and extracted with toluene. The toluene layer was dried and evaporated to give the free base of **8** as an oil. The oily base was converted to the hydrobromide or oxalate, which was recrystallized from the solvent shown in Table II to give **8a—h**. The results are summarized in Table II.

General Procedure for Preparation of the Esters (9a—e, 10a—f)——A solution of **6** (1 g, 4.3 mmol) or **7** (1 g, 4.1 mmol) and thionyl chloride (5 eq) in dry toluene (30 ml) was stirred for 1 h at 100 °C. After evaporation of the

TABLE II. Physicochemical Properties of the Esters **8**, **9**, and **10**

Compd. No.	Salt	mp (°C) (Recrystn. solvent[a])	Yield (%)	Formula	Analysis (%) Calcd (Found)			
					C	H	Br or I	N
8a	HBr	133—135 (A–E)	77	$C_{21}H_{30}N_2O_3 \cdot HBr$	57.40 (57.39	7.11 7.15	18.19 18.06	6.37 6.61)
8b	HBr	128—130 (A–E)	88	$C_{22}H_{32}N_2O_3 \cdot HBr$	58.28 (58.46	7.34 7.36	17.62 17.92	6.18 5.90)
8c	HBr	119—121 (A–E)	47	$C_{23}H_{34}N_2O_3 \cdot HBr$	59.10 (58.86	7.55 7.32	17.09 17.09	5.99 5.93)
8d	HBr	136—138 (A–E)	57	$C_{22}H_{32}N_2O_3 \cdot HBr$	58.28 (58.47	7.34 7.43	17.62 17.59	6.18 6.16)
8e	HBr	153—156 (A–E)	78	$C_{24}H_{36}N_2O_3 \cdot HBr$	59.87 (59.57	7.75 7.84	16.60 16.39	5.82 5.73)
8f	HBr	199—201 (A–E)	73	$C_{21}H_{28}N_2O_3 \cdot HBr \cdot 1/3\,H_2O$[b]	56.89 (56.87	6.74 6.60	18.02 18.04	6.32 6.46)
8g	Ox	103—106 (A–E)	11	$C_{22}H_{30}N_2O_3 \cdot C_2H_2O_4 \cdot 1/2\,H_2O$	61.39 (61.29	7.09 7.11		5.97 5.96)
8h	Ox	179—181 (A–E)	56	$C_{22}H_{30}N_2O_3 \cdot C_2H_2O_4$	62.59 (62.37	7.00 7.07		6.08 6.12)
8i		118—124 (A–E)	81[c]	$C_{22}H_{33}IN_2O_3$	52.80 (52.88	6.65 6.54	25.36 25.58	5.60 5.62)
8j		88—91 (A)	35[c]	$C_{23}H_{35}IN_2O_3 \cdot 3/4\,H_2O$	52.32 (52.16	6.87 6.82	24.04 23.91	5.32 5.32)
8k		103—104 (THF)	20[c]	$C_{22}H_{31}IN_2O_3 \cdot 1/3\,C_4H_8O$[d]	53.61 (53.34	6.51 6.63	24.31 24.04	5.37 5.10)
9a	HBr	150—151 (A–E)	73	$C_{19}H_{26}N_2O_3 \cdot HBr$	55.48 (55.50	6.62 6.75	19.43 19.69	6.81 6.60)
9b	HBr	124—126 (A–E)	30	$C_{20}H_{28}N_2O_3 \cdot HBr$	56.47 (56.25	6.87 6.90	18.79 19.07	6.59 6.57)
9c	HBr	139—141 (A–E)	44	$C_{20}H_{28}N_2O_3 \cdot HBr$	56.47 (56.74	6.87 6.75	18.79 18.98	6.59 6.88)
9d	Ox	157—160 (A–E)	23	$C_{19}H_{24}N_2O_3 \cdot C_2H_2O_4 \cdot 3/4\,H_2O$	58.39 (58.59	6.42 6.19		6.48 6.34)
9e	HBr	163—165 (A–E)	78	$C_{20}H_{26}N_2O_3 \cdot HBr$	56.74 (56.85	6.43 6.68	18.87 18.68	6.62 6.65)
9f		138—141 (A–E)	86[c]	$C_{20}H_{29}IN_2O_3$	50.85 (50.82	6.19 6.16	26.87 26.60	5.93 6.00)
9g		173—178 (A–E)	80[c]	$C_{20}H_{27}IN_2O_3$	51.07 (50.81	5.79 5.82	26.98 27.01	5.96 6.04)
9h		146—149 (Ac–E)	76[c]	$C_{21}H_{29}IN_2O_3$	52.07 (52.35	6.03 6.10	26.20 26.47	5.78 5.77)
10a	HBr	177—179 (A–E)	64	$C_{20}H_{28}N_2O_3 \cdot HBr$	56.47 (56.40	6.87 6.94	18.79 18.95	6.58 6.59)
10b	HBr	122—124 (A–E)	33	$C_{21}H_{30}N_2O_3 \cdot HBr \cdot 1/4\,H_2O$	56.88 (56.91	7.16 7.13		6.32 6.27)
10c	HBr	150—152 (A–E)	59	$C_{21}H_{30}N_2O_3 \cdot HBr$	57.40 (57.43	7.11 7.04	18.19 18.31	6.37 6.64)
10d	Ox	126—130 (A–E)	43	$C_{20}H_{26}N_2O_3 \cdot C_2H_2O_4 \cdot 3/2\,H_2O$	57.50 (57.34	6.79 6.58		6.10 6.09)
10e	Ox	156—158 (A–E)	14	$C_{20}H_{26}N_2O_3 \cdot C_2H_2O_4$	61.10 (60.84	6.53 6.69		6.48 6.61)
10f	HBr	188—190 (A–E)	22	$C_{21}H_{28}N_2O_3 \cdot HBr$	57.67 (57.82	6.68 6.61	18.27 18.26	6.40 6.21)
10g		130—133 (A–E)	78[c]	$C_{21}H_{31}IN_2O_3$	51.86 (51.56	6.42 6.41	26.09 26.35	5.76 5.79)
10h		239—242 (A–W)	55[c]	$C_{21}H_{29}IN_2O_3$	52.07 (51.81	6.03 5.84	26.20 26.04	5.78 5.77)

a) A = EtOH, E = ether, Ac = acetone, THF = tetrahydrofuran, W = water. Ox = oxalate. *b*) Compounds obtained as hydrates showed the presence of water in their IR and ^{1}H-NMR spectra even after being dried at 80—90 °C for 6—8 h under reduced pressure. *c*) Yields of quaternization. *d*) Tetrahydrofuran.

solvent, dry toluene (10 ml), triethylamine (5 eq) and an appropriate aminoalkanol (1.2 eq) were added to the residue. The mixture was stirred at 100 °C for 1 h and worked up in a similar manner to that used for the preparation of **8a—h**. Recrystallization of the hydrobromide or oxalate from the solvent shown in Table II gave **9a—e** and **10a—f**. The results are summarized in Table II.

General Procedure for Preparation of the Quaternary Salts (8i—k, 9f—h, 10g—h)——A solution of the free base of **8**, **9** or **10** and an excess of methyl iodide in acetone was stirred at room temperature for 2 d. After evaporation of the solvent, the residue was recrystallized from the solvent shown in Table II to give **8i—k**, **9f—h**, and **10g—h**. The results are summarized in Table II.

Pharmacology

Method for Assaying the Musculotropic (anti-KCl) Activity——From male, Hartley strain guinea pigs, weighing 300—350 g, sections of the taenia-coli about 3 cm in length were prepared and mounted in physiological solution kept at 35 °C and oxygenated with 95% O_2–5% CO_2. The physiological solution used had the following composition (mM): NaCl, 137; KCl, 2.7; $MgCl_2$, 1.0; $CaCl_2$, 1.80; NaH_2PO_4, 0.42; $NaHCO_3$, 11.9; glucose, 5.55. The contractile responses were recorded isometrically. Each preparation was adjusted to a tension of 1 g and was allowed to equilibrate for 30 min. Test compounds were applied 5 min before adding KCl (40 mM). Each compound was tested at three different concentrations. On the basis of the percent inhibition at each concentration, the ID_{50} value was determined by the usual graphical method. The results are shown in Table I.

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Synthesis of 6β-[^{131}I](2'-Iodo)ethyl-19-norcholest-5(10)-en-3β-ol and Its Tissue Distribution in Mice

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Iodine-131 labeled 6β-(2'-iodo)ethyl-19-norcholest-5(10)-en-3β-ol has been prepared by isotopic exchange using the hydrothermal melt method. A tissue distribution study in mice showed a rather selective localization of radioactivity in the adrenal gland, but the adrenal-to-liver concentration ratios were much lower than those previously observed with the iodine-131 labeled 6β-iodomethyl analog.

Keywords——6β-[^{131}I](2'-iodo)ethyl-19-norcholest-5(10)-en-3β-ol; isotopic exchange; tissue distribution; adrenal gland

Several radiolabeled steroids based on the cholesterol nucleus have been developed and evaluated as potential adrenocortical imaging agents.[1] These include 19-[^{131}I]iodocholest-5-en-3β-ol (CL-19-^{131}I),[2] 6β-[^{131}I]iodomethyl-19-norcholest-5(10)-en-3β-ol (NCL-6-^{131}I),[3] 6-[^{131}I]iodocholesterol,[4] and 6β-(methyl-[^{75}Se]seleno)methyl-19-norcholest-5(10)-en-3β-ol.[5] NCL-6-^{131}I has found wide clinical use in diagnosing adrenal disease. However, the adrenal can not be imaged until several days after injection, since sufficient hepatic clearance is necessary to obtain a favorable target-to-background radioactivity ratio. In earlier publications we and others have described the preparation and tissue uptake characteristics of structurally modified cholesterol and 19-norcholesterol analogs labeled with various radionuclides,[6-9] with the aim of determining structural factors which may affect adrenal affinity and accelerate the parameters of imaging. As part of a continuing effort to assess the influence of lengthening the carbon chain at the C_6 position in the 19-norcholesterol structure on adrenal specificity, the synthesis of 6β-[^{131}I] (2'-iodo)ethyl-19-norcholest-5(10)-en-3β-ol (NCL-6-^{131}I-E) and the result of a preliminary study of its biodistribution in mice are described in this paper.

Radioiodination was readily achieved by isotopic exchange of NCL-6-I-E with Na^{131}I in the hydrothermal melt state in a manner similar to that previously employed for 6-[^{131}I]iodocholesterol.[10] The exchange reaction at 150 °C proceeded rapidly and the yield reached 95% at 30 min. Since the product at this point was contaminated by only 5% of inorganic radioiodide, the resulting ethanol solution was used for the subsequent animal study; no effort was made to remove the free iodide.

The distribution of radioactivity in the tissues of mice was determined at time intervals from 1 to 7 d following i.v. administration of NCL-6-^{131}I-E, and the result is summarized in Table I. NCL-6-^{131}I-E showed selective accumulation in the adrenal gland (maximum at 3 d) persisting throughout the 7 d of the study; the average concentration in the adrenal gland ranged from 170 to 218% dose/g. This adrenal affinity is slightly greater than that obtained with NCL-6-^{131}I in rats,[11] although the concentration of radioactivity in the adrenal of mice

TABLE I. Tissue Distribution of 6β-[^{131}I](2′-Iodo)ethyl-19-norcholest-5(10)-en-3β-ol at Various Time Intervals in Mice[a]

Tissue	Days after administration			
	1	3	5	7
Adrenal	192±14	218±26	190±6	170±21
Liver	13.8±1.8 (14)[b]	6.4±0.9 (34)[b]	3.9±0.4 (48)[b]	1.9±0.3 (89)[b]
Kidney	4.8±0.4 (40)	3.3±0.3 (66)	1.6±0.4 (118)	0.7±0.1 (242)
Lung	9.8±0.5 (19)	4.6±0.3 (47)	2.1±0.2 (90)	0.7±0.1 (242)
Spleen	16.1±0.7 (12)	14.1±0.6 (15)	4.5±0.3 (42)	0.9±0.4 (188)
Heart	3.5±0.3 (54)	3.3±0.3 (66)	2.6±0.1 (73)	0.6±0.1 (283)
Blood	4.1±0.3 (47)	1.6±0.2 (136)	0.6±0.1 (316)	0.3±0.0 (566)

a) Number of mice; $n=3$. Values represent mean percent administered dose per gram of tissue with the standard deviation of mean. *b*) Values in parentheses represent adrenal-to-tissue concentration ratios.

after injection of NCL-6-^{131}I has not been described in the literature. On the other hand, the concentration of radioactivity in the liver is nearly an order of magnitude higher than that for either NCL-6-^{131}I or CL-19-^{131}I in rats over a 7-d period. Furthermore, the relatively high level of uptake observed in other tissues at 1 d diminished very slowly. Because of this slow hepatic clearance of radioactivity, the adrenal-to-liver concentration ratios for NCL-6-^{131}I-E were 48 and 89 at 5 and 7 d, respectively, markedly lower than those observed with NCL-6-^{131}I in mice.[12] Thus, the introduction of an iodoethyl group in place of the iodomethyl group in NCL-6-I gives a slightly higher adrenal uptake but does considerably raise the liver uptake. As a result of the above preliminary study, we considered that NCL-6-^{131}I-E does not appear to warrant further investigation as an adrenal imaging agent.

Experimental

6β-(2′-Iodo)ethyl-19-norcholest-5(10)-en-3β-ol (NCL-6-I-E) was synthesized by the method previously reported.[13] Aqueous carrier-free Na^{131}I was provided by the Institute of Atomic Energy, Beijing.

The steroid (0.2 mg) and dry ethanol (100 μl) were placed in a vial containing aqueous Na^{131}I (13.3 μl, 140 – 150 μCi). The vial was sealed and the exchange reaction was allowed to proceed at 150 °C in an oil bath. Thin layer chromatography (TLC) (silica gel; solvent system, benzene–ethyl acetate (9 : 1 v/v) indicated the formation of NCL-6-^{131}I-E ($Rf=0.64$), which comigrated with an authentic nonradioactive sample. The labeling yield reached 95% at 30 min as determined from the radioactive peaks shown by radiochromatogram scanning. The resulting ethanolic solution was directly used for the animal study without any treatment because the product at this point was contaminated by only 5% of inorganic radioiodide ($Rf=0$) as determined by TLC. The specific activity was estimated to be 669.7 μCi/mg. The change of purity of the labeled steroid solution during storage at 20 °C was checked by TLC, showing that only 5—6% of deiodination occurred in 14 d.

The solution was administered to male mice (20—25 g) by injection through the tail vein, each mouse receiving dose of 5—6 μCi of NCL-6-^{131}I-E. The mice were sacrificed at 1, 3, 5 and 7 d after injection, three at each time point. The major organs including adrenals were excised, weighed, placed in small counting vials and counted in a gamma-well counter. After correction for radioactivity decay, the concentration in each tissue was expressed as a percentage of injected dose per gram.

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Interaction between 7-Anilino-4-methylcoumarin-3-acetic Acid and Human Serum Albumin

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The fluorescence characteristics of 7-anilino-4-methylcoumarin-3-acetic acid (AMCA) were examined in human serum albumin (HSA) solution. The fluorescence of AMCA was significantly enhanced in the presence of HSA. Scatchard plots and continuous variation plots indicated that one molecule of AMCA binds to HSA. The binding parameters of AMCA were also estimated. Specific binding sites on HSA were examined in connection with Sudlow's classification. The primary binding site of AMCA was found to be site III (digitoxin site) from the results of competitive displacement studies with several drugs. Furthermore, it was suggested from the fluorescence behavior in the presence of tryptophan-, tyrosine-, and lysine-modified HSAs that the binding site of AMCA lies in a region near a tryptophan residue on HSA. The present data indiate that AMCA is useful as a site III marker.

Keywords——7-anilino-4-methylcoumarin-3-acetic acid; human serum albumin; modified serum albumin; protein binding fluorescence probe; coumarincarboxylic acid binding site

In the previous paper[2] we reported that 7-anilino-4-methylcoumarin-3-acetic acid (AMCA) is useful as a fluorescence probe for assessing the binding of steroids, including estrogens, to α-globulin.

The present paper deals with the interaction of AMCA with human serum albumin (HSA) to examine further the utility of AMCA as a probe in drug–protein binding studies.

Experimental

Materials——AMCA was synthesized according to the method described in our earlier paper[3] and purified as follows. Crude AMCA was dissolved in 5% aqueous NaOH and neutralized with 1 N HCl. Yellow precipitates obtained was well washed with water, dried and repeatedly recrystallized from EtOH until no spot of 7-anilino-4-methyl-3-acetate was observed on a thin layer chromatographic plate (benzene : ethyl acetate = 4 : 1, v/v). HSA (Lot. No. A-2386) was purchased from Sigma Chemical Company, St. Louis, Mo. Tryptophan (Trp)-, tyrosine (Tyr)-, and lysine (Lys)-modified HSAs were prepared by known methods using 2-hydroxy-5-nitrobenzyl bromide,[4] *N*-acetylimidazole,[5] and pyridoxal-5′-phosphate,[6] respectively. The degree of modification was estimated by measuring the ultraviolet absorbance of modified HSAs and control samples according to the cited methods. Phenylbutazone (Ciba-Geigy Co.), ibuprofen (Kakenyaku Kako Co.), and digitoxin (Mitsubishi Yuka Pharmaceutical Co.) were gifts of the manufacturers. All other chemicals used were of reagent grade, and deionized and distilled water was used throughout.

Apparatus and Methods——Fluorescence spectra were measured with a Hitachi 650-60 fluorescence spectrophotometer. All HSA and AMCA solutions were prepared in 0.1 M phosphate buffer of pH 7.4 at 25±1 °C. HSA solutions of 1.0×10^{-6}—1.0×10^{-4} M (M.W. 69000) were used, together with a wide concentration range of 0.5×10^{-7}—1.0×10^{-5} M AMCA.

Fluorometric Titrations: HSA solution of appropriate concentration was titrated by successive additions of a solution of AMCA (to give a final concentration of 1.0×10^{-5} M) and the fluorescence intensity was measured (excitation at 370 nm and emission at 460 nm).

Data Treatment: The fraction of AMCA, X, was usually determined by using Eq. 1,

$$X=\frac{F_p-F_a}{F_b-F_a} \tag{1}$$

where F_p and F_a are the fluorescence intensities of a given concentration of AMCA in a solution of low HSA concentration and in a solution without HSA, and F_b is the fluorescence of the same concentration of fully bound AMCA. To determine the values of F_b for a given concentration of AMCA, fluorescence titrations were carried out at several HSA concentrations. F_b is taken to be the fluorescence intensity of the substrate in the presence of excess HSA. After values for the fraction of bound AMCA had been found for all points along the titration curve, the results were plotted according to the Scatchard equation[7]:

$$r/D_f=nK-rK \tag{2}$$

where r is the number of mol of AMCA bound per mol of protein, n is the number of binding sites, K is the binding constant and D_f is the concentration of free AMCA.

Results and Discussion

The fluorescence behavior of AMCA in the presence of HSA in pH 7.4 phosphate buffer was first examined (Fig. 1). As shown in Fig. 1, the fluorescence intensity of AMCA was greatly enhanced with increasing HSA concentration and the emission maximum was slightly shifted toward the blue. This suggests that AMCA binds to HSA, forming a complex. To estimate the binding parameters for AMCA–HSA interaction, fluorometric titration was done by the method described in the previous paper[2] (Fig. 2). The linearity of Scatchard plots for the AMCA–HSA system obtained from the titration curves in Fig. 2 indicates that AMCA binds to one class of sites on HSA (Fig. 3). The binding constant, K, and the n value estimated from the Scatchard plots were $3.86\times10^5\ \mathrm{M}^{-1}$ and 1.24, respectively. To determine the maximum number of binding sites, Job's plots[8] were also prepared for the AMCA–HSA system by keeping the total concentration of AMCA and HSA at 1.0×10^{-6} M (Fig. 4). The

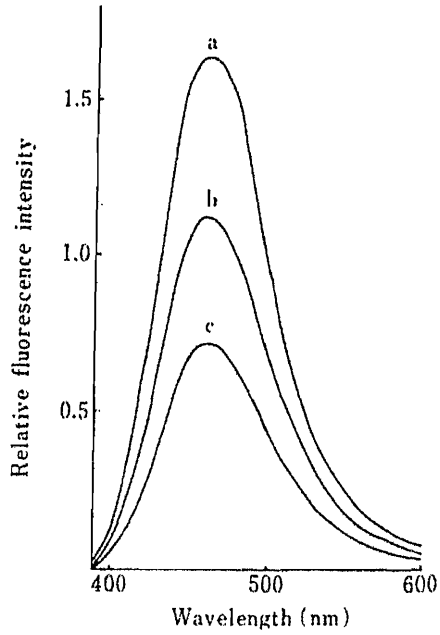


Fig. 1. Fluorescence Spectra of AMCA (1.0×10^{-6} M) in the Presence of HSA

HSA concentration: a, 5.0×10^{-6} M; b, 2.0×10^{-6} M; c, 1.0×10^{-6} M.

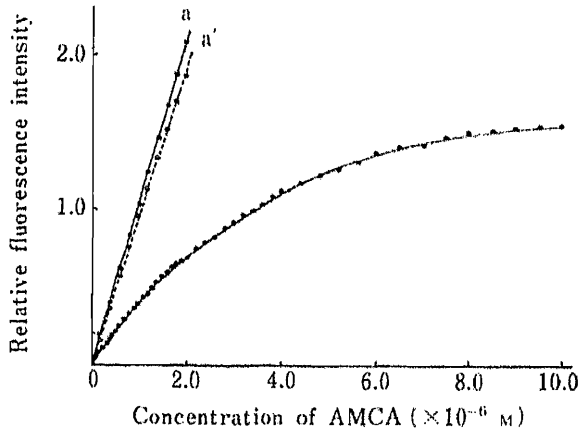


Fig. 2. Relative Fluorescence Intensity for the AMCA–HSA Interaction as a Function of the Concentration of AMCA

HSA concentration: a', 0.8×10^{-4} M and 1.0×10^{-4} M; b, 1.6×10^{-6} M. The straight line a is obtained after correcting the fluorescence intensities in line a' for the absorbance effect.

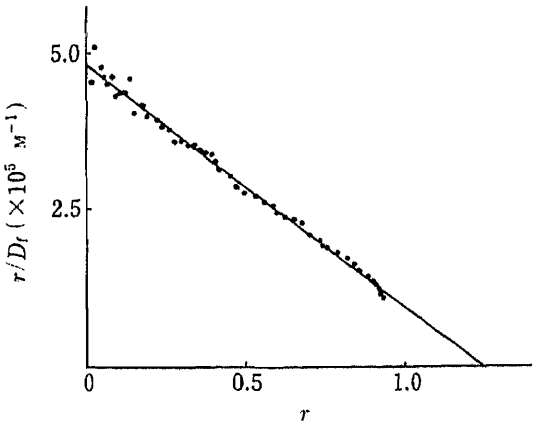


Fig. 3. Scatchard Plots of AMCA–HSA Interaction

AMCA concentration, 0.5×10^{-7}—1.0×10^{-5} M; HSA concentration, 1.6×10^{-6} M.

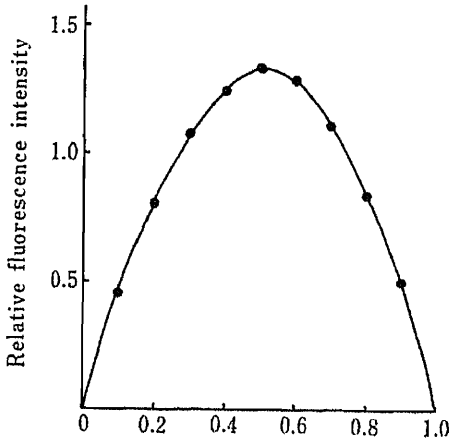


Fig. 4. Job's Plots of Relative Fluorescence Intensity for the AMCA–HSA Interaction

The total concentration of [HSA]+[AMCA] was kept at 1.0×10^{-6} M.

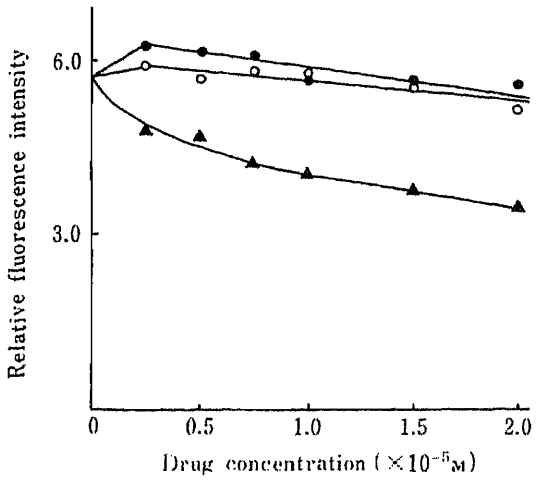


Fig. 5. Effects of Drugs on the Fluorescence Intensity of AMCA (1.0×10^{-7} M) in the Presence of HSA (1.0×10^{-6} M)

●, phenylbutazone; ○, ibuprofen; ▲, digitoxin.

TABLE I. Binding Constants of AMCA to Modified HSA

Compound	K_a (M^{-1})
Trp-modified HSA	1.65×10^5
Tyr-modified HSA	2.69×10^5
Lys-modified HSA	3.01×10^5

inflection point for the plots is near 0.5, the value expected for 1 : 1 complex formation. The n value estimated from the Scatchard plots was in fair agreement with that from Job's plots. Therefore, it is reasonable to conclude that the maximum number of binding sites contributing to the fluorescence in the AMCA–HSA interaction is one. The specific drug-binding sites on HSA have been established as sites I, II, and III by Sudlow *et al.*[9] and Sjoholm *et al.*[10] To identify the AMCA binding site on HSA in relation to Sudlow's classification,[9] the competitive displacements by various drugs were examined. Figure 5 shows the change in the fluorescence of the AMCA–HSA system on addition of various drugs. As shown in Fig. 5, AMCA was not displaced by phenylbutazone (site I drug) or ibuprofen (site II drug), whereas digitoxin (site III drug) caused significant displacement of AMCA at small molar ratios of drug to HSA. It is suggested on the basis of these results that the primary binding site of AMCA on HSA is site III, as is also the case for an analogous compound, 7-anilinocoumarin-

4-acetic acid (ACA), reported in a previous paper.[3]

In order to characterize further the AMCA-binding site on HSA, the interaction between AMCA and modified HSAs (Trp-, Tyr-, and Lys-modified HSAs) was examined. The modification of these residues on HSA gave 114 (Trp), 20 (Tyr), and 55% (Lys) yields, which were in fair agreement with the known values. Binding constants (K_a) of AMCA–modified HSA systems obtained from the results of fluorometric titrations and Scatchard plots are summarized in Table I. As shown in Table I, the AMCA–modified HSA systems in comparison with the native HSA system ($3.86 \times 10^5\ \mathrm{M}^{-1}$) showed decreases in K_as (1.65—$3.01 \times 10^5\ \mathrm{M}^{-1}$). This may involve the effect of conformational change of HSA accompanying the modification, but a decrease in the K_a value of the AMCA–Trp modified HSA system was found as a specific effect of Trp modification. On the other hand, the modification did not change the number of binding sites for AMCA. Therefore, the present data suggest that the AMCA-binding site on HSA lies in a broad region near a Trp residue. Further investigations by use of the energy transfer technique[11] together with the development of new probes will be necessary to elucidate the contribution of Trp residue to site III. The fluorescence quantum yield ($\phi = 0.14$)[12] of AMCA in the presence of HSA under the same conditions as in the case of ACA was larger than that of ACA ($\phi = 0.06$),[3] suggesting that a wide range of AMCA concentrations can be employed in protein binding investigations.

Thus, the limited data obtained here suggest that AMCA should be useful as an additional fluorescence probe for site III on HSA.

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Conformational Changes of the Two Different Conformers of Grifolan in Sodium Hydroxide, Urea or Dimethylsulfoxide Solution

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Grifolan is an antitumor (1→3)-β-D-glucan with a β-glucosyl substitution at C-6 of every three main chain units, and is produced by *Grifola frondosa*. Grifolan possesses two different solid-state conformations, N (native) and H (helix) (N. Ohno *et al.*, *Chem. Pharm. Bull.*, **34**, 2555 (1986)), and both form a gel in an aqueous neutral media. In this study, the gel-to-sol or sol-to-gel transition of these forms in sodium hydroxide, urea and dimethylsulfoxide solutions were examined by carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. The N gel showed ^{13}C-signals in sodium hydroxide of more than 0.15 N, and the H gel showed them at more than 0.05 N. Gel-to-sol transitions of the N and H gels in urea were induced at 8 and 2 M, respectively. Sol-to-gel transitions of the glucans in dimethylsulfoxide were induced by the addition of more than 20% of water. These results suggested firstly that the architecture of the two gels is different because the gel-to-sol transition occurred more easily in the case of the H gel than the N gel, and secondly that the structure of the two glucans in the sol state is essentially the same.

Keywords——*Grifola frondosa*; (1→3)-β-D-glucan; gel; conformation; ^{13}C-NMR

Many kinds of antitumor glucans have been investigated, and lentinan from *Lentinus edodes* and schizophyllan from *Schizophyllum commune*, both of which are branched (1→3)-β-D-glucans, have been applied clinically in Japan.[1] It has been suggested that a triple helix structure is required for the antitumor activity of these polysaccharides. In the case of pachyman, antitumor activity is known to be induced by treatment with urea which causes a conformational change in the glucan.[2]

Recently, we have found that grifolan possesses two kinds of solid-state structure dependent on the extraction and purification procedures.[3~5] One was suggested to be a helix because the chemical shift of the C-3 carbon was similar to those of curdlan and lentinan, which are helices. Both forms showed antitumor activity and formed a gel.

The gel structures of several antitumor glucans, such as curdlan, lentinan and schizophyllan, can be degraded into sol by the addition of sodium hydroxide or urea.[6] Conformations of segments are changed from helix to random coil concomitantly. The transition is characteristic for each glucan. The gel of curdlan was changed to a sol quite sharply, but the transitions of lentinan and schizophyllan were broad. These differences are presumably due to differences in the gel networks.

To elucidate the gel network of grifolan, we examined the gel-to-sol and sol-to-gel transitions using carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.

Materials and Methods

Preparation of Polysaccharide Fractions——Grifolan NMF-5N was purified from NMF-5, a cold alkali extract of the matted mycelium of *Grifola frondosa*, by diethylaminoethyl (DEAE)-Sephadex A-25 chromatography and ethanol precipitation.[4] LELFD was prepared by incubation of the liquid-cultured mycelium with a buffer composed

of glucose and citric acid.[3] LELFD (8 M) was prepared by treatment of LELFD with 8 M urea solution at 100 °C and dialysis.[5] Curdlan was purchased from Wako Chemical Co., Ltd.

^{13}C-NMR Spectra——^{13}C-NMR spectra were measured with JEOL FX-200 instruments. Solution NMR spectra were measured using 10 i.d. sampling tubes at room temperature for aqueous solution and at 60 °C for dimethylsulfoxide-d_6 (DMSO-d_6) solution. All spectra were obtained from 5000 to 100000 scans.

Results and Discussion

Gel-to-Sol Transition

Several antitumor glucans, such as curdlan, schizophyllan, scleroglucan and lentinan, are known to form gel structures. These gel structures are degraded into sols by the addition of sodium hydroxide or urea.[6] To examine the architecture of the gel network of the N and H forms of grifolan, a ^{13}C-NMR study of sodium hydroxide- or urea-induced conformational changes was performed. (1→3)-β-D-Glucan is known to show no signals or only a few in the gel state. This is because of the lowered mobility of glucan chains resulting from gelation (physical cross-links). Degradation of the gel state to sol by the addition of sodium hydroxide resulted in the appearance of ^{13}C-NMR signals.[6]

LELFD, which was obtained from liquid-cultured mycelium,[3] was used as the N form of grifolan, and grifolan NMF-5N, which was obtained from the alkaline extract of the matted mycelium,[4] and LELFD (8 M),[5] which was prepared by the treatment of LELFD with 8 M urea, were used as the H form of grifolan. ^{13}C-NMR spectra of LELFD at various sodium hydroxide concentrations are shown in Fig. 1B. No signals were observed at 0.05 N or 0.1 N sodium hydroxide but signals appeared at concentrations of more than 0.15 N sodium hydroxide. The chemical shifts of the C-1 and C-3 signals were slightly different depending on the sodium hydroxide concentration. The chemical shifts of C-1 were 105 and 103 ppm at 0.15 and 0.2 N, respectively, and changed to 104 ppm at 0.25 N. Those of C-3 were 88 ppm at 0.15 and 0.2 N, and 86 ppm at 0.25 N. The observation that the ^{13}C-chemical shifts (C-1 and C-3) changed depending on the sodium hydroxide concentration is similar to that in the case of gel-forming (1→3)-β-D-glucans, lentinan and curdlan (Fig. 1A).[6] Saito *et al.*[6] suggested that the observed ^{13}C-signals of linear (1→3)-β-D-glucans in neutral and dilute alkaline solutions were due to segments of single helical conformation. It is most likely that the signals at 105 ppm (C-1) and 88 ppm (C-3) in 0.15 and 0.2 N sodium hydroxide are attributable to the main chain moiety of single helical segments, and these signals disappeared as a result of the further conformational change in 0.25 N sodium hydroxide. Compared with that of lentinan, the signal of the single helical segments (105 ppm) was narrower and clearly separated from the signals at 103 ppm, which included signals of the branching points and random coil segments. In 0.2 N sodium hydroxide, the signals at 105 and 88 ppm were broader than those at 0.15 N, indicating the induction of helix-to-coil transition to a small extent. Thus, it is likely that (1) segmental motion of LELFD was highly restricted in 0 to 0.1 N sodium hydroxide, (2) in 0.15 N sodium hydroxide, some physical cross-links were degraded to form some single helical regions, and (3) in more than 0.25 N sodium hydroxide, a random-coiled sol was formed.

In the case of LELFD (8 M), clear ^{13}C-NMR signals were obtained at lower sodium hydroxide concentrations than for LELFD (Fig. 1C). Under neutral conditions, weak but significant signals were observed in LELFD (8 M), and these signals were assumed to be due to side chain glucose units.[6] At more than 0.05 N sodium hydroxide, LELFD (8 M) showed sharp signals. From 0.05 to 0.2 N sodium hydroxide, single helical segments existed, because the chemical shifts of C-1 and C-3 were similar to those of curdlan and LELFD at the same alkaline concentrations. At 0.25 N sodium hydroxide, the signals at 105 and 88 ppm were moved to 104 and 86 ppm, presumably as a result of helix-coil transition. In the case of grifolan NMF-5N, signals appeared at more than 0.1 N sodium hydroxide, and the helix-to-coil transition occurred at about 0.2 N sodium hydroxide (Fig. 1D). These results suggested

that the rigidity of the gel network of the H form is less than that of the N form.

As described above, gel-to-sol transition proceeded in two stages. The first stage was the appearance of single helical segments which showed NMR signals. This occurred at 0.15 N in the case of LELFD, at 0.05 N for LELFD (8 M) and at 0.1 N for grifolan NMF-5N. The second stage was the conformational change of helix to random coil, which was revealed by the disappearance of the 105 and 88 ppm signals and the appearance of 104 and 86 ppm signals. This occurred at 0.25 N in LELFD and LELFD (8 M) and 0.2 N in grifolan NMF-5N. Most of the single helical segment was changed to random coil at about 0.25 N sodium hydroxide in LELFD and LELFD (8 M) because the chemical shift of the C-3 signal appeared at 86 ppm only in 0.25 N sodium hydroxide, but the observations that the C-1 and C-3 signals were a little broader at 0.2 N sodium hydroxide indicated that a small but significant amount of conformational change occurred in about 0.2 N sodium hydroxide. It is not known at present what level of conformational change from single helical segments to random coil occurred in 0.2 N sodium hydroxide because we can not distinguish the signal of C-1 of the side chain unit from that of the random coil segment. In the case of grifolan NMF-5N, the second stage occurred at lower concentration than those of LELFD and LELFD (8 M), *i.e.*, at about 0.2 N sodium hydroxide, because most of the C-1 signals were observed at 104 ppm and the C-3

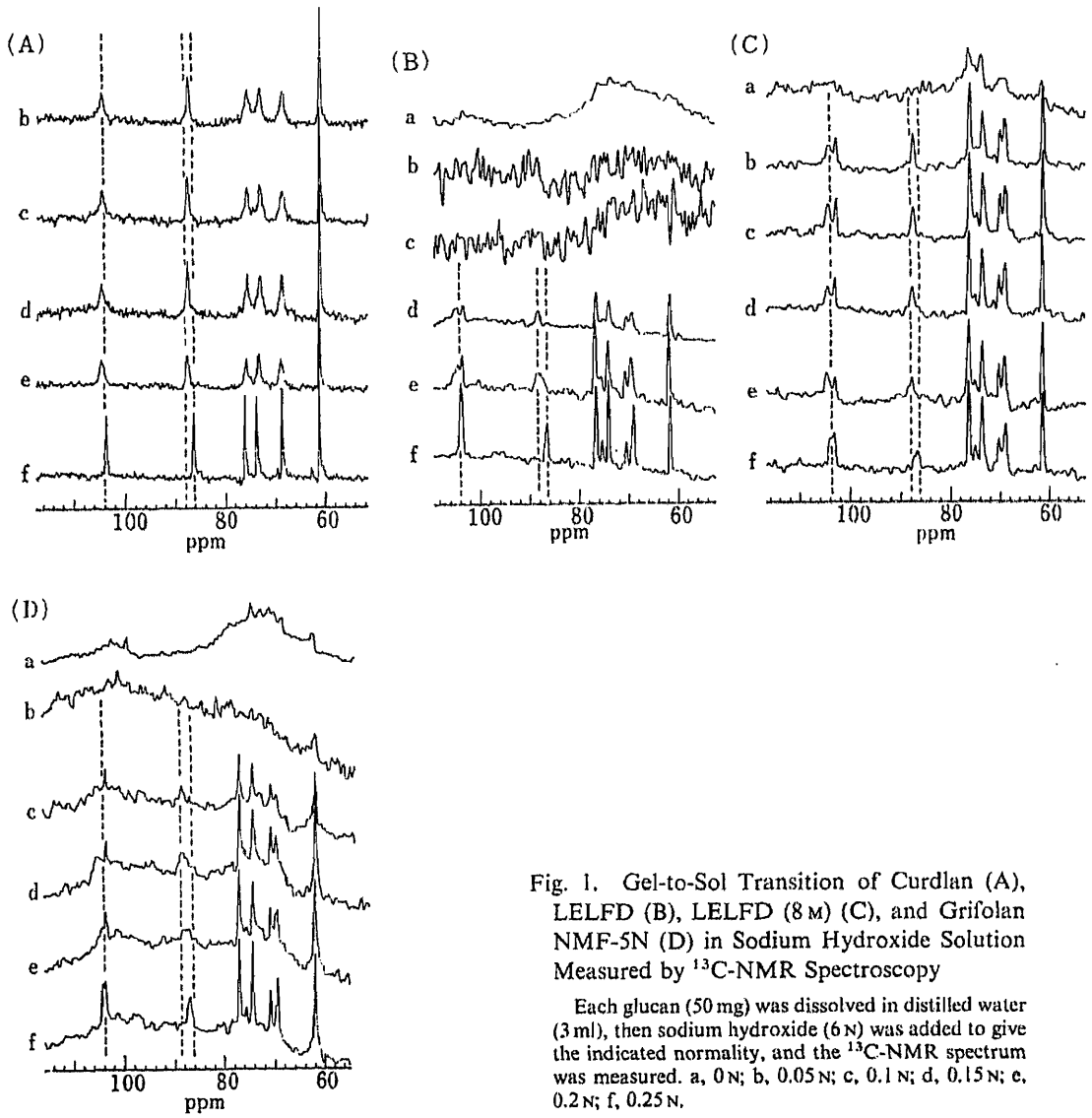


Fig. 1. Gel-to-Sol Transition of Curdlan (A), LELFD (B), LELFD (8 M) (C), and Grifolan NMF-5N (D) in Sodium Hydroxide Solution Measured by ^{13}C-NMR Spectroscopy

Each glucan (50 mg) was dissolved in distilled water (3 ml), then sodium hydroxide (6 N) was added to give the indicated normality, and the ^{13}C-NMR spectrum was measured. a, 0 N; b, 0.05 N; c, 0.1 N; d, 0.15 N; e, 0.2 N; f, 0.25 N.

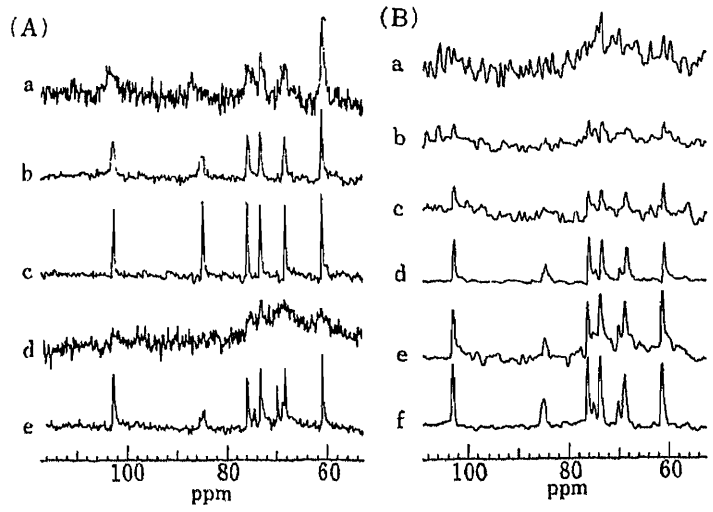


Fig. 2. Gel-to-Sol Transition of Curdlan (A), LELFD (B), LELFD (8 M) (B), and Grifolan NMF-5N (A) in Urea Solution Measured by ^{13}C-NMR Spectroscopy

Each glucan (50 mg) was dissolved in the indicated molarity of urea (3 ml) and the ^{13}C-NMR spectrum was measured. (A) a—c, curdlan; d, e, grifolan NMF-5N; a, d, 2 M; b, e, 4 M; c, 6 M. (B) a—d, LELFD; e, f, LELFD (8 M); a, e, 2 M; b, f, 4 M; c, 6 M; d, 8 M.

signal was observed between 88 and 86 ppm. Previously, we suggested that the helix-coil transition of grifolan NMF-5N occurred at about 0.15 N sodium hydroxide, based on the results of viscosity measurement and Congo Red binding.[4] These results suggested that the majority of the helix-to-coil transition took place between 0.15 and 0.2 N sodium hydroxide. The differences in gel stability of LELFD (8 M) and grifolan NMF-5N depend not on the conformation but on the microheterogeneity of the primary structure between grifolan NMF-5N and LELFD (8 M), which we have not examined yet. In a comparison of the C-1 and C-3 signals of these three glucans at 0.25 N sodium hydroxide, LELFD showed only one signal and LELFD (8 M) and grifolan NMF-5N showed broad signals. This difference does not necessarily suggest differences in the critical point of helix-to-coil transition between the N and H forms because the molarity of sodium hydroxide probably included some experimental error.

The gel stability of LELFD, LELFD (8 M) and grifolan NMF-5N was further studied by ^{13}C-NMR spectroscopy in various concentrations of urea (Fig. 2). Carbon-13 signals of curdlan were seen at more than 2 M urea and the line width was sharper at 6 and 8 M urea, suggesting that gel-to-sol transition occurred at near 4 M urea. Signals of LELFD, LELFD (8 M) and grifolan NMF-5N appeared at 8, 2, and 4 M urea, respectively. These results showed that the gel-to-sol transition of each gel occurred near the indicated molarity of urea. These properties also suggested that the gel stability differed for each glucan and that of LELFD, N form, was the greatest.

Sol-to-Gel Transition

In dimethylsulfoxide, (1→3)-β-D-glucan showed a random-coiled, disordered structure. In the case of curdlan, the addition of several non-solvents, such as water, 2-chloroethanol or dioxane, to the glucan in dimethylsulfoxide solution induce an ordered structure.[7] It was of interest to examine the sol-to-gel transition of grifolan in dimethylsulfoxide solution. Thus, the sol-to-gel transition was measured by using ^{13}C-NMR spectroscopy during the stepwise addition of water to the sol conformation of grifolan in DMSO-d_6 (Fig. 3). In the case of LELFD, addition of 400 μl of water (17%) caused only a slight decrease in NMR signals, but addition of 500 μl (20%) clearly decreased the glucan signals (about 50% intensity) relative to

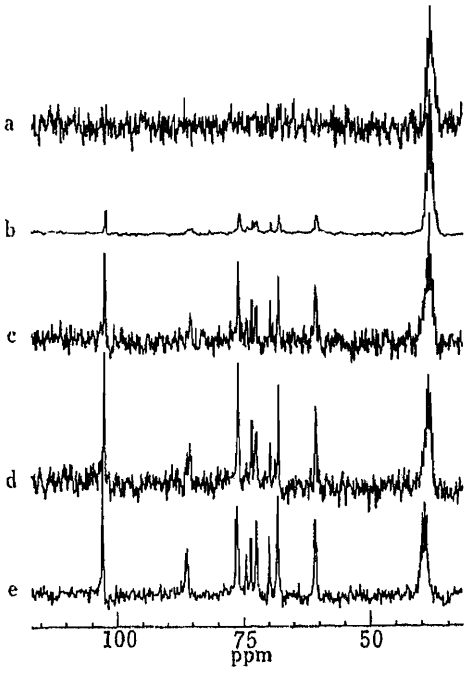


Fig. 3. Sol-to-Gel Transition of LELFD in Dimethylsulfoxide Caused by the Addition of Water Measured by ^{13}C-NMR Spectroscopy

LELFD (30 mg) was dissolved in DMSO-d_6 (2 ml) and distilled water was added to the indicated volume. The ^{13}C-NMR spectrum was measured at 60 °C. The solvent signal appears at 39.5 ppm as a multiplet. a, 700 μl; b, 600 μl; c, 500 μl; d, 400 μl; e, 0 μl.

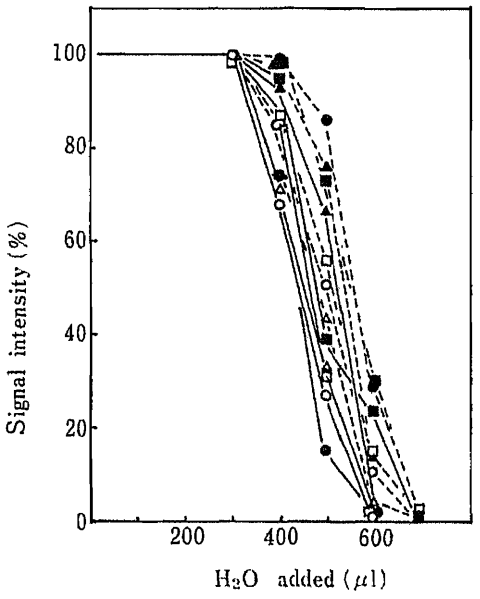


Fig. 4. Signal Intensities of Each Glucan during Sol-to-Gel Transition

Each glucan (30 mg) was dissolved in DMSO-d_6 (2 ml) and distilled water was added to the indicated volume, then the ^{13}C-NMR spectrum was measured at 60 °C. Signal intensity was plotted against that in 100% DMSO-d_6. ○●, C-1; △▲, C-3; □■, C-6; ○ (△□) ——, curdlan; ● (▲■) ——, grifolan NMF-5N; ○ (△□) – – –, LELFD; ● (▲■) – – –, LELFD (8 M).

the signals in 100% DMSO-d_6, and 600 μl (23%) caused the glucan signals to disappear. This observation showed that the sol-to-gel transition of LELFD in dimethylsulfoxide solution occurs when more than about 20% of water is present. The signal intensities of C-1, C-3 and C-6 *versus* those in 100% DMSO-d_6 are plotted in Fig. 4. As with LELFD, the signal intensities of curdlan, LELFD (8 M), and grifolan NMF-5N decreased concentration-dependently. These findings suggested that physical cross-links were regenerated at a similar percentage of water in all cases, and that formation of the gel network was similar to that of curdlan. During the addition of water, a C-3 signal appeared at 86 ppm and did not change until the signals disappeared. It is suggested that, in this system, single helical segments are rarely formed during sol-to-gel transition.

Conclusion

It is concluded that the two conformers of grifolan show different characteristics in gel-to-sol transition. Gel formation is thought to be due to physical cross links in which a major factor is the hydrogen bonding between the sugar units and segments. The data presented in this paper suggest that the hydrogen bonding is more rigid in the case of the N form.

As regards the H form glucans, LELFD (8 M) and grifolan NMF-5N showed different properties during helix-to-coil transition. Previously, we showed that grifolan NMF-5N contained the (1→3)-β-D-glucanase-resistant core part.[4] It was assumed that the microheterogeneity of the primary structure of the glucan also affects the gel stability of grifolan.

Recently, Saito *et al.* reported that the gel structures of glucans of form I (curdlan, lentinan) and form II (HA-glucan) were indistinguishable by 75 MHz ^{13}C-NMR spectroscopy in 0.05 M sodium hydroxide.[8] In contrast, the native form of LELFD showed no signals in 0.05 M sodium hydroxide. The concentration of glucans should not be a factor, because we used a lower concentration (17 mg/ml) than theirs (20 mg/ml). Thus, it is suggested that the gel structure of LELFD is more rigid than those of other glucans, such as curdlan, lentinan, and HA-glucan.

We showed that the N form of grifolan possesses an ordered structure other than the helix structure in the solid state.[5] The data presented in this paper (*i.e.*, that LELFD, N form, contained a certain amount of single helical segments between 0.15 to 0.25 N) indicated that rapid conformational change occurred at about 0.15 N sodium hydroxide from an ordered structure to single helical segments through the cleavage of physical cross-links.

We reported that the LELFD fraction contained a small amount of protein (—9%).[3] It is still possible that the protein moiety modulated the solution behavior of LELFD and LELFD (8 M). However, purification of the LELFD is not possible without 8 M urea treatment, which induces the conformational change of native to helix, because (1) (1→3)-β-D-glucan is not amenable to chromatographic techniques under neutral-gel-producing conditions, and (2) impurities included in the gel matrix can not be removed without degradation of the gel structure. Thus, we are able to characterize the solution behavior of the native form LELFD only by using untreated material.

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A New Technique for *in Vivo* Percutaneous Absorption

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One method to evaluate the amount of a drug that is percutaneously absorbed is to recover and measure the amount of the drug that remains on the skin. Wiping the skin with cotton soaked in an ointment base is the method ordinarily used to recover the unabsorbed drug. The recovery rate with this method varies widely depending on the ointment base. In general, it is difficult to quantitatively remove the drug vehicle and the drug from the skin except with certain vehicles such as water. In order to improve the method of drug recovery, a simple and reproducible *in vivo* method using rats was developed. The test drug was applied within an overflow barrier designed to maintain a constant drug application area, and five hours later the skin was excised without recovering the applied ointment and the level of residual drug determined. The recovery rate was nearly 100% when the skin was totally excised without wiping immediately after drug application. The overflow of drug from the ointment base was prevented by the overflow barrier, and the application area was kept constant. This method should be useful to evaluate percutaneous absorption of drugs quantitatively.

Keywords——skin; corticosteroid; percutaneous absorption; hydrocortisone butyrate propionate; rat; *in vivo*

Recently, the use of dermatological preparations has been extended beyond simple dermatological treatment to the field of transdermal therapy, where a drug applied cutaneously functions systemically. Therefore, it is essential to develop a simple, more reliable method for estimating transcutaneous drug absorption. Many *in vitro* experiments[1-3] have been devised to study the release and diffusion rates of topically administered drugs through artificial membranes or through excised skin. The results from *in vitro* and *in vivo* experiments are not always in agreement, because transcutaneous drug absorption is influenced by pharmaceutical and physiological factors: the partition coefficient of the drug, the viscosity and composition of the vehicle, blood flow and skin hydration.

To establish the importance of physiological factors, *in vivo* methods are essential, because they allow an assessment of the actual magnitude of drug absorption. Because most drugs permeate the skin in relatively minute amounts, many methods depend on the use of radiolabeled compounds to measure blood and urine drug concentrations; however, suitable radiolabeled compounds may not always be available.

Although direct measurement of the unabsorbed drugs on the skin is useful particularly for drugs that are very poorly absorbed, it is difficult to recover the vehicles and the drug to measure quantitatively the amount of drug remaining. Further, if more than one vehicle is spread over the test area, or if the vehicle spreads to the surrounding area, it is difficult to assess transcutaneous absorption in the applied region.

In this study a novel *in vivo* method, the total excision method, was developed, in which the skin is excised without recovering the administered ointment, and the entire amount of the unabsorbed drug is determined. In addition, an improved overflow-barrier method was developed in order to maintain an application area of constant size.

Experimental

Materials——Hydrocortisone 17-butyrate 21-propionate (HBP) and hydrocortisone 17-butyrate (HB) were prepared from hydrocortisone (HC) according to the method of Sota *et al.*[4] The HC was JP grade commercial material. Carboxyvinyl polymer (CVP: HIVISWAKO® 104) was obtained from Wako Pure Chemical Industries, Ltd. Plastibase was obtained from Japan Squibb Co., Ltd. All other reagents used were of an analytical grade.

Preparation of Test Vehicles——Various additives were included in the topical preparation, which contained 0.02% of HBP, HB or HC: gel (CVP, 1%; ethanol, 48.5%; distilled water, 48.5%; a solution of 5% ammonia in distilled water, 2%), plastibase and hydrophilic ointment (JPX). In addition, the pure liquid base was used. The concentration of all tested drugs was 10 μg/6 μl.

Animals——Male rats weighing 200—250 g were anesthetized with urethane. Abdominal hair was removed with electric hair clippers and the skin was cleansed with 70% ethanol.

Preparation of Overflow Barrier and Absorption Experiment——A circular 2 cm^2 area was delineated with a 1-mm-wide strip of 5% CVP gel which did not contain drugs; the overflow barrier was functional when the gel had dried. The test vehicles, 50 mg of ointment or 6 μl of a liquid base, were applied on the 2 cm^2 area by the use of a microinfusion pump (Furue Science Co., Ltd., model JP). The animals were sacrified after appropriate intervals. In the case of excision up to the dermis, the skin with the overflow barrier was carefully cut up to the dermis with scissors, and subcutaneous tissue was stripped off with forceps. In the case of skin excision up to the subcutaneous tissue and muscle, the skin with the overflow barrier was cut up to the subcutaneous tissue and muscle, respectively, with scissors. The skin sample in the surrounding region was obtained by excising up to the subcutaneous tissue.

Extraction of HBP, HB and HC——Acetonitrile (10 ml) was added to the excised skin and the mixture was homogenized with a Hiscotron homogenizer, type NS-10. After addition of 5 g of Na_2SO_3 to the homogenate, the mixture was centrifuged and 8 ml of the organic phase was withdrawn. After reextraction with 10 ml of acetonitrile, the combined organic phase was evaporated to dryness. The residue was dissolved in an high performance liquid chromatography (HPLC) mobile phase. In the control experiment, the test area was wiped three times with cotton soaked in a drug-free base. The cotton wipings were extracted with acetonitrile in the manner described above.

Instrumentation——The HBP, HB and HC levels were analyzed by HPLC (Shimadzu LC-3A pump, and SPD-2A UV monitor) with a 150 × 4 mm i.d. column containing TSK-Gel LS 410 (5 μm). The flow rate was 1 ml·min^{-1}, the ultraviolet (UV) wavelength was 245 nm, and the column temperature was set at 50 °C. As a mobile phase, a mixture of methanol, water and acetic acid was used. The composition of the mixture was 65.5 : 35 : 0.5 for HBP and for HB, and 49 : 50.5 : 0.5 for HC.

Measurement of Overflow of Vehicles——Various ointments containing 0.025% of lipophilic dye (Sudan III) and hydrophilic dye (Amaranth) were applied to a 2 cm^2 area of rat skin by the above method. Five hours after application, the abdominal skin including the application site was excised and spread on a glass plate. Dye overflow beyond the application area was observed visually.

Results and Discussion

Effect of Skin Excision Method

Tables I and II show the amount of residual drug on the skin after a 5 h application; the skin was excised at various thicknesses. Excision to the subcutaneous tissue gave the same results as excision to the dermis for both HBP and HC. It is possible that HBP penetrates more deeply than HC, since a slight increase in the amount of HBP was noticed when the skin was

Table I. Effect of Excision Method on Residual Percent at 5 h after Topical Application of HBP Gel Containing 3% IPM

Excision method	n	Residual percent[a]
Treated part I[b]	6	66.5±5.6
Treated part II[c]	6	65.0±3.0
Treated part III[d]	4	69.8±4.2
Surrounding part II	4	0

a) Values are the means±S.D. *b*) I=epidermis+dermis. *c*) II=I+subcutaneous tissue. *d*) III=II+muscle. IPM=isopropyl myrystate.

Table II. Effect of Excision Method on Residual Percent at 5 h after Topical Application of HC Gel Containing 3% IPM

Excision method	n	Residual percent[a]
Treated part I[b]	4	84.2±2.4
Treated part II[c]	4	82.3±1.9
Treated part III[d]	4	82.3±3.5
Surrounding part II	4	0

a) Values are the means±S.D. *b*) I=epidermis+dermis. *c*) II=I+subcutaneous tissue. *d*) III=II+muscle.

Table III. Effect of Overflow Barrier on Dye (Sudan III and Amaranth) Overflow and HBP Residual Percent at 5 h after Topical Application of Various Vehicles ($n=2$)

Vehicle[a]	Overflow barrier[b]	Overflow dye[c]	HBP residual percent[d] at 5 h	
			Administration part	Surrounding part
Gel	+	−	86.7	0
	−	−	86.2	0.2
Gel (5% IPM)	+	−	62.9	0
	−	+	54.0	12.3
Gel (5% squalane)	+	−	80.9	0
	−	+	63.3	5.2
Gel (5% liquid paraffin)	+	−	85.3	0
	−	±	67.9	1.3
Plastibase (5% IPM)	−	+	—	—
Hydrophilic ointment (5% IPM)	−	−	—	—

a) All vehicles contained 0.025% of Sudan III and Amaranth. *b*) +, with overflow barrier; −, without overflow barrier. *c*) The degree of dye overflow was visually judged: +, clearly visible; ±, slightly visible; −, not visible. *d*) Values are the means of two experiments.

excised to the muscle. This enhanced permeability is thought to be due to the highly lipophilic character of HBP, a diester derivative of HC. The major barrier to drug diffusion through the skin is the stratum corneum,[5] which plays an important role as a reservoir.[6] Therefore, when measuring the residual drugs on the skin to estimate drug absorption, the estimate should be little affected by the depth of the skin excision even when drug distribution in subcutaneous tissue differs. Excision to the subcutaneous tissue was adopted in this study since it is a little difficult to separate the dermis from the subcutaneous tissue in rats.

Effect of Overflow Barrier

When quantitatively measuring percutaneous absorption, it is important to maintain a constant drug application area. There are a variety of diffusion cells already in use to maintain a constant area when applying liquid samples, but a method to prevent ointment overflow has not been adopted. After the application of ointment to the skin, base ingredients such as ethanol and water evaporate rapidly, and/or the system breaks down as a result of interaction with the skin. After the system breaks down it is presumed that the liquid oleaginous bases which were surrounded by base ingredients such as water and ethanol are able to spread over the skin.

Table III shows the state of the dye after application of the dye-containing gels. In the absence of an overflow barrier, the dye was confined to the application area when the gel was applied without additives. When the gel was applied with liquid non-volatile matter, the dye spread over the skin.

In some cases of dye overflow, the test area and the surrounding area were excised and analyzed. The drug was always found in the surrounding tissue to which it had not been applied. It became apparent that the prevention of drug overflow required a circular 1-mm-wide strip of 5% CVP gel overflow barrier.

Effect of Recovery Methods

Takeda and Kukita[7] pointed out that wiping by organic solvents such as alcohols rids the stratum corneum of the drug. Malkinson and Kirschenbaum[8] have shown that use of ethanol as a recovery solvent, on the contrary, forces the drug to penetrate due to alteration of the stratum corneum barrier. These results suggest that it is generally difficult to quantitatively

TABLE IV. Comparison of Recovery Methods Immediately after Topical Application of Various Vehicles

Vehicle	Drug	Recovery percent[a]		
		Vehicle	Total excision method	Vehicle recovery method
Gel	HBP	99.0±1.3	99.9±3.8	90.6± 1.1
Gel (3% IPM)	HBP	99.7±1.2	100.2±5.1	101.0± 2.7
Plastibase	HBP	100.3±1.8	98.2±1.3	91.1± 8.0
Plastibase (3% IPM)	HBP	100.3±0.5	99.2±0.5	95.2± 4.1
Hydrophilic ointment	HBP	101.1±1.2	99.0±3.1	93.5± 4.4
IPM (100%)	HBP	100.0±1.7	103.7±0.9	89.3± 2.9
PG (100%)	HBP	99.7±1.6	98.5±0.7	95.8± 3.4
DID (100%)	HBP	99.9±1.9	103.7±0.9	90.6± 2.4
Hydrophilic ointment	HC	98.2±0.5	101.6±0.1	86.1± 8.3
Plastibase	HC	98.5±1.1	101.6±2.3	74.2±10.0

a) Values are the means±S.D. of 3—4 experiments. PG = propylene glycol. DID = diisopropyl adipate.

TABLE V. Percutaneous Absorption at 24h after Topical Application of Gel Containing 3% IPM

Drug	n	Percent absorbed[a]
HBP	4	77.2±5.8
HB	3	43.2±8.2
HC	3	20.5±7.3

a) Values are the means±S.D.

recover the vehicle on the skin and the drug except when certain vehicles such as water are used.

Table IV shows the drug recovery rate when two different methods were used for recovery after the application of various vehicles in the presence of an overflow barrier. Nearly 100% recovery was achieved when the skin was totally excised immediately after drug application. The recovery rate was comparatively low, and varied widely between bases, when the skin was immediately wiped with cotton soaked with a drug-free ointment base.

Urakubo[9] studied the percutaneous absorption of ^{113}Sn-labeled triphenyltin acetate that was applied to the skin on the backs of guinea pigs. The amount of the compound remaining on the tissue after wiping was extremely large, corresponding to 12 to 16% even 2d after application. Since the amount absorbed into the body varied from about 19% (including the drug that remained on the skin) to 3—8% (when the drug that remained on the skin was not included), Urakubo[9] found it difficult to determine which measurement method should be adopted. In our previous studies[10,11] on percutaneous absorption of corticosteroids from aqueous solutions in rats and dogs, about 90% of the corticosteroid absorbed into the skin was localized in the stratum corneum; however, tissue drug contents do not always reach 100% of that lost from an ointment base. When this is the case and recovery is incomplete, it is inaccurate to assume that the amount of drug that has disappeared from the base is equal to the amount absorbed.

Generally with *in vitro* experiments,[3] the drug functions as a solute and penetrates through the entire skin, going into solution at the receptor side. The amount of residual drug in the *in vivo* method corresponds to both the amount that penetrated the skin and the amount

that was unabsorbed in the *in vitro* method. Percutaneous gel absorption of HC and its mono- and diester derivatives, HB and HBP, was studied by using the total excision method, and the results are given in Table V. The absorption rates for HBP, HB and HC were 77.2, 43.2 and 20.5%, respectively. The results in the present study agree with those obtained in a previous study using aqueous solutions.[8]

The findings in this study suggest that it is possible to use a simplified procedure with a gel overflow barrier and skin excision to the subcutaneous tissue to measure quantitatively transcutaneous drug absorption.

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Communications to the Editor

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PAF ANTAGONISTIC ACTIVITY OF SOME THIENO[3,2-f][1,2,4]TRIAZOLO[4,3-a][1,4]DIAZEPINES

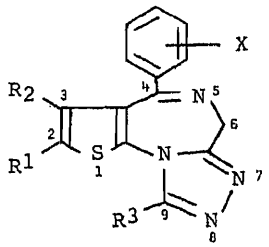
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Some thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepines were antagonistic to PAF-induced platelet aggregation in rabbit PRP and bronchoconstriction in guinea pigs. A study of structure-activity relationships demonstrates that the fused triazolo ring with a lower alkyl group is crucial for the activity. Among these compounds, etizolam was the most potent and specific antagonist of PAF.

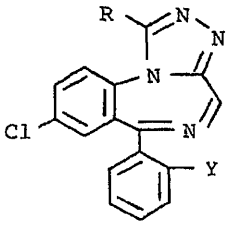
KEYWORDS —— thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine; PAF antagonistic activity; anti-platelet aggregation; anti-bronchoconstriction; etizolam

Platelet activating factor (PAF) is a potent mediator of anaphylaxis and inflammation released from stimulated neutrophils, basophils, platelets and macrophages.[1] Since the structure of PAF was identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholines,[2] the structural requirement for the PAF function has been intensively investigated.[3] While the biological functions of PAF may be mediated through a receptor process,[4] its structural flexibility makes it difficult to identify an active conformer. On the other hand, it has been reported that some naturally occurring[5] and synthetic compounds[6] are potent PAF antagonists. Recently, a triazolobenzodiazepine class of compounds has been found to be a specific and competitive antagonist of PAF.[7] Such rigid compounds seem to be useful in analyzing the active conformation of PAF interacted with the receptor site. We have previously reported that etizolam (9) is a potent antagonist of PAF-induced platelet aggregation and bronchoconstriction, and it is also a competitive inhibitor of [^{3}H]-PAF binding.[8] Here we describe the PAF antagonism of several 6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]-diazepines related to etizolam.

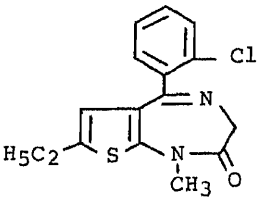
The compounds tested were previously synthesized in our laboratory[9] and evaluated for their ability to antagonize PAF-induced platelet aggregation (rabbit PRP) and bronchoconstriction (guinea pig). The biological results are shown in Table I. The structure-activity relationships are briefly as follows. Optimal activity was obtained by substituting a halogen atom or a methoxy group at the ortho position in the phenyl ring on the C-4 position. But the activity was decreased by introduing a methyl group on the ortho position and a chlorine atom or a methoxy group on the para position. The antagonism was enhanced by substituting a lower alkyl group, a chlorine atom, or an acetyl moiety at the C-2 position, but it was reduced by introducing a hydroxyethyl group at the C-2 position or an alkylene linkage between C-2 and C-3 positions. The substitution on the triazolo ring seems to be critical for



1∿23
(9; etizolam)



24; R=CH_3, Y-Cl, triazolam
25; R=Y=H, estazolam



26; clotiazepam

Table I.

Compd.	X	R^1	R^2	R^3	anti-platelet aggregation[a] IC_{50}(μg/ml)	anti-broncho-constriction[b] inhibition %
1	H	H	H	H	>30	——
2	H	C_2H_5	H	H	>30	——
3	H	C_2H_5	H	CH_3	14.8	69.4
4	H	H	H	CH_3	5.1	——
5	o-Cl	H	H	CH_3	4.9	97.2
6	o-Cl	CH_3	H	H	>30	——
7	o-Cl	CH_3	H	CH_3	1.8	100
8	o-Cl	C_2H_5	H	H	>30	——
9	o-Cl	C_2H_5	H	CH_3	1.3	100
10	o-Cl	C_2H_5	H	C_2H_5	1.5	100
11	o-Cl	C_2H_5	H	C_3H_7	8.9	——
12	o-Cl	CH_3	CH_3	CH_3	3.2	45.5
13	o-Cl	$-(CH_2)_4-$		H	>30	——
14	o-F	C_2H_5	H	CH_3	3.8	65.4
15	o-Br	C_2H_5	H	CH_3	3.8	——
16	o-Cl	C_2H_5	H	C_6H_5	>30	——
17	o-CH_3O	C_2H_5	H	CH_3	2.2	92.4
18	o-CH_3	C_2H_5	H	CH_3	25.4	——
19	o-Cl	$COCH_3$	H	CH_3	3.1	64.3
20	o-Cl	$CH(OH)CH_3$	H	CH_3	40.0	0
21	p-Cl	C_2H_5	H	CH_3	13.5	——
22	p-CH_3O	C_2H_5	H	CH_3	15.0	——
23	o-Cl	Cl	H	CH_3	2.2	100
24	triazolam				13	——
25	estazolam				>100	——
26	clotiazepam				>100	——

a) Platelet aggregation was measured at 37°C with a turbidimetric device by using a modification[10] of the method of Born and Cross.[11] The test solution or vehicle was added to rabbit platelet-rich plasma (PRP) 2 min before the addition of PAF (0.1 μg/ml). The inhibition concentration was assessed by comparing the maximum change in drug-treated PRP with that in vehicle-treated PRP.

b) Bronchoconstriction was provoked with PAF (0.3 μg/kg, i.v.) in anesthetized guinea pigs according to the method of Vargaftig et al.[12] Resistance to lung inflation was measured by the overflow technique of Konzett and Rössler[13] with the modification[14] for electrical recording. Test compounds (0.1 mg/kg, i.v.) were given 2 min before the treatment of PAF. The inhibition rate was calculated by the following equation:

$$\% \text{ inhibition} = 100 - \frac{\% \text{ peak bronchoconstriction in the test group}}{\% \text{ peak bronchoconstriction in the vehicle group}} \times 100$$

the antagonism. The unsubstituted compounds (1,2,6,8,13) were practically inactive, while the analogues (9,10), in which a methyl or an ethyl group had been attached at the C-9 position, had increased potency. The substitutional effect was decreased by replacing the methyl and ethyl groups with more bulky groups, such as a propyl group or a phenyl moiety. The most potent compound tested was etizolam which has been marketed in Japan as an anxiolytic drug. Interestingly, clotiazepam, lacking a triazolo moiety, had no effect on platelet aggregation at a concentration of 100 μg/ml. These facts indicate that the fused triazolo ring with the lower alkyl group is essential for the antagonism.

A comparison of clotiazepam with etizolam affords another insight into the mode of action. Clotiazepam has a great affinity for the benzodiazepine (BZP) receptor comparable to that of etizolam,[15] but it does not have the anti-platelet aggregation activity, as described above. This shows that the PAF antagonism of these compounds may not be mediated through the BZP receptor. Also, these compounds, at a concentration of 100 μg/ml, were not effective against arachidonic acid- and adenosine diphosphate-induced platelet aggregation.

In conclusion, these results indicate that some of the compounds (7,9,10,23) are specific and competitive antagonists of PAF. Further studies are necessary to explain how these derivatives compete with PAF at the receptor site, and their molecular rigidity may help in investigating the PAF-active conformations, and their modification may aid in designing a new PAF antagonist.

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Communications to the Editor

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NEW FUNGAL METABOLITES AVELLANINS A AND B FROM HAMIGERA AVELLANEA, WITH PRESSOR EFFECT

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Two new fungal metabolites with pressor effect, avellanins A and B, were isolated from Hamigera avellanea and their structures were found to be cyclo-(N-methyl-D-phenylalanyl-D-alanyl-L-isoleucyl-2-aminobenzoyl-L-prolyl) and cyclo-(N-methylphenylalanyl-alanyl-valyl-2-aminobenzoyl-prolyl), respectively.

KEYWORDS——avellanin; fungal metabolite; Hamigera avellanea; cyclic peptide; pressor effect; mycotoxin; X-ray analysis

In a series of investigations to find out new biologically active fungal metabolites, we recently discovered that the ethyl acetate extract of a teleomorphic fungus, Hamigera avellanea Stolk et Samson, is lethally toxic to mice. Repeated chromatographic separations of the extract on silica gel columns were tested for toxicity to mice. This afforded two new compounds named avellanins A and B. The avellanins led to the death of mice and rats after a great increase in blood pressure.

Avellanin A (1) had the following physical characteristics: colorless plates (from acetone-n-hexane), mp 202-204°C, $[\alpha]_D^{24}$ +161.5°(c=0.5, $CHCl_3$), $C_{31}H_{39}N_5O_5$(EI-High MS, M^+: calcd., m/z 561.2948, found, m/z 561.2923), IR_{max}^{KBr} cm^{-1}: 3360, 1680, 1635, 1620, 1590, 1419, 1303, UV λ_{max}^{MeOH}nm(log ε): 210(4.54, end absorption), 250(4.21, shoulder), 292(3.45).

On acid hydrolysis with 6N HCl (110°C, 24 h), 1 afforded five amino acids which were identified by cellulose thin-layer chromatography as authentic 2-aminobenzoic acid, N-methylphenylalanine, isoleucine, proline, and alanine. The presence of these five amino acids were also supported by the ^{1}H-NMR and ^{13}C-NMR spectra as follows: ^{1}H-NMR data for 1[3]: 0.93(3H, t, J=7.7 Hz), 0.97(3H, d, J=6.8 Hz), 1.18(1H, m), 1.46(1H, m), 1.48(1H, m), 1.52(3H, d, J=6.8 Hz), 1.88(1H, m), 2.00(1H, m), 2.10(1H, m), 2.30(1H, m), 2.97(1H, dd, J_1=16.2, J_2=12.8 Hz), 3.02(3H, s), 3.50-3.70(2H, m), 3.83(1H, dd, J_1=16.2, J_2=4.7 Hz), 4.69(1H, dd, J_1=9.4, J_2=3.4 Hz), 4.72(1H, dd, J_1=7.3, J_2=2.6 Hz), 4.89(1H, m), 5.78(1H, dd, J_1=12.8, J_2=4.7 Hz), 6.47(1H, d, J=9.4 Hz, -NH-), 7.08(1H, t, J=7.7 Hz), 7.18-7.36(6H, m), 7.43(1H, d, J=7.7 Hz, -NH-), 7.44(1H, t, J=7.7 Hz), 8.45(1H, d, J=7.7 Hz), 9.79(1H, broad s, -NH-); ^{13}C-NMR data for 1[3]: 12.2(q), 16.3(q), 18.1(q), 24.4(t), 25.0(t), 28.6(t), 31.1(q), 33.1(t), 36.2(d), 48.5(d), 51.4(t), 56.4(d), 57.5(d), 58.5(d), 121.4(d), 123.0(d), 123.4(s), 126.8(d), 127.3(d), 128.2(2C, d), 128.6(2C,

d), 131.7(d), 136.6(s), 137.1(s), 169.5(2C, s), 169.7(s), 172.0(s), 174.0(s).

For X-ray crystallographic analysis of 1 (monoclinic, space group $P2_1$, lattice constants a=10.325(5), b=18.994(11), c=7.982(2)Å, β=107.51(3)°, V=1493.0(12)Å^3, Z=2, $D_{obsd.}$=1.26g/cm^3 for $D_{calcd.}$=1.25g/cm^3), the data on 2125 independent reflections ($|Fo|>2\sigma(|Fo|)$) within a range of $2°\leqq 2\theta \leqq 130°$ collected with a Rigaku automated four-circle diffractometer using CuKα radiation, were solved directly by the MULTAN program,[4] and the solution was refined by the block-diagonal least-squares method with isotropic and anisotropic temperature factors of all non-hydrogen atoms to give a final R-value of 0.0756, excluding the contributions of all hydrogen atoms in 1. The solution showed that 1 is a cyclic pentapeptide whose structure is cyclo-(N-methyl-D-phenylalanyl-D-alanyl-L-isoleucyl-2-aminobenzoyl-L-prolyl) or its enantiomorph (Fig. 1).

Isoleucine obtained from 1 on acid hydrolysis with 6N HCl afforded (+)-plain curve (solvent: 0.2N HCl) which was similar to that of authentic L-isoleucine on measurement of optical rotatory dispersion and was completely consumed by treatment (pH 7.9, 37°C, 4 h) with L-amino acid oxidase (crude Crotalus adamanteus venom type I, Sigma Chemical Co.). Accordingly, the absolute stereostructure of 1 was deduced to be as shown below.

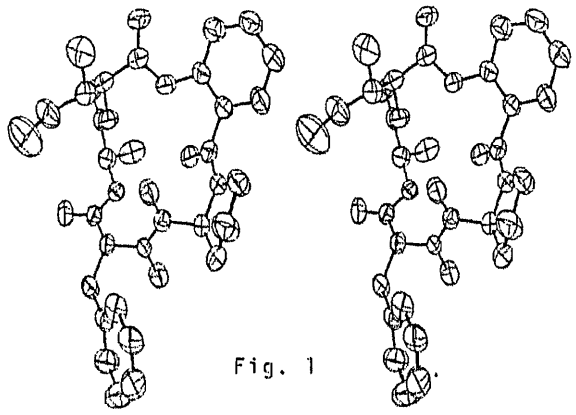
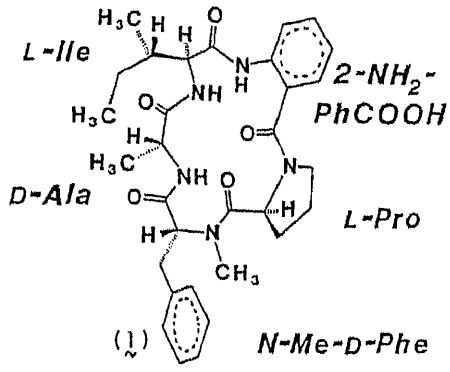


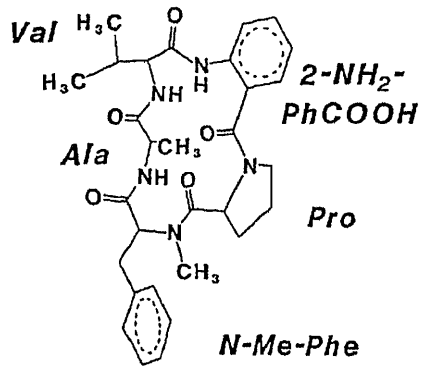
Fig. 1



(1)

Avellanin B(2) had the following physical characteristics: colorless amorphous, $[\alpha]_D^{24}$ +281.9°(c=0.17, $CHCl_3$), $C_{30}H_{37}N_5O_5$(EI-High MS, M^+: calcd. m/z 547.2791, found m/z 547.2774), IR_{max}^{KBr} cm^{-1}: 3360, 1680, 1640, 1620, 1590, 1415, 1305, UV λ_{max}^{MeOH}nm(log ε): 210(4.50, end absorption), 250(4.16, shoulder), 292(3.37).

Comparison of ^{13}C-NMR and ^{1}H-NMR spectra of 2 with the corresponding spectra of 1[3] indicated that all of the spectral signals of 2 are quite similar to those of 1 except that the signals of CH_3-CH_2-$CH(CH_3)$- group(^{13}C-NMR: 12.2(q), 16.3(q), 24.4(t), 36.2(d); ^{1}H-NMR: 0.93(3H, t, J=7.7 Hz), 0.97 (3H, d, J=6.8 Hz), 1.18(1H, m), 1.46(1H, m),[5] 2.30(1H, m)) are replaced by the signals of CH_3-$CH(CH_3)$- group(^{13}C-NMR: 16.6(q), 19.5(q), 29.1(d); ^{1}H-NMR: 0.93(3H, d, J=6.8 Hz), 0.99(3H, d, J=7.7 Hz), 2.61(1H, m)) in 2. This indicates that 2 has the same structure as 1 except for an isopropyl group instead of the sec-butyl group in 1. Accordingly, the structure of 2 was deduced to be cyclo-(N-methylphenylalanyl-alanyl-valyl-2-aminobenzoyl-prolyl) as shown below.



(2)

Intravenous administration of 1 mg/kg of 1 immediately led to a pressor effect in rats. Administration of 12.5-50 mg/kg of 1 caused remarkable exophtalmos in mice, which may be ascribed to its pressor effect. After that, some of the mice died. The LD_{50} of 1 on intravenous administration to mice was found by the up and down method to be 30.2 mg/kg. Details of the pharmacological study of avellanins will be published elsewhere in near future.

Aspercolorin[6] and cycloaspeptides[7] have been isolated from the anamorphic fungi Aspergillus versicolor and Aspergillus sp., respectively, as cyclic peptides containing 2-aminobenzoic acid. Some of the component amino acids of these peptides are the same as those of avellanins, but their amino acid sequences are quite different from those of our avellanins.

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Communications to the Editor

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ISOLATION AND STRUCTURE OF REGELIN AND REGELINOL, NEW ANTITUMOR URSENE-TYPE TRITERPENOIDS FROM TRIPTERYGIUM REGELII

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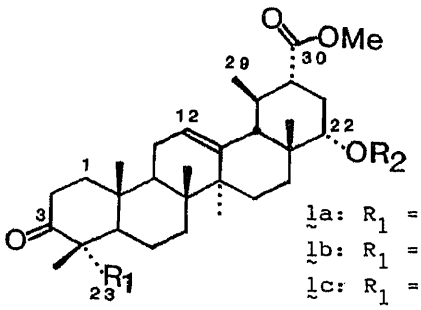
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Regelin (1a), a new antitumor ursene-type triterpenoid, the major principle, and another new ursene-type regelinol (1c) were isolated together with oleanene-type regelide (2) from the roots of Tripterygium regelii. The respective structures of 1a and 1c were determined, by ^{1}H- and ^{13}C-NMR spectrometric studies and X-ray analysis of 1a, to be the methyl ester of 3-oxo-22α-hydroxy-urs-12-en-30-oic acid and its 23-hydroxy analogue. 2 was found to be identical with wilforlide A or abruslactone A by comparing their physico-chemical data.

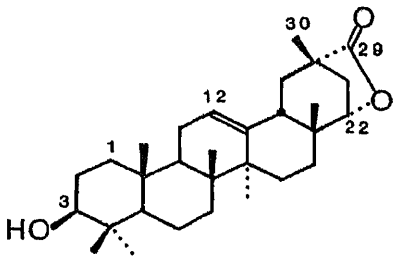
KEYWORDS ——— regelin; regelinol; wilforlide A; Tripterygium regelii; ursene; triterpenoid; ^{1}H-NMR; ^{13}C-NMR; X-ray diffraction; antitumor activity

The plants of genus Tripterygium are perennial twining vines, commonly called Lei Kung Teng or thunder god vine in China. Of the three Chinese species of the genus Tripterygium, T. wilfordii Hook. f. (雷公藤) is grown in the south-eastern area of China, T. hypoglaucum (Levl.) Hutch (昆明山海棠) occurs in south-western China, and T. regelii Sprague et Tak. (黑蔓or東北雷公藤) is found in north-east of China.[2)] The roots of these plants have been extensively used since ancient times to kill garden insects.[3)] They have also been used as folk medicines for cancer and inflammatory diseases.[4)] Since antileukemic diterpenoids were found in T. wilfordii,[5)] and the same components as above were isolated from T. hypoglaucum,[6)] a number of studies have been focused in China on the chemistry and pharmacology of the genus Tripterygium. Certain triterpenoids isolated from T. wilfordii have recently been investigated.[7,8)] This prompted us to conduct further chemical investigations of bioactive constituents in the roots of T. regelii. The present paper deals with the isolation and structure of the new triterpenoid constituents from this plant, regelin (1a) and regelinol (1c), which have an antitumoral activity.

The ethanol extract of the dried roots of the plant collected at Chang Bai Shan Mountain in Fusong Prefecture, Jilin Province, China was percolated



1a: $R_1 = CH_3$, $R_2 = H$
1b: $R_1 = CH_3$, $R_2 = COCH_3$
1c: $R_1 = CH_2OH$, $R_2 = H$
1d: $R_1 = CH_2OCOCH_3$, $R_2 = COCH_3$



2

successively with n-hexane, ethyl acetate and n-butanol. Column chromatography of the ethyl acetate fraction resulted in the isolation of three triterpenes, regelin (1a), regelinol (1c) and regelide (2). First of all, crystallization of 2 from dichloromethane gave colourless prisms of mp 320-322°C. The empirical formula $C_{30}H_{46}O_3$ was determined by high-resolution MS (h.r.MS): m/z (M^+) 454.3435 (Theor. 454.3445). Its IR, ^{1}H-NMR and MS spectra were coincident with those of triptotriterpenoid lactone A.[8,9]

Crystallization of 1a from dichloromethane/methanol gave chunky crystals of mp 221-223°C, $[\alpha]_D^{27.6}$ +20° (C, 0.9; $CHCl_3$). The empirical formula $C_{31}H_{48}O_4$ was determined by h.r.MS: m/z (M^+) 484.3523 (Theor. 484.3550). The IR spectrum showed a hydroxy band at 3550 cm^{-1}, and two carbonyl bands at 1728 and 1700 cm^{-1}, which are attributable to a methyl ester and a six-membered ring carbonyl group, respectively. Acetylation of 1a gave the monoacetate (1b), $C_{33}H_{50}O_5$, h.r.MS m/z (M^+) 526.3673 (Theor. 526.3658), indicating the presence of one hydroxyl group. The four oxygen functions in the molecule were thus characterized. The MS spectrum of 1a revealed that it should be assigned to a triterpenoid belonging to either the urs-12-ene or olean-12-ene type. In consideration of the MS fragmentations of m/z 278 and 205, typical of the retro Diels-Alder spalling,[10] the hydroxyl group and methoxycarbonyl group seem to be located in ring D and/or ring E, and the carbonyl group appears to be derived by the oxidation of 3β-hydroxyl group. The ^{1}H-NMR spectrum of 1a showed five sharp signals of singlets at δ 0.99 (3H), 1.07 (6H), 1.08 (3H), 1.10 (3H) and 1.14 (3H), for six tertiary methyl groups, and a doublet signal at δ 0.76 (3H) for a secondary methyl group. The secondary methyl signal on ring E provides a most useful indicator for the presence of an urs-12-ene skeleton.[11] The presence of only one doublet signal indicates that the remaining methyl group of the two in ring E must be biologically oxidized to the carboxylic acid. The triplet signal at δ 5.24 (J = 4, 1H) for the C(12) olefinic proton indicates that 1a should be one of the 3-oxo-urs-12-en-30-oic acid series of triterpenoids. An inspection of Dreiding models suggests that the C(29) carboxylic function could deshield the proton at C(12). In fact, the downfield signal appears at δ 5.50.[12] It remained, therefore, to determine the exact location of the hydroxyl group in either ring D or E of 1a. A broad triplet at δ 3.38 (J = 8, 1H) was attributed to the proton attached to the carbon bearing a hydroxyl group. The base peak at m/z 246, $(C_{16}H_{22}O_2)^+$, is attributable to the lactonization of a retro Diels-Alder fragment ion m/z 278 caused by the loss of methanol.[13] Hence, this hydroxyl group must be located at C(22), probably with the α-configuration. The structure of the new

triterpenoid constituent, regelin (1a), has now been confirmed as methyl 3-oxo-22α-hydroxy-urs-12-en-30-oate.

The other new constituent, regelinol (1c) was crystallized from methanol to afford chunky crystals, $C_{31}H_{48}O_5$, mp 210-212°, $[\alpha]_D^{26}$+14° (c = 0.5, $CHCl_3$), FD-MS: m/z(M^+) 500, positive Liebermann test. The IR spctrum of 1c showed peaks at ν_{max}^{KBr} 3470, 1710 and 1740 cm^{-1} for a hydroxyl group, a six-membered ketone and a carbomethoxy group, respectively. The existence of two hydroxyl groups in 1c was found by acetylation of 1c with 4-dimethylaminopyridine and acetic anhydride at room temperature yielding a diacetate (1d): $C_{35}H_{52}O_7$, h.r.MS: m/z (M^+) 584.3722 (Theor. 584.3712). In the EI-MS of 1c, the molecular ion peak was not observed, but there was a weak peak at m/z 470 (M^+-CH_2O), indicating the existence of a primary hydroxyl group. The most abundant ions at m/z 278 and 246 corresponded to the fragmentation identical with those observed in 1a (cf. Δ^{12}-triterpenoids[10]). Furthermore, in the ^{13}C-NMR spectrum of 1c, the signals at δ 138.3 (s, C-13) and δ 125.4 (d, C-12) indicate an ursene-type skeleton[14] the same as in 1a. The ^{1}H-NMR spectrum of 1c showed sharp singlets at δ 0.99 (3H), 1.04 (3H), 1.08 (3H), 1.13 (3H), and 1.18 (3H) for five tertiary methyl groups and at δ 3.69 (3H) for a C(30) carbomethoxy group,[12,15] as well as a doublet at δ 0.75 (3H) for one secondary methyl gorup. A triplet centered at δ 5.19 (J = 3.3) was assigned to C(12) olefinic proton. A pair of doublets at δ 3.66 and 3.44 (J = 11.3) were attributed to AB system methylene protons assigned to the $-CH_2OH$ group at C(4α) (cf. those for $-CH_2OH$ of C(4β) at δ 3.98 and 3.41[11] and C(10) at 4.26 and 3.95[16]). On the basis of the above findings and from the biogenetic point of view, regelinol (1c) can be represented as methyl 3-oxo-22α,23-dihydroxy-urs-12-en-30-oate.

To confirm their stereostructures unequivocally, 1a and 2 were studied by X-ray diffraction. The stereoview of the molecular structure of 1a is shown in Fig. 1. The crystal data of both regelide (2) and wilforlide A[17] were congruous with those of abruslactone A.[13,18] The crystal data of 1a are as follows: $C_{31}H_{48}O_4$; MW = 484.7; orthorhombic; space group $P2_12_12_1$; lattice constants, a = 12.198(6), b = 28.827(15), c = 7.743(4) Å, U = 2723 $Å^3$; D_{calc} = 1.182 gcm^{-3}; Z = 4. Intensities of 2823 reflections were measured at the 2σ(I) level, out of the 3320 theoretically possible ones, in the 2θ range of 6° through 156° using graphite monochromated CuKα radiation. The crystal structure was solved by the

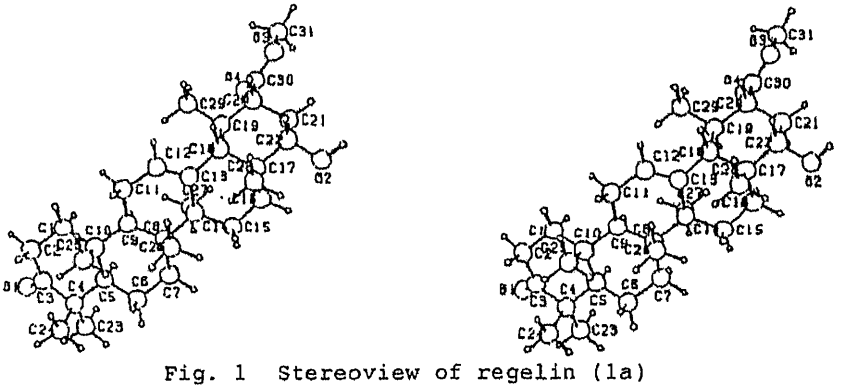


Fig. 1 Stereoview of regelin (1a)

direct method and refined by the block-diagonal matrix least-squares calculations. All 48 hydrogen atoms were located and refined by assuming isotropic thermal vibrations. The final R value was 0.048.[19] The novel ursene types, regelin

(1a), regelinol (1c) and an oleanene type of triterpenoid, wilforlide A (2), were isolated from T. regelii for the first time. The antitumor activities of 1a, 1c and 2, against KB cell lines were ED_{50} 4.2, 10.6 and >100 μg/ml, respectively, according to the screening test of the NCI (USA) protocol.

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Communications to the Editor

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STRUCTURE OF THEONELLAPEPTOLIDE IE, A NEW TRIDECAPEPTIDE LACTONE FROM AN OKINAWAN MARINE SPONGE, THEONELLA SP. (THEONELLIDAE)

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A new tridecapeptide lactone named theonellapeptolide Ie (2), isolated from an Okinawan marine sponge of Theonella sp. (Theonellidae) together with theonellapeptolide Id (1) and some other minor peptide lactones, has been determined on the basis of chemical and physicochemical evidence. A new HPLC analysis method for N-methyl amino acids has been developed to analyze the amino acid composition of theonellapeptolide Id (1) and Ie (2). Ie (2) contains one N-methyl-β-alanine and two β-alanine moieties among thirteen constituent amino acids whereas Id (1) contains three β-alanine moieties.

KEYWORDS —— Theonella sp.; marine sponge; Theonellidae; theonellapeptolide Ie; peptolide; N-methyl amino acid HPLC analysis; D amino acid; N-methyl amino acid; peptide FAB-MS

As a continuing study on bioactive marine natural products,[1] we recently reported the isolation of five peptide lactones named theonellapeptolide Ia, Ib, Ic, Id, and Ie from an Okinawan marine sponge of Theonella sp. (Theonellidae) and elucidated the structure of the major peptide lactone, Id (1).[2] These peptide lactones were characterized by their inhibition of the development of the fertilized eggs of the sea urchin Hemicentrotus pulcherrimus. In this paper, we describe the structure of theonellapeptolide Ie (2), using a new HPLC analysis method for N-methyl amino acids.

Theonellapeptolide Ie (2), colorless crystals, mp 153–155°C (MeOH), $C_{71}H_{127}O_{16}N_{13}$, $[\alpha]_D^{23}$ -62° (MeOH), FAB-MS[3] m/z 1419 $(M+H)^+$, possesses amide groups (3350, 3030, 1675, 1545 cm^{-1}) and a lactone group (1735 cm^{-1}) as shown by its IR spectrum. The ^1H NMR spectrum (500 MHz, $CDCl_3$-CD_3OD=10:1) of 2 showed signals assignable to seven amide protons (δ 8.32, 1H br d, J=ca. 8.6 Hz; δ 8.15, 1H d, J=9.2 Hz; δ 8.00, 1H d, J=8.9 Hz; δ 7.31, 1H br d, J=ca. 8.9 Hz; δ 7.19, 2H d, J=9.2 Hz; δ 6.93, 1H d, J=6.1 Hz) and six N-methyl groups (δ 3.30, 3.26, 3.23, 3.16, 2.76, 2.74, all 3H s). The ^{13}C NMR spectrum (125 MHz, CD_3OD) of 2 showed signals due to thirteen amide carbons and one lactone carbon (δc 169.8, 171.3, 171.5, 171.6, 172.1, 172.5, 172.8, 173.0, 173.5, 173.6, 175.1, 175.3, 175.6, 176.0, all s). Thus, theonellapeptolide Ie (2) was revealed to be a tridecapeptide containing six N-methyl amino acids.

To clarify the amino acid composition including the N-methyl amino acids, we have developed a new HPLC analysis method. Theonellapeptolide Ie (2) was hydrolyzed completely with 6N HCl at 110°C for 24 h and the hydrolysate was subjected to reversed phase HPLC [Cosmosil $5C_{18}$, 10 mm ID x 500 mm, 205 nm, elution with H_2O (2 ml/min)] to give the chromatogram shown in Fig. 1. The retention time and the peak area indicate an amino acid composition. In addition, the CD spectra (in 0.5N HCl) of the amino acids eluted from the column show their absolute structures to be βAla (2), L-Thr (1), MeβAla (1), L-MeAla (1), L-Val (1), L-MeVal (1), D-aIle (1), D-Leu (2), D-MeaIle (1), L-MeIle (1), and D-MeLeu (1).[4]

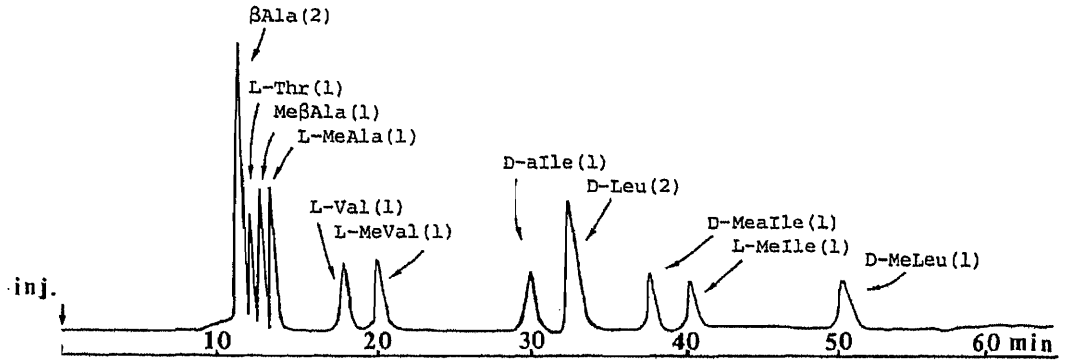


Fig. 1 The HPLC Chromatogram of the Complete Acidic Hydrolysate of Theonellapeptolide Ie (2).

Theonellapeptolide Ie (2) was negative in the Ninhydrin test, but was positive for the Dragendorff reagent. The ^1H NMR spectrum of 2 showed signals attributable to a methoxyacetyl group [δ 3.43, 3H s; δ 3.95, 3.88, each 1H d, J=15.0 Hz] as was found in co-occurring theonellapeptolide Id (1).[2] Therefore, the N-terminal amino acid of Ie (2) apparently was protected with the methoxyacetyl moiety. Furthermore, theonellapeptolide Ie (2) was unaffected by diazomethane treatment and the IR spectrum of Ie (2) showed a lactone absorption band. These findings indicate that the C-terminal of Ie (2) is esterified with the hydroxyl group of L-Thr. Thus, theonellapeptolide Ie (2) is shown to be a tridecapeptolide comprising six N-methyl amino acids and seven amino acids.

To determine the amino acid sequence, theonellapeptolide Ie (2) was subjected to partial hydrolysis with 30% aq. trifluoroacetic acid (TFA) (110°C, 40 min) and the resulting hydrolysates were preparatively separated by HPLC [Zorbax ODS, 0.25 m x 9.6 φ; eluent: solvent A=0.1% TFA in isoPrOH-CH_3CN (7:3), solvent B= 0.1% TFA in H_2O; gradient: A (0% → 25%) and B (100% → 75%) in 30 min, A (25% → 70%) and B (75% → 30%) in 70 min, and A (70% → 100%) and B (30% → 0%) in 120 min] to afford Fr.8 ∿ Fr.32 and Fr.39. These hydrolysates were then analyzed by combination of the above-described HPLC method, the dansyl (DNS) derivation method, and the FAB-MS (positive and negative).

The major hydrolysate Fr.39 (13), m/z 1139 $(M+H)^+$, was shown to comprise ten amino acids [Val (1), MeLeu (1), Thr (1), βAla (1), MeβAla (1), Leu (2), MeaIle (1), MeIle (1), MeAla (1)], among which MeAla was the newly formed N-terminal as proved by the DNS derivation method. Comparison of Fr.32 (3), m/z 1010 $(M-H)^-$, Fr.26 (4), m/z 940 $(M+H)^+$, and Fr.29 (5), m/z 897 $(M-H)^-$, led us to determine a

Fr.32 (3)[5]: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu
O ← MeaIle ← Leu ← βAla ← MeAla

Fr.26 (4)[5]: MeLeu ← Val
Thr → MeβAla → Leu
O ← MeaIle ← Leu ← βAla ← MeAla

Fr.29 (5)[5]: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla
O ← MeaIle ← Leu ← βAla ← MeAla

A (6)[6]: (MeLeu / Thr, MeβAla) ← Val-$COCH_2OCH_3$ → Leu → MeIle
O ← (MeaIle, Leu, βAla) ← MeAla

Fr.21 (7)[5]: Thr → MeβAla → Leu
O ← MeaIle ← Leu ← βAla ← MeAla

Fr.24 (8)[5]: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu

B (9)[6]: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu → MeIle
O ← (MeaIle, Leu, βAla) ← MeAla

Fr.36 (10)[5]: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu → MeIle
O ← MeaIle ← Leu ← βAla

Fr.31 (11)[5]: MeLeu ← Val
Thr → MeβAla → Leu
O ← MeaIle ← Leu

Fr.9 (12): Leu ← βAla ← MeAla

Fr.39 (13): MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu → MeIle
O ← MeaIle ← Leu ← βAla ← MeAla

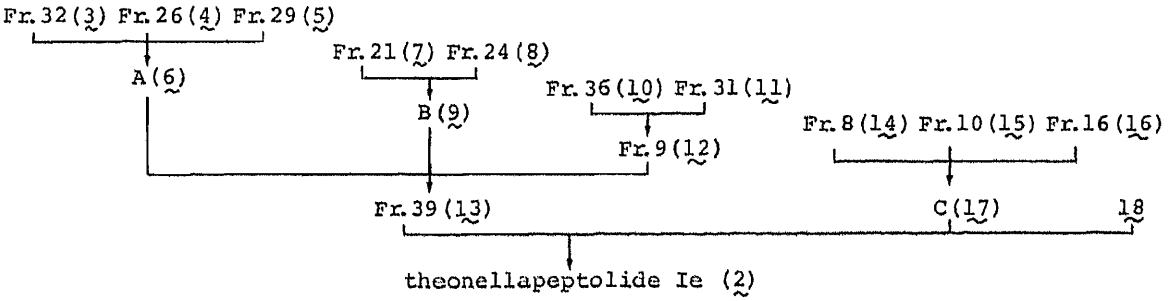
Fr.8 (14): βAla → aIle

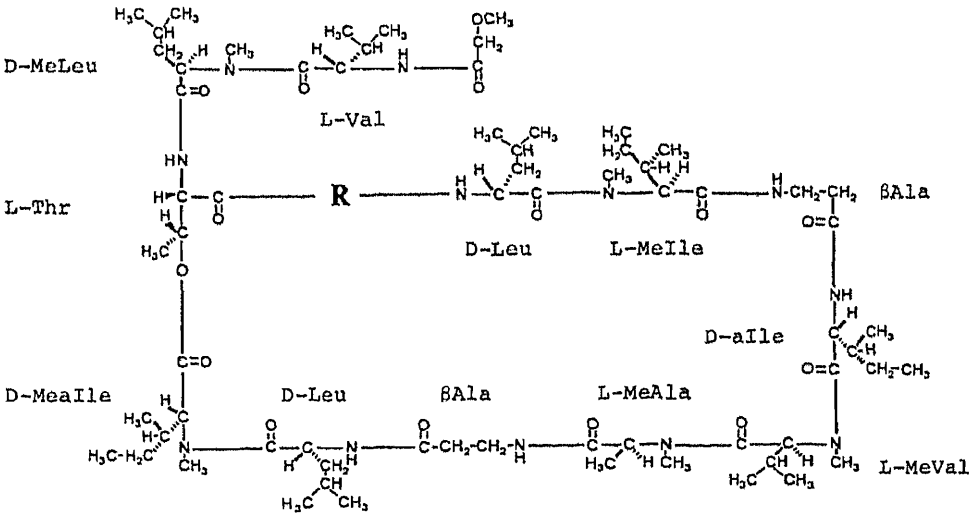
Fr.10 (15): βAla → aIle → MeVal

Fr.16 (16): Leu → MeIle → βAla → aIle

C (17): Leu → MeIle → βAla → aIle → MeVal

18: MeLeu ← Val-$COCH_2OCH_3$
Thr → MeβAla → Leu → MeIle → βAla → aIle
OH MeVal
H_3CO-C(=O)-MeaIle ← Leu ← βAla ← MeAla





R = βAla : theonellapeptolide Id (1)
R = MeβAla : theonellapeptolide Ie (2)

partial structure A (6) in Fr.39 (13).[5,6] Furthermore, comparison of the partial structure A (6) with Fr.21 (7), m/z 714 $(M+H)^+$ and Fr.24 (8), m/z 616 $(M+H)^+$, led us to extend the partial structure of Fr.39 (13) from A (6) to B (9).[5,6] On the other hand, comparative analysis of Fr.36 (10), m/z 1054 $(M+H)^+$ and Fr.31 (11), m/z 784 $(M+H)^+$, supported the amino acid sequence of Fr.9 (12), m/z 288 $(M+H)^+$.[5] Combined evidence for Fr.9 (12) and B (9) disclosed the amino acid sequence of Fr.39 (13) as shown.

Similarly, comparative analysis of Fr.8 (14), m/z 203 $(M+H)^+$, Fr.10 (15), m/z 316 $(M+H)^+$, and Fr.16 (16), m/z 443 $(M+H)^+$, disclosed the presence of another partial structure C (17) in theonellapeptolide Ie (2).

Treatment of theonellapeptolide Ie (2) with CH_3ONa-CH_3OH furnished the methyl ester (18), FAB-MS m/z 1451 $(M+H)^+$. The ^1H NMR spectrum (500 MHz, CD_3OD) of 18 showed a one-proton multiplet at δ 4.08 assignable to the proton geminal to the hydroxyl moiety of Thr. The chemical shift indicated that the hydroxyl moiety of Thr in theonellapeptolide Ie (2) was esterified, since the signal due to the corresponding geminal proton of Ie was observed at δ 5.26 (m). These findings led us to presume that Fr.39 (13) and the partial structure C (17) may overlap at the Leu → MeIle moiety. The formation of the methyl ester (18) ascertained the presence of the MeVal → MeAla sequence in theonellapeptolide Ie (2).

Based on the above evidence, the whole structure of theonellapeptolide Ie (2) has been determined as shown. Theonellapeptolide Ie (2) is a tridecapeptolide having an amino acid sequence very similar to that of theonellapeptolide Id (1). Ie (2) differs from Id (1) in the fourth amino acid constituent from the N-terminal (next to Thr), Ie (2) contains MeβAla while Id (1) contains βAla.

ACKNOWLEDGEMENT The authors are grateful to Prof. T. Momose, Faculty of Pharmaceutical Sciences, Kinki University, for measuring the FAB-MS. They also thank the Ministry of Education, Science, and Culture of Japan for financial support.

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6) The sequences of the amino acids given in the parentheses were not ascertained at this stage of discussion. They were clarified by combination of the evidence given later.

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Communications to the Editor

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3-CAFFEOYL-4-SINAPOYLQUINIC ACID, A NOVEL LIPOXYGENASE INHIBITOR FROM GARDENIAE FRUCTUS

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A new 5-lipoxygenase inhibitor, 3-O-caffeoyl-4-O-sinapoyl-quinic acid (2), was isolated from Gardeniae Fructus. The structure was elucidated on the basis of spectral data and the result of partial methanolysis of its tetramethylate (3).

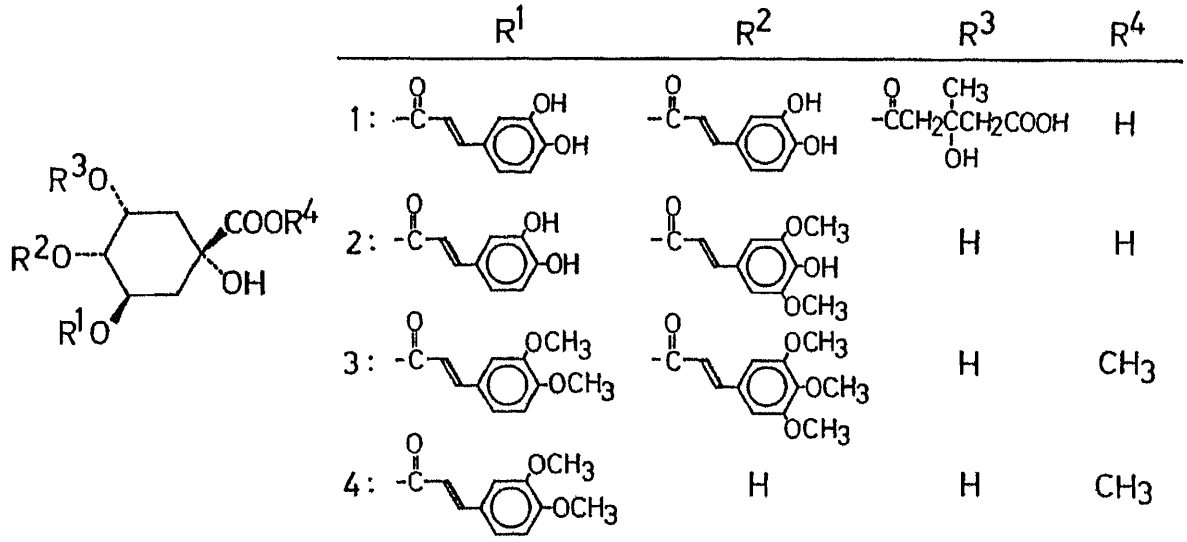
KEYWORDS ——— Gardeniae Fructus; Gardenia jasminoides; Rubiaceae; lipoxygenase inhibitor; quinic acid; caffeic acid; sinapic acid

Gardeniae Fructus (fruit of Gardenia jasminoides ELLIS, Rubiaceae) is a crude drug which has been used as a diuretic, a cholagogue and an anti-inflammatory in China and Japan. Gardeniae Fructus was known to contain a number of iridoid glycosides, e.g. geniposide,[1] genipin gentiobioside,[2] and yellow pigments[3] (crocin and crocetin). In the previous communication,[4] we reported the isolation and structural elucidation of a lipoxygenase inhibitor, 3,4-di-O-caffeoyl-5-O-(3-hydroxy-3-methyl)-glutaroylquinic acid (1) from this crude drug. Caffeic acid and other hydroxycinnamic acid derivatives are known to inhibit lipoxygenase activity,[4,5] so we have continued the studies on hydroxycinnamic acid derivatives in Gardeniae Fructus. In this paper, we report the isolation and structural elucidation of a new hydroxycinnamic acid derivative which inhibits the lipoxygenase activity.

The powdered Gardeniae Fructus was extracted with 50% aqueous acetone three times at room temperature. After evaporation of the acetone under reduced pressure, the solution was extracted with hexane, ethyl acetate and n-butanol, successively. The new compound (2), [a pale yellow amorphous powder, $[\alpha]_D^{20}$ -252° (c=0.54, MeOH), IR ν_{max}^{nujol} cm^{-1}: 3380(OH), 1690(br,COO), UV λ_{max}^{MeOH} nm (ε): 328 (40300), 239(28800), 220(31800), NMR data see note[6]] was isolated from the n-butanol extract by column chromatography (Diaion HP-20 and HP-40, MeOH-H_2O gradient elution), and then preparative HPLC (Fuji gel ODS RQ-2, MeOH-H_2O-HCOOH or

CH_3CN-H_2O-HCOOH).

The compound (2) showed a molecular ion peak at m/z 560 (M^+) in its FD-MS spectrum. The ^{1}H-NMR spectrum (270MHz) of 2 showed two pairs of the signals at δ 7.61, 7.54, 6.40 and 6.22 (each 1H, d, J=15.9Hz) due to trans olefins, a set of signals due to 1,3,4-trisubstituted aromatic protons, a singlet at δ 6.98(2H), and a signal of aromatic methoxy groups (6H). This indicated the presence of caffeoyl and sinapoyl moieties. In the aliphatic proton region, three methine protons each bearing an oxygen function appeared at δ 5.68(1H, ddd), 5.16(1H, dd) and 4.45(1H, br.d) together with four methylene protons. The coupling constants and the chemical shifts of the methine protons bearing an oxygen function indicated that 2 is a 3,4-di-O-acylated quinic acid.



Treatment of 2 with diazomethane gave a mixture of methylates which were further methylated with dimethyl sulfate-potassium carbonate to give a tetramethyl derivative (3) [$[\alpha]_D^{20}$ -128° (c=0.37,$CHCl_3$), IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500(OH), 1725sh., 1700 (COO). EI-MS m/z: 616(M^+), 238(methylsinapoyl), 191(dimethylcaffeoyl), NMR data see note[7)]. The ^{1}H-NMR spectrum of 3 showed the presence of five aromatic methoxy groups and one carboxy methyl group. On the partial methanolysis with anhydrous methanolic HCl, 3 gave the permethylates of sinapic acid[8)] and chlorogenic acid (4),[9)] which were identified by comparing their spectral data with those of authentic samples. Based on these results, the structure of 2 was determined to be 3-O-caffeoyl-4-O-sinapoylquinic acid.

The inhibitory effects of 2 on 5-lipoxygenase were shown to be 100% at 10^{-4}M and 89% at 10^{-5}M; thus 2 is a more potent inhibitor than 1.[4)] There have been many reports on the sinapoyl esters of D-glucose,[10)] flavonoids[11)] and anthocyans.[12)] However, 3-sinapoylquinic acid[13)] has only been reported as the sinapoyl ester of quinic acid. This is the first report of the sinapoyl and caffeoyl ester of quinic acid.

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6) ^{1}H-NMR(acetone-d_6-D_2O, 270MHz) δ: 7.61(1H,d,J=15.9), 7.54(1H,d,J=15.9), 7.13 (1H,d,J=2.0), 7.01(1H,dd,J=2.0,8.3), 6.98(2H,s), 6.84(1H,d,J=8.3), 6.40(1H,d, J=15.9), 6.22(1H,d,J=15.9), 5.68(1H,ddd,J=4.8,9.7,10.6), 5.16(1H,dd,J=2.9,9.7), 4.45(1H,br.d,J=2.9), 3.89(6H,s), 2.39(1H,ddd,J=2.5,4.8,13.2), 2.29(2H,m), 2.18 (1H,dd,J=11.0,13.2). ^{13}C-NMR(acetone-d_6-D_2O, 22.5MHz) δ: 167.7, 167.4, 163.9, 155.1, 152.9, 149.4(2C), 147.2, 147.0, 146.8, 127.7, 126.5, 123.1, 116.7, 116.2, 115.5(2C), 107.4(2C), 78.1, 76.1, 69.7, 68.8, 57.2(2C), 40.3, 38.5.

7) ^{1}H-NMR($CDCl_3$,270MHz)δ: 7.63(1H,d,J=15.8), 7.59(1H,d,J=15.8), 7.03(1H,dd,J=1.8, 8.4), 7.00(1H,d,J=1.8), 6.84(1H,d,J=8.4), 6.72(2H,s), 6.41(1H,d,J=15.8), 6.24 (1H,d,J=15.8), 5.76(1H,ddd,J=4.8,10.3,11.3), 5.20(1H,dd,J=2.9,10.3), 4.40(1H, m), 3.895(3H,s), 3.886(3H,s), 3.859(3H,s), 3.853(6H,s), 3.84(3H,s), 2.47(1H, br.dd,J=5.0,13.3), 2.24(2H,m), 2.08(1H,dd,J=11.3,13.3). ^{13}C-NMR($CDCl_3$, 22.5MHz) δ: 174.5, 166.5, 166.4, 153.5(2C), 151.5, 149.4, 145.8, 145.6, 130.4, 129.8, 127.3, 122.9, 116.9, 115.3, 111.2, 109.9, 105.6(2C), 77.3, 75.7, 69.1, 61.0, 56.2(2C), 56.0(2C), 53.5, 39.5, 37.4.

8) EI-MS m/z: 252(M^+), 237. ^{1}H-NMR($CDCl_3$, 100MHz) δ: 7.61(1H,d,J=15.6), 6.76(2H, s), 6.35 (1H,d,J=15.6), 3.89(9H,s), 3.81(3H,s).

9) $[\alpha]_D^{20}$ -38°(c=0.26,$CHCl_3$), IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3450(OH), 1710(COO). EI-MS m/z: 396 (M^+),208,191,181,164. ^{1}H-NMR($CDCl_3$, 100MHz) δ: 7.67(1H,d,J=16.1), 7.12(1H,dd, J=2.0,8.3), 7.06(1H,d,J=2.0), 6.86(1H,d,J=8.3), 6.34(1H,d,J=16.1), 5.40(1H,ddd, J=4.6,9.8,11.7), 4.23(1H,br.dd,J=3.0,6.0), 3.90(6H,s), 3.81(3H,s), 3.69(1H,dd, J=3.0,9.8), 1.82-2.48(4H,m).

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Communications to the Editor

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SYNTHESIS OF DIBEKACIN (3',4'-DIDEOXYKANAMYCIN B) FROM D-GLUCOSAMINE AND D-GLUCOSE

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An aminoglycoside antibiotic, dibekacin (=3',4'-dideoxykanamycin B) (22), has been synthesized from D-glucosamine and D-glucose using an oxidative decarboxylation reaction with lead tetraacetate and a reductive deacetoxylation reaction with sodium borohydride as key-reactions.

KEYWORDS —— dibekacin; 3',4'-dideoxykanamycin B; aminoglycoside antibiotic; aminocyclitol; D-glucosamine; D-glucose; nitromethane cyclization; oxidative decarboxylation; reductive deacetoxylation; selective glycosidation

Using an oxidative decarboxylation reaction with lead tetraacetate and a reductive deacetoxylation reaction with sodium borohydride as key-reactions, we have been developing a versatile new synthetic method for synthesizing aminocyclitols and their glycosides from monosaccharides.[1] As an application of this method, we reported a synthesis of ribostamycin, an aminoglycoside antibiotic, which comprises two monosaccharide residues attached one each to C-4 and C-5 hydroxyl groups of the central 2-deoxystreptamine moiety.[2] In this paper, we report a synthesis of dibekacin (=3',4'-dideoxykanamycin B) (22) using D-glucosamine and D-glucose as the starting materials.[3] Dibekacin is a clinically important aminoglycoside antibiotic prepared by the chemical modification of kanamycin B.[4] It comprises two monosaccharide residues attached to C-4 and C-6 hydroxyls of the 2-deoxystreptamine moiety.

N-Carbobenzyloxy(Cbz)-D-glucosamine (1)[5] was converted to 2 (an anomeric mixture in a 3:2 ratio) by successive treatment with *p*-anisylchlorodiphenylmethane (MMTrCl) and methanesulfonyl chloride, both in pyridine.[6] Two mesyloxy residues in 2 were removed with Zn-NaI and the resulting olefin was subjected to detritylation and benzoylation to provide 3 quantitatively from 2. 3 thus obtained was a 3:2 mixture of α-anomer, $C_{22}H_{23}NO_6$,[7] mp 152-154°C, $[\alpha]_D$ -18°, and β-anomer, $C_{22}H_{23}NO_6$, mp 149-151°C, $[\alpha]_D$ -40°. Catalytic hydrogenation (PtO_2, H_2 3.5 atm) of 3 and subsequent treatment of the product with 2,4-dinitrofluorobenzene (DNFB) and Na_2CO_3 furnished 4 (88%): a mixture of α-anomer, $C_{20}H_{27}N_3O_8$, yellow oil, $[\alpha]_D$ +53° and β-anomer, $C_{20}H_{27}N_3O_8$, yellow oil, $[\alpha]_D$ +14°. Alkaline deacylation of 4 followed by acidic hydrolysis (70°C), acetylation, and anomeric bromination (5°C, 5 min), provided α-bromide (5) (73% from 4).

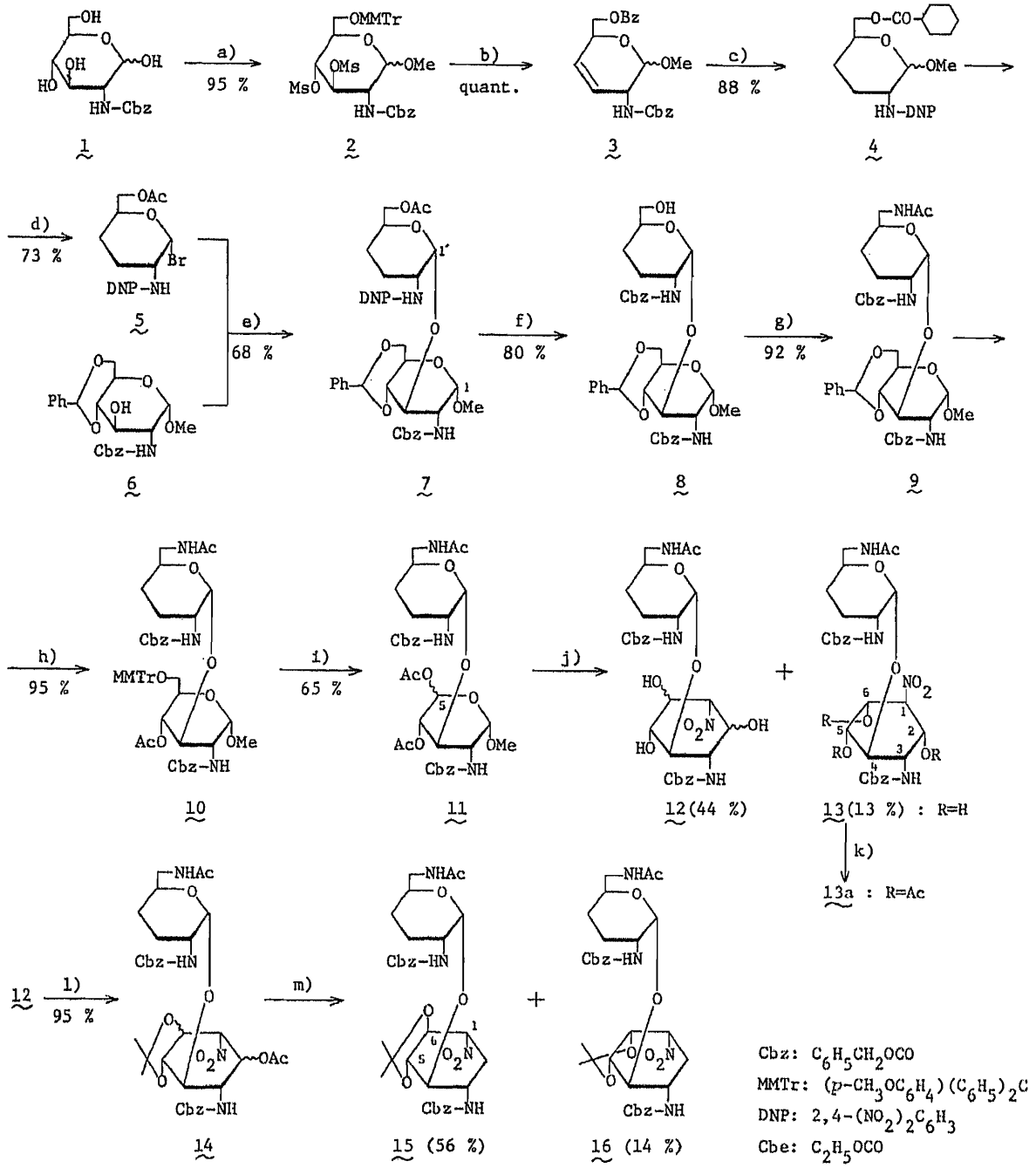
Glycosidation of 6 (0.9 mmole)[1b,5] with 5 (1.8 mmole) in the presence of $AgClO_4$ and *sym*-collidine in benzene-dioxane (2:1) (27°C, 15 min) furnished 7 (68%),[8] $C_{36}H_{40}N_4O_{14}$, yellow oil, $[\alpha]_D$ +22°, with a recovery of 6 (23%). The ^{13}C NMR spectrum (22.5 MHz, $CDCl_3$) of 7 showed signals due to two α-anomeric carbons [δc 100.3 (1-C), 97.9 (1'-C)]. Removal of the DNP and acetyl residues in 7 with Dowex 1x2 (OH^-) (25°C) and subsequent carbobenzyloxylation (2°C) yielded 8, $C_{36}H_{42}N_2O_{11}$, mp 182-183°C, $[\alpha]_D$ +26° (80% from 7). Tosylation of 8 followed by azide formation, reduction, and acetylation furnished 9, $C_{38}H_{45}N_3O_{11}$, mp 187-189°C, $[\alpha]_D$ +38° (93% from 8). Removal of the benzylidene group in 9 and subsequent treatment with MMTrCl and acetylation, gave 10, $C_{53}H_{59}N_3O_{13}$, colorless oil, $[\alpha]_D$ +33° (95% from 9).

Treatment of 10 with BF_3-etherate followed by Jones oxidation provided a uronide, which, by decarboxylative acetoxylation with $Pb(OAc)_4$ in benzene-pyridine (5:1) (reflux for 1.5 h),[1] was converted to 11, a 1:3 mixture of 5α- and 5β-OAc isomers (65% from 10). Treatment of 11 with 1.7% NaOMe-MeOH / CH_3NO_2 (1:1) (26°C, 48 h) furnished 12 (44%) (a mixture of *scyllo* and *myo* isomers) and 13 (13%, *muco*), $C_{30}H_{38}N_4O_{12}$, white powder, $[\alpha]_D$ +15°, which, on acetylation with Ac_2O-BF_3 etherate, was converted to the acetate (13a). The ^1H NMR spectrum (500 MHz, $CDCl_3$) of 13a substantiated the structure with signals due to three O-acetyl groups [δ 1.93 (6H), 1.98(3H)] and protons attached to the nitrocyclitol moiety: δ 4.82 (dd, J=9.5, 9.5 Hz, 1-H), 5.31 (dd, J=4.8, 9.5 Hz, 2-H), 3.99 (ddd, J=4.5, 4.8, 10.0 Hz, 3-H), 3.92 (dd, J=4.3, 4.5 Hz, 4-H), 5.52 (dd, J=4.3, 4.8 Hz, 5-H), 5.26 (dd, J=4.8, 9.5 Hz, 6-H). Thus, the *muco* configuration of 13 was ascertained.

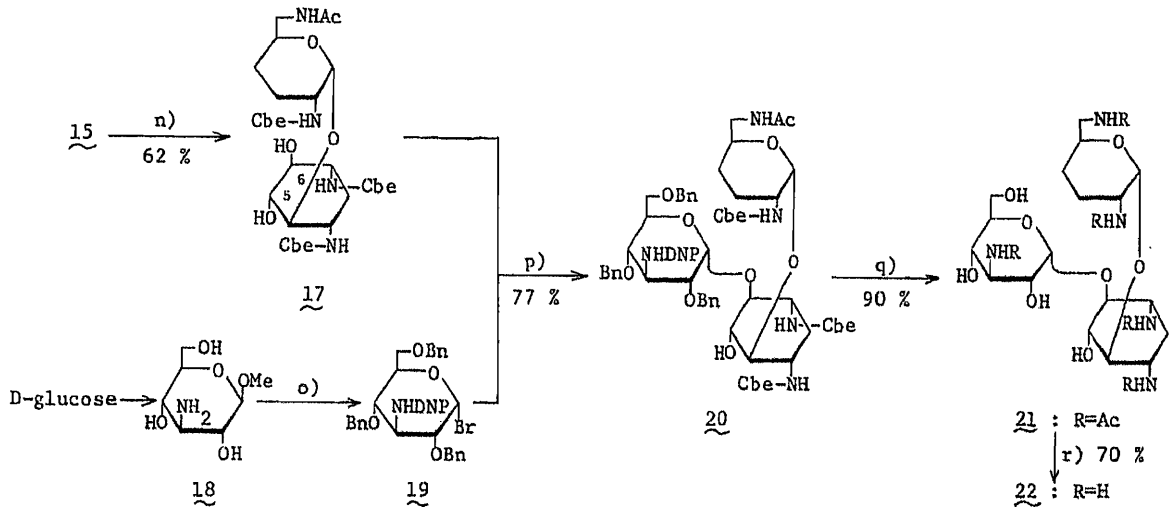
Treatment of 12 with isopropenyl methyl ether in DMF in the presence of *d*-10-camphorsulfonic acid (CSA) and subsequent acetylation, afforded 14. $NaBH_4$ reduction of 14 in 95% EtOH (5°C, 30 min) yielded 15 (56%), $C_{33}H_{42}N_4O_{11}$, colorless oil, $[\alpha]_D$ +32°, and 16 (14%), $C_{33}H_{42}N_4O_{11}$, colorless oil, $[\alpha]_D$ +19°. The ^1H NMR spectrum (500 MHz, $CDCl_3$) of 15 corroborated the structure with signals due to protons on the cyclitol moiety: δ 4.63 (ddd, J=4.3, 9.8, 9.8 Hz, 1-H), 3.92 (dd, J=9.8, 9.8 Hz, 6-H), and 3.47 (dd, J=9.8, 9.8 Hz, 5-H). Comparison in detail of the ^1H and ^{13}C NMR data for 15 and 16 revealed that 16 was a 6-epimer of 15. Catalytic hydrogenation [Raney Ni (T-4), H_2 1 atm] of 15 followed by carboethoxylation and removal of the isopropylidene group, furnished 17 (62%), $C_{23}H_{40}N_4O_{11}$, white powder, $[\alpha]_D$ +33°.

On the other hand, a bromide (19) was prepared from 18[9] *via* DNP protection, O-benzylation, acidic hydrolysis, and anomeric bromination. Glycosidation of 17 (0.08 mmole) with 19 (0.16 mmole) in the presence of $AgClO_4$ and *sym*-collidine in benzene-dioxane (2:1) (24°C, 30 min) furnished 20 (77%), $C_{56}H_{71}N_7O_{19}$, yellow powder, $[\alpha]_D$ +75°. The ^{13}C NMR spectrum (22.5 MHz, d_5-pyridine) of 20 showed the presence of two α-linkages with signals due to anomeric carbons (δc 97.9, 97.0). Furthermore, comparison of the ^{13}C NMR data for 17 and 20 indicated a glycosidation shift of the C-6 signal of 20. Thus, it became clear that selective glycosidation at the C-6-OH of 17 probably occurred due to the steric congestion on the C-5-OH of 17 caused by the neighboring C-4 glycosyloxy moiety.

Removal of the DNP group and the Cbe group (100°C, 4 h) of 20 and subsequent N-acetylation and catalytic hydrogenation (5% Pd-C, H_2 2.5 atm), yielded penta-N-acetyldibekacin (21, 90%), which was found to be identical with the authentic



(a) MMTrCl / Py.; MsCl / Py. (b) Zn / NaI / DMF (100°C, 4.5 h); *p*-TsOH·H_2O / Et_2O; BzCl / Py. (c) H_2 / PtO_2 / AcOH; 2,4-dinitrofluorobenzene / Na_2CO_3 / H_2O-acetone (1:2) (d) 1% NaOMe-MeOH; 6N.aq.HCl-AcOH (1:4, 70°C); Ac_2O / Py.; 25% HBr-AcOH / $CHCl_3$ (5°C) (e) $AgClO_4$ / *sym*-collidine / benzene-dioxane (2:1) (f) Dowex 1x2 (OH^-form) / acetone-H_2O (10:1); carbobenzoxy chloride / $NaHCO_3$ / dioxane-H_2O (2:1) (g) *p*-TsCl / Py.; NaN_3 / DMF (80°C); Zn / 90% aq.AcOH / DMF (2°C); Ac_2O / Py. (h) 60% $HClO_4$-acetone (1:60); MMTrCl / Py.; Ac_2O / 4-dimethylaminopyridine / Py. (i) BF_3-Et_2O / THF; Jones oxid.; $Pb(OAc)_4$ / benzene-Py. (5:1) (j) CH_3NO_2 / 1.7% NaOMe-MeOH (k) Ac_2O / BF_3-Et_2O (l) isopropenyl methyl ether / CSA / DMF; Ac_2O / NaOAc (m) $NaBH_4$ / 95% EtOH (5°C)



(n) H_2 / Raney Ni (T-4); ethyl chloroformate / Na_2CO_3 / H_2O-dioxane; 80% aq.AcOH (60°C) (o) 2,4-dinitrofluorobenzene / Na_2CO_3 / H_2O-acetone (4:1); BnBr / BaO / $Ba(OH)_2 \cdot 8H_2O$ / DMF; 6N.aq.HCl-AcOH (90°C); 25% HBr-AcOH / $CHCl_3$ (5°C) (p) $AgClO_4$ / *sym*-collidine / benzene-dioxane (2:1) (q) Dowex 1x2 (OH^-form) / acetone-H_2O (10:1); $Ba(OH)_2 \cdot 8H_2O$ / dioxane-H_2O (2:1); Ac_2O / MeOH; H_2 / Pd-C / AcOH (r) 80% aq.$NH_2NH_2 \cdot H_2O$

sample[10] by mixed mp determination and by $[\alpha]_D$, TLC, IR (KBr), and ^{13}C NMR (CD_3OD) comparisons. Finally, deacetylation of 21 with 80% aq. NH_2NH_2 in a sealed tube (100°C, 72 h) furnished dibekacin (22, 70%) identical with the authentic sample[4] [TLC, IR (KBr), $[\alpha]_D$].

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8) The yield was calculated on the basis of 6.
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10) Prepared from authentic dibekacin[4], mp 251-252°C, $[\alpha]_D$ +128°.

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SYNTHESIS OF (-)-SWAINSONINE AND OPTICALLY ACTIVE 3,4-DIHYDROXY-2-HYDROXYMETHYLPYRROLIDINES

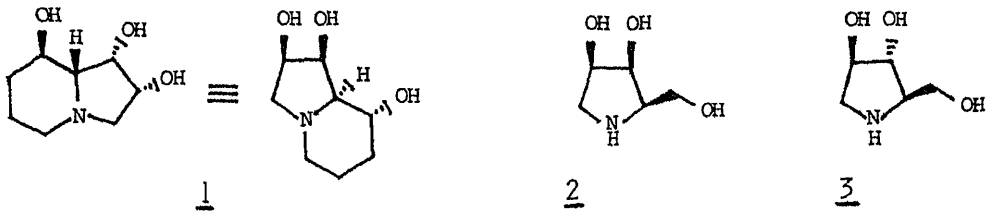
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(-)-Swainsonine, (2R,3S,4R)- and (2R,3R,4R)-3,4-dihydroxy-2-hydroxymethylpyrrolidine (2 and 3) were synthesized stereoselectively starting from (R)- and (S)-glutamic acid derivatives, respectively.

KEYWORDS ——(-)-swainsonine; polyhydroxylated pyrrolidine; cis-dihydroxylation; glutamic acid; diastereoselective allylation

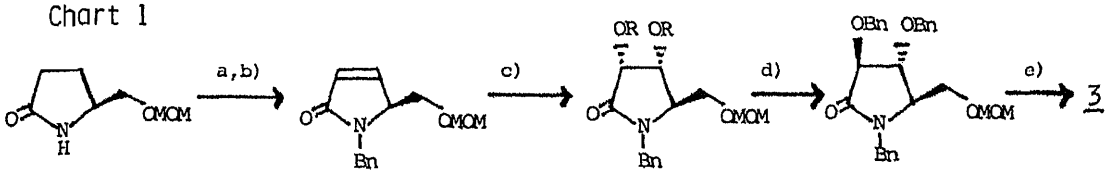
(-)-Swainsonine (1),[1] a trihydroxylated indolizidine alkaloid, exhibits a potent α-mannosidase inhibitory activity. Being interested in the remarkable physiological effects including an immunoregulative activity,[2] the synthesis of (-)-1[3,4] and its stereoisomers[5] from D-mannose or D-glucose, and by asymmetric synthesis[4] have been achieved recently. On the other hand, polyhydroxylated pyrrolidines such as optically active 3,4-dihydroxy-2-hydroxymethylpyrrolidines (2 and 3)[6,7] were reported to be potent α-galactosidase or α-glucosidase inhibitors. As a continuation of our work on the utility of optically active glutamic acid derivatives for the asymmetric synthesis[8] and natural product synthesis, we describe here the versatile synthesis of (-)-swainsonine, (-)-8-epi-swainsonine, and optically active polyhydroxylated pyrrolidines (2 and 3) using (S)- or (R)-glutamic acid derivative as a starting material.

As shown in Chart 1, (S)-pyroglutamic acid derivative (4)[8] was converted in 53% yield into the unsaturated lactam (5)[9] ($[\alpha]_D^{20}$ -28.7°(c 1.8, $CHCl_3$)) by N-benzylation followed by the selenenylation-deselenenylation procedure.[10] cis-Dihydroxylation of 5 with catalytic OsO_4 and N-methylmorpholine N-oxide (NMO) in aqueous acetone[11] gave the diol (6) ($[\alpha]_D^{20}$ +89.1°(c 0.5, $CHCl_3$)) in 65% yield and the other stereoisomer of 6 was not detected. O-Benzylation of 6 with benzyl bromide in DMF-THF in the presence of sodium hydride afforded the di-O-benzyl derivative (7) in 85% yield. Epimerization of 7 with sodium methoxide in THF-MeOH at room temperature afforded 8 ($[\alpha]_D^{20}$ +16.7°(c 1.1, $CHCl_3$)) in 65% yield. Reduction of 8 with borane-dimethylsulfide complex followed by deprotection of the methoxymethyl and benzyl groups furnished 3,[12] isolated as its hydrochloride in 60% yield, mp 113°C; $[\alpha]_D^{20}$ +36.3°(c 0.3, H_2O). It was identical in every respect with previously published data for the hydrochloride of 3.[6,13]



1 2 3

Chart 1

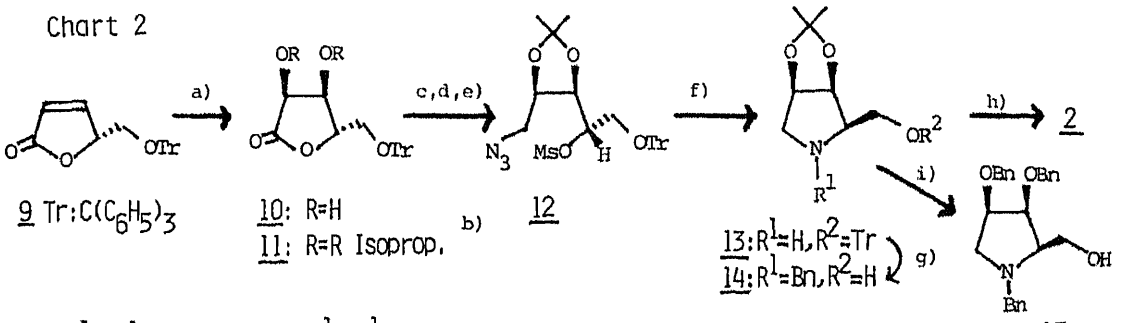


4 MOM: CH_2OCH_3 5 Bn: $CH_2C_6H_5$ 6: R=H 7: R=Bn 8

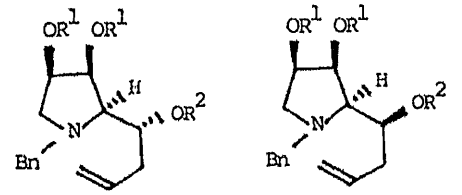
Conditions

a) BnBr,NaH/DMF-THF. b) i,LDA,PhSeBr,THF,-78°C; ii,30%H_2O_2,AcOEt. c) cat.OsO_4,NMO,aq. acetone. d) NaOMe/MeOH-THF. e) i,$BH_3{\cdot}Me_2S$,THF,reflux; ii,aq.HCl; iii,H_2-10%Pd/C,HCl/EtOH.

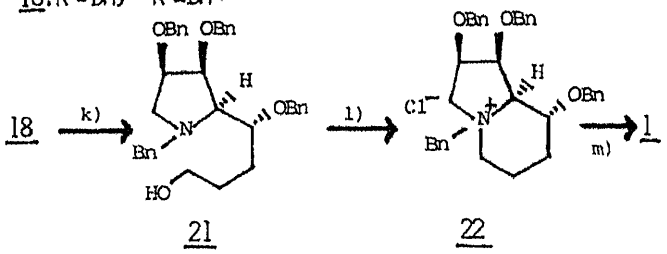
Chart 2



9 Tr: $C(C_6H_5)_3$ 10: R=H 11: R=R Isoprop. 12 13: R^1=H, R^2=Tr 14: R^1=Bn, R^2=H 15



16: R^1+R^1=Isoprop., R^2=H 17: R^1=Bn, R^2=H 18: R^1=Bn, R^2=Bn 19: R^1+R^1=Isoprop., R^2=H 20: R^1=Bn, R^2=H



21 22

Conditions

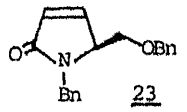
a) cat.OsO_4,NMO,aq.acetone. b) 2,2-dimethoxypropane,acetone,p-TsOH. c) $LiAlH_4$,THF. d) MsCl(2.2eq.), py. e) NaN_3(2eq.),DMF,130°C. f) H_2-Pd black EtOH. g) BnBr,K_2CO_3,acetone,then conc. HCl/MeOH(1:99). h) 10%HCl/MeOH, 70°C. i) i,MOMCl,N,N-diethylaniline; ii,10% HCl/MeOH,40°C; iii,BnBr,NaH/DMF-THF; iv, 10%HCl/MeOH,70°C. j) BnBr,NaH/DMF-THF. k) BH_3,THF, then 30%H_2O_2,12%NaOH. l) MsCl(1.2eq.),triethylamine,CH_2Cl_2. m) H_2-10%Pd/C,HCl/EtOH.

On the other hand, as shown in Chart 2, cis-dihydroxylation of optically active butenolide (9), analogously prepared from (R)-glutamic acid according to the reported procedure,[14] with catalytic OsO_4 and NMO in aqueous acetone afforded the diol (10)[15] (mp 168-170°C; $[\alpha]_D^{20}$ -53.1°(c 1,CH_2Cl_2), lit.[16] for the antipode of 10, mp 170-172°C; $[\alpha]_D^{20}$ +50°(c 3.8, CH_2Cl_2)) in 52% yield with 10% recovery of 9. After protection of the diol in 10 with an isopropylidene group, successive treatments of 11 with $LiAlH_4$, mesyl chloride, and sodium azide provided the azido-mesylate (12) ($[\alpha]_D^{20}$ +18.2°(c 1, $CHCl_3$)) in 31% yield. Catalytic hydrogenation of the azido group in 12 with palladium black in EtOH gave the pyrrolidine derivative (13) with intramolecular S_N2 displacement in 65% yield, from which the hydrochloride of 2 (mp 155-157°C; $[\alpha]_D^{20}$ +21.4°(c 0.25, H_2O), lit.[6] mp 157-159°C; $[\alpha]_D^{20}$ +18.8°(c 0.16, H_2O)) was obtained in 80% yield by acid hydrolysis. N-Benzylation followed by cleavage of the trityl group in 13 afforded 14[17] ($[\alpha]_D^{20}$ -76.0°(c 1.2, $CHCl_3$)) in 80% yield. The three-carbon unit required for the indolizidine skeleton was introduced by oxidation of 14 followed by diastereoselective allylation. Oxidation of 14 by the method of Swern[18] furnished the sensitive aldehyde. Without isolation, this aldehyde was condensed with allylmagnesium chloride in THF at -78°C to furnish a 1:1.3 ratio of 16 and 19 in 80% yield, while the reaction with an organocopper reagent derived from allylmagnesium chloride and copper(I) iodide in THF-dimethylsulfide[19] at -78°C afforded a 1:3.2 ratio of 16 and 19 in 71% yield. Since the compound 16, which could be transformed into (-)-swainsonine, was obtained as a minor isomer in the allylation of the aldehyde derived from 14 having the isopropylidene protecting group, 14 was converted to the di-O-benzyl derivative (15) ($[\alpha]_D^{20}$ -58.3°(c 1.6, $CHCl_3$)) in 38% yield, and the diastereoselective allylation was examined. The allylation of the aldehyde derived from 15 with Grignard reagent gave an 85% yield of the alcohols 17 and 20 in a 3:1 ratio, while the reverse selectivity (1:3.8=17/20) occurred when organocopper reagent was used.[19] The configuration of the newly formed asymmetric carbon in the compounds (16, 17, 19 and 20) was determined by conversion of these compounds into (-)-1 or (-)-8-epi-1. After protection of the hydroxy group in 17 as a benzyl ether, 18 was converted into the alcohol (21) ($[\alpha]_D^{20}$ -40.0°(c 1.2, $CHCl_3$)) by hydroboration-oxidation. Mesylation of 21 leads directly to a bicyclic compound (22), which was debenzylated followed by treatment with Dowex 50W-X8 (H^+ form) to furnish (-)-swainsonine (1) (mp 141-142°C; $[\alpha]_D^{20}$ -85.1°(c 0.6, MeOH), lit.[1c] mp 144-145°C; $[\alpha]_D^{20}$ -87.2°(c 2.1, MeOH)) in 41% yield from 17. Its spectral data (IR, ^1H and ^{13}C NMR) were identical with those of natural (-)-swainsonine. The same reaction sequence transformed 16 and 20 into (-)-1 and (-)-8-epi-swainsonine (mp 92-93°C; $[\alpha]_D^{20}$ -22.1°(c 0.25, MeOH), lit.[5b] mp 93-95°C; $[\alpha]_D^{20}$ -24.8°(c 0.67, MeOH)) in 35% and 33% yields, respectively.

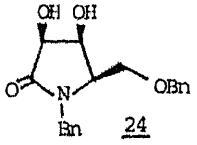
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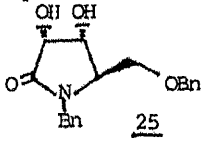
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23



24



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Communications to the Editor

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THE PHARMACOLOGICAL ACTIVITY OF CANNABINOL AND ITS MAJOR METABOLITE, 11-HYDROXYCANNABINOL

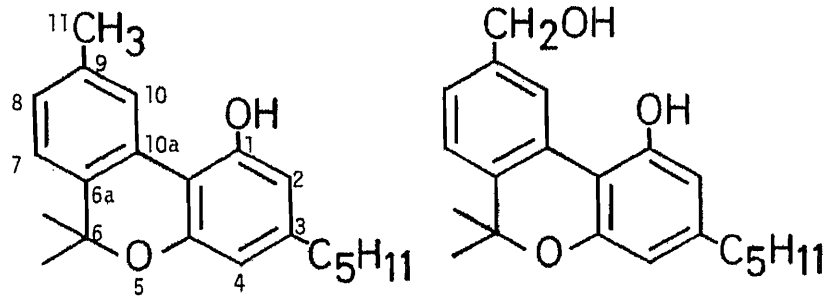
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School of Pharmacy, Hokuriku University,[a] 3-Ho, Kanagawa-machi, Kanazawa 920-11, Japan, and Faculty of Pharmaceutical Sciences, Kyushu University,[b] 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

The pharmacological effects (catalepsy, hypothermia, pentobarbital-induced sleep prolongation and effect on locomotor activity) of cannabinol and its major metabolite, 11-hydroxycannabinol were studied in mice. 11-Hydroxycannabinol was more potent than cannabinol in the pharmacological indices examined. The results indicate that 11-hydroxycannabinol can be categorized as an active metabolite of cannabinol.

KEYWORDS ——— cannabinol; 11-hydroxycannabinol; active metabolite; catalepsy; hypothermia; pentobarbital-induced sleep; locomotor activity

Δ^9-Tetrahydrocannabinol (Δ^9-THC) has been considered to be responsible for most of the psychotropic effects of marihuana. Another cannabinoid, cannabinol (CBN) has been reported to be inactive on the basis of animal and human experiments,[1] although some workers described marginal activity of CBN.[2] In connection with the metabolic activation at the 11-position of THC,[3] the present communication describes the pharmacological effects of 11-hydroxy-CBN (11-OH-CBN), which is a major metabolite of CBN (Fig. 1).[4]



CBN 11-OH-CBN

Fig. 1. Structures of Cannabinoids

11-OH-CBN was prepared according to the method of Ben-Zvi and Mechoulam.[5] Male ddN mice (22-28 g) were used in pharmacological experiments. CBN and 11-OH-CBN were suspended in isotonic saline containing 1% (v/v) Tween 80 and injected into mice intravenously (i.v.) through the tail vein (10 ml/kg body weight). The pharmacological effects (catalepsy, hypothermia, pentobarbital-induced sleep prolongation and effect on locomotor activity) were measured by the methods described previously.[6]

CBN and 11-OH-CBN exhibited THC-like pharmacological effects. The ED_{50}s (mg/kg, i.v.) for the cataleptogenic effect of CBN and 11-OH-CBN were 22.5 and 9.6, respectively (Table I). These values were 8.7 and 3.7 times higher than the ED_{50} of Δ^9-THC reported previously.[7] The hypothermic effect of CBN and 11-OH-CBN is shown in Fig. 2. Both cannabinoids exerted a significant hypothermic effect. The maximal hypothermia produced by CBN and 11-OH-CBN (10 mg/kg, i.v.) were -0.66 and -1.35 °C, respectively, at 15 min after the administrations. The duration of the effect of 11-OH-CBN (30 min) was longer than that of CBN (15 min). Both cannabinoids (10 and 15 mg/kg, i.v.) significantly prolonged pentobarbital-induced sleeping time 1.6- and 2.5-fold (CBN), and 3.4- and 4.4-fold (11-OH-CBN) as compared with the control (14 $\pm$ 2 min, sodium pentobarbital 40 mg/kg, i.p.)(Table II). CBN (10 mg/kg, i.v.) significantly decreased horizontal and vertical motor activities of mice for 1 h after the administration (Fig. 3). 11-OH-CBN was more potent than CBN in decreasing the vertical activity.

The present study demonstrates that CBN, which has been considered to be a non-psychoactive cannabinoid, has some pharmacological activity. It is well known that Δ^9-THC is easily converted to CBN in aging conditions.[8] We also reported that Δ^9-THC in cannabis is mainly converted to CBN upon strage.[9] This indicates that CBN, especially aged samples, may play some role in the pharmacological and toxicological effects of marihuana. Although much attention has been directed to the metabolic activation of THC, little is known at present about CBN. Consroe et al.[10] and Mechoulam et al.[11] only described preliminary results on some pharmacological effects of 11-OH-CBN. The present study clearly demonstrated that the pharmacological effects of 11-OH-CBN are stronger than those of CBN. The results indicate that 11-OH-CBN can be categorized as an active metabolite of CBN that contributes to the pharmacological effects of marihuana.

ACKNOWLEDGMENT The authors are grateful to Prof. I. Nishioka and Assoc. Prof. Y. Shoyama, Faculty of Pharmaceutical Sciences, Kyushu University, for supplying cannabis leaves, which are starting materials for preparing CBN and 11-OH-CBN.

Table I. Cataleptogenic Effect of CBN and 11-OH-CBN

	ED_{50} (mg/kg, i.v.)[a]
CBN	22.5 (19.7-28.6)
11-OH-CBN	9.6 (6.8-13.6)

a) The values were calculated by the method of Litchfield and Wilcoxon.[12]

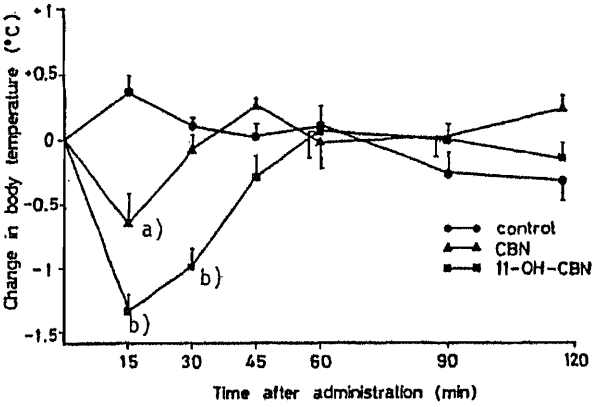


Fig. 2. Hypothermic Effect of CBN and 11-OH-CBN

Both cannabinoids were administered at a dose of 10 mg/kg i.v. a) Significantly different from control ($p < 0.05$). b) Significantly different from control ($p < 0.01$).

Table II. Effects of CBN and 11-OH-CBN on Pentobarbital-Induced Sleeping Time

	Dose (mg/kg, i.v.)	Mean Sleeping Time ± S.E. (min)	
Control	—	14 ± 2	(16)[a]
CBN	10	23 ± 4*	(11)
	15	35 ± 4**	(8)
11-OH-CBN	10	48 ± 5**	(14)
	15	61 ± 10**	(8)

Both cannabinoids were administered 20 min prior to the injection of pentobarbital (40 mg/kg, i.p.). a) Numbers in parentheses represent the numbers of animals used. * Significantly different from control ($p < 0.05$). ** Significantly different from control ($p < 0.01$).

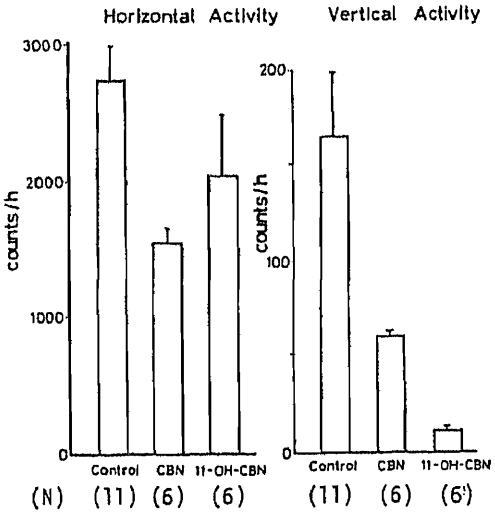


Fig. 3. Effects of CBN and 11-OH-CBN on Locomotor Activity

Both cannabinoids were administered at a dose of 10 mg/kg i.v.

a) Significantly different from control ($p < 0.05$).

b) Significantly different from control ($p < 0.01$).

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Communications to the Editor

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TRIALKYLTIN RADICAL USED TO CATALYZE THE O,S-REARRANGEMENT OF CYCLIC THIONOCARBONATES. A NEW ENTRY TO THIO-SUGARS[1)]

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The cyclic thionocarbonates give O,S-rearrangement products, the thiol-cabonates, in appreciable yields, when treated with a catalytic amount of tributyltin hydride and α,α-azobisisobutyronitrile. This reaction opens a new route to regioselective preparation of thio-sugars under very mild conditions.

KEYWORDS——cyclic thionocarbonate; cyclic thiol-carbonate; O,S-rearrangement; tributyltin hydride; thio-sugar; regioselective thiolation; carbohydrate

Sometimes when cyclic thionocarbonates are deoxygenated by tributyltin hydride several by-products are produced such as the methylidene derivative (C), the carbonate (D), and the thiol-carbonate (F), (Chart 1).[2)] Formation of the thiocarbonate (F), an O,S-rearrangement product, is particularly interesting because it indicates that the intermediary radical (E) is re-cyclized with a loss of the tributyltin radical, instead of being trapped by a hydride. In this pathway the tributyltin radical may be playing the role of a catalyst, since it is regenerated. This idea prompted us to investigate a new radical-catalyzed O,S-rearrangement reaction of the thionocarbonates.

After several attempts, we found that the rearrangement proceeded smoothly and became to be the major path when the cyclic thionocarbonates were treated with 0.3 mol eq of tributyltin hydride in the presence of a sufficient amount of α,α-azobisisobutyronitrile (AIBN). A typical experimental procedure is as follows, and the other examples are shown in Table I.

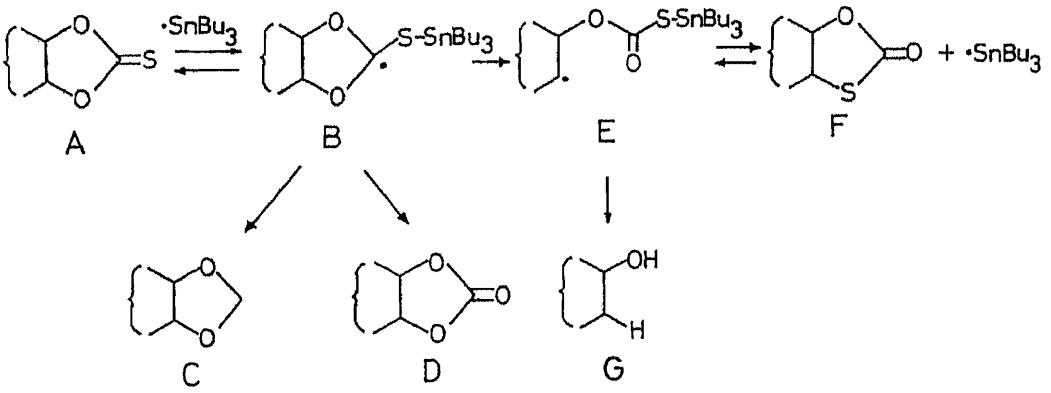
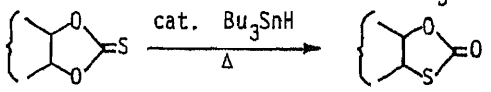


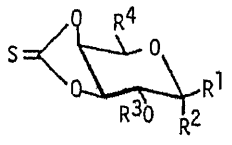
Chart 1

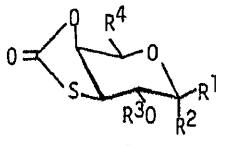
Table I. Reaction of Some Thionocarbonates with Bu_3SnH[a]

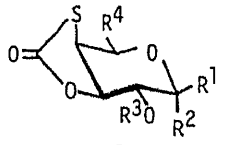


Entry	Compd. (A)	Condition (°C)	(h)	Product[b] O,S-Rearrangement(F) Yield(%)	2 : 3	Carbonate(D) Yield(%)	Deoxy deriv.(G) Yield(%)	S.M.(A) Recovered(%)
1	1a	110[c]	3.5	60(72)[d]	56 : 44	23(28)	t[e]	17
2	1b	90	3	69(78)	60 : 40	20(22)	t	11
3	1c	90	2.5	51[f]	80 : 20	21[f]	25[f]	0
4	1d	90	3	79	43 : 56	t	21	t
5	1e	90	3	57(76)	52 : 48	8(11)	10(13)	25
6	4	110[c]	3	61(73)	[80 : 20][g]	10(11)	5(7)	24

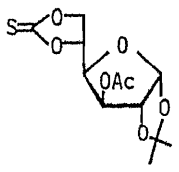
a) Reaction: Thionocarbonate (1 eq); Bu_3SnH (0.3 eq); AIBN (0.3 eq) in benzene.
b) Yields are based on GLC peak areas.
c) Toluene was used as a solvent.
d) Parenthetical values indicate net yield.
e) t: trace.
f) Isolation yield after HPLC.
g) Ratio of 5-thiol derivative (5)(5:3 mixture of the gluco- and ido-forms) and 6-thiol derivative (6).

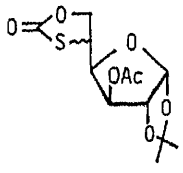

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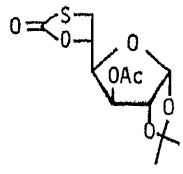

2


3

	R^1	R^2	R^3	R^4
a	H	OMe	Ac	H
b	H	OMe	H	H
c	OPh	H	H	H
d	H	OMe	Ac	CH_2OAc
e	OMe	H	Ac	CH_2OAc


4


5


6

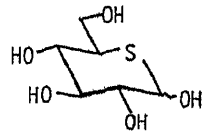

7

Chart 2

Table II. ^{13}C-NMR Data of O,S-Rearrangement Products in Pyridine-d_5

Compd.	C-1	C-2	C-3	C-4	C-5	C-6	OMe	O,S-C=O
2a	96.3	73.5	47.0	80.6	57.5		55.5	171.5
3a	97.5	69.3	78.0	48.2	57.3		55.6	171.6
2b	100.0	71.9	51.1	81.1	57.5		55.5	172.5
3b	100.7	67.9	82.2	48.0	58.1		55.7	172.4
2c	102.1	63.6	53.2	79.8	73.9			172.4
3c	99.5	67.3	82.1	44.7	60.8			172.5
2d	97.3	73.1	47.4	79.9	65.3	64.6	55.3	170.0
3d	96.5	69.3	78.1	51.1	63.2	64.0	55.4	170.3
2e	101.9	73.7	50.5	79.5	72.1	63.1	56.8	170.0
3e	101.5	69.7	80.8	51.2	70.5	64.5	56.3	170.2
5[a] gluco-	105.3	83.2	76.3	80.8	45.5	70.7		172.4
ido-	104.7	83.5	75.9	79.1	46.8	69.4		171.5
6[a]	104.9	83.4	75.9	78.0	75.9	34.0		171.9

a) Data in $CDCl_3$.

Example——Phenyl α-L-arabinopyranoside 3,4-thionocarbonate **1c** (100 mg) and AIBN (18 mg, 0.3 eq) in benzene (25 ml) were stirred until all compounds dissolved completely. To this mixture freshly distilled Bu_3SnH (32 mg, 0.3 eq) was injected and the mixture was heated under reflux for 2.5 h with stirring in an argon atmosphere. The cooled mixture was subjected to column chromatography eluting with benzene to remove tin compound(s). The following ethylacetate eluate was separated by preparative HPLC (solvent, benzene:AcOEt=1:1) to yield the 3-thiol-3,4-carbonate **2c** (42 mg, mp 201-202 C), the 4-thiol-3,4-carbonate **3c** (9.4 mg, mp 142-143 C), the carbonate (20 mg, mp 188-189 C), and a mixture of the 3-deoxy and 4-deoxy derivatives (19.5mg).

The structure of each rearrangement product was determined, after isolation as described in the Example, by spectral comparison with **2a** and **3a** whose structures were already definitely established,[2] and by desulfurization to the known deoxy compounds.[3] The ^{13}C-NMR spectra are listed in Table II.

Apparently the reaction is radical-catalyzed, because in the absence of a radical source (tributyltin hydride) or radical initiator (AIBN) neither the deoxygenation[4] nor the rearrangement proceeded.

Care must be taken for the reaction: Air (oxygen) should be avoided, otherwise the carbonate may be formed. Benzene or toluene was the most suitable solvent. When the solvent was a possible source of hydrogen, such as dioxane, the ratio of deoxygenation product(s) increased.

Allyltributyltin and bis-tributyltin can also be used as radical sources, although the reaction condition is not optimized.

As seen in Table I, the stereochemistry of the product from the thionocarbonates between two secondary hydroxyl groups (Entry 1-5) was always cis, though the regiochemistry was not always controlled. However, in the case of the thionocarbonate between primary and secondary hydroxyls (Entry 6), the secondary thiol derivative was produced exclusively, as expected, although the stereochemistry of the C-S linkage was not controlled in this case.

The above method, when combined under very mild conditions with the regioselective preparation of cyclic thionocarbonates,[5] makes it possible to regioselectively convert a carbohyrate hydroxyl group to a thiol group. For example, the 5-thio derivative **5** derived from the 5,6-thionocarbonate of isopropylidene-α-D-glucofuranose **4** should be a useful intermediate to thioglucose **7**. Contrary to this radical reaction, the iodide catalyzed O,S-rearrangement of the same substrate **4** produces the 6-thiol derivative **6** exclusively.[6]

ACKNOWLEDGEMENT The authors wish to thank Mr. K. Tsubono, Toyama Medical and Pharmaceutical University, for measurements of NMR COSY spectra.

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Communications to the Editor

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GREATER GUEST BINDING ABILITY OF β-CYCLODEXTRIN HAVING BOTH A CAP AND FLOOR

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β-Cyclodextrin having both 2-naphthalenesulfonyl and tosyl moieties as the cap and floor has a greater ability to bind guests than β-cyclodextrin which has a cap only.

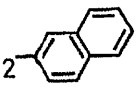
KEYWORDS——β-cyclodextrin; inclusion complex; circular dichroism; naphthalene; binding constant; host-guest

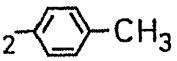
Cyclodextrins can bind a variety of guests in their molecular cavities.[1] The host-guest complexation occurs usually in aqueous solution mainly due to hydrophobic interaction between ether-like cyclodextrin walls and lipophilic guests. Consequently, the binding is stronger when the environment around the included guest is made more hydrophobic by modifying the native cyclodextrins with hydrophobic moieties (cap or floor).[2,3] Heretofore, such modification has been performed at one side of the cyclodextrins, that is, at either side of the primary or secondary hydroxyls. In this study, based on the recent establishment[4] of a secondary site-modification method for β-cyclodextrin, we have prepared a β-cyclodextrin (cycloheptaamylose) derivative **1**, which bears two different aromatic moieties as the cap and floor at opposite sides of the cavity. This system enables guest molecules to be totally encapsulated in the hydrophobic cavity of **1** by co-operation of the cyclodextrin wall, cap, and floor.

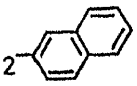
Compound **1** was prepared by treating 2-naphthalenesulfonyl chloride with one equiv. of β-cyclodextrin-2-O-tosylate[4] in pyridine at room temperature for 1 h. Evaporation of the reaction solution followed by column chromatography on Sephadex LH-20 with N,N-dimethylformamide/water (1:1) as the eluting solvent afforded the product in 10% yield. Compound **2**, which bears a 2-naphthalenesulfonyl moiety at the primary side of β-cyclodextrin, was also prepared by treating 2-naphthalenesulfonyl chloride with β-cyclodextrin in pyridine as the reference compound of **1**. Both compounds were identified by ^{1}H-NMR, UV, IR, and elemental analyses.[5]

The circular dichroism (CD) spectrum of **2** reveals positive peaks around 227 nm ([θ]=+26700) and 275 nm ([θ]=+3700) associated with the naphthalene ^1B_b (ε_{229} =112000) and ^1L_a (ε_{277}=8140) transitions, respectively. The intensities of the

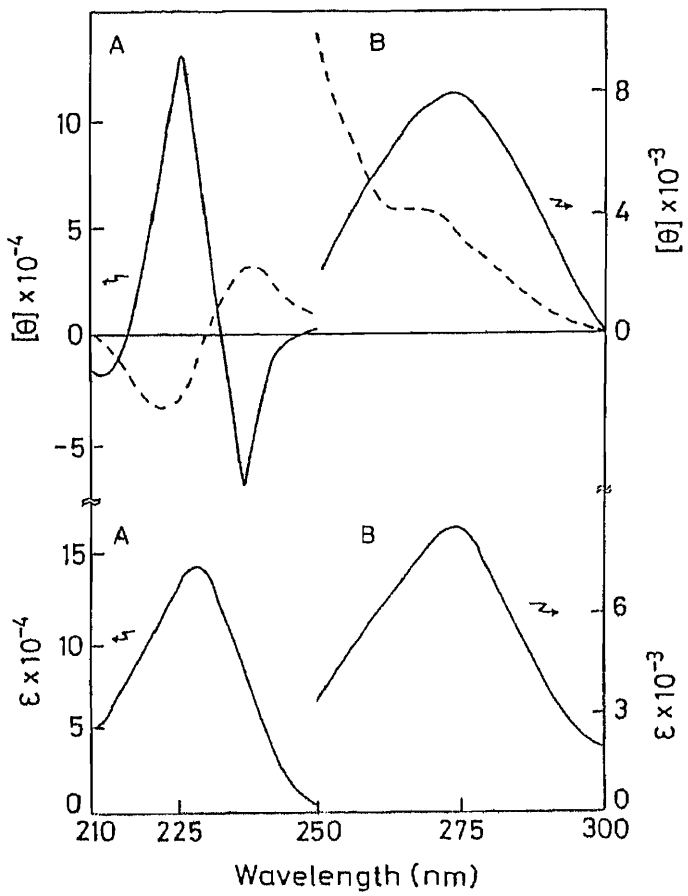


1 : X= -O-SO_2- 

Y= -O-SO_2-  -CH_3

2 : X= -O-SO_2- 

Y= -OH



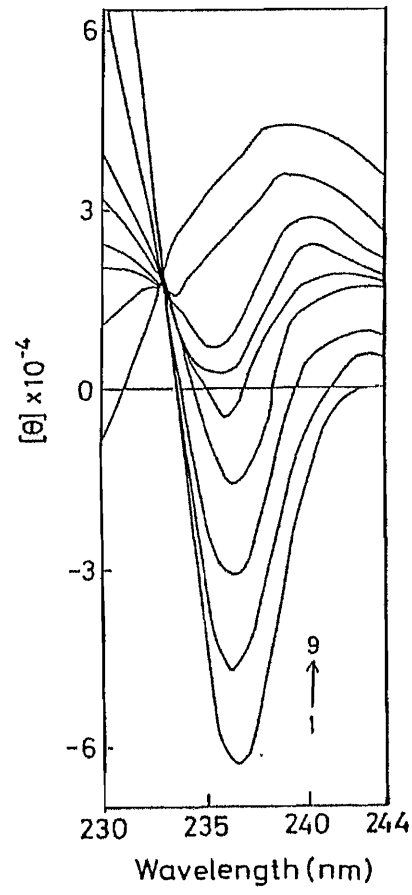


Fig. 1 (left). CD Spectra (top) and Absorption Spectra (bottom) of 1, Alone (——, A: 7.5 x 10^{-6} mol l^{-1}; B: 1.51 x 10^{-4} mol l^{-1}) or in the Presence of Cyclohexanol (- - - -, A, B: 4.80 x 10^{-2} mol l^{-1}).

Fig. 1 (right). Variations of the CD Pattern of 1 Induced by Cyclohexanol at 25 °C (1, 0; 2, 1.60 x 10^{-3}; 3, 3.20 x 10^{-3}; 4, 6.40 x 10^{-3}; 5, 1.20 x 10^{-2}; 6, 1.60 x 10^{-2}; 7, 2.24 x 10^{-2}; 8, 3.20 x 10^{-2}; 9, 4.80 x 10^{-2} mol l^{-1}).

Table I. Binding Constants of 1 and 2 for Various Guests in water at 25 °C[a)]

Guest	Host	K / mol^{-1} l
(-)-Borneol	1	4070 ± 90
	2	1020 ± 60
Cyclohexanol	1	138 ± 9
	2	67 ± 4
t-BuOH	1	7.8 ± 0.8
	2	3.3 ± 0.2
n-BuOH	1	5.0 ± 0.3
	2	3.7 ± 0.7

a) The values were obtained by the analysis of the CD intensities at 237 and 275 nm for 1 and 2, respectively.

CD bands are reduced in the presence of guests, indicating that the naphthalenesulfonyl moiety, which is inserted into the cyclodextrin cavity, is extruded when a guest molecule enters the cavity. Figure 1 (left) shows the absorption and CD spectra of **1** in water. The CD patterns of **1** in the 210-250 nm region are quite different from the simple positive band of **2**: a sinusoidal CD curve with a change in sign from minus to plus from the longer wavelength side appears in the absence of a guest, while the CD curve with an opposite change in sign occurs in the presence of an excess guest. These sinusoidal CD curves, which were observed for **1** and not for **2**, are likely to result from the exciton coupling[6)] between the two aromatic rings of the cap and floor. The guest-induced CD variations therefore indicate that the positional relationship between the cap and floor changes by guest accommodation. It is reasonably thought that **1** is a mixture of several isomers which are different in the mutual positions of the tosyl and naphthalenesulfonyl moieties. However, attempts to identify the mixtures of **1** have not been successful. Furthermore, we observed an isoellipticity point at 233 nm in the CD spectra of the solutions with different guest concentrations (Fig. 1 (right)). Such an isoellipticity point usually appears in the system of 1:1 host-guest complexation of modified cyclodextrins.[7)] The present CD variations of **1** can also be analyzed based on a simple 1:1 stoichiometry of complexation[7)] using the CD intensities at 237 nm. Table I shows the binding constants of **1** and **2** for various guests. The values are in the order of n-BuOH< t-BuOH<cyclohexanol<(-)-borneol for both **1** and **2**. For any of the guests, the binding ability of **1** is larger than that of **2** with 4.0, 2.1, 2.4, and 1.4-fold stronger binding for (-)-borneol, cyclohexanol, t-BuOH, and n-BuOH, respectively. The strongest binding of **1** observed for (-)-borneol may be attributed to the largest contact area of the guest to both cap and floor. From these data, it has been shown that both the cap and floor of **1** co-operate in producing the improved hydrophobic environment around the guest molecule in **1** by adjusting their locations.

Another aspect of compound **1**, which has two reactive sulfonyl units, is that it can be used as a starting material to construct unique hosts or enzyme models with two appropriate functional moieties at the opposite sides of β-cyclodextrin. A study along this line is now under way.

This work was partly supported by Grant-in-Aid (No. 61470152, No. 61550585) for Special Project Research from the Ministry of Education, Science and Culture.

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5) Data of **1**: Rf 0.58 (n-BuOH:EtOH:water 5:4:3 by volume); IR (KBr) cm^{-1}: 1355, 1180; ^{1}H-NMR (DMSO-d_6, 60 °C) δ: 1.20 (3H, s, CH_3), 3.0-4.0 (br, m, cyclodextrin protons other than C_1H and OH), 4.2 (6H, br, m, O_6H), 4.8 (7H, br, m, C_1H), 5.55 (13H, br, s, O_2H, O_3H), 7.6-8.3 (11H, m, Ar-H); Anal. Calcd for $C_{59}H_{82}O_{39}S_2.5H_2O$: C, 45.15; H, 5.91; S, 4.09. Found: C, 45.28; H, 5.89; S, 4.42.
Data of **2**: Rf 0.55 (n-BuOH:EtOH:water 5:4:3 by volume); IR (KBr) cm^{-1}: 1355, 1180; ^{1}H-NMR (DMSO-d_6, 60 °C) δ: 3.0-4.0 (br, m, cyclodextrin-H other than C_1H and OH), 4.2 (6H, br, m, O_6H), 4.8 (7H, br, m, C_1H), 5.55 (14H, br, m, O_2H, O_3H), 7.6-8.3 (7H, m, Ar-H); Anal. Calcd for $C_{52}H_{76}O_{37}S.H_2O$: C, 46.50; H, 5.85; S, 2.39. Found: C, 46.76; H, 6.43; S, 2.53.
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Communications to the Editor

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PENTACO-ORDINATE SILICON COMPOUNDS IN SYNTHESIS: REGIOSPECIFIC ALLYLATION OF ALDEHYDES USING TRIALKOXY-SUBSTITUTED ALLYLSILANE, PYROCATECHOL, AND AN AMINE

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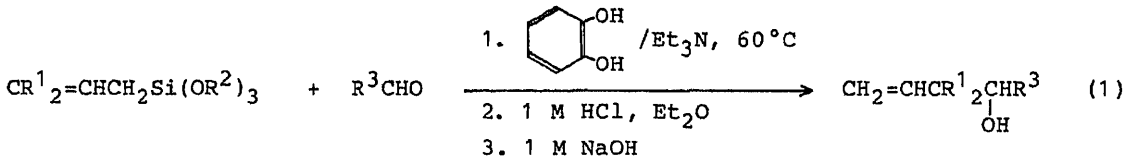
Aliphatic and aromatic aldehydes are allylated with trialkoxy-substituted allylsilane to give the corresponding homoallyl alcohols regiospecifically in the presence of pyrocatechol and triethylamine without solvent at 60°C or in tetrahydrofuran at reflux. The allylation reaction takes place even in a protic solvent such as methanol and ethanol.

KEYWORDS ——— regiospecific allylation; pentaco-ordinate silicon compound; allylsilicate; allyltrialkoxysilane; homoallyl alcohol

The allylation of carbonyl compounds is one of the most fundamental and important synthesis reactions, so efficient methods are highly desirable. We have reported that allyltrialkylsilane is an excellent reagent for the regiospecific allylation of various electrophiles activated by a Lewis acid.[1] Also we have reported the reduction of carbonyl compounds by the pentaco-ordinate hydrosilane using trialkoxy-substituted silane-dilithium pinacolate.[2] In an extension of our studies on the pentaco-ordinate organosilanes in synthesis,[3] we now report that an active species derived from a trialkoxy-substituted allylsilane (1),[4] pyrocatechol and triethylamine, reacts with aldehydes (2) smoothly without solvent at 60°C or in tetrahydrofuran at reflux temperature to give the corresponding homoallyl alcohols (3) in quite high yield as illustrated in eq. 1. The allylation takes place in the regiospecific mode.

The reaction of allyltriethoxysilane (1a) with benzaldehyde (2a) in the presence of pyrocatechol and triethylamine proceeds smoothly for 12 h at 60°C without solvent to afford 1-phenyl-3-buten-1-ol (3a) in 85% yield after acid hydrolysis. Similarly, 3b-3d were obtained when 1a reacted with substituted benzaldehydes (2b-2d) in 98, 87 and 97% yields, respectively. It is significant that the allylation occurs even in the presence of the nitro function. Aliphatic and α,β-unsaturated aldehydes such as 2e-2g can also react with 1a to afford 3e-3g in 68, 95 and 62% yields, respectively.

The reaction did not proceed at all when triethylamine and pyrocatechol



$CR^1_2=CHCH_2Si(OR^2)_3$ + R^3CHO → $CH_2=CHCR^1_2CHR^3$ (OH) (1)

1a, R^1=H; R^2=Et
b, R^1=Me; R^2=Me

2a, $R^3=C_6H_5$
b, R^3=p-$CH_3C_6H_4$
c, R^3=p-ClC_6H_4
d, R^3=m-$NO_2C_6H_4$
e, $R^3=C_6H_5CH=CH$
f, R^3=1-C_3H_7
g, R^3=1-C_7H_{15}

3a, R^1=H; $R^3=C_6H_5$: 85%
b, R^1=H; R^3=p-$CH_3C_6H_4$: 98%
c, R^1=H; R^3=p-ClC_6H_4: 87%
d, R^1=H; R^3=m-$NO_2C_6H_4$: 97%
e, R^1=H; $R^3=C_6H_5CH=CH$: 62%
f, R^1=H; R^3=1-C_3H_7: 68%
g, R^1=H; R^3=1-C_7H_{15}: 95%
h, R^1=Me; $R^3=C_6H_5$: 75%

were used independently, and it was found that, among various diols or monools and bases examined, a combination of these two was the most expedient and effective in promoting the allylation of aldehydes. The allylation takes place in tetrahydrofuran and acetonitrile, though rather slowly. Moreover it is of interest from the standpoint of organic synthesis that the reaction proceeds in a protic solvent such as methanol and ethanol, and exclusively in the 1,2-addition mode to **2e**. Unfortunately, aliphatic, alicyclic and aromatic ketones such as 2-octanone, cyclohexanone, and acetophenone did not give satisfactory results in the present reaction system.

It should be emphasized that prenyltrimethoxysilane (**1b**) reacts with **2a** in a completely regiospecific mode to afford 2,2-dimethyl-1-phenyl-3-buten-1-ol (**3h**) in 75% yield. That there was no contamination of the regioisomer in the reaction mixture was confirmed by the spectroscopic and gaschromatographic analyses. Since the regiospecificity could not be attained by the fluoride ion-promoted allylation of carbonyl compounds with allyltrimethylsilane,[5] the mechanisms of the two reactions are apparently different. From the mechanistic viewpoint it is important to state that the present reaction conditions are almost similar to those for the synthesis of pentaco-ordinate organosilicates (**4**, Z = organic group) reported by C. L. Frye,[6] except for the solvent and the addition of the aldehyde. Thus, in addition to this, the realization of the regiospecificity in the allylation strongly suggests that the present reaction proceeds via the pentaco-ordinate allylsilicate (**4**, Z = allyl), generated in situ, as a key intermediate of the allylation.

$$[M]^+[Z\text{-}Si(OCR_2CR_2O)_2]^-$$

4

In a typical procedure allyltriethoxysilane (**1a**) (1.5 mmol) was added to a mixture of an aldehyde (**2**) (1.0 mmol), pyrocatechol (3.0 mmol) and triethylamine (1.5 mmol) and the resulting mixture was stirred for 12 h at 60°C. Diethyl ether (20 ml) and hydrochloric acid (1 M, 5 ml) were then added and the mixture was stirred for 1 h. The usual work-up after treatment with aqueous sodium hydroxide (1M, 10 ml) and separation by the preparative thin layer chromato-

graphy on silica gel gave the pure homoallyl alcohol (3).

Although the phenylation with the isolated 4 (M = Et_3NH, Z = Ph) does not occur successfully at present under the similar conditions employed here, additional aspects of this class of reactions including isolation of 4 (Z = allyl or other organic groups), asymmetric induction, and stereo-controlled allylation in the acyclic system are currently under active investigation. In addition, pentaco-ordinate silicon compounds are interesting from the viewpoint of biological activities, since among those derivatives the silatranes reveal high mammalian toxicity and other bioactivities.[7)]

ACKNOWLEDGMENT The work was supported in part by the Ministry of Education, (Grant-in-Aids for Scientific Research), the Research Foundation for Pharmaceutical Sciences and the Houan-sha, and Yamada Science Foundation.

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Communications to the Editor

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SYNTHESIS OF SAFRAMYCINS. I.
TOTAL SYNTHESIS OF (±)-SAFRAMYCIN B AND ITS CONGENERS.

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We describe the total synthesis of the dimeric isoquinolinequinone antibiotic saframycin B (1) from tricyclic lactam 3. The key step is the stereoselective intramolecular cyclization of the aminoacetal 6. The structure of the pentacyclic intermediate 8 was confirmed by X-ray crystallography.

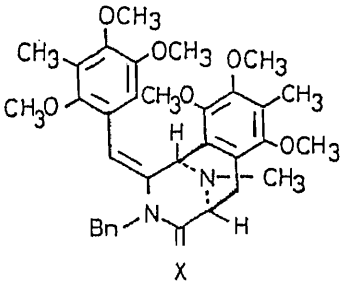
KEYWORDS —— dimeric isoquinolinequinone; saframycin B; aminoacetal; total synthesis; X-ray crystallography; oxidative demethylation

For some time we have studied the synthesis of the antitumor isoquinolinequinone antibiotics, saframycin B (1) and its closely related congeners.[1] The structure of 1 was elucidated by comparing its spectroscopic data with those of saframycin C (2) whose structure has been determined by X-ray crystallography.

Although several chemists[2] have been trying to synthesize this molecule, only one successful total synthesis of 1 has been reported, by Fukuyama and his co-worker.[3] We have reported an efficient synthesis of the tricyclic lactam 3.[4]

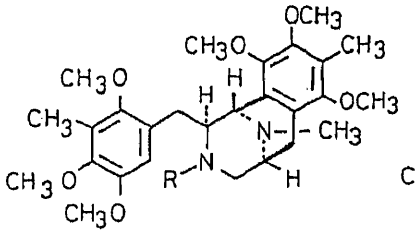
In this paper, we wish to report the utilization of 3 for an alternative synthesis of 1 and the syntheses of the monoquinones 21 and 23, which are particularly interesting for their structural resemblance to saframycins D,[5a] R,[5b] Mx-1,[5c] Mx-2[5c] and safracins.[5d]

Reduction of 3 (AlH_3, THF, 0 °C, 1 h) gave in 93% yield the unstable enamine 4, which was catalytically hydrogenated (4 atm H_2, 20% Pd-C, EtOH, 80 °C, 24 h)[6] to afford the amine 5 [mp 121-122 °C] in 99% yield. The reduction occurred cleanly from the α-face accompanied by the debenzylation. The problem of constructing the pentacyclic carbon skeleton was solved by using an aminoacetal intermediate.[7] The reaction of 5 with a large excess of n-butyl glyoxylate[8] (K_2CO_3, n-BuOH, 25 °C, 24 h) gave the aminoacetal 6, which was subsequently treated with trifluoroacetic acid (25 °C, 1 h) to provide the desired pentacyclic product 7 [mp 111-112 °C; ^{1}H-NMR δ 4.56 (1H, s, 9-H)] in 70% overall yield. The stereochemistry of 7 was undetermined at this stage. n-Butyl ester 7 was then transformed to the ethyl ester 8 [mp 209.5-211 °C; ^{1}H-NMR δ 4.56 (1H, s, 9-H),

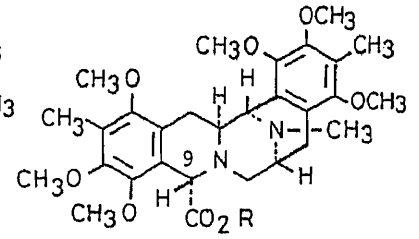


X

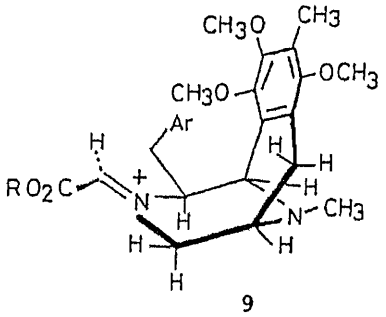
3 X = O
4 X = H_2



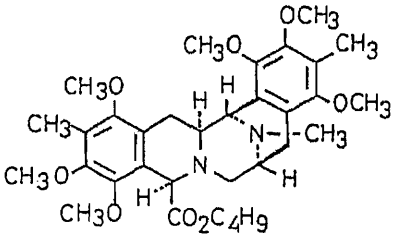
5 R = H
6 R = $CH(OC_4H_9)CO_2C_4H_9$



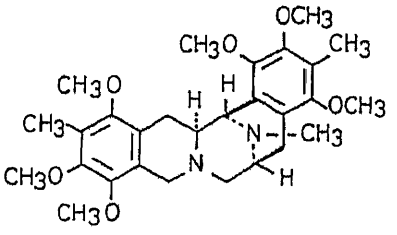
7 R = C_4H_9
8 R = C_2H_5



9



10



11

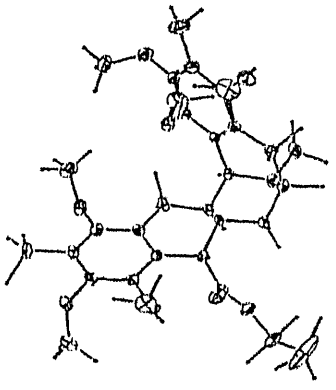
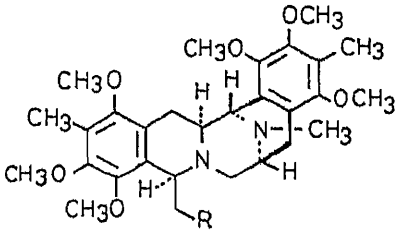
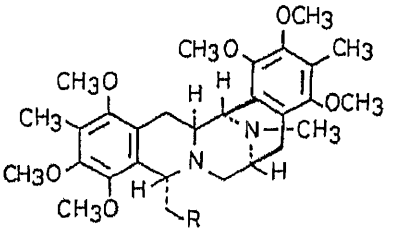


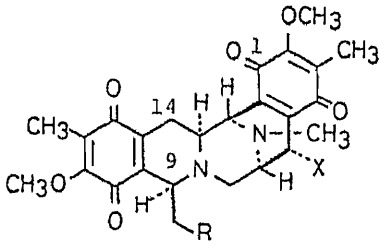
Fig. 1 ORTEP STRUCTURE OF COMPOUND 8



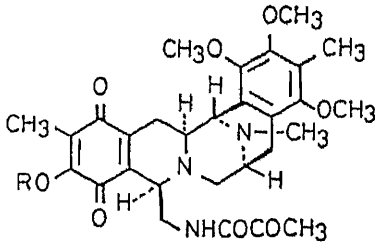
12 R = OH
13 R = NPht
14 R = NH_2
15 R = $NHCOCOCH_3$
20 R = $OCOCH_3$



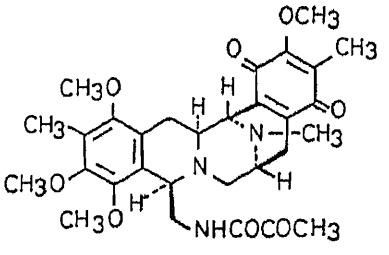
16 R = OH
17 R = NPht
18 R = NH_2
19 R = $NHCOCOCH_3$



1 R = $NHCOCOCH_3$, X = H
2 R = $NHCOCOCH_3$, X = OCH_3
24 R = $OCOCH_3$, X = H



21 R = CH_3
22 R = H



23

72%] in the usual manner (EtOH, H_2SO_4, reflux). Its structure was determined by X-ray crystallography (Fig. 1)[9] which revealed that the stereochemistry of C-9 was epimeric with that of saframycins. Comparison of the ^{1}H-NMR and ^{13}C-NMR spectra of **7** and **8** indicated that their stereochemistries were identical with each other. The stereochemical course of this reaction could be rationalized by assuming (E)-imminium isomer **9**. Thus, this stereoselective cyclization would proceed from the α-face. Epimerization of C-9 in **7** with excess mercury acetate (5% AcOH-H_2O, 90°C, 2 h)[10] followed by reduction ($NaBH_4$, EtOH, 25°C, 1 h) afforded the desired ester **10** [mp 174-176°C; ^{1}H-NMR δ 4.09 (1H, s, 9-H); 71%] along with the decarbobutoxylated compound **11** [mp 158.5-160°C; 13%], which was synthesized alternatively from **5** in two steps [(1. HCHO, K_2CO_3, EtOH; 2. CF_3CO_2H); 81%].[7] Reduction of **10** ($LiAlH_4$, THF, reflux, 2 h) afforded the alcohol **12** [mp 152.5-154°C] in 77% yield. Facile conversion of the alcohol **12** to the amine **14** was carried out by using Mitsunobu's procedure.[11] Treatment of **12** with diethylazodicarboxylate (DEAD), Ph_3P, and phthalimide (THF, 25°C, 3 h) to give the imide **13**, followed by cleavage of the phthaloyl group (NH_2NH_2-H_2O, EtOH, reflux, 2 h) to afford the amine **14**, which was acylated with pyruvoyl chloride[12] (DMAP, Et_3N, CH_2Cl_2, 25°C, 1 h) to give the pyruvamide **15** [mp 135.5-137°C] in 76% overall yield. In a similar manner, the C-9 epi alcohol **16** [mp 200-201.5°C], which was obtained from **7** by reduction ($LiAlH_4$, THF, reflux, 2 h; 84%), was converted into the pyruvamide **19** [mp 170-171.5°C] in 77% overall yield.

Many attempts to oxidatively demethylate **15** failed,[13] but it was found that oxidation with 10 N nitric acid[14] (25°C, 1 h) gave **1** (1.5%) along with the monoquinones **21** [mp 203-205°C (dec.); ^{1}H-NMR $J_{9\text{-}14\beta}$ = 3.0 Hz;[15] 12%] and **22** [^{1}H-NMR $J_{9\text{-}14\beta}$ = 2.9 Hz;[15] 27%]. Methylation of **22** with CH_2N_2 yielded a methoxy derivative, identical with **21** (81%). When treatment of **15** with excess boron tribromide (CH_2Cl_2, -78°C, 1 h) followed by oxidation with ceric ammonium nitrate (CAN) afforded **1** (17%) and the monoquinone **23** [amorphous powder; ^{1}H-NMR $J_{9\text{-}14\beta}$ = 0 Hz;[15] 45%]. Subsequently, demethylation of **15** (BBr_3, CH_2Cl_2, -78°C, 1 h) followed by oxidation with 10 N nitric acid (25°C, 1 h) afforded (±)-saframycin B (**1**) [mp 175-180°C (dec.)] in 41% overall yield. The synthetic saframycin B was identical with the natural one on comparison of spectroscopic ^{1}H-NMR, ^{13}C-NMR, IR, UV, MS, and TLC data. On the other hand, the oxidative demethylation of the epi pyruvamide **19** afforded only polar polymeric material. Finally, the acetate **20** [mp 160-161°C], which was obtained from **12** (Ac_2O, pyridine, 25°C; 68%), gave the quinone **24**[16] [mp 144-147°C (dec.); 33%] when a similar demethylation and oxidation sequence was applied.

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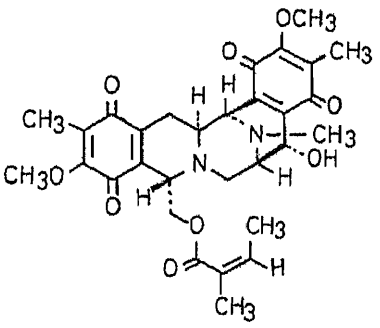
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15) In the ^{1}H-NMR spectra of **1**, **21**, **22**, and **24**, the diagnostic homoallylic coupling between 9-H and 14-βH over five bonds was observed. On the other hand, this coupling was negligible in the spectra of the pentacyclic methoxyarenes (**7**-**20**) and the monoquinone **23**.

16) **24**: MS m/z (%): 510 (M^+, 10), 439 (46), 437 (27), 234 (44), 220 (100), 218 (39); UV λ_{max}^{MeOH} nm (log ε): 270 (4.36), 368 (3.14); IR ν_{max}^{KBr} cm^{-1}: 1715, 1655, 1645; ^{1}H-NMR ($CDCl_3$) δ: 1.28 (1H, ddd, J = 17.1, 11.2, 2.7 Hz, 14-βH), 1.75 (3H, s), 1.94 (3H, s), 1.96 (3H, s), 2.25 (3H, s), 2.35 (1H, d, J = 21.0 Hz, 5-βH), 2.69 (1H, dd, J = 21.0, 7.1 Hz, 5-αH), 2.69 (1H, ddd, J = 11.2, 2.7, 2.7 Hz, 14a-H), 2.78 (1H, dd, J = 10.7, 2.7 Hz, 7-αH), 2.82 (1H, dd, J = 17.1, 2.7 Hz, 14-αH), 3.04 (1H, dd, J = 10.7, 2.4 Hz, 7-βH), 3.11 (1H, m, 6-H), 3.61 (1H, ddd, J = 3.7, 2.9, 2.7 Hz, 9-H), 3.87 (1H, dd, J = 11.2, 2.9 Hz, OCH), 3.96 (1H, dd, J = 2.7, 0.5 Hz, 15-H), 3.98 (3H, s), 4.00 (3H, s), 4.49 (1H, dd, J = 11.2, 3.7 Hz, OCH).
The chemical shifts and coupling constants were very similar to that of renieramycin A (**25**),[17] which was isolated from a marine sponge Reniera sp.



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