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Reproduction of *ab Initio* Electrostatic Potential with Classical Fractional Point Charges for Biological Molecules: Dopamine, Gamma-Aminobutyric Acid, and Acetylcholine

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To describe electrostatic potentials, new sets of fractional point charges are presented for medium-sized neurotransmitters; dopamine, gamma-aminobutyric acid (GABA) and acetylcholine. The charge set consists of the original STO-3G Mulliken net atomic charges and new additional charges. The new additional charge sets were determined to correct electrostatic potential (ESP) values on the van der Waals molecular surface based upon Mulliken net atomic charges from STO-3G calculation.

The additional charge sets were obtained first for a variety of small molecules (water, ammonia, methane, ethane, benzene, formic acid anion, acetic acid, methylammonium, trimethylammonium, tetramethylammonium, and methyl acetate). Lone-pair regions of oxygen and nitrogen atoms showed remarkable improvement. The new additional charge sets were transferred as they were to Mulliken net atomic charge sets of the larger neurotransmitter molecules.

The present method is useful for large molecules of the types often considered in the fields of biological and pharmaceutical sciences; the approach gives approximately the same qualities to classical fractional point charge ESP's as can be obtained with complex *ab initio* calculations.

Keywords —— electrostatic potential; van der Waals molecular surface; molecular orbital; quantum chemistry; *ab initio*; dopamine; gamma-aminobutyric acid; acetylcholine

Introduction

If the final step of drug action is considered at the microscopic level, it may be reduced to a problem of chemicophysical interaction between a drug molecule and a receptor. It is, therefore, necessary to analyze precisely what sorts of forces operate in drug–receptor binding. They may be categorized into electrostatic interaction, dipole–dipole interaction, polarization interaction, dispersion interaction, charge transfer interaction, hydrogen bonding, hydrophobic interaction, and so on. Among them, in particular, electrostatic interaction often plays an important role in biological systems.[1,2]

If one wants to understand and describe electrostatic interactions quantitatively, accurate expression of the electrostatic potential (ESP) of a molecule is necessary, and this is possible, if one calculates ESP by applying *ab initio* molecular orbital theory. Many studies along this line

have been reported.[3] However, this theoretical approach requires extremely long computational times and consequently its applications are limited to small-sized compounds.

In the biological field, interest is directed toward rather large and complex molecules, to which the *ab initio* approach is not easily applied. On this account, a more convenient and simpler means of ESP evaluation is needed. The most popular one is to describe electron distribution by the use of 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 not likely that they represent the real electrostatic situation to a satisfactory approximation.

To solve this problem with Mulliken charges, several methodologies have been reported at the semiempirical level and at the *ab initio* level. At the semiempirical level, Pepe *et al.*[5] studied ESP of amino acids and a protein using the method of Del Re. At the *ab initio* level, studies have been done by Smit *et al.*,[6] Cox and Williams,[7] Momany,[8] Singh and Kollman[9] and Kubodera *et al.*[10] Cox and Williams[7] and Momany[8] evaluated ESP at grid points in the surrounding region of the van der Waals molecular surface, and optimized the fractional point charges to get the best least-squares fit with the *ab initio* method. Singh and Kollman[9] used the Connolly surface and optimized fractional point charges at points determined with the Connolly algorithm to reproduce *ab initio* ESP.

Kubodera *et al.*[10] developed 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 the van der Waals molecular surface was adopted 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 was to introduce new fractional point charges into a set of STO-3G[11] Mulliken charges to make a new set of charges which reproduced well the *ab initio* STO-3G ESP. The reason for the choice of STO-3G was discussed in their paper.

Here we tried to reproduce *ab initio* ESP for large molecules with new charges obtained for small-sized fundamental molecules by following our previous method,[10] with some modifications, for the simulation of *ab initio* ESP. New additional fractional point charges thus obtained for small-sized molecules were applied to similar portions of other compounds. For instance, the fractional point charges determined for the lone pairs of oxygen in the water molecule were transferred to oxygen atoms in phenolic hydroxyl groups of dopamine.

In the present paper, we will deal first with ESP's of a series of fundamental molecules; water, ammonia, methane, ethane, benzene, formic acid anion, acetic acid, methylammonium, trimethylammonium and tetramethylammonium ions, and methyl acetate. Then the charge sets obtained will be put together to reproduce the ESP's of larger biological molecules; dopamine, gamma-aminobutyric acid (GABA) and acetylcholine. In the case of acetylcholine, some information about the dependence of the accuracy of reproduction upon the selection of fundamental molecules selected as parts of the neurotransmitter was obtained.

The present method is useful for large molecules of the types commonly considered in the biological field; the approach gives approximately the same qualities to classical fractional point charge ESP's as can be obtained by means of *ab initio* calculations.

Method

The molecular surface was represented by an assembly of atom spheres. Van der Waals radii of spheres used were 1.2 Å for hydrogens, 1.7 Å for carbons, 1.6 Å for nitrogens and 1.5 Å for oxygens.[12] The sphere surfaces were divided into small patches by latitudes and longitudes.[2] The centers of the patches were used as points for evaluating

ESP's.

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

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

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

ESP due to Mulliken charges is simply written as;

$$\mathrm{ESP}^{\mathrm{M}}_{\mathrm{MLKN}}=\sum_{\mathrm{A}}\frac{q^{\mathrm{MLKN}}_{\mathrm{A}}}{r_{\mathrm{AM}}} \tag{2}$$

where $q^{\mathrm{MLKN}}_{\mathrm{A}}$ = Mulliken charge of atom A, and r_{AM} = distance between nucleus A and point M.

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

$$\mathrm{ESP}^{\mathrm{M}}_{\mathrm{NEWCH}}=\sum_{\mathrm{A}}\frac{q^{\mathrm{MLKN}}_{\mathrm{A}}}{r_{\mathrm{AM}}}+\sum_{\mathrm{B}}\frac{q^{\mathrm{NEWCH}}_{\mathrm{B}}}{r_{\mathrm{BM}}} \tag{3}$$

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, $\{q^{\mathrm{NEWCH}}_{\mathrm{B}}\}$, was determined by minimizing the value;

$$\mathrm{S.D.}=\sqrt{\sum_{\mathrm{i}}(\Delta E_{\mathrm{i}}-\overline{\Delta E})^2 s_{\mathrm{i}}/\sum_{\mathrm{i}} s_{\mathrm{i}}}$$

$$\Delta E_{\mathrm{i}}=\mathrm{ESP}^{\mathrm{i}}_{\mathrm{NEWCH}}-\mathrm{ESP}^{\mathrm{i}}_{\mathrm{ABIN}},\quad \overline{\Delta E}=\sum_{\mathrm{i}}\Delta E_{\mathrm{i}} s_{\mathrm{i}}/\sum_{\mathrm{i}} s_{\mathrm{i}} \tag{4}$$

where s_{i} is a weight factor of the area of the i-th patch determined by a pair of latitudes and longitudes. The strategy employed here is that the total sum of deviation between *ab initio* ESP and Mulliken charge ESP is dispersed over all the van der Waals molecular surface to eliminate the distortion of ESP distribution. The result of the strategy was almost the same as in our previous study.[10] It is reasonable to employ a common computer program routine to evaluate the 'usual' standard deviation, because the total of deviation between *ab initio* ESP and Mulliken charge ESP is apt to be almost zero, positive and negative deviations canceling each other out.

The new fractional point charges were added according to three patterns as in Fig. 1 of our previous paper.[10] Type 1: Three points are given and they define a plane and an angle. Two new points are added on the bisecting plane of the angle so as to be 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, and parameters (charges, angles, and lengths) being changed, the new charges were optimized.

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

Optimization was done iteratively by our original program DRAG-PMM, which estimated the 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 should be adequate for the purpose of proper treatment of electrostatic intermolecular interactions.

Ab initio calculations to obtain molecular orbitals, two-electron integrals, and Mulliken charges were done with the GAUSSIAN GENERAL program.[13] *Ab initio* ESP's were calculated with our original GAUESP program. The above calculations were done on the HITAC M-280H at the computer center of the University of Tokyo. Among all the calculations above, only the graphical part employed the TERAS routine.[14]

As for water dimer, its stable conformation was determined by a simple random walk procedure as described in our previous paper.[10] The calculation was done with a handmade program on a PC-9801M microcomputer (Nippon Electric Corporation).

The additional charges newly obtained for small-sized molecules were collected and transferred as they were without any change to larger-sized molecules; dopamine, GABA, and acetylcholine. For dopamine, for instance, the "building materials" are two water molecules, benzene, ethane, and ammonia. The geometries of the small molecules were taken from experimental data.[15] The structure of GABA was taken from an X-ray diffraction study.[16] The geometries of dopamine and acetylcholine were taken to be the same as those of specific portions of apomorphine[17]

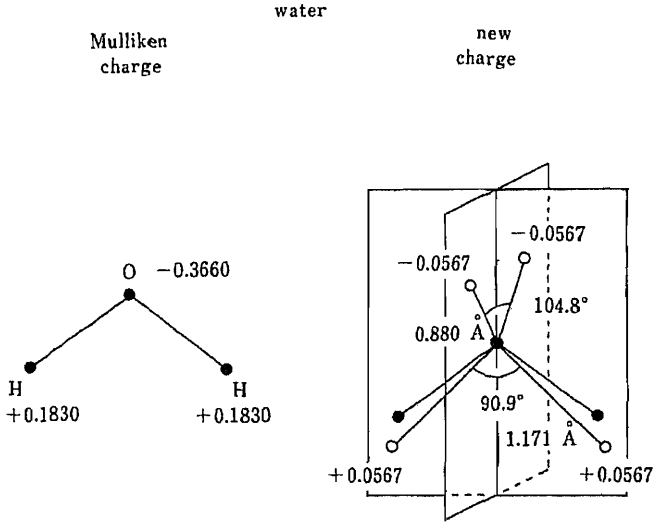


Fig. 1. Mulliken Charges (STO-3G) and Additional New Charges for Water

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

TABLE I. Optimization of S.D.

	Mulliken charge (kcal/mol)	New charge (kcal/mol)		Mulliken charge (kcal/mol)	New charge (kcal/mol)
Water	11.5	1.8	Acetic acid	8.0	3.2
Ammonia	13.2	1.3	Methyl acetate	8.0	3.6
Methane	3.4	0.5	Methylammonium	6.1	2.4
Ethane	2.5	0.9	Trimethylammonium	8.5	3.3
Benzene	3.4	1.6	Tetramethylammonium	8.0	2.8
Formic acid	8.8	2.7			

Probe radius is 0.0 Å, which corresponds to the van der Waals surface.

and pancuronium,[18] respectively (see Results and Discussion). Details of the "building materials" are as follows.

Water——The new fractional point charges are presented in Fig. 1. The new charge set "new charge" was determined by minimization of S.D. The negative charges located above the oxygen atom represent two lone pairs of electrons. The other additional positive charges maintain the neutrality of the total point charge set.

Because the optimization procedure adopted here was similar to that used in our previous report,[10] the results were similar too. The additional charge magnitude was 0.0577 e in the previous case, while that in the present work was 0.0567 e. The difference of 0.0010 e is, however, quite small compared with Mulliken charge magnitude of oxygen (0.3660 e) and therefore the two results are essentially the same. As for angles and lengths, the results were also similar to the first decimal places in degree unit for angles, and to the second decimal places in angstrom unit for length.

The standard deviation difference in ESP, between ESP_{MLKN} and ESP_{ABIN}, was reduced from 11.5 to 1.8 kcal/mol (Table I). The improvement is seen graphically to be quite similar to that in the previous report.[10] The ESP difference on the molecular surface was reduced drastically in the region around the lone-pair electrons of oxygen.

The number of fractional point charges employed here to reproduce the STO-3G ESP of water was seven. Three Mulliken charges were used as they were, and four new fractional point charges were introduced. Inclusion of those additional charges in random walk procedure brought about a better reproduction of water dimer structure, as was reported in our previous study.[10] The angle theta (50 °) was in good agreement with the values obtained by *ab initio* molecular orbital calculations[19-22] and experiments.[23,24]

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

ESP difference, $ESP_{MLKN} - ESP_{ABIN}$, on the molecular surface was reduced, especially around the region of the lone pair. The numerical improvement was from 13.2 to 1.3 kcal/mol (Table I).

Methane and Ethane——As nonpolar typical aliphatic hydrocarbons, methane and ethane molecules were examined. ESP differences ($ESP_{MLKN} - ESP_{ABIN}$) had a negative region around the hydrogens, which indicated that the electrical distribution along C–H bonds deviated toward the carbon atom(s). To represent this effect, positive charges were introduced on the extension of the C–H bonds (Figs. 3 and 4).

Numerically, standard deviations (S.D.'s) were reduced from 3.4 to 0.5 kcal/mol for methane, and from 2.5 to

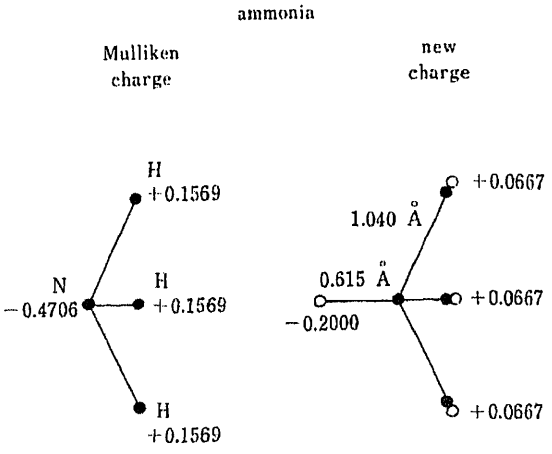


Fig. 2. Mulliken Charges (STO-3G) and Additional New Charges for Ammonia

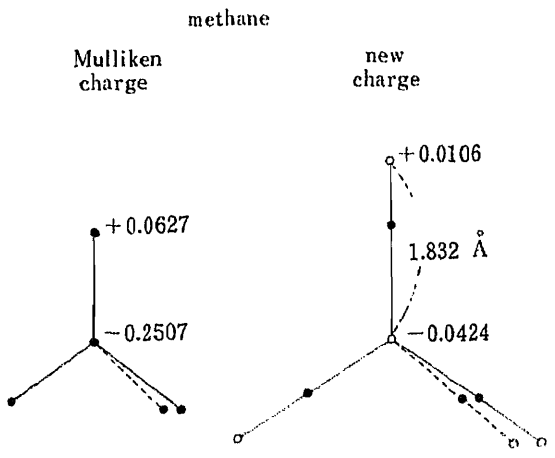


Fig. 3. Mulliken Charges (STO-3G) and Additional New Charges for Methane

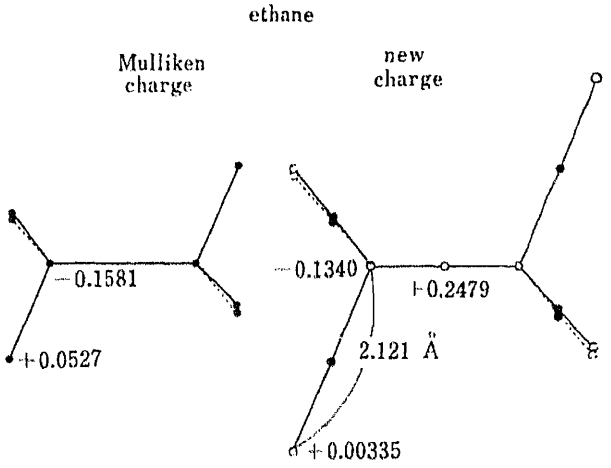


Fig. 4. Mulliken Charges (STO-3G) and Additional New Charges for Ethane

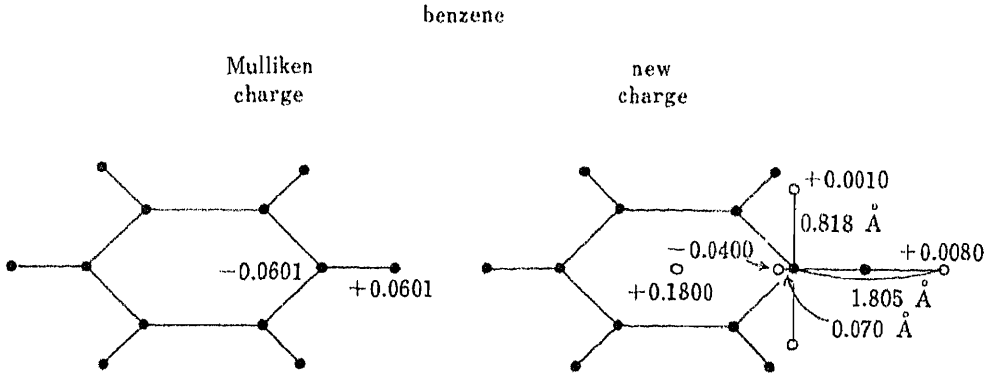


Fig. 5. Mulliken Charges (STO-3G) and Additional New Charges for Benzene

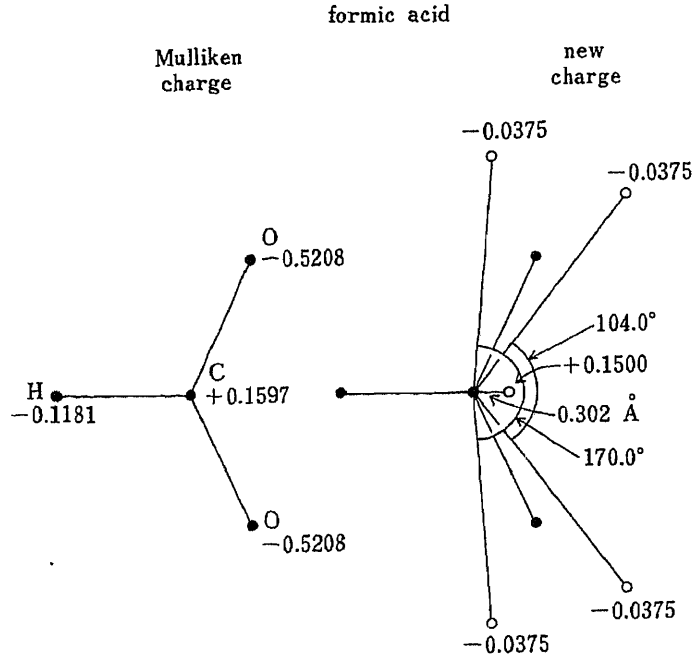


Fig. 6. Mulliken Charges (STO-3G) and Additional New Charges for Formic Acid

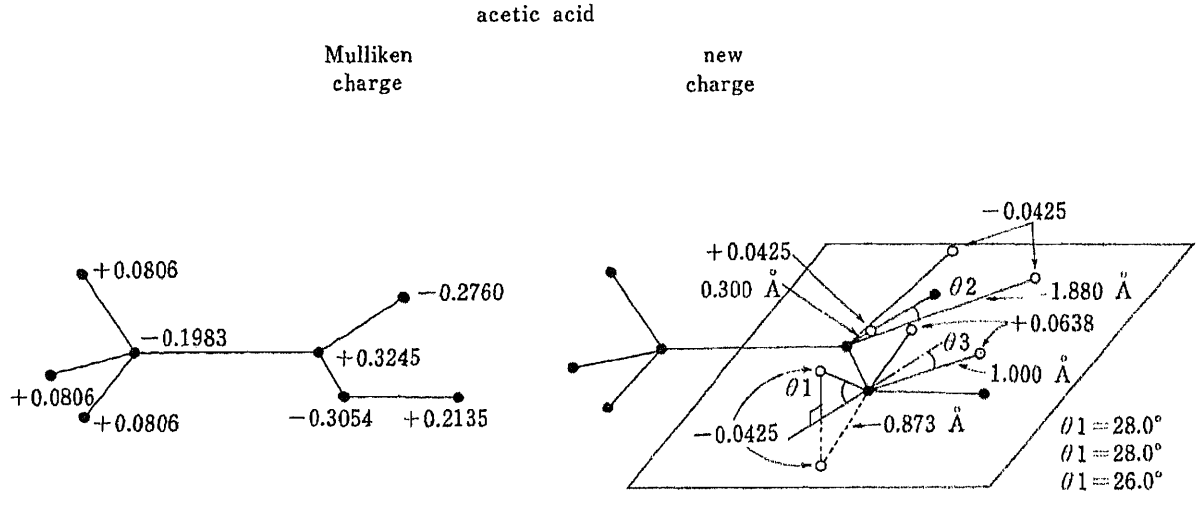


Fig. 7. Mulliken Charges (STO-3G) and Additional New Charges for Acetic Acid

0.9 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. 5. The location of positive charges above the molecular plane may seem strange, because *p* orbital lobes are supposed to be in that region. However, ESP is not determined only by pi electrons but by all the electrons of the molecule, and the total electron density is not greatest around the molecular surface region.[10] Standard deviation S.D. for "new charge" was 1.6 kcal/mol, while that for Mulliken charges was 3.4 kcal/mol (Table I).

Formic Acid Anion and Acetic Acid——The results of new charge set optimization are shown in Figs. 6 and 7 for formic acid anion and neutral acetic acid, respectively. Each lone pair of oxygen was represented by a negative fractional point charge as was the case with the warter molecule. The directions of lone pairs, however, are not necessarily the same as those of water. For formic acid anion, the negative charges newly added to express lone pairs were all located in the molecular plane. This was also the case with one oxygen atom (double-bonded) in the acetic acid molecule. For the other oxygen atom in the molecule, however, two negative point charges were located out of the molecular plane. Consequently, oxygen atoms in carboxylic acids should be treated in different ways depending on whether they are protonated or not, because the electron distributions around the atoms are altered. As for the

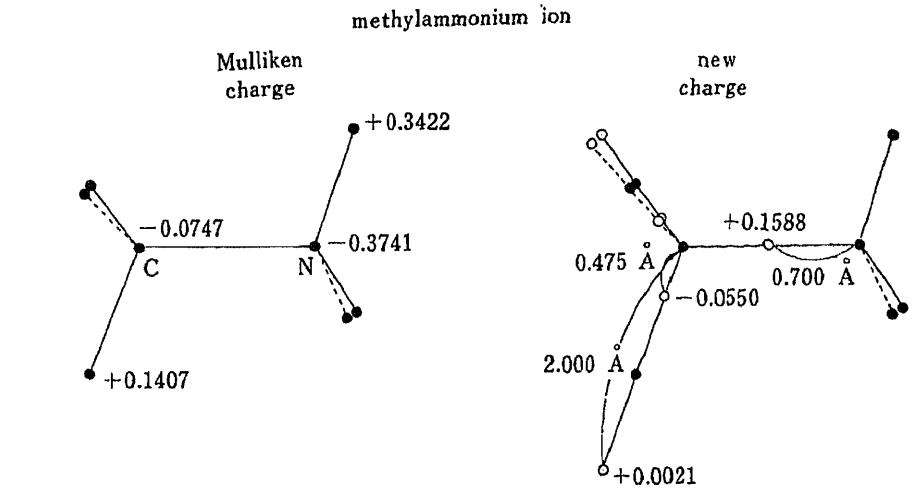


Fig. 8. Mulliken Charges (STO-3G) and Additional New Charges for Methylammonium

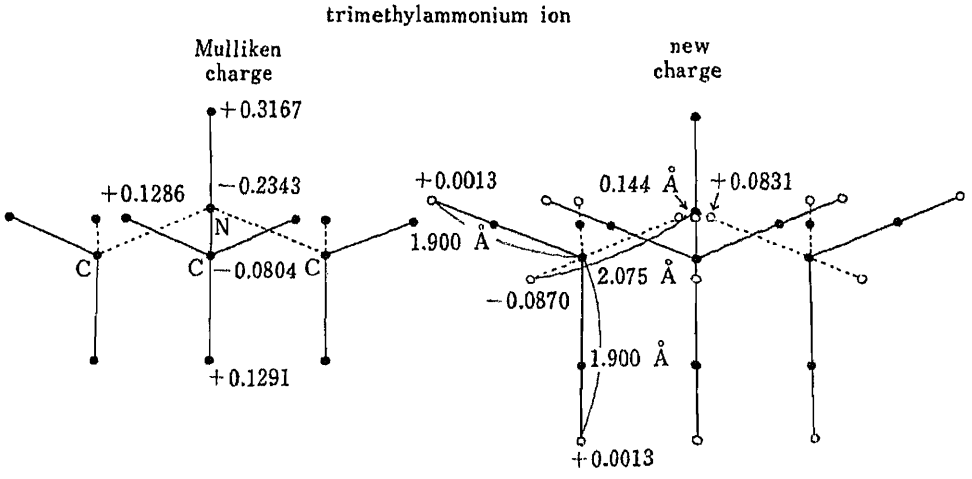


Fig. 9. Mulliken Charges (STO-3G) and Additional New Charges for Trimethylammonium

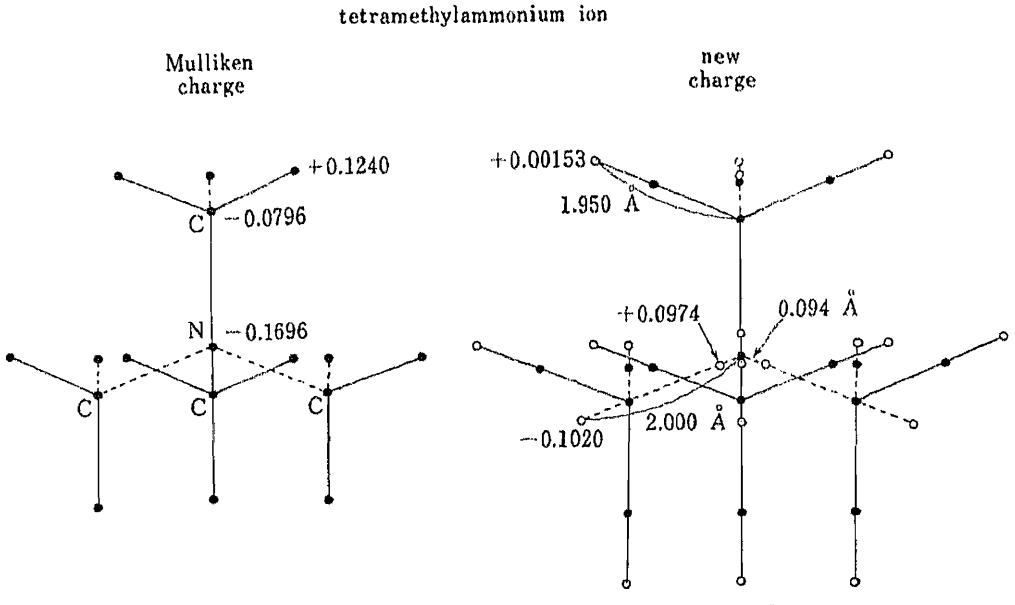


Fig. 10. Mulliken Charges (STO-3G) and Additional New Charges for Tetramethylammonium

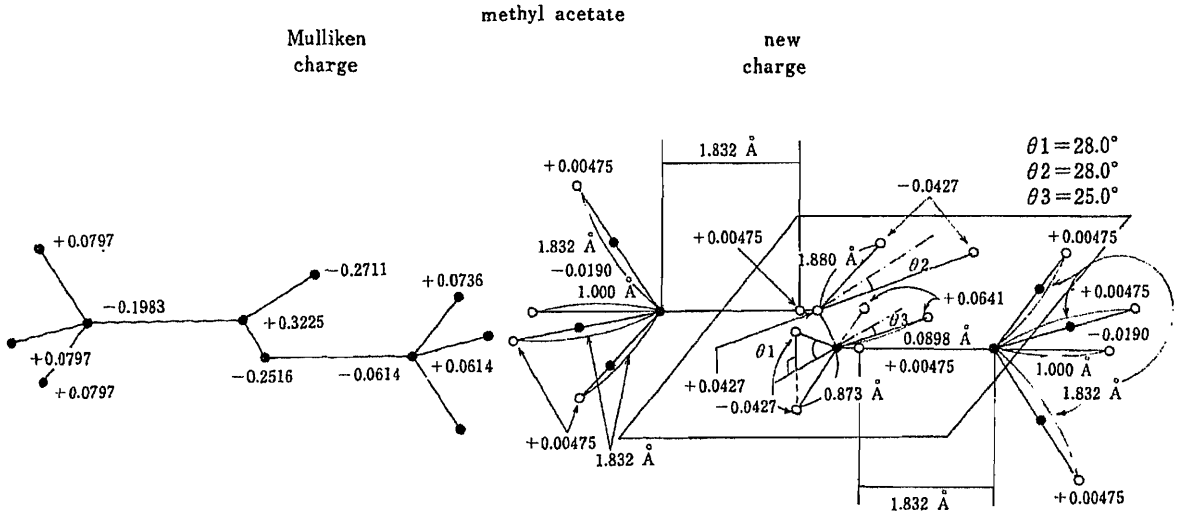


Fig. 11. Mulliken Charges (STO-3G) and Additional New Charges for Methyl Acetate

parts of the molecules other than the regions near oxygen lone pairs, no additional charges were introduced. That is because the main contribution to the difference ($ESP_{MLKN} - ESP_{ABIN}$) is the lack of a lone-pair distribution effect in the Mulliken charge set, and the remaining parts of the molecules have only minor effects.

Numerically, S.D.'s decreased from 8.8 to 2.7 kcal/mol and from 8.0 to 3.2 kcal/mol for formic acid anion and acetic acid, respectively (Table I).

Methylammonium, Trimethylammonium, Tetramethylammonium——For these three types of ammonium cations, a weak positive charge was added on the extension of each C–H bond, with negative charges being located around the carbon atoms. Positive fractional point charges were located near the nitrogen atom to adjust the overestimated negativity of the atom (Figs. 8—10). In the case of the methylammonium ion, several patterns of adding fractional point charges were tested, but patterns other than that presented here did not give better agreement.

These locations of fractional point charges reduced the Mulliken charge ESP deviations from 6.1 to 2.4 kcal/mol for methylammonium, 8.5 to 3.3 kcal/mol for trimethylammonium, and 8.0 to 2.8 kcal/mol for tetramethylammonium (Table I).

Methyl Acetate——As an example of an ester molecule, methyl acetate is presented. The ESP difference ($ESP_{MLKN} - ESP_{ABIN}$) indicates the inadequacy of the Mulliken charge representation (the situation is a combination of those for acetic acid and methane). New additional charges were added employing the principles described for methane and acetic acid. The result is summarized in Table I (S.D. dropped from 8.0 to 3.6 kcal/mol) and shown graphically in Fig. 11.

Results and Discussion

Dopamine

It is not difficult to obtain *ab initio* ESP's for small molecules such as water, ammonia, and so forth. In the biological field, however, molecules of interest are rather large and the procedures employed for small molecules are not directly applicable. However, if a specific group in a molecule is transferable to another molecule with no change of the properties of the group, then it is only necessary to calculate a suitable charge set for the group in one small molecule and to apply the set to a corresponding portion in any other molecule. Here we tried combining the results obtained previously (*vide supra*) in an attempt to reproduce the ESP of a medium-sized neurotransmitter molecule, dopamine.

Since the skeleton of dopamine molecule can be considered as a combination of ammonia, ethane, benzene, and two water molecules (Fig. 12), its ESP should be approximately described by superimposing the ESP's of these 'components.' Accordingly, by addition of the new fractional point charges obtained above to Mulliken charges of dopamine, ESP_{MLKN} of dopamine should be improved to give a good agreement with ESP_{ABIN}.

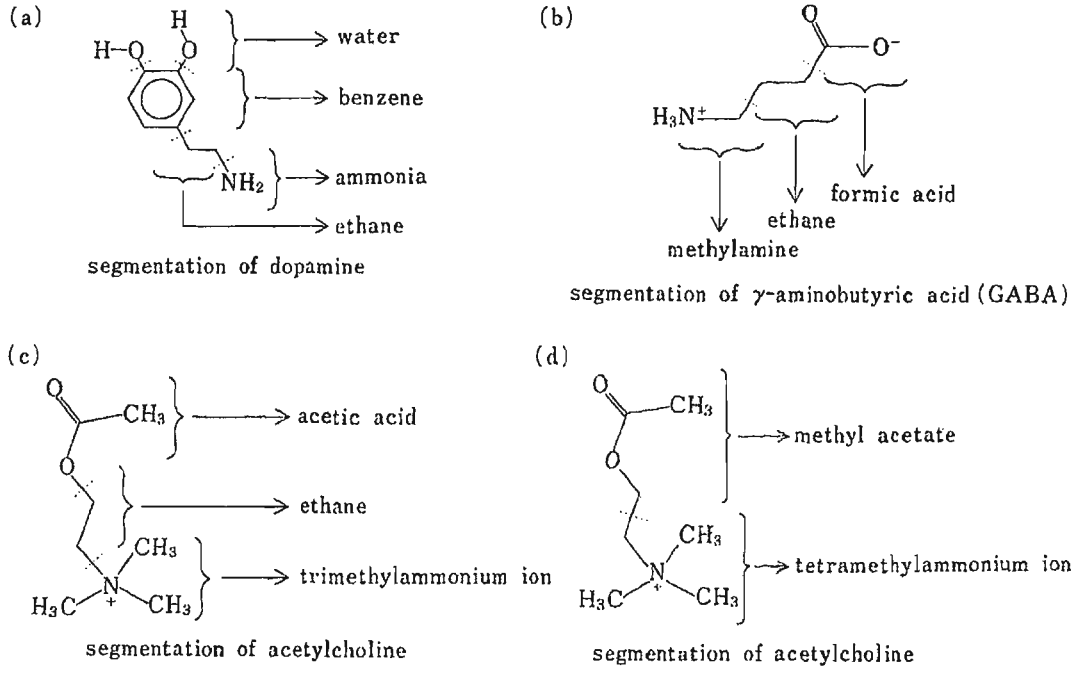


Fig. 12. Segmentation of Neurotransmitters

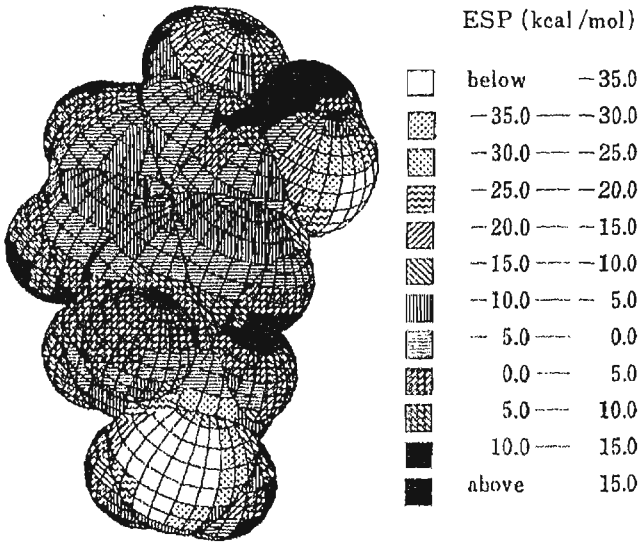


Fig. 13. ESP on van der Waals Surface of Dopamine
The STO-3G basis set was used.

In the first place, the *ab initio* ESP of dopamine is depicted in Fig. 13. The location of the lone-pair distribution is clear from the figure. The nitrogen has the strongest negative region and the oxygens have the next strongest negative region. A strong positive region is seen around the hydrogen atoms of the hydroxyl groups. The surface of the benzene ring has a slightly negative potential. The two methylene groups are almost neutral. If the dopamine molecule binds to the same receptor as apomorphine (*vide infra*) then the lone-pair regions may be significant for hydrogen bonding.

ESP difference ($ESP_{MLKN} - ESP_{ABIN}$) on the van der Waals surface is presented in Fig. 14. Actual application of the method of transferring components gave the result in Fig. 15. The improvement is satisfactory considering that the additional point charges were just superimposed and that no optimizing procedure was employed. Numerical reduction of standard

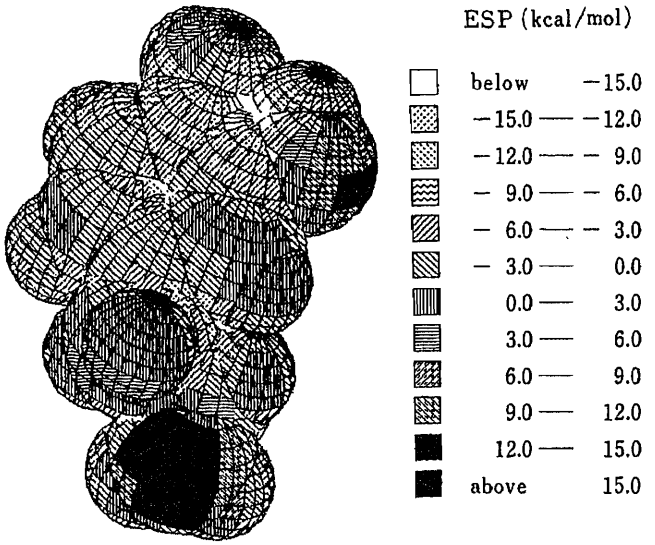


Fig. 14. ESP Difference ($ESP_{MLKN} - ESP_{ABIN}$) on van der Waals Surface of Dopamine

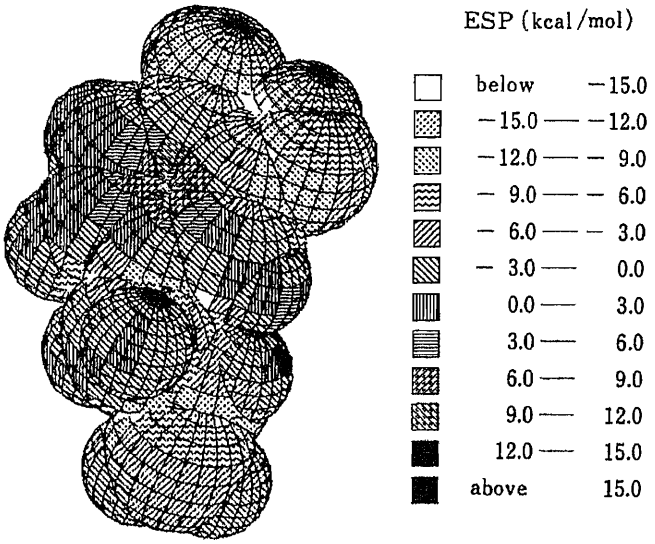


Fig. 15. ESP Difference ($ESP_{NEWCH} - ESP_{ABIN}$ on van der Waals Surface of Dopamine

deviation is tabulated in Table II (from 8.5 to 5.8 kcal/mol). If the improvement on specific atoms is looked at, the nitrogen atom changed dramatically (Figs. 14—16, and Table III), due to the existence of the lone pair which can not be described well by the Mulliken charge. The situation is similar for the oxygen atoms numbered 10 and 11 in Fig. 16.

The situation on the solvent-accessible surface 1.5 Å distant from the van der Waals surface is similar to that stated above. It is clear from the ESP maps (not shown) and numerical values in Table II (change from 2.5 to 1.7 kcal/mol) that the charge set obtained with attention to the ESP on the van der Waals surface improved the ESP on the solvent-accessible surface, too. The degree of improvement is largest on the nitrogen atom again. This is plausible because of the simplicity of the shape of the point charge set potential.

The above discussion substantiates the view that "functional groups" of a molecule, which have characteristic contributions to the ESP, can be transferred to some other molecule without modification. This transferability is encouraging indeed for researchers who aim at understanding drug-receptor interactions, empirical force field studies, and so on.

Dopamine is a neurotransmitter which is important in relation to Parkinsonism, schizophrenia, control of pituitary hormone secretion (*e.g.* prolactin), and so forth. Recently, furthermore, it was proved to play an important role in the cardiovascular system and

TABLE II. Optimization of S.D.

	Probe[a] (Å)	Mulliken charge (kcal/mol)	New charge (kcal/mol)
Dopamine	0.0	8.5	5.8
	1.5	2.5	1.7
GABA	0.0	7.6	5.4
	1.5	2.5	1.3
Acetylcholine	0.0	9.5	5.0
	1.5	1.2	1.1

a) Probe radius corresponds to distance from the van der Waals surface.

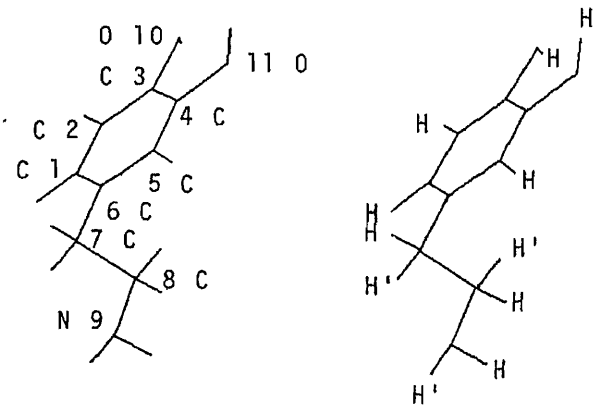


Fig. 16. Stereo View of Dopamine
Notations correspond to Table III.

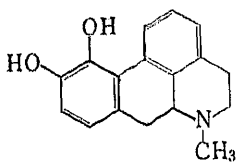
TABLE III. Optimization of ESP of Dopamine

No.	Atom[b]	0.0 Å[a] Mulliken charge (kcal/mol)	0.0 Å[a] New charge (kcal/mol)	Difference (kcal/mol)
1	C	3.5	3.8	0.3
	H	1.5	1.5	0.0
2	C	6.3	4.4	−1.8
	H	3.2	2.4	−0.8
3	C	7.9	9.3	1.4
4	C	6.1	8.5	2.4
5	C	4.3	5.7	1.4
	H	2.1	2.6	0.5
6	C	5.2	6.4	1.2
7	C	6.8	4.7	−2.1
	H	2.1	2.1	0.0
	H′	4.4	3.6	−0.8
8	C	8.5	5.7	−2.9
	H	3.7	2.5	−1.2
	H′	2.5	4.7	2.2
9	N	15.4	4.0	−11.3
	H	5.3	3.1	−2.2
	H′	6.3	3.5	−2.8
10	O	8.7	4.3	−4.4
	H	4.4	3.2	−1.2
11	O	8.5	4.0	−4.5
	H	4.3	3.5	−0.9
Total		8.5	5.8	−2.7

12 × 24 mesh for each atom. S.D. was optimized. *a*) Probe radius corresponding to the van der Waals surface. *b*) See Fig. 16.

digestive system. Like other neurotransmitters, dopamine is excreted from nerve termini and binds to a specific receptor in the cellular membrane, to give rise to a certain physiological function.

Dopamine receptors in the central nerve system can be categorized into D_1, D_2 and D_3. Receptors in the peripheral nerve system can be classified into DA_1 and DA_2. Among drugs active at the dopamine D_2 receptor, apomorphine has a fragment analogous to the dopamine skeleton in its structure. The geometry of the fragment, furthermore, is fixed rigidly, whilst the geometry of dopamine is so flexible that it is very hard to specify its active conformation for binding to the D_2 receptor. Therefore, apomorphine gives a clue to the active dopamine conformation for D_2 receptor binding.



apomorphine and dopamine-like structure (thick line)

For this reason, in performing calculations, the dopamine geometry was chosen from the skeleton of apomorphine, which was determined by X-ray diffraction analysis.[17] Now that we have a rather precise ESP of dopamine, we have a good basis to discuss the electronic

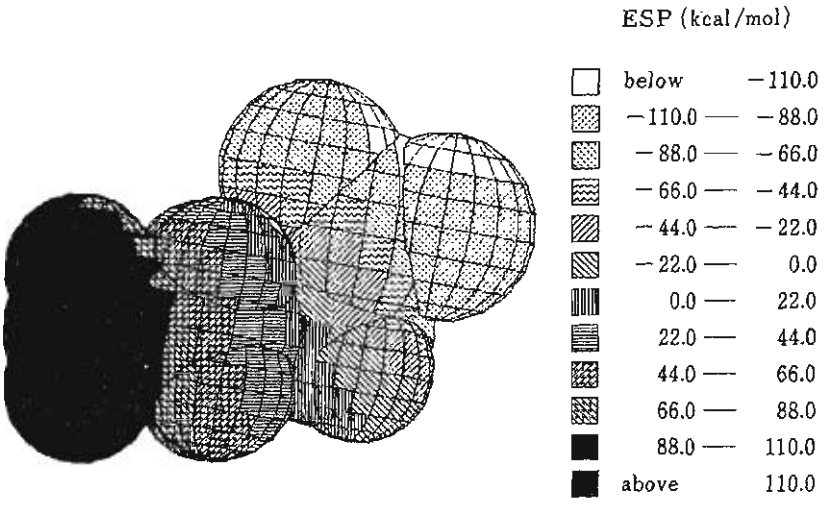


Fig. 17. ESP on van der Waals Surface of GABA
The STO-3G basis set was used.

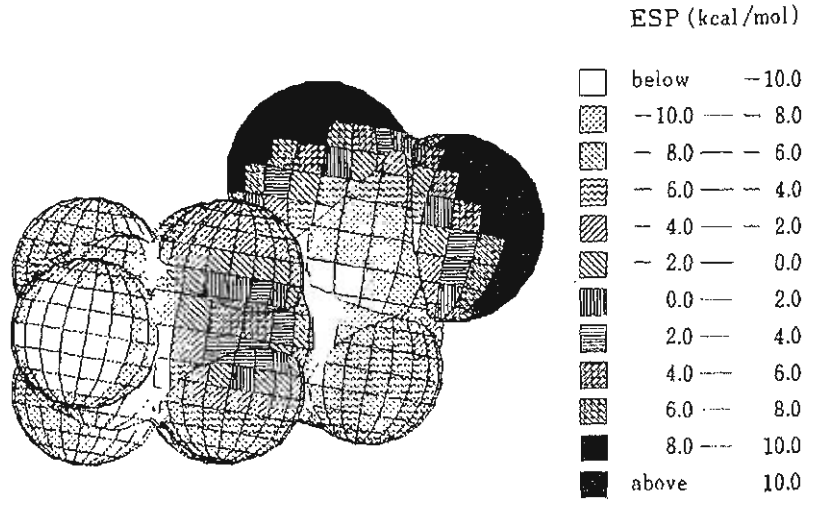


Fig. 18. ESP Difference ($ESP_{MLKN} - ESP_{ABIN}$) on van der Waals Surface of GABA

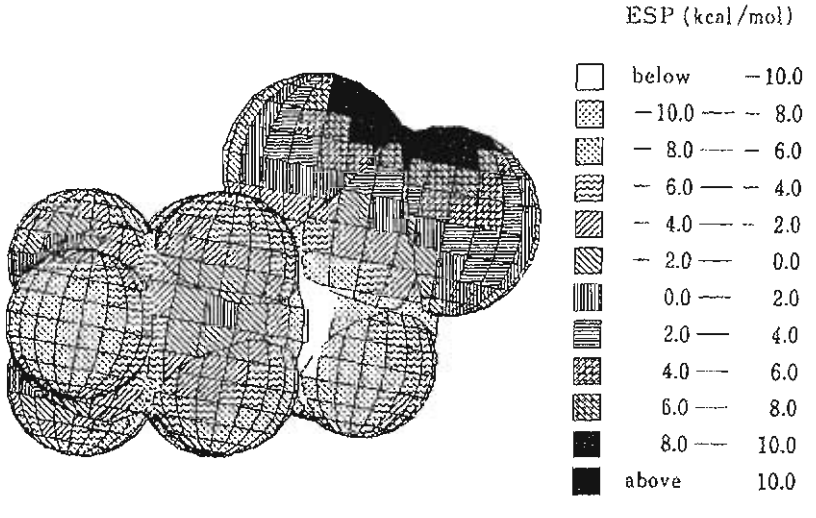


Fig. 19. ESP Difference ($ESP_{NEWCH} - ESP_{ABIN}$) on van der Waals Surface of GABA

structure and possible hydrogenbonding site of the D_2 receptor, namely, we can carry out receptor mapping.

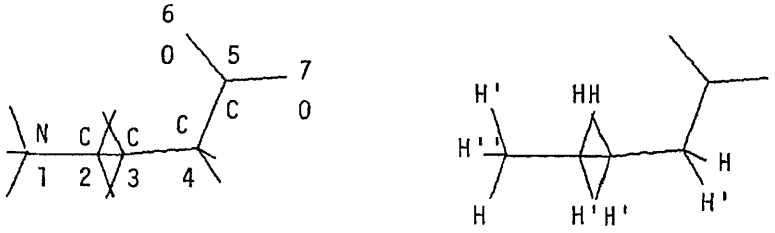


Fig. 20. Stereo View of GABA

Notations correspond to Table IV.

TABLE IV. Optimization of ESP of GABA

No.	Atom[b]	0.0 Å[a]		
		Mulliken charge (kcal/mol)	New charge (kcal/mol)	Difference (kcal/mol)
1	N	4.1	4.6	0.5
	H	3.3	4.0	0.7
	H′	2.6	3.7	1.1
	H″	2.0	2.6	0.6
2	C	6.8	5.3	−1.5
	H	3.5	2.6	−0.9
	H′	3.1	2.3	−0.8
3	C	5.2	5.4	0.2
	H	3.3	3.1	−0.2
	H′	2.8	3.1	0.3
4	C	4.8	4.5	−0.2
	H	2.3	1.6	−0.7
	H′	2.2	2.2	0.0
5	C	3.5	5.3	1.8
6	O	6.3	5.4	−0.9
7	O	6.0	4.6	−1.4
Total		7.6	5.4	−2.2

10 × 20 mesh for each atom. S.D. was optimized. *a*) Probe radius corresponding to the van der Waals surface. *b*) See Fig. 20.

Gamma-Aminobutyric Acid (GABA)

Following the procedure taken for dopamine, the ESP of GABA was reproduced. The *ab initio* ESP of GABA is shown in Fig. 17. The zwitter-ionic characteristic can be seen clearly from the figure. The ESP gradually decreases from the amino group to the carboxyl group. Segmentation of this molecule is shown in Fig. 12(b); it is considered to be a combination of methylamine, ethane and formic acid. With new fractional point charges for these components, ESP_{MLKN} on the van der Waals surface of GABA was corrected. ESP difference was reduced from that ($ESP_{MLKN} - ESP_{ABIN}$) shown in Fig. 18 to that ($ESP_{NEWCH} - ESP_{ABIN}$) in Fig. 19. The difference between ESP_{MLKN} and ESP_{ABIN} (Fig. 18) shows strong negative potential around the amino group and positive potential around the carboxyl group, because the polar character of the molecule is not described well by the Mulliken charges. Furthermore, the pattern around three methylene groups in the central part of the molecules is rather complicated and is again indicative of the deficiency of the Mulliken charges. The

situation is corrected in Fig. 19. The degree of improvement can also be monitored numerically as in Tables II (from 7.6 to 5.4 kcal/mol) and IV (*cf.* Fig. 20). The atoms which showed the greatest improvement are one oxygen of the carboxyl group and the carbon adjacent to the amino group. The change is a little complicated, but as a whole, the ionic termini of the molecule showed the largest improvement (see Table IV and Fig. 20).

The improvement was also achieved at the solvent-accessible surface 1.5 Å distant from the van der Waals surface. The standard deviation was reduced from 2.5 to 1.3 kcal/mol (Table II). The transferability of the new additional fractional point charges is thus confirmed.

Acetylcholine

The *ab initio* ESP of acetylcholine is depicted in Fig. 21. Because the molecule has one unit of positive charge, the ESP is mostly positive. The only exception is around the lone-pair regions of the oxygens. The trimethylammonium group has a strongly positive ESP.

For acetylcholine, two types of segmentation were considered. The first was to decompose the molecule into acetic acid, ethane and trimethylammonium, while the other into methyl acetate and tetramethylammonium (Fig. 12c, d).

The ESP differences ($ESP_{MLKN} - ESP_{ABIN}$) are presented in Fig. 22. The regions around

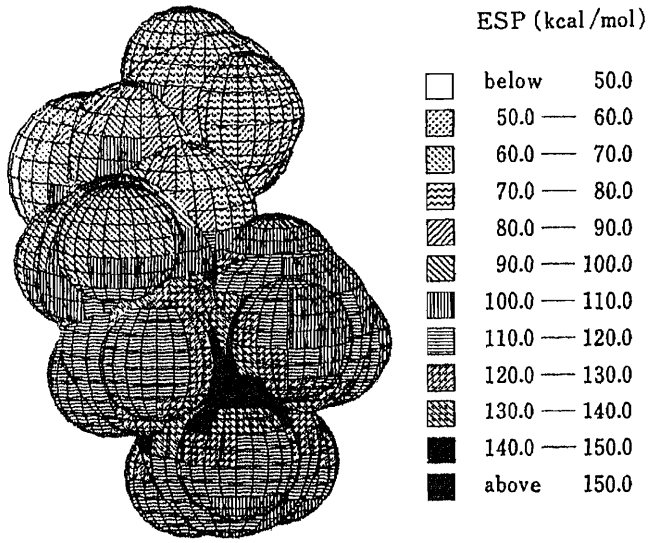


Fig. 21. ESP on van der Waals Surface of Acetylcholine
The STO-3G basis set was used.

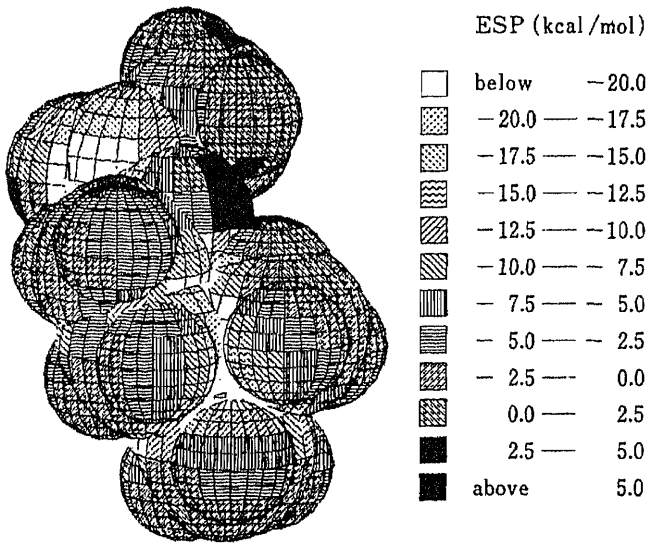


Fig. 22. ESP Difference ($ESP_{MLKN} - ESP_{ABIN}$) on van der Waals Surface of Acetylcholine

the lone pairs of the two oxygen atoms have large positive deviations, while the carbon atoms adjacent to the oxygens, and nitrogen atom surrounded by three methyl groups have negative deviations.

When the former segmentation was employed, the difference in ESP on the van der Waals surface became as shown in Fig. 23. Although overall numerical agreement was improved, the deviation around two methylene groups was worsened. Furthermore, the numerical result on the solvent-accessible surface 1.5 Å distant from the van der Waals surface showed no improvement.

When the second segmentation was employed, the result on the van der Waals surface was improved graphically and numerically, as shown in Fig. 24 and Table II (from 9.5 to 5.0 kcal/mol). On the solvent-accessible surface good agreement was also found graphically (not shown) and numerically (Table II, 1.2 to 1.1 kcal/mol). In the second segmentation, the most drastic improvement was obtained around the three carbon atoms of the trimethylammonium group, where the positive charge distribution is corrected. Oxygen atoms, where the lone-pair distributions exist, were also improved (*cf.* Table V and Fig. 25).

Thus, the charge distribution should be carefully considered upon segmentation of a molecule; in the present case, positive charge distribution is considered to reside around the

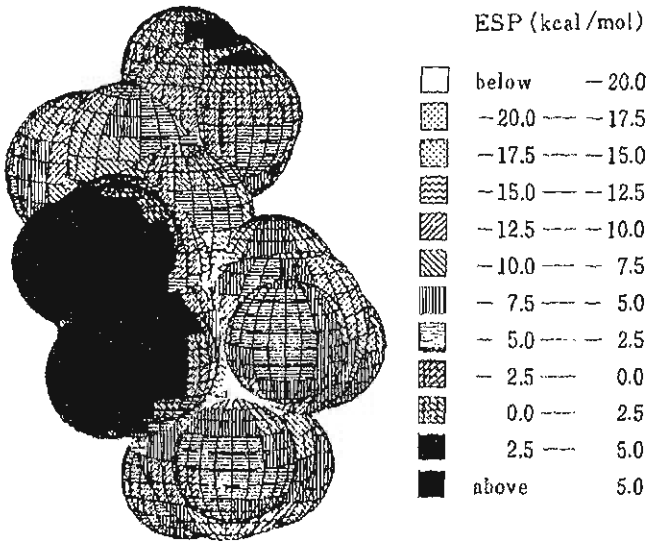


Fig. 23. ESP Difference ($ESP_{NEWCH} - ESP_{ABIN}$) on van der Waals Surface of Acetylcholine

The segmentation in Fig. 12c was used.

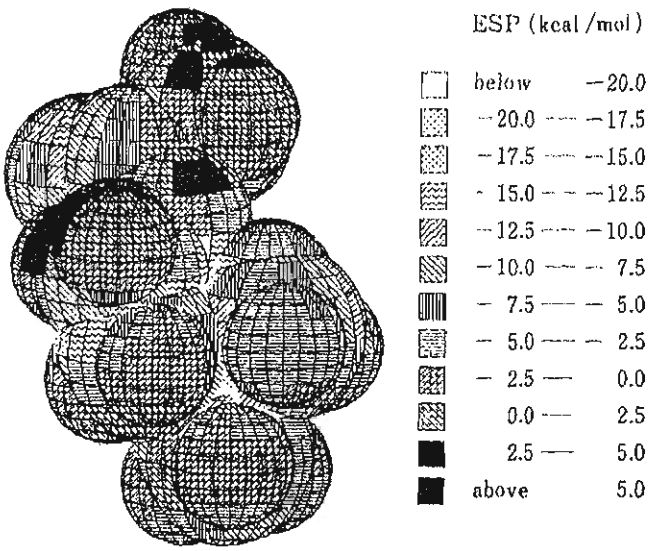


Fig. 24. ESP Difference ($ESP_{NEWCH} - ESP_{ABIN}$) on van der Waals Surface of Acetylcholine

The segmentation in Fig. 12d was used.

ammonium group with tailing into the surrounding region to some extent. If a small-sized molecule such as trimethylammonium is selected as a model of this group, the positive charge distribution is limited spatially to a narrow region. On the other hand, if tetramethylammonium is chosen, the distribution is broadened to reduce positive charge–positive charge

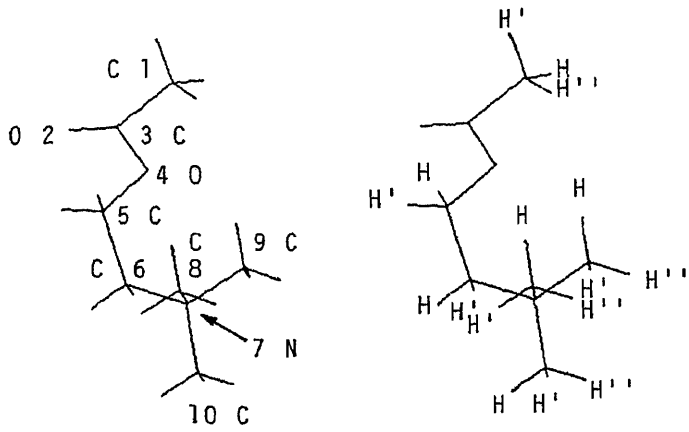


Fig. 25. Stereo View of Acetylcholine
Notations correspond to Table V.

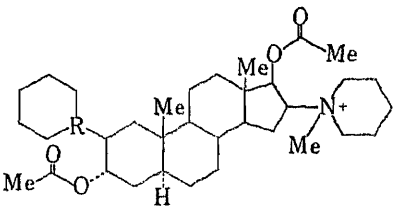
TABLE V. Optimization of ESP of Acetylcholine

No.	Atom[b]	0.0 Å[a]		
		Mulliken charge (kcal/mol)	New charge (kcal/mol)	Difference (kcal/mol)
1	C	5.4	3.5	−1.9
	H	1.7	1.5	−0.2
	H′	2.3	1.7	−0.5
	H″	2.7	1.4	−1.2
2	O	8.6	3.9	−4.7
3	C	6.2	3.9	−2.3
4	O	10.7	6.1	−4.6
5	C	11.3	7.6	−3.6
	H	2.3	2.7	0.4
	H′	4.9	3.4	−1.5
6	C	10.2	6.0	−4.2
	H	6.6	3.2	−3.4
	H′	6.0	2.9	−3.1
7	N	2.6	3.2	0.7
8	C	11.5	4.4	−7.1
	H	7.2	3.2	−4.0
	H′	6.8	2.4	−4.4
	H″	6.6	2.5	−4.2
9	C	14.5	5.9	−8.6
	H	7.6	3.8	−3.8
	H′	6.9	2.3	−4.6
	H″	6.9	2.3	−4.6
10	C	13.1	5.3	−7.8
	H	7.6	2.6	−5.0
	H′	7.0	2.5	−4.6
	H″	7.1	2.3	−4.7
Total		9.5	5.0	−4.5

12 × 24 mesh for each atom. S.D. was optimized. Segmentation into methyl acetate and tetramethylammonium was used. See Fig. 12d. *a*) Probe radius corresponding to the van der Waals surface. *b*) See Fig. 25.

repulsive force. The situation in the remaining region is analogous, except that negative charge distribution is now in question.

As a biological application of the present work, we may consider the two types of acetylcholine receptors, nicotinic and muscarinic receptors. The former receptor is known to be composed of two alpha, beta, gamma, and delta subunits, whose sequences are known, and the quaternary structure has been clarified by electron microscopy.[25] The tertiary structure, however, has not been determined yet. Competitive blocking agents bind to the nicotinic receptor competitively with acetylcholine, and prevent the nervous information from reaching the receptor, thus keeping the muscle relaxed. Among such agents is pancuronium, which bears two acetylcholine-like fragments in the A and D rings. Since pancuronium has a very rigid structure, it may provide a clue to the active conformation of acetylcholine when bound to the receptor. Furthermore, vecuronium, which has only one quarternary amino group in the D ring, has activity of the same order as pancuronium.[26] Only the D ring is rigid in this compound, and this part is very similar conformationally to the corresponding portion of pancuronium. Therefore the D ring part appears to be responsible for the pharmacological activity, and should be a good analogue of the active acetylcholine conformation.



pancuronium (R=N$^+$–Me) and vecuronium (R=N), and acetylcholine-like structure (thick line)

In the present paper, the geometry of acetylcholine was taken as that of the D ring of pancuronium.[18] Therefore, the ESP is considered to reflect the electronic three-dimensional structure of the nicotinic receptor, and should provide a basis for discussing the structure and function of the receptor.

Conclusion

In the present paper, for a series of small fundamental molecules, we present fractional point charge sets which overcome the defects of the STO-3G Mulliken charges. Several similar studies having the same purpose have been reported,[5-9] but the present approach seems to be more accurate and convenient for graphical comprehension.[10]

The new charge sets thus obtained were applied directly to larger molecules. Although no optimization process was included in this application, ESP based on the charge set derived for small molecules showed a good fit to *ab initio* ESP. In the field of biology and biophysics, or in the development of new drugs, the molecules of interest are rather large in size in general, and are far beyond the reach of a nonempirical quantum-chemical approach. Accordingly, new charge sets can not be determined for such molecules in the same way as for small molecules. To cope with such a situation, the technique presented here to treat large-sized molecules as an assembly of smaller parts would be of practical value.

If the ESP of a drug is characterized appropriately, we can discuss what structure the receptor of the drug has, and to which part of the receptor the drug binds. Although the number of the receptors whose three-dimensional structures are elucidated is not large, it is steadily increasing. Therefore the present method provides a useful approach for understanding electrostatic interaction between drugs and receptors.

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Quantitative Drug Design Studies. VI.[1] Quantitative Structure–Activity Relationships of Ionizable Substances: Antibacterial Activities of Phenols

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The antibacterial potencies of phenols were analyzed by using empirical and theoretical quantitative structure–activity relationships equations which take account of the influence of ionization. The lowest unoccupied molecular orbital energy term of the neutral form related to the drug-receptor interaction process always had a negative coefficient and was highly significant, while the hydrophobic term related to the drug transport process also made a significant contribution to the potency. The equilibrium model-based equation with one aqueous, one nonaqueous, and one receptor compartments gave the best result in analysis of the antibacterial assay data at pH 7.4. However, in general, the highest correlation was obtained from the neutral form-based Hansch equation, and the distribution coefficient-based Hansch equation offered the most helpful information for the design of drugs with selective antibacterial activity. The mechanism of antibacterial action of phenols is also discussed from the frontier electronic standpoint, and a drug-receptor interaction model of the charge-transfer type is proposed.

Keywords——antibacterial activity; phenol; ionization; QSAR; Hansch equation; model-based equation; LUMO; transport process; mechanism; charge-transfer interaction

Drug molecules often ionize in part at physiological pH. In order to establish precise quantitative structure–activity relationships (QSAR) of such compounds, it is necessary to take into account the influence of ionization. Two types of approaches, that is, empirical and theoretical, have been proposed so far.[2]

The former approach tries to solve the problem within the framework of the Hansch equation, by one of two methods. The first treats ionization as a modification to be applied to the true partition coefficient (P), that is, the partition coefficient of the neutral form.[3] Thus, the distribution coefficient (D), namely the ion-uncorrected apparent partition coefficient is used as the hydrophobic term instead of P. In this case, it is assumed that active transport takes part in the membrane penetration process.[4] On the other hand, the second method is to assume that the total concentration should be replaced with that of the neutral form from the standpoint that only the neutral form can reach the receptor site to contribute to the onset of action.[5]

The latter approach is based on the equilibrium model.[6] According to this model, the biological system is simulated by combining compartments representing the aqueous, nonaqueous, and receptor phases. The concentration of drug molecules which reach the receptor site is estimated by considering the equilibrium between these compartments.

In the present investigation, the author took phenol derivatives as an example of ionizable molecules, and analyzed their antibacterial data by applying the above approaches. It has been reported that fairly high correlations exist between the antibacterial potencies of phenols and the lowest unoccupied molecular orbital (LUMO) energies of the neutral form (E_{LUMO}) calculated by using the MINDO/3 method.[7] However, a detailed treatment

considering the transport process of drug molecules has not been carried out as yet.

Experimental

Biological Data——The antibacterial data of phenols reported in Ref. 7 were taken as the subject of the investigation. Potencies towards 8 kinds of strains (gram-positives, 4; gram-negatives, 4) were available for each of 11 compounds. The experimental pH was 7.4, and results at pH 6, 7, and 8 were also available for some compounds. The inoculum size of the antibacterial assays at pH 6, 7, and 8 was slightly different from that of pH 7.4. However, no corrections were made to the biological data, since preliminary analysis revealed that this difference had no effect upon the result of QSAR.

Physicochemical Parameters——log P, pK_a, and E_{LUMO} estimated by the MINDO/3 method were examined as physicochemical parameters. The values reported in the literature[7] were used as they were. For the log P of *p*-cyanophenol and *p*-acetylphenol, values of 1.66 and 1.35 were used, respectively.[8]

Computers——Data processing was performed on personal computers—NEC PC-8801 mkII/model 30 and NEC PC-9801VM2.

QSAR Analyses——The multiple regression and principal component analysis were carried out by using the Program Package for Multivariate Analyses written by Tanaka *et al.*[9] The equilibrium model-based equation was formulated by applying the nonlinear least-squares algorithm (Marquardt method).[10]

Molecular Orbital Calculations——The frontier electronic states of phenols and amino acids were estimated by using the CNDO/2 method.[11] In the case of amino acids, $-CH(NH_2)COOH$ groups were replaced by hydrogen atoms to reduce the computation time. The molecular geometries were modeled by using GPQDD/PC (version: PC-9801VM2).[12]

Results and Discussion

Principal Component Analysis

Figure 1 shows the result of the principal component analysis applied to the antibacterial data of phenols at pH 7.4. The scores of the first principal component [eigenvalue (λ_1), 6.95] corresponding to the overall potency based on 8 kinds of assay data are plotted on the abscissa. The potency becomes increasingly strong toward the right. On the other hand, the scores of the second principal component [eigenvalue (λ_2), 0.57] representing the selectivity of action are plotted on the ordinate. The compounds become increasingly selective against the gram-negatives toward the upper direction, and *vice versa* against the gram-positives. Figure 1 shows that 2,5-dinitrophenol, for example, has the strongest overall potency, and is effective against both gram-positives and gram-negatives.

QSAR Equations for the Data at pH 7.4

The results obtained by applying the above-mentioned empirical and theoretical approach to the antibacterial data at pH 7.4 will be described below.

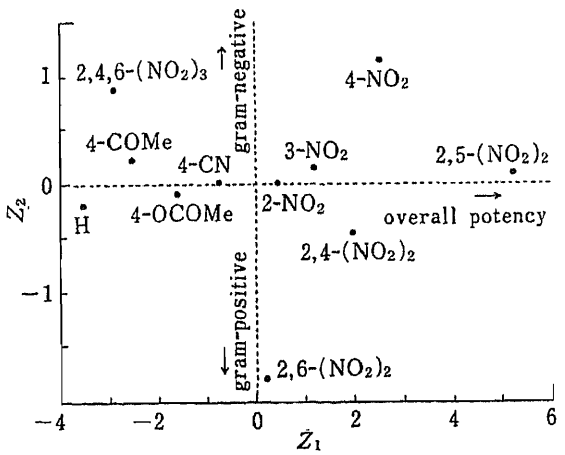

Fig. 1. Two-Dimensional Principal Component Space Derived from the Antibacterial Data of Phenols at pH 7.4

Z_1 represents the score of the first principal component and Z_2 represents that of the second principal component.

Empirical Approaches——log D-Based Hansch Equation: Equation 1 shows the general formula for the equations examined.

$$\log(1/C)=a(\log D)^2+b\log D+cE_{\mathrm{LUMO}}+\text{constant} \quad (1)$$

The distribution coefficient (D) can be obtained by multiplying the true partition coefficient (P) by the fraction unionized ($1-\alpha$), as follows:

$$D=P\cdot(1-\alpha) \quad (2)$$

where

$$1-\alpha=1/(10^{\mathrm{pH-p}K_a}+1) \quad (3)$$

In Eq. 1, $(\log D)^2$ and $\log D$ are closely associated with the pharmacokinetic phase or the drug transport process, whereas E_{LUMO} is related to the pharmacodynamic phase or the drug-receptor interaction process. The reason why E_{LUMO} was used as the pharmacodynamic parameter was that it has been suggested that the strength of the drug-receptor interaction of phenols depends on the LUMO energy level of the neutral form.[7]

The results are summarized in Table I. SA1, SA2, *etc.* in the first column stand for the types of bacterial strains used in the assays. The top four are the gram-positives, while the next four are the gram-negatives. The coefficients (a, b, and c) of the equation are standard partial regression coefficients to facilitate comparison of the contributions to the potency. The log D-dependence of the potency is clearly different between the gram-positives and the gram-negatives. This result may reflect the properties of the cell membrane and the cell wall of the two types of organisms. Thus, in the gram-positives, the drug with $\log D=0$ shows the highest permeability through the cell membrane and the cell wall, while in the gram-negatives, the permeability increases monotonously within the log D range examined (-5.77—1.96), and the optimum could be attained beyond the upper limit of 1.96. By using this result, it is possible to design a drug with the desired selectivity against the gram-positives or the gram-negatives. Since the sign of the coefficient c is always negative, it may be pointed out that a drug with a lower E_{LUMO} value interacts more strongly with the receptor. The fact that the coefficient a (or b) is almost equal to c in absolute value indicates that the drug transport and drug-receptor interaction process contribute similarly to the potency.

Neutral Form-Based Hansch Equation: Equation 4 shows the general formula for the equations examined.

$$\log(1/C)-\log(1-\alpha)=a(\log P)^2+b\log P+cE_{\mathrm{LUMO}}+\text{constant} \quad (4)$$

Table I. Summary of the Distribution Coefficient-Based Hansch Equations[a]

Strain[b]	a	b	c	r[c]	s[d]	F[e]
SA1	-0.816[f]	0	-0.956[f]	0.927	0.311	24.42 ($p<0.005$)
SA2	-0.895[f]	0	-0.945[f]	0.955	0.238	41.67 ($p<0.005$)
ML	-0.856[f]	0	-0.802[f]	0.861	0.337	11.47 ($p<0.005$)
BS	-0.878[f]	0	-0.889[f]	0.917	0.261	21.02 ($p<0.005$)
EC	0	0.803[i]	-1.220[f]	0.826	0.341	8.58 ($p<0.025$)
KP	0	0.946[h]	-1.164[g]	0.787	0.315	6.50 ($p<0.025$)
ST	0	0.980[f]	-1.317[f]	0.885	0.336	14.44 ($p<0.005$)
PV	0	1.204[f]	-1.188[f]	0.862	0.372	11.56 ($p<0.005$)

a) See Eq. 1 in the text; pH = 7.4; No. of data points = 11. *b*) SA1, *S. aureus* 209P; SA2, *S. aureus* U9NO; ML, *M. luteus*; BS, *B. subtilis*; EC, *E. coli*; KP, *K. pneumoniae*; ST, *S. typhimurium*; PV, *P. vulgaris*. *c*) Correlation coefficient. *d*) Standard deviation. *e*) F-value (p, significance level in F-test). *f*) $p<0.005$ (p, significance level in partial F-test). *g*) $p<0.01$. *h*) $p<0.025$. *i*) $p<0.05$.

TABLE II. Summary of the Neutral Form-Based Hansch Equations[a]

Strain[b]	a	b	c	r[c]	s[d]	F[e]
SA1	0	−0.321[h]	−0.836[f]	0.942	0.935	31.47 ($p<0.005$)
SA2	0	−0.306[h]	−0.850[f]	0.949	0.851	35.92 ($p<0.005$)
ML	0	−0.295[i]	−0.836[f]	0.930	0.938	25.71 ($p<0.005$)
BS	0	−0.367[g]	−0.813[f]	0.944	0.876	32.46 ($p<0.005$)
EC	0	−0.324[h]	−0.816[f]	0.925	1.035	23.77 ($p<0.005$)
KP	0	−0.321[i]	−0.802[f]	0.911	1.078	19.39 ($p<0.005$)
ST	0	−0.266[i]	−0.850[f]	0.930	0.994	25.67 ($p<0.005$)
PV	0	−0.284[i]	−0.849[f]	0.937	0.861	28.82 ($p<0.005$)

a) See Eq. 4 in the text; pH=7.4; No. of data points=11. *b*) See footnote *b*) of Table I. *c*) Correlation coefficient. *d*) Standard deviation. *e*) F-value (p, significance level in F-test). *f*) $p<0.005$ (p, significance level in partial F-test). *g*) $p<0.025$. *h*) $p<0.05$. *i*) $p<0.1$.

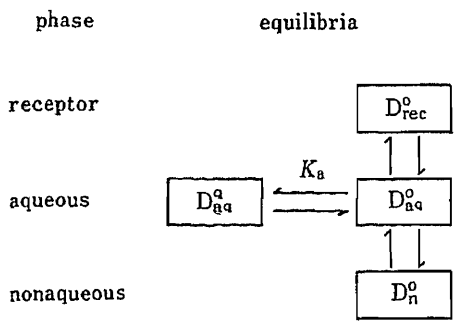

Fig. 2. Equilibrium Model with One Aqueous, One Nonaqueous, and One Receptor Compartments

Only the neutral form of the drug (D) binds to the receptor. The superscripts indicate the charge and the subscripts indicate the phase.

In Eq. 4, $(\log P)^2$ and $\log P$ are associated with the drug transport process, and E_{LUMO} represents the contribution from the drug-receptor interaction process.

The results are summarized in Table II. The values of a, b, and c are standard partial regression coefficients to facilitate comparison of the contributions to the potency. It can be seen that the correlations are much higher than those of the $\log D$-based equation. The coefficients of E_{LUMO} are always negative and highly significant, while for the coefficients of $\log P$, only the terms of the first power are significant at less than 10% level and the squared terms are not. These results indicate that the phase which makes the major contribution to the potency is that of the drug-receptor interaction, though the contribution from the transport phase can not be neglected. It is expected that the optimum value of $\log P$ could be attained beyond the lower limit of the data range (1.25—2.00), probably near zero. No appreciable difference in the form of the equation could be observed between the two types of organisms.

Theoretical Approach——Equation 5 shows the general formula for the equations examined.

$$\log(1/C) = -\log[1 + dP^{e} + 1/\{aP^{b}(1-\alpha)\}] + cE_{LUMO} + f \quad (5)$$

In this case, the equilibrium model with one aqueous, one nonaqueous, and one receptor compartment, where only the neutral form binds to the receptor, was applied (Fig. 2). According to the model, the drug ionizes in the aqueous compartment, and only the neutral form is in equilibrium with the receptor and nonaqueous compartments, assuming the equilibrium constants to be proportional to $\log P$. The logarithmic term of the right-hand side of Eq. 5 represents the concentration of the neutral form which can reach the receptor compartment, and E_{LUMO} is related to the intrinsic activity of the drug at the receptor site. Though this model requires the approximation that the intra- and extracellular aqueous phases are combined as a single aqueous compartment, the correlations are excellent in most

TABLE III. Summary of the Equilibrium Model-Based Equations[a]

Strain[b]	log a	b	c	log d	e	f	r[c]	s[d]
SA1	4.995	0	−4.759	6.290	−0.756	5.162	0.943	0.318
SA2	4.860	0	−4.089	5.588	−0.720	5.354	0.975	0.206
ML	5.284	0	−6.832	9.570	−0.547	5.235	0.981	0.149
BS	5.212	0	−0.201	0	−0.536	5.313	0.935	0.248
EC	6.013	0	−14.692	19.297	−0.591	5.356	0.907	0.294
KP	6.234	0	−2.033	3.279	−0.331	5.430	0.835	0.325
ST	5.601	0	−4.410	6.552	−0.612	5.453	0.954	0.249
PV	4.995	0	−17.765	23.528	−0.613	5.391	0.950	0.265

a) See Eq. 5 in the text; pH=7.4; No. of data points=11. *b*) See footnote *b*) of Table I. *c*) Correlation coefficient. *d*) Standard deviation.

TABLE IV. Comparison of Correlation Coefficients Obtained from the Three Types of Equations (pH=7.4)[a]

Strain[b]	A[c]	B[d]	C[e]
SA1	0.927	0.942	0.943[f]
SA2	0.955	0.949	0.975
ML	0.861	0.930	0.981
BS	0.917	0.944	0.935
EC	0.826	0.925	0.907
KP	0.787	0.911	0.835
ST	0.885	0.930	0.954
PV	0.862	0.937	0.950

a) No. of data points=11. *b*) See footnote *b*) of Table I. *c*) Distribution coefficient-based Hansch equation. *d*) Neutral form-based Hansch equation. *e*) Equilibrium model-based equation with one aqueous, one nonaqueous, and one receptor compartments. *f*) The best result is underscored for each of the assay data.

TABLE V. Comparison of Correlation Coefficients Obtained from the Three Types of Equations (pH=7.4, 6, 7, and 8)[a]

Strain[b]	A[c]	B[d]	C[e]
SA1	0.867	0.948[f]	0.853
SA2	0.851	0.944	0.822
ML	0.834	0.920	0.890
BS	0.833	0.954	0.817
EC	0.790	0.925	0.760
KP	0.821	0.889	0.804
ST	0.886	0.930	0.865
PV	0.861	0.935	0.835

a) No. of data points=29. *b*) See footnote *b*) of Table I. *c*) Distribution coefficient-based Hansch equation. *d*) Neutral form-based Hansch equation. *e*) Equilibrium model-based equation with two aqueous, one nonaqueous, and one receptor compartments. *f*) The best result is underscored for each of the assay data.

cases, as shown in Table III. Coefficients of E_{LUMO} are highly significant and their signs are always negative. Since the values of log a and b of Eq. 5 are not unique, but depend on each other, we can obtain equations with the same precision when either of the two terms varies while the other is kept constant. Table III summarizes the results obtained under the condition that the equilibrium constant between the receptor and aqueous compartment is assumed to be constant ($b=0$) regardless of the chemical structure of the molecule.

Comparison of Results——Table IV summarizes the results of analyzing the antibacterial data at pH 7.4 as described above. A, B, and C show the correlation coefficients obtained from the log D-based, neutral form-based, and equilibrium model-based equations, respectively. It can be seen from Table IV that C gives the best results for 5 kinds of assays, followed by B. This result indicates that the approximation of combining the intra- and extracellular aqueous phases as a single aqueous compartment in the equilibrium model-based equation is adequate to explain the data, and that the intracellular pH can be assumed to be roughly equal to the extracellular pH, 7.4.

QSAR Equations for the Data at pH 7.4, 6, 7, and 8

For 3-nitro-, 4-nitro-, 2,4-dinitro-, 2,5-dinitro-, 2,6-dinitro-, and 2,4,6-trinitrophenol, antibacterial data at pH 6, 7, and 8 have been reported in addition to those at pH 7.4.[7] Table

V summarizes the results of fitting all the data at pH 7.4, 6, 7, and 8. Columns A, B, and C show the correlation coefficients obtained from the $\log D$-based, neutral form-based, and equilibrium model-based equation, respectively. In the case of the equilibrium model-based equation, the model with two aqueous, one nonaqueous, and one receptor compartments (Eq. 6) was adopted from the standpoint that separate aqueous compartments should be assigned to the intra- and extracellular aqueous phases, since the extracellular pH was variable.

$$\log(1/C) = -\log\left[1 + dP^c + \sum_{i=1}^{2} 1/\{a_i P^b (1-\alpha_i)\}\right] + eE_{\mathrm{LUMO}} + f \qquad (6)$$

In Eq. 6, the pH of the intracellular aqueous compartment was assumed to be 7.4 in the light of the above results at pH 7.4.

It can be seen from Table V that B gave the largest correlation coefficient in every case examined, while C gave the poorest result. This means that more complex models should be investigated to improve the result of the equilibrium model-based approach.

Mechanism of the Drug-Receptor Interaction

The LUMO coefficients of the neutral form of phenols were calculated by using the CNDO/2 method to investigate the mechanism of the drug-receptor interaction. For instance, Fig. 3 depicts the results for *m*-nitrophenol and 2,5-dinitrophenol. Large LUMO coefficients are found on the phenyl ring and nitro group of both molecules. The other phenols also show a similar tendency. Collectively, it was found that all the compounds examined have large LUMO coefficients and a common phase pattern on the phenyl ring. This observation suggests that charge-transfer interaction takes place between the LUMO π-orbital of the phenyl ring of phenols and the highest occupied molecular orbital (HOMO) of the electron-donating group at the receptor site to modify the secondary or tertiary structure of the receptor protein, and that the strength of this interaction determines the intrinsic antibacterial activity of phenols. In this connection, amino acid residues containing an aromatic moiety complementary to the phenyl ring of phenols seemed to be candidates for the electron donor.

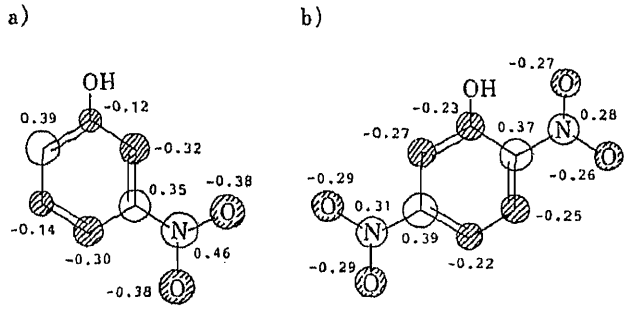

Fig. 3. LUMO Coefficients of Selected Phenols (CNDO/2)

a) *m*-Nitrophenol. b) 2,5-Dinitrophenol.

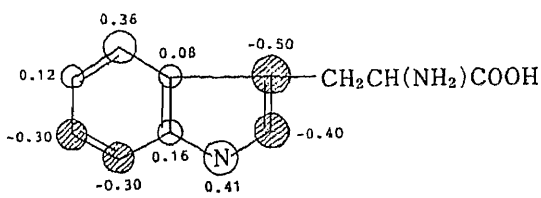

Fig. 4. HOMO Coefficients of Tryptophan (CNDO/2)

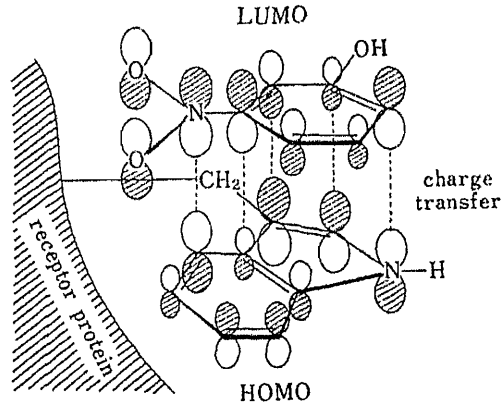

Fig. 5. Charge–Transfer Interaction Model between *m*-Nitrophenol and a Tryptophan Residue of the Receptor Protein

Hence, their HOMO energies were estimated by using the CNDO/2 method. The results were as follows: tryptophan, −10.39 eV; histidine, −11.24 eV; tyrosine, −12.10 eV; phenylalanine, −12.90 eV. Thus, tryptophan has the highest HOMO energy. This finding is in harmony with that of Pullman and Pullman.[13] Figure 4 shows the HOMO coefficients of tryptophan. Inspection of Figs. 3 and 4 reveals that tryptophan has large HOMO coefficients and the most suitable phase pattern on the pyrrole part of the indole ring. These results suggest that the tryptophan residue should be the best electron donor for the charge-transfer interaction with phenols, and lead to a model in which LUMO of phenols interacts with HOMO of the pyrrole part of the indole ring of tryptophan in such a manner as to maximize the bonding overlap between the two frontier orbitals. Figure 5 illustrates the charge-transfer interaction model for *m*-nitrophenol as an example.

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Asymmetric Synthesis of (+)- and (−)-Batyl Alcohol, a Key Synthetic Intermediate for Platelet-Activating Factor, by Using Biocatalysts

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Asymmetric synthesis of platelet-activating factor (PAF) and its enantiomer by using biocatalysts was studied. Microbial reduction of the pro-chiral α-ketoester (**3**) afforded (+)-**4** (>99% ee), which could be converted to (+)- and (−)-batyl alcohol (**12**), a key synthetic intermediate for PAF. Compound (−)-**4** (96% ee), with the requisite configuration for the synthesis of natural PAF, could also be obtained by enzyme-catalyzed enantioselective hydrolysis of (±)-**15**.

Keywords——platelet-activating factor; batyl alcohol; asymmetric reduction; microbial reduction; enantioselective hydrolysis; enzymic hydrolysis; kinetic resolution

Platelet-activating factor (PAF, **1**) was first discovered as a stimulator of rabbit platelets, and its structure was shown by Hanahan *et al.*[1] to be 1-*O*-hexadecyl (or octadecyl)-2-acetyl-*sn*-glycero-3-phosphorylcholine. PAF is able to provoke platelet and neutrophil activation, hypotension and broncho-constriction.[2-4]

As a part of our attempts to develop a simple synthetic route to PAF, we previously reported the asymmetric synthesis[5,6] of PAF intermediates by the (*S*)-BINAL-H[7] reduction of octadecyloxymethyl (*E*)-2-cyclohexylvinyl ketone and by enzyme-catalyzed hydrolysis of a *meso* compound, 1,3-di-*O*-acetyl-2-*O*-benzylglycerol. However, in each case, the optical purity (40—80% ee) was unsatisfactory.

Therefore, we designed a new chiral synthon ((*S*)-**4**) for natural PAF as shown in Chart 1. This sequence starts with the synthesis of methyl 2-oxo-4,4-propylenedithiopentanoate (**3**), followed by microbial reduction. Regioselective protection of the ketone function in

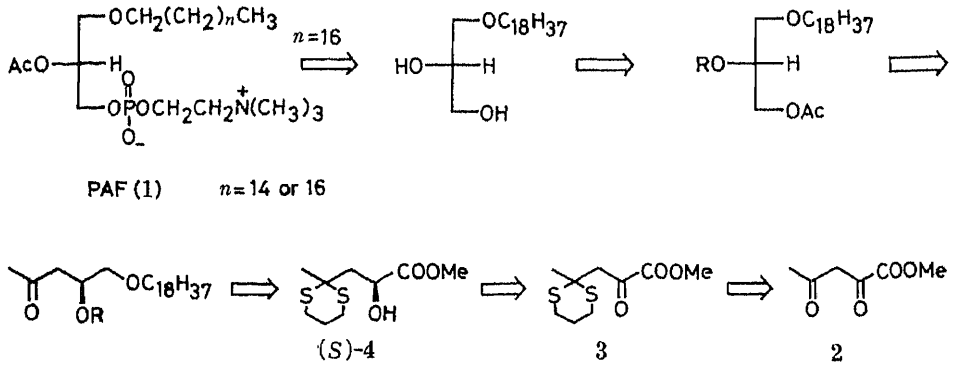
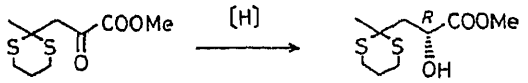


Chart 1

TABLE I. Asymmetric Reduction of **3** with Yeast



3 (+)-**4**

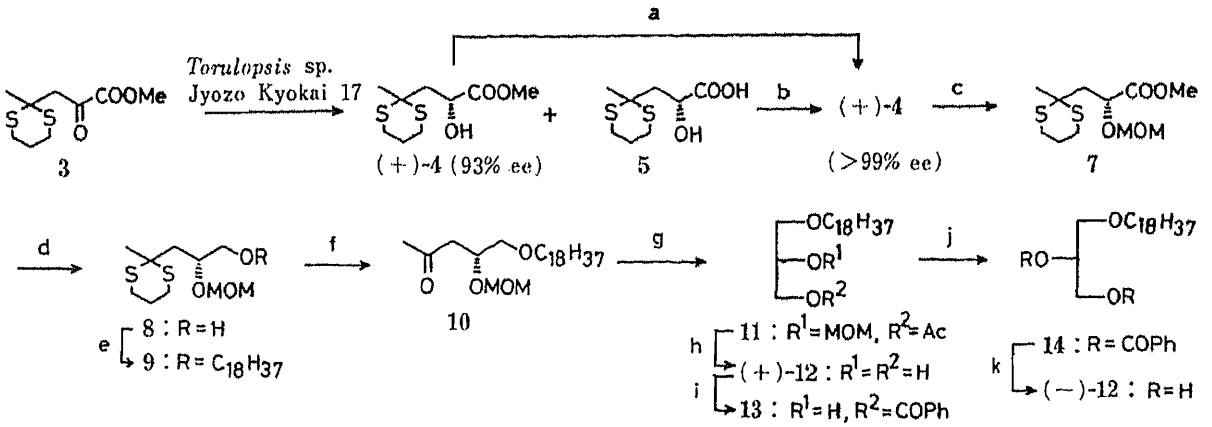
Run	Microorganism	Chemical yield as (+)-MTPA ester (%)	Optical purity (% ee)	Absolute configuration
1	*Hansenula anomala* NI-7572	15	64	*R*
2	*Pichia membranaefaciens*	34	32	*S*
3	*Saccharomyces acidifaciens*	31	50	*R*
4	*Saccharomyces delbrueckii*	33	49	*R*
5	*Saccharomyces fermentati* IFO-0422	37	88	*R*
6	*Saccharomyces tokyo* Jyozo Kyokai 2013	41	85	*R*
7	*Schizosaccharomyces octosporus*	41	23	*R*
8	*Torulopsis famata* NI-7550	19	81	*R*
9	*Torulopsis* sp. Jyozo Kyokai 17	15	>99	*R*
10	*Saccharomyces cerevisiae* Kitasato Inst.	22	89	*R*
11	*Trichosporon fermentans* IFO-1199	34	71	*R*
12	*Candida utilis* IFO-0619	25	80	*R*
13	*Saccharomyces cerevisiae*[a] (baker's yeast)	80[b]	64	*R*

a) The reaction was performed on a preparative scale (500 mg of **3**). *b*) Isolated yield of (+)-**4**.

methyl 2,4-dioxopentanoate (**2**) was performed by treatment with 1,3-propanedithiol–BF_3 etherate in CH_2Cl_2 to afford the monoacetal (**3**) in 61% yield.

Thin layer chromatographic screening of forty strains of yeast[8] for ability to reduce **3** indicated that thirteen strains afforded the hydroxy ester (**4**). Secondary screening on a larger scale (30 mg of substrate) was performed using these strains, and the results are summarized in Table I.

The enantiomeric excess (ee) of the reduction products was determined from the 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectra after conversion to the (+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA)[9] esters by treatment with (+)-MTPA chloride. The absolute stereochemistry was finally determined by conversion into optically active batyl alcohol ((+)-**12**).



a) enhancement of optical purity b) CH_2N_2 c) MOMCl, $(iso\text{-}Pr)_2NEt$ d) $LiAlH_4$
e) $C_{18}H_{37}OMs$, KH f) $Tl(NO_3)_3$ g) CF_3CO_3H, Na_2HPO_4 h) aq. HCl, MeOH
i) PhCOCl, Py j) EtOOCN=NCOOEt, Ph_3P, PhCOOH k) NaOH, MeOH

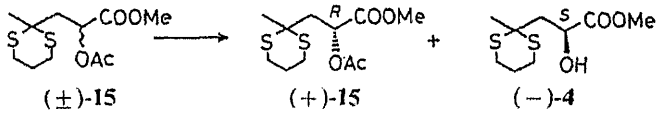
Chart 2

In most cases, (+)-**4**, which was finally found to have *R* configuration, was predominantly obtained except for run 2 (*Pichia membranaefaciens*) in Table I. This result is in harmony with that of microbial reduction of other α-ketoesters.[10] Among the tested yeasts in Table I, *Torulopsis* sp. Jyozo Kyokai 17 (run 9 in Table I) afforded optically pure (+)-**4** (>99% ee). A large-scale reaction (substrate **3**, 1.0 g × 13) using this strain of yeast afforded (+)-**4** (40% yield, 93% ee) and the hydroxyacid (**5**), which was purified after esterification with CH_2N_2 to afford (+)-**4** (33% yield from **3**, >99% ee). The optical purity of (+)-**4** (93% ee) was increased to >99% ee by a single recrystallization of the corresponding 3,5-dinitrobenzoate (**6**) from MeOH.

Optically pure (+)-**4** obtained in this manner was used as a starting material for the synthesis of PAF. The alcohol function in (+)-**4** was protected as the methoxymethyl (MOM) ether by treatment with MOM chloride–*N*,*N*-diisopropylethylamine, and subsequent reduction with $LiAlH_4$ yielded the alcohol (**8**) in 88% yield from (+)-**4**. The octadecyl ether function in **9** was introduced in 78% yield by the reaction with $C_{18}H_{37}OMs$ in the presence of KH.[11] Compound **9** has three different functional groups: dithioacetal, MOM ether and alkyl ether. The selective deprotection of the dithioacetal function in **9** was achieved by treatment with thallium(III) trinitrate to afford the ketone (**10**) in 90% yield. Subsequent Baeyer–Villiger oxidation of **10** with CF_3CO_3H–Na_2HPO_4 yielded the acetate (**11**) in 69% yield, although oxidation with *m*-chloroperbenzoic acid did not proceed at all. Concurrent hydrolysis of the MOM ether and acetate in **11** with HCl–MeOH afforded (+)-batyl alcohol ((+)-**12**) ($[\alpha]_D^{25}$ +2.41° (*c*=2.42, tetrahydrofuran (THF)); reported value $[\alpha]_D$ +2.28° (*c*=3.5, THF)).[12] Based on the sign of the specific rotation of (+)-**12**, the absolute stereochemistry of the starting material (+)-**4** was determined to be *R*.

(−)-Batyl alcohol, required for the synthesis of natural PAF, was synthesized as follows. After protection of the primary alcohol in (+)-**12** as the benzoate (**13**), inversion of the secondary alcohol was performed by the Mitsunobu method[13] to afford the dibenzoate (**14**) in 78% yield from (+)-**12**. Methanolysis of **14** gave (−)-**12**[14] in 90% yield. The optical

Table II. Enzyme-Catalyzed Hydrolysis of (±)-**15**



(±)-**15** (+)-**15** (−)-**4**

Run	Lipase	(+)-**15**		(−)-**4**	
		Chemical yield (%)	Optical purity (% ee)[a]	Chemical yield (%)	Optical purity (% ee)[a]
1	*Candida cylindracea* Meito "OF-360"	17	33	43	12
2	*Candida cylindracea* Meito "MY-30"	56	20	24	25
3	*Candida cylindracea* Sigma	50	18	30	22
4	*Pseudomonas fluorescens* Amano "P"	41	97	26	96
5	Porcine pancreas Amano	3	36	28	87
6	*Aspergillus niger* Amano "A"	18	14	60	0
7	*Aspergillus niger* Amano "A-6"	4	0	71	16[b]
8	*Rhizopus niveus* Amano "F"	53	58	17	92
9	*Rhizopus japonicus* Saiken "Lilipase A-10"	62	28	14	92
10	*Rhizopus japonicus* Nagase	56	19	11	61
11	*Rhizopus javanicus* Amano "F-AP-15"	42	46	18	90

a) The optical purities of (+)-**15** and (−)-**4** were determined from the 400 MHz ^{1}H-NMR spectra after conversion into the corresponding (+)-MTPA esters. *b*) Compound (+)-**4** was obtained as the hydrolyzed product.

purities of the obtained (+)- and (−)-**12** were confirmed to be >99% ee from the 400 MHz ^{1}H-NMR spectra after conversion into di-(+)-MTPA esters.[15] These findings suggest that racemization did not occur during the synthetic route from (+)-**4** to (+)-**12** and the inversion process from (+)-**12** to (−)-**12**.

In attempts to prepare (−)-**4** as a chiral synthon, inversion of (+)-**4** (>99% ee) using Ikegami's method [i) mesylation, ii) AcOCs–18-crown-6, iii) K_2CO_3][16] afforded partially racemized (−)-**4** (88% ee), and the Mitsunobu method afforded a complex mixture. To develop a synthetic route that would give optically pure (−)-**4**, the racemic acetate ((±)-**15**) derived from **3** was submitted to enzyme-catalyzed enantioselective hydrolysis. Enzymic hydrolyses of (±)-**15** were performed in 0.1 M phosphate buffer solution (pH 7.25) at 33 °C for 48 h, and the results are summarized in Table II.

Among the eleven enzymes tested, lipase "Amano-P" from *Pseudomonas fluorescens* (run 4 in Table II) afforded (+)-**15** (41% yield, 97% ee) as the recovered substrate and (−)-**4** (26% yield, 96% ee) as the hydrolyzed product. Lipase "Amano F" from *Rhizopus niveus* and lipase "Saiken-Lilipase A-10" from *Rhizopus japonicus* (runs 8 and 9 in Table II) also afforded (−)-**4** (17% yield, 92% ee and 14% yield, 92% ee, respectively). This (−)-**4**, formed in high enantiomeric excess, could be converted to (−)-**12** for the synthesis of natural PAF in a manner similar to that described for the sequence from (+)-**4** to (+)-**12**.

This is the first report of the asymmetric synthesis of enantiomerically pure batyl alcohol.

Experimental

Infrared (IR) spectra were measured with a JASCO A-202 spectrometer. ^{1}H-NMR spectra were measured on a JEOL LNP-PS-100 spectrometer unless otherwise stated. Mass spectra (MS) were taken on a JEOL JMS-D 300 spectrometer. Specific rotations were measured on a JASCO DIP-4 polarimeter. For column chromatography, silica gel (Merck, Kieselgel 60, 70—230 mesh) was used. All organic solvent extracts were washed with brine and dried on anhydrous sodium sulfate.

Methyl 2-Oxo-4,4-propylenedithiopentanoate (3)——1,3-Propanedithiol (30 ml, 0.299 mol) in CH_2Cl_2 (20 ml) was added dropwise to a stirred mixture of methyl 2,4-dioxopentanoate (**2**, 35 g, 0.243 mol) and BF_3 etherate (10 ml) in CH_2Cl_2 (60 ml) at 0 °C, and the whole was stirred at room temperature for 23 h, then poured into ice-water (100 ml), and extracted with ether. The ether extract was successively washed with 5% aq. $NaHCO_3$ and brine, then dried. Removal of the solvent *in vacuo* afforded an oily residue, which was subjected to column chromatography on silica gel (300 g). The fraction eluted with 2—5% AcOEt in hexane (v/v) afforded **3** (34.8 g, 61%) as a pale yellow solid, mp 38.9—39.5 °C. IR (Nujol): 1725, 1265, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, s, CH_3), 3.52 (2H, s, CH_2CO), 3.86 (3H, s, $COOCH_3$). MS *m/z*: 234 (M^+), 147, 133.

Screening of Yeasts——i) Preliminary Screening: The microorganisms in a previous paper[8] were examined for ability to reduce **3**. Test tubes (25×200 mm) containing 10 ml of the culture medium (5% glucose, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% urea, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract and tap water (pH 7.0)) were inoculated with microorganisms and cultured at 30 °C for 3 d with continuous shaking. Then the substrate (*ca.* 5 mg of compound **3**) was added to the test tube, which was further incubated for 3 d under the same conditions. The mixture was extracted with AcOEt. The AcOEt extract was dried and concentrated *in vacuo*. Monitoring of the residue by thin layer chromatography (TLC) indicated that thirteen strains of yeast (listed in Table I) were effective for the reduction of **3**.

ii) Secondary Screening: Reduction with these effective microorganisms using 30—40 mg of **3** in 100 ml of culture was carried out under essentially the same conditions as noted above. In order to examine the stereochemistry and optical purity, the reduction products were converted into (+)-MTPA esters. 400 MHz ^{1}H-NMR ($CDCl_3$) δ: (+)-MTPA ester of *R*-(+)-**4**: 3.756 (3H, s, OCH_3), 3.814 (3H, s, $COOCH_3$); (+)-MTPA ester of *S*-(−)-**4**: 3.532 (3H, s, OCH_3), 3.785 (3H, s, $COOCH_3$).

Asymmetric Reduction of 3 on a Preparative Scale——The above-mentioned seed culture of *Torulopsis* sp. Jyozo Kyokai 17 (1 ml) was transferred to 800 ml of the same culture medium. After cultivation at 30 °C for 3 d, the substrate **3** (1 g) was added to this seed culture, and the cultivation was continued for a further 3 d under the same conditions. Similar reduction of **3** on a preparative scale (1 g×13) was carried out. The reaction mixture was filtered with the aid of celite and the filtrate was extracted with AcOEt. The AcOEt extract was washed with brine and dried. Removal of the solvent *in vacuo* gave an oily residue, which was chromatographed on silica gel (150 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded (+)-**4** (5.25 g, 40%, 93% ee). The optical purity of (+)-**4** was enhanced by recrystallization from MeOH after conversion to the corresponding 3,5-dinitrobenzoate (**6**) in the usual manner, followed by methanolysis with K_2CO_3 in MeOH to afford optically pure (+)-**4** (4.2 g, 32% from **3**, >99%

ee) as a colorless oil. The fraction eluted with AcOEt afforded the crude hydroxyacid (**5**), which was purified after conversion to (+)-**4** (4.33 g, 33% from **3**, >99% ee) by treatment with CH_2N_2. (+)-**4**: $[\alpha]_D^{25}$ +8.02° (c=5.12, $CHCl_3$). IR (neat): 3460, 1740, 1375, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.67 (3H, s, CH_3), 3.81 (3H, s, $COOCH_3$), 4.51 (1H, m, CH). MS m/z: 236 (M^+), 147, 133. **6**: Yellow needles, mp 114.0—114.5 °C, recrystallized from MeOH. $[\alpha]_D^{18}$ +34.4° (c=1.16, $CHCl_3$). IR (Nujol): 1765, 1750, 1548, 1465, 1350 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.69 (3H, s, CH_3), 3.83 (3H, s, $COOCH_3$), 5.70 (1H, dd, J=4, 8 Hz, CH), 9.22 (3H, m, Ar-H). MS m/z: 430 (M^+), 230, 216, 212. **5**: ^{1}H-NMR ($CDCl_3$) δ: 1.62 (1H, s, CH_3), 2.31 (1H, dd, J=9, 15 Hz, C_3-H), 2.66 (1H, dd, J=2, 15 Hz, C_3-H), 4.56 (1H, dd, J=2, 9 Hz, C_2-H), 5.54 (2H, br, COOH, OH).

Methyl (*R*)-2-Methoxymethoxy-4,4-propylenedithiopentanoate (7)——MOM chloride (1.92 g) was added to a stirred solution of (+)-**4** (820 mg) in *N*,*N*-diisopropylethylamine (5.3 g) at 0 °C. The whole was stirred at room temperature for 3 h, poured into 2% aq. HCl, and then extracted with ether. The ether extract was successively washed with 5% aq. $NaHCO_3$ and brine, then dried. The solvent was removed *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (24 g). The fraction eluted with 7.5% AcOEt in hexane (v/v) gave **7** (924 mg, 95%) as a pale yellow oil. $[\alpha]_D^{24}$ −1.86° (c=6.48, $CHCl_3$). IR (neat): 1740, 1435, 1365, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, s, CH_3), 3.41 (3H, s, OCH_3), 3.76 (3H, s, $COOCH_3$), 4.34 (1H, dd, J=4, 8 Hz, CH), 4.68, 4.71 (1H each, d, J=10 Hz, OCH_2O). MS m/z: 280 (M^+), 235, 147.

(*R*)-2-Methoxymethoxy-4,4-propylenedithio-1-pentanol (8)——Compound **7** (915 mg) in ether (10 ml) was added dropwise with stirring to a suspension of $LiAlH_4$ (136 mg) in ether (4 ml) at 0 °C, and the mixture was stirred for 10 min. The usual work-up afforded a crude oil, which was subjected to column chromatography on silica gel (30 g). The fraction eluted with 20—25% AcOEt in hexane (v/v) afforded **8** (767 mg, 93%) as a colorless oil. $[\alpha]_D^{24}$ −69.5° (c=0.965, $CHCl_3$). IR (neat): 3440, 1440, 1375, 1035 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.64 (3H, s, CH_3), 3.34 (1H, br, OH), 3.44 (3H, s, OCH_3), 3.58 (2H, m, CH_2OH), 3.86 (1H, m, CH), 4.72, 4.76 (1H each, d, J=11 Hz, OCH_2O). MS m/z: 252 (M^+), 220, 147.

(*R*)-2-Methoxymethoxy-1-octadecyloxy-4,4-propylenedithiopentane (9)——A dispersion of KH in mineral oil (35% w/v, 1 ml, 10 mmol) was added to a stirred solution of **8** (750 mg) in benzene (10 ml) at 0 °C under an Ar atmosphere. After 10 min, octadecyl methanesulfonate (1.61 g) in benzene (20 ml) was added dropwise, then the whole was refluxed for 15 min. The reaction mixture was diluted with hexane (10 ml), quenched with EtOH (2 ml) and H_2O (5 ml), then extracted with ether. The ether extract was successively washed with 1% aq. HCl, 5% aq. $NaHCO_3$ and brine, then dried. The solvent was removed *in vacuo* to give an oily residue, which was chromatographed on silica gel (25 g). The fraction eluted with 2% AcOEt in hexane (v/v) gave **9** (1.16 g, 78%) as a pale yellow oil. $[\alpha]_D^{23}$ −10.4° (c=4.01, $CHCl_3$). IR (neat): 1460, 1345, 1165 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7 Hz, CH_3), 1.20—1.70 (32H, m, $CH_2 \times 16$), 1.64 (3H, s, CH_3), 3.38 (3H, s, OCH_3), 3.44—3.52 (4H, m, CH_2OCH_2), 3.94 (1H, m, CH), 4.72, 4.80 (1H each, d, J=10 Hz, OCH_2O). MS m/z: 504 (M^+), 442, 133.

(*R*)-4-Methoxymethoxy-5-octadecyloxy-2-pentanone (10)——Thallium (III) trinitrate trihydrate (2.42 g) in MeOH (2 ml) was added at 0 °C to a stirred mixture of **9** (1.15 g), MeOH (6 ml) and ether (2 ml), and the mixture was stirred for 15 min at room temperature. After removal of the resulting precipitate by filtration, the filtrate was concentrated *in vacuo*, diluted with brine, and then extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried and concentrated *in vacuo* to afford an oily residue, which was subjected to column chromatography on silica gel (30 g). The fraction eluted with 7% AcOEt in hexane (v/v) afforded **10** (852 mg, 90%) as a colorless oil. $[\alpha]_D^{24}$ +7.20° (c=4.30, $CHCl_3$). IR (neat): 1720, 1105, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, t, J=7 Hz, CH_3), 2.18 (3H, s, $COCH_3$), 2.71 (2H, d, J=7 Hz, $COCH_2$), 3.34 (3H, s, OCH_3), 3.41—3.48 (4H, m, CH_2OCH_2), 4.15 (1H, m, CH), 4.68 (2H, s, OCH_2O). MS m/z: 353 ($M^+ - OCH_2OCH_3$), 310, 131. *Anal.* Calcd for $C_{25}H_{50}O_4$: C, 72.41; H, 12.15. Found: C, 72.59; H, 12.10.

1-*O*-Acetyl-2-*O*-methoxymethyl-3-*O*-octadecyl-*sn*-glycerol (11)——CF_3CO_3H [freshly prepared from $(CF_3CO)_2O$ (42 ml), 60% H_2O_2 (5 ml) and Na_2HPO_4 (74 g) in CH_2Cl_2 (100 ml)] was added dropwise to a well-stirred suspension of **10** (830 mg) and Na_2HPO_4 (12 g) in CH_2Cl_2 (24 ml) at 0 °C. The whole was stirred for 7 h at room temperature, diluted with 5% aq. $NaHCO_3$ and then extracted with CH_2Cl_2. The CH_2Cl_2 extract was successively washed with 2% aq. KI, 5% aq. $Na_2S_2O_3$, and brine, then dried. Removal of the solvent gave an oily residue, which was purified by column chromatography on silica gel (30 g). The fraction eluted with 3% AcOEt in hexane (v/v) afforded **11** (595 mg, 69%) as a colorless oil. $[\alpha]_D^{26}$ +10.61° (c=3.08, $CHCl_3$). IR (neat): 1740, 1235, 1115 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7 Hz, CH_3), 1.20—1.70 (32H, m, $CH_2 \times 16$), 2.08 (3H, s, $OCOCH_3$), 3.39 (3H, s, OCH_3), 3.44—3.54 (4H, m, CH_2OCH_2), 3.96 (1H, m, CH), 4.20 (2H, m, CH_2OCO), 4.72 (2H, s, OCH_2). MS m/z: 431 ($M^+ + 1$), 399, 385, 369. *Anal.* Calcd for $C_{25}H_{50}O_5$: C, 69.72; H, 11.70. Found: C, 69.77; H, 11.83.

3-*O*-Octadecyl-*sn*-glycerol ((+)-Batyl Alcohol) ((+)-12)——Solution of **11** (313 mg) in MeOH (10 ml) was heated at 50 °C for 3 h in the presence of 35% aq. HCl (0.1 ml). After additon of 5% aq. $NaHCO_3$ (2 ml), the whole was diluted with brine, and extracted with ether. The ether extract was dried and concentrated *in vacuo* to give a colorless solid, which was purified by column chromatography on silica gel (9 g). The fraction eluted with 20% AcOEt in hexane (v/v) gave **12** (225 mg, 90%) as colorless needles, mp 70.8—71.2 °C, recrystallized from AcOEt–hexane. $[\alpha]_D^{25}$ +2.41° (c=2.42, THF). IR (Nujol): 3370, 1460, 1368, 1120 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, J=7 Hz, CH_3), 1.20—1.70 (32H, m, $CH_2 \times 16$), 2.34, 2.73 (1H each, br, $OH \times 2$), 3.39—3.92 (7H, m). MS m/z: 345 ($M^+ + 1$), 313, 253. High-MS

for $C_{21}H_{44}O_3$ (M^+): Calcd *m/z* 344.32893; Found 344.32841.

1-*O*-Benzoyl-3-*O*-octadecyl-*sn*-glycerol (13)——Benzoyl chloride (64 mg) in CH_2Cl_2 (1 ml) was added to a mixture of (+)-**12** (156 mg) and pyridine (0.1 ml) in CH_2Cl_2 (3 ml) at 0 °C, and the mixture was stirred for 30 min at room temperature. The whole was diluted with 5% aq. HCl and extracted with ether. The ether extract was successively washed with 5% $NaHCO_3$ and brine, then dried. Removal of the solvent *in vacuo* gave a crystalline residue, which was chromatographed on silica gel (2 g). The fraction eluted with 10% AcOEt in hexane (v/v) afforded **13** (148 mg, 73%), mp 48.5—49.5 °C, recrystallized from hexane, and the 2-*O*-benzoate of (+)-**12** (19 mg, 9%) as a colorless solid. Compound (+)-**12** (22 mg, 14%) was also recovered from the eluate with 20% AcOEt–hexane (v/v). **13**: $[\alpha]_D^{25}$ −0.54° (*c*=3.09, $CHCl_3$). IR (Nujol): 3470, 1690, 1600, 1582, 1295 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=7 Hz, CH_3), 2.60 (1H, br, OH), 3.42—3.58 (4H, m, CH_2OCH_2), 4.14 (1H, m, CH), 4.40 (2H, d, *J*=5 Hz, CH_2OCO), 7.36—7.58 (3H, m, Ar-H), 8.00—8.10 (2H, m, Ar-H). MS *m/z*: 448 (M^+), 375, 325. 2-*O*-Benzoate of (+)-**12**: IR (Nujol): 3470, 1687, 1600, 1581, 1285 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=7 Hz, CH_3), 3.38 (1H, br, OH), 3.50 (2H, t, *J*=6 Hz, $OC\underline{H}_2C_{17}H_{35}$), 3.77 (2H, d, *J*=5 Hz, CH_2O-alkyl), 3.94 (2H, d, *J*=5 Hz, $C\underline{H}_2OH$), 5.26 (1H, tt, *J*=5, 5 Hz, CH), 7.34—7.70 (3H, m, Ar-H), 8.02—8.15 (2H, m, Ar-H).

2,3-Di-*O*-benzoyl-1-*O*-octadecyl-*sn*-glycerol (14)——Diethyl azodicarboxylate (62 mg) in ether (2 ml) was added to a mixture of **13** (107 mg), triphenylphosphine (95 mg) and benzoic acid (44 mg) in ether (5 ml). The reaction mixture was stirred for 2 h at 0 °C, diluted with ether, and washed with brine, then dried. The solvent was removed *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (4 g). The fraction eluted with 2.5% AcOEt in hexane (v/v) afforded **14** (125 mg, 95%) as a colorless oil. $[\alpha]_D^{26}$ −9.57° (*c*=2.16, $CHCl_3$). IR (neat): 1720, 1600, 1260, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t, *J*=7 Hz, CH_3), 3.50 (2H, t, *J*=6 Hz, $OC\underline{H}_2C_{17}H_{35}$), 3.76 (2H, d, *J*=6 Hz, CH_2O-alkyl), 4.65 (2H, d, *J*=7 Hz, CH_2OCO), 5.60 (1H, m, CH). MS *m/z*: 552 (M^+), 479, 430.

1-*O*-Octadecyl-*sn*-glycerol ((−)-Batyl Alcohol) ((−)-12)——A mixture of **14** (63 mg) and NaOH (34 mg) in MeOH (3 ml) was stirred for 1 h at room temperature. Usual work-up afforded a crystalline residue, which was chromatographed on silica gel (1.5 g). The fraction eluted with 20% AcOEt in hexane (v/v) afforded (−)-**12** (35 mg, 90%), mp 70.8—71.4 °C, recrystallized from AcOEt–hexane. $[\alpha]_D^{29}$ −2.35° (*c*=2.10, THF). High-MS for $C_{21}H_{44}O_3$ (M^+): Calcd *m/z* 344.32893; Found 344.32813.

Di-*O*-(+)-MTPA Esters of (+)- and (−)-12——Compounds (+)- and (−)-**12**[15] were converted into the corresponding di-*O*-(+)-MTPA esters in the usual manner. For determination of the enantiomeric excess, the following signals in the 400 MHz ^{1}H-NMR spectra ($CDCl_3$) were examined. 1,2-Di-*O*-(+)-MTPA ester of (+)-**12**: δ: 3.424, 3.494 (3H each, $OCH_3\times2$), 4.367 (1H, dd, *J*=4.88, 12.21 Hz, $CH_a\underline{H}_bOMTPA$), 4.623 (1H, dd, *J*=3.18, 12.21 Hz, $CH_a\underline{H}_bOMTPA$), 2,3-Di-*O*-(+)-MTPA ester of (−)-**12**: δ: 3.399, 3.482 (3H each, $OCH_3\times2$), 4.429 (1H, dd, *J*=6.45, 12.20 Hz, $C\underline{H}_a H_bOMTPA$), 4.733 (1H, dd, *J*=2.93, 12.20 Hz, $CH_a\underline{H}_bOMTPA$).

Methyl (±)-2-Hydroxy-4-oxopentanoate Propylene 1,3-Dithioacetal ((±)-4)——$NaBH_4$ (626 mg) was added portionwise to a stirred mixture of **3** (3.52 g) and $CeCl_3$ (6.17 g) in MeOH (50 ml) at −78 °C, and the whole was stirred for 1 h at −78 °C. The reaction mixture was quenched with acetone (3 ml) and diluted with brine, then extracted with AcOEt. The AcOEt extract was dried and concentrated *in vacuo* to afford an oily residue, which was subjected to column chromatography on silica gel (80 g). Compound **3** (492 mg, 14%) was recovered from the fraction eluted with 10% AcOEt in hexane (v/v). The fraction eluted with 10—20% AcOEt in hexane (v/v) afforded (±)-**4** (2.49 g, 70%) as a colorless oil.

Methyl (±)-2-Acetoxy-4-oxopentanoate Propylene 1,3-Dithioacetal ((±)-15)——Ac_2O (1.8 ml) was added to a solution of (±)-**4** (2.26 g) in pyridine (10 ml) at 0 °C. The reaction mixture was stirred for 2 h at room temperature and poured into 3% aq. HCl, then extracted with ether. The ether extract was washed successively with 5% aq. $NaHCO_3$ and brine, then dried. The solvent was removed *in vacuo* to afford an oily residue, which was purified by column chromatography on silica gel (60 g). The fraction eluted with 7.5% AcOEt in hexane (v/v) afforded (±)-**15** (2.44 g, 92%) as a colorless solid, mp 86.0—86.2 °C. IR (Nujol): 1755, 1740, 1435, 1380, 1225 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.58 (3H, s, CH_3), 2.14 (3H, s, $OCOCH_3$), 2.38 (1H, dd, *J*=8, 15 Hz, C_3-H), 2.68 (1H, dd, *J*=3, 15 Hz, C_3-H), 3.76 (3H, s, $COOCH_3$), 5.30 (1H, dd, *J*=3, 8 Hz, CH). MS *m/z*: 278 (M^+), 216, 112.

General Procedure for Enzyme-Catalyzed Hydrolysis of (±)-15——An enzyme (100 mg) was added to a stirred suspension of substrate ((±)-**15**, 200 mg) in phosphate buffer (pH 7.25, 0.1 M, 40 ml). The reaction mixture was stirred for 48 h at 33 °C, and extracted with AcOEt (100 ml×2), and the combined extracts were dried. After removal of the solvent *in vacuo*, the crude product was purified by column chromatography on silica gel (8 g). The results are summarized in Table II. Specific rotations of (+)-**15** and (−)-**4** obtained by the use of lipase "Amano P" (run 4 in Table II) were as follows. (+)-**15**: $[\alpha]_D^{24}$ +4.44° (*c*=3.68, $CHCl_3$). (−)-**4**: $[\alpha]_D^{25}$ −8.01° (*c*=4.38, $CHCl_3$).

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Studies on Pyrimidine Derivatives. XXXIX.[1] Site-Selectivity in the Reaction of 5-Substituted and 4,5-Disubstituted Pyrimidine *N*-Oxides with Trimethylsilyl Cyanide

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The site-selectivity in the modified Reissert–Henze reaction of 5-substituted and 4,5-disubstituted pyrimidine 1-oxides with trimethylsilyl cyanide was examined. The reaction of 5-substituted 4-methoxypyrimidine 1-oxides with trimethylsilyl cyanide gave exclusively 2-pyrimidinecarbonitriles in good yields without exception. On the other hand, the other 5-substituted and 4,5-disubstituted pyrimidine 1-oxides gave mainly 6-pyrimidinecarbonitriles.

Keywords——site-selectivity; pyrimidine *N*-oxide; trimethylsilyl cyanide; Reissert–Henze reaction; pyrimidinecarbonitrile

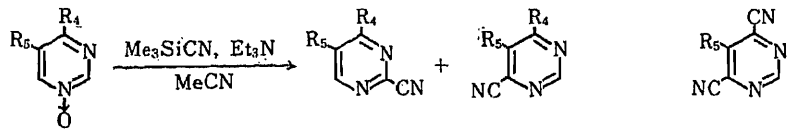
As reported previously, when pyrimidine 1-oxides substituted with an electron-donating group at position 4 were treated with trimethylsilyl cyanide (TMSCN),[2] significant site-selectivity due to substituent effects was observed, and 2-pyrimidinecarbonitriles were formed predominantly.[3] For example, the reaction of 4-methylpyrimidine 1-oxide with TMSCN gave 4-methyl-2-pyrimidinecarbonitrile as a sole product, while the reaction of pyrimidine 1-oxide itself under the same conditions gave a 2:3 mixture of 4-pyrimidinecarbonitrile and 2-pyrimidinecarbonitrile.

On the other hand, the reaction of 3-halopyridine 1-oxides with TMSCN was reported to give 3-halo-2-pyridinecarbonitriles selectively.[4] Similar results were observed in the reaction of 3-methoxy- and 3-dimethylaminopyridine 1-oxides with the same reagent. The orienting effect of these substituents on the cyanation has been explained by assuming a cyclic mechanism due to the interaction between the substituents and TMSCN.[4]

Our interest was next focussed on the comparison of these two different orienting effects in the same ring system. The present paper deals with the reaction of 4,5-disubstituted pyrimidine 1-oxides with TMSCN, because such pyrimidine 1-oxides are considered to be easily available substrates having favorable structures for the comparison of these effects in a single molecule.

In advance of the main investigation, the reaction of 5-substituted pyrimidine 1-oxides with TMSCN was carried out. When 5-phenyl- (**1a**), 5-methyl- (**1b**), 5-methoxy- (**1c**), 5-chloro- (**1d**), and 5-bromopyrimidine 1-oxide (**1e**) were treated with TMSCN under the reported conditions,[3,4] the corresponding 5-substituted 4-pyrimidinecarbonitriles (**5'a—e**) were formed predominantly. The formation ratio of the isomers determined by gas-chromatographic analysis are listed in Table I.

Based on the results described above, it is concluded that the reaction of 5-substituted pyrimidine 1-oxides tends to give 4-pyrimidinecarbonitriles, although the orienting effect due to the interaction between 5-substituents and TMSCN is not sharply observed as in the case of 3-substituted pyridine 1-oxides.



1a—e (R_4 = H) **5a—e** **5'a—e** **9a, d**
2a—e (R_4 = Ph) **6a—e** **6'a—e**
3a—e (R_4 = Me) **7a—e** **7'a—e**
4a—e (R_4 = OMe) **8a—e** —

a: R_5 = Ph **b**: R_5 = Me **c**: R_5 = OMe **d**: R_5 = Cl **e**: R_5 = Br

Chart 1

TABLE I. Reaction of Pyrimidine 1-Oxides with Trimethylsilyl Cyanide

No.	Pyrimidine 1-oxide		Reaction temp. (°C)	Reaction time (h)	Ratio of isomer		Isolated yield (%)	
	R_4	R_5			2-Isomer	6-Isomer	2-Isomer	6-Isomer
1a	H	Ph	82	3	11	89	9[a]	64[a]
1b	H	Me	Room temp.	3	0	100	0	75
1c	H	OMe	Room temp.	12	0	100	0	64
1d	H	Cl	0	0.5	13	87	2[b]	14[b]
1e	H	Br	Room temp.	0.5	2	98	<1	22
2a	Ph	Ph	Room temp.	3	19	81	15	67
2b	Ph	Me	82	1	5	95	<4	89
2c	Ph	OMe	82	3	24	76	<2	60
2d	Ph	Cl	Room temp.	2	3	97	<1	88
2e	Ph	Br	82	2	5	95	<2	73
3a	Me	Ph	82	10	32	68	25	46
3b	Me	Me	82	2	31	69	25	57
3c	Me	OMe	82	6	71	29	38[c]	<1
3d	Me	Cl	Room temp.	2	3	97	<2	77
3e	Me	Br	Room temp.	4	3	97	<2	72
4a	OMe	Ph	Room temp.	3	100	0	92	0
4b	OMe	Me	Room temp.	5	100	0	77	0
4c	OMe	OMe	Room temp.	8	100	0	95	0
4d	OMe	Cl	82	1	100	0	53	0
4e	OMe	Br	Room temp.	1	100	0	72	0

a) **9a**: 7%. *b*) **9d**: 14%. *c*) Yield of the mixture of **7c** and **7'c** was 63%.

In the cases of 5-chloropyrimidine 1-oxide (**1d**), formation of the monocarbonitrile (**5d** and **5'd**) was disturbed by the concomitant formation of 5-chloro-4,6-pyrimidinedicarbonitrile (**9d**), which was probably derived from further 1,6-addition of TMSCN to **5'd** followed by spontaneous oxidation of the dihydro intermediate. Similar 1,6-addition of TMSCN seems to occur in the reaction of 5-bromopyrimidine 1-oxide (**1e**), although 5-bromo-4,6-pyrimidinedicarbonitrile was not isolated.[5]

Next, the reaction of 4,5-disubstituted pyrimidine 1-oxides (**2**—**4**) with TMSCN was investigated. When 5-substituted 4-phenyl- (**2a**—**e**) and 4-methylpyrimidine 1-oxides (**3a**, **b**, **d**, **e**) were treated with TMSCN in acetonitrile, the corresponding 5,6-disubstituted 4-pyrimidinecarbonitriles (**6'a**—**e** and **7'a**, **b**, **d**, **e**) were obtained predominantly, while the reaction of 5-methoxy-4-methylpyrimidine 1-oxide (**3c**) gave 5-methoxy-4-methyl-2-pyrimidinecarbonitrile (**7c**) together with a small amount of the positional isomer, 5-methoxy-6-methyl-4-pyrimidinecarbonitrile (**7'c**).

As reported previously,[3] the reaction of pyrimidine 1-oxide itself gave a mixture of 2-

and 4-pyrimidinecarbonitriles (2 : 3, total yield 75%), the reaction of 4-phenylpyrimidine 1-oxide gave a mixture of 4-phenyl-2-pyrimidinecarbonitrile and 6-phenyl-4-pyrimidinecarbonitrile (55 and 21%),[6] and the reaction of 4-methylpyrimidine 1-oxide gave 4-methyl-2-pyrimidinecarbonitrile as a sole product (74%). Accordingly, the present results suggest that, in 4,5-disubstituted pyrimidine 1-oxides, the 5-substituents (such as phenyl and methyl groups) play a more important role than the 4-substituents in influencing the orientation of the cyanation with TMSCN.

In contrast to the above, the reaction of 5-substituted 4-methoxypyrimidine 1-oxides, irrespective of the kind of 5-substituents, gave the corresponding 2-pyrimidinecarbonitriles exclusively, as shown in Table I.

The following results relating to the exclusive formation of **8a—e** from **4a—e** were obtained. 4-Methoxy-6-methylpyrimidine 1-oxide reacted with TMSCN smoothly to give 4-

Table II. Pyrimidine *N*-Oxides

No.	R_4	R_5	Method	Yield (%)	mp (°C)	^{1}H-NMR ($CDCl_3$) δ (ppm)
1a	H	Ph	A	37	105—107	7.57 (5H, s), 8.47 (1H, d, J=2 Hz), 8.63 (1H, dd, J=2 Hz), 9.00 (1H, d, J=2 Hz)
1b	H	Me	A	44	114—115.5[a]	2.37 (3H, s), 8.10 (1H, brs), 8.30 (1H, brs), 8.90 (1H, brs)
1d	H	Cl	B	13	155—156	8.20 (1H, d, J=2 Hz), 8.43 (1H, dd, J=2 Hz), 8.90 (1H, d, J=2 Hz)
1e	H	Br	A	15	161.5—163[b]	8.30 (1H, d, J=2 Hz), 8.57 (1H, dd, J=2 Hz), 8.90 (1H, d, J=2 Hz)
2a	Ph	Ph	A	26	128	7.0—7.6 (10H, m), 8.07 (1H, d, J=2 Hz), 9.02 (1H, d, J=2 Hz)
2b	Ph	Me	A	32	149.5—150[c]	2.40 (3H, s), 7.3—7.8 (5H, m), 8.30 (1H, brs), 8.93 (1H, brs)
2c	Ph	OMe	A	75	178—178.5[d]	3.98 (3H, s), 7.3—7.8 (3H, m), 7.8—8.2 (2H, m), 8.20 (1H, d, J=2 Hz)
2d	Ph	Cl	B	41	199—200 (dec.)	7.4—7.7 (3H, m), 7.7—8.1 (2H, m), 8.50 (1H, d, J=2 Hz), 8.95 (1H, d, J=2 Hz)
3a	Me	Ph	A	9	Viscous oil	2.46 (3H, s), 7.2—7.7 (5H, m), 8.25 (1H, d, J=2 Hz), 8.90 (1H, d, J=2 Hz)
3'a	Me	Ph	A	18	86.5—88	2.50 (3H, s), 7.2—7.7 (5H, m), 8.10 (1H, s), 9.05 (1H, s)
3b	Me	Me	A	23	139—140	2.27 (3H, s), 2.47 (3H, s), 8.20 (1H, brs), 8.77 (1H, brs)
3'b	Me	Me	A	36	Hygroscopic semi-solid	2.33 (3H, s), 2.50 (3H, s), 7.98 (1H, s), 8.85 (1H, s)
3c	Me	OMe	A	30	206—209 (dec.)	2.40 (3H, s), 3.90 (3H, s), 8.05 (1H, d, J=2 Hz), 8.60 (1H, d, J=2 Hz)
3'c	Me	OMe	A	22	139—139.5	2.43 (3H, s), 3.97 (3H, s), 7.80 (1H, s), 8.63 (1H, s)
3d	Me	Cl	B	22	141.5—142.5	2.57 (3H, s), 8.40 (1H, d, J=2 Hz), 8.78 (1H, d, J=2 Hz)
3'd	Me	Cl	B	34	70.5—72	2.65 (3H, s), 8.20 (1H, s), 8.90 (1H, s)
3e	Me	Br	B	20	133—133.5 (dec.)	2.60 (3H, s), 8.55 (1H, d, J=2 Hz), 8.83 (1H, d, J=2 Hz)
3'e	Me	Br	B	30	106—107.5	2.67 (3H, s), 8.22 (1H, s), 8.87 (1H, s)
4a	OMe	Ph	A	69	166—167	4.05 (3H, s), 7.55 (5H, s), 8.37 (1H, d, J=2 Hz), 8.73 (1H, d, J=2 Hz)
4b	OMe	Me	A	68	156—157	2.37 (3H, s), 4.00 (3H, s), 8.10 (1H, brs), 8.67 (1H, brs)
4c	OMe	OMe	A	30	203—205 (dec.)	4.00 (3H, s), 4.12 (3H, s), 8.20 (1H, d, J=2 Hz), 8.50 (1H, d, J=2 Hz)
4d	OMe	Cl	B	37	165—165.5	4.07 (3H, s), 8.33 (1H, d, J=2 Hz), 8.57 (1H, d, J=2 Hz)
4e	OMe	Br	B	36	158—158.5 (dec.)	4.05 (3H, s), 8.47 (1H, d, J=2 Hz), 8.63 (1H, d, J=2 Hz)

a) Lit.[17] mp 113—116 °C. *b*) Lit.[18] mp 166—167 °C. *c*) Lit.[19] mp 151—153 °C. *d*) Lit.[16] mp 173—176 °C.

methoxy-6-methyl-2-pyrimidinecarbonitrile in good yield, whereas 4-methoxy-2-methylpyrimidine 1-oxide was recovered unchanged after the reaction with TMSCN.[3] Accordingly, it is clear that the presence of the 4-methoxyl group decreases the reactivity of position 6 to nucleophilic reagents.

In many substitution reactions of pyrimidine derivatives, an orienting effect of a 4(or 6)-methoxyl group to the 2-position has been reported.[3,7] Further, the 4-methoxyl group was confirmed to enhance the relative reactivity of the 2-methyl group in 4-methoxy-2,6-dimethylpyrimidine systems.[8-10] The results obtained in the present investigation represent an additional example of such findings.

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, dd=double doublet, m=multiplet, and br=broad.

5-Bromo-4-phenylpyrimidine——A mixture of 5-bromo-4-chloro-6-phenylpyrimidine[11] (8.93 g, 33 mmol), tosylhydrazine (12.34 g, 66 mmol), and $CHCl_3$ (100 ml) was refluxed for 12 h. The resulting precipitate was filtered off and added portionwise to 10% K_2CO_3 (100 ml) at 90 °C. The mixture was refluxed for 30 min and extracted with $CHCl_3$. The crude product obtained from the $CHCl_3$ extract was recrystallized from ether to give colorless scales, mp 91.5—93.5 °C. Lit.[11] mp 89—90 °C. Yield 5.37 g (70%).

5-Methoxy-4-phenylpyrimidine——A mixture of 5-bromo-4-phenylpyrimidine (2.34 g, 10 mmol) and methanolic sodium methoxide [prepared from Na (0.25 g, 11 mmol) and dry MeOH (50 ml)] was heated in a sealed tube at 120 °C for 20 h. After removal of the MeOH, the residue was diluted with H_2O and extracted with $CHCl_3$. The crude product obtained from the $CHCl_3$ extract was distilled under reduced pressure to give a colorless liquid, bp 150 °C/3 mmHg. Picrate (acetone–hexane): pale yellow needles, mp 147—148.5 °C. Yield 1.25 g (62%). ^{1}H-NMR (CCl_4): 3.93 (3H, s), 7.2—7.5 (3H, m), 7.9—8.2 (2H, m), 8.30 (1H, s), 8.73 (1H, s). *Anal.* Calcd for $C_{17}H_{13}N_5O_8$ (picrate): C, 49.16; H, 3.15;

Table III. Analytical Data for Pyrimidine *N*-Oxides

No.	R_4	R_5	Formula	Analysis (%) Calcd			Found		
				C	H	N	C	H	N
1a	H	Ph	$C_{10}H_8N_2O$	69.75	4.68	16.27	69.73	4.53	16.41
1b	H	Me	$C_5H_6N_2O$	54.54	5.49	25.44	54.62	5.34	25.42
1d	H	Cl	$C_4H_3ClN_2O$	36.81	2.32	21.46	37.02	2.20	21.54
2a	Ph	Ph	$C_{16}H_{12}N_2O$	77.40	4.87	11.28	77.18	4.66	11.01
2d	Ph	Cl	$C_{10}H_7ClN_2O$	58.12	3.44	13.55	58.48	3.44	13.55
3a	Me	Ph	$C_{17}H_{13}N_5O_8$[a]	49.16	3.15	16.86	49.39	2.92	16.86
3'a	Me	Ph	$C_{11}H_{10}N_2O$	70.95	5.41	15.04	70.67	5.43	14.94
3b	Me	Me	$C_6H_8N_2O$	58.05	6.49	22.56	58.09	6.47	22.73
3'b	Me	Me	$C_{12}H_{11}N_5O_8$[b]	40.80	3.14	19.83	40.71	2.98	19.63
3c	Me	OMe	$C_6H_8N_2O_2$	51.42	5.75	19.99	51.29	5.54	19.69
3'c	Me	OMe	$C_6H_8N_2O_2$	51.42	5.75	19.99	51.27	5.84	19.77
3d	Me	Cl	$C_5H_5ClN_2O$	41.54	3.49	19.38	41.41	3.29	19.38
3'd	Me	Cl	$C_5H_5ClN_2O$	41.54	3.49	19.38	41.41	3.26	19.36
3e	Me	Br	$C_5H_5BrN_2O$	31.77	2.66	14.82	31.91	2.60	14.98
3'e	Me	Br	$C_5H_5BrN_2O$	31.77	2.66	14.82	31.75	2.49	14.81
4a	OMe	Ph	$C_{11}H_{10}N_2O_2$	65.33	4.98	13.86	65.19	4.92	13.74
4b	OMe	Me	$C_6H_8N_2O_2$	51.42	5.75	19.99	51.15	5.60	19.97
4c	OMe	OMe	$C_6H_8N_2O_3$	46.15	5.16	17.94	45.98	4.98	18.18
4d	OMe	Cl	$C_5H_5ClN_2O_2$	37.40	3.13	17.44	36.97	3.11	17.88
4e	OMe	Br	$C_5H_5BrN_2O_2$	29.29	2.45	13.66	29.37	2.27	13.63

a) Picrate: mp 132—133 °C (dec.) (acetone–hexane). *b*) Picrate: mp 124—126 °C (AcOEt–ether).

N, 16.86. Found: C, 49.22; H, 2.98; N, 17.14.

4,5-Dichloro-6-phenylpyrimidine——A 30% hydrogen peroxide solution (6 ml) was added to a solution of 4-phenyl-6(1*H*)-pyrimidinone[11] (8.60 g, 50 mmol) in conc. HCl (50 ml) at 30—40 °C, and the mixture was stirred at 30—40 °C overnight. After evaporation of the solvent, the residue was refluxed with $POCl_3$ (50 ml) for 3 h, and the excess $POCl_3$ was removed under reduced pressure. The residue was poured into ice-water and extracted with $CHCl_3$. The residue obtained from the $CHCl_3$ extract was purified through a short Al_2O_3 column using C_6H_6 as an eluent. The product obtained from the C_6H_6 eluate was recrystallized from hexane to give colorless scales, mp 71.5—73 °C. Lit.[12] mp 72—74 °C. Yield 7.38 g (66%).

5-Chloro-4-phenylpyrimidine——When treated according to the procedure used for the preparation of 5-bromo-4-phenylpyrimidine, 4,5-dichloro-6-phenylpyrimidine (900 mg, 4 mmol) gave colorless prisms, mp 87.5—88.5 °C, which were recrystallized from hexane. Yield 440 mg (58%). ^{1}H-NMR ($CDCl_3$): 7.3—7.6 (3H, m), 7.6—8.0 (2H, m), 8.72 (1H, s), 9.25 (1H, s). *Anal.* Calcd for $C_{10}H_7ClN_2$: C, 63.01; H, 3.70; N, 14.70. Found: C, 63.06; H, 3.56; N, 14.70.

5-Bromo-4-methylpyrimidine——When treated according to the procedure used for the preparation of 5-bromo-4-phenylpyrimidine, 5-bromo-4-chloro-6-methylpyrimidine (10.4 g, 50 mmol) gave a colorless liquid, bp 90 °C/

Table IV. Pyrimidinecarbonitriles

No.	R_4	R_5	mp (°C) [bp/mmHg]	IR ($CHCl_3$) cm^{-1}	^{1}H-NMR ($CDCl_3$) δ (ppm)
5a	H	Ph	126—127	2240	7.60 (5H, s), 9.05 (2H, s)
5'a	H	Ph	89—91	2220	7.60 (5H, s), 9.03 (1H, s), 9.33 (1H, s)
9a	H	Ph	143—143.5	2240	7.62 (5H, s), 9.40 (1H, s)
5'b	H	Me	[120/20]	2240	2.57 (3H, s), 8.80 (1H, s), 9.10 (1H, s)
5'c	H	OMe	83—84	2240	4.10 (3H, s), 8.67 (1H, s), 8.95 (1H, s)
5d	H	Cl	83.5—84.5[a]	—	8.87 (2H, s)
5'd	H	Cl	[120/24]	2240	8.93 (1H, s), 9.18 (1H, s)
9d	H	Cl	99.5—100	2240	9.35 (1H, s)
5e	H	Br	116—117[b]	—	8.95 (2H, s)
5'e	H	Br	35—36 [135/19]	2240	9.08 (1H, s), 9.27 (1H, s)
6a	Ph	Ph	105.5—106.5	2250	7.3—7.6 (10H, m), 8.78 (1H, s)
6'a	Ph	Ph	150.5—151.5	2240	7.3—7.6 (10H, m), 9.35 (1H, s)
6b	Ph	Me	89—90	2230	2.52 (3H, s), 7.57 (5H, br s), 8.68 (1H, s)
6'b	Ph	Me	101—102	2240	2.60 (3H, s), 7.55 (6H, s), 9.17 (1H, s)
6c	Ph	OMe	123—123.5	2240	4.10 (3H, s), 7.4—7.6 (3H, m), 8.0—8.3 (2H, m), 8.50 (1H, s)
6'c	Ph	OMe	54.5—55	2240	3.98 (3H, s), 7.4—7.7 (3H, m), 8.0—8.3 (2H, m), 9.05 (1H, s)
6d	Ph	Cl	92—93	—	7.4—7.7 (3H, m), 7.8—8.1 (2H, m), 8.83 (1H, s)
6'd	Ph	Cl	83—84	2240	7.5—7.7 (3H, m), 7.7—8.0 (2H, m), 9.23 (1H, s)
6e	Ph	Br	102.5—103	—	7.4—7.6 (3H, m), 7.7—8.0 (2H, m), 8.97 (1H, s)
6'e	Ph	Br	97.5—98	—	7.4—7.7 (3H, m), 7.7—8.0 (2H, m), 9.23 (1H, s)
7a	Me	Ph	105—106	2240	2.60 (3H, s), 7.3—7.7 (5H, m), 8.65 (1H, s)
7'a	Me	Ph	82—83	2240	2.50 (3H, s), 7.3—7.7 (5H, m), 9.20 (1H, s)
7b	Me	Me	70—70.5	2240	2.37 (3H, s), 2.57 (3H, s), 8.50 (1H, s)
7'b	Me	Me	56—57	2230	2.53 (3H, s), 2.60 (3H, s), 9.00 (1H, s)
7c	Me	OMe	67—67.5	2240	2.50 (3H, s), 4.02 (3H, s), 8.27 (1H, s)
7'c	Me	OMe	52.5—53.5	2230	2.57 (3H, s), 4.20 (3H, s), 8.83 (1H, s)
7d	Me	Cl	[110/24]	—	2.68 (3H, s), 8.68 (1H, s)
7'd	Me	Cl	57.5—58.5	2240	2.75 (3H, s), 9.07 (1H, s)
7e	Me	Br	67—67.5	—	2.72 (3H, s), 8.83 (1H, s)
7'e	Me	Br	84.5—85.5	—	2.75 (3H, s), 9.07 (1H, s)
8a	OMe	Ph	129—129.5	2250	4.08 (3H, s), 7.52 (5H, br s), 8.53 (1H, s)
8b	OMe	Me	50—50.5	2250	2.23 (3H, s), 4.07 (3H, s), 8.33 (1H, s)
8c	OMe	OMe	86.5—87.5	2240	4.03 (3H, s), 4.12 (3H, s), 8.10 (1H, s)
8d	OMe	Cl	45—46	—	4.15 (3H, s), 8.55 (1H, s)
8e	OMe	Br	147—149 (dec.)	—	4.17 (3H, s), 8.63 (1H, s)

a) Lit.[21] mp 85—86 °C. *b*) Lit.[21] mp 115—118 °C.

23 mmHg. Yield 4.4 g (51%). ^{1}H-NMR (CCl_4): 2.60 (3H, s), 8.62 (1H, s), 8.83 (1H, s). *Anal.* Calcd for $C_5H_5BrN_2$: C, 34.71; H, 2.91; N, 16.19. Found: C, 34.70; H, 2.78; N, 16.38.

5-Methoxy-4-methylpyrimidine——When treated according to the procedure described for the preparation of 5-methoxy-4-phenylpyrimidine, 5-bromo-4-methylpyrimidine (10.38 g, 60 mmol) gave a colorless liquid, bp 100 °C/20 mmHg. Lit.[13] bp 90—92 °C/15 mmHg. Yield 3.19 g (43%).

4,5-Dichloro-6-methylpyrimidine——When treated according to the procedure used for the preparation of 4,5-dichloro-6-phenylpyrimidine, 6-methyl-4(3*H*)-pyrimidinone (44.0 g, 0.4 mol) gave a colorless liquid, bp 100 °C/18 mmHg. Yield 26.7 g (41%). ^{1}H-NMR ($CDCl_3$): 2.67 (3H, s), 8.68 (1H, s). *Anal.* Calcd for $C_5H_4Cl_2N_2$: C, 36.84; H, 2.67; N, 17.19. Found: C, 36.51; H, 2.26; N, 17.32.

5-Chloro-4-methylpyrimidine——When treated according to the procedure used for the preparation of 5-bromo-4-phenylpyrimidine, 4,5-dichloro-6-methylpyrimidine (16.3 g, 0.1 mol) gave a colorless liquid, bp 90 °C/19 mmHg. Lit.[14] mp 49—50 °C. Picrate (ether–hexane): yellow prisms, mp 110—111 °C. Yield 5.93 g (47%). ^{1}H-NMR (CCl_4): 2.57 (3H, s), 8.48 (1H, s), 8.82 (1H, s). *Anal.* Calcd for $C_{11}H_8ClN_5O_7$ (picrate): C, 36.94; H, 2.25; N, 19.58. Found: C, 37.04; H, 2.09; N, 19.65.

4,5-Dimethoxypyrimidine——A mixture of 5-methoxy-4(3*H*)-pyrimidinone[15] (25.2 g, 0.2 mol) and $POCl_3$ (150 ml) was refluxed for 30 min. After removal of the excess $POCl_3$, the residue was made alkaline with NH_4OH and extracted with $CHCl_3$. An MeONa–MeOH solution [prepared from Na (9.2 g, 0.4 mol) and dry MeOH (200 ml)] was

Table V. Analytical Data for Pyrimidinecarbonitriles

No.	R_4	R_5	Formula	Analysis (%) Calcd			Found		
				C	H	N	C	H	N
5a	H	Ph	$C_{11}H_7N_3$	72.91	3.89	23.19	73.04	3.83	23.27
5'a	H	Ph	$C_{11}H_7N_3$	72.91	3.89	23.19	72.76	3.75	23.40
9a	H	Ph	$C_{12}H_6N_4$	69.89	2.93	27.17	70.08	2.77	27.13
5'b	H	Me	$C_6H_5N_3$	60.50	4.20	35.29	60.49	4.20	35.53
5'c	H	OMe	$C_6H_5N_3O$	53.33	3.72	31.09	53.40	3.48	31.34
5'd	H	Cl	$C_5H_2ClN_3$	43.03	1.44	30.11	43.38	1.47	29.81
9d	H	Cl	C_6HClN_4	43.79	0.61	34.04	43.93	0.58	34.35
5e	H	Br	$C_5H_2BrN_3$	32.62	1.09	22.83	32.79	1.09	22.91
5'e	H	Br	$C_5H_2BrN_3$	32.62	1.09	22.83	32.82	1.09	22.75
6a	Ph	Ph	$C_{17}H_{11}N_3$	79.36	4.31	16.33	79.61	4.03	16.53
6'a	Ph	Ph	$C_{17}H_{11}N_3$	79.36	4.31	16.33	79.55	4.28	16.30
6b	Ph	Me	$C_{12}H_9N_3$	73.82	4.64	21.52	74.08	4.51	21.33
6'b	Ph	Me	$C_{12}H_9N_3$	73.82	4.64	21.52	74.07	4.51	21.65
6c	Ph	OMe	$C_{12}H_9N_3O$	68.23	4.29	19.89	68.43	4.37	19.97
6'c	Ph	OMe	$C_{12}H_9N_3O$	68.23	4.29	19.89	68.47	4.39	20.03
6d	Ph	Cl	$C_{11}H_6ClN_3$	61.26	2.80	19.48	61.56	2.57	19.66
6'd	Ph	Cl	$C_{11}H_6ClN_3$	61.26	2.80	19.48	61.63	2.68	19.62
6e	Ph	Br	$C_{11}H_6BrN_3$	50.79	2.32	16.15	51.16	2.34	16.39
6'e	Ph	Br	$C_{11}H_6BrN_3$	50.79	2.32	16.15	50.79	2.15	16.05
7a	Me	Ph	$C_{12}H_9N_3$	73.82	4.64	21.52	74.07	4.56	21.58
7'a	Me	Ph	$C_{12}H_9N_3$	73.82	4.64	21.52	73.97	4.59	21.60
7b	Me	Me	$C_7H_7N_3$	63.14	5.30	31.56	63.19	5.20	31.50
7'b	Me	Me	$C_7H_7N_3$	63.14	5.30	31.56	63.23	5.25	31.73
7c	Me	OMe	$C_7H_7N_3O$	56.37	4.73	28.18	56.38	4.59	28.39
7'c	Me	OMe	$C_7H_7N_3O$	56.37	4.73	28.18	56.19	4.56	28.08
7d	Me	Cl	$C_6H_4ClN_3$	46.92	2.62	27.36	47.03	2.56	27.64
7'd	Me	Cl	$C_6H_4ClN_3$	46.92	2.62	27.36	46.91	2.35	27.57
7e	Me	Br	$C_6H_4BrN_3$	36.36	2.02	21.21	36.40	2.28	21.29
7'e	Me	Br	$C_6H_4BrN_3$	36.36	2.02	21.21	36.34	1.80	21.50
8a	OMe	Ph	$C_{12}H_9N_3O$	68.23	4.29	19.89	68.51	4.04	19.87
8b	OMe	Me	$C_7H_7N_3O$	56.37	4.73	28.18	56.55	4.67	28.29
8c	OMe	OMe	$C_7H_7N_3O_2$	50.90	4.27	25.44	51.02	4.14	25.66
8d	OMe	Cl	$C_6H_4ClN_3O$	42.49	2.37	24.78	42.71	2.28	25.02
8e	OMe	Br	$C_6H_4BrN_3O$	33.64	1.86	19.62	33.68	1.72	19.78

added to the residue obtained from the $CHCl_3$ extract, and the mixture was refluxed for 1 h. After evaporation of the MeOH, H_2O was added to the residue and the mixture was extracted with $CHCl_3$. The residue obtained from the $CHCl_3$ extract was passed through a short Al_2O_3 column using C_6H_6 as an eluent. The C_6H_6 eluate gave colorless scales, mp 76—77 °C (ether). Yield 12.34 g (44%). ^{1}H-NMR (CCl_4): 3.87 (3H, s), 3.98 (3H, s), 7.92 (1H, s), 8.25 (1H, s). *Anal.* Calcd for $C_6H_8N_2O_2$: C, 51.42; H, 5.75; N, 19.99. Found: C, 51.05; H, 5.81; N, 19.86.

5-Chloro-4-methoxypyrimidine——Chlorine gas was introduced into a solution of 4(3*H*)-pyrimidinone (9.6 g, 0.1 mol) in AcOH (50 ml) at 50 °C for 30 min. After evaporation of the AcOH, the residue was heated with $POCl_3$ (150 ml) for 1 h. The excess $POCl_3$ was evaporated off, and the residue was made alkaline with NH_4OH and extracted with $CHCl_3$. The crude product obtained from the $CHCl_3$ extract was dissolved in dry MeOH (50 ml). The MeOH solution was added to an MeONa–MeOH solution [prepared from Na (4.6 g, 0.2 mol) and dry MeOH (150 ml)], and the mixture was refluxed for 30 min. After evaporation of the MeOH, the residue was partitioned with $CHCl_3$–H_2O. The residue obtained from the $CHCl_3$ layer was passed through a short Al_2O_3 column using $CHCl_3$ as an eluent. The $CHCl_3$ eluate gave colorless needles, mp 39—40 °C (hexane), bp 77—78 °C/17 mmHg. Yield 9.0 g (62%). ^{1}H-NMR (CCl_4): 4.03 (3H, s), 8.35 (1H, s), 8.50 (1H, s). *Anal.* Calcd for $C_5H_5ClN_2O$: C, 41.54; H, 3.48; N, 19.37. Found: C, 41.50; H, 3.37; N, 19.31.

Pyrimidine *N*-Oxides

Except for 5-bromo-4-phenylpyrimidine 1-oxide,[16] starting pyrimidine *N*-oxides were prepared by the following methods.

General Procedure A——A solution of a pyrimidine (1.0 eq) and *m*-chloroperbenzoic acid (1.5 eq) in $CHCl_3$ was allowed to stand at room temperature for 24 h. The mixture was washed with 30% K_2CO_3, and the $CHCl_3$ was evaporated off. The residue was purified by Al_2O_3 or SiO_2 column chromatography, and the product was recrystallized.

General Method B——A mixture of a pyrimidine (10—20 mmol), MoO_3 (1.1 eq), 30% H_2O_2 (4.0 eq), and AcOH (2 ml) was heated at 40 °C for 16 h. The mixture was made alkaline with 3 N NaOH and extracted with $CHCl_3$. The crude product was purified as above.

The structure of pyrimidine 1-oxide or 3-oxide was determined by the reported method.[20]

Reaction of Pyrimidine *N*-Oxides with Trimethylsilyl Cyanide (General Procedure)——A mixture of a pyrimidine 1-oxide (1.0 eq), trimethylsilyl cyanide (3.0 eq), Et_3N (2.0 eq), and MeCN was allowed to react under the conditions shown in Table I. After removal of the solvent, the residue was diluted with H_2O and extracted with CH_2Cl_2 or $CHCl_3$. The residue obtained from the extract was purified by SiO_2 column chromatography, recrystallization or distillation under reduced pressure.

References and Notes

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6) In the table in the previous paper,[3] the yields of 4-phenyl-2-pyrimidine- and 6-phenyl-4-pyrimidinecarbonitrile were shown to be 21% and 55% as a result of a printing error. The yields should be revised to 55% and 21%, respectively.
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Tannins and Related Compounds. LVIII.[1] Novel Gallotannins Possessing an α-Glucose Core from *Nuphar japonicum* DC.

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Together with 6-*O*- and 2,3,4,6-tetra-*O*-galloylglucoses, two unusual gallotannins possessing an α-glucopyranose core have been isolated from the rhizomes of *Nuphar japonicum* DC. (Nymphaeaceae), and their structures have been established as 1,2,4-tri-*O*-galloyl-α-D-glucose (**3**) and 1,2,3,4,6-penta-*O*-galloyl-α-D-glucose (**6**) on the basis of chemical and spectroscopic evidence.

Keywords——*Nuphar japonicum*; Nymphaeaceae; hydrolyzable tannin; gallotannin; α-glucose; gallic acid; HPLC

The rhizomes of *Nuphar japonicum* DC. (Nymphaeaceae) have been used, mixed with other crude drugs in most cases, as a tonic and a diuretic, and also to treat bleeding, a blood-stasis syndrome and menstrual disorder. Because of these pharmacological activities, this crude drug is regarded in Japan as important especially for women pre- and post-partum. With regard to the constituents of this drug, many investigators have focused their attention on analysis of alkaloids, and have isolated several novel sesquiterpene alkaloids, such as nupharidine, nupharamine, *etc.*[3] Our earlier brief report described the presence of hydrolyzable tannins in this drug and showed them to contain an unusual α-glucose core.[4] In this paper, we wish to present details of the isolation and structural elucidation of the component gallotannins.

A preliminary examination of the ethyl acetate-soluble portion of the aqueous acetone extract by normal-phase high-performance liquid chromatography (HPLC)[5] showed the presence of three major gallotannins corresponding to tri-, tetra- and pentagalloylglucoses (Fig. 1). The large-scale extraction of the freeze-dried material with aqueous acetone gave an extract which was chromatographed on Sephadex LH-20 with water containing increasing amounts of ethanol to yield individual fractions containing mono-, tri-, tetra- and pentagallates. The mono- and trigallate fractions were each almost homogeneous on reverse-phase HPLC,[5] and were further purified by Sephadex LH-20 and MCI-gel CHP 20P chromatog-

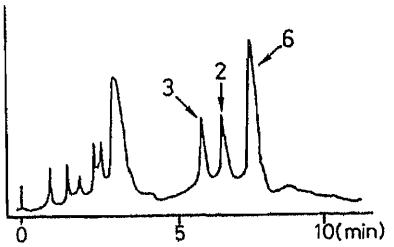
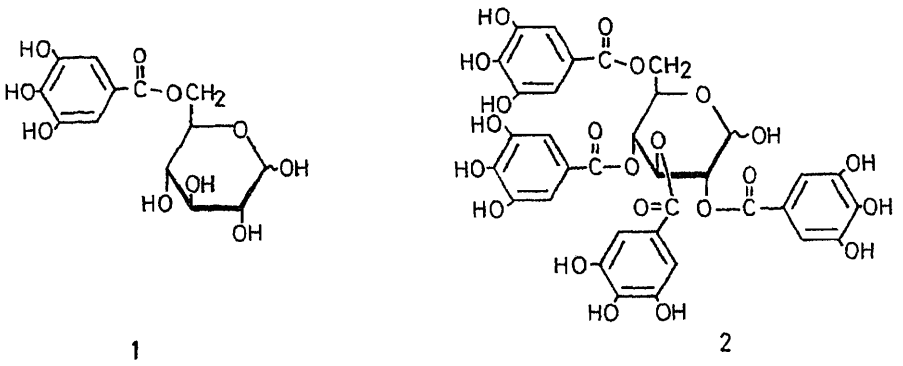


Fig. 1. HPLC of Tannins in Nupharis Rhizoma

Column: Nucleosil 50-10 (4 × 300 mm). Solvent: *n*-Hexane–MeOH–THF–formic acid (55 : 33 : 11 : 1) (oxalic acid 1.0 g/l). Flow rate: 1.8 ml/min. Detection: 280 nm.



1 2

Chart I

TABLE I. ^{1}H-NMR Data for Compounds **3**, **5**, **6** and **7** (δ Values)[a,b]

	3	5[c]	6	7
Glucose moiety				
H-1	6.59 (d, J=4)	6.04 (d, J=8)	6.75 (d, J=3)	6.35 (d, J=8)
H-2	5.19 (dd, J=4, 9)	5.21 (t, J=9)	5.50 (dd, J=3, 9)	5.62 (t, J=9)
H-3	4.57 (t, J=9)	4.32 (t, J=9)	6.21 (t, J=9)	6.03 (t, J=9)
H-4	5.29 (t, J=9)	5.35 (t, J=9)	5.80 (t, J=9)	5.66 (t, J=9)
H-5	4.18 (dt-like, J=4, 9)	3.92 (m)	4.52 (m)	4.60 (m)
H-6	4.66 (2H, d, J=4)	3.68 (2H, m)	4.40 (dd, J=3, 12) 4.70 (d-like, J=12)	4.50 (2H, m)
Galloyl moiety	7.10, 7.19, 7.20 (each 2H, s)	7.09 (4H, s) 7.16 (2H, s)	7.00, 7.01, 7.08, 7.20, 7.28 (each 2H, s)	6.97, 7.02, 7.06, 7.12, 7.18 (each 2H, s)

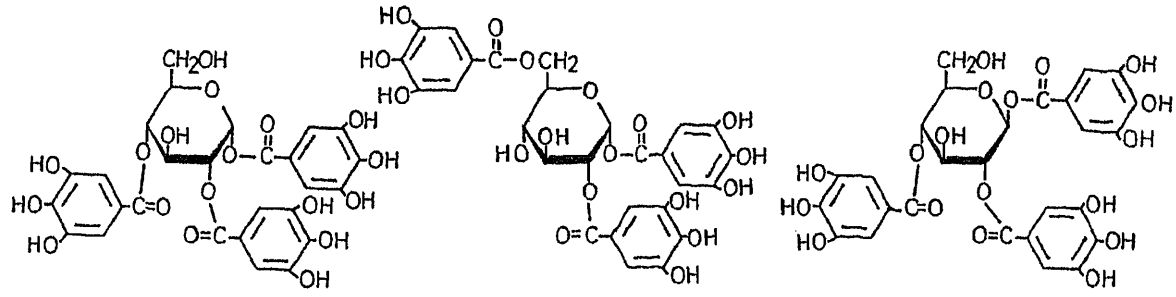
a) Spectra were measured in acetone-d_6 at 100 MHz. b) J values are expressed in Hz. c) Compound **5**: 1,2,4-tri-*O*-galloyl-β-D-glucose.[9]

raphies to furnish compounds **1** and **3**, respectively. On the other hand, the tetra- and pentagallate fractions were shown to be complex mixtures by reverse-phase HPLC. Repeated chromatography of these fractions yielded compounds **2** and **6**, and several ellagitannins.

Detailed examinations of the proton nuclear magnetic resonance (^{1}H-NMR) spectra of the mono- and tetragallates (**1** and **2**) led us to conclude that **1** and **2** are identical with 6-*O*- and 2,3,4,6-tetra-*O*-galloylglucoses which were previously isolated from commercial rhubarb[6] and the underground part of *Sanguisorba officinalis* L.,[7] respectively.

The major gallotannin **3** formed colorless fine needles when treated with water. Acid hydrolysis (1 N H_2SO_4) afforded gallic acid and glucose. The presence of three gallic acid ester groups in **3** was confirmed by observation of three two-proton aromatic singlets at δ 7.10, 7.19 and 7.20 in its ^{1}H-NMR spectrum, and also by field desorption mass spectrometry (FD-MS) (M^+: m/z 752) of the nonamethyl ether (**3a**) prepared from **3** by methylation with dimethyl sulfate and potassium carbonate in dry acetone.

The ^{1}H-NMR spectrum of **3** showed, in association with the three galloyl peaks, three lowfield signals due to glucose methine protons geminal to the galloyloxy group at δ 6.59 (d, J=4 Hz), 5.29 (t, J=9 Hz) and 5.19 (dd, J=4, 9 Hz). Based on the coupling modes of these signals, the two signals at δ 6.59 and 5.19 were readily assignable to the C(1) and C(2) protons, respectively. The assignment of the remaining triplet was achieved by spin-decoupling techniques. On irradiation at the frequency (δ 5.19) of the C(2) proton, an upfield triplet signal (δ 4.57) changed into a doublet (the C(1) proton signal at δ 6.59 likewise changed to a singlet), thus indicating that this upfield triplet is due to the C(3) proton. Next, irradiation of this C(3) proton caused a change of the triplet at δ 5.29, as well as the C(2) proton signal. These results



3 4 5

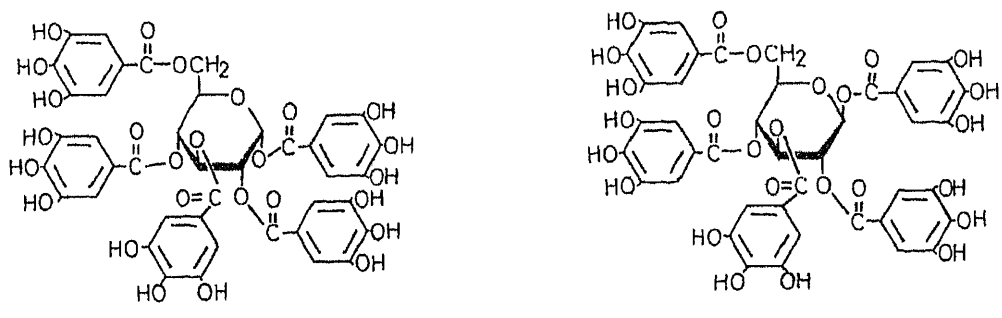
Chart 2

indicated that the gallic acid ester groups are located at the C(1), C(2) and C(4) positions in the glucose moiety.

Since it is clear from the ^{1}H-NMR data (Table I) that the glucopyranose ring adopts $^4C_1$ conformation, the coupling constant ($J=4$ Hz) of the above-mentioned anomeric proton signal clearly indicated that the mode of linkage at the anomeric center is α [*cf.* β-form (5), C(1)-H, $J=8$ Hz], thus permitting the assignment of the structure for this compound as 1,2,4-tri-*O*-galloyl-α-D-glucose.

In a previous communication,[4] we proposed the structure **4** for this trigalloylglucose, based on the fact that methanolysis of the permethyl ether prepared by two steps of methylation, first with dimethyl sulfate and potassium carbonate in dry acetone and then with silver oxide and methyl iodide in dimethyl formamide (the Kuhn method), afforded methyl 3,4-di-*O*-methylglucoside. Careful re-examination of these reactions showed that on prolonged heating in the first methylation step, the galloyl group originally located at the glucose C(4) position migrates to both the neighboring C(6) and C(3) positions, yielding 1,2,6- and 1,2,3-tri-*O*-trimethylgalloyl-α-D-glucoses. The production of 1,2,6-tri-*O*-trimethylgalloyl-α-D-glucose had thus led us to assign the wrong structure **4**.

The gallotannin **6** failed to crystallize, and was obtained as an off-white amorphous powder. The ^{1}H-NMR spectrum showed the presence of five galloyl and well-defined glucose proton signals (Table I). The large coupling constants in the glucose C(2)–C(5) proton signals indicated that the ring adopts the $^4C_1$ conformation, while the small one ($J=3$ Hz) of the C(1) proton signal showed the anomeric center to have α-configuration. Comparison of the ^{13}C-chemical shift (δ 90.2) of the C(1) atom in **6** with that (δ 93.3) in the β-anomer (**7**)[8] also confirmed the mode of the linkage to be α. The lowfield shifts of all of the glucose protons, combined with the fact that the infrared (IR) spectrum of the pentadecamethyl ether of **6** displayed no hydroxyl absorption band, indicated that all of the glucose hydroxyls are acylated. On the basis of these chemical and spectroscopic findings, **6** was characterized as



6 7

Chart 3

1,2,3,4,6-penta-*O*-galloyl-α-D-glucose.

This is the first report on the isolation of gallotannins possessing an α-glucopyranose core from a natural source. It is interesting from the viewpoint of plant physiology that in contrast to ubiquitous plant glucosides which invariably contain a β-linkage, the gallotannins in the rhizomes of *Nuphar japonicum* are based on the configurationally less unstable α-glucose core as long as an acyl group is attached to the anomeric center. We have also isolated the accompanying monomeric, dimeric and trimeric ellagitannins which similarly contain the α-glucose core, and their structures will be reported elsewhere.

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were taken with a JASCO DIP-4 digital polarimeter. IR spectra were obtained with a JASCO DS-301 spectrophotometer. FD-MS were measured with a JEOL JMS-DX 300 instrument using glycerol as the matrix at the accelerating voltage of 2 kV and emitter current of 24—25 mA. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL PS-100 and JEOL FX-100 spectrometers, respectively, with tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet, dd, double doublet. Column chromatography was carried out on Sephadex LH-20 (25—100 μm, Pharmacia Fine Chemical Co., Ltd.) and MCI-gel CHP 20P (75—150 μm, Mitsubishi Chemical Industries, Ltd.). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck) with benzene–ethyl formate–formic acid (1 : 7 : 1 or 2 : 7 : 1) for free phenolics and with benzene–acetone (3 : 1 or 5 : 1) for methyl ethers, and on Avicel SF cellulose plates (Funakoshi) with 2% acetic acid. Spots on TLC were detected under ultraviolet (UV) light or by spraying the plates with 1% ferric chloride solution. HPLC was performed on a Hitachi model 638 liquid chromatograph equipped with a Hitachi variable-wavelength spectrophotometric detector. A Nucleosil 50-10 (Macherey-Nagel) column (3 mm i.d. × 300 mm, glass) was used for normal-phase HPLC, and the mobile phase was prepared by dissolving 1.0 g of oxalic acid in 1 l of *n*-hexane–methanol–tetrahydrofuran (THF)–formic acid (55 : 33 : 11 : 1). A Nucleosil 5 C_{18} column (4 mm i.d. × 250 mm) was used for reverse-phase HPLC, and the mobile phase was prepared by dissolving 2.0 g of oxalic acid in 1 l of acetonitrile–water (21 : 79).

HPLC Analysis of Gallotannins——A finely powdered commercial sample (5 g) of *Nuphar japonicum* was extracted with 50% aqueous acetone (20 ml) at room temperature for 2 h. After removal of insoluble materials by centrifugation, the supernatant was treated with saturated aqueous sodium chloride solution (20 ml) and extracted twice with ethyl acetate (20 ml each). The ethyl acetate-soluble portion was analyzed by HPLC.

Isolation of Gallotannins——The freeze-dried rhizomes (4.3 kg) of *N. japonicum*, which were collected near Sapporo in Hokkaido during the autumn, were extracted three times with 80% aqueous acetone. Concentration of the extracts under reduced pressure afforded dark brown precipitates, which were removed by filtration. The filtrate was chromatographed on a Sephadex LH-20 column. Elution with water containing increasing amounts of ethanol yielded fractions consisting of mono-, tri-, tetra- and pentagallates. The mono- and trigallate fractions were separately purified by Sephadex LH-20 chromatography with water and MCI-gel CHP 20P chromatography with a mixture of water and methanol to yield compounds **1** (140 mg) and **3** (990 mg), respectively. The tetragallate fraction was repeatedly chromatographed over Sephadex LH-20 with ethanol and MCI-gel CHP 20P with water–methanol to give compound **2** (252 mg). Similar chromatographic separation of the pentagallate fraction yielded compound **6** (180 mg) and several ellagitannins. The mono- and tetragallates (**1** and **2**) were shown to be identical with 6-*O*- and 2,3,4,6-tetra-*O*-galloylglucoses, respectively, by comparison of their physical and spectral data with those of authentic samples.

Compound 3——Colorless fine needles (H_2O), mp 208—210 °C, $[\alpha]_D^{31}$ +73.5° (c=1.6, acetone). *Anal.* Calcd for $C_{27}H_{24}O_{18}\cdot 2H_2O$: C, 47.86; H, 4.16. Found: C, 47.58; H, 4.38. UV λ_{max}^{EtOH} nm (ε): 278 (21200). ^{13}C-NMR (acetone-d_6): 61.9 (glc. C-6), 70.3, 72.1, 73.7, 74.3 (glc. C-2, C-3, C-4, C-5), 90.4 (glc. C-1), 110.3 (galloyl C-2, C-6), 120.8, 121.0, 121.3 (galloyl C-1), 139.2, 139.5 (galloyl C-5), 145.9, 146.1, 146.2 (galloyl C-3, C-5), 165.0, 166.4 (-COO-).

Methylation of 3——a) A mixture of **3** (150 mg), dimethyl sulfate (1.5 ml) and anhydrous potassium carbonate (1.8 g) in dry acetone (30 ml) was heated under reflux for 2.5 h. After removal of the inorganic precipitates by filtration, the filtrate was concentrated to dryness under reduced pressure. The residue was chromatographed over silica gel, and elution with benzene–acetone (3 : 1) afforded the nonamethyl ether (**3a**) as a white amorphouse powder (84 mg), $[\alpha]_D^{21}$ +59.6° (c=0.6, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 2.50 (1H, t, OH of glc. C-6, disappeared on addition of D_2O), 3.02 (1H, d, J=5 Hz, OH of glc. C-3, disappeared on addition of D_2O), 3.7—3.9 (OCH_3), 4.20 (1H, m, glc. 5-H), 4.60 (1H, sextet, J=5, 9 Hz, glc. 3-H, changed into triplet, J=9 Hz on addition of D_2O), 5.38 (1H, t, J=9 Hz, glc. 4-H), 5.41 (1H, dd, J=9, 3 Hz, glc. 2-H), 6.67 (1H, d, J=3 Hz, glc. 1-H), 7.16, 7.29, 7.35 (each 2H, s, galloy H). FD-MS m/z: 752 (M^+). b) Heating of a mixture of **3** (480 mg), dimethyl sulfate (1 ml) and potassium carbonate (1.2 g) in dry acetone (30 ml) for 4 h, followed by similar chromatographic separation, afforded the nonamethyl ether

(**3a**) (121 mg), 1,2,3-tri-*O*-trimethylgalloyl-α-D-glucose (32 mg) as a white amorphous powder, $[\alpha]_D^{20}$ +151.8° (*c*=0.85, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 3.63—3.96 (OCH_3), 5.47 (1H, dd, *J*=4, 9 Hz, glc. 2-H), 5.91 (1H, t-like, *J*=9 Hz, glc. 3-H), 6.68 (1H, d, *J*=4 Hz, glc. 1-H), 7.07, 7.22, 7.36 (each 2H, s, galloyl H). FD-MS *m/z*: 752 (M^+). And 1,2,6-tri-*O*-trimethylgalloyl-α-D-glucose (33 mg) as a white amorphous powder, $[\alpha]_D^{20}$ +62.0° (*c*=0.8, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): 3.68—3.96 (OCH_3), 4.42 (1H, dd, *J*=2, 12 Hz, glc. 6-H), 4.94 (1H, dd, *J*=3, 12 Hz, glc. 6-H), 5.26 (1H, dd, *J*=4, 9 Hz, glc. 2-H), 6.60 (1H, d, *J*=4 Hz, glc. 1-H), 7.12, 7.28, 7.29 (each 2H, s, galloyl H). FD-MS *m/z*: 752.

Acid Hydrolysis of 3——A solution of **3** (10 mg) in 1 N H_2SO_4 (1 ml) was heated at 90 °C for 2 h. After cooling, the reaction mixture was extracted with ethyl acetate. TLC examination of the ethyl acetate layer showed the presence of gallic acid. The aqueous layer was neutralized with barium carbonate, and analyzed by cellulose TLC [solvent, *n*-BuOH–pyridine–H_2O (6 : 2 : 1); detection, aniline–hydrogen-phthalate reagent]. A spot corresponding to glucose was detected.

Compound 6——An off-white amorphous powder, $[\alpha]_D^{29}$ +134.5° (*c*=0.5, acetone). *Anal.* Calcd for $C_{41}H_{32}O_{26}\cdot 5H_2O$: C, 47.78; H, 4.11. Found: C, 47.88; H, 3.83. ^{13}C-NMR (acetone-d_6): 62.7 (glc. C-6), 68.9 (glc. C-4), 71.0, 71.3 (×2) (glc. C-2, C-3, C-5), 90.2 (glc. C-1), 110.1 (galloyl C-2, C-6), 119.5, 119.8, 120.7 (galloyl C-1), 139.2, 139.6, 139.9 (galloyl C-5), 145.9, 146.2 (galloyl C-3, C-5), 165.4, 166.2, 166.3, 166.9 (×2) (–COO–).

Methylation of 6——A solution of **6** (25 mg) in methanol was treated five times with ethereal diazomethane. The reaction product was purified by silica gel chromatography with benzene–acetone (9 : 1) to yield the pentadecamethyl ether (**6a**) as a white amorphous powder (20 mg), $[\alpha]_D^{25}$ +67.3° (*c*=0.18, acetone). IR ν_{max}^{Nujol} cm^{-1}: 1725 (–COO–), 1585, 1500 (C=O), no OH band. Electron impact mass spectrometry (EI-MS) *m/z*: 1150 (M^+), 406, 195.

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New Laxative Constituents of Rhubarb. Isolation and Characterization of Rheinosides A, B, C and D[1)]

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Four new laxative constituents, rheinosides A, B, C and D, were isolated from commercial rhubarb (Rhei Rhizoma). On the basis of high-resolution nuclear magnetic resonance spectral data and chemical correlation with rhein, the structures were characterized as stereoisomers of 8-*O*-*β*-D-glucosyl-10-hydroxy-10-*C*-*β*-D-glucosyl rhein-9-anthrone (rheinosides A and B), and 8-*O*-*β*-D-glucosyl-10-*C*-*β*-D-glucosyl rhein-9-anthrone (rheinosides C and D).

Keywords——rhubarb; Rhei Rhizoma; Polygonaceae; rheinoside; *C*-glucosyl anthrone; rhein anthrone; laxative activity

Rhubarb (Rhei Rhizoma) has been used as an important traditional crude drug in China and Japan, and as a laxative medicine in European countries. Many investigators have studied the constituents of rhubarb, and it has been shown to contain anthraquinones,[3)] dianthrones (sennosides),[4)] naphthalenes,[5)] stilbenes,[6,7)] phenylbutanones,[7-9)] chromones,[10)] *etc.* Recently, we have reported the isolation and characterization of highly water-soluble polyphenolic compounds (rhatannin)[8)] which decreased urea nitrogen concentration in rat serum, and other tannin-related compounds.[8,9,11,12)] Chemical and pharmacological studies of rhubarb have revealed that sennosides A—F are active principles.[4)] Our systematic investigation on the water-soluble fraction of rhubarb, however, has resulted in the isolation of four new laxative *C*-glucosides. In this paper, we report the characterization and laxative activity of these new principles.

A commercial rhubarb (Gaoh, produced in Sichuan province in China) obtained in Osaka market was extracted with 50% aqueous acetone, and the extract was partitioned between ethyl acetate and water. The water-soluble portion containing a mixture of

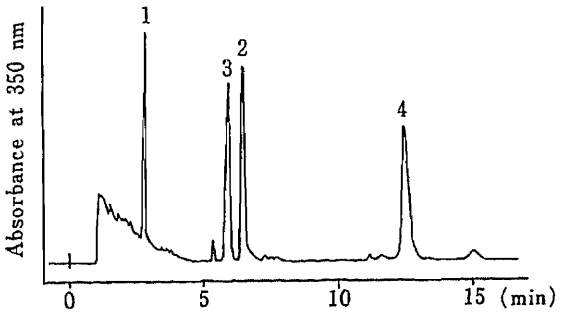


Fig. 1. High-Performance Liquid Chromatogram of Rheinosides

Conditions: column, Nucleosil $5C_{18}$ (4 i.d. × 250 mm); solvent, $CH_3CN:H_2O:HCOOH = 12:88:1$; flow rate, 1.2 ml/min; detection, 350 nm.
1, rheinoside A; 2, rheinoside B; 3, rheinoside C; 4, rheinoside D.

polyphenolics, sennosides and *C*-glucosides, was applied to a Sephadex LH-20 column. The column was eluted with water, and then 50% aqueous acetone. The polyphenolic compounds were efficiently adsorbed on the Sephadex column, and a mixture of *C*-glycosides was obtained from the water eluate. High-performance liquid chromatographic (HPLC) analysis of the mixture of *C*-glycosides showed four major peaks in the chromatogram (Fig. 1). These *C*-glycosides, named rheinosides A (**1**), B (**2**), C (**3**) and D (**4**), were isolated by repeated preparative liquid chromatography on a reversed-phase column using two solvent systems (acetonitrile–water–acetic acid and methanol–water–acetic acid), as pale yellow amorphous powders.

All of these compounds showed blue colorations with the $FeCl_3$ reagent and were soluble in dilute aqueous NaOH, giving yellow solutions which showed intense yellow fluorescence under ultraviolet (UV) light (360 nm). The infrared (IR) spectra of these compounds showed absorptions at 1640 cm^{-1} (ketone) and 1700 cm^{-1} (carboxylic acid), and the UV spectra showed absorption maxima around 268, 295 and 330 nm which closely resemble those of barbaloin (aloe-emodin anthrone 10-*C*-*β*-D-glucoside).[13]

The field desorption mass spectra (FD-MS) of **1** and **2** showed the same $(M+Na)^+$ ion peak at *m/z* 633, and the elemental analyses established the molecular formulae to be

Table I. ^{13}C-NMR Data

	1	2	3	4
Anthrone moiety				
C-1	160.8 s	161.4 s	160.9 s	161.3 s
C-2	120.4 d	120.4 d	119.1 d	116.6 d
C-3	137.9 s	138.6 s	137.8 s	137.9 s
C-4	119.7 d	120.5 d	121.6 d	122.9 d
C-4a	147.6 s	149.7 s	145.9 s	147.8 s
C-5	123.3 d	123.0 d	126.2 d	125.8 d
C-6	138.0 d	138.4 d	137.8 d	138.4 d
C-7	118.4 d	120.2 d	117.4 d	119.6 d
C-8	159.3 s	159.0 s	159.5 s	159.8 s
C-8a	124.5 s[a]	123.5 s[a]	125.2 s	125.4 s[a]
C-9	191.6 s	192.0 s	192.7 s	193.0 s
C-9a	123.6 s[a]	123.5 s[a]	125.2 s	125.1 s[a]
C-10	77.6 s	77.7 s	47.0 d	46.7 d
C-10a	147.8 s	146.0 s	144.0 s	141.9 s
COOH	171.5 s	175.7 s	171.4 s	171.9 s
C-Glucose moiety				
C-1′	84.7 d	84.6 d	85.6 d	85.4 d
C-2′	75.6 d	74.0 d	73.1 d	72.9 d
C-3′	80.0 d	79.0 d	80.4 d	80.4 d
C-4′	71.8 d	71.8 d[b]	72.0 d	72.2 d[b]
C-5′	82.0 d	82.1 d	82.0 d	82.1 d
C-6′	63.7 t	63.7 t	63.5 t	63.7 t
O-Glucose moiety				
C-1″	103.3 d	104.5 d	103.1 d	105.0 d
C-2″	75.6 d	75.7 d	75.6 d	75.7 d
C-3″	78.3 d	77.7 d	78.4 d	77.9 d
C-4″	74.1 d	72.0 d[b]	72.0 d	72.1 d[b]
C-5″	78.8 d	79.0 d	78.9 d	79.0 d
C-6″	63.2 t	63.3 t	63.2 t	63.4 t

a, *b*) Assignments in each column may be interchanged. Multiplicities were determined by the INEPT method. Assignments in rheinoside A (**1**) were made by ^1H–^{13}C shift correlation spectroscopy (^1H–^{13}C COSY).

$C_{27}H_{30}O_{16}$. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of **1** and **2** showed the presence of two aromatic rings, a carbonyl carbon, a carboxyl carbon, and a quaternary carbon bearing an oxygen function, together with two sugar moieties (see Table I). The proton nuclear magnetic resonance (^{1}H-NMR) spectra showed the presence of 14 protons assignable to sugar moieties and 5 aromatic protons.

On hydrolysis with 0.5 N H_2SO_4, **1** afforded a sugar and a pale yellow compound (**1a**), mp 230 °C, $[\alpha]_D^{25}$ +34° (c=0.50, methanol). EI-MS m/z: 448 (M^+). The sugar was identified as D-glucose by HPLC on Nucleosil 5NH_2 and from its optical rotation. On the other hand, oxidative degradation of **1** with 2 N H_2SO_4 in the presence of $FeCl_3$ afforded rhein, D-glucose and D-arabinose. Hay and Haynes[13] have already reported that treatment of barbaloin with $FeCl_3$ in 50% H_2SO_4 gave aloe-emodin and D-arabinose, and that D-arabinose was artificially formed from the *C*-glucosyl moiety by the retro-Prince reaction.[14] Our results, combined with these findings, suggested that **1** was a compound analogous to barbaloin, having a rhein anthrone skeleton with both *O*- and *C*-glucosyl moieties.

All signals in the ^{1}H-NMR spectrum of **1** were assigned by measurement of two-dimensional shift correlation (COSY, Fig. 2) and two-dimensional *J*-resolved (2D-*J*) spectra. The anomeric protons of *O*-glucosyl and *C*-glucosyl moieties were observed at δ 5.19 (J= 7.9 Hz) and δ 3.34 (J=9.8 Hz), respectively, and there was no signal due to the C-10 proton. In the ^{13}C-NMR spectrum of **1**, a quaternary carbon bearing a hydroxy group was observed at δ 77.6 which was assigned to the C-10 carbon of the rhein anthrone moiety. These

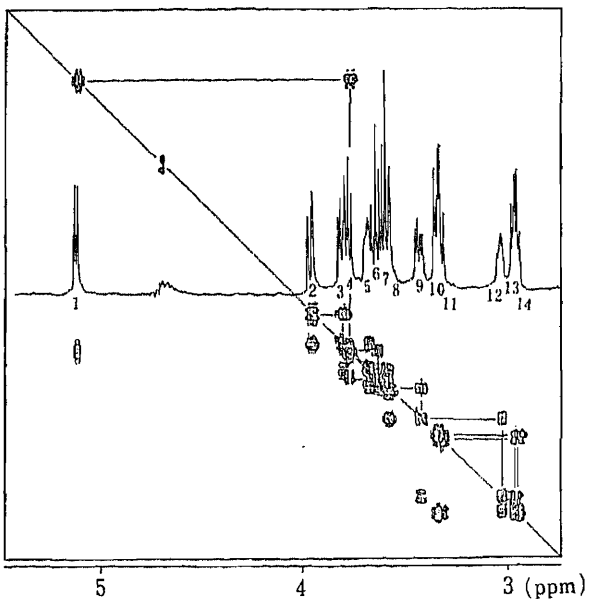


Fig. 2. Two-Dimensional Shift Correlation (COSY) Spectrum of Rheinoside A (Sugar Proton Region)

1, $C_{1''}$-H; 2, $C_{6''}$-H; 3, $C_{6''}$-H'; 4, $C_{2''}$-H; 5, $C_{5''}$-H; 6, $C_{4''}$-H; 7, $C_{3''}$-H; 8, $C_{6'}$-H; 9, $C_{6'}$-H'; 10, $C_{1'}$-H; 11, $C_{3'}$-H; 12, $C_{5'}$-H; 13, $C_{4'}$-H; 14, $C_{3'}$-H.

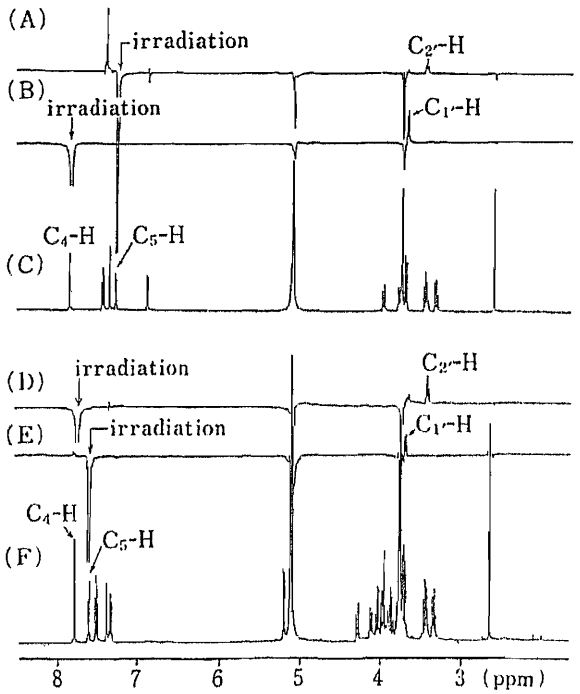


Fig. 3. Nuclear Overhauser Effect (NOE) Differential Spectra of **1a** and **2**

(A) NOE differential spectrum of **1a** irradiated at C_5-H (7.35 ppm).
(B) NOE differential spectrum of **1a** irradiated at C_4-H (7.75 ppm).
(C) Normal spectrum of **1a**.
(D) NOE differential spectrum of **2** irradiated at C_4-H' (7.82 ppm).
(E) NOE differential spectrum of **2** irradiated at C_5-H (7.65 ppm).
(F) Normal spectrum of **2**.

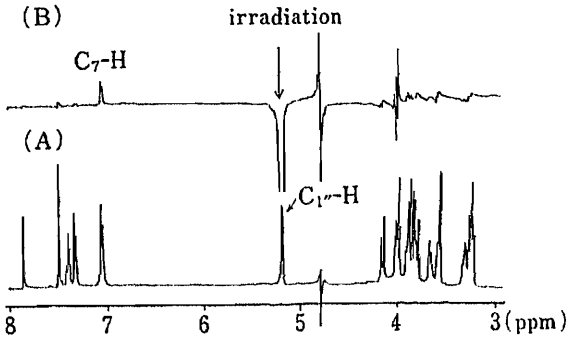


Fig. 4. NOE Differential Spectrum of **1** Irradiated at $C_{1''}$-H (5.19 ppm)

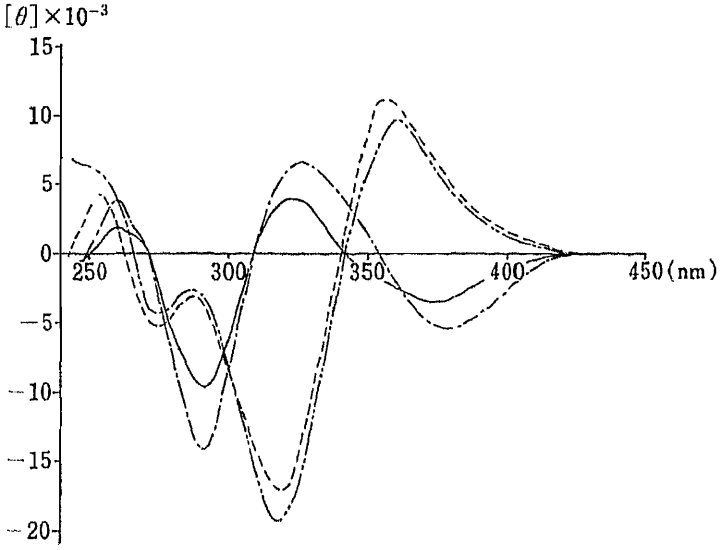


Fig. 5. CD Spectra of **1** (——), **2** (------), **3** (– – – –) and **4** (— — — —)

observations suggested that the *C*-glucosyl linkage was located at the C-10 position of the rhein anthrone moiety. This was further confirmed by nuclear Overhauser effect (NOE) experiments on **1** and **1a**. In the NOE differential spectra of **1a**, irradiation of the C-4 and C-5 protons of the rhein anthrone moiety showed NOE effects at the C-1′ and C-2′ protons, respectively (Fig. 3). The NOE effect was also observed at the C-7 proton of the rhein anthrone moiety in **1** when the C-1″ proton was irradiated (Fig. 4). Moreover, the fact that the signal (δ 6.95, d, J=8.4 Hz) of the C-7 proton in **1a** appeared at 0.39 ppm higher field compared with that of **1** indicated that the *O*-glucosyl group was attached to the C-8 hydroxy group of the rhein anthrone moiety. Therefore, **1** was characterized as 8-*O*-*β*-D-glucosyl-10-*C*-*β*-D-glucosyl-10-hydroxy rhein-9-anthrone.

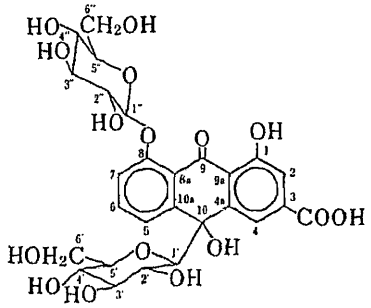
The spectral and chemical properties of rheinoside B (**2**) resembled those of **1** except for the results of NOE experiments and circular dichroism (CD) data. The CD spectrum of **1** showed a negative Cotton effect at 374 nm ($[\theta] = 3.60 \times 10^3$) and positive one at 321 nm ($[\theta] = 3.96 \times 10^3$), whereas that of **2** showed a positive Cotton effect at 355 nm ($[\theta] = 11.0 \times 10^3$) and negative one at 321 nm ($[\theta] = 17.2 \times 10^3$) as shown in Fig. 5. In the NOE differential spectra of **2** obtained by irradiation of the C-5 and C-4 protons, the NOE effects were observed at the C-1′ and C-2′ protons, respectively (Fig. 3), which was opposite to those in **1**. These observations indicated that **1** and **2** were configurational isomers with respect to the C-10 position of the rhein anthrone framework.

Rheinosides C (**3**) and D (**4**) showed the same $(M+Na)^+$ ion peak at m/z 617 in their FD-MS, which was 16 mass units less than that of **1** and **2**. Combined with this data, the molecular

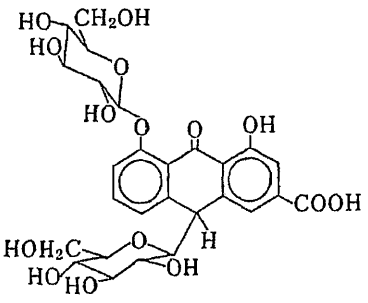
formulae of **3** and **4** were determined to be $C_{27}H_{30}O_{15}$ by elemental analyses. The ^{1}H-NMR spectra of **3** and **4** resembled those of **1** and **2** except for the presence of doublets at δ 4.24 (J= 1.8 Hz for **3**) and δ 4.12 (J=0.5 Hz for **4**). The ^{13}C-NMR spectra of **3** and **4** also resembled those of **1** and **2**, although methine carbon signals were observed at δ 47.0 (**3**) and δ 46.7 (**4**) instead of the quaternary carbons (δ 77.6 for **1** and δ 77.7 for **2**). These results implied the absence of the hydroxy group at C-10 of the rhein anthrone framework.

On oxidative degradation in the presence of $FeCl_3$, **3** gave rhein, D-glucose and D-arabinose. In the NOE differential spectra of **3**, irradiation of the C-1″ proton showed an NOE effect at the C-7 proton of rhein anthrone moiety. Irradiation of the C-1′ proton showed NOE effects at the C-4 and C-10 protons, and irradiation of the C-2′ proton showed NOE effects at the C-5 and C-10 protons. These results indicated that the *O*- and *C*-glucosyl moieties were located at the C-8 and C-10 positions of rhein anthrone, respectively.

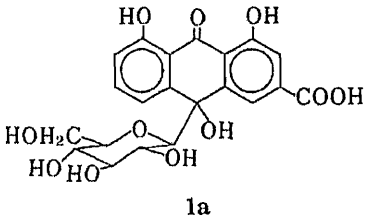
The NOE experiment on **4** also showed the presence of the *O*-glucosyl moiety at the C-8 position. In the NOE differential spectra of **4**, irradiation of C-1′ proton showed NOE effects at the C-5 and C-10 protons, and irradiation of the C-2′ proton showed NOE effects at the C-4 and C-10 protons. In the CD spectra, **3** and **4** showed almost opposite Cotton effects around 365 and 320 nm (Fig. 5), indicating that **3** and **4** differ only in the configuration at the C-10 position. Therefore, **3** and **4** were characterized as a pair of stereoisomers at the C-10 position of 8-*O*-β-D-glucosyl-10-*C*-β-D-glucosyl rhein-9-anthrone.



rheinosides A (1) and B (2)



rheinosides C (3) and D (4)



1a

Chart 1

The laxative effect of rheinoside A was tested in Wistar rats, and ED_{50} was determined to be 15.6 mg/kg. In some commercial rhubarbs, the total rheinosides content exceeded 10 mg/g, being comparable to that of sennosides. Therefore, rheinosides may also play an important role in the laxative activity of rhubarb. Elucidation of the absolute structures of the rheinosides is in progress.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus (model MS-S3) and are uncorrected. UV and IR spectra were obtained with Hitachi model 200-10 and model 260-10 spectrometers, respectively. EI and FD-MS were measured with a Shimadzu 2000-DF spectrometer. ^{1}H- and ^{13}C-NMR spectra were taken with JEOL GX-500 and FX-200 spectrometers using sodium 3-trimethylsilylpropane sulfonic acid (DSS) as an internal standard, and chemical shifts are given in δ (ppm). Optical rotations were determined with a JASCO DIP-4 digital polarimeter and CD spectra with a JASCO CD J-500 spectropolarimeter in methanol solution (1.0 mg/20 ml). HPLC was performed on a Hitachi model 655 liquid chromatograph equipped with a Nucleosil $5C_{18}$ (Macherey-Nagel) column (4 mm i.d. × 250 mm) and a variable-wavelength spectrophotometric detector operated at 280 and 350 nm. The mobile phase was prepared by mixing 1 ml of formic acid with 100 ml of water containing acetonitrile (10—20%). Column chromatographies were carried out on Sephadex LH-20 (20—100 μ, Pharmacia Fine Chemical Co., Ltd.) with a water–acetone system, and Fuji gel ODS-Q3 (30—50 μ, Fuji gel Hanbai Co., Ltd.) with a solvent system similar to that employed for HPLC.

Isolation of Rheinosides——The powdered Rhei Rhizoma (710 g, Gaoh [雅黃] produced in Sichuan province of

China) was extracted twice with 2 l each of 50% aqueous acetone at room temperature. The extracts were concentrated under reduced pressure below 40 °C, and then extracted with 1 l of ethyl acetate. The aqueous layer was concentrated to 500 ml. Acidification with 10 ml of acetic acid afforded dark brown precipitates, which were removed by decantation after standing at room temperature for 12 h. The supernatant was, after concentration, passed through a column of Sephadex LH-20 (24 i.d. × 200 mm) with water and then 50% aqueous acetone. The water eluate was repeatedly chromatographed on Sephadex LH-20 with the same solvent system, yielding a fraction (38.4 g) containing a mixture of rheinosides, which was treated with methanol. The methanol-soluble portion was dissolved in water and applied to a column of ODS-Q3 with a solvent system of 5—15% acetonitrile in water containing acetic acid (10 ml/l), to give rheinosides A (14.18 g), B (3.64 g), C (2.64 g) and D (4.56 g).

Rheinoside A (1)——A pale yellow amorphous powder, $[\alpha]_D^{25}$ −53° (c=0.5, MeOH), IR ν_{max}^{Nujol} cm^{-1}: 3600—3100 (OH), 1700 (COOH), 1640 (C=O). UV λ_{max}^{MeOH} nm (ε): 267 (6300), 296 (8800), 346 (7000). FD-MS m/z: 633 $(M+Na)^+$. CD (c=0.005, MeOH) $[\theta]^{25}$ (nm): -3.6×10^3 (374), 4.0×10^3 (321), 9.6×10^3 (292). ^{1}H-NMR (D_2O) δ: 7.88 (s, C_4-H), 7.75 (dd, J=7.2, 7.6, C_6-H), 7.67 (d, J=7.6, C_5-H), 7.38 (s, C_2-H), 7.34 (d, J=7.3, C_7-H), 5.19 (d, J=7.9, $C_{1''}$-H), 3.98 (d, J=12.5, $C_{6''}$-H), 3.82 (dd, J=5.5, 12.5, $C_{6''}$-H'), 3.79 (dd, J=7.9, 9.1, $C_{2''}$-H), 3.71 (m, $C_{5''}$-H), 3.65 (dd, J=8.9, 9.1, $C_{3''}$-H), 3.60 (dd, J=8.9, 9.1, $C_{4''}$-H), 3.60 (br d, J=12.2, $C_{6'}$-H), 3.43 (dd, J=5.5, 12.2, $C_{6'}$-H'), 3.34 (d, J=9.8, $C_{1'}$-H), 3.33 (t, J=9.2, $C_{3'}$-H), 3.03 (m, $C_{5'}$-H), 2.95 (t, J=9.2, $C_{4'}$-H), 2.94 (dd, J=9.2, 9.8, $C_{2'}$-H). *Anal.* Calcd for $C_{27}H_{30}O_{16}\cdot2H_2O$: C, 50.15; H, 4.99. Found: C, 50.19; H, 4.96.

Hydrolysis of Rheinoside A——A solution of rheinoside A (300 mg) in 5 ml of 0.5 N H_2SO_4 was refluxed for 2 h. After cooling, the reaction mixture was extracted with 50 ml each of ethyl acetate three times. The combined ethyl acetate layers were washed with water, dried over Na_2SO_4, and then concentrated to dryness. The product was purified on a column of ODS-Q2 using acetonitrile (15—20%)–water–acetic acid (1%) as an eluent to yield the *C*-glucoside **1a** (114 mg, 52%). After neutralization with $Ba(OH)_2$, the aqueous layer was passed through a Sep-pak ODS (Waters Associates Co., Ltd.). D-Glucose ($[\alpha]_D^{25}$ +33.5°, c=0.8 water) was separated from the water eluate by preparative HPLC on a Nucleosil 10 NH_2 column.

1a: Pale yellow crystals, mp 230 °C, $[\alpha]_D^{25}$ +34° (c=0.5, MeOH). EI-MS m/z: 448 (M^+). ^{1}H-NMR (D_2O) δ: 7.75 (d, J=1.2, C_4-H), 7.63 (dd, J=8.1, 8.4, C_6-H), 7.39 (d, J=1.2, C_2-H), 7.35 (d, J=8.1, C_5-H), 6.95 (d, J=8.4, C_7-H), 3.66 (dd, J=1.8, 12.1, $C_{6''}$-H), 3.46 (dd, J=6.2, 12.1, $C_{6''}$-H'), 3.52 (d, J=9.5, $C_{1''}$-H), 3.22 (dd, J=8.8, 9.2, $C_{3''}$-H), 3.08 (m, $C_{5''}$-H), 2.88 (dd, J=8.8, 9.5, $C_{4''}$-H), 2.63 (dd, J=9.2, 9.5, $C_{2''}$-H). ^{13}C-NMR (D_2O) δ: 194.7 (C_9, C=O), 171.1 (COOH), 163.2 (s), 162.3 (s), 148.2 (s), 147.1 (s), 139.5 (d), 138.8 (s), 121.2 (d, 2C), 120.6 (s), 120.6 (d), 120.1 (d), 118.2 (s), 86.1 (d), 82.1 (d), 80.0 (d), 77.0 (s), 73.7 (d), 71.9 (s), 63.8 (t). *Anal.* Calcd for $C_{21}H_{20}O_{11}\cdot1.5H_2O$: C, 53.05; H, 4.88. Found: C, 52.84; H, 4.58.

Oxidative Degradation of Rheinoside A——A solution of 10 mg of rheinoside A and 50 mg of $FeCl_3$ in 5 ml of 2 N H_2SO_4 was refluxed for 30 min. After cooling, the reaction mixture was diluted with water (50 ml) and extracted with ethyl acetate (30 ml × 3). The combined ethyl acetate layers were dried over Na_2SO_4 and concentrated. HPLC showed a peak identical with that of rhein. The aqueous layer was neutralized with $Ba(OH)_2$ and passed through a Sep-pak ODS. The fraction eluted with water was analyzed by HPLC on a column of Nucleosil 10NH_2 (mobile phase: $CH_3CN:H_2O$=70:30), and showed peaks corresponding to glucose and arabinose.

Rheinoside B (2)——A pale yellow amorphous powder, $[\alpha]_D^{25}$ −43° (c=0.5, MeOH). IR ν_{max}^{Nujol} (cm^{-1}): 3600—3100 (OH), 1700 (C=O), 1640 (COOH). UV λ_{max}^{MeOH} nm (ε): 269 (6200), 298 (9100), 345 (6600). FD-MS m/z: 633 $(M+Na)^+$. CD (c=0.005, MeOH) $[\theta]^{25}$ (nm): $+11.0\times10^3$ (355), -17.2×10^3 (321), -5.6×10^3 (275). ^{1}H-NMR (D_2O) δ: 7.82 (d, J=1.2, C_4-H), 7.71 (dd, J=7.6, 8.2, C_6-H), 7.65 (d, J=7.6, C_5-H), 7.42 (d, J=1.2, C_2-H), 7.37 (d, J=8.2, C_7-H), 5.20 (d, J=7.6, $C_{1''}$-H), 3.96 (dd, J=1.8, 12.5, $C_{6''}$-H), 3.80 (dd, J=5.5, 12.5, $C_{6''}$-H'), 3.74 (dd, J=7.6, 9.2, $C_{2''}$-H), 3.68 (dd, J=9.2, 9.5, $C_{3''}$-H), 3.66 (ddd, J=1.8, 5.5, 9.5, $C_{5''}$-H), 3.58 (t, J=9.2, $C_{4''}$-H), 3.56 (br d, J=12.2, $C_{6'}$-H), 3.44 (dd, J=5.5, 12.2, $C_{6'}$-H'), 3.38 (d, J=9.8, $C_{1'}$-H), 3.34 (t, J=9.2, $C_{3'}$-H), 3.03 (ddd, J=1.9, 7.9, 9.8, $C_{5'}$-H), 2.93 (t, J=9.5, $C_{4'}$-H), 2.89 (dd, J=9.2, 9.8, $C_{2'}$-H). *Anal.* Calcd for $C_{27}H_{30}O_{16}\cdot H_2O$: C, 51.59; H, 5.27. Found: C, 51.27; H, 5.13.

Rheinoside C (3)——A pale yellow amorphous powder, $[\alpha]_D^{25}$ −40° (c=0.5, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3600—3100 (OH), 1700 (C=O), 1640 (COOH). UV λ_{max}^{MeOH} nm (ε): 268 (7500), 292 (10500), 335 (7500). FD-MS m/z: 617 $(M+Na)^+$. CD (c=0.005, MeOH) $[\theta]^{25}$ (nm): -5.6×10^3 (378), $+6.3\times10^3$ (325), -14.0×10^3 (298). ^{1}H-NMR (D_2O) δ: 7.60 (dd, J=7.6, 8.2, C_6-H), 7.24 (d, J=8.2, C_5-H), 7.23 (d, J=1.5, C_4-H), 7.08 (d, J=7.6, C_7-H), 7.03 (d, J=1.5, C_2-H), 5.21 (d, J=7.9, $C_{1''}$-H), 4.24 (d, J=1.8, C_{10}-H), 3.99 (dd, J=2.2, 12.5, $C_{6''}$-H), 3.833 (dd, J=5.5, 12.5, $C_{6''}$-H'), 3.827 (d, J=7.9, 9.5, $C_{2''}$-H), 3.731 (m, $C_{5''}$-H), 3.727 (t, J=9.5, $C_{4''}$-H), 3.63 (t, J=9.5, $C_{3''}$-H), 3.49 (dd, J=1.8, 12.2, $C_{6'}$-H), 3.38 (dd, J=5.2, 12.2, $C_{6'}$-H'), 3.31 (t, J=8.9, $C_{3'}$-H), 3.26 (dd, J=1.8, 9.8, $C_{1'}$-H), 2.88—2.98 (m, $C_{2'}$-H, $C_{4'}$-H, $C_{5'}$-H). *Anal.* Calcd for $C_{27}H_{30}O_{15}\cdot2H_2O$: C, 51.43; H, 5.44. Found: C, 51.71; H, 5.31.

Oxidative Degradation of Rheinoside C——A solution of 10 mg of rheinoside C and 50 mg of $FeCl_3$ in 5 ml of 2 N H_2SO_4 was refluxed for 30 min, and treated as mentioned above. The HPLC analyses showed peaks corresponding to rhein, glucose and arabinose.

Rheinoside D (4)——A pale yellow amorphous powder, $[\alpha]_D^{25}$ −56° (c=0.5, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3600—3100 (OH), 1700 (C=O), 1640 (COOH). UV λ_{max}^{MeOH} nm (ε): 270 (7100), 295 (9800), 327 (7600). FD-MS m/z: 617 $(M+Na)^+$. CD (c=0.005, MeOH) $[\theta]^{25}$ (nm): $+9.6\times10^3$ (361), -19.4×10^3 (319), -4.5×10^3 (275). ^{1}H-NMR

(D_2O) δ: 7.65 (t, J=8.5, C_6-H), 7.34 (d, J=8.5, C_5-H), 7.25 (d, J=1.6, C_4-H), 7.17 (d, J=1.6, C_2-H), 7.16 (d, J=8.5, C_7-H), 5.09 (d, J=7.6, $C_{1''}$-H), 4.12 (d, J=0.5, C_{10}-H), 4.02 (br d, J=12.0, $C_{6''}$-H), 3.88 (dd, J=5.2, 12.0, $C_{6''}$-H'), 3.73 (dd, J=7.6, 8.9, $C_{2''}$-H), 3.61—3.68 (m, $C_{3''}$-H, $C_{4''}$-H, $C_{5''}$-H), 3.46 (br d, J=12.2, $C_{6'}$-H), 3.32 (br d, J=9.5, $C_{1'}$-H), 3.31 (dd, J=5.5, 12.2, $C_{6'}$-H'), 2.85—2.93 (m, $C_{3'}$-H, $C_{4'}$-H, $C_{5'}$-H), 2.81 (t, J=9.5, $C_{2'}$-H). *Anal.* Calcd for $C_{27}H_{30}O_{15}\cdot 2.5H_2O$: C, 50.70; H, 5.52. Found: C, 50.70; H, 5.23.

Bioassey——Laxative activity of rheinoside A was determined according to Ishii *et al.*[15] Doses of 21.0, 14.2, 10.2 and 7.1 mg/kg of rheinoside A were administered to groups of five Wistar rats with an average body weight of 120 g, and the activities were measured after 20 h. The ED_{50} value was calculated by the Litchfield–Wilcoxon method.

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Ketene-*S*,*N*-acetals as Synthetic Intermediates for Heterocycles. New Synthesis of Multisubstituted Pyridine-2-thiones

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Ketene-*S*,*N*-acetals reacted with isothiocyanates to give *β*-aminothiocarbonyl-*α*-methylthioenamines, which were transformed into enaminonitriles by carbon–carbon bond formation with malononitriles. Treatment of the enaminonitriles with base afforded pyridine-2-thiones. In addition, bis-lithio-ketene-*S*,*N*-acetals, generated from the enaminonitriles and *n*-BuLi, reacted with alkyl halides to give 3-alkylpyridine-2-thiones.

Keywords——ketene-*S*,*N*-acetal; thioamide; pyridine-2-thione; *α*-methylthioenamine; enaminonitrile; bis-lithioenamine

Ketene-*S*,*N*-acetals are attractive *α*-alkylthioenamines[1] as synthetic intermediates for nitrogen-[2-6] and oxygen-containing heterocycles[7] and undergo to the addition of nucleophiles at the *α*-position followed by selective elimination of alkylthiol (addition–elimination reaction) (Chart 1). Recently we described a novel synthesis of multisubstituted pyridine-2-thiones *via* carbon–carbon bond formation between *β*-aminothiocarbonyl-*α*-methylthioenamines readily available from ketene-*S*,*N*-acetals and malononitrile as a carbon nucleophile.[8] Here we present the full details of that work, together with some extension of its scope.

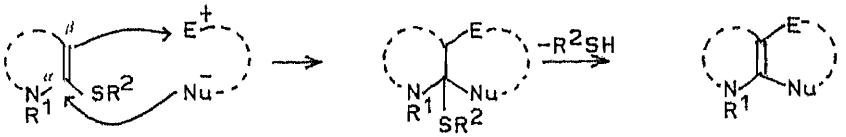

Chart 1

Results and Discussion

Addition of ketene-*S*,*N*-acetals **1**—**3** to aryl isothiocyanates **4a**—**c** has been reported[3,5] to provide the 1 : 1 adducts **5a**—**c**, **6a**—**c**, and **7a**—**c**, which are attractive synthetic equivalents of 1,3-dicarbonyls.[4,5] Although acyclic *β*-arylaminothiocarbonyl-*α*-methylthioenamines **5a**—**c** were obtained quantitatively, the cyclic compounds **6a**—**c** and **7a**—**c** were formed in only moderate yields (Chart 2). First, we examined the addition–elimination reaction of **5a**, **6a**, and **7a** with malonic acid derivative [malononitrile (**8**), ethyl cyanoacetate (**9**), ethyl malonate (**10**), and Meldrum's acid (**11**)] as carbon nucleophiles (Chart 3). Although the treatment of **5a** or **6a** with **8** in acetonitrile at room temperature gave the enaminonitrile **12a** (82%) or **13a** (80%), the reaction of **7a** with **8** afforded the amide **14** in 67% yield.[9] While the reaction of **9** with **6a** gave **15** in 65% yield, its reaction with **5a** or **7a** afforded no product. Under similar conditions, the reaction of **10** or **11** with **5a**, **6a**, and **7a** failed to give the desired compounds. It was found

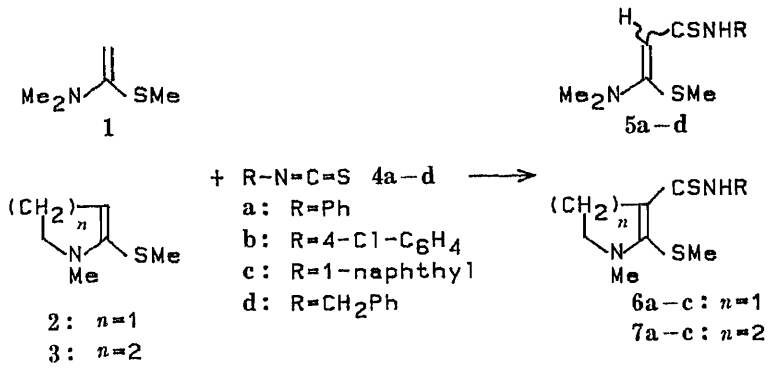

Chart 2

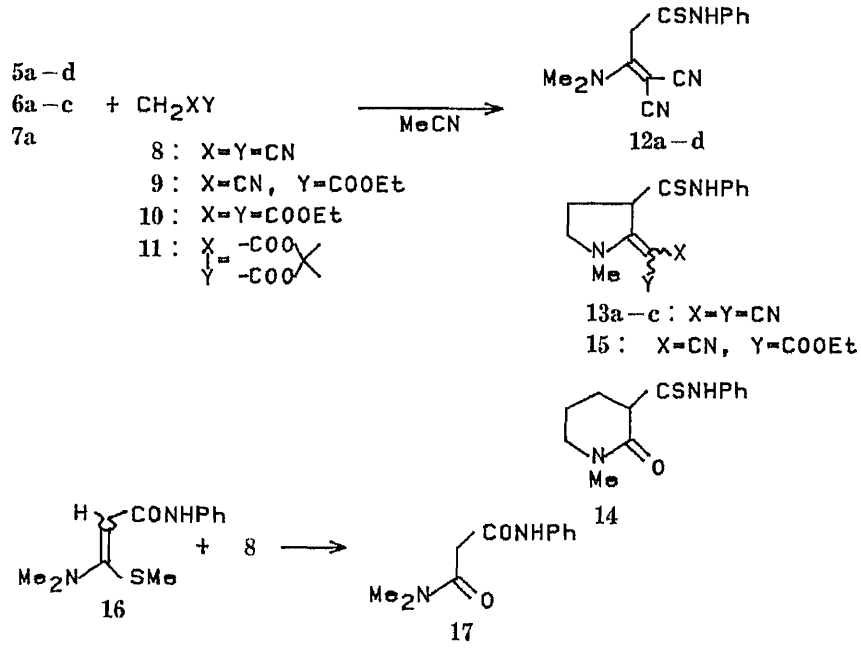

Chart 3

that **8** was more reactive than the other nucleophiles **9**—**11** toward the thioenamines **5a**, **6a**, and **7a** at the α-position. When β-aminocarbonyl-α-methylthioenamine **16** was treated with **8**, the amide **17** was obtained homogeneously in 73% yield.[9] Based on these results, **8** was chosen as the carbon nucleophile. Compounds **5b**, **c** or **6b**, **c** underwent addition–elimination with **8** as expected to give the corresponding enaminonitriles **12b**, **c** or **13b**, **c**, respectively (Chart 3 and Table I). The addition of **4d** to **1** provided the **5d**, which was then converted to **12d** without isolation.

Annulation of the enaminonitriles **12a**—**d** or **13a**—**c** thus prepared was then examined. Compounds **12a**—**d** or **13a**—**c** smoothly underwent cyclization with NaOEt to give the pyridine-2-thiones **8a**—**d** or **19a**—**c**, respectively, in good yields (Table II). In addition, compounds **18a**—**c** were directly obtained by the reaction of **5a**—**c** with **8** in the presence of NaOEt (Chart 4 and Table II).

Bis-lithio-ketene-*S*,*N*-acetals derived from secondary thioamides are regarded as interesting metallo enamines[10,11] and have been used as synthetic intermediates for heterocycles.[12] Carbon–carbon bond-forming reaction of the bis-lithio-ketene-*S*,*N*-acetals **20a**—**d** (generated from **12a**—**d** with 2 eq of *n*-BuLi) with alkyl halides **21**—**23** as electrophiles followed by cyclization yielded 3-alkylpyridine-2-thiones **24a**—**d**, **25a**—**d**, and **26a**—**d**,

TABLE I. The Enaminonitriles **12a**—**d** and **13a**—**c**

Compd.	Yield (%)	mp (°C) (Solvent)	IR ν_{max}^{Nujol} cm^{-1}	NMR ($CDCl_3$) δ	Formula	Analysis (%) Calcd (Found)		
						C	H	N
12a	82[a]	119—121 (CH_2Cl_2–iso-Pr_2O)	3200, 2220, 2180, 1580	3.37 (6H, s, NMe_2)[b] 4.10 (2H, s, CH_2)	$C_{14}H_{14}N_4S$	62.19 (61.99)	5.22 5.38	20.73 20.46)
12b	83	138—140 (CH_2Cl_2–iso-Pr_2O)	3260, 2220, 2180, 1580	3.40 (6H, s, NMe_2)[b] 4.07 (2H, s, CH_2)	$C_{14}H_{13}ClN_4S$	55.17 (55.06)	4.30 4.46	17.73 17.98)
12c	89	146—148 (CH_2Cl_2–iso-Pr_2O)	3230, 2210, 2180, 1595	3.27 (6H, s, NMe_2) 4.07 (2H, s, CH_2)	$C_{18}H_{16}N_4S$	67.48 (67.18)	5.04 5.06	17.49 17.04)
12d	71[a]	148—150 ($CHCl_3$–MeOH)	3200, 2200, 2180, 1570	3.27 (6H, s, NMe_2) 3.93 (2H, s, CH_2) 4.77 (2H, s, CH_2Ph)	$C_{15}H_{16}N_4S$	63.35 (63.24)	5.67 5.60	19.70 19.92)
13a	80	153—155 (AcOEt–pet. ether)	3320, 2200, 2180, 1620	3.37 (3H, s, NMe) 4.57 (1H, dd, J=9, 2.5 Hz, C_3-H)	$C_{15}H_{14}N_4S$	63.80 (63.61	5.00 5.01	19.84 19.66)
13b	79	168—170 (CH_2Cl_2–iso-Pr_2O)	3300, 2200, 2180, 1610	3.40 (3H, s, NMe) 4.57 (1H, dd, J=9, 2.5 Hz, C_3-H)	$C_{15}H_{13}ClN_4S$	56.88 (57.16	4.14 4.18	17.69 17.93)
13c	88	192—194 (AcOEt–iso-Pr_2O)	3260, 2200, 2180, 1620	3.33 (3H, s, NMe) 4.70 (1H, dd, J=9, 2.5 Hz, C_3-H)	$C_{19}H_{16}N_4S$	68.66 (68.56	4.85 4.82	16.86 16.93)

a) Overall yield from **1**. *b*) Taken in $CDCl_3$ + DMSO-d_6.

TABLE II. The Pyridine-2-thiones **18a**—**d** and **19a**—**c**

Compd.	Yield (%)	mp (°C)[a]	IR ν_{max}^{Nujol} cm^{-1}	NMR ($CDCl_3$ + DMSO-d_6) δ	Formula	Analysis (%) Calcd (Found)		
						C	H	N
18a	98 82[b]	230—232	3320, 2220, 1610, 1575	3.20 (6H, s, NMe_2) 5.13 (2H, br s, NH_2) 6.61 (1H, s, C_3-H)	$C_{14}H_{14}N_4S \cdot 3/10\,H_2O$	61.00 (61.29	5.30 5.29	20.33 19.98)
18b	85 80[b]	220—222	3330, 2200, 1620, 1580	3.23 (6H, s, NMe_2) 4.83 (2H, br s, NH_2) 6.63 (1H, s, C_3-H)	$C_{14}H_{13}ClN_4S$	55.16 (55.03	4.30 4.25	18.38 18.61)
18c	84 86[b]	328—330	3320, 2200, 1620, 1580	3.28 (6H, s, NMe_2) 5.02 (2H, br s, NH_2) 6.88 (1H, s, C_3-H)	$C_{18}H_{16}N_4S \cdot 9/10\,H_2O$	64.24 (64.55	5.29 5.02	16.65 16.27)
18d	85	237—238[c]	3240, 2200, 1620, 1570	3.18 (6H, s, NMe_2) 6.13 (2H, s, CH_2Ph) 6.60 (1H, s, C_3-H) 6.80 (2H, br s, NH_2)	$C_{15}H_{16}N_4S \cdot 1/2\,H_2O$	61.40 (61.45	5.84 5.49	19.09 18.83)
19a	80	296—298	3440, 2200, 2180, 1610, 1570	3.20 (3H, s, NMe) 6.47 (2H, br s, NH_2)	$C_{15}H_{14}N_4S$	63.82 (63.66	5.00 5.03	19.85 20.14)
19b	75	252—254	3440, 2240, 2210, 1650, 1580	3.30 (3H, s, NMe) 6.50 (2H, br s, NH_2)	$C_{15}H_{13}ClN_4S$	56.86 (57.10	4.14 4.15	17.68 17.39)
19c	77	318—320	3480, 2220, 2200, 1630, 1580	3.25 (3H, s, NMe) 6.62 (2H, br s, NH_2)	$C_{19}H_{16}N_4S$	68.66 (68.78	4.85 4.91	16.86 16.62)

a) Recrystallized from CH_2Cl_2–iso-Pr_2O. *b*) One-pot yield from **5**. *c*) Recrystallized from MeOH–$CHCl_3$.

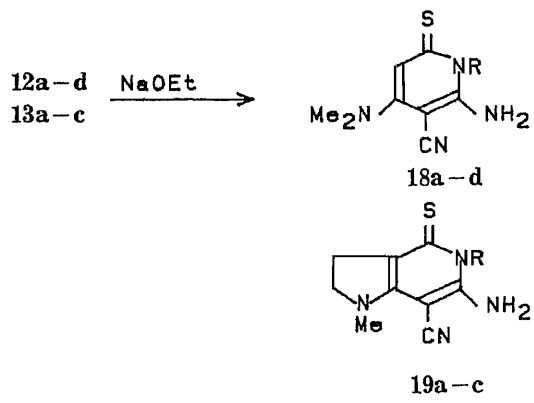

Chart 4

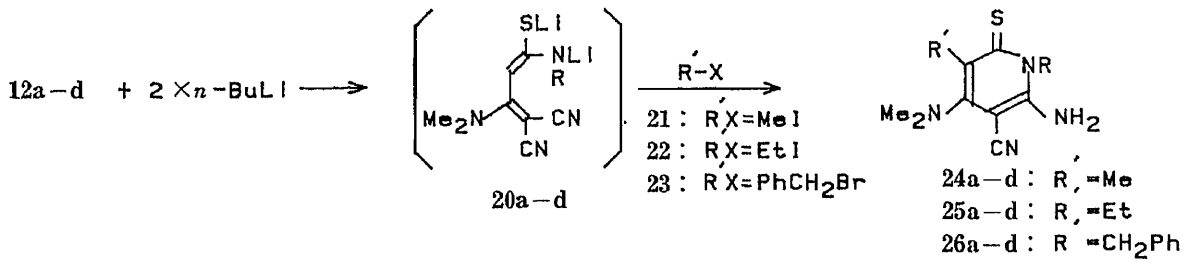

Chart 5

respectively (Chart 5 and Table III). The reaction of **20a** with benzoyl chloride as an electrophile quantitatively provided bis-benzoyl-ketene-*S*,*N*-acetal **27**. Its proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a vinyl proton signal at δ 6.73 as a singlet. The presence of the vinyl proton was also confirmed by carbon-13 nuclear magnetic resonance (^{13}C-NMR), *i.e.*, C-4 signal appeared at δ 116 as a doublet. Due to the "hard" character of benzoyl chloride compared with alkyl halides, harder N and S anion centers instead of the carbanion might be attacked.[13] Next, benzoyl cyanide as (a less hard electrophile) was used,

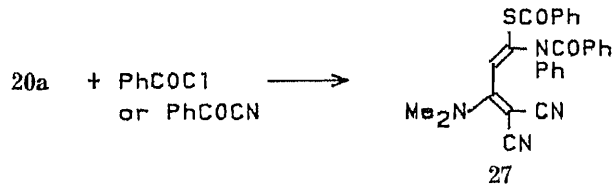

Chart 6

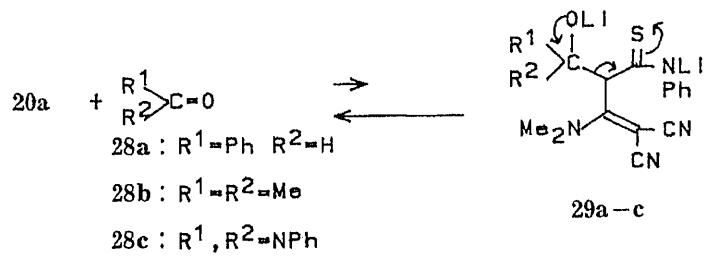

Chart 7

TABLE III. The 3-Alkylpyridine-2-thiones **24a—d**, **25a—d**, and **26a—d**

Compd.	Yield (%)	mp (°C) (Solvent)	IR ν_{max}^{Nujol} cm^{-1}	NMR ($CDCl_3$) δ	Formula	Analysis (%) Calcd (Found)		
						C	H	N
24a	84	184—186 (CH_2Cl_2–iso-Pr_2O)	3400, 2190, 1590	2.32 (3H, s, Me) 3.30 (6H, s, NMe_2) 4.60 (2H, brs, NH_2)	$C_{15}H_{16}N_4S$	63.35 (63.01	5.67 5.70	19.70 19.58)
24b	89	223—225 (CH_2Cl_2–iso-Pr_2O)	3370, 2220, 1600	2.33 (3H, s, Me) 3.30 (6H, s, NMe_2) 4.47 (2H, brs, NH_2)	$C_{15}H_{15}ClN_4S$	56.51 (56.16	4.74 4.69	17.57 17.40)
24c	89	194—196 (CH_2Cl_2–iso-Pr_2O)	3370, 2200, 1605	2.20 (3H, s, Me) 3.33 (6H, s, NMe_2) 4.27 (2H, brs, NH_2)	$C_{19}H_{18}N_4S\cdot$ 1/5 H_2O	67.51 (67.71	5.48 5.40	16.58 16.21)
24d	29	166—168 (AcOEt–$CHCl_3$)	3350, 2150, 1560	2.47 (3H, s, Me) 3.30 (6H, s, NMe_2) 5.51 (2H, s, CH_2Ph) 5.72 (2H, brs, NH_2)	$C_{16}H_{18}N_4S$	64.40 (64.37	6.08 5.99	18.78 18.56)
25a	85	172—174 (CH_2Cl_2–iso-Pr_2O)	3400, 2180, 1590	1.26 (3H, t, J=7.5 Hz, Me) 2.76 (2H, q, J=7.5 Hz, CH_2) 3.27 (6H, s, NMe_2) 5.10 (2H, brs, NH_2)	$C_{16}H_{18}N_4S\cdot$ 1/2 H_2O	62.51 (62.53	6.23 5.82	18.22 17.91)
25b	94	167—169 (CH_2Cl_2–iso-Pr_2O)	3400, 2180, 1590	1.30 (3H, t, J=8 Hz, Me) 2.78 (2H, q, J=8 Hz, CH_2) 3.28 (6H, s, NMe_2) 4.58 (2H, brs, NH_2)	$C_{16}H_{17}ClN_4S$	57.73 (58.01	5.15 5.09	16.83 16.47)
25c	99	213—215 (CH_2Cl_2–iso-Pr_2O)	3400, 2180, 1590	1.18 (3H, t, J=7 Hz, Me) 2.78 (2H, q, J=7 Hz, CH_2) 3.33 (6H, s, NMe_2) 4.00 (2H, brs, NH_2)	$C_{20}H_{20}N_4S\cdot$ 1/2 H_2O	67.20 (67.45	5.92 5.70	15.69 15.61)
25d	76	190—191 (AcOEt–$CHCl_3$)	3350, 2155, 2155	1.33 (3H, t, J=8 Hz, Me) 2.87 (2H, q, J=8 Hz, CH_2) 3.25 (6H, s, NMe_2) 5.52 (2H, s, CH_2Ph)	$C_{17}H_{20}N_4S$	65.35 (65.19	6.45 6.48	17.93 17.67)
26a	88	119—121 (CH_2Cl_2–iso-Pr_2O)	3430, 2200, 1580	3.18 (6H, s, NMe_2) 4.07 (2H, s, CH_2Ph) 5.50 (2H, brs, NH_2)	$C_{21}H_{20}N_4S$	69.97 (69.83	5.59 5.54	15.54 15.71)
26b	90	118—120 (CH_2Cl_2–iso-Pr_2O)	3400, 2190, 1590	3.17 (6H, s, NMe_2) 4.02 (2H, s, CH_2Ph) 5.65 (2H, brs, NH_2)	$C_{21}H_{19}ClN_4S$	63.87 (64.00	4.85 4.83	14.19 14.07)
26c	91	133—135 (CH_2Cl_2–iso-Pr_2O)	3400, 2180, 1580	3.13 (6H, s, NMe_2) 3.90 (2H, s, CH_2Ph) 4.83 (2H, brs, NH_2)	$C_{25}H_{22}N_4S\cdot$ H_2O	70.06 (70.02	5.64 5.36	13.07 12.73)
26d	45	167—168 (AcOEt–$CHCl_3$)	3300, 2220, 1565	3.18 (6H, s, NMe_2) 4.00 (2H, brs, NH_2) 4.18 (2H, s, CH_2Ph) 5.72 (2H, s, NCH_2Ph)	$C_{22}H_{22}N_4S$	70.56 (70.55	5.92 5.87	14.96 14.76)

but unfortunately gave **27** (16%) together with **18a** (22%) (Chart 6). We tried the reaction of some electrophiles containing no leaving groups, such as benzaldehyde (**28a**), acetone (**28b**), and phenyl isocyanate (**28c**) with **20a**, but the desired compounds were not obtained and the starting material **12a** was recovered, presumably due to the thermodynamically favored dissociation of the carbon–carbon bond-formed products **29a—c** into **28a—c** and **20a** (Chart 7).

In summary, this method provides a new access to multisubstituted pyridine-2-thiones

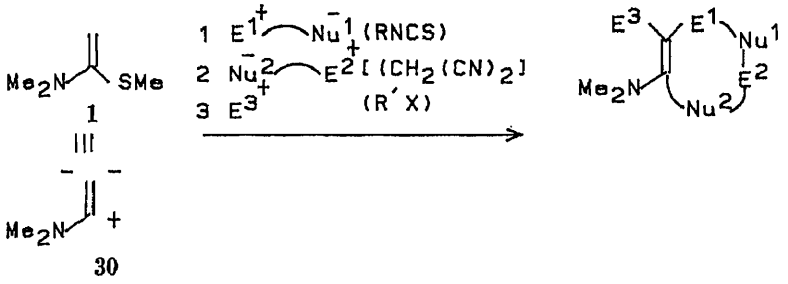

Chart 8

bearing an *o*-aminonitrile moiety, convertible into a variety of condensed heterocycles.[14] In these sequences, as shown in Chart 8, **1** is synthetically equivalent to **30**.

Experimental

NMR spectra were measured with a JEOL PMX-60 or a Varian XL-200 in $CDCl_3$ form (with tetramethylsilane as an internal reference), mass spectra with a JEOL JMS-D200 machine, and infrared (IR) spectra in Nujol (unless otherwise noted) on a JASCO A-102 spectrophotometer. All melting points were determined on a Yanaco micro melting point apparatus and are uncorrected. Column chromatography was performed on alumina with CH_2Cl_2–MeOH (A) or on Silica gel 60 (230—400 mesh) under medium pressure with *n*-hexane–ethyl acetate (B).

General Preparation of *N*-Alkyl-4,4-dicyano-3-dimethylamino-3-butenethioamides (12a—d) or 3-(*N*-Arylthiocarbamoyl)-2-dicyanomethylene-1-methylpyrrolidine (13a—c)——A mixture of a *β*-aminothiocarbonyl-*α*-methylthioenamine (**5a—d** or **6a—c**) (1 mmol), prepared according to the reported method,[3-5] and malononitrile (1 mmol) in acetonitrile (5 ml) was stirred at room temperature for 3 h. After evaporation of the solvent, the residue was crystallized with ether to give the corresponding product (**12a—d, 13a—c**) (Table I).

1-Methyl-3-(*N*-phenylthiocarbamoyl)piperidin-2-one (14)——By using the same procedure as described above, a mixture of **7a** (1 mmol) and malononitrile (1 mmol) in acetonitrile (5 ml) afforded **14** (166 mg, 67%). mp 181—183 °C (diisopropyl ether–CH_2Cl_2). IR: 3180, 3130, 1610, 1590 cm^{-1}. ^{1}H-NMR δ: 3.03 (3H, s, N-Me). *Anal.* Calcd for $C_{13}H_{16}N_2OS$: C, 62.87; H, 6.49; N, 11.28. Found: C, 62.63; H, 6.54; N, 11.50.

2-(Cyanoethoxycarbonylmethylene)-1-methyl-3-(*N*-phenylcarbamoyl)pyrrolidin-2-one (15)——A mixture of **9a** (1 mmol) and ethyl cyanoacetate (1 mmol) in acetonitrile (5 ml) was stirred at room temperature for 10 h. After evaporation of the solvent, the residue was chromatographed (A) to give **15a** (167 mg, 49%). mp 196—198 °C (diisopropyl ether–CH_2Cl_2). IR: 3240, 3200, 3130, 2200, 1690, 1590 cm^{-1}. ^{1}H-NMR δ: 1.31 (3H, t, J=7 Hz, $COOCH_2Me$), 3.52 (3H, s, N-Me), 4.22 (2H, q, J=7 Hz, $COOCH_2Me$). *Anal.* Calcd for: $C_{18}H_{19}N_3O_2S$: C, 61.98; H, 5.81; N, 12.76. Found: C, 62.26; H, 5.77; N, 12.40.

***N*-Phenyl-(*N*,*N*-dimethylamino)malonamide (17)**——A mixture of **16** (1 mmol) and malononitrile (1 mmol) in acetonitrile (5 ml) was stirred at room temperature for 10 h. After evaporation of the solvent, the residue was crystallized with ether to give **17** (151 mg, 73%). mp 114—116 °C (diisopropyl ether–CH_2Cl_2). IR: 3250, 3200, 3130, 1650, 1625, 1590 cm^{-1}. ^{1}H-NMR δ: 3.03 (3H, s, N-Me), 3.12 (3H, s, N-Me), 3.46 (2H, s, CH_2). *Anal.* Calcd for $C_{11}H_{14}N_2O_2$: C, 64.06; H, 6.84; N, 13.58. Found: C, 64.01; H, 6.79; N, 13.63.

General Procedure for 1-Alkyl-6-amino-5-cyano-3-(*N*,*N*-dimethylamino)pyridine-2-thiones (18a—d) or 3-Amino-2-aryl-4-cyano-5-methyl-1-thioxo-1,2,6,7-tetrahydropyrrolo[3,2-*c*]pyridines (19a—c)——Procedure A: One of **12a—d** and **13a—c** (0.5 mmol) was added to a solution of sodium ethoxide [prepared by dissolving sodium (11.5 mg), 0.5 mg-atom] in ethanol (5 ml)] and the mixture was stirred at room temperature for 1 h. After evaporation of the solvent, CH_2Cl_2 was added to the residue. Insoluble material was filtered off and the filtrate was evaporated to give a solid, which was recrystallized from diisopropyl ether–CH_2Cl_2 to afford the corresponding product (**18a—d** or **19a—c**) (Table II).

Procedure B: Malononitrile (0.5 mmol) was added to a solution of sodium ethoxide (0.5 mmol) in ethanol (5 ml). Subsequently one of **5a—d** (0.5 mmol) was added to the mixture, which was stirred at room temperature for 1 h. According to the procedure described under procedure A, the corresponding product (**18a—d**) was obtained (Table II).

General Procedure for 1-Aryl-3-alkyl-6-amino-5-cyano-4-(*N*,*N*-dimethylamino)pyridine-2-thiones (24a—d, 25a—d, and 26a—d)——A 15% solution of *n*-BuLi in hexane (0.7 ml, 1.1 mmol) was added to a stirred solution of one of **12a—d** (0.5 mmol) in tetrahydrofuran (4 ml) under argon at −70 °C. After stirring at the same temperature for 0.5 h, an alkyl halide (**21—23**) (0.55 mmol) was added to the reaction mixture. The resulting mixture was gradually warmed to 0 °C, stirred for an additional hour, quenched with saturated ammonium chloride aqueous solution, and extracted

with ethyl acetate. The organic layers were combined, dried, and evaporated. The residue was chromatographed (A) to yield the corresponding product (**24a—d**, **25a—d**, and **26a—d**) (Table III).

5-Benzoyloxy-5-(*N*,*N*-benzoylphenylamino)-2-cyano-3-(*N*,*N*-dimethylamino)-penta-2,4-dienenitrile (27)——In the same manner as described above, **12a** (0.5 mmol) was treated with *n*-BuLi (1.1 mmol) and benzoyl chloride (0.5 mmol) was injected into the resulting mixture. Work-up (B) yielded **27** (120 mg, 100%). mp 218—220 °C (AcOEt). IR: 2200, 1660 cm^{-1}. ^{1}H-NMR δ: 3.26 (6H, s, N–Me_2), 6.73 (1H, s, C_4-H). ^{13}C-NMR δ: 116 (d, C_4). *Anal.* Calcd for $C_{28}H_{22}N_4O_2S$: C, 70.27; H, 4.63; N, 11.70. Found: C, 70.01; H, 4.73; N, 11.52. Analogous treatment of **20a** (1.5 mmol) with benzoyl cyanide (1.5 mmol) gave **27** (57 mg, 16%) and **18a** (59 mg, 22%), which were found to be identical with authentic samples by comparison of their spectral data.

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The Chemistry of Indoles. XXXIX.[1] A Facile Synthetic Method for 7-Substituted Indoles

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A simple four-step synthetic method for 7-iodo-, 7-bromo- and 7-chloroindole was established with high overall yield starting from 2,3-dihydroindole. Several 7-substituted indoles carrying a carbon side chain and 7-methoxyindole were also synthesized.

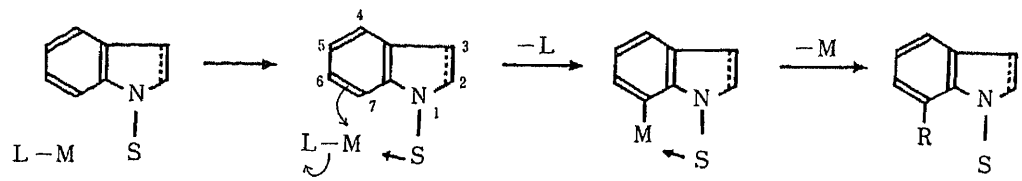
Keywords——thallation; 7-substituted indole; regioselective metalation; 7-iodoindole; 7-bromoindole; 7-chloroindole; 7-methoxyindole; methyl 3-(indol-7-yl)acrylate; 4-(indol-7-yl)-2-methyl-3-buten-2-ol; Heck reaction

For one possible approach to the construction of various types of structurally and biologically interesting indole alkaloids having a substituent at the 7-position,[2] 7-halogenoindoles are required as common building blocks. However, little work has been done on their syntheses and consequently they are available only through a laborious multistep route[3] with poor overall yield. In our continuing studies on regioselective functionalization of indoles,[4,5] we have elaborated a facile and regioselective synthetic method for 7-halogenoindoles, as reported in the preliminary communication.[6] In this report, we describe in detail these results and the syntheses of various 7-substituted indoles.

Regioselective Syntheses of 7-Halogenoindoles

In the previous paper, we reported that the thallation–palladation method[4] was a useful synthetic reaction for 4-substituted indoles with high regioselectivity. It was also shown that the regioselectivity was dramatically influenced by introduction of an extra substituent into the 2 or 3 position of the indole nucleus.[5] Based on these results, we designed the strategy shown in Chart 1. If a suitable ligand (S) is introduced at the indole (or 2,3-dihydroindole) nitrogen, the S group can coordinate to a metal reagent (metal (M)–leaving group (L)) putting the metal close to the 7-position. Consequently, metallation would occur regioselectively at the carbon-7 making the carbon susceptible to various functionalizations.

During examination of the feasibility of the strategy as planned, we soon recognized that thallation of 1-acyl-3-unsubstituted indoles with thallium tris-trifluoroacetate (TTFA) was unsuccessful because the compounds were quite sensitive to acids, and formed polymers.



S, ligand; M, metal; L, leaving group; R, suitable functional group.

Chart 1. Strategy for the Synthesis of 7-Substituted Indoles

However, in the 2,3-dihydroindole series, we could actualize our strategy by using TTFA and the acetyl group as M–L and S, respectively, though a methoxycarbonyl group could not function as the S group.

1-Acetyl-2,3-dihydroindole[7] (**2**) was produced in quantitative yield by the reaction of 2,3-dihydroindole (**1**) with refluxing acetic anhydride (Chart 2). Thallation of **2** was carried out with 1.6 mol eq of TTFA in trifluoroacetic acid[8] (TFA) at room temperature to give crude (1-acetyl-2,3-dihydroindol-7-yl)thallium bis(trifluoroacetate) (**3**) as a crystalline residue, which was exposed to a vacuum to remove a trace amount of TFA. Although **3** could be isolated as a colorless crystalline powder, the crude residue (**3**) was subjected to the following reactions without further purification since we hoped to establish a simple one-pot procedure. When the residue was reacted with aqueous potassium iodide (KI), 1-acetyl-2,3-dihydro-7-iodoindole[9] (**4a**) was produced in 74% yield together with 1-acetyl-2,3-dihydro-5-iodoindole[10] (**5**) in 5% yield. It should be noted that when iodination was carried out in the mixed solvent of TFA and water (1 : 3, v/v) with KI, the yield of **5** significantly increased to 21%, whereas the yield of **4a** decreased to 42%.

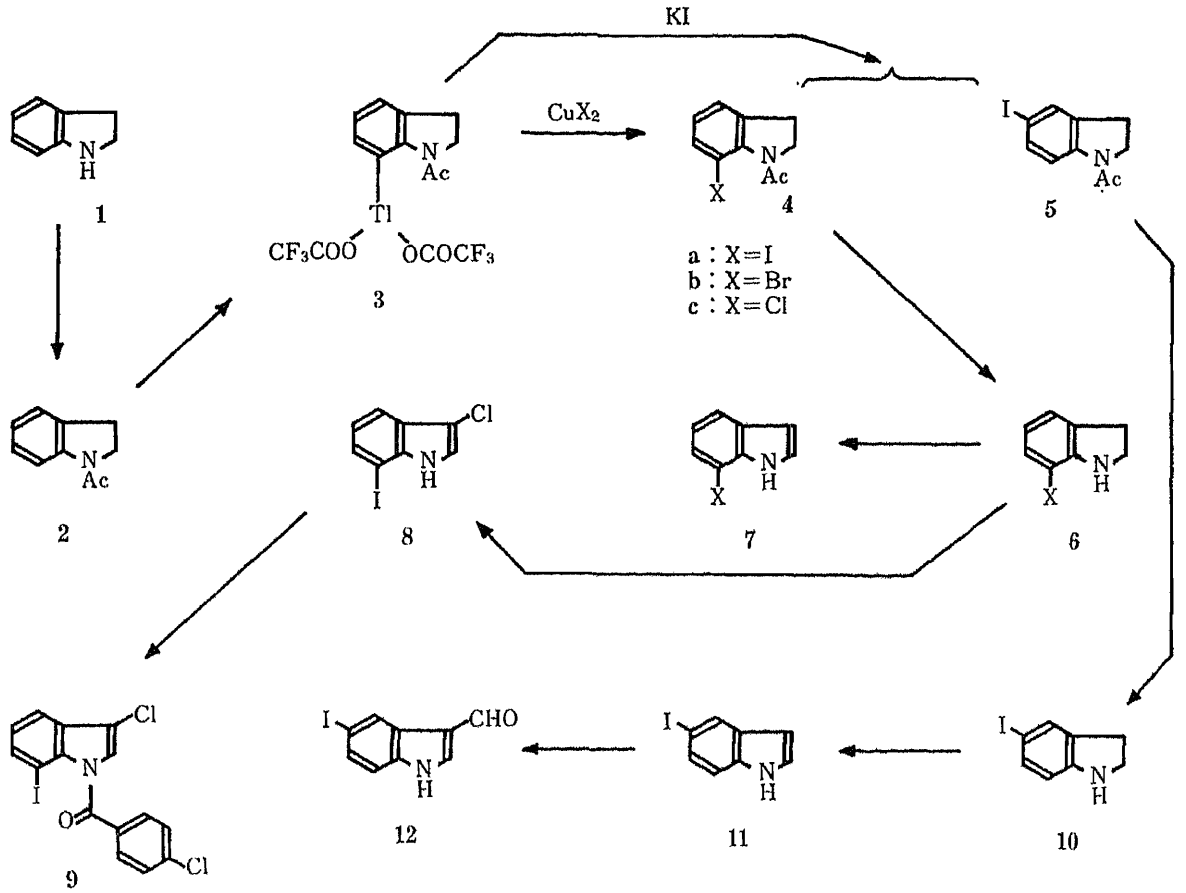


Chart 2

Although the reaction mechanism is unknown, these results of acidic iodination suggest that iodination should be carried out after removal of contaminating TFA from the thallated crystalline residue (**3**) as completely as possible.

Reaction of the thallated crystalline residue (**3**) with either cupric bromide or cupric chloride in *N*,*N*-dimethylformamide (DMF) afforded 1-acetyl-2,3-dihydro-7-bromo-[9] (**4b**) or -7-chloroindole (**4c**) in 62% and 42% yields, respectively, without the formation of the corresponding 5-halogenated compounds. Hydrolysis of 1-acetyl-2,3-dihydro-7-halo-

genoindoles (**4a**, **4b**, and **4c**) with aqueous sodium hydroxide afforded the corresponding 2,3-dihydro-7-halogenoindoles (**6a**, **6b**,[11] and **6c**) in 98%, 96%, and 93% yields, respectively.

Next, oxidation of the 2,3-dihydroindole (**6a**) to indole was examined by using active manganese dioxide (MnO_2),[12] *N*-chlorosuccinimide (NCS),[13] and *tert*-butylhypochlorite.[14] The reaction of **6a** with active MnO_2 in methylene chloride afforded the desired 7-iodoindole (**7a**) in only 13% yield, with the predominant formation of an unknown dimeric product (66%), whose mass spectrum (MS) showed the molecular ion peak at *m/z* 486. Oxidation of **6a** with NCS or *tert*-butylhypochlorite in the presence of triethylamine (NEt_3) afforded 3-chloro-7-iodoindole (**8**) together with recovery of **6a**; their ratios varied depending on the amount of the chlorinating reagent, but the formation of **7a** was not detected in the reaction mixtures. To our surprise, compound **8** was stable and its sodium salt, prepared by the action of sodium hydride in absolute DMF, was demonstrated to react with 4-chlorobenzoyl chloride, affording 3-chloro-1-(4-chlorobenzoyl)-7-iodoindole (**9**) in 62% yield. Further functionalization of **8** is in progress.

Finally, treatment of **6a** with oxygen in the presence of a catalytic amount of salcomine[15] in methanol at room temperature was found to afford **7a** cleanly in 77% yield. Under similar reaction conditions, **6b** and **6c** were successfully converted to the desired 7-bromo-[3a] (**7b**) and 7-chloroindole[3b] (**7c**) in 72% and 70% yields, respectively. Thus, 7-halogenoindoles are now readily available with high overall yield in four steps starting from **1**.

The structure of **5** was established as follows. Hydrolysis of **5** gave an 86% yield of 2,3-dihydro-5-iodoindole (**10**), which was converted to 5-iodoindole[16] (**11**) in 71% yield by salcomine-catalyzed oxidation with oxygen. Vilsmeier reaction of **11** with phosphorus oxychloride and DMF gave 5-iodo-3-indolecarbaldehyde (**12**) in 89% yield. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **5**, the C-7 proton signal appeared as a broad doublet (J=8.4 Hz) at a lower magnetic field than usual at δ 7.85 due to the anisotropic effect of the 1-acetyl group. In the compound **12**, the C-4 proton was deshielded by the 3-formyl group and resonated at a lower magnetic field (δ 8.88) as a doublet (J=1.6 Hz). These results clearly established the 5-substituted indole structure.

Syntheses of Various 7-Substituted Indoles

Since selective thallation of **2** was established as discussed above, we next examined direct introduction of a carbon side chain into the 7-position according to our thallation–palladation method.[4] Thus, **2** was thallated and the resulting crude residue (**3**) was treated with methyl acrylate in the presence of a catalytic amount of palladium acetate ($Pd(OAc)_2$) in DMF to afford methyl 3-(1-acetyl-2,3-dihydroindol-5-yl)acrylate (**13**) and methyl 3-(1-acetyl-2,3-dihydroindol-7-yl)acrylate (**14**) in 2% and 4% yields, respectively (Chart 3).

The structure of **13** was readily proved by the fact that the palladium-catalyzed Heck reaction[17] of **5** with methyl acrylate gave **13** in 96% yield. On the other hand, the structure of **14** was proved as follows. First, **6a** was led to methyl 3-(2,3-dihydroindol-7-yl)acrylate (**15**) in 90% yield by means of the Heck reaction with methyl acrylate. Treatment of **15** with acetic anhydride and pyridine afforded **14** in 94% yield. Subsequent catalytic hydrogenation of **15** over 10% palladium on carbon at ordinary atmospheric pressure gave an 84% yield of 4*H*-1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinoline-4-one (**16**), which was identical with an authentic sample prepared by the cyclization of 1-(3-chloropropionyl)-2,3-dihydroindole.[18] For further structural confirmation, **15** was subjected to salcomine-catalyzed oxidation with oxygen to afford in 71% yield the known methyl 3-(indol-7-yl)acrylate (**17**),[5] which had been alternatively prepared starting from methyl 3-methyl-2-nitrobenzoate (**18**) *via* 7-indolecarbaldehyde (**19**).

It is notable that the thallation–palladation reaction of **2** afforded a poor result, while the Heck reaction of **6a** gave a satisfactory result. The formation of **3** was unambiguously proved

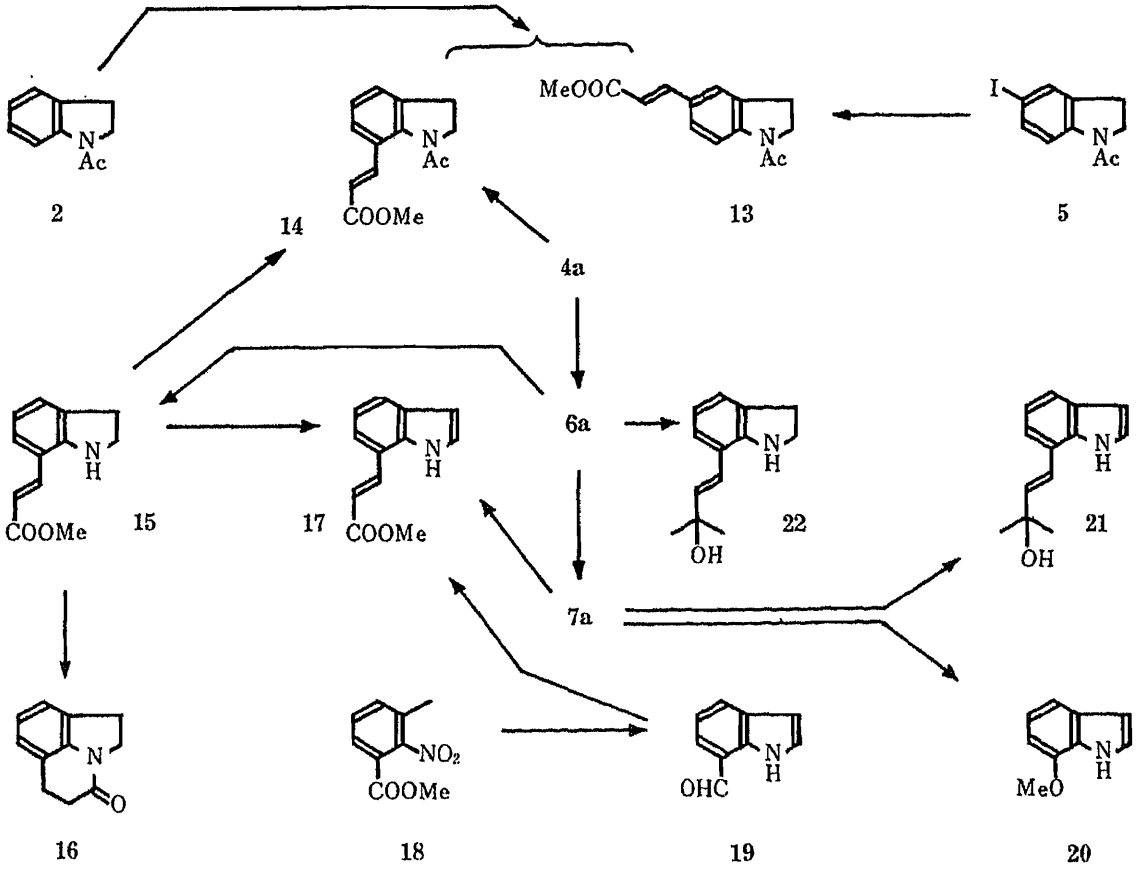


Chart 3

by its high-yield conversion to the corresponding 7-iodo compound (**4a**), as discussed above. Therefore, the above results suggest that the thallium–palladium exchange can not occur effectively at the 7-position of **3**, probably because palladium requires a strictly square-planar ligand field. The acetyl group on the sp^3 nitrogen can neither satisfy this requirement nor function as a ligand, but acts only as a sterically congesting group making the formation of the corresponding palladium complex unfavorable. Support for these assumptions has been furnished by the result of the Heck reaction of **4a** with methyl acrylate, providing **14** and **2**, and recovery of **4a** in 5%, 14%, and 60% yields, respectively, while **6a** successfully afforded **15** under similar reaction conditions.

The versatility of 7-halogenoindoles was shown by the following reactions using **7a** as a representative substrate. Thus, the treatment of **7a** with sodium methoxide in DMF in the presence of cuprous iodide[19] afforded 7-methoxyindole[20] (**20**) in 76% yield. The structure of **20** was unequivocally established by an alternative synthesis starting from 3-methoxy-2-nitrotoluene by the use of the improved Leimgruber–Batcho method.[21] The synthesis of 8-methoxy-1-oxo-1,2,3,4-tetrahydro-β-carboline starting from **20** has already been reported.[22] On the other hand, the Heck reaction of **7a** with methyl acrylate successfully afforded **17** in 90% yield. When 2-methyl-3-buten-2-ol was used as an olefin component, **7a** produced 4-(indol-7-yl)-2-methyl-3-buten-2-ol (**21**) in 48% yield, together with recovery of **7a** in 33% yield. Similarly, the Heck reaction of **6a** with 2-methyl-3-buten-2-ol proceeded smoothly to give 4-(2,3-dihydroindol-7-yl)-2-methyl-3-buten-2-ol (**22**) in 74% yield.

In conclusion, simple 7-substituted indoles are now readily accessible from 2,3-dihydroindole (**1**). With these compounds in hand, we are currently investigating the syntheses of 7-substituted natural indole alkaloids.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were determined with a Shimadzu IR-420 spectrophotometer, and ^{1}H-NMR spectra with a JEOL JNM-PMX60 or FX100S spectrometer with tetramethylsilane as an internal standard. Mass spectra (MS) were recorded on a Hitachi M-80 spectrometer. Preparative thin-layer chromatography (p-TLC) was performed on Merck Kieselgel GF_{254} (Type 60) (SiO_2). Column chromatography was performed on silica gel (SiO_2, 100—200 mesh, from Kanto Chemical Co., Inc.) throughout the present study.

1-Acetyl-2,3-dihydro-7-iodoindole (4a) and 1-Acetyl-2,3-dihydro-5-iodoindole (5) from 1-Acetyl-2,3-dihydroindole (2)——A 0.88 M solution of TTFA (32.8 ml, 1.6 mol eq) in TFA[8] was added to a solution of **2** (2.908 g) in TFA (29.0 ml) and stirring was continued at room temperature for 3 h. After evaporation of the solvent under reduced pressure, the residue was dried *in vacuo* at room temperature. By this work-up, the oily residue was transformed into a crystalline residue (**3**). [By the addition of a small amount of TFA to the residue, followed by filtration and washing with 1,2-dichloromethane, (1-acetyl-2,3-dihydroindol-7-yl)thallium bis(trifluoroacetate) (**3**) could be isolated as a colorless crystalline powder. **3**: mp 135—140 °C. IR (KBr): 1670 (br), 1613, 1211, 1137, 837, 805, 723 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.25 (3H, d, J=12 Hz), 3.09 (2H, dt, J=76, 8 Hz), 4.12 (2H, br s), 7.09 (1H, dt, J=268, 7 Hz), 7.27 (1H, dd, J=1050, 7 Hz), 7.29 (1H, dd, J=112, 7 Hz).] A solution of KI (23.912 g) in H_2O (160 ml) was added to the crystalline residue (**3**) and stirring was continued at room temperature for 2 h. After addition of CH_2Cl_2–MeOH (95 : 5, v/v) to the reaction mixture, the whole was filtered through SiO_2 to remove solid precipitates. The organic layer was separated and the water layer was extracted with CH_2Cl_2. The combined organic layer was washed successively with 5% aqueous sodium thiosulfate and brine, and dried over Na_2SO_4. After evaporation of the solvent, the residue was subjected to column chromatography on SiO_2 with AcOEt–*n*-hexane (1 : 1, v/v) as an eluent. From the early fractions, **4a** (3.843 g, 74%) was obtained. From the later fractions, **5** (254.5 mg, 5%) was obtained.

4a: mp 128.0—128.5 °C (lit.[9] mp 119—120 °C, colorless prisms, recrystallized from AcOEt). IR (KBr): 1654, 1589, 1570 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.23 (3H, s), 2.98 (2H, t, J=7.4 Hz), 4.05 (2H, t, J=7.4 Hz), 6.62 (1H, t, J=7.2 Hz), 7.06 (1H, br d, J=7.2 Hz), 7.51 (1H, br d, J=7.2 Hz). MS *m/z*: 287 (M^+). *Anal.* Calcd for $C_{10}H_{10}INO$: C, 41.84; H, 3.51; N, 4.88. Found: C, 41.74; H, 3.50; N, 4.73.

5: mp 142.5—143.5 °C (lit.[10] mp 139.5—140.5 °C, colorless prisms, recrystallized from AcOEt). IR (KBr): 1650, 1581 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.17 (3H, br s), 3.09 (2H, t, J=7.8 Hz), 3.98 (2H, br t, J=7.8 Hz), 7.26—7.48 (2H, m), 7.85 (1H, br d, J=8.4 Hz). MS *m/z*: 287 (M^+). *Anal.* Calcd for $C_{10}H_{10}INO$: C, 41.84; H, 3.51; N, 4.88. Found: C, 42.00; H, 3.52; N, 4.69.

1-Acetyl-2,3-dihydro-7-bromoindole (4b) from 2——A 0.88 M solution of TTFA (17.1 ml, 1.6 mol eq) in TFA was added to a solution of **2** (1.504 g) in TFA (15.0 ml) and stirring was continued at room temperature for 3 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in DMF (20.0 ml). A solution of cupric bromide (8.358 g) in DMF (55.0 ml) was added to the above solution and the whole was stirred at room temperature for 3 h. After evaporation of the solvent under reduced pressure, CH_2Cl_2–MeOH (95 : 5, v/v) and brine were added to the residue and the whole was filtered to remove precipitates. The organic layer was separated, washed with brine, and dried over Na_2SO_4. After evaporation of the solvent, the residue was dissolved in benzene. The resulting benzene solution was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave a crystalline solid, which was purified by column chromatography on SiO_2 with CH_2Cl_2–AcOEt (95 : 5, v/v) as an eluent to give **4b** (1.381 g, 62%). mp 115.0—115.5 °C (lit.[9] mp 116—117 °C, colorless prisms, recrystallized from AcOEt–*n*-hexane). IR (KBr): 1666, 1451, 1383 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.24 (3H, s), 2.99 (2H, t, J=7.2 Hz), 4.13 (2H, t, J=7.2 Hz), 6.79 (1H, t, J=7.2 Hz), 7.06 (1H, br d, J=7.2 Hz), 7.26 (1H, br d, J=7.2 Hz). MS *m/z*: 241, 239 (M^+). *Anal.* Calcd for $C_{10}H_{10}BrNO$: C, 50.02; H, 4.20; N, 5.83. Found: C, 50.30; H, 4.18; N, 5.93.

1-Acetyl-2,3-dihydro-7-chloroindole (4c) from 2——A 0.88 M solution of TTFA (45.0 ml, 2.1 mol eq) in TFA was added to a solution of **2** (3.000 g) in TFA (30.0 ml) and stirring was continued at 45—55 °C for 4 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in DMF (20.0 ml). A solution of cupric chloride (10.178 g) in DMF (100 ml) was added to the above solution and the whole was stirred at 45—55 °C for 4.5 h. After evaporation of the solvent under reduced pressure, CH_2Cl_2–MeOH (95 : 5, v/v) and brine were added to the residue and the whole was filtered to remove precipitates. The organic layer was separated, washed with brine, and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was subjected to column chromatography on SiO_2 with AcOEt–CH_2Cl_2–*n*-hexane (1 : 2 : 5, v/v) as an eluent to give **4c** (1.511 g, 42%). mp 99.0—100.0 °C (colorless prisms, recrystallized from *n*-hexane). IR (KBr): 1666, 1595 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.23 (3H, s), 2.96 (2H, t, J=7.2 Hz), 4.10 (2H, t, J=7.2 Hz), 6.70—7.20 (3H, m). MS *m/z*: 197, 195 (M^+). *Anal.* Calcd for $C_{10}H_{10}ClNO$: C, 61.39; H, 5.15; N, 7.16. Found: C, 61.14; H, 5.11; N, 7.05.

2,3-Dihydro-7-iodoindole (6a) from 4a——A 40% aqueous NaOH solution (2.0 ml) was added to a solution of **4a** (199.7 mg) in MeOH (2.0 ml) and the whole was heated under reflux for 30 min with stirring. After cooling of the reaction mixture, water was added and the whole was extracted with CH_2Cl_2–MeOH (95 : 5, v/v). The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave an oil, which was purified by column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as an eluent to give **6a** (167.0 mg, 98%) as a

colorless oil. IR (film): 3360, 1605, 1571 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.92—3.29 (2H, m), 3.40—3.75 (3H, m, on addition of D_2O, 1H disappeared), 6.30 (1H, t, J=7.4 Hz), 6.91 (1H, brd, J=7.4 Hz), 7.22 (1H, brd, J=7.4 Hz). High resolution MS *m/z*: Calcd for C_8H_8IN: 244.9702. Found: 244.9727.

7-Bromo-2,3-dihydroindole (6b) from 4b——A 40% aqueous NaOH solution (2.0 ml) was added to a solution of **4b** (106.3 mg) in MeOH (2.0 ml) and the whole was heated under reflux for 30 min with stirring. After cooling of the reaction mixture, H_2O was added and the whole was extracted with CH_2Cl_2. The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave an oil, which was purified by column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as an eluent to give **6b** (84.5 mg, 96%) as a colorless oil. IR (film): 3380, 1608, 1575 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.02—3.24 (2H, m, A_2 part of A_2B_2), 3.51—3.72 (2H, m, B_2 part of A_2B_2), 3.70 (1H, brs, disappeared on addition of D_2O), 6.54 (1H, dd, J=8.0, 7.2 Hz), 7.01 (1H, dd, J=7.2, 1.2 Hz), 7.14 (1H, dd, J=8.0, 1.2 Hz). High resolution MS *m/z*: Calcd for C_8H_8BrN: 196.9840 and 198.9821. Found: 196.9857 and 198.9845.

7-Chloro-2,3-dihydroindole (6c) from 4c——A 40% aqueous NaOH solution (1.0 ml) was added to a solution of **4c** (105.9 mg) in MeOH (1.0 ml) and the whole was heated under reflux for 30 min with stirring. After cooling of the reaction mixture, H_2O was added and the whole was extracted with CH_2Cl_2. The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave an oil, which was purified by column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as an eluent to give **6c** (77.6 mg, 93%) as a colorless oil. IR (film): 3380, 1610, 1583 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.70 (1H, brs, disappeared on addition of D_2O), 2.98—3.21 (2H, m, A_2 part of A_2B_2), 3.52—3.73 (2H, m, B_2 part of A_2B_2), 6.60 (1H, t, J=7.7 Hz), 6.92—7.08 (2H, m). High resolution MS *m/z*: Calcd for C_8H_8ClN: 153.0345. Found: 153.0363.

7-Iodoindole (7a) from 6a——Oxygen was bubbled into a solution of **6a** (268.8 mg) and salcomine (36.6 mg) in MeOH (55.0 ml) at room temperature for 5 h with stirring. After evaporation of the solvent under reduced pressure, the residue was subjected to column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 3, v/v) as an eluent to give **7a** (205.3 mg, 77%). mp 55.0—56.0 °C (colorless plates, recrystallized from hexane). IR (KBr): 3390, 1606, 1554 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 6.46 (1H, d, J=3.0 Hz), 6.65 (1H, t, J=7.5 Hz), 7.15 (1H, d, J=3.0 Hz), 7.32 (1H, dd, J=7.5, 1.2 Hz), 7.43 (1H, dd, J=7.5, 1.2 Hz). MS *m/z*: 243 (M^+). *Anal.* Calcd for C_8H_6IN: C, 39.53; H, 2.49; N, 5.76. Found: C, 39.63; H, 2.41; N, 5.52.

7-Bromoindole (7b) from 6b——Oxygen was bubbled into a solution of **6b** (61.1 mg) and salcomine (10.4 mg) in MeOH (12.0 ml) at room temperature for 11 h with stirring. After evaporation of the solvent under reduced pressure, the residue was subjected to p-TLC on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as a developing solvent to give **7b** (44.4 mg, 72%). mp 45.0—45.5 °C (lit.[3a] mp 42—43 °C, colorless prisms, recrystallized from *n*-hexane). IR (KBr): 3400, 1613, 1559 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.63 (1H, dd, J=3.2, 2.2 Hz), 6.99 (1H, t, J=7.7 Hz), 7.25 (1H, dd, J=3.2, 2.2 Hz), 7.35 (1H, dd, J=7.7, 0.7 Hz), 7.58 (1H, brd, J=7.7 Hz), 8.28 (1H, brs). MS *m/z*: 197, 195 (M^+). *Anal.* Calcd for C_8H_6BrN: C, 49.01; H, 3.08; N, 7.14. Found: C, 48.89; H, 2.99; N, 7.01.

7-Chloroindole (7c) from 6c——Oxygen was bubbled into a solution of **6c** (46.7 mg) and salcomine (10.1 mg) in MeOH (10.0 ml) at room temperature for 4 h with stirring. After evaporation of the solvent under reduced pressure, the residue was subjected to p-TLC on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as a developing solvent to give **7c** (32.1 mg, 70%). mp 59.0—59.5 °C (lit.[3b] mp 57—58 °C, colorless plates, recrystallized from *n*-hexane). IR (KBr): 3400, 1620, 1570 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.57 (1H, dd, J=3.3, 2.1 Hz), 7.02 (1H, t, J=7.6 Hz), 7.18 (1H, dd, J=7.6, 1.2 Hz), 7.25 (1H, dd, J=3.3, 2.1 Hz), 7.53 (1H, dd, J=7.6, 1.2 Hz), 8.31 (1H, brs). *Anal.* Calcd for C_8H_6ClN: C, 63.38; H, 3.99; N, 9.24. Found: C, 63.23; H, 3.96; N, 9.23.

3-Chloro-7-iodoindole (8) from 6a——A solution of NCS (3.052 g) in CH_2Cl_2 (50.0 ml) was added to a solution of **6a** (1.397 g) in CH_2Cl_2 (10.0 ml) and NEt_3 (8.0 ml), and the mixture was stirred at room temperature for 1 h. The whole was washed with 2 N HCl, then with brine, and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residual oil was purified by column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as an eluent to give **8** (1.030 g, 65%). mp 58.0—58.5 °C (colorless prisms, recrystallized from CCl_4). IR (KBr): 3380, 1608, 771, 733 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 6.76 (1H, dd, J=8.1, 7.0 Hz), 7.18 (1H, s), 7.40 (1H, dd, J=8.1, 1.3 Hz), 7.42 (1H, dd, J=7.0, 1.3 Hz). MS *m/z*: 279, 277 (M^+). *Anal.* Calcd for C_8H_5ClIN: C, 34.63; H, 1.82; N, 5.05. Found: C, 34.46; H, 1.68; N, 4.99.

3-Chloro-1-(4-chlorobenzoyl)-7-iodoindole (9) from 8——A solution of **8** (198.8 mg) in absolute DMF (2.0 ml) was added to NaH (prepared by washing 50% NaH (41.5 mg, 1.2 mol eq) with absolute benzene) with stirring at room temperature. Stirring was continued for 5 min, then a solution of 4-chlorobenzoyl chloride (164.0 mg) in benzene (1.0 ml) was added and the whole was stirred at room temperature for 14.5 h. Aqueous saturated $NaHCO_3$ was added and the whole was extracted with benzene. The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave a crystalline solid, which was subjected to column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (4 : 6, v/v) as an eluent. From the early fractions, the starting material (52.5 mg, 27%) was recovered. From the later fractions, **9** (185.9 mg, 62%) was obtained. mp 149.0—150.0 °C (colorless prisms, recrystallized from CH_2Cl_2–*n*-hexane). IR (KBr): 1703, 1590 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.98 (1H, t, J=7.4 Hz), 7.12 (1H, s), 7.40 (2H, d, J=8.0 Hz), 7.46—7.64 (1H, m), 7.68—7.89 (1H, m), 7.76 (2H, d, J=8.0 Hz). MS *m/z*: 419, 417, 415 (M^+). *Anal.* Calcd for $C_{15}H_8Cl_2INO$: C, 43.30; H, 1.94; N, 3.37. Found: C, 43.49; H, 1.81; N, 3.31.

2,3-Dihydro-5-iodoindole (10) from 5——A 40% aqueous NaOH solution (1.0 ml) was added to a solution of **5** (27.4 mg) in MeOH (1.0 ml) and the mixture was heated under reflux for 30 min under an argon atmosphere with stirring. After the reaction mixture had cooled, the whole was extracted with CH_2Cl_2. The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave an oil, which was purified by column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1 : 1, v/v) as an eluent to give **10** (20.1 mg, 86%) as a colorless oil. IR (film): 3380, 1600 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.89 (2H, t, J=8.3 Hz), 3.41 (2H, t, J=8.3 Hz), 6.33 (1H, d, J=8.1 Hz), 7.17 (1H, br d, J=8.1 Hz), 7.27 (1H, br s). MS *m/z*: 245 (M^+). High resolution MS *m/z*: Calcd for C_8H_8IN: 244.9702. Found: 244.9754.

5-Iodoindole (11) from 10——Oxygen was bubbled into a solution of **10** (33.4 mg) and salcomine (48.0 mg) in MeOH (6.0 ml) at room temperature for 5 h with stirring. After evaporation of the solvent, the residue was subjected to p-TLC on SiO_2 with CH_2Cl_2–*n*-hexane (7 : 3, v/v) as a developing solvent. Under ultraviolet (UV) light, two bands were detected on the whole luminescent plate. Extraction of the band at *Rf* 0.88—0.77 with CH_2Cl_2–MeOH (95 : 5, v/v) afforded **11** (23.6 mg, 71%). Extraction of the band at *Rf* 0.32—0.24 with the same mixed solvent afforded the starting material (3.1 mg, 9%). **11**: mp 103.0—103.5 °C (colorless prisms, recrystallized from *n*-hexane). IR (film): 3410, 796, 761 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 6.45—6.51 (1H, m), 7.13—7.25 (2H, m), 7.44 (1H, dd, J=8.6, 1.6 Hz), 7.98 (1H, br s), 8.16 (1H, br s). MS *m/z*: 243 (M^+). *Anal.* Calcd for C_8H_6IN: C, 39.53; H, 2.49; N, 5.76. Found: C, 39.60; H, 2.45; N, 5.62.

5-Iodo-3-indolecarbaldehyde (12) from 11——A solution of **11** (23.2 mg) in absolute DMF (0.6 ml) was added to stirred Vilsmeier reagent, prepared by mixing $POCl_3$ (26.7 mg) with ice-cooled absolute DMF (0.5 ml), and stirring was continued at room temperature for 17 h. Ice and H_2O were added and the whole was made alkaline by adding 2 N NaOH, and then extracted with CH_2Cl_2–MeOH (95 : 5, v/v). The extract was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to leave a crystalline solid, which was purified by column chromatography on SiO_2 with CH_2Cl_2–MeOH (95 : 5, v/v) as an eluent to give **12** (23.0 mg, 89%). mp 237.0—238.0 °C (colorless prisms, recrystallized from MeOH). IR (KBr): 3060, 1619 cm^{-1}. ^{1}H-NMR (pyridine-d_5) δ: 7.19 (1H, d, J=8.4 Hz), 7.49 (1H, dd, J=8.4, 1.6 Hz), 7.99 (1H, s), 8.88 (1H, d, J=1.6 Hz), 9.98 (1H, s). MS *m/z*: 271 (M^+). *Anal.* Calcd for C_9H_6INO: C, 39.88; H, 2.23; N, 5.17. Found: C, 39.89; H, 2.21; N, 4.88.

Methyl 3-(1-Acetyl-2,3-dihydroindol-5-yl)acrylate (13) and Methyl 3-(1-Acetyl-2,3-dihydroindol-7-yl)acrylate (14) from 2——A 0.88 M solution of TTFA (1.13 ml, 1.6 mol eq) in TFA was added to a solution of **2** (99.6 mg) in TFA (1.0 ml) and stirring was continued at room temperature for 3 h. After removal of the solvent under reduced pressure, the residue was dissolved in DMF (2.0 ml). $Pd(OAc)_2$ (11.5 mg) and a solution of freshly distilled methyl acrylate (159.4 mg) were added, and the whole was heated at 120 °C for 30 min with stirring. After evaporation of the solvent under reduced pressure, the residue was subjected to column chromatography on SiO_2 with AcOEt–CH_2Cl_2–*n*-hexane (1 : 1 : 2, v/v) as an eluent. From the early fractions, the starting material (32.8 mg, 33%) was recovered. From the later fractions, a mixture of **13** and **14** was obtained. The mixture was separated by p-TLC on SiO_2 with CH_2Cl_2–Et_2O (9 : 1, v/v) as a developing solvent. Under UV light, two bands were detected. Extraction of the upper band at *Rf* 0.61—0.57 with CH_2Cl_2–MeOH (95 : 5, v/v) afforded **14** (5.8 mg, 4%). Extraction of the lower band at *Rf* 0.52—0.44 with the same mixed solvent afforded **13** (3.5 mg, 2%).

13 from 5——A solution of **5** (48.5 mg), freshly distilled methyl acrylate (51.3 mg), $Pd(OAc)_2$ (5.3 mg), and NEt_3 (0.5 ml) in DMF (3.0 ml) was heated at 110 °C for 30 min with stirring. After cooling of the reaction mixture, AcOEt was added and the whole was filtered to remove solid precipitates. The filtrate was washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to leave a crystalline solid, which was purified by p-TLC on SiO_2 with CH_2Cl_2–MeOH (95 : 5, v/v) as a developing solvent to give **13** (39.9 mg, 96%). mp 179.5—180.5 °C (colorless prisms, recrystallized from MeOH). IR (KBr): 1711, 1659, 1628 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.23 (3H, s), 3.19 (2H, t, J=8.3 Hz), 3.78 (3H, s), 4.09 (2H, t, J=8.3 Hz), 6.30 (1H, d, J=15.9 Hz), 7.26—7.41 (2H, br m), 7.61 (1H, d, J=15.9 Hz), 8.16 (1H, br d, J=8.8 Hz). MS *m/z*: 245 (M^+). *Anal.* Calcd for $C_{14}H_{15}NO_3$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.57; H, 6.19; N, 5.70.

14 from 4a——A solution of **4a** (99.1 mg), freshly distilled methyl acrylate (91.2 mg), $Pd(OAc)_2$ (10.2 mg), and NEt_3 (0.5 ml) in DMF (3.0 ml) was heated at 115 °C for 30 min with stirring. After cooling of the reaction mixture, solid precipitates were removed by filtration. The filtrate was concentrated under reduced pressure to leave an oil, which was subjected to p-TLC on SiO_2 with CH_2Cl_2–Et_2O (95 : 5, v/v) as a developing solvent. Under UV light, three dark bands were detected. Extraction of the upper band at *Rf* 0.51—0.40 with CH_2Cl_2–MeOH (95 : 5, v/v) afforded the starting material (59.2 mg, 60%). Extraction of the middle band at *Rf* 0.37—0.31 with the same mixed solvent afforded **2** (7.6 mg, 14%). Extraction of the lower band at *Rf* 0.21—0.14 with the same mixed solvent afforded **14** (4.6 mg, 5%). Compound **14** was identical with the sample prepared by the acetylation of **15**.

14 from Methyl 3-(2,3-Dihydroindol-7-yl)acrylate (15)——Acetic anhydride (0.5 ml) was added to a solution of **15** (20.5 mg) in pyridine (1.0 ml) and the mixture was stirred at room temperature for 17 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in CH_2Cl_2–MeOH (95 : 5, v/v). The solution was washed successively with aqueous saturated $NaHCO_3$ and brine, and dried over Na_2SO_4. Evaporation of the solvent under reduced pressure left an oil, which was purified by p-TLC on SiO_2 with CH_2Cl_2–MeOH (95 : 5, v/v) as a developing solvent to give **14** (23.2 mg, 94%). mp 123.0—124.0 °C (colorless needles, recrystallized from Et_2O–*n*-hexane). IR

(KBr): 1712, 1655, 1626 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.24 (3H, brs), 3.04 (2H, t, J=7.6 Hz), 3.79 (3H, s), 4.17 (2H, t, J=7.6 Hz), 6.31 (1H, d, J=16.1 Hz), 7.10 (1H, t, J=7.3 Hz), 7.24 (1H, br d, J=7.3 Hz), 7.43 (1H, br d, J=7.3 Hz), 7.69 (1H, d, J=16.1 Hz). MS m/z: 245 (M^+). *Anal.* Calcd for $C_{14}H_{15}NO_3$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.60; H, 6.17; N, 5.66.

15 from 6a——A solution of **6a** (53.7 mg), freshly distilled methyl acrylate (58.7 mg), $Pd(OAc)_2$ (5.1 mg), and NEt_3 (0.5 ml) in DMF (3.0 ml) was heated at 120 °C for 30 min with stirring. After cooling, AcOEt was added to the reaction mixture and the whole was filtered to remove solid precipitates. The filtrate was washed with brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was purified by p-TLC on SiO_2 with CH_2Cl_2 as a developing solvent to give **15** (40.0 mg, 90%). mp 86.0—87.0 °C (yellow prisms, recrystallized from ether–*n*-hexane). IR (KBr): 3370, 1690, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.06 (2H, t, J=8.5 Hz), 3.39—4.08 (1H, br s, disappeared on addition of D_2O), 3.64 (2H, t, J=8.5 Hz), 3.79 (3H, s), 6.27 (1H, d, J=16.1 Hz), 6.68 (1H, t, J=7.6 Hz), 7.03—7.21 (2H, m), 7.70 (1H, d, J=16.1 Hz). MS m/z: 203 (M^+). *Anal.* Calcd for $C_{12}H_{13}NO_2$: C, 70.91; H, 6.45; N, 6.89. Found: C, 70.71; H, 6.45; N, 6.76.

4*H*-1,2,5,6-Tetrahydropyrrolo[3,2,1-*ij*]quinoline-4-one (16) from 15——A solution of **15** (795.0 mg) in MeOH (50.0 ml) was hydrogenated over 10% Pd–C (203.2 mg) at room temperature and atmospheric pressure for 1.75 h. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure to leave a crude product, which was purified by column chromatography on SiO_2 with ether as an eluent to give **16** (567.3 mg, 84%). mp 111.0—112.0 °C (lit.[17] mp 112—113 °C, colorless prisms, recrystallized from *n*-hexane). IR (KBr): 1649, 1485, 1394 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 2.63 (2H, t, J=7 Hz), 2.96 (2H, t, J=7 Hz), 3.17 (2H, t, J=8 Hz), 4.00 (2H, t, J=8 Hz), 6.76—7.12 (3H, m). MS m/z: 173 (M^+). *Anal.* Calcd for $C_{11}H_{11}NO$: C, 76.27; H, 6.40; N, 8.09. Found: C, 76.42; H, 6.43; N, 7.88.

Methyl 3-(Indol-7-yl)acrylate (17) from 15——Oxygen was bubbled into a solution of **15** (50.0 mg) and salcomine (8.0 mg) in MeOH (10.0 ml) at room temperature for 4 h with stirring. After evaporation of the solvent under reduced pressure, the residue was subjected to p-TLC on SiO_2 with CH_2Cl_2–*n*-hexane (7:3, v/v) as a developing solvent. Under UV light, two dark bands were detected on the whole luminescent plate. Extraction of the band at *Rf* 0.48—0.22 with CH_2Cl_2–MeOH (95:5, v/v) afforded **17** (35.0 mg, 71%). Extraction of the band at *Rf* 0.19—0.10 with the same mixed solvent afforded the recovered starting material (7.5 mg, 15%). **17**: mp 97.0—98.0 °C (pale yellow prisms, recrystallized from ether–*n*-hexane). Spectral data were identical with those of the authentic sample reported in our previous paper.[5]

Methyl 3-(Indol-7-yl)acrylate (17) from 7a——A solution of **7a** (50.8 mg), freshly distilled methyl acrylate (60.9 mg), $Pd(OAc)_2$ (5.6 mg), and NEt_3 (0.5 ml) in DMF (3.0 ml) was heated at 108 °C for 30 min with stirring. After cooling of the reaction mixture, AcOEt was added and the whole was filtered to remove solid precipitates. The filtrate was washed with brine, dried over Na_2SO_4, and evaporated to leave a crystalline solid, which was subjected to p-TLC on SiO_2 with CH_2Cl_2 as a developing solvent to give **17** (37.7 mg, 90%) as pale yellow prisms. Compound **17** was identical with a sample prepared from 7-indolecarbaldehyde[5] (**19**) by means of the Wittig reaction.

7-Methoxyindole (20) from 7a——A solution of **7a** (36.9 mg) in absolute DMF (3.0 ml) and cuprous iodide (58.4 mg) were added to an absolute methanol solution of sodium methoxide, prepared by dissolving sodium (57.6 mg) in absolute MeOH (1.0 ml). The whole was heated at 120 °C for 1 h with stirring. After evaporation of the solvent under reduced pressure, CH_2Cl_2–MeOH (95:5, v/v) and water were added to the residual oil and the whole was filtered through SiO_2 to remove precipitates. The organic layer was separated, washed with brine, and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, the residue was purified by p-TLC on SiO_2 with CH_2Cl_2–*n*-hexane (2:1, v/v) as a developing solvent to give **20**[19] (16.9 mg, 76%) as a colorless oil. IR (film): 3405, 1628, 1584 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.86 (3H, s), 6.36—6.46 (1H, m), 6.47 (1H, dd, J=7.8, 1.8 Hz), 6.87 (1H, t, J=7.8 Hz), 6.93—7.05 (1H, m), 7.12 (1H, dd, J=7.8, 1.8 Hz), 8.10 (1H, br s). High resolution MS m/z: Calcd for C_9H_9NO: 147.0684. Found: 147.0702.

4-(Indol-7-yl)-2-methyl-3-buten-2-ol (21) from 7a——A mixture of **7a** (200.7 mg), freshly distilled 2-methyl-3-buten-2-ol (213.9 mg), $Pd(OAc)_2$ (18.7 mg), NEt_3 (1.0 ml), and tetra-*n*-butylammonium bromide (53.3 mg) in DMF (5.0 ml) was heated at 100 °C for 30 min with stirring. After cooling of the reaction mixture, AcOEt was added and the whole was filtered to remove solid precipitates. The filtrate was washed with brine, dried over Na_2SO_4, and concentrated to leave an oil, which was subjected to column chromatography on SiO_2 with CH_2Cl_2–*n*-hexane (1:1, v/v) as an eluent. From the early fractions, the starting material (65.8 mg, 33%) was recovered. From the later fractions, **21** (80.3 mg, 48%) was obtained. **21**: mp 123.0—127.0 °C (colorless needles, recrystallized from CH_2Cl_2–*n*-hexane). IR (KBr): 3480, 3240, 1604, 1117 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.44 (6H, s), 6.41 (1H, d, J=3.2 Hz), 6.42 (1H, d, J=16.2 Hz), 6.93 (1H, t, J=7.5 Hz), 6.94 (1H, d, J=16.2 Hz), 7.18 (1H, br d, J=7.5 Hz), 7.19 (1H, d, J=3.2 Hz), 7.40 (1H, dd, J=7.5, 1.3 Hz). *Anal.* Calcd for $C_{13}H_{15}NO$: C, 77.58; H, 7.51; N, 6.96. Found: C, 77.41; H, 7.47; N, 6.94.

4-(2,3-Dihydroindol-7-yl)-2-methyl-3-buten-2-ol (22) from 6a——A mixture of **6a** (2.264 g), freshly distilled 2-methyl-3-buten-2-ol (3.838 g), $Pd(OAc)_2$ (692.0 mg), NEt_3 (20.0 ml), and tetra-*n*-butylammonium bromide (1.896 g) in DMF (20.0 ml) was heated at 100—110 °C for 30 min with stirring. After cooling of the reaction mixture, AcOEt was added and the whole was filtered to remove solid precipitates. The filtrate was washed with brine, dried over Na_2SO_4,

and concentrated to leave an oil, which was subjected to column chromatography on SiO_2 with CH_2Cl_2 and then ether as eluents to give **22** (1.382 g, 74%). mp 85.0—86.5 °C (colorless prisms, recrystallized from ether). IR (KBr): 3290, 1598, 1451 cm^{-1}. ^1H-NMR (CD_3OD) δ: 1.38 (6H, s), 2.96 (2H, t, J=8.0 Hz), 3.46 (2H, t, J=8.0 Hz), 6.16 (1H, d, J=16.0 Hz), 6.52 (1H, d, J=16.0 Hz), 6.60 (1H, t, J=7.5 Hz), 6.91 (1H, brd, J=7.5 Hz), 6.01 (1H, brd, J=7.5 Hz). MS *m/z*: 203 (M^+). *Anal.* Calcd for $C_{13}H_{17}NO$: C, 76.81; H, 8.43; N, 6.89. Found: C, 76.57; H, 8.31; N, 6.92.

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Chemical Transformation of Protoberberines. XIV.[1] Acid-Catalyzed Cleavage of 8-Alkyl-8,14-cycloberbines. A Simple Method for the Preparation of *N*-Unsubstituted Spirobenzylisoquinolines[2]

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On treatment with an acid, 8-alkyl-8,14-cycloberbines (**9**) afforded the *N*-unsubstituted spirobenzylisoquinolines (**10**, **11**, and **12**) through regioselective C_8–N bond cleavage in contrast to the 8-unsubstituted 8,14-cycloberbine (**9d**), which gave the benzindenoazepine (**19**, R=H) through regioselective C_{14}–N bond cleavage. Reduction of **9** with $NaBH_4$ or $LiAlH(OBu^t)_3$ yielded stereoselectively the alcohol (**20** or **21**, respectively) as the main product. Acidic treatment of the isomeric alcohols (**20** and **21**) effected regioselective C_8–N bond cleavage, resulting in the *N*-unsubstituted spirobenzylisoquinolines (**22**—**26**).

Keywords——8-alkyl-8,14-cycloberbine; spirobenzylisoquinoline; regioselective C–N bond cleavage; stereoselective reduction; hydrochloric acid; trifluoroacetic acid; lithium aluminum tri-*tert*-butoxyhydride; sodium borohydride; oxazolidinone; oxazolidine

Berberinephenolbetaines (**2**), derived from protoberberines (**1**) by successive lithium aluminum hydride reduction and oxidation with *m*-chloroperbenzoic acid, are susceptable to photo-induced valence isomerization to produce the unique 8,14-cycloberbines (**3**),[3] which were shown to be key intermediates in the formation of related alkaloids from **1**. For example, the spirobenzylisoquinolines (**4** and **5**) were obtained from **3** (R=H) and the 8-alkyl congeners (**3**, R=alkyl), respectively, through a regioselective C_8–N bond cleavage with ethyl chloroformate. This reaction has been successfully applied to a stereoselective synthesis of (±)-fumaricine (**6**).[4] On the other hand, **3** (R=H) underwent a regioselective C_{14}–N bond fission[5-7] to yield the benzindenoazepines (**7**) on exposure to acid. Benzindenoazepine and rhoeadine alkaloids such as *cis*-alpinigenine (**8**)[7] have been synthesized from **1** according to this method. Thus, it seemed to be of great interest to check whether acid treatment of **3** (R=alkyl) effects either C_8–N or C_{14}–N bond fission leading to spirobenzylisoquinolines or benzindenoazepines, respectively. This paper describes a simple method for the synthesis of *N*-unsubstituted spirobenzylisoquinolines.

The 8-methyl-8,14-cycloberbine (**9a**)[3] was heated in 10% hydrochloric acid at 70—80 °C (method I) for 2 h to furnish the 8-hydroxyspirobenzylisoquinoline (**10a**) accompanied with the unsaturated spirobenzylisoquinoline (**12a**) in 74 and 21% yields, respectively, through a C_8–N bond cleavage. The corresponding benzindenoazepine derived through a C_{14}–N bond fission could not be detected. Similar treatment of the 8-ethyl- and 8-allyl-8,14-cycloberbine (**9b** and **9c**)[3] also gave the 8-hydroxyspirobenzylisoquinolines (**10b**, 80% and **10c**, 56%) along with the unsaturated spirobenzylisoquinolines (**12b**, 17% and **12c**, 33%), respectively (Table I).

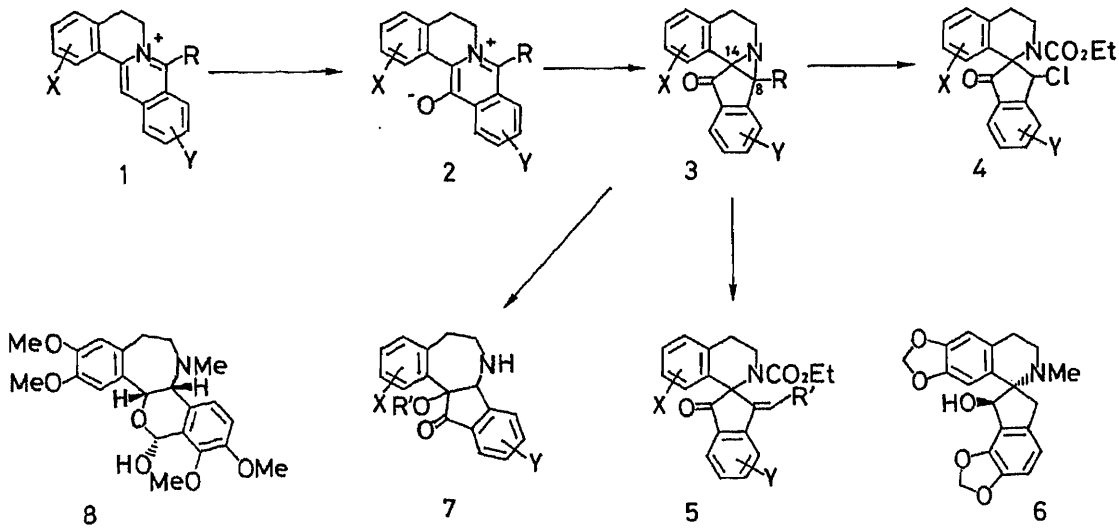

Chart 1

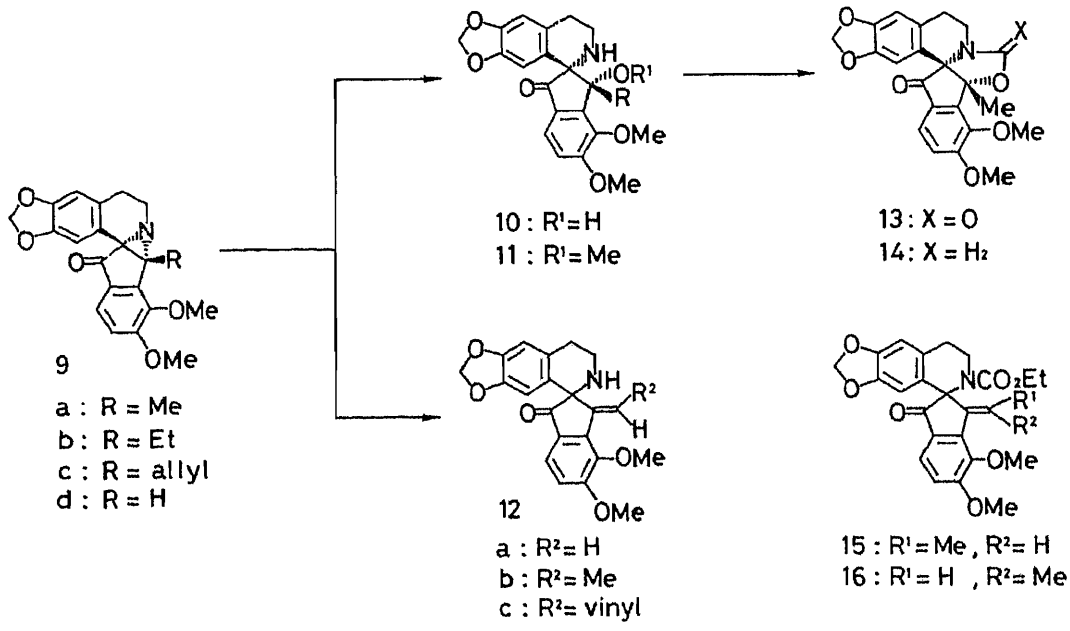

Chart 2

TABLE I. Solvolysis of 8,14-Cycloberbines (**9**)

Compd.	R	Method[a]	Product (yield, %)		
			10	**11**	**12**
9a	Me	I	74		21
9a	Me	II		76	
9b	Et	I	80		17
9b	Et	II		83	12
9c	Allyl	I	56		33
9c	Allyl	II		56	31

a) I, 10% HCl; II, CF_3CO_2H–MeOH.

The spirobenzylisoquinoline structures of these products were apparent from spectral data. The salient feature in the proton nuclear magnetic resonance (^{1}H-NMR) spectra of **10** and **12** is the H-1 signals, which appeared at relatively high field (5.94—6.21 ppm), characteristic of a spirobenzylisoquinoline skeleton.[8] In order to establish the stereochemistry of **10**, the methyl derivative (**10a**) was treated with ethyl chloroformate[4] or formaldehyde[9,10] resulting in the oxazolidinone (**13**, 63%) or the oxazolidine (**14**, 85%), respectively. Their structures were assigned from the spectral data, especially a characteristic band at 1740 cm^{-1} in the infrared (IR) spectrum of **13** and an AB quartet at 4.56 and 4.13 ppm due to the methylene of the oxazolidine ring in the ^{1}H-NMR spectrum of **14**. The *cis* relationship between C_{14}–N and the hydroxy group in **10a** was thus chemically determined. The *Z*-configuration of **12b** and **12c** was confirmed by the following features in the ^{1}H-NMR spectra. The *exo*-olefinic protons of **12b** and **12c** resonated at 7.04 and 7.41 ppm, respectively, and the downfield shift may be attributed to the deshielding effect of the benzene ring (ring D) as well as steric repulsion between the olefinic proton and the methoxy group at the C-9 position. This stereochemistry is well supported by the fact that the chemical shifts of the olefinic protons of **12b** and **12c** are similar to that of **15** (7.09 ppm) rather than that of **16** (5.91 ppm), both of which have established stereochemistry.[3]

When the cycloberbines (**9a**, **b**, and **c**) were stirred in methanol in the presence of a catalytic amount of trifluoroacetic acid at room temperature (method II) for 3.5 h, the 8-methoxyspirobenzylisoquinolines (**11a**, **b**, and **c**) were obtained in 76, 83, and 56% yields, respectively, together with the unsaturated spirobenzylisoquinolines (**12a**, **b**, and **c**) in 0, 12, and 31% yields, respectively. Upon treatment with *p*-toluenesulfonic acid instead of trifluoroacetic acid, **9b** similarly afforded **11b** and **12b** in 75 and 6% yields, respectively. The stereochemistry of **10b**, **10c**, and **11** is probably the same as that of **10a**, assuming an analogous attack of the solvent from the less-hindered side.

Thus, it appeared that acid treatment of the 8-alkyl-8,14-cycloberbines (**9**) effected exclusively C_8–N bond fission leading to the spirobenzylisoquinolines, and these results are contrary to that in the case of 8-unsubstituted 8,14-cycloberbine (**9d**), which gave the benzindenoazepine (**19**, R=H)[5-7] through a C_{14}–N bond cleavage. The introduction of an alkyl group at the C-8 position was found to alter dramatically the regioselectivity in C–N bond cleavage of the aziridine ring.

The above intriguing observation can presumably be interpreted as follows. An S_N1-type cleavage of the aziridine ring with acid produces the carbocation (**17** or **18**). The former cation, leading to the spirobenzylisoquinolines, might be more stable than the latter, leading to the benzindenoazepines, because the latter has a carbonyl group adjacent to the cation.

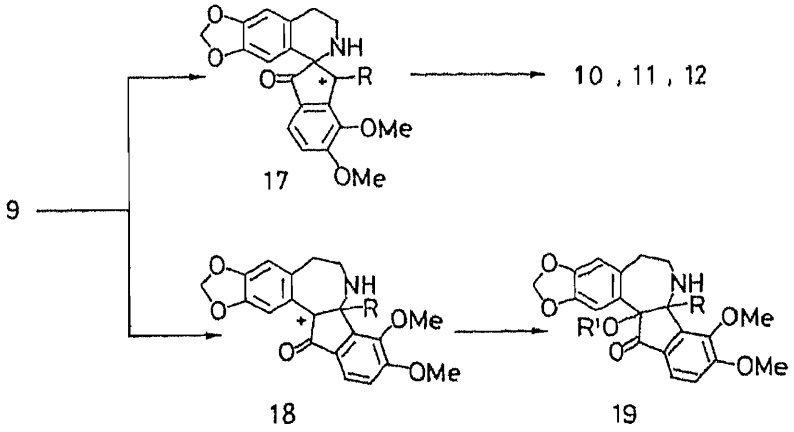

Chart 3

Consequently the reaction proceeded *via* the more stable carbocation (**17**) to the spirobenzylisoquinolines. In the case of the 8-unsubstituted 8,14-cycloberbine (**9d**), however, reaction would proceed *via* the tertiary carbocation (**18**, R=H) rather than the secondary carbocation (**17**, R=H). If this explanation is correct, the carbonyl group adjacent to the carbocation plays a crucial role in determining the reaction pathway. Therefore we next investigated the solvolysis of the 13-hydroxy-8,14-cycloberbines (**20** and **21**).

A solution of **9** in methanol was reduced with sodium borohydride ($NaBH_4$)[4] at room temperature to afford predominantly the alcohol (**20**) accompanied with the diastereoisomeric alcohol (**21**), whereas the latter was obtained as the main product when the reduction was carried out with lithium aluminum tri-*tert*-butoxyhydride [$LiAlH(OBu^t)_3$][11] in tetrahydro-

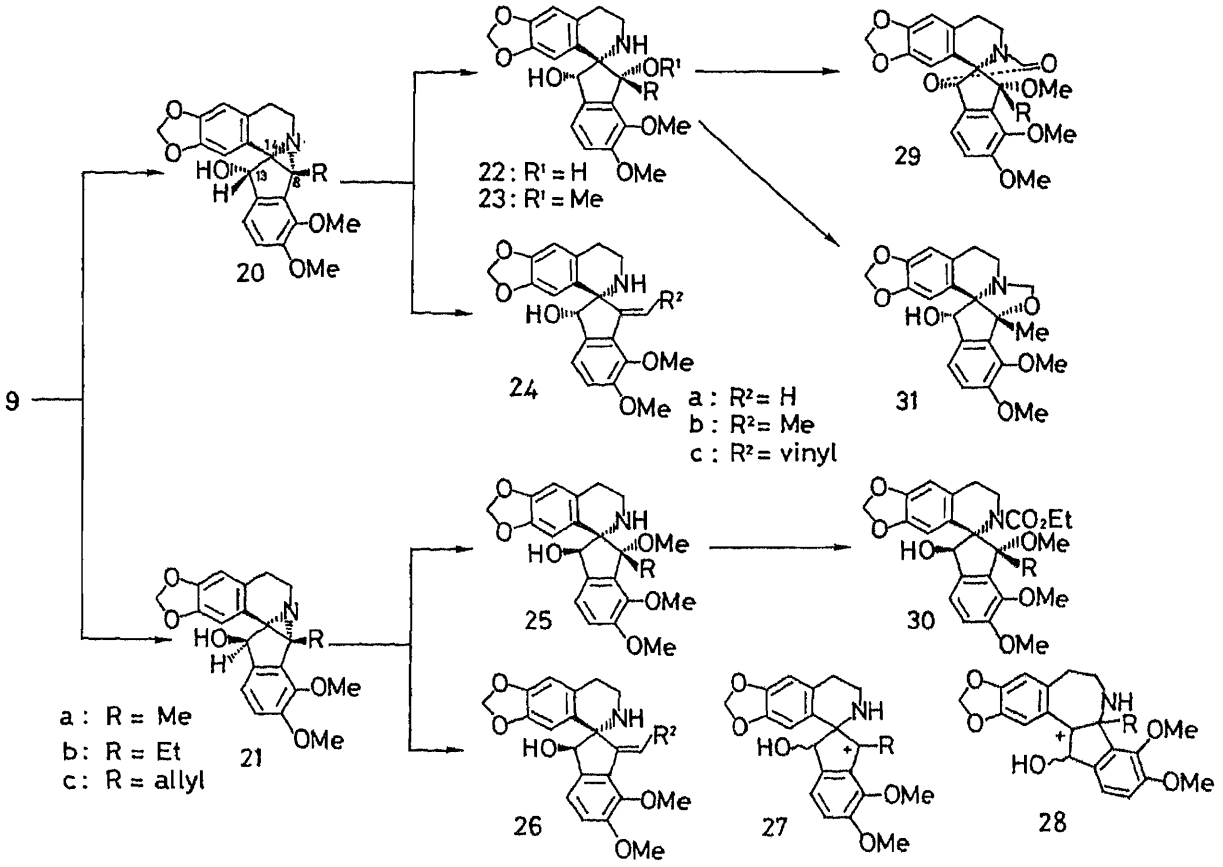

Chart 4

TABLE II. Reduction of 8,14-Cycloberbines (**9**)

Compd.	R	Reagent[a]	Product (**20**)		Product (**21**)		Product ratio (**20**/**21**)	Chemical shift difference[c]
			Yield (%)	H-13[b]	Yield (%)	H-13[b]		
9a	Me	A	71	5.21	13	4.77	5.5/1.0	0.44
9a	Me	B	14		77		1.0/5.5	
9b	Et	A	88	5.22	9	4.74	9.5/1.0	0.48
9b	Et	B	12		88		1.0/7.5	
9c	Allyl	A	80	5.20	12	4.68	6.5/1.0	0.52
9c	Allyl	B	13		85		1.0/6.5	

a) A, $NaBH_4$; B, $LiAlH(OBu^t)_3$. *b*) Chemical shift δ (ppm). *c*) $\Delta\delta$ (**20**−**21**).

furan (THF) at refluxing temperature. The results are summarized in Table II. The stereochemical relationship between the hydroxy group and the C_{14}–N bond in **20** and **21** was clarified from the ^{1}H-NMR spectral data. The C-13 proton signal of **20** appeared at lower field than that of **21**, in the range of 0.44—0.52 ppm, and the downfield shift may be ascribed to the deshielding effect of the benzene ring (ring A). Examination of a molecular model indicated that the C-13 proton of **20**, *cis* to ring A, lies on nearly the same plane as ring A and is strongly deshielded, whereas such a deshielding effect does not occur in **21**. Accordingly the relative stereochemistry of the C_{13}–OH and C_{14}–N bond in **20** is *cis* and that in **21**, *trans*.

The reverse stereoselectivity depending on the reducing agent used can be plausibly rationalized in terms of both steric hindrance and the reactivity of the reagents. In contrast to the case of $NaBH_4$ reduction, in which the hydride attacks the carbonyl group from the sterically less hindered side, producing the alcohol (**20**), less reactive $LiAlH(OBu^t)_3$, that does not react at all at room temperature, predominantly forms at first a complex with the nitrogen of the aziridine ring and then this complex reduces the carbonyl group intramolecularly from the same side as the nitrogen to provide the alcohol (**21**).

Acid cleavage of the 13-hydroxy-8,14-cycloberbines (**20** and **21**) was next investigated. The alcohols (**20** and **21**) were treated with 10% hydrochloric acid (method I) or trifluoroacetic acid in methanol (method II) as described for the reaction of the ketones (**9**) to afford exclusively the spirobenzylisoquinolines (**22**—**26**). The structures of these products were elucidated from spectral evidence, and the yields are summarized in Table III. Exclusive formation of spirobenzylisoquinolines in these reactions can be interpreted in terms of the intermediacy of the tertiary carbocation (**27**) which might still be more stable than the tertiary carbocation (**28**) destabilized by the inductive effect of the vicinal polar hydroxy group.

On treatment with ethyl chloroformate, 8-methoxyspirobenzylisoquinolines (**23**), derived from **20**, gave the oxazolidinones (**29**), which exhibited characteristic bands at 1730—1740 cm^{-1} in their IR spectra. On the other hand, the carbamates (**30**) were obtained on exposure of **25**, derived from **21**, to ethyl chloroformate. The carbamates (**30**) showed absorptions at 1670—1680 cm^{-1} in their IR spectra. These results confirmed unambiguously the aforementioned stereochemistry of the *cis*- and *trans*-alcohols (**20** and **21**). Treatment of **22a** with formaldehyde afforded the oxazolidine (**31**), which was identical with the product obtained from the reaction of **20a** with formaldehyde.[12] The formation of **31** established the

Table III. Solvolysis of 13-Hydroxy-8,14-cycloberbines (**20** and **21**)

Compd.	R	Method[a]	Product (yield, %)				
			22	**23**	**24**	**25**	**26**
20a	Me	I	45		36		
20a	Me	II		71			
20b	Et	I	25		54		
20b	Et	II		83			
20c	Allyl	I	49		22		
20c	Allyl	II		60	16		
21a	Me	I					79
21a	Me	II				70	
21b	Et	I					70
21b	Et	II				68	
21c	Allyl	I					74
21c	Allyl	II				64	

a) I, 10% HCl; II, CF_3CO_2H–MeOH.

TABLE IV. Physical and Spectral Data for Spirobenzylisoquinolines

Compd.	mp (°C) (Solvent)[a]	Formula	Analysis Calcd (Found) C	H	N	IR (cm^{-1}) ($CHCl_3$)	MS *m/z* (%)
10a	173—174 (B–H)	$C_{21}H_{21}NO_6$	65.78 (65.91	5.52 5.64	3.65 3.65)	3300, 1700[c]	383 (M^+, 100), 365 (44), 350 (37), 176 (62)
10b	163—164 (B–H)	$C_{22}H_{23}NO_6$	66.49 (66.61	5.83 5.77	3.52 3.46)	3300, 1700	397 (M^+, 100), 379 (45), 364 (47), 176 (65)
10c	157—159 (I)	$C_{23}H_{23}NO_6$	67.46 (67.66	5.66 5.69	3.42 3.70)	3450, 3300, 1705	409 (M^+, 72), 391 (38), 368 (100), 176 (35)
11a	190—191 (M)	$C_{22}H_{23}NO_6$	66.49 (66.23	5.83 5.84	3.52 3.47)	3350, 1710	397 (M^+, 100), 366 (73), 364 (30), 174 (16)
11b	143—144 (M)	$C_{23}H_{25}NO_6$	67.14 (66.91	6.12 5.92	3.40 3.51)	3400, 1700[c]	411 (M^+, 100), 380 (68), 378 (48)
11c	Amorphous	$C_{24}H_{25}NO_6$	423.1680[b] (423.1680)			1710, 1640	423 (M^+, 100), 392 (38), 382 (87), 367 (57), 352 (3)
12a	152—153 (B–H)	$C_{21}H_{19}NO_5$	69.03 (69.20	5.24 5.23	3.83 3.83)	3400, 1710	365 (M^+, 100), 336 (23), 320 (31), 306 (48), 290 (19), 149 (19)
12b	217—218 (B–H)	$C_{22}H_{21}NO_5$	69.64 (69.90	5.58 5.59	3.69 3.77)	3350, 1680[c]	379 (M^+, 100), 364 (45), 350 (59), 320 (43)
12c	213—214 (M)	$C_{23}H_{21}NO_5$	70.57 (70.42	5.41 5.43	3.58 3.56)	1700, 1630	391 (M^+, 100), 362 (50), 332 (22)
20a	189—190 (M)	$C_{21}H_{21}NO_5$	68.65 (68.46	5.76 5.78	3.81 3.77)	3550	367 (M^+, 47), 350 (45), 338 (21), 308 (100)
20b	197—198 (B–H)	$C_{22}H_{23}NO_5$	69.27 (69.24	6.08 6.01	3.67 3.88)	3300[c]	381 (M^+, 1.9), 364 (100), 334 (35), 320 (5.7)
20c	181—182.5 (B–H)	$C_{23}H_{23}NO_5$	70.21 (70.11	5.89 5.88	3.56 3.63)	3400, 1640	393 (M^+, 3.4), 376 (100), 360 (9.4), 346 (21), 330 (5.7)
21a	186—187 (I)	$C_{21}H_{21}NO_5$	68.65 (68.67	5.76 5.84	3.81 3.83)	3550	368 ($M^+ + 1$, 58),[d] 350 (100)
21b	167—168 (B–H)	$C_{22}H_{23}NO_5$	69.27 (69.23	6.08 6.08	3.67 3.57)	3600	381 (M^+, 2.3), 364 (100), 334 (21)
21c	197—198 (EA–H)	$C_{23}H_{23}NO_5$	70.21 (70.34	5.89 5.92	3.56 3.77)	3500, 3350, 1640	393 (M^+, 3.9), 376 (100), 360 (8.5), 346 (21), 344 (5.4), 330 (5.3)
22a	181.5—182.5 (B–H)	$C_{21}H_{23}NO_6$	65.44 (65.52	6.02 6.02	3.63 3.64)	3550, 3300	385 (M^+, 1.5), 367 (100), 352 (64), 338 (16), 308 (57)
22b	156—157 (M)	$C_{22}H_{25}NO_6$	66.15 (65.94	6.31 6.30	3.51 3.56)	3350	399 (M^+, 39), 381 (82), 176 (100)
22c	173.5—175 (M)	$C_{23}H_{25}NO_6$	67.14 (67.27	6.12 6.09	3.40 3.51)	3550, 3350, 1630	411 (M^+, 6.9), 393 (99), 364 (35), 352 (97), 189 (99), 176 (100)
23a	139—141 (I)	$C_{22}H_{25}NO_6$	66.15 (65.85	6.31 6.17	3.51 3.39)	3300	399 (M^+, 15), 367 (50), 352 (42), 338 (20), 308 (100), 176 (19)
23b	83—85 (M)	$C_{23}H_{27}NO_6 \cdot$ MeOH (64.70	7.01	3.14		3500, 3400[c]	413 (M^+, 14), 381 (84), 364 (24), 352 (100), 322 (93), 189 (18), 176 (37)
23c	62—64 (M)	$C_{24}H_{27}NO_6 \cdot$ MeOH (65.72	6.83	3.06		3350, 1640	425 (M^+, 15), 393 (35), 364 (18), 352 (100), 334 (16), 189 (51), 176 (35)
24a	192—194 (A)	$C_{21}H_{21}NO_5$	68.65 (68.54	5.76 5.82	3.81 3.96)	3300, 1635[c]	367 (M^+, 38), 338 (20), 308 (100)
24b	142—144 (A)	$C_{22}H_{23}NO_5 \cdot H_2O$ (66.15	6.31	3.51		3350	381 (M^+, 100), 366 (40), 321 (68)
24c	186.5—188 (M–E)	$C_{23}H_{23}NO_5 \cdot$ 1/2 MeOH (68.73	6.15	3.42		3350, 1620	393 (M^+, 100), 364 (30), 352 (50), 334 (23), 189 (98), 176 (33)
25a	144.5—145.5 (I)	$C_{22}H_{25}NO_6 \cdot H_2O$ (64.32	6.34	3.26		3550, 3300	399 (M^+, 13), 367 (47), 352 (41), 328 (21), 308 (100), 176 (18)
25b	139—140 (I)	$C_{23}H_{27}NO_6$	66.81 (66.77	6.58 6.52	3.39 3.28)	3550, 3350	413 (M^+, 13), 381 (70), 364 (15), 352 (96), 322 (100), 189 (18), 176 (40)

TABLE IV. (continued)

Compd.	mp (°C) (Solvent)[a]	Formula	Analysis Calcd (Found) C H N	IR (cm^{-1}) ($CHCl_3$)	MS *m/z* (%)
25c	143.5—145 (M–I)	$C_{24}H_{27}NO_6$	67.75 6.40 3.29 (67.47 6.51 3.29)	3550, 3350, 1660	425 (M^+, 11), 393 (79), 352 (100), 176 (29)
26a	Amorphous	$C_{21}H_{21}NO_5$	367.1418[b] (367.1423)	3550, 3300, 1635	367 (M^+, 49), 338 (21), 308 (100)
26b	98—100 (A)	$C_{22}H_{23}NO_5$	381.1574[b] (381.1600)	3550	381 (M^+, 100), 364 (45), 322 (76), 189 (26), 176 (25)
26c	97—99 (M)	$C_{23}H_{23}NO_5$	70.21 5.89 3.56 (70.09 5.75 3.69)	3550, 3300, 1625	393 (M^+, 2.6), 376 (100), 346 (68)

a) A, ethanol; B, benzene; E, ethyl ether; EA, ethyl acetate; H, hexane; I, isopropyl ether; M, methanol. *b*) High-resolution MS. *c*) KBr. *d*) Chemical ionization MS.

cis relationship between C_{14}–N and the hydroxy group at C-8 in **22a** and suggested the same stereochemistry at C-8 of the other products (**22**, **23**, and **25**), as depicted.

Thus, we have found that the 8-alkyl-8,14-cycloberbines (**9**) and their 13-hydroxy derivatives (**20** and **21**) undergo exclusive C_8–N bond cleavage on acid treatment to give the *N*-unsubstituted spirobenzylisoquinolines, which could be led to various modified derivatives by further elaboration. In combination with an easy preparation of the cycloberbines, this simple transformation reaction provides an efficient method for the synthesis of *N*-unsubstituted spirobenzylisoquinolines possessing substituents at C-8 and C-13 on the five-membered ring from protoberberines.

Experimental

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

General Procedure for Reaction of the 8-Alkyl-8,14-cycloberbines (9, 20, and 21) with 10% Hydrochloric Acid A solution of the cycloberbine (**9**, **20**, or **21**; 1.2 mmol) in 10% hydrochloric acid (60 ml) was heated at 70—80 °C for 2 h. After cooling, the reaction mixture was made alkaline with solid potassium carbonate, and then extracted with methylene chloride. The extract was washed with water and brine, dried, and concentrated. Chromatography of the residue on silica gel with ethyl acetate–benzene (1 : 1) (in the case of **9**) or ethyl acetate (in the case of **20** and **21**) gave the products. The results and the physical data of the products are summarized in Tables I and III—V.

General Procedure for Reaction of the 8-Alkyl-8,14-cycloberbines (9, 20, and 21) with Trifluoroacetic Acid in Methanol——A solution of the cycloberbine (**9**, **20**, or **21**; 0.6 mmol) in methanol (10 ml) was stirred for 3.5 h in the presence of trifluoroacetic acid (2 drops) at room temperature. Methanol was evaporated off and the residue was made alkaline with 10% aq. potassium carbonate, and then extracted with methylene chloride. The extract was washed with water and brine, dried, and concentrated. Chromatography of the residue on alumina with methylene chloride–benzene (2 : 1) (in the case of **9**) or on silica gel with ethyl acetate (in the case of **20** and **21**) gave the products. The results and the physical data of the products are summarized in Tables I and III—V.

General Procedure for Reduction of 9 with $NaBH_4$——$NaBH_4$ (20 mmol) was added portionwise to a solution of **9** (4 mmol) in methanol (60 ml) and the reaction mixture was stirred for 1 h at room temperature. After evaporation of the methanol, water was added to the residue and the mixture was extracted with methylene chloride. The extract was washed with water and brine, dried, and concentrated. Chromatography of the residue on alumina with ethyl acetate–hexane (3 : 1) gave the alcohols (**20** and **21**). The results and the physical data of the products are summarized in Tables II, IV, and V.

General Procedure for Reduction of 9 with $LiAlH(OBu^t)_3$——$LiAlH(OBu^t)_3$ (10 mmol) was added to a solution of **9** (1 mmol) in dry THF (100 ml) and the reaction mixture was heated under reflux for 1 h, then allowed to cool. Water

TABLE V. ^{1}H-NMR Spectral Data for Spirobenzylisoquinolines

Compd.	Chemical shift (δ: ppm, J in Hz, $CDCl_3$)							
	H-1	H-4	H-11	H-12	H-13	OCH_2O	OMe	Others
10a	5.94 s	6.58 s	7.07 (AB-q, J=9)	7.61	—	5.84, 5.80 (AB-q, J=2)	4.00 s 3.98 s	1.64 s (3H)
10b	6.04 s	6.58 s	7.09 (AB-q, J=8)	7.59	—	5.86, 5.81 (AB-q, J=2)	3.99 s 3.96 s	1.92 q (2H, J=7) 0.72 t (3H, J=7)
10c	6.00 s	6.53 s	7.08 (AB-q, J=9)	7.60	—	5.83, 5.80 (AB-q, J=2)	4.00 s 3.98 s	4.92—4.36 m (2H)
11a	5.75 s	6.58 s	7.12 (AB-q, J=9)	7.66	—	5.81, 5.77 (AB-q, J=1)	4.02 s 3.90 s 3.13 s	1.56 s (3H)
11b	5.85 s	6.58 s	7.12 (AB-q, J=8)	7.62	—	5.83, 5.78 (AB-q, J=2)	4.01 s 3.91 s 3.09 s	1.87 q (2H, J=7) 0.85 t (3H, J=7)
11c	5.83 s	6.54 s	7.13 (AB-q, J=9)	7.64	—	5.80, 5.78 (AB-q, J=2)	4.02 s 3.93 s 3.09 s	6.16—5.72 m (1H) 5.04—4.55 m (2H)
12a	6.12 s	6.60 s	7.05 (AB-q, J=9)	7.61	—	5.85, 5.81 (AB-q, J=2)	3.99 s 3.94 s	6.38 s (1H) 5.38 s (1H)
12b	6.21 s	6.62 s	6.98 (AB-q, J=8)	7.57	—	5.86, 5.81 (AB-q, J=1)	3.98 s 3.90 s	7.04 q (1H, J=7) 1.63 d (3H, J=7)
12c	6.16 s	6.63 s	7.01 (AB-q, J=9)	7.57	—	5.84, 5.80 (AB-q, J=2)	3.98 s 3.94 s	7.41 d (1H, J=10) 6.72—6.31 m (1H) 5.43—5.12 m (2H)
20a	6.74 s	6.64 s	6.82 (AB-q, J=8)	7.09	5.21 brs	5.93 s	3.88 s 3.86 s	1.50 s (3H)
20b	6.78 s	6.65 s	6.87 (AB-q, J=8)	7.11	5.22 d[a] (J=12)	5.96, 5.94 (AB-q, J=2)	3.90 s 3.88 s	1.30 q (2H, J=7) 0.99 t (3H, J=7)
20c	6.74 s	6.64 s	6.84 (AB-q, J=8)	7.08	5.20 d[a] (J=12)	5.94, 5.92 (AB-q, J=1.5)	3.89 s 3.85 s	5.97—5.67 m (1H) 5.32—4.80 m (2H)
21a	7.00 s	6.67 s	6.81 (AB-q, J=9)	7.14	4.77 s	5.93, 5.91 (AB-q, J=2)	3.86 s 3.85 s	1.50 s (3H)
21b	7.07 s	6.68 s	6.86 (AB-q, J=8)	7.14	4.74 s	5.94, 5.92 (AB-q, J=2)	3.94 s 3.92 s	1.25 q (2H, J=9) 1.04 t (3H, J=9)
21c	7.03 s	6.67 s	6.82 (AB-q, J=9)	7.12	4.68 s	5.91, 5.89 (AB-q, J=2)	3.87 s 3.85 s	6.14—5.66 m (1H) 5.12—4.87 m (2H)
22a	5.93 s	6.55 s	6.96 (AB-q, J=8)	7.25	4.56 s	5.80 s	3.90 s 3.89 s	1.34 s (3H)
22b	6.24 s	6.58 s	6.97 (AB-q, J=8)	7.21	4.70 s	5.84, 5.83 (AB-q, J=1)	3.90 s (6H)	2.26 q (2H, J=8) 0.77 t (3H, J=8)
22c	6.12 s	6.54 s	6.98 (AB-q, J=9)	7.24	4.64 s	5.85, 5.82 (AB-q, J=2)	3.90 s (6H)	5.92—5.48 m (1H) 5.00—4.72 m (2H)
23a	5.80 s	6.55 s	7.02 (AB-q, J=9)	7.34	4.59 s	5.80 s	3.92 s 3.85 s 3.13 s	1.35 s (3H)
23b	5.90 s	6.53 s	7.02 (AB-q, J=8)	7.28	4.50 s	5.83, 5.82 (AB-q, J=2)	3.92 s 3.86 s 3.13 s	1.63 q (2H, J=7) 0.81 t (1H, J=7)
23c	5.87 s	6.49 s	7.04 (AB-q, J=9)	7.30	4.47 s	5.80, 5.79 (AB-q, J=2)	3.92 s 3.88 s 3.14 s	6.00—5.60 m (1H) 4.94—4.48 m (2H)
24a	6.06 s	6.56 s	6.95 (AB-q, J=9)	7.22	4.98 s	5.87, 5.86 (AB-q, J=1)	3.89 s 3.88 s	6.48 s (1H) 5.01 s (1H)
24b	6.44 s	6.55 s	6.89 (AB-q, J=8)	7.16	4.67 s	5.88, 5.85 (AB-q, J=1)	3.88 s 3.84 s	6.86 q (1H, J=8) 1.57 d (3H, J=8)

TABLE V. (continued)

Compd.	Chemical shift (δ: ppm, J in Hz, $CDCl_3$)							
	H-1	H-4	H-11	H-12	H-13	OCH_2O	OMe	Others
24c	6.41 s	6.57 s	6.92 (AB-q, J=8)	7.18	4.72 s	5.85, 5.84 (AB-q, J=1)	3.89 s (6H)	7.33 d (1H, J=12) 6.51—6.08 m (1H) 5.39—4.98 m (2H)
25a	5.74 s	6.58 s	6.96 (AB-q, J=9)	7.13	5.08 s	5.79, 5.78 (AB-q, J=2)	3.91 s 3.84 s 3.20 s	1.50 s (3H)
25b	5.80 s	6.60 s	6.98 (AB-q, J=8)	7.08	5.14 s	5.82, 5.79 (AB-q, J=2)	3.91 s 3.88 s 3.16 s	1.82 q (2H, J=7) 0.82 t (3H, J=7)
25c	5.79 s	6.58 s	6.99 (AB-q, J=8)	7.14	5.26 br s	5.84, 5.82 (AB-q, J=2)	3.92 s 3.90 s 3.22 s	6.12—5.82 m (1H) 5.04—4.56 m (2H)
26a	6.16 s	6.59 s	6.93 (AB-q, J=9)	7.17	4.93 s	5.86, 5.84 (AB-q, J=2)	3.89 s 3.88 s	6.41 s (1H) 5.17 s (1H)
26b	6.44 s	6.60 s	6.91 (AB-q, J=8)	7.15	4.91 s	5.86, 5.85 (AB-q, J=1)	3.89 s 3.84 s	6.85 q (1H, J=8) 1.50 d (3H, J=8)
26c	6.35 s	6.61 s	6.91 (AB-q, J=9)	7.16	4.93 s	5.85 s	3.89 s (6H)	7.30 d (1H, J=11) 6.50—6.16 m (1H) 5.36—4.92 m (2H)

a) Changed to s on addition of D_2O.

was added, and the precipitates were filtered off. The filtrate was concentrated to leave the residue, which was taken up in methylene chloride. The methylene chloride solution was washed with water and brine, dried, and concentrated. Chromatography of the residue on alumina with ethyl acetate–hexane (3 : 1) gave the alcohols (**20** and **21**). The results and the physical data of the products are summarized in Tables II, IV, and V.

General Procedure for Reaction of the Spirobenzylisoquinolines (10, 23, and 25) with Ethyl Chloroformate—A solution of the spirobenzylisoquinoline (**10a**, **23**, or **25**; 0.15 mmol) and ethyl chloroformate (0.75 mmol) in chloroform (5 ml) was refluxed for 5 h (in the case of **10a**, 26 h) in the presence of trimethylamine (0.75 mmol). After cooling, the solution was washed with 10% aq. potassium carbonate, water, and brine, dried, and concentrated. Chromatography of the residue on silica gel with ethyl acetate–benzene (1 : 2 or 1 : 3) gave the product.

rel-(8*R*,14*R*)-9,10-Dimethoxy-8-methyl-2,3-methylenedioxy-13-oxonorochotensane-7,8-carbolactone[13] (**13**): 63%. mp 234—236 °C (EtOH). IR ν_{max} cm^{-1}: 1740 (C=O), 1710 (C=O). ^{1}H-NMR δ: 7.90, 7.00 (2H, AB-q, J=8.5 Hz, H-12 and H-11), 6.67 (1H, s, H-4), 6.06 (1H, s, H-1), 5.92, 5.89 (2H, AB-q, J=1 Hz, OCH_2O), 4.02, 4.00 (each 3H, each s, OMe×2), 1.74 (3H, s, Me). MS *m/z* (%): 409 (M^+, 31), 365 (100), 350 (84), 320 (57), 306 (15). *Anal.* Calcd for $C_{22}H_{19}NO_7$: C, 64.54; H, 4.68; N, 3.42. Found: C, 64.75; H, 4.66; N, 3.31.

rel-(8*R*,13*S*,14*S*)-8,9,10-Trimethoxy-8-methyl-2,3-methylenedioxynorochotensane-7,13-carbolactone (**29a**): 78%. mp 151—153 °C (MeOH). IR ν_{max} cm^{-1}: 1740 (C=O). ^{1}H-NMR δ: 7.33, 7.06 (2H, AB-q, J=9 Hz, H-12 and H-11), 6.64 (1H, s, H-4), 5.99 (1H, s, H-1), 5.88 (2H, s, OCH_2O), 5.35 (1H, s, H-13), 3.94, 3.88, 3.16 (each 3H, each s, OMe×3), 1.40 (3H, s, Me). MS *m/z* (%): 425 (M^+, 36), 350 (100), 320 (13). High-resolution mass calcd for $C_{23}H_{23}NO_7$: 425.1473. Found: 425.1446.

rel-(8*R*,13*S*,14*S*)-8-Ethyl-8,9,10-trimethoxy-2,3-methylenedioxynorochotensane-7,13-carbolactone (**29b**): 79%. mp 145—147 °C (MeOH). IR ν_{max} cm^{-1}: 1730 (C=O). ^{1}H-NMR δ: 7.31, 6.97 (2H, AB-q, J=8 Hz, H-12 and H-11), 6.55 (1H, s, H-4), 5.81, 5.79 (2H, AB-q, J=2 Hz, OCH_2O), 5.77, 5.71 (each, 1H, each s, H-1 and H-13), 3.93, 3.87, 3.20 (each 3H, each s, OMe×3), 1.68 (2H, q, J=7 Hz, CH_2CH_3), 0.90 (3H, J=7 Hz, CH_2CH_3). MS *m/z* (%): 439 (M^+, 5.6), 380 (19), 366 (61), 364 (100). *Anal.* Calcd for $C_{24}H_{25}NO_7 \cdot 1/2MeOH$: C, 64.60; H, 5.98; N, 3.07. Found: C, 64.83; H, 6.12; N, 3.00.

rel-(8*R*,13*S*,14*S*)-8-Allyl-8,9,10-trimethoxy-2,3-methylenedioxynorochotensane-7,13-carbolactone (**29c**): 82%. mp 195—198 °C (MeOH). IR ν_{max} cm^{-1}: 1730 (C=O). ^{1}H-NMR δ: 7.31, 7.10 (2H, AB-q, J=8 Hz, H-12 and H-11), 6.55 (1H, s, H-4), 6.01 (1H, s, H-1), 5.89, 5.88 (2H, AB-q, J=2 Hz, OCH_2O), 5.80—5.38 (1H, m, $\overset{H}{>}$=), 5.25 (1H, s, H-13), 5.02—4.70 (2H, m, $=\overset{H}{<}_{H}$), 3.95, 3.91, 3.16 (each 3H, each s, OMe×3). MS *m/z* (%): 451 (M^+, 30), 392 (29),

376 (26), 366 (100), 351 (29), 336 (37). *Anal.* Calcd for $C_{25}H_{25}NO_7$: C, 66.51; H, 5.58; N, 3.10. Found: C, 66.59; H, 5.56; N, 3.08.

rel-(8*R*,13*R*,14*S*)-7-Ethoxycarbonyl-13-hydroxy-8,9,10-trimethoxy-8-methyl-2,3-methylenedioxynorochotensane (**30a**): 61%. mp 169—170 °C (MeOH). IR ν_{max} cm^{-1}: 3450 (OH), 1670 (C=O). ^{1}H-NMR δ: 7.28, 7.06 (2H, AB-q, J=8 Hz, H-12 and H-11), 6.53 (1H, s, H-4), 6.40 (1H, s, H-1), 6.00 (1H, brs, H-13), 5.80 (2H, s, OCH_2O), 5.02 (1H, brs, OH), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$). 3.90, 3.81, 3.16 (each 3H, each s, OMe×3), 1.44 (3H, s, Me), 1.30 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$). MS *m/z* (%): 439 (M^+ −MeOH, 100), 366 (30), 350 (21). *Anal.* Calcd for $C_{26}H_{29}NO_8$: C, 64.58; H, 6.05; N, 2.90. Found: C, 64.79; H, 6.11; N, 2.99.

rel-(8*R*,13*R*,14*S*)-7-Ethoxycarbonyl-8-ethyl-13-hydroxy-8,9,10-trimethoxy-2,3-methylenedioxynorochotensane (**30b**): 62%. mp 148—149 °C (MeOH). IR ν_{max} cm^{-1}: 3450 (OH), 1675 (C=O). ^{1}H-NMR δ: 7.31, 7.07 (2H, AB-q, J= 8 Hz, H-12 and H-11), 6.53 (1H, s, H-4), 6.50 (1H, s, H-1), 5.97 (1H, d, J=4 Hz, H-13), 5.79 (2H, s, OCH_2O), 4.97 (1H, d, J=4 Hz, OH), 4.14 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 3.90, 3.82, 3.11 (each 3H, each s, OMe×3), 1.29 (3H, t, J= 7 Hz, $OCH_2C\underline{H}_3$), 0.31 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$). MS *m/z* (%): 453 (M^+ −MeOH, 76), 435 (50), 380 (100), 335 (24), 248 (38). *Anal.* Calcd for $C_{26}H_{31}NO_8$: C, 64.31; H, 6.44; N, 2.89. Found: C, 64.57; H, 6.20; N, 3.00.

rel-(8*R*,13*R*,14*S*)-8-Allyl-7-ethoxycarbonyl-13-hydroxy-8,9,10-trimethoxy-2,3-methylenedioxynorochotensane (**30c**): 60%. mp 154—155 °C (MeOH). IR ν_{max} cm^{-1}: 3450 (OH), 1670 (C=O). ^{1}H-NMR δ: 7.31, 7.10 (2H, AB-q, J= 7 Hz, H-12 and H-11), 6.52 (1H, s, H-4), 6.43 (1H, s, H-1), 5.99 (1H, d, J=4 Hz, H-13), 5.77 (2H, brs, OCH_2O), 5.52—5.06 (1H, m, $\overset{H}{\diagdown}\!=$), 5.00 (1H, d, J=4 Hz, OH), 4.50—4.09 (2H, m, $=\!\!<^{H}_{H}$), 4.16 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.90, 3.83, 3.13 (each 3H, each s, OMe×3), 1.32 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$). MS *m/z*: (%): 465 (M^+ −MeOH, 100), 392 (36), 352 (15), 336 (19), 248 (81). *Anal.* Calcd for $C_{27}H_{31}NO_8$: C, 65.18; H, 6.28; N, 2.82. Found: C, 65.23; H, 6.24; N, 2.98.

***rel*-(8*R*,14*R*)-8,7-Epoxymethano-9,10-dimethoxy-8-methyl-2,3-methylenedioxynorochotensan-13-one (14)**——Aqueous formaldehyde solution (38%, 3 ml) was added to a solution of **10a** (74 mg, 0.2 mmol) in MeOH (10 ml) and the mixture was refluxed for 30 min. The precipitate was collected by filtration and recrystallized from ethyl acetate to give **14** (61 mg, 85%). mp 256—258 °C. IR ν_{max} cm^{-1}: 1710 (C=O). ^{1}H-NMR δ: 7.64, 7.13 (2H, AB-q, J=8.5 Hz, H-12 and H-11), 6.64 (1H, s, H-4), 5.99 (1H, s, H-1), 5.89, 5.84 (2H, AB-q, J=1.5 Hz, OCH_2O), 4.56, 4.13 (2H, AB-q, J=8 Hz, NCH_2O), 4.00, 3.98 (each 3H, each s, OMe×2), 1.72 (3H, s, Me). MS *m/z* (%): 395 (M^+, 63), 366 (53), 352 (100), 324 (64). *Anal.* Calcd for $C_{22}H_{21}NO_6$: C, 66.84; H, 5.32; N, 3.54. Found: C, 66.47; H, 5.18; N, 3.56.

***rel*-(8*R*,13*S*,14*S*)-8,7-Epoxymethano-13-hydroxy-9,10-dimethoxy-8-methyl-2,3-methylenedioxynorochotensane (31)**——Aqueous formaldehyde solution (38%, 3 ml) was added to a solution of **22a** (70 mg, 0.2 mmol) in MeOH (10 ml), and the mixture was refluxed for 1 h and concentrated. The residue was made alkaline with sat. aq. potassium carbonate and extracted with methylene chloride. The extract was washed with water, dried, and concentrated. Chromatography of the residue on alumina with ethyl acetate–methylene chloride (1 : 4) gave **31** (60 mg, 83%). mp 153—155 °C (MeOH) (lit.[12] 155—157 °C). The product was identical with an authentic sample in IR and NMR spectra and thin-layer chromatographic behavior.

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Studies on Diazepines. XXV.[1] Syntheses of Fully Unsaturated 1,4-Oxazepines and 1*H*-1,4-Diazepines Using Photochemical Valence Isomerization of Tricycloheptene Systems

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3-Azatricyclo[4.1.0.0^{2,5}]hept-3-ene derivatives prepared from pyridines *via* five steps were found to be useful synthons for fully unsaturated monocyclic seven-membered heterocyclic rings. Photolysis of the 7-oxa derivatives **15** resulted in valence isomerization with ring opening to give the 1,4-oxazepines (**19**), which are the first examples of fully conjugated 1,4-oxazepines. Similarly, the 7-aza derivatives **16**, upon irradiation, afforded the novel 1*H*-1,4-diazepines (**20**).

The 2,7-dimethyl-1,4-oxazepine (**19c**) and 2,7-dimethyl-1,4-diazepine (**20c**) underwent thermal ring conversion, giving rise to the 1,3-oxazepine (**22**) and 1,3-diazepine (**24**), respectively.

Keywords——1,4-oxazepine; 1,4-diazepine; 7-oxa-3-azatricyclo[4.1.0.0^{2,5}]hept-3-ene; 3,7-diazatricyclo[4.1.0.0^{2,5}]hept-3-ene; photolysis; valence isomerization; thermolysis; 1,3-oxazepine; 1,3-diazepine

There is considerable current interest in the synthesis of new seven-membered rings with two heteroatoms.[2] We have already reported the syntheses of 1,2-,[2d,3] 1,3-,[2e,4] 2,3-,[5] and 2,4-benzodiazepines,[6] and related fused diazepines condensed with aromatic heterocyclic rings. With regard to fully unsaturated monocyclic compounds, diheteroepines, 1,2-diazepines (**1**)[2c] have been prepared mainly by the photochemical rearrangement of pyridine *N*-imides, and 1,3-diazepines (**2**)[2e,7] can be synthesized from 1,2-diazepines by thermal isomerization, but as for 1,4-diazepines, only highly substituted 6*H*-1,4-diazepines[8] had been reported prior to the present work. On the other hand, 1,3-oxazepines (**3**) can be prepared from pyridine *N*-oxides,[9] pyrylium salts,[10] or 2-oxa-3-azabicyclo[3.2.0]hept-3,6-dienes.[11] However, 1,4-oxazepines (**4a**) have not been reported, although perhydro derivatives[2b] are known.

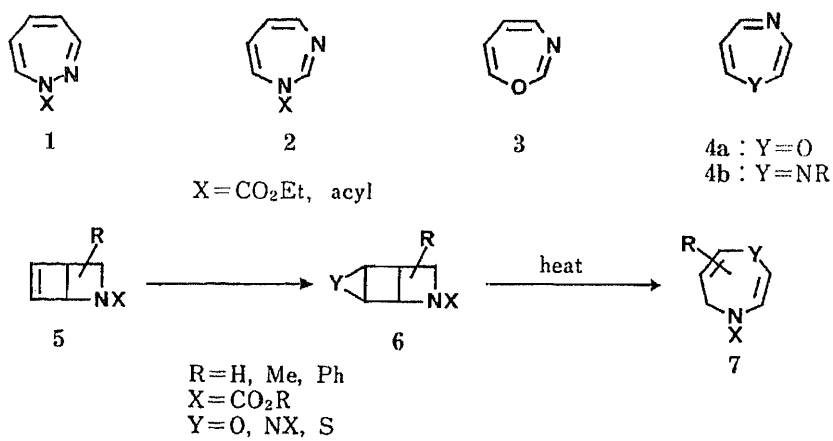


Chart 1

Therefore, we were interested in the synthesis of 1,4-dihetero seven-membered rings, and have recently shown[12] that the highly strained tricyclic compounds **6**, prepared readily from pyridines *via* the azabicyclohexanes (**5**), can be used as new useful synthons for novel seven-membered heterocyclic rings such as the dihydro-1,4-diheteroepines (**7**). We report here the syntheses of the first examples of fully conjugated 1,4-oxazepines[13] and 1*H*-1,4-diazepines by photo-induced valence isomerization of 3-azatricyclo[4.1.0.$0^{2,5}$]hept-3-enes with a nitrogen or an oxygen atom in the 7-position.

The synthetic route to the key tricyclic compounds **15** and **16** used in the present reaction is shown in Chart 2. 3-Benzyloxycarbonyl-4-phenyl-7-oxa-3-azatricyclo[4.1.0.$0^{2,5}$]heptanes (**11a, b**) and their 7-aza analogues **12a, b** were prepared from the corresponding pyridines (**8**) *via* the dihydropyridines (**9**) and 2-azabicyclo[2.2.0]hex-5-enes (**10**) by the reported method.[12] The new compounds **11c** and **12c** were synthesized by the same route as used for the **a** and **b** series. 3,4-Lutidine (**8c**) was treated with phenylmagnesium bromide in the presence of benzyl chloroformate to give 1-benzyloxycarbonyl-4,5-dimethyl-2-phenyl-1,2-dihydropyridine (**9c**), which, on irradiation (400 W, high-pressure Hg lamp), cyclized to afford the 2-azabicyclohexene (**10c**). Treatment of **10c** with *m*-chloroperbenzoic acid (*m*-CPBA) gave the oxirane compound **11c**. The reaction of **10c** with ethoxycarbonylnitrene generated from *N*-ethoxycarbonyl-*p*-nitrobenzenesulfonamide[14] by treatment with benzyltriethylammonium bromide and sodium hydrogencarbonate afforded the aziridine compound **12c**. Catalytic hydrogenation of the *N*-benzyloxycarbonyl tricyclic compounds **11a—c** and **12a—c** thus obtained gave the corresponding *N*-free compounds **13** and **14** in moderate yields. The *N*-free compounds **13** and **14** were successively treated[15] with *tert*-butyl hypochlorite and 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU) in dimethylformamide, giving rise to the corresponding tricycloheptenes (**15** and **16**) in 70—85% yields by dehydrogenation. The 4-unsubstituted tricycloheptanes (**17**) were also prepared, but they did not react under similar conditions, indicating that a phenyl group in the 4-position is needed for the dehydrogenation.

The tricyclic compounds **13—16** were characterized by their spectral data, particularly the proton nuclear magnetic resonance (^{1}H-NMR) spectra (Table I). In the ^{1}H-NMR spectra

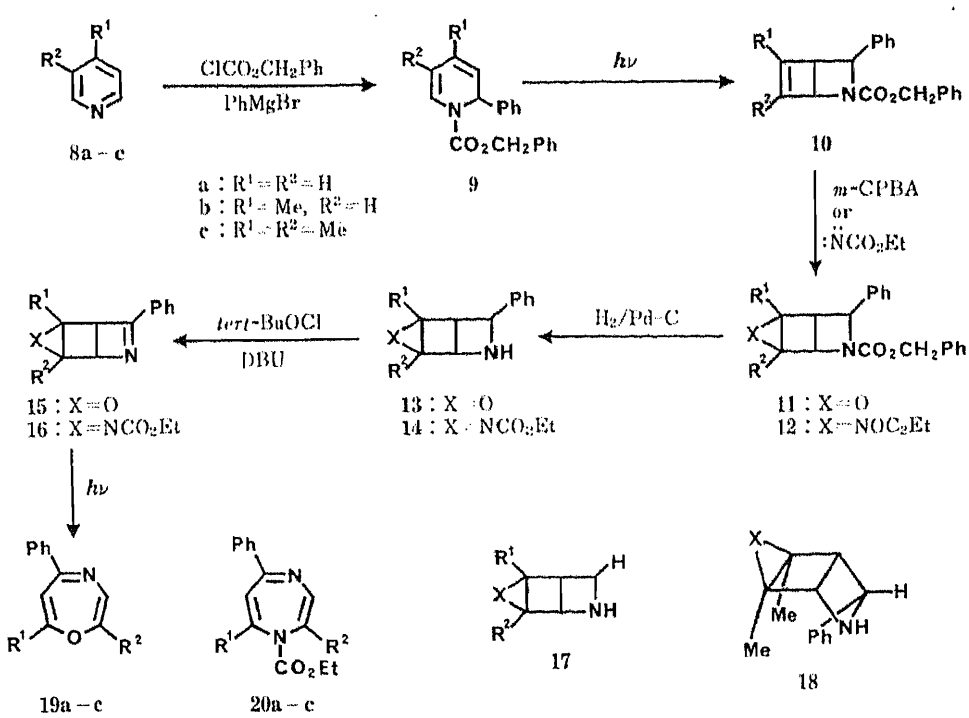
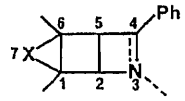


Chart 2

TABLE I. ^{1}H-NMR Spectral Data for the Tricyclic Compounds **13**—**16**



13a	3.0 (1H, br, NH), 3.30 (1H, ddd, J=8, 3.5, 3, 5-H), 3.58 (1H, dd, J=4, 2, 6-H), 4.04 (1H, dd, J=3.5, 2, 1-H), 4.08 (1H, dd, J=4, 3, 2-H), 5.18 (1H, d, J=8, 4-H), 7.1—7.4 (5H, m, Ph-H)
13b	0.80 (3H, s, 6-Me), 2.4 (1H, br, NH), 3.31 (1H, ddd, J=8, 3.5, 3, 5-H), 4.09 (1H, d, J=3, 2-H), 4.14 (1H, d, J=3.5, 1-H), 5.32 (1H, d, J=8, 4-H), 7.1—7.4 (5H, m, Ph-H)
13c	0.72 (3H, s, 6-Me), 1.58 (3H, s, 1-Me), 2.6 (1H, br, NH), 3.23 (1H, dd, J=8, 3, 5-H), 3.95 (1H, d, J=3, 2-H), 5.16 (1H, d, J=8, 4-H), 7.1—7.4 (5H, m, Ph-H)
14a	2.6 (1H, br, NH), 2.80 (1H, dd, J=4, 2, 6-H), 3.27 (1H, m, 5-H), 3.30 (1H, brs, 1-H), 4.03 (1H, m, 2-H), 5.13 (1H, brd, J=6, 4-H), 6.9—7.3 (5H, m, Ph-H), 1.23 and 4.03 (3H, t, and 2H, q, CO_2Et)
14b	0.75 (3H, s, 6-Me), 2.6 (1H, br, NH), 3.32 (1H, m, 5-H), 3.35 (1H, brs, 1-H), 4.05 (1H, m, 2-H), 5.24 (1H, brd, J=6, 4-H), 7.0—7.4 (5H, m, Ph-H), 1.25 and 4.06 (3H, t, and 2H, q, CO_2Et)
14c	0.59 (3H, s, 6-Me), 1.47 (3H, s, 1-Me), 2.7 (1H, br, NH), 3.26 (1H, dd, J=8, 3, 5-H), 3.93 (1H, d, J=3, 2-H), 5.11 (1H, d, J=8, 4-H), 7.0—7.4 (5H, m, Ph-H), 1.22 and 4.03 (3H, t, and 2H, q, CO_2Et)
15a	3.95 (1H, dd, J=3.5, 1, 5-H), 4.15 (1H, dd, J=4, 2, 6-H), 4.22 (1H, dd, J=3.5, 2, 1-H), 4.52 (1H, dd, J=4, 1, 2-H), 7.2—7.8 (5H, m, Ph-H)
15b	1.57 (3H, s, 6-Me), 3.96 (1H, dd, J=3.5, 1, 5-H), 4.18 (1H, d, J=3.5, 1-H), 4.41 (1H, d, J=1, 2-H), 7.2—7.8 (5H, m, Ph-H)
15c	1.50 and 1.57 (each 3H, s, 1- and 6-Me), 3.90 (1H, d, J=1, 5-H), 4.47 (1H, d, J=1, 2-H), 7.2—7.8 (5H, m, Ph-H)
16a	3.48 (1H, dd, J=3.5, 2.5, 6-H), 3.58 (1H, dd, J=3.5, 2.5, 1-H), 3.94 (1H, dd, J=3.5, 1, 5-H), 4.49 (1H, dd, J=3.5, 1, 2-H), 7.1—7.8 (5H, m, Ph-H), 1.32 and 4.15 (3H, t, and 2H, q, CO_2Et)
16b	1.47 (3H, s, 6-Me), 3.47 (1H, d, J=3, 1-H), 4.00 (1H, d, J=3, 5-H), 4.41 (1H, brs, 2-H), 7.1—7.8 (5H, m, Ph-H), 1.31 and 4.14 (3H, t, and 2H, q, CO_2Et)
16c	1.40 (3H, s, 1-Me), 1.49 (3H, s, 6-Me), 3.99 (1H, brs, 5-H), 4.51 (1H, brs, 2-H), 7.1—7.8 (5H, m, Ph-H), 1.30 and 4.18 (3H, t, and 2H, q, CO_2Et)

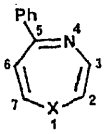
δ ($CDCl_3$), J=Hz.

of **13c** and **14c**, the signal of 6-Me appeared at higher field (δ 0.72 for **13c**; 0.59 for **14c**) than that of 1-Me (δ 1.58 for **13c**; 1.47 for **14c**). This difference in chemical shifts may arise from the shielding effect of the phenyl group on the azetidine ring, and thus the tricyclic compounds are considered to be *anti-endo* stereostructures **18** shown in Chart 2. Consequently **15** and **16** also seem to have *anti*-structures. The infrared (IR) spectra of **15** and **16** showed a strong band at 1560 cm^{-1}; this suggested the presence of a C=N function in the four-membered ring.[16]

Irradiation (30W, low-pressure Hg lamp) of the oxirane compounds **15a**—**c** in acetonitrile for 10—15 min resulted in valence isomerization with ring opening to give the desired 1,4-oxazepines (**19a**—**c**) in 90—95% yields. The products **19** are the first examples of fully unsaturated monocyclic 1,4-oxazepines. Under similar photolytic conditions, the aziridine compounds **16a**—**c** also afforded the novel 1*H*-1,4-diazepines (**20a**—**c**) in 65—85% yields. As was expected, the 1,4-oxazepines (**19**) are relatively unstable, presumably owing to their *anti*-aromatic character due to 8π-electrons, by analogy with the 1,3-oxazepines already reported,[2a,9-11] and thus they are susceptible to decomposition in a silica gel or alumina column. Therefore, the photolyzed solution was evaporated and the residue was extracted with *n*-hexane–isopropyl ether to give nearly pure **19**, which, however, gradually formed resinous materials on standing even in a refrigerator. The 1,4-diazepines (**20**) are stable and can be purified by chromatography, because they are stabilized by the electron-withdrawing ethoxycarbonyl group on the nitrogen atom, analogous to 1-acylazepines,[2c] and 1-acyl-1*H*-1,2-diazepines.[2a]

The structures of the new diheteroepines (**19** and **20**) were elucidated from their spectral

TABLE II. ^{1}H-NMR Spectral Data for 1,4-Oxazepines (**19**) and 1,4-Diazepines (**20**)



19a	5.20 (1H, d, J=4, 2-H), 5.71 (1H, d, J=6, 6-H), 5.81 (1H, d, J=6, 7-H), 6.62 (1H, d, J=4, 3-H), 7.0—7.6 (5H, m, Ph-H)
19b	1.96 (3H, d, J=0.8, 7-Me), 5.47 (1H, d, J=4, 2-H), 5.63 (1H, q, J=0.8, 6-H), 6.38 (1H, d, J=4, 3-H), 7.0—7.6 (5H, m, Ph-H)
19c	1.84 and 1.95 (each 3H, d, J=0.8, 2- and 7-Me), 5.64 (1H, q, J=0.8, 6-H), 6.25 (1H, q, J=0.8, 3-H), 7.0—7.6 (5H, m, Ph-H)
20a	5.49 (1H, d, J=6, 2-H), 5.69 (1H, d, J=8, 6-H), 6.33 (1H, d, J=6, 3-H), 6.38 (1H, d, J=8, 7-H), 7.1—7.7 (5H, m, Ph-H), 1.27 and 4.15 (3H, t, and 2H, q, CO_2Et)
20b	2.16 (3H, brs, 7-Me), 5.57 (1H, d, J=6, 2-H), 5.86 (1H, brs, 6-H), 6.60 (1H, d, J=6, 3-H), 7.1—7.7 (5H, m, Ph-H), 1.25 and 4.07 (3H, t, and 2H, q, CO_2Et)
20c	2.20 (3H, brs, 2-Me), 2.13 (3H, brs, 7-Me), 5.95 (1H, brs, 6-H), 6.46 (1H, brs, 3-H), 7.1—7.7 (5H, m, Ph-H), 1.21 and 4.01 (3H, t, and 2H, q, CO_2Et)

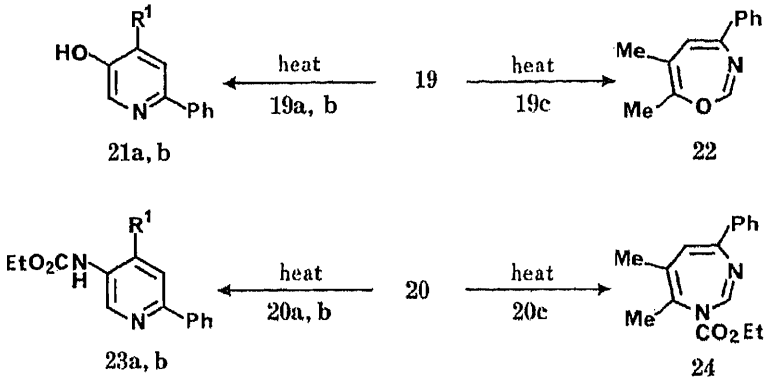


Chart 3

data and the results of the following thermal study. For example, in the ^{1}H-NMR spectra of **19a** and **20a** (Table II), signals due to the four heterocyclic ring protons lie in the olefinic range (δ 5.2—6.6 for **19a**; 5.5—6.4 for **20a**) as two pairs of doublets. It is known[17] that the geminal coupling constants have an approximately linear dependence on the electronegativity of the substituents in vinyl compounds. Therefore, the small coupling constant ($J_{2,3}$ = 4 Hz) for the O-CH=CH-N function in **19a** seems to be reasonable. In addition, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of **19a** and **20a** showed no signal due to sp^3 carbon except for the ethoxy carbons in **20a**.

Heating the oxazepines (**19a, b**: R^2 = H) in refluxing benzene for 1 h gave the rearrangement products, 5-hydroxypyridines (**21**), in 50—60% yields, whereas the 2,7-dimethyl-1,4-oxazepine (**19c**), upon heating at 80 °C for 30 min, underwent ring conversion to afford the 1,3-oxazepine (**22**) in 70% yield. Similarly, thermolysis of the 1,4-diazepines (**20a, b**: R^2 = H) gave the 5-aminopyridines (**23**) in *ca.* 20% yields and that of the diazepine (**20c**: $R^1 = R^2$ = Me) gave the 1,3-diazepine (**24**) in 50% yield. However, somewhat more drastic conditions, heating in *o*-dichlorobenzene at 160 °C for 4 h (for **20a, b**) or 12 h (for **20c**), were required for the thermolysis of **20**.

Possible mechanisms for the photochemical and thermal isomerizations reported are outlined in Chart 4. The formation of the 1,4-diheteroepines (**19** and **20**) from the tricycloheptenes (**15** and **16**) may involve the aza-norcaradiene intermediates **25**, which then

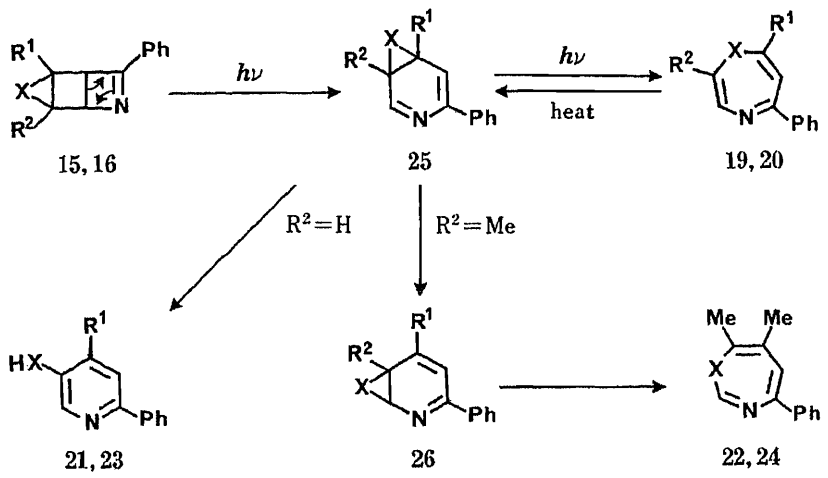


Chart 4

isomerize to give the ring expansion products **19** and **20**. Aza- and oxa-norcaradienes are well known[2] to undergo ring expansion to seven-membered heterocyclic rings. Other possible mechanisms *via* initial homolytic or ionic fission of the oxirane or aziridine ring seem unlikely, because the fully saturated tricycloheptanes (**11**—**14**) did not undergo such photochemical ring opening.

The thermolysis of **19** and **20** may proceed by initial reversion to the key norcaradiene intermediates **25**. The reason why the thermolysis of the diazepines (**20**) required a higher temperature and a longer time than that of the oxazepines (**19**) may be that oxepines revert to norcaradiene forms more readily than 1*H*-azepines.[2a] In the case of $R^2 = H$, the intermediates **25** would be converted into the pyridines (**21** and **23**) by C_4–O or C_4–N bond fission followed by hydrogen atom transfer, analogous to the thermolysis of various diazepines and 1,3-oxazepines.[2] In contrast, in the case of $R^2 = Me$, the intermediate **25c** might undergo a walk rearrangement to form another norcaradiene (**26**), which then gives the 1,3-diheteroepines (**22** and **24**) by ring opening. Similar walk processes have been widely observed in reactions involving norcaradiene intermediates having an oxirane[18] or aziridine ring[2d,4,7] and substituents on either side of the three-membered ring.

Compound **24** was characterized by spectral comparison with the 1,3-diazepines already reported.[7] Compound **22** is the first example of 2-unsubstituted 1,3-oxazepines, and its structure was confirmed by the following reaction. Treatment of **22** with hydrogen chloride in tetrahydrofuran at room temperature resulted in the formation of the *N*-formylpyrrole (**29**) in 90% yield. This acid-catalyzed ring contraction may proceed by initial ring opening to **27**, which would then cyclize to give the product **29** *via* **28**. This result is analogous to those for 2-phenyl-1,3-oxazepines[19] and 3,1-benzoxazepines.[18]

In addition, the formation of the 1,4-diheteroepines (**19** and **20**) was also observed in the thermolysis of **15** and **16**, but resinous complex mixtures containing pyridine derivatives **22** or **24** were obtained, and thus the yields of **19** and **20** were very low.

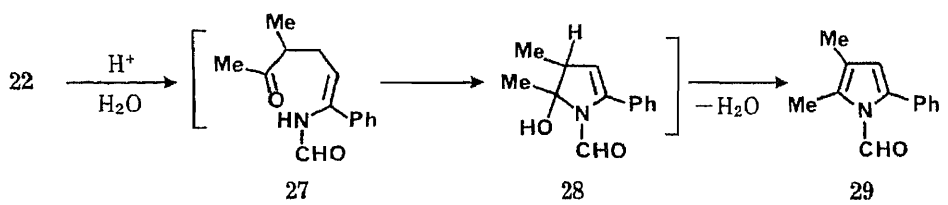


Chart 5

Experimental

Melting points were measured on a Yanagimoto micro melting point hot stage apparatus and are uncorrected. IR spectra were determined with a Hitachi 270-30 spectrometer and mass spectra (MS) were measured with a JEOL DX-300 instrument. Ultraviolet (UV) spectra were recorded on a Shimadzu UV-3000 spectrometer in ethanol solutions. ^{1}H-NMR spectra were recorded on a JEOL JNM-MH100 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard unless otherwise stated; spectral assignments were confirmed by spin-decoupling experiments and, in the case of NH protons, by exchange with D_2O. ^{13}C-NMR spectra were recorded on a JEOL FX-100 spectrometer. Microanalyses were performed in the Microanalytical Laboratory of this school by Mrs. R. Igarashi. Photolyses were carried out under a nitrogen atmosphere in an immersion apparatus equipped with a 400 W high-pressure or 30 W low-pressure Hg lamp, which was cooled internally with running water.

Starting Materials——3-Benzyloxycarbonyl-4-phenyl-7-oxa-3-azatricyclo[4.1.0.0^{2,5}]heptanes (**11a, b**) and 3-benzyloxycarbonyl-7-ethoxycarbonyl-3,7-diazatricyclo[4.1.0.0^{2,5}]heptanes (**12a, b**) were prepared from the pyridines (**8a, b**) *via* **9a, b** and **10a, b** by the reported procedures,[12] which were applied for the syntheses of the new compounds **11c** and **12c**.

1-Benzyloxycarbonyl-4,5-dimethyl-2-phenyl-1,2-dihydropyridine (9c)——A solution of 3,4-lutidine (**8c**: 10.7 g, 0.1 mol) in tetrahydrofuran (THF: 20 ml) was added dropwise to a stirred soluion of PhMgBr (0.1 mol) in THF (100 ml) in an ice bath. A solution of benzyl chloroformate (0.1 mol) in THF (100 ml) was then added dropwise to the above stirred mixture at below -5 °C. The reaction mixture was stirred for an additional 2 h at -5 °C, allowed to warm to room temperature, and then stirred for a further 1 h. After addition of water (100 ml), the reaction mixture was extracted with ether. The extract was successively washed with satd. $NaHCO_3$ and satd. NaCl, dried over $MgSO_4$, and concentrated *in vacuo* to give **9c** (pale yellow oil), the identity of which was confirmed by the ^{1}H-NMR spectrum of the resulting residue: δ: 1.70 and 1.78 (each 3H, s, 4- and 5-Me), 5.0—5.3 (3H, m, 2-H and C$\underline{H}_2$Ph), 5.41 (1H, br, 3-H), 6.90 (1H, s, 6-H), 7.0—7.6 (10H, m, Ph-H). However, **9c** was unstable and readily decomposed during isolation by chromatography, so the residue was used in the following photolysis without purification.

2-Benzyloxycarbonyl-5,6-dimethyl-2-azabicyclo[2.2.0]hex-5-ene (10c)——A solution of the residue (12.5 g containing **9c** obtained by the above procedure in CH_2Cl_2 (400 ml) was irradiated (400 W, high-pressure Hg lamp; Pyrex filter) until the signals of **9c** were no longer apparent in the ^{1}H-NMR spectrum; the photolysis was complete in 20—22 h. After removal of the solvent *in vacuo*, the residue was chromatographed on silica gel using *n*-hexane–ether (4 : 1) as an eluent to give **10c**: 3.02 g, 24% yield from **8c**, mp *ca.* 35 °C, colorless prisms [from isopropyl ether (IPE)]. MS *m/z*: 319 (M^+). IR ($CHCl_3$): 1695 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.03 (3H, s, 5-Me), 1.78 (3H, br s, 6-Me), 3.46 (1H, dd, J=7, 3 Hz, 4-H), 4.74 (1H, d, J=3 Hz, 1-H), 5.10 (2H, br s, OC$\underline{H}_2$Ph), 5.27 (1H, d, J=7 Hz, 3-H), 7.0—7.4 (10H, m, Ph-H). *Anal.* Calcd for $C_{21}H_{21}NO_2$: C, 78.97; H, 6.63; N, 4.39. Found: C, 78.76; H, 6.60; N, 4.18.

3-Benzyloxycarbonyl-1,6-dimethyl-4-phenyl-7-oxa-3-azatricyclo[4.1.0.0^{2,5}]heptane (11c)——A solution of *m*-chloroperbenzoic acid (2.20 g, 1.5 mol eq) in CH_2Cl_2 (10 ml) was added dropwise with stirring to a solution of **10c** (2.17 g) in CH_2Cl_2 (20 ml). After being stirred for an additional 2 d at room temperature, the reaction mixture was diluted with CH_2Cl_2 (100 ml). The solution was successively washed with satd. $NaHCO_3$ and satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on alumina using *n*-hexane–ether (1 : 1) as an eluent to give **11c**: 2.14 g, 94% yield, mp 100.5—102 °C, colorless prisms (from IPE). MS *m/z*: 335 (M^+). IR (KBr): 1710 (C=O) cm^{-1}. ^{1}H-NMR δ: 0.63 (3H, s, 6-Me), 1.67 (3H, s, 1-Me), 3.14 (1H, dd, J=8, 3 Hz, 5-H), 4.44 (1H, d, J=3 Hz, 2-H), 5.11 (2H, br s, OC$\underline{H}_2$Ph), 5.44 (1H, d, J=8 Hz, 4-H), 7.1—7.5 (10H, m, Ph-H). *Anal.* Calcd for $C_{21}H_{21}NO_3$: C, 75.20; H, 6.31; N, 4.18. Found: C, 75.18; H, 6.33; N, 4.01.

3-Benzyloxycarbonyl-7-ethoxycarbonyl-1,6-dimethyl-3,7-diazatricyclo[4.1.0.0^{2,5}]heptane (12c)——Benzyltriethylammonium bromide (0.5 g, 0.1 mol eq) and aq. 0.5 N $NaHCO_3$ (90 ml, 3 mol eq) were added with stirring to a solution of **10c** (5.72 g) in CH_2Cl_2 (110 ml). *N*-Ethoxycarbonyl-*p*-nitrobenzenesulfonamide (10.5 g, 2 mol eq) was added in small portions over a 1 h period to the above mixture with vigorous stirring in an ice bath. The reaction mixture was stirred for a further 5 h at room temperature and diluted with CH_2Cl_2 (100 ml). The organic layer was separated, washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using *n*-hexane–ether (4 : 1) as an eluent to give the starting compound **10c** (3.4 g) and **12c**, successively. **12c**: 0.9 g, 30% yield, mp 104—105.5 °C, colorless prisms (from IPE). MS *m/z*: 406 (M^+). IR (KBr): 1716 (C=O) cm^{-1}. ^{1}H-NMR δ: 0.55 (3H, s, 6-Me), 1.52 (3H, s, 1-Me), 3.31 (1H, dd, J=8, 3 Hz, 5-H), 4.55 (1H, d, J=3 Hz, 2-H), 5.53 (1H, d, J=8 Hz, 4-H), 1.33 and 4.16 (3H, t, and 2H, q, CO_2Et), 5.12 (2H, s, OC$\underline{H}_2$Ph), 7.0—7.4 (10H, m, Ph-H). *Anal.* Calcd for $C_{24}H_{26}N_2O_4$: C, 70.91; H, 6.45; N, 6.89. Found: C, 70.85; H, 6.44; N, 6.78.

3-Unsubstituted 7-Oxa-3-azatricyclo[4.1.0.0^{2,5}]heptanes (13a—c)——General Procedure: Compound **11** (5—8 mmol) was hydrogenated over 5% Pd–C (100—150 mg) in ethyl acetate (50—80 ml) at room temperature under atmospheric pressure for 2—4 h. The catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel using ether as an eluent to give the starting compound **11** (18—33%) and **13**, successively. ^{1}H-NMR spectral data of **13a—c** are collected in Table I.

13a: 46% yield, colorless oil. MS *m/z*: 173 (M^+). IR ($CHCl_3$): 3350 (NH) cm^{-1}.

13b: 58% yield, mp 33—35 °C, colorless prisms (from IPE). MS *m/z*: 187 (M^+). IR ($CHCl_3$): 3350 (NH) cm^{-1}.

13c: 73% yield, mp 70—71 °C, colorless prisms (from IPE). MS *m/z*: 201 (M^+). IR ($CHCl_3$): 3350 (NH) cm^{-1}. *Anal.* Calcd for $C_{13}H_{15}NO$: C, 77.58; H, 7.51; N, 6.96. Found: C, 77.65; H, 7.32; N, 6.69.

When the reaction was carried out until the spot of the starting compound **11** was no longer apparent on silica gel thin-layer chromatography (TLC), the yield of **13** decreased.

3-Unsubstituted 3,7-Diazatricyclo[4.1.0.$0^{2,5}$]heptanes (14a—c)——Compounds **12a—c** (4—5 mmol) were hydrogenated and worked up as described for **13** to give **14a—c**. ^{1}H-NMR spectra data of **14a—c** are collected in Table I.

14a: 64% yield, colorless oil. MS *m/z*: 244 (M^+). IR (neat): 3300 (NH), 1710 (C=O) cm^{-1}.

14b: 76% yield, colorless oil. MS *m/z*: 258 (M^+). IR (neat): 3350 (NH), 1710 (C=O) cm^{-1}.

14c: 83% yield, colorless oil. MS *m/z*: 272 (M^+). IR (neat): 3350 (NH), 1715 (C=O) cm^{-1}.

7-Oxa-3-azatricyclo[4.1.0.$0^{2,5}$]hept-3-enes (15a—c)——General Procedure: *tert*-Butyl hypochlorite (2 mol eq) was added dropwise over a 5 min period to a stirred solution of **13** (2—3 mmol) and DBU (3 mol eq) in dry ether (5—10 ml) in an ice bath. After stirring for a further 30 min at 0—5 °C, dimethylformamide (10—20 ml) was added to the reaction mixture, and then the mixture was stirred for 7—8 h at room temperaure and concentrated *in vacuo* at below 35 °C. The residue was chromatographed on silica gel using *n*-hexane–ether (1 : 3) as an eluent to give **15**. ^{1}H-NMR spectral data of **15a—c** are collected in Table I.

15a: 67% yield, mp 32—35 °C, colorless prisms (from IPE). MS *m/z*: 171 (M^+). IR (KBr): 1560 (C=N) cm^{-1}. UV λ_{max} nm (ε): 253 (15000). *Anal.* Calcd for $C_{11}H_9NO$: C, 77.17; H, 5.30; N, 8.18. Found: C, 76.95; H, 5.28; N, 8.20.

15b: 80% yield, mp 71—72.5 °C, colorless prisms (from IPE). MS *m/z*: 185 (M^+). IR (KBr): 1560 (C=N) cm^{-1}. UV λ_{max} nm (ε): 253 (16000). *Anal.* Calcd for $C_{12}H_{11}NO$: C, 77.81; H, 5.99; N, 7.56. Found: C, 77.80; H, 5.82; N, 7.51.

15c: 73% yield, mp 77—80 °C, colorless prisms (from IPE). MS *m/z*: 199 (M^+). IR (KBr): 1560 (C=N) cm^{-1}. UV λ_{max} nm (ε): 253 (15000). *Anal.* Calcd for $C_{13}H_{13}NO$: C, 78.36; H, 6.58; N, 7.03. Found: C, 78.33; H, 6.47; N, 6.99.

3,7-Diazatricyclo[4.1.0.$0^{2,5}$]hept-3-enes (16a—c)——Compounds **14a—c** (2—4 mmol) were successively treated with *tert*-butyl hypochlorite and DBU, and worked up as described for **15** to give **16a—c**. ^{1}H-NMR spectral data of **16a—c** are collected in Table I.

16a: 82% yield, mp 73—74 °C, colorless prisms (from IPE). MS *m/z*: 242 (M^+). IR ($CHCl_3$): 1720 (C=O), 1560 (NH) cm^{-1}. UV λ_{max} nm (ε): 254 (13000). *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.40; H, 5.83; N, 11.56. Found: C, 69.27; H, 5.83; N, 11.32.

16b: 86% yield, colorless oil. MS *m/z*: 256 (M^+). IR ($CHCl_3$): 1720 (C=O), 1560 (C=N) cm^{-1}. UV λ_{max} nm (ε): 255 (12000). *Anal.* Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 69.95; H, 6.11; N, 10.81.

16c: 81% yield, colorless oil. MS *m/z*: 270 (M^+). IR ($CHCl_3$): 1720 (C=O), 1560 (C=N) cm^{-1}. UV λ_{max} nm (ε): 254 (12000). *Anal.* Calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.18; H, 6.54; N, 10.14.

1,4-Oxazepines (19a—c)——General Procedure: A solution of **15** (0.5—1 mmol) in acetonitrile (200 ml) was irradiated (30 W, low-pressure Hg lamp) in an ice bath. The photolysis was followed in terms of the disappearance of the spot of the starting **15** on silica gel TLC, and was complete in 10—15 min. After removal of the solvent *in vacuo*, the residue was treated with active charcoal in *n*-hexane–IPE (1 : 1) and the mixture was filtered. The filtrate was evaporated *in vacuo* to give **19** in a nearly pure state. The oxazepines (**19**) thus obtained were relatively unstable and readily decomposed in a column (silica gel, alumina, Sephadex LH-20, or kieselguhr), so they could not be further purified. ^{1}H-NMR spectral data of **19a—c** are collected in Table II.

19a: 91% yield, orange oil. IR (neat): 1650 (C=N) cm^{-1}. UV λ_{max} nm (ε): 253 (12000). High-resolution MS *m/z*: M^+ Calcd for $C_{11}H_9NO$: 171.0684. Found: 171.0680.

19b: 96% yield, orange oil. IR (neat): 1650 (C=N) cm^{-1}. UV λ_{max} nm (ε): 254 (10000). High-resolution MS *m/z*: M^+ Calcd for $C_{12}H_{11}NO$: 185.0841. Found: 185.0833.

19c: 93% yield, orange oil. IR (neat): 1660 (C=N) cm^{-1}. UV λ_{max} nm (ε): 250 (13000). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{13}NO$: 199.0997. Found: 199.0994.

1,4-Diazepines (20a—c)——General Procedure: A solution of **16** (0.5—1.5 mmol) in acetonitrile (300 ml) was irradiated (30 W, low-pressure Hg lamp) for 10—15 min in an ice bath. After removal of the solvent *in vacuo*, the residue was chromatographed on silica gel using *n*-hexane–ether (1 : 1) as an eluent to give **20**. ^{1}H-NMR spectral data of **20a—c** are collected in Table II.

20a: 84% yield, mp 109—110.5 °C, yellow needles (from IPE). IR (KBr): 1718 (C=O) cm^{-1}. UV λ_{max} nm (ε): 257 (23000). High-resolution MS *m/z*: M^+ Calcd for $C_{14}H_{14}N_2O_2$: 242.1055. Found: 242.1056. *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.40; H, 5.83; N, 11.56. Found: C, 69.34; H, 5.84; N, 11.46.

20b: 65% yield, mp 75—76.5 °C, yellow prisms (from IPE). IR ($CHCl_3$): 1710 (C=O) cm^{-1}. UV λ_{max} nm (ε): 257 (24000). High-resolution MS *m/z*: M^+ Calcd for $C_{15}H_{16}N_2O_2$: 256.1212. Found: 256.1201.

20c: 72% yield, mp 118—119 °C, yellow prisms (from IPE). IR (KBr): 1712 (C=O) cm^{-1}. UV λ_{max} nm (ε): 257 (27000). High-resolution MS *m/z*: M^+ Calcd for $C_{16}H_{18}N_2O_2$: 270.1369. Found: 270.1370. *Anal.* Calcd for $C_{16}H_{18}N_2O_2$: C, 71.09; H, 6.71; N, 10.36. Found: C, 71.00; H, 6.73; N, 10.22.

Thermolysis of 19a, b——A solution of a 1,4-oxazepine (**19a**, 40 mg; **19b**, 64 mg) in benzene (3 ml) was refluxed for 1 h and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–MeOH (50 : 1) as an eluent to give the 5-hydroxy-2-phenylpyridine (**21**).

21a: 25 mg, 63% yield, mp 190—191.5 °C, colorless needles (from benzene). IR (KBr): 3200 (OH) cm^{-1}. MS *m/z*: 171 (M^+). ^{1}H-NMR δ: 4.7 (1H, br, OH), 7.12 (1H, dd, J=8, 2.5 Hz, 4-H), 7.48 (1H, d, J=8 Hz, 3-H), 7.96 (1H, d, J=2.5 Hz, 6-H), 7.1—7.7 (5H, m, Ph-H). *Anal.* Calcd for $C_{11}H_9NO$: C, 77.17; H, 5.30; N, 8.18. Found: C, 77.14; H, 5.19; N, 8.05.

21b: 33 mg, 52% yield, mp 177—178 °C, colorless needles (from benzene). IR (KBr): 3200 (OH) cm^{-1}. MS *m/z*: 185 (M^+). ^{1}H-NMR δ: 2.27 (3H, s, 4-Me), 4.56 (1H, br, OH), 7.36 (1H, s, 3-H), 8.19 (1H, s, 6-H), 7.2—7.8 (5H, m, Ph-H). *Anal.* Calcd for $C_{12}H_{11}NO$: C, 77.81; H, 5.99; N, 7.56. Found: C, 77.82; H, 5.94; N, 7.47.

Thermolysis of 19c: Conversion into 1,3-Oxazepine (22)——A solution of **19c** (48 mg) in benzene (2 ml) was refluxed for 30 min and then evaporated *in vacuo*. The residue was chromatographed on silica gel using *n*-hexane–ether (4 : 1) as an eluent to give 6,7-dimethyl-4-phenyl-1,3-oxazepine (**22**): 33 mg, 69% yield, pale yellow oil. IR (neat): 1640 (C=N) cm^{-1}. UV λ_{max} nm (ε): 223 sh (15000), 262 (14000), 312 sh (10000). ^{1}H-NMR δ: 1.83 (3H, s, 7-Me), 2.01 (3H, d, J=0.5 Hz, 6-Me), 6.16 (1H, q, J=0.5 Hz, 5-H), 6.35 (1H, s, 2-H), 7.1—7.6 (5H, m, Ph-H). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{13}NO$: 199.0997. Found: 199.0995.

Thermolysis of 20a, b——A solution of a 1,4-diazepine (**20a**, 80 mg; **20b**, 110 mg) in *o*-dichlorobenzene (0.5 ml) was heated at 160 °C for 4 h and then evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–ether (4 : 1) as an eluent to give the 5-(ethoxycarbonylamino)-2-phenylpyridine (**23**).

23a: 15 mg, 18% yield, mp 175—177 °C, colorless needles (from benzene). MS *m/z*: 242 (M^+). IR (KBr): 3400 (C=N), 1735 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.32 and 4.23 (3H, t, and 2H, q, CO_2Et), 6.8 (1H, br, NH), 7.67 (1H, d, J= 9 Hz, 3-H), 8.04 (1H, dd, J=9, 2.5 Hz, 4-H), 8.56 (1H, d, J=2.5 Hz, 6-H), 7.3—8.0 (5H, m, Ph-H). *Anal.* Calcd for $C_{14}H_{14}N_2O_2$: C, 69.40; H, 5.83; N, 11.56. Found: C, 69.49; H, 5.69; N, 11.53.

23b: 21 mg, 20% yield, mp 120.5—122 °C, colorless needles (from benzene). MS *m/z*: 256 (M^+). IR (KBr): 3450 (NH), 1735 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.33 and 4.25 (3H, t, and 2H, q, CO_2Et), 2.32 (3H, s, 4-Me), 6.4 (1H, br, NH), 7.51 (1H, brs, 3-H), 8.91 (1H, brs, 6-H), 7.3—8.0 (5H, m, Ph-H). *Anal.* Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.08; H, 6.27; N, 10.77.

Thermolysis of 20c: Conversion into 1,3-Diazepine (24)——A solution of **20c** (85 mg) in *o*-dichlorobenzene (0.6 ml) was heated at 160 °C for 12 h and then concentrated *in vacuo*. The residue was chromatographed on silica gel using *n*-hexane–ether (4 : 1) as an eluent to give 1-ethoxycarbonyl-6,7-dimethyl-4-phenyl-1,3-diazepine (**24**): 42 mg, 49% yield, pale yellow oil. IR (neat): 1710 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.28 and 4.18 (3H, t, and 2H, q, CO_2Et), 1.91 (3H, s, 6-Me), 2.09 (3H, s, 7-Me), 6.27 (1H, s, 5-H), 6.87 (1H, s, 2-H), 7.2—7.7 (5H, m, Ph-H). High-resolution MS *m/z*: M^+ Calcd for $C_{16}H_{18}N_2O_2$: 270.1368. Found: 270.1372.

Hydrolysis of 22——A mixture of **22** (22 mg), aq. 0.2 N HCl (0.4 ml), and THF (1 ml) was stirred for 1.5 h at room temperature and then diluted with ether (50 ml). The mixture was successively washed with satd. $NaHCO_3$ and satd. NaCl, dried, and evaporated to dryness *in vacuo*. The solid residue was purified by preparative TLC [silica gel; *n*-hexane–ether (1 : 1)] to give 1-formyl-2,3-dimethyl-5-phenylpyrrole (**29**): 20 mg, 91% yield, mp 40—41.5 °C, colorless prisms (from *n*-hexane). IR (KBr): 1715 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.04 (3H, s, 3-Me), 2.52 (3H, s, 2-Me), 6.12 (1H, s, 4-H), 7.3—7.5 (5H, m, Ph-H), 9.08 (1H, s, CHO). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{13}NO$: 199.0997. Found: 199.0991.

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Studies on Diazepines. XXVI.[1] Syntheses of 6*H*-1,4-Diazepines and 1-Acyl-1*H*-1,4-diazepines from 4-Pyridyl Azides

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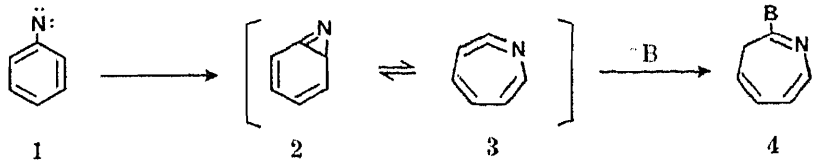
Photolysis of 4-azidopyridines (**7a—e**) in the presence of methoxide ions resulted in ring expansion to give 5-methoxy-6*H*-1,4-diazepines (**10a—e**), presumably *via* the azirine intermediates **8** derived from the initially formed singlet pyridylnitrenes. Treatment of the 6*H*-1,4-diazepine (**10a**) with benzoyl chloride, acetyl chloride, or ethyl chloroformate in pyridine afforded the corresponding 1-acyl-1*H*-1,4-diazepines (**21a—c**), whose structures were confirmed by means of thermal and photochemical reactions.

Keywords——4-azidopyridine; 6*H*-1,4-diazepine; 1*H*-1,4-diazepine; photolysis; ring-expansion; pyridylnitrene; azirine intermediate; thermolysis

Among the three possible fully unsaturated diazepine isomers due to the isomeric positions of the two nitrogen atoms, 1,2-[2] and 1,3-diazepines[3] are known, but as regards 1,4-diazepines, only highly substituted 6*H*-1,4-diazepines[4] had been reported prior to our recent work. Therefore, we were interested in the synthesis of 1,4-diazepines as part of our continuing studies on diazepines, and in the preceding paper,[1] we reported the synthesis of novel 1*H*-1,4-diazepines by the photo-induced valence isomerization of 3,7-diazatricyclo-[4.1.0.0^{2,5}]hept-3-enes. This paper describes the synthesis of less substituted 6*H*-1,4-diazepines from 4-pyridyl azides by irradiation in the presence of methoxide ions and the conversion of the 6*H*-1,4-diazepines into 1-acyl-1*H*-1,4-diazepines.[5,6]

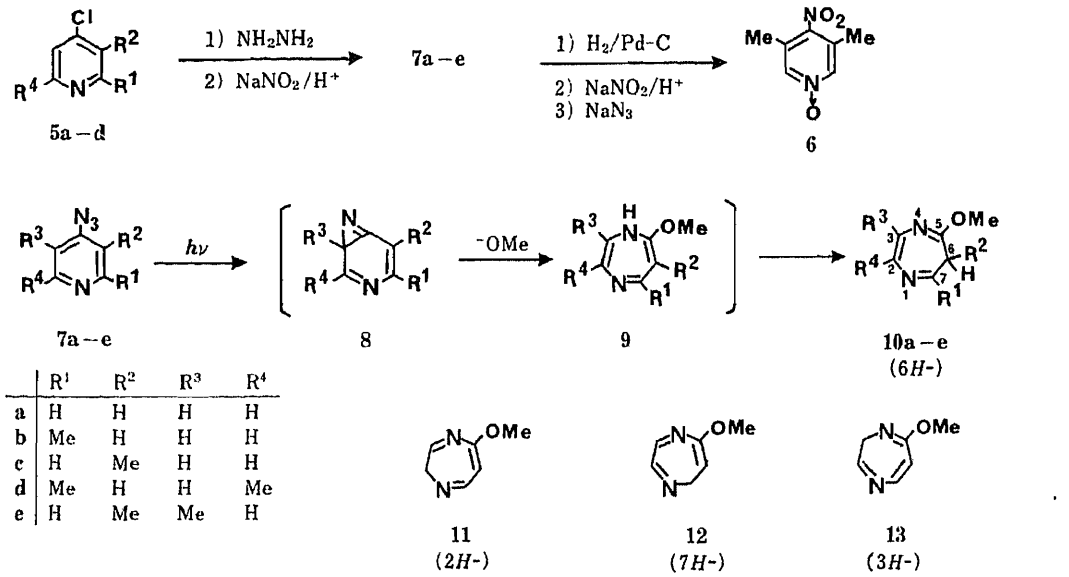
The singlet phenylnitrenes (**1**), generated from azido-,[7] nitro-, and nitroso-benzenes,[8] are known to undergo ring-expansion to give the 3*H*-azepines (**4**) *via* the azirine (**2**) or the azacycloheptatetraene (**3**) intermediates, upon photolysis or thermolysis in the presence of bases such as alkoxides and amines. However, such a reaction of monocyclic pyridyl azides is little known,[6] although the photolysis of benzopyridyl azides has been reported.[9,10] Therefore, we examined the photochemical behavior of monocyclic 4-pyridyl azides under basic conditions.

The starting 4-azidopyridines (**7a—d**) were prepared from the corresponding 4-chloropyridines (**5a—d**) by treatment with hydrazine hydrate followed by diazotization, according to the procedure reported for the preparation of 4-azido-2-methylpyridine (**7b**).[11] 4-Azido-3,5-dimethylpyridine (**7e**) was also obtained from 4-chloro-3,5-dimethylpyridine, but in a very low yield. Therefore, **7e** was prepared by the following route. 4-Amino-3,5-dimethylpyridine



1 2 3 4

Chart 1



	R^1	R^2	R^3	R^4
a	H	H	H	H
b	Me	H	H	H
c	H	Me	H	H
d	Me	H	H	Me
e	H	Me	Me	H

Chart 2

1-oxide, obtained from the 4-nitropyridine 1-oxide (**6**)[12] by catalytic reduction, was diazotized and then treated with sodium azide to give 4-azido-3,5-dimethylpyridine 1-oxide, which afforded **7e** on deoxygenation with phosphorus trichloride in 62% yield from **6**.

Irradiation (400 W, high-pressure Hg lamp; Pyrex filter) of the azides (**7a**—**e**: *ca.* 1 g) in methanol–dioxane (1 : 1)[13] containing sodium methoxide for 3—4 h resulted in the formation of the desired 6*H*-1,4-diazepines (**10a**—**e**) in 35—70% yields, as the sole ring-expansion products. However, irradiation of **7** in the presence of an amine such as ethylamine or diethylamine gave no ring-expansion products. The 1,4-diazepines (**10**) are extremely susceptible to decomposition in a silica gel or alumina column. Thus, the photolyzed solution was evaporated and the resulting residue was extracted with *n*-hexane to give nearly pure **10**, which could be further purified only by Sephadex or kieselguhr chromatography. The diazepines (**10**) thus obtained as oils are relatively unstable and gradually decomposed on standing even in a refrigerator.

The structures of the new diazepines (**10**) were elucidated mainly from their spectral data. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **10a** showed signals assignable to 5-OMe (δ 3.68, s), 2-H (δ 6.64, dd, J=6, 1 Hz), 3-H (δ 6.94, d, J=6 Hz), 6-H_2 (δ 2.92, d, J=5 Hz), and 7-H (δ 6.70, td, J=5, 1 Hz); indicating that the 7-H signal couples with the 6-H_2 (J=5 Hz) and 2-H (J=1 Hz) signals. The carbon-13 nuclear (^{13}C-NMR) spectrum of **10a** showed signals due to two sp^3 carbons at δ 38.65 (t, 6-C) and 55.0 (q, OMe-C), and four sp^2 carbons at δ 127.66 and 128.36 (each d, 2- and 3-C), 139.36 (d, 7-C), and 146.30 (s, 5-C). These NMR spectral data strongly suggest the presence of a –CH=CH–N=CH–CH_2–C function in the ring and thus eliminate the other possible 2*H*- (**11**) and 7*H*- (**12**) structures. The 3*H*-structure **13** is also ruled out by the ^{1}H-NMR spectra of **10b** and **10c**; the methyl signal of **10c** (R^2=Me) was observed as a doublet (J=6 Hz) at δ 1.66 and the methylene signal in **10b** (R^1=Me) appeared as a singlet at δ 2.92. In addition, it is known[7,8] that the 2-alkoxy-3*H*-azepines such as **4** are the most stable form among the six possible CH-tautomers.

The formation of the diazepines (**10**) from **7** may involve ring expansion of the azirine intermediates **8** derived from the initially formed singlet pyridylnitrenes to the unstable *anti*-aromatic NH-diazepines (**9**), which tautomerize to the more stable CH-form **10**, by analogy with the formation of 2-alkoxy-3*H*-azepines from phenylnitrenes.[7,8] Unsymmetrical azides

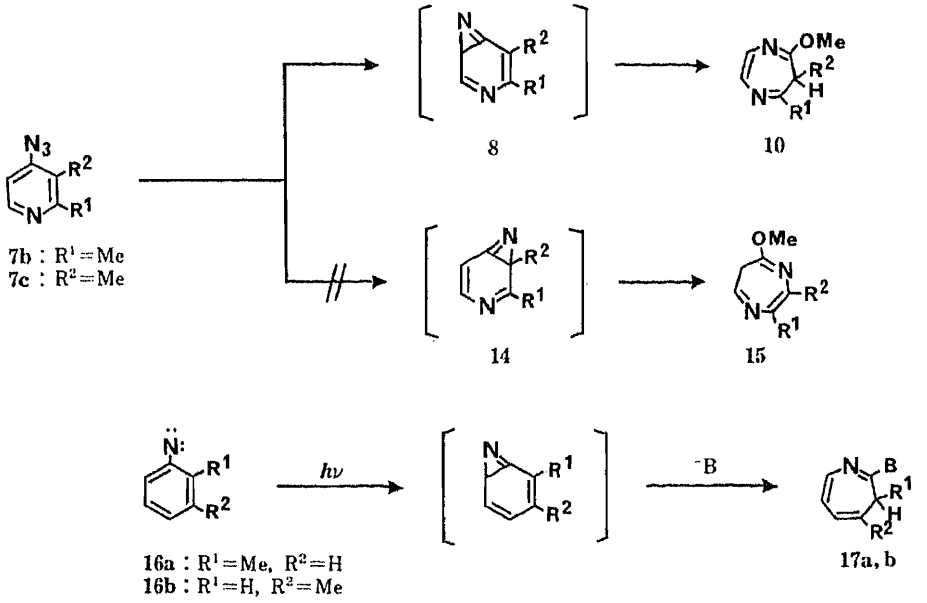


Chart 3

(**7b** and **7c**) may ring-close in either of two directions to give two isomeric azirine intermediates **8** and **14**, each of which may react with methoxide ion to afford the 1,4-diazepines (**10** and **15**).[6] However, in the present photolysis, the formation of **15** could not be observed, although the isolated yields of the sole ring-expansion products **10b**, **c** were relatively low (45—55%). This behavior is similar to the cases of 2-methyl- (**16a**) and 3-methylphenylnitrene (**16b**), which cyclize predominantly at the 6-position, giving rise to the 7-methyl- (**17a**) and 6-methyl-3*H*-azepine (**17b**), respectively.[8]

In order to obtain 1-acyl-1*H*-1,4-diazepines, the following reactions were carried out. When **10a** was treated with benzoyl chloride in the presence of a base such as an alkoxide, no reaction occurred. Treatment of **10a** with benzoyl chloride in potassium hydroxide solution gave no 1*H*-1,4-diazepine, but afforded the ring-opened dibenzoyl compound **19** in 21% yield, presumably *via* the dihydro intermediate **18**, which may undergo ring fission followed by further benzoylation. Similarly, treatment of **10a** with benzoyl chloride in the presence of potassium cyanide afforded the nitrile compound **20** in 32% yield. However, treatment of **10a** with benzoyl chloride, acetyl chloride, or ethyl chloroformate in pyridine resulted in acylation with tautomerization to give the corresponding desired 1-acyl-1*H*-1,4-diazepines (**21a**—**c**) in 20—65% yields. This result is analogous to those of the acylation of 4*H*-1,2-diazepines[14] and 5*H*-2,3-benzodiazepines.[15]

The ^{1}H-NMR spectra of **21a**—**c** showed two AB pairs of doublets in the olefinic range, in addition to the methoxy and ethoxycarbonyl proton signals; *e.g.*, in **21c**, $J=6$ Hz, at δ 5.58 (2-H) and 5.90 (3-H), and $J=8$ Hz, at δ 5.14 (6-H) and 6.58 (7-H). These data are similar to those for the 1-ethoxycarbonyl-5-phenyl-1*H*-1,4-diazepines reported in the preceding paper,[1] but they did not eliminate the other possible *N*-benzoyl structure **21'**. However, the result of the following thermolysis is consistent with the proposed structure **21**. Heating **21a** in xylene at 120—130 °C for 10 h resulted in isomerization to give 5-benzoylamino-2-methoxypyridine (**23**) in 95% yield, probably *via* the azirine intermediate **22**. This thermal behavior, including the mode of the azirine ring fission and the substituent effect, is similar to those observed in the thermolysis of 1,2- and 1,3-diazepines.[16] If the structure of the *N*-benzoyldiazepine was **21'**, the thermolysis product would be 3-benzoylamino-4-methoxypyridine (**23'**).

Irradiation (400 W, high-pressure Hg lamp) of **21a** for 30 h afforded 2-benzoyl-3-

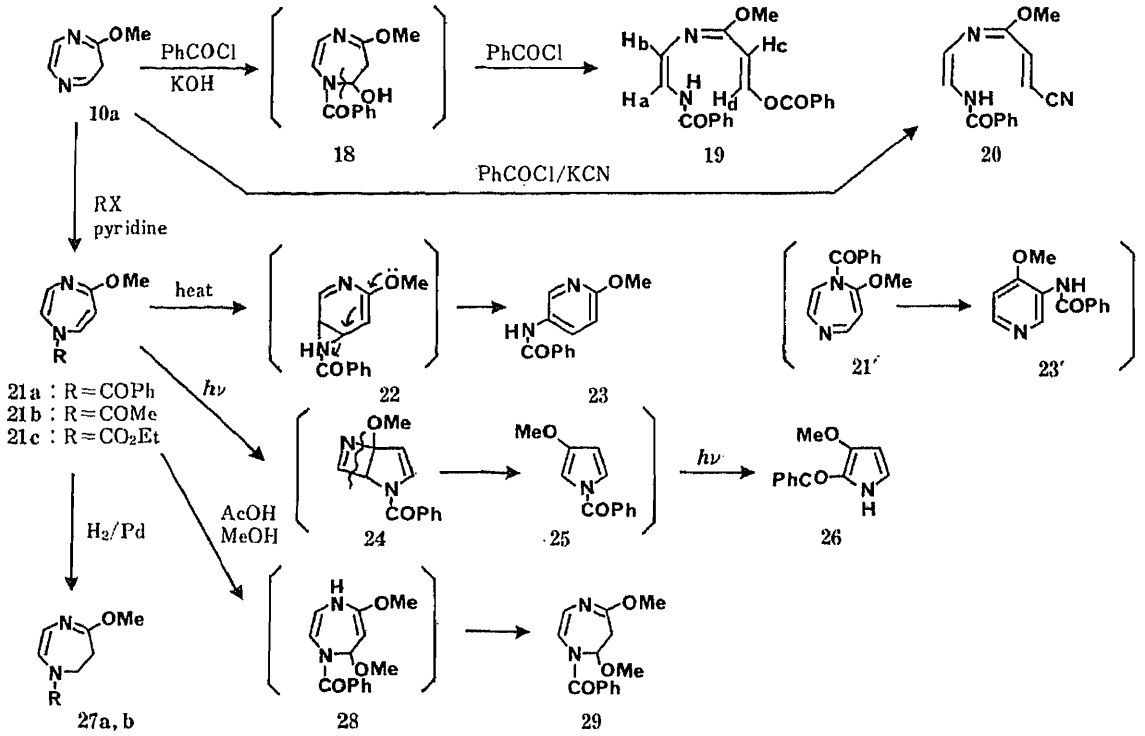


Chart 4

methoxypyrrole (**26**) in 15% yield. The pyrrole (**26**) may be derived from the initially formed bicyclic valence isomer **24**, which gives the 1-benzoylpyrrole (**25**) by extrusion of HCN. The *N*-benzoyl group in **25** may migrate to the 2-position, giving the product **26**. Similar migration of acyl groups from nitrogen to the α-position has been widely observed in the photolysis of *N*-acylpyrroles.[17] In addition, the catalytic hydrogenation of **21a, b** over Pd–C gave the 6,7-dihydrodiazepines (**27a, b**) in high yields. Treatment of **21a** with acetic acid in methanol afforded the solvent adduct **29** in 60% yield. This acid-catalysed reaction may proceed by initial 1,4-addition of methanol to give the 4,7-dihydrodiazepine (**28**), which might then undergo 1,3-shift of hydrogen, giving rise to the product **29**.

Experimental

The general experimental procedures were the same as in Part XXV.[1] Photolyses were carried out under a nitrogen atmosphere in an immersion apparatus equipped with a 400 W high-pressure Hg lamp and a Pyrex filter, which was cooled internally with running water.

Starting Materials——4-Azidopyridine (**7a**)[11,18] and 4-azido-2-methylpyridine (**7b**)[11] were prepared from the corresponding 4-chloropyridines (**5**) by the reported methods.

4-Azido-3-methylpyridine (7c)——The same procedure as used for the preparation of **7b** was employed. A mixture of 4-chloro-3-methylpyridine[19] (**5c**, 6 g), 95% hydrazine hydrate (7.8 g), and EtOH (20 ml) was heated at 140 °C for 15 h in a sealed tube, and then evaporated *in vacuo*. The residue containing 4-hydrazino-3-methylpyridine thus formed was used in the following reaction without isolation. A solution of $NaNO_2$ (4.8 g) in water (60 ml) was added dropwise with stirring to a solution of the above residue in 5% HCl (120 ml), cooled at 0 °C in an ice-salt bath. The reaction mixture was stirred for a further 30 min at 0 °C, then made alkaline with Na_2CO_3, and extracted with CH_2Cl_2. The extract was washed with water, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on alumina using benzene as an eluent to give **7c**: 5.0 g, 77% yield from **5c**, viscous oil. MS *m/z*: 134 (M^+). IR (neat): 2140 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 2.20 (3H, s, 3-Me), 7.20 (1H, d, J=6 Hz, 5-H), 8.40 (1H, s, 2-H), 8.46 (1H, d, J=6 Hz, 6-H). *Anal.* Calcd for $C_6H_6N_4$: C, 53.72; H, 4.51; N, 41.77. Found: C, 53.87; H, 4.31; N, 41.49.

4-Azido-2,6-dimethylpyridine (7d)——A mixture of 4-chloro-2,6-dimethylpyridine[20] (**5d**, 14 g), 95% hydrazine hydrate (15.9 g), and EtOH (40 ml) was heated and then diazotized as described for **7c** to give **7d**: 6.2 g, 42% yield

from **5d**, viscous oil. MS *m/z*: 148 (M^+). IR (neat): 2150 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 2.48 (6H, s, 2- and 6-Me), 6.62 (2H, s, 3- and 5-H). *Anal.* Calcd for $C_7H_8N_4$: C, 56.74; H, 5.44; N, 37.82. Found: C, 56.61; H, 5.18; N, 37.57.

4-Azido-3,5-dimethylpyridine (7e)——3,5-Dimethyl-4-nitropyridine 1-oxide[12] (**6**, 7.4 g) was hydrogenated over 5% Pd–C (400 mg) in EtOH (150 ml) with stirring at room temperature under atmospheric pressure. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The resulting solid residue was recrystallized from CH_2Cl_2–isopropyl ether to give 4-amino-3,5-dimethylpyridine 1-oxide: 5.5 g, 91% yield, mp 220—225 °C (dec.), colorless needles. MS *m/z*: 138 (M^+). ^{1}H-NMR δ: 2.12 (6H, s, 3- and 5-Me), 7.76 (2H, s, 2- and 6-H). *Anal.* Calcd for $C_7H_{10}N_2O$: C, 60.87; H, 7.25; N, 20.29. Found: C, 60.55; H, 7.27; N, 20.13. A solution of $NaNO_2$ (3.7 g) in water (6 ml) was added dropwise with stirring to a solution of the 4-aminopyridine (5.2 g) obtained above in 60% H_2SO_4 (15 ml), cooled at −5—0 °C in an ice-salt bath. Stirring was continued for an additional 10 min, then a solution of sodium azide (3.75 g) in water (10 ml) was added dropwise with stirring at *ca.* 0 °C. The reaction mixture was stirred for a further 1 h at room temperature, then made alkaline with Na_2CO_3, and extracted with CH_2Cl_2. The extract was washed, dried, and evaporated to dryness *in vacuo*. The crystalline residue was recrystallized from benzene to give 4-azido-3,5-dimethylpyridine 1-oxide: 4.4 g, 71% yield, mp 147—149 °C. MS *m/z*: 168 (M^+). IR (KBr): 2150 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 2.62 (6H, s, 3- and 5-Me), 8.48 (2H, s, 2- and 6-H). *Anal.* Calcd for $C_7H_8N_4O$: C, 50.00; H, 4.76; N, 33.33. Found: C, 49.92; H, 4.85; N, 33.30. PCl_3 (30 ml) was added dropwise with stirring to a solution of the 4-azidopyridine 1-oxide (4.8 g) in $CHCl_3$ (70 ml), cooled in an ice bath, and then the mixture was stirred for a further 18 h at room temperature. The reaction mixture was poured into ice-water (*ca.* 150 ml) and the aqueous mixture was made alkaline with Na_2CO_3, then extracted with CH_2Cl_2. The extract was washed with water, dried, and concentrated *in vacuo*. The residue was chromatographed on alumina using CH_2Cl_2 as an eluent to give **7e**: 4.1 g, 95% yield, viscous oil. MS *m/z*: 148 (M^+). IR (neat): 2140 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 2.44 (6H, s, 3- and 5-Me), 8.55 (2H, s, 2- and 6-H). *Anal.* Calcd for $C_7H_8N_4$: C, 56.74; H, 5.44; N, 37.82. Found: C, 56.51; H, 5.48; N, 37.49.

Photolysis of the Azides (7a—e): Formation of 5-Methoxy-6*H*-1,4-Diazepines (10a—e)——General Procedure: A mixture of **7** (0.9—1.0 g), 28% sodium methoxide in MeOH (10 ml), MeOH (65 ml), and dioxane (75 ml) was irradiated. The photolysis was followed in terms of the disappearance of the spot of the starting **7** on silica gel thin-layer chromatography and was complete in 3—4 h. After removal of the solvents *in vacuo*, ice-water (30—50 ml) was added to the residue and the aqueous mixture was extracted with *n*-hexane or benzene. The extract was dried and evaporated *in vacuo* to give the diazepine (**10**) as an oil in a nearly pure state. The product was further purified by chromatography on Sephadex or kieselguhr using benzene as an eluent to give an analytical sample.

10a: 36% yield. IR (neat): 1640 (C=N) cm^{-1}. UV λ_{max} nm (ε): 280 (4400). ^{1}H-NMR δ: 2.92 (2H, d, J=5 Hz, 6-H_2), 3.68 (3H, s, 5-OMe), 6.64 (1H, dd, J=6, 1 Hz, 2-H), 6.70 (1H, td, J=5, 1 Hz, 7-H), 6.94 (1H, d, J=6 Hz, 3-H). ^{13}C-NMR δ: 38.65 (t, 6-C), 55.00 (q, OMe), 127.66 and 128.36 (each d, 2- and 3-C), 139.36 (d, 7-C), 146.30 (s, 5-C). High-resolution MS *m/z*: M^+ Calcd for $C_6H_8N_2O$: 124.0637. Found: 124.0634.

10b: 45% yield. IR (neat): 1620 (C=N) cm^{-1}. ^{1}H-NMR δ: 2.16 (3H, s, 7-Me), 2.92 (2H, br s, 6-H_2), 3.74 (3H, s, 5-OMe), 6.58 (1H, d, J=7 Hz, 2-H), 6.80 (1H, d, J=7 Hz, 3-H). ^{13}C-NMR δ: 25.53 (q, 7-Me), 41.36 (t, 6-C), 54.42 (q, 5-OMe), 126.13 and 127.42 (each d, 2- and 3-C), 146.72 and 148.66 (each s, 5- and 7-C). High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0795.

10c: 55% yield. IR (neat): 1620 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.66 (3H, d, J=6 Hz, 6-Me), 1.84—2.04 (1H, m, 6-H), 3.74 (3H, s, 5-OMe), 6.40 (1H, d, J=4 Hz, 7-H), 6.70 (1H, d, J=5 Hz, 2-H), 6.98 (1H, d, J=5 Hz, 3-H). ^{13}C-NMR δ: 11.23 (q, 6-Me), 43.12 (d, 6-C), 55.13 (q, 5-OMe), 127.19 and 128.01 (each d, 2- and 3-C), 144.23 (d, 7-C), 147.36 (s, 5-C). High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 138.0793. Found: 138.0794.

10d: 68% yield. IR (neat): 1620 (C=N) cm^{-1}. ^{1}H-NMR δ: 2.04 (3H, s, 2-Me), 2.12 (3H, s, 7-Me), 2.90 (2H, br s, 6-H_2), 3.66 (3H, s, 5-OMe), 6.46 (1H, s, 3-H). ^{13}C-NMR δ: 21.43 (q, 2-Me), 25.28 (q, 7-Me), 41.60 (t, 6-C), 54.69 (q, 5-OMe), 122.99 (d, 3-C), 136.03 (s, 2-C), 146.34 (s, 7-C), 147.66 (s, 5-C). High-resolution MS *m/z*: M^+ Calcd for $C_7H_{10}N_2O$: 152.0950. Found: 152.0939.

10e: 35% yield. IR (neat): 1620 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.60 (3H, d, J=6 Hz, 6-Me), 1.82—2.10 (1H, m, 6-H), 2.06 (3H, s, 3-Me), 3.68 (3H, s, 5-OMe), 6.28 (1H, d, J=4 Hz, 7-H), 6.82 (1H, s, 2-H). ^{13}C-NMR δ: 11.24 (q, 6-Me), 22.59 (q, 3-Me), 42.77 (d, 6-C), 54.88 (q, 5-OMe), 125.12 (d, 2-C), 136.42 (s, 3-C), 142.48 (d, 7-C), 145.83 (s, 5-C). High-resolution MS *m/z*: M^+ Calcd for $C_8H_{12}N_2O$: 152.0950. Found: 152.0947.

Treatment of 10a with Benzoyl Chloride in the Presence of KOH——Benzoyl chloride (1.4 g) was added in small portions with stirring to a mixture of a solution of **10a** (607 mg) in CH_2Cl_2 (10 ml) and 10% NaOH (10 ml) in an ice bath. The reaction mixture was stirred for a further 20 h at room temperature and then diluted with CH_2Cl_2 (30 ml). The organic layer was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–AcOEt (20:1) as an eluent to give the dibenzoyl compound **19**: 350 mg, 21% yield, mp 162—164 °C, pale yllow needles (from isopropyl ether). MS *m/z*: 350 (M^+). IR (KBr): 1735 and 1660 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.86 (3H, s, OMe), 6.38 (1H, d, 12 Hz, H_c), 6.32 (1H, d, J=6 Hz, H_b), 6.84 (1H, dd, J=10, 6 Hz, H_a), 7.32—8.16 (10H, m, Ph-H), 8.32 (1H, d, J=12 Hz, H_d), 8.88 (1H, br d, J=10 Hz, NH). *Anal.* Calcd for $C_{20}H_{18}N_2O_4$: C, 68.57; H, 5.14; N, 7.99. Found: C, 68.34; H, 5.21; N, 7.77.

Treatment of 10a with Benzoyl Chloride in the Presence of KCN——Benzoyl chloride (420 mg) was added in small portions with stirring to a mixture of a solution of **10a** (309 mg) and a solution of KCN (0.5 g) in water (5 ml) in an ice

bath. The reaction mixture was stirred for a further 20 h at room temperature and then diluted with CH_2Cl_2 (20 ml). The organic layer was washed with water, dried, and evaporated *in vacuo*. The residue was chromatographed on alumina using benzene–CH_2Cl_2 (1 : 2) as an eluent to give the nitrile compound **20**: 200 mg, 32% yield, mp 147—149 °C, pale yellow needles (from isopropyl ether). MS *m/z*: 255 (M^+). IR (KBr): 3400 (NH), 2225 (CN), 1655 (C=O) cm^{-1}. ^{1}H-NMR δ: 6.16 (1H, d, J=16 Hz, H_c), 6.40 (1H, d, J=6 Hz, H_b), 6.96 (1H, dd, J=10, 6 Hz, H_a), 7.12 (1H, d, J=16 Hz, H_d), 7.50—7.82 (5H, m, Ph-H), 8.80 (1H, br d, J=10 Hz, NH). *Anal.* Calcd for $C_{14}H_{13}N_3O_2$: C, 65.88; H, 5.10; N, 16.47. Found: C, 65.88; H, 4.98; N, 16.73.

1-Benzoyl-5-methoxy-1*H*-1,4-diazepine (21a)——A solution of **10a** (386 mg) and benzoyl chloride (550 mg) in pyridine (5 ml) was allowed to stand for 2 d in a refrigerator and then diluted with ice-cold water (15 ml). The aqueous mixture was extracted with CH_2Cl_2, and the extract was washed with water, dried, and concentrated *in vacuo*. The residue was chromatographed on alumina using benzene as an eluent to give **21a**: 420 mg, 65% yield, viscous oil. IR (neat): 1640 (C=O) cm^{-1}. UV λ_{max} nm (ε): 215 (22800), 270 (6800). ^{1}H-NMR δ: 3.72 (3H, s, 5-OMe), 5.34 (1H, d, J=8 Hz, 6-H), 5.70 (1H, d, J=6 Hz, 2-H), 6.00 (1H, d, J=6 Hz, 3-H), 6.82 (1H, d, J=8 Hz, 7-H), 7.3—7.8 (5H, m, Ph-H). ^{13}C-NMR δ: 53.51 (q, 5-OMe), 110.93 (d, 6-C), 119.87 (d, 2-C), 129.19 (d, 3-C), 143.31 (d, 7-C), 165.32 (s, 5-C), 168.75 (s, C=O), Ph-C [127.39 (d), 128.86 (d), 132.03 (d), 133.98 (s)]. High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{12}N_2O_2$: 228.0899. Found: 228.0892.

1-Acetyl-5-methoxy-1*H*-1,4-diazepine (21b)——A solution of **10a** (220 mg) and acetyl chloride (90 ml) in pyridine (2 ml) was worked up as described for **21a** to give **21b**: 56 mg, 21% yield, viscous oil. IR (neat): 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.10 (3H, s, COMe), 3.66 (3H, s, 5-OMe), 5.36 (1H, d, J=8 Hz, 6-H), 5.54 (1H, d, J=6 Hz, 2-H), 5.80 (1H, d, J=6 Hz, 3-H), 6.84 (1H, d, J=8 Hz, 7-H). ^{13}C-NMR δ: 21.24 (q, COMe), 53.22 (q, 5-OMe), 111.37 (d, 6-C), 118.02 (d, 2-C), 129.15 (d, 3-C), 142.67 (d, 7-C), 165.19 (s, 5-C), 169.38 (s, C=O). High-resolution MS *m/z*: M^+ Calcd for $C_8H_{10}N_2O_2$: 166.0742. Found: 166.0741.

1-Ethoxycarbonyl-5-methoxy-1*H*-1,4-diazepine (21c)——A solution of **10a** (200 mg) and ethyl chloroformate (250 mg) in pyridine (2 ml) was worked up as described for **21a** to give **21c**: 36 mg, 18% yield, viscous oil. IR (neat): 1620 cm^{-1}. ^{1}H-NMR δ: 1.30 and 4.24 (3H, t, and 2H, q, CO_2Et), 3.66 (3H, s, 5-OMe), 5.14 (1H, d, J=8 Hz, 6-H), 5.58 (1H, d, J=6 Hz, 2-H), 5.90 (1H, d, J=6 Hz, 3-H), 6.58 (1H, d, J=8 Hz, 7-H). High-resolution MS *m/z*: M^+ Calcd for $C_9H_{12}N_2O_3$: 196.0848. Found: 196.0844.

Thermolysis of 21a——A solution of **21a** (200 mg) in xylene (5 ml) was heated at 120—130 °C for 10 h in a sealed tube and then evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–AcOEt (10 : 1) as an eluent to give 5-benzoylamino-2-methoxypyridine (**23**): 190 mg, 95% yield, mp 139—140 °C, colorless prims (from isopropyl ether). MS *m/z*: 228 (M^+). IR (KBr): 3340 (NH), 1650 (C=O) cm^{-1}. ^{1}H-NMR δ ($CDCl_3$–CD_3OD): 3.84 (3H, s, 2-OMe), 6.72 (1H, d, J=8 Hz, 3-H), 7.36—7.60 (3H, m, Ph-H), 7.8—8.0 (3H, m, Ph-H and 4-H), 8.32 (1H, d, J=2 Hz, 6-H). *Anal.* Calcd for $C_{13}H_{12}N_2O_2$: C, 68.42; H, 5.26; N, 12.28. Found: C, 68.37; H, 5.37; N, 12.26. This product **23** was identical with an authentic sample prepared from 5-amino-2-methoxypyridine by treatment with benzoyl chloride.

Photolysis of 21a——A solution of **21a** (250 mg) in benzene (150 ml) was irradiated for 30 h under ice cooling. After removal of the solvent *in vacuo*, the residue was chromatographed on alumina using benzene–AcOEt (10 : 1) as an eluent to give 2-benzoyl-3-methoxypyrrole (**26**): 33 mg, 15% yield, mp 88—90 °C, colorless leaflets (from *n*-hexane–isopropyl ether). IR ($CHCl_3$): 3300 (NH), 1600 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.70 (3H, s, OMe), 5.92 (1H, br t, J=3 Hz, 4-H), 6.94 (1H, br t, J=3 Hz, 5-H), 7.3—7.9 (5H, m, Ph-H), 9.30 (1H, br d, J=3 Hz, NH). High-resolution MS *m/z*: M^+ Calcd for $C_{12}H_{11}NO_2$: 201.0790. Found: 201.0763.

1-Benzoyl-5-methoxy-6,7-dihydro-1*H*-1,4-diazepine (27a)——The diazepine (**21a**, 68 mg) was hydrogenated over 5% Pd–C (20 mg) in EtOH (5 ml) under atmospheric pressure at room temperature for 3 h. The catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residue was chromatographed on alumina using benzene as an eluent to give **27a**: 45 mg, 66% yield, viscous oil. IR (neat): 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.84 (2H, t, J=7 Hz, 6-H_2), 3.72 (3H, s, 5-OMe), 4.15 (2H, t, J=7 Hz, 7-H_2), 5.72 (1H, d, J=6 Hz, 2-H), 6.12 (1H, br d, J=6 Hz, 3-H), 7.34—7.68 (5H, m, Ph-H). High-resolution MS *m/z*: M^+ Calcd for $C_{13}H_{14}N_2O_2$: 230.1055. Found: 230.1024.

1-Acetyl-5-methoxy-6,7-dihydro-1*H*-1,4-diazepine (27b)——The diazepine (**21b**, 32 mg) was hydrogenated and worked up as described for **27a** to give **27b**: 31 mg, 96% yield, viscous oil. IR (neat): 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.20 (3H, s, COMe), 2.68 (2H, t, J=7 Hz, 6-H_2), 3.68 (3H, s, 5-OMe), 3.96 (2H, t, J=7 Hz, 7-H_2), 5.80 (1H, d, J=6 Hz, 2-H), 6.12 (1H, d, J=6 Hz, 3-H). ^{13}C-NMR δ: 22.24 (q, COMe), 32.65 (t, 6-C), 44.30 (t, 7-C), 53.53 (q, 5-OMe), 117.24 (d, 2-C), 118.95 (d, 3-C), 167.83 and 168.89 (each s, 5-C and C=O). High-resolution MS *m/z*: M^+ Calcd for $C_8H_{12}N_2O_2$: 168.0899. Found: 168.0915.

Treatment of 21a with Acetic Acid in MeOH——A mixture of **21a** (72 mg), AcOH (0.5 ml), and MeOH (20 ml) was stirred for 24 h at room temperature. After removal of the solvent *in vacuo*, the residue was dissolved in CH_2Cl_2 (30 ml) and the solution was successively washed with 5% Na_2CO_3 and water, dried, and concentrated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–AcOEt (10 : 1) as an eluent to give 1-benzoyl-5,7-dimethyl-6,7-dihydro-1*H*-1,4-diazepine (**29**): 50 mg, 61% yield, viscous oil. IR (neat): 1650 cm^{-1}. ^{1}H-NMR δ: 2.56 (1H, dd, J=12, 1 Hz, one of 6-H_2), 3.06 (1H, dd, J=12, 8 Hz, one of 6-H_2), 3.32 (3H, s, 7-OMe), 3.68 (3H, s, 5-OMe), 5.64 (1H, d, J=8 Hz, 3-H), 6.04 (1H, d, J=8 Hz, 2-H), 6.05 (1H, dd, J=8, 1 Hz, 7-H), 7.14—7.40 (5H, m, Ph-H). ^{13}C-NMR δ:

37.77 (t, 6-C), 53.89 (q, 7-OMe), 56.30 (q, 5-OMe), 82.95 (d, 7-C), 115.83 (d), 117.83 (d), 128.36 (d), 128.65 (d), 131.01 (d), 134.95 (s), 164.01 (s), 170.89 (s). High-resolution MS *m/z*: M^+ Calcd for $C_{14}H_{16}N_2O_3$: 260.1161. Found: 260.1163.

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Studies on Diazepines. XXVII.[1] Syntheses of Fully Unsaturated 1*H*- and 3*H*-1,4-Benzodiazepines from 4-Quinolyl Azides

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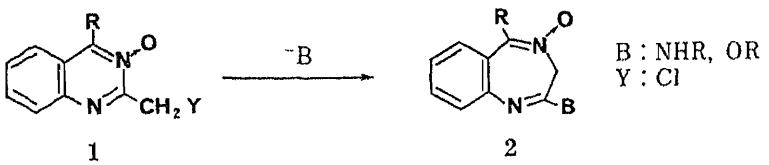
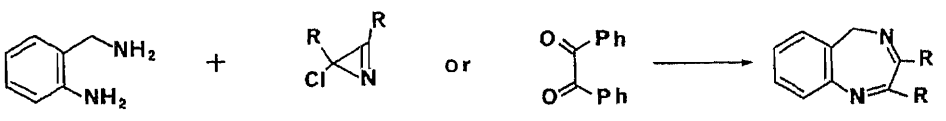
Irradiation of the 4-azidoquinolines (**7a—d**) in a 1:1 mixture of methanol and dioxane containing sodium methoxide resulted in ring expansion to form the unstable fully unsaturated 1*H*-1,4-benzodiazepines (**9a—d**), which were tautomerized to the stable 3*H*-1,4-benzodiazepines (**10a—d**) by further treatment with sodium methoxide. Acetylation of the 3*H*-diazepines (**10a** and **10d**) resulted in tautomerization to afford the 1-acetyl compounds **13a** and **13d**, which are the first isolated examples of 1*H*-1,4-benzodiazepines. From these fully unsaturated diazepines (**10** and **13**), the 2,3-dihydro-1*H*-1,4-benzodiazepines (**14—18**) and their 5-oxo derivatives (**19** and **20**) were prepared.

Keywords——4-azidoquinoline; 1*H*-1,4-benzodiazepine; 3*H*-1,4-benzodiazepine; 2,3-dihydro-1*H*-1,4-benzodiazepine; 1,4-benzodiazepin-5-one; photolysis; ring expansion; tautomerization

Much effort has recently been devoted to the synthesis of new fully unsaturated seven-membered heterocyclic rings.[2] As for benzodiazepines, 1,2-,[3,4] 1,3-,[5] and 2,3-benzodiazepines[6,7] and related fused diazepines condensed with aromatic heterocyclic rings have been prepared mainly by the photo-induced rearrangement of fused pyridine *N*-imides[3,5,6] or by the thermal cyclization of *o*-substituted styrene derivatives.[4,7] Very recently, we have reported the synthesis of the first examples of 2,4-benzodiazepines by the photochemical ring expansion of 4-azidoisoquinolines.[8] Among the six benzodiazepine isomers, the 1,4-benzodiazepines have been most widely investigated owing to their biological activities, but surprisingly, there are only a few examples of fully unsaturated compounds. The 3*H*-1,4-benzodiazepines (**2**)[9] are prepared by the base-induced ring enlargement of the quinazoline 3-oxides (**1**), and the 5*H*-1,4-benzodiazepines (**6**)[10] are obtained by the reaction of *o*-



1 2



3 4 5 6 : R=Ph, Me

Chart 1

aminobenzylamine (**3**) with the chloroazirines (**4**) or the 1,2-diketones (**5**), but no 1*H*-isomers had been reported. Therefore, we were interested in finding new synthetic routes to 1,4-benzodiazepines, and we report here that 4-azidoquinolines prepared readily from 4-chloroquinolines can be used as new synthons for some novel 1*H*- and 3*H*-1,4-benzodiazepines.[11]

The starting 4-azidoquinoline (**7a**)[12] and 4-azido-7-chloroquinoline (**7d**)[13] were prepared from the corresponding 4-chloroquinolines by the reported methods. 4-Azido-2-methylquinoline (**7b**) was obtained by the deoxygenation of its 1-oxide[13] with phosphorus trichloride. 4-Azido-2-phenylquinoline (**7c**) was prepared from 4-chloro-2-phenylquinoline[14] by treatment with sodium azide.

Irradiation (400 W, high-pressure Hg lamp; Pyrex filter) of the azides (**7a—d**: 0.5—0.6 g) in methanol–dioxane (1 : 1) containing sodium methoxide (3—4 mol eq) for 20—30 min under ice cooling resulted in ring expansion to yield the desired 5-methoxy-1*H*-1,4-benzodiazepines (**9a—d**) as yellow oils in high yields, presumably *via* the azirine intermediates **8** by analogy with the photolysis of pyridyl[1] and isoquinolyl azides.[8] The products (**9**) are the first examples of 1*H*-1,4-benzodiazepines,[15] but they are extremely unstable. The photolyzed solution was evaporated and the residue was extracted with ether to give nearly pure **9**, which, however, readily decomposed during purification by chromatography or on standing even in a refrigerator. Therefore, the structures of **9a—d** were confirmed only by their proton nuclear magnetic resonance (^{1}H-NMR) spectral data (Table I). For example, the ^{1}H-NMR spectrum of **9a** showed an AB pair of doublets (δ 5.46 and 5.66, J=6 Hz) assignable to 2-H and 3-H, and a broad NH signal at δ 5.10 in addition to the methoxy (δ 3.72, s) and aromatic (δ 7.4—8.4, m) proton signals. These spectral data are consistent with the proposed 1*H*-1,4-benzodiazepine structure **9**.

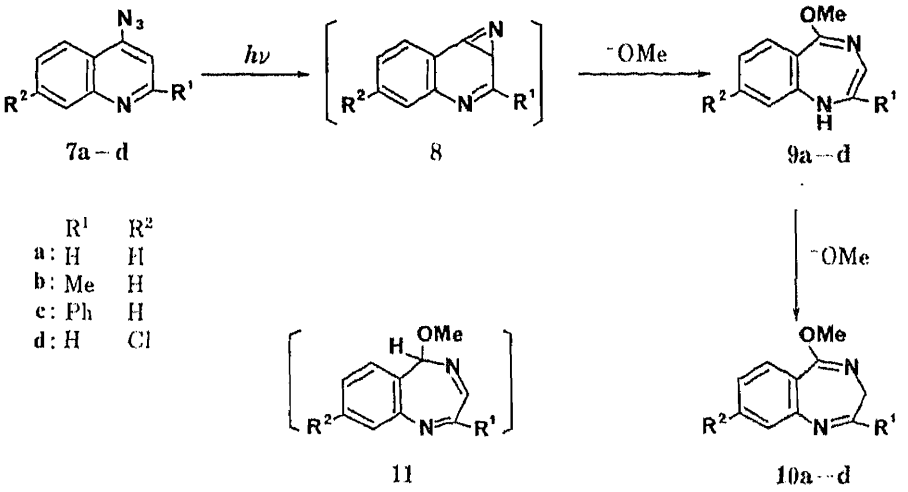


Chart 2

However, it should be noted that the unstable 1*H*-diazepines (**9**) were found to undergo tautomerization, giving rise to the relatively stable 3*H*-isomers **10**, on further treatment with sodium methoxide in methanol. Thus, after addition of a further 1—2 g of sodium methoxide, the photolyzed solution containing **9** (*ca.* 0.5 g) was stirred for 7—8 h to give the 5-methoxy-3*H*-1,4-benzodiazepines (**10a—d**), which could be isolated by chromatography in 35—65% yields calculated from the starting azides (**7**). In all cases, the formation of the possible 5*H*-isomer (**11**) was not observed. The ^{1}H-NMR of **10a—d** (Table I) showed methylene signals at δ 3.6—3.9 and no NH signals. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **10a** showed a signal due to a methylene carbon at δ 45.42 (t). These spectral data

and the results of the following reactions are consistent with the proposed 3*H*-1,4-benzodiazepine structures.

Hydrolysis of **10a** and **10b** either by refluxing in water–dioxane (1 : 1) for 2 h or treatment with 5% hydrochloric acid at room temperature resulted in decomposition of the ring to give methyl anthranilate (**12**) in high yields. This result strongly supports the 3*H*-1,4-benzodiazepine structure. Treatment of the 1*H*-diazepines (**10a** and **10d**) with acetyl chloride in pyridine resulted in tautomerization with acetylation to give the 1-acetyl-1*H*-1,4-benzodiazepines (**13a** and **13d** respectively) in *ca.* 20% yields; these products are the first isolated examples of 1*H*-1,4-benzodiazepines. The ^{1}H-NMR spectra of **13** (Table I) indicate

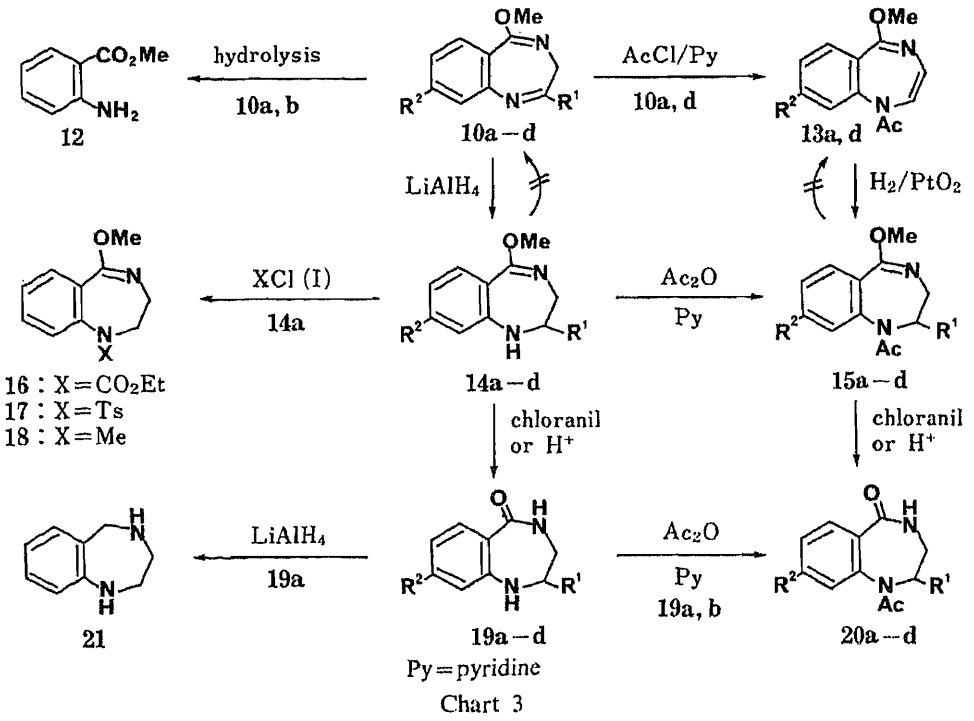


Chart 3

TABLE I. ^{1}H-NMR Spectral Data for the 1,4-Benzodiazepines (**9**, **10**, and **13**)

9a[a]	3.72 (3H, s, 5-OMe), 5.46 (1H, d, J=6, 3-H), 5.66 (1H, d, J=6, 2-H), 5.10 (1H, br, NH), 7.4—8.4 (4H, m, Ph-H)
9b[a]	2.55 (3H, s, 2-Me), 3.75 (3H, s, 5-OMe), 5.05 (1H, br, NH), 6.70 (1H, s, 3-H), 7.4—8.4 (4H, m, Ph-H)
9c[a]	3.64 (3H, s, 5-OMe), 4.8 (1H, br, NH), 7.0—8.0 (10H, m, 3-H and Ph-H)
9d[a]	3.68 (3H, s, 5-OMe), 4.7 (1H, br, NH), 5.30 (1H, d, J=6, 3-H), 5.60 (1H, d, J=6, 2-H), 6.50 (1H, d, J=2, 9-H), 6.7—6.8 (1H, m, 7-H), 7.15 (1H, d, J=9, 6-H)
10a[b]	3.62 (2H, d, J=4, 3-H_2), 3.85 (3H, s, 5-OMe), 7.2—7.6 (3H, m, Ph-H), 7.85 (1H, dd, J=8, 1, 6-H), 8.01 (1H, t, J=4, 2-H)
10b[b]	2.43 (3H, s, 2-Me), 3.66 (2H, s, 3-H_2), 3.88 (3H, s, 5-OMe), 7.2—7.6 (3H, m, Ph-H), 7.83 (1H, d, J=8, 6-H)
10c[b]	3.70 (3H, s, 5-OMe), 3.97 (2H, brs, 3-H_2), 6.9—7.4 (8H, m, Ph-H), 7.60 (1H, d, J=8, 6-H)
10d[b]	3.60 (2H, d, J=4, 3-H_2), 3.84 (3H, s, 5-OMe), 7.20 (1H, dd, J=8, 2, 7-H), 7.37 (1H, d, J=2, 9-H), 7.55 (1H, d, J=8, 6-H), 7.96 (1H, t, J=4, 2-H)
13a[b]	1.96 and 2.16 (3H, each s, intensity ratio 2 : 3, 1-Ac), 3.84 (3H, s, 5-OMe), 5.84 and 6.06 (1H, each d, J=5, intensity ratio 2 : 3, 2-H), 6.30 and 6.32 (1H, each d, J=5, intensity ratio 2 : 3, 3-H), 7.0—7.7 (4H, m, Ph-H)
13d[b]	2.03 and 2.22 (3H, each s, intensity ratio 2 : 3, 1-Ac), 3.86 (3H, s, 5-OMe), 5.90 and 6.11 (1H, each d, J=5, intensity ratio 2 : 3, 2-H), 6.40 (1H, d, J=5, 3-H), 7.0—7.7 (4H, m, Ph-H)

a) δ (CD_3OD), J=Hz; compounds **9** were readily decomposed in $CDCl_3$. *b*) δ ($CDCl_3$), J=Hz.

that the compounds **13** exist as mixtures of two rotatory isomers[16] due to the amide moiety in a ratio of 2 : 3, as shown by the dual signals of the acetyl methyl, 2-H, and 3-H protons; *e.g.*, in **13a**, 1-Ac (δ 1.96 and 2.16, each s, intensity ratio 2 : 3), 2-H (δ 5.84 and 6.06, each d, J=5 Hz, intensity ratio 2 : 3), and 3-H (δ 6.30 and 6.32, each d, J=5 Hz, intensity ratio 2 : 3).

However, the 2-substituted 3*H*-diazepines (**10b** and **10c**) did not react with acetyl chloride under similar conditions, probably because of their steric hindrance. In addition, in order to obtain 1-acyl-1*H*-1,4-benzodiazepines, the *N*-free 1*H*-diazepines (**9**) were treated with various acylating reagents such as acetyl chloride, benzoyl chloride, and ethyl chloroformate to give only decomposition products, methyl *N*-acylanthranilates, in very low yields, and the expected compounds were not formed.

$LiAlH_4$ reduction of **10a—d** afforded the 1,2-dihydro compounds **14a—d** in 40—60% yields. Treatment of **14a—d** with acetic anhydride in pyridine gave the 1-acetyl-2,3-dihydro-1,4-benzodiazepines (**15a—d**) in 65—85% yields. Compound **15a** was also obtained from **13a** by catalytic hydrogenation in 43% yield. Similarly, **14a** was treated with ethyl chloroformate or *p*-toluenesulfonyl chloride (TsCl) in pyridine, or with methyl iodide in the presence of *n*-butyl lithium to give the corresponding 1-substituted 2,3-dihydro-1*H*-diazepines (**16**, **17**, and **18**) in 35—45% yields, respectively. Attempts to transform the dihydro compounds (**14** and **15**) back into the fully unsaturated diazepines (**10** and **13**) by dehydrogenation with various reagents such as 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) and Pd–C were unsuccessful. However, treatment of **14a—d** and **15a—d** with chloranil in xylene unexpectedly gave the corresponding 5-oxo compounds (**19a—d** (45—50% yields) and **20a—d** (60—80% yields), respectively, although the expected dehydrogenation products (**10** and **13**) could not be obtained. To our knowledge, such a reaction with chloranil has not been reported, and thus studies on the detailed mechanism of the reaction and on applications of this result to other alkoxy compounds are in progress. The 5-oxo compounds (**19** and **20**) were also obtained by the hydrolysis of **14a—d** and **15a—d** in water–dioxane containing acetic acid, but in lower yields (**19a—d**, 20—30%; **20a—d**, 30—40% yields). Acetylation of **19a** and **19b** with acetic anhydride in pyridine gave **20a** and **20b**, respectively.

Finally, treatment of the 5-oxo compound (**19a**) with $LiAlH_4$ resulted in the formation of the tetrahydro-1,4-benzodiazepine (**21**, 82% yield), which was identical with an authentic sample prepared from methyl anthranilate by the reported method.[17]

In conclusion, the present results provide new synthetic routes to fully unsaturated 1*H*- and 3*H*-1,4-benzodiazepines, and their dihydro and oxo derivatives.

Experimental

The general experimental procedures were the same as in Part XXVI.[1]

Starting Materials——4-Azidoquinoline (**7a**)[12] and 4-azido-7-chloroquinoline (**7d**)[13] were prepared from the corresponding 4-chloroquinolines by the reported methods.

4-Azido-2-methylquinoline (7b)——A solution of 4-azido-2-methylquinoline 1-oxide[13] (25 g) and phosphorus trichloride (40 g) in $CHCl_3$ (500 ml) was refluxed for 1.5 h and then concentrated *in vacuo*. The residue was made alkaline with ice-cooled satd. $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2 as an eluent to give **7b**: 7.8 g, 34% yield, mp 52—54 °C, pale yellow prisms (from *n*-hexane). MS *m/z*: 184 (M^+). IR (KBr): 2240 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 2.34 (3H, s, 2-Me), 6.15 (1H, s, 3-H), 6.9—7.4 and 7.60 (3H, m, and 1H, d, J=8 Hz, Ph-H). *Anal.* Calcd for $C_{10}H_8N_4$: C, 65.22; H, 4.35; N, 30.43. Found: C, 65.01; H, 4.42; N, 30.57.

4-Azido-2-phenylquinoline (7c)——A solution of 4-chloro-2-phenylquinoline[14] (11.98 g, 0.05 mol) and sodium azide (6.5 g, 0.1 mol) in dimethyl sulfoxide (100 ml) was heated at 100 °C for 3 h with stirring. After cooling, the mixture was poured into water (100 ml) and the aqueous mixture was extracted with CH_2Cl_2. The extract was washed with water, dried, and evaporated to dryness *in vacuo*. The resulting solid residue was recrystallized from acetone–*n*-hexane to give **7c**: 9.96 g, 81% yield, mp 120—122 °C, pale yellow prisms. MS *m/z*: 246 (M^+). IR (KBr): 2200 ($-N_3$) cm^{-1}. ^{1}H-NMR δ: 7.4—7.8 and 7.9—8.2 (6H, m, and 4H, m, Ar-H). *Anal.* Calcd for $C_{15}H_{10}N_4$: C, 73.17; H, 4.07; N, 22.76. Found: C, 73.28; H, 4.03; N, 22.78.

Photolysis of the Azides (7a—d): Formation of 5-Methoxy-1*H*-1,4-benzodiazepines (9a—d)——General Procedure: A solution of **7** (0.5—0.6 g) and sodium methoxide (300—350 mg) in MeOH–dioxane (1 : 1, 150 ml) was irradiated with a 400 W high-pressure Hg lamp for 20—30 min under a nitrogen atmosphere. After removal of the solvent *in vacuo*, ice-water (*ca.* 50 ml) was added to the residue and the aqueous mixture was extracted with ether. The extract was washed with satd. NaCl, dried, and evaporated *in vacuo* to give **9** in a nearly pure state in 70—80% yield. However, the products (**9**) were unstable and gradually decomposed during isolation, and thus they were used in the following isomerization reaction without isolation. Their structures were elucidated from only the ^{1}H-NMR spectral data, which are collected in Table I.

Isomerization of 9a—d into 5-Methoxy-3*H*-1,4-benzodiazepines (10a—d)——General Procedure: i) A solution of crude **9** obtained above in MeOH (10 ml) containing sodium methoxide (1 g) was stirred for 7—8 h at room temperature and then concentrated *in vacuo*. Ice-water (*ca.* 30 ml) was added to the residue and the aqueous mixture was extracted with ether. The extract was washed with satd. NaCl, dried, and concentrated *in vacuo*. The residue was chromatographed on silica gel using ether as an eluent to give **10**. ii) Sodium methoxide (1—2 g) was further added to the photolyzed solution obtained in the photolysis of **7**, and the mixture was stirred for 7—8 h at room temperature, and worked up as described for i) to give **10**. ^{1}H-NMR spectral data of **10a—d** are collected in Table I and the yields (method ii) were calculated from the azides (**7**).

10a: 35—40% yield, pale yellow oil. MS *m/z*: 174 (M^+). IR (neat): 1650 (C=N) cm^{-1}. ^{13}C-NMR δ: 45.42 (t, 3-C), 54.06 (q, OMe), 161.01 (d, 2-C), 163.71 (s, 5-C), Ph-C [124.18 (s), 125.54 (d), 127.01 (d), 128.36 (d), 131.19 (d), 147.54 (s)]. *Anal.* Calcd for $C_{10}H_{10}N_2O$: C, 68.97; H, 5.75; N, 16.09. Found: C, 69.15; H, 5.49; N, 15.81.

10b: *ca.* 60% yield, pale yellow oil. MS *m/z*: 188 (M^+). IR (neat): 1650 (C=N) cm^{-1}. *Anal.* Calcd for $C_{11}H_{12}N_2O$: C, 70.21; H, 6.38; N, 14.89. Found: C, 69.98; H, 6.38; N, 15.09.

10c: 65—70% yield, pale yellow oil. MS *m/z*: 250 (M^+). IR (neat): 1630 (C=N) cm^{-1}. *Anal.* Calcd for $C_{16}H_{14}N_2O$: C, 76.80; H, 5.60; N, 11.20. Found: C, 76.71; H, 5.80; N, 10.96.

10d: *ca.* 40% yield, pale yellow oil. MS *m/z*: 208, 210 (M^+). IR (neat): 1650 (C=N) cm^{-1}. *Anal.* Calcd for $C_{10}H_9ClN_2O$: C, 57.55; H, 4.32; N, 13.43. Found: C, 57.45; H, 4.07; N, 13.68.

Hydrolysis of 10a, b——A solution of **10** (100 mg) in dioxane–water (1 : 1, 10 ml) was refluxed for 2 h. After cooling, the reaction solution was extracted with CH_2Cl_2 and the extract was washed with satd. NaCl, dried, and concentrated *in vacuo*. The residue was chromatographed on silica gel using *n*-hexane–CH_2Cl_2 (1 : 1) as an eluent to give methyl anthranilate (**12**: 86 mg, from **10a**, 99% yield; 77 mg from **10b**, 96% yield), which was identical with an authentic sample obtained from Tokyo Kasei Kogyo Co., Ltd.

1-Acetyl-5-methoxy-1*H*-1,4-benzodiazepines (13a, d)——Acetyl chloride (110 mg, 1.4 mmol) was added dropwise to a solution of **10** (**10a**, 250 mg; **10d**, 290 mg, 1.4 mmol) in pyridine (2 ml) with stirring in an ice bath. The reaction mixture was stirred for a further 2 h at room temperature and ice-water (*ca.* 20 ml) was added to the mixture. The aqueous mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with water, dried, and concentrated *in vacuo*. The residue was chromatographed on silica gel using ether as an eluent to give **13**, which was recrystallized from acetone–*n*-hexane. ^{1}H-NMR spectral data for **13a** and **13d** are collected in Table I.

13a: 68 mg, 22% yield, mp 62—65 °C, colorless needles. MS *m/z*: 216 (M^+). IR (KBr): 1670 (C=O), 1630 (C=N) cm^{-1}. *Anal.* Calcd for $C_{12}H_{12}N_2O_2$: C, 66.67; H, 5.56; N, 12.96. Found: C, 66.42; H, 5.81; N, 12.88.

13d: 70 mg, 20% yeild, mp 84—86 °C, cololress needles. MS *m/z*: 250, 252 (M^+). IR (KBr): 1680 (C=O), 1630 (C=N) cm^{-1}. *Anal.* Calcd for $C_{12}H_{11}ClN_2O_2$: C, 57.49; H, 4.39; N, 11.18. Found: C, 57.71; H, 4.55; N, 11.00.

5-Methoxy-2,3-dihydro-1*H*-1,4-benzodiazepines (14a—d)——General Procedure: $LiAlH_4$ (100 mg) was added in small portions to a solution of **10** (0.5—1.0 mmol) in anhydrous ether (15 ml) with stirring in an ice bath. The reaction mixture was stirred for a further 1 h at room temperature. The excess reagent was decomposed with water and the resulting precipitate was filtered off. The filtrate was dried and evaporated *in vacuo*. The residue was chromatographed on silica gel using ether as an eluent to give **14**.

14a: 40% yield, pale yellow oil. MS *m/z*: 176 (M^+). IR (neat): 3400 and 3250 (NH), 1650 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.5—3.9 (4H, m, 2- and 3-H_2), 3.79 (3H, s, 5-OMe), 4.2 (1H, br, NH), 6.50 (1H, d, J=8 Hz, 9-H), 6.66 (1H, dd, J=8, 8 Hz, 7-H), 7.14 (1H, ddd, J=8, 8, 2 Hz, 8-H), 7.64 (1H, dd, J=8, 2 Hz, 6-H). *Anal.* Calcd for $C_{10}H_{12}N_2O$: C, 68.18; H, 6.82; N, 15.91. Found: C, 67.92; H, 6.81; N, 16.02.

14b: 57% yield, mp 92—93 °C, pale yellow prisms (from CH_2Cl_2–*n*-hexane). MS *m/z*: 190 (M^+). IR (KBr): 3300 (NH), 1640 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.25 (3H, d, J=7 Hz, 2-Me), 3.4—4.0 (3H, m, 2-H and 3-H_2), 3.95 (3H, s, 5-OMe), 4.12 (1H, br, NH), 6.80 (1H, d, J=8 Hz, 9-H), 6.94 (1H, dd, J=8, 8 Hz, 7-H), 7.44 (1H, ddd, J=8, 8, 2 Hz, 8-H), 7.93 (1H, dd, J=8, 2 Hz, 6-H). *Anal.* Calcd for $C_{11}H_{14}N_2O$: C, 69.47; H, 7.37; N, 14.74. Found: C, 69.41; H, 7.58; N, 14.57.

14c: 45% yield, mp 109—110 °C, pale yellow prisms (from CH_2Cl_2–*n*-hexane). MS *m/z*: 252 (M^+). IR (KBr): 3400 and 3300 (NH), 1650 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.6—4.1 (2H, m, 3-H_2), 3.95 (3H, s, 5-OMe), 4.25 (1H, br, NH), 5.02 (1H, dd, J=8, 3 Hz, 2-H), 6.86 (1H, d, J=8 Hz, 9-H), 7.03 (1H, dd, J=8, 8, 7-H), 7.4—7.6 (6H, m, 8-H and 2-Ph-H), 7.84 (1H, dd, J=8, 2 Hz, 6-H). *Anal.* Calcd for $C_{16}H_{16}N_2O$: C, 76.19; H, 6.35; N, 11.11. Found: C, 76.20; H, 6.37; N, 10.95.

14d: 58% yield, mp 99—101 °C, pale yellow prisms (from CH_2Cl_2–*n*-hexane). MS *m/z*: 210, 212 (M^+). IR (KBr):

3400 and 3250 (NH), 1650 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.5—3.9 (4H, m, 2- and 3-H_2), 3.81 (3H, s, 5-OMe), 4.4 (1H, br, NH), 6.60 (1H, d, J=2 Hz, 9-H), 6.71 (1H, dd, J=9, 2 Hz, 7-H), 7.66 (1H, d, J=9 Hz, 6-H). *Anal.* Calcd for $C_{10}H_{11}ClN_2O$: C, 57.01; H, 5.23; N, 13.30. Found: C, 57.01; H, 5.28; N, 13.37.

1-Acetyl-5-methoxy-2,3-dihydro-1*H*-1,4-benzodiazepines (15a—d)——i) From **14a—d**: General Procedure: A mixture of **14** (60—70 mg), Ac_2O (1 ml), and pyridine (2 ml) was stirred for 12 h at room temperature and then ice-water (*ca.* 5 ml) was added. The aqueous mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using ether–MeOH (100 : 1) as an eluent to give **15**, which was recrystallized from CH_2Cl_2–*n*-hexane.

15a: 80% yield, mp 109—110 °C, colorless prisms. MS *m/z*: 218 (M^+). IR (KBr): 1660 (C=O), 1640 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.94 (3H, s, 1-Ac), 3.0—4.0 (3H, m, 3-H_2 and one of 2-H_2), 4.8—5.2 (1H, m, one of 2-H_2), 4.04 (3H, s, 5-OMe), 7.4—7.6 and 7.8—8.0 (1H, m, and 3H, m, Ph-H). *Anal.* Calcd for $C_{12}H_{14}N_2O_2$: C, 66.06; H, 6.42; N, 12.84. Found: C, 66.11; H, 6.56; N, 12.74.

15b: 68% yield, mp 106—108 °C, colorless prisms. MS *m/z*: 232 (M^+). IR (KBr): 1660 (C=O), 1640 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.15 (3H, d, J=7 Hz, 2-Me), 1.72 (3H, s, 1-AC), 2.60 (1H, dd, J=12, 12 Hz, one of 3-H_2), 3.48 (1H, dd, J=12, 5 Hz, one of 3-H_2), 3.78 (3H, s, 5-OMe), 4.8—5.2 (1H, m, 2-H), 7.0—7.1 and 7.3—7.5 (1H, m, and 3H, m, Ph-H). *Anal.* Calcd for $C_{13}H_{16}N_2O_2$: C, 67.24; H, 6.90; N, 12.07. Found: C, 67.25; H, 7.00; N, 11.75.

15c: 82% yield, mp 114—115 °C, colorless prisms. MS *m/z*: 294 (M^+). IR (KBr): 1660 (C=O), 1650 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.84 (3H, s, 1-Ac), 3.32 (1H, dd, J=13, 13 Hz, one of 3-H_2), 3.90 (1H, dd, J=13, 4 Hz, one of 3-H_2), 4.04 (3H, s, 5-OMe), 6.20 (1H, dd, J=13, 4 Hz, 2-H), 7.4—7.7 and 7.8—8.0 (6H, m, and 3H, m, Ph-H). *Anal.* Calcd for $C_{18}H_{18}N_2O_2$: C, 73.47; H, 6.12; N, 9.52. Found: C, 73.21; H, 6.23; N, 9.42.

15d: 66% yield, mp 141—142 °C, colorless prisms. MS *m/z*: 252, 254 (M^+). ^{1}H-NMR δ: 1.96 (3H, s, 1-Ac), 3.0—3.9 (3H, m, 3-H_2 and one of 2-H_2), 4.6—5.2 (1H, m, one of 2-H_2), 4.00 (3H, s, 5-OMe), 7.5—7.6 and 7.7—7.9 (1H, m, and 2H, m, Ph-H). *Anal.* Calcd for $C_{12}H_{13}ClN_2O_2$: C, 57.03; H, 5.15; N, 11.09. Found: C, 57.05; H, 5.13; N, 11.01.

ii) From **13a**: A solution of **13a** (72 mg, 0.3 mmol) in EtOH (10 ml) was hydrogenated over PtO_2 (40 mg) with stirring at room temperature under atmospheric pressure. After uptake of *ca.* 0.3 mmol of hydrogen, the reaction was stopped. The catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (10 : 1) as an eluent to give **15a**: 31 mg, 43% yield.

1-Ethoxycarbonyl-5-methoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (16)——Ethyl chloroformate (1 ml) was added dropwise to a solution of **14a** (176 mg) in pyridine (3 ml) with stirring in an ice bath. The reaction mixture was stirred for a further 12 h at room temperature and then poured into ice-water (*ca.* 10 ml). The aqueous mixture was extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (5 : 1) as an eluent to give **16**: 100 mg, 45% yield, colorless oil. MS *m/z*: 248 (M^+). IR ($CHCl_3$): 1710 (C=O), 1660 (C=N) cm^{-1}. ^{1}H-NMR δ: 1.20 and 4.16 (3H, t, and 2H, q, J= 7 Hz, CO_2Et), 3.3—3.5 (2H, m, 3-H_2), 3.87 (3H, s, 5-OMe), 3.9—4.2 (2H, m, 2-H_2), 7.2—7.6 (4H, m, Ph-H). *Anal.* Calcd for $C_{13}H_{16}N_2O_3$: C, 62.90; H, 6.45; N, 11.29. Found: C, 62.81; H, 6.70; N, 11.22.

1-(*p*-Toluenesulfonyl)-5-methoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (17)——A solution of *n*-butyl lithium (15% in hexane, 0.65 ml, 1 mmol) was added dropwise to a solution of **14a** (176 mg, 1 mmol) in tetrahydrofuran (THF, 10 ml) with stirring at −75 °C. Stirring was continued for a further 1 h at −75 °C, then a solution of TsCl (1 mmol) in THF (10 ml) was added dropwise to the reaction mixture. The whole was stirred for 1 h at −70——50 °C, then warmed to room temperature, and diluted with water (30 ml). The aqueous mixture was extracted with CH_2Cl_2 and the extract was washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (20 : 1) as an eluent to give **17**: 112 mg, 34% yield, mp 151—153 °C, colorless prisms (from acetone–*n*-hexane). MS *m/z*: 330 (M^+). IR (KBr): 1660 (C=N), 1360, 1330, and 1180 ($-SO_2-$) cm^{-1}. ^{1}H-NMR δ: 2.41 (3H, s, Ts-Me), 3.34 (3H, s, 5-OMe), 3.2—3.4 (2H, m, 3-H_2), 4.0—4.2 (2H, m, 2-H_2), 7.1—7.7 (8H, m, Ph-H). *Anal.* Calcd for $C_{17}H_{18}N_2O_3S$: C, 61.81; H, 5.45; N, 8.48. Found: C, 62.01; H, 5.73; N, 8.20.

1-Methyl-5-methoxy-2,3-dihydro-1*H*-1,4-benzodiazepine (18)——Compound **14a** (176 mg, 1 mmol) was successively treated with *n*-butyl lithium (1 mmol) and methyl iodide (1 mmol), and worked up as described for **17** to give **18**: 80 mg, 42% yield, colorless oil. MS *m/z*: 190 (M^+). IR ($CHCl_3$): 1660 (C=N) cm^{-1}. ^{1}H-NMR δ: 3.03 (3H, s, N-Me), 3.8—3.9 (4H, m, 2- and 3-H_2), 4.15 (3H, s, 5-OMe), 7.4—7.7 and 7.9—8.2 (each 2H, m, Ph-H). *Anal.* Calcd for $C_{11}H_{14}N_2O$: C, 69.47; H, 7.37; N, 14.74. Found: C, 69.18; H, 7.42; N, 14.51.

2,3,4,5-Tetrahydro-1*H*-1,4-benzodiazepin-5-ones (19a—d)——General Procedure: i) Treatment of **14** with Chloranil: A solution of **14** (0.5 mmol) and chloranil (0.6 mmol) in xylene (15 ml) was refluxed for 2—3 h with stirring and then evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (1 : 1) as an eluent to give **19**, which was recrystallized from acetone–*n*-hexane.

19a: 50% yield, mp 135—136 °C, colorless prisms. MS *m/z*: 162 (M^+). IR (KBr): 3150 (NH), 1610 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.4—3.7 (4H, m, 2- and 3-H_2), 4.2—4.6 (1H, br, NH), 6.56 (1H, d, J=8 Hz, 9-H), 6.75 (1H, dd, J=8, 8 Hz, 7-H), 7.1—7.4 (2H, m, 8-H and NH), 7.96 (1H, dd, J=8, 2 Hz, 6-H). *Anal.* Calcd for $C_9H_{10}N_2O$: C, 66.67; H, 6.17; N, 17.28. Found: C, 66.42; H, 6.17; N, 17.20.

19b: 43% yield, mp 165—167 °C, colorless prisms. MS *m/z*: 176 (M^+). IR (KBr): 3300 and 3200 (NH), 1630 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.29 (3H, d, J=7 Hz, 2-Me), 3.1—3.3 (3H, m, 2-H and 3-H_2), 4.8 (2H, br, 2×NH), 6.6—

6.8 (2H, m, 7- and 9-H), 7.19 (1H, ddd, J=8, 8, 2 Hz, 8-H), 7.66 (1H, dd, J=9, 2 Hz, 6-H). *Anal.* Calcd for $C_{10}H_{12}N_2O$: C, 68.18; H, 6.82; N, 15.91. Found: C, 67.82; H, 6.79; N, 15.66.

19c: 47% yield, mp 181—182 °C, colorless prisms. MS *m/z*: 238 (M^+). IR (KBr): 3300 and 3200 (NH), 1620 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.4—3.6 (2H, m, 3-H_2), 4.2 (1H, br, NH), 4.75 (1H, br t, J=6 Hz, 2-H), 6.62 (1H, dd, J=8, 2 Hz, 9-H), 6.81 (1H, dd, J=8, 8 Hz, 7-H), 7.1—7.4 (7H, m, 8-H, 2-Ph-H and NH), 7.78 (1H, dd, J=8, 2 Hz, 6-H). *Anal.* Calcd for $C_{15}H_{14}N_2O$: C, 75.63; H, 5.88; N, 11.76. Found: C, 75.52; H, 5.97; N, 11.65.

19d: 46% yield, mp 179—180 °C, colorless prisms. MS *m/z*: 196, 198 (M^+). IR (KBr): 3300 and 3200 (NH), 1610 (C=O) cm^{-1}. ^{1}H-NMR δ: 3.2—3.4 (4H, m, 2- and 3-H_2), 4.7 (2H, br, 2 × NH), 6.36 (1H, dd, J=8, 2 Hz, 7-H), 6.46 (1H, d, J=2 Hz, 9-H), 7.50 (1H, d, J=8 Hz, 6-H). *Anal.* Calcd for $C_9H_9ClN_2O$: C, 54.96; H, 4.58; N, 14.25. Found: C, 54.91; H, 4.59; N, 13.98.

ii) Hydrolysis of **14**: A mixture of **14** (1 mmol), dioxane (5 ml), water (5 ml), and AcOH (1 drop) was refluxed for 12 h. After cooling, the reaction mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (1 : 1) as an eluent to give **19**. **19a**, 21%; **19b**, 28%; **19c**, 32%; and **19d**, 26% yield.

1-Acetyl-2,3,4,5-tetrahydro-1*H*-1,4-benzodiazepin-5-ones (20a—d)——General Procedure: i) From **15**: Compounds **15a—d** (50—100 mg) were treated with chloranil in xylene and worked up in the same manner as described for the preparation of **19** from **14** to give **20a—d**, which were recrystallized from acetone–*n*-hexane.

20a: 73% yield, mp 172—173 °C, colorless prisms. MS *m/z*: 204 (M^+). IR (KBr): 3200 (NH), 1615 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.90 (3H, s, N-Ac), 3.2—3.5 (3H, m, 3-H_2 and one of 2-H_2), 4.7—5.1 (1H, m, one of 2-H_2), 7.4—8.1 (5H, m, NH and Ph-H). *Anal.* Calcd for $C_{11}H_{12}N_2O_2$: C, 64.71; H, 5.88; N, 13.73. Found: C, 64.40; H, 5.91; N, 13.42.

20b: 62% yield, mp 181—182 °C, colorless prisms. MS *m/z*: 218 (M^+). IR (KBr): 3300 (NH), 1620 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.08 (3H, d, J=7 Hz, 2-Me), 1.78 (3H, s, N-Ac), 2.5—2.8 and 3.1—3.4 (each 1H, m, 3-H_2), 4.8—5.1 (1H, m, 2-H), 7.0—7.7 (4H, m, Ph-H), 8.1 (1H, br, NH). *Anal.* Calcd for $C_{12}H_{14}N_2O_2$: C, 66.06; H, 6.42; N, 12.84. Found: C, 66.30; H, 6.55; N, 12.87.

20c: 80% yield, mp 200—202 °C, colorless prisms. MS *m/z*: 280 (M^+). IR (KBr): 3300 (NH), 1670 and 1630 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.78 (3H, s, N-Ac), 3.2—3.4 (2H, m, 3-H_2), 5.84 (1H, dd, J=12, 6 Hz, 2-H), 7.0—7.3 and 7.5—7.7 (6H, m, and 4H, m, Ph-H and NH). *Anal.* Calcd for $C_{17}H_{16}N_2O_2$: C, 72.86; H, 5.71; N, 10.00. Found: C, 72.80; H, 5.81; N, 9.90.

20d: 76% yield, mp 203—204 °C, colorless prisms. MS *m/z*: 238, 240 (M^+). IR (KBr): 3200 (NH), 1650 (C=O) cm^{-1}. ^{1}H-NMR δ: 1.92 (3H, s, N-Ac), 3.1—3.5 (3H, m, 3-H_2 and one of 2-H_2), 4.6—5.0 (1H, m, one of 2-H_2), 7.1—7.4 (2H, m, 9-H and NH), 7.54 (1H, dd, J=8, 2 Hz, 7-H), 7.80 (1H, d, J=8 Hz, 6-H). *Anal.* Calcd for $C_{11}H_{11}ClN_2O_2$: C, 55.35; H, 4.61; N, 11.74. Found: C, 55.26; H, 4.74; N, 11.62.

Compounds **20a—d** were also obtained from **15a—d** by hydrolysis in dioxane–water containing AcOH followed by work-up as described for the preparation of **19** from **14**. **20a**, 33%; **20b**, 28%; **20c**, 40%; and **20d**, 36% yield.

ii) From **19a, b**: A mixture of **19** (100 mg), Ac_2O (2 ml), and pyridine (4 ml) was stirred for 6 h at room temperature and then poured into ice-water (*ca.* 15 ml). The aqueous mixture was made alkaline with $NaHCO_3$ and extracted with CH_2Cl_2. The extract was washed with satd. NaCl, dried, and evaporated *in vacuo*. The residue was chromatographed on silica gel using CH_2Cl_2–acetone (50 : 1) as an eluent to give **20**, **20a**, 55% and **20b**, 65% yield.

2,3,4,5-Tetrahydro-1*H*-1,4-benzodiazepine (21)——$LiAlH_4$ (100 mg) was added to a solution of **19a** (70 mg) in THF (15 ml) with stirring in an ice bath and then the reaction mixture was refluxed for 6 h. After cooling, the excess reagent was decomposed with water and the resulting precipitate was filtered off. The filtrate was dried and evaporated *in vacuo*. The residue was chromatographed on alumina using CH_2Cl_2–MeOH (100 : 1) as an eluent to give **21**: 53 mg, 82% yield, colorless oil. This compound was identical with an authentic sample prepared from methyl anthranilate by the reported method.[17]

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New-Type Inducers of Differentiation of HL-60 Leukemia Cells Suppress c-*myc* Expression

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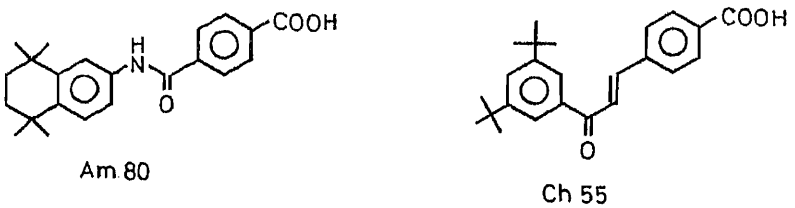
New-type inducers of differentiation of human promyelocytic leukemia cells HL-60, *i.e.*, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am 80) and (*E*)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch 55), suppress c-*myc* expression as all-*trans*-retinoic acid (RA) does. The decreased level of c-*myc* expression was detected within 5—14 h after the start of treatment, and preceded morphological differentiation and functional differentiation measured in terms of nitroblue tetrazolium (NBT) reduction. Treatment of HL-60 cells with 0.1 μM Am 80, Ch 55 or RA for 96 h caused more than 90% suppression of c-*myc* expression and around 10-fold increase in the percentage of NBT-positive cells.

Keywords——terminal differentiation; c-*myc*; retinoic acid; HL-60; 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am 80); (*E*)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch 55)

Introduction

Induction of differentiation of cancer cells may have implications in cancer chemotherapy. The human myelogeneous leukemia cell line HL-60, eastablished by Gallo *et al.*,[1] is known to be induced to differentiate terminally *in vitro* by various compounds such as dimethyl sulfoxide (DMSO), vitamin D_3, retinoic acid, *etc.*[2] Recently, we reported the synthesis of two new-type inducers of differentiation of HL-60 cells, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid (Am 80)[3] and (*E*)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch 55).[4] (Fig. 1). Both Am 80 and Ch 55 possess very strong activity in inducing differentiation of HL-60 cells to mature granulocytes.[3,4] These compounds with novel structures are also potent inhibitors of the induction of ornithine decarboxylase (ODC) in mouse skin by tumor promotors,[5] and are potent inducers of the expression of the membrane receptors for epidermal growth factor (EGF).[6]

On the other hand, it has been reported that the terminal differentiation of myelogeneous cells is closely related to the regulation of oncogene expression, especially the suppression of c-*myc* expression.[7-10] All-*trans*-retinoic acid (RA) is known to suppress the expression of c-*myc* in HL-60 cells,[7] which are very sensitive to induction of differentiation by RA. The HL-



Am 80 Ch 55

Fig. 1. Structures of Am 80 and Ch 55

60 genome contains several oncogenes: among them, c-*myc* is significantly amplified and transcribed at a high level.[11] It is presumed that excessive expression of c-*myc* is correlated with the excessive proliferation and arrested differentiation of the HL-60 cells, though the specific molecular function of the nuclear protein encoded by the c-*myc* gene has not yet been elucidated. In this paper, we describe the regulation of c-*myc* expression in HL-60 cells during chemically induced differentiation with the new synthetic compounds, Am 80 and Ch 55.

Materials and Methods

Chemicals——Am 80 and Ch 55 were prepared as described previously[3,4]

Cell Culture——HL-60 cells[1] were maintained in RPMI1640 medium supplemented with fetal calf serum (FCS) (10%, v/v) in 5% carbon dioxide at 37 °C. For induction experiments, cells were resuspended at a concentration of 8×10^4 cells/ml in the same medium in the absence or presence of 0.1 μM Am 80, Ch 55 or RA.

NBT Reduction——Nitroblue tetrazolium (NBT) reduction was assayed as described[12] in the presence of tetradecanoylphorbol 13-acetate (TPA).

Ribonucleic Acid (RNA) Isolation——Cellular RNA was isolated from 100 ml of culture medium after incubation for 0—96 h as described[13] in the presence of 20 mM vanadyl ribosides. The amount of the isolated RNA was calculated from the absorbance at 254 nm of the diluted stock solution and checked by agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

Northern Analysis——Samples of cellular RNA (10 μg) were denatured with formaldehyde and fractionated by electrophoresis on 1% formaldehyde-agarose gels as described.[13] The RNA was transferred to nitrocellulose paper with 20 × sodium saline citrate (SSC). The blots were baked in a vacuum oven for 5 h at 80 °C. The blots were prehybridized for 12 h at 42 °C and hybridyzed for 20 h at 42 °C on 50% (v/v) formamide solution containing 0.1 M Pipes–NaOH (pH 6.8), 0.65 M NaCl, 5 × Denhardt's solution, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecylsulfate (SDS), 10% dextran sulfate, 100 μg/ml heat-denatured salmon testis deoxyribonucleic acid (DNA) and 5×10^7 cpm/ml of ^{32}P-labeled probe with a specific activity of more than 2×10^9 cpm/μg. Filters were washed 4 times at 52 °C for 30 min with 2 × SSC containing 0.05% SDS and 20 mM sodium pyrophosphate, and then exposed to Kodak XAR film at −70 °C. The ^{32}P-labeled c-*myc*-specific probe DNA[14] was prepared by using the Amersham Multiprime DNA Labeling System (RPN 1601).

Results and Discussion

The effects of Am 80, Ch 55 and RA on HL-60 cell growth, on terminal differentiation measured in terms of NBT reduction (morphological changes of the cells are parallel with the percentage of NBT-positive cells), and on expression of c-*myc* were investigated. All three compounds showed marked inhibition of cell growth (Fig. 3a, inset), induction of terminal differentiation measured in terms of NBT reduction (more than 10-fold increase in the percentage of NBT-positive cells after 96 h of incubation), and suppression of c-*myc* expression (more than 90% after 96 h of incubation). Incubation in the absence of these compounds caused neither differentiation of the cells nor suppression of c-*myc* expression (Figs. 2d and 3a).

A kinetic study showed that the suppression of c-*myc* expression by Am 80, Ch 55 and RA preceded growth inhibition and differentiation of the cells; a decreased level of c-*myc* expression was detected within 5—14 h, though no significant growth inhibition or appearance of NBT-positive cells was detected within 14—24 h (Figs. 2a—c and 3b—d). After treatment of the cells with these compounds for 24 h, the c-*myc* expression was reduced to around 50% of the control level, though the percentage of NBT-positive cells was within the control range. The order of the suppressing effects of Am 80, Ch 55 and RA (at 0.1 μM) on the c-*myc* expression and that on the increase of NBT-positive cells were the same, but the response to RA was slightly faster than those to the other two compounds (Figs. 2 and 3). Other sets of independent experiments gave essentially the same results (data not shown).

As mentioned above, our compounds, Am 80 and Ch 55, cause terminal differentiation of HL-60 cells to mature granulocytes and dramatic suppression of c-*myc* expression at the

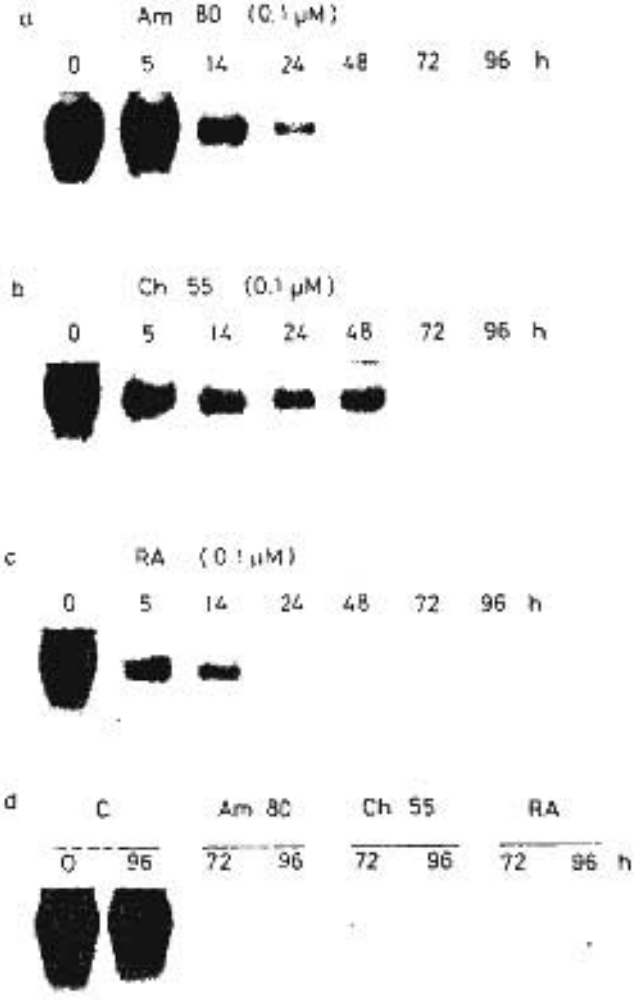


Fig. 2. Northern Analyses of c-*myc* Expression

(a)—(c), northern blot of cellular RNA from HL-60 cells treated with 0.1 μM (a) Am 80, (b) Ch 55 or (c) RA for 0—96 h; (d), northern blot of cellular RNA from the initial cell culture (C h), culture incubated for 96 h without chemical inducers (96 h), and culture incubated for 96 h with 0.1 μM Am 80, Ch 55 or RA. Autoradiography (exposure time): 15 h for (a)—(c) and 40 h for (d) at −70 °C.

concentration of 0.1 μM, as RA dose. The latter effect is of interest in view of the widely discussed hypothesis of carcinogenesis which states that cell transformation and malignancy may result from the elevated expression of an oncogene locus.[15] The finding that the suppression of c-*myc* expression precedes the terminal differentiation induced by Am 80, Ch 55 and RA suggests that the suppression of c-*myc* expression may be one of the basic actions of these compounds.

The compounds, Am 80, Ch 55 and RA, cause quite similar specific biological responses, as far as investigated to date: (1) induction of terminal differentiation of human promyelocytic leukemia cell HL-60 and mouse teratocarcinoma cell F9, (2) suppression of c-*myc* expression, (3) enhancement of epidermal growth factor (EGF) binding to its membrane receptors,[6] (4) inhibition of ornithine decarboxylase (ODC) induction by tumor promoters in mouse skin,[5] *etc.* Thus, Am 80 and Ch 55 can be classified into the family of retinoids; it has been proposed to define a retinoid as a substance that can elicit specific biological responses by virtue of binding to and activating a specific receptor or a set of receptors.[16,17] The simple structures of these compounds and the relatively simple organic chemistry involved in their

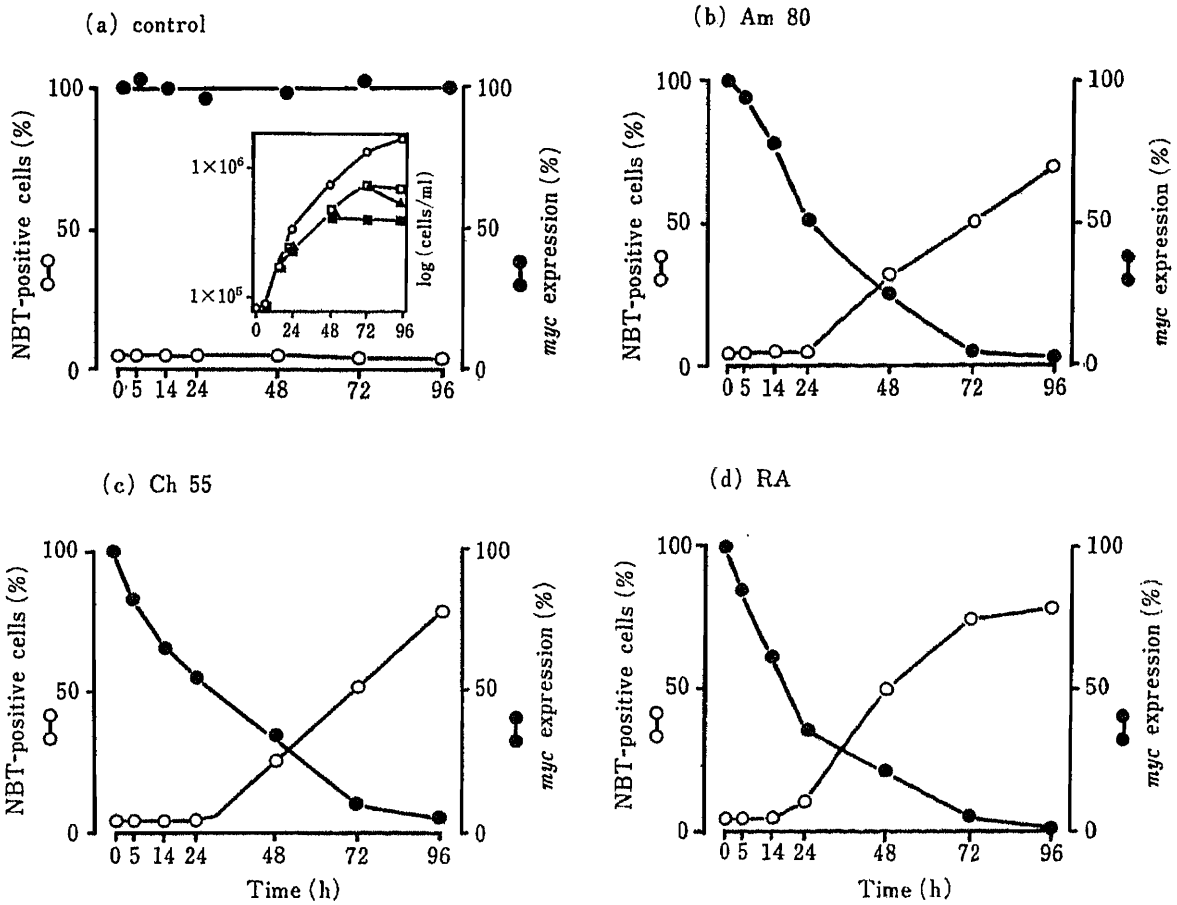


Fig. 3. Effects of Am 80, Ch 55 and RA on HL-60 Cell Growth, Differentiation Measured in Terms of NBT Reduction and c-*myc* Expression

Graphic representation of the time courses of percentage of NBT-positive cells and expression of c-*myc* (from densitometry of autoradiograms).
(a) HL-60 cells incubated without differentiation inducers. The inset shows growth curves of HL-60 cells incubated in the absence of chemical differentiation inducers (○), and in the presence of 0.1 μM Am 80 (□), Ch-55 (▲) or RA (■). (b)—(d) HL-60 cells incubated in the presence of 0.1 μM Am 80 (b), Ch-55 (c) or RA (d).

synthesis (and that of related molecules), as well as their greater stability to heat, light, acids, and oxidation compared with RA, strongly suggest the usefulness of these new-type retinoids for investigation of the molecular mechanism of retinoidal action. These compounds are also considered to be promising agents for application in the chemoprevention of human cancer, in the treatment of human leukemia, or in clinical dermatology.

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Studies on Uricosuric Diuretics. I. 6,7-Dichloro-5-sulfamoyl-2,3-dihydrobenzofuran-2-carboxylic Acids

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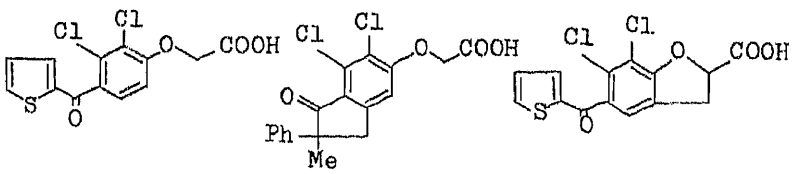
2,3-Dihydrobenzofuran derivatives having various sulfamoyl groups at the 5-position were synthesized and tested for oral diuretic and saluretic activities in rats and mice. Intraperitoneal uricosuric activity was also tested by a clearance method using oxonate-treated rats. Structure–activity relationships are presented. The 6,7-dichloro-5-*N*,*N*-disubstituted sulfamoyl-2,3-dihydrobenzofuran-2-carboxylic acids (**9ab**, **ac**, **13a** and **b**) having lower alkyl substituents showed the most potent diuretic and saluretic activities among the compounds synthesized. Hyperuricosuric activity was observed in 6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic acids and 2-hydroxymethyl-6,7-dichloro-2,3-dihydrobenzofurans having a 5-sulfamoyl group, with relatively small substituents (**9aa**—**ac**, **af**, **ak**, **al**, **an**, **ao** and **16a**—**c**). The saluretic activity of **9ab** showed a high-ceiling profile. Examination of the enantiomers of **9ab** revealed that the (−)-enantiomer is responsible for most of the diuretic and saluretic activities, while the (+)-enantiomer is responsible for most of the uricosuric activity.

Keywords——diuretic activity; saluretic activity; uricosuric activity; antihypertensive activity; 2,3-dihydrobenzofuran-5-sulfonamide; structure–activity relationship; S-8666

Diuretics are widely used as the agents of choice in hypertension therapy. Thiazide drugs, which are most frequently used other than antialdosterone-type compounds, accelerate excretion of Na and Cl by restraining their reabsorption at the nephron: this action is favorable with respect to the diuretic effect, but frequently causes side effects such as hypokalemia, glycohemia and hyperuricemia. Loop diuretics, which cause potent but



1 tienilic acid 2 indacrinone 3

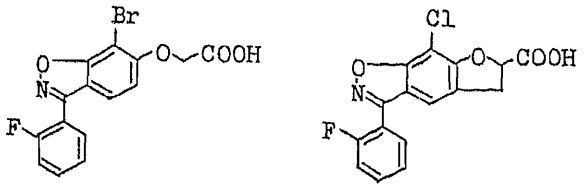


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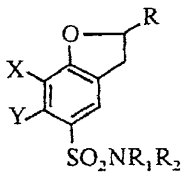
Chart 1

TABLE I. Substituted 2,3-Dihydrobenzofuran-5-sulfonamides



No.	X	Y	R_1	R_2	R	Yield (%)	mp (°C)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found) C	H	Cl	N	S
9aa	Cl	Cl	H	H	COOH	91	227—229	EA–E	$C_9H_7Cl_2NO_5S$	34.63	2.26	22.72	4.49	10.27
										(34.73	2.49	22.78	4.45	10.09)
9ab	Cl	Cl	CH_3	CH_3	COOH	94	155—156	D–E–H	$C_{11}H_{11}Cl_2NO_5S$	38.84	3.26	20.84	4.12	9.43
										(38.64	2.99	20.92	4.31	9.66)
(−)-**9ab**							130—131	EA–H		(38.87	3.32	20.79	4.17	9.28)
(+)-**9ab**							130—131	EA–H		(39.07	3.43	20.55	4.03	9.17)
9ac	Cl	Cl	C_2H_5	C_2H_5	COOK	96	235—236	EL–W	$C_{13}H_{14}Cl_2KNO_5S \cdot H_2O$	36.79	3.80	16.70	3.30	7.56
										(36.64	3.81	16.92	3.29	7.89)
9ad	Cl	Cl	*n*-C_3H_7	*n*-C_3H_7	COOH	84	120—121	EA–E	$C_{15}H_{19}Cl_2NO_5S$	45.46	4.83	17.89	3.53	8.09
										(45.36	4.80	17.83	3.25	8.12)
9ae	Cl	Cl	iso-C_3H_7	iso-C_3H_7	COOH	80	176—177	EA–E	$C_{15}H_{19}Cl_2NO_5S$	45.46	4.83	17.89	3.53	8.09
										(45.23	4.75	17.95	3.48	7.91)
9af	Cl	Cl	CH_3	*n*-C_4H_9	COOH	76	92—93	EA–H	$C_{14}H_{17}Cl_2NO_5S$	43.99	4.48	18.55	3.66	8.39
										(43.85	4.35	18.43	3.77	8.33)
9ag	Cl	Cl	$PhCH_2$	$PhCH_2$	COOH	72	127—128	EA–E	$C_{23}H_{19}Cl_2NO_5S$	56.11	3.89	14.40	2.84	6.51
										(56.01	3.81	14.62	2.83	6.31)
9ah	Cl	Cl	CH_3	$PhCH_2$	COOH	80	148—149	EA–E	$C_{17}H_{15}Cl_2NO_5S$	49.05	3.63	17.03	3.36	7.70
										(48.90	3.72	17.20	3.42	7.56)
9ai	Cl	Cl	CH_3	Ph	COOK	80	194—195	EL–W	$C_{16}H_{12}Cl_2KNO_5S \cdot H_2O$	41.93	3.08	15.46	3.05	6.99
										(41.73	3.14	15.75	3.30	7.06)
9aj	Cl	Cl	CH_3	*cyclo*-C_6H_{11}	COOH	52	148—149	D–E–H	$C_{16}H_{19}Cl_2NO_5S$	47.07	4.69	17.37	3.43	7.85
										(47.34	4.73	17.12	3.41	7.50)
9ak	Cl	Cl	$-(CH_2)_4-$		COOH	99	181—182	EA–E	$C_{13}H_{13}Cl_2NO_5S$	42.64	3.58	19.36	3.82	8.76
										(42.44	3.59	19.20	3.77	8.56)
9al	Cl	Cl	$-(CH_2)_5-$		COOH	99	199—200	EA–E	$C_{14}H_{15}Cl_2NO_5S$	44.22	3.98	18.65	3.68	8.43
										(44.17	3.95	18.57	3.71	8.23)
9am	Cl	Cl	$-(CH_2)_2O(CH_2)_2-$		COOH	89	228—229	EA–E	$C_{13}H_{13}Cl_2NO_6S$	40.85	3.43	18.55	3.66	8.39
										(40.43	3.39	18.37	3.60	8.28)
9an	Cl	Cl	H	CH_3	COOH	88	215—216	EA–E	$C_{10}H_9Cl_2NO_5S$	36.83	2.78	21.74	4.29	9.83
										(36.93	3.04	21.09	4.17	9.51)
9ao	Cl	Cl	H	*n*-C_3H-	COOH	95	200—201	EA–E	$C_{12}H_{13}Cl_2NO_5S$	40.69	3.70	20.02	3.95	9.05
										(40.52	3.73	19.96	3.91	8.82)

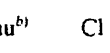
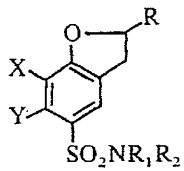
9ap	Cl	Cl	H	iso-C_3H_7-	COOH	95	184—185	EA-E	$C_{12}H_{13}Cl_2NO_5S$	40.69	3.70	20.02	3.95	9.05
										(40.53	3.68	19.72	4.00	8.89)
9aq	Cl	Cl	H	$PhCH_2$	COOH	85	199—200	EA-E	$C_{16}H_{13}Cl_2NO_5S$	47.78	3.26	17.63	3.48	7.97
										(47.90	3.47	17.68	3.40	7.59)
9ar	Cl	Cl	H	Ph	COOH	86	201—202	EA-E	$C_{15}H_{11}Cl_2NO_5S$	46.41	2.86	18.26	3.61	8.26
										(46.31	2.92	17.96	3.61	8.06)
9as	Cl	Cl	H	4-Cl-Ph	COOH	94	189—190	EA-E	$C_{15}H_{10}Cl_3NO_5S$	42.63	2.38	25.16	3.31	7.59
										(42.93	2.71	24.55	3.20	7.42)
9at	Cl	Cl	H	4-CH_3O-Ph	COOH	91	173—174	E-H	$C_{16}H_{13}Cl_2NO_6S$	45.95	3.13	16.95	3.35	7.67
										(45.99	3.23	16.74	3.34	7.52)
9au[b]	Cl	Cl	H		COOH	87	250—251	W	$C_{15}H_{18}Cl_2N_2O_6S\cdot$ HCl	39.01	4.15		6.07	6.94
										(38.96	4.17		6.06	7.07)
9av	Cl	H	CH_3	CH_3	COOH	97	164—166	A-H	$C_{11}H_{12}ClNO_5S$	43.21	3.96	11.60	4.58	10.49
										(43.01	4.00	11.51	4.57	10.21)
9aw	Cl	H	C_2H_5	C_2H_5	COOH	91	164—165	A-H	$C_{13}H_{16}ClNO_5S$	46.78	4.83	10.62	4.20	9.60
										(46.59	4.85	10.30	4.21	9.34)
9ax	H	Cl	CH_3	CH_3	COOH	94	145	A-H	$C_{11}H_{12}ClNO_5S$	43.21	3.96	11.60	4.58	10.49
										(42.85	4.02	11.75	4.59	10.30)
9ay	H	Cl	C_2H_5	C_2H_5	COOH	87	83—85	A-H	$C_{13}H_{16}ClNO_5S\cdot$ 3/4 H_2O	44.96	5.08	10.21	4.03	9.23
										(44.92	4.93	10.17	4.32	9.49)
9az	Br	H	CH_3	CH_3	COOH	93	194—195	A-H	$C_{11}H_{12}BrNO_5S\cdot$ 3/4 H_2O	37.73	3.45	22.82[c]	4.00	9.16
										(37.73	3.57	22.97[c]	3.87	9.20)
9ba	Br	H	C_2H_5	C_2H_5	COOH	99	161—162	A-H	$C_{13}H_{16}BrNO_5S\cdot$ 3/4 H_2O	41.28	4.26	21.13[c]	3.70	8.48
										(41.26	4.37	20.42[c]	3.78	8.35)
9bb	CH_3	CH_3	H	H	COOH	92	229—230	EA-E	$C_{11}H_{13}NO_5S$	48.70	4.83		5.16	11.82
										(48.41	4.85		5.19	11.18)
9bc	CH_3	CH_3	H	CH_3	COOH	87	179—180	EA-E	$C_{12}H_{15}NO_5S$	50.52	5.30		4.91	11.24
										(50.43	5.23		4.92	11.16)
9bd	CH_3	CH_3	CH_3	CH_3	COOH	87	127—128	EA-E	$C_{13}H_{17}NO_5S$	52.16	5.72		4.68	10.71
										(52.11	5.57		4.73	10.62)
10a	Cl	Cl	CH_3	CH_3	COOPht[d]	56	152—154	EA-H	$C_{19}H_{15}Cl_2NO_7S\cdot$ 1/3 H_2O	47.71	3.45	14.82	2.92	6.70
										(47.88	3.26	14.61	2.99	6.50)
10b	Cl	Cl	CH_3	CH_3	COOPOM[e]	69	113—114	EA-E	$C_{17}H_{21}Cl_2NO_7S\cdot$ 1/3 H_2O	44.94	4.66	15.61	3.08	7.06
										(44.93	4.65	15.73	3.14	6.98)
10c	Cl	Cl	CH_3	CH_3	$COOCH_2COOH$	85	149—150	A-E-H	$C_{13}H_{13}Cl_2NO_7S$	39.21	3.29	17.81	3.52	8.05
										(39.16	3.42	17.65	3.60	7.98)
10d	Cl	Cl	C_2H_5	C_2H_5	$COOCH_2COOH$	70	102—103	A-E-H	$C_{15}H_{17}Cl_2NO_7S$	42.27	4.02	16.63	3.29	7.52
										(42.03	4.04	16.80	3.32	7.45)
11a	Cl	Cl	CH_3	CH_3	$CONH_2$	97	209—210	EA-E	$C_{11}H_{12}Cl_2N_2O_4S$	38.95	3.57	20.90	8.26	9.45
										(38.70	3.62	21.16	8.22	9.52)
11b	Cl	Cl	CH_3	CH_3	$CONHCH_3$	98	182—184	EL-E	$C_{12}H_{14}Cl_2N_2O_4S$	40.80	4.00	20.07	7.93	9.08
										(40.59	4.01	20.03	7.91	8.96)

TABLE I. (continued)



No.	X	Y	R_1	R_2	R	Yield (%)	mp (°C)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found) C	H	Cl	N	S
11c	Cl	Cl	CH_3	CH_3	$CON(CH_3)_2$	70	171—172	A–E	$C_{13}H_{16}Cl_2N_2O_4S \cdot 1/4H_2O$	41.95 (42.26	4.28 4.48		7.54 7.53	8.63 8.62)
12	Cl	Cl	CH_3	$COOC_2H_5$	COOH	63	199—201	A–E–H	$C_{13}H_{13}Cl_2NO_7S$	39.21 (39.40	3.29 3.43	17.81 17.66	3.52 3.54	8.05 7.92)
13a	Cl	Cl	CH_3	C_2H_5	COONa	76	176—177	EL–W	$C_{12}H_{12}Cl_2NNaO_5S \cdot H_2O$	36.56 (36.58	3.58 3.47		3.55 3.41	8.13 8.37)
13b	Cl	Cl	CH_3	n-C_3H_7	COONa	73	162—163	EL–W	$C_{13}H_{14}Cl_2NNaO_5S \cdot 2H_2O$	36.63 (36.70	4.26 4.15	16.63 16.76	3.29 3.28	7.52 7.74)
16a	Cl	Cl	H	H	CH_2OH	99	183—186	EL–EA–E	$C_9H_9Cl_2NO_4S$	36.26 (36.09	3.06 3.12	23.78 23.48	4.70 4.70	10.75 10.57)
16b	Cl	Cl	CH_3	CH_3	CH_2OH	89	133—134	EA–E	$C_{11}H_{13}Cl_2NO_4S$	40.50 (40.32	4.02 3.98	21.74 21.56	4.29 4.21	9.83 9.71)
16c	Cl	Cl	CH_3	$PhCH_2$	CH_2OH	76	73—74	EA–E	$C_{17}H_{17}Cl_2NO_4S \cdot 1/4H_2O$	50.19 (50.11	4.34 4.33		3.44 3.62	7.88 7.76)
16d	Cl	Cl	CH_3	Ph	CH_2OH	87	117—118	EA–E	$C_{16}H_{15}Cl_2NO_4S$	49.50 (49.46	3.89 3.79	18.26 18.18	3.61 3.50	8.26 8.26)
16e	Cl	Cl	H	CH_3	CH_2OH	78	118—119	EA–E	$C_{10}H_{11}Cl_2NO_4S$	38.48 (38.36	3.55 3.50	22.71 22.72	4.49 4.41	10.27 10.00)
16f	Cl	Cl	H	$PhCH_2$	CH_2OH	91	55—56	A–E	$C_{16}H_{15}Cl_2NO_4S \cdot 1/4H_2O$	48.93 (49.06	3.98 4.09	18.05 17.60	3.57 3.45	8.16 7.60)
16g	Cl	Cl	H	Ph	CH_2OH	81	161—162	A–E	$C_{15}H_{13}Cl_2NO_4S$	48.14 (48.10	3.50 3.58	18.95 19.07	3.74 3.69	8.57 8.31)
17a	Cl	Cl	H	H	CH_2Cl	66	195—196	EA–E	$C_9H_8Cl_3NO_3S$	34.14 (34.09	2.55 2.71	33.60 33.22	4.42 4.40	10.13 10.20)
17b	Cl	Cl	CH_3	CH_3	CH_2Cl	63	77—78	D–H	$C_{11}H_{12}Cl_3NO_3S$	38.34 (38.09	3.51 3.54	30.86 30.63	4.06 4.10	9.30 9.22)
18a	Cl	Cl	H	H	$CH_2OC_2H_5$	24	148—149	EA–E	$C_{11}H_{13}Cl_2NO_4S$	40.50 (40.37	4.02 3.95	21.74 21.50	4.29 4.22	9.83 9.79)
18b	Cl	Cl	CH_3	CH_3	$CH_2OC_2H_5$	43	91—92	A–E	$C_{13}H_{17}Cl_2NO_4S$	44.08 (44.19	4.84 4.87	20.02 20.13	3.95 4.05	9.05 8.82)

a) A = acetone, D = dichloromethane, E = ether, EA = ethyl acetate, EL = ethanol, H = hexane, W = water. *b*) HCl salt. *c*) Br. *d*) Pht = phthalidyl. *e*) POM = pivaloyloxymethyl.

temporary diuresis, occasionally fail to decrease blood pressure because of their short action time. Recently, however, they have been used in sustained-release preparations in order to avoid the side effects caused by thiazides.

One large family of modern diuretics, first recognized with the discovery of ethacrynic acid, is that of aryloxyacetates.[1] Recently, many new compounds having interesting pharmacological actions, including uricosuric and diuretic activities have been reported from this family, *e.g.*, tienilic acid (**1**),[1] indacrinone (**2**),[1,2] **3**,[1,3] HP-522 (**4**),[4] and **5**[5] (Chart 1).

We tried to create a new type of uricosuric diuretic which would display temporary diuretic action and inhibit reabsorption of uric acid by renal tubules. The structure of 5-acyl-2,3-dihydrobenzofuran-2-carboxylic acid, found in compounds **3**—**5**, seemed to be a developed form of 4-acylphenoxyacetic acid. A promising substituent was considered to be the sulfamoyl group, which is found in thiazide diuretics and probenecid, a uricosuric drug. We therefore synthesized some dihydrobenzofuran derivatives with 5-sulfamoyl substituents and found that they display both actions.

Chemistry

The compounds prepared for this study are shown in Table I and their syntheses are outlined in Charts 2 and 3. The starting materials (**7a** and **14**,[3] **7b**, **c** and **e**,[6] **d**[7]) are described in the literature.

5-Sulfamoyl-2,3-dihydrobenzofuran-2-carboxylic acids (**9aa**—**az** and **9ba**—**bd**) were obtained by chlorosulfonation of the corresponding esters (**7**) with chlorosulfonic acid and thionyl chloride followed by aminolysis and hydrolysis. The esters **10a** and **b** were prepared by alkylation of the potassium salt of **9ab** with phthalidyl bromide or iodomethyl pivalate. The esters **10c** and **d** were prepared by the reaction of diphenylmethyl glycolate with acid chlorides

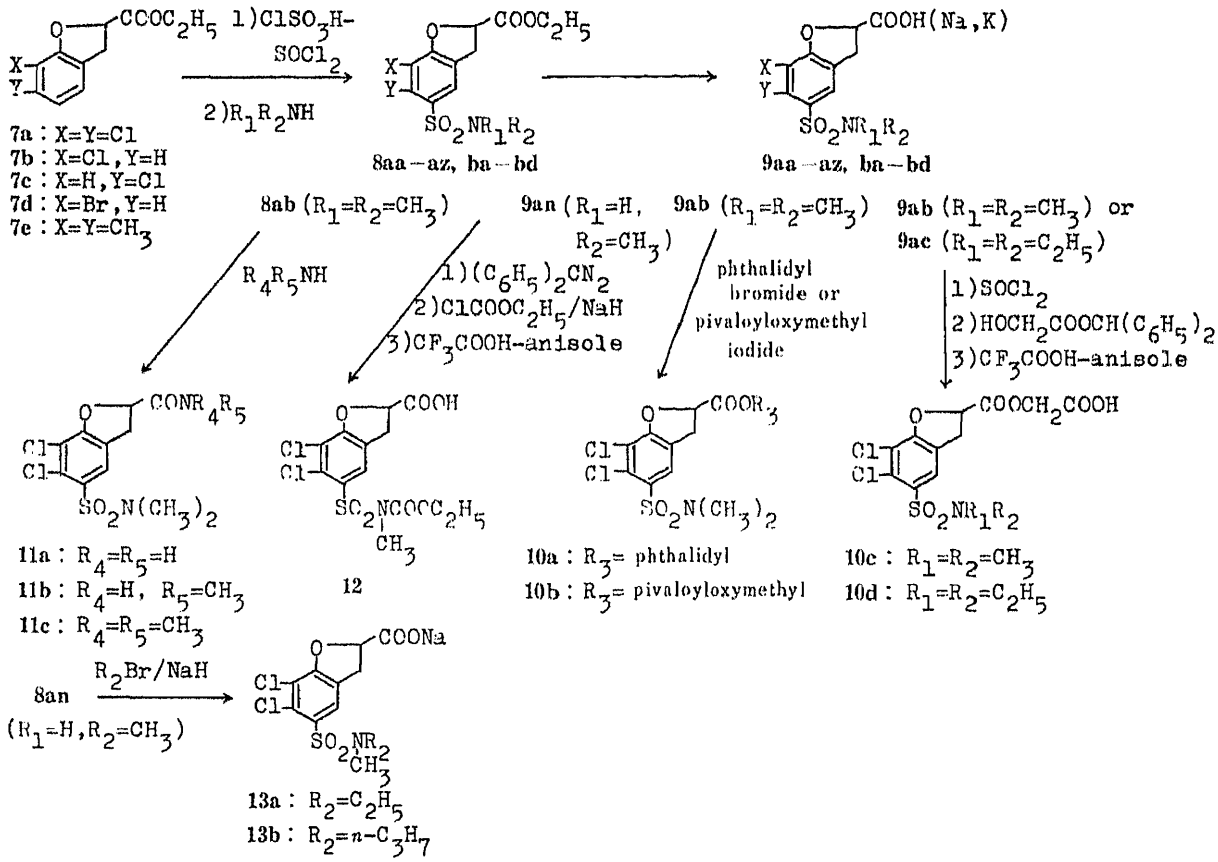


Chart 2

of **9ab** and **9ac** followed by hydrolysis with trifluoroacetic acid. The 2-carboxamides (**11a—c**) were obtained by aminolysis of the ethyl esters (**8ab**). The urethane (**12**) was prepared by ethoxycarbonylation of **9an** benzhydryl ester with ethyl chloroformate and sodium hydride followed by hydrolysis with trifluoroacetic acid and anisole. The 5-(*N*-ethyl-*N*-methyl)- and 5-(*N*-methyl-*N*-propyl)sulfamoyl derivatives (**13a** and **b**) were prepared by alkylation of **8an** with the corresponding alkyl halides in the presence of sodium hydride followed by alkaline hydrolysis (Chart 2).

The 2-hydroxymethyl-2,3-dihydrobenzofuran-5-sulfonamide derivatives (**16**) were prepared by sulfamoylation of the acetate of **14** followed by alkaline hydrolysis through a procedure similar to that described for the preparation of **9** (Chart 3). 2-Chloromethyl derivatives (**17a** and **b**) were obtained by chlorination of **16a** and **b**, and 2-ethoxymethyl derivatives (**18a** and **b**) were synthesized by sulfamoylation of the ethoxy derivative of **14** or by direct ethylation of **16b**, respectively. Intermediates to the above compounds are described in the experimental section.

Optical resolution of **9ab** was carried out as outlined in Chart 4. The diastereoisomeric amides (**19**) were obtained by the reaction of the acid chloride of **9ab** with L-proline *tert*-butyl ester in the presence of triethylamine and 4-*N*,*N*-dimethylaminopyridine in dry benzene under ice-cooling. One isomer (**19a**) was readily crystallized from the mixture and the resultant mother liquor was separated by silica gel column chromatography. The separated diastereoisomers (**19a** and **b**) were hydrolyzed in aqueous sulfuric acid–dioxane under reflux to give the optically active enantiomers [(−)- and (+)-**9ab**], which showed the same melting points and optical rotations (except for the sign).

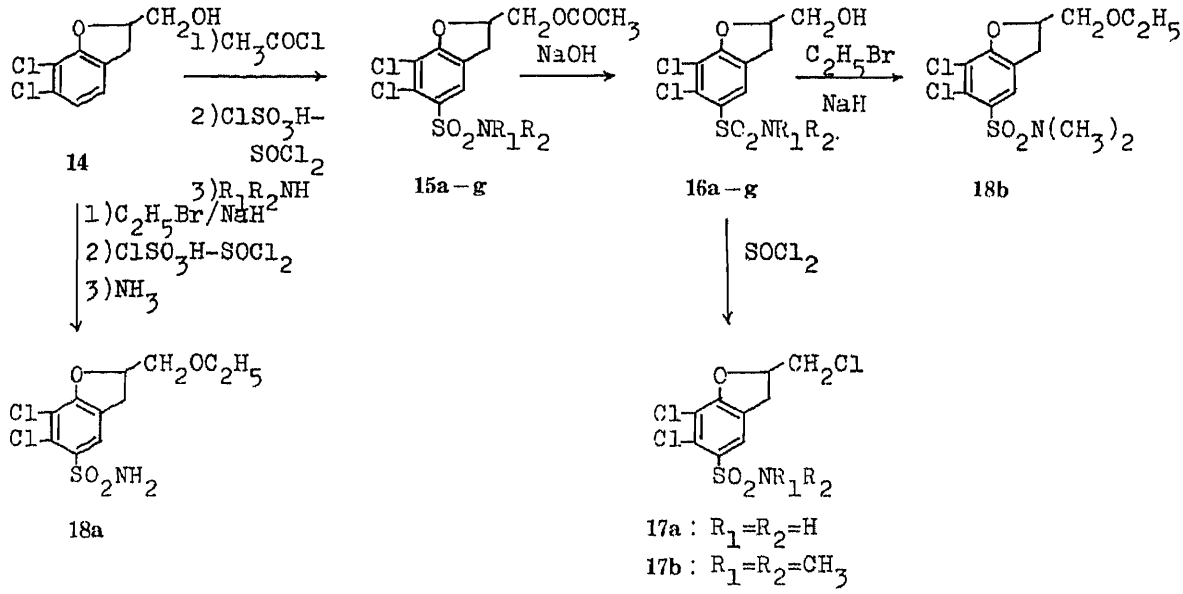


Chart 3

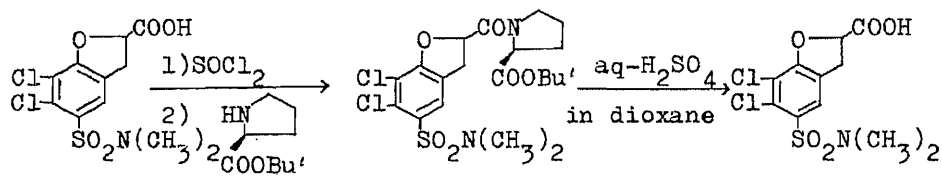


Chart 4

TABLE II. Oral Diuretic and Saluretic Activities[a,b] in Rats and Mice

No.	Rats				Mice			
	Dose mg/kg	Urine volume ml/kg B.W.	Na meq/kg B.W.	K meq/kg B.W.	Dose mg/kg	Urine volume ml/kg B.W.	Na meq/kg B.W.	K meq/kg B.W.
9aa	50	26 (N)	0.88 (N)	0.28 (1.5)	30	27 (N)	0.83 (N)	0.92 (1.7)
9ab	50	39 (1.4)	3.0 (4.9)	0.87 (4.5)	30	48 (1.9)	4.7 (9.1)	1.2 (2.2)
(−)-9ab	50	47 (2.0)	3.8 (8.3)	1.2 (5.0)	30	73 (3.3)	7.8 (15)	1.8 (3.9)
(+)-9ab	50	28 (1.2)	0.53 (N)	0.32 (N)	30	28 (N)	1.3 (2.6)	0.85 (1.8)
9ac	50	40 (1.6)	3.0 (6.0)	1.1 (4.5)	30	85 (2.9)	9.3 (11)	2.1 (3.0)
9ad	50	28 (1.3)	1.3 (2.8)	0.37 (N)	30	41 (2.0)	3.8 (4.9)	1.0 (1.5)
9ae	50	25 (N)	0.77 (N)	0.23 (N)	30	29 (N)	2.3 (3.0)	0.96 (1.4)
9af	50	33 (1.4)	1.8 (3.6)	0.63 (2.5)	30	61 (2.3)	6.3 (8.0)	2.0 (2.5)
9ag	50	23 (N)	0.72 (1.5)	0.25 (N)	30	31 (N)	1.3 (N)	1.0 (N)
9ah	50	36 (1.6)	2.0 (4.1)	0.58 (2.5)	30	64 (2.3)	6.3 (7.5)	1.7 (2.1)
9ai	50	39 (1.6)	2.5 (5.1)	0.73 (2.9)	30	44 (1.7)	4.3 (5.4)	1.5 (1.7)
9aj	50	25 (N)	0.72 (N)	0.32 (N)	30	29 (N)	1.3 (N)	0.7 (N)
9ak	50	35 (1.6)	1.7 (3.5)	0.58 (N)	30	58 (2.1)	5.8 (6.8)	1.5 (1.9)
9al	50	29 (N)	1.3 (2.7)	0.50 (2.1)	30	66 (2.3)	6.7 (7.9)	1.9 (2.3)
9am	50	25 (N)	0.67 (1.5)	0.35 (N)	30	39 (1.4)	3.5 (4.1)	1.2 (1.5)
9an	50	30 (1.2)	1.3 (2.0)	0.54 (2.6)	30	34 (1.3)	2.7 (2.8)	1.0 (N)
9ao	50	28 (N)	1.1 (1.7)	0.42 (2.0)	30	47 (1.7)	4.3 (4.6)	1.2 (1.5)
9ap	50	26 (1.2)	1.0 (1.7)	0.38 (N)	30	38 (1.3)	2.6 (3.4)	1.2 (1.7)
9aq	50	24 (N)	0.71 (N)	0.26 (N)	30	26 (N)	0.79 (N)	0.84 (N)
9ar	50	26 (N)	0.75 (N)	0.30 (N)	30	39 (N)	2.9 (3.3)	0.97 (N)
9as	50	22 (N)	0.51 (N)	0.23 (N)	30	37 (1.3)	2.1 (2.4)	0.93 (N)
9at	50	26 (N)	0.92 (1.6)	0.40 (N)	30	36 (1.3)	1.8 (2.3)	0.88 (N)
9au	50	23 (N)	0.55 (N)	0.25 (N)				
9av	50	27 (N)	0.54 (N)	0.46 (N)	30	24 (N)	0.94 (N)	0.60 (N)
9aw	50	29 (N)	0.91 (1.8)	0.40 (1.5)	30	31 (1.6)	2.0 (2.4)	0.85 (1.4)
9ax	50	30 (N)	1.4 (2.3)	0.62 (2.2)	30	24 (N)	1.5 (2.4)	0.62 (N)
9ay	50	29 (N)	1.1 (2.1)	0.46 (1.8)	30	26 (1.4)	1.5 (2.4)	0.71 (1.9)
9az	50	29 (N)	0.78 (N)	0.25 (N)	30	29 (N)	1.2 (N)	0.91 (N)
9ba	50	27 (N)	0.75 (N)	0.29 (N)	30	28 (N)	1.6 (2.4)	1.1 (2.0)
9bb	50	26 (N)	0.51 (N)	0.27 (N)	30	27 (N)	0.8 (N)	0.62 (N)
9bc	50	27 (N)	0.72 (N)	0.36 (N)	30	30 (N)	1.5 (N)	0.83 (N)
9bd	50	32 (N)	1.5 (1.9)	0.49 (1.8)	30	36 (1.3)	2.4 (2.1)	0.96 (N)
10a	50	38 (N)	2.4 (3.1)	1.2 (1.7)	30	29 (1.2)	1.0 (1.6)	0.41 (2.0)
10b	50	34 (N)	1.4 (1.8)	0.74 (N)	30	29 (N)	0.81 (1.5)	0.30 (1.6)
10c	50	39 (1.7)	2.5 (4.1)	0.86 (4.1)	30	56 (1.6)	5.2 (5.9)	1.5 (2.2)
10d	50	39 (1.7)	2.8 (4.7)	1.1 (5.3)	30	82 (2.4)	8.3 (9.4)	1.9 (2.7)
11a	50	35 (1.5)	2.2 (5.4)	0.81 (3.7)	30	22 (N)	2.0 (2.5)	0.75 (1.3)
11b	50	31 (1.3)	2.6 (1.4)	0.51 (1.8)	30	26 (N)	1.5 (1.8)	1.1 (N)
11c	50	25 (N)	1.3 (3.2)	0.34 (N)				
12	50	32 (1.4)	1.7 (2.8)	0.67 (3.2)	30	35 (N)	1.8 (2.4)	0.92 (N)
13a	50	47 (1.6)	3.3 (5.0)	1.1 (3.7)	30	75 (3.8)	8.2 (9.6)	1.9 (3.1)
13b	50	44 (1.5)	3.2 (4.2)	0.85 (2.9)	30	75 (3.8)	8.2 (9.6)	1.9 (3.2)
16a	50	43 (1.6)	3.3 (5.3)	0.74 (4.4)	30	35 (1.3)	2.6 (4.6)	1.3 (2.1)
16b	50	37 (1.2)	2.4 (3.7)	0.56 (2.4)	30	26 (N)	3.3 (4.7)	0.90 (1.9)
16c	50	32 (1.5)	1.9 (4.3)	0.48 (1.6)	30	47 (1.9)	4.9 (5.9)	1.4 (1.9)
16d	50	20 (N)	0.27 (N)	0.21 (N)	30	25 (N)	1.1 (N)	0.57 (N)
16e	50	23 (N)	1.2 (2.9)	0.36 (1.6)	30	28 (N)	3.8 (4.3)	1.27 (N)
16f	50	31 (1.3)	1.7 (3.1)	0.68 (2.4)	30	31 (N)	1.2 (N)	0.97 (1.4)
16g	50	22 (N)	0.25 (N)	0.26 (N)	30	22 (N)	1.1 (N)	0.79 (N)
17a	50	26 (N)	0.93 (1.7)	0.23 (N)	30	30 (N)	1.6 (2.2)	1.1 (N)
17b	50	26 (N)	1.2 (2.2)	0.43 (2.1)	30	28 (N)	1.1 (N)	0.75 (N)
18a	50	35 (1.5)	2.0 (4.1)	0.69 (3.6)	30	46 (1.3)	2.9 (3.3)	1.6 (2.3)
18b	50	29 (1.6)	1.6 (3.0)	0.39 (1.6)	30	30 (N)	2.7 (3.3)	1.0 (N)
Tienilic acid	100	39 (1.8)	2.2 (1.7)	1.3 (5.7)	30	36 (2.4)	3.9 (5.4)	1.2 (1.9)
Indacrinone	50	34 (1.2)	1.3 (2.3)	0.5 (2.0)	30	72 (2.5)	6.4 (8.4)	1.9 (2.8)

a) The procedures used for the diuretic and saluretic tests are described in Experimental. *b*) Ratio to the control is shown in parenthesis; N indicates that the difference from the control was not statistically significant.

Biological Activities

Saluresis and Diuresis——The compounds shown in Table I were evaluated for oral diuretic and saluretic activities in rats and mice. The results are listed in Table II. Tienilic acid and indacrinone were used as reference compounds. Diuretic and kaliuretic activities paralleled the natriuretic activity. Structural requirements for natriuretic activity within these compounds were evaluated at four positions (2, 5, 6 and 7 positions) of the 2,3-dihydrobenzofuran ring system. In variants of the X and Y groups at the 6- and 7-positions, the highest activity was found when X=Y=Cl, with lesser activities for X=H, Y=Cl (**9ax** and **ay**) and X=Y=CH_3 (**9bb**—**bd**). Among derivatives having the 2-COOH group, alkyl variants of the 5-sulfonamide group (R_1 and R_2) showed the most potent activities in the cases of the lower alkyl groups (**9ab**, **ac**, **13a** and **b**), while the unsubstituted and monoalkyl-substituted sulfonamides (**9aa** and **9an**—**au**) had completely lost the activities or exhibited only slight activities. Among derivatives having a 5-*N*,*N*-dimethylsulfamoyl substituent ($R_1=R_2=CH_3$), variants of the R group at the 2-position influenced the natriuretic activities. As shown in Fig. 1, compounds having a carboxyl group or substituent having a carboxyl group (**9ab** and **10c**) showed potent activities in rats when given orally. The relative effectiveness of other substituents at the 2-position in producing natriuretic activity was $CH_2OH \simeq CONH_2 > CH_2OC_2H_5 > CH_2Cl >$ esters.

The natriuretic dose response curves of a representative compound, **9ab**, in male rats and female mice after oral administration are shown in Figs. 2 and 3. Compound **9ab** displayed a typical high-ceiling curve like that of furosemide in both models, in contrast to tri-

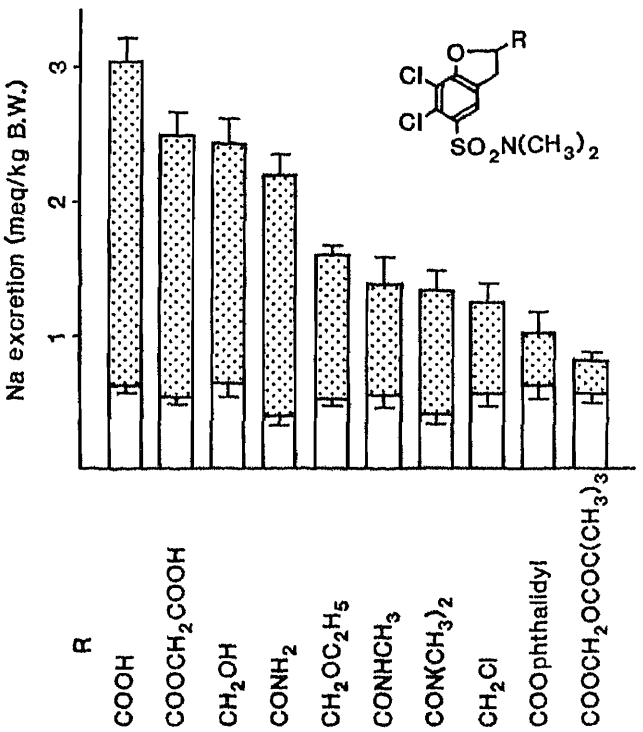


Fig. 1. Effect of the 2-Substituent in 6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran on Na Excretion during a 5-h Period after Oral Administration (50 mg/kg) to Male Rats

Each column represents the mean±S.E. of 8 rats that received the test compound (shaded column) or the vehicle only (open column).

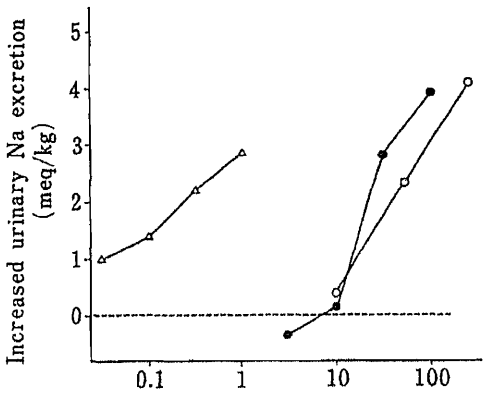


Fig. 2. Increase of Na Excretion during a 5-h Period after Oral Administration of **9ab**, Furosemide or Trichlormethiazide (TCM) to Male Rats

Excretion level of the control is shown by the dotted line.
△—△, TCM; ●—●, furosemide; ○—○, **9ab**.

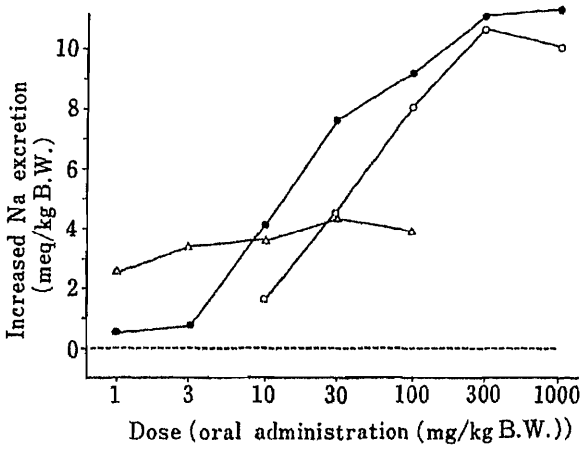


Fig. 3. Increase of Na Excretion during a 4-h Period after Oral Administration of **9ab**, Furosemide or TCM to Female Mice

Each point is the value of five mice/cage. Excretion level of the control is shown by the dotted line.
△--△, TCM; ●—●, furosemide; ○—○, **9ab**.

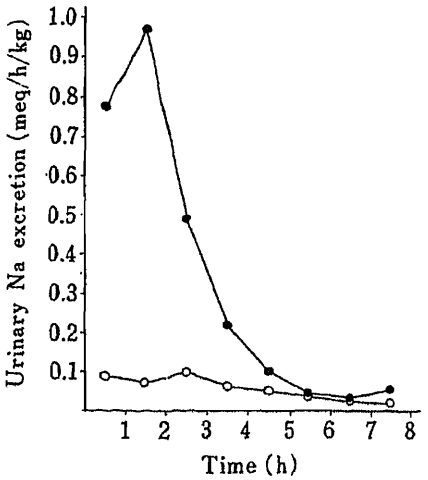


Fig. 4. Na Excretion after Oral Administration (50 mg/kg) of **9ab** to Male Rats

●—●, **9ab**; ○—○, control.

TABLE III. Uricosuric Effect of 5-Sulfamoyl-6,7-dichloro-2,3-dihydrobenzofurans in Intraperitoneally Oxonate-Treated Rats

No.	Dose mg/kg	Increase of UuaV mg/kg min	Increase of FEua
9aa	50	0.154	0.421
9ab	50	0.107	0.212
(−)-**9ab**	50	0.026	−0.040
(+)-**9ab**	50	0.018	0.123
9ac	50	0.047	0.092
9ad	50	0.046	=
9af	50	0.085	0.065
9ah	50	0.032	=
9ak	50	0.075	0.122
9al	50	0.110	0.202
9an	50	0.100	0.302
9ao	50	0.090	0.339
16a	50	0.058	0.172
16b	50	0.137	0.204
16c	50	0.045	0.115
Probenecid	50	0.124	0.070
Tienilic acid	100	0.123	0.055
Indacrinone	50	0.063	=
Furosemide	50	0.028	−0.124

Increases of UuaV and FEua were calculated as the average value for 80 min after dosing. The symbol = means that there was no difference compared with the control.

chlormethiazide. The time course of natriuretic activity in rats administered **9ab** orally is shown in Fig. 4. Compound **9ab** showed temporary natriuresis. These data suggest that the main site of action of **9ab** inducing diuresis may be in the loop of Henle.

Optical isomers of **9ab**, resolved as shown in Chart 4, were tested in rats and mice in comparison with the racemate. The (−)-enantiomer possesses most of the saluretic activity

and its activity is approximately twice that of the racemate.

Uricosuric Activity——Mammals which excrete uric acid as an end product of purine metabolism are the Cebus monkey, chimpanzee and man. Thus, the activity usually cannot be tested in commonly used experimental animals such as rodents. Tienilic acid and indacrinone have been shown to have a hyperuricosuric character through inhibition of urate reabsorption by renal tubules in man and chimpanzee, respectively.

A method has been developed[8] to test uricosuric activity using rats treated with potassium oxonate which is known to be a uricase inhibitor. The test compounds were administered intraperitoneally and the uricosuric activity was evaluated in terms of the increase in fractional excretion of uric acid (FEua) and urine-excreted amounts of uric acid (UuaV) values. The results are listed in Table III.

Probenecid and tienilic acid, which were used as reference compounds, showed hyperuricosuric activities with increased FEua and UuaV values. Indacrinone, however, showed only an increase in UuaV. Furosemide showed a decrease of FEua, suggesting the possibility of hypouricosuric action.

Among 6,7-dichloro-2,3-dihydrobenzofuran derivatives having either a 2-carboxyl or a 2-hydroxymethyl group, marked increases of both FEua and UuaV were observed in variants having unsubstituted and mono- or disubstituted sulfonamides (**9aa—ac**, **af**, **ak**, **al**, **an**, **ao**, and **16a—c**) in which the substituents were lower alkyl groups. Accordingly, these compounds are expected to show a hyperuricosuric character in higher animals, including man.

The resolved enantiomers of **9ab** were tested for uricosuric activity. The (+) enantiomer had this activity, while the (−) enantiomer showed an increase in UuaV with a slight decrease of FEua.

Indacrinone (**2**) has been reported to show urate-retaining activity as a result of potent and long-lasting diuresis in clinical trials.[9] The diuretic potency of **9ab** seems to be intermediate between those of **2**—**5** and tienilic acid (**1**).[1,3,5] Thus, it should show both uricosuric and moderate diuretic actions, which would allow its use in clinical antihypertensive therapy. We therefore selected **9ab** for clinical evaluation, and further investigation is in progress.

Experimental

Melting points were determined on a Yanagimoto hot plate micro melting point apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken on a Varian EM-390 spectrometer with tetramethylsilane (TMS) as an internal standard. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet), br (broad), m (multiplet). Chemical shifts are expressed in δ values and the coupling constants in Hz. Abbreviations are as follows: Ph, phenyl; arom. H, aromatic proton(s). For column chromatography, Silica gel 60 (E. Merck, 0.063—0.200 mm) was used.

Ethyl 6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-carboxylate (8ab)——Chlorosulfonic acid (1.25 g, 0.0107 mol) was added dropwise to a solution of **7a** (1.0 g, 0.0038 mol) in thionyl chloride (2.5 ml) under ice-cooling, and the mixture was allowed to react at room temperature for 2 h. The reaction mixture was poured into ice water then extracted with ethyl acetate (60 ml). The organic layer was dried and evaporated *in vacuo*. A solution of the residue dissolved in dichloromethane (8 ml) was cooled to −20——10 °C, then 30% ethanolic dimethylamine (0.518 g, 3 × 0.0038 mol) was added dropwise, and the reaction was allowed to proceed for 1 h. The completion of the reaction was confirmed by thin layer chromatography (TLC) (silica gel/dichloromethane), then the reaction mixture was adjusted to about pH 5 and extracted with dichloromethane. When the organic layer was dried and evaporated *in vacuo*, it left an oily residue, which was chromatographed. Elution with dichloromethane gave **8ab** (1.0 g, 71%).

Compounds **8aa**, **8ac—az** and **8ba—bd** were obtained in a similar manner (Table IV). ^{1}H-NMR spectral data are given in Table V.

6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-carboxylic Acid (9ab)——A 15% aqueous potassium carbonate solution (14 ml) was added to a solution of **8ab** (1.0 g, 0.0029 mol) in tetrahydrofuran (9 ml), and the mixture was stirred for 72 h at room temperature. Concentration of the reaction mixture *in vacuo* left a residue,

which was acidified to pH 5, then extracted with ethyl acetate. The organic layer was dried and evaporated *in vacuo*, leaving an oil, which, when treated with ether, gave **9ab** (0.868 g, 94%).

Compounds **9aa**, **9ac**—**az** and **9ba**—**bd** were obtained in a similar manner. ^{1}H-NMR spectral data for **9aa**—**az** and **9ba**—**bd** are listed in Table VI.

Phthalidyl 6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-carboxylate (10a)——Potassium carbonate (0.425 g, 2.1 × 0.0029 mol) was added to a solution of **9ab** (1.0 g, 0.0029 mol) in acetonitrile (10 ml), and the mixture was stirred for 1 h at room temperature. After confirmation by TLC [silica gel; dichloromethane/ethanol (10 : 1)/1% acetic acid] that no free acid was left, the precipitated crystals (1.35 g) were collected by filtration. The crystals were dissolved in acetonitrile (20 ml), then cooled to −20 °C. Next, 1-phthalidyl bromide(1,3-dihydro-3-oxo-1-isobenzofuranyl bromide, 1.32 g, 2.1 × 0.0029 mol) was added, and the mixture was stirred at room temperature. *N*,*N*-Dimethylformamide (DMF) (6 ml) was added, and the resulting mixture was refluxed for 30 min. After removal of the insoluble matter by filtration, the reaction mixture was concentrated *in vacuo*, and the residue was extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated *in vacuo*. Chromatography of the resulting residue using dichloromethane as the eluant gave **10a** (0.77 g, 56%), mp 152—154 °C (ethyl acetate–hexane), which was a diastereoisomeric mixture according to its ^{1}H-NMR spectrum. ^{1}H-NMR spectral data are summarized in Table VI.

Pivaloyloxymethyl 6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-carboxylate (10b)——Pivaloyloxymethyl iodide (1.5 g, 2.1 × 0.0029 mol) was added dropwise to a solution of the potassium salt of **9ab** (1.0 g, 0.0029 mol) in acetonitrile (10 ml) at 20 °C, and the mixture was refluxed for 2.5 h. The insoluble matter was removed by filtration, and the reaction solution was extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated *in vacuo*, giving a crystalline residue. Chromatography of this residue using dichloromethane as the eluant gave **10b** (0.911 g, 69%). ^{1}H-NMR spectral data are given in Table VI.

6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-yl-carbonyloxyacetic Acid (10c)——The acid chloride of **9ab**, which was formed by treatment of **9ab** (1.0 g, 0.0029 mol) with thionyl chloride (1.5 ml) in benzene (5 ml) under reflux, was dissolved in benzene (8 ml). This solution was added dropwise at 0 °C to a solution of benzhydryl glycolate (0.855 g, 0.0035 mol), triethylamine (0.3 g) and 4-*N*,*N*-dimethylaminopyridine (35 mg, 0.0003 mol) in benzene (5 ml). The mixture was stirred for 1 h under ice-cooling and then extracted with dichloromethane. The organic layer was washed with water, dried and evaporated *in vacuo*, giving a residue. Chromatography of this residue using dichloromethane as the eluant gave diphenylmethoxycarbonylmethyl 5-(*N*,*N*-dimethylsulfamoyl)-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (1.35 g, 85%), mp 56—58 °C (ethyl acetate–hexane). *Anal.* Calcd for $C_{26}H_{23}Cl_2NO_7S$: C, 55.33; H, 4.11; Cl, 12.56; N, 2.48; S, 5.68. Found: C, 55.82; H, 4.22; Cl, 12.29; N, 2.60; S, 5.51. ^{1}H-NMR (in $CDCl_3$) δ: 2.82 (6H, s, 2 × C$\underline{H}_3$), 3.10—3.90 (2H, m, C$\underline{H}_2$), 4.67 and 4.92 (2H, ABq, J = 16.0, O–C$\underline{H}_2$–CO), 5.43 (1H, 2 × d, J = 10.0, 6.3, O–C$\underline{H}$), 6.87 (1H, s, O–C$\underline{H}$), 7.27 (10H, s, arom. H), 7.75 (1H, t, J = 1.0, arom. H). Trifluoroacetic acid (1 ml) was added to a stirred suspension of this compound (0.85 g) in anisole (1 ml) under ice-cooling. After being stirred for 1 h at room temperature, the reaction mixture was evaporated *in vacuo*. The residue was recrystallized from hexane–ether, giving **10c** (0.599 g, 100%).

Compound **10d** was obtained in a similar manner; yield 70%. ^{1}H-NMR spectral data for **10a**—**d** are given in Table VI.

6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-carboxamide (11a)——A solution of **8ab** (0.500 g, 0.0016 mol) in 20% ethanolic ammonia (50 ml) was stirred for 17 h at room temperature. Evaporation of the reaction mixture *in vacuo* gave a crystalline residue, which was washed with ether, giving **11a** (0.448 g, 97%).

Compounds **11b** and **11c** were obtained in a similar manner. Compound **11b**, yield 98%. Compound **11c**, yield 70%. ^{1}H-NMR spectral data for **11a**—**c** are listed in Table VI.

6,7-Dichloro-5-(*N*-ethoxycarbonyl-*N*-methylsulfamoyl)-2,3-dihydrobenzofuran-2-carboxylic Acid (12)——Diphenyl diazomethane (1.3 g, 0.0067 mol) was gently added to a solution of **9an** (1.5 g, 0.0046 mol) in dichloromethane (20 ml) under ice-cooling. The mixture was stirred for 2 h at room temperature, then the remaining reagent was decomposed by adding 10% hydrochloric acid. The mixture was extracted with dichloromethane (60 ml), then the organic layer was washed with water, dried and evaporated *in vacuo*. The residue was chromatographed using dichloromethane as the eluant to give benzhydryl 6,7-dichloro-5-*N*-methylsulfamoyl-2,3-dihydrobenzofuran-2-carboxylate (2.06 g, 91%), mp 132—133 °C (hexane–ether). Sodium hydride (50% suspension in oil, 0.180 g, 0.0038 mol) was gently added to a solution of the ester (1.655 g, 0.0034 mol) in DMF (20 ml) at 4 °C, and the mixture was stirred for 1 h at room temperature. Next, ethyl chloroformate (0.41 g, 0.0038 mol) was added, and the reaction was allowed to continue for 1 h. The reaction mixture was then poured into water and extracted with ether (300 ml). The organic layer was dried and evaporated *in vacuo*, giving an oily residue. Chromatography of this residue using dichloromethane as the eluant gave benzhydryl 6,7-dichloro-5-(*N*-ethoxycarbonyl-*N*-methylsulfamoyl)-2,3-dihydrobenzofuran-2-carboxylate (1.314 g, 69%), mp 111—112 °C (ethyl acetate–hexane). *Anal.* Calcd for $C_{26}H_{23}Cl_2NO_7S$: C, 55.33; H, 4.11; Cl, 12.56; N, 2.48; S, 5.58. Found: C, 55.50; H, 4.17; Cl, 12.54; N, 2.43; S, 5.61. ^{1}H-NMR (in $CDCl_3$) δ: 1.07 (3H, t, J = 7.0, C$\underline{H}_3$), 3.20—3.90 (2H, m, C$\underline{H}_2$), 3.42 (3H, s, N–C$\underline{H}_3$), 4.05 (2H, q, J = 7.0, O–C$\underline{H}_2$), 5.48 (1H, 2 × d, J = 10.0, 7.0, O–C$\underline{H}$), 6.93 (1H, s, C$\underline{H}$), 7.30 (5H, s, arom. H), 7.36 (5H, s, arom. H), 7.96 (1H, t, J = 1.0, 4-H). Trifluoroacetic acid (2.5 ml) was added dropwise to a solution of 1.314 g (0.0023 mol) of the urethane

TABLE IV. Compounds **8** and **15**

No.	X	Y	R_1	R_2	Yield (%)	mp (°C)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found)				
									C	H	Cl	N	S
8aa	Cl	Cl	H	H	50	167—168	A–E–H	$C_{11}H_{10}Cl_2NO_5S \cdot 1/2\,H_2O$	37.94 (38.27	3.18 3.09		4.09 4.13	9.21 9.18)
8ab	Cl	Cl	CH_3	CH_3	71	124—125	E–H	$C_{13}H_{15}Cl_2NO_5S$	42.40 (42.11	4.11 4.19	19.26 19.28	3.80 3.82	8.61 8.61)
8ac	Cl	Cl	C_2H_5	C_2H_5	70	Oil		$C_{15}H_{19}Cl_2NO_5S$	45.46 (45.08	4.83 4.76	17.89 18.56	3.53 3.58	8.09 8.21)
8ad	Cl	Cl	*n*-C_3H_7	*n*-C_3H_7	75	87—88	E–H	$C_{17}H_{23}Cl_2NO_5S$	48.12 (47.98	5.46 5.41	16.71 16.81	3.30 3.40	7.64 7.64)
8ae	Cl	Cl	iso-C_3H_7	iso-C_3H_7	28	Oil		$C_{17}H_{23}Cl_2NO_5S \cdot 1/2\,H_2O$	47.11 (47.24	5.58 5.36		3.23 3.38	7.40 7.45)
8af	Cl	Cl	CH_3	*n*-C_4H_9	85	Oil		$C_{16}H_{21}Cl_2NO_5S$	46.84 (46.77	5.16 5.21	17.28 17.47	3.41 3.32	7.81 7.54)
8ag	Cl	Cl	$PhCH_2$	$PhCH_2$	75	Oil		$C_{25}H_{23}Cl_2NO_5S \cdot 1/2\,H_2O$	56.71 (56.96	4.57 4.51		2.65 2.72	6.06 6.02)
8ah	Cl	Cl	CH_3	$PhCH_2$	76	102—103	E–H	$C_{19}H_{19}Cl_2NO_5S$	51.36 (51.11	4.31 4.18	15.96 16.13	3.15 3.21	7.22 7.38)
8ai	Cl	Cl	CH_3	Ph	50	Oil		$C_{18}H_{17}Cl_2NO_5S \cdot H_2O$	48.22 (48.47	4.27 3.95		3.12 3.24	7.15 6.97)
8aj	Cl	Cl	CH_3	*cyclo*-C_6H_{11}	73	Oil		$C_{18}H_{23}Cl_2NO_5S$	49.55 (49.37	5.31 5.28	16.25 16.24	3.21 3.29	7.35 7.17)
8ak	Cl	Cl	–$(CH_2)_4$–		56	135—136	E–H	$C_{15}H_{17}Cl_2NO_5S$	45.70 (45.40	4.35 4.27	17.98 18.24	3.55 3.52	8.13 7.95)
8al	Cl	Cl	–$(CH_2)_5$–		67	131—132	E–H	$C_{16}H_{19}Cl_2NO_5S$	47.07 (46.96	4.69 4.67	17.37 17.50	3.43 3.31	7.85 7.65)
8am	Cl	Cl	–$(CH_2)_2O(CH_2)_2$–.		70	141—142	EA–H	$C_{15}H_{17}Cl_2NO_6S$	43.91 (43.45	4.18 4.03		3.41 3.48	7.82 7.76)
8an	Cl	Cl	H	CH_3	75	140—141	EA–E	$C_{12}H_{13}Cl_2NO_5S$	40.69 (40.62	3.70 3.70	20.02 20.13	3.95 3.96	9.05 9.00)
8ao	Cl	Cl	H	*n*-C_3H_7	70	102—103	EA–E	$C_{14}H_{17}Cl_2NO_5S$	43.99 (43.88	4.48 4.46	18.55 18.77	3.66 3.69	8.39 8.24)
8ap	Cl	Cl	H	iso-C_3H_7	80	169—170	EA–H	$C_{14}H_{17}Cl_2NO_5S$	43.99 (43.60	4.48 4.41	18.55 19.03	3.66 3.67	8.39 8.17)
8aq	Cl	Cl	H	$PhCH_2$	73	168—169	EA–E	$C_{18}H_{17}Cl_2NO_5S$	50.24 (49.96	3.98 3.98	16.48 16.80	3.26 3.27	7.45 7.33)

8ar	Cl	Cl	H	Ph	64	141—142	EA–E	$C_{17}H_{15}Cl_2NO_5S$	49.05	3.63	17.03	3.36	7.70
									(48.93	3.66	17.37	3.43	7.61)
8as	Cl	Cl	H	4-Cl–Ph	58	164—165	EA–E	$C_{17}H_{14}Cl_3NO_5S$	45.30	3.13	23.60	3.11	7.11
									(44.91	3.13	23.89	2.94	7.05)
8at	Cl	Cl	H	4-CH_3O–Ph	80	109—110	EA–H	$C_{18}H_{17}Cl_2NO_6S$	48.44	3.84	15.89	3.14	7.18
									(48.33	3.84	15.92	3.15	7.11)
8au	Cl	Cl	H	O⌒N(CH_2)$_2$	53	Oil		$C_{17}H_{22}Cl_2N_2O_6S \cdot 1/2\,H_2O$	44.16	5.04		6.05	
									(44.04	4.87		5.88)	
8av	Cl	H	CH_3	CH_3	81	74—75	EA–E–H	$C_{13}H_{16}ClNO_5S$	46.78	4.83	10.62	4.20	9.60
									(46.74	4.80	10.39	4.06	9.84)
8aw	Cl	H	C_2H_5	C_2H_5	76	Oil		$C_{15}H_{20}ClNO_5S$	49.79	5.57	9.80	3.87	8.86
									(49.62	5.56	9.99	3.85	8.72)
8ax	H	Cl	CH_3	CH_3	36	42—43	EA–E–H	$C_{13}H_{16}ClNO_5S$	46.78	4.83	10.62	4.20	9.61
									(46.69	4.61	10.80	4.22	9.59)
8ay	H	Cl	C_2H_5	C_2H_5	37	Oil		$C_{15}H_{20}ClNO_5S$	49.79	5.57	9.80	3.87	8.86
									(49.66	5.29	9.96	3.89	8.84)
8az	Br	H	CH_3	CH_3	81	97—98	EA–H	$C_{13}H_{16}BrNO_5S$	41.28	4.26	21.13[b]	3.70	8.48
									(41.19	4.19	21.04[b]	3.78	8.40)
8ba	Br	H	C_2H_5	C_2H_5	79	Oil		$C_{15}H_{20}BrNO_5S$	44.34	4.96	19.67[b]	3.45	7.89
									(44.34	4.96	20.00[b]	3.53	7.76)
8bb	CH_3	CH_3	H	H	87	154—155	EA–E	$C_{13}H_{17}NO_5S$	52.16	5.72		4.68	10.71
									(52.22	5.66		4.70	10.49)
8bc	CH_3	CH_3	H	CH_3	68	80—81	EA–E	$C_{14}H_{19}NO_5S$	53.66	6.11		4.47	10.23
									(53.47	6.03		4.65	10.06)
8bd	CH_3	CH_3	CH_3	CH_3	89	110—111	EA–E	$C_{15}H_{21}NO_5S$	55.03	6.47		4.28	9.79
									(54.76	6.41		4.27	9.61)
15a	Cl	Cl	H	H	51	164—166	EA–E	$C_{11}H_{11}Cl_2NO_5S$	38.84	3.26	20.84	4.12	9.43
									(38.64	3.11	21.03	4.07	9.25)
15b	Cl	Cl	CH_3	CH_3	82	159—160	E	$C_{13}H_{15}Cl_2NO_5S$	42.40	4.11	19.26	3.80	8.71
									(42.27	4.05	19.49	3.67	8.52)
15c	Cl	Cl	CH_3	$PhCH_2$	70	106—107	E	$C_{19}H_{18}Cl_2NO_5S \cdot 1/4\,H_2O$	51.16	4.34	15.92	3.24	7.16
									(50.96	4.16	15.83	3.13	7.16)
15d	Cl	Cl	CH_3	Ph	51	Oil		$C_{18}H_{17}Cl_2NO_5S$	50.24	3.98		3.26	7.45
									(49.98	3.99		3.16	7.25)
15e	Cl	Cl	H	CH_3	51	121—122	EA–E	$C_{12}H_{13}Cl_2NO_5S$	40.69	3.70	20.02	3.95	9.05
									(40.43	3.71	20.34	3.95	8.89)
15f	Cl	Cl	H	$PhCH_2$	51	122—123	EA–E	$C_{18}H_{17}Cl_2NO_5S$	50.24	3.98	16.48	3.26	7.45
									(49.95	4.09	16.87	3.27	7.26)
15g	Cl	Cl	H	Ph	86	169—170	EA–E	$C_{17}H_{15}Cl_2NO_5S \cdot 1/2\,H_2O$	48.01	3.79		3.29	7.54
									(47.88	3.57		3.18	7.42)

a) See ref. *a* in Table I for abbreviations for the solvents used. *b*) Br.

TABLE V. ^{1}H-NMR Data for **8** and **15** in $CDCl_3$ Solution

8aa[a]	1.25 (3H, t, J=7.2, CH_3), 3.45—4.05 (2H, m, Ph-CH_2-CH), 4.21 (2H, q, J=7.2, CH_2), 5.51 (1H, 2×d, J=10.1, 6.5, O-CH), 6.62 (2H, brs, NH_2), 7.87 (1H, t, J=1.2, 4-H)
8ab	1.33 (3H, t, J=7.0, CH_3), 2.90 (6H, s, 2×CH_3), 3.30—3.90 (2H, m, Ph-CH_2-CH), 4.31 (2H, q, J=7.2, CH_2), 5.40 (1H, 2×d, J=10.0, 7.0, O-CH), 7.88 (1H, t, J=1.0, 4-H)
8ac	1.12 (6H, t, J=7.0, 2×CH_3), 1.30 (3H, t, J=7.0, CH_3), 3.32 (4H, q, J=7.0, 2×CH_2), 3.10—3.90 (2H, m, Ph-CH_2-CH), 4.25 (2H, q, J=7.0, CH_2), 5.35 (1H, 2×d, J=10.0, 7.0, O-CH), 7.86 (1H, t, J=1.0, 4-H)
8ad	0.83 (6H, t, J=7.0, 2×CH_3), 1.31 (3H, t, J=7.0, CH_3), 1.40—1.80 (4H, m, 2×CH_2), 3.24 (4H, t, J=7.0, 2×CH_2), 3.00—4.00 (2H, m, Ph-CH_2-CH), 4.30 (2H, q, J=7.0, CH_2), 5.38 (1H, 2×d, J=10.0, 7.0, O-CH), 7.92 (1H, t, J=1.0, 4-H)
8ae	1.28 (12H, d, J=7.0, 4×CH_3), 1.30 (3H, t, J=7.0, CH_3), 3.25—4.00 (4H, m, Ph-CH_2-CH, 2×CH), 4.26 (2H, q, J=7.0, CH_2), 5.35 (1H, 2×d, J=10.0, 7.0, O-CH), 7.90 (1H, t, J=1.0, 4-H)
8af	0.90 (3H, t, J=7.0, CH_3), 1.31 (3H, t, J=7.0, CH_3), 1.10—1.75 (4H, m, 2×CH_2), 2.85 (3H, s, CH_3), 3.22 (2H, t, J=7.0, CH_2), 3.30—3.85 (2H, m, Ph-CH_2-CH), 4.28 (2H, q, J=7.0, CH_2), 5.37 (1H, 2×d, J=10.0, 7.0, O-CH), 7.88 (1H, t, J=1.0, 4-H)
8ag	1.31 (3H, t, J=7.0, CH_3), 3.20—3.80 (2H, m, Ph-CH_2-CH), 4.26 (2H, q, J=7.0, CH_2), 4.37 (4H, s, 2×Ph-CH_2), 5.35 (1H, 2×d, J=10.0, 7.0, O-CH), 7.00—7.36 (10H, m, arom. H), 7.78 (1H, t, J=1.0, 4-H)
8ah	1.32 (3H, t, J=7.0, CH_3), 2.75 (3H, s, CH_3), 3.26—3.93 (2H, m, Ph-CH_2-CH), 4.30 (2H, q, J=7.0, O-CH_2), 4.42 (2H, s, Ph-CH_2), 5.38 (1H, 2×d, J=10.0, 7.0, O-CH), 7.35 (5H, s, arom. H), 7.92 (1H, t, J=1.0, 4-H)
8ai	1.28 (3H, t, J=7.0, CH_3), 3.13—3.80 (2H, m, Ph-CH_2-CH), 3.38 (3H, s, CH_3), 4.24 (2H, q, J=7.0, O-CH_2), 5.32 (1H, 2×d, J=10.0, 7.0, O-CH), 7.25 (5H, s, arom. H), 7.63 (1H, t, J=1.0, 4-H)
8aj	1.30 (3H, t, J=7.0, CH_3), 0.80—1.90 (10H, m, 5×CH_2), 2.77 (3H, s, CH_3), 3.20—3.86 (3H, m, Ph-CH_2-CH, CH), 4.25 (2H, q, J=7.0, O-CH_2), 5.35 (1H, 2×d, J=10.0, 7.0, O-CH), 7.86 (1H, t, J=1.0, 4-H)
8ak	1.31 (3H, t, J=7.0, CH_3), 1.80—2.10 (4H, m, 2×CH_2), 3.30—3.90 (6H, m, Ph-CH_2-CH, 2×N-CH_2), 4.28 (2H, q, J=7.0, O-CH_2), 5.37 (1H, 2×d, J=10.0, 7.0, O-CH), 7.88 (1H, t, J=1.0, 4-H)
8al	1.33 (3H, t, J=7.0, CH_3), 1.59 (6H, br, 3×CH_2), 3.26 (4H, br, 2×N-CH_2), 3.30—3.93 (2H, m, Ph-CH_2-CH), 4.30 (2H, q, J=7.0, O-CH_2), 5.38 (1H, 2×d, J=10.0, 7.0, O-CH), 7.86 (1H, t, J=1.0, 4-H)
8am	1.32 (3H, t, J=7.0, CH_3), 3.20—4.00 (10H, m, 5×CH_2), 4.30 (2H, q, J=7.0, O-CH_2), 5.40 (1H, 2×d, J=10.0, 7.0, O-CH), 7.84 (1H, t, J=1.0, 4-H)
8an	1.31 (3H, t, J=7.0, CH_3), 2.63 (3H, d, J=5.0, NH-CH_3), 3.30—3.93 (2H, m, Ph-CH_2-CH), 4.30 (2H, q, J=7.0, O-CH_2), 5.03 (1H, q, J=5.0, NH), 5.41 (1H, 2×d, J=10.0, 7.0, O-CH), 7.88 (1H, t, J=1.0, 4-H)
8ao	0.88 (3H, t, J=7.0, CH_3), 1.33 (3H, t, J=7.0, CH_3), 1.20—1.70 (2H, m, CH_2), 2.78 (2H, q, J=7.0, CH_2), 3.30—3.90 (2H, m, Ph-CH_2-CH), 4.30 (2H, q, J=7.0, O-CH_2), 5.05 (1H, t, J=7.0, NH), 5.41 (1H, 2×d, J=10.0, 7.0, O-CH), 7.90 (1H, t, J=1.0, 4-H)
8ap[a]	1.06 (6H, d, J=7.0, 2×CH_3), 1.26 (3H, t, J=7.0, CH_3), 3.20—4.00 (3H, m, Ph-CH_2-CH, CH), 4.25 (2H, q, J=7.0, O-CH_2), 5.56 (1H, 2×d, J=10.0, 7.0, O-CH), 6.53 (1H, brd, J=7.0, NH), 7.92 (1H, t, J=1.0, 4-H)
8aq	1.33 (3H, t, J=7.0, CH_3), 3.23—3.86 (2H, m, Ph-CH_2-CH), 4.10 (2H, d, J=5.0, Ph-CH_2), 4.31 (2H, q, J=7.0, O-CH_2), 5.35 (1H, t, J=5.0, NH), 5.38 (1H, 2×d, J=10.0, 7.0, O-CH), 7.25 (5H, s, arom. H), 7.80 (1H, t, J=1.0, 4-H)
8ar[a]	1.23 (3H, t, J=7.0, CH_3), 3.30—3.97 (2H, m, Ph-CH_2-CH), 4.23 (2H, q, J=7.0, O-CH_2), 5.53 (1H, 2×d, J=10.0, 7.0, O-CH), 6.90—7.50 (5H, m, arom. H), 7.95 (1H, t, J=1.0, 4-H)
8as[a]	1.26 (3H, t, J=7.0, CH_3), 3.20—4.00 (2H, m, Ph-CH_2-CH), 4.20 (2H, q, J=7.0, O-CH_2), 5.52 (1H, 2×d, J=10.0, 7.0, O-CH), 7.25 (4H, s, arom. H), 7.91 (1H, t, J=1.0, 4-H)
8at[a]	1.23 (3H, t, J=7.0, CH_3), 3.20—3.95 (2H, m, Ph-CH_2-CH), 3.70 (3H, s, O-CH_3), 4.18 (2H, q, J=7.0, O-CH_2), 5.50 (1H, 2×d, J=10.0, 7.0, O-CH), 6.75, 7.14 (4H, ABq, J=9.0, arom. H), 7.77 (1H, t, J=1.0, 4-H), 8.90 (1H, br, NH)
8au	1.31 (3H, t, J=7.0, CH_3), 2.20—2.60 (6H, m, 3×N-CH_2), 3.00 (2H, t, J=5.0, N-CH_2), 3.25—4.00 (6H, m, Ph-CH_2-CH, 2×O-CH_2), 4.30 (2H, q, J=7.0, O-CH_2), 5.42 (1H, 2×d, J=10.0, 7.0, O-CH), 5.85 (1H, br, NH), 7.87 (1H, t, J=1.0, 4-H)
8av	1.31 (3H, t, J=7.0, CH_3), 2.71 (6H, s, 2×CH_3), 3.46 (1H, 2×d, J=16.6, 7.5, Ph-CH_2-CH), 3.71 (1H, 2×d, J=16.6, 9.8, Ph-CH_2-CH), 4.28 (2H, q, J=7.0, O-CH_2), 5.36 (1H, 2×d, J=9.8, 7.5, O-CH), 7.49 (1H, brs, 6- or 4-H), 7.64 (1H, brs, 4- or 6-H)
8aw	1.13 (6H, t, J=7.2, 2×CH_3), 1.30 (3H, t, J=7.1, CH_3), 3.22 (4H, q, J=7.2, 2×CH_2), 3.44 (1H, 2×d, J=17.4, 7.5, Ph-CH_2-CH), 3.70 (1H, 2×d, J=17.4, 10.0, Ph-CH_2-CH), 4.28 (2H, q, J=7.1, O-CH_2), 5.34 (1H, 2×d, J=10.0, 7.5, O-CH), 7.51 (1H, brs, 6- or 4-H), 7.67 (1H, brs, 4- or 6-H)

TABLE V. (continued)

8ax	1.30 (3H, t, J=7.2, CH_3), 2.86 (6H, s, 2×CH_3), 3.20—3.80 (2H, m, Ph–CH_2–CH), 4.27 (2H, q, J=7.2, O–CH_2), 5.30 (1H, 2×d, J=10.2, 7.0, O–CH), 7.01 (1H, s, 7-H), 7.88 (1H, t, J=1.2, 4-H)
8ay	1.11 (6H, t, J=7.1, 2×CH_3), 1.30 (3H, t, J=7.1, CH_3), 3.17—3.75 (6H, m, Ph–CH_2–CH, 2×CH_2), 4.25 (2H, q, J=7.1, O–CH_2), 5.27 (1H, 2×d, J=10.2, 7.5, O–CH), 6.97 (1H, s, 7-H), 7.91 (1H, t, J=1.2, 4-H)
8az	1.31 (3H, t, J=7.1, CH_3), 2.72 (6H, s, 2×CH_3), 3.49 (1H, 2×d, J=16.3, 7.2, Ph–CH_2–CH), 3.75 (1H, 2×d, J=16.3, 9.9, Ph–CH_2–CH), 4.28 (2H, q, J=7.1, O–CH_2), 5.36 (1H, 2×d, J=9.9, 7.2, O–CH), 7.53 (1H, brs, 6- or 4-H), 7.79 (1H, brs, 4- or 6-H)
8ba	1.13 (6H, t, J=7.0, 2×CH_3), 1.30 (3H, t, J=7.2, CH_3), 3.22 (4H, q, J=7.0, 2×CH_2), 3.47 (1H, 2×d, J=16.2, 7.3, Ph–CH_2–CH), 3.72 (1H, 2×d, J=16.2, 9.9, Ph–CH_2–CH), 4.28 (2H, q, J=7.2, O–CH_2), 5.34 (1H, 2×d, J=9.9, 7.3, O–CH), 7.55 (1H, brs, 6- or 4-H), 7.81 (1H, brs, 4- or 6-H)
8bb	1.28 (3H, t, J=7.0, CH_3), 2.20, 2.54 (2×3H, 2×s, 2×CH_3), 3.10—3.75 (2H, m, Ph–CH_2–CH), 4.22 (2H, q, J=7.0, O–CH_2), 4.90 (2H, br, NH_2), 5.18 (1H, 2×d, J=10.5, 7.5, O–CH), 7.72 (1H, brs, 4-H)
8bc	1.28 (3H, t, J=7.0, CH_3), 2.20, 2.50 (2×3H, 2×s, 2×CH_3), 2.56 (3H, d, J=7.0, NH–CH_3), 3.10—3.80 (2H, m, Ph–CH_2–CH), 4.24 (2H, q, J=7.0, O–CH_2), 4.47 (1H, d, J=7.0, NH), 5.21 (1H, 2×d, J=10.5, 7.5, O–CH), 7.70 (1H, brs, 4-H)
8bd	1.28 (3H, t, J=7.0, CH_3), 2.20, 2.48 (2×3H, 2×s, 2×CH_3), 2.74 (6H, s, 2×CH_3), 3.10—3.75 (2H, m, Ph–CH_2–CH), 4.23 (2H, q, J=7.0, O–CH_2), 5.20 (1H, 2×d, J=10.5, 7.5, O–CH), 7.65 (1H, brs, 4-H)
15a[a]	2.00 (3H, s, $COCH_3$), 3.07—3.77 (2H, m, Ph–CH_2–CH), 4.15—4.56 (2H, m, O–CH_2), 5.16—5.53 (1H, m, O–CH), 6.64 (2H, brs, NH_2), 7.87 (1H, t, J=1.2, 4-H)
15b	1.97 (3H, s, $COCH_3$), 2.83 (6H, s, 2×CH_3), 2.95—3.70 (2H, m, Ph–CH_2–CH), 4.10—4.65 (2H, m, O–CH_2), 5.17—5.53 (1H, m, O–CH), 7.85 (1H, t, J=1.2, 4-H)
15c	2.07 (3H, s, $COCH_3$), 2.75 (3H, s, CH_3), 2.95—3.70 (2H, m, Ph–CH_2–CH), 4.35 (2H, d, J=5.0, O–CH_2), 4.32 (2H, s, Ph–CH_2), 5.05—5.45 (1H, m, O–CH), 7.35 (5H, s, arom. H), 7.91 (1H, t, J=1.0, 4-H)
15d	2.03 (3H, s, $COCH_3$), 3.30 (3H, s, CH_3), 2.80—3.60 (2H, m, Ph–CH_2–CH), 4.27 (2H, d, J=5.0, O–CH_2), 5.00—5.37 (1H, m, O–CH), 7.25 (5H, s, arom. H), 7.64 (1H, t, J=1.0, 4-H)
15e	2.07 (3H, s, $COCH_3$), 2.60 (3H, d, J=5.0, NH–CH_3), 3.00—3.70 (2H, m, Ph–CH_2–CH), 4.34 (2H, d, J=5.0, O–CH_2), 5.00 (1H, q, J=5.0, NH), 5.00—5.43 (1H, m, O–CH), 7.86 (1H, t, J=1.0, 4-H)
15f	2.08 (3H, s, $COCH_3$), 2.90—3.60 (2H, m, Ph–CH_2–CH), 4.10 (2H, d, J=6.0, NH–CH_2), 4.33 (2H, d, J=5.0, O–CH_2), 5.05—5.45 (2H, m, O–CH, NH), 7.24 (5H, s, arom. H), 7.80 (1H, t, J=1.0, 4-H)
15g	1.97 (3H, s, $COCH_3$), 2.85—3.60 (2H, m, Ph–CH_2–CH), 4.25 (2H, d, J=5.0, O–CH_2), 4.95—5.30 (1H, m, O–CH), 6.80—7.50 (1H, br, NH), 7.13 (5H, s, arom. H), 7.73 (1H, t, J=1.0, 4-H)

a) In acetone-d_6 solution.

compound obtained above in anisole (3 ml) under ice-cooling, and the mixture was stirred at room temperature for 1 h. Evaporation of the reaction mixture *in vacuo* gave a residue, which, when treated with hexane, gave **12** (0.942 g, 100%). Recrystallization from hexane–ether–acetone gave colorless crystals, mp 199—201 °C, ^{1}H-NMR spectral data of which are given in Table VI.

6,7-Dichloro-5-(*N*-ethyl-*N*-methylsulfamoyl)-2,3-dihydrobenzofuran-2-carboxylic Acid (13a)——Sodium hydride 50%; (0.150 g, 0.0031 mol) was added to a solution of **8an** (1.0 g, 0.0031 mol) in anhydrous DMF (10 ml) and the mixture was stirred for 30 min. After addition of ethyl bromide (0.40 g, 0.0036 mol) under ice-cooling, the mixture was stirred for 30 min under ice-cooling and then for another 30 min at room temperature. Next, 10% hydrochloric acid (1.0 ml) and water (10 ml) were added to the reaction mixture, which was then extracted with ether (80 ml). The ether layer was dried and evaporated *in vacuo*, giving a residue. A solution of the residue in ether was combined with an ethereal solution of diazomethane and allowed to react for 30 min. Evaporation of the reaction mixture *in vacuo* gave a residue, which, when chromatographed using dichloromethane–ether as the eluant, gave ethyl 6,7-dichloro-5-(*N*-ethyl-*N*-methylsulfamoyl)-2,3-dihydrobenzofuran-2-carboxylate (0.905 g, 84%), mp 80—81 °C (hexane–ether). *Anal.* Calcd for $C_{14}H_{17}Cl_2NO_5S$: C, 43.99; H, 4.48; Cl, 18.55; N, 3.66; S, 8.39. Found: C, 43.77; H, 4.47; Cl, 18.71; N, 3.84; S, 8.41. ^{1}H-NMR (in $CDCl_3$) δ: 1.16 (3H, t, J=7.0, CH_3), 1.30 (3H, t, J=7.0, CH_3), 2.86 (3H, s, N–CH_3), 3.27 (2H, q, J=7.0, N–CH_2), 3.20—3.90 (2H, m, CH_2), 4.28 (2H, q, J=7.0, O–CH_2), 5.38 (1H, 2×d, J=10.0, 7.0, O–CH), 7.87 (1H, t, J=1.0, arom. H). A 1 N sodium hydroxide solution (1.65 ml) was added to a solution of the ester (0.60 g, 0.0016 mol) obtained above in acetonitrile (2 ml), and the mixture was stirred for 30 min. Evaporation of the reaction mixture *in vacuo* gave a residue, which was acidified with 10% hydrochloric acid and extracted with ethyl acetate (50 ml). The ethyl acetate layer was dried and evaporated, giving a viscous residue. The residue was dissolved in acetonitrile (10 ml), then combined with 1 N sodium hydroxide (1.6 ml), and the mixture was stirred for 30 min under ice-cooling. The precipitated crystals were collected by filtration, then recrystallized from aqueous ethanol, giving **13a**

TABLE VI. ^{1}H-NMR Data for Compounds **9**—**13** and **16**—**18** in d_6-Acetone Solution

9aa	3.27—4.55 (2H, m, Ph-CH$_2$-CH), 5.55 (1H, 2×d, J=10.2, 6.6, O-CH), 6.4 (1H, br, COOH), 6.67 (2H, brs, NH$_2$), 7.90 (1H, t, J=1.2, 4-H)
9ab	2.85 (6H, s, 2×CH$_3$), 3.30—4.00 (2H, m, Ph-CH$_2$-CH), 5.55 (1H, 2×d, J=10.0, 7.0, O-CH), 7.87 (1H, t, J=1.0, 4-H), 8.40 (1H, br, COOH)
9ac[a]	1.03 (6H, t, J=7.0, 2×CH$_3$), 3.26 (4H, q, J=7.0, 2×CH$_2$), 3.00—3.70 (2H, m, Ph-CH$_2$-CH), 4.95 (1H, 2×d, J=10.0, 7.0, O-CH), 7.71 (1H, t, J=1.0, 4-H)
9ad	0.80 (6H, t, J=7.0, 2×CH$_3$), 1.00—1.80 (4H, m, 2×CH$_2$), 3.26 (4H, t, J=7.0, 2×CH$_2$), 3.25—4.20 (2H, m, Ph-CH$_2$-CH), 5.57 (1H, 2×d, J=10.0, 7.0, O-CH), 7.94 (1H, t, J=1.0, 4-H), 8.93 (1H, br, COOH)
9ae	1.27 (12H, d, J=7.0, 4×CH$_3$), 3.40—4.10 (4H, m, Ph-CH$_2$-CH, 2×CH), 5.57 (1H, 2×d, J=10.0, 7.0, O-CH), 8.00 (1H, t, J=1.0, 4-H)
9af	0.87 (3H, t, J=7.0, CH$_3$), 1.05—1.80 (4H, m, 2×CH$_2$), 2.85 (3H, s, CH$_3$), 3.25 (2H, t, J=7.0, CH$_2$), 3.40—4.10 (2H, m, Ph-CH$_2$-CH), 5.60 (1H, 2×d, J=10.0, 7.0, O-CH), 7.93 (1H, t, J=1.0, 4-H), 8.00—9.80 (1H, br, COOH)
9ag	3.30—4.05 (2H, m, Ph-CH$_2$-CH), 4.44 (4H, s, 2×Ph-CH$_2$), 5.55 (1H, 2×d, J=10.0, 7.0, O-CH), 7.00—7.35 (10H, s, arom. H), 7.85 (1H, t, J=1.0, 4-H), 7.30—8.50 (1H, br, COOH)
9ah	2.73 (3H, s, CH$_3$), 3.30—4.05 (2H, m, Ph-CH$_2$-CH), 4.43 (2H, s, Ph-CH$_2$), 5.57 (1H, 2×d, J=10.0, 7.0, O-CH), 7.32 (5H, s, arom. H), 7.95 (1H, t, J=1.0, 4-H), 9.37 (1H, br, COOH)
9ai[a]	3.00—3.70 (2H, m, Ph-CH$_2$-CH), 3.28 (3H, s, CH$_3$), 4.96 (1H, 2×d, J=10.0, 7.0, O-CH), 7.10—7.50 (5H, m, arom. H), 7.62 (1H, t, J=1.0, 4-H)
9aj	0.70—1.90 (10H, m, 5×CH$_2$), 2.80 (3H, s, CH$_3$), 3.00—4.10 (3H, m, Ph-CH$_2$-CH, CH), 5.51 (1H, 2×d, J=10.0, 7.0, O-CH), 6.10 (1H, br, COOH), 7.91 (1H, brs, 4-H)
9ak	1.65—2.15 (4H, m, 2×CH$_2$), 3.10—4.25 (6H, m, Ph-CH$_2$-CH, 2×N-CH$_2$), 5.56 (1H, 2×d, J=10.0, 7.0, O-CH), 7.90 (1H, t, J=1.0, 4-H), 8.86 (1H, br, COOH)
9al[a]	1.50 (6H, br, 3×CH$_2$), 3.15 (4H, br, 2×N-CH$_2$), 3.10—3.85 (2H, m, Ph-CH$_2$-CH), 5.05 (1H, 2×d, J=10.0, 7.0, O-CH), 7.76 (1H, t, J=1.0, 4-H)
9am[a]	2.80—3.40 (4H, m, 2×CH$_2$), 3.49—4.10 (6H, m, 3×CH$_2$), 5.52 (1H, 2×d, J=10.0, 7.0, O-CH), 7.83 (1H, t, J=1.0, 4-H)
9an	2.57 (3H, d, J=5.0, NH-CH$_3$), 3.35—4.20 (2H, m, Ph-CH$_2$-CH), 5.57 (1H, 2×d, J=10.0, 7.0, O-CH), 6.50 (1H, q, J=5.0, NH), 7.92 (1H, t, J=1.0, 4-H), 7.90—8.95 (1H, br, COOH)
9ao	0.83 (3H, t, J=7.0, CH$_3$), 1.25—1.70 (2H, m, CH$_2$), 2.86 (2H, q, J=7.0, CH$_2$), 3.30—4.05 (2H, m, Ph-CH$_2$-CH), 5.55 (1H, 2×d, J=10.0, 7.0, O-CH), 6.85 (1H, brt, NH), 7.90 (1H, t, J=1.0, 4-H)
9ap	1.06 (6H, d, J=7.0, 2×CH$_3$), 3.20—4.10 (3H, m, Ph-CH$_2$-CH, CH), 5.60 (1H, 2×d, J=10.0, 7.0, O-CH), 6.52 (1H, brd, J=7.0, NH), 7.95 (1H, t, J=1.0, 4-H), 9.25—10.05 (1H, br, COOH)
9aq	3.25—4.10 (2H, m, Ph-CH$_2$-CH), 4.15 (2H, d, J=6.0, Ph-CH$_2$), 5.52 (1H, 2×d, J=10.0, 7.0, O-CH), 7.17 (5H, s, arom. H), 7.75 (1H, t, J=1.0, 4-H), 7.30—9.10 (1H, br, COOH)
9ar	3.30—4.00 (2H, m, Ph-CH$_2$-CH), 5.51 (1H, 2×d, J=10.0, 7.0, O-CH), 6.85—7.35 (5H, m, arom. H), 7.93 (1H, t, J=1.0, 4-H), 8.60 (1H, br, COOH), 9.20 (1H, brs, NH)
9as	3.35—4.10 (2H, m, Ph-CH$_2$-CH), 5.56 (1H, 2×d, J=10.0, 7.0, O-CH), 7.27 (4H, s, arom. H), 7.93 (1H, t, J=1.0, 4-H), 7.50—9.30 (1H, br, COOH), 9.40 (1H, br, NH)
9at	3.30—4.20 (2H, m, Ph-CH$_2$-CH), 3.67 (3H, s, O-CH$_3$), 5.53 (1H, 2×d, J=10.0, 7.0, O-CH), 6.77, 7.27 (4H, ABq, J=9.0, arom. H), 7.82 (1H, t, J=1.0, 4-H), 8.75 (1H, br, COOH), 8.92 (1H, brs, NH)
9au[a]	2.80—3.40 (8H, m, 4×N-CH$_2$), 3.40—4.10 (6H, m, Ph-CH$_2$-CH, 2×O-CH$_2$), 5.53 (1H, 2×d, J=10.0, 7.0, O-CH), 7.86 (1H, t, J=1.0, 4-H), 8.10 (1H, t, J=5.0, NH)
9av	2.70 (6H, s, 2×CH$_3$), 3.58 (1H, 2×d, J=16.8, 6.9, Ph-CH$_2$-CH), 3.90 (1H, 2×d, J=16.8, 10.2, Ph-CH$_2$-CH), 5.56 (1H, 2×d, J=10.2, 6.9, O-CH), 7.63 (2H, s, 4-H, 6-H), 7.6—9.3 (1H, br, COOH)
9aw	1.11 (6H, t, J=7.0, 2×CH$_3$), 3.23 (4H, q, J=7.0, 2×CH$_2$), 3.55 (1H, 2×d, J=16.5, 6.8, Ph-CH$_2$-CH), 3.89 (1H, 2×d, J=16.5, 10.5, Ph-CH$_2$-CH), 5.53 (1H, 2×d, J=10.5, 6.8, O-CH), 7.66 (2H, s, 4-H, 6-H)
9ax	2.80 (6H, s, 2×CH$_3$), 3.25—3.93 (2H, m, Ph-CH$_2$-CH), 5.43 (1H, 2×d, J=10.2, 6.8, O-CH), 7.03 (1H, s, 7-H), 7.88 (1H, t, J=1.2, 4-H)
9ay	1.08 (6H, t, J=7.2, 2×CH$_3$), 3.20—3.90 (6H, m, Ph-CH$_2$-CH, 2×CH$_2$), 4.2—5.3 (br, COOH), 5.43 (1H, 2×d, J=10.5, 7.0, O-CH), 7.00 (1H, s, 7-H), 7.91 (1H, t, J=1.2, 4-H)
9az	2.70 (6H, s, 2×CH$_3$), 3.60 (1H, 2×d, J=16.6, 7.0, Ph-CH$_2$-CH), 3.93 (1H, 2×d, J=16.6, 10.5, Ph-CH$_2$-CH), 5.54 (1H, 2×d, J=10.5, 7.0, O-CH), 7.66 (1H, m, 6- or 4-H), 7.75 (1H, m, 4- or 6-H), 7.2—8.5 (1H, br, COOH)
9ba	1.10 (6H, t, J=7.1, 2×CH$_3$), 3.22 (4H, q, J=7.1, 2×CH$_2$), 3.57 (1H, 2×d, J=16.5, 6.8, Ph-CH$_2$-CH), 3.90 (1H, 2×d, J=16.5, 10.5, Ph-CH$_2$-CH), 5.52 (1H, 2×d, J=10.5, 6.8, O-CH), 7.70 (1H, m, 6- or 4-H), 7.79 (1H, m, 4- or 6-H), 7.2—8.5 (1H, br, COOH)

TABLE VI. (continued)

9bb	2.20, 2.56 (2×3H, 2×s, 2×C$\underline{H}_3$), 3.20—3.90 (2H, m, Ph-C$\underline{H}_2$-CH), 5.38 (1H, 2×d, J=10.8, 7.0, O-C$\underline{H}$), 6.33 (2H, br, N$\underline{H}_2$), 7.74 (1H, brs, 4-H)
9bc	2.20, 2.50 (2×3H, 2×s, 2×C$\underline{H}_3$), 2.50 (3H, d, J=5.0, NH-C$\underline{H}_3$), 3.20—4.00 (2H, m, Ph-C$\underline{H}_2$-CH), 5.37 (1H, 2×d, J=10.8, 7.0, O-C$\underline{H}$), 6.10 (1H, br, N$\underline{H}$), 7.71 (1H, brs, 4-H)
9bd	2.20, 2.50 (2×3H, 2×s, 2×C$\underline{H}_3$), 2.70 (6H, s, 2×C$\underline{H}_3$), 3.25—4.00 (2H, m, Ph-C$\underline{H}_2$-CH), 5.38 (1H, 2×d, J=10.8, 7.0, O-C$\underline{H}$), 7.67 (1H, brs, 4-H)
10a	2.83 (6H, s, 2×C$\underline{H}_3$), 3.15—4.15 (2H, m, Ph-C$\underline{H}_2$-CH), 5.60—5.90 (1H, m, O-C$\underline{H}$), 7.54 (1H, s, O-C$\underline{H}$-O), 7.60—8.00 (5H, arom. H)
10b	1.16 (9H, s, 3×C$\underline{H}_3$), 2.84 (6H, s, 2×C$\underline{H}_3$), 3.10—4.10 (2H, m, Ph-C$\underline{H}_2$-CH), 5.62 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 5.84 (2H, s, O-C$\underline{H}_2$-O), 7.88 (1H, t, J=1.0, 4-H)
10c	2.85 (6H, s, 2×C$\underline{H}_3$), 3.30—4.15 (2H, m, Ph-C$\underline{H}_2$-CH), 4.67, 4.87 (2H, ABq, J=16.0, O-C$\underline{H}_2$-CO), 5.67 (1H, 2×d, J=10.0, 6.3, O-C$\underline{H}$), 7.88 (1H, t, J=1.0, 4-H)
10d	1.10 (6H, t, J=7.0, 2×C$\underline{H}_3$), 3.35 (4H, q, J=7.0, 2×C$\underline{H}_2$), 3.40—4.20 (2H, m, Ph-C$\underline{H}_2$-CH), 4.67, 4.86 (2H, ABq, J=15.0, O-C$\underline{H}_2$-CO), 5.68 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 7.93 (1H, t, J=1.0, 4-H)
11a	2.86 (6H, s, 2×C$\underline{H}_3$), 3.25—3.96 (2H, m, Ph-C$\underline{H}_2$-CH), 5.45 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 6.50—7.50 (2H, br, N$\underline{H}_2$), 7.90 (1H, t, J=1.0, 4-H)
11b[b]	2.85 (6H, s, 2×C$\underline{H}_3$), 2.87 (3H, d, J=7.0, NH-C$\underline{H}_3$), 3.30—4.00 (2H, m, Ph-C$\underline{H}_2$-CH), 5.35 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 6.60 (1H, br, N$\underline{H}$), 7.84 (1H, t, J=1.0, 4-H)
11c[b]	2.78 (6H, s, 2×C$\underline{H}_3$), 3.05, 3.25 (2×3H, 2×s, 2×NC$\underline{H}_3$), 3.20—4.15 (2H, m, Ph-C$\underline{H}_2$-CH), 5.67 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 7.77 (1H, t, J=1.0, 4-H)
12	1.07 (3H, t, J=7.0, C$\underline{H}_3$), 3.40 (3H, s, C$\underline{H}_3$), 3.40—4.05 (2H, m, Ph-C$\underline{H}_2$-CH), 4.05 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.62 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 8.03 (1H, t, J=1.0, 4-H), 9.30—10.20 (1H, br, COO$\underline{H}$)
13a[a]	1.06 (3H, t, J=7.0, C$\underline{H}_3$), 2.80 (3H, s, C$\underline{H}_3$), 3.20 (2H, q, J=7.0, N-C$\underline{H}_2$), 3.20—3.75 (2H, m, Ph-C$\underline{H}_2$-CH), 5.06 (1H, 2×d, J=11.0, 7.0, O-C$\underline{H}$), 7.76 (1H, t, J=1.0, 4-H)
13b[a]	0.80 (3H, t, J=7.0, C$\underline{H}_3$), 1.25—1.80 (2H, m, C$\underline{H}_2$), 2.77 (3H, s, C$\underline{H}_3$), 3.12 (2H, t, J=7.0, N-C$\underline{H}_2$), 3.10—3.75 (2H, m, Ph-C$\underline{H}_2$-CH), 5.04 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 7.74 (1H, t, J=1.0, 4-H)
16a	3.13—4.17 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.21 (1H, 2×d, J=6.2, 5.3, O$\underline{H}$), 5.00—5.35 (1H, m, O-C$\underline{H}$), 6.62 (2H, brs, N$\underline{H}_2$), 7.83 (1H, t, J=1.2, 4-H)
16b	2.83 (6H, s, 2×C$\underline{H}_3$), 3.13—4.10 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.20 (1H, 2×d, J=6.3, 5.9, O$\underline{H}$), 5.00—5.33 (1H, m, O-C$\underline{H}$), 7.80 (1H, t, J=1.2, 4-H)
16c[b]	2.35 (3H, s, C$\underline{H}_3$), 3.00—4.15 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.38 (2H, s, Ph-C$\underline{H}_2$), 4.38 (1H, t, J=7.0, O$\underline{H}$), 4.95—5.35 (1H, m, O-C$\underline{H}$), 7.35 (5H, s, arom. H), 7.87 (1H, t, J=1.0, 4-H)
16d	3.20—4.00 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 3.37 (3H, s, C$\underline{H}_3$), 4.21 (1H, t, J=6.0, O$\underline{H}$), 5.00—5.35 (1H, m, O-C$\underline{H}$), 7.31 (5H, s, arom. H), 7.68 (1H, t, J=1.0, 4-H)
16e	2.56 (3H, d, J=6.0, NH-C$\underline{H}_3$), 3.10—4.00 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.20 (1H, t, J=7.0, O$\underline{H}$), 5.00—5.33 (1H, m, O-C$\underline{H}$), 6.42 (1H, br, N$\underline{H}$), 7.83 (1H, t, J=1.0, 4-H)
16f	3.00—4.30 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.25 (2H, d, J=7.0, Ph-C$\underline{H}_2$), 5.00—5.35 (1H, m, O-C$\underline{H}$), 7.21 (5H, s, arom. H), 7.73 (1H, t, J=1.0, 4-H)
16g	3.15—4.05 (4H, m, Ph-C$\underline{H}_2$-CH, O-C$\underline{H}_2$), 4.90—5.40 (1H, m, O-C$\underline{H}$), 6.70—7.40 (5H, m, arom. H), 7.90 (1H, t, J=1.0, 4-H), 8.40—9.80 (1H, br, N$\underline{H}$)
17a	3.15—4.20 (4H, m, Ph-C$\underline{H}_2$-CH, Cl-C$\underline{H}_2$), 5.15—5.60 (1H, m, O-C$\underline{H}$), 6.67 (2H, brs, N$\underline{H}_2$), 7.87 (1H, t, J=1.0, 4-H)
17b[b]	2.87 (6H, s, 2×C$\underline{H}_3$), 3.05—3.85 (2H, m, Ph-C$\underline{H}_2$-CH), 3.81 (2H, d, J=5.0, Cl-C$\underline{H}_2$), 5.10—5.45 (1H, m, O-C$\underline{H}$), 7.85 (1H, t, J=1.0, 4-H)
18a	1.12 (3H, t, J=7.0, C$\underline{H}_3$), 3.05—3.75 (2H, m, Ph-C$\underline{H}_2$-CH), 3.71 (2H, d, J=5.0, O-C$\underline{H}_2$), 5.05—5.45 (1H, m, O-C$\underline{H}$), 6.60 (2H, br, N$\underline{H}_2$), 7.82 (1H, t, J=1.0, 4-H)
18b[b]	1.20 (3H, t, J=7.0, C$\underline{H}_3$), 2.87 (6H, s, 2×C$\underline{H}_3$), 3.10—3.55 (2H, m, Ph-C$\underline{H}_2$-CH), 3.60 (2H, q, J=7.0, O-C$\underline{H}_2$), 3.70 (2H, d, J=5.0, O-C$\underline{H}_2$), 5.00—5.35 (1H, m, O-C$\underline{H}$), 7.83 (1H, t, J=1.0, 4-H)

a) In DMSO-d_6 solution. *b*) In $CDCl_3$ solution.

(0.591 g, 91%).

Compound **13b** was obtained in a similar manner. Ethyl 6,7-dichloro-5-(*N*-methyl-*N*-propylsulfamoyl)-2,3-dihydrobenzofuran-2-carboxylate; yield 85%, mp 70—71 °C (hexane-ether). *Anal.* Calcd for $C_{15}H_{19}Cl_2NO_5S$: C, 45.46; H, 4.83; Cl, 17.89; N, 3.53; S, 8.09. Found: C, 45.41; H, 4.75; Cl, 18.10; N, 3.63; S, 8.10. ^{1}H-NMR (in $CDCl_3$) δ: 0.87 (3H, t, J=7.0, C$\underline{H}_3$), 1.30 (3H, t, J=7.0, C$\underline{H}_3$), 1.30—1.90 (2H, m, C$\underline{H}_2$), 2.84 (3H, s, N-C$\underline{H}_3$), 3.19 (2H, q, J=7.0, N-C$\underline{H}_2$), 3.20—3.85 (2H, m, C$\underline{H}_2$), 4.27 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.37 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 7.87

(1H, t, $J=1.0$, arom. H). Compound **13b**, yield 86%. ^{1}H-NMR spectral data for **13a, b** are given in Table VI.

6,7-Dichloro-5-*N*,*N*-dimethylsulfamoyl-2,3-dihydrobenzofuran-2-ylmethyl Acetate (15b)——Acetyl chloride (5.37 g, 1.5 × 0.0457 mol) was added dropwise to a solution of **14** (10.0 g, 0.0457 mol) and 4-*N*,*N*-dimethylaminopyridine (11.13 g, 2 × 0.0457 mol) in dichloromethane (100 ml) under ice-cooling, and the reaction mixture was stirred for 1 h. Next, dichloromethane (100 ml) was added, and the organic layer was washed several times with water, then dried and evaporated. The residue was chromatographed and eluted with dichloromethane, giving oily 6,7-dichloro-2,3-dihydrobenzofuran-2-ylmethyl acetate (11.04 g, 93%). ^{1}H-NMR (in $CDCl_3$) δ: 2.06 (3H, s, $COC\underline{H}_3$), 3.01 (1H, 2 × d, $J=16.0$, 7.5, Ph–C$\underline{H}_2$–CH), 3.35 (1H, 2 × d, $J=16.0$, 9.5, Ph–C$\underline{H}_2$–CH), 4.09—4.45 (2H, m, O–C$\underline{H}_2$), 4.93—5.30 (1H, m, O–C$\underline{H}$), 6.93 (2H, s, arom. H). Chlorosulfonic acid (1.25 ml) was added to a solution of this oily acetate (1.0 g, 0.0038 mol) in thionyl chloride (2 ml) under ice-cooling, and the mixture was stirred at room temperature for 2 h. The reaction mixture was poured into ice water and extracted with ether. The ethereal layer was washed with water, then dried and evaporated *in vacuo*. A 30% ethanolic solution of dimethylamine (1.8 ml, 3 × 0.0038 mol) was added dropwise to a solution of this oily chlorosulfonate in dichloromethane (10 ml) at −30 °C, and the mixture was stirred for 2 h. Next, dichloromethane was added, then the reaction mixture was washed with 10% hydrochloric acid. The organic layer was dried, evaporated *in vacuo* and treated with ether, giving **15b** (1.24 g, 88%).

Compounds **15a** and **15c—g** were obtained in a similar manner (Table IV). ^{1}H-NMR spectral data for **15a—g** are given in Table V.

6,7-Dichloro-2-hydroxymethyl-*N*,*N*-dimethyl-2,3-dihydrobenzofuran-5-sulfonamide (16b)——A solution of **15b** (2.94 g, 0.0080 mol) in tetrahydrofuran (15 ml) was stirred with 5% sodium hydroxide (7.5 ml) at room temperature for 2 h. The reaction mixture became transparent. After removal of tetrahydrofuran by evaporation *in vacuo*, the reaction mixture was acidified and extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated *in vacuo*. The residue was treated with ether–ethyl acetate, giving **16b** (2.15 g, 89%).

Compounds **16a** and **16c—g** were obtained in a similar manner. ^{1}H-NMR spectral data for **16a—g** are given in Table VI.

2-Chloromethyl-6,7-dichloro-*N*,*N*-dimethyl-2,3-dihydrobenzofuran-5-sulfonamide (17b)——Thionyl chloride (1 ml) was added dropwise to a solution of **16b** (1.0 g, 0.0031 mol) in pyridine (10 ml) at 4 °C, and the mixture was stirred at room temperature for 21 h. Next, 10% hydrochloric acid was added, and the mixture was extracted with ethyl acetate (200 ml). The organic layer was washed with 10% hydrochloric acid and then water, dried and evaporated *in vacuo*. Chromatography of the residue using dichloromethane as the eluant gave **17b** (0.663 g, 63%).

Compound **17a** was obtained in a similar manner. Yield 66%. ^{1}H-NMR spectral data for **17a, b** are given in Table VI.

6,7-Dichloro-2-ethoxymethyl-2,3-dihydrobenzofuran-5-sulfonamide (18a)——Sodium hydride 50%; (0.790 g, 0.0165 mol) was added to a solution of **14** (2.88 g, 0.0132 mol) in DMF (22 ml), and the mixture was stirred at room temperature for 30 min. Next, ethyl bromide (1.73 g, 0.0159 mol) was added, and the mixture was allowed to react at room temperature for 17 h. After decomposition of sodium hydride by addition of water, the reactant was extracted with ether. The ether layer was washed with water, dried and evaporated *in vacuo*, giving a residue, which, when chromatographed with dichloromethane, gave oily 6,7-dichloro-2-ethoxymethyl-2,3-dihydrobenzofuran (2.0 g, 62%).

Chlorosulfonic acid (3.0 g, 0.0258 mol) was added dropwise to a solution of this compound (2.0 g, 0.0081 mol) in thionyl chloride (5 ml) under ice-cooling. The reaction mixture was stirred at room temperature for 2 h, poured into ice water, and then extracted with ethyl acetate. The organic layer was washed with water, dried and evaporated *in vacuo*, giving an oil, which was then dissolved in dichloromethane (30 ml). After introduction of gaseous ammonia at −30——20 °C, the mixture was allowed to stand overnight at room temperature. The reaction mixture was evaporated *in vacuo* and the residue was dissolved in ethyl acetate. The solution was washed with water, dried and evaporated *in vacuo*, leaving an oily residue, which, when chromatographed with dichloromethane–acetone (20 : 1), gave **18a** (0.900 g, 24%).

6,7-Dichloro-*N*,*N*-dimethyl-2-ethoxymethyl-2,3-dihydrobenzofuran-5-sulfonamide (18b)——Sodium hydride 50%; (0.200 g, 0.0031 mol) was added to a solution of **16b** (1.0 g, 0.0031 mol) in DMF (8 ml), and the mixture was stirred at room temperature for 30 min. After addition of ethyl bromide (0.450 g, 1.3 × 0.0031 mol), the reaction mixture was stirred at room temperature for 24 h and then combined with water and ether. The organic layer was washed twice with water, dried and evaporated *in vacuo*, leaving an oily residue, which, when chromatographed with dichloromethane–acetone (20 : 1), gave **18b** (0.464 g, 43%). ^{1}H-NMR spectral data for **18a, b** are given in Table VI.

Resolution of the Optical Isomers of 9ab——Thionyl chloride (2.5 ml) was added to a stirred solution of **9ab** (1.50 g, 0.0044 mol) in absolute benzene (10 ml), and the solution was refluxed for 1 h, then evaporated *in vacuo*. The resulting oily acid chloride was dissolved in benzene (5 ml), and a solution of L-proline *tert*-butyl ester (0.90 g, 0.0053 mol), triethylamine (0.88 g, 0.0088 mol) and 4-*N*,*N*-dimethylaminopyridine (0.053 g, 0.0004 mol) in benzene (10 ml) was added dropwise with stirring at 4 °C. The mixture was allowed to react for 1 h, then evaporated *in vacuo*. The residue was dissolved in ethyl acetate (150 ml), and the solution was washed successively with 10% hydrochloric acid and water, dried and evaporated *in vacuo*. Treatment of the oily residue with ether gave colorless crystals of a diastereomer, **19a** (0.924 g, 44%). The mother liquor was chromatographed with a silica gel Lobar column (E. Merck, size B), which was eluted with dichloromethane–acetone (20 : 1). After separation of small amounts of **19a**, collection

of the subsequent fractions gave oily **19b** (0.89 g, 42%), which crystallized in a refrigerator at −20 °C. Compound **19a**: mp 177—178 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{20}H_{26}Cl_2N_2O_6S$: C, 48.69; H, 5.31; Cl, 14.37; N, 5.68; S, 6.50. Found: C, 48.72; H, 5.22; Cl, 14.44; N, 5.68; S, 6.47. ^{1}H-NMR (in $CDCl_3$) δ: 1.31 and 1.46 (9H, 2×s, 3×C$\underline{H}_3$), 1.80—2.45 (4H, m, 2×C$\underline{H}_2$), 2.86 (6H, s, 2×C$\underline{H}_3$), 3.30—4.20 (4H, m, N–C$\underline{H}_2$, Ph–C$\underline{H}_2$–CH), 4.20—5.00 (1H, m, N–C$\underline{H}$), 5.60 (1H, 2×d, J=10.0, 7.0, O–C$\underline{H}$), 7.84 (1H, t, J=1.2, arom. H). Compound **19b**: mp 96—98 °C (hexane–ether). *Anal.* Calcd for $C_{20}H_{26}Cl_2N_2O_6S$: C, 48.69; H, 5.31; Cl, 14.37; N, 5.68; S, 6.50. Found: C, 48.28; H, 5.19; Cl, 14.59; N, 5.78; S, 6.37. ^{1}H-NMR (in $CDCl_3$) δ: 1.40 and 1.50 (9H, 2×s, 3×C$\underline{H}_3$), 1.70—2.50 (4H, m, 2×C$\underline{H}_2$), 2.88 (6H, s, 2×C$\underline{H}_3$), 3.20—4.10 (4H, m, N–C$\underline{H}_2$, Ph–C$\underline{H}_2$–CH), 4.30—4.90 [1H, 2 sets of m and 2×d (J=7.5, 4.5), N–C$\underline{H}$], 5.43 and 5.55 (1H, 2 sets of 2×d, J=10.2, 6.8, O–C$\underline{H}$), 7.75 (1H, br s, arom. H).

A solution of **19a** (11.8 g, 0.0239 mol) in water (10 ml), concentrated sulfuric acid (25 g) and dioxane (225 ml) was heated at 90 °C with stirring for 17 h. After addition of water (200 ml), the solution was extracted with ethyl acetate (800 ml), then the organic layer was washed with water three times, dried and evaporated *in vacuo*. The residue was dissolved in a solution of sodium hydroxide (95%, 1.1 g, 0.0261 mol) and water (20 ml), and then acetonitrile (40 ml) was added. The precipitated crystalline material was collected by filtration and suspended in 10% hydrochloric acid. The suspension was extracted with ethyl acetate and the organic layer was washed with water, dried and evaporated *in vacuo*. Treatment of the residue with hexane–ethyl acetate gave (−)-**9ab** (7.43 g, 91%), mp 130—131 °C (ethyl acetate–hexane). *Anal.* Calcd for $C_{11}H_{11}Cl_2NO_5S$: C, 38.84; H, 3.26; Cl, 20.84; N, 4.12; S, 9.43. Found: C, 38.87; H, 3.32; Cl, 20.79; N, 4.17; S, 9.28. ^{1}H-NMR (in acetone-d_6) δ: 2.83 (6H, s, 2×C$\underline{H}_3$), 3.53 (1H, 3×d, J=16.0, 7.0, 1.0, Ph–C$\underline{H}_2$–CH), 3.86 (1H, 3×d, J=16.0, 10.0, 1.0, Ph–C$\underline{H}_2$–CH), 5.55 (1H, 2×d, J=10.0, 7.0, O–C$\underline{H}$), 7.10—8.20 (1H, br, COO$\underline{H}$), 7.86 (1H, t, J=1.0, 4-$\underline{H}$). $[\alpha]_D$ −18.7±0.6° (c=1.0, acetone). Compound (+)-**9ab** was obtained from **19b** in a similar manner. Yield 86%, mp 130—131 °C. *Anal.* Calcd for $C_{11}H_{11}Cl_2NO_5S$: C, 38.84; H, 3.26; Cl, 20.84; N, 4.12; S, 9.43. Found: C, 39.07; H, 3.43; Cl, 20.55; N, 4.03; S, 9.17. The ^{1}H-NMR spectrum in acetone-d_6 was the same as that of (−)-**9ab**, except for the signal of COOH, which appeared at 5.70—6.80 as a broad signal. $[\alpha]_D$ +17.6±0.6° (c=1.0, acetone).

Diuretic Effect on Rats——Male Sprague-Dawley rats, weighing about 250 g, at 8 weeks of age, were used in this test. A few lumps of sugar in place of ordinary diet were given on the morning of the day before the test day and 20 ml/kg of 5% glucose solution was given orally at approximately 4 p.m. On the morning of the test day, a suspension or solution of the test compound in 2% gum arabic was orally administered to each rat at a dose of 20 ml/kg. The control group received only 2% gum arabic orally at 20 ml/kg. Immediately after the administration, the test animals were put in plastic cages for the metabolic tests and urine samples were collected for 5 h. The cumulative urine volume, urinary sodium, and urinary potassium were quantitated.

Diuretic Effect on Mice——Female ddY mice, weighing about 20 g, were used for the test. The mice were fasted overnight, but were allowed free access to water. On the morning of the test day, a suspension or solution of the test compound in 2% gum arabic was orally administered to each mouse at 30 ml/kg. The control mice received only the vehicle. Immediately after the administration, five mice of the treated group were put together in a plastic cage for the metabolic tests and urine was collected for 4 h. The cumulative urine volume, urinary sodium, and urinary potassium were quantitated.

Uricosuric Effect on Rats——Nine-week-old male rats were employed for the test. Potassium oxonate was intraperitoneally administered to the animals at a dose of 250 mg/kg to measure uric acid clearance and inulin clearance. Within 2 h after the administration of the potassium oxonate, cannulae were placed in the right femoral artery, left femoral vein, and urinary bladder of each animal under pentobarbital anesthesia for blood collection, drug infusion, and urine collection, respectively. At 2 h after the first administration, potassium oxonate was administered again at the same dosage and then 60% urethane (2 ml/kg) and 15% inulin (4 ml/kg) were subcutaneously injected. A mixture of 4% mannitol–1.5% inulin–0.9% saline was infused at the flow rate of 0.1 ml/min into each animal on a plate kept at 30 °C. The animal was allowed 40 min to reach an equilibrium state, then arterial blood (0.2 ml each) samples were collected six times at 20-min intervals, and five 20-min urine samples were collected. Immediately after the collection of each blood sample, the serum was separated. The serum and urine samples were stored in a refrigerator.

Immediately after collection of the first urine sample, a test compound suspended in 1% gum arabic was intraperitoneally administered at 2 ml/kg. Uric acid levels in the serum and the urine were quantitated by the method of Yonetani *et al.*[10] Inulin was measured essentially by the method of Vurek and Pegram.[11] To analyze uric acid, 0.1 ml of a diluted solution of deproteinized serum or urine was admixed with a 1% dimedone–phosphoric acid solution and the resulting mixture was heated for 5 min. The mixture was then cooled in iced water and combined with 2.0 ml of acetic acid. The fluorescence was measured at 410 nm with excitation at 360 nm.

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Studies on Uricosuric Diuretics. II.[1] 6,7-Dichloro-4-nitro-, 6,7-Dichloro-4-sulfamoyl- and 6,7-Dichloro-4-acyl-2,3-dihydrobenzofuran-2-carboxylic Acids

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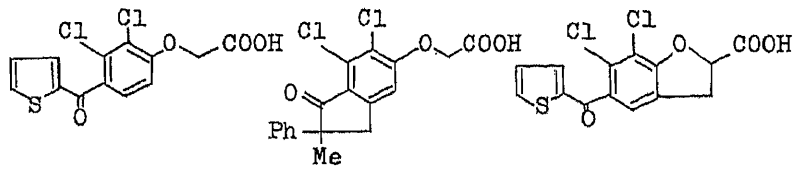
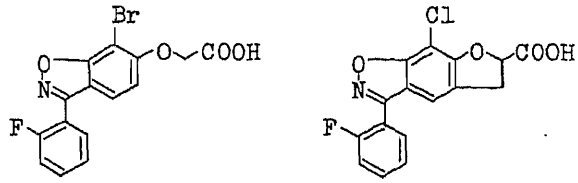
2,3-Dihydrobenzofuran-2-carboxylic acids substituted with electronegative nitro, acyl and sulfamoyl groups at the 4-position were synthesized and tested for oral diuretic and saluretic activities in rats and mice. The intraperitoneal uricosuric activity was also tested by a clearance method using oxonate-treated rats. The 4-nitro compounds (**11b**, **12b**, **13b** and **14b**) showed more potent saluretic activity than the corresponding 5-nitro compounds (**7b**, **18b**, **19b** and **20**). Although the 5-acyl compounds were reported to show potent saluretic activities, the 4-acyl compounds (**41a** and **b**) had much lower activities. On the other hand, the saluretic activities of the 4-sulfamoyl compounds (**22a—e**) were as potent as those of the 5-sulfamoyl compounds reported previously. Uricosuric activity was found in **14b** and **22a**.

Keywords——diuretic activity; saluretic activity; uricosuric activity; 4-nitro-2,3-dihydrobenzofuran-2-carboxylic acid; 4-sulfamoyl-2,3-dihydrobenzofuran-2-carboxylic acid; 4-acyl-2,3-dihydrobenzofuran-2-carboxylic acid; structure-activity relationship

Diuretics are widely used in hypertension therapy. Thiazide diuretics have been used safely and efficiently in long-term administration, but recently, various side effects, such as hypokalemia, glycohemia and hyperuricemia have been reported. Loop diuretics, which display potent but temporary action, are rarely used. However, their value as antihypertensive diuretics without the side effects caused by thiazides has been recognized following the development of sustained-release preparations.



1 tienilic acid 2 indacrinone 3



4 HP-522 5

Chart 1

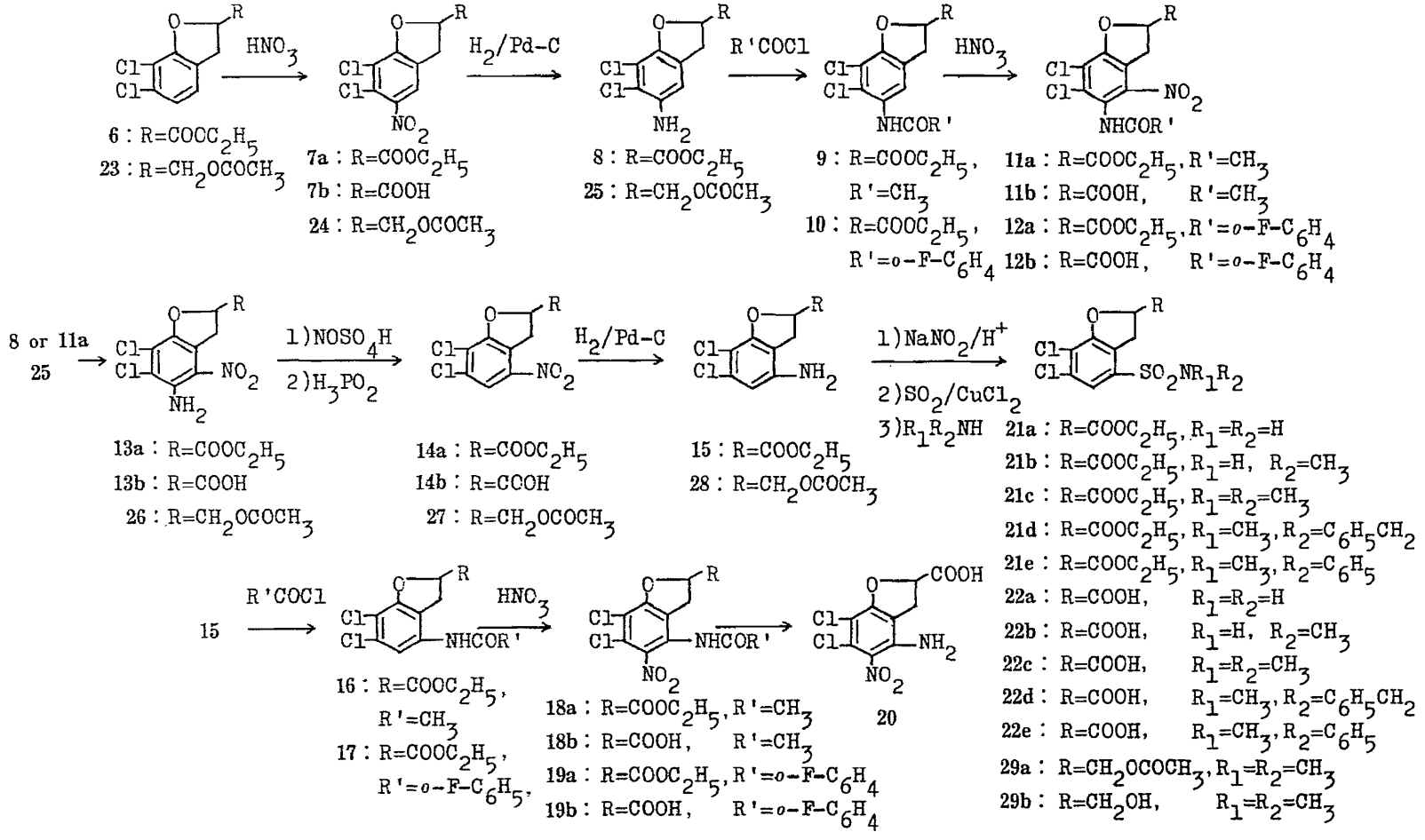

HNO3
H2/Pd-C
R'COCl
HNO3
6 : R=COOC2H5
23 : R=CH2OCOCH3
7a : R=COOC2H5
7b : R=COOH
24 : R=CH2OCOCH3
8 : R=COOC2H5
25 : R=CH2OCOCH3
9 : R=COOC2H5, R'=CH3
10 : R=COOC2H5, R'=o-F-C6H4
11a : R=COOC2H5, R'=CH3
11b : R=COOH, R'=CH3
12a : R=COOC2H5, R'=o-F-C6H4
12b : R=COOH, R'=o-F-C6H4
8 or 11a
25
1)NOSO4H
2)H3PO2
H2/Pd-C
1)NaNO2/H+
2)SO2/CuCl2
3)R1R2NH
13a : R=COOC2H5
13b : R=COOH
26 : R=CH2OCOCH3
14a : R=COOC2H5
14b : R=COOH
27 : R=CH2OCOCH3
15 : R=COOC2H5
28 : R=CH2OCOCH3
21a : R=COOC2H5, R1=R2=H
21b : R=COOC2H5, R1=H, R2=CH3
21c : R=COOC2H5, R1=R2=CH3
21d : R=COOC2H5, R1=CH3, R2=C6H5CH2
21e : R=COOC2H5, R1=CH3, R2=C6H5
22a : R=COOH, R1=R2=H
22b : R=COOH, R1=H, R2=CH3
22c : R=COOH, R1=R2=CH3
22d : R=COOH, R1=CH3, R2=C6H5CH2
22e : R=COOH, R1=CH3, R2=C6H5
29a : R=CH2OCOCH3, R1=R2=CH3
29b : R=CH2OH, R1=R2=CH3
15
R'COCl
HNO3
16 : R=COOC2H5, R'=CH3
17 : R=COOC2H5, R'=o-F-C6H5
18a : R=COOC2H5, R'=CH3
18b : R=COOH, R'=CH3
19a : R=COOC2H5, R'=o-F-C6H4
19b : R=COOH, R'=o-F-C6H4
20

Chart 2

Attempts to develop new types of uricosuric diuretics to avoid hyperuricemia led to the discovery of tienilic acid (**1**),[2] indacrinone (**2**)[2,3] and **3**,[2,4] HP-522 (**4**)[5] and **5**[6] in the 1970's (Chart 1).

We also tried to create a new type of uricosuric diuretic having temporary diuretic action and inhibiting reabsorption of uric acid by renal tubules. We found some dihydrobenzofuran-2-carboxylic acids with electronegative substituents that display both actions. In this paper, we discuss these 4-substituted-2,3-dihydrobenzofuran-2-carboxylic acids.

Chemistry

Compounds having 4-nitro or 4-sulfamoyl substituents were synthesized by the route shown in Chart 2. 6,7-Dichloro-2,3-dihydrobenzofuran-2-carboxylic acid ethyl ester (**6**)[4] or 6,7-dichloro-2,3-dihydrobenzofuran-2-ylmethyl acetate (**23**) was nitrated then reduced to **8** and **25**. The acetate (**9**) and *o*-fluorobenzoate (**10**) of **8** were nitrated to **11a** and **12a**. Hydrolysis of **11a** or direct nitration of **8** gave **13a**. Similar treatment of **25** gave **26**. Compounds **13a** and **26** were diazotized with nitrosylsulfuric acid then deaminated by reduction of the diazonium salts with hypophosphorus acid to obtain the 4-nitro compounds (**14** and **27**). After reduction of **14a** and **27**, the 4-amino substituents of the resultant compounds **15** and **28** were substituted with sulfamoyl groups *via* diazotization. Intermediates (**15**) were acetylated or *o*-fluorobenzoylated, then nitrated to obtain **18** and **19**. Compound **20** was obtained by hydrolysis of **18a**. The esters of the 2-substituents were hydrolyzed, then the free acids and alcohols obtained were tested for biological activities.

The synthetic pathway to the compounds with 4-acyl derivatives is shown in Chart 3. Nitration, methylation, then hydrogenation of 2,3-dichlorophenol (**30**) gave an anisidine derivative (**33**), which was acylated using boron trichloride–aluminum trichloride/benzonitrile or propionitrile, then hydrolyzed according to Sugasawa *et al.*[7] Deamination and demethylation of the aminoacyl compounds (**34**) gave phenols (**36**). Allylation of **36** and

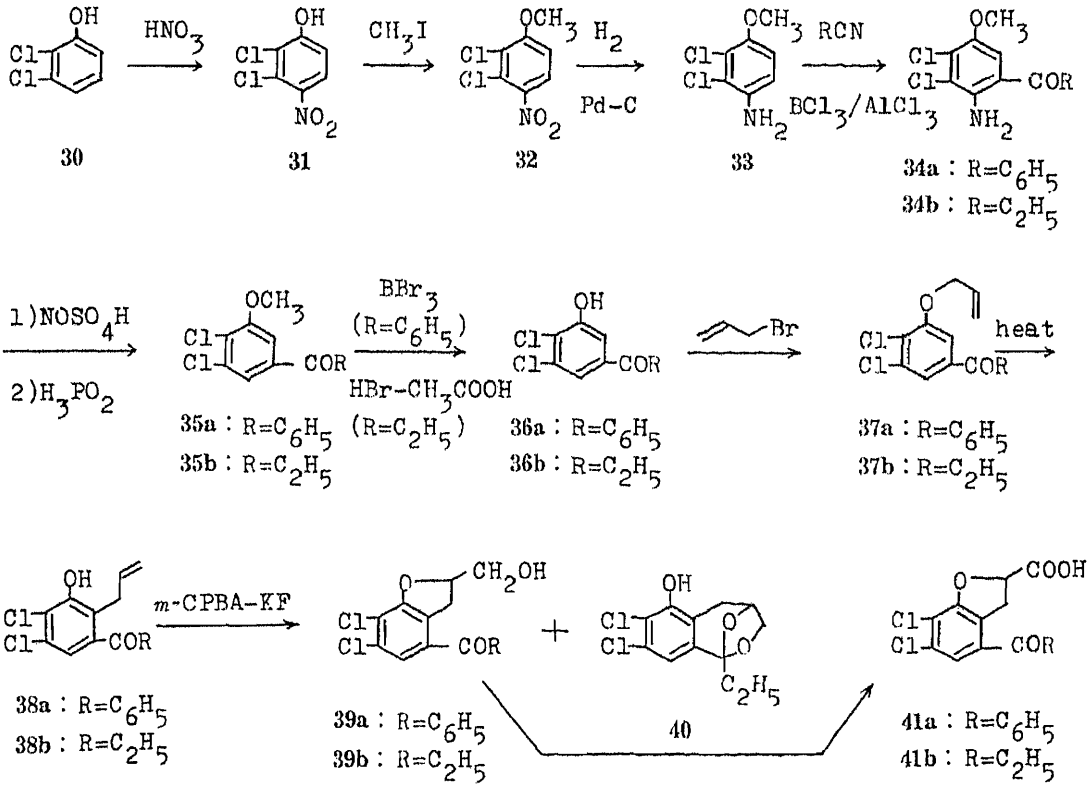
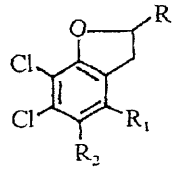


Chart 3

TABLE I. Substituted 6,7-Dichloro-2,3-dihydrobenzofurans



No.	R	R_1	R_2	mp (°C)	Recrystn. solvent[a]	Formula	Analysis (%) Calcd (Found) C	H	Cl	N	F or S
7b	COOH	H	NO_2	200—203	D	$C_9H_5Cl_2NO_5$	38.89 (38.79	1.81 2.09	25.50 25.21	5.04 5.02)	
11b	COOH	NO_2	$NHCOCH_3$	250—252 (dec.)	EA–E	$C_{11}H_8Cl_2N_2O_6$	39.43 (39.42	2.41 2.48	21.16 20.91	8.36 8.22)	
12b	COOH	NO_2	$NHCOC_6H_4$-*o*-F	227—230	EA–E	$C_{16}H_9Cl_2FN_2O_6$	46.29 (46.04	2.19 2.44	17.08 17.11	6.75 6.73	F=4.58 F=4.59)
13b	COOH	NO_2	NH_2	242—243 (dec.)	EA–H	$C_9H_6Cl_2N_2O_5$	36.89 (36.92	2.06 2.27	24.19 24.12	9.56 9.34)	
14b	COOH	NO_2	H	169—170	EA–H	$C_9H_5Cl_2NO_5$	38.88 (39.05	1.81 2.22	25.50 25.13	5.04 5.03)	
18b	COOH	$NHCOCH_3$	NO_2	248 (dec.)	EA–E	$C_{11}H_8Cl_2N_2O_6$	39.43 (39.22	2.41 2.49	21.16 21.25	8.36 8.30)	
19b	COOH	$NHCOC_6H_4$-*o*-F	NO_2	209—210	EA–E	$C_{16}H_9Cl_2FN_2O_6$	46.29 (46.20	2.19 2.54	17.08 16.92	6.75 6.51	F=4.58 F=4.66)
20	COOH	NH_2	NO_2	206—207 (dec.)	E–H	$C_9H_6Cl_2N_2O_5$	36.89 (37.26	2.06 2.44	24.19 24.01	9.56 8.95)	
22a	COOH	SO_2NH_2	H	237—238	EA–E	$C_9H_7Cl_2NO_5S \cdot 1/2H_2O$	33.66 (33.36	2.51 2.73	22.08 21.70	4.36 4.30	S=9.98 S=9.66)
22b	COOH	SO_2NHCH_3	H	225—226	EA–E	$C_{10}H_9Cl_2NO_5S$	36.83 (36.62	2.78 2.98	21.74 21.53	4.29 4.34	S=9.83 S=9.61)
22c	COOH	$SO_2N(CH_3)_2$	H	207—208	EA–E	$C_{11}H_{11}Cl_2NO_5S$	38.84 (38.60	3.26 3.35	20.84 20.85	4.12 4.14	S=9.43 S=9.30)
22d	COOH	$SO_2N(CH_3)CH_2Ph$	H	178—180	EA–E	$C_{17}H_{15}Cl_2NO_5S$	49.05 (49.00	3.63 3.70	17.03 17.02	3.36 3.38	S=7.70 S=7.58)
22e	COOH	$SO_2N(CH_3)Ph$	H	194—195	EA–E	$C_{16}H_{13}Cl_2NO_5S$	47.78 (47.62	3.26 3.39	17.63 17.37	3.48 3.51	S=7.97 S=7.77)
29b	CH_2OH	$SO_2N(CH_3)_2$	H	114	EA–E	$C_{11}H_{13}Cl_2NO_4S$	40.50 (40.77	4.02 4.06	21.74 21.40	4.29 4.28	S=9.83 S=9.85)
41a	COOH	COPh	H	152—154	D–H	$C_{16}H_{10}Cl_2O_4$	57.00 (56.70	2.99 3.14	21.03 21.36)		
41b	COOH	COC_2H_5	H	198—200	D	$C_{12}H_{10}Cl_2O_4$	49.85 (49.60	3.49 3.50	24.52 24.62)		

a) D=dichloromethane, E=ether, EA=ethyl acetate, H=hexane.

subsequent Claisen rearrangement gave **38**. Epoxidation of **38a** with *m*-chloroperbenzoic acid resulted in recovery of the starting materials, but the use of potassium fluoride/*m*-chloroperbenzoic acid according to Camps *et al.*,[8] followed by alkaline treatment successfully gave 2,3-dihydrobenzofurans. In the case of **38b**, the intramolecular ketal (**40**) was obtained as a by-product. Jones oxidation of **39a** and **b** gave the 2-carboxylic acids (**41a** and **b**).

The products used in this study are listed in Table I.

Biological Activities

Saluresis and Diuresis——Diuretic and saluretic activities on rats and mice of the compounds listed in Table I are shown in Table II. Tienilic acid and indacrinone were used as reference compounds. Indacrinone showed more potent activity than tienilic acid in mice. Diuretic and kaliuretic activities paralleled the natriuretic activity. The diuretic–saluretic activities of 5-nitro substituted 2,3-dihydrobenzofuran-2-carboxylic acid derivatives (**7b**, **18b**, **19b**, **20**) were negative or weak, while those of 4-nitro compounds (**11b**, **12b**, **13b**, **14b**) were equivalent to or more potent than those of the reference compounds in rats and were similar to those of tienilic acid in mice. 4-Sulfamoyl compounds were more potent than the reference compounds. In these compounds, the activities of **22b** and **c** were equivalent even in mice. On the other hand, the activities of compounds with 4-acyl derivatives were weak, although the 5-propionyl derivative was reported to show very potent activities.[2] Thus, the diuretic–saluretic activities varied markedly according to the substituents and their positions. For compounds with the nitro, sulfamoyl and acyl groups, the potencies of diuretic-saluretic activities in relation to the position of substitution were $4 \gg 5$, $4 \geqq 5$, and $4 \ll 5$, respectively.

Uricosuric Activity——Uricosuric activity was evaluated in terms of the fractional excretion of uric acid (FEua) and urine-excreted amounts of uric acid (UuaV) values using potassium oxonate-treated rats.[9] The results are shown in Table III.

TABLE II. Diuretic and Saluretic Activities[a,b] in Rats and Mice (Oral Administration)

No.	Rats				Mice			
	Dose mg/kg	Urine volume ml/kg B.W.	Na meq/kg B.W.	K meq/kg B.W.	Dose mg/kg	Urine volume ml/kg B.W.	Na meq/kg B.W.	K meq/kg B.W.
7b	100	33 (N)	1.3 (1.8)	0.36 (N)	30	25 (N)	0.49 (N)	0.55 (N)
11b	100	40 (1.3)	2.3 (3.8)	0.64 (3.5)	30	45 (1.7)	3.7 (5.2)	1.2 (1.5)
12b	100	48 (1.6)	3.4 (3.9)	0.93 (4.9)	30	42 (1.4)	2.8 (4.5)	1.3 (2.1)
13b	100	47 (1.5)	3.0 (5.0)	0.93 (5.1)	30	35 (1.3)	3.0 (4.1)	1.3 (1.6)
14b	50	38 (1.4)	2.6 (3.8)	0.96 (N)	30	36 (1.4)	2.4 (3.2)	0.78 (1.3)
18b	50	33 (N)	0.87 (N)	0.25 (N)	30	30 (N)	1.5 (1.8)	0.67 (N)
19b	50	33 (N)	0.86 (N)	0.29 (N)	30	26 (N)	1.2 (2.4)	0.64 (N)
20	50	30 (N)	1.6 (N)	0.49 (N)	30	37 (N)	2.4 (4.6)	0.91 (1.7)
22a	50	35 (1.5)	2.3 (4.4)	0.68 (3.2)	30	44 (1.5)	4.1 (3.7)	1.3 (2.0)
22b	50	42 (1.8)	3.3 (6.3)	1.0 (4.8)	30	61 (2.2)	6.4 (5.8)	1.8 (2.6)
22c	50	48 (1.7)	4.2 (6.5)	1.4 (5.6)	30	60 (2.6)	6.8 (7.7)	2.0 (2.5)
22d	50	34 (1.4)	1.9 (3.7)	0.51 (2.4)	30	39 (N)	3.6 (5.2)	1.2 (1.8)
22e	50	27 (1.1)	1.2 (2.3)	0.26 (N)	30	34 (1.4)	2.7 (3.9)	1.1 (1.6)
29b	50	28 (1.2)	2.0 (2.7)	0.52 (2.2)	30	47 (2.0)	4.6 (7.7)	1.1 (3.2)
41a	50	26 (N)	0.63 (N)	0.23 (N)	30	28 (1.3)	1.2 (1.8)	0.78 (1.6)
41b	50	31 (1.4)	1.8 (3.2)	0.57 (2.7)	30	37 (N)	2.2 (2.5)	0.89 (N)
Tienilic acid	100	39 (1.8)	2.2 (1.7)	1.3 (5.7)	30	36 (2.4)	3.9 (5.4)	1.2 (1.9)
Indacrinone	50	34 (1.2)	1.3 (2.3)	0.5 (2.0)	30	72 (2.5)	6.4 (8.4)	1.9 (2.8)

a) The experimental details are discribed in the experimental section. *b*) Ratio to the control (treated/control value) is shown in parenthesis; N indicates that the difference from the control is not statistically significant.

TABLE III. Uricosuric Effect of 5-Sulfamoyl-6,7-dichloro-2,3-dihydrobenzofurans in Intraperitoneally Oxonate-Treated Rats

No.	Dose mg/kg	Increase of UuaV mg/kg min	Increase of FEua
11b	50	−0.006	−0.188
12b	50	=	−0.128
14b	50	0.126	0.259
22a	50	0.029	0.097
22c	50	0.048	−0.106
22d	50	0.032	=
41b	50	0.030	=
Probenecid	50	0.124	0.070
Tienilic acid	100	0.123	0.055
Indacrinone	50	0.063	=
Furosemide	50	0.028	−0.124

Increases of UuaV and FEua were calculated as the average values for 80 min after dosing. The symbol = represents no change compared with the control.

Probenecid and tienilic acid, used as positive reference compounds, showed hyperuricosuric activities with increases in both FEua and UuaV values. Indacrinone, however, showed only an increase of UuaV. Furosemide showed a decrease in FEua, suggesting the possibility of hypouricosuric action. Among the 4-nitro compounds, **11b** and **12b** showed marked decrease of both FEua and UuaV, and thus have a hypouricosuric character. Compound **14b** was hyperuricosuric because both values increased, but its diuretic character is not potent enough to allow its use as a diuretic agent. Among the 4-sulfamoyl compounds, only **22a** showed hyperuricosuric activity, and **22c** and **d** increased the UuaV values but not the FEua values. The observed increase of FEua in **22a** was only temporary.

As reported in the previous paper, some 5-sulfamoyl-2,3-dihydrobenzofuran derivatives showed both diuretic and uricosuric characteristics. However, among the 4-substituted-2,3-dihydrobenzofurans used in this study, none showed a good balance of diuretic and uricosuric actions, although compounds **14b** and **22a** showed both activities.

Experimental

Melting points were determined on a Yanagimoto hot plate micro melting point apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken on a Varian EM-390 spectrometer with tetramethylsilane (TMS) as an internal standard. Signal multiplicities are represented by s (singlet), d (doublet), t (triplet), q (quartet), br (broad), m (multiplet). Chemical shifts are expressed in δ values and coupling constants are given in Hertz. Abbreviations are as follows: Ph, phenyl; arom. H, aromatic proton(s). For column chromatography, Silica gel 60 (E. Merck, 0.063—0.200 mm) was used.

Ethyl 6,7-Dichloro-5-nitro-2,3-dihydrobenzofuran-2-carboxylate (7a)——Fuming nitric acid (d=1.50, 25 ml) was added dropwise to a solution of ethyl 6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (10 g, 0.038 mol) in dichloromethane (150 ml) at 4 °C with stirring. After 1.5 h, the reactant was poured into ice water, then extracted with dichloromethane, and the extract was dried and evaporated *in vacuo*. Chromatography of the residue using dichloromethane as the eluant gave **7a** (9.4 g, 80%), mp 111—113 °C (hexane–ether). *Anal.* Calcd for $C_{11}H_9Cl_2NO_5$: C, 43.16; H, 2.96; Cl, 23.16; N, 4.58. Found: C, 42.96; H, 2.99; Cl, 23.07; N, 4.62. ^{1}H-NMR (in $CDCl_3$) δ: 1.33 (3H, t, J=7.2, C$\underline{H}_3$), 3.48 (1H, 3×d, J=17.3, 7.2, 1.1, Ph–C$\underline{H}_2$–CH), 3.73 (1H, 3×d, J=17.3, 7.2, 1.1, Ph–C$\underline{H}_2$–CH), 4.30 (2H, q, J=7.2, O–C$\underline{H}_2$–CH$_3$), 5.42 (1H, 2×d, J=10.2, 7.2, O–C$\underline{H}$), 7.72 (1H, t, J=1.1, 4-H).

Compound **24** was obtained in a similar manner starting from **23**, which was obtained by acetylation of 6,7-dichloro-2,3-dihydrobenzofuran-2-ylmethanol.[4] ^{1}H-NMR (in $CDCl_3$) δ: 2.08 (3H, s, COC$\underline{H}_3$), 3.16 (1H, 3×d, J=16.5, 7.5, 1.2, Ph–C$\underline{H}_2$–CH), 3.50 (3H, 3×d, J=16.5, 9.3, 1.2, Ph–C$\underline{H}_2$–CH), 4.16—4.54 (2H, m, C$\underline{H}_2$–OCOCH$_3$), 5.10—5.45 (1H, m, O–C$\underline{H}$), 7.72 (1H, t, J=1.2, 4-H).

6,7-Dichloro-5-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (7b)——A mixture of **7a** (0.500 g, 0.0016 mol) and

5% aqueous sodium hydroxide (20 ml) in tetrahydrofuran (THF) (10 ml) was stirred for 1 h, then concentrated *in vacuo*. The alkaline solution was acidified, then extracted with ethyl acetate and the organic layer was dried and evaporated. The residue was treated with dichloromethane and gave **7b** (0.440 g, 97%). ^{1}H-NMR (in acetone-d_6) δ: 3.57 (1H, 3 × d, J=17.3, 6.5, 1.2, Ph-C$\underline{H}_2$-CH), 3.88 (1H, 3 × d, J=17.3, 10.5, 1.2, Ph-C$\underline{H}_2$-CH), 5.58 (1H, 2 × d, J=10.5, 6.5, O-C$\underline{H}$), 7.07 (1H, br, COO$\underline{H}$), 7.90 (1H, t, J=1.2, 4-H).

Ethyl 5-Amino-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (8)——A solution of **7a** (1.44 g, 0.0047 mol) in ethyl acetate (50 ml) was hydrogenated over 10% palladium carbon catalyst (0.1 g). The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*, giving **8** (1.3 g, 100%), which was used for the next reaction without further purification.

Compound **25** was prepared in a similar manner. Yield 95%, mp 126—127 °C (ethyl acetate–ether).

Ethyl 5-Acetamido-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (9)——Acetyl chloride (1.53 g, 0.019 mol) was added to a solution of **8** (3.59 g, 0.013 mol) and 4-*N*,*N*-dimethylaminopyridine (1.90 g, 0.016 mol) in dichloromethane (40 ml) at 3 °C with stirring, and the mixture was allowed to react at room temperature for 0.5 h, then washed, dried and evaporated. Treatment of the residue with ether gave **9** (3.95 g, 96%), mp 169—172 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{13}H_{13}Cl_2NO_4$: C, 49.08; H, 4.12; Cl, 22.29; N, 4.40. Found: C, 48.77; H, 4.19; Cl, 22.52; N, 4.53. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.1, C$\underline{H}_3$), 2.22 (3H, s, COC$\underline{H}_3$), 3.37 (1H, 3 × d, J=15.8, 8.7, 1.2, Ph-C$\underline{H}_2$-CH), 3.63 (1H, 3 × d, J=15.8, 10.2, 1.2, Ph-C$\underline{H}_2$-CH), 4.24 (2H, q, J=7.1, O-C$\underline{H}_2$), 5.25 (1H, 2 × d, J=10.2, 8.7, O-C$\underline{H}$), 7.47 (1H, br s, N$\underline{H}$), 8.00 (1H, t, J=1.2, 4-H).

Compound **10** was obtained in a similar manner. Yield 91%, mp 141—143 °C (ether). *Anal.* Calcd for $C_{18}H_{14}Cl_2FNO_4$: C, 54.19; H, 3.54; Cl, 17.81; F, 4.77; N, 3.52. Found: C, 54.10; H, 3.82; Cl, 17.83; F, 4.75; N, 3.54. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.0, C$\underline{H}_3$), 3.41 (1H, 3 × d, J=16.2, 6.9, 1.2, Ph-C$\underline{H}_2$-CH), 3.67 (1H, 3 × d, J=16.2, 10.0, 1.2, Ph-C$\underline{H}_2$-CH), 4.24 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.27 (1H, 2 × d, J=10.0, 6.9, O-C$\underline{H}$), 6.93—7.66 (3H, m, arom. H), 8.14 (1H, d, t, J=2.3, 7.8, arom. H), 8.29 (1H, t, J=1.2, 4-H), 8.95 (1H, d, J=16.5, N$\underline{H}$).

Ethyl 5-Acetamido-6,7-dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylate (11a)——Fuming nitric acid (d=1.50, 20 ml) was added to a solution of **9** (3.85 g, 0.012 mol) in dichloromethane (100 ml), and the mixture was allowed to react for 1.5 h at 4 °C with stirring. The reactant was then poured into ice water, and the dichloromethane layer was separated. The aqueous layer and the precipitated materials were extracted with ethyl acetate. The dichloromethane and ethyl acetate extracts were combined, dried, and evaporated. Treatment of the residue with ether gave **11a** (4.00 g, 91%), mp 169—172 °C (ethyl acetate–hexane). *Anal.* Calcd for $C_{13}H_{12}Cl_2N_2O_6$: C, 43.00; H, 3.33; Cl, 19.52; N, 7.71. Found: C, 42.85; H, 3.52; Cl, 19.68; N, 7.84. ^{1}H-NMR (in $CDCl_3$) δ: 1.32 (3H, t, J=7.1, C$\underline{H}_3$), 2.19 (3H, s, COC$\underline{H}_3$), 3.65 (1H, 2 × d, J=18.0, 7.2, Ph-C$\underline{H}_2$-CH), 3.94 (1H, 2 × d, J=18.0, 10.2, Ph-C$\underline{H}_2$-CH), 5.35 (1H, 2 × d, J=10.2, 7.2, O-C$\underline{H}$), 7.47 (1H, br s, N$\underline{H}$).

Compound **12a** was obtained in a similar manner. Yield 92%, mp 154—155 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{18}H_{13}Cl_2FN_2O_6$: C, 48.78; H, 2.96; Cl, 16.00; F, 4.29; N, 6.32. Found: C, 48.83; H, 3.00; Cl, 15.83; F, 4.47; N, 6.42. ^{1}H-NMR (in $CDCl_3$) δ: 1.32 (3H, t, J=7.0, C$\underline{H}_3$), 3.69 (1H, 2 × d, J=18.0, 7.0, Ph-C$\underline{H}_2$-CH), 3.99 (1H, 2 × d, J=18.0, 9.8, Ph-C$\underline{H}_2$-CH), 4.28 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.40 (1H, 2 × d, J=9.8, 7.0, O-C$\underline{H}$), 6.80—7.75 (3H, arom. H), 8.11 (1H, d, t, J=1.8, 7.8, arom. H), 8.77 (1H, d, J=15.8, N$\underline{H}$).

5-Acetamido-6,7-dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (11b)——A mixture of **11a** (0.40 g, 0.0011 mol), 7.5% aqueous potassium carbonate (10 ml), and THF (5 ml) was stirred for 20 h, then concentrated *in vacuo*. The reactant was diluted with water, washed with dichloromethane, acidified with 20% hydrochloric acid, then extracted with ethyl acetate. After evaporation of the ethyl acetate, the residue was treated with ether to give **11b** (0.34 g, 92%), mp 250—252 °C (dec.) (ethyl acetate–ether). ^{1}H-NMR (in acetone-d_6) δ: 2.10 (3H, s, COC$\underline{H}_3$), 3.70 (1H, 2 × d, J=17.4, 6.3, Ph-C$\underline{H}_2$-CH), 4.04 (1H, 2 × d, J=17.4, 10.2, Ph-C$\underline{H}_2$-CH), 5.57 (1H, 2 × d, J=10.2, 6.3, O-C$\underline{H}$), 9.16 (1H, br s, N$\underline{H}$).

Compound **12b** was obtained in a similar manner. Yield 88%. ^{1}H-NMR (in acetone-d_6) δ: 3.77 (1H, 2 × d, J=18.0, 6.9, Ph-C$\underline{H}_2$-CH), 4.10 (1H, 2 × d, J=18.0, 10.1, Ph-C$\underline{H}_2$-CH), 5.62 (1H, 2 × d, J=10.1, 6.9, O-C$\underline{H}$), 7.15—7.77 (3H, m, arom. H), 7.89 (1H, d, t, J=1.8, 7.5, arom. H), 9.34 (1H, d, J=7.5, NH).

Ethyl 5-Amino-6,7-dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylate (13a)——Fuming nitric acid (d=1.50, 18 ml) was added to a solution of **8** (8.963 g, 0.032 mol) in dichloromethane (180 ml), and the mixture was allowed to react at 4—6 °C for 2 h with stirring. The reactant was poured into ice water, then extracted with dichloromethane. The organic layer was dried and evaporated. The residue was chromatographed and eluted with dichloromethane, giving **13a** (7.542 g, 72%), which was used for the next reaction without further purification. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.2, C$\underline{H}_3$), 3.83 (1H, 2 × d, J=18.7, 7.1, Ph-C$\underline{H}_2$-CH), 4.12 (1H, 2 × d, J=18.7, 7.1, Ph-C$\underline{H}_2$-CH), 4.27 (2H, q, J=7.2, O-C$\underline{H}_2$), 5.25 (1H, 2 × d, J=9.8, 7.1, O-C$\underline{H}$), 6.35 (2H, br s, N$\underline{H}_2$).

Compound **26** was obtained in a similar manner. Dichloromethane–ethyl acetate (30 : 1) was used for the chromatographic separation. Yield 77%, mp 168—169 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{11}H_{10}Cl_2N_2O_5$: C, 41.14; H, 3.14; Cl, 22.08; N, 8.72. Found: C, 41.15; H, 3.30; Cl, 22.09; N, 8.73. ^{1}H-NMR (in $CDCl_3$) δ: 2.10 (3H, s, COC$\underline{H}_3$), 3.53 (1H, 2 × d, J=17.0, 7.0, Ph-C$\underline{H}_2$-CH), 3.90 (1H, 2 × d, J=17.0, 9.0, Ph-C$\underline{H}_2$-CH), 4.31 (2H, d, J=6.0, O-C$\underline{H}_2$), 4.90—5.35 (1H, m, O-C$\underline{H}$), 6.30 (2H, br, N$\underline{H}_2$).

5-Amino-6,7-dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (13b)——A mixture of **11a** (3.05 g,

0.0084 mol), 20% hydrochloric acid (25 ml) and dioxane (25 ml) was refluxed for 1 h, then concentrated at atmospheric pressure. Precipitates formed by addition of water were filtered off and dried at room temperature to obtain **13b** (1.92 g, 78%). ^{1}H-NMR (in DMSO-d_6) δ: 3.68 (1H, 2×d, J=18.0, 6.8, Ph-CH$_2$-CH), 4.03 (1H, 2×d, J=18.0, 10.5, Ph-CH$_2$-CH), 5.33 (1H, 2×d, J=10.5, 6.8, O-CH), 6.90 (2H, br s, NH$_2$), 13.2 (1H, br, COOH).

Ethyl 6,7-Dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylate (14a)——A 45% nitrosylsulfuric acid solution in sulfuric acid (22 g) was added to a solution of **13a** (10.47 g, 0.033 mol) in THF (440 ml) at −25——18 °C over 1 h with stirring, then the reaction temperature was raised to −5 °C for 2.5 h. Next, 50% aqueous hypophosphorus acid (230 ml) was added at −14—15 °C over 1 h, then the reaction mixture was extracted with ether. The extract was dried and evaporated. The residue was chromatographed and eluted with dichloromethane to obtain **14a** (6.584 g, 66%), mp 104 °C (ether). *Anal.* Calcd for $C_{11}H_9Cl_2NO_5$: C, 43.16; H, 2.96; Cl, 23.16; N, 4.59. Found: C, 43.06; H, 3.16; Cl, 23.78; N, 4.46. ^{1}H-NMR (in $CDCl_3$) δ: 1.33 (3H, t, J=7.0, CH$_3$), 3.83 (1H, 2×d, J=18.9, 7.5, Ph-CH$_2$-CH), 4.08 (1H, 2×d, J=18.9, 9.8, Ph-CH$_2$-CH), 4.27 (2H, q, J=7.0, O-CH$_2$), 5.39 (1H, 2×d, J=9.8, 7.5, O-CH), 7.85 (1H, s, 5-H).

Compound **27** was obtained in a similar manner. Yield 77%, mp 100—101 °C (hexane–ether). *Anal.* Calcd for $C_{11}H_9Cl_2NO_5$: C, 43.16; H, 2.96; Cl, 23.16; N, 4.58. Found: C, 43.07; H, 3.09; Cl, 23.41; N, 4.63. ^{1}H-NMR (in $CDCl_3$) δ: 2.06 (3H, s, CH$_3$), 3.53 (1H, 2×d, J=18.0, 7.5, Ph-CH$_2$-CH), 3.90 (1H, 2×d, J=18.0, 10.5, Ph-CH$_2$-CH), 4.15—4.60 (2H, m, O-CH$_2$), 5.10—5.43 (1H, m, O-CH), 7.86 (1H, s, 5-H).

6,7-Dichloro-4-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (14b)——A mixture of **14a** (1.90 g, 0.0062 mol), THF (20 ml) and 7.5% aqueous potassium carbonate solution (20 ml) was stirred for 20 h, then concentrated *in vacuo*. The residue was acidified, then extracted with ethyl acetate. The extract was treated with ether to obtain **14b** (1.527 g, 88%), mp 169—170 °C (ethyl acetate–hexane).

Ethyl 4-Amino-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (15) and 4-Amino-6,7-dichloro-2,3-dihydrobenzofuran-2-ylmethyl Acetate——Compounds **15** and **28** were obtained by a procedure similar to that described for **8**. Compound **15**: Yield 92%, mp 134—135 °C (ether). *Anal.* Calcd for $C_{11}H_{11}Cl_2NO_3$: C, 47.85; H, 4.02; Cl, 25.68; N, 5.07. Found: C, 47.64; H, 3.97; Cl, 25.74; N, 5.08. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.2, CH$_3$), 3.17 (1H, 2×d, J=15.5, 7.2, Ph-CH$_2$-CH), 3.42 (1H, 2×d, J=15.5, 10.1, Ph-CH$_2$-CH), 3.0—3.7 (2H, br, NH$_2$), 4.26 (2H, q, J=7.2, O-CH$_2$), 5.28 (1H, 2×d, J=10.1, 7.2, O-CH), 6.36 (1H, s, 5-H). Compound **28**: Yield 45%, mp 96—97 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{11}H_{11}Cl_2NO_3$: C, 47.85; H, 4.02; Cl, 25.68; N, 5.07. Found: C, 47.95; H, 4.06; Cl, 25.21; N, 5.22. ^{1}H-NMR (in $CDCl_3$) δ: 2.07 (3H, s, COCH$_3$), 2.81 (1H, 2×d, J=15.0, 7.5, Ph-CH$_2$-CH), 3.16 (1H, 2×d, J=15.0, 9.0, Ph-CH$_2$-CH), 3.0—4.0 (2H, br, NH$_2$), 4.30 (2H, d, J=6.0, O-CH$_2$), 4.95—5.35 (1H, m, O-CH), 6.35 (1H, s, 5-H).

Ethyl 4-Acetamido-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylate (16) and Ethyl 6,7-Dichloro-4-(*o*-fluorobenzoylamino)-2,3-dihydrobenzofuran-2-carboxylate (17)——Compounds **16** and **17** were obtained by a procedure similar to that described for **9** and **10**. Compound **16**: Yield 96%, mp 171—173 °C (ether). *Anal.* Calcd for $C_{13}H_{13}Cl_2NO_4$: C, 49.08; H, 4.12; Cl, 22.29; N, 4.40. Found: C, 48.71; H, 3.90; Cl, 22.13; N, 4.30. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.2, CH$_3$), 2.15 (3H, s, COCH$_3$), 3.25 (1H, 2×d, J=16.5, 7.5, Ph-CH$_2$-CH), 3.53 (1H, 2×d, J=16.5, 10.2, Ph-CH$_2$-CH), 4.23 (2H, q, J=7.2, O-CH$_2$), 5.26 (1H, 2×d, J=10.2, 7.5, O-CH), 7.14 (1H, br, NH), 7.30 (1H, s, 5-H). Compound **17**: Yield 97%. ^{1}H-NMR (in DMSO-d_6) δ: 1.22 (3H, t, J=7.0, CH$_3$), 3.40 (1H, 2×d, J=16.5, 6.8, Ph-CH$_2$-CH), 3.70 (1H, 2×d, J=16.5, 10.2, Ph-CH$_2$-CH), 4.20 (2H, q, J=7.0, O-CH$_2$), 5.55 (1H, 2×d, J=10.2, 6.8, O-CH), 7.23—7.83 (5H, m, 5-H, arom. H), 10.25 (1H, s, NH).

Ethyl 4-Acetamido-6,7-dichloro-5-nitro-2,3-dihydrobenzofuran-2-carboxylate (18a) and Ethyl 6,7-Dichloro-4-(*o*-fluorobenzoylamino)-5-nitro-2,3-dihydrobenzofuran-2-carboxylate (19a)——Compounds **18a** and **19a** were obtained by a procedure similar to that described for **11a** and **12a**. Compound **18a**: Yield 76%, mp 143 °C (hexane–ether). *Anal.* Calcd for $C_{13}H_{12}Cl_2N_2O_6$: C, 43.00; H, 3.33; Cl, 19.52; N, 7.71. Found: C, 43.01; H, 3.25; Cl, 19.80; N, 7.59. ^{1}H-NMR (in $CDCl_3$) δ: 1.31 (3H, t, J=7.0, CH$_3$), 2.15 (3H, s, COCH$_3$), 3.31 (1H, 2×d, J=17.0, 7.0, Ph-CH$_2$-CH), 3.66 (1H, 2×d, J=17.0, 10.2, Ph-CH$_2$-CH), 4.26 (2H, q, J=7.0, O-CH$_2$), 5.36 (1H, 2×d, J=10.2, 7.0, O-CH), 8.00 (1H, br s, NH).

Compound **19a**: Yield 55%, mp 126—128 °C (ethyl acetate–ether). *Anal.* Calcd for $C_{18}H_{13}Cl_2FN_2O_6$: C, 48.78; H, 2.96; Cl, 16.00; F, 4.29; N, 6.32. Found: C, 48.73; H, 3.01; Cl, 15.98; F, 4.27; N, 6.41. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.1, CH$_3$), 3.38 (1H, 2×d, J=17.4, 6.8, Ph-CH$_2$-CH), 3.79 (1H, 2×d, J=17.4, 10.5, Ph-CH$_2$-CH), 4.27 (2H, q, J=7.1, O-CH$_2$), 5.41 (1H, 2×d, J=10.5, 6.8, O-CH), 7.05—7.75 (3H, m, arom. H), 8.10 (1H, d, t, J=2.0, 8.1, arom. H), 9.07 (1H, d, J=15.3, NH).

4-Acetamido-6,7-dichloro-5-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (18b) and 6,7-Dichloro-4-(*o*-fluorobenzoylamino)-5-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (19b)——Compounds **18b** and **19b** were obtained by a procedure similar to that described for **11b** and **12b**. Compound **18b**: Yield 95%. ^{1}H-NMR (in acetone-d_6) δ: 2.10 (3H, s, COCH$_3$), 3.43 (1H, 2×d, J=17.1, 6.8, Ph-CH$_2$-CH), 3.76 (1H, 2×d, J=17.1, 10.4, Ph-CH$_2$-CH), 5.56 (1H, 2×d, J=10.4, 6.8, O-CH), 9.17 (1H, br, NH).

Compound **19b**: Yield 91%. ^{1}H-NMR (in acetone-d_6) δ: 3.57 (1H, 2×d, J=17.1, 6.8, Ph-CH$_2$-CH), 3.88 (1H, 2×d, J=17.1, 10.1, Ph-CH$_2$-CH), 5.65 (1H, 2×d, J=10.1, 6.8, O-CH), 7.15—7.80 (3H, m, arom. H), 7.89 (1H, d, t, J=2.3, 7.5, arom. H), 9.48 (1H, d, J=7.5, NH).

4-Amino-6,7-dichloro-5-nitro-2,3-dihydrobenzofuran-2-carboxylic Acid (20)——A mixture of **18a** (0.47 g, 0.0013 mol), 20% hydrochloric acid (10 ml) and THF (10 ml) was refluxed for 4 h. After cooling, the reaction mixture was extracted with ethyl acetate, then the organic layer was extracted with an aqueous sodium bicarbonate solution. The aqueous layer was acidified, then extracted with ethyl acetate, and the organic layer was dried and evaporated. The residue was treated with hexane–ether to obtain **20** (0.23 g, 63%). ^{1}H-NMR (in DMSO-d_6) δ: 3.22 (1H, 2×d, J= 16.5, 6.8, Ph-C$\underline{H}_2$-CH), 3.50 (1H, 2×d, J= 16.5, 10.5, Ph-C$\underline{H}_2$-CH), 5.46 (1H, 2×d, J= 10.5, 6.8, O-C$\underline{H}$), 6.30 (3H, br, N$\underline{H}_2$, COO$\underline{H}$).

Ethyl 6,7-Dichloro-4-sulfamoyl-2,3-dihydrobenzofuran-2-carboxylate (21a)——Sodium nitrite (0.376 g, 0.0054 mol) was added to a solution of **15** (1.0 g, 0.0036 mol), concentrated hydrochloric acid (15 ml) and acetic acid (15 ml) at −20 °C with stirring, then the reaction temperature was raised gradually to 0 °C over 2 h. The reaction mixture was cooled to −20 °C, then liquid sulfur dioxide (10 g) and a solution of cupric chloride (1.4 g, 0.0104 mol) in water (2 ml) and acetic acid (20 ml) were added. The reaction mixture was stirred at 0 °C for 20 min, at room temperature for 30 min, and finally at 50 °C for 1 h to drive out the sulfur dioxide. It was then poured into ice water and extracted with dichloromethane. The organic layer was washed with chilled water, dried and evaporated *in vacuo*. The residue was dissolved in dichloromethane (20 ml), and ammonia gas was passed through the solution at room temperature for 3 h. Next, the solution was evaporated *in vacuo*, and the resultant oily material was dissolved in ethyl acetate. This solution was washed with water, dried and evaporated. Ethereal diazomethane was added to the residue until evolution of nitrogen gas ceased. Evaporation and treatment with hexane–ether gave **21a** (0.81 g, 66%), mp 174—175 °C (acetone–hexane). *Anal.* Calcd for $C_{11}H_{11}Cl_2NO_5S$: C, 38.84; H, 3.26; Cl, 20.84; N, 4.12; S, 9.42. Found: C, 38.89; H, 3.46; Cl, 20.63; N, 4.28; S, 9.19. ^{1}H-NMR (in acetone-d_6) δ: 1.25 (3H, t, J=7.0, C$\underline{H}_3$), 3.66 (1H, 2×d, J=17.0, 7.0, Ph-C$\underline{H}_2$-CH), 3.97 (1H, 2×d, J=17.0, 10.0, Ph-C$\underline{H}_2$-CH), 4.21 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.52 (1H, 2×d, J=10.0, 7.0, O-C$\underline{H}$), 6.80 (2H, br, N$\underline{H}_2$), 7.48 (1H, s, 5-$\underline{H}$).

Compounds **21b**—**e** and **29a** were obtained in a similar manner *via* reactions of methanol solutions of the corresponding amines at −20——10 °C instead of ammonia gas. For the synthesis of **29a**, the diazomethane treatment was omitted.

Compound **21b**: Yield 71%, mp 135—137 °C (hexane–ether). *Anal.* Calcd for $C_{12}H_{13}Cl_2NO_5S$: C, 40.69; H, 3.70; Cl, 20.02; N, 3.95; S, 9.05. Found: C, 40.55; H, 3.77; Cl, 19.98; N, 4.00; S, 8.95. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.0, C$\underline{H}_3$), 2.68 (3H, d, J=5.0, NHC$\underline{H}_3$), 3.64 (1H, 2×d, J=17.4, 7.5, Ph-C$\underline{H}_2$-CH), 3.90 (1H, 2×d, J=17.4, 10.5, Ph-C$\underline{H}_2$-CH), 4.27 (2H, q, J=7.0, O-C$\underline{H}_2$), 4.75 (1H, br, N$\underline{H}$), 5.35 (1H, 2×d, J=10.5, 7.5, O-C$\underline{H}$), 7.47 (1H, s, 5-$\underline{H}$).

Compound **21c**: Yield 68%, mp 93—94 °C (hexane–ether). *Anal.* Calcd for $C_{13}H_{15}Cl_2NO_5S$: C, 42.40; H, 4.11; Cl, 19.26; N, 3.80; S, 8.71. Found: C, 42.43; H, 4.09; Cl, 19.54; N, 3.78; S, 8.56. ^{1}H-NMR (in $CDCl_3$) δ: 1.30 (3H, t, J=7.0, C$\underline{H}_3$), 2.80 (6H, s, 2×N-C$\underline{H}_3$), 3.66 (1H, 2×d, J=18.0, 7.1, Ph-C$\underline{H}_2$-CH), 3.93 (1H, 2×d, J=18.0, 10.2, Ph-C$\underline{H}_2$-CH), 4.27 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.35 (1H, 2×d, J=10.2, 7.1, O-C$\underline{H}$), 7.39 (1H, s, 5-H).

Compound **21d**: Yield 44%, mp 108—109 °C (hexane–ether). *Anal.* Calcd for $C_{19}H_{19}Cl_2NO_5S$: C, 51.36; H, 4.31; Cl, 15.96; N, 3.15; S, 7.22. Found: C, 51.23; H, 4.35; Cl, 15.84; N, 3.15; S, 7.13. ^{1}H-NMR (in $CDCl_3$) δ: 1.28 (3H, t, J=7.0, C$\underline{H}_3$), 2.67 (3H, s, N-C$\underline{H}_3$),3.66 (1H, 2×d, J=18.0, 7.5, Ph-C$\underline{H}_2$-CH), 3.93 (1H, 2×d, J=18.0, 9.6, Ph C$\underline{H}_2$-CH), 4.25 (2H, s, Ph-C$\underline{H}_2$), 4.28 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.33 (1H, 2×d, J=9.6, 7.5, O-C$\underline{H}$), 7.33 (5H, s, arom. H), 7.41 (1H, s, 5-H).

Compound **21e**: Yield 67%, mp 145—146 °C (hexane–ether). *Anal.* Calcd for $C_{18}H_{17}Cl_2NO_5S$: C, 50.24; H, 3.98; Cl, 16.48; N, 3.26; S, 7.45. Found: C, 50.02; H, 4.06; Cl, 16.60; N, 3.32; S, 7.34. ^{1}H-NMR (in $CDCl_3$) δ: 1.26 (3H, t, J=7.0, C$\underline{H}_3$), 2.72 (1H, 2×d, J=18.0, 7.1, Ph-C$\underline{H}_2$-CH), 3.14 (1H, 2×d, J=18.0, 10.5, Ph-C$\underline{H}_2$-CH), 3.23 (3H, s, NC$\underline{H}_3$), 4.21 (2H, q, J=7.0, O-C$\underline{H}_2$), 5.05 (1H, 2×d, J=10.5, 7.1, O-C$\underline{H}$), 7.0—7.5 (5H, m, arom. H), 7.38 (1H, s, 5-H).

Compound **29a**: Yield 50%, mp 113—114 °C (ether). *Anal.* Calcd for $C_{13}H_{15}Cl_2NO_5S$: C, 42.40; H, 4.11; Cl, 19.26; N, 3.80; S, 8.71. Found: C, 42.35; H, 4.11; Cl, 19.26; N, 3.77; S, 8.58. ^{1}H-NMR (in $CDCl_3$) δ: 2.06 (3H, s, COC$\underline{H}_3$), 2.80 (6H, s, 2×N-C$\underline{H}_3$), 3.35 (1H, 2×d, J=18.0, 7.5, Ph-C$\underline{H}_2$-CH), 3.70 (1H, 2×d, J=18.0, 9.0, Ph C$\underline{H}_2$-CH), 4.15—4.55 (2H, m, O-C$\underline{H}_2$), 5.00—5.40 (1H, m, O-C$\underline{H}$), 7.35 (1H, s, 5-H).

6,7-Dichloro-4-sulfamoyl-2,3-dihydrobenzofuran-2-carboxylic Acid (22a)——A mixture of **21a** (0.34 g, 0.0011 mol), 1 N sodium hydroxide solution (1.5 ml) and acetonitrile (5 ml) was stirred for 1 h, then evaporated *in vacuo*. The residue was mixed with 10% hydrochloric acid (1.5 ml) and this mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried and evaporated. Treatment of the residue with ether gave **22a** (0.23 g, 73%). ^{1}H-NMR (in acetone-d_6) δ: 3.67 (1H, 2×d, J=18.0, 7.0, Ph-C$\underline{H}_2$-CH), 4.00 (1H, 2×d, J=18.0, 9.6, Ph-C$\underline{H}_2$-CH), 5.52 (1H, 2×d, J=9.6, 7.0, O-C$\underline{H}$), 6.83 (2H, br, N$\underline{H}_2$), 7.47 (1H, s, 5-H).

Compounds **22b**—**e** and **29b** were obtained in a similar manner.

Compound **22b**: Yield 97%. ^{1}H-NMR (in acetone-d_6) δ: 2.62 (3H, d, J=5.0, N-C$\underline{H}_3$), 3.67 (1H, 2×d, J=18.0, 7.2, Ph-C$\underline{H}_2$-CH), 3.99 (1H, 2×d, J=18.0, 10.5, Ph-C$\underline{H}_2$-CH), 5.52 (1H, 2×d, J=10.5, 7.2, O-C$\underline{H}$), 6.60 (1H, br, N$\underline{H}$), 7.44 (1H, s, 5-H).

Compound **22c**: Yield 79%. ^{1}H-NMR (in acetone-d_6) δ: 2.82 (6H, s, 2×N-C$\underline{H}_3$), 3.73 (1H, 2×d, J=18.0, 6.8, Ph-C$\underline{H}_2$-CH), 4.05 (1H, 2×d, J=18.0, 10.0, Ph-C$\underline{H}_2$-CH), 5.55 (1H, 2×d, J=10.0, 6.8, O-C$\underline{H}$), 7.40 (1H, s, 5-H).

Compound **22d**: Yield 94%. ^{1}H-NMR (in acetone-d_6) δ: 2.72 (3H, s, N–C$\underline{H}_3$), 3.76 (1H, 2×d, J=18.0, 7.2, Ph–C$\underline{H}_2$–CH), 4.07 (1H, 2×d, J=18.0, 9.8, Ph–C$\underline{H}_2$–CH), 4.33 (2H, s, Ph–C$\underline{H}_2$), 5.54 (1H, 2×d, J=9.8, 7.2, O–C$\underline{H}$), 7.33 (5H, s, arom. H), 7.46 (1H, s, 5-H).

Compound **22e**: Yield 99%. ^{1}H-NMR (in acetone-d_6) δ: 3.31 (3H, s, N–C$\underline{H}_3$), 2.77 (1H, 2×d, J=17.4, 7.0, Ph–C$\underline{H}_2$–CH), 3.30 (1H, 2×d, J=17.4, 10.5, Ph–C$\underline{H}_2$–CH), 5.28 (1H, 2×d, J=10.5, 7.0, O–C$\underline{H}$), 7.1—7.5 (6H, m, 5-H, arom. H).

Compound **29b**: Yield 90%. ^{1}H-NMR (in $CDCl_3$) δ: 2.04 (1H, t, J=7.0, O$\underline{H}$), 2.80 (6H, s, 2×N–C$\underline{H}_3$), 3.42 (1H, 2×d, J=16.0, 7.5, Ph–C$\underline{H}_2$–CH), 3.68 (1H, 2×d, J=16.0, 9.0, Ph–C$\underline{H}_2$–CH), 3.70—4.20 (2H, m, O–C$\underline{H}_2$), 4.90—5.30 (1H, m, O–C$\underline{H}$), 7.35 (1H, s, 5-H).

2,3-Dichloro-4-nitrophenol (31)——A solution of 2,3-dichlorophenol (**30**, 48 g, 0.245 mol) in acetic anhydride (100 ml) was added to a solution of nitric acid (d=1.38, 200 ml) in acetic acid (100 ml) dropwise at 4—8 °C with stirring. The mixture was kept for 1 h at the same temperature, then poured into ice water. The precipitated crystalline material was collected by filtration and washed with dichloromethane to obtain **31** as pale yellow crystals (25.6 g, 42%), mp 154 °C (dichloromethane–ether). *Anal.* Calcd for $C_6H_3Cl_2NO_3$: C, 34.65; H, 1.45; Cl, 34.09; N, 6.73. Found: C, 34.61; H, 1.54; Cl, 33.73; N, 6.85. ^{1}H-NMR (in acetone-d_6) δ: 3.3—5.5 (br, O$\underline{H}$), 7.18 and 7.93 (2H, AB, J=9.0, arom. H).

2,3-Dichloro-4-nitroanisole (32)——A mixture of **31** (11.9 g, 0.0572 mol), methyl iodide (32.5 g, 0.229 mol), and potassium carbonate (8 g, 0.058 mol) in *N,N*-dimethylformamide (DMF) (50 ml) was refluxed for 40 min, then evaporated *in vacuo*. The residue was dissolved in dichloromethane and the solution was washed, dried, and evaporated. The residue was chromatographed. Elution with dichloromethane gave **32** (12.38 g, 97%), which was used for the next reaction without further purification.

2,3-Dichloro-4-methoxyaniline (33)——A solution of **32** (25.17 g, 0.113 mol) in ethyl acetate (300 ml) was hydrogenated over 10% palladium–carbon catalyst (1.1 g). The catalyst was removed by filtration, and the filtrate was washed with 5% sodium hydroxide and brine, then dried and evaporated *in vacuo*. The residue was used for the next reaction without further purification.

2-Amino-3,4-dichloro-5-methoxybenzophenone (34a)——A solution of **33** (1.92 g, 0.01 mol) in dichloromethane (6 ml) and a solution of benzonitrile (1.53 g, 0.0149 mol) in dichloromethane (2 ml) were added dropwise to 2.0 mol of boron trichloride in dichloromethane (6.3 ml). Next, solid aluminum trichloride (1.5 g, 0.0112 mol) was added at 5-12 °C with stirring. The mixture was stirred at room temperature for 30 min, refluxed for 2 h, then left standing overnight. Next, 20% hydrochloric acid (10 ml) was added, and the mixture was hydrolyzed at 70 °C for 10 min, then filtered. The filtrate was extracted with dichloromethane and the organic layer was dried, then evaporated *in vacuo*. Chromatography of the residue with dichloromethane as the eluant gave **34a** (0.677 g, 23%), mp 81—83 °C (ether). *Anal.* Calcd for $C_{14}H_{11}Cl_2NO_2$: C, 56.78; H, 3.74; Cl, 23.94; N, 4.73. Found: C, 56.47; H, 3.92; Cl, 23.73; N, 4.89. ^{1}H-NMR (in $CDCl_3$) δ: 3.67 (3H, s, O–C$\underline{H}_3$), 6.1 (2H, br, N$\underline{H}_2$), 7.00 (1H, s, arom. H), 7.30—7.75 (5H, m, arom. H).

Compound **34b** was obtained in a similar manner with heating at 75 °C for 90 h using propionitrile instead of refluxing for 7 h with benzonitrile. Compound **34b**: Yield 34%, mp 86 °C (hexane–ether). *Anal.* Calcd for $C_{10}H_{11}Cl_2NO_2$: C, 48.41; H, 4.47; Cl, 28.58; N, 5.65. Found: C, 48.24; H, 4.46; Cl, 28.40; N, 5.74. ^{1}H-NMR (in $CDCl_3$) δ: 1.22 (3H, t, J=7.1, C$\underline{H}_3$), 2.95 (2H, q, J=7.1, COC$\underline{H}_2$), 3.85 (3H, s, O–C$\underline{H}_3$), 6.66 (2H, br, N$\underline{H}_2$), 7.25 (2H, s, arom. H).

3,4-Dichloro-5-methoxybenzophenone (35a)——A solution of 45% nitrosylsulfuric acid in sulfuric acid (2.0 g) was added dropwise to a solution of **34a** (0.975 g, 0.0033 mol) in THF (15 ml) at −25——16 °C over 7 min with stirring, then the reaction temperature was raised gradually to 0 °C over 1.5 h. Next, 45—50% aqueous hypophosphorus acid (25 ml) was added at 0—9 °C over 30 min and the solution was stirred at 6—10 °C for 2 h. Dichloromethane was added to the reactant, then the mixture was extracted, dried and evaporated *in vacuo*. Chromatography of the residue using hexane–dichloromethane (1 : 4) as the eluant gave **35a** (0.868 g, 94%), mp 85 °C (hexane–ether). *Anal.* Calcd for $C_{14}H_{10}Cl_2O_2$: C, 59.81; H, 3.59; Cl, 25.22. Found: C, 59.83; H, 3.58; Cl, 25.50. ^{1}H-NMR (in $CDCl_3$) δ: 3.96 (3H, s, O–C$\underline{H}_3$), 7.25—7.87 (7H, m, arom. H).

Compound **35b** was obtained in a similar manner. Compound **35b**: Yield 93%, mp 85 °C (hexane–ether). *Anal.* Calcd for $C_{10}H_{10}Cl_2O_2$: C, 51.53; H, 4.32; Cl, 30.42. Found: C, 51.31; H, 4.34; Cl, 30.38. ^{1}H-NMR (in $CDCl_3$) δ: 1.22 (3H, t, J=7.2, C$\underline{H}_3$), 2.95 (2H, q, J=7.2, C$\underline{H}_2$), 3.95 (3H, s, O–C$\underline{H}_3$), 7.42 and 7.61 (2H, AB, J=1.8, arom. H).

3,4-Dichloro-5-hydroxybenzophenone (36a)——Boron tribromide (2.40 g, 0.0096 mol) was added to a solution of **35a** (1.627 g, 0.0058 mol) in dichloromethane (10 ml) at 5 °C with stirring. The mixture was maintained at the same temperature for 1 h, than allowed to react at room temperature for 20 min. The reactant was poured into ice-cooled 4% hydrochloric acid (50 ml), then extracted with dichloromethane. The organic layer was washed with 4% hydrochloric acid then brine, dried and evaporated *in vacuo*. Treatment of the residue with dichloromethane gave **36a** (1.513 g, 98%), mp 177 °C (dichloromethane). *Anal.* Calcd for $C_{13}H_8Cl_2O_2 \cdot 1/2H_2O$: C, 56.55; H, 3.29; Cl, 25.68. Found: C, 56.69; H, 3.17; Cl, 25.55. ^{1}H-NMR (in DMSO-d_6) δ: 7.30 and 7.34 (2H, ABq, J=1.8, arom. H), 7.45—7.85 (5H, m, arom. H), 11.13 (1H, s, O$\underline{H}$).

3',4'-Dichloro-5'-hydroxypropiophenone (36b)——A solution of **35b** (1.54 g, 0.0062 mol) in 30% hydrogen bromide in acetic acid (35 ml) was refluxed for 48 h, then evaporated *in vacuo*. The residue was treated with hexane–

ether to obtain **36b** (1.28 g, 88%), mp 133—134 °C (ether). *Anal.* Calcd for $C_9H_8Cl_2O_2$: C, 49.35; H, 3.68; Cl, 32.39. Found: C, 48.81; H, 3.69; Cl, 32.44. ^{1}H-NMR (in acetone-d_6) δ: 1.10 (3H, t, J=7.1, CH_3), 3.00 (2H, q, J=7.1, CH_2), 7.53 and 7.63 (2H, AB, J=1.8, arom. H), 9.0—10.0 (1H, br, OH).

3,4-Dichloro-5-allyloxybenzophenone (37a)——A mixture of **36a** (1.433 g, 0.0054 mol), potassium carbonate (0.753 g, 0.0055 mol), DMF (15 ml) and allyl bromide (1.85 g, 0.0153 mol) was allowed to react with stirring at room temperature. The reaction mixture was evaporated *in vacuo*, and the residue was dissolved in ether. This solution was washed with 5% aqueous sodium hydroxide, 5% hydrochloric acid, then brine, and dried and evaporated *in vacuo*. Treatment of the residue with hexane–ether gave **37a** (1.596 g, 97%), mp 78 °C (hexane–ether). *Anal.* Calcd for $C_{16}H_{12}Cl_2O_2$: C, 62.56; H, 3.94; Cl, 23.08. Found: C, 62.43; H, 3.91; Cl, 22.89. ^{1}H-NMR (in $CDCl_3$) δ: 4.66 (2H, d, t, J=4.8, 1.5, O–CH_2), 5.23—5.60 (2H, m, CH_2), 5.85—6.32 (1H, m, CH), 7.23—7.88 (7H, m, arom. H).

Compound **37b** was obtained in a similar manner. Compound **37b**: Yield 94%, mp 50 °C (hexane–ether). *Anal.* Calcd for $C_{12}H_{12}Cl_2O_2$: C, 55.62; H, 4.67; Cl, 27.36. Found: C, 55.64; H, 4.53; Cl, 27.36. ^{1}H-NMR (in $CDCl_3$) δ: 1.21 (3H, t, J=7.1, CH_3), 2.93 (2H, J=7.1, O–CH_2), 4.65 (2H, d, t, J=5.1, 1.5, O–CH_2), 5.22—5.60 (2H, m, CH_2), 5.83—6.30 (1H, m, CH), 7.41 and 7.62 (2H, AB, J=1.8, arom. H).

2-Allyl-4,5-dichloro-3-hydroxybenzophenone (38a)——**37a** (1.535 g, 0.005 mol) was heated on an oil bath at 235 °C for 8 min. After cooling, it was chromatographed and eluted with hexane–dichloromethane (1 : 1) to obtain **38a** (1.028 g, 67%), mp 105 °C (hexane–ether). *Anal.* Calcd for $C_{16}H_{12}Cl_2O_2$: C, 62.56; H, 3.94; Cl, 23.08. Found: C, 62.34; H, 3.89; Cl, 23.31. ^{1}H-NMR (in $CDCl_3$) δ: 3.42 (2H, d, t, J=6.0, 1.5, CH_2), 4.75—5.05 (2H, m, CH_2), 5.60—6.00 (1H, m, CH), 6.03 (1H, s, OH), 7.02 (1H, s, arom. H), 7.33—7.90 (5H, m, arom. H).

Compound **38b** was obtained in a similar manner. Compound **38b**: Yield 59%, mp 80 °C (hexane–ether). *Anal.* Calcd for $C_{12}H_{12}Cl_2O_2$: C, 55.62; H, 4.67; Cl, 27.36. Found: C, 55.30; H, 4.58; Cl, 27.13. ^{1}H-NMR (in $CDCl_3$) δ: 1.16 (3H, t, J=7.1, CH_3), 2.80 (2H, q, J=7.1, CH_2), 3.51 (2H, d, t, J=6.2, 1.5, CH_2), 4.82—5.15 (2H, m, CH_2), 5.65—6.10 (1H, m, CH), 6.12 (1H, s, OH), 7.15 (1H, s, arom. H).

6,7-Dichloro-2-hydroxymethyl-2,3-dihydrobenzofuran-4-yl Phenyl Ketone (39a)——*m*-Chloroperbenzoic acid (*m*-CPBA) (1.39 g, 0.0081 mol) and potassium fluoride (0.373 g, 0.0064 mol) were added to a solution of **38a** (0.986 g, 0.0032 mol) in dichloromethane (40 ml) with stirring, and the reaction was allowed to proceed for 17 h. The reactant was washed with 5% sodium hydroxide, then brine, and dried then evaporated *in vacuo*. The residue was chromatographed and eluted with dichloromethane–acetone (20 : 1) to obtain **39a** (0.793 g, 76%), mp 102—104 °C (hexane–ether). *Anal.* Calcd for $C_{16}H_{12}Cl_2O_3$: C, 59.47; H, 3.74; Cl, 21.94. Found: C, 59.35; H, 3.83; Cl, 21.90. ^{1}H-NMR (in $CDCl_3$) δ: 2.20 (1H, br, OH), 3.28 (1H, 2×d, J=17.3, 8.3, Ph–CH_2–CH), 3.55 (1H, 2×d, J=17.3, 9.0, Ph–CH_2–CH), 3.60—4.15 (2H, m, O–CH_2), 4.93—5.30 (1H, m, O–CH), 7.20 (1H, s, arom. H), 7.37—7.90 (5H, m, arom. H).

6,7-Dichloro-2-hydroxymethyl-2,3-dihydrobenzofuran-4-yl Ethyl Ketone (39b)——*m*-CPBA (1.78 g, 0.0103 mol) and potassium fluoride (0.48 g, 0.008 mol) were added to a soltuion of **38b** (1.055 g, 0.004 mol) in dichloromethane (25 ml) with stirring, which was continued for 20 h at room temperature. The reaction mixture was washed with an aqueous potassium carbonate solution, dried and evaporated *in vacuo*. The residue was chromatographed and eluted with dichloromethane–acetone (20 : 1). The first fractions were treated with ether to obtain **40** (0.500 g, 47%), mp 174 °C (ether). *Anal.* Calcd for $C_{12}H_{12}Cl_2O_3$: C, 52.39; H, 4.40; Cl, 25.77. Found: C, 52.15; H, 4.30; Cl, 25.61. ^{1}H-NMR (in acetone-d_6) δ: 0.99 (3H, t, J=7.3, CH_3), 2.19 (2H, q, J=7.3, CH_2), 2.68 (1H, d, J=18.0, Ph–CH_2), 3.13 (1H, 3×d, J=18.0, 4.5, 1.8, Ph–CH_2), 3.66 (1H, 2×d, J=7.5, 2.0, O–CH_2), 3.97 (1H, 3×d, J=7.5, 6.0, 1.8, O–CH_2), 4.93 (1H, br t, J=5.5, O–CH), 7.04 (1H, s, arom. H), 8.47 (1H, br s, OH). The subsequent fractions eluted with the same solvent gave **39b** (0.494 g, 36%), mp 91—93 °C (ether). *Anal.* Calcd for $C_{12}H_{12}Cl_2O_3$: C, 52.39; H, 4.40; Cl, 25.77. Found: C, 52.13; H, 4.26; Cl, 25.71. ^{1}H-NMR (in $CDCl_3$) δ: 1.16 (3H, t, J=7.1, CH_3), 2.53 (1H, br, OH), 2.91 (2H, q, J=7.1, CH_2), 3.38 (1H, 2×d, J=18.0, 7.8, Ph–CH_2–CH), 3.66 (1H, 2×d, J=18.0, 9.0, Ph–CH_2–CH), 3.74 (1H, 2×d, J=12.3, 5.6, O–CH_2), 3.95 (1H, 2×d, J=12.3, 3.3, O–CH_2), 4.90—5.25 (1H, m, O–CH), 7.47 (1H, s, arom. H).

4-Benzoyl-6,7-dichloro-2,3-dihydrobenzofuran-2-carboxylic Acid (41a)——Jones reagent[10] (1.5 ml) was added to a solution of **39a** (0.682 g, 0.0021 mol) in acetone (12 ml) at 15 °C with stirring, which was continued for 7.5 h at the same temperature. The insoluble chromium salts were collected by filtration and washed with acetone. The washings and the filtrates were combined, then evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with water, then extracted three times with 5% aqueous sodium hydroxide. The alkaline solution was acidified with concentrated hydrochloric acid, then extracted with ethyl acetate. The organic layer was washed with brine, dried, then evaporated *in vacuo*. The residue was chromatographed and eluted with dichloromethane–methanol–acetic acid (400 : 25 : 2) to obtain **41a** (0.385 g, 54%, from hexane–dichloromethane). ^{1}H-NMR (in acetone-d_6) δ: 3.55 (1H, 2×d, J=17.8, 6.6, Ph–CH_2–CH), 3.88 (1H, 2×d, J=17.8, 10.2, Ph–CH_2–CH), 5.50 (1H, 2×d, J=10.2, 6.6, O–CH), 5.0—6.5 (br, COOH), 7.26 (1H, s, arom. H), 7.42—7.90 (5H, m, arom. H).

Compound **41b** was obtained in a similar manner. Compound **41b**: Yield 76%. ^{1}H-NMR (in acetone-d_6) δ: 1.11 (3H, t, J=7.1, CH_3), 3.01 (2H, q, J=7.1, CH_2), 3.67 (1H, 2×d, J=18.0, 7.1, Ph–CH_2–CH), 3.99 (1H, 2×d, J=18.0, 10.2, Ph–CH_2–CH), 5.45 (1H, 2×d, J=10.2, 6.7, 1.0, O–CH), 7.0—9.5 (br, COOH), 7.66 (1H, s, arom. H).

Biological Activities——Diuretic, saluretic and uricosuric activities were evaluated by the methods described in

the previous paper.[1]

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Synthesis and Biological Evaluation of Phosphonopyrimidine and Phosphonopurine Ribonucleosides[1)]

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Treatment of lithiated 2′,3′,5′-tri-*O*-protected uridine and 6-chloropurine ribonucleoside with diethyl chlorophosphate, followed by deblocking (and amination) and hydrolysis, provided 5- and 6-phosphonouridine (IV and VII), and 8-phosphonoadenosine (Xb), respectively. The Arbuzov reaction of 2′,3′,5′-tri-*O*-protected 4-chloro-2(1*H*)-pyrimidinone ribonucleoside and triethyl phosphite afforded the diethyl 4-phosphonate derivative (XII). Compounds IV, VII and Xb, and their respective diethyl esters (IIb and VIb) and monoethyl ester (Xa) were inactive *in vitro* as antiviral and cytostatic agents, but the diethyl 8-phosphonate derivative (IXb) of 6-chloro-9-(β-D-ribofuranosyl)purine (VIIIa) showed some antiviral and cytostatic activities, which were comparable in all respects to those of VIIIa.

Keywords——phosphonopyrimidine ribonucleoside; phosphonopurine ribonucleoside; Arbuzov reaction; phosphonylation; antiviral activity; cytostatic activity

The potential of pyrimidine and purine nucleoside analogs as chemotherapeutic agents in the treatment of virus infections and cancer has long been recognized.[2)] The biochemistry of phosphono compounds has also been reviewed.[3)] The occurrence of antibiotics (*e.g.*, phosphonomycin and N-1409) and phosphonolipids has raised intriguing questions about the biological role of these compounds in nature. Some phosphono nucleosides have been reported in which the phosphono group is linked to the sugar.[4)] However, no report has ever appeared on phosphono nucleosides which contain a phosphono group attached to the base moiety. This paper deals with the synthesis and biological activity of such novel nucleoside analogs.

Synthesis

In a previous paper,[5)] we reported the synthesis of some phosphono derivatives of pyrimidine and purine bases, based upon a halogen–metal or proton–metal exchange reaction of bromopyrimidine or purine followed by phosphonylation. Thus, the reaction of 5-bromouridine (Ia) with dihydropyran[6)] in dimethylformamide (DMF) in the presence of *p*-toluenesulfonic acid afforded 5-bromo-2′,3′,5′-tri-*O*-(tetrahydro-2-pyranyl)uridine (Ib) in a quantitative yield. Successive treatment of Ib with *n*-butyllithium and with diethyl chlorophosphate in tetrahydrofuran (THF) at −78 °C under argon gas provided, after work-up, two products, which were separated by silica gel column chromatography. The major product was isolated in 38% yield. Its ultraviolet (UV) absorption maximum was shifted by 10 nm to longer wavelength, and the proton nuclear magnetic resonance (^{1}H-NMR) spectrum revealed the absence of the C-5 proton signal and the presence of signals due to methyl and methylene protons in the diethyl phosphonate group. The compound was thus confirmed to be the 5-diethyl phosphonate derivative (IIa).[7)] The minor product, which was isolated in 26% yield,

had a UV absorption spectrum similar to that of uridine. The ^{1}H-NMR spectrum showed the presence of C-5 and C-6 proton signals. The product was identified as 2′,3′,5′-tri-*O*-(tetrahydro-2-pyranyl)uridine (III), which may have been formed by hydrolysis of the lithiouridine derivative.[8] Deblocking of the tetrahydropyranyl group in IIa with pyridinium *p*-toluenesulfonate[9] (PPTS) gave white needles in 51% yield. This product was confirmed to be diethyl 5-uridinylphosphonate (IIb) by elemental analysis and ^{1}H-NMR spectroscopy. Hydrolysis of the ethyl phosphonate in IIb with iodotrimethylsilane[10] in acetonitrile gave, after work-up, a white powder in 76% yield. This product was proved to be pure by high-performance liquid chromatography (HPLC) and migrated similarly to uridine 5′-monophosphate on paper electrophoresis (PE). The ^{1}H-NMR spectrum disclosed the absence of signals due to ethyl protons. The compound was thus identified as 5-phosphonouridine (IV) (Chart 1).

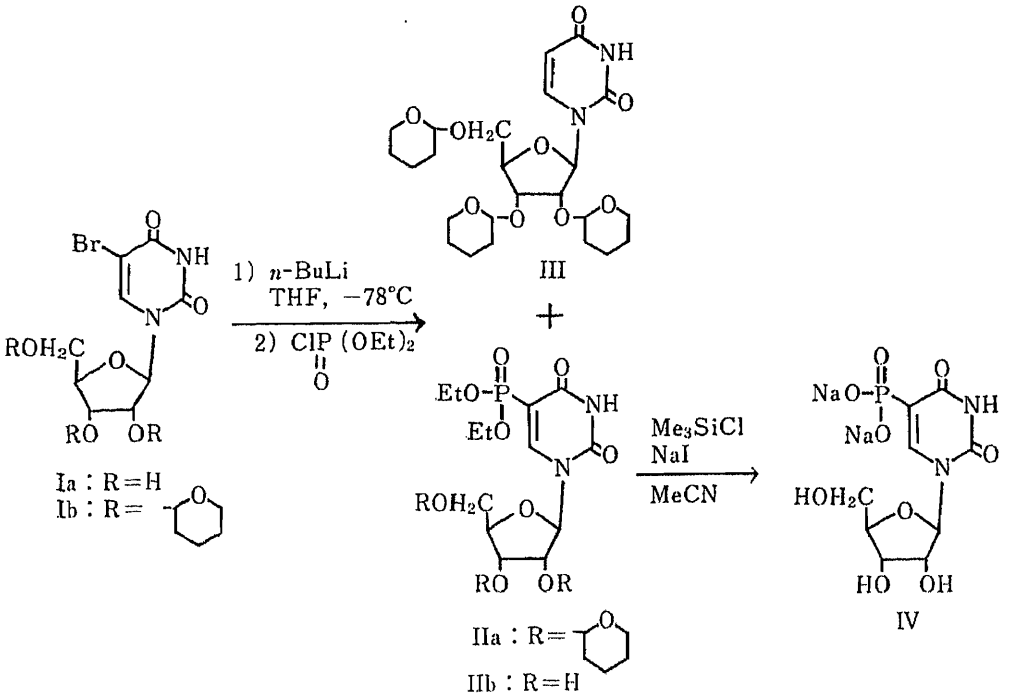


Chart 1

The reaction of 2′,3′-*O*-isopropylideneuridine (Va) with 2,3-dihydrofuran provided the 5′-*O*-(tetrahydro-2-furanyl) derivative (Vb). Successive treatment of Vb with lithium diisopropylamide[11a–c] and with diethyl chlorophosphate, followed by removal of the tetrahydrofuranyl group with PPTS, afforded, after purification by silica gel chromatography, the diethyl phosphonate derivative (VIa) in an overall yield of 51% relative to Va, and recovered Va in 19% yield. Removal of the isopropylidene group of VIa with 80% trifluoroacetic acid provided diethyl 6-uridinylphosphonate (VIb). Hydrolysis of the diethyl phosphonate group in VIb, as described for IIb, resulted in cleavage of the glycosidic bond so as to release 6-phosphonouracil, as proven by ^{1}H-NMR spectroscopy. However, a modified hydrolysis with the addition of a small amount of pyridine provided a white solid in 89% yield. The structure was confirmed to be 6-phosphonouridine (VII) by UV and ^{1}H-NMR spectroscopies as well as PE (Chart 2).

A sequence of reactions starting from 6-chloro-9-(β-D-ribofuranosyl)purine (VIIIa), similar to that starting from Ia, provided diethyl 6-chloro-9-(β-D-ribofuranosyl)-8-purinylphosphonate (IXb)[11d] *via* the 2′,3′,5′-tri-*O*-(tetrahydro-2-pyranyl) derivative (VIIIb)

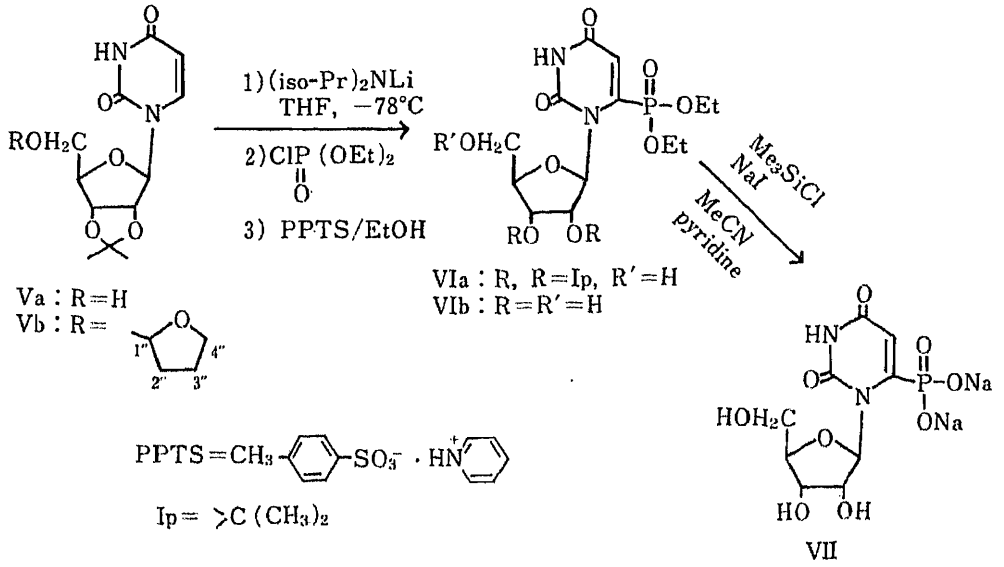


Chart 2

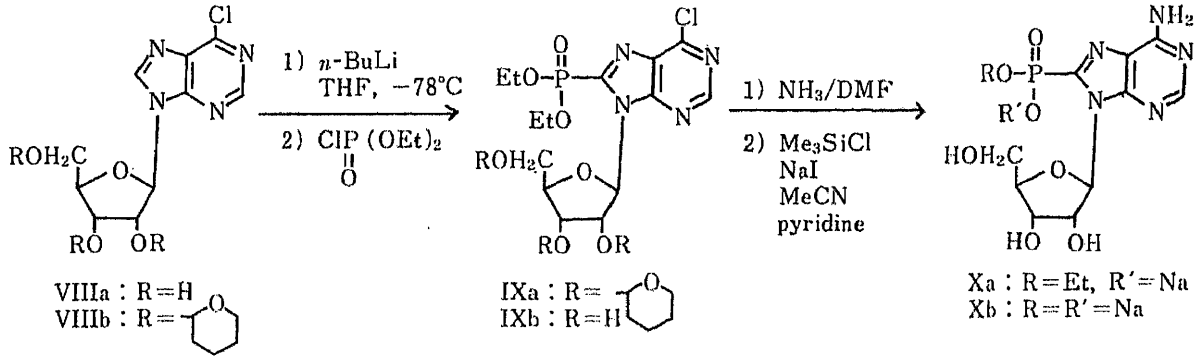


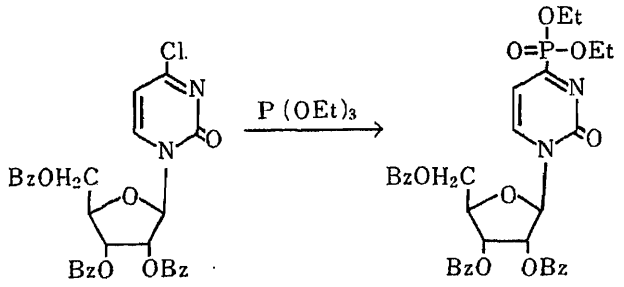
Chart 3

and the diethyl phosphonate derivative (IXa). Treatment of IXb with DMF saturated with ammonia afforded, after work-up including repeated purification by DE 23 column chromatography, a white powder in 82% yield; this product was ethyl 8-adenosinylphosphonate (Xa). It showed a single UV-absorbing spot of $M_{5'\text{-AMP}}$[12]=0.44 on PE. Compound IXb was treated with DMF saturated with ammonia and the product was allowed to react, without isolation, with iodotrimethylsilane in acetonitrile containing a small amount of pyridine. The product (a white powder) showed a single UV-absorbing spot of $M_{5'\text{-AMP}}$=0.94 on PE and was assigned the 8-phosphonoadenosine (Xb) structure from the results of UV and ^{1}H-NMR spectroscopies as well as elemental analysis (Chart 3).

Finally, we subjected 1-(2',3',5'-tri-*O*-benzoyl-β-D-ribofuranosyl)-4-chloro-2(1*H*)-pyrimidinone (XI) to the Arbuzov reaction[13] (treatment with triethyl phosphite at 125 °C). It gave a white crystalline 4-diethyl phosphonate derivative (XII) in 75% yield. The structure of XII was proven by elemental analysis and ^{1}H-NMR spectroscopy (Chart 4).

Biological Activity

Compounds IIb, IV, VIb, VII, VIIIa, IXb, Xa and Xb were evaluated for biological activity in a variety of antiviral and cytostatic assay systems according to previously established procedures.[14,15] The antiviral assays were run in primary rabbit kidney (PRK) cells (herpes simplex virus type 1 (strain KOS), herpes simplex virus type 2 (strain G), vaccinia virus,



XI XII

Chart 4

TABLE I. Antiviral and Cytostatic Activities of VIIIa and IXb

Assay system	MIC_{50} (μg/ml)[a]	
	VIIIa	IXb
Antiviral activity		
Herpes simplex virus type 1 (KOS)/PRK	>200	>100
Herpes simplex virus type 2 (G)/PRK	≥100	>100
Vaccinia virus/PRK	7	20
Vesicular stomatitis virus/PRK	7	20
Vesicular stomatitis virus/HeLa	2	10
Coxsackie B4 virus/HeLa	70	200
Poliovirus type 1/HeLa	150	200
Reovirus type 1/Vero	40	100
Parainfluenza virus type 3/Vero	150	300
Sindbis virus/Vero	200	>400
Semliki forest virus/Vero	300	>400
Coxsackie B4 virus/Vero	20	10
Cytostatic activity		
Murine leukemia (L1210)	15	27
Murine mammary carcinoma (FM3A)	138	54
Human B-lymphoblast (Raji)	85	38
Human T-lymphoblast (Molt-4F)	286	284

a) Minimum inhibitory concentration required to reduce virus-induced cytopathogenicity or tumor cell count by 50%.

vesicular stomatitis virus), HeLa cells (vesicular stomatitis virus, Coxsackie B4 virus, poliovirus type 1) or Vero (African green monkey kidney) cells (reovirus type 1, parainfluenza virus type 3, Sindbis virus, Semliki forest virus, Coxsackie B4 virus). Cytostatic activity was assessed with murine leukemia (L1210), murine mammary carcinoma (FM3A), human B-lymphoblast (Raji) and human T-lymphoblast (Molt/4F) cells.

Compounds IIb, IV, VIb, VII, Xa and Xb proved inactive as antiviral and cytostatic agents at concentrations up to 400 and 1000 μg/ml, respectively (data not shown). Compound IXb showed some antiviral activity, *i.e.* against vaccinia virus (in PRK cells), vesicular stomatitis virus (in PRK and HeLa cells) and Coxsackie B4 virus (in Vero cells). Compound IXb was also inhibitory to the proliferation of tumor cells (Table I). It is evident, however, that the biological activity of compound IXb is due to its 6-chloro group rather than its 8-diethylphosphonate group, since compound VIIIa showed antiviral and cytostatic activities that were comparable in all respects to those of IXb.

Discussion

Substitution of a phosphonate or diethyl phosphonate group at C-5 or C-6 of the uracil ring did not endow uridine with either antiviral or cytostatic activity. Nor did adenosine acquire any antiviral or cytostatic activity upon substitution of a phosphonate or diethyl phosphonate group at C-8 of the adenine moiety. The only phosphonyl derivative that was found to be biologically active was the 6-chloropurine ribonucleoside in which a diethyl phosphonate group was substituted at C-8. However, the activity of this compound was comparable to that of the 6-chloropurine ribonucleoside itself, suggesting that the chlorine, and not the diethyl phosphonate, group was responsible for its biological effects.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus (hot stage type) and are uncorrected. HPLC was conducted with a Shimadzu LC-2 apparatus using a column packed with Nucleosil 10DMA (10 μ) and a mobile phase of 10 mM phosphate buffer (pH 5.3). The UV spectra were recorded with a Shimadzu UV-190 digital spectrometer. The ^{1}H-NMR spectra were recorded with a JEOL GX-400 (400 MHz) spectrometer in $CDCl_3$ or dimethyl sulfoxide (DMSO)-d_6 with tetramethylsilane as an internal standard and in D_2O with sodium 3-(trimethylsilyl)propionate as an internal standard, respectively. PE was carried out at 22 V/cm using 0.01 M phosphate buffer (pH 7.5).

5-Bromo-2′,3′,5′-tri-*O*-(tetrahydro-2-pyranyl)uridine (Ib)——*p*-Toluenesulfonic acid (4.0 g) was added to a cooled solution of 5-bromouridine (5.30 g, 16.4 mmol) in a mixture of DMF (28 ml) and dihydropyran (12 ml), and the solution was kept at 4 °C for 15 h. Triethylamine (3.5 ml) was added to the reaction mixture and the solvent was evaporated off *in vacuo* to give a residue, which was partitioned between $CHCl_3$ (200 ml) and H_2O (200 ml). The organic phase was dried over $MgSO_4$, concentrated to a small volume, and chromatographed over a column of Silica gel G (4.0 × 25 cm) using a gradient (2 l) of 0—4% EtOH in $CHCl_3$ to obtain a syrup (8.90 g, 94%), which showed a single UV-absorbing spot on thin layer chromatography (TLC) with $CHCl_3$–EtOH (25 : 1). MS *m/z*: 490, 492 ($M^+ - C_5H_8O$). UV λ_{max}^{MeOH} nm: 278. ^{1}H-NMR ($CDCl_3$) δ: 9.90 (1H, d-like, N^3-H), 8.23 (1H, q-like, H-6), 6.0—6.4 (1H, m, H-1′), 3.3—5.2 (14H, H-2′, H-3′, H-4′, H-5′, –OC<u>H</u>(–O–)CH_2– and –O<u>C</u>H_2(CH_2)$_3$–). 1.2—2.2 (18H, –OCH(–O–)C<u>H</u>$_2$C<u>H</u>$_2$C<u>H</u>$_2$C<u>H</u>$_2$–).

Diethyl 2′,3′,5′-Tri-*O*-(tetrahydro-2-pyranyl)uridine-5-phosphonate (IIa)——A solution of Ib (5.75 g, 10 mmol) in THF (120 ml) was cooled at −78 °C under argon, then *n*-butyllithium (11.8 ml of a 1.7 M solution in hexane, 20 mmol) was added dropwise for 5 min. The brownish solution was stirred at −78 °C for 1 h, and then diethyl chlorophosphate (4.14 g, 24 mmol) was added dropwise. The mixture was stirred for 5 h, and warmed to room temperature, then 20% ammonium formate (30 ml) and pyridine (2 ml) were added. The residue obtained after removal of the solvents was partitioned between benzene (100 ml) and H_2O (50 ml). The organic layer was washed twice with water (50 ml), dried over $MgSO_4$, and concentrated to a small volume. Toluene was added, and the azeotropic mixture was distilled off. The residue was taken up in $CHCl_3$ (30 ml) and the solution was chromatographed over a column of Silica gel G (4.0 × 30 cm) with $CHCl_3$–EtOH (50 : 1) to give two main fractions. The first fraction was evaporated to dryness to give a caramel (2.40 g, 38%). UV λ_{max}^{MeOH} nm: 264.5. ^{1}H-NMR ($CDCl_3$) δ: 9.8 (1H, brs, N^3-H), 8.25 (1H, m, H-6), 5.9 (1H, m, H-1′), 4.2 (4H, m, –C<u>H</u>$_2$CH$_3$), 1.28 (6H, t, –CH$_2$C<u>H</u>$_3$).

2′,3′,5′-Tri-*O*-(tetrahydro-2-pyranyl)uridine (III)——Evaporation of the other main fraction in the preceding section gave a caramel (1.28 g, 26%). UV λ_{max}^{MeOH} nm: 260. ^{1}H-NMR ($CDCl_3$) δ: 7.95 (1H, m, H-6), 6.05 (1H, m, H-1′), 5.64 (1H, d-like, H-5), 3.3—5.1 (H-2′, H-5′, –OC<u>H</u>(–O–)(CH_2)$_3$C<u>H</u>$_2$–), 1.3—2.0 (–OCH(–O–)C<u>H</u>$_2$C<u>H</u>$_2$C<u>H</u>$_2$C<u>H</u>$_2$–).

Diethyl 5-Uridinylphosphonate (IIb)——PPTS (244 mg, 0.97 mmol) was added to a solution of IIa (1.60 g, 2.53 mmol) in EtOH (25 ml). The mixture was kept at 50 °C for 5 h and concentrated to dryness. The residue was dissolved in CH_2Cl_2 (15 ml) and the solution was chromatographed over a column of Silica gel G (3.0 × 35 cm) with a gradient (1 l) of 5—25% EtOH in $CHCl_3$. The residue obtained after removal of the solvents was triturated from EtOH (20 ml) to give a white crystalline product (490 mg, 51%). mp 161—163 °C. *Anal.* Calcd for $C_{13}H_{21}N_2O_9P$: C, 41.06; H, 5.57; N, 7.38. Found: C, 41.32; H, 5.69; N, 7.22. UV $\lambda_{max}^{0.1\,N\,HCl}$ nm: (ε): 265.5 (11400), $\lambda_{max}^{H_2O}$ nm (ε): 265 (11600), $\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε): 264 (7500). ^{1}H-NMR (DMSO-d_6) δ: 11.63 (1H, d, J_{HNCCP} = 4.58 Hz, N^3-H), 8.35 (1H, d, J_{HCCP} = 13.19 Hz, H-6), 5.79 (1H, d, $J_{1',2'}$ = 4.58 Hz, H-1′), 5.44 (1H, d, $J_{2'OH,2'}$ = 5.31 Hz, H-2′OH), 5.09 (1H, d, $J_{3'OH,3'}$ = 5.13 Hz, H-3′OH), 5.06 (1H, t, $J_{5'-OH,5'}$ = 4.68 Hz, H-5′OH), 4.04 (5H, m, –C<u>H</u>$_2$CH$_3$, and H-2′), 3.95 (1H, q, $J_{3',2'} = J_{3',4'}$ = 4.53 Hz, H-3′), 3.90 (1H, m, H-4′), 3.63 (1H, m, $J_{5a',4'}$ = 2.7 Hz, H-5′a), 3.55 (1H, m, $J_{5'b,4'}$ = 3.05 Hz, H-5′b), 1.23 (6H, t, –CH$_2$C<u>H</u>$_3$).

5-Phosphonouridine 2Na (IV)——Chlorotrimethylsilane (0.63 ml, 5.0 mmol) was added to a solution of IIb (150 mg, 0.39 mmol) and NaI (750 mg, 5.0 mmol) in acetonitrile (5 ml). The mixture was stirred at room temperature for 1 h (NaCl precipitated immediately). The supernatant was concentrated and the residue was dissolved in MeOH

(5 ml). This solution was adjusted to pH 7—9 with conc. NH_4OH to afford a white solid (109 mg, 74%), which showed a single UV-absorbing spot and a single peak on PE and HPLC, respectively. mp 204—210 °C. *Anal.* Calcd for $C_9H_{11}N_2Na_2O_9P \cdot H_2O$: C, 27.99; H, 3.39; N, 7.25. Found: C, 27.84; H, 3.39; N, 7.22. UV $\lambda_{max}^{0.1\,N\,HCl}$ nm (ε): 264 (12500), $\lambda_{max}^{H_2O}$ nm (ε): 264 (12100), $\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε): 261.5 (8700). ^{1}H-NMR (D_2O) δ: 7.91 (1H, d, J_{HCCP}=12.09 Hz, H-6), 5.81 (1H, d, $J_{1',2'}$=4.95 Hz, H-1'), 4.23 (1H, t, $J_{2',3'}$=5.31 Hz, H-2'), 4.10 (1H, t, $J_{3',4'}$=5.14 Hz, H-3'), 3.99 (1H, sestet, H-4'), 3.76 (1H, q, $J_{5'a,4'}$=2.93 Hz, $J_{5'a,5'b}$=12.87 Hz, H-5'a), 3.66 (1H, q, $J_{5'b,4'}$=4.86 Hz, H-5'b).

5'-*O*-(Tetrahydro-2-furanyl)-2',3'-*O*-isopropylideneuridine (Vb)——2,3-Dihydrofuran (1.4 ml) and PPTS (244 mg, 0.97 mmol) were added to a solution of 2',3'-*O*-isopropylideneuridine (Va) (2.77 g, 9.75 mmol) in CH_2Cl_2 (50 ml). After standing at room temperature overnight, the mixture was concentrated and chromatographed over a column of Silica gel G (3.2 × 30 cm) with a gradient (800 ml × 2) of 0—6.5% EtOH in $CHCl_3$. The combined fractions was evaporated to dryness to give a white crystalline product (2.90 g, 84%). mp 94—97 °C. *Anal.* Calcd for $C_{16}H_{22}N_2O_7$: C, 54.23; H, 6.26; N, 7.91. Found: C, 54.21; H, 6.13; N, 7.74. MS *m/z*: 339 ($M^+ - CH_3$), 383 ($M^+ - C_4H_7O$). UV λ_{max}^{MeOH} nm (ε): 259.5 (9500). ^{1}H-NMR ($CDCl_3$) δ: 7.60 (1H, d, $J_{6,5}$=8.06 Hz, H-6), 7.59 (1H, d, $J_{6,5}$=8.06 Hz, H-6), 5.90 (1H, d, $J_{1',2'}$=1.47 Hz, H-1'), 5.87 (1H, d, $J_{1',2'}$=2.20 Hz, H-1'), 5.692 (1H, d, H-5), 5.686 (1H, d, H-5), 5.13 (2H, m, H-2'), 4.72—4.78 (4H, m, H-3' and H-1''), 4.44 (1H, m, H-4'), 4.38 (1H, m, H-4'), 3.99 (1H, q, $J_{5'a,4'}$=2.93 Hz, $J_{5'a,5'b}$=10.99 Hz, H-5'a), 3.86—3.91 (5H, m, H-5'b and H-4''), 3.67 (1H, q, $J_{5'a,4'}$=2.20 Hz, $J_{5'a,5'b}$=10.99 Hz, H-5'a), 3.55 (1H, q, $J_{5'b,4'}$=4.39 Hz, H-5'b), 1.76—2.00 (8H, m, H-2'', H-3''), 1.59, 1.36 (each 6H, s, $>C(CH_3)_2$). These data indicate that Va is a mixture of two diastereoisomers.

Diethyl 2',3'-*O*-Isopropylideneuridine-6-phosphonate (VIa)——A solution of Vb (10.0 g, 28.2 mmol) and diisopropylamine (9.8 ml, 70 mmol) in THF (130 ml) was cooled at −78 °C under argon for 1 h, and *n*-butyllithium (4.1 ml of 1.7 M solution in hexane, 70 mmol) was added. The mixture was stirred for 1 h, then diethyl chlorophosphate (16.2 g, 94 mmol) was added dropwise. The solution was stirred for 6 h and chromatographed in a manner similar to that described in the case of IIa. TLC ($CHCl_3$–EtOH, 20:1) of the crude products showed two spots of *Rf* 0.28 and *Rf* 0.44. The product was dissolved in EtOH (50 ml), and PPTS (700 mg, 2.79 mmol) was added. After standing at 40 °C for 6 h, the mixture was evaporated to dryness. The residue was dissolved in a small amount of $CHCl_3$ and chromatographed over a column of Silica gel G (3.2 × 30 cm) with a gradient (2 l) of 5—15% EtOH in $CHCl_3$ to afford the two main fractions. The first fraction was evaporated to dryness to yield a caramel (VIb) (4.91 g, 51%). UV λ_{max}^{MeOH} nm: 268. ^{1}H-NMR ($CDCl_3$) δ: 9.87 (1H, brs, N^3-H), 6.47 (1H, d, J_{HCCP}=13.92 Hz, H-5), 6.15 (1H, d, $J_{1',2'}$=2.56 Hz, H-1'), 5.27 (1H, q, $J_{2',3'}$=6.78 Hz, H-2'), 5.06 (1H, q, $J_{3',4'}$=4.40 Hz, H-3'), 4.31 (5H, m, H-5'OH and $-CH_2CH_3$), 4.22 (1H, m, H-4'), 3.91 (1H, q, $J_{5'a,4'}$=2.93 Hz, $J_{5'a,5'b}$=12.09 Hz, H-5'a), 3.80 (1H, q, $J_{5'b,4'}$=4.03 Hz, H-5'b), 1.56, 1.35 (each 3H, s, $>C(CH_3)_2$, 1.42 (6H, sestet, $-CH_2CH_3$). The residue obtained from the other main fraction was crystallized from AcOEt, giving colorless prisms (Va) (1.38 g, 19%). mp 161—163 °C (lit.[16] 159—160 °C).

Diethyl 6-Uridinylphosphonate (VIb)——Compound VIa (320 mg, 0.76 mmol) was treated with 80% trifluoroacetic acid (2 ml) at 0 °C for 30 min and the solution was evaporated to dryness. EtOH (5 ml) was added to the residue and the azeotropic mixture was distilled off. The resulting syrup was dissolved in CH_2Cl_2 and the solution was purified by column chromatography (Silica gel G) to give a foam (280 mg). TLC ($CHCl_3$–EtOH, 9:1), *Rf* 2.90. UV $\lambda_{max}^{0.05\,N\,HCl}$ nm: 265.5, $\lambda_{max}^{H_2O}$ nm: 265, $\lambda_{max}^{0.05\,N\,NaOH}$ nm: 262. ^{1}H-NMR (DMSO-d_6) δ: 11.63 (1H, d, N^3-H), 6.15 (1H, q, J_{HCCP}=13.92 Hz, J_{HCCNH}=1.47 Hz, H-5), 5.69 (1H, d, $J_{1',2'}$=2.74 Hz, H-1'), 5.04 (1H, d, $J_{2'OH,2'}$=4.95 Hz, H-2'OH), 4.93 (1H, d, $J_{3'OH,3'}$=6.77 Hz, H-3'OH), 4.60 (1H, t, $J_{5'-OH,5'}$=5.77 Hz, H-5'OH), 4.50 (1H, m, H-2') 4.21—4.08 (6H, m, H-3', H-4', and $-CH_2CH_3$), 3.62 (1H, m, H-5'a), 3.43 (1H, m, H-5'b), 1.31 (6H, sestet, J_{HCCP}=7.23 Hz, $-CH_2CH_3$).

6-Phosphonouridine 2Na (VII)——Chlorotrimethylsilane (1.92 ml, 15.0 mmol) was added to a solution of VIb (350 mg, 0.91 mmol), NaI (2.25 g, 15.0 mmol) and pyridine (0.5 ml) in acetonitrile (15 ml). The mixture was stirred at room temperature for 1 h (NaCl precipitated immediately). The supernatant was treated in a manner similar to that described for IV to provide a white solid, which was passed through a column of Amberlite IR120B (Na^+ form, 1.8 × 10 cm). Evaporation of the eluate gave a caramel (186 mg, 51%), which showed a single UV-absorbing spot at the same position as that of uridine 5'-monophosphate on PE. mp > 300 °C. *Anal.* Calcd for $C_9H_{11}N_2Na_2O_9P \cdot H_2O$: C, 27.99; H, 3.39; N, 7.24. Found: C, 27.66; H, 3.37; N, 7.14. UV $\lambda_{max}^{0.1\,N\,HCl}$ nm (ε): 266.5 (9300), $\lambda_{max}^{H_2O}$ nm (ε): 268 (9500), $\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε): 268 (7600). ^{1}H-NMR (D_2O) δ: 6.02 (1H, d, $J_{1',2'}$=2.93 Hz, H-1'), 5.74 (1H, d, J_{HCCP}=10.63 Hz, H-5), 4.16 (1H, q, $J_{2',3'}$=6.60 Hz, H-2'), 3.83 (1H, t, $J_{3',4'}$=7.32 Hz, H-3'), 3.43 (1H, sestet, H-4'), 3.34 (1H, q, $J_{5a',4'}$=2.94 Hz, $J_{5'a,5'b}$=12.09 Hz, H-5'a), 3.20 (1H, q, $J_{5'b,4'}$=6.22 Hz, H-5'b).

6-Chloro-9-(2',3',5'-tri-*O*-(tetrahydro-2-pyranyl)-β-D-ribofuranosyl)purine (VIIIb)——*p*-Toluenesulfonic acid (6.0 g, dried over P_2O_5) was added to an ice-cooled solution of 6-chloro-9-(β-D-ribofuranosyl)purine (VIIIa) (4.0 g, 13.9 mmol) in a mixture of DMF (20 ml) and 2,3-dihydropyran (20 ml). The reaction mixture was allowed to stand at 0—5 °C for 6 h and treated in a manner similar to that described for Ib to give a syrup (6.44 g, 86%), which showed a single UV-absorbing spot on TLC (C_6H_6–AcOEt, 2:1). UV λ_{max}^{MeOH} nm: 263. ^{1}H-NMR ($CDCl_3$) δ: 8.5—8.8 (2H, m, H-2 and H-8), 6.35 (1H, m, H-1'), 3.30—5.0 (14H, H-2', H-3', H-4', H-5', $-OCH(-O-)CH_2-$ and $-OCH_2(CH_2)_3-$), 1.2—2.2 (18H, $-OCH(-O-)CH_2CH_2CH_2CH_2-$).

Diethyl 6-Chloro-9-(2',3',5'-tri-*O*-(tetrahydro-2-pyranyl)-β-D-ribofuranosyl)purine-8-phosphonate (IXa)——A solution of VIIIb (1.21 g, 2.24 mmol) in THF (25 ml) was cooled at −78 °C under argon and treated with *n*-butyllithium

(5.75 mmol) in a similar manner to that described for VIa. After 1 h, diethyl chlorophosphate (1.0 ml, 6.9 mmol) was added and the mixture was stirred for 30 min and treated in a manner similar to that described for IIa to obtain a foam (1.04 g, 69%), which showed a single UV-absorbing spot on TLC (C_6H_6–AcOEt, 5:1). UV λ_{max}^{MeOH} nm: 270, 255 (sh). ^{1}H-NMR ($CDCl_3$) δ: 8.74 (1H, s, H-2), 6.72 (1H, m, H-1'), 5.61 (1H, m, H-2'), 4.30 (4H, m, $-CH_2CH_3$), 1.45 (6H sestet, $-CH_2CH_3$).

Diethyl 6-Chloro-9-(β-D-ribofuranosyl)purine-8-phosphonate (IXb)——PPTS (800 mg, 3.5 mmol) was added to a solution of IXa (6.95 g, 10.3 mmol) in EtOH (35 ml), and the solution was kept at 50 °C for 6 h. The reaction mixture was worked up to obtain pale yellowish crystals (2.41 g, 55%). mp 119—121.5 °C. *Anal.* Calcd for $C_{14}H_{20}ClN_4O_7P$: C, 39.77; H, 4.77; N, 13.27. Found: C, 39.73; H, 4.71; N, 13.38. UV $\lambda_{max}^{0.1\,N\,HCl}$ nm (ε): 270 (13800), $\lambda_{max}^{H_2O}$ nm (ε): 270 (13500). ^{1}H-NMR (DMSO-d_6) δ: 8.95 (1H, s, H-2), 6.42 (1H, d, $J_{1',2'}$ = 5.74 Hz, H-1'), 5.38 (1H, d, $J_{2'OH,2'}$ = 5.74 Hz), 5.24 (1H, d, $J_{3'OH,3'}$ = 5.64 Hz, H-3'OH), 5.12 (1H, q, $J_{2',3'}$ = 5.49 Hz, H-2'), 4.83 (1H, t, $J_{5'-OH,5'}$ = 5.74 Hz, H-5'OH), 4.27 (4H, m, $-CH_2CH_3$), 3.95 (1H, q, H-4'), 3.72 (1H, m, $J_{5a',4'}$ = 4.88 Hz, $J_{5'a,5'b}$ = 11.84 Hz, H-5'a), 3.56 (1H, m, H-5'b), 1.33 (6H, q, $-CH_2CH_3$).

Ethyl Adenosine-8-phosphonate Na (Xa)——A solution of IXb (350 mg, 0.83 mmol) in DMF (20 ml) was saturated with ammonia at 0 °C and the mixture was heated at 100 °C overnight in a stainless steel bomb, and then cooled to 0 °C. The solvent was evaporated off *in vacuo* and the residue was dissolved in water (10 ml). The solution was chromatographed over a column of diethyl amino ethyl (DEAE) cellulose (bicarbonate form, 2.1 × 25 cm) with a gradient (1 l) of 0—0.05 M triethylammonium bicarbonate (TEAB) as the eluent. The required fraction was evaporated to dryness. Water (10 ml) was added to the residue, and the azeotropic mixture was distilled off. The residue was dissolved in water (20 ml) and the solution was passed through an Amberlite IR 120B column (Na^+, 1.6 × 25 cm). The flow-through fraction was evaporated to dryness and the residue was triturated with EtOH, giving a white solid (269 mg, 82%). PE $M_{5'\text{-AMP}}$ = 0.44. mp > 300 °C. UV $\lambda_{max}^{0.05\,N\,HCl}$ nm: 266, $\lambda_{max}^{H_2O}$ nm: 268, $\lambda_{max}^{0.05\,N\,NaOH}$ nm: 268. ^{1}H-NMR (D_2O) δ: 8.01 (1H, s, H-2), 6.41 (1H, d, $J_{1',2'}$ = 7.08 Hz, H-1'), 4.91 (1H, t, $J_{2',3'}$ = 4.64 Hz, H-2'), 4.35 (1H, m, H-3'), 4.15 (1H, s-like, H-4'), 3.84 (2H, m, H-5'), 3.74 (2H, q, $-CH_2CH_3$), 1.10 (3H, t-like, $-CH_2CH_3$).

8-Phosphonoadenosine 2Na (Xb)——A solution of IXb (250 mg, 0.59 mmol) in DMF (15 ml) was hydrolyzed with ammonia in a manner similar to that described for Xa. The reaction mixture was evaporated *in vacuo* and the residue was dissolved in a mixture of acetonitrile (25 ml) and pyridine (1 ml). Chlorotrimethylsilane (3.25 ml, 25 mmol) and NaI (3.63 g, 24.2 mmol) were added to the solution, and the mixture was stirred for 1 h (NaCl precipitated immediately). The supernatant was treated in a manner similar to that described for IV to give a white solid (106 mg, 43%). mp > 300 °C. *Anal.* Calcd for $C_{10}H_{12}N_5Na_2O_7P \cdot 1.7H_2O$: C, 28.46; H, 3.68; N, 16.60. Found: C, 28.80; H, 3.87; N, 7.12. UV $\lambda_{max}^{0.1\,N\,HCl}$ nm (ε): 265.5 (20300), $\lambda_{max}^{H_2O}$ nm (ε): 267 (15900), $\lambda_{max}^{0.1\,N\,NaOH}$ nm (ε): 267.5 (16900). ^{1}H-NMR (D_2O) δ: 8.02 (1H, s, H-2), 6.58 (1H, d, $J_{1',2'}$ = 6.96 Hz, H-1'), 4.85 (1H, t, $J_{2',3'}$ = 5.49 Hz, H-2'), 4.38 (1H, q, $J_{3',4'}$ = 2.20 Hz, H-3'), 4.19 (1H, d-like, H-4'), 3.82 (1H, q, $J_{5a',4'}$ = 1.47 Hz, $J_{5'a,5'b}$ = 12.82 Hz, H-5'a), 3.73 (1H, q, $J_{5'b,4'}$ = 2.93 Hz, H-5'b).

Diethyl 1-(2',3',5'-tri-*O*-benzoyl-β-D-ribofuranosyl)-2(1*H*)-pyrimidinone-4-phosphonate (XII)——A mixture of 1-(2',3',5'-tri-*O*-benzoyl-β-D-ribofuranosyl)-4-chloro-2(1*H*)-pyrimidinone (XI) (2.35 g, 4.09 mmol) and triethyl phosphite (5 ml) was heated at 125 °C for 4 h, and then cooled to room temperature. Ether (50 ml) was added to give a white crystalline product (2.08 g, 75%). mp 162—164 °C. *Anal.* Calcd for $C_{34}H_{33}N_2O_{11}P$: C, 60.36; H, 4.92; N, 4.14. Found: C, 60.02; H, 4.67; N, 4.17. UV λ_{max}^{MeOH} nm (ε): 328 (3340), 281 (3090), 274.5 (3420), 234 (15900). ^{1}H-NMR ($CDCl_3$) δ: 8.3—7.2 (16H, m, C_6H_5–, H6), 6.73 (1H, q, J_{HCCP} = 4 Hz, $J_{5,6}$ = 11 Hz, H5), 6.35 (1H, d, $J_{1',2'}$ = 3 Hz, H1'), 5.88 (2H, m, H2', H3'), 4.81 (3H, m, H4', H5'), 4.27 (4H, pentet, $-CH_2CH_3$), 1.35 (6H, t-like, $-CH_2CH_3$).

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Studies on Dihydropyridines. II.[1] Synthesis of 4,7-Dihydropyrazolo[3,4-*b*]-pyridines with Vasodilating and Antihypertensive Activities

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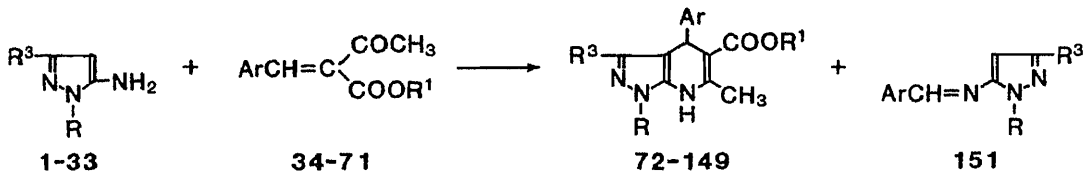
A series of 4-aryl-4,7-dihydropyrazolo[3,4-*b*]pyridine-5-carboxylate derivatives (**72**—**149**) was prepared and the compounds were tested for Ca-blocking activity in isolated guinea pig portal vein, antihypertensive activity in spontaneously hypertensive rats, and coronary vasodilating effect in isolated guinea pig heart. A number of derivatives had potent antihypertensive and coronary vasodilating activities. The structure–activity relationships of the series indicated that a 3-cyclopentyl or 3-cyclohexyl substituent and a hydrophobic 5-ester moiety with moderate bulkiness were effective for increasing the pharmacological potencies.

Keywords——pyrazolo[3,4-*b*]pyridine; calcium antagonist; antihypertensive activity; vasodilating activity

The interesting biological properties of nifedipine, 2,6-dimethyl-3,5-dimethoxycarbonyl-4-(2-nitrophenyl)-1,4-dihydropyridine,[2] have stimulated a variety of studies on the chemistry and pharmacology of the 1,4-dihydropyridines, as well as on the preparation of more potent analogues.[3] A number of 4-aryl-1,4-dihydropyridine-3,5-dicarboxylate derivatives have been prepared and tested for cardiovascular activity. Some of them have been found to possess potent vasodilating activity due to their calcium (Ca)-blocking effect, and are now in clinical trials or therapeutic use for the treatment of cardiovascular diseases, such as several kinds of hypertension, angina, and cerebrovascular insufficiency. In trying to prepare new types of 1,4-dihydropyridine derivatives superior to nifedipine in biological activity, we synthesized a number of 4,7-dihydropyrazolo[3,4-*b*]pyridine derivatives, having a modified 1,4-dihydropyridine system with a fused pyrazole nucleus, and screened their antihypertensive and coronary vasodilating activities. The Ca-blocking activities of these compounds were estimated from their inhibitory effect on K-contracture of isolated guinea pig portal vein. Some of the compounds were found to be promising cardiovascular agents. This paper deals with the synthesis and biological activities of the title compounds.

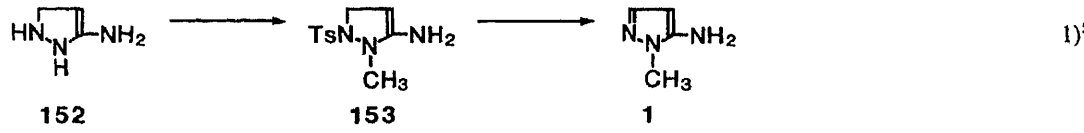
Synthesis

A number of 4-aryl-4,7-dihydropyrazolo[3,4-*b*]pyridine-5-carboxylate derivatives (**72**—**149**) were prepared by Michael addition of 5-aminopyrazoles (**1**—**33**) to α,β-unsaturated ketones (**34**—**71**), followed by cyclocondensation[4] (Chart 1). The requisite 5-aminopyrazole derivatives for the synthesis of the desired compounds were prepared by the method of Dorn *et al.*[5] (1) in Chart 2) and by cyclocondensation of alkyl and phenylhydrazines with acylacetonitriles (**154**)[6] (2) and 4) in Chart 2), as well as by the reaction of methylhydrazine sulfate with methyl cyanopyruvate sodium salt (**155**) (3) in Chart 2). Thirty-three 5-aminopyrazoles (**1**—**33**) were prepared by these methods. Thirty-six benzylideneacetoacetates (**34**—**69**) and two pyridylmethylideneacetoacetates (**70** and **71**) were readily obtainable by

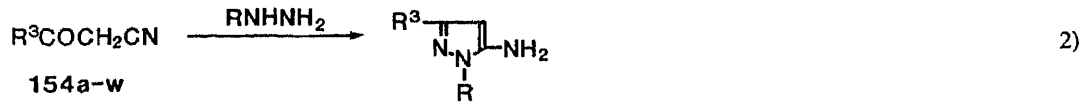


1-33 34-71 72-149 151

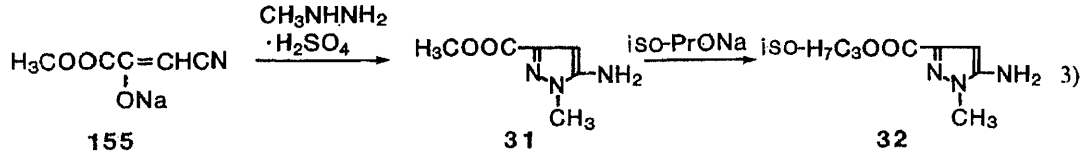
Chart 1



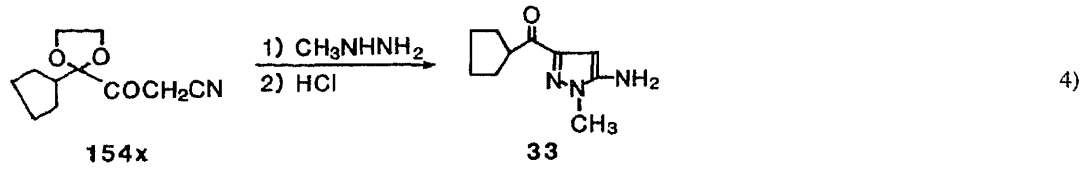
152 153 1 1)[5]



154a-w 2-30 2)

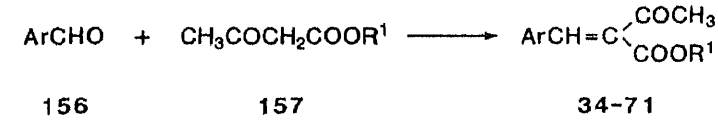


155 31 32 3)



154x 33 4)

Chart 2



156 157 34-71

Chart 3

means of the Knoevenagel reaction[7] from the corresponding aldehydes (**156**) and alkyl acetoacetates (**157**), prepared by the reaction of diketene with alcohols[8] (Chart 3). Heating a solution of 5-amino-1-methylpyrazole (**1**) with methyl 2-nitrobenzylideneacetoacetate (**34**) in *tert*-butanol afforded methyl 4,7-dihydro-1,6-dimethyl-4-(2-nitrophenyl)pyrazolo[3,4-*b*]-pyridine-5-carboxylate (**72**) in 42.1% yield, together with a small amount of 1-methyl-5-(2-nitrobenzylidene)aminopyrazole (**151a**).[9] Similar treatment of 5-amino-3-cyclopentyl-1-methylpyrazole (**14**) with methyl 3-nitrobenzylideneacetoacetate (**58**) gave the 3-cyclopentyl-4-(3-nitrophenyl) derivative (**76**) in 94.0% yield. The compounds listed in Tables I and II were prepared from the corresponding 5-aminopyrazoles and acetoacetate derivatives in the same manner as described for the preparation of **72**. Reduction of **76** yielded the 4-(3-aminophenyl) derivative (**150**). Compounds **72**—**150** were characterized as having the 4-aryl-4,7-dihydro-6-methylpyrazolo[3,4-*b*]pyridine structure on the basis of their proton nuclear magnetic resonance (^{1}H-NMR) and infrared (IR) spectra, as well as carbon-13 nuclear magnetic resonance (^{13}C-NMR) data in some cases. However, the data did not completely rule out

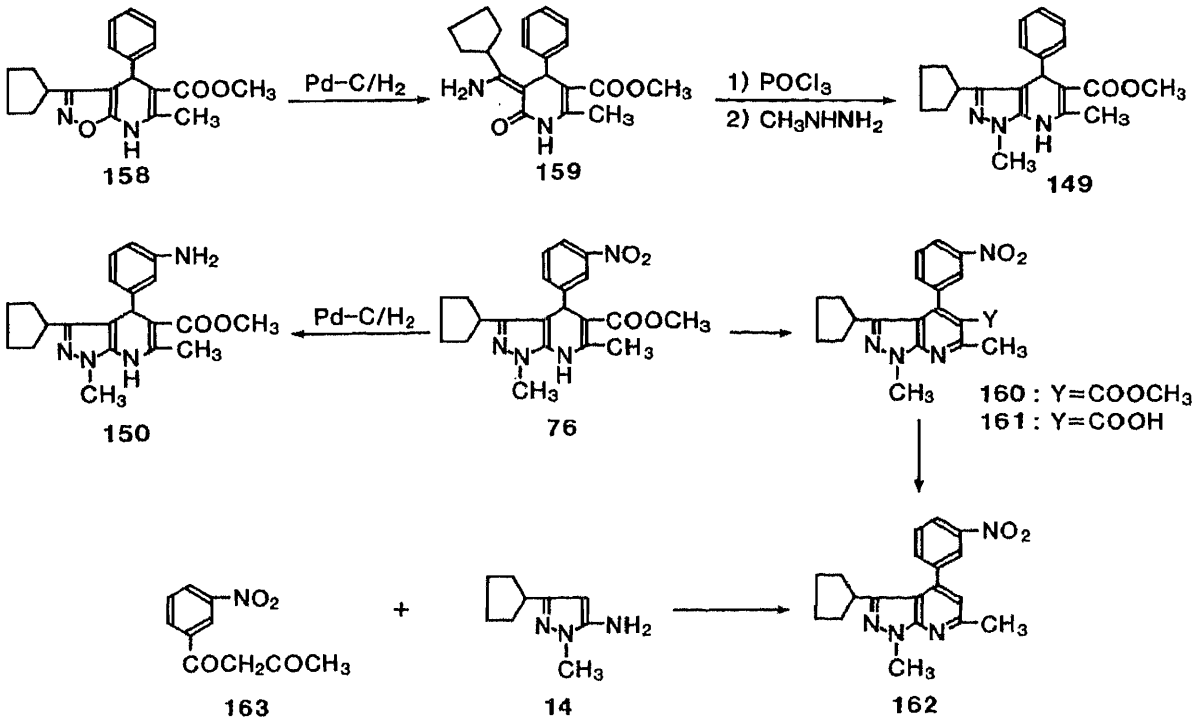


Chart 4

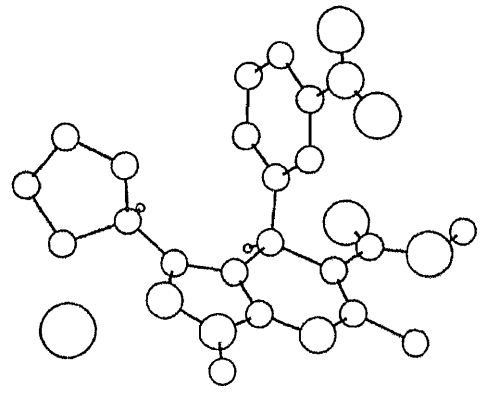


Fig. 1. X-Ray Crystallographic Structure of Methyl 3-Cyclopentyl-4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate Hydrochloride (**76**)

another structure, 6-aryl-1,2-dihydro-4-methylpyrazolo[3,4-*b*]pyridine form. In order to unequivocally establish the structure, the synthesis of **149** was successfully conducted *via* an alternative pathway starting from a known compound (**158**),[1a] as shown in Chart 4. Further, the product (**162**), which had been derived from **76** *via* oxidation followed by hydrolysis and decarboxylation, was identical with 3-cyclopentyl-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine, obtained by the reaction of **14** with 3-nitrobenzoylacetone (**163**).[10] Further confirmation of the structure came from an X-ray analysis of **76** hydrochloride[11] (Fig. 1).

Pharmacological Results and Discussion

The Ca-blocking activity of each test compound was evaluated in terms of its inhibitory effect on the K-contracture of isolated guinea pig portal vein.[12] The values of the concentration required for 50% relaxation of the contracture (RC_{50}) by the compounds and two reference Ca-blockers, nifedipine and nicardipine hydrochloride, were calculated from

TABLE I. Alkyl (R^1) 4-Aryl-1-R-3-R^3-4,7-dihydro-

Compd. No.	Ar	R^3	R^1	R	mp (°C)	Recrystn. solvent	Yield (%)
72	2-NO_2-C_6H_4	H	CH_3	CH_3	213—214	iso-Pr_2O	42.1
73	3-NO_2-C_6H_4	CH_3	CH_3	CH_3	186—188	iso-Pr_2O	80.5
74	3-NO_2-C_6H_4	iso-C_3H_7	C_2H_5	CH_3	217—220	MeOH	92.6
75	3-NO_2-C_6H_4	*n*-C_4H_9	CH_3	CH_3	129—132	Et_2O	96.7
76	3-NO_2-C_6H_4	Cyclopentyl	CH_3	CH_3	172—173	iso-PrOH	94.0
77	3-NO_2-C_6H_4	Cyclopentyl	C_2H_5	CH_3	170—173	Acetone	78.4
78	3-NO_2-C_6H_4	Cyclopentyl	iso-C_3H_7	CH_3	190 (dec.)	iso-PrOH	68.2
79	2-NO_2-C_6H_4	Cyclopentyl	CH_3	CH_3	212—213	EtOH	77.6
80	2-NO_2-C_6H_4	Cyclopentyl	$CH_2CH_2OCH_3$	CH_3	197—198	EtOH	68.6
81	3-NO_2-C_6H_4	Cyclohexyl	CH_3	CH_3	230 (dec.)	MeOH	71.7
82	2-NO_2-C_6H_4	Cyclohexyl	CH_3	CH_3	217—219	MeCN	79.6
83	3-NO_2-C_6H_4	Cyclopentyl	CH_3	Cyclopentyl	175—176	EtOH	71.8
84	3-NO_2-C_6H_4	C_6H_5	C_2H_5	CH_3	157—158	CH_2Cl_2–Et_2O	83.7
85	3-NO_2-C_6H_4	C_6H_5	C_2H_5	C_6H_5	214—215	EtOAc	79.2
86	3-NO_2-C_6H_4	CO_2CH_3	CH_3	CH_3	208—209	EtOAc	59.8
87	3-NO_2-C_6H_4	CO_2-iso-C_3H_7	CH_3	CH_3	181—182	EtOAc	56.5
88	2-Cl-C_6H_4	Cyclopentyl	C_2H_5	CH_3	170 (dec.)	MeOH–acetone	59.0
89	2,6-Cl_2-C_6H_3	Cyclopentyl	C_2H_5	CH_3	147—148	iso-PrOH	15.7
	Nifedipine						
	Nicardipine hydrochloride						

Abbreviations used are: anti-HT activity, antihypertensive activity; SBP, systolic blood pressure; CVD effect, coronary vasodilating effect; CPF, coronary perfusion flow. *a*) RC_{50} values are the concentrations required for 50% relaxation of the contracture of isolated guinea-pig portal veins by KCl at 5×10^{-2} M. Usually 4 to 6 preparations were used for the determination of RC_{50} of test compounds except in the cases of **80** and **82** (2 preparations for each). *b*) For the determination of antihypertensive activities, test compounds were intraperitoneally administered at a dose of 3 mg/kg in 2 SHRs. Four SHRs were used for nifedipine

the concentration–response curves (Table I). Compound **72**, methyl 4,7-dihydro-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate, had no Ca-blocking effect, but the introduction of a 3-alkyl substituent into it increased its potency. Compounds **75**—**82** exhibited potent Ca-blocking activity, though they were less active than nifedipine and nicardipine hydrochloride. Similarly, the 3-phenyl and 3-isopropoxycarbonyl derivatives (**84** and **87**) as well as the 3-alkyl-4-(2-chlorophenyl) derivative (**88**) showed moderate potency.

6-methylpyrazolo[3,4-*b*]pyridine-5-carboxylates (**72**—**89**)

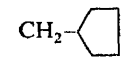
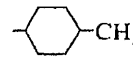
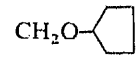
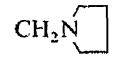
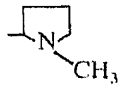
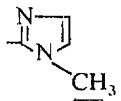
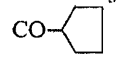
Formula	Analysis (%) Calcd (Found)			Ca-blocking activity[a] RC_{50} ($\times 10^{-9}$ M)	Anti-HT activity[b] max. change of SBP (mmHg)	CVD effect[c] max. change of CPF (%)	Acute toxicity[d] LD_{50} (mg/kg)
	C	H	N				
$C_{16}H_{16}N_4O_4$	58.53 (58.68	4.91 4.89	17.07 17.14)	>10000	0	0	
$C_{17}H_{18}N_4O_4$	59.64 (59.66	5.30 5.16	16.37 16.31)	—[e]	−15	+11.3	
$C_{20}H_{25}ClN_4O_4$[f]	57.07 (56.93	5.99 5.97	13.31 13.36)	30	−5	+21.2	
$C_{20}H_{24}N_4O_4$	62.48 (62.52	6.29 6.31	14.58 14.46)	13	−46	+50.0 (5)	
$C_{21}H_{24}N_4O_4$	63.62 (63.42	6.10 6.08	14.13 14.07)	11	−66	+47.1 (20)	524
$C_{22}H_{27}ClN_4O_4$[f]	59.12 (58.90	6.09 6.10	12.54 12.57)	11	−42	+69.4 (9)	>1000
$C_{23}H_{29}ClN_4O_4$[f]	59.93 (59.92	6.34 6.00	12.16 12.03)	32	−16	+56.3 (6)	
$C_{21}H_{24}N_4O_4$	63.62 (63.50	6.10 6.15	14.13 14.10)	16	−90	0	239
$C_{23}H_{28}N_4O_5$	62.71 (62.69	6.41 6.20	12.72 12.82)	12.5	−45	+59.1 (8)	
$C_{22}H_{27}ClN_4O_4$[f]	59.13 (58.76	6.09 6.09	12.54 12.50)	26	−65	+49.7 (20)	
$C_{22}H_{26}N_4O_4$	64.37 (64.21	6.39 6.31	13.65 13.52)	20.5	−76	0	292
$C_{25}H_{30}N_4O_4$	66.65 (66.68	6.71 6.68	12.44 12.44)	>10000	0	0	
$C_{23}H_{22}N_4O_4$	66.01 (65.94	5.30 5.16	13.39 13.33)	50	0	+15.7	
$C_{28}H_{24}N_4O_4$	69.99 (70.28	5.03 5.09	11.66 11.68)	>10000	0	0	
$C_{18}H_{18}N_4O_6$	55.95 (55.91	4.70 4.71	14.50 14.40)	120	−19	0	
$C_{20}H_{22}N_4O_6$	57.96 (57.73	5.35 5.36	13.52 13.30)	39	−30	0	
$C_{22}H_{27}Cl_2N_3O_2$[f]	60.55 (60.63	6.24 6.31	9.63 9.75)	44	−4	+59.4 (6)	
$C_{22}H_{25}Cl_2N_3O_2 \cdot 1/2\,H_2O$	59.60 (59.56	5.91 6.04	9.47 9.41)	70	0	+50.1 (7)	
				4.2	−45	+70.4 (7)	562
				4.8	−53	+91.0 (13)	

and **76**. *c*) The test compounds were intravascularly administered at a dose of 0.1 μg. Two to 4 preparations for each compound were used for the determination of CVD effect. The values in parentheses are the times (min) required for 50% recovery of the maximum change of CPF. *d*) LD_{50} values were determined after the oral administration of test compounds to 12 to 40 male slc. ddY mice. However, small number of mice was employed in some cases to obtain a rough indication of toxicity: **77**, **93**, **116**, **117**, **119**, **123** and **131**. *e*) Not tested. *f*) Hydrochloride.

Replacements with 1-phenyl and 1-cyclopentyl substituents resulted in a decrease of potency as seen in compounds **85** and **83**.

Antihypertensive activity was evaluated in conscious spontaneously hypertensive rats (SHR). Systolic blood pressure (SBP), recorded indirectly from the tail, was determined before dosing and at various time intervals during the ensuing 6 h after intraperitoneal administration of a test compound.[13] As can be seen in Table I, the compounds with potent

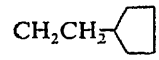
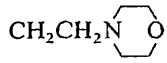
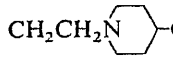
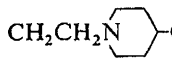
TABLE II. Alkyl (R^1) 4-Aryl-3-R^3-4,7-dihydro-1,6-

Compd. No.	Ar	R^1	R^3	mp (°C)	Recrystn. solvent
90	3-NO_2-C_6H_4	CH_3	iso-C_4H_9	114—115	EtOAc
91	3-NO_2-C_6H_4	CH_3	*tert*-C_4H_9	153—154	EtOAc
92	3-NO_2-C_6H_4	CH_3	$CH_2CH=CH_2$	102—105	Et_2O
93	2-NO_2-C_6H_4	CH_3		159—160	EtOAc–hexane
94	3-NO_2-C_6H_4	CH_3	$CH_2C_6H_5$	125—126	CH_2Cl_2–Et_2O
95	3-NO_2-C_6H_4	CH_3	Cyclopropyl	101—102	Et_2O
96	3-NO_2-C_6H_4	CH_3	Cyclobutyl	183—185	EtOAc
97	3-NO_2-C_6H_4	CH_3	Cycloheptyl	199—200	Et_2O
98	3-NO_2-C_6H_4	CH_3		197—198	iso-PrOH
99	3-NO_2-C_6H_4	CH_3		181—183	iso-PrOH
100	3-NO_2-C_6H_4	CH_3		196—197	EtOAc
101	2-NO_2-C_6H_4	CH_3		216—217	EtOH
102	3-NO_2-C_6H_4	CH_3	$CH_2CH_2OCH_3$	157—158	EtOAc
103	3-NO_2-C_6H_4	CH_3		174—175	EtOAc
104	3-NO_2-C_6H_4	CH_3		230 (dec.)	MeOH
105	3-NO_2-C_6H_4	CH_3		158—160	EtOAc
106	3-NO_2-C_6H_4	CH_3		146—150	MeOH
107	3-NO_2-C_6H_4	CH_3	C_6H_3-3,5-Cl_2	263—264	THF–EtOH
108	3-NO_2-C_6H_4	CH_3	2-Pyridyl	213—214	iso-PrOH
109	3-NO_2-C_6H_4	CH_3	3-Furyl	197—198	EtOH
110	2-NO_2-C_6H_4	CH_3	2-Thienyl	185—188	EtOH
111	3-NO_2-C_6H_4	CH_3		207—208	MeOH
112	2-NO_2-C_6H_4	CH_3		204—205	EtOH
113	2-NO_2-C_6H_4	C_2H_5	Cyclopentyl	149—150	EtOAc

dimethylpyrazolo[3,4-*b*]pyridine-5-carboxylates (**90**—**150**)

Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	Anti-HT activity[a] max. change of SBP (mmHg)	CVD effect[b] max. change of CPF (%)		Acute toxicity[c] LD_{50} (mg/kg)
59.0	$C_{20}H_{24}N_4O_4$	62.48 (62.12)	6.29 (6.12)	14.58 (14.76)	−55	+36.5	(7)	
38.8	$C_{20}H_{24}N_4O_4 \cdot 1/2\,H_2O$	61.06 (61.25)	6.40 (6.52)	14.24 (13.88)	−33	+21.6		
81.3	$C_{19}H_{20}N_4O_4$	61.94 (61.85)	5.47 (5.68)	15.21 (14.94)	−22	+15.5		
81.9	$C_{22}H_{26}N_4O_4$	64.37 (64.44)	6.39 (6.39)	13.65 (13.62)	−30	+71.4	(13)	1000
71.4	$C_{23}H_{23}ClN_4O_4 \cdot 1/2\,H_2O$[e]	59.54 (59.35)	5.22 (5.36)	12.07 (11.83)	0	0		
73.7	$C_{19}H_{20}N_4O_4$	61.94 (61.84)	5.47 (5.56)	15.21 (15.05)	−9	+20.3		
68.5	$C_{20}H_{22}N_4O_4$	62.81 (62.63)	5.80 (5.83)	14.65 (14.58)	−64	+36.9	(7)	720
95.2	$C_{23}H_{28}N_4O_4$	65.07 (65.13)	6.65 (6.77)	13.20 (13.10)	−38	+59.7	(14)	
53.2	$C_{23}H_{28}N_4O_4$	65.07 (65.09)	6.65 (6.60)	13.20 (13.05)	−2	+10.4		
78.7	$C_{21}H_{22}N_4O_4$	63.94 (64.01)	5.62 (5.50)	14.21 (13.93)	−60	+20.1		
81.7	$C_{22}H_{24}N_4O_4$	64.69 (64.61)	5.92 (5.70)	13.72 (13.74)	−40	+27.1		
70.1	$C_{22}H_{24}N_4O_4$	64.69 (64.61)	5.92 (5.82)	13.72 (13.50)	−65	0		
54.2	$C_{19}H_{22}N_4O_5$	59.06 (58.91)	5.74 (5.71)	14.50 (14.39)	0	0		
82.7	$C_{22}H_{26}N_4O_5$	61.96 (61.80)	6.15 (6.17)	13.14 (12.93)	−13	+23.8		
79.3	$C_{21}H_{26}ClN_5O_4$[e]	56.31 (56.12)	5.85 (5.83)	15.64 (15.59)	0	0		
21.5	$C_{21}H_{25}N_5O_4$	61.30 (61.19)	6.12 (6.26)	17.02 (16.89)	−9	0		
33.0	$C_{19}H_{21}ClN_4O_4S_2$[e]	48.66 (48.66)	4.51 (4.81)	11.95 (12.01)	−38	0		
84.5	$C_{22}H_{18}Cl_2N_4O_4$	55.82 (55.80)	3.83 (3.82)	11.84 (11.74)	0	0		
75.7	$C_{21}H_{10}N_5O_4$	62.21 (61.99)	4.72 (4.90)	17.28 (17.10)	−3	+15.5		
77.7	$C_{20}H_{18}N_4O_5$	60.91 (60.85)	4.60 (4.70)	14.21 (14.08)	−41	0		
53.5	$C_{20}H_{18}N_4O_4S \cdot C_2H_5OH$	57.88 (57.77)	5.30 (5.13)	12.27 (12.17)	−40	+15.4		
76.8	$C_{20}H_{20}N_6O_4 \cdot CH_3OH$	57.26 (57.10)	5.49 (5.48)	19.08 (19.05)	−19	0		
24.7	$C_{22}H_{24}N_4O_5$	62.25 (62.04)	5.70 (5.54)	13.20 (13.13)	−17	+29.6		
65.2	$C_{22}H_{27}ClN_4O_4$[e]	59.12 (58.86)	6.09 (6.03)	12.54 (12.37)	−58	+101.5	(14)	325

TABLE II.

Compd. No.	Ar	R^1	R^3	mp (°C)	Recrystn. solvent
114	2-NO_2–C_6H_4	C_2H_5	Cyclohexyl	148—151	EtOAc
115	2-NO_2–C_6H_4	*n*-C_5H_{11}	Cyclopentyl	112—113	EtOAc
116	2-NO_2–C_6H_4	CH_2CH_2– 	Cyclopentyl	147—150	Acetone
117	2-NO_2–C_6H_4	$CH_2CH_2C_6H_5$	Cyclopentyl	181—182	Acetone
118	2-NO_2–C_6H_4	$CH_2CH_2C_6H_4$-4-Cl	Cyclopentyl	166—170	Acetone
119	3-NO_2–C_6H_4	$CH_2CH_2C_6H_4$-4-Cl	Cyclopentyl	137—140	Acetone
120	2-NO_2–C_6H_4	$CH_2CH_2C_6H_4$-4-Br	Cyclopentyl	199—200	Acetone
121	2-NO_2–C_6H_4	$CH_2CH_2C_6H_4$-3-CF_3	Cyclopentyl	148—149	Acetone
122	2-NO_2–C_6H_4	$CH_2CH_2C_6H_3$-3,4-$(OCH_3)_2$	Cyclopentyl	129—130	Acetone
123	3-NO_2–C_6H_4	$CH_2CH_2C_6H_3$-3,4-$(OCH_3)_2$	Cyclopentyl	151—152	EtOH
124	2-NO_2–C_6H_4	$CH_2CH_2C_6H_3$-3,4-Cl_2	Cyclopentyl	125—126	Acetone
125	2-NO_2–C_6H_4	Cyclohexyl	Cyclopentyl	152—155	Acetone
126	2-NO_2–C_6H_4	$CH_2CH_2OC_6H_5$	Cyclopentyl	172—173	CH_2Cl_2
127	2-NO_2–C_6H_4	$CH_2CH_2OC_6H_5$	Cyclohexyl	127—130	Acetone
128	2-NO_2–C_6H_4	$CH_2CH_2OC_6H_4$-4-Cl	Cyclopentyl	166—167	Acetone
129	2-NO_2–C_6H_4	$CH_2CH_2CH_2O$iso-C_3H_7	Cyclopentyl	102—103	iso-Pr_2O
130	2-NO_2–C_6H_4	CH_2CH_2O– 	Cyclopentyl	119—121	Acetone
131	2-NO_2–C_6H_4	$CH_2CH_2SCH_3$	Cyclopentyl	125—126	iso-Pr_2O
132	2-NO_2–C_6H_4	CH_2CH_2Siso-C_3H_7	Cyclopentyl	152—155	Acetone
133	2-NO_2–C_6H_4	CH_2CH_2S– 	Cyclopentyl	149—150	Acetone
134	2-NO_2–C_6H_4	$CH_2CH_2SC_6H_5$	Cyclopentyl	126—127	Acetone
135	2-NO_2–C_6H_4	$CH_2CH_2CH_2N(CH_3)_2$	Cyclopentyl	135—136	Acetone
136	2-NO_2–C_6H_4	CH_2CH_2– 	Cyclopentyl	164—165	Acetone
137	2-NO_2–C_6H_4	CH_2CH_2– 	Cyclopentyl	169—170	EtOH
138	3-NO_2–C_6H_4	CH_2CH_2– 	Cyclopentyl	112—113	Acetone–Et_2O

(continued)

Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	Anti-HT activity[a] max. change of SBP (mmHg)	CVD effect[b] max. change of CPF (%)	Acute toxicity[c] LD_{50} (mg/kg)
72.5	$C_{23}H_{29}ClN_4O_4$[e]	59.92 (59.55)	6.34 (6.25)	12.16 (12.37)	−38	+59.6 (20)	
72.8	$C_{25}H_{33}ClN_4O_4$[e]	61.40 (61.21)	6.80 (6.97)	11.46 (11.45)	−74	+64.0 (80)	
72.8	$C_{27}H_{35}ClN_4O_4$[e]	62.96 (62.64)	6.85 (6.97)	10.88 (10.80)	−64	+54.1 (60)	300
67.2	$C_{30}H_{32}N_4O_8 \cdot H_2O$[f]	60.60 (60.60)	5.76 (5.44)	9.42 (9.33)	−75	+118.7 (120)	>1000
76.0	$C_{28}H_{30}Cl_2N_4O_4$[e]	60.33 (60.10)	5.42 (5.52)	10.05 (9.85)	−114	+154.1 (>120)	65
73.2	$C_{28}H_{30}Cl_2N_4O_4$[e]	60.32 (60.20)	5.42 (5.65)	10.05 (9.68)	−74	+48.4 (>120)	>500
70.8	$C_{28}H_{30}BrClN_4O_4$[e]	55.82 (55.62)	4.98 (4.90)	9.30 (9.30)	−106	+160.0 (>120)	
67.8	$C_{29}H_{30}ClF_3N_4O_4$[e]	58.93 (58.78)	5.12 (4.96)	9.48 (9.40)	−102	+92.7 (>120)	
74.8	$C_{30}H_{35}ClN_4O_6$[e]	61.80 (61.52)	6.05 (6.20)	9.61 (9.32)	−119	+49.4 (>120)	64
56.6	$C_{30}H_{35}ClN_4O_6$[e]	61.80 (61.77)	6.05 (5.95)	9.61 (9.54)	−86	+33.2 (60)	>500
58.2	$C_{28}H_{29}Cl_3N_4O_4$[e]	56.81 (56.66)	4.94 (5.32)	9.47 (8.98)	−110	+158.0 (>120)	
64.3	$C_{28}H_{33}ClN_4O_4$[e]	62.33 (62.10)	6.64 (6.46)	11.18 (11.25)	−25	+12.0	
81.9	$C_{30}H_{32}N_4O_6 \cdot H_2O$[f]	59.01 (58.79)	5.61 (5.70)	9.18 (8.94)	−74	+65.9 (120)	129
81.7	$C_{29}H_{33}ClN_4O_5$[e]	62.98 (62.59)	6.01 (5.99)	10.13 (9.86)	−92	+119.8 (>120)	100
34.9	$C_{30}H_{31}ClN_4O_6 \cdot H_2O$[f]	55.86 (55.62)	5.16 (4.83)	8.69 (8.37)	−91	+147.1 (>120)	
75.4	$C_{26}H_{34}N_4O_5$	64.71 (64.89)	7.10 (7.07)	11.61 (11.53)	−53	+100.0 (30)	>1000
77.0	$C_{27}H_{35}ClN_4O_5$[e]	61.07 (59.75)	6.64 (6.55)	10.55 (10.28)	−53	+103.7 (100)	1000
31.5	$C_{25}H_{30}N_4O_8S$[f]	54.94 (54.68)	5.53 (5.38)	10.25 (10.05)	−20	+105.4 (30)	>1000
77.5	$C_{25}H_{33}ClN_4O_4S$[e]	57.63 (57.43)	6.38 (6.43)	10.75 (10.63)	−69	+121.3 (120)	
77.8	$C_{27}H_{35}ClN_4O_4S$[e]	59.28 (59.30)	6.45 (6.66)	10.24 (10.21)	−70	+68.3 (>120)	
71.4	$C_{28}H_{31}ClN_4O_4S$[e]	60.58 (60.60)	5.63 (5.91)	10.09 (9.73)	−82	+112.5 (100)	185
42.3	$C_{27}H_{35}N_5O_8 \cdot 2H_2O$[f]	54.63 (54.73)	6.62 (6.29)	11.80 (11.87)	−17	0	
66.9	$C_{26}H_{35}Cl_2N_5O_5 \cdot H_2O$[e]	53.24 (52.95)	6.36 (6.36)	11.94 (11.88)	−24	0	
43.8	$C_{33}H_{41}Cl_2N_5O_4$[e]	61.68 (61.43)	6.43 (6.73)	10.90 (10.56)	−103	+63.9 (>120)	
43.0	$C_{33}H_{39}N_5O_4$	69.57 (69.31)	6.90 (6.90)	12.30 (11.92)	−68	+64.3 (75)	

TABLE II.

Compd. No.	Ar	R^1	R^3	mp (°C)	Recrystn. solvent
139	2-NO_2-C_6H_4	CH_2CH_2- 	Cyclopentyl	140—141	Acetone
140	2-NO_2-C_6H_4	$CH_2CH_2C_6H_5$	2-Thienyl	144—145	EtOH–Et_2O
141	2-NO_2-C_6H_4	$CH_2CH_2C_6H_4$-4-Cl	2-Thienyl	183—184	EtOAc
142	2-NO_2-C_6H_4	CH_2CH_2O- 	2-Thienyl	158—160	EtOH
143	2-NO_2-C_6H_4	$CH_2CH_2C_6H_4$-4-Cl	CO- 	205—206	EtOH
144	3-CF_3-C_6H_4	CH_3	Cyclopentyl	183 (dec.)	MeOH
145	2,3-$(CH_3O)_2C_6H_3$	CH_3	Cyclopentyl	182—184	EtOH
146	2,3-Cl_2-C_6H_3	$CH_2CH_2C_6H_4$-4-Cl	2-Thienyl	143—144	EtOAc
147	2-Pyridyl	CH_3	Cyclopentyl	217 (dec.)	MeOH
148	3-Pyridyl	CH_3	Cyclopentyl	225—227	MeOH
149	C_6H_5	CH_3	Cyclopentyl	201—202	iso-PrOH
150	3-NH_2-C_6H_4	CH_3	Cyclopentyl	106—110	EtOH

a—e) See footnotes *b—f* in Table I. *f*) Oxalate. *g*) The yield from **76**.

Ca-blocking activity showed a marked antihypertensive effect. Maximal decreases in SBP for compounds **76**, **79**, **81** and **82** observed at 1—2 h post administration were greater than those caused by nifedipine.

Coronary vasodilating activity was measured in isolated guinea pig heart by Langendorff's method.[14] The values shown in Table I were obtained by measuring the volume of the coronary arterial perfusates after intracoronary administration of the test compounds. Increases in coronary arterial flow were induced by the 4-(3-nitrophenyl) derivatives (**76** and **81**), in accordance with their Ca-blocking and antihypertensive activities, and the durations of action of these compounds were longer than those of the reference drugs. On the other hand, the 4-(2-nitrophenyl) derivatives (**79** and **82**), which showed stronger antihypertensive activity than **76** and nifedipine, did not exhibit a coronary vasodilating effect. Potent effects were observed with **80**, a 5-(2-methoxy)ethoxycarbonyl analogue of **79**, and also with the 4-(2-chlorophenyl) derivative (**88**), which had only weak potency for lowering SBP.

On the basis of these findings on the structure–activity relationships, pyrazolo[3,4-*b*]pyridines were further modified to improve the pharmacological effects. First, 3-alkyl and 3-aryl substituents were introduced into compound **72**. Table II lists a series of methyl 3-substituted-4,7-dihydro-1,6-dimethyl-4-(2- or 3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylates (**90**—**112**) and their antihypertensive and coronary vasodilating activities. Compounds **90**, **96**, **99** and **101**, which have 3-isobutyl, 3-cyclobutyl, 3-cyclopentenyl and 3-

(continued)

Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	Anti-HT activity[a] max. change of SBP (mmHg)	CVD effect[b] max. change of CPF (%)		Acute toxicity[c] LD_{50} (mg/kg)
81.1	$C_{26}H_{29}ClN_4O_4S$[e]	59.03 (58.73)	5.53 (5.20)	10.59 (10.55)	−88	+97.1	(50)	316
67.7	$C_{27}H_{24}N_4O_4S$	64.78 (64.74)	4.83 (4.79)	11.19 (11.11)	−43	+37.7	(24)	
47.4	$C_{27}H_{23}ClN_4O_4S$	60.61 (60.68)	4.33 (4.41)	10.47 (10.41)	−73	+22.2		
61.5	$C_{26}H_{28}N_4O_5S$	61.40 (61.28)	5.54 (5.48)	11.01 (11.01)	−36	+60.0	(16)	
24.7	$C_{29}H_{29}ClN_4O_5$	63.44 (63.30)	5.32 (5.16)	10.21 (10.07)	−75	+124.0	(120)	>1000
88.0	$C_{22}H_{25}ClF_3N_3O_2$[e]	57.96 (57.86)	5.53 (5.45)	9.22 (9.34)	−8	0		
18.4	$C_{23}H_{30}ClN_3O_4$[e]	61.67 (61.55)	6.75 (6.78)	9.38 (9.38)	0	0		
35.5	$C_{29}H_{24}Cl_3N_3O_6S$[f]	53.68 (53.29)	3.73 (3.80)	6.48 (6.26)	−63	0		
51.0	$C_{20}H_{24}N_4O_2$	68.16 (68.12)	6.86 (6.84)	15.90 (15.80)	−10	0		
84.2	$C_{20}H_{24}N_4O_2$	68.16 (67.96)	6.86 (7.07)	15.90 (15.63)	−17	0		
75.0	$C_{21}H_{20}ClN_3O_2$[e]	65.02 (64.97)	6.76 (6.85)	10.83 (10.61)	—[d]	—[d]		
84.1[g]	$C_{21}H_{20}N_4O_2$	68.83 (68.58)	7.15 (7.30)	15.29 (14.92)	0	0		

cyclohexenyl substituents, respectively, showed relatively potent antihypertensive activity. The 3-cyclopentylmethyl derivative (**93**) showed potent coronary vasodilating activity, comparable to that of nifedipine, but its antihypertensive effect was weak. The second modification focused on the 5-ester group in **76**, **79** and **82**, which showed potent Ca-blocking and antihypertensive activities. Table II lists a series of 3-cyclopentyl- or 3-cyclohexyl-4,7-dihydro-1,6-dimethyl-4-(2- or 3-nitrophenyl)pyrazolo[3,4-*b*]pyridine derivatives (**113**—**139**) having various 5-ester substituents and their biological activities. Most of these compounds exhibited potent antihypertensive and coronary vasodilating activities with long-lasting actions. Compounds **117**—**124**, which had phenethyloxycarbonyl or substituted phenethyloxycarbonyl substituents at the C-5 position, showed remarkably strong potencies with long durations of action. Some 5-aryloxyethyl esters (**126**—**128**), as well as 5-isopropoxy- and 5-cyclopentyloxyethyl esters (**129** and **130**) also showed strong potencies. On the other hand, **125**, which had a bulkier cyclohexyloxycarbonyl group at the C-5 position, showed lesser potency. Table II also includes compounds having other substituents at the C-3 and C-4 positions of the 4,7-dihydropyrazolo[3,4-*b*]pyridine system and their pharmacological activities. Compound **143** showed good potency, comparable to the reference compounds. However, compounds **144**—**148** and **150**, which had other substituents in place of the 2- or 3-nitrophenyl groups at the C-4 position, showed lesser potencies, probably because of their chemical instability.

Although a precise relationship can not yet be established between chemical structure

and biological activity, fusion of the pyrazole nucleus to 1,4-dihydropyridine seems to satisfy the structure requirements for enhancing the Ca-blocking activity. The results also indicate that the potency is enhanced in those compounds which have a 3-cycloalkyl substituent and a hydrophobic 5-ester moiety with moderate bulkiness. The nitro group on the 4-phenyl substituent seems to be necessary not only to increase the pharmacological activities but also to increase the stability of the compounds.

Finally, the acute toxicity in mice was determined for several compounds which showed potent antihypertensive and coronary vasodilating activities. The LD_{50} values were calculated by the Bliss method[15] for the 24 h after oral administration (Tables I and II).

On the basis of these results, five compounds (**76**, **117**, **129**, **130** and **143**), which showed potent antihypertensive and coronary vasodilating actions and were less toxic in mice, were selected as promising agents. Further pharmacological evaluations of these compounds are in progress.

Experimental

All melting points and boiling points are uncorrected. IR spectra were measured on a Hitachi 260-10 spectrometer. ^{1}H-NMR spectra were recorded with a Varian EM390 spectrometer in the indicated solvents. Chemical shifts are represented by δ-values using tetramethylsilane as an internal standard and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Mass spectra (MS) were obtained on an RMU-8GN mass spectrometer. After the reactions were run as indicated, thin-layer chromatography (TLC) was conducted on Merck Silica gel F_{254} plates. Standard work-up procedures were as follows: the reaction mixture was partitioned between the indicated solvent and water, and the organic extract was washed successively with water, $NaHCO_3$ solution (aq. $NaHCO_3$), NaOH solution (aq. NaOH) and hydrochloric acid (aq. HCl), and then dried over $MgSO_4$, filtered and evaporated *in vacuo*. Chromatographic separation was carried out on Merck Silica gel 60 using the indicated eluents.

Alkanoylacetonitriles and Aroylacetonitriles (**154a—w**) were prepared by means of the literature method.[16]

2-Cyanoacetyl-2-cyclopentyl-1,3-dioxolane (154x)——Methyl methylsulfinylmethyl sulfide (FAMSO) (4.95 g, 39.9 mmol) was added dropwise to a mixture of *tert*-BuOK (8.95 g, 79.8 mmol) and tetrahydrofuran (THF) (45 ml) under cooling at 0 °C. After stirring of the mixture for 15 min, a solution of ethyl cyclopentylcarboxylate (67 g, 39.9 mmol) in THF (15 ml) was added dropwise. The reaction mixture was stirred at 20 °C for 14 h, and then cooled, quenched with aq. HCl, and extracted with CH_2Cl_2. The extract, after removal of the solvent, was chromatographed on silica gel with CH_2Cl_2–Et_2O (1 : 1) to give cyclopentylcarbonyl-FAMSO (6.85 g, 78.0%) as a mixture of both isomers in the ratio of 4 : 6. ^{1}H-NMR ($CDCl_3$) δ: 1.77 (8H, m), 2.15, 2.21 (3H, s), 2.62, 2.77 (3H, s), 3.22 (1H, m), 4.41, 4.52 (1H, s). A mixture of the product (5.4 g, 24.5 mmol), $CuCl_2 \cdot 2H_2O$ (5.03 g, 24.5 mmol) and EtOH (54 ml) was stirred for 21 h at 25 °C. The solvent was evaporated off, and the residue was extracted with benzene. After removal of the solvent, the extract was chromatographed on silica gel with hexane–ethyl acetate (EtOAc) (20 : 1) to give ethyl cyclopentylpyruvate (2.75 g, 65.8%) as a colorless liquid. ^{1}H-NMR ($CDCl_3$) δ: 1.83 (8H, m), 1.34 (3H, t, J=7 Hz), 3.49 (1H, m), 4.32 (2H, q, J=7 Hz). A solution of the pyruvate (1.58 g, 9.3 mmol), ethyleneglycol (0.7 g, 11.3 mmol) and boron trifluoride diethylether complex (44 ml) in benzene (6 ml) was stirred at 25 °C for 20 h. The mixture was quenched with aq. $NaHCO_3$, washed with NaCl, dried over $MgSO_4$, filtered, and evaporated. The residue was dissolved in MeCN (0.7 g, 17 mmol)–THF (8 ml), and the solution was added to a mixture of *tert*-BuOK (1.9 g, 17 mmol) and THF (10 ml) under cooling at 0 °C. The mixture, after stirring for 6 h at 25 °C, was quenched with aq. HCl, and extracted with benzene. Chromatography of the extract on silica gel with CH_2Cl_2 gave **154x** (1.02 g, 60.1%) as a colorless liquid. ^{1}H-NMR ($CDCl_3$) δ: 1.56 (8H, m), 2.37 (1H, m), 3.67 (2H, s), 3.99 (4H, m).

5-Amino-1-methyl-3-R^3-pyrazoles (1—28)——**1** (R^3=H),[5] **2** (R^3=Me),[6c] **3** (R^3=iso-Pr),[6d] **4** (R^3=Ph),[6e] and **5** (R^3=2-pyridyl)[6f] were prepared by the literature methods. The pyrazoles (**6—28**) were prepared by the following general procedure. A solution of **154** (20 mmol) and methylhydrazine (0.92 g, 20 mmol) in EtOH (15 ml) was heated under reflux for 3—5 h, and then evaporated. The residue was chromatographed on silica gel with CH_2Cl_2–MeCN (19 : 1) to give the product. Futher purification was done by recrystallization from the appropriate solvents. **6** (R^3=*n*-Bu): 89.1% yield, liquid. ^{1}H-NMR ($CDCl_3$) δ: 1.58 (9H, m), 3.51 (2H, br s), 3.53 (3H, s), 5.29 (1H, s). **7** (R^3=iso-Bu): 55.1% yield, mp 102—104 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (6H, d, J=7 Hz), 1.86 (1H, m), 2.35 (2H, d, J=6 Hz), 3.55 (5H, br s). **8** (R^3=*tert*-Bu): 79.4% yield, mp 156—157 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 1.25 (9H, m), 3.52 (2H, br s), 3.60 (3H, s), 5.38 (1H, s). **9** (R^3=allyl): 16.3% yield, mp 52—55 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 3.23 (2H, m), 3.57 (3H, s), 3.62 (2H, br s), 5.09 (2H, m), 5.33 (1H, s), 5.99 (1H, m). **10** (R^3=cyclopentylmethyl): 64.9% yield, mp 106—107 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 1.63 (9H, m), 2.47 (2H, m), 3.51 (3H, s), 3.60 (2H, br s), 5.28 (1H, s). **11** (R^3=benzyl): 89.2% yield, mp 130—131 °C from iso-Pr_2O. ^{1}H-NMR

($CDCl_3$) δ: 3.40 (2H, brs), 3.53 (3H, s), 3.78 (2H, s), 5.22 (1H, s), 7.23 (5H, s). **12** (R^3=cyclopropyl): 53.6% yield, mp 123—125 °C from Et_2O. ^{1}H-NMR ($CDCl_3$) δ: 0.78 (4H, m), 1.85 (1H, m), 3.52 (5H, brs), 5.12 (1H, s). **13** (R^3=cyclobutyl): 68.2% yield, mp 117—118 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 2.42 (7H, m), 3.54 (3H, s), 3.60 (2H, brs), 5.38 (1H, s). **14** (R^3=cyclopentyl): 74.4% yield, mp 149—150 °C from PrOH. ^{1}H-NMR ($CDCl_3$) δ: 1.77 (8H, m), 2.63 (1H, m), 3.57 (5H, brs), 5.32 (1H, s). **15** (R^3=cyclohexyl): 71.7% yield, mp 173—174 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 1.84 (11H, m), 3.43 (2H, brs), 3.57 (3H, s), 5.32 (1H, s). **16** (R^3=cycloheptyl): 82.9% yield, mp 162—163 °C from Et_2O. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (13H, m), 3.48 (2H, brs), 3.55 (3H, s), 5.30 (1H, s). **17** (R^3=4-methylcyclohexyl): 60.4% yield, mp 170—175 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 1.72 (13H, m), 3.50 (2H, brs), 3.57 (3H, s), 5.33 (1H, s). **18** (R^3=3-cyclopentenyl): 59.3% yield, mp 102—105 °C from iso-Pr_2O–hexane. ^{1}H-NMR ($CDCl_3$) δ: 3.03 (7H, m), 3.57 (3H, s), 5.35 (1H, s), 5.71 (2H, m). **19** (R^3=3-cyclohexenyl): 75.0% yield, mp 146—148 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 1.99 (6H, m), 2.75 (1H, m), 3.58 (3H, s), 3.72 (2H, s), 5.36 (1H, s), 5.72 (2H, m). **20** (R^3=2-methoxyethyl): 80.7% yield, liquid. ^{1}H-NMR ($CDCl_3$) δ: 2.73 (2H, t, J=6 Hz), 3.47 (2H, brs), 3.55 (3H, s), 3.60 (5H, m), 5.35 (1H, s). **21** (R^3=cyclopentyloxymethyl): 24.6% yield, mp 112—114 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 1.64 (8H, m), 3.47 (2H, brs), 3.60 (3H, s), 4.00 (1H, m), 4.31 (2H, s), 5.55 (1H, s). **22** (R^3=pyrrolidinomethyl): 49.5% yield, liquid. ^{1}H-NMR ($CDCl_3$) δ: 1.83 (4H, m), 3.05 (4H, m), 3.62 (3H, s), 3.85 (2H, s), 5.68 (1H, s), 6.87 (2H, brs). **23** (R^3=1,3-dithiolan-2-yl): 47.5% yield, mp 77—78 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.47 (6H, m), 3.56 (3H, s), 5.33 (1H, s), 5.60 (1H, s). **24** (R^3=1-methylpyrrolidin-2-yl): 54.7% yield, mp 138—139 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 1.98 (9H, m), 3.10 (1H, m), 3.53 (2H, brs), 3.58 (3H, s), 5.47 (1H, s). **25** (R^3=3,5-Cl_2-Ph): 99.1% yield, mp 155—156 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 3.63 (5H, brs), 5.72 (1H, s), 7.34 (3H, m). **26** (R^3=3-furyl): 14.2% yield, mp 118—120 °C from iso-Pr_2O. ^{1}H-NMR ($CDCl_3$) δ: 3.52 (2H, brs), 3.65 (3H, s), 5.63 (1H, s), 7.19 (3H, m). **27** (R^3=2-thienyl): 58.1% yield, mp 135—140 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.58 (3H, s), 3.60 (2H, brs), 5.68 (1H, s), 7.08 (3H, m). **28** (R^3=1-methylimidazol-2-yl): 51.6% yield, mp 164—165 °C from EtOAc. ^{1}H-NMR ($CDCl_3$) δ: 3.60 (2H, brs), 3.67 (3H, s), 3.93 (3H, s), 6.03 (1H, s), 6.83 (1H, d, J=1 Hz), 7.00 (1H, d, J=1 Hz).

Methyl 5-Amino-1-methylpyrazole-3-carboxylate (31)——A solution of methyl cyanopyruvate sodium-enolate[17] (**155**, 10.0 g, 60 mmol) with methylhydrazine sulfate (9.0 g, 60 mmol) was stirred for 72 h at room temperature, and then evaporated. The residue was extracted with $CHCl_3$, and the extract was washed with aq. NaCl, dried over $MgSO_4$, filtered, and evaporated. The residue was chromatographed on silica gel with EtOAc to give **31** (6.54 g, 70.3%). Recrystallization from EtOH gave colorless needles, mp 101—102 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.71 (3H, s), 3.90 (2H, brs), 3.86 (3H, s), 6.05 (1H, s).

iso-Propyl 5-Amino-1-methylpyrazole-3-carboxylate (32)——A solution of **31** (2.0 g, 12.9 mmol) and iso-PrONa (0.1 g) in iso-PrOH (40 ml) was heated under reflux for 20 h. After removal of the solvent, the residue was extracted with $CHCl_3$, and the extract was washed with water, dried over $MgSO_4$, filtered, and evaporated. The crystalline residue was recrystallized from iso-PrOH to give **32** (1.72 g, 72.9%) as colorless needles, mp 86—87 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (6H, d, J=6 Hz), 3.72 (3H, s), 3.75 (2H, brs), 5.23 (1H, s).

5-Amino-3-cyclopentylcarbonyl-1-methylpyrazole (33)——A solution of **154x** (2.35 g, 11.2 mmol) and methylhydrazine (0.52 g, 11.3 mmol) in MeOH (20 ml) was stirred at 25 °C for 7 h, and then evaporated. The residue was chromatographed on silica gel with CH_2Cl_2–EtOH (20:1) to give **33**-ethyleneketal (1.45 g, 54.5%) as colorless crystals, mp 158—159 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.64 (8H, m), 3.53 (2H, brs), 3.62 (3H, s), 3.96 (4H, m), 5.49 (1H, s). A solution of the ketal (3.88 g, 16.3 mmol) and 10% aq. HCl (40 ml) in dioxane (40 ml) was stirred at room temperature for 72 h, and then evaporated. The residue was extracted with CH_2Cl_2, and the extract was washed with aq. $NaHCO_3$, then dried over $MgSO_4$, filtered, and evaporated. The residue was chromatographed on silica gel with CH_2Cl_2–Et_2O (1:1) to give **33** (2.29 g, 72.6%) as a pale yellow liquid. ^{1}H-NMR ($CDCl_3$) δ: 1.73 (8H, m), 3.68 (3H, s), 3.76 (1H, m), 3.85 (2H, brs), 5.94 (1H, s).

5-Amino-1,3-diphenylpyrazole (29)——**29** was prepared by a literature method.[10]

5-Amino-1,3-dicyclopentylpyrazole (30)——A solution of cyclopentylcarbonylacetonitrile (**154h**, 0.69 g, 5 mmol) and cyclopentylhydrazine[18] (0.5 g, 5 mmol) in EtOH (5 ml) was stirred for 16 h at 25 °C and then evaporated. The residue was chromatographed on silica gel with benzene–EtOAc (19:1) to give **30** (0.79 g, 71.5%) as pale yellow crystals. Recrystallization from iso-Pr_2O–hexane gave colorless prisms, mp 92—93 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.76 (16H, m), 2.97 (1H, m), 3.42 (2H, brs), 4.35 (1H, m), 5.33 (1H, s).

Alkyl (R^1) 2-Nitrobenzylideneacetoacetates (34—57)——**34**—**57** were prepared by the following general procedure.[7a] A solution of 2-nitrobenzaldehyde (**156a**, 15.0 g, 0.1 mol), alkyl (R^1) acetoacetate[8] (**157**, 0.1 mol), AcOH (3 ml) and piperidine (0.8 ml) in benzene (40 ml) was stirred for 24 h at 30—45 °C. After cooling, the mixture was washed with aq. $NaHCO_3$, followed by aq. NaOH, then dried over $MgSO_4$, filtered, and evaporated. The residue was chromatographed on silica gel with CH_2Cl_2–MeCN (9:1) to give the product as a mixture of the *cis* and *trans* isomers. **34** (R^1=Me)[7b]: 90% yield. **35** (R^1=Et)[7b]: 19.2% yield. **36** (R^1=pentyl): 89.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.32 (9H, m), 2.20, 2.47 (3H, s), 3.98, 4.27 (2H, t, J=7 Hz), 7.81 (5H, m). **37** (R^1=2-cyclopentylethyl): 97.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.56 (14H, m), 3.98, 4.27 (2H, t, J=7 Hz), 7.77 (5H, m). **38** (R^1=phenethyl): 88.2% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.12, 2.40 (3H, s), 2.68, 3.00 (2H, t, J=7 Hz), 4.22, 4.47 (2H, t, J=7 Hz), 7.53 (10H, m). **39** (R^1=4-chlorophenethyl): 96.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.10, 2.43 (3H, s), 2.68, 3.00 (2H, t, J=7 Hz), 4.20, 4.48 (2H, t, J=7 Hz), 7.58 (9H, m). **40** (R^1=4-bromophenethyl): 76.5% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.12, 2.40 (3H, s), 2.63, 2.95

(2H, t, J=7 Hz), 4.18, 4.43 (2H, t, J=7 Hz), 7.50 (9H, m). **41** (R^1=3-trifluoromethylphenethyl): 54.1% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.10, 2.40 (3H, s), 2.77, 3.08 (2H, t, J=7 Hz), 4.23, 4.48 (2H, t, J=7 Hz), 7.68 (9H, m). **42** (R^1=3,4-dimethoxyphenethyl): 96.8% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.08, 2.40 (3H, s), 2.62, 2.93 (2H, t, J=7 Hz), 3.78, 3.83, 3.85 (6H, s), 4.18, 4.45 (2H, t, J=7 Hz), 7.16 (8H, m). **43** (R^1=3,5-dichlorophenethyl): 93.1% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.12, 2.43 (3H, s), 2.67, 2.97 (2H, t, J=7 Hz), 4.20, 4.45 (2H, t, J=7 Hz), 7.49 (8H, m). **44** (R^1=cyclohexyl): 97.3% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.50 (10H, m), 2.22, 2.47 (3H, s), 4.87 (1H, m), 7.78 (5H, m). **45** (R^1=2-methoxyethyl)[7b]: 90.2% yield. **46** (R^1=2-phenoxyethyl): 94.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.18, 2.42 (3H, s), 4.20 (4H, m), 7.42 (10H, m). **47** [R^1=2-(4-chloro)phenethyl]: 95.2% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.17, 2.47 (3H, s), 4.23 (4H, m), 7.39 (9H, m). **48** (R^1=3-isopropoxypropyl): 50.7% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.08 (6H, d, J=6 Hz), 1.79 (2H, m), 2.47 (3H, s), 3.80 (5H, m), 7.76 (5H, m). **49** (R^1=2-cyclopentyloxyethyl): 84.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (8H, m), 2.23, 2.49 (3H, s), 3.82 (5H, m), 7.84 (5H, m). **50** (R^1=2-methylthioethyl): 86.7% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.00, 2.17 (3H, s), 2.22, 2.48 (3H, s), 2.45, 2.83 (2H, t, J=7 Hz), 4.17, 4.45 (2H, t, J=7 Hz), 7.77 (5H, m). **51** (R^1=2-isopropylthioethyl): 93.2% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.20, 1.32 (6H, d, J=6 Hz), 2.23, 2.50 (3H, s), 2.51, 2.87 (2H, t, J=7 Hz), 2.97 (1H, m), 4.15, 4.42 (2H, t, J=7 Hz), 7.78 (5H, m). **52** (R^1=2-cyclopentylthioethyl): 92.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.59 (8H, m), 2.23, 2.48 (3H, s), 2.52, 2.87 (2H, t, J=7 Hz), 3.03 (1H, m), 4.17, 4.40 (2H, t, J=7 Hz), 7.82 (5H, m). **53** (R^1=2-phenylthioethyl): 84.2% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.17, 2.42 (3H, s), 2.82, 3.18 (2H, t, J=7 Hz), 4.12, 4.37 (2H, t, J=7 Hz), 7.62 (10H, m). **54** (R^1=3-dimethylaminopropyl): 53.1% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.86 (4H, m), 2.13 (6H, s), 2.47 (3H, s), 4.15, 4.33 (2H, t, J=7 Hz), 7.79 (5H, m). **55** (R^1=2-morpholinoethyl): 85.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.22, 2.49 (3H, s), 2.53 (6H, m), 3.67 (4H, m), 4.15, 4.42 (2H, t, J=7 Hz), 7.82 (5H, m). **56** [R^1=2-(4-phenylpiperidino)ethyl]: 54.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.36 (14H, m), 4.15, 4.41 (2H, t, J=7 Hz), 7.69 (9H, m). **57** [R^1=2-(2-thienyl)ethyl]: 94.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.12, 2.43 (3H, s), 2.73, 3.07 (2H, t, J=7 Hz), 4.23, 4.48 (2H, t, J=7 Hz), 7.52 (8H, m).

Alkyl (R^1) 3-Nitrobenzylideneacetoacetates (58—63)——**58—63** were prepared in a similar manner to that described for **34**. **58** (R^1=Me)[7c]: 80.2% yield. **59** (R^1=Et)[7c]: 87.5% yield. **60** (R^1=iso-Pr)[7c]: 32.4% yield. **61** (R^1=4-chlorophenethyl): 98.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.25, 2.37 (3H, s), 2.95 (2H, m), 4.46 (2H, m), 7.60 (9H, m). **62** (R^1=3,5-dimethoxyphenethyl): 90.4% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.27, 2.38 (3H, s), 2.91 (2H, m), 3.77, 3.80, 3.83, 3.87 (6H, s), 4.46 (2H, m), 7.43 (8H, m). **63** [R^1=2-(4-phenylpiperidino)ethyl]: 17.8% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (14H, m), 4.43, 4.47 (2H, t, J=7 Hz), 7.73 (10H, m).

The acetoacetates (**64—71**) were prepared in a similar manner to that described for **34**. Ethyl 2-Chlorobenzylideneacetoacetate (**64**)[7d]: 78.4% yield. 2-Chlorophenethyl 2,3-Dichlorobenzylideneacetoacetate (**65**): 94.5% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.12, 2.40 (3H, s), 2.76, 2.97 (2H, t, J=7 Hz), 4.30, 4.42 (2H, t, J=7 Hz), 7.35 (8H, m). Ethyl 2,6-Dichlorobenzylideneacetoacetate (**66**): 83.5% yield. ^{1}H-NMR ($CDCl_3$) δ: 1.00, 1.38 (3H, t, J=7 Hz), 2.33, 2.50 (3H, s), 4.08, 4.33 (2H, q, J=7 Hz), 7.18 (5H, m). Methyl 2-Trifluoromethylbenzylideneacetoacetate (**67**)[7d]: 94.0% yield. Methyl 2,3-Dimethoxybenzylideneacetoacetate (**68**): 93.0% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (3H, s), 3.78 (6H, s), 3.87 (3H, s), 7.43 (5H, m). Methyl 2-Pyridylmethylideneacetoacetate (**70**): 52.1% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.43, 2.51 (3H, s), 3.84, 3.90 (3H, s), 7.93 (5H, m). Methyl 3-Pyridylmethylideneacetoacetate (**71**): 91.7% yield. ^{1}H-NMR ($CDCl_3$) δ: 2.44 (3H, s), 3.87 (3H, s), 8.01 (5H, m). Methyl Benzylideneacetoacetate (**69**)[7a]: 90.5% yield.

Alkyl (R^1) 4-Aryl-1-R-3-R^3-4,7-dihydro-6-methylpyrazolo-[3,4-*b*]pyridine-5-carboxylates (72—149)——**72—149**, prepared by the following general procedure, are listed in Tables I and II. A solution of a 5-aminopyrazole (**1—33**, 5 mmol) and an alkyl arylmethylideneacetoacetate (**34—71**, 5 mmol) in *tert*-BuOH (10 ml) was heated at 80 °C for 24 h under N_2. After removal of the solvent, the residue was chromatographed on silica gel using benzene–EtOAc (2 : 1) and EtOAc as the eluants. The benzene–EtOAc eluate was evaporated to obtain the Schiff base (**151**). The product from the EtOAc eluate was recrystallized from the indicated solvents to obtain the corresponding 4,7-dihydropyrazolo[3,4-*b*]pyridine (**72—149**). When an oily product was obtained, it was converted into the hydrochloride or oxalate in the usual manner. The IR and ^{1}H-NMR spectra of the products are shown in Table III.

Methyl 4-(3-Aminophenyl)-3-cyclopentyl-4,7-dihydro-1,6-dimethylpyrazolo[3,4-*b*]pyridine-5-carboxylate (150)——A solution of **76** (1.0 g, 2.52 mmol) in MeOH (10 ml) was hydrogenated in the presence of 10% Pd–C (0.1 g) under atmospheric pressure. After absorption of H_2 (166 ml), the reaction was worked up, and the mixture was filtered and evaporated. The residue was chromatographed on silica gel with EtOAc and gave **150** (0.78 g, 84.1%). Recrystallization from EtOH gave colorless prisms, mp 106—110 °C (Table II). IR (Nujol): 3430, 3300, 1690 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.02 (12H, m), 3.35 (2H, br s), 3.45 (3H, s), 3.55 (3H, s), 5.00 (1H, s), 6.68 (4H, m), 7.58 (1H, br s).

Conversion of Methyl 3-Cyclopentyl-4,7-dihydro-6-methyl-4-phenylisoxazolo[5,4-*b*]pyridine-5-carboxylate (158) into 149—A solution of **158**[1] (2.67 g, 8.0 mmol) in EtOAc (25 ml) was hydrogenated over PtO_2 (0.3 g) in H_2 at room temperature for 6 h. The precipitated crystals were dissolved by addition of CH_2Cl_2. The solution, after removal of the catalyst by filtration, was evaporated and the residue was crystallized from EtOAc, giving **159** (2.5 g, 93.6%) as colorless prisms, mp 222—224 °C. IR (Nujol): 3450, 1695, 1605 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53 (8H, m), 2.27 (3H, s), 3.09 (1H, m), 3.70 (3H, s), 4.98 (1H, s), 7.19 (5H, m). *Anal.* Calcd for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.39; H, 7.00; N, 8.13. $POCl_3$ (6 ml) was added dropwise to a solution of **159** (2.0 g, 6.84 mmol) in CH_2Cl_2 (6 ml) under cooling, and the solution was stirred at 25 °C for 20 h. After removal of the solvent, the residue was

TABLE III. IR and ^{1}H-NMR Data for 4,7-Dihydropyrazolo[3,4-*b*]pyridines (**72**—**149**)

Compd. No.	IR (Nujol) (cm^{-1}) NH	CO	NO_2	^{1}H-NMR (in $CDCl_3$) δ
72	3280	1680	1350	2.45 (3H, s), 3.40 (3H, s), 3.70 (3H, s), 5.70 (1H, s), 7.34 (6H, m)
73	3275	1645	1352	1.87 (3H, s), 2.42 (3H, s), 3.57 (3H, s), 3.65 (3H, s), 5.20 (1H, s), 7.59 (5H, m)
74	3290	1690	1350	1.00 (6H, d, J=7 Hz), 1.21 (3H, t, J=7 Hz), 2.40 (3H, s), 2.52 (1H, m), 3.71 (3H, s), 4.05 (2H, q, J=7 Hz), 5.32 (1H, s), 7.54 (5H, m)
75	3350	1693	1345	1.03 (7H, m), 2.20 (2H, m), 2.40 (3H, s), 3.59 (3H, s), 3.68 (3H, s), 5.25 (1H, s), 7.68 (5H, m)
76	3375	1700	1380	2.00 (12H, m), 3.58 (3H, s), 3.67 (3H, s), 5.25 (1H, s), 7.40 (5H, m)
77	3270	1690	1350	1.90 (12H, m), 2.38 (3H, s), 3.67 (3H, s), 4.03 (2H, q, J=7 Hz), 5.25 (1H, s), 7.62 (5H, m)
78	2560	1693	1353[a]	1.14 (6H, d, J=7 Hz), 2.07 (12H, m), 3.65 (3H, s), 4.90 (2H, q, J=7 Hz), 5.23 (1H, s), 7.67 (5H, m)[b]
79	3290	1673	1355	1.99 (12H, m), 3.32 (3H, s), 3.67 (3H, s), 5.62 (1H, s), 7.47 (5H, m)
80	3260	1690	1360	1.50 (8H, m), 2.33 (3H, s), 2.92 (1H, m), 3.27 (3H, s), 3.51 (2H, m), 3.63 (3H, s), 4.13 (2H, m), 5.92 (1H, s), 7.20 (5H, m)
81	2495	1699	1352[a]	3.17 (14H, m), 3.62 (3H, s), 3.70 (3H, s), 5.31 (1H, s), 7.64 (5H, m)
82	3320	1685	1360	1.79 (14H, m), 3.50 (3H, s), 3.67 (3H, s), 5.87 (1H, s), 7.42 (5H, m)
83	3350	1695	1340	1.71 (16H, m), 2.40 (3H, s), 2.62 (1H, m), 3.58 (3H, s), 4.35 (1H, m), 5.25 (1H, s), 7.20 (5H, m)
84	3280	1678	1350	1.18 (3H, t, J=7 Hz), 2.43 (3H, s), 3.77 (3H, s), 4.07 (2H, q, J=7 Hz), 5.50 (1H, s), 7.41 (10H, m)
85	3360	1698	1345	1.13 (3H, t, J=7 Hz), 2.43 (3H, s), 4.02 (2H, q, J=7 Hz), 5.62 (1H, s), 7.47 (15H, m)[b]
86	3320	1700	1355	2.39 (3H, s), 3.58 (3H, s), 3.75 (3H, s), 3.78 (3H, s), 5.50 (1H, s), 7.57 (5H, m)
87	3370	1693	1350	1.26 (6H, d, J=6 Hz), 2.38 (3H, s), 3.63 (3H, s), 3.78 (3H, s), 5.17 (1H, m), 5.58 (1H, s), 7.71 (5H, m)
88	2360	1701[a]		1.17 (3H, t, J=7 Hz), 2.20 (12H, m), 3.52 (3H, s), 4.02 (2H, q, J=7 Hz), 5.62 (1H, s), 7.12 (5H, m)
89	3280	1670		1.07 (3H, t, J=7 Hz), 2.04 (12H, m), 3.57 (3H, s), 3.99 (2H, q, J=7 Hz), 6.08 (1H, s), 7.12 (5H, m)
90	3220	1690	1347	0.77 (6H, d, J=6 Hz), 1.82 (3H, m), 2.40 (3H, s), 3.57 (3H, s), 3.67 (3H, s), 5.20 (1H, s), 7.08 (1H, brs), 7.62 (4H, m)
91	3300	1700	1350	1.08 (9H, s), 2.42 (3H, s), 3.72 (3H, s), 3.78 (3H, s), 5.45 (1H, s), 6.57 (1H, brs), 7.67 (4H, m)
92	3220	1690	1348	2.39 (3H, s), 2.99 (2H, m), 3.58 (3H, s), 3.68 (3H, s), 4.92 (2H, m), 5.20 (1H, s), 5.74 (1H, m), 7.59 (4H, m), 7.80 (1H, brs)
93	3350	1700	1350	1.42 (9H, m), 2.19 (2H, m), 2.40 (3H, s), 3.57 (3H, s), 3.66 (3H, s), 5.22 (1H, s), 7.48 (5H, m)
94	3200	1690	1350	2.30 (3H, s), 3.52 (3H, s), 3.60 (5H, s), 5.00 (1H, s), 7.48 (10H, m)
95	3225	1695	1350	0.97 (5H, m), 2.40 (3H, s), 3.57 (3H, s), 3.60 (3H, s), 5.28 (1H, s), 7.54 (5H, m)
96	3375	1705	1350	2.59 (10H, m), 3.56 (3H, s), 3.65 (3H, s), 5.16 (1H, s), 7.64 (5H, m)
97	3355	1700	1350	1.85 (16H, m), 3.58 (3H, s), 3.67 (3H, s), 5.25 (1H, s), 7.54 (5H, m)
98	3340	1695	1340	1.56 (16H, m), 3.59 (3H, s), 3.68 (3H, s), 5.27 (1H, s), 7.47 (5H, m)
99	3380	1700	1345	2.63 (7H, m), 3.59 (3H, s), 3.68 (3H, s), 3.08 (1H, m), 5.23 (1H, s), 5.60 (2H, s), 7.52 (5H, m)
100	3340	1695	1345	1.86 (7H, m), 2.39 (3H, s), 3.59 (3H, s), 3.69 (3H, s), 5.28 (1H, s), 5.66 (2H, m), 7.52 (5H, m)
101	3310	1670	1355	1.66 (6H, m), 2.36 (3H, s), 2.85 (1H, m), 3.48 (3H, s), 3.68 (3H, s), 5.61 (2H, m), 5.86 (1H, s), 7.30 (5H, m)
102	3270	1690	1345	2.40 (3H, s), 3.24 (3H, s), 3.58 (3H, s), 3.65 (3H, s), 2.49 (2H, t, J=6 Hz), 3.40 (2H, m), 5.27 (1H, s), 7.00 (1H, brs), 7.73 (4H, m)
103	3225	1690	1345	1.59 (8H, m), 2.43 (3H, s), 3.54 (3H, s), 3.66 (3H, s), 3.86 (1H, m), 4.01 (2H, m), 5.30 (1H, s), 6.52 (1H, brs), 7.74 (4H, m)
104	3430	1690	1350	1.72 (4H, m), 2.38 (7H, m), 3.18 (2H, m), 3.53 (3H, s), 3.65 (3H, s), 5.28 (1H, s), 7.63 (5H, m)

TABLE III. (continued)

Compd. No.	IR (Nujol) (cm^{-1}) NH	CO	NO_2	^{1}H-NMR (in $CDCl_3$) δ
105	3240	1685	1350	1.60 (4H, m), 2.07 (3H, s), 2.39 (3H, s), 3.00 (3H, m), 3.54 (3H, s), 3.67 (3H, s), 5.28 (1H, s), 7.16 (5H, m)
106	3420	1690	1350	2.37 (3H, s), 3.27 (4H, m), 3.59 (3H, s), 3.69 (3H, s), 5.23 (1H, s), 7.70 (5H, m)
107	3355	1683	1350	2.40 (3H, s), 3.53 (3H, s), 3.83 (3H, s), 5.47 (1H, s), 7.60 (8H, m)
108	3360	1695	1355	2.45 (3H, s), 3.70 (3H, s), 3.85 (3H, s), 5.80 (1H, s), 7.75 (9H, m)
109	3360	1701	1350	2.39 (3H, s), 3.64 (3H, s), 3.74 (3H, s), 5.36 (1H, s), 7.57 (8H, m)
110	3330	1695	1345	2.30 (3H, s), 3.51 (3H, s), 3.72 (3H, s), 6.21 (1H, s), 7.29 (8H, m)
111		1667	1347	2.65 (3H, s), 3.18 (3H, s), 3.44 (3H, s), 3.60 (3H, s), 5.38 (1H, s), 7.73 (7H, m)
112	3270	1685	1350	1.54 (8H, m), 2.30 (3H, s), 3.51 (3H, s), 3.68 (1H, m), 3.77 (3H, s), 6.31 (1H, s), 7.28 (5H, m)
113	2510	1700	1373[a]	2.07 (12H, m), 2.37 (3H, s), 3.68 (3H, s), 3.93 (2H, q, J=7 Hz), 5.68 (1H, s), 7.59 (5H, m)
114	2200	1700	1355[a]	1.82 (17H, m), 3.67 (3H, s), 3.95 (2H, q, J=7 Hz), 5.93 (1H, s), 7.63 (5H, m)
115	2570	1697	1357[a]	1.97 (21H, m), 3.63 (3H, s), 3.91 (2H, m), 5.92 (1H, s), 7.36 (5H, m)
116	2520	1700	1358[a]	1.94 (23H, m), 3.45 (2H, m), 3.62 (3H, s), 5.90 (1H, s), 7.28 (5H, m)
117	2300	1700	1375[a]	1.55 (8H, m), 2.27 (3H, s), 2.92 (3H, m), 3.62 (3H, s), 4.23 (2H, m), 5.93 (1H, s), 7.64 (9H, m)
118	2370	1705	1360[a]	2.15 (12H, m), 2.73 (2H, t, J=7 Hz), 3.67 (3H, s), 4.13 (2H, m), 5.88 (1H, s), 7.24 (9H, m)
119	3270	1685	1350	1.59 (8H, m), 2.35 (3H, s), 2.60 (1H, m), 2.82 (2H, m), 3.66 (3H, s), 4.21 (2H, m), 5.12 (1H, s), 7.18 (9H, m)
120	2380	1713	1355[a]	1.54 (8H, m), 2.27 (3H, s), 2.72 (2H, t, J=7 Hz), 2.95 (1H, m), 3.62 (3H, s), 5.87 (1H, s), 7.33 (8H, m)
121	2590	1715	1338[a]	1.60 (8H, m), 2.25 (3H, s), 2.78 (1H, m), 2.83 (2H, t, J=7 Hz), 3.62 (3H, s), 4.17 (2H, t, J=7 Hz), 5.87 (1H, s), 7.40 (8H, m)
122	2360	1699	1348[a]	2.15 (9H, m), 2.70 (2H, t, J=7 Hz), 3.30 (3H, s), 3.64 (3H, s), 3.82 (6H, s), 4.11 (2H, m), 5.90 (1H, s), 6.72 (3H, s), 7.12 (5H, m)
123	3280	1685	1350	1.43 (8H, m), 2.36 (3H, s), 2.58 (1H, m), 2.83 (2H, m), 3.67 (3H, s), 3.85 (6H, s), 4.24 (2H, m), 5.20 (1H, s), 7.34 (8H, m)
124	2670	1700	1375[a]	2.19 (14H, m), 3.67 (3H, s), 4.17 (2H, t, J=7 Hz), 5.90 (1H, s), 7.55 (8H, m)
125	2525	1702	1355[a]	1.97 (22H, m), 3.63 (3H, s), 4.62 (1H, m), 6.03 (1H, s), 7.33 (5H, m)
126	2300	1700	1378[a]	1.49 (8H, m), 2.33 (3H, s), 2.88 (1H, m), 3.63 (3H, s), 4.12 (4H, m), 5.95 (1H, s), 7.24 (10H, m)
127	2590	1679	1357[a]	1.80 (14H, m), 3.63 (3H, s), 4.00 (2H, m), 4.33 (2H, m), 5.91 (1H, s), 7.23 (10H, m)
128	3280	1685	1355	1.46 (8H, m), 2.32 (3H, s), 2.94 (1H, m), 3.61 (3H, s), 4.17 (4H, m), 5.91 (1H, s), 7.17 (9H, m)
129	3240	1692	1355	1.49 (16H, m), 2.37 (3H, s), 2.89 (1H, m), 3.33 (3H, m), 3.67 (3H, s), 4.04 (2H, t, J=7 Hz), 5.90 (1H, s), 7.23 (5H, m)
130	3430	1690	1355	1.57 (16H, m), 2.36 (3H, s), 2.89 (1H, m), 3.66 (3H, s), 3.81 (3H, m), 5.91 (1H, s), 7.02 (5H, m)
131	2300	1702	1378[a]	2.10 (17H, m), 3.65 (3H, s), 4.08 (2H, m), 5.90 (1H, s), 7.52 (4H, m)
132	2670	1705	1363[a]	2.10 (21H, m), 3.67 (3H, s), 4.08 (2H, m), 5.90 (1H, s), 7.38 (5H, m)
133	2480	1701	1359[a]	2.17 (23H, m), 3.68 (3H, s), 4.10 (2H, m), 5.91 (1H, s), 7.33 (5H, m)
134	2700	1694	1352[a]	1.47 (8H, m), 2.30 (3H, s), 2.95 (3H, m), 3.60 (3H, s), 4.08 (2H, m), 5.85 (1H, s), 7.45 (9H, m)
135	3430	1695	1355	2.04 (22H, m), 3.70 (3H, s), 3.94 (3H, m), 5.91 (1H, s), 7.38 (4H, m)
136	2660	1712	1352[a]	2.09 (18H, m), 3.60 (3H, s), 3.65 (4H, m), 4.07 (2H, m), 5.92 (1H, s), 7.39 (5H, m)
137	3300	1695	1355	2.12 (23H, m), 3.64 (3H, s), 4.09 (2H, m), 5.93 (1H, s), 7.39 (10H, m)
138	3260	1680	1349	2.23 (23H, m), 3.70 (3H, s), 4.18 (2H, t, J=7 Hz), 5.28 (1H, s), 7.10 (10H, m)
139	2520	1703	1370[a]	1.68 (8H, m), 2.28 (3H, s), 2.98 (3H, m), 3.62 (3H, s), 4.17 (2H, m), 5.90 (1H, s), 7.30 (8H, m)

TABLE III. (continued)

Compd. No.	IR (Nujol) (cm^{-1}) NH	CO	NO_2	^{1}H-NMR (in $CDCl_3$) δ
140	3275	1670	1345	2.23 (3H, s), 2.83 (2H, t, J=7 Hz), 3.73 (3H, s), 4.20 (2H, m), 6.26 (1H, s), 7.25 (13H, m)
141	3280	1685	1345	2.24 (3H, s), 2.80 (2H, t, J=7 Hz), 3.74 (3H, s), 4.17 (2H, t, J=7 Hz), 6.23 (1H, s), 7.21 (12H, m)
142	3230	1673	1350	1.57 (11H, m), 3.73 (3H, s), 3.92 (5H, m), 6.27 (1H, s), 7.32 (8H, m)
143	3350	1675	1355	1.61 (8H, m), 2.38 (3H, s), 2.76 (2H, t, J=7 Hz), 3.62 (1H, m), 3.77 (3H, s), 4.17 (4H, t, J=7 Hz), 6.24 (1H, s), 7.21 (9H, m)
144	3430	1690		1.47 (8H, m), 2.32 (3H, s), 2.47 (1H, m), 3.53 (3H, s), 3.58 (3H, s), 5.17 (1H, s), 7.22 (5H, m)
145	3430	1690		1.54 (8H, m), 2.38 (3H, s), 2.80 (1H, m), 3.53 (3H, s), 3.70 (3H, s), 3.80 (3H, s), 5.66 (1H, s), 6.69 (4H, m)
146	3290	1695		2.23 (3H, s), 2.78 (2H, t, J=7 Hz), 3.58 (3H, s), 4.18 (2H, m), 5.68 (1H, s), 7.31 (11H, m)
147	3220	1655		1.44 (8H, m), 2.00 (3H, s), 2.58 (1H, m), 3.11 (3H, s), 3.54 (3H, s), 5.36 (1H, s), 7.96 (5H, m)
148	3240	1665		1.63 (8H, m), 2.33 (3H, s), 2.62 (1H, m), 3.59 (3H, s), 3.61 (3H, s), 5.14 (1H, s), 7.81 (5H, m)
149	2460	1698[a]		1.63 (8H, m), 2.29 (3H, s), 2.62 (1H, m), 3.42 (3H, s), 3.55 (3H, s), 5.12 (1H, s), 7.13 (5H, s)

a) Hydrochloride. *b*) In DMSO-d_6.

dissolved in CH_2Cl_2 (10 ml) and then methylhydrazine (1 ml) was added dropwise under cooling. The mixture was stirred at 25 °C for 20 h, and then refluxed for 4 h. After removal of the solvent, the residue was chromatographed on silica gel with CH_2Cl_2–EtOAc (10 : 1) to give a yellow liquid (0.4 g, 40%), which, when treated with EtOH–HCl, gave colorless crystals. This product was identified as **149** (Table II) by comparison of the IR spectrum with that of an authentic sample.

Methyl 3-Cyclopentyl-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylate (160)——$NaNO_2$ (0.26 g) was added portionwise to a solution of **76** (0.5 g, 1.26 mmol) in acetic acid (5 ml) at 20 °C. After stirring for 5 min, the mixture was poured into ice-water, and the precipitated crystalline solid was collected by filtration. Recrystallization from EtOH gave **160** (0.33 g, 66.4%) as colorless needles, mp 133—134 °C. IR (Nujol): 1722, 1345 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.84 (9H, m), 2.72 (3H, s), 3.57 (3H, s), 4.11 (3H, s), 8.03 (4H, m). *Anal.* Calcd for $C_{21}H_{22}N_4O_4$: C, 63.94; H, 5.62; N, 14.21. Found: C, 64.00; H, 5.45; N, 14.22.

Hydrolysis of 160——A solution of **160** (0.5 g, 1.27 mmol) and KOH (0.13 g) in 70% aq. MeOH (10 ml) was refluxed for 43 h. After evaporation of the solvent, the residue was dissolved in water. This solution was washed with ether, then acidified with aq. HCl. A crystalline solid which precipitated was obtained by filtration and recrystallized from MeOH, giving 3-cyclopentyl-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine-5-carboxylic acid (**161**, 0.44 g, 91.7%) as yellow prisms, mp 284—286 °C. IR (Nujol): 2540, 1710, 1345 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.98 (9H, m), 2.68 (3H, s), 4.00 (3H, s), 8.10 (4H, m). *Anal.* Calcd for $C_{20}H_{20}N_4O_4$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.02; H, 5.08; N, 14.73.

3-Cyclopentyl-1,6-dimethyl-4-(3-nitrophenyl)pyrazolo[3,4-*b*]pyridine (162)——A mixture of **161** (0.25 g, 0.66 mmol), $CuCO_3$ (0.03 g, 0.13 mmol) and quinoline (1.5 ml) was heated at 220 °C for 0.5 h under stirring. After cooling, the mixture was diluted with ether and the insoluble materials were filtered off. The filtrate was washed with aq. HCl and water, dried over $MgSO_4$, filtered and evaporated. The residue was chromatographed on silica gel with CH_2Cl_2–MeCN (9 : 1), giving **162** (0.18 g, 80.1%), which was recrystallized from EtOH. Yellow plates, mp 116—117 °C. MS m/z: 336 (M^+). IR (Nujol): 1353 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.13 (12H, m), 4.10 (3H, s), 6.87 (1H, s), 7.99 (4H, m). *Anal.* Calcd for $C_{19}H_{20}N_4O_2$: C, 67.84; H, 5.99; N, 16.66. Found: C, 67.99; H, 6.11; N, 16.58.

Reaction of 3-Nitrobenzoylacetone (163)[10] with 14——A solution of **163** (0.414 g, 2 mmol) with **14** (0.33 g, 2 mmol) in diphenylether (1 ml) was heated at 180 °C for 5 h, then cooled, and chromatographed on silica gel with CH_2Cl_2–EtOAc (4 : 1). The yellow crystalline product (0.5 g, 74.7%) obtained was identified as **162** by comparison of the IR spectrum with that of an authentic sample.

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References and Notes

1) *a*) Part I: T. Yamamori, Y. Hiramatsu, K. Sakai, and I. Adachi, *Tetrahedron*, **41**, 913 (1985); *b*) A part of this work was presented at the 105th Annual Meeting of the Pharmaceutical Society of Japan, Kanazawa, April 1985.
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Synthesis of 2-Phenylthiazolidine Derivatives as Cardiotonic Agents. V.[1] Modification of the Thiazolidine Moiety of 2-(Phenylpiperazinylalkoxyphenyl)thiazolidine-3-thiocarboxamides and the Corresponding Carboxamides

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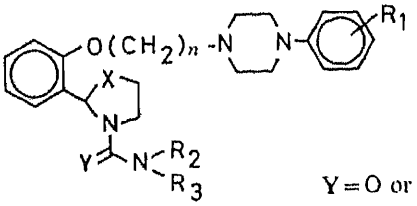
The carba (**2**) and oxa analogues (**3**) of 2-(phenylpiperazinylalkoxyphenyl)thiazolidine-3-carboxamide (**1**, Y=O) and -thiocarboxamide (**1**, Y=S) were synthesized and tested for cardiotonic activity. These analogues (**2** and **3**) were prepared from the aldehydes (**4**) through several intermediates (**7**, **10**, and **13**). In a series of the *N*-methylcarboxamides, positive inotropic activity in anesthetized dogs decreased in the following order: the thiazolidine (**1a**)≫oxazolidine (**3a**)>pyrrolidine (**2a**). In the corresponding thiocarboxamide series, however, the oxazolidine (**3b**) was the most potent, followed by the thiazolidine (**1b**) and the pyrrolidine (**2c**).

Keywords——2-arylthiazolidine-3-thiocarboxamide; 2-arylthiazolidine-3-carboxamide; 2-arylpyrrolidine-3-thiocarboxamide; 2-arylpyrrolidine-3-carboxamide; 2-aryloxazolidine-3-thiocarboxamide; 2-aryloxazolidine-3-carboxamide; cardiotonic agent; positive inotropic activity; structure-activity relationship

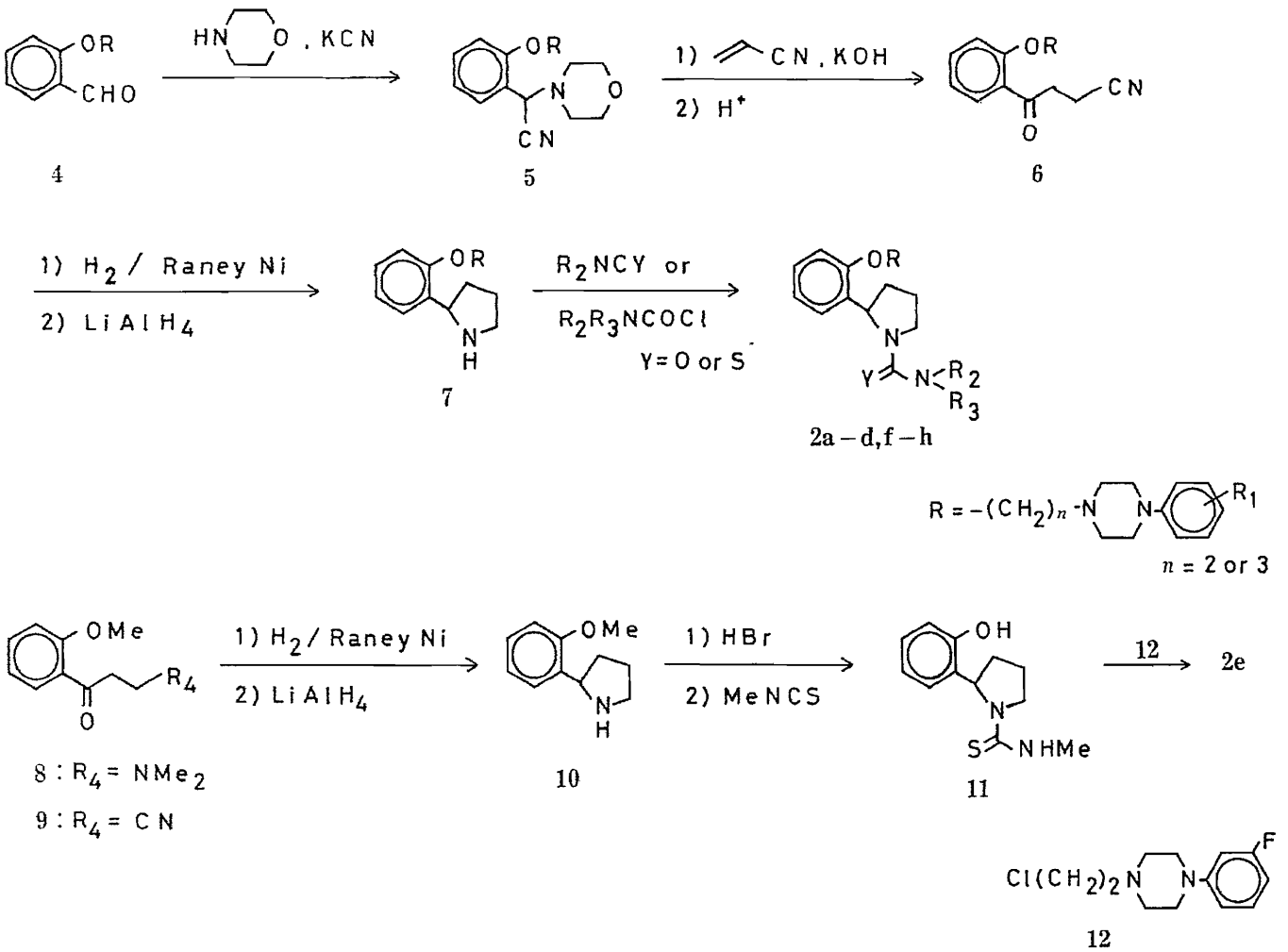
Previous papers of this series[1-4] described the synthesis and cardiotonic activity of a series of new 2-phenylthiazolidine-3-carboxamides and thiocarboxamides (**1**). These compounds exhibited marked and sustained positive inotropic activity without producing significant effects on heart rate or blood pressure in anesthetized dogs. Although the effect of various substituents in structure **1** on the activity became apparent through those studies, the effect of modification of the thiazolidine ring remained unclear. Our continued interest in the structure–activity relationships (SAR) of this class of compounds as a new class of cardiotonic agents led us to attempt replacement of the sulfur atom of the thiazolidine ring of **1** with carbon and oxygen. In this report we describe the synthesis and cardiotonic activity of the pyrrolidine (**2**) and oxazolidine (**3**), which are carba and oxa analogues of **1**, respectively.



Y=O or S

1: X=S
2: X=CH_2
3: X=O

Chart 1



4 5 6

7

2a – d,f – h

8 : R_4 = NMe_2

9 : R_4 = CN

10

11

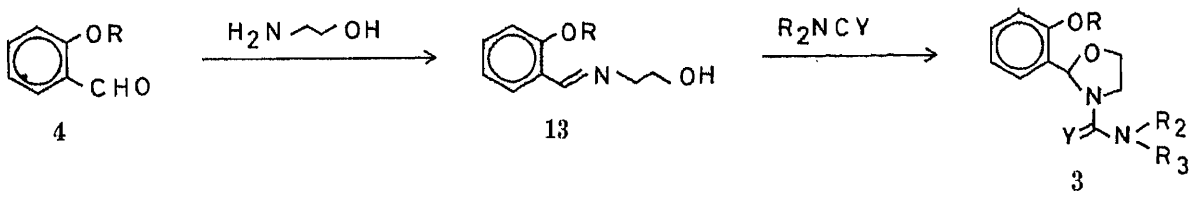
12

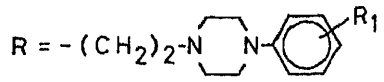
Chart 2

Chemistry

Synthesis of the pyrrolidine analogue (**2**) was carried out through the sequence of reactions outlined in Chart 2. Reaction of the benzaldehyde (**4**)[3] with morpholine and potassium cyanide gave the aminonitrile (**5**). Cyanoethylation of **5** followed by acidic hydrolysis[5] readily gave the ketonitrile (**6**). Catalytic hydrogenation of **6** over Raney Ni and reduction of the resulting pyrroline with lithium aluminum hydride ($LiAlH_4$)[6] gave the pyrrolidine (**7**). Carbamoylation or thiocarbamoylation of **7** by the usual method gave the carboxamide or thiocarboxamide derivatives (**2a—d, f—h**) listed in Table I.

As an alternative route to **2**, introduction of an aminoalkyl side chain into the phenolic thiocarboxamide (**11**) was attempted. The aminoketone (**8**)[7] was converted to the ketonitrile (**9**), which, on hydrogenation over Raney Ni and $LiAlH_4$ reduction, gave the pyrrolidine (**10**). *O*-Demethylation of **10** followed by treatment with methyl isothiocyanate (MeNCS) gave the phenolic thiocarboxamide (**11**). Alkylation of **11** with the aminoethyl chloride (**12**)[3] gave the aminoether (**2e**) (Table I) in only low yield (6.4%), probably due to concomitant *S*-alkylation of the thiocarboxamide group.





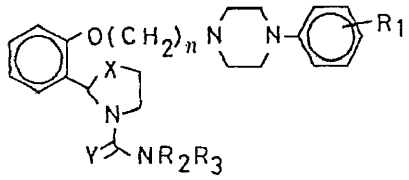
a: Y=O, $R_1=R_3=H$, $R_2=Me$
b: Y=S, $R_1=R_3=H$, $R_2=Me$
c: Y=S, R_1=3-F, $R_2=Me$, $R_3=H$
d: Y=O, $R_1=R_2=R_3=H$
e: Y=O, $R_1=R_3=H$, $R_2=COCH_3$

Chart 3

The oxazolidine analogue (**3**) was also synthesized from the benzaldehyde (**4**) (Chart 3). Condensation of aromatic aldehydes with ethanolamine has been claimed to give oxazolidines directly[8] or Schiff bases[9] without definite structural elucidation. When the aldehyde (**4**) was heated in benzene with continuous removal of water, the Schiff base (**13**) was obtained in quantitative yield. The spectroscopic data of **13** both in solution and in the solid state were in good agreement with the iminocarbinol structure and are given in the experimental section. The structure of **13** ($R_1=H$) was further confirmed by X-ray crystallographic analysis (Fig. 1). On treatment with methyl isocyanate in tetrahydrofuran, the Schiff base (**13**, $R_1=H$) gave the carbamoylated oxazolidine (**3a**) in 82.6% yield. The presence of a singlet at δ 6.36 in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum and absence of an OH absorption in the infrared (IR) spectrum are consistent with the assigned structure. Similar formation of *N*-acyl-2-aryl oxazolidines from benzylideneaminoethanol by treatment with acylating agents has been reported by several workers.[10] Treatment of **13** with MeNCS similarly gave the oxazolidine-3-thiocarboxamide (**3b, c**). The unsubstituted 3-carboxamide (**3d**) was obtained from **13** by treatment with trimethylsilyl isocyanate (TMSNCO)[11] followed by hydrolytic work-up.

Acetylation of **3d** with acetyl chloride gave the diacetate of **3d** in 86.8% yield. Hydrolysis of the diacetate with aq. sodium hydroxide solution gave the *N*-acetylurea (**3e**). The physical properties of **3a—e** are summarized in Table I. These oxazolidines were quite susceptible to

TABLE I. 2-(2-(Phenylpiperazinoalkoxy)phenyl)pyrrolidines and Oxazolidines (**2** and **3**)



Compd. No.	n	X	Y	R_1	R_2	R_3	Yield (%)	Salt	mp (°C) (Recrystn. solvent)[b]	Analysis (%) Calcd (Found)			Myocardial contractility[g] Anesthetized dog		
										C	H	N	Dose mg/kg i.v.	LVdP/dt_{max} Δ%	Duration (min)
2a	2	CH_2	O	H	CH_3	H	65.4	—	151.5—154 (A)						
								Ox[a]	165—168[c] (B)	$C_{24}H_{32}N_4O_2 \cdot C_2H_2O_4$			0.3	16	17
										62.64	6.87	11.24			
										(62.73	6.84	11.20)			
2b	2	CH_2	O	3-F	CH_3	H	78.8	—	159—163 (A)						
								Ox	180.5—182.5[c] (C)	$C_{24}H_{31}FN_4O_2 \cdot C_2H_2O_4 \cdot H_2O$			0.03	22	20
										58.42	6.60	10.48			
										(58.36	6.32	10.26)			
2c	2	CH_2	S	H	CH_3	H	59.5	—	100—103 (A)						
								Ox	163—168[c] (D)	$C_{24}H_{32}N_4OS \cdot C_2H_2O_4$			0.1	27	25
										60.68	6.66	10.89			
										(60.62	6.83	11.08)			
2d	3	CH_2	O	H	CH_3	H	78.8	—	105—108 (A)						
								Ox	118—123[c] (E)	$C_{25}H_{34}N_4O_2 \cdot C_2H_2O_4 \cdot 0.5H_2O$			0.1	23	15
										62.17	7.15	10.74			
										(62.50	7.01	10.56)			
2e	2	CH_2	S	3-F	CH_3	H	6.4[d]	—	94—99 (A)						
								Ox	149—152[c] (F)	$C_{24}H_{31}FN_4OS \cdot C_2H_2O_4$			0.03	39	40
										58.63	6.24	10.52			
										(58.51	6.21	10.34)			

2f	2	CH_2	O	H	CH_3CO	H	76.0	—	130—131 (A)				
								Ox	169.5—170[c] (F)	$C_{25}H_{32}N_4O_3 \cdot C_2H_2O_4$	0.03	30	27
										61.59 6.51 10.64			
										(61.86 6.67 10.69)			
2g	2	CH_2	O	H	H	H	75.3	—	122—124 (A)				
								Ox	136—138[c] (F)	$C_{23}H_{30}N_4O_2 \cdot C_2H_2O_4 \cdot 0.33H_2O$	0.03	26	13
										61.21 6.71 11.42			
										(61.25 6.96 11.31)			
2h	2	CH_2	O	H	CH_3	CH_3	85.6	—	Oil —				
								Ox	156—158[c] (F)	$C_{25}H_{34}N_4O_2 \cdot C_2H_2O_4$	0.03	27	21
										63.26 7.08 10.93			
										(63.02 7.04 10.92)			
3a	2	O	O	H	CH_3	H	82.6	—	129—132 (A)	$C_{23}H_{30}N_4O_3$	0.1	25	21
										67.29 7.37 13.65			
										(67.25 7.35 13.71)			
3b	2	O	S	H	CH_3	H	76.5	—	125—127 (G)	$C_{23}H_{30}N_4O_2S$	0.03	20	21
										67.76 7.09 13.13			
										(67.89 7.19 13.01)			
3c	2	O	S	3-F	CH_3	H	57.5	—	83—88 (H)	$C_{23}H_{29}FN_4O_2S$	0.01	19	45
										62.14 6.57 12.60			
										(62.33 6.62 12.47)			
3d	2	O	O	H	H	H	30.3	—	165—168 (I)	$C_{22}H_{28}N_4O_3$	0.1	32	29
										66.65 7.12 14.13			
										(66.35 7.25 14.38)			
3e	2	O	O	H	CH_3CO	H	55.4[e]	—	123—125 (A)	$C_{24}H_{30}N_4O_4$	0.03	27	30
										65.73 6.90 12.78			
										(65.99 6.71 12.84)			
1a[f]	2	S	O	H	CH_3	H					0.003	30	30
1b[f]	2	S	S	H	CH_3	H					0.1	27	20
Amrinone											0.3	25	24

a) Ox represents oxalate. *b*) A=AcOEt–hexane, B=acetone–MeOH–Et_2O, C=acetone–hexane, D=MeOH, E=EtOH–Et_2O, F=acetone, G=AcOEt–Et_2O, H=iso-PrOH–iso-Pr_2O, I=EtOH. *c*) With decomposition. *d*) Yield from 11. *e*) Yield from 3d. *f*) See reference 3. *g*) Myocardial contractility was examined with the salts. For methodology, see reference 12.

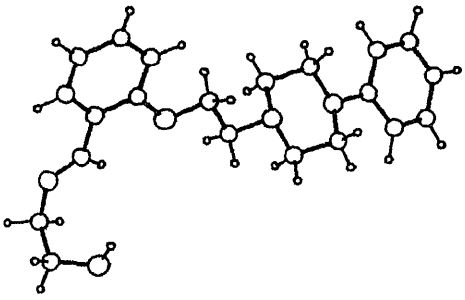


Fig. Perspective Drawing of the Molecule of **13**

acidic hydrolysis and decomposed to the aldehyde (**4**) even during salt formation with oxalic acid. After examination of the stability of various salts of **3**, the D-glucuronate was found to be the most stable salt. The oxazolidines (**3a**—**e**) were, therefore, converted to their D-glucuronates in ether, and the precipitated amorphous powder was used for assessment of cardiotonic activity without purification.

Pharmacology and Structure–Activity Relationships

The positive inotropic activity of the pyrrolidine (**2a**—**h**) and oxazolidine (**3a**—**e**) analogues prepared in the present study was determined by measuring the increase in the maximum derivative of left ventricular pressure ($LVdP/dt_{max}$) after i.v. administration to anesthetized dogs.[12] The results are included in Table I together with comparative data for the corresponding thiazolidine derivatives (**1a**, **b**) and amrinone.

Generally, the pyrrolidine and oxazolidine analogues (**2** and **3**) exhibited stronger positive inotropy than amrinone. The thiocarboxamides (**2c** and **3b**) were more potent than the corresponding carboxamides (**2a** and **3a**) in both the pyrrolidine and oxazolidine series. This constitutes a major deviation from the SAR in the thiazolidine series,[3] where the carboxamide (**1a**) was much more potent than the thiocarboxamide (**1b**). As in the previous case,[3] introduction of a fluoro group onto the benzene ring of the piperazine moiety produced a significant increase in activity (**2a** *vs.* **2b**, **2c** *vs.* **2e**, and **3b** *vs.* **3c**). As for the effect of the substituent on the (thio)carboxamide group, the *N*-acetyl derivatives (**2f** and **3e**) were more active than the corresponding *N*-methyl derivatives (**2a** and **3a**). This tendency was more pronounced in the pyrrolidine series, where the *N*-dimethyl (**2h**) and unsubstituted (**2g**) derivatives also showed potent activity. Finally, the effects of the conversion of the thiazolidine to the oxazolidine and pyrrolidine rings on the activity varied with the nature of the *N*-substituents. Looking at a series of the *N*-methylcarboxamides, one observes that positive inotropic activity decreases in the following order: the thiazolidine (**1a**) ≫ oxazolidine (**3a**) > pyrrolidine (**2a**). In the corresponding thiocarboxamide series, however, the oxazolidine (**3b**) was the most potent, followed by the thiazolidine (**1b**) and pyrrolidine (**2c**). This is in sharp contrast with the SAR in a series of simple 2-phenylthiazolidine-3-thiocarboxamides,[4] where the conversion of the thiazolidine ring to the oxazolidine ring caused a marked fall in activity. These results suggest an important role of the phenylpiperazine group in the appearance of positive inotropy in this series of compounds.

Experimental

All melting points are uncorrected. IR spectra were recorded on a Hitachi IR-215 spectrometer. ^{1}H-NMR spectra were taken in $CDCl_3$, unless otherwise noted, at 60 MHz on a JEOL PMX-60 spectrometer with tetramethylsilane (TMS) as an internal reference. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument.

Analytical data are given in Table I, unless otherwise noted.

α-(2-(2-(4-Phenylpiperazinyl)ethoxy)phenyl)-4-morpholineacetonitrile (5, $n=2$, $R_1=H$)——A mixture of mor-

pholine (120 g, 1.38 mol), *p*-toluenesulfonic acid (hydrate, 10.1 g, 0.0531 mol), 2-(2-(4-phenylpiperazinyl)-ethoxy)benzaldehyde[3] (15 g, 0.0484 mol), and tetrahydrofuran (THF) (50 ml) was heated at 80 °C for 1.5 h. Then, a solution of KCN (4.7 g, 0.0722 mol) in H_2O (50 ml) was added to the mixture, and the whole was heated at 100 °C for 1.5 h. After cooling, the reaction mixture was extracted with AcOEt. The AcOEt extracts were washed with water, dried, and evaporated. The residue was digested with a mixture of AcOEt–hexane and filtered to give 18.3 g (93.1%) of **5** ($n=2$, $R_1=H$), mp 108—110 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 1600. MS *m/z*: 406 (M^+), 379, 321, 186. ^{1}H-NMR δ: 2.53—2.94 (10H, m), 3.13—3.29 (4H, m), 3.60—3.75 (4H, m), 4.19 (2H, t, $J=6.2$ Hz), 5.13 (1H, s, >CHCN), 6.82—7.50 (9H, m). *Anal.* Calcd for $C_{24}H_{30}N_4O_2$: C, 70.91; H, 7.44; N, 13.78. Found: C, 71.15; H, 7.49; N, 13.64.

The 3-fluorophenyl analogue (**5**, $n=2$, $R_1=3$-F) was similarly obtained in a quantitative yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 1610, 1580. MS *m/z*: 424 (M^+), 397, 339, 204.

The propyloxy analog (**5**, $n=3$, $R_1=H$) was similarly obtained in 98.6% yield and had mp 109—110 °C (from Et_2O–hexane). *Anal.* Calcd for $C_{25}H_{32}N_4O_2$: C, 71.40; H, 7.67; N, 13.32. Found: C, 71.58; H, 7.60; N, 13.53.

3-(2-(2-(4-Phenylpiperazinyl)ethoxy)benzoyl)propionitrile (6, $n=2$, $R_1=H$)——A methanolic KOH solution (30%, 1 ml) was added to a stirred solution of **5** ($n=2$, $R_1=H$; 35 g, 0.086 mol) in THF (200 ml). A solution of acrylonitrile (6.85 g, 0.129 mol) in THF (50 ml) was then added to the mixture, and the whole was stirred at room temperature for 3 h. The reaction mixture was concentrated *in vacuo* and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. The residue was dissolved in a solution of AcOH (100 ml), H_2O (50 ml), and THF (200 ml) and allowed to stand at room temperature for 20 h. The solution was evaporated, made basic with aq. 10% K_2CO_3, and extracted with AcOEt. The extracts were washed with H_2O, dried, and evaporated. The residue was purified by SiO_2 chromatography (benzene–AcOEt (1 : 1)) and recrystallized from AcOEt–hexane to give 22.6 g (72.2%) of **6** ($n=2$, $R_1=H$), mp 98—100 °C. IR ν_{max}^{Nujol} cm^{-1}: 2240, 1660, 1590. MS *m/z*: 363 (M^+), 189, 175. ^{1}H-NMR δ: 2.58—2.99 (8H, m), 3.08—3.62 (6H, m), 4.24 (2H, t, $J=6.2$ Hz), 6.80—7.95 (9H, m). The oxalate was recrystallized from MeOH and had mp 173.5—174.5 °C (dec.). *Anal.* Calcd for $C_{24}H_{27}N_3O_6$: C, 63.57; H, 6.00; N, 9.27. Found: C, 63.50; H, 5.99; N, 9.36.

The 3-fluorophenyl analogue (**6**, $n=2$, $R_1=3$-F) was similarly obtained in 63.7% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 2220, 1660, 1600, 1580. MS *m/z*: 381 (M^+), 207, 193.

The propyloxy analogue (**6**, $n=3$, $R_1=H$) was similarly obtained in 57.3% yield and had mp 70—72 °C (from AcOEt–hexane). *Anal.* Calcd for $C_{23}H_{27}N_3O_2$: C, 73.18; H, 7.21; N, 11.13. Found: C, 73.31; H, 7.29; N, 11.23.

2-(2-(2-(4-Phenylpiperazinyl)ethoxy)phenyl)pyrrolidine (7, $n=2$, $R_1=H$)——A mixture of **6** ($n=2$, $R_1=H$; 10 g, 0.0275 mol), Raney Ni (40 ml), and EtOH (400 ml) was hydrogenated at room temperature and atmospheric pressure. After 5 h, the catalyst and solvent were removed, and the residue was dissolved in THF (100 ml). The solution was added to a stirred suspension of $LiAlH_4$ (2 g, 0.0527 mol) in THF (20 ml), and the whole was refluxed for 1.5 h. The mixture was decomposed by addition of moist Et_2O, and inorganic materials were filtered off. The filtrate was diluted with AcOEt, washed with H_2O, dried, and evaporated. The residue was purified by chromatography on SiO_2 ($CHCl_3$–MeOH–Et_3N (23 : 1 : 1)) to give 7.93 g (82.1%) of **7** ($n=2$, $R_1=H$) as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300, 1590. MS *m/z*: 351 (M^+), 350, 349, 219, 217. ^{1}H-NMR δ: 1.52—2.24 (4H, m), 2.30—2.96 (8H, m), 3.05—3.49 (5H, m), 4.18 (2H, t, $J=6$ Hz), 3.95—4.55 (1H, m, CH-N), 6.50—7.60 (9H, m).

The 3-fluorophenyl analogue (**7**, $n=2$, $R_1=3$-F) was similarly obtained in 53.8% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300, 1600, 1580. MS *m/z*: 369 (M^+), 259, 245, 233, 219.

The propyloxy analogue (**7**, $n=3$, $R_1=H$) was similarly obtained in 47.3% yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300, 1590, 1490, 750. MS *m/z*: 365 (M^+), 364, 233, 231, 173, 162.

***N*-Methyl-2-(2-(2-(4-phenylpiperazinyl)ethoxy)phenyl)pyrrolidine-1-carboxamide (2a)**——According to the method[4] described previously, the title compound (**2a**) was obtained from **7** ($n=2$, $R_1=H$; 1.50 g, 0.00427 mol) and methyl isocyanate (0.29 g, 0.00508 mol) in a yield of 1.14 g (65.4%) as colorless needles. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1630. MS *m/z*: 409, 408 (M^+), 407, 406, 393, 302, 290, 289, 277, 271, 219. ^{1}H-NMR δ: 1.57—2.50 (4H, m), 2.60—3.00 (9H, m), 3.17—3.32 (4H, m), 3.67 (2H, brt, $J=5.2$ Hz), 4.11 (2H, t, $J=8$ Hz), 3.82—4.40 (1H, m), 5.06 (1H, dd, $J=2$, 7 Hz), 6.50—7.50 (9H, m).

Compounds **2b—d**, **f—h** were also prepared from **7** by the reported procedure.[4]

3-(2-Methoxybenzoyl)propionitrile (9)——A mixture of **8**·hydrochloride (28.5 g, 0.117 mol) and potassium cyanide (25 g, 0.384 mol) in H_2O (200 ml) was refluxed for 15 min with stirring. After being cooled, the mixture was made acidic with 10% aq. HCl and extracted with AcOEt. The extract was washed with H_2O, dried, and evaporated. Chromatographic purification of the residue (SiO_2, benzene) gave 7.90 g (35.7%) of **9** as an oil. IR ν_{max}^{liquid} cm^{-1}: 2240, 1665, 1595, 755. MS *m/z*: 189 (M^+), 162, 135. ^{1}H-NMR δ: 2.57—2.80 (2H, m), 3.26—3.50 (2H, m), 3.90 (3H, s), 6.89—7.85 (4H, m).

2-(2-Methoxyphenyl)pyrrolidine (10)——According to the method described for the preparation of **7**, the title compound (**10**) was obtained from **9** (7.90 g, 0.0418 mol) in a yield of 5.96 g (80.6%) as an oil. IR ν_{max}^{liquid} cm^{-1}: 3300 (br), 1590, 750. MS *m/z*: 177 (M^+), 176, 160, 148, 137. ^{1}H-NMR δ: 1.55—2.25 (5H, m), 2.65—3.35 (2H, m), 3.80 (3H, s), 4.23—4.47 (1H, m), 6.74—7.45 (4H, m).

2-(2-Hydroxyphenyl)-*N*-methylpyrrolidine-1-thiocarboxamide (11)——A mixture of **10**·HCl (1.53 g, 0.0072 mol)

and 48% aq. HBr (10 ml) was heated at 120 °C for 4 h and then evaporated. The residue was made basic with aq. $NaHCO_3$ solution and extracted with AcOEt. The extract was dried and evaporated, and the residue was dissolved in EtOH (25 ml). Methyl isothiocyanate (0.7 g, 0.0096 mol) was added to the solution, and the mixture was refluxed for 2 h then evaporated. The residue was purified by SiO_2 chromatography ($CHCl_3$–EtOH (40:1)) to give, after recrystallization from MeOH–Et_2O, 0.69 g (40.8%) of **11**, mp 200—202 °C. IR ν_{max}^{Nujol} cm^{-1}: 3320, 3200, 1590, 1530. MS *m/z*: 236 (M^+), 219, 162. ^{1}H-NMR ($CDCl_3$–DMSO-d_6): δ: 1.40—2.30 (4H, m), 3.00 (3H, d, J=5.0 Hz), 3.80—4.15 (2H, m), 5.25—5.40 (1H, m), 5.90 (1H, br), 6.75—7.30 (4H, m), 9.15 (1H, s). *Anal.* Calcd for $C_{12}H_{16}N_2OS$: C, 60.99; H, 6.82; N, 11.85; S, 13.57. Found: C, 61.14; H, 6.78; N, 11.71; S, 13.39.

2-(2-(2-(4-(3-Fluorophenyl)piperazinyl)ethoxy)phenyl)-*N*-methylpyrrolidine-1-thiocarboxamide (2e)——A mixture of **11** (0.64 g, 0.0027 mol), 1-(2-chloroethyl)-4-(3-fluorophenyl)piperazine (**12**) hydrochloride[3] (0.76 g, 0.0027 mol), K_2CO_3 (0.37 g, 0.0027 mol), and NaI (0.405 g, 0.0027 mol) in dimethylformamide (DMF) (10 ml) was heated at 90 °C for 10 h. A further amount of **12**·HCl (0.38 g, 0.0014 mol) was added to the mixture, and the whole was heated at 90 °C for 8 h. The reaction mixture was concentrated, diluted with H_2O, and extracted with AcOEt. The extract was washed with H_2O, dried and evaporated. The residue was purified by chromatography on SiO_2 ($CHCl_3$–Me_2CO (5:1)) to give 0.075 g (6.4%) of **2e**, mp 94—99 °C (from AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 3300, 1600, 1550, 1480, 750. MS *m/z*: 442 (M^+), 368, 292, 236, 219, 193, 162, 150.

2-(2-(2-(4-Phenylpiperazinyl)ethoxy)benzylideneamino)ethanol (13)——A solution of **4** (n=2, R_1=H[3]; 10.24 g, 0.033 mol) and ethanolamine (2.12 g, 0.035 mol) in benzene (200 ml) was refluxed for 6 h with continuous removal of H_2O. The solution was washed with sat. NaCl solution, dried, and evaporated. The residue was digested with iso-Pr_2O and filtered to give 11.5 g (98.6%) of **13** (n=2, R_1=H), mp 106—112 °C. The analytical sample was recrystallized from CCl_4 and had mp 115—118 °C. IR ν_{max}^{Nujol} cm^{-1}: 3200 (br), 1630, 1590. MS *m/z*: 353 (M^+), 308, 221, 188, 132, 119. ^{1}H-NMR δ: 2.50—3.26 (11H, m), 3.62—3.77 (4H, m), 4.13 (2H, t, J=6.0 Hz), 6.81—7.49 (8H, m), 7.85—7.99 (1H, m), 8.74 (1H, s). *Anal.* Calcd for $C_{21}H_{27}N_3O_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.52; H, 7.79; N, 11.74.

The 3-fluorophenyl analogue (**13**, n=2, R_1=3-F) was similarly obtained in quantitative yield as an oil. IR ν_{max}^{liquid} cm^{-1}: 3400—3200, 1630, 1610, 1580, 1490, 750. MS *m/z*: 371 (M^+), 326, 221, 206, 204, 193, 191, 150, 137, 122. ^{1}H-NMR δ: 2.45—2.96 (7H, m), 3.13—3.28 (4H, m), 3.56—3.95 (4H, m), 4.18 (2H, t, J=5.8 Hz), 6.41—7.60 (7H, m), 7.89—8.02 (1H, m), 8.77 (1H, s).

***N*-Methyl-2-(2-(2-(4-phenylpiperazinyl)ethoxy)phenyl)oxazolidine-3-carboxamide (3a)**——A mixture of **13** (R_1=H; 1.98 g, 0.0056 mol), methyl isocyanate (0.48 g, 0.0084 mol), and THF (50 ml) was heated at 50 °C for 3.5 h. Then, a further amount of methyl isocyanate (0.32 g, 0.0056 mol) was added to the mixture, and the whole was heated at 50 °C for 30 min. The reaction mixture was evaporated and the residue was purified by chromatography on SiO_2 to give, after recrystallization from AcOEt–hexane, 1.9 g (82.6%) of **3a**, mp 129—132 °C. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1620. MS *m/z*: 410 (M^+), 395, 353, 278, 221, 132. ^{1}H-NMR δ: 2.66—4.34 (19H, m), 4.59—4.65 (1H, br s), 6.36 (1H, s), 6.80—7.40 (9H, m).

The thiocarboxamides (**3b, c**) were prepared from **13** in the same manner as described above.

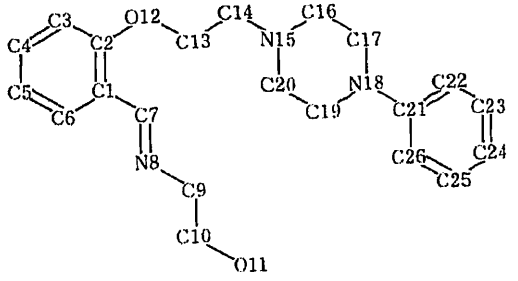
2-(2-(2-(4-Phenylpiperazinyl)ethoxy)phenyl)oxazolidine-3-carboxamide (3d)——A solution of TMSNCO[11] (2.94 g, 0.0255 mol) in CH_2Cl_2 (6 ml) was added to a stirred solution of **13** (R_1=H; 6 g, 0.017 mol) in CH_2Cl_2 (70 ml) under ice-cooling, and the mixture was stirred at room temperature for 2 d. A further amount (1.96 g, 0.017 mol) of TMSNCO was added to the mixture, and the whole was stirred for 24 h. The reaction mixture was washed with H_2O, dried, and evaporated. The residue was purified by chromatography on SiO_2 ($CHCl_3$–MeOH (30:1)) to give 2.04 g (30.3%) of **3d**, mp 165—168 °C (from EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3440, 3420, 3140, 1660. MS *m/z*: 396 (M^+), 381, 353, 221, 188, 175, 132, 119. ^{1}H-NMR δ: 2.64—4.34 (16H, m), 5.02 (2H, br s), 6.44 (1H, s), 6.76—7.50 (9H, m).

***N*-Acetyl-2-(2-(2-(4-phenylpiperazinyl)ethoxy)phenyl)oxazolidine-3-carboxamide (3e)**——Acetyl chloride (0.776 g, 0.0099 mol) was added to a solution of **3d** (0.979 g, 0.0025 mol), Et_3N (1 ml), THF (30 ml), and toluene (20 ml), and the mixture was heated at 80 °C for 45 min. The mixture was evaporated, and the residue was diluted with H_2O and extracted with AcOEt. The extract was washed with H_2O, dried, and evaporated. The residue was purified by chromatography on SiO_2 ($CHCl_3$–MeOH (50:1)) to give 1.03 g (86.6%) of the diacetate of **3d** as an amorphous powder. MS *m/z*: 480 (M^+), 465, 352, 189, 175, 132. A solution of NaOH (0.257 g, 0.0064 mol) in H_2O (2.5 ml) was added to a stirred solution of the diacetate (1.03 g, 0.0021 mol) in THF (10 ml) and EtOH (8 ml) under ice-cooling. The mixture was stirred under ice-cooling for 30 min. After addition of sat. NaCl solution (80 ml), the reaction mixture was adjusted to pH 7.0 with 10% aq. HCl solution and extracted with AcOEt. The extract was washed with sat. NaCl solution, dried, and evaporated. The residue was purified by chromatography on SiO_2 ($CHCl_3$–MeOH (50:1)) to give, after recrystallization from AcOEt–hexane, 0.6 g, (64%) of **3e**, mp 123—125 °C. IR ν_{max}^{Nujol} cm^{-1}: 3270, 1700, 1680, 1600. MS *m/z*: 438 (M^+), 353, 308, 221, 186, 132. ^{1}H-NMR δ: 2.37 (3H, s), 2.64—4.40 (16H, m), 6.51 (1H, s), 7.22—7.55 (9H, m), 8.02 (1H, m).

Crystal Data for 13——$C_{21}H_{27}N_3O_2$, M_r=353.47, monoclinic, a=21.635 (1), b=8.264 (1), c=11.249 (1) Å, β=104.409 (4)°, V=1948.0 (2) Å^3, D_c=1.205 g/cm^3, space group $P2_1/a$.

X-Ray Analysis——A single crystal of **13** was obtained from CCl_4. The diffraction intensities were measured on a four-circle diffractometer (Rigaku AFC-5) using graphite-monochromated CuK_α radiation. The intensities of 3315 independent reflections were collected and used in the structure determination. The structure was solved by the direct

TABLE II. Fractional Coordinates ($\times 10^4$) and Isotropic Thermal Parameters ($Å^2$) for **13**



Atom	x	y	z	B_{eq}	Atom	x	y	z	B_{eq}
C(1)	4974 (1)	3857 (3)	3215 (2)	4.9 (0.1)	C(14)	4532 (1)	1065 (4)	6495 (3)	5.6 (0.1)
C(2)	4488 (1)	3055 (3)	3596 (2)	4.7 (0.1)	N(15)	4103 (1)	358 (3)	7183 (2)	5.0 (0.1)
C(3)	3873 (1)	2949 (4)	2812 (3)	5.1 (0.1)	C(16)	3723 (2)	1627 (4)	7593 (3)	11.1 (0.2)
C(4)	3759 (2)	3625 (4)	1658 (3)	5.6 (0.1)	C(17)	3286 (2)	907 (5)	8309 (3)	12.4 (0.2)
C(5)	4236 (2)	4414 (4)	1264 (3)	6.7 (0.2)	N(18)	3647 (1)	3 (3)	9364 (2)	7.7 (0.1)
C(6)	4834 (2)	4518 (3)	2041 (3)	6.4 (0.1)	C(19)	4046 (1)	−1224 (3)	8993 (2)	6.4 (0.1)
C(7)	5619 (1)	3942 (3)	4035 (2)	5.0 (0.1)	C(20)	4474 (1)	−467 (4)	8281 (2)	6.2 (0.1)
N(8)	6102 (1)	4288 (3)	3644 (2)	6.1 (0.1)	C(21)	3352 (1)	−354 (3)	10315 (2)	4.9 (0.1)
C(9)	6717 (1)	4289 (4)	4546 (3)	5.0 (0.2)	C(22)	2764 (1)	314 (4)	10360 (3)	5.4 (0.2)
C(10)	7117 (1)	2876 (4)	4307 (3)	5.9 (0.2)	C(23)	2520 (2)	73 (5)	11366 (3)	6.0 (0.2)
O(11)	6873 (1)	1369 (3)	4586 (2)	6.0 (0.1)	C(24)	2836 (2)	−844 (5)	12354 (3)	6.3 (0.2)
O(12)	4662 (1)	2381 (3)	4736 (2)	5.7 (0.1)	C(25)	3406 (2)	−1536 (5)	12307 (3)	8.1 (0.2)
C(13)	4184 (1)	1580 (4)	5214 (3)	5.3 (0.1)	C(26)	3668 (2)	−1322 (4)	11315 (3)	7.7 (0.2)

Isotropic thermal parameters are in the form $B_{eq}=4/3\sum_i\sum_j \beta_{ij}\boldsymbol{a}_i\cdot\boldsymbol{a}_j$.

method using MULTAN and refined by the block-diagonal least-squares method. The final R index was 0.054. Fractional coordinates and thermal parameters are listed in Table II.

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Synthesis of 1,2,3,4-Tetrahydro-β-carboline Derivatives as Hepatoprotective Agents. II. Alkyl 1,2,3,4-Tetrahydro-β-carboline-2-carbodithioates

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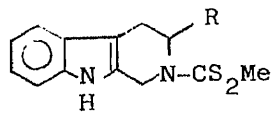
A large number of alkyl 1,2,3,4-tetrahydro-β-carboline-2-carbodithioates (**2** and **5**) with 3-hydroxycarbonyl and 3-hydroxymethyl groups were synthesized and tested for hepatoprotective activity against CCl_4-induced liver damage in mice. Structure–activity relationships were investigated. Lengthening of the alkyl group in **2** and **5** tends to adversely affect the activity. Both enantiomers of the methyl derivatives (**2a** and **5a**), the most potent compounds in this series, were synthesized, and no difference in hepatoprotective activity was observed. Apparent neighboring group participation was observed in the treatment of **5a** with base or acid, giving the cyclized product (**6**) or the rearranged products (**7**, **8**, and **9**).

Keywords——tetrahydro-β-carboline; structure–activity relationship; carbon tetrachloride-induced liver damage; hepatoprotective activity; dithiocarbamate; optical isomer; neighboring group participation

The preceding paper of this series[1] disclosed the synthesis and hepatoprotective activity of *N*-(methylthio)thiocarbonyl derivatives of several α-amino acids. Among them, 1,2,3,4-tetrahydro-2-(methylthio)thiocarbonyl-β-carboline-3-carboxylic acid (**2a**) exhibited the most potent activity as determined in terms of protection against acutely CCl_4-induced liver damage in mice. In view of the novelty of this class of compounds as hepatoprotective agents, we commenced the synthesis of a large number of derivatives in an effort to establish structure–activity relationships (SAR). In the present study, both enantiomers of **2a** and the corresponding 3-hydroxymethyl derivative (**5a**) were synthesized, and their hepatoprotective activity was examined. The effects of varying the alkyl group in the dithiocarbamate moiety of **2** and **5** are also presented.



2a: R=CO_2H
5a: R=CH_2OH

Chart 1

Chemistry

The optical isomers of **2a** were readily synthesized according to the procedure described previously for the racemate[1] (Chart 2). The Pictet–Spengler cyclization of L- and D-tryptophan with formalin according to the method reported by Brossi *et al.*[2] gave (*S*)- and (*R*)-

1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (**1**), respectively. Reaction of (*S*)- and (*R*)-**1** with carbon disulfide (CS_2) in the presence of potassium hydroxide followed by alkylation with methyl iodide gave the dithiocarbamates [(*S*)- and (*R*)-**2a**], respectively (Table I). The 3-hydroxymethyl derivatives [(*RS*)-, (*S*)-, and (*R*)-**4**][3] were obtained by sodium borohydride reduction[4] of the 3-methoxycarbonyl-β-carbolines[5] [(*RS*)-, (*S*)-, and (*R*)-**3**].

Apparent neighboring group participation was observed in the reaction of **4** with CS_2. When treated with CS_2 and then with methyl iodide in the presence of sodium hydroxide in the usual manner,[1] (*S*)-**4** gave, after chromatographic separation, the dithiocarbamate [(*S*)-**5a**] and the cyclized product (**6**) in 41 and 40% yields, respectively. Replacement of sodium hydroxide with triethylamine in the reaction, however, gave (*S*)-**5a** as a sole product in 90.2% yield. Compounds (*RS*)-**5a** and (*R*)-**5a** were similarly prepared, and their physical properties are listed in Table I.

The cyclized product (**6**) showed the molecular ion peak at *m/z* 244 in its mass spectrum (MS), and no carbonyl band appeared in the infrared (IR) spectrum, in accordance with the fused oxazolidine-2-thione structure. On treatment with aqueous potassium hydroxide in ethanol at room temperature, the dithiocarbamate [(*S*)-**5a**] readily underwent cyclization, apparently *via* intermediate A, giving **6** in 88.9% yield (Chart 3). Heating of (*S*)-**5a** with 10% HCl in ethanol caused precipitation of the hydrochloride (**7**) in 82% yield. Compound **7** was analyzed for $C_{14}H_{16}N_2OS_2 \cdot HCl$ and showed the molecular ion peak at *m/z* 292 in its MS, indicating that **7** is the HCl salt of a compound having a molecular formula identical with that of **5a**. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, **7** showed a singlet

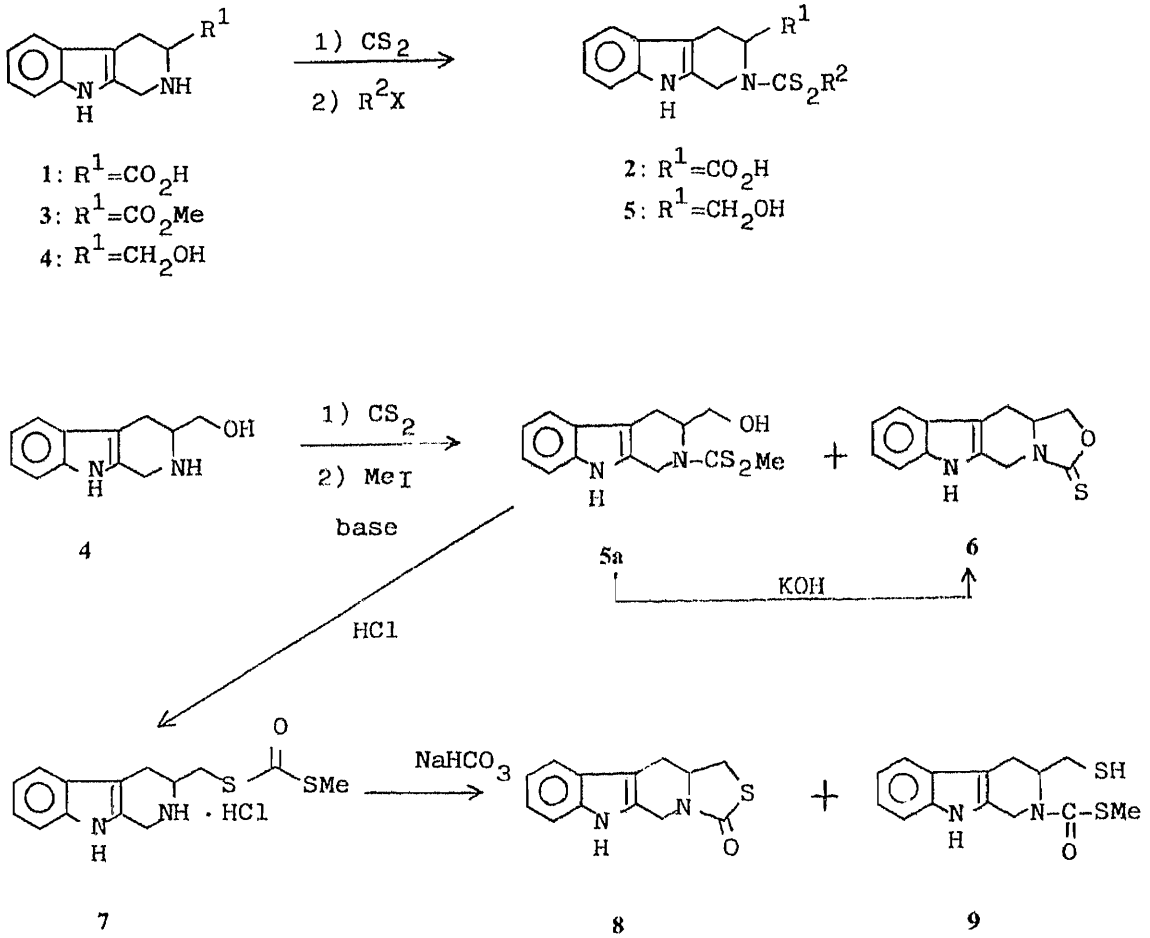


Chart 2

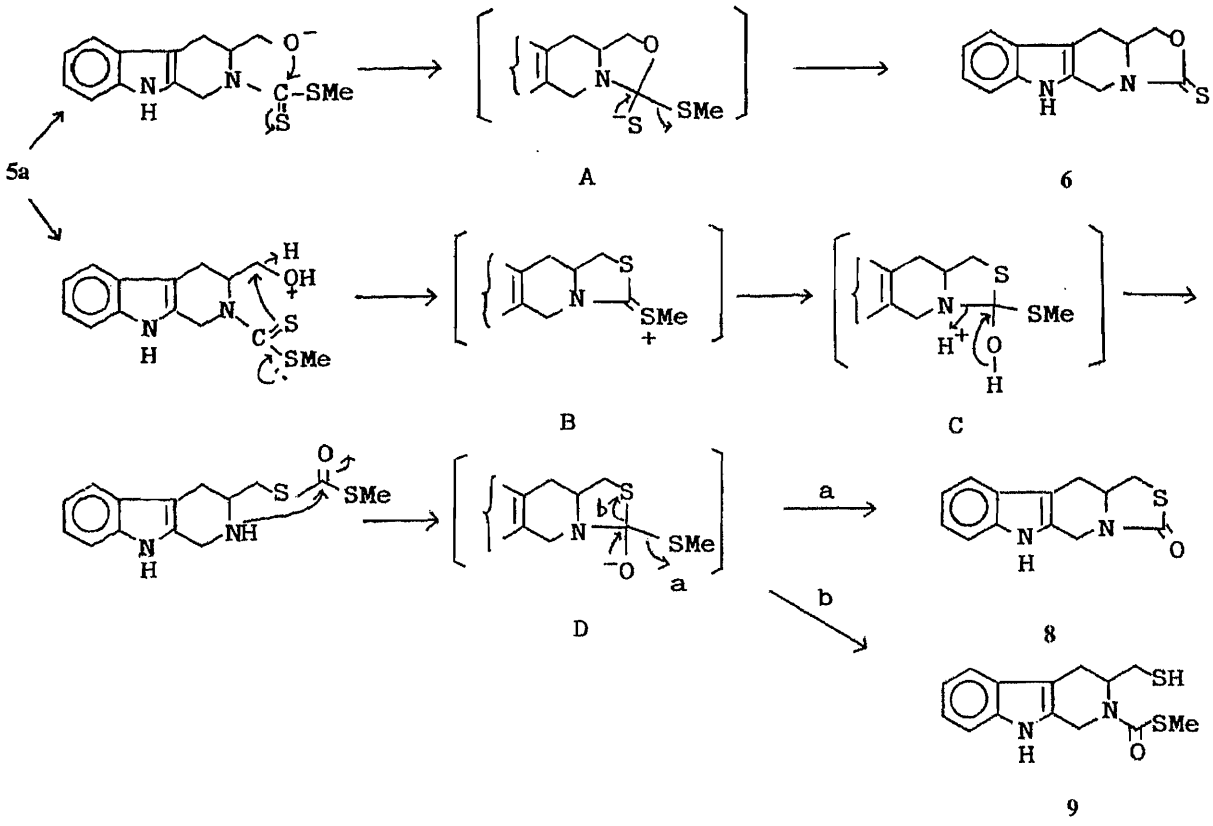


Chart 3

assignable to the SMe group at δ 2.48 ppm. The IR spectrum of 7 exhibited the carbonyl absorption at 1640 cm^{-1} together with NH_2^+ absorption at 2330—2730 cm^{-1}. On the basis of these spectroscopic data, the dithiocarbonate structure (7) was assigned for this compound. Upon alkalinization with aqueous sodium bicarbonate, 7 gave, after chromatographic separation, two compounds (**8** and **9**) in 43 and 11.2% yields, respectively. Compound **8** (M^+: *m/z* 244), which is isomeric with the oxazolidine-2-thione (**6**), was assigned the fused 2-thiazolidinone structure based on its carbonyl absorption at 1640 cm^{-1}. On the other hand, the MS of **9** showed the molecular ion peak at *m/z* 292, indicating that this compound is isomeric with **5a** or 7. The presence of the SH group in **9** was confirmed by an absorption at 2550 cm^{-1}, and the thiol proton resonance at δ 1.47 ppm (t, J=8 Hz, disappeared on addition of D_2O). The IR and NMR spectra of **9** also showed the presence of a methylthiocarbonyl group (carbonyl absorption at 1640 cm^{-1} and a singlet at δ 2.41 ppm for the SMe group). On the basis of these data, the 3-mercaptomethyl-2-(methylthio)carbonyl structure was assigned for **9**.

Formation of 7, **8**, and **9** may be rationalized in terms of the sequence of reactions outlined in Chart 3. Protonation at the hydroxyl group of **5a** followed by intramolecular attack of the thiocarbonyl group would give intermediate B. Cleavage of the C–N bond by protonation at the nitrogen in intermediate C would lead to 7. Upon alkalinization, 7 would give intermediate D, from which **8** or **9** could be formed by expulsion of the SMe group or by cleavage of the C–S bond in the thiazolidine ring, respectively.

To examine the effect of varying the alkyl group in the dithiocarbamate moiety on hepatoprotective activity, a number of derivatives (**2b—p** and **5b—l**) listed in Tables II and III were synthesized. Since no difference of activity between the optical isomers (**2a** and **5a**) was observed (see below), the choice of the chirality of starting materials (*S*, *R*, or *RS*) was

TABLE I. Optical Isomers of Tetrahydro-2-methylthiothiocarbonyl-β-carboline Derivatives (**2a** and **5a**)

Compd. No.	mp (°C) (Recryst. solvent)	Yield (%)	$[\alpha]_D^{20}$ (°), $c=1.0$ (Solvent)	Formula	Analysis (%) Calcd (Found)				Hepatoprotective activity[b]	
					C	H	N	S	100 mg/kg	10 mg/kg
(*S*)-**2a**	103—105 (dec.) (aq. EtOH)	73	+196.4 ($CHCl_3$)	$C_{14}H_{14}N_2O_2S \cdot 1/2\,H_2O$	53.31 (53.25	4.79 4.63	8.88 8.63	20.33 20.12)	AA	AA
(*R*)-**2a**	103—106 (dec.) (aq. EtOH)	60	−196.0 ($CHCl_3$)	$C_{14}H_{14}N_2O_2S_2 \cdot 1/2\,H_2O$	(53.18	4.76	8.76	20.19)	AA	AA
(*RS*)-**2a**[a]									AA	AA
(*S*)-**5a**	114—116 (aq. EtOH)	90	+159.0 (MeOH)	$C_{14}H_{16}N_2OS_2$	57.50 (57.44	5.52 5.49	9.58 9.60	21.93 21.80)	AA	AA
(*R*)-**5a**	106—108 (aq. EtOH)	88	−158.6 (MeOH)	$C_{14}H_{16}N_2OS_2$	(57.21	5.75	9.53	21.89)	AA	AA
(*RS*)-**5a**	172—173 (aq. EtOH)	90	—	$C_{14}H_{16}N_2OS_2$	(57.58	5.46	9.61	21.99)	AA	AA

a) See reference 1. *b*) AA=significantly effective; A, B, and C=effective; D=ineffective. For criteria, see reference 1.

arbitrary.

Pharmacology and Structure–Activity Relationships

The dithiocarbamates (**2** and **5**) prepared in the present study were tested for hepatoprotective activity against CCl_4-induced liver damage in mice after oral administration by the method reported previously.[1] The results were evaluated according to the criteria defined previously[1] and are included in Tables I—III.

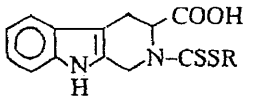
As can be seen in Table I, no difference of hepatoprotective activity between optical isomers of **2a** was observed. This is also the case for the corresponding 3-hydroxymethyl derivative (**5a**), which exhibited potent activity comparable to that of **2a**.

The effect of varying the alkyl group in the dithiocarbamate moiety was examined next for a series of the carboxylic acids (**2b—p**) and the hydroxymethyl derivatives (**5b—l**) (Tables II and III). Lengthening of the alkyl group (R) in **2** tends to adversely affect the activity. Thus, a change from methyl (**2a**) to decyl (**2f**) resulted in a gradual decrease in hepatoprotective activity. This is also the case for the corresponding 3-hydroxymethyl derivatives (**5a—f**) (Table III). These results suggest that the hepatoprotective activity of a series of the alkyl dithiocarbamates (**2** and **5**) decreases with increasing hydrophobicity of the alkyl group. The presence of a secondary alkyl group caused a marked fall in activity, as exemplified by the isopropyl derivative (**2d**).

Since some of the benzyl dithiocarbamates were significantly active, the activity of several aralkyl derivatives (**2g—p** and **5g—l**) was examined. In a series of substituted benzyl derivatives (**2g—l**), activity increased with increasing electron-donating ability of the substituent, as shown by the 4-NH_2 (**2l**), 4-OMe (**2i**), and 4-Me (**2k**) derivatives. In contrast, the presence of electron-withdrawing groups such as 4-NO_2 (**2j**) and 4-Cl (**2h**) caused a marked decrease in activity (Table II). In a series of the corresponding 3-hydroxymethyl derivatives (**5g—k**), however, no clear SAR could be deduced with respect to the effect of substituents on the benzene ring (Table III). Thienyl dithiocarbamates exhibited potent activity both in the carboxylic acid (**2m**) and in the 3-hydroxymethyl (**5l**) series.

Further studies on the SAR of new dithiocarbamates of β-carboline as hepatoprotective agents are being continued.

TABLE II. Various Dithiocarbamate Derivatives of Tetrahydro-β-carboline-3-carboxylic Acid

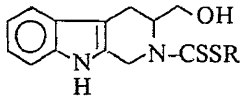


2

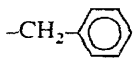
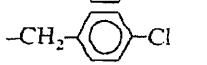
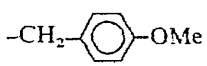
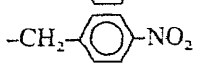
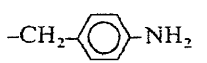
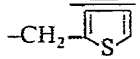
Compd. No.	R	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found)					Hepatoprotective activity[b]	
					C	H	Cl	N	S	100 mg/kg	10 mg/kg
(*RS*)-**2b**	Et	177—178 (aq. EtOH)	69	$C_{15}H_{16}N_2O_2S_2 \cdot 1/5\,C_2H_5OH$	56.11 (55.97	5.25 5.09		8.50 8.59	19.45 19.72)	AA	A
(*RS*)-**2c**	Pr	180—182 (dec.) (EtOH)	69	$C_{16}H_{18}N_2O_2S_2$	57.46 (57.30	5.42 5.43		8.38 8.22	19.17 18.98)	A	
(*RS*)-**2d**	iso-Pr	188—190 (EtOH)	31	$C_{16}H_{18}N_2O_2S_2 \cdot C_2H_5OH$	56.82 (56.70	6.36 6.37		7.36 7.37	16.85 16.92)	D	
(*RS*)-**2e**	Bu	163—164 ($CHCl_3$)	36	$C_{17}H_{20}N_2O_2S_2$	58.59 (58.31	5.78 5.63		8.04 7.82	18.40 18.17)	B	
(*RS*)-**2f**	Decyl	152—154 (dec.) (hexane)	55	$C_{23}H_{32}N_2O_2S_2$	63.85 (64.17	7.46 7.48		6.47 6.52	14.82 14.67)	B	
(*RS*)-**2g**	$-CH_2-C_6H_5$	Powder	50	$C_{20}H_{18}N_2O_2S_2$	62.80 (62.63	4.74 4.72		7.32 7.18	16.77 16.54)	B	
(*RS*)-**2h**	$-CH_2-C_6H_4-Cl$	Powder	65	$C_{20}H_{17}ClN_2O_2S_2$	57.61 (57.79	4.11 4.26	8.50 8.70	6.72 6.71	15.38 15.08)	D	
(*RS*)-**2i**	$-CH_2-C_6H_4-OMe$	195—196 (iso-Pr_2O)	79	$C_{21}H_{20}N_2O_3S_2$	61.14 (61.05	4.89 4.82		6.79 6.83	15.55 15.34)	A	
(*RS*)-**2j**	$-CH_2-C_6H_4-NO_2$	Powder	54	$C_{20}H_{17}N_3O_4S_2 \cdot 1/4\,CH_3CO_2C_2H_5$	56.11 (56.41	4.26 4.16		9.35 9.27	14.27 14.40)	C	
(*RS*)-**2k**	$-CH_2-C_6H_4-Me$	188—190 (dec.) (aq. EtOH)	39	$C_{21}H_{20}N_2O_2S_2 \cdot 1/2\,C_2H_5OH$	63.29 (63.26	5.31 5.11		6.87 6.87	15.72 15.72)	A	
(*R*)-**2l**[a]	$-CH_2-C_6H_4-NH_2$	Powder	36	$C_{20}H_{19}N_3O_2S_2 \cdot 1/2\,CH_3CO_2C_2H_5$	60.01 (59.75	5.12 5.06		9.84 9.82	15.02 15.46)	AA	B
(*RS*)-**2m**	$-CH_2-$(2-thienyl)	172—173 (dec.) (EtOH)	40	$C_{18}H_{16}N_2O_2S_3 \cdot 1/5\,C_2H_5OH$	55.56 (55.52	4.35 4.19		7.04 7.03	24.18 24.34)	AA	A
(*R*)-**2n**[a]	$-CH_2-$(2-furyl)	Powder	14	$C_{18}H_{16}N_2O_3S_2 \cdot 1/2\,CH_3CO_2C_2H_5$	58.80 (58.55	4.93 4.91		6.86 6.60	15.70 15.57)	B	
(*RS*)-**2o**	$-CH_2-$(3-pyridyl)	Powder	36	$C_{19}H_{17}N_3O_2S_2$	59.51 (59.29	4.47 4.63		10.96 10.76	16.72 16.51)	D	
(*RS*)-**2p**	$-CH_2CH_2-C_6H_5$	Powder	47	$C_{21}H_{20}N_2O_2S_2$	63.61 (63.39	5.08 5.05		7.06 6.93	16.17 16.30)	C	

a) (*R*)-**21**, $[\alpha]_D^{20}$ −118.0° (*c*=0.5, EtOH); (*R*)-**2n**, $[\alpha]_D^{20}$ −151.2° (*c*=1.0, MeOH). *b*) See footnote *b* in Table I.

TABLE III. Various Dithiocarbamate Derivatives of Tetrahydro-3-hydroxymethyl-β-carboline



5

Compd. No.	R	mp (°C) (Recryst. solvent)	Yield (%)	$[\alpha]_D^{20}$ (°), $c=1.0$ (Solvent)	Formula	Analysis (%) Calcd (Found)					Hepatoprotective activity[a]	
						C	H	Cl	N	S	100 mg/kg	10 mg/kg
(*S*)-5b	Et	74—76 (EtOH)	65	+146.0 (MeOH)	$C_{15}H_{18}N_2OS_2$	58.79 (58.54	5.92 6.05		9.14 8.90	20.93 21.00)	AA	A
(*S*)-5c	Pr	130—131 (aq. EtOH)	78	+141.8 (MeOH)	$C_{16}H_{20}N_2OS_2$	59.97 (60.11	6.29 6.30		8.74 8.72	20.01 20.30)	AA	A
(*S*)-5d	Bu	60—63 (EtOH)	60	+133.6 (MeOH)	$C_{17}H_{22}N_2OS_2$	61.04 (60.96	6.63 6.71		8.31 8.25	19.17 19.07)	D	
(*S*)-5e	Hexyl	102—106 (Et_2O–hexane)	73	+130.0 (MeOH)	$C_{19}H_{26}N_2OS_2$	62.94 (62.73	7.23 7.21		7.72 7.63	17.69 17.54)	A	
(*S*)-5f	Decyl	115—118 (Et_2O–hexane)	59	+113.0 (MeOH)	$C_{23}H_{34}N_2OS_2$	65.98 (66.14	8.18 8.20		6.69 6.71	15.32 15.47)	B	
(*S*)-5g		Powder	70	+117.3 (MeOH)	$C_{20}H_{20}N_2OS_2$	65.19 (64.97	5.47 5.46		7.60 7.50	17.40 17.39)	AA	D
(*S*)-5h		175—176 (dec.) (AcOEt–hexane)	67	+94.4 (MeOH)	$C_{20}H_{19}ClN_2OS_2$	59.61 (59.64	4.75 4.81	8.80 8.91	6.95 6.76	15.91 16.09)	AA	D
(*RS*)-5i		Powder	71	—	$C_{21}H_{22}N_2O_2S_2$	63.29 (63.11	5.56 5.50		7.03 6.92	16.09 15.65)	AA	A
(*RS*)-5j		193—194 (MeOH)	71	—	$C_{20}H_{19}N_3O_3S_2$	58.09 (57.82	4.63 4.69		10.16 10.33	15.51 15.61)	C	
(*S*)-5k		Powder	50	+123.6 (DMF)	$C_{20}H_{21}N_3OS_2$	59.82 (60.04	5.77 5.76		10.46 10.75	15.97 15.88)	B	
(*S*)-5l		Powder	55	+108.4 (MeOH)	$C_{18}H_{18}N_2OS_3$	57.72 (57.48	4.84 4.81		7.48 7.64	25.68 25.40)	AA	C

a) See footnote *b* in Table 1.

Experimental

All melting points are uncorrected. IR spectra were recorded on a Hitachi IR-215 spectrometer. ^{1}H-NMR spectra were taken on a JEOL-60 instrument. Chemical shifts are given as δ values from tetramethylsilane as an internal standard. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. MS were measured with a Hitachi RMU-6M instrument. Optical rotations were determined on a Union PM-201 automatic digital polarimeter.

(3*S*), (3*R*), and (3*RS*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic Acid (1)——These compounds were synthesized from L-, D-, and DL-tryptophan according to the method reported by Brossi *et al.*[2]

(3*S*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic Acid[2] [(*S*)-1]——mp 291—294 °C (dec.), $[\alpha]_D^{20}$ −63.3° (*c*=1.0, 1 N HCl : MeOH = 1 : 1) [*lit.*[2] mp 315 °C, $[\alpha]_D^{25}$ −49.6° (*c*=1.0, 1 N HCl : MeOH = 1 : 1)].

(3*R*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic Acid [(*R*)-1]——mp 290—293 °C (dec.), $[\alpha]_D^{20}$ +62.4° (*c*=1.0, 1 N HCl : MeOH = 1 : 1).

(3*RS*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic Acid [(*RS*)-1]——mp 286—288 °C (dec.).

Methyl (3*S*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylate [(*S*)-3] Hydrochloride[4]——L-Tryptophan methyl ester hydrochloride (5.09 g, 20 mmol) was dissolved in MeOH (60 ml), and formalin (35%, 1.89 g, 22 mmol) was added to the solution. The mixture was stirred at room temperarture overnight, and the solvent was evaporated off. The residual solid was crystallized from MeOH to yield (*S*)-3·HCl (4.42 g, 82.9%), mp 250—253 °C, $[\alpha]_D^{20}$ −67.2° (*c*=1.0, MeOH). Compounds (*R*)-3 and (*RS*)-3 were prepared in a similar manner [lit.[4] mp 284—285 °C, $[\alpha]_D^{20}$ −62.5° (*c*=0.4, EtOH)].

Methyl (3*R*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylate [(*R*)-3] Hydrochloride——mp 247—249 °C (MeOH), 79%, $[\alpha]_D^{20}$ +69.6° (*c*=1.0, MeOH).

Methyl (3*RS*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxylate [(*RS*)-3] Hydrochloride[4]——mp 218—222 °C (MeOH), 75% (lit.[4] mp 228—230 °C).

(3*S*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-β-carboline [(*S*)-4]——$NaBH_4$ (10 g, 0.264 mol) was added to a mixture of (*S*)-3·HCl (21.0 g, 0.079 mol), EtOH (400 ml), and water (40 ml) under ice-cooling. The mixture was stirred at room temperature for 2 h and then refluxed for 3 h. Insoluble material was filtered off, and washed with hot EtOH, and the filtrate and the washing were concentrated. Water was added to the residue, and a solid was collected, washed with water, and dried. Recrystallization from EtOH gave (*S*)-4 (12.1 g, 76%), mp 192—193 °C, $[\alpha]_D^{20}$ −83.0° (*c*=1.0, MeOH). Compounds (*R*)-4 and (*RS*)-4 were prepared in a similar manner.

(3*R*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-β-carboline [(*R*)-4]——mp 192—194 °C (EtOH–hexane), 63% $[\alpha]_D^{20}$ +81.9° (*c*=1.0, MeOH).

(3*RS*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-β-carboline[5] [(*RS*)-4]——mp 187—188 °C (iso-PrOH), 64% (lit.[5] mp 168 °C).

(3*R*)-1,2,3,4-Tetrahydro-2-(methylthio)thiocarbonyl-β-carboline-3-carboxylic Acid [(*R*)-2a]——CS_2 (1.82 ml, 30 mmol) was added to a solution of (*R*)-1 (6.49 g, 30 mmol) and KOH (3.5 g, 60 mmol) in 50% aqueous EtOH (110 ml) under cooling in an ice-bath, and the whole was stirred at room temperature for 1 h. MeI (5.11 g, 36 mmol) was added to the mixture, and the whole was stirred at room temperature for 4 h. After removal of the solvent, the residue was dissolved in H_2O and extracted with ether. The aqueous layer was acidified with 10% HCl and extracted with AcOEt. The extracts were washed with H_2O and dried over $MgSO_4$. The solvent was removed and the residue was purified by chromatography on silica gel using $CHCl_3$–MeOH (97 : 3) as the eluent to give (*R*)-2a (5.5 g, 60%), mp 103—106 °C (dec.) (aq. EtOH). IR ν_{max}^{Nujol} cm^{-1}: 1713. ^{1}H-NMR ($CDCl_3$) δ: 2.65 (3H, s). MS *m/z*: 306 (M^+), 258 (M^+ − MeSH). The corresponding enantiomer [(*S*)-2a] was similarly prepared and its properties are listed in Table I. Various derivatives (2b—p) listed in Table II were also synthesized in the same manner.

Methyl (3*S*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-β-carboline-2-carbodithioate [(*S*)-5a]——CS_2 (11.65 g, 0.15 mol) was added to a solution of (*S*)-4 (30.3 g, 0.15 mol) and Et_3N (15.5 g, 0.15 mol) in MeOH (300 ml)–H_2O (80 ml), and the whole was stirred at room temperature for 30 min. MeI (21.73 g, 0.15 mol) was added, and the mixture was stirred at room temperature for 1.5 h. After removal of the solvent, the residue was dissolved in AcOEt, washed with 5% HCl and H_2O, and dried over Na_2SO_4. The solvent was evaporated off to give a solid, which was recrystallized from aqueous EtOH to yield (*S*)-5a (42.0 g, 90.2%), mp 114—116 °C. IR ν_{max}^{Nujol} cm^{-1}: 3370, 1630. ^{1}H-NMR ($CDCl_3$) δ: 2.62 (3H, s). MS *m/z*: 292 (M^+), 244 (M^+ − MeSH). The corresponding (*R*) and (*RS*) isomers were similarly prepared and their physical properties are listed in Table I. Various derivatives (5b—l) listed in Table III were also synthesized in essentially the same manner.

Methyl (3*S*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-β-carboline-2-carbodithioate [(*S*)-5a] and (11a*S*)-5,5a,11,11a-Tetrahydro-1*H*,3*H*-oxazolo[4′,3′ : 6,1]pyrido[3,4-*b*]indole-3-thione (6)——A solution of (*S*)-4 (2.02 g, 10 mmol), 2 N NaOH (15 ml, 30 mmol), and CS_2 (2.33 g, 30 mmol) in 80% EtOH (50 ml) was stirred at 0 °C for 30 min, and then MeI (4.26 g, 30 mmol) was added. After being stirred at 0 °C for 2 h, the mixture was concentrated under reduced pressure. The residue was dissolved in AcOEt, and this solution was washed with water, and then dried over Na_2SO_4. The solvent was evaporated off to leave an oil, which was chromatographed on silica gel with $CHCl_3$–AcOEt (19 : 1) as the eluent. The oxazolidine (6) was eluted first, (980 mg, 40%), mp 236—237 °C (dec.) (AcOEt). IR ν_{max}^{KBr} cm^{-1}: 3400, 3320.

^{1}H-NMR (DMSO-d_6) δ: 2.66 (1H, dd, J=15.0, 9.7 Hz), 3.11 (1H, dd, J=15.0, 3.7 Hz), 4.12 (1H, d, J=17.0 Hz), 5.55 (1H, d, J=17.0 Hz). MS m/z: 244 (M^+), 211, 184. $[\alpha]_D^{20}$ −210.8° (c=1.0, tetrahydrofuran (THF)). *Anal.* Calcd for $C_{13}H_{12}N_2OS$: C, 63.91; H, 4.95; N, 11.47; S, 13.12. Found: C, 63.69; H, 4.89; N, 11.47; S, 12.95. The next fraction gave (*S*)-**5a** (1.20 g, 41%), mp 114—116 °C (aq. EtOH).

Treatment of (*S*)-5a with KOH——A suspension of (*S*)-**5a** (2.92 g, 10 mmol), 10% KOH (20 ml, 36 mmol), and 80% aqueous EtOH (100 ml) was stirred at room temperature for 3 h. The solvent was evaporated off under reduced pressure, and water was added to the residue. A solid was collected by filtration, washed with water, dried, and recrystallized from AcOEt to give **6** (2.17 g, 88.9%), mp 236—238 °C (dec.). Its spectral data were identical with those of **6** obtained from (*S*)-**4**.

***S*-Methyl *S*-((3*S*)-1,2,3,4-Tetrahydro-β-carbolin-3-yl)-methyl Dithiocarbonate Hydrochloride (7)**——A mixture of (*S*)-**5a** (10.0 g, 34 mmol), 10% HCl (500 ml) and EtOH (300 ml) was refluxed for 3 h and concentrated under reduced pressure. The residual solid was washed with water, dried, and recrystallized from EtOH to give **7** (8.72 g, 82%), mp 221—223 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3230, 2330—2730, 1640. NMR ($CDCl_3$–DMSO-d_6) δ: 2.48 (3H, s), 4.42 (2H, s), 10.79 (1H, s). MS m/z: 292 (M^+), 244, 143. *Anal.* Calcd for $C_{14}H_{16}N_2OS_2 \cdot HCl$: C, 51.13; H, 5.21; Cl, 10.78; N, 8.52; S, 19.50. Found: C, 51.47; H, 5.21; Cl, 11.08; N, 8.69; S, 19.78.

(11a*S*)-5,5a,11,11a-Tetrahydro-1*H*,3*H*-thiazolo[4′,3′:6,1]pyrido[3,4-*b*]indol-3-one (8) and *S*-Methyl (3*S*)-1,2,3,4-Tetrahydro-3-mercaptomethyl-β-carboline-2-carbothioate (9)——A mixture of **7** (2.0 g, 6.4 mmol), sat. aqueous $NaHCO_3$ solution (40 ml), and $CHCl_3$ (30 ml) was stirred at room temperature for 10 min. The $CHCl_3$ layer was separated, washed with water, and dried over $MgSO_4$. The solvent was evaporated off to leave an oil, which was purified by chromatography on silica gel with $CHCl_3$ as the eluent. The thiazolidinone (**8**) was eluted first (0.68 g, 43.3%), mp 228—231 °C (dec.) (EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3300 (br), 1640. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 4.10 (1H, m), 4.26 (1H, d, J=17.0 Hz), 5.03 (1H, d, J=17.0 Hz). MS m/z: 244 (M^+), 143. *Anal.* Calcd for $C_{13}H_{12}N_2OS$: C, 63.91; H, 4.95; N, 11.47; S, 13.12. Found: C, 63.73; H, 4.89; N, 11.57; S, 12.97. The next fraction afforded **9** (0.21 g, 11.2%), mp 157—159 °C (AcOEt–hexane). IR ν_{max}^{Nujol} cm^{-1}: 3370, 2550, 1640. NMR ($CDCl_3$–DMSO-d_6) δ: 1.47 (1H, t, J=8.0 Hz), 2.41 (3H, s) 3.03 (2H, brs). MS m/z: 292 (M^+), 244, 143. *Anal.* Calcd for $C_{14}H_{16}N_2OS_2$: C, 57.50; H, 5.52; N, 9.58; S, 21.93. Found: C, 57.32; H, 5.42; N, 9.54; S, 22.21.

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Benzylpiperazine Derivatives. IV.[1] Syntheses and Cerebral Vasodilating Activities of 1-Benzyl-4-diphenylmethylpiperazine Derivatives

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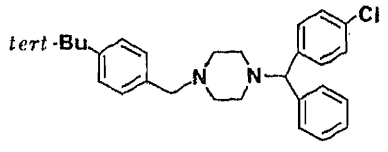
A series of 1-benzyl-4-diphenylmethylpiperazines was prepared and tested for cerebral vasodilating activity. Among the compounds with stronger potency and longer-lasting action than papaverine, the most potent analog **19** (KB-2796) was selected for further study. Some pharmacological properties of **19** are presented.

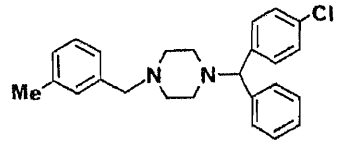
Keywords——benzylpiperazine; diphenylmethylpiperazine; 1-benzyl-4-diphenylmethylpiperazine; Leuckart–Wallach reaction; cerebral vasodilator; calcium antagonistic activity

A considerable body of literature exists on the biological activity of compounds containing the piperazine moiety. In the field of 1-benzyl-4-diphenylmethylpiperazines, antihistaminics,[2–4] tranquillizers,[5,6] and vasodilators[7] have been reported. Some of them, such as buclizine (**1**), meclizine (**2**) and medibazine (**3**), have been used clinically. More recently, 1-diphenylmethyl-4-(4-hydroxybenzyl)piperazines (**4**) were disclosed to be useful in the treatment of disturbance of cerebral blood flow.[8]

In the course of our studies aimed at finding novel cerebral vasodilators,[9] 1-diphenylmethyl-4-(2,3,4-trimethoxybenzyl)piperazine (**5**) was found to possess considerable activity. This compound was described in the patent literature but nothing about its medical



1



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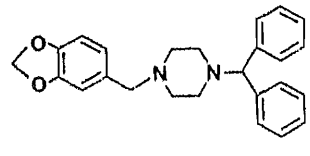

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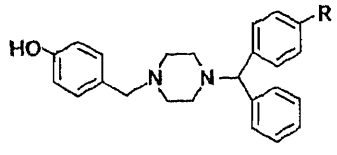
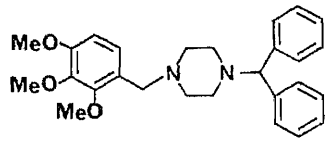


4a : R=H

4b : R=Cl



5

Chart 1

utility was disclosed.[10]

This paper is concerned with the syntheses of novel analogs of **5** and their evaluation as potential cerebral vasodilators.

Chemistry

Most of the compounds were synthesized by three general methods according to Chart 2. In method A, a substituted benzyl chloride was allowed to react with a diphenylmethylpiperazine derivative in the presence of triethylamine. In method B, which is an application of the Leuckart–Wallach reaction,[11] a substituted benzaldehyde was condensed reductively with a diphenylmethylpiperazine derivative by the use of formic acid without a solvent. In method C, 1-(2,3,4-trimethoxybenzyl)piperazine (X=2,3,4-tri-OMe; trimetazidine)[12] was allowed to react with diphenylmethyl chloride derivatives, which were prepared from diphenylmethanols[13] by chlorination with thionyl chloride.

Compounds **15**, **17** and **35** were obtained by acetylation of **13**, **4a** and **31**, respectively, and **13** was obtained from **16** by reduction with $SnCl_2$–HCl. Diphenylmethylpiperazine derivatives were prepared according to the literature.[14]

All the compounds prepared were converted to acid addition salts and purified by recrystallization.

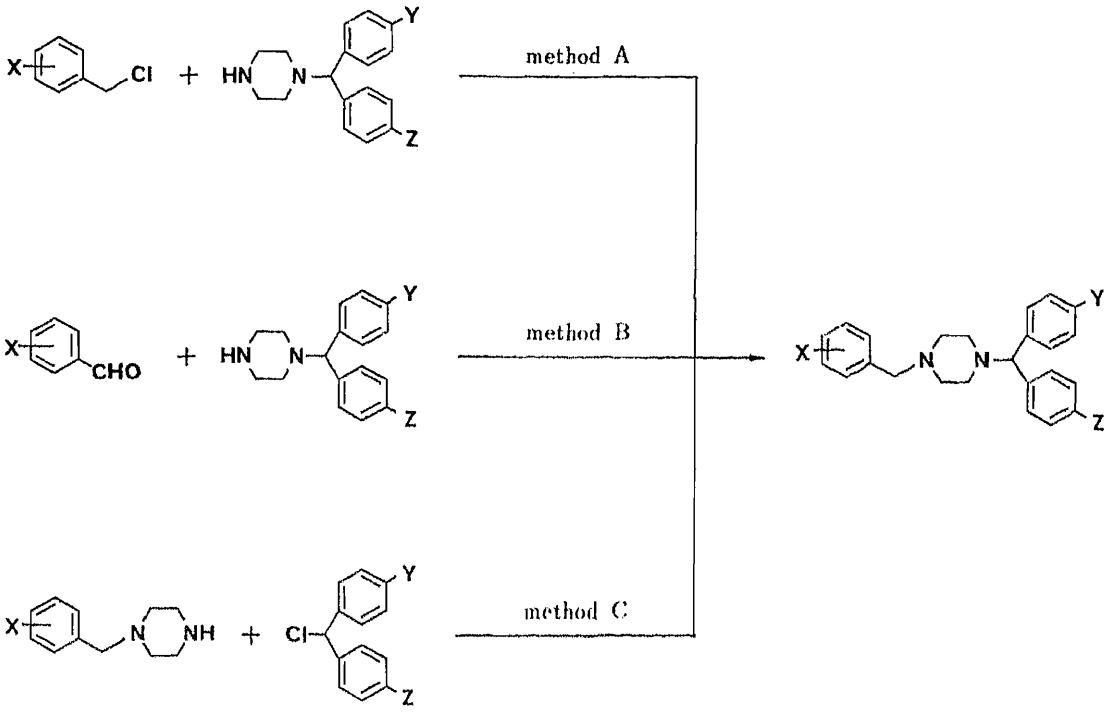


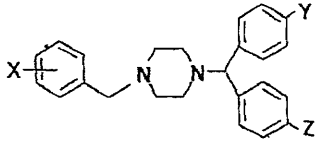
Chart 2

Results and Discussion

All the compounds listed in Table I except for **16**, which was practically insoluble, were tested for cerebral vasodilating activity by the method reported previously.[9]

In the case Y=Z=H (**3**—**18**), the potency decreased in the following order: **7** (X=2,4,6-tri-OMe) >**5** (X=2,3,4-tri-OMe) >**4a** (X=OH). These compounds did not exhibit long-lasting effects (a compound was judged to be long-acting when it exhibited more than 20 times longer duration of action than papaverine). Duration of action and cerebrovascular specificity are considered to be important for cerebral vasodilators.[15] Other compounds were less potent

TABLE I. 1-Benzyl-4-diphenylmethylpiperazines



Compd. No.	X	Y	Z	Yield (%)	mp (°C)	Recrystn.[a] solvent	Formula[b]	Analysis (%) Calcd (Found)			Potency[c]
								C	H	N	
3[d]	3,4-OCH_2O	H	H	37	238—242 (dec.)	E–W	$C_{25}H_{26}N_2O_2 \cdot 2HCl$	65.36 (65.63)	6.14 6.22	6.10 6.08)	0.74
4a[e]	4-OH	H	H	30	223—227 (dec.)	E	$C_{24}H_{26}N_2O \cdot 2HCl \cdot H_2O$	64.14 (64.14)	6.73 6.72	6.23 6.10)	1.00
5[f]	2,3,4-$(OMe)_3$	H	H	48	196—199 (dec.)	E–A	$C_{27}H_{32}N_2O_3 \cdot 2HCl \cdot H_2O$	61.95 (61.96)	6.93 6.80	5.35 5.46)	1.18
6	3,4,5-$(OMe)_3$	H	H	42	240—242 (dec.)	E–W	$C_{27}H_{32}N_2O_3 \cdot 2HCl$	64.16 (64.35)	6.78 6.82	5.54 5.43)	0.64
7	2,4,6-$(OMe)_3$	H	H	58	216—218 (dec.)	E	$C_{27}H_{32}N_2O_3 \cdot FA$	67.87 (67.72)	6.61 6.67	5.11 5.19)	1.42
8[g]	3,4-$(OMe)_2$	H	H	21	241—244 (dec.)	E–W	$C_{26}H_{30}N_2O_2 \cdot 2HCl$	65.68 (65.94)	6.78 6.91	5.89 5.84)	0.62
9	2,4-$(OMe)_2$	H	H	44	210—212 (dec.)	E	$C_{26}H_{30}N_2O_2 \cdot FA$	69.48 (69.35)	6.61 6.66	5.40 5.39)	0.99 (D)
10[h]	H	H	H	28	221—222 (dec.)	E	$C_{24}H_{26}N_2 \cdot 2HCl \cdot 0.5H_2O$	67.92 (67.82)	6.89 6.67	6.60 6.63)	0.62
11[i]	4-Me	H	H	21	215—218 (dec.)	IP	$C_{25}H_{28}N_2 \cdot 2HCl \cdot H_2O$	67.11 (67.09)	7.21 6.92	6.26 6.29)	0.84 (D)
12[j]	4-Cl	H	H	41	228—230 (dec.)	M	$C_{24}H_{25}ClN_2 \cdot 2HCl$	64.08 (64.12)	6.05 6.00	6.23 6.24)	0.57
13	4-NH_2	H	H	53	177—178	M	$C_{24}H_{27}N_3 \cdot FA \cdot 0.75H_2O$	69.05 (68.98)	6.73 6.91	8.63 8.45)	0.81
14	4-$N(Me)_2$	H	H	21	202—204 (dec.)	M	$C_{26}H_{31}N_3 \cdot FA$	71.83 (72.01)	7.03 7.11	8.38 8.46)	0.69 (D)
15	4-NHAc	H	H	33	200—202 (dec.)	E–W	$C_{26}H_{29}N_3O \cdot MA$	69.88 (69.64)	6.45 6.17	8.15 8.22)	0.60
16	4-NO_2	H	H	54	248—249 (dec.)	M–C	$C_{24}H_{25}N_3O_2 \cdot HCl$	68.00 (68.12)	6.18 6.13	9.91 9.95)	N.D.
17	4-OAc	H	H	46	204—206 (dec.)	E	$C_{26}H_{28}N_2O_2 \cdot FA$	69.75 (69.62)	6.24 6.00	5.42 5.46)	0.61 (D)
18	4-F	H	H	42	207—211 (dec.)	E	$C_{24}H_{25}FN_2 \cdot FA \cdot 1.25H_2O$	67.39 (67.51)	6.36 6.11	5.61 5.67)	0.58

19	2,3,4-$(OMe)_3$	F	F	40	204—207 (dec.)	AN	$C_{27}H_{30}F_2N_2O_3 \cdot 2HCl$	59.89 (59.89	5.96 6.14	5.17 5.22)	1.23 (D)
20	2,3,4-$(OMe)_3$	Cl	Cl	21	180—188 (dec.)	IP–Et	$C_{27}H_{30}Cl_2N_2O_3 \cdot 2HCl \cdot H_2O$	54.74 (54.46	5.78 5.92	4.73 4.95)	0.65
21	2,3,4-$(OMe)_3$	Me	Me	12	160—168	E–W	$C_{29}H_{36}N_2O_3 \cdot 2HCl \cdot H_2O$	63.15 (62.97	7.31 7.47	5.08 5.10)	1.05
22	2,3,4-$(OMe)_3$	OMe	OMe	60	174—177 (dec.)	M	$C_{29}H_{36}N_2O_5 \cdot FA \cdot H_2O$	63.25 (63.35	6.75 6.60	4.47 4.41)	0.38
23	2,3,4-$(OMe)_3$	H	F	14	204—205 (dec.)	E	$C_{27}H_{31}FN_2O_3 \cdot FA$	65.71 (65.84	6.23 6.19	4.94 5.08)	1.04 (D)
24	2,3,4-$(OMe)_3$	H	Cl	13	208—209	E	$C_{27}H_{31}ClN_2O_3 \cdot FA$	63.86 (63.65	6.05 6.04	4.80 4.91)	0.71 (D)
25	2,3,4-$(OMe)_3$	H	Me	26	194—195	E	$C_{28}H_{34}N_2O_3 \cdot FA \cdot 0.25H_2O$	67.77 (67.55	6.84 6.93	4.94 5.00)	0.48
26	2,3,4-$(OMe)_3$	H	OMe	39	186—188 (dec.)	E	$C_{28}H_{34}N_2O_4 \cdot FA \cdot 0.25H_2O$	65.91 (65.86	6.65 6.59	4.80 4.94)	0.27
27	3,4,5-$(OMe)_3$	F	F	33	223—229 (dec.)	E–W	$C_{27}H_{30}F_2N_2O_3 \cdot 2HCl \cdot 5H_2O$	58.91 (58.70	6.04 6.25	5.09 5.15)	0.60
28	2,4,6-$(OMe)_3$	F	F	30	182—184	E	$C_{27}H_{30}F_2N_2O_3 \cdot 2HCl \cdot H_2O$	57.96 (57.69	6.13 6.32	5.01 5.19)	1.05
29	2,4-$(OMe)_2$	F	F	23	159—161	E	$C_{26}H_{28}F_2N_2O_2 \cdot 2HCl \cdot 2H_2O$	57.04 (57.25	6.26 6.18	5.12 5.38)	1.05 (D)
30	3,4-OCH_2O	F	F	20	233—238 (dec.)	M–W	$C_{25}H_{24}F_2N_2O_2 \cdot 2HCl$	60.61 (60.61	5.29 5.23	5.66 5.56)	0.71
31[k]	4-OH	F	F	21	206—210 (dec.)	E–W	$C_{24}H_{24}F_2N_2O \cdot 2HCl \cdot 0.5H_2O$	60.51 (60.21	5.71 5.78	5.88 5.77)	0.82 (D)
32	H	F	F	50	212—218 (dec.)	E	$C_{24}H_{34}F_2O_2 \cdot 2HCl \cdot H_2O$	61.41 (61.22	6.01 5.92	5.97 6.03)	0.58
33	4-Me	F	F	39	221—225	E	$C_{25}H_{26}F_2N_2 \cdot 2HCl$	64.52 (64.26	6.06 6.24	6.02 6.14)	0.61 (D)
34	4-$N(Me)_2$	F	F	29	195—198 (dec.)	M	$C_{26}H_{29}F_2N_3 \cdot FA$	67.03 (66.79	6.19 6.33	7.82 7.97)	1.16 (D)
35	4-OAc	F	F	39	192—194 (dec.)	E	$C_{26}H_{26}F_2N_2O_2 \cdot FA$	65.21 (65.02	5.47 5.55	5.07 5.21)	0.63
36	2,4,6-$(OMe)_3$	H	F	29	209—211 (dec.)	E	$C_{27}H_{31}FN_2O_3 \cdot FA$	65.71 (65.87	6.23 6.23	4.94 5.04)	0.92
37	2,4-$(OMe)_2$	H	F	13	198—203 (dec.)	E–Et	$C_{26}H_{29}FN_2O_2 \cdot FA$	67.15 (67.08	6.20 6.15	5.22 5.31)	0.96 (D)
38	3,4,5-$(OMe)_3$	H	F	15	234—235 (dec.)	E–W	$C_{27}H_{31}FN_2O_3 \cdot 2HCl \cdot 0.5H_2O$	60.90 (60.90	6.44 6.56	5.26 5.56)	0.65
39	3,4-OCH_2O	H	F	28	234—235	E–W	$C_{25}H_{25}FN_2O_2 \cdot 2HCl$	62.90 (62.97	5.70 5.78	5.87 5.95)	0.58
40	4-$N(Me)_2$	H	F	12	190—191 (dec.)	E	$C_{26}H_{30}FN_3 \cdot FA$	69.35 (69.32	6.60 6.62	8.09 8.10)	1.04 (D)

a) A = AcOEt, AN = MeCN, C = $CHCl_3$, E = EtOH, Et = Et_2O, IP = iso-PrOH, M = MeOH, W = water. *b*) FA and MA stand for fumaric acid and maleic acid, respectively. *c*) The potency is expressed as the ratio of cerebral vasodilating activity to that of papaverine taken as 1; (D) stands for duration of the action. *d*) Ref. 7*a* mp 228 °C. *e*) Ref. 8 mp 225 °C. *f*) Ref. 10 mp 195—198 °C. *g*) Ref. 2*b* mp 230 °C. *h*) Ref. 2*b* mp 225 °C. *i*) Ref. 2*b* mp 218 °C. *j*) Ref. 2*b* mp 232 °C. *k*) For free base of **31**, see T. Raabe, B. Heinz, M. P. Anton, S. Josef and N. Rolf-Eberhard, Ger. Offen. 2900810 (1979) [*Chem. Abstr.*, **93**, 186413*k* (1980)].

Table II. Comparative Pharmacological Effects of **19** and Flunarizine

Pharmacological effects	**19**	Flunarizine
Cerebral blood flow ED_{30}[a] (mg/kg, i.v.)	0.048	0.15
Coronary blood flow ED_{30}[b] (mg/kg, i.v.)	0.77	0.45
Femoral blood flow ED_{30}[c] (mg/kg, i.v.)	0.41	0.82
Acute toxicity LD_{50} (mg/kg, mice, *p.o*)	300	285
Ca antagonistic activity (pA_2 value[d])	7.68	7.55

a, *b*) and *c*) The doses causing 30% increase of control values of vertebral, coronary and femoral blood flow in anesthetized dogs, respectively. *d*) In the depolarized taenia caecum of guinea pig preparation.

than papaverine.

When X is 2,3,4-tri-OMe and Y=Z (**5**, **19**—**22**), **5** (Y=H), **19** (Y=F) and **21** (Y=Me) were found to be more potent than papaverine, and the potency decreased in the following order: **19** > **5**> **21** > **20** >**22**. As for the compounds in which X is 2,3,4-tri-OMe and Y is H (**23**—**26**), the potency decreased in the following order: **23** (F) >**24** (Cl) >**25** (Me) >**26** (OMe). These results indicate that a bulky substituent on the diphenylmethyl moiety decreases the activity. Among these compounds, **19**, **23** and **24** exhibited long-lasting actions.

In the case of Y=Z=F (**19**, **27**—**35**), the potency decreased in the following order: **19** (2,3,4-tri-OMe) >**34** (N-di-Me) >**29** (2,4-di-OMe) =**28** (2,4,6-tri-OMe). Other derivatives were less potent than papaverine. Among the compounds in which Y=H and Z=F (**23**, **36**—**40**), **23** (2,3,4-tri-OMe) and **40** (N-di-Me) were more potent than papaverine. Compounds **19**, **29**, **31**, **33**, **34**, **37** and **40** exhibited prolonged duration of action.

In summary, from the standpoint of stronger activity as well as longer-lasting effect than papaverine, five compounds (**19**, **23**, **29**, **34** and **40**) were found. However, acute toxicity data indicated that **34** (LD_{50}=60 mg/kg) is harmful. Among the remaining four compounds, the most potent **19** (KB-2796) was selected for further study. Some pharmacological properties of **19** are given in Table II and compared with those of flunarizine.

Table II indicates that **19** is a more selective and potent cerebral vasodilator than flunarizine. In both cases, the cerebral vasodilating activities are due to the calcium antagonistic activities. The results of detailed pharmacological characterization of **19** will be reported elsewhere.

Experimental

Melting points were determined on a Yamato capillary melting point apparatus, model MP-21, and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a Hitachi R-24A NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Silica gel 60 F_{254} (Merck) TLC plates were used for thin layer chromatography. For column chromatography Silica gel 60 (Merck) was used.

Typical Procedures of Method A: 1-Benzyl-4-diphenylmethylpiperazine Dihydrochloride 0.5 Hydrate (10) ——Benzyl chloride (1.48 g), diphenylmethylpiperazine (2.52 g), triethylamine (2.18 g) and benzene (50 ml) were refluxed for 2 h. The reaction mixture was washed with water and dried over $MgSO_4$. Evaporation of the solvent gave the free base of **10**, which was diluted with EtOH (20 ml). Concentrated HCl (2 ml) was added to the solution. A small amount of Et_2O was then added and the precipitated solid was collected. Recrystallization from EtOH gave **10** (1.17 g).

Compounds **11**, **12**, **16** and **32** were obtained in the same manner as described for **10**. The yield, melting point and elementary analysis data are given in Table I.

Typical Procedures of Method B: 1-Diphenylmethyl-4-(3,4,5-trimethoxybenzyl)piperazine Dihydrochloride (6) ——3,4,5-Trimethoxybenzaldehyde (2.0 g) and diphenylmethylpiperazine (2.5 g) were melted in an oil bath at 120 °C and formic acid (0.47 g) was added dropwise. The mixture was stirred for 1 h under heat, and then allowed to cool to room temperature. Then the mixture was diluted with AcOEt (20 ml), washed with water and dried over $MgSO_4$. After removal of the solvent, conc. HCl (2 ml) in EtOH (20 ml) was added and the deposited solid was collected. Recrystallization from EtOH–water (1 : 1) gave **6** (2.2 g).

Compounds **3**—**5**, **7**—**9**, **14**, **18**, **19**, **23**—**25**, **27**—**31**, **33**, **34** and **36**—**40** were obtained in the same manner as described for **6**. The yield, melting point and elementary analysis data are given in Table I.

Typical Procedures of Method C: 1-[Bis(4-chlorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl)piperazine Dihydrochloride Hydrate (20)——Trimetazidine·2HCl (2.4 g), bis(4-chlorophenyl)methyl chloride (2.0 g), triethylamine (3.7 g) and xylene (50 ml) were refluxed for 9 h. The mixture was washed with water and dried over $MgSO_4$. After removal of the solvent, the residue was diluted with EtOH (30 ml) then conc. HCl (1.5 ml) was added to the solution. A small amount of Et_2O was added and the precipitated solid was collected. Recrystallization from iso-PrOH–Et_2O gave **20**.

Compounds **21**, **22** and **26** were obtained in the same manner as described for **20**. The yield, melting point and elementary analysis data are given in Table I.

1-(4-Acetoxybenzyl)-4-diphenylmethylpiperazine Fumarate (17)——**4a** (2.0 g) was converted to the free base in the usual manner and dissolved in Ac_2O (2.0 ml). The mixture was stirred for 2 h at room temperature then poured into ice-water. The solution was adjusted to pH 8—9 by adding 20% NaOH then extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. After removal of the solvent, the residue was diluted with MeOH (20 ml) and fumaric acid (1.1 g) in MeOH (20 ml) was added to the solution. The precipitated solid was collected and recrystallized from EtOH to give **17** (1.1 g).

Compounds **15** and **35** were obtained in the same manner as described for **17** from **13** and **31**, respectively. The yield, melting point and elementary analysis data are given in Table I.

1-(4-Aminobenzyl)-4-diphenylmethylpiperazine Fumarate 0.75 Hydrate (13)——**16** (4.4 g) was dissolved in conc. HCl (66 ml) and MeOH (44 ml). The solution was warmed at 60 °C while $SnCl_2$ was added portionwise over 2 h. The solution was cooled to 0 °C, the pH was adjusted to 10 by adding 20% NaOH, and the solution was extracted with AcOEt. The organic layer was washed with water and dried over $MgSO_4$. After removal of the solvent, the residue was diluted with EtOH (20 ml) and fumaric acid (2.3 g) in EtOH (30 ml) was added to the solution. The precipitated solid was collected and recrystallized from MeOH to give **13** (2.4 g). The yield, melting point and elementary analysis data are given in Table I.

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Studies on Hypolipidemic Agents. IV.[1] Syntheses and Biological Activities of *trans*- and *cis*-2-(4-Alkylcyclohexyl)-2-oxoethyl Arenesulfonates

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trans- and *cis*-2-Diazo-1-(4-alkylcyclohexyl)-1-ethanones were reacted with arenesulfonic acids to afford the corresponding 2-(4-alkylcyclohexyl)-2-oxoethyl arenesulfonates. The esterase-inhibitory activity and hypolipidemic effect of the arenesulfonates were examined, and it was found that in most cases, the *trans*-isomers were more active than the corresponding *cis*-isomers.

Stereoselective syntheses of several biologically potent *trans*-isomers (*trans*-**3**) were also developed.

Keywords——diazoketone; *trans*-arenesulfonate; *cis*-arenesulfonate; 4-alkylcyclohexyl methyl-ketone; α-bromoketone; α-hydroxyketone; esterase-inhibitory activity; chymotrypsin-inhibitory activity; hypolipidemic activity; structure–activity relationship

We have previously reported the synthesis and esterase-inhibitory activity as well as hypolipidemic effect of 2-oxoalkyl arenesulfonates.[1] In the preceding paper,[1c] we also found that stereoisomeric mixtures of several 2-(4-alkylcyclohexyl)-2-oxoethyl arenesulfonates possess considerable activities. In general, the pharmaceutical activities of stereoisomers are considerably different.[2,3] Thus, stereoselective synthesis or separation of the stereoisomers of arenesulfonates (**3**) is important for pharmaceutical evaluation. In this paper, we wish to report preparations and pharmaceutical evaluations of both stereoisomers of the arenesulfonates (**3**), as well as stereoselective synthesis of the *trans*-arenesulfonates (*trans*-**3**).

Chemistry

Catalytic hydrogenation of 4-isopropylbenzoic acid over platinum oxide catalyst afford-

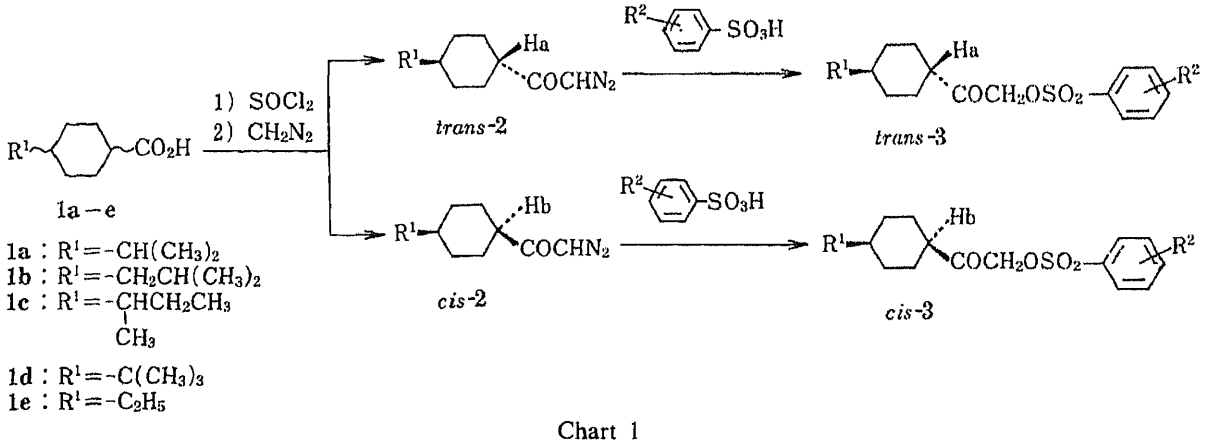
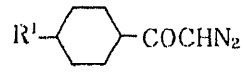


Chart 1

ed a stereoisomeric mixture of *trans*- and *cis*-4-isopropylcyclohexanecarboxylic acid (**1a**),[4] whose separation by distillation was difficult. However, the *cis*- and *trans*-isomers (ratio *ca.* 3 : 1) of 2-diazo-1-(4-isopropylcyclohexyl)-1-ethanone (**2b**),[4] which were obtained by treating a stereoisomeric mixture of 4-isopropylcyclohexane-carbonyl chloride with diazomethane, could be separated into *trans*- (*trans*-**2b**) and *cis*-isomers (*cis*-**2b**) by column chromatography.

The stereochemistry of the separated *trans*-isomer (*trans*-**2b**) and *cis*-isomer (*cis*-**2b**) was confirmed as follows. The proton nuclear magnetic resonance (^{1}H-NMR) spectra of *trans*-**2b** and *cis*-**2b** in $CDCl_3$ showed the signal of the methine proton (Ha and Hb) at the 1-position of the cyclohexane ring at δ 2.18 pm (1H, t, J = 12 Hz) and δ 2.46 ppm (1H, m, $W_{1/2}$ = 13 Hz), respectively. The former signal can be assigned as the axial proton and the latter, the equatorial proton based on comparisons with the data reported by Jensen *et al.*[5] Although the signal of Ha overlapped partially with the signals of protons at other positions on the cyclohexane ring in the 100 MHz ^{1}H-NMR spectrum of *trans*-**2b**, it was clearly isolated in the 400 MHz ^{1}H-NMR spectrum at δ 2.16 ppm (1H, tt, J = 3.5 and 12 Hz). Furthermore, there is no difference between the chemical shifts of the two methyls on the isopropyl group of *trans*-**2b** (δ 0.85 ppm, d, J = 6.5 Hz) and those of *cis*-**2b** (δ 0.85 ppm, d, J = 6.5 Hz). These results indicate that the isopropyl group in both *trans*-**2b** and *cis*-**2b** is equatorial. From the above spectral observations, *trans*-**2b** and *cis*-**2b** were clearly assigned as *trans*-isomer and *cis*-isomer, respectively. Stereoisomeric mixtures of other diazoketones (**2a** and **2c**—**e**), which were obtained from the corresponding acyl halides of **1b**—**e** by treatment with diazomethane, were also separated into the *trans*-isomer (*trans*-**2a** and *trans*-**2c**—**e**) and the *cis*-isomer (*cis*-**2a** and *cis*-**2c**—**e**) in the same manner as described for *trans*-**2b** and *cis*-**2b**. The structure of *trans*-**2a**, *trans*-**2c**—**e**, *cis*-**2a** and *cis*-**2c**—**e** were similarly confirmed. The physical data for *trans*-**2** and *cis*-**2** are listed in Table I.

TABLE I. Physical Data for *trans*-**2** and *cis*-**2**

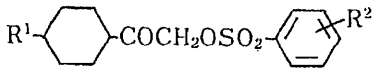


trans-**2**, *cis*-**2**

Compd.[a] No.	R^1	mp (°C)	MS (M^+)	^{1}H-NMR ($CDCl_3$) δ ppm
trans-**2a**	$-C_2H_5$	Oil	180	0.60—2.40 (14H, m), 2.18 (1H, t, J = 12 Hz), 5.27 (1H, s)
cis-**2a**	$-C_2H_5$	Oil	180	0.60—2.04 (14H, m), 2.38 (1H, br m), 5.33 (1H, s)
trans-**2b**	$-CH(CH_3)_2$	34—35	194	0.85 (6H, d), 0.96—2.36 (10H, m), 2.18 (1H, t, J = 12 Hz), 5.24 (1H, s)[b]
cis-**2b**	$-CH(CH_3)_2$	Oil	194	0.85 (6H, d), 0.95—2.15 (10H, m), 2.46 (1H, br m), 5.31 (1H, s)
trans-**2c**	$-CH(CH_3)CH_2CH_3$	Oil	208	0.60—2.35 (18H, m), 2.18 (1H, t, J = 12 Hz), 5.24 (1H, s)
cis-**2c**	$-CH(CH_3)CH_2CH_3$	Oil	208	0.65—2.14 (18H, m), 2.40 (1H, br m), 5.33 (1H, s)
trans-**2d**	$-CH_2CH(CH_3)_2$	36—37	208	0.89 (6H, d), 0.70—2.38 (12H, m), 2.20 (1H, t, J = 12 Hz), 5.27 (1H, s)
cis-**2d**	$-CH_2CH(CH_3)_2$	Oil	208	0.85 (6H, d), 1.00—2.04 (12H, m), 2.38 (1H, br m), 5.32 (1H, s)
trans-**2e**[c]	$-C(CH_3)_3$	58—59	208	0.84 (9H, s), 0.60—2.32 (9H, m), 2.18 (1H, t, J = 12 Hz), 5.25 (1H, s)
cis-**2e**[c]	$-C(CH_3)_3$	32—33	208	0.82 (9H, s), 0.65—2.28 (9H, m), 2.38 (1H, br m), 5.36 (1H, s)

a) All compounds were light yellowish oils. All IR spectra of these compounds in $CHCl_3$ showed the presence of a diazo group and a carbonyl group at 2100 and 1630 cm^{-1}, respectively. *b*) ^{1}H-NMR (400 MHz) spectrum at 50 °C in $CDCl_3$: 0.86 (6H, d, J = 6.8 Hz), 0.92—1.15 (3H, m), 1.30—1.65 (3H, m), 1.80 (2H, br d, J = 11.2 Hz), 1.89 (2H, br d, J = 11.2 Hz), 2.16 (1H, tt, J = 3.5, 12 Hz), 5.20 (1H, s). *c*) Lit.,[6]: *trans*-isomer: mp 61.5—63 °C, *cis*-isomer: mp 34—36 °C.

TABLE II. Inhibitory Activities on Enzymes, and Hypolipidemic Effect of *trans*-3 and *cis*-3



trans-3, *cis*-3

Compd. No.	R^1	R^2	Yield[a] (%)	mp (°C)	Recryst.[b] solv.	Inhibitions		Reduction[e]
						Esterase[c] IC_{50} (μM)	Chymotry.[d] (1×10^{-4} M)	Trigly.[f]
trans-3a	$-C_2H_5$	H	76	58—59	Et	0.0012	65	—[g]
cis-3a	$-C_2H_5$	H	65	39—40	E	0.02	16	—[g]
trans-3b	$-CH(CH_3)_2$	H	78	48—49	Et–W	0.065	62	90
cis-3b	$-CH(CH_3)_2$	H	84	42—43	E	0.7	16	60
trans-3c	$-CH(CH_3)_2$	$4\text{-}OC_2H_5$	69	80—81	Et	2.0	35	76
cis-3c	$-CH(CH_3)_2$	$4\text{-}OC_2H_5$	73	71—72	Et	11.0	14	50
trans-3d	$-CH(CH_3)_2$	$2,4,6\text{-}(CH_3)_3$	78	66—67	Et	0.13	—[g]	—[g]
cis-3d	$-CH(CH_3)_2$	$2,4,6\text{-}(CH_3)_3$	75	45—46	PE–E	1.2	—[g]	—[g]
trans-3e	$-\underset{\displaystyle CH_3}{\overset{\displaystyle |}{C}}HCH_2CH_3$	H	72	Oil[h]	—	0.15	19	76
cis-3e	$-\underset{\displaystyle CH_3}{\overset{\displaystyle |}{C}}HCH_2CH_3$	H	68	29—30	PE–E	0.56	13	61
trans-3f	$-CH_2CH(CH_3)_2$	H	77	50—51	Et–W	0.07	43	79
cis-3f	$-CH_2CH(CH_3)_2$	H	74	36—37	Et	0.4	30	57
trans-3g	$-C(CH_3)_3$	H	79	73—74	Et	4.6	0	72
cis-3g	$-C(CH_3)_3$	H	75	79—80	Et	4.6	0	52

a) Yields from the corresponding diazoketones *trans*-2 or *cis*-2. *b*) Recrystallization solvents: Et = ethanol, E = diethyl ether, PE = petroleum ether, W = H_2O. *c*) Methyl butyrate was used as a substrate. *d*) ATEE was used as a substrate. The activity was expressed as percentage inhibition of chymotrypsin-inhibitory activity at 10^{-4} M. *e*) The activity was expressed as percentage deviation from the control value. Dose: 0.3 mmol/kg, *p.o.* in rats. See also the experimental section. *f*) Plasma triglyceride. *g*) Not tested. *h*) This compound was purified by column chromatography.

Treatment of the *trans*- (*trans*-2) and *cis*-diazoketones (*cis*-2) with arenesulfonic acids afforded the corresponding *trans*- (*trans*-3) and *cis*-arenesulfonates (*cis*-3) in fairly good yields, respectively.

Enzyme-Inhibitory Activity (*in Vitro* Experiments)

Methyl butyrate and *N*-acetyltyrosine ethyl ester (ATEE) were used as substrates for the determination of esterase[7] and chymotrypsin[7] activities, respectively (Table II).

Pharmacological Examination (*in Vivo* Experiments)

Male Wistar rats (7 weeks old) were used, with five animals in each experimental group. A test compound (0.3 mmol) was mixed with 5 ml of olive oil and the mixture was orally administered to rats at the dose of 0.3 mmol per kg. Blood for the determination of plasma triglyceride was taken from the orbital vein of the rats at 2 h after the administration. Plasma triglyceride was analyzed by using a commercially available analysis kit (Determiner TG-S Kyowa[8]). Decreases of the triglyceride were expressed as percentage values with respect to the control value obtained for animals given olive oil containing no test compound.

Results and Discussion

The physical and biological data for the *trans*- (*trans*-3) and *cis*-arenesulfonates (*cis*-3) are listed in Tables II and III. As shown in Table II, in most cases, except for *trans*-3g and *cis*-3g, the *trans*-isomers (*trans*-3) exhibited 4 to 20 times more potent esterase-inhibitory activity

TABLE III. Physical Data for *trans*-3 and *cis*-3

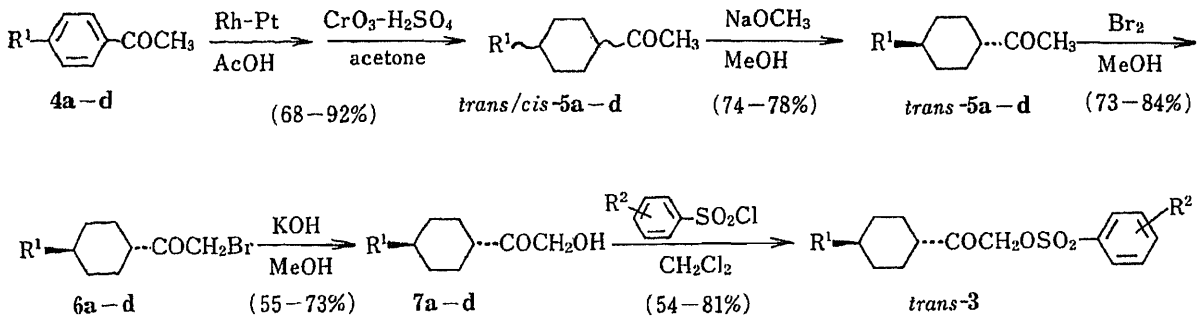
Compd. No.	Formula	Analysis (%) Calcd (Found) C	H	^{1}H-NMR ($CDCl_3$) δ ppm
trans-3a	$C_{16}H_{22}O_4S$	61.91	7.14	0.87 (3H, t), 1.00—2.00 (11H, m), 2.46 (1H, tt, J=4, 12 Hz), 4.64
		(61.87	6.98)	(2H, s), 7.40—7.82 (3H, m), 7.82—8.10 (2H, m)
cis-3a	$C_{16}H_{22}O_4S$	61.91	7.14	0.84 (3H, t), 1.00—2.00 (11H, m), 2.64 (1H, br m), 4.65 (2H, s),
		(62.17	7.22)	7.40—7.80 (3H, m), 7.80—8.10 (2H, m)
trans-3b	$C_{17}H_{24}O_4S$	62.94	7.46	0.85 (6H, d), 0.94—2.00 (10H, m), 2.46 (1H, tt, J=4, 12 Hz), 4.63
		(62.78	7.39)	(2H, s), 7.50—8.10 (5H, m)
cis-3b	$C_{17}H_{24}O_4S$	62.94	7.46	0.82 (6H, d), 0.94—2.10 (10H, m), 2.70 (1H, br m), 4.65 (2H, s),
		(62.76	7.51)	7.50—8.10 (5H, m)
trans-3c	$C_{19}H_{28}O_5S$	61.93	7.66	0.86 (6H, d), 1.45 (3H, t), 0.95—2.00 (10H, m), 2.48 (1H, tt, J=4,
		(61.84	7.65)	12 Hz), 4.11 (2H, q), 4.58 (2H, s), 6.99 (2H, d), 7.85 (2H, d)
cis-3c	$C_{19}H_{28}O_5S$	61.93	7.66	0.82 (6H, d), 1.45 (3H, t), 0.94—2.10 (10H, m), 2.72 (1H, br m), 4.11
		(61.92	7.77)	(2H, q), 4.59 (2H, s), 6.99 (2H, d), 7.86 (2H, d)
trans-3d	$C_{20}H_{30}O_4S$	65.54	8.25	0.85 (6H, d), 0.95—2.04 (10H, m), 2.32 (3H, s), 2.50 (1H, tt, J=4,
		(65.48	8.34)	12 Hz), 2.64 (6H, s), 4.54 (2H, s), 6.99 (2H, s)
cis-3d	$C_{20}H_{30}O_4S$	65.54	8.25	0.82 (6H, d), 0.95—2.10 (10H, m), 2.31 (3H, s), 2.64 (6H, s), 2.70
		(65.20	8.20)	(1H, br m), 4.56 (2H, s), 6.98 (2H, s)
trans-3e	$C_{18}H_{26}O_4S$	63.88	7.74	0.70—2.10 (18H, m), 2.46 (1H, tt, J=4, 12 Hz), 4.63 (2H, s),
		(63.97	7.85)	7.45—8.10 (5H, m)
cis-3e	$C_{18}H_{26}O_4S$	63.88	7.74	0.60—2.10 (18H, m), 2.68 (1H, br m), 4.65 (2H, s), 7.44—8.10
		(63.68	8.05)	(5H, m)
trans-3f	$C_{18}H_{26}O_4S$	63.88	7.74	0.84 (6H, d), 0.64—2.00 (12H, m), 2.48 (1H, tt, J=4, 12 Hz), 4.64
		(64.20	7.97)	(2H, s), 7.42—8.10 (5H, m)
cis-3f	$C_{18}H_{26}O_4S$	63.88	7.74	0.83 (6H, d), 0.95—2.00 (12H, m), 2.66 (1H, br m), 4.66 (2H, s),
		(63.89	7.98)	7.45—8.10 (5H, m)
trans-3g	$C_{18}H_{26}O_4S$	63.88	7.74	0.84 (9H, s), 0.60—2.04 (9H, m), 2.44 (1H, tt, J=4, 12 Hz), 4.63
		(63.64	7.85)	(2H, s), 7.50—8.08 (5H, m)
cis-3g	$C_{18}H_{26}O_4S$	63.88	7.74	0.79 (9H, s), 0.78—2.30 (9H, m), 2.70 (1H, br m), 4.66 (2H, s),
		(63.66	8.03)	7.48—8.08 (5H, m)

than the *cis*-isomers (*cis*-3). On the other hand, alkyl substituents on the cyclohexane ring of *trans*-3 increased the esterase-inhibitory activity in the following order: ethyl > isopropyl ≧ isobutyl > *sec*-butyl > *tert*-butyl. An ethoxy substituent on the benzene ring as in 3e decreased the activity. Chymotrypsin-inhibitory activity of the *trans*-isomers (*trans*-3) was also more potent than that of the *cis*-isomers (*cis*-3). Further, in the tests of plasma triglyceride-reducing effect *in vivo*, the *trans*-isomers (*trans*-3) showed a more potent hypolipidemic action than the *cis*-isomers (*cis*-3).

Stereoselective Synthesis of *trans*-Isomers (*trans*-3)

Biological tests of the *trans*- (*trans*-3) and the *cis*-arenesulfonates (*cis*-3) showed that *trans*-3 exhibited more potent esterase-inhibitory activity as well as greater hypolipidemic effect than *cis*-3. Thus, we devised a synthetic scheme for a facile synthesis of *trans*-3, which might be applicable to large-scale preparation (Chart 2).

Catalytic hydrogenation of 4-alkylacetophenones (4) over rhodium–platinum (3 : 1) was carried out at room temperature under a pressure of 50—60 atm in acetic acid to afford mixtures of the acetylcyclohexanes (5) and a small quantity of the cyclohexyl alcohols. The mixtures were subjected to Jones oxidation[9] to afford stereoisomeric mixtures (5). Equilibration of the stereoisomeric mixtures (5) by refluxing in methanol in the presence of sodium methoxide[10] proceeded successfully to give the *trans*-isomers (*trans*-5) in 74—78% yield. Bromination of the active methyl group of *trans*-5 was performed according to the



a : R^1=-$CH(CH_3)_2$ b : R^1=-$CH_2CH(CH_3)_2$ c : R^1=-$CHCH_2CH_3$ (CH_3) d : R^1=-$C(CH_3)_3$

Chart 2

TABLE IV. Conversion of 2-Bromo-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (**6b**) into 2-Hydroxy-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (**7b**)

Entry[a]	Substrate (**6b**) (mmol)	Solvent MeOH (ml)	Basic conditions	Temp. (°C)	Time (h)	Yield[b] (%)
1	9.6	20	KOH (1.1 eq)/H_2O (6 ml)	5	1	51
2	9.6	20	KOH (1.2 eq)/MeOH (25 ml)	5	1	58
3	30.8	60	NaOMe (1.3 eq)/MeOH (30 ml)	5	0.5	51
4	19.2	50	KOH (1.3 eq)/MeOH (15 ml)	10	1	66
5	19.2	50	*tert*-BuOK (1.2 eq)	20	2	52
6	19.2	50	HCO_2K (1.5 eq), NaI (0.1 eq)	40	16	63
7	19.2	50	HCO_2K (1.5 eq), NaI (0.5 eq)	40	16	26

a) In the cases of entries 1—4, a solution of the base was added dropwise to a solution of **6b** in MeOH. In the cases of entries 5—7, the base was added in one portion. *b*) Isolated yield, recrystallized from hexane-ether.

procedure of Bettahar *et al.*[11] to give the bromoacetylcyclohexanes (**6**) in 73—84% yield. Hydrolysis of 2-bromo-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (**6b**) into 2-hydroxyl-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (**7b**) under various basic conditions was examined as shown in Table IV in order to find optimum conditions. In this reaction, the yield was almost independent of the kind of bases (entries 1—6) and the reaction temperature (entries 1—5) except in the case of potassium formate as a base, which required a long reaction time at 40 °C (entry 6). Addition of a larger excess of sodium iodide than that used in entry 6 rather lowered the yield (entry 7). Thus, the conditions of entry 4 are recommended as a general method for the preparation of **7** starting from **6**.

Esterification of **7** into the *trans*-arenesulfonates (*trans*-**3**) was performed by the same method as described in the preceding paper.[1c] The properties of the products were identical with those of the *trans*-arenesulfonates (*trans*-**3**) obtained from the *trans*-diazoketones (*trans*-**2**). The synthetic route to *trans*-**3** starting from **4** seems to be suitable for large-scale operation.

Conclusion

We prepared the *trans*- (*trans*-**3**) and the *cis*-arenesulfonates (*cis*-**3**) from the pure *trans*- (*trans*-**2**) and the *cis*-diazoketones (*cis*-**2**), respectively, and their esterase-inhibitory activity and hypolipidemic effect were evaluated. The *trans*-isomers (*trans*-**3**) were synthesized in six steps starting from the acetophenone derivatives (**4**) and the synthetic route seems to be

suitable for large-scale operation. The biological activities of the *trans*-isomers (*trans*-**3**) were found to be more potent than those of the *cis*-isomers (*cis*-**3**). Among the effective *trans*-isomers (*trans*-**3**), we consider that *trans*-**3a**, *trans*-**3b** and *trans*-**3f** may be favorable as hypolipidemic agents, and these compounds are now undergoing pre-clinical studies.

Experimental

All melting point were recorded with Yanagimoto micromelting point apparatus and are uncorrected. Spectral data were obtained as follows: infrared (IR) spectra with a Hitachi 260-50 spectrophotometer; mass spectra (MS) with a JEOL JMS-01G-2 spectrometer; ^{1}H-NMR spectra with JEOL JMN-FX 100 and Bruker WH-400 spectrometers (using tetramethylsilane as an internal standard). Chemical shifts of ^{1}H-NMR spectra are given in δ values (ppm).

Starting Materials——Stereoisomeric mixtures of the 4-alkylcyclohexanecarboxylic acids (**1a—e**) were prepared by catalytic hydrogenation of the corresponding 4-alkylbenzoic acids over platinum oxide according to the procedure described in the preceding paper.[1c] **1a**[4]: bp 133—134 °C/1 mmHg. **1b**: bp 128—130 °C/3 mmHg (lit.[12] bp 118—119 °C/0.8 mmHg). **1c**[13]: bp 173 °C/20 mmHg. MS *m/z*: 184 (M^+). ^{1}H-NMR ($CDCl_3$): 0.75—1.90 (18H, m), 2.00—2.75 (1H, m), 11.10 (1H, br). **1d**: mp 92—94 °C (lit.[14] mp 111 °C). MS *m/z*: 184 (M^+). ^{1}H-NMR ($CDCl_3$): 0.75—2.15 (18H, m), 2.20—2.75 (1H, m), 11.70 (1H, br). **1e**[4]: bp 120 °C/1 mmHg. The 4-alkylacetophenones (**4a—d**) were prepared from alkylbenzene and acetyl chloride by means of the Friedel-Crafts reaction according to the procedure of Allen.[15] **4a**: bp 94—96 °C/1 mmHg (lit.[15] bp 118 °C/13 mmHg). **4b**: bp 98—99 °C/2 mmHg (lit.[12] bp 110 °C/3 mmHg). **4c**: bp 98—101 °C/1 mmHg (lit.[16] bp 133—136 °C/2 mmHg). **4d**: bp 100—102 °C/2 mmHg (lit.[17] bp 134—135 °C/11 mmHg).

2-Diazo-1-(*trans*-4-ethylcyclohexyl)-1-ethanone (*trans*-2a) and 2-Diazo-1-(*cis*-4-ethylcyclohexyl)-1-ethanone (*cis*-2a)——Typical procedure for the syntheses of the stereoisomeric mixtures (**2a—e**) and for the separation into the *trans*-isomers (*trans*-**2a—e**) and the *cis*-isomers (*cis*-**2a—e**). A mixture of thionyl chloride (30 ml) and 4-ethylcyclohexanecarboxylic acid (**1e**)[4] (1.7 g) was stirred for 2 h under reflux, and then the reaction mixture was evaporated under reduced pressure. The residue (4-ethylcyclohexylcarbonyl chloride) was added dropwise to an ethereal solution (100 ml) of diazomethane (obtained from 7.0 g of nitrosomethylurea) under stirring with ice-cooling. After being stirred for 1 h, the reaction mixture was evaporated under reduced pressure to give **2a**[4] as a crude oil (stereoisomeric mixture, *cis*: *trans* = *ca.* 3 : 1). The crude oil (**2a**) (2.0 g) was chromatographed on a long silica gel column with chloroform as an eluent. From the first eluate, the *cis*-isomer (*cis*-**2a**) was obtained as a yellowish oily product. Yield, 1.0 g (51%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2100 (N_2), 1630 (CO). From the second eluate, the *trans*-isomers (*trans*-**2a**) was obtained as a yellowish oily product. Yield, 0.3 g (15%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2100 (N_2), 1630 (CO). Similar procedures were used for the preparations of the other stereoisomeric mixtures (**2b—e**) and for the separations into the *trans*-isomers (*trans*-**2b—e**) and the *cis*-isomers (*cis*-**2b—e**). Other data are listed in Table I.

2-(*trans*-4-Ethylcyclohexyl)-2-oxoethyl Benzenesulfonate (*trans*-3a) and 2-(*cis*-4-Ethylcyclohexyl)-2-oxoethyl Benzenesulfonate (*cis*-3a)——Typical procedure for the syntheses of the *trans*-isomers (*trans*-**3a—g**) and the *cis*-isomers (*cis*-**3a—g**). Benzenesulfonic acid monohydrate (1.5 g) was added to an ethereal solution (50 ml) of the *trans*-diazoketone (*trans*-**2a**) (0.5 g) under ice-cooling. After being stirred for 1 h at room temperature, the reaction mixture was washed with water and dried over sodium sulfate. The ethereal layer was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with chloroform as an eluent, followed by recrystallization from ethanol to give the *trans*-arenesulfonate (*trans*-**3a**). Yield, 0.65 g (76%). A similar procedure was used for the preparations of the *trans*- (*trans*-**3b—g**) and *cis*-arenesulfonates (*cis*-**3a—g**). Other data are listed in Tables II and III.

1-(4-Isobutylcyclohexyl)-1-ethanone (5b)——Typical procedure for the syntheses of **5a—d**. Hydrogenation of 4-isobutylacetophenone (**4b**) (40.0 g) in AcOH (70 ml) was carried out in the presence of a catalytic amount of rhodium-platinium (1.5 g) under a pressure of 50 atm at room temperature (about 4 h). The reaction mixture was evaporated under reduced pressure to give a crude mixture of the acetylcyclohexane (**5b**) and the cyclohexyl alcohol. Jones reagent (120 ml) (consisting of CrO_3; 31 g, conc. H_2SO_4; 27 ml and water) was added dropwise to a solution of the mixture in acetone (100 ml) under ice-cooling. The whole was stirred for 5 h at room temperature, then water (200 ml) was added and the separated material was extracted with ether (200 ml × 2). The ethereal layer was washed with water (50 ml), dried over sodium sulfate, and evaporated under reduced pressure to give **5b** as a crude oily product, which was purified by distillation. Yield, 32.0 g (77%). bp 120—130 °C/24 mmHg. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1695 (CO). MS *m/z*: 182 (M^+). ^{1}H-NMR ($CDCl_3$): 0.83, 0.86 (6H, d, J = 6.5 Hz), 0.75—2.65 (13H, m), 2.15, 2.16 (3H, s). A similar procedure was used for the syntheses of **5a**, **5c** and **5d**. **5a**: Yield, 77%. bp 105—115 °C/18 mmHg. (lit.[18] bp 59—60 °C/0.8 mmHg). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1695 (CO). MS *m/z*: 168 (M^+). ^{1}H-NMR ($CDCl_3$): 0.84, 0.86 (6H, d, J = 6.5 Hz), 0.80—2.60 (11H, m), 2.13, 2.14 (3H, s). **5c**: Yield, 68%. bp 110—125 °C/18 mmHg. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1700 (CO). MS *m/z*: 182 (M^+). **5d**: Yield, 92%. bp 125—130 °C/24 mmHg. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1700 (CO). MS *m/z*: 182 (M^+).

***trans*-1-(4-Isobutylcyclohexyl)-1-ethanone (*trans*-5b)**——Typical procedure for the syntheses of *trans*-**5a—d**. A

solution of sodium methoxide (9.9 g) in MeOH (350 ml) was added to the ketone (**5b**) (32.0 g). After being stirred for 4 h under refluxing, the reaction mixture was evaporated under reduced pressure, water (100 ml) was added and the mixture was extracted with benzene (200 ml). The benzene layer was washed with water, dried over sodium sulfate and evaporated under reduced pressure to give *trans*-**5b** as a crude oily product, which was purified by distillation. Yield, 25.0 g (78%). bp 125—128 °C/22 mmHg. ^{1}H-NMR ($CDCl_3$): 0.86 (6H, d, J=6.5 Hz), 0.78—2.45 (13H, m), 2.11 (3H, s). A similar procedure was used for the syntheses of *trans*-**5a**, *trans*-**5c** and *trans*-**5d**. *trans*-**5a**: Yield, 75%. bp 109—113 °C/19 mmHg. ^{1}H-NMR ($CDCl_3$): 0.86 (6H, d, J=6.5 Hz), 0.60—2.50 (11H, m), 2.13 (3H, s). *trans*-**5c**: Yield, 76%. bp 127—130 °C/18 mmHg. ^{1}H-NMR ($CDCl_3$): 0.60—2.10 (18H, m), 2.10—2.50 (1H, br), 2.13 (3H, s). *trans*-**5d**[19]: Yield, 74%. bp 127—131 °C/22 mmHg. ^{1}H-NMR ($CDCl_3$): 0.85 (9H, s), 0.70—2.45 (10H, m), 2.11 (3H, s).

2-Bromo-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (6b)——Typical procedure for the syntheses of **6a—d**. Bromine (14.0 g) was added in one portion to a solution of *trans*-**5b** (15.0 g) in MeOH (250 ml) at room temperature. After being stirred for 4 h, the reaction mixture was evaporated under reduced pressure. Water (20 ml) and benzene (100 ml) were added to the residue. The benzene layer was washed with water, dried over sodium sulfate and evaporated under reduced pressure to give **6b** as a crude oily product, which was purified by distillation. Yield, 18.0 g (84%). bp 124—127 °C/1.5 mmHg. ^{1}H-NMR ($CDCl_3$): 0.70—2.10 (18H, m), 2.46—2.86 (1H, br), 3.97 (2H, s). *Anal.* Calcd for $C_{12}H_{21}BrO$: C, 54.97; H, 8.07. Found: C, 55.35; H, 8.32. A similar procedure was used for the syntheses of **6a**, **6c** and **6d**. **6a**: Yield, 73%. bp 113—115 °C/2 mmHg. ^{1}H-NMR ($CDCl_3$): 0.75—2.15 (16H, m), 2.40—2.85 (1H, br), 3.95 (2H, s). *Anal.* Calcd for $C_{11}H_{19}BrO$: C, 53.45; H, 7.46. Found: C, 53.47; H, 7.75. **6c**: Yield, 75%. bp 130—133 °C/2 mmHg. ^{1}H-NMR ($CDCl_3$): 0.60—2.20 (18H, m), 2.44—2.84 (1H, br), 3.96 (2H, s). *Anal.* Calcd for $C_{12}H_{21}BrO$: C, 54.97; H, 8.07. Found: C, 54.59; H, 7.89. **6d**[9]: Yield, 81%. bp 117—119 °C/1 mmHg. ^{1}H-NMR ($CDCl_3$): 0.50—2.16 (18H, m), 2.44—2.82 (1H, br), 3.97 (2H, s). *Anal.* Calcd for $C_{12}H_{21}BrO$: C, 54.97; H, 8.07. Found: C, 54.90; H, 8.12.

2-Hydroxy-1-(*trans*-4-isobutylcyclohexyl)-1-ethanone (7b)——Typical procedure for the syntheses of **7a—d**. The reaction conditions of entry 4 in Table IV were used for the synthesis of **7b**. A solution of KOH (5.0 g) in MeOH (50 ml) was added dropwise to a solution of **6b** (18.0 g) in MeOH (180 ml) below 10 °C. After being stirred for 1 h at the same temperature, the reaction mixture was concentrated to about 50 ml under reduced pressure. Water (30 ml) was added and the mixture was extracted with ether (100 ml × 2). The ethereal layer was washed with water, dried over sodium sulfate and evaporated under reduced pressure to give **7b** as a crude oily product, which was recrystallized from hexane–ether. Yield, 9.3 g (68%). mp 69—70 °C. ^{1}H-NMR ($CDCl_3$): 0.86 (6H, d, J=6.6 Hz), 0.80—2.10 (12H, m), 2.34 (1H, tt, J=4, 12 Hz), 3.15 (1H, t, J=5 Hz), 4.30 (2H, d, J=5 Hz). *Anal.* Calcd for $C_{12}H_{22}O_2$: C, 72.68; H, 11.18. Found: C, 72.47; H, 11.30. Compound **7b** was also obtained by hydrolysis of **6b** under various basic conditions as shown in Table IV (entries 1—7). Reaction conditions and data are listed in Table IV. A similar procedure was used for the syntheses of **7a**, **7c** and **7d**. **7a**: Yield, 70%. mp 43—44 °C. ^{1}H-NMR ($CDCl_3$): 0.88 (6H, d, J=6.5 Hz), 0.70—2.10 (10H, m), 2.34 (1H, tt, J=4, 12 Hz), 3.16 (1H, t, J=4.5 Hz), 4.30 (2H, d, J=4.5 Hz). *Anal.* Calcd for $C_{11}H_{20}O_2$: C, 71.70; H, 10.94. Found: C, 71.51; H, 10.92. **7c**: Yield, 55%. bp 117—123 °C/2 mmHg. ^{1}H-NMR ($CDCl_3$): 0.85 (6H, d, J=6.5 Hz), 0.60—2.00 (12H, m), 2.32 (1H, tt, J=4, 12 Hz), 3.16 (1H, t, J=4.5 Hz), 4.30 (2H, d, J=4.5 Hz). **7d**: Yield, 73%. mp 64—65.5 °C. ^{1}H-NMR ($CDCl_3$): 0.85 (9H, s), 0.70—2.10 (9H, m), 2.32 (1H, tt, J=4, 12 Hz), 3.16 (1H, t, J=4.5 Hz), 4.30 (2H, d, J=4.5 Hz). *Anal.* Calcd for $C_{12}H_{22}O_2$: C, 72.68; H, 11.18. Found: C, 72.54; H, 11.45.

2-(*trans*-4-Isobutylcyclohexyl)-2-oxoethyl Benzenesulfonate (*trans*-3f)——Typical procedure for the syntheses of *trans*-**3b**, *trans*-**3e**, *trans*-**3f** and *trans*-**3g** from **7a—d**; Triethylamine (11 ml) was added dropwise to a solution of benzenesulfonyl chloride (12.0 g) and **7b** (15.0 g) in dichloromethane (20 ml) at 0—5 °C. After being stirred for 2 h, the reaction mixture was extracted with ether (200 ml) and washed with 1 N HCl (50 ml × 2). The ethereal layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was recrystallized from aqueous ethanol to give white crystals. The product was identical with *trans*-**3f**, which was obtained from the *trans*-diazoketone (*trans*-**2d**). Yield, 20.7 g (81%). A similar procedure was used for the syntheses of *trans*-**3b**, *trans*-**3e** and *trans*-**3g** in 60%, 54% and 73% yields, respectively.

Enzyme-Inhibitory Activities——The esterase- and chymotrypsin-inhibitory activities were determined by the method described in the previous paper.[1]

Pharmacology——The triglyceride level in plasma was measured by the same method as described in the preceding paper.[1c]

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Synthesis of 1,2,3,4-Tetrahydro-β-carboline Derivatives as Hepatoprotective Agents. III. Introduction of Substituents onto Methyl 1,2,3,4-Tetrahydro-β-carboline-2-carbodithioate

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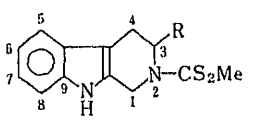
Dithiocarbamates of various substituted tetrahydro-β-carbolines were synthesized and tested for hepatoprotective activity against carbon tetrachloride (CCl_4)-induced liver damage in mice. Structure-activity relationships were investigated. Some neighboring group participation of the 3-substituent with the dithiocarbamate group appeared to be important for the manifestation of activity. The compounds (**1a**, **2a**, and **3i**) with hydrophilic substituents at the 3 poisition exhibited significant activity. Substitution at the 9 position of the 3-carboxylic acid (**1a**) lowered the activity.

Keywords——dithiocarbamate; substituted tetrahydro-β-carboline; hepatoprotective activity; carbon tetrachloride-induced liver damage; structure-activity relationship; neighboring group participation

In our previous paper,[1] 1,2,3,4-tetrahydro-2-methylthiothiocarbonyl-β-carboline-3-carboxylic acid (**1a**) was found to exhibit potent hepatoprotective activity against acutely carbon tetrachloride (CCl_4)-induced liver damage in mice. This observation was followed by the findings[2] that the corresponding 3-hydroxymethyl derivative (**2a**) is equipotent to **1a** and that the methyl dithiocarbamate exhibits the most potent activity in a series of alkyl congeners. Our continued interest in the structure-activity relationships (SAR) of **1a** and related compounds as new hepatoprotective agents led us to examine further the effects of substitution on C_3, the indole nitrogen, and the benzene ring of β-carboline. This paper describes the synthesis and hepatoprotective activity of various 3-substituted derivatives (**3a**—**i**) and several derivatives of **1a** and **2a** bearing substituents at the 6, 8, or 9 position.

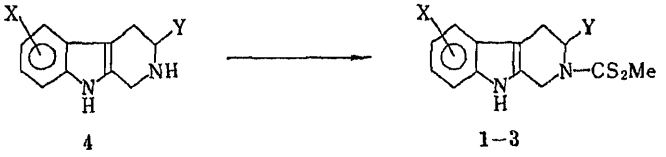
Chemistry

Since no difference between the activities of the optical isomers of **1a** and **2a** was observed,[2] the choice of the starting material (3*S*, 3*R*, or 3*RS*) was arbitrary in the present study. Conversion of the 2-benzyloxycarbonyl (Cbz)-3-carboxylic acid [(*RS*)-**5**][3] to (*RS*)-**6**, **7**, followed by reductive removal of the Cbz group gave the amides[4] [(*RS*)-**4a**, **b**]. Dehydration of (*RS*)-**6** with phosphorus oxychloride in pyridine gave the nitrile [(*RS*)-**8**]. Reaction of (*RS*)-

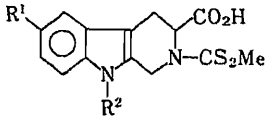


1a : R = CO_2H
2a : R = CH_2OH

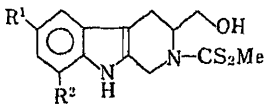
Chart 1



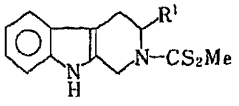
4 1–3



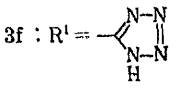
1a : R^1, R^2=H
1b : R^1=H, R^2=Me
1c : R^1=H, R^2=CH_2Ph
1d : R^1=Cl, R^2=H
1e : R^1=OH, R^2=H
1f : R^1=OMe, R^2=H
1g : R^1=Me, R^2=H



2a : R^1, R^2=H
2b : R^1=F, R^2=H
2c : R^1=Cl, R^2=H
2d : R^1=Me, R^2=H
2e : R^1=H, R^2=Me



3a : R^1=H
3b : R^1=CO_2Me
3c : R^1=CH_2CO_2H
3d : R^1=$CONH_2$
3e : R^1=$CONMe_2$



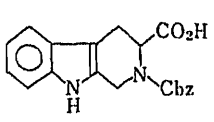
3f : R^1=

3g : R^1=CH_2OMe

3h : R^1=

3i : R^1=CH_2CH_2OH

Chart 2



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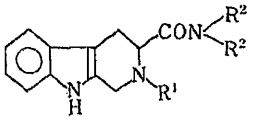
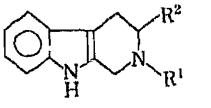
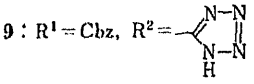


6 : R^1=Cbz, R^2=H
4a : R^1, R^2=H

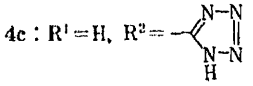


7 : R^1=Cbz, R^2=Me
4b : R^1=H, R^2=Me

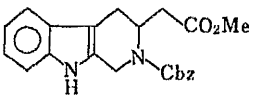
8 : R^1=Cbz, R^2=CN



9 : R^1=Cbz, R^2=



4c : R^1=H, R^2=



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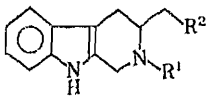
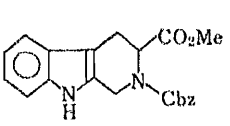


11 : R^1=Cbz, R^2=CO_2H
4d : R^1=H, R^2=CO_2H

12 : R^1=Cbz, R^2=CH_2OH
4e : R^1=H, R^2=CH_2OH



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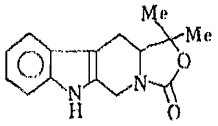

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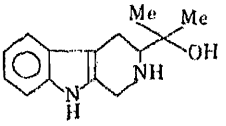
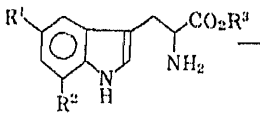
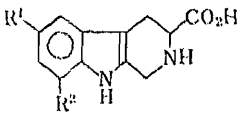


4f

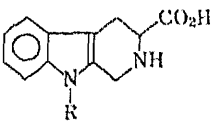


15 : R^3=H
16 : R^3=Me

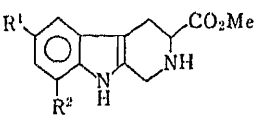


4g : R^1=Cl, R^2=H
4h : R^1=OH, R^2=H

4i : R^1=OMe, R^2=H
4j : R^1=Me, R^2=H



18a : R=Me
18b : R=CH_2Ph



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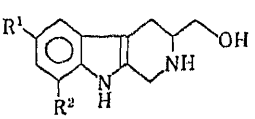


4k : R^1=F, R^2=H
4l : R^1=Cl, R^2=H

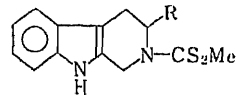
4m : R^1=Me, R^2=H
4n : R^1=H, R^2=Me

Chart 3

8 with sodium azide followed by removal of the Cbz group gave the 3-(5-tetrazolyl) derivative [(*RS*)-**4c**]. Homologenation of the 2-Cbz-3-carboxylic acid [(*R*)- and (*S*)-**5**] was effected by means of the Arndt–Eistert reaction in the usual manner, giving the homoester [(*R*)- and (*S*)-**10**]. Alkaline hydrolysis of (*S*)-**10** followed by reductive removal of the Cbz group gave the 3-acetic acid [(*S*)-**4d**]. The 3-ethanol derivative [(*R*)-**4e**] was also obtained from [(*R*)-**10**] by sodium borohydride ($NaBH_4$) reduction and subsequent hydrogenolysis. Grignard reaction of the 2-Cbz-3-methoxycarbonyl derivative [(*RS*)-**13**] with methylmagnesium iodide (MeMgI) gave the fused oxazolidinone [(*RS*)-**14**], which gave, on alkaline hydrolysis, the *gem*-dimethylcarbinol [(*RS*)-**4f**]. Various 3-substituted *β*-carbolines (**4**) thus obtained were allowed to react with carbon disulfide in the presence of potassium hydroxide or triethylamine and treated with methyl iodide, giving the corresponding methylthiothiocarbonyl derivatives (**3a—f, h, i**) listed in Table I. The 3-methoxymethyl derivative (**3g**) was prepared by the methylation of **2a** with diazomethane in the presence of silica gel.[5]

Pictet–Spengler cyclization[6] of the substituted tryptophans (**15**)[7] gave the *β*-carboline-3-carboxylic acids (**4g—j**), which were converted to the corresponding dithiocarbamates (**1d—g**, Table II) in the usual manner. The 9-substituted *β*-carboline-3-carboxylic acids[8] (**18a, b**) were similarly converted to the dithiocarbamates (**1b, c**, Table II). The 3-hydroxymethyl-*β*-carbolines (**4k—n**) bearing substituents at the 6 or 8 position were prepared from the substituted tryptophan methyl esters (**16**)[7] through the usual sequence of reactions (Chart 3) and converted to the dithiocarbamates (**2b—e**) listed in Table III.

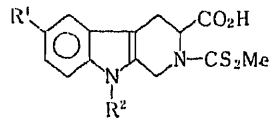
TABLE I. Dithiocarbamate Derivatives of 3-Substituted Tetrahydro-*β*-carbolines



Compd. No.	R	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found)				Hepatoprotective activity[d]	
					C	H	N	S	100 mg/kg	10 mg/kg
1a[a]	CO_2H								AA	AA
2a[b]	CH_2OH								AA	AA
(*RS*)-**3a**	H	145—146 (EtOH)	30	$C_{13}H_{14}N_2S_2$	59.51 (59.45	5.38 5.40	10.68 10.69	24.44 24.26)	B	
(*RS*)-**3b**	CO_2Me	132—134 (Et_2O–hexane)	66	$C_{15}H_{16}N_2O_2S_2$	56.22 (56.46	5.03 5.05	8.74 8.67	20.01 20.01)	AA	D
(*S*)-**3c**[c]	CH_2CO_2H	219—220 ($EtOH$–H_2O)	12	$C_{15}H_{16}N_2O_2S_2 \cdot H_2O$	53.23 (53.36	5.36 5.29	8.28 8.05	18.95 18.78)	AA	D
(*RS*)-**3d**	$CONH_2$	224—225 (AcOEt)	41	$C_{14}H_{15}N_3OS_2$	55.19 (55.29	4.95 4.99	13.76 13.88	21.00 20.91)	A	
(*RS*)-**3e**	$CONMe_2$	211—213 (AcOEt)	29	$C_{16}H_{19}N_3OS_2$	57.63 (57.54	5.74 5.73	12.60 12.55	19.23 19.23)	AA	D
(*RS*)-**3f**	5-Tetrazolyl	203—205 (dec.) ($CHCl_3$)	27	$C_{14}H_{14}N_6S_2$	50.89 (50.78	4.27 4.24	25.43 25.27	19.41 19.13)	AA	C
(*RS*)-**3g**	CH_2OMe	162—164 (AcOEt–hexane)	21	$C_{15}H_{18}N_2OS_2$	58.79 (58.58	5.92 5.90	9.14 8.97	20.93 20.81)	AA	D
(*RS*)-**3h**	$C(Me)_2OH$	184—186 ($EtOH$–H_2O)	11	$C_{16}H_{20}N_2OS_2 \cdot 0.5H_2O$	58.33 (58.44	6.42 6.13	8.50 8.44	19.46 19.37)	B	
(*R*)-**3i**[c]	CH_2CH_2OH	166—167 (AcOEt–hexane)	38	$C_{15}H_{18}N_2OS_2$	58.79 (58.84	5.92 6.04	9.14 8.98	20.93 20.78)	AA	AA

a) See reference 1. *b*) See reference 2. *c*) (*S*)-**3c**, $[\alpha]_D^{20}$ +184.0° (*c*=1.0, MeOH); (*R*)-**3i**, $[\alpha]_D^{20}$ −178.0° (*c*=1.0, MeOH). *d*) AA=significantly effective; A, B, and C=effective; D=ineffective. For criteria, see reference 1.

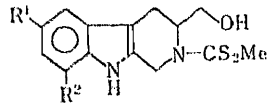
TABLE II. Dithiocarbamate Derivatives of 6-, and 9-Substituted Tetrahydro-β-carboline-3-carboxylic Acid



Compd. No.	R^1	R^2	mp (°C) (Recryst. solvent)	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	S	Hepatoprotective activity[b] 100 mg/kg	10 mg/kg
(*RS*)-**1b**	H	Me	212—213 (EtOH)	87	$C_{15}H_{16}N_2O_2S_2$	56.21 (56.23)	5.03 (5.01)	8.80 (8.67)	20.01 (19.91)	A	
(*RS*)-**1c**	H	CH_2Ph	Powder	52	$C_{21}H_{20}N_2O_2S_2$	63.61 (63.53)	5.08 (5.01)	7.06 (6.98)	16.17 (15.88)	A	
(*R*)-**1d**[a]	Cl	H	Powder	25	$C_{14}H_{13}ClN_2O_2S_2$	49.33 (49.23)	3.84 (3.63)	8.22 (8.11)	18.81 (18.83)	AA	AA
(*RS*)-**1e**	OH	H	Powder	26	$C_{14}H_{14}N_2O_3S_2 \cdot H_2O$	47.39 (47.58)	5.00 (4.85)	7.89 (7.79)	18.07 (18.13)	B	
(*S*)-**1f**[a]	OMe	H	Powder	52	$C_{15}H_{16}N_2O_3S_2$	53.55 (53.58)	4.79 (4.92)	8.33 (8.03)	19.06 (18.97)	AA	B
(*S*)-**1g**[a]	Me	H	200—201 (EtOH–H_2O)	66	$C_{15}H_{16}N_2O_2S_2$	56.23 (56.34)	5.03 (5.03)	8.74 (8.70)	20.01 (20.13)	AA	AA

a) **1d**, $[\alpha]_D^{20}$ −210.8° (*c*=0.5, MeOH); **1f**, $[\alpha]_D^{20}$ +228.6° (*c*=1.0, MeOH); **1g**, $[\alpha]_D^{20}$ +240.8° (*c*=0.8, MeOH). *b*) See footnote *d*) in Table I.

TABLE III. Dithiocarbamate Derivatives of 6-, and 8-Substituted Tetrahydro-3-hydroxymethyl-β-carbolines



Compd. No.	R^1	R^2	mp (°C) (Recryst. solvent)	Yield (%)	$[\alpha]_D^{20}$ (°) in MeOH (*c*)	Formula	Analysis (%) Calcd (Found) C	H	N	S	Hepatoprotective activity[a] 100 mg/kg	10 mg/kg
(*S*)-**2b**	F	H	Powder	95	+132.0 (0.5)	$C_{14}H_{15}FN_2OS_2$	54.17 (53.88)	4.87 (4.79)	9.02 (8.93)	20.66 (20.41)	AA	AA
(*S*)-**2c**	Cl	H	180—184 (EtOH–H_2O)	86	+98.0 (1.0)	$C_{14}H_{15}ClN_2OS_2$	51.44 (51.46)	4.63 (4.65)	8.57 (8.77)	19.62 (19.67)	AA	C
(*S*)-**2d**	Me	H	170—172 (EtOH)	78	+108.0 (1.0)	$C_{15}H_{12}N_2OS_2$	58.79 (58.50)	5.92 (5.68)	9.14 (8.97)	20.93 (20.63)	AA	A
(*S*)-**2e**	H	Me	Powder	83	+199.2 (1.0)	$C_{15}H_{18}N_2OS_2$	58.79 (58.61)	5.92 (5.93)	9.14 (9.06)	20.92 (20.72)	AA	A

a) See footnote *d*) in Table I.

Pharmacology and SAR

The dithiocarbamates (**1**, **2**, and **3**) were tested for hepatoprotective activity against CCl_4-induced liver damage in mice after oral administration by the method reported previously.[1] The results were evaluated according to the criteria defined previously[1] and are included in Tables I—III.

With regard to the effect of modifying the 3-substituent (Table I), most of the derivatives (**3b**, **c**, **3e—g**, **3i**) displayed significant activity (AA) at 100 mg/kg orally. Two exceptions of

significant interest are the unsubstituted derivative (**3a**) and the sterically hindered *gem*-dimethylcarbinol (**3h**), which showed much reduced activity. These results appear to suggest that some neighboring group participation of the 3-substituent with the dithiocarbamate group is important for the appearance of activity. Looking at the results at 10 mg/kg, one observes that the presence of a hydrophilic substituent at the 3-position has a favorable effect. The 3-carboxylic acid (**1a**), 3-hydroxymethyl derivative (**2a**) and its homologue (**3i**) thus exhibit significant activity (AA). Neither the corresponding ester (**3b**) nor the methyl ether (**3g**) was effective at this dose.

Substitution at the 9 position of **1a** lowered the activity, as exemplified by the methyl (**1b**) and benzyl (**1c**) derivatives (Table II). The effects of substitution on the benzene ring of **1a** and **2a** were examined in a series of derivatives listed in Tables II and III. Although no clear SAR can be deduced, 6-Cl(**1d**), 6-Me(**1g**), and 6-F(**2b**) derivatives retain the activity of the parent compounds (**1a** and **2a**). Other substitutions on the benzene ring resulted in a decrease of the activity.

Further studies on the synthesis and hepatoprotective activity of new β-carbolines are in progress.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi IR-215 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken on a JEOL model 60 instrument. Chemical shifts are given as δ values from tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, and br = broad. Mass spectra (MS) were measured with a Hitachi RMU-6M instrument. Optical rotations were determined on a Union PM-201 automatic digital polarimeter.

(3*RS*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-carboxamide [(*RS*)-6]——Ethyl chloroformate (6.6 g, 60 mmol) was added dropwise to a solution of (*RS*)-**5** (21.0 g, 60 mmol) and Et_3N (8.4 ml, 60 mmol) in tetrahydrofuran (THF) (320 ml), and the whole was stirred at -10 °C for 40 min. After addition of 28% NH_4OH (5.9 ml), the mixture was stirred at 0 °C for 3 h. Insoluble material was filtered off, and the filtrate was concentrated under reduced pressure. The residue was extracted with AcOEt, and the extract was washed with sat. aqueous $NaHCO_3$ and water, dried over $MgSO_4$, and evaporated. The residue was triturated with hexane to give (*RS*)-**6** (17.9 g, 85%) as a powder. IR ν_{max}^{Nujol} cm^{-1}: 3430, 3280, 1680. NMR ($CDCl_3$–dimethylsulfoxide-d_6 (DMSO-d_6)) δ: 5.17 (2H, s). MS *m/z*: 349 (M^+). *Anal.* Calcd for $C_{20}H_{19}N_3O_3 \cdot 0.25CH_3CO_2C_2H_5$: C, 67.91; H, 5.70; N, 11.31. Found: C, 68.16; H, 5.63; N, 11.30.

(3*RS*)-1,2,3,4-Tetrahydro-β-carboline-3-carboxamide [(*RS*)-4a]——The amide [(*RS*)-**6**] (2.47 g, 7 mmol) was hydrogenated over 10% Pd–C in EtOH (350 ml) containing AcOH (2 drops) under ordinary pressure and temperature. After removal of Pd–C and the solvent, the resulting foam was crystallized from ether to give (*RS*)-**4a** (1.21 g, 79%), mp 212—216 °C (dec.). (lit.[4] mp 216—219 °C).

(3*RS*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-*N*,*N*-dimethylcarboxamide [(*RS*)-7]——A mixture of (*RS*)-**5** (21 g, 60 mmol), 1-hydroxybenzotriazole (8.1 g, 60 mmol), $HNMe_2 \cdot HCl$ (4.9 g, 60 mmol), Et_3N (8.4 ml, 60 mmol), dicyclohexylcarbodiimide (DCC, 12.4 g, 60 mmol) and THF (200 ml) was stirred at 5 °C for 1 h and then at room temperature for 16 h. The solvent was evaporated off under reduced pressure, and AcOEt (500 ml) was added to the residue. Insoluble material was filtered off, and the filtrate was washed with 10% HCl, water, sat. aqueous $NaHCO_3$, and water. After removal of the solvent, the residue was crystallized from AcOEt to give (*RS*)-**7** (15.0 g, 67%), mp 179—180 °C. IR ν_{max}^{Nujol} cm^{-1}: 3250, 1700, 1640. NMR ($CDCl_3$) δ: 2.90 (3H, br s), 3.10 (3H, br s), 5.20 (2H, s). MS *m/z*: 377 (M^+), 305. *Anal.* Calcd for $C_{22}H_{23}N_3O_3$: C, 70.01; H, 6.14; N, 11.13. Found: C, 70.47; H, 6.15; N, 11.01.

(3*RS*)-1,2,3,4-Tetrahydro-β-carboline-3-*N*,*N*-dimethylcarboxamide [(*RS*)-4b]——This compound was prepared from (*RS*)-**7** in a manner similar to that described for (*RS*)-**4a**. mp 199—200 °C (dec.), 91%. IR ν_{max}^{Nujol} cm^{-1}: 3400, 1655. NMR ($CDCl_3$) δ: 2.91 (3H, s), 2.99 (3H, s). MS *m/z*: 243 (M^+). *Anal.* Calcd for $C_{14}H_{17}N_3O$: C, 69.11; H, 7.04; N, 17.27. Found: C, 68.88; H, 7.01; N, 17.30.

(3*RS*)-2-Benzyloxycarbonyl-3-cyano-1,2,3,4-tetrahydro-β-carboline [(*RS*)-8]——$POCl_3$ (0.37 ml, 4 mmol) was added to a solution of (*RS*)-**6** (1.05 g, 3 mmol) in pyridine (6 ml) at -5 °C, and the mixture was stirred at room temperature for 2 h. The mixture was poured onto ice-water and extracted with AcOEt. The extract was washed with 10% HCl and water, and then dried over $MgSO_4$. The solvent was evaporated off to give (*RS*)-**8** (810 mg, 82%) as a powder. IR ν_{max}^{Nujol} cm^{-1}: 2210, 1690. MS *m/z*: 331 (M^+), 240, 196. *Anal.* Calcd for $C_{20}H_{17}N_3O_2$: C, 72.49; H, 5.17; N, 12.68. Found: C, 72.42; H, 5.12; N, 12.46.

(3*RS*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-3-(1*H*-tetrazol-5-yl)-β-carboline [(*RS*)-9]——A mixture of (*RS*)-**8** (663 mg, 2 mmol), NaN_3 (143 mg, 2.2 mmol), NH_4Cl (118 mg, 2.2 mmol), and dimethylformamide (DMF) (2 ml) was heated at 95—100 °C for 6 h and poured onto ice-water. The mixture was acidified with 10% HCl and extracted with AcOEt. The extract was washed with water, dried, and concentrated. The residue was crystallized from AcOEt to yield (*RS*)-**9** (358 mg, 48%), mp 218—219 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3380, 1700. MS *m/z*: 374 (M^+), 329, 240. *Anal.* Calcd for $C_{20}H_{18}N_6O_2$: C, 64.16; H, 4.85; N, 22.45. Found: C, 63.95; H, 4.75; N, 22.28.

(3*RS*)-1,2,3,4-Tetrahydro-3-(1*H*-tetrazol-5-yl)-β-carboline [(*RS*)-4c]·Hydrobromide——A 25% HBr–AcOH solution (2 ml) was added to a solution of (*RS*)-**9** (300 mg, 0.8 mmol) in AcOH (1 ml), and the whole was stirred at room temperature for 20 min. Ether was added to the mixture, and a precipitated solid was filtered off, washed with ether, and then dried. The tetrazole [(*RS*)-**4c**]·hydrobromide (257 mg, quantitative yield) was obtained as a pale yellow powder. IR ν_{max}^{Nujol} cm^{-1}: 3240, 1630. MS *m/z*: 240 (M^+), 171. *Anal.* Calcd for $C_{12}H_{12}N_6$·HBr: C, 44.88; H, 4.08; Br, 24.88; N, 26.17. Found: C, 45.12; H, 4.19; Br, 24.99; N, 26.01.

Methyl (3*R*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-acetate [(*R*)-10]——*N*-Methylmorpholine (0.30 g, 3 mmol) and isobutyl chloroformate (0.39 ml, 3 mmol) were added to a solution of (*R*)-**5** (1.05 g, 3 mmol) in THF (50 ml) at −5 °C, and the whole was stirred at −5 °C for 40 min. An ethereal solution of diazomethane [prepared from *N*-methylnitrosourea (1.55 g, 15 mmol) and 40% KOH (4.5 ml) in ether (15 ml)] was added dropwise to the mixture, and the whole was stirred at the same temperature for 4.5 h. After removal of the solvent, the residue was dissolved in MeOH (40 ml), and a solution of silver benzoate (90 mg) in Et_3N (0.9 ml) was added. The whole was stirred for 1 h, and insoluble material was filtered off. The filtrate was concentrated under reduced pressure. The residue was dissolved in AcOEt, washed with 10% HCl, water, sat. aqueous $NaHCO_3$, and water, and dried over $MgSO_4$. The solvent was evaporated off to give a pale yellow oil, which was purified by thin layer chromatography (AcOEt : hexane = 4 : 6) to provide (*R*)-**10** (345 mg, 30%), mp 173—174 °C. IR ν_{max}^{Nujol} cm^{-1}: 3380, 1735, 1680. NMR ($CDCl_3$) δ: 3.53 (3H, s), 5.15 (2H, s). MS *m/z*: 378 (M^+), 347, 305, 287, 243. $[\alpha]_D^{23}$ −54.6° (*c* = 1.0, THF). *Anal.* Calcd for $C_{22}H_{22}N_2O_4$: C, 69.83; H, 5.86; N, 7.40. Found: C, 69.54; H, 5.83; N, 7.26.

Methyl (3*S*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-acetate [(*S*)-10]——The ester [(*S*)-**10**] was similarly prepared from (*S*)-**5** in 42% yield. mp 174—176 °C. IR ν_{max}^{Nujol} cm^{-1}: 3380, 1735, 1680. NMR ($CDCl_3$) δ: 3.55 (3H, s), 5.17 (2H, s). MS *m/z*: 378 (M^+), 305, 287, 243. $[\alpha]_D^{22}$ +54.4° (*c* = 1.0, THF). *Anal.* Calcd for $C_{22}H_{22}N_2O_4$: C, 69.83; H, 5.86; N, 7.40. Found: C, 69.71; H, 5.77; N, 7.40.

(3*S*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-β-carboline-3-acetic Acid [(*S*)-11]——A mixture of (*S*)-**10** (870 mg, 2.3 mmol), 1 N NaOH (5 ml), and THF (12 ml) was stirred at room temperature for 16 h and then concentrated under reduced pressure. The residue was dissolved in water and extracted with AcOEt. The aqueous phase was acidified with 10% HCl, and extracted with AcOEt. The extract was washed with water, dried, and then evaporated to give an oil. Trituration of this oil with hexane yielded (*S*)-**11** (630 mg, 75%) as a pale yellow powder. IR ν_{max}^{Nujol} cm^{-1}: 3360, 1700, 1670. NMR ($CDCl_3$) δ: 5.17 (2H, s). MS *m/z*: 364 (M^+), 273, 229. $[\alpha]_D^{20}$ +68.0° (*c* = 1.0, MeOH). *Anal.* Calcd for $C_{21}H_{20}N_2O_4$: C, 69.22; H, 5.53; N, 7.69. Found: C, 69.09; H, 5.42; N, 7.48.

(3*S*)-1,2,3,4-Tetrahydro-β-carboline-3-acetic Acid [(*S*)-4d]·Hydrochloride——A solution of (*S*)-**11** (550 mg, 1.5 mmol) in 80% EtOH (12 ml)–10% HCl (3 ml) was hydrogenated in the presence of 10% Pd–C (500 mg) under ordinary pressure and temperature. After removal of Pd–C and the solvent, the resulting solid was recrystallized from AcOEt to give (*S*)-**4d**·hydrochloride (290 mg, 72%), mp 233—235 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3100—3420, 1680. MS *m/z*: 230 (M^+), 169, 143. $[\alpha]_D^{20}$ −16.0° (*c* = 1.0, H_2O). *Anal.* Calcd for $C_{13}H_{14}N_2O_2$·HCl: C, 58.54; H, 5.67; Cl, 13.29; N, 10.50. Found: C, 58.38; H, 5.53; Cl, 13.35; N, 10.47.

(3*R*)-2-Benzyloxycarbonyl-1,2,3,4-tetrahydro-3-(2-hydroxyethyl)-β-carboline [(*R*)-12]——A solution of $NaBH_4$ (347 mg, 9.2 mmol) in 80% EtOH (5 ml) was added dropwise to a solution of (*R*)-**10** (580 mg, 1.53 mmol) in 80% EtOH (10 ml) under cooling in an ice-bath, and the mixture was stirred at room temperature for 16 h. After removal of the solvent, the residue was diluted with water, and extracted with AcOEt. The extract was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized from AcOEt–hexane to give (*R*)-**12** (481 mg, 90%), mp 125—127 °C. IR ν_{max}^{Nujol} cm^{-1}: 3500, 3360, 1670. NMR ($CDCl_3$) δ: 5.20 (2H, s). MS *m/z*: 350 (M^+), 259, 215. $[\alpha]_D^{20}$ −39.2° (*c* = 1.0, $CHCl_3$). *Anal.* Calcd for $C_{21}H_{22}N_2O_3$: C, 71.98; H, 6.33; N, 7.99. Found: C, 71.82; H, 6.30; N, 7.88.

(3*R*)-1,2,3,4-Tetrahydro-3-(2-hydroxyethyl)-β-carboline [(*R*)-4e]——A mixture of (*R*)-**12** (440 mg, 1.25 mmol) in EtOH (10 ml) was shaken with H_2 in the presence of 10% Pd–C (200 mg) under ordinary pressure and temperature. The catalyst was filtered off, and the filtrate was concentrated. The residue was treated with hexane to afford (*R*)-**4e** (205 mg, 75%) as a powder. IR ν_{max}^{Nujol} cm^{-1}: 3150—3280 (br). MS *m/z*: 216 (M^+), 169, 143. *Anal.* Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.03; H, 7.45; N, 12.88.

(11a *RS*)-5,5a,11,11a-Tetrahydro-1,1-dimethyl-1*H*,3*H*-oxazolo[4′,3′ : 6,1]pyrido[3,4-*b*]indol-3-one[(*RS*)-14]——A solution of (*RS*)-**13**[4] (1.82 g, 5 mmol) in THF (10 ml) was added dropwise to a solution of MeMgI [prepared from Mg (0.49 g, 20 matm) and MeI (1.25 ml, 20 mmol) in ether (10 ml)–THF (20 ml)], and the mixture was stirred at room temperature for 1 h and then refluxed for 3 h. After removal of the solvent, 5% NH_4Cl was added to the residue. The mixture was extracted with AcOEt, and the extract was washed with water, dried over $MgSO_4$, and then concentrated. The residue was dissolved in AcOEt and allowed to stand at room temperature to give (*RS*)-**14**

(410 mg, 32%) as colorless needles, mp 204—206 °C. IR ν_{max}^{Nujol} cm^{-1}: 1740, 1685 (sh). NMR ($CDCl_3$) δ: 1.50 (3H, s), 1.55 (3H, s). MS *m/z*: 256 (M^+), 241, 223. *Anal.* Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.17; H, 6.11; N, 10.85.

(3*RS*)-1,2,3,4-Tetrahydro-3-(2-hydroxypropan-2-yl)-β-carboline [(*RS*)-4f]——A mixture of (*RS*)-**14** (128 mg, 0.5 mmol), 10 N NaOH (2 ml, 20 mmol), and MeOH (4 ml) was refluxed for 22 h. After cooling, the mixture was made acidic with 10% HCl. Insoluble material was filtered off, and the filtrate was basified with 10% NaOH. The precipitate was filtered off, washed with water, and dried to give (*RS*)-**4f** (61 mg, 54%) as a powder. IR ν_{max}^{Nujol} cm^{-1}: 3350 (br). NMR (DMSO-d_6) δ: 1.22 (6H, s). MS *m/z*: 230 (M^+), 171. *Anal.* Calcd for $C_{14}H_{18}N_2O$: C, 73.01; H, 7.88; N, 12.16. Found: C, 72.92; H, 7.65; N, 12.03.

(3*R*)-6-Chloro-1,2,3,4-tetrahydro-β-carboline-3-carboxylic Acid [(*R*)-4g]——A solution of D-5-chlorotryptophan[7] (**15**, R^1=Cl, R^2=R^3=H) (0.61 g, 2.56 mmol), 35% formalin (0.27 g, 3 mmol), and 0.1 N H_2SO_4 (4 ml) in H_2O (1.4 ml)–EtOH (2 ml) was stirred at room temperature for 18 h. The resulting solid was filtered off, washed with water, and dried to give (*R*)-**4g** (0.64 g, 99%), mp 272—274 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3280, 1640. MS *m/z*: 252, 250 (M^+). $[\alpha]_D^{20}$ +89.6° (*c*=0.5, 0.1 N, NaOH). *Anal.* Calcd for $C_{12}H_{11}ClN_2O_2 \cdot 1.5H_2O$: C, 51.90; H, 5.08; Cl, 12.77; N, 10.09. Found: C, 52.19; H, 4.92; Cl, 13.05; N, 9.87. The following compounds were similarly synthesized from DL-5-hydroxy-, L-5-methoxy- and L-5-methyltryptophan,[7] respectively.

(3*RS*)-1,2,3,4-Tetrahydro-6-hydroxy-β-carboline-3-carboxylic Acid [(*RS*)-4h]——mp 269 °C (dec.), 91%. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1650 (sh), 1620. MS *m/z*: 232 (M^+) 215, 184, 159. *Anal.* Calcd for $C_{12}H_{12}N_2O_3 \cdot 1.5H_2O$: C, 55.59; H, 5.83; N, 10.80. Found: C, 55.81; H, 5.50; N, 10.74.

(3*S*)-1,2,3,4-Tetrahydro-6-methoxy-β-carboline-3-carboxylic Acid [(*S*)-4i]——mp 285—286 °C (dec.), 68%. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1640. MS *m/z*: 246 (M^+). *Anal.* Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.37. Found: C, 63.13; H, 5.57; N, 11.36.

(3*S*)-1,2,3,4-Tetrahydro-6-methyl-β-carboline-3-carboxylic Acid [(*S*)-4j]——mp 298—300 °C (dec.), 86%. IR ν_{max}^{Nujol} cm^{-1}: 3280, 1635. MS *m/z*: 230 (M^+). $[\alpha]_D^{20}$ −119.8° (*c*=1.0, 0.1 N NaOH). *Anal.* Calcd for $C_{13}H_{14}N_2O_2 \cdot 0.25H_2O$: C, 66.51; H, 6.23; N, 11.93. Found: C, 66.51; H, 6.02; N, 11.83.

Methyl (3*S*)-6-Fluoro-1,2,3,4-tetrahydro-β-carboline-3-carboxylate [(*S*)-17, R^1=F, R^2=H]·Hydrochloride——A mixture of L-5-fluorotryptophan methyl ester hydrochloride [(*S*)-**15**·HCl, R^1=F, R^2=H] (0.927 g, 3.4 mmol), 35% formalin (0.52 g, 6 mmol), and MeOH (14 ml) was stirred for 20 h at room temperature and concentrated. The residual solid was crystallized from MeOH–ether to give (*S*)-**17** (R^1=F, R^2=H)·HCl (0.66 g, 68%), mp 246—248 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3230, 1745. $[\alpha]_D^{20}$ −66.0° (*c*=0.2, MeOH). *Anal.* Calcd for $C_{13}H_{13}FN_2O_2 \cdot HCl$: C, 52.64; H, 5.21; Cl, 11.95; F, 6.40; N, 9.44. Found: C, 52.79; H, 5.11; Cl, 11.87; F, 6.68; N, 9.37. The esters [(*S*)-**17** (R^1=Cl, R^2=H), (*S*)-**17** (R^1=Me, R^2=H), and (*S*)-**17** (R^1=H, R^2=Me)] were prepared in a similar manner.

Methyl (3*S*)-6-Chloro-1,2,3,4-tetrahydro-β-carboline-3-carboxylate [(*S*)-17, (R^1=Cl, R^2=H)]·Hydrochloride——mp 248—250 °C (dec.) (MeOH–ether), 56%. IR ν_{max}^{Nujol} cm^{-1}: 3230, 1755. $[\alpha]_D^{20}$ −47.8° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{13}H_{13}ClN_2O_2 \cdot HCl$: C, 51.85; H, 4.69; Cl, 23.54; N, 9.30. Found: C, 52.09; H, 4.64; Cl, 23.32; N, 9.42.

Methyl (3*S*)-1,2,3,4-Tetrahydro-6-methyl-β-carboline-3-carboxylate [(*S*)-17, (R^1=Me, R^2=H)]·Hydrochloride——mp 284—285 °C (dec.) (MeOH), 80%. IR ν_{max}^{Nujol} cm^{-1}: 3250, 1760. $[\alpha]_D^{20}$ −57.2° (*c*=0.5, MeOH). *Anal.* Calcd for $C_{14}H_{16}N_2O_2 \cdot HCl$: C, 59.89; H, 6.10; Cl, 12.63; N, 9.98. Found: C, 59.91; H, 6.04; Cl, 12.87; N, 9.93.

Methyl (3*S*)-1,2,3,4-Tetrahydro-8-methyl-β-carboline-3-carboxylate [(*S*)-17, (R^1=H, R^2=Me)]——mp 146—148 °C ($CHCl_3$), 63%. IR ν_{max}^{Nujol} cm^{-1}: 1710. $[\alpha]_D^{20}$ −52.0° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{14}H_{16}N_2O_2 \cdot 0.5CHCl_3$: C, 57.29; H, 5.47; Cl, 17.49; N, 9.22. Found: C, 57.03; H, 5.45; Cl, 17.59; N, 9.18.

(3*S*)-6-Fluoro-1,2,3,4-tetrahydro-3-hydroxymethyl-β-carboline [(*S*)-4k]——$NaBH_4$ (0.32 g, 8.4 mmol) was added to a solution of [(*S*)-**17**, (R^1=F, R^2=H)]·HCl (0.577 g, 2 mmol) in EtOH (20 ml)–H_2O (10 ml) at 5 °C, and the whole was stirred at room temperature for 21 h. After removal of the solvent, water was added, and the precipitate was filtered off, washed with water, and dried to afford (*S*)-**4k** (0.286 g, 65%), mp 210—211 °C (dec.). IR ν_{max}^{Nujol} cm^{-1}: 3230. $[\alpha]_D^{20}$ −76.0° (*c*=0.5, MeOH). *Anal.* Calcd for $C_{12}H_{13}FN_2O \cdot 0.5H_2O$: C, 62.87; H, 6.16; F, 8.29; N, 12.22. Found: C, 62.65; H, 5.93; F, 8.26; N, 12.18. The following compounds were prepared in a similar manner.

(3*S*)-6-Chloro-1,2,3,4-tetrahydro-3-hydroxymethyl-β-carboline [(*S*)-4l]——mp 225—227 °C (dec.) (aq. EtOH), 52%. IR ν_{max}^{Nujol} cm^{-1}: 3300. $[\alpha]_D^{20}$ −67.6° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{12}H_{13}ClN_2O_2 \cdot 0.25H_2O$: C, 59.76; H, 5.64; N, 11.61. Found: C, 59.68; H, 5.42; N, 11.53.

(3*S*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-6-methyl-β-carboline [(*S*)-4m]——mp 206—207 °C (iso-PrOH), 73%. IR ν_{max}^{Nujol} cm^{-1}: 3290. $[\alpha]_D^{20}$ −72.8° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.39; H, 7.35; N, 13.08.

(3*S*)-1,2,3,4-Tetrahydro-3-hydroxymethyl-8-methyl-β-carboline [(*S*)-4n]——mp 246—248 °C (EtOH), 63%. IR ν_{max}^{Nujol} cm^{-1}: 3240. $[\alpha]_D^{20}$ −87.4° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.11; H, 7.44; N, 12.97.

(3*R*)-9-Benzyl-1,2,3,4-tetrahydro-2-(methylthiothiocarbonyl)-β-carboline-3-carboxylic Acid [(*R*)-1c]——A solution of (*R*)-**18b** (1.225 g, 4 mmol), 10 N NaOH (0.8 ml, 8 mmol), and CS_2 (0.24 ml, 4 mmol) in DMSO (20 ml) was stirred at room temperature for 10 min, and then methyl iodide (MeI) (0.29 ml, 4.4 mmol) was added. After being stirred for 20 min, the mixture was diluted with H_2O (100 ml), acidified with 10% HCl and extracted with AcOEt. The

extract was washed with water, dried over $MgSO_4$, and evaporated. The residue was purified by chromatography on silica gel using $CHCl_3$–MeOH (90 : 5) as the eluent to yield (*R*)-**1c** (0.89 g, 52%) as a pale yellow powder. IR ν_{max}^{Nujol} cm^{-1}: 1700. NMR ($CDCl_3$) δ: 2.65 (3H, s), 5.17 (2H, s). MS *m/z*: 396 (M^+), 348 ($M^+ - CH_3SH$). Compounds **1b**, **1d**—**g**, and **3c** were prepared in a similar manner and are listed in Tables I and II.

Methyl (3*S*)-6-Chloro-1,2,3,4-tetrahydro-3-hydroxymethyl-β-carboline-2-carbodithioate [(*S*)-2c]——CS_2 (0.13 ml, 2.2 mmol) was added to a solution of (*S*)-**4l** (0.473 g, 2 mmol) and Et_3N (0.31 ml, 2.2 mmol) in DMSO (5 ml), and the whole was stirred for 30 min. After addition of MeI (0.14 ml, 2.2 mmol), the mixture was stirred at room temperature for 5 h, diluted with water, and extracted with AcOEt. The extract was washed with water, dried over $MgSO_4$, and evaporated. The residue was crystallized from aq. EtOH to give (*S*)-**2c** (560 mg, 86%), mp 180—184 °C. IR ν_{max}^{Nujol} cm^{-1}: 3300—3370, 1590. NMR ($CDCl_3$–DMSO-d_6) δ: 2.70 (3H, s), 3.65 (2H, d, J=7.2 Hz). MS *m/z*: 328, 326 (M^+), 280, 278 ($M^+ - CH_3SH$). Compounds **2b**, **2d**—**e**, **3a**—**b**, and **3d**—**i** were similarly prepared and are listed in Tables I and III.

Methyl (3*S*)-1,2,3,4-Tetrahydro-3-methoxymethyl-β-carboline-2-carbodithioate [(*S*)-3g]——An ethereal CH_2N_2 solution [prepared from *N*-methylnitrosourea (20.6 g, 200 mmol) and 40% KOH (63 ml) in ether (200 ml)] was added dropwise to a suspension of (*S*)-**2a** (1.46 g, 5 mmol) and silica gel (10 g) in ether (50 ml) at 5 °C, and the whole was stirred at the same temperature for 2 h. The same amount of a solution of CH_2N_2 was added to the mixture, and the suspension was stirred for 1 h. The silica gel was filtered off, and washed with AcOEt, and the filtrate and washing were combined. After removal of the solvent, the residue was dissolved in AcOEt and allowed to stand at room temperature to give (11a*S*)-5,5a,11,11a-tetrahydro-1*H*,3*H*-oxazolo[4′,3′ : 6,1]pyrido[3,4-*b*]indol-3-one (270 mg, 24%), mp 227—229 °C. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1750, 1730. MS *m/z*: 228 (M^+), 213, 167, 143. $[\alpha]_D^{20}$ −125.2° (*c*=1.0, dioxane). *Anal.* Calcd for $C_{13}H_{12}N_2O_2$: C, 68.41; H, 5.30; N, 12.27. Found: C, 68.18; H, 5.25; N, 12.09. The mother liquor (AcOEt) was evaporated, and the residue was purified by chromatography on silica gel with hexane–AcOEt (8 : 2) to give (*S*)-**3g** (315 mg, 21%), mp 162—164 °C. NMR ($CDCl_3$) δ: 2.72 (3H, s), 3.30 (3H, s). MS *m/z*: 306 (M^+), 291, 274, 259, 227. $[\alpha]_D^{20}$ +114.4° (*c*=1.0, $CHCl_3$).

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Studies on the Constituents of the European Mistletoe, *Viscum album* L.

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Four new flavonoid glycosides, 2'-hydroxy-3,4',6'-trimethoxychalcone-4-*O*-glucoside (VII), 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-[apiosyl(1→2)]glucoside (VIII), (2*R*)-5,7-dimethoxyflavanone-4'-*O*-glucoside (IX) and (2*S*)-3',5,7-trimethoxyflavanone-4'-*O*-glucoside (X), and a known flavonoid glucoside, 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-glucoside (VI), were isolated from the twigs and leaves of *Viscum album* L. (Loranthaceae). These structures were established on the basis of the spectral and chemical data. β-Amyrin acetate, oleanolic acid, betulinic acid, phytosterol, phytosterol-β-D-glucoside and syringin were also isolated.

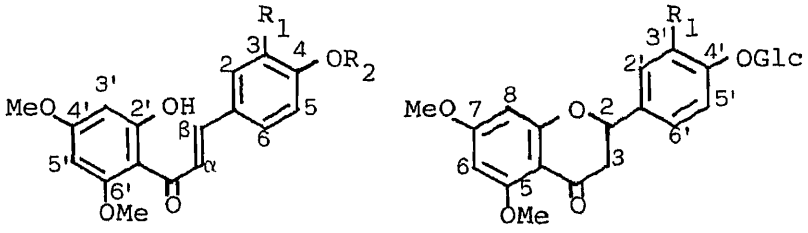
Keywords——*Viscum album*; mistletoe; Loranthaceae; flavonoid; 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-glucoside; 2'-hydroxy-3,4',6'-trimethoxychalcone-4-*O*-glucoside; 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-[apiosyl(1→2)]glucoside; (2*R*)-5,7-dimethoxyflavanone-4'-*O*-glucoside; (2*S*)-3',5,7-trimethoxyflavanone-4'-*O*-glucoside

The European mistletoe, *Viscum album* L. (Loranthaceae), is evergreen parasitic plant widely distributed throughout Europe except in northern areas. It was firstly used for the treatment of epilepsy and dermatitis in Europe. Later, it was believed to have hypotensive, vasodilator, cardiac depressive, sedative, antispasmodic and anticancer activities.[1-3] A number of its constituents have been reported.[2-12] In the present paper, we report the isolation of four new flavonoid glycosides (VII—X) together with several other compounds from the twigs and leaves of *V. album* L.

MeOH extract of *V. album* L. (dried commercial product) was extracted with *n*-hexane and $CHCl_3$. Each extract was concentrated and chromatographed on silica gel and Sephadex LH-20. Four new flavonoid glycosides (VII—X) and 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-glucoside (VI)[6] were isolated from the $CHCl_3$ extract. In addition, we isolated β-amyrin acetate (I), phytosterol (II) and oleanolic acid (III) from the *n*-hexane extract, and III, betulinic acid (IV), phytosterol-β-D-glucoside (V), and syringin (XI) were isolated from the $CHCl_3$ extract.[4a,6,9]

Compound VI was obtained as yellowish needles, mp 166—168 °C. The absorption maximum at 356 nm in the ultraviolet (UV) spectrum and the signals of α-H and β-H (δ 7.65 and 7.69, $J=15.7$ Hz) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum suggested VI to be a chalcone.[6,13,14] Upon hydrolysis of VI, glucose was identified by thin layer chromatography (TLC). Compound VI was identified as 2'-hydroxy-4',6'-dimethoxychalcone-4-*O*-glucoside by comparing various spectral data with those in the literature.[6]

Compound VII was obtained as a pale yellowish powder, mp 223—226 °C, and its infrared (IR) spectrum suggested the presence of hydroxyl (3450 cm^{-1}) and carbonyl



VI : R_1= H, R_2= Glc

VII : R_1= OMe, R_2= Glc

VIII : R_1= H, R_2= Glc(2→1)apiose

IX : R_1= H

X : R_1= OMe

Chart 1

(1655 cm^{-1}) groups. The absorption maximum at 362 nm in the UV spectrum and the signals of α-H and β-H (δ 7.69, 7.81, J=15.6 Hz) in the ^{1}H-NMR spectrum suggested compound VII to be a chalcone.[13,14] The ^{1}H-NMR spectrum of VII exhibited singlet signals due to three methoxyl groups (δ 3.85, 3.91, 3.95), doublets at δ 6.12 and 6.14 (each 1H, J=1.9 Hz) due to *meta* coupling of two protons of the A-ring, and doublets at δ 7.19 and 7.27 (each 1H, J=8.5 Hz) due to *ortho* coupling and a singlet at δ 7.30 (1H) arising from the protons of the B-ring. The electron impact-mass spectrum (EI-MS) of VII showed ion peaks at *m/z* 493 (M^++1), 207 (M−B-ring) and 181 (A_1+H), suggesting the presence of two methoxyl groups in the A-ring and one methoxyl group and the sugar in the B-ring.[14] After the hydrolysis of VII, glucose was identified by TLC. The UV absorption of the aglycone showed a band I bathochromic shift ($\Delta\lambda_{max}$=+88 nm) with NaOMe which suggested the presence of a 4-hydroxyl group on the B-ring.[13] On the basis of the above results, compound VII was identified as 2′-hydroxy-3,4′,6′-trimethoxychalcone-4-*O*-glucoside. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data supported the structure of VII (Tables I, II).[15]

Compound VIII was obtained as a yellowish powder, mp 215—218 °C and its IR, UV, MS and ^{1}H-NMR spectral data were similar to those of compound VI. Thus, the aglycone moiety of VIII was assumed to have the same structure as in VI. In addition, the ^{13}C-NMR spectrum of the sugar moiety of VIII exhibited five more signals than that of VI. On the basis of a comparison of the ^{13}C-chemical shifts of the sugar moiety of VIII with those of apiin[15] (*i.e.* apigenin-7-*O*-[apiosyl(1→2)] glucoside), the sugar moiety of VIII was identified as apiosyl(1→2) glucoside (Table II). On the basis of the above results, the structure of compound VIII was established as 2′-hydroxy-4′,6′-dimethoxychalcone-4-*O*-[apiosyl(1→2)] glucoside.

Compound IX was obtained as colorless needles, mp 184—187 °C, and gave a positive Mg–HCl test. The IR spectrum suggested the presence of hydroxyl (3370 cm^{-1}) and carbonyl (1645 cm^{-1}) groups. The absorption maxima at 313 (sh) and 281 nm in the UV spectrum and the signals of 2-H at δ 5.48 (1H, dd, J=2.8, 12.5 Hz), 3-H *cis* at δ 2.62 (1H, dd, J=2.8, 16.3 Hz) and 3-H *trans* at δ 3.05 (1H, dd, J=12.5, 16.3 Hz) in the ^{1}H-NMR spectrum suggested IX to be a flavanone.[13,14] The ^{1}H-NMR spectrum of IX exhibited signals of two methoxyl groups (δ 3.78, 3.80), doublets at δ 6.20 and 6.22 (each 1H, J=2.3 Hz) due to *meta* coupling of two protons of the A-ring and doublets at δ 7.06 and 7.43 (each 2H, J=8.7 Hz) due to *ortho* coupling of four protons of the B-ring. The MS of IX showed ion peaks at *m/z* 462 (M^+), 207 (M−B-ring) and 181 (A_1+H), suggesting the presence of two methoxyl groups in the A-ring and the sugar in the B-ring.[14] After the hydrolysis of IX, glucose was detected as

the sugar component of IX by TLC. The circular dichroism (CD) spectrum of IX exhibited a negative Cotton effect at 330 nm and a positive Cotton effect at 283 nm. Therefore, C-2 was assigned the *R* configuration.[16] On the basis of the above results, compound IX was identified as (2*R*)-5,7-dimethoxyflavanone-4′-*O*-glucoside. The ^{13}C-NMR spectral data supported the structure of IX (Tables I and II).

Compound X was obtained as a colorless powder, mp 146—149 °C and gave a positive Mg–HCl test. The absorption maxima at 314 (sh) and 280 nm in UV spectrum and the signals of 2-H at δ 5.44 (1H, dd, J=2.8, 12.5 Hz), 3-H *cis* at δ 2.71 (1H, dd, J=2.8, 16.5 Hz) and 3-H *trans* at δ 3.07 (1H, dd, J=12.5, 16.5 Hz) in the ^{1}H-NMR spectrum suggested X to be a flavanone.[13,14] The ^{1}H-NMR spectrum of X exhibited signals of three methoxyl groups (δ

Table I. ^{13}C-NMR Chemical Shifts[a] (Aglycone Moieties)

Compd. No.	VI	VII	VIII	Compd. No.	IX	X
C-α	142.3	142.7	142.3	C-2	77.8	78.2
C-β	125.3	125.6	125.3	C-3	44.6	44.7
C=O	192.1	192.2	192.1	C-4	187.7	187.8
C-1′	106.2	106.4	106.2	C-4a	105.3	105.3
C-2′	161.8	161.7	161.9	C-5	164.2[b]	164.2[b]
C-3′	93.8	93.8	93.8	C-6	92.8	92.8
C-4′	165.5[b]	165.2[b]	165.6[b]	C-7	165.3[b]	165.3[b]
C-5′	91.0	91.0	91.0	C-8	93.6	93.7
C-6′	165.4[b]	165.1[b]	165.4[b]	C-8a	161.7	161.7
C-1	128.4	128.6	128.5	C-1′	132.1	132.5
C-2	130.1	111.8	130.1	C-2′	127.8	111.2
C-3	116.6	149.1	116.5	C-3′	116.1	148.8
C-4	159.2	148.6	159.0	C-4′	157.3	146.5
C-5	116.6	115.1	116.5	C-5′	116.1	115.1
C-6	130.1	122.1	130.1	C-6′	127.8	118.9
4′-OMe	55.6	55.6	55.6	5-OMe	55.8[c]	55.8[c]
6′-OMe	56.1	56.1	56.1	7-OMe	55.6[c]	55.6[c]
3-OMe		56.1		3′-OMe		55.7[c]

a) Spectra run at 100 MHz in DMSO-d_6. *b*, *c*) Assignments may be interchanged in each column.

Table II. ^{13}C-NMR Chemical Shifts[a] (Sugar Moieties)

Compd. No.	Apiin[b]	VIII	VI	VII	IX	X
Glucose						
C-1	99.7	98.3	100.0	99.6	100.2	99.9
C-2	76.8	76.9	73.1	73.1	73.1	73.1
C-3	76.6[c]	75.8[c]	76.5[c]	76.8[c]	76.5[c]	76.8[c]
C-4	70.2	69.8	69.6	69.5	69.7	69.6
C-5	77.2[c]	76.8[c]	77.0[c]	77.0[c]	77.0[c]	77.0[c]
C-6	60.9	60.5	60.6	60.5	60.6	60.6
Apiose						
C-1	109.0	108.7				
C-2	76.5[c]	76.0[c]				
C-3	79.1	79.2				
C-4	74.0	73.9				
C-5	64.4	64.2				

a) Spectra run at 100 MHz in DMSO-d_6. *b*) Apigenin-7-*O*-[apiosyl(1→2)] glucoside. *c*) Assignments may be interchanged in each column.

3.84, 3.84, 3.87), doublets at δ 6.21 and 6.22 (each 1H, J=2.1 Hz) due to *meta* coupling of two protons of the A-ring and doublets at δ 7.04 and 7.18 (each 1H, J=8.5 Hz) due to *ortho* coupling and a singlet at δ 7.16 (1H) arising from protons of the B-ring. The MS of X showed ion peaks at *m/z* 493 ($M^+ + 1$), 207 (M − B-ring) and 181 (A_1 + H), suggesting the presence of two methoxyl groups in the A-ring and one methoxyl group and the sugar in the B-ring.[14] After the hydrolysis of X, glucose was identified by TLC. The UV absorption of the aglycone showed a band I bathochromic shift ($\Delta\lambda_{max}$ = +74 nm) with NaOMe which suggested the presence of a 4′-hydroxyl group in the B-ring.[13] The CD spectrum of X exhibited a positive Cotton effect at 330 nm and a negative Cotton effect at 283 nm. Therefore, C-2 was assigned the *S* configuration.[16] On the basis of the above results, compound X was identified as (2*S*)-3′,5,7-trimethoxyflavanone-4′-*O*-glucoside.

The isolation and structural elucidation of the components of the butanol extract are under investigation.

Experimental

All melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. Spectral data were obtained with the following instruments; UV on a Hitachi 220A, IR on a Hitachi 285, optical rotation on a JASCO DIP-181, CD on a JASCO J-500C, and MS on a Hitachi M80 or JEOL DX300. ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Bruker AM400 and chemical shifts are given as δ (ppm) with tetramethylsilane (TMS) as an internal standard (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet). Gas liquid chromatography (GLC) was run on a Hewlett Packard 5730 with a flame ionization detector, using a glass column (4 mm i.d. × 183 cm) packed with 2% SE-30 on Uniport HP (60—80 mesh); column temperature, 260 °C; carrier gas, He (60 ml/min). TLC was carried out on precoated 0.25 mm Kieselgel 60 F_{254} (Merck) plates. Spots were detected by exposure to UV light (254, 365 nm) and by spraying 10% H_2SO_4 and anisaldehyde sulfate reagent followed by heating. Column chromatography was carried out with Wakogel C-200 (Wako Pure Chemical Ind. Lrd.) and Sephadex LH-20 (Pharmacia Fine Chemicals). High-performance liquid chromatography (HPLC) was carried out on a CIG column system (22 i.d. × 300 mm, 50 μ silica gel, Kusano Scientific Co.).

Extraction and Isolation——The twigs and leaves, cut into pieces, of *Viscum album* L. (10 kg) from West Germany were purchased from Iwase-Kenjiro Shoten and were extracted with MeOH (36 l) three times. The MeOH extract (2.75 kg) was partitioned between water (1.6 l) and *n*-hexane (8 l) with a separatory funnel and then between water and $CHCl_3$ (12 l) and further between water and water-saturated *n*-BuOH (15 l), yielding 280 g of *n*-hexane, 520 g of $CHCl_3$ and 740 g of *n*-BuOH extracts.

The *n*-hexane extract (280 g) was subjected to column chromatography on silica gel (2 kg) with *n*-hexane–EtOAc (95 : 5—0 : 100). β-Amyrin acetate (I) was eluted with *n*-hexane–EtOAc (95 : 5) and phytosterol (II) with *n*-hexane–EtOAc (9 : 1).

The $CHCl_3$ extract (170 g) was subjected to column chromatography on silica gel (4 kg) with $CHCl_3$–MeOH (98 : 2—1 : 1). Oleanolic acid (III) and betulinic acid (IV) were eluted with $CHCl_3$–MeOH (98 : 2) and phytosterol-β-D-glucoside (V) with $CHCl_3$–MeOH (95 : 5). Further elution with $CHCl_3$–MeOH (4 : 1) gave 2′-hydroxy-4′,6′-dimethoxychalcone-4-*O*-glucoside (VI), new flavonoid glycoside (VII—X) and syringin (XI).

β-Amyrin Acetate (I)——Colorless needles, 11 g. mp 236—239 °C. IR ν_{max}^{KBr} cm^{-1}: 2940, 1732, 1460, 1383, 1365, 1250, 1023, 1000. EI-MS *m/z* (%): 468 (M^+; 2), 218 (100). ^{1}H-NMR ($CDCl_3$) δ: 0.83, 0.86, 0.87, 0.88, 0.88, 0.96, 0.97, 1.13 (each s, *tert*-CH_3 × 8), 2.04 (s, CH_3COO–). ^{13}C-NMR ($CDCl_3$) δ: 15.6 (q), 16.7 (q), 16.8 (q), 18.3 (t), 21.3 (q), 23.5 (t), 23.6 (t), 23.7 (q), 26.0 (q), 26.2 (t), 27.0 (t), 28. 0 (q), 28.4 (q), 31.1 (s), 32.5 (s), 32.6 (t), 33.3 (q), 34.8 (t), 37.2 (t), 37.7 (s), 38.1 (t), 39.8 (s), 41.7 (s), 46.8 (t), 47.3 (d), 47.6 (d), 55.3 (d), 80.9 (d), 121.7 (d), 145.2 (s), 170.9 (s).

Phytosterol (II)——Colorless plates, 285 mg. mp 131—134 °C. IR ν_{max}^{KBr} cm^{-1}: 3430, 2940, 2480, 1630 br, 1465, 1385, 1055, 970. GLC: t_R = 10.6 (stigmasterol), 11.9 (β-sitosterol), 13.4 (unknown) min (1 : 3 : 1).

Oleanolic Acid (III)——Colorless powder, 1.5 g. mp 306—309 °C. IR ν_{max}^{KBr} cm^{-1}: 3430, 2940, 1690, 1460, 1390, 1365, 1265, 1180, 1025, 995. III was identified as oleanolic acid by comparison of the melting point, TLC behavior and IR spectral data with those of an authentic sample.

Betulinic Acid (IV)——Colorless needles, 54 mg. mp 293—296 °C. IR ν_{max}^{KBr} cm^{-1}: 3430, 2940, 1685, 1640, 1450, 1390, 1380, 1235, 1185, 1040, 880. EI-MS *m/z* (%): 456 (M^+, 36), 438 (15), 248 (66), 189 (100), 135 (41). ^{1}H-NMR (CD_3CD + $CDCl_3$) δ: 0.75, 0.85, 0.95, 0.96, 1.00, 1.69 (each s, *tert*-CH_3 × 6), 4.59 (1H, dd, J=1.4, 2.2 Hz), 4.71 (1H, d, J=2.2 Hz). IV was identified as betulinic acid by comparison of the melting point, TLC behavior and IR spectral data with those of an authentic sample.

Phytosterol-β-D-glucoside (V)——Colorless powder, 430 mg. mp 284—290 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3400, 2940, 2870, 1630 br, 1465, 1380, 1080, 1025.

Hydrolysis of V——V (2 mg) was dissolved in EtOH–5% HCl (1 : 1) (20 ml) and refluxed for 1.5 h. After removal of the EtOH under reduced pressure, the genin was identified by GLC: $t_R = 10.5$ (stigmasterol), 11.8 (β-sitosterol), 13.2 (unknown) min (2 : 7 : 1). Glucose was detected as the sugar component of V by TLC (*n*-BuOH–AcOH–H_2O = 4 : 1 : 1, anisaldehyde reagent).

2′-Hydroxy-4′,6′-dimethoxychalcone-4-*O*-glucoside (VI)——Yellowish needles, 41 mg. mp 166—168 °C. UV λ_{max}^{EtOH} nm (log ε): 356 (4.66), 234 (sh); [after hydrolysis] λ_{max}^{EtOH} nm (log ε): 367 (3.78), 314 (sh), 282 (4.20). IR ν_{max}^{KBr} cm^{-1}: 3380, 2900, 1625, 1560, 1510, 1425, 1345, 1290, 1220, 1175, 1160, 1085, 830. EI-MS *m/z* (%): 300 (100), 272 (10), 207 (28), 181 (50). CI-MS *m/z*: 463 ($M^+ + 1$). ^{1}H-NMR (DMSO-d_6) δ: 3.82, 3.90 (each 3H, each s, $-OCH_3 \times 2$), 4.96 (1H, d, $J = 7.3$ Hz, anomeric proton of glucose), 6.12 (1H, d, $J = 2.3$ Hz, 3′-H), 6.16 (1H, d, $J = 2.3$ Hz, 5′-H), 7.09 (2H, d, $J = 8.8$ Hz, 3- and 5-H), 7.69 (2H, d, $J = 8.8$ Hz, 2- and 6-H), 7.65 (1H, d, $J = 15.7$ Hz, α-H), 7.69 (1H, d, $J = 15.7$ Hz, β-H).

2′-Hydroxy-3,4′,6′-trimethoxychalcone-4-*O*-glucoside (VII)——Pale yellowish powder, 7.3 mg. mp 223—226 °C. UV λ_{max}^{EtOH} nm (log ε): 362 (4.15), 246 sh (4.28); [after hydrolysis] λ_{max}^{EtOH} nm (log ε): 380 (3.92), 314 sh (4.02), 282 (4.41); $\lambda_{max}^{EtOH+NaOMe}$ nm (log ε): 468 (4.02), 282 (4.41). IR ν_{max}^{KBr} cm^{-1}: 3450, 2970, 1655, 1610, 1590, 1540, 1450, 1290, 1240, 1190, 1120. EI-MS *m/z* (%): 493 ($M^+ + 1$), 330 (100), 207 (36), 181 (88), CI-MS *m/z*: 493 ($M^+ + 1$). ^{1}H-NMR (DMSO-d_6) δ: 3.85, 3.91, 3.95 (each 3H, each s, $-OCH_3 \times 3$), 5.01 (1H, d, $J = 6.8$ Hz, anomeric proton of glucose), 6.12 (1H, d, $J = 1.9$ Hz, 3′-H), 6.14 (1H. d, $J = 1.9$ Hz, 5′-H), 7.19 (1H, d, $J = 8.5$ Hz, 5-H), 7.27 (1H, d, $J = 8.5$ Hz, 6-H), 7.30 (1H, s, 2-H), 7.69 (1H, $J = 15.6$ Hz, α-H), 7.81 (1H, d, $J = 15.6$ Hz, β-H).

2′-Hydroxy-4′,6′-dimethoxychalcone-4-*O*-[apiosyl(1→2)]glucoside (VIII)——Yellowish powder, 230 mg. mp 215—218 °C.UV λ_{max}^{EtOH} nm (log ε): 355 (4.32), 290 (sh), 228 (sh). IR ν_{max}^{KBr} cm^{-1}: 3400, 2940, 1630, 1580, 1515, 1420, 1345, 1220, 1160, 1115, 1070, 830. EI-MS *m/z* (%): 462, 300 (84), 207 (48), 181 (100). ^{1}H-NMR (DMSO-d_6) δ: 3.82, 3.90 (each 3H, each s, $-OCH_3 \times 2$), 5.04 (1H, d, $J = 7.4$ Hz, anomeric proton of glucose, tentative), 5.36 (1H, $J = 1.0$ Hz, anomeric proton of apiose), 6.12 (1H, d, $J = 2.3$ Hz, 3′-H), 6.16 (1H, d, $J = 2.3$ Hz, 5′-H), 7.07 (2H, d, $J = 8.8$ Hz, 3- and 5-H), 7.68 (2H, d, $J = 8.8$ Hz, 2- and 6-H), 7.65 (1H, d, $J = 15.3$ Hz, α-H), 7.69 (1H, d, $J = 15.3$ Hz, β-H).

(2*R*)-5,7-Dimethoxyflavanone-4′-*O*-glucoside (IX)——Colorless needles, 120 mg. mp 184—187 °C. CD ($c = 3.2 \times 10^{-4}$, MeOH) $[\theta]^{25}$ (nm): -10000 (330) (negative maximum), $+14400$ (283) (positive maximum). $[\alpha]_D^{25}$ $-25°$ ($c = 0.32$, MeOH–acetone). UV λ_{max}^{EtOH} nm (log ε): 313 (sh), 281 (4.26); [after hydrolysis] λ_{max}^{EtOH} nm (log ε): 365 (3.45), 314 (sh), 281 (4.14). EI-MS *m/z* (%): 462 (M^+, 2), 300 (100), 272 (9), 207 (27), 181 (44). CI-MS *m/z*: 463 ($M^+ + 1$). IR ν_{max}^{KBr} cm^{-1}: 3370, 2920, 1645, 1605, 1570, 1515, 1470, 1420, 1215, 1115, 1070, 830. ^{1}H-NMR (DMSO-d_6) δ: 2.62 (1H, dd, $J = 2.8$, 16.3 Hz, 3-H *cis*), 3.05 (1H, dd, $J = 12.5$, 16.3 Hz, 3-H *trans*), 3.78, 3.80 (each 3H, each s, $-OCH_3 \times 2$), 4.89 (1H, d, $J = 7.2$ Hz, anomeric proton of glucose), 5.48 (1H, dd, $J = 2.8$, 12.5 Hz, 2-H), 6.20 (1H, d, $J = 2.3$ Hz, 6-H), 6.22 (1H, d, $J = 2.3$ Hz, 8-H), 7.06 (2H, d, $J = 8.7$ Hz, 3′- and 5′-H), 7.43 (2H, d, $J = 8.7$ Hz, 2′- and 6′-H).

(2*S*)-3′,5,7-Trimethoxyflavanone-4′-*O*-glucoside (X)——Colorless powder, 19.6 mg. mp 146—149 °C. CD ($c = 2.2 \times 10^{-4}$, MeOH) $[\theta]^{25}$ (nm): $+6360$ (330) (positive maximum), -36400 (283) (negative maximum). $[\alpha]_D^{25}$ $-43°$ ($c = 0.30$, MeOH–acetone). UV λ_{max}^{EtOH} nm (log ε): 314 (sh), 280 (4.32); [after hydrolysis] λ_{max}^{EtOH} nm (log ε): 386 (3.55), 314 (sh), 281 (4.29); $\lambda_{max}^{EtOH+NaOMe}$ nm (log ε): 460 (3.52), 314 (sh), 281 (4.29). EI-MS *m/z* (%): 493 ($M^+ + 1$), 330 (100), 207 (34), 181 (85). CI-MS *m/z*: 493 ($M^+ + 1$). IR ν_{max}^{KBr} cm^{-1}: 3350, 2930, 1665, 1615, 1575, 1520, 1470, 1430, 1270, 1225, 1160, 1115, 1075, 825. ^{1}H-NMR (DMSO-d_6) δ: 2.71 (1H, dd, $J = 2.8$, 16.5 Hz, 3-H *cis*), 3.07 (1H, dd, $J = 12.5$, 16.5 Hz, 3-H *trans*), 3.84 (6H, s, $-OCH_3 \times 2$), 3.87 (3H, s, $-OCH_3$), 4.89 (1H, d, $J = 7.2$ Hz, anomeric proton of glucose), 5.44 (1H, dd, $J = 2.8$, 12.5 Hz, 2-H), 6.21 (1H, d, $J = 2.1$ Hz, 6-H), 6.22 (1H, d, $J = 2.1$ Hz, 8-H), 7.04 (1H, d, $J = 8.5$ Hz, 5′-H), 7.16 (1H, s, 2′-H), 7.18 (1H, d, $J = 8.5$ Hz, 6′-H).

Hydrolysis of VI—X——A sample (1 mg) was dissolved in EtOH–5% HCl (1 : 1) (10 ml) and refluxed for 1 h. The reaction solution was evaporated to dryness and analyzed by UV spectroscopy. Glucose was detected as the sugar component of VI—X by TLC (*n*-BuOH–AcOH–H_2O = 4 : 1 : 1, anisaldehyde reagent).

Syringin (XI)——Colorless needles, 46 mg. mp 180—184 °C. UV λ_{max}^{EtOH} nm (log ε): 265 (3.98), 220 (4.29). IR ν_{max}^{KBr} cm^{-1}: 3380, 2930, 1585, 1505, 1465, 1420, 1340, 1240, 1130, 1070, 1020, 630. EI-MS *m/z* (%): 372 (M^+), 300 (58), 210 (100). ^{1}H-NMR (DMSO-d_6) δ: 3.86, 3.87 (each 3H, each s, $-OCH_3 \times 2$), 4.22 (2H, d, $J = 5.4$ Hz), 6.32 (1H, dt, $J = 5.4$, 15.8 Hz), 6.55 (1H, d, $J = 15.8$ Hz), 6.75 (2H, d, $J = 3.9$ Hz).

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Diarylheptanoids from the Rhizomes of *Curcuma xanthorrhiza* and *Alpinia officinarum*

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Five diarylheptanoids including two new compounds were isolated from the rhizomes of *Curcuma xanthorrhiza* (Zingiberaceae). The structures of the new compounds were determined to be octahydrocurcumin ((3*S*,5*S*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-diol) (Ia) and (1ξ)-1-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione (II) on the basis of spectral and chemical evidence.

The absolute configurations of Ia and a new similar diarylheptanoid, (3*R*,5*R*)-1-(4-hydroxyphenyl)-7-phenylheptane-3,5-diol (VIa), isolated from the rhizomes of *Alpinia officinarum* (Zingiberaceae), were established by application of the exciton chirality rule.

Keywords——diarylheptanoid; *Curcuma xanthorrhiza*; *Alpinia officinarum*; Zingiberaceae; (3*S*,5*S*)-1,7-bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-diol; (1ξ)-1-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione; (3*R*,5*R*)-1-(4-hydroxyphenyl)-7-phenylheptane-3,5-diol; exciton chirality rule; CD

The rhizomes of Temu Lawak, *Curcuma xanthorrhiza* (Zingiberaceae), have been utilized as a tonic in Indonesia and as a choleretic drug in Europe. As regards the chemical components of this plant, two diarylheptanoids have been reported.[1] In the present paper, we wish to report the isolation and structural elucidation of two new diarylheptanoids (Ia and II) and three known diarylheptanoids (dihydrocurcumin (III), hexahydrocurcumin (IV) and curcumin (V)). In addition to these compounds, we wish to describe a new diarylheptanoid (VIa), obtained from the rhizomes of *Alpinia officinarum* (Zingiberaceae).

Each crude drug was extracted with methanol, and the extract was separated into the *n*-hexane- and chloroform-soluble fractions. Each chloroform-soluble fraction was chromatographed on silica gel to give Ia, II—V and VIa.

Compound Ia was obtained as colorless needles from benzene–chloroform, mp 132—134 °C, $[\alpha]_D$ −18.5° (c=0.26, EtOH). The molecular formula was established by the high-resolution mass spectrum (MS). The infrared (IR) spectrum showed a strong and broad hydroxyl absorption at 3310 cm^{-1}. The typical benzenoid absorption (282 nm), showing a bathochromic shift on addition of alkali, in the ultraviolet (UV) spectrum, and the positive coloration with diazo reagent, suggested the presence of a phenolic group. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, the signals of two methoxyl groups (δ 3.81) and 1,2,4-substituted benzene rings (2H each at δ 6.64, dd, J=8, 2 Hz; 6.70, d, J=8 Hz; 6.81, d, J=2 Hz) were observed. These findings and the base peak at m/z 137 in the MS indicated the presence of 4-hydroxy-3-methoxyphenyl groups and was confirmed by comparing the carbon-13 nuclear magnetic resonance (^{13}C-NMR) data with those of analogous compounds.[2,3] Furthermore, the signals due to five methylenes (δ 1.58, 2H, t, J=6 Hz, H-4;

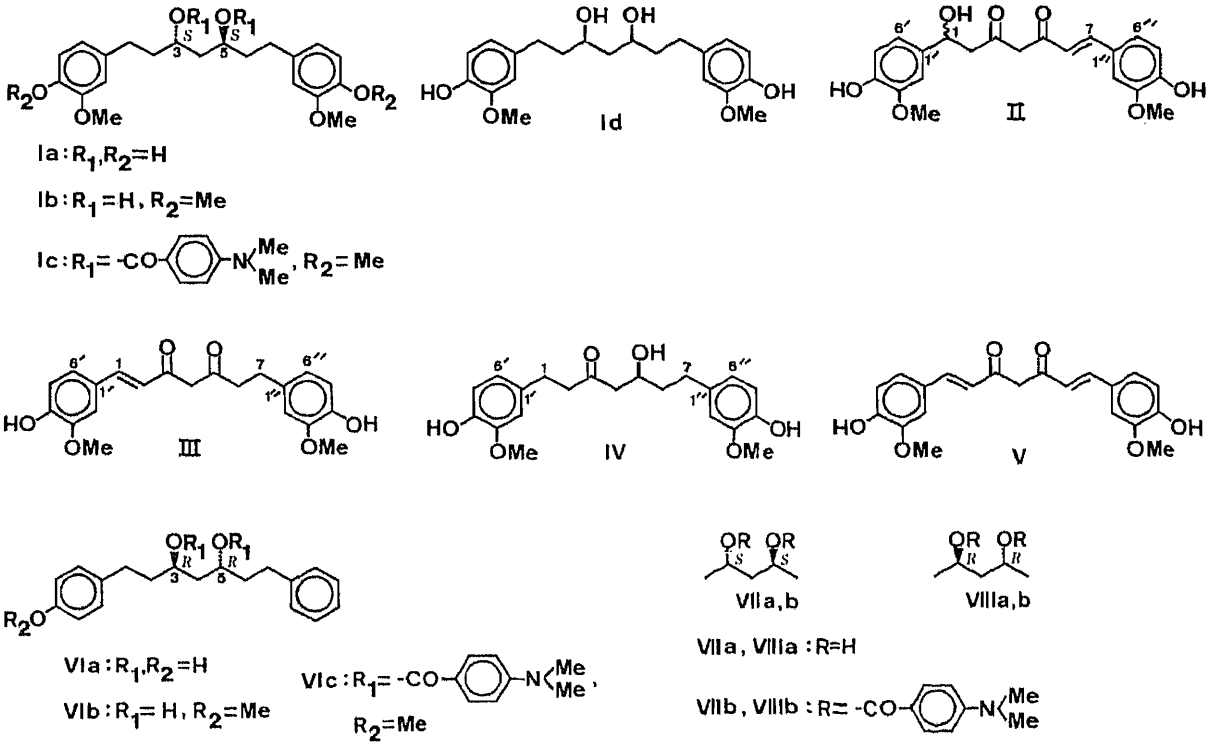

Chart 1. The Chemical Structures of Ia—VIIIb

TABLE I. ^{13}C-NMR Data for Ia—VIa

C No.	Ia	II[a]	III[a]	IV[3]	V[a]	VIa[3]
1	32.3 t	80.9 d	140.0 d	29.3 t	141.3 d	31.2[o] t
2	40.8 t	43.2 t	120.2[g] d	45.4 t	122.3 d	39.2[p] t
3	68.3 d	192.9 d	177.8 s	211.3 s	184.1 s	69.0[q] d
4	44.7 t	105.5 d	100.0 d	49.4 t	101.6 d	42.5 t
5	68.3 d	168.9 s	198.8 s	67.0 d	184.1 s	68.9[q] d
6	40.8 t	122.5 d	41.1 t	38.3 t	122.3 d	39.0[p] t
7	32.3 t	137.7 d	30.2 t	31.4 t	141.3 d	32.1[o] t
1′	134.9 s	130.4 s	126.3 s	132.6 s	128.2 s	133.6 s
1″	134.9 s	127.8 s	131.5 s	133.7 s	128.2 s	141.8 s
2′	113.1 d	109.3[h] d	111.2 d	111.1[k] d	111.6 d	129.4 d
2″	113.1 d	109.1[h] d	112.5 d	111.2[k] d	111.6 d	128.4 d
3′	148.2 s	146.9[c] s	149.1 s	146.5 s	150.0 s	115.4 d
3″	148.2 s	147.6 s	147.9[h] s	146.5 s	150.0 s	128.4 d
4′	145.2 s	146.3[c] s	147.3[h] s	143.8[l] s	148.8 s	154.0 s
4″	145.2 s	146.8[c] s	144.6 s	144.0[l] s	148.8 s	125.9 d
5′	115.7 d	114.9[d] d	115.2[i] d	114.5[m] d	116.2 d	115.4 d
5″	115.7 d	114.6[d] d	115.6[i] d	114.4[m] d	116.2 d	128.4 d
6′	121.4 d	119.9[e] d	122.8 d	120.8[n] d	123.8 d	129.4 d
6″	121.4 d	119.0[e] d	119.6[g] d	120.9[n] d	123.8 d	128.4 d
3′-OMe	56.3 q	56.1[f] q	55.5[j] q	55.9 q	56.3 q	
3″-OMe	56.3 q	56.0[f] q	55.6[j] q	55.9 q	56.3 q	

The measurements were made on a JEOL FX-270 spectrometer in acetone-d_6 (Ia, V and VIa), $CDCl_3$ (II and IV) or DMSO-d_6 (III) with tetramethylsilane as an internal reference, and are expressed in terms of ppm. *a*) Only the data of enol form are shown. *b*—*q*) The assignments may be reversed.

1.62—1.78, 4H, m, H-2, 6; 2.41—2.76, 4H, m, H-1, 7), and two methine protons adjacent to a hydroxyl group (δ 3.85—3.97, 2H, m, H-3, 5) were observed in the ^{1}H-NMR spectrum. The above data and the additivity[4] of substituent group effects in the ^{13}C-NMR spectrum indicate the position of two hydroxyl groups to be at C-3 and C-5. On the other hand, catalytic hydrogenation of curcumin (V) afforded octahydrocurcumin (Id). Compound Id was considered to be a mixture of *dl* form and *meso* type on the basis of ^{13}C-NMR spectral analysis[5] and the presence of identical signals of Ia suggested that Ia is one of the stereoisomers of Id. On the basis of these chemical and spectral data, the structure of Ia was determined to be octahydrocurcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-diol).

The absolute configuration of the 3,5-hydroxyl groups was determined by application of the exciton chirality rule[6] to the circular dichroism (CD) spectrum of the bis-*p*-dimethylaminobenzoate of a derivative of Ia. According to the exciton chirality rule, a pair of enantiomeric derivatives gives mirror-image CD curves and the absolute configuration of the enantiomers can be assigned from the sign of the maximum at longer wavelength. A positive sign indicates a clockwise spatial relationship between the interacting benzoate chromophores and *vice versa*. Prior to the examination, (2*S*,4*S*)-(+)- and (2*R*,4*R*)-(−)-pentanediol (VIIa and VIIIa) were used as model compounds. They were converted into the bis-*p*-dimethylaminobenzoates (VIIb and VIIIb) in the usual manner. The CD spectra of VIIb and VIIIb provided mirror-image curves. That of VIIb showed a strong positive interaction peak at 318 nm crossing through zero at 305 nm, indicating the absolute configuration to be (2*S*,4*S*) (Fig. 1). The configuration of VIIIb was thus (2*R*,4*R*). Hence, it has become apparent that the exciton chirality rule can be applied to determine the absolute configurations of these 1,3-dihydroxy compound.

In order to protect the phenolic hydroxyl groups, Ia was methylated with methyl iodide (Ib), and then converted into the bis-*p*-dimethylaminobenzoate (Ic). The CD spectrum of Ic showed a strong positive interaction peak at 318 nm crossing through zero at 307 nm (Fig. 1). Therefore, it is apparent that the 3,5-dihydroxyl groups of Ia are situated in clockwise spatial relationship and the absolute configuration of Ia is concluded to be (3*S*,5*S*).

Compound II was obtained as a yellow powder, mp 92.0—96.0 °C, $[\alpha]_D$ +12.2° (c=0.06, EtOH). The MS of II was similar to that of V. Dehydration of II gave V. The IR spectrum had a prominent band at 3525 cm^{-1} (OH). The UV absorption (370 nm), which shows a bathochromic shift on addition of alkali, suggested the presence of a phenolic group. In the ^{13}C-NMR spectrum of II, the signal of 80.9 (d) showed the existence of an aliphatic secondary hydroxyl group. The ^{1}H-NMR spectrum of II showed a 1,2,4-substituted benzene ring (δ 7.03,

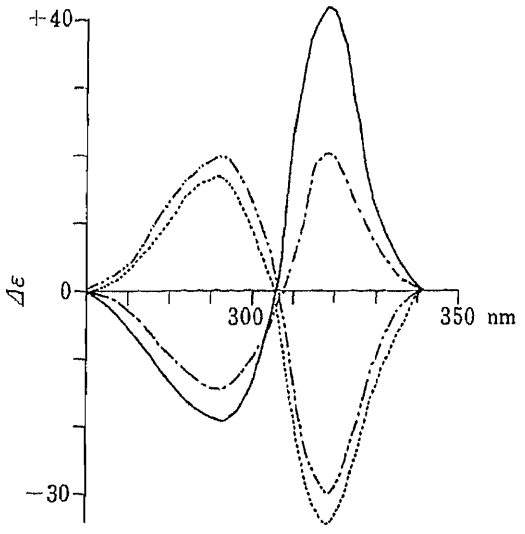

Fig. 1. Circular Dichroism (CD) Spectra of the Bis-*p*-dimethylaminobenzoate Derivatives of Ib (– – – –), VIb (– – – – –), VIIa (——) and VIIIa (------)

1H, dd, J=8, 2 Hz; 6.90, 1H, d, J=8 Hz; 7.00, 4H, br s), a pair of *trans*-olefinic protons (δ 7.30, 1H, d, J=16 Hz; 6.44, 1H, d, J=16 Hz), the proton on the central carbon of a β-diketone in its enol form (δ 5.60) and two aromatic methoxyl groups (δ 3.93 and 3.95). The methine proton adjacent to a hydroxyl group (δ 5.39, 1H, dd, J=14, 3.5 Hz) was assigned to H-1, because this proton was coupled with the protons at δ 2.93 (1H, dd, J=17, 14 Hz) and 2.65 (1H, dd, J=17, 3.5 Hz) which were assigned as methylene protons at H-2. Thus, the position of the hydroxyl group was assigned as C-1. On the basis of these chemical and spectral properties, the structure of II was determined to be 1-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione. The absolute configuration of the hydroxyl group is now under investigation.

Compound III was obtained as yellow needles, mp 178.0 °C, and was identified as dihydrocurcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1-heptene-3,5-dione), which has already been isolated from *Curcuma longa*, by comparing its spectral data with those of an authentic specimen.[7] The structure of this compound was confirmed by the catalytic hydrogenation of V to III.

Compound IV was obtained as a colorless oil and was identified as hexahydrocurcumin (5-hydoxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone), which has already been isolated from *Zingiber officinale* and *Alpinia officinarum*, by comparing its spectral data with those of an authentic specimen.[8-10] The structure of this compound was confirmed by the catalytic hydrogenation of V to IV. Since IV is optically inactive, it seems to be a racemate.

Compound V was obtained as yellow needles, mp 183.0 °C, and was identified as curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which has already been isolated from this plant,[1] by direct comparison with an authentic sample, isolated from *Curcuma longa*.

Compound VIa, colorless needles, mp 109.0—111.0 °C, $[\alpha]_D$ +8.3° (c=0.39, $CHCl_3$), obtained from the rhizomes of *Alpinia officinarum*, was isolated as a compound similar to Ia, as judged from the spectral properties. The molecular formula was established by high-resolution MS. The IR spectrum had a prominent band at 3600 cm^{-1} (OH). The typical benzenoid UV absorption (275 nm), showing a bathochromic shift on addition of alkali, suggested the presence of a phenolic group. Furthermore, the signals of three carbons (δ 68.9, 69.0, each d; 154.0 s) in ^{13}C-NMR showed the existence of two aliphatic secondary hydroxyl groups and one phenolic hydroxyl group. As regards the substitution pattern of the two aromatic rings, a *p*-substituted benzene ring and a monosubstituted benzene ring were indicated by the ^{1}H-NMR signals at δ 6.73 (2H, d, J=8.5 Hz), 7.03 (2H, d, J=8.5 Hz) and 7.18—7.29 (5H, m). These findings and the MS fragment peaks at m/z 91 and 107 suggested the presence of phenyl and 4-hydroxyphenyl groups. The signals due to five methylenes (δ 1.59, 2H, t, J=6 Hz, H-4; 1.66—1.79, 4H, m, H-2, 6; 2.50—2.87, 4H, m, H-1, 7) and two methine protons adjacent to hydroxyl groups (δ 3.84—3.95, 2H, m, H-3, 5) were observed in the ^{1}H-NMR spectrum. The two aliphatic secondary hydroxyl groups assigned to C-3 and C-5 were confirmed by comparing the ^{13}C-NMR data of VIa with those of Ia. On the basis of these spectral data, VIa was concluded to be 1-(4-hydroxyphenyl)-7-phenylheptane-3,5-diol.

The absolute configurations of the 3,5-hydroxyl groups were determined in the same manner as with Ia. Compound VIa was methylated with methyl iodide (VIb), and then converted into the bis-*p*-dimethylaminobenzoate (VIc). The CD spectrum of VIc showed a strong negative interaction peak at 318 nm crossing through zero at 306 nm (Fig. 1). Therefore, it became apparent that the 3,5-hydroxyl groups of VIa are situated in a counterclockwise spatial relationship and the absolute configuration of VIa is concluded to be (3*R*,5*R*).

Many diarylheptanoids have been isolated from *Curcuma xanthorrhiza* and *Alpinia officinarum*.[1-3,10,11] We have now added three new diarylheptanoids to this group.

Experimental

All melting points were recorded on a Yazawa micro melting point apparatus and are uncorrected. Spectral data were obtained on the following instruments; UV spectra on a Hitachi 320, optical rotation on a JASCO DIP-181, CD on a JASCO J-500C, IR on a Hitachi EPI-G3 and MS on a JEOL JMS D-300 (at an ionizing potential of 70 eV). ^{1}H- and ^{13}C-NMR spectra were measured on a JEOL FX-270 (at 269.65 MHz and 67.8 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard. (2*S*,4*S*)-(+)-Pentanediol was purchased from Aldrich Co. and (2*R*,4*R*)-(−)-pentanediol from Wako Pure Chemical Industries Co. Diazobenzenesulfonic acid in water was used as the diazo reagent. *p*-Dimethylaminobenzoyl chloride was prepared as described in the literature.[12] Column chromatography was carried out on Silica gel 60 (230—400 mesh ASTM, Merck) unless otherwise stated. Thin layer chromatography (TLC) and preparative TLC were performed on Silica gel 60 F_{254} precoated plate (Merck). The developing solvent for TLC was benzene–AcOEt (1 : 1) unless otherwise stated, and detection was carried out by UV irradiation (254 nm) and spraying 10% H_2SO_4 followed by heating.

Extraction and Isolation——*Curcuma xanthorrhiza*: The rhizomes of *Curcuma xanthorrhiza* were collected in 1982, in Ciandur, Indonesia. The crude drug (400 g) was extracted three times with methanol. The concentrated methanol extract (60 g) was diluted with water to about 10% aq. MeOH and then successively partitioned with *n*-hexane and $CHCl_3$ three times each. Evaporation of the $CHCl_3$-soluble fraction left a brown oil (16 g). The $CHCl_3$ extract was subjected to column chromatography on silica gel (silica gel for dry column chromatography, ICN) with a $CHCl_3$–MeOH gradient system. Repeated chromatography of each fraction (silica gel column chromatography and preparative TLC) afforded Ia (20 mg), II (10 mg), III (10 mg), IV (50 mg) and V (250 mg).

Alpinia officinarum: The rhizomes (10 kg) of *Alpinia officinarum* were extracted three times with methanol. The methanol extract was diluted with water to about 10% aq. MeOH and partitioned with *n*-hexane. The aq. MeOH layer was further concentrated and partitioned with $CHCl_3$. Evaporation of the $CHCl_3$-soluble fraction left a brown oil (518 g). The $CHCl_3$ extract (100 g) was subjected to column chromatography on silica gel (silica gel for dry column chromatography, ICN) with a $CHCl_3$–MeOH gradient system. Repeated chromatography of each fraction (silica gel column chromatography and preparative TLC) afforded VI (100 mg).

(3*S*,5*S*)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-diol (Ia): Colorless needles. mp 132—134 °C, $[\alpha]_D$ −18.5° (c=0.26, EtOH). UV λ_{max}^{EtOH} nm (ε): 225 (21000), 282 (8600). UV $\lambda_{max}^{EtOH+NaOH}$ nm: 243, 296 (bathochromic shift). Diazo reagent: positive (orange). MS *m/z* (%): 376 (M^+, 7, Calcd for $C_{21}H_{28}O_6$, 376.1883; Found 376.1858), 358 (11), 340 (3), 151 (7), 150 (5), 138 (24), 137 (100). IR (KBr) cm^{-1}: 3460, 3310 (br), 2940, 1600, 1515, 1460, 1430, 1350, 1270, 1225, 1200, 1155, 1070, 1040. ^{1}H-NMR (acetone-d_6): 1.58 (2H, t, J=6 Hz), 1.62—1.78 (4H, m), 2.41—2.76 (4H, m), 3.81 (6H, s), 3.85—3.97 (2H, m), 6.64 (2H, dd, J=8, 2 Hz), 6.70 (2H, d, J=8 Hz), 6.81 (2H, d, J=2 Hz), 7.45 (1H, br s, disappeared on addition of D_2O). TLC: *Rf* 0.1.

Methylation of Ia to Ib[13]——A solution of Ia (3 mg) in dry acetone (1 ml) with anhydrous potassium carbonate (10 mg) and methyl iodide (0.5 ml) was refluxed at 60—70 °C for about 6 h. The reaction mixture was filtered, and the residue was washed thoroughly with acetone. The combined filtrate was evaporated *in vacuo* and the residue was chromatographed on a silica gel column (benzene–AcOEt = 1 : 1) and further purified by preparative TLC. The purified product was recrystallized from benzene to give colorless needles (Ib, 2 mg), mp 95—96 °C, $[\alpha]_D$ −9.4° (c=0.1, EtOH). MS *m/z* (%): 404 (M^+, 8), 386 (14), 368 (9), 177 (12), 165 (7), 152 (31), 151 (100), 137 (8). ^{1}H-NMR ($CDCl_3$) δ: 1.68 (2H, t, J=5.5 Hz), 1.70—1.91 (4H, m), 2.27 (2H, br s, disappeared on addition of D_2O), 2.50—2.79 (4H, m), 3.85 (6H, s), 3.87 (6H, s), 3.94—4.06 (2H, br m), 6.72 (2H, d, J=2 Hz), 6.73 (2H, dd, J=8, 2 Hz), 6.80 (2H, d, J=8 Hz).

Bis-*p*-dimethylaminobenzoate (Ic) of Ib——The mixture of Ib (2 mg) and excess *p*-dimethylaminobenzoyl chloride in 1 ml of dry pyridine was heated for 30 min under reflux. After complete removal of pyridine *in vacuo*, the residue was purified by column chromatography (benzene : AcOEt = 5 : 1) and preparative TLC (benzene : $CHCl_3$: CH_3CN = 2 : 1 : 1) to give Ic as a colorless oil (1 mg), $[\alpha]_D$ +36° (c=0.07, EtOH). UV λ_{max}^{EtOH} nm (ε): 229 (30000), 311 (50000). CD (c=0.0004, EtOH): $\Delta\varepsilon$ = +20 (318) (positive maximum), $\Delta\varepsilon$=0 (307), $\Delta\varepsilon$ = −15 (292) (negative maximum). MS *m/z* (%): 698 (M^+, 4), 533 (7), 368 (17), 204 (27), 177 (26), 165 (43), 164 (47), 151 (80), 148 (100). ^{1}H-NMR ($CDCl_3$) δ: 1.80—2.17 (6H, m), 2.50—2.72 (4H, m), 3.01 (12H, s), 3.79 (6H, s), 3.82 (6H, s), 5.25 (2H, br m), 6.57 (4H, d, J=9 Hz), 6.60—6.70 (4H, m), 6.73 (2H, d, J=8.5 Hz), 7.83 (4H, d, J=9 Hz).

Bis-*p*-dimethylaminobenzoates (VIIb and VIIIb) of VIIa and VIIIa——The method described above was employed with 100 mg of VIIa and VIIIa. Each product was purified by column chromatography (benzene : AcOEt = 5 : 1) and recrystallized from EtOH to give VIIb and VIIIb as colorless needles.

VIIb: mp 125—126 °C, $[\alpha]_D$ +349° (c=0.205, EtOH). UV λ_{max}^{EtOH} nm (ε): 311 nm (53100). CD (c=0.00025, EtOH): $\Delta\varepsilon$ = +41.4 (318) (positive maximum), $\Delta\varepsilon$=0 (305), $\Delta\varepsilon$ = −19.5 (292) (negative maximum). MS *m/z* (%): 398 (M^+, 13), 234 (18), 233 (19), 166 (14), 165 (100), 164 (89), 149 (16), 148 (64). ^{1}H-NMR ($CDCl_3$) δ: 1.37 (6H, d, J=6 Hz), 2.05 (2H, t, J=6 Hz), 3.01 (12H, s), 5.24 (2H, tq, J=6, 6 Hz), 6.59 (4H, d, J=9 Hz), 7.85 (4H, d, J=9 Hz).

VIIIb: mp 123—125 °C, $[\alpha]_D$ −302° (c=0.194, EtOH). UV λ_{max}^{EtOH} nm (ε): 311 (54100). CD (c=0.00023, EtOH): $\Delta\varepsilon$ = −34.8 (318) (negative maximum), $\Delta\varepsilon$=0 (305), $\Delta\varepsilon$ = +16.8 (292) (positive maximum). The MS and ^{1}H-NMR data for VIIIb were identical with those for VIIb.

(1ξ)-1-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione (II): Yellow powder, mp 92.0—96.0 °C, $[\alpha]_D$ +12.2° (c=0.06, EtOH). UV λ_{max}^{EtOH} nm (ε): 370 (56000), 283 (18000), 260 (21000), 230 (sh). UV $\lambda_{max}^{EtOH+NaOH}$ nm: 452, 286, 252 (bathochromic shift). MS m/z (%): 368 (M^+-18, 33), 350 ($M^+-18\times2$, 26, Calcd for $C_{21}H_{18}O_5$, 350.1152; Found 350.1147), 191 (30), 190 (43), 177 (100), 150 (37), 145 (49), 137 (50). IR ($CHCl_3$) cm^{-1}: 3525, 2900, 2850, 1650, 1620, 1600, 1560, 1460, 1375, 1325, 1260, 1120, 1000, 965. ^{1}H-NMR ($CDCl_3$) δ: 2.65 (1H, dd, J=17, 3.5 Hz), 2.93 (1H, dd, J=17, 14 Hz), 3.93 (3H, s), 3.95 (3H, s), 5.39 (1H, dd, J=14, 3.5 Hz), 5.60 (1H, s), 5.80 (1H, br s, disappeared on addition of D_2O), 5.86 (1H, br s, disappeared on addition of D_2O), 6.44 (1H, d, J=16 Hz), 6.90 (1H, d, J=8 Hz), 7.00 (4H, br s), 7.03 (1H, dd, J=8, 2 Hz), 7.30 (1H, d, J=16 Hz). TLC: Rf 0.2.

Dehydration of II to V——II (2 mg) was heated at 130 °C under reduced pressure (<15 mmHg) for 10 h. After cooling, it was subjected to preparative TLC to give yellow needles (1.5 mg), which were identical with V on the basis of mixed melting point determination and TLC, MS and ^{1}H-NMR comparisons.

Dihydrocurumin (III): Yellow needles, mp 178.0 °C. UV λ_{max}^{EtOH} nm (ε): 376 (32000), 285 (7500), 260 (7900), 230 (sh). MS m/z (%): 370 (M^+, 11, Calcd for $C_{21}H_{22}O_6$, 370.1415; Found 370.1426), 352 (10), 233 (6), 219 (11), 191 (24), 193 (4), 177 (56), 150 (32), 137 (100). IR (KBr) cm^{-1}: 3400, 1635, 1610, 1515, 1435, 1410, 1280, 1240, 1200, 1160, 1120, 1035, 860, 820. ^{1}H-NMR (DMSO-d_6) δ: 2.68 (2H, m), 2.78 (2H, m), 3.74 (3H, s), 3.82 (3H, s), 5.78 (1H, s), 6.65 (3H, m), 6.81 (2H, m), 7.11 (1H, d, J=7.5 Hz), 7.29 (1H, s), 7.48 (1H, d, J=15.5 Hz), 8.69 (1H, s, disappeared on addition of D_2O), 9.60 (1H, s, disappeared on addition of D_2O). TLC: Rf 0.5.

Hexahydrocurcumin (IV): Colorless oil, $[\alpha]_D$ 0° (c=0.2, $CHCl_3$). UV λ_{max}^{EtOH} nm (ε): 282 (7400), 228 (15600). MS m/z (%): 374 (M^+, 6, Calcd for $C_{21}H_{26}O_6$, 374.1727; Found 374.1706), 356 (9), 138 (17), 151 (4), 150 (7), 137 (100). IR ($CHCl_3$) cm^{-1}: 3540, 3005, 2940, 2400, 1705, 1610, 1510, 1460, 1430, 1370, 1270, 1150, 1125, 1040, 930. ^{1}H-NMR ($CDCl_3$) δ: 1.50—1.87 (2H, m), 2.40—2.88 (8H, m), 3.85 (3H, s), 3.86 (3H, s), 3.92—4.08 (1H, m), 5.55 (2H, br s, disappeared on addition of D_2O), 6.60—6.74 (4H, m), 6.82 (2H, d, J=8 Hz). TLC: Rf 0.3.

Curcumin (V): Yellow needles, mp 183 °C. UV λ_{max}^{EtOH} nm (ε): 420 (57000). MS m/z (%): 368 (M^+, 25), 350 (M^+-18, 32, Calcd for $C_{21}H_{18}O_5$, 350.1152: Found 350.1152), 191 (37), 190 (57), 177 (100), 150 (40), 145 (82), 137 (58). IR (KBr) cm^{-1}: 3450, 1630, 1605, 1510, 1430, 1280, 1235, 1205, 1185, 1160, 1120, 1030, 963. ^{1}H-NMR (acetone-d_6): 3.92 (6H, s), 5.97 (1H, s), 6.70 (2H, d, J=16 Hz), 6.89 (2H, d, J=8 Hz), 7.18 (2H, dd, J=8, 2 Hz), 7.32 (2H, d, J=2 Hz), 7.60 (2H, d, J=16 Hz), 8.25 (2H, br s, disappeared on addition of D_2O). TLC: Rf 0.4.

Hydrogenation of Curcumin (V) to Dihydrocurcumin (III), Hexahydrocurcumin (IV) and Octahydrocurcumin (Id)——A solution of V (50 mg) in MeOH (5 ml) was stirred with PtO_2 (20 mg) for 20 min at room temperature under an H_2 atmosphere, then the catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The product was subjected to preparative TLC to give yellow needles (5 mg), which were identical with III on the basis of mixed melting point determination and TLC, MS and ^{1}H-NMR comparisons.

Further hydrogenation of V (50 mg) for 9 h under the above conditions gave a mixture of oily products, which gave two spots on TLC. Separation of this mixture by preparative TLC afforded a colorless oil (20 mg), identical with Id on the basis of MS data, as the more polar component. In the ^{13}C-NMR spectrum of Id, some carbons appeared as a pair of signals with the same multiplicity (the signal with stronger intensity is indicated in parentheses) as follows: ^{13}C-NMR (acetone-d_6) δ: 32.3 (31.9) (t), 40.8 (41.1) (t), 44.7 (44.2) (t), 56.3 (q), 68.3 (71.3) (d), 113.1 (d), 115.7 (d), 121.5 (d), 134.9 (134.8) (s), 145.2 (s), 148.2 (s). $[\alpha]_D$ 0° (c=1.0, EtOH). UV λ_{max}^{EtOH} nm (ε): 225, 282. UV $\lambda_{max}^{EtOH+NaOH}$ nm: 243, 296. MS m/z (%): 376 (M^+, 7), 358 (9), 340 (3), 151 (9), 150 (7), 138 (24), 137 (100). TLC: Rf 0.1.

The less polar colorless oil (20 mg), $[\alpha]_D$ 0° (c=1.0, EtOH), was identical with IV on the basis of TLC, MS, ^{1}H- and ^{13}C-NMR comparisons.

(3R,5R)-1-(4-Hydroxyphenyl)-7-phenylheptane-3,5-diol (VIa): Colorless needles, mp 109—111 °C, $[\alpha]_D$ +8.3° (c=0.39, $CHCl_3$). UV λ_{max}^{EtOH} nm (ε): 215 (8000), 225 (8500), 275 (1500). UV $\lambda_{max}^{EtOH+NaOH}$ nm: 218, 235, 290. MS m/z (%): 300 (M^+, 0.3, Calcd for $C_{19}H_{24}O_3$ 300.1724; Found 300.1749), 282 (7), 264 (0.7), 121 (6), 120 (16), 107 (100), 105 (10), 91 (70), 77 (23). IR ($CHCl_3$) cm^{-1}: 3600, 2940, 1615, 1600, 1520, 1500, 1455, 1430, 1380, 1330, 1255, 1170. ^{1}H-NMR (acetone-d_6) δ: 1.59 (2H, t, J=6 Hz), 1.66—1.79 (4H, m), 2.50—2.87 (4H, m), 3.84—3.95 (2H, m), 6.73 (2H, d, J=8.5 Hz), 7.03 (2H, d, J=8.5 Hz), 7.18—7.29 (5H, m), 8.01 (1H, br s, disappeared on addition of D_2O). TLC: Rf 0.3.

Methylation of VIa to VIb——The method described above was employed with 2 mg of VIa. The product was recrystallized from benzene-ethyl acetate to give VIb as colorless needles (1.5 mg), mp 73—76 °C. MS m/z (%): 314 (0.02), 296 (0.9), 278 (8), 135 (3), 134 (10), 121 (100), 105 (5), 91 (38), 77 (12). ^{1}H-NMR ($CDCl_3$) δ: 1.67 (2H, t, J=5.5 Hz), 1.70—1.91 (4H, m), 2.56—2.82 (4H, m), 3.78 (3H, s), 3.94—4.02 (2H, m), 6.83 (2H, d, J=8.5 Hz), 7.11 (2H, d, J=8.5 Hz), 7.19 (3H, d, J=7.5 Hz), 7.29 (2H, d, J=7.5 Hz).

Bis-*p*-dimethylaminobenzoate (VIc) of VIb——The method described above was employed with 1 mg of VIb. The product was purified by column chromatography (benzene:AcOEt=5:1) and preparative TLC (benzene:$CHCl_3$:CH_3CN=2:1:1) to give VIc as a colorless oil (0.5 mg), $[\alpha]_D$ −80° (c=0.03, EtOH). UV λ_{max}^{EtOH} nm (ε): 225 (35000), 311 (50000). CD (c=0.0008, EtOH): $\Delta\varepsilon$=−30 (318) (negative maximum), $\Delta\varepsilon$=0 (306), $\Delta\varepsilon$=+20 (292) (positive maximum). MS m/z (%): 608 (M^+, 1.2), 443 (7.5), 278 (19), 174 (31), 165 (96), 164 (63), 148 (71), 147 (26), 134 (35), 121 (100), 91 (34). ^{1}H-NMR ($CDCl_3$) δ: 1.89—2.13 (6H, m), 2.59—2.78 (4H, m), 3.02 (12H, s), 3.75 (3H, m), 5.23 (2H, br s), 6.57 (4H, d, J=9 Hz), 6.79 (2H, d, J=8 Hz), 7.05 (2H, d, J=8 Hz), 7.1—7.2 (5H, m), 7.83 (4H,

d, $J=9$ Hz).

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Studies on the Alkaloids from *Picrasma quassioides* BENNET. IX.[1] Structures of Two β-Carboline Dimeric Alkaloids, Picrasidines-G and -S

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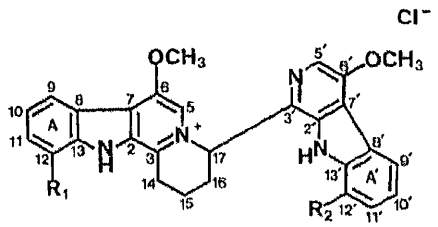
Two new β-carboline dimeric alkaloids, (±)-picrasidine G (**1**) and (±)-picrasidine-S (**2**), have been isolated from the root bark of *Picrasma quassioides* BENNET (Simaroubaceae). The structures were determined on the basis of spectral analysis.

Keywords——*Picrasma quassioides*; nigaki; Simaroubaceae; root bark; β-carboline; β-carbolinium; dimeric alkaloid; (±)-picrasidine-G; (±)-picrasidine-S

In our previous study,[1] we obtained a novel alkaloid named (±)-picrasidine-F from the root bark of *Picrasma quassioides* BENNET (Japanese name: nigaki) (Simaroubaceae). The alkaloid was confirmed to be a racemic compound by single crysytal X-ray diffraction analysis.[1] We have recently isolated two new dimeric alkaloids named picrasidines-G and -S from the root bark of the plant.

This paper deals with the structure elucidation by spectroscopic analyses of (±)-picrasidine-G hydrochloride (**1**) and (±)-picrasidine-S hydrochloride (**2**).



1: $R_1 = R_2 = H$
2: $R_1 = R_2 = OCH_3$
3: $R_1 = H$, $R_2 = OCH_3$

Chart 1

Picrasidine-G hydrochloride (**1**) was isolated as pale yellow needles, mp 272—273 °C (dec.). $[\alpha]_D^{22}$ 0° ($c = 1.0$, MeOH). Picrasidine-S hydrochloride (**2**) was isolated as pale yellow needles, mp 215—217 °C (dec.), $[\alpha]_D^{22}$ 0° ($c = 1.0$, MeOH). Compounds **1** and **2** were determined to have the molecular formulae $C_{28}H_{24}N_4O_2 \cdot HCl$ and $C_{30}H_{28}N_4O_4 \cdot HCl$, respectively, by high-resolution mass spectroscopy. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table I) of **1** in deuteriodimethylsulfoxide (DMSO-d_6), analyzed by the use of the ^{1}H–^{1}H shift correlated spectrum (COSY), showed two methoxyl signals at δ 4.01 and 4.04 (each 3H, s), two lowest-field singlets at δ 12.03 and 12.29 (each 1H, s, disappeared on addition of deuterium oxide) which are assignable to NH protons, two aromatic proton singlets at δ 7.82 and 8.29 (each 1H, s), and two sets of four adjacent aromatic proton signals

at δ 8.32 (1H, dd), 7.45 (1H, td), 7.77 (1H, td), and 7.86 (1H, dd) (each J=8.0 and 1.0 Hz), and δ 8.20 (1H, dd), 7.30 (1H, td), 7.59 (1H, td), and 7.72 (1H, dd) (each J=8.0 and 1.0 Hz). Comparison of the ^{1}H-NMR spectrum of **1** with that of the β-carbolinium moiety of **3** revealed that the chemical shifts of **1** are similar to those of corresponding protons of **3**.

The ^{1}H-NMR spectrum of picrasidine-S hydrochloride (**2**) in DMSO-d_6, analyzed by the use of ^{1}H–^{1}H COSY, showed four methoxyl signals at δ 4.01, 4.04, 4.11, and 4.14 (each 3H, s), two lowest-field singlets at δ 12.13 and 12.93 (each 1H, s, disappeared on addition of deuterium oxide), which are assignable to NH protons, two aromatic proton singlets at δ 7.85 and 8.18 (each 1H, s) and two sets of ABX aromatic protons at δ 7.83 (1H, dd, J=8.0 and 1.0 Hz), 7.36 (1H, t, J=8.0 Hz), and 7.31 (1H, dd, J=8.0 and 1.0 Hz), and δ 7.83 (1H, dd, J=8.0 and 1.0 Hz), 7.25 (1H, t, J=8.0 Hz), and 7.20 (1H, dd, J=8.0 and 1.0 Hz). Comparison of the ^{1}H-NMR spectrum of **2** with that of the β-carboline moiety of **3** revealed similar chemical shifts.

The pronounced deshielding of H-5 (δ 8.29 for **1** and δ 8.18 for **2**) compared with H-5′ (δ 7.82 for **1** and δ 7.85 for **2**) indicated that the proton at the C-5 position suffered a caused larger downfield shift than the proton at the C-5′ position due to the neighboring aromatic quaternary nitrogen atom.[1)]

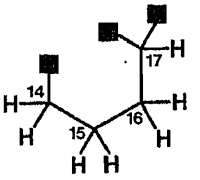
Therefore, the two methoxyl groups (δ 4.01 and 4.04) of **1** were unambiguously placed at C-6 and C-6′, respectively,[2)] and the four methoxyl groups (δ 4.01, 4.14, 4.04, and 4.14) of **2** were unambiguously placed at C-6, C-12, C-6′, and C-12′, respectively.[2)]

The remaining seven aliphatic proton signals in the ^{1}H-NMR spectra of **1** and **2** (Table I), assigned on the basis of double resonance, triple resonance, and ^{1}H–^{1}H COSY experiments,

Table I. ^{1}H-NMR Spectral Data for **1**, **2**, and **3**

Proton	1	2	3
H-1	12.29 (s)[j)]	12.93 (s)[j)]	12.32 (s)[j)]
H-5	8.29 (s)	8.18 (s)	8.11 (s)
H-9	8.32 (dd)[a)]	7.83 (dd)[a)]	8.30 (dd)[a)]
H-10	7.45 (td)[a)]	7.36 (t)[b)]	7.43 (td)[a)]
H-11	7.77 (td)[a)]	7.31 (dd)[a)]	7.75 (td)[a)]
H-12	7.86 (dd)[a)]		7.87 (dd)[a)]
H-14a	3.60 (ddd)[c)]	3.62 (ddd)[c)]	3.62 (ddd)[c)]
H-14b	3.79 (ddd)[d)]	3.85 (ddd)[d)]	3.79 (ddd)[d)]
H-15a	1.79 (ddddd)[e)]	1.85 (ddddd)[e)]	1.79 (ddddd)[e)]
H-15b	2.00 (ddddd)[f)]	2.23 (ddddd)[f)]	2.23 (ddddd)[f)]
H-16a	2.57 (dddd)[g)]	2.60 (dddd)[g)]	2.57 (dddd)[g)]
H-16b	2.68 (dddd)[h)]	2.67 (dddd)[h)]	2.68 (dddd)[h)]
H-17	6.94 (dd)[i)]	7.14 (dd)[i)]	7.06 (dd)[i)]
H-1′	12.03 (s)[j)]	12.13 (s)[j)]	11.92 (s)[j)]
H-5′	7.82 (s)	7.85 (s)	7.83 (s)
H-9′	8.20 (dd)[a)]	7.83 (dd)[a)]	7.81 (dd)[a)]
H-10′	7.30 (td)[a)]	7.25 (t)[b)]	7.23 (t)[b)]
H-11′	7.59 (td)[a)]	7.20 (dd)[a)]	7.18 (dd)[a)]
H-12′	7.72 (dd)[a)]		
6-OCH_3	4.01 (s)	4.01 (s)	3.99 (s)
6′-OCH_3		4.14 (s)	
12-OCH_3	4.04 (s)	4.04 (s)	4.03 (s)
12′-OCH_3		4.11 (s)	4.08 (s)

The spectra were measured with a JEOL JNM-GX-400 spectrometer (400 MHz) in DMSO-d_6 with tetramethylsilane as an internal reference. Coupling constants: *a*) J=8.0, 1.0 Hz; *b*) J=8.0 Hz; *c*) J=18.0, 10.0, 8.0 Hz; *d*) J=18.0, 7.0, 2.0 Hz; *e*) J=15.0, 12.0, 10.0, 7.0, 4.0 Hz; *f*) J=15.0, 8.0, 3.0, 2.0, 1.0 Hz; *g*) J=16.0, 4.0, 2.0, 1.0 Hz; *h*) J=16.0, 12.0, 4.0, 3.0 Hz; *i*) J=4.0, 2.0 Hz. *j*) Disappeared with D_2O.



A

Fig. 1. Partial Structure of 1 and 2

TABLE II. Partial ^{13}C-NMR Spectral Data for **1**, **2**, and **3**

Carbon	1	2	3
C-14	24.23	24.40	24.48
C-15	13.21	13.22	13.41
C-16	26.60	27.01	27.21
C-17	64.74	64.54	64.56

The spectra were measured with a JEOL JNM-GX-400 spectrometer (100 MHz) in DMSO-d_6 with TMS as an internal reference.

revealed the presence of a partial structure A (Fig. 1). The shape of the C–H network in the partial structure of **1** and **2** was deduced by comparison of the ^{1}H-NMR data (Table I) and partial carbon-13 nuclear magnetic resonance (^{13}C-NMR) data (Table II) of **1** and **2** with those of **3**.[1] Thus the C-14 position of the partial structure A is most likely linked to the C-3 position of the β-carbolinium structure and the C-17 position of the structure is linked to the N-4 position of the β-carbolinium structure and the C-3′ position of the β-carboline structure.

From these results, the structures of (±)-picrasidine-G hydrochloride and (±)-picrasidine-S hydrochloride were concluded to be represented by formulae **1** and **2**, respectively.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. The ultraviolet (UV) and infrared (IR) spectra were recorded with Hitachi 340 and Hitachi 260-30 spectrophotometers, respectively. The ^{1}H- and ^{13}C-NMR spectra were recorded with a JEOL GX-400 spectrometer (^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz). Chemical shifts are given on the δ scale (ppm) with tetramethylsilane (TMS) as an internal standard and coupling constants are given in Hz. Field desorption-mass spectrum (FD-MS), electron ionization (EI)-MS, and high-resolution MS were measured with a JEOL JMS DX-300 mass spectrometer. Column chromatography was carried out on silica gel (BW-820 MH, Fuji Devison Co., Ltd.). Medium-pressure preparative chromatography was performed on a silica gel column (24 mm i.d. × 360 mm). Thin-layer chromatography (TLC) was performed on Silica gel 60 GF_{254} (Merck) plates and spots were detected with Dragendorff's reagent or by UV illumination.

Extraction and Isolation — Dried root-bark (5.5 kg) of *Picrasma quassioides*, collected at Funabashi city, Chiba prefecture, in August 1984, was extracted with MeOH (72 l) at 40 °C for 48 h. The extract was evaporated to dryness and the residue was partitioned between $CHCl_3$ and water. The $CHCl_3$ solution was dried over Na_2SO_4 and concentrated to give a $CHCl_3$-soluble fraction (530 g), which was applied to a column of silica gel (1.5 kg) and eluted successively with $CHCl_3$, $CHCl_3$–MeOH (99 : 1, 49 : 1, 19 : 1, 9 : 1 and 1 : 1), and MeOH. The $CHCl_3$–MeOH (19 : 1) fraction (15 g) was repeatedly chromatographed on silica gel to afford the crude alkaloid fraction (3.2 g). The alkaloid fraction was dissolved in MeOH, and 10% HCl solution was added. The hydrochloric acid salt that precipitated was further purified by medium-pressure preparative chromatography on a silica gel column (24 mm i.d. × 360 mm) with $CHCl_3$–MeOH (19 : 1) as an eluent (1 ml/min) to give (±)-picrasidine-G hydrochloride (**1**, 15 mg) and (±)-picrasidine-S hydrochloride (**2**, 760 mg).

(±)-Picrasidine-G Hydrochloride (1)——Pale yellow needles (MeOH), mp 272—273 °C (dec.), $[\alpha]_D^{22}$ 0° (c = 1.0, MeOH). UV λ_{max}^{EtOH} nm (log ε): 230 (sh, 4.24), 248 (4.35), 302 (sh, 4.00), 316 (3.76), 342 (3.57), 372 (3.30). UV $\lambda_{max}^{EtOH+NaOH}$ nm (log ε): 240 (4.28), 279 (4.45), 328 (3.68), 400 (3.24). IR ν_{max}^{KBr} cm^{-1}: 3450, 1623, 1585, 1455, 1325, 1150, 1030. ^{1}H-NMR: see Table I. ^{13}C-NMR (δ in DMSO-d_6 at 50 °C): 13.21 (t), 24.23 (t), 26.60 (t), 55.86 (q), 57.30 (q), 64.74 (d), 111.22 (d), 112.31 (d), 117.03 (s), 117.17 (d), 118.31 (s), 119.35 (d), 119.55 (s), 119.65 (d), 120.62 (s), 121.23 (d), 122.67 (d), 123.65 (d), 126.91 (d), 129.61 (s), 129.68 (d), 133.52 (s), 134.26 (s), 137.36 (s), 139.30 (s), 141.71 (s), 150.05 (s), 150.51 (s). FD-MS m/z: 448 $(M-HCl)^+$. EI-MS m/z (%): 448 $[(M-HCl)^+$, 30], 250 (9), 238 (15), 237 (18), 224 (60), 223 (20), 212 (14), 209 (14), 181 (20), 52 (100). High-resolution MS: Calcd for $C_{28}H_{24}N_4O_2$ $[(M-HCl)^+]$ m/z: 448.1899. Found m/z: 448.1953.

(±)-Picrasidine-S Hydrochloride (2)——Pale yellow needles (MeOH), mp 215—217 °C (dec.), $[\alpha]_D^{22}$ 0° (c = 1.0, MeOH). UV λ_{max}^{EtOH} nm (log ε): 236 (4.35), 256 (4.42), 280 (sh, 3.87), 314 (3.74), 344 (3.67). UV $\lambda_{max}^{EtOH+NaOH}$ nm (log ε):

236 (4.35), 276 (4.44), 328 (3.68), 404 (3.20). IR ν_{max}^{KBr} cm^{-1}: 3430, 1635, 1587, 1548, 1285, 1145, 1055, 1045. ^{1}H-NMR: see Table I. ^{13}C-NMR (δ in DMSO-d_6 at 50 °C): 13.22 (t), 24.40 (t), 27.01 (t), 55.42 (q), 55.64 (q), 55.85 (q), 57.24 (q), 64.54 (d), 107.60 (d), 109.52 (d), 114.90 (d), 115.27 (d), 116.83 (d), 117.67 (s), 119.57 (s), 119.64 (d), 119.73 (s), 120.06 (d), 120.65 (s), 121.98 (d), 129.69 (s), 132.26 (s), 132.49 (s), 133.30 (s), 134.71 (s), 138.37 (s), 145.42 (s), 145.64 (s), 149.98 (s), 150.51 (s). FD-MS *m/z*: 508 $(M-HCl)^+$. EI-MS *m/z* (%): 508 [$(M-HCl)^+$, 14], 478 (89), 463 (22), 267 (100), 255 (20), 241 (31), 237 (27), 225 (19), 212 (31), 193 (67), 169 (6). High-resolution MS: Calcd for $C_{30}H_{28}N_4O_4$ [$(M-HCl)^+$], *m/z*: 508.2111. Found *m/z*: 508.2098.

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References and Notes

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2) T. Ohmoto and K. Koike, *Chem. Pharm. Bull.*, **30**, 1204 (1982); *idem*, *ibid.*, **31**, 3198 (1983); K. Koike and T. Ohmoto, *ibid.*, **34**, 2090 (1986).

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New Phenylethanoid Glycosides from *Cistanche tubulosa* (SCHRENK) HOOK. f. I.

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Four new phenylethanoid glycosides, tubulosides A (II), B (VI), C (VII) and D (VIII), have been isolated from *Cistanche tubulosa* (SCHRENK) HOOK. f. (Orobanchaceae), together with four known phenylethanoid glycosides, echinacoside (I), acteoside (III), acteoside isomer (IV) and 2′-acetylacteoside (V). The structures of II, VI, VII and VIII were established on the basis of chemical evidence and spectral data. Compounds VII and VIII possess a triacetylrhamnosyl moiety as the terminal sugar.

Keywords——*Cistanche tubulosa*; Orobanchaceae; parasitic plant; phenylethanoid glycoside; tubuloside A; tubuloside B; tubuloside C; tubuloside D

In our series of investigations on the chemical constituents of *Cistanche* spp. (Orobanchaceae), the phenylethanoid glycosides[1–3] and iridoids[4] from *Cistanche salsa* (C. A. MEY.) G. BECK have been reported. The present paper deals with the phenylethanoid glycosides from *Cistanche tubulosa* (SCHRENK) HOOK. f. collected in Pakistan. *Cistanche tubulosa* (SCHRENK) HOOK. f.[5] is a parasitic plant growing on the roots of *Salvadora* and *Calotropis* spp., and occurs widely in North Africa, Arabia, West Asia to Pakistan and India. The whole plant is used medicinally in Pakistan as a remedy for diarrhea and sores.[6]

We now wish to report the isolation of four new phenylethanoid glycosides, named tubulosides A (II), B (VI), C (VII) and D (VIII), as well as four known phenylethanoid glycosides, echinacoside (I), acteoside (III), acteoside isomer (IV) and 2′-acetylacteoside (V). The structures of these compounds were determined on the basis of chemical evidence and spectroscopic studies.

The ethanolic extract of the whole plants was suspended in water. This suspension was extracted with ethyl acetate and then with *n*-butanol saturated with water. The *n*-butanol-soluble fraction was chromatographed on polyamide and silica gel columns and subjected to high-performance liquid chromatography (HPLC) successively, to give eight phenylethanoid glycosides (I—VIII).

Compounds I, III, IV and V were isolated as amorphous powders, showing similar spectra to those of echinacoside,[1] acteoside,[1] acteoside isomer[7] and 2′-acetylacteoside,[2] respectively, and were identified by direct comparison with authentic samples [thin layer chromatography (TLC), infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR), and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra].

Tubuloside A (II) was isolated as an amorphous powder, $[\alpha]_D$ −103.7° (MeOH), $C_{37}H_{48}O_{21}\cdot 3/2\,H_2O$. The IR spectrum suggested the presence of hydroxyl groups

(3440 cm^{-1}), a conjugated ester (1705 cm^{-1}), a double bond (1634 cm^{-1}) and aromatic rings (1608, 1522 cm^{-1}), and the ultraviolet (UV) spectrum showed absorption maxima at 220, 250 sh, 292 sh and 334 nm. The ^{1}H-NMR spectrum of II showed signals of a methyl group of rhamnose [δ 1.07 (3H, d, J=6 Hz)], a methyl signal of an acetoxyl group [δ 1.98 (3H, s)], benzylic methylene protons [δ 2.70 (2H, t, J=7 Hz)], two glucose-anomeric protons [δ 4.32, 4.54 (1H each, d, J=8 Hz)], a rhamnose-anomeric proton [δ 5.11 (1H, br s)], two *trans* olefinic protons [δ 6.25, 7.64 (1H each, d, J=16 Hz)] and aromatic protons [δ 6.5—7.2 (6H)]. On acetylation, II afforded the undecaacetate (IIa), which was identical with the dodecaacetate[1] of echinacoside (I). The ^{13}C-NMR spectrum of II was almost identical with that of I, except for the signals due to the glucose bonded directly to the aglycone and the acetoxyl group [δ 20.9 (CH_3), 171.5 (C=O)], suggesting that the acetoxyl group is attached to the glucose moiety. In the ^{13}C-NMR spectrum of II, the acylation shifts[8] [−2.3 (C-1), −0.9 (C-2) and −1.0 (C-3)] were observed at C-1, C-2 and C-3 of the inner glucose by detailed comparison with the spectrum of I, indicating that the acetoxyl group is linked to the C-2 hydroxyl group of the glucose moiety in II. On methanolysis of II with acetyl chloride in methanol, methyl caffeate and 3,4-dihydroxyphenethyl alcohol were detected by TLC and HPLC. Acid hydrolysis of II with 10% sulfuric acid afforded glucose and rhamnose in a ratio of 2 to 1.

On the basis of the above-mentioned evidence, the structure of tubuloside A was determined to be 2-(3,4-dihydroxyphenyl)ethyl *O*-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-*O*-caffeoyl)-2-*O*-acetyl-β-D-glucopyranoside (II).

Tubuloside B (VI) was isolated as an amorphous powder, $[\alpha]_D$ −39.0° (MeOH), $C_{31}H_{38}O_{16}$, whose ^{1}H-NMR spectrum showed the presence of an aliphatic acetoxyl group [δ 1.98 (3H, s)]. The ^{13}C-NMR spectrum of VI was very similar to that of acteoside isomer (IV), but differed slightly in the signals due to the glucose moiety and the presence of the acetoxyl group [δ 20.9 (CH_3), 171.6 (C=O)]. The location of the acetoxyl group in the glucose moiety of VI was determined from its ^{13}C-NMR spectrum by detailed comparison with that of IV.

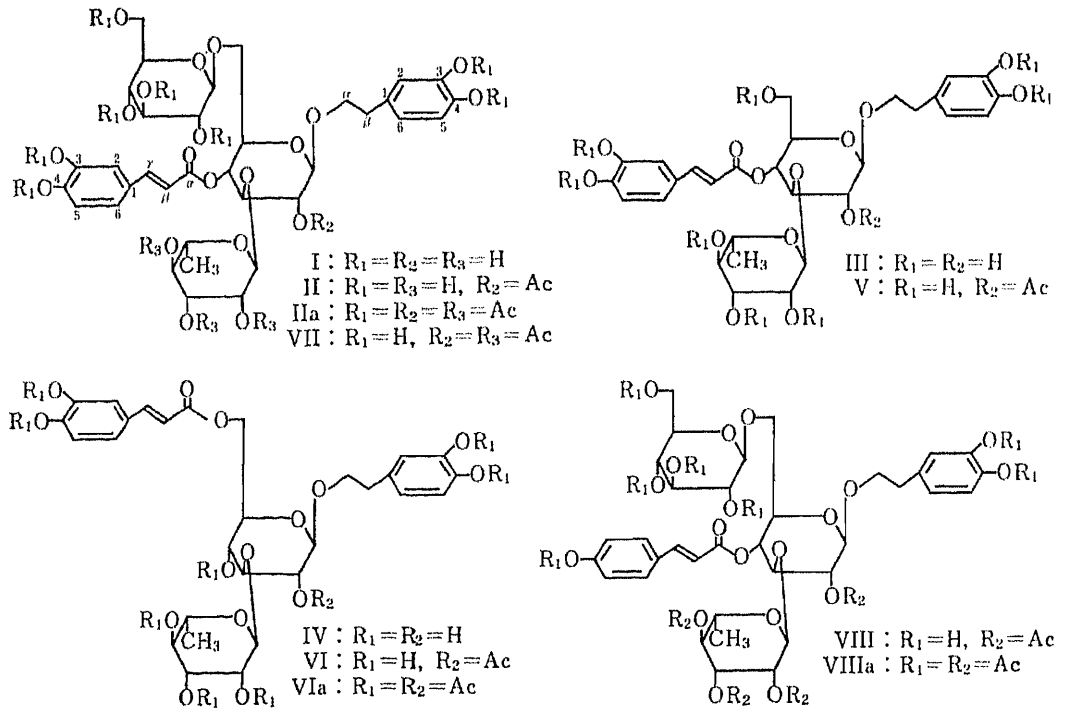

Chart 1

TABLE I. ^{13}C-NMR Chemical Shifts of I—VIII in Methanol-d_4

	Carbon No.	I	II	III	IV	V	VI	VII	VIII
Aglycone moiety	1	131.4	131.8	131.4	131.4	131.9	131.7	131.8	131.7
	2	117.0	117.2	117.1	117.1	117.2	117.1	117.1	117.1
	3	145.8	145.9	145.9	146.0	145.9	145.9	145.9	145.9
	4	144.4	144.5	144.4	144.6	144.4	144.4	144.4	144.4
	5	116.3	116.3	116.3	116.3	116.4	116.3	116.3	116.3
	6	121.2	121.4	121.3	121.3	121.4	121.3	121.4	121.3
	α	72.0	72.5	72.2	72.3	72.5	72.4	71.8	71.9
	β	36.3	36.2	36.4	36.6	36.2	36.3	36.2	36.2
Caffeic acid moiety	1	127.5	127.6	127.6	127.7	127.7	127.7	127.4	126.8
(*p*-Coumaric)	2	115.2	115.4	115.3	115.1	115.5	115.2	115.3	116.9
	3	146.6	146.7	146.6	146.7	146.6	146.7	146.8	131.4
	4	149.5	149.8	149.5	149.5	149.6	149.5	149.9	161.5
	5	116.3	116.6	116.5	116.5	116.6	116.5	116.6	131.4
	6	123.1	123.3	123.2	123.1	123.2	123.1	123.2	116.9
	α	168.2	168.2	168.2	169.1	168.1	169.0	168.0	168.1
	β	114.6	114.6	114.6	114.9	114.7	114.9	114.3	114.4
	γ	148.1	148.3	147.9	147.2	148.1	147.2	148.4	148.0
Glucose moiety	1	103.9	101.6	104.0	104.3	101.6	101.8	101.6	101.6
(Inner)	2	75.9	75.0	75.8	75.4	75.1	74.8	75.5	75.0
	3	81.5	80.5	81.6	84.0	80.3	82.6	80.1	80.3
	4	70.2[a]	70.7	70.3[c]	70.0[d]	70.7	70.3[f]	70.5	70.7
	5	74.5	74.8	76.0	75.6	76.0	75.4	74.6	74.6
	6	69.2	69.2	62.3	64.6	62.2	64.4	69.1	69.1
Rhamnose moiety	1	102.8	103.9	102.8	102.7	103.1	102.8	99.5	99.6
	2	72.2	71.9[b]	72.0	72.3	72.0[e]	72.1[g]	70.0	70.1
	3	72.2	71.4[b]	72.0	72.3	71.7[e]	71.8[g]	71.2[h]	71.2[i]
	4	73.6	73.6	73.7	74.0	73.6	73.7	71.4[h]	71.4[i]
	5	70.4[a]	70.7	70.5[c]	70.4[d]	70.7	70.5[f]	68.2	68.2
	6	18.3	18.5	18.4	17.8	18.4	17.8	18.0	18.0
Glucose moiety	1	104.4	104.6					104.6	104.6
(Terminal)	2	74.9	75.0					74.6	74.6
	3	77.6	77.8					77.7	77.7
	4	71.2	71.9					71.8	71.9
	5	77.6	77.8					77.7	77.7
	6	62.5	62.6					62.6	62.6
OAc	CH_3		20.9			20.9	20.9	20.4	20.4
								20.9	20.9
	CO		171.5			171.5	171.6	171.2	171.2
								171.5	171.5

a—*i*) Assignments may be interchanged in each column.

The signals of C-1, C-2 and C-3 of the glucose moiety showed acylation shifts [−2.5 (C-1), −0.6 (C-2) and −1.4 (C-3)], as in the case of II, indicating that the acetoxyl group is linked to the C-2 hydroxyl group of the glucose moiety in VI. On acetylation, VI afforded the octaacetate (VIa) which was identical with the nonaacetate[7] of IV. On methanolysis of VI with acetyl chloride in methanol, methyl caffeate and 3,4-dihydroxyphenethyl alcohol were detected by TLC and HPLC. Acid hydrolysis of VI with 10% sulfuric acid afforded glucose and rhamnose in a ratio of 1 to 1. These results led us to conclude that the structure of tubuloside B is 2-(3,4-dihydroxyphenyl)ethyl *O*-α-L-rhamnopyranosyl-(1→3)-(6-*O*-caffeoyl)-2-*O*-acetyl-β-D-glucopyranoside (VI).

Tubuloside C (VII) was isolated as an amorphous powder, $[\alpha]_D$ −104.8° (MeOH),

$C_{43}H_{54}O_{24}\cdot H_2O$, whose ^{1}H-NMR spectrum showed the presence of four aliphatic acetoxyl groups [δ 1.80, 1.92, 1.95 and 2.08 (3H each, s)]. The ^{13}C-NMR spectrum of VII was almost identical with that of tubuloside A (II), which possesses an aliphatic acetoxyl group in the inner glucose, except for the signals due to the rhamnose moiety. Furthermore, in the ^{13}C-NMR spectrum of VII, acylation shifts[9] were observed in the signals due to C-2, C-3 and C-4 of the rhamnose moiety by detailed comparison with the data for II. Consequently, the locations of the four acetoxyl groups were determined to be C-2 of the inner glucose and C-2, C-3, C-4 of the rhamnose moiety in VII. On acetylation, VII afforded the octaacetate which was identical with tubuloside A undecaacetate (IIa). On methanolysis of VII with acetyl chloride in methanol, methyl caffeate and 3,4-dihydroxyphenethyl alcohol were detected by TLC and HPLC. Acid hydrolysis of VII with 10% sulfuric acid afforded glucose and rhamnose in a ratio of 2 to 1.

From the above results, the structure of tubuloside C was determined to be 2-(3,4-dihydroxyphenyl)ethyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-*O*-caffeoyl)-2-*O*-acetyl-β-D-glucopyranoside (VII).

Tubuloside D (VIII) was isolated as an amorphous powder, $[\alpha]_D$ −91.4° (MeOH), $C_{43}H_{54}O_{23}\cdot H_2O$, whose ^{1}H-NMR spectrum showed the presence of four aliphatic acetoxyl groups [δ 1.81, 1.93, 1.96 and 2.09 (3H each, s)]. On acetylation, VIII afforded the heptaacetate (VIIIa), whose ^{1}H-NMR spectrum showed eight aliphatic [δ 1.87, 1.94, 1.96, 1.99, 2.10 (3H each, s) and 2.02 (9H, s)] and three aromatic [δ 2.27, 2.30 and 2.32 (3H each, s)] acetoxyl methyl signals. The ^{13}C-NMR spectrum of VIII was almost identical with that of tubuloside C (VII), except for the signals due to the *p*-coumaric acid moiety. On methanolysis of VIII with acetyl chloride in methanol, methyl *p*-coumarate and 3,4-dihydroxyphenethyl alcohol were detected by TLC and HPLC. Acid hydrolysis of VIII with 10% sulfuric acid afforded glucose and rhamnose in a ratio of 2 to 1.

On the basis of these results, the structure of tubuloside D was determined to be 2-(3,4-dihydroxyphenyl)ethyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-*O*-*p*-coumaryl)-2-*O*-acetyl-β-D-glucopyranoside (VIII).

Many phenylethanoid glycosides such as forsythoside A,[10] leucosceptoside A,[7] isomartynoside[11] and so on, having a rhamnose moiety as the terminal sugar, have been reported. In these cases, the rhamnose moiety is not acetylated. Tubulosides C (VII) and D (VIII) contain an acetylated rhamnose moiety and are the first naturally occurring compounds having a triacetylrhamnose moiety to be reported.

Experimental

Melting points were determined on a Mitamura micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. IR spectra were recorded with Hitachi 270-30 infrared spectrophotometer and UV spectra with a Hitachi 200-20 spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a JEOL FX-90Q machine (89.55 and 22.5 MHz, respectively). Chemical shifts are given on the δ (ppm) scale with tetramethylsilane (TMS) as an internal standard (s, singlet; d, doublet; t, triplet; br, broad). Gas-liquid chromatography (GC) was run on a Shimadzu GC-4CM apparatus with a flame ionization detector. HPLC was performed on a Hitachi 655A-11 machine. Silica gel (Wako gel C-300, Wako Pure Chemical) was used for column chromatography. Kieselgel 60 F_{254} (Merck) precoated plates were used for TLC and detection was carried out by spraying 10% H_2SO_4 followed by heating.

Isolation——Fresh whole plants of *C. tubulosa* (22 kg), collected in December 1984, in Karachi, Pakistan, were extracted with EtOH. The ethanolic extract was suspended in H_2O, and extracted with EtOAc and then with *n*-BuOH saturated with H_2O. The *n*-BuOH extract (99.1 g) was absorbed on a Diaion HP-20 (Nippon Rensui Co.) column and the resin was eluted with MeOH after being washed with H_2O. The MeOH eluate (15.4 g) was chromatographed on a polyamide C-200 (Wako Pure Chemical) column using H_2O and then MeOH. The fraction eluted with MeOH was concentrated to give a residue (phenolic crude glycosides) (8.0 g). After repeated chromatography of the residue on silica gel with $CHCl_3$–MeOH–H_2O (70 : 30 : 5) and HPLC with a H_2O–CH_3CN or H_2O–MeOH solvent system, eight glycosides (I—VIII) were isolated. I, 230 mg; II, 200 mg; III, 240 mg; IV, 60 mg; V, 210 mg; VI, 100 mg; VII, 60 mg;

VIII, 65 mg. Conditions for HPLC: column, Develosil ODS-10 (20×250 mm); solvent, I, II (17% CH_3CN), III, IV (20% CH_3CN), V (22% CH_3CN), VI (25% CH_3CN), VII (53% MeOH), VIII (55% MeOH); detector (UV), 220 nm; flow rate, 6.9 ml/min.

Echinacoside (I)——Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3400, 1690, 1625, 1600, 1518. ^{1}H-NMR (methanol-d_4) δ: 1.09 (3H, d, J=6 Hz, CH_3 of rhamnose), 2.79 (2H, t, J=7 Hz, Ar-CH_2-), 4.29, 4.37 (1H each, d, J=8 Hz, H-1 of glucose), 5.16 (1H, d, J=1 Hz, H-1 of rhamnose), 6.26 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.4—7.1 (6H, aromatic H), 7.59 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Tubuloside A (II)——Amorphous powder, $[\alpha]_D^{23}$ −103.7° (c=1.08, MeOH). *Anal.* Calcd for $C_{37}H_{48}O_{21}\cdot 3/2\,H_2O$: C, 51.93; H, 6.01. Found: C, 51.80; H, 5.81. IR ν_{max}^{KBr} cm^{-1}: 3440, 1732, 1705, 1634, 1608, 1522. UV λ_{max}^{MeOH} nm (log ε): 220 (4.14), 250 sh (3.85), 292 sh (3.95), 334 (4.13). ^{1}H-NMR (methanol-d_4): see text. ^{13}C-NMR: Table I.

Acteoside (III)——Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3420, 1696, 1634, 1606, 1520. ^{1}H-NMR (methanol-d_4) δ: 1.10 (3H, d, J=6 Hz, CH_3 of rhamnose), 2.78 (2H, t, J=7 Hz, Ar-CH_2-), 4.36 (1H, d, J=8 Hz, H-1 of glucose), 5.17 (1H, d, J=1 Hz, H-1 of rhamnose), 6.25 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.4—7.1 (6H, aromatic H), 7.58 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Acteoside Isomer (IV)——Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3280, 1686, 1624, 1602, 1512. ^{1}H-NMR (methanol-d_4) δ: 1.26 (3H, d, J=6 Hz, CH_3 of rhamnose), 2.77 (2H, t, J=7 Hz, Ar-CH_2-), 4.33 (3H, d, J=8 Hz, H-1 of glucose), 5.18 (1H, br s, H-1 of rhamnose), 6.28 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.4—7.1 (6H, aromatic H), 7.54 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

2'-Acetylacteoside (V)——Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450, 1735, 1705, 1640, 1610, 1535. ^{1}H-NMR (methanol-d_4) δ: 1.07 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.99 (3H, s, OAc), 2.69 (2H, t, J=7 Hz, Ar-CH_2-), 4.50 (1H, d, J=8 Hz, H-1 of glucose), 5.16 (1H, br s, H-1 of rhamnose), 6.25 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.5—7.2 (6H, aromatic H), 7.59 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Tubuloside B (VI)——Amorphous powder, $[\alpha]_D^{23}$ −39.0° (c=1.05, MeOH). *Anal.* Calcd for $C_{31}H_{38}O_{16}$: C, 55.85; H, 5.75. Found: C, 55.91; H, 6.00. IR ν_{max}^{KBr} cm^{-1}: 3420, 1734, 1696, 1634, 1608, 1522. UV λ_{max}^{MeOH} nm (log ε): 220 (4.32), 246 sh (4.09), 292 sh (4.21), 340 (4.31). ^{1}H-NMR (methanol-d_4) δ: 1.24 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.98 (3H, s, OAc), 2.69 (2H, t, J=7 Hz, Ar-CH_2-), 4.48 (1H, d, J=8 Hz, H-1 of glucose), 6.32 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.5—7.2 (6H, aromatic H), 7.61 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Tubuloside C (VII)——Amorphous powder, $[\alpha]_D^{25}$ −104.8° (c=1.86, MeOH). *Anal.* Calcd for $C_{43}H_{54}O_{24}\cdot H_2O$: C, 53.08; H, 5.80. Found: C, 53.28; H, 5.68. IR ν_{max}^{KBr} cm^{-1}: 3440, 1748, 1634, 1608, 1522. UV λ_{max}^{MeOH} nm (log ε): 220 sh (4.06), 250 sh (3.72), 292 sh (3.88), 333 (4.04). ^{1}H-NMR (methanol-d_4) δ: 1.02 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.80, 1.92, 1.95, 2.08 (3H each, s, OAc), 2.70 (2H, t, J=7 Hz, Ar-CH_2-), 4.32, 4.56 (1H each, d, J=8 Hz, H-1 of glucose), 5.02 (1H, br s, H-1 of rhamnose), 6.30 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.5—7.2 (6H, aromatic H), 7.66 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Tubuloside D (VIII)——Amorphous powder, $[\alpha]_D^{25}$ −91.4° (c=1.85, MeOH). *Anal.* Calcd for $C_{43}H_{54}O_{23}\cdot H_2O$: C, 54.00; H, 5.90. Found: C, 54.10; H, 5.75. IR ν_{max}^{KBr} cm^{-1}: 3440, 1750, 1634, 1608, 1518. UV λ_{max}^{MeOH} nm (log ε): 228 (4.16), 292 sh (4.21), 302 sh (4.26), 318 (4.35). ^{1}H-NMR (methanol-d_4) δ: 1.00 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.81, 1.93, 1.96, 2.09 (3H each, s, OAc), 2.70 (2H, t, J=7 Hz, Ar-CH_2-), 4.32, 4.53 (1H each, d, J=8 Hz, H-1 of glucose), 5.03 (1H, br s, H-1 of rhamnose), 6.38 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.52—6.75 (3H, aromatic H), 6.84 (2H, d, J=9 Hz, H-3, H-5 of *p*-coumaric acid), 7.54 (2H, d, J=9 Hz, H-2, H-6 of *p*-coumaric acid), 7.74 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). ^{13}C-NMR: Table I.

Acetylation of II and VII——Treatment of II or VII (30 mg) with Ac_2O (1 ml) and pyridine (1 ml) at room temperature overnight followed by the usual work-up afforded a crude acetate, which was purified by chromatography on silica gel with benzene–acetone (5:1) to give the undecaacetate (IIa) (25 mg) from II or the octaacetate (22 mg) from VII, as colorless needles from MeOH, mp 130—131 °C. IR ν_{max}^{KBr} cm^{-1}: 1775, 1660, 1523, 1450. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.89, 1.96, 1.97, 2.01, 2.11 (3H each, s, OAc), 2.03 (9H, s, OAc×3), 2.29 (3H, s, Ar-OAc), 2.31 (9H, s, Ar-OAc×3), 2.88 (2H, t, J=7 Hz, Ar-CH_2-), 6.35 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 7.0—7.4 (6H, aromatic H), 7.66 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). These products were found to be identical with the dodecaacetate of echinacoside (I) by direct comparison (TLC, mixed mp, IR and ^{1}H-NMR).

Acetylation of VI——Compound VI (40 mg) was acetylated in the same manner as described for II and the reaction product was purified by chromatography on silica gel with benzene–acetone (9:1) to give the octaacetate (VIa) (30 mg) as an amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1742, 1630, 1498. ^{1}H-NMR ($CDCl_3$) δ: 1.14 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.95, 2.03, 2.05, 2.09, 2.13 (3H each, s, OAc), 2.27, 2.31 (6H each, s, Ar-OAc×2), 2.87 (2H, t, J=7 Hz, Ar-CH_2-), 6.42 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 6.9—7.5 (6H, aromatic H), 7.64 (1H, d, J=16 Hz, Ar-C<u>H</u>=CH-). VIa was found to be identical with the nonaacetate of acteoside isomer (IV) by direct comparison (TLC, IR and ^{1}H-NMR).

Acetylation of VIII——Compound VIII (35 mg) was acetylated in the same manner as described for II to give the heptaacetate (VIIIa) (30 mg) as an amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 1760, 1638, 1604, 1510. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, d, J=6 Hz, CH_3 of rhamnose), 1.87, 1.94, 1.96, 1.99, 2.10 (3H each, s, OAc), 2.02 (9H, s, OAc×3), 2.27, 2.30, 2.32 (3H each, s, Ar-OAc), 2.88 (2H, t, J=7 Hz, Ar-CH_2-), 6.36 (1H, d, J=16 Hz, Ar-CH=C<u>H</u>-), 7.0—7.2

(3H, aromatic H), 7.25 (2H, d, J=9 Hz, H-3, H-5 of *p*-coumaric acid), 7.57 (2H, d, J=9 Hz, H-2, H-6 of *p*-coumaric acid), 7.72 (1H, d, J=16 Hz, Ar-CH=CH-).

Methanolysis of II, VI, VII and VIII——Compound II, VI, VII or VIII (*ca.* 1 mg) was refluxed with methanolic 5% CH_3COCl (2 ml) for 30 min, and then the reagents were evaporated off. The presence of methyl caffeate and 3,4-dihydroxyphenethyl alcohol in the residue of II, VI and VII, and methyl *p*-coumarate and 3,4-dihydroxyphenethyl alcohol in that of VIII, was demonstrated by TLC [$CHCl_3$–MeOH (20 : 1)] and HPLC [column, TSK GEL LS-410AK (4 × 300 mm); solvent, H_2O–MeOH (4 : 6); detector (UV), 250 nm; flow rate, 1.0 ml/min]. Methyl caffeate [*Rf* 0.20, t_R (min) 10.8], methyl *p*-coumarate [*Rf* 0.40, t_R (min) 15.6], 3.4-dihydroxyphenethyl alcohol [*Rf* 0.06, t_R (min) 2.8].

Acid Hydrolysis of II, VI, VII and VIII——A solution of a glycoside (*ca.* 2 mg) in 10% H_2SO_4 (1 ml) was heated in a boiling water bath for 30 min. The solution was passed through an Amberlite IR-45 column and the eluate was concentrated to give a residue, which was reduced with sodium borohydride (*ca.* 3 mg) for 1 h. The reaction mixture was passed through an Amberlite IR-120 column and concentrated to dryness. Boric acid was removed by distillation with MeOH and the residue was acetylated with Ac_2O (1 drop) and pyridine (1 drop) at 100 °C for 1 h. The reagents were evaporated off. Glucitol acetate and rhamnitol acetate were detected in a ratio of 2 to 1 from II, VII and VIII, and 1 to 1 from VI by GC. t_R (min): 2.0 (rhamnitol acetate), 5.5 (glucitol acetate). Conditions for GC: column, 1.5% OV-17 (3 mm × 1.5 m); column temp., 180 °C; carrier gas, N_2 (30 ml/min).

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New 2,5-Bis-aryl-3,4-dimethyltetrahydrofuran Lignans from the Aril of *Myristica fragrans*[1)]

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Several new tetrahydrofuran lignans, named fragransins A_2 (II), B_1 (IV), B_2 (V), B_3 (VI), C_1 (VII), C_2 (VIII), C_{3a} (IX) and C_{3b} (X) were isolated from the methanolic extract of the aril of *Myristica fragrans* HOUTT. (Myristicaceae), along with nectandrin B (I) and verrucosin (III). The structures of these compounds were elucidated by spectroscopic methods.

Keywords——fragransin; mace; *Myristica fragrans*; Myristicaceae; nectandrin B; tetrahydrofuran lignan; verrucosin

In preceding papers, we have reported the isolation of various *threo* and *erythro* acyclic bis-phenylpropanoids from mace (the aril of *Myristica fragrans* HOUTT., Myristicaceae),[2,3)] as well as the antibacterial action of the major phenolic components, dehydrodiisoeugenol and 5′-methoxydehydrodiisoeugenol, against a cariogenic bacterium, *Streptococcus mutans*.[3)] In the present paper, we wish to report the isolation of ten 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans, which have not been previously isolated from mace.

Results and Discussion

The phenolic fraction of the methanolic extract of mace was repeatedly chromatographed on a silica gel column in the usual manner. These procedures led to the isolation of new minor components, I—X, which gave intense green and blue colors on thin layer plates in iodine vapor, along with 4-propenylphenols and cyclic and acyclic neolignans.[2,3)] The structures of the new components were elucidated by spectroscopic methods as described below.

Compound I, $[\alpha]_D$ 0, was isolated as an oily substance with the molecular formula $C_{20}H_{24}O_5$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum and mass spectrum (MS; Fig. 1 and Table I) were characteristic of a structurally symmetrical tetrahydrofuran lignan (galgravin type)[4)] with two 4-hydroxy-3-methoxyphenyl groups. From a comparison of the spectroscopic data with reported values, I was identified as nectandrin B (Fig. 2), which had been isolated previously from *Nectandra rigida* NESS (Lauraceae).[5)]

Compound II (named fragransin A_2), $[\alpha]_D$ +79.0°, was isolated as colorless crystals, mp 200—202 °C, with the molecular formula $C_{20}H_{24}O_5$ (a stereoisomeric form of I). The ^{1}H-NMR spectrum suggested the presence of a magnetically symmetric element in the molecule, as in I; signals due to two *sec*-methyls (δ 1.04), two methines (δ 1.78, m), two benzylic methines substituted by oxygen (δ 4.63, d, J=9.2 Hz), two methoxyls, two hydroxyls and two aromatic ABX-type protons were seen. The chemical shifts of the methyl, methine and benzylic methine protons indicate that II has the same steric configuration as the known compound galbelgin (a galbelgin type of tetrahydrofuran lignan)[6)] and the structure was

concluded to be *r*-2,*t*-5-bis-(4-hydroxy-3-methoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran (a new natural product). The ^{1}H-NMR spectral data were in good agreement with those of the synthetic racemate as reported by Sarkanen and Wallis.[7]

Compound III, $[\alpha]_D$ +14.8°, was isolated as an oily substance with the molecular formula $C_{20}H_{24}O_5$, isomeric with I and II. The ^{1}H-NMR spectrum was indicative of a veraguensin type[8] of tetrahydrofuran lignan but with two 4-hydroxy-3-methoxyphenyl groups. The relative structure seemed to be identical with that of verrucosin, *r*-2,*c*-5-bis-(4-hydroxy-3-methoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran, which had been isolated from *Urbanodendron verrucosum* (NEES) MEZ. (Lauraceae),[9] based on a comparison of the ^{1}H-NMR spectral data with the published values.[7,9] Proton–proton shift correlation (^{1}H–^{1}H COSY), carbon-13–proton shift correlation (^{13}C–^{1}H COSY), long-range ^{13}C–^{1}H COSY and

Table I. Mass Spectral Data for Tetrahydrofuran Lignans (I—X)

Compounds	M^+	Mass fragments				
		A	B	C	E	F
I	344 (35)	164 (25)	180 (10)	192 (100)	177 (50)	151 (18)
II	344 (68)	164 (65)	180 (32)	192 (100)	177 (85)	151 (32)
III	344 (100)	164 (30)	180 (10)	192 (100)	177 (100)	151 (30)
IV	404 (100)	194 (100)	210 (95)	222 (100)	207 (95)	181 (40)
V	404 (50)	194 (90)	210 (50)	222 (90)	207 (25)	181 (25)
VI	404 (38)	194 (28)	210 (10)	222 (100)	207 (20)	181 (20)
VII	374 (100)	164 (90)	180 (15)	192 (100)	177 (88)	151 (90)
		194 (68)	210 (10)	222 (100)	207 (30)	181 (30)
VIII	374 (50)	164 (18)	180 (10)	192 (100)	177 (40)	151 (16)
		194 (75)	210 (10)	222 (80)	207 (20)	181 (12)
IX	374 (100)	164 (25)	180 (6)	192 (100)	177 (100)	151 (25)
		194 (30)	210 (6)	222 (100)	207 (20)	181 (22)
X	374 (100)	164 (20)	180 (8)	192 (100)	177 (80)	151 (15)
		194 (40)	210 (8)	222 (100)	207 (20)	181 (20)

a) See Fig. 1.

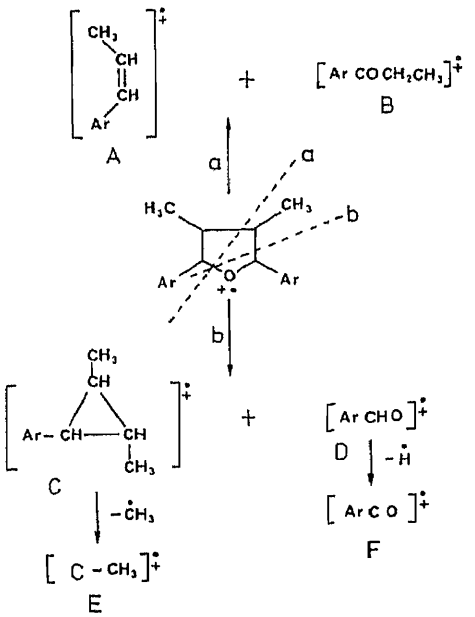
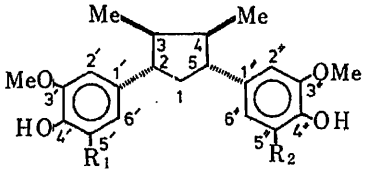
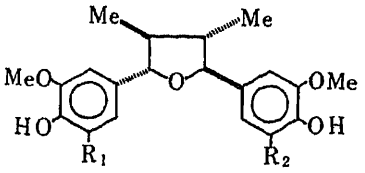


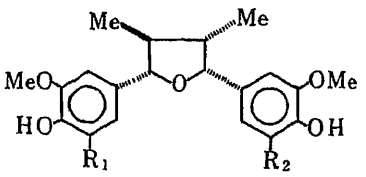
Fig. 1. Mass Fragmentation Pattern for Tetrahydrofuran Lignans (I—X)



I: $R_1=H$, $R_2=H$
IV: $R_1=OMe$, $R_2=OMe$
VII: $R_1=OMe$, $R_2=H$



II: $R_1=H$, $R_2=H$
V: $R_1=OMe$, $R_2=OMe$
VIII: $R_1=OMe$, $R_2=H$



III: $R_1=H$, $R_2=H$
VI: $R_1=OMe$, $R_2=OMe$
IX: $R_1=OMe$, $R_2=H$
X: $R_1=H$, $R_2=OMe$

Fig. 2. Structures of Tetrahydrofuran Lignans (I—X)

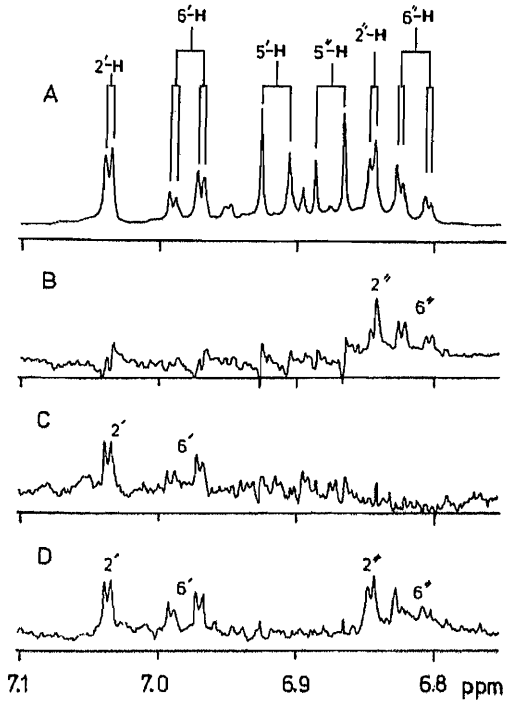


Fig. 3. Difference NOE Spectrum for III

A) Enlarged ^{1}H-NMR spectrum of the low-field region. B) The difference NOE spectrum on irradiation at δ 5.11 (5-H). C) On irradiation at δ 4.40 (2-H). D) On irradiation at δ 1.78 (3-H).

nuclear Overhauser effect (NOESY and difference-NOE) spectroscopic experiments confirmed the structure of III and enabled us to assign all the proton and carbon signals (Table II); Fig. 3 shows the enlarged difference-NOE spectrum of the low-field region. Signals of 2′-H and 6′-H as well as those of 2″-H and 6″-H were readily assignable on the basis of 2-H or 5-H irradiated NOE enhancements. The long-range ^{13}C–^1H correlation spectrum indicated that appreciable long-range spin coupling was present between C-5 (carbon-13 nuclei) and 2″-H/6″-H or between C-2 and 2′-H/6′-H, and quaternary carbons, C-1′, C-1″, C-3′, C-3″, C-4′ and C-4″, were readily assignable (Fig. 4, in the δ 3.7—7.2 region; Table II).

Compound IV (named fragransin B_1), $[\alpha]_D$ 0, was obtained as colorless crystals, mp 100—102 °C, with the molecular formula $C_{22}H_{28}O_7$. The ^{1}H-NMR spectrum showed the presence of two *sec*-methyls (δ 1.06, d), four methoxyls (δ 3.88, s), two hydroxyls, two methines (δ 2.34, m), two benzylic methines substituted by oxygen (δ 4.51, d, J=6.2 Hz) and four magnetically equivalent aromatic protons (δ 6.67, s). The signals due to the methyl and methine and benzylic methine protons were essentially similar in chemical shifts and coupling constants to those of a galgravin-type lignan.[4] The ^{13}C-NMR spectrum showed the presence of two 4-hydroxy-3,5-dimethoxyphenyl groups (Table II). The structure was thus concluded to be *r*-2,*c*-5-bis-(4-hydroxy-3,5-dimethoxyphenyl)-*t*-3,*t*-4-dimethyltetrahydrofuran (a new natural

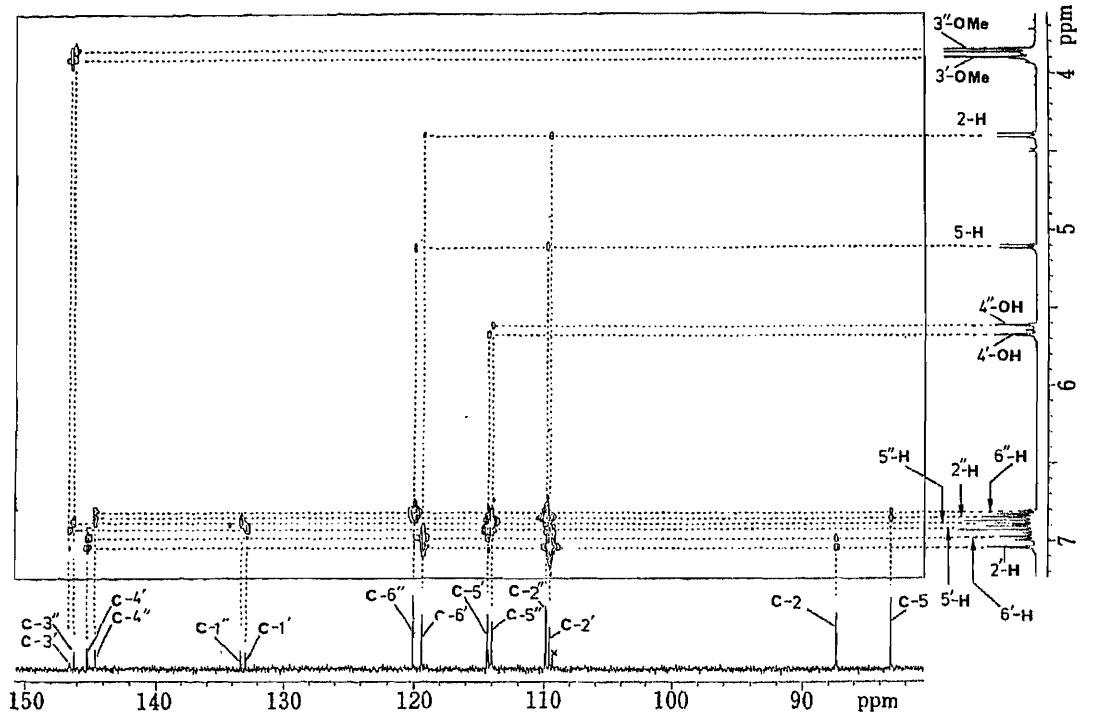


Fig. 4. Long-Range ^{13}C–^{1}H Shift Correlation Spectrum for III

TABLE II. ^{13}C-NMR Spectral Data for Tetrahydrofuran Lignans (I—X)

Carbon No.	I	II	III[a]	IV	V	VI[a]	VII[b]	VIII	IX[a]	X[a]
C-1′	134.1	134.3	132.8	133.3	128.2	132.0	133.5	128.5	132.2	132.8
C-2′	109.2	108.6	109.5	103.4	103.1	103.5	103.3	103.0	103.5	109.6
C-3′	146.4	146.6	146.5	147.0	147.0	147.1	147.0	147.0	147.1	146.6
C-4′	145.0	145.1	145.2	134.3	130.0	134.4	134.2	133.4	134.5	145.3
C-5′	114.1	114.0	114.1	147.0	147.0	147.1	147.0	147.0	147.1	114.3
C-6′	119.2	119.3	119.3	103.4	103.1	103.5	103.3	103.0	103.5	119.3
C-2	87.2	88.3	87.3	87.4	88.6	87.4	87.6	88.5	87.6	87.4
C-3	44.2	50.9	47.8	44.2	50.9	47.8	44.5	50.9	48.1	47.6
C-4	44.2	50.9	46.0	44.2	50.9	46.0	44.0	50.9	46.1	46.1
C-5	87.2	88.3	83.1	87.4	88.6	83.3	87.3	88.5	83.2	83.4
Me-3	12.8	13.8	14.9	12.9	13.8	15.2	13.0	14.0[d]	15.8	15.1
Me-4	12.8	13.8	15.0	12.9	13.8	14.8	12.7	13.7[d]	15.0	14.9
C-1″	134.1	134.3	132.2	133.3	128.2	132.2	134.1	133.1	133.2	132.4
C-2″	109.2	108.6	109.8	103.4	103.1	104.0	109.3	108.6	109.9	104.0
C-3″	146.4	146.6	146.2	147.0	147.0	146.8	146.5	146.9	146.3	146.8
C-4″	145.0	145.1	144.6	134.3	130.0	133.9	145.2	143.8	144.8	133.9
C-5″	114.1	114.0	113.8	147.0	147.0	146.8	114.2	113.9	114.0	146.8
C-6″	119.2	119.3	119.9	103.4	103.1	104.0	119.3	119.2	120.1	104.6
MeO–	55.8 (2)	55.9 (2)	55.8 (2)	56.2 (4)	56.2 (4)	56.3 (4)[c]	55.8 (1) 56.3 (2)	56.3 (3)	55.9 (1) 56.4 (2)	55.9 (1) 56.3 (2)

a) Assignments were done on the basis of ^{13}C–^{1}H COSY and long-range ^{13}C-^{1}H COSY experiments. *b*) Assignments were done by ^{13}C–^{1}H COSY. *c*) δ 56.33 for 3′,5′-OMe and 56.27 for 3″,5″-OMe. *d*) Assignments may be interchanged. Compounds III, VI, VII, IX and X were measured in $CDCl_3$ at 100 MHz, and others were measured at 22.5 MHz.

product). This compound has been synthesized from (*Z*)-2,6-dimethoxy-4-propenylphenol with hydrogen peroxide-peroxidase but was isolated only as the dimethylether by Sarkanen and Wallis.[7]

Compound V (named fragransin B_2), $[\alpha]_D$ 0, had the molecular formula $C_{22}H_{28}O_7$, isomeric with IV. The ^{1}H-NMR spectrum, however, slightly differed in chemical shifts and coupling constants from that of IV; two methine signals (3-H and 4-H) appeared at higher field, δ 1.78, and two benzylic methine signals (2-H and 5-H) at lower field, δ 4.63, as compared with those of IV (δ 2.34 and 4.51, respectively). The chemical shifts and coupling constants of the methyl, methine and benzylic methine protons indicate that V is a galbelgin type lignan[6] with two 4-hydroxy-3,5-dimethoxyphenyl groups. The structure of V was thus concluded to be *r*-2,*t*-5-bis-(4-hydroxy-3,5-dimethoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran. In contrast to naturally occurring galbelgin type compounds including II and VIII (see below), this compound seems to be a racemate on the basis of its $[\alpha]_D$ value. Although V has been synthesized by Sarkanen and Wallis,[7] this is the first time that it has been isolated from a natural source.

Compound VI (named fragransin B_3), $[\alpha]_D$ +12.5°, had the molecular formula $C_{22}H_{28}O_7$, isomeric with IV and V. In contrast to the ^{1}H-NMR spectra of IV and V, two *sec*-methyls (4-Me and 3-Me) appeared at δ 0.68 and 1.09 as two doublets, two methines (3-H and 4-H) at δ 1.78 and 2.25 as two multiplets, and two benzylic methines (2-H and 5-H) at δ 4.40 and 5.10 as two doublets (J=9.3 and 8.3 Hz, respectively) in the spectrum of VI. On irradiation at δ 0.68 or 1.09, the multiplets at δ 2.25 (4-H) or 1.78 (3-H) became a double doublet ($J_{4,5}$=8.3 Hz, $J_{3,4}$=*ca.* 8—9 Hz, $J_{2,3}$=9.3 Hz). On irradiation at δ 1.78, the two doublets at 1.09 (3-Me) and 4.40 (2-H) became two singlets. On irradiation at δ 2.25, the two doublets at δ 0.68 (4-Me) and 5.10 (5-H) became two singlets. These decoupling experiments led to the structure of *r*-2,*c*-5-bis-(4-hydroxy-3,5-dimethoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran for VI. This structure was confirmed by ^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, long-range ^{13}C–^{1}H COSY and NOE experiments. In the long-range ^{13}C–^{1}H shift correlation spectrum, appreciable long-range spin coupling was observed between C-5 (carbon-13 nuclei) and 2″-H, 6″-H (both at δ 6.57), 3-H and 4-Me protons, or between C-2 and 2′-H, 6′-H (both at 6.67), 4-H and 3-Me protons (not shown). This compound has been synthesized as the racemate by Sarkanen and Wallis,[7] but this is the first isolation in an optically active form from a natural source.

Compound VII (named fragransin C_1), $[\alpha]_D$ +3.8°, had the molecular formula $C_{21}H_{26}O_6$. The mass spectrum showed a molecular ion peak at *m/z* 374 and intense fragment ions at *m/z* 192 and 222, ascribed to [Ar–ĊH–CH(Me)–ĊH–Me]$^+$ (Ar=hydroxy-methoxyphenyl and Ar=hydroxy-dimethoxyphenyl, respectively; Table I). In addition, the ^{13}C-NMR spectrum showed the presence of 4-hydroxy-3-methoxyphenyl and 4-hydroxy-3,5-dimethoxyphenyl groups (Table II). The ^{1}H-NMR chemical shifts of methyl, methine and benzylic methine protons were similar to those of a galgravin-type lignan.[9] The signal patterns, however, were rather complex due to the presence of two different aryl substituents in the molecule; signals due to two *sec*-methyls (δ 1.04 and 1.06), two methines (δ *ca.* 2.33), and two benzylic methines substituted by oxygen (δ 4.50 and 4.51) were seen. On irradiation at δ *ca.* 1.04—1.06, the multiplets at δ 2.32 and 2.34 (3-H and 4-H, respectively) became two doublets. On irradiation at δ *ca.* 2.32—2.34, the two doublets at δ 1.04 and 1.06 (3-Me and 4-Me) became two singlets, and the two doublets at δ 4.50 and 4.51 (2-H and 5-H) became two singlets. Furthermore, appreciable NOE was observed between 2-H (δ 4.50) and 2′-H/6′-H/5-H and between 5-H (δ 4.51) and 2″-H/2-H by NOESY. Based on these findings, VII was concluded to be *r*-2-(4′-hydroxy-3′,5′-dimethoxyphenyl)-*c*-5-(4″-hydroxy-3″-methoxyphenyl)-*t*-3,*t*-4-dimethyltetrahydrofuran (a new natural product). The ^{1}H–^{1}H COSY and ^{13}C–^{1}H COSY experiments enabled us to assign the ^{1}H- and ^{13}C-signals.

Compound VIII (named fragransin C_2), $[\alpha]_D$ +20.2°, had the molecular formula $C_{21}H_{26}O_6$, isomeric with VII. The ^{1}H-NMR spectrum showed signals of two *sec*-methyls (4-Me and 3-Me) at δ 1.05 as doublets (J=6.1 Hz), two methines (3-H and 4-H) at δ 1.78 as

multiplets, and two benzylic methines substituted by oxygen (2-H and 5-H) at δ 4.62 and 4.64 as two doublets (J=9.3 and 9.0 Hz, respectively), as well as those ascribed to 4-hydroxy-3-methoxyphenyl and 4-hydroxy-3,5-dimethoxyphenyl groups. The chemical shifts and their coupling constants were similar to those of a galbelgin-type lignan.[6] This led to the structure of *r*-2-(4′-hydroxy-3′,5′-dimethoxyphenyl)-*t*-5-(4″-hydroxy-3″-methoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran (a new natural product).

Compound IX (named fragransin C_{3a}), $[\alpha]_D$ +19.6°, was obtained as an oily substance with the molecular formula $C_{21}H_{26}O_6$, isomeric with VII and VIII. The ^{1}H-NMR spectrum was indicative of a veraguensin-type lignan.[8] The two-dimensional NOE correlation spectrum showed appreciable NOE between 5-H and 2″-H/2-H/4-H, and between 2-H and 2′-H/6′-H/5-H/3-Me, indicating that a 4″-hydroxyl-3″-methoxyphenyl group is attached to C-5 of the tetrahydrofuran ring and a 4′-hydroxy-3′,5′-dimethoxyphenyl group to C-2. These findings led to the structure of *r*-2-(4′-hydroxy-3′,5′-dimethoxyphenyl)-*c*-5-(4″-hydroxy-3″-methoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran for IX. In addition, ^{13}C–^{1}H COSY, long-range ^{13}C–^{1}H COSY experiments allowed a complete assignment of all the ^{13}C- and ^{1}H-NMR signals.

Compound X (named fragransin C_{3b}), $[\alpha]_D$ +7.2°, was obtained as an oily substance with the molecular formula $C_{22}H_{28}O_7$. The ^{1}H-NMR spectrum was quite similar to that of IX, suggesting that X is a substitutional variant of IX. Contrary to the case of IX, ^{13}C–^{1}H COSY and NOESY (Fig. 5) experiments provided evidence for a *trans* relationship between the 4′-hydroxy-3′-methoxyphenyl and the adjacent methyl (3-Me) groups, and for a *cis* relationship between the 4″-hydroxy-3″,5″-dimethoxyphenyl and the adjacent methyl (4-Me) groups. The structure of X was thus concluded to be *r*-2-(4′-hydroxy-3′-methoxyphenyl)-*c*-5-(4″-hydroxy-3″,5″-dimethoxyphenyl)-*t*-3,*c*-4-dimethyltetrahydrofuran. ^{1}H- and ^{13}C-NMR signals were completely assigned on the basis of two dimentional NMR (2D-NMR) experi-

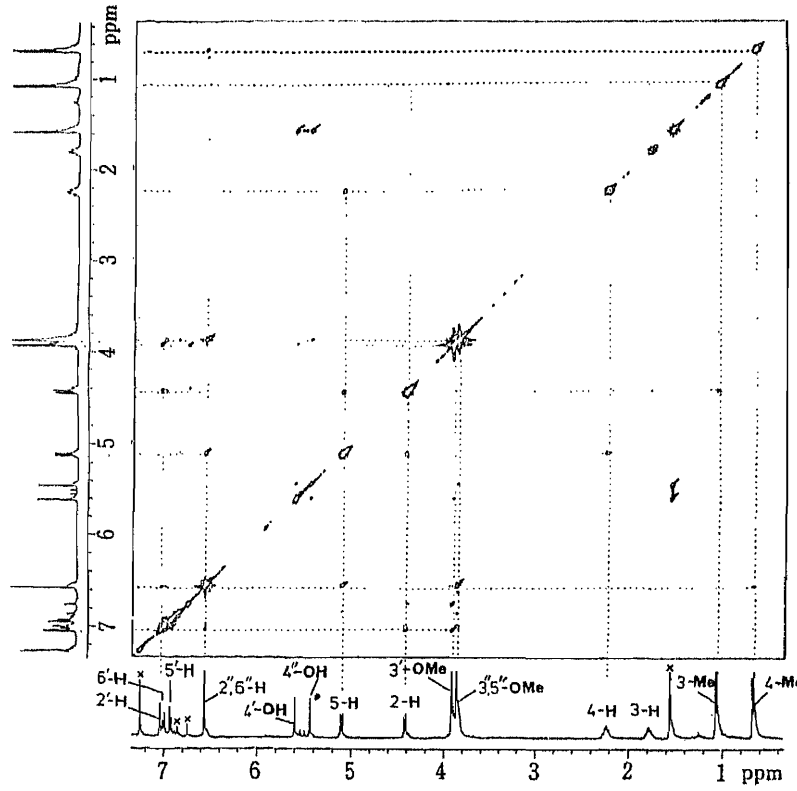


Fig. 5. 2D-NOE Correlation Spectrum of Fragransin C_{3b} (X)

ments including long-range ^{13}C–^{1}H COSY.

^{1}H-NMR signals of 2-H, 3-H, 4-H and 5-H, as well as those of 3-Me and 4-Me protons, are used as diagnostic signals for the determination of relative configuration in the 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans, but ^{13}C-NMR signals (Table II) also provide useful diagnostic information on the stereochemistry of such lignans; ^{13}C-signals of C-3 and C-4 appeared at δ *ca.* 44, 51 and 46—48, and those of 3-Me and 4-Me appeared at δ *ca.* 13, 14 and 15 for the 2,3-*trans*/3,4-*cis*/4,5-*trans* (I, IV and VII), 2,3-*trans*/3,4-*trans*/4,5-*trans* (II, V and VIII) and 2,3-*trans*/3,4-*trans*/4,5-*cis* (III, VI, IX and X) forms, respectively. In the former two forms, signals of pairs of carbons, C-2/C-5, C-3/C-4 and 3-Me/4-Me, appeared at the same or quite similar positions, but in the last form, at separate positions. This generalization is also true for a series of 2-(4-hydroxy-3-methoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-3,4-dimethyltetrahydrofurans and 5-(4-hydroxy-3-methoxyphenyl)-2-(3,4-methylenedioxyphenyl)-3,4-dimethyltetrahydrofurans which have also been isolated from mace, as will be reported elsewhere.[10]

In conclusion, we have isolated several tetrahydrofuran lignans, fragransins A_2 (II), B_1 (IV), B_2 (V), B_3 (VI), C_1 (VII), C_2 (VIII), C_{3a} (IX) and C_{3b} (X) as new natural products, together with the known compounds, nectandrin B (I) and verrucosin (III), from the aril of *Myristica fragrans*. This is the first report on the occurrence of tetrahydrofuran lignans in this plant. Though compounds II, IV, V and VI have already been synthesized by Sarkanen and Wallis,[7] compounds II, IV and V have been isolated for the first time as optically active forms, and compound V as a racemate.

Experimental

Apparatus——Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were measured with JEOL GX-270 (^{1}H, 270 MHz), JEOL GX-400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) and JEOL FX-90Q (^{13}C, 22.5 MHz) spectrometers, with tetramethylsilane as an internal standard. The multiplicities of signals are represented by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, br, broad; dd, double doublet; ddq, double double quartet. Mass spectra (MS) were measured with a JMS-D 300 mass spectrometer at an ionization voltage of 70 eV. Optical rotations, $[\alpha]_D$, were taken on a Jasco model DIP-4 automatic polarimeter at 25 °C. High-performance liquid chromatography (HPLC) was carried out on a Tri Rotar SR-1 (JASCO) HPLC system using a preparative HPLC column.

Isolation——The MeOH extract (290 g) of mace was partitioned between 95% MeOH and *n*-hexane. The 95% MeOH-soluble (176.5 g) was then separated into acidic, phenolic and basic fractions as usual. The phenolic fraction (31.5 g) was chromatographed on a silica gel column. Elution with benzene–EtOAc (0—50%) gave various cyclic and acyclic lignans[2,3] along with a mixture of unidentified compounds. The mixture was purified to give 10 compounds, I (30 mg as the pure sample), II (3 mg), III (40 mg), IV (20 mg), V (3 mg), VI (40 mg), VII (8 mg), VIII (3 mg), IX (6 mg) and X (5 mg), by preparative thin layer chromatography (Merck Kieselgel 60 F_{254} or Merck PSC 60 F_{254} plate) and preparative HPLC (Chemosorb 5Si, column size, 10 mm i.d. × 500 mm, Chemco Co.; solvent system, hexane–dichloroethane–ethanol, 19 : 4 : 2).

Nectandrin B (I)——Colorless oil; $[\alpha]_D$ 0 (c=0.37, $CHCl_3$). High-resolution MS *m/z*: 344.1610 (Calcd for $C_{20}H_{24}O_5$: 344.1622). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.03 (6H, d, J=6.6 Hz, 3- and 4-Me), 2.32 (2H, m, 3- and 4-H), 3.88 (6H, 3′- and 3″-OMe), 4.49 (2H, d, J=6.4 Hz, 2- and 5-H), 5.58 (2H, s, 4′- and 4″-OH), 6.87—6.95 (6H, m, 2′-, 5′-, 6′-, 2″-, 5″- and 6″-H).

Fragransin A_2 (II)——Colorless crystals; mp 200—202 °C; $[\alpha]_D$ +79.0° (c=0.84, $CHCl_3$). High-resolution MS *m/z*: 344.1633 (Calcd for $C_{20}H_{24}O_5$: 344.1622). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.04 (6H, d, J=6.0 Hz, 3- and 4-Me), 1.78 (2H, m, 3- and 4-H), 3.92 (6H, s, 3′- and 3″-OMe), 4.63 (2H, d, J=9.2 Hz, 2- and 5-H), 5.57 (2H, s, 4′- and 4″-OH), 6.84—6.95 (6H, m, 2′-, 5′-, 6′-, 2″-, 5″- and 6″-H).

Verrucosin (III)——Colorless oil; $[\alpha]_D$ +14.8° (c=1.38, $CHCl_3$). High-resolution MS *m/z*: 344.1676 (Calcd for $C_{20}H_{24}O_5$: 344.1622). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.66 (3H, d, J=7.0 Hz, 4-Me), 1.05 (3H, d, J=6.4 Hz, 3-Me), 1.78 (1H, ddq, 3-H), 2.24 (1H, ddq, 4-H), 3.84 (3H, s, 3″-OMe), 3.89 (3H, s, 3′-OMe), 4.40 (1H, d, J=9.4 Hz, 2-H), 5.11 (1H, d, J=8.5 Hz, 5-H), 5.61 (1H, s, 4″-OH), 5.68 (1H, s, 4′-OH), 6.82 (1H, dd, J=2.4, 8.4 Hz, 6″-H), 6.84 (1H, d, J=2.4 Hz, 2″-H), 6.88 (1H, d, J=8.4 Hz, 5″-H), 6.92 (1H, d, J=8.4 Hz, 5′-H), 6.98 (1H, dd, J=2.4, 8.4 Hz, 6′-H), 7.04 (1H, d, J=2.4 Hz, 2′-H).

Fragransin B_1 (IV)——Colorless crystals; mp 100—102 °C; $[\alpha]_D$ 0 (c=0.11, $CHCl_3$). High-resolution MS *m/z*:

404.1835 (Calcd for $C_{22}H_{28}O_7$: 404.1833). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.06 (6H, d, J=6.6 Hz, 3- and 4-Me), 2.34 (2H, m, 3- and 4-H), 3.88 (12H, s, 3'-, 5'-, 3''- and 5''-OMe), 4.51 (2H, d, J=6.2 Hz, 2- and 5-H), 5.47 (2H, s, 4'- and 4''-OH), 6.67 (4H, s, 2'-, 6'-, 2''- and 6''-H).

Fragransin B_2 (V)——Colorless oil (lit.[7] crystals, mp 157—159 °C); $[\alpha]_D$ 0 (c=0.09, $CHCl_3$). High-resolution MS m/z: 404.1861 (Calcd for $C_{22}H_{28}O_7$: 404.1833). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.06 (6H, d, J=6.8 Hz, 3- and 4-Me), 1.78 (2H, m, 3- and 4-H), 3.91 (12H, s, 3'-, 5'-, 3''- and 5''-OMe), 4.63 (2H, d, J=9.0 Hz, 2- and 5-H), 5.51 (2H, s, 4'- and 4''-OH), 6.64 (4H, s, 2'-, 6'-, 2''- and 6''-H).

Fragransin B_3 (VI)——Colorless oil; $[\alpha]_D$ +12.5° (c=1.02, $CHCl_3$). High-resolution MS m/z: 404.1831 (Calcd for $C_{22}H_{28}O_7$: 404.1833). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 0.68 (3H, d, J=7.3 Hz, 4-Me), 1.09 (3H, d, J=6.3 Hz, 3-Me), 1.78 (1H, m, 3-H), 2.25 (1H, m, 4-H), 3.87 (6H, s, 3''- and 5''-OMe), 3.91 (6H, s, 3'- and 5'-OMe), 4.40 (1H, d, J=9.3 Hz, 2-H), 5.10 (1H, d, J=8.3 Hz, 5-H), 5.45 (1H, s, 4''-OH), 5.50 (1H, s, 4'-OH), 6.57 (2H, s, 2''- and 6''-H), 6.67 (2H, s, 2'- and 6'-H).

Fragransin C_1 (VII)——Colorless oil; $[\alpha]_D$ +3.8° (c=0.60, $CHCl_3$). High-resolution MS m/z: 374.1715 (Calcd for $C_{21}H_{26}O_6$: 374.1728). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.04 (3H, d, J=6.7 Hz, 3-Me), 1.06 (3H, d, J=6.7 Hz, 4-Me), 2.32 (1H, m, 3-H), 2.34 (1H, m, 4-H), 3.88 (9H, s, 3'-, 3''- and 5'-OMe), 4.50 (1H, d, J=5.5 Hz, 2-H), 4.51 (1H, d, J=5.8 Hz, 5-H), 5.47 (1H, s, 4'-OH), 5.58 (1H, s, 4''-OH), 6.65 (2H, 2'- and 6'-H), 6.90 (1H, d, J=7.9 Hz, 5''-H), 6.93 (1H, dd, J=1.5, 7.9 Hz, 6''-H), 6.96 (1H, d, J=1.5 Hz, 2''-H).

Fragransin C_2 (VIII)——Colorless oil; $[\alpha]_D$ +20.2° (c=0.11, $CHCl_3$). High-resolution MS m/z: 374.1715 (Calcd for $C_{21}H_{26}O_6$: 374.1728). ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.05 (6H, d, J=6.1 Hz, 3- and 4-Me), 1.78 (2H, m, 3- and 4-H), 3.92 (6H, s, 3'- and 5'-OMe), 3.88 (3H, s, 3''-OMe), 4.62 (1H, d, J=9.3 Hz, 2- or 5-H), 4.64 (1H, d, J=9.0 Hz, 5- or 2-H), 5.47, 5.57 (each 1H, s, OH), 6.63 (2H, s, 2'- and 6'-H), 6.85—6.98 (3H, 2''-, 5''- and 6''-H).

Fragransin C_{3a} (IX)——Colorless oil; $[\alpha]_D$ +19.6° (c=0.29, $CHCl_3$). High-resolution MS m/z: 374.1690 (Calcd for $C_{21}H_{26}O_6$: 374.1728). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.67 (3H, d, J=7.0 Hz, 4-Me), 1.08 (3H, d, J=6.4 Hz, 3-Me), 1.77 (1H, m, 3-H), 2.24 (1H, m, 4-H), 3.86 (3H, 3''-OMe), 3.91 (6H, s, 3'- and 5'-OMe), 4.39 (1H, d, J=9.2 Hz, 2-H), 5.12 (1H, d, J=8.5 Hz, 5-H), 5.50 (1H, s, OH), 5.54 (1H, s, OH), 6.74 (2H, s, 2'- and 6'-H), 6.84 (1H, br dd, J=*ca.* 1, 7.9 Hz, 6''-H), 6.85 (1H, br d, J=*ca.* 1 Hz, 2''-H), 6.89 (1H, d, J=7.9 Hz, 5''-H).

Fragransin C_{3b} (X)——Colorless oil; $[\alpha]_D$ +7.2° (c=0.24, $CHCl_3$). High-resolution MS m/z: 374.1778 (Calcd for $C_{21}H_{26}O_6$: 374.1728). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.68 (3H, d, J=7.0 Hz, 4-Me), 1.07 (3H, d, J=6.7 Hz, 3-Me), 1.78 (1H, m, 3-H), 2.25 (1H, m, 4-H), 3.86 (6H, s, 3''- and 5''-OMe), 3.91 (3H, s, 3'-OMe), 4.42 (1H, d, J=9.5 Hz, 2-H), 5.10 (1H, d, J=8.6 Hz, 5-H), 5.44 (1H, s, 4''-OH), 5.60 (1H, s, 4'-OH), 6.56 (2H, s, 2''- and 6''-H), 6.93 (1H, d, J=8 Hz, 5'-H), 7.00 (1H, dd, J=2, 8 Hz, 6'-H), 7.04 (1H, d, J=2 Hz, 2'-H).

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Histochemistry. X.[1] Distributions of Aluminum, Phosphorus, and Other Elements in *Bupleurum falcatum* Root Cultivated in Japan

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The concentrations and distributions of nine elements and three saikosaponins in *Bupleurum falcatum* root cultivated in Japan were histochemically examined by means of an electron microprobe X-ray microanalyzer (EXMA), inductively coupled plasma spectrometry (ICP) and high-performance liquid chromatography. Point and line analyses by semi-quantitative EXMA coupled with quantitative ICP proved to be a convenient and selective method for histochemical analysis of the tissue-specific distributions of the elements in plant roots. The present histochemical examination showed that aluminum and manganese along with three saikosaponins a, c and d occur exclusively in the outermost peripheral layer, consisting of a cork layer and pericycle, of the root, and that phosphorus occurs in the innermost xylem layer at a high concentration.

Keywords——X-ray microanalyzer; plasma spectrometer; HPLC; histochemistry; *Bupleurum falcatum*; saikosaponin; aluminum; phosphorus; Chinese crude drug

Bupleuri Radix (saiko in Japanese) is a Chinese crude drug widely used in Kampo prescriptions such as sho-saiko-to, kami-shoyo-san and hocyu-ekki-to. According to the XIth Japanese Pharmacopoeia, the crude drug is a dried root of some *Bupleurum* species (Umbelliferae). In the course of our histochemical analyses of crude drugs, we have examined the distribution of the saikosaponins, the active constituents of the drug, in the root of *B. falcatum*.[1]

To further clarify the nature of Bupleuri Radix, we examined the distribution profiles of various elements, including aluminum, potassium, calcium and phosphorus, in the *B. falcatum* root in relation to those of saikosaponins. Although these elements have not been studied in connection with the biological activities of the plant, they are considered to be related to the growth conditions, which influence the quality of the crude drug prepared from the plant.

Experimental

Material—— Fresh roots of 1-year-old *B. falcatum* plants cultivated in Kumamoto (harvested in December 1982) and Ibaraki (in November 1982) Prefectures were used.

Sample Preparation——Fresh main roots of the plant washed free of soil particles with deionized and distilled water obtained from an automatic still (AS-20S, Iwaki-garasu) were cross-sectioned, as described previously[1] to obtain thin slices for observation with a microscope (Vanox, Olympus) equipped with a microphotographic camera (PM-10, Olympus). Relatively thick (about 2 mm) cross-sections were also prepared and subjected to both line and point analyses by the use of an electron microprobe X-ray microanalyzer (EXMA, an energy-dispersive type, 8500E, Horiba).

For semi-quantitative analysis with EXMA and quantitative histochemical analyses of elements and saikosaponins, fresh main roots were cut into about 3 cm pieces, which were divided concentrically into three tissues: the outermost peripheral tissue (ph2) consisting of cork layer (kl) and pericycle (pr), the inner phloem tissue (ph1) consisting of parenchyma cells, secretory canals (sec) and phloem (ph) between the pericycle and cambium (c), and the innermost xylem tissue (xy) consisting of vessels (v), wood fibers (wf) and medullary rays. To calculate the weight ratio of the three tissues, they were dried naturally at room temperature and weighed using an electronic balance (ED-200, Shimadzu).

The dividing procedures to obtain the three tissues were repeated three times using five roots, and three quantitative analyses were carried out to obtain the means and the standard deviations (mean $\pm$ S.D.) of the observed values.

Qualitative Analysis of Elements——Point analysis at five points in the cross-section of a main root as shown in Fig. 1 was performed by means of EXMA at an accelerating voltage of 30 kV, a sample current of 3×10^{-11} A, a vertical scale of 512 counts and an integrated pulse counting time of 100 s. The electron beam scanning image at each point showing the distributions of nine elements: magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca) and iron (Fe) was analyzed (Fig. 1).

Line profile analysis using the same slice by EXMA progressing along the line in the scanning transmission image (Fig. 1) was also carried out to evaluate the aluminum distribution in the slices. The operating conditions were as follows: energy range of 1.48 keV (Al K_α), a vertical full scale of 500 counts and a counting time constant of 0.3 s.

Furthermore, the three dried tissues separated as above were pulverized in an agate mortar and each powder (50 mg each) was formed into a pellet 13 mm in diameter under a pressure of 560 kg/cm^2 by means of an evacuable KBr die (Shimadzu, for infrared spectroscopy). The point analysis using three pellets was performed by EXMA under the following conditions: vertical scale of 1000 counts, a counting time of 200 s and an electron beam spot of 5.4×4 mm^2 of the scanning transmission image at a magnification of 25 times.

Quantitative Analysis of Elements——One hundred milligrams of each of the dried and pulverized tissues was accurately weighed into a quartz dish and treated for 24 h to obtain a dry ash in a plasma asher (ASH-302, Hitachi) at a high-frequency power of 150 W and oxygen gas flow rate of 50 ml/min. The ash thus obtained was dissolved in an acid mixture (3 ml) of concentrated HCl and HNO_3 (3 : 1) and then made up to 100 ml with deionized water.

The sample solutions were diluted to suitable concentrations as follows: for Ca, 100 to 500 times; Mg and Al, 100 times; and K, P, Fe, manganese (Mn) and strontium (Sr), 5 times. Standard element solutions were also prepared for $CaCl_2$, KCl, Na_2HPO_4, $MgCl_2$, $AlCl_3$, $FeCl_3$, $MnCl_2$, and $Sr(NO_3)_2$. The sample solution was mixed with two concentrations of the standard solutions: Ca, Al, Mn and Sr, 0.3 and 0.6 ppm; K and P, 10 and 20 ppm; Fe 0.1 and 0.2 ppm; and Mg, 1 and 2 ppm. The blank solution (100 ml) contained 3 ml of the concentrated HCl–HNO_3 mixture and water.

The four solutions obtained above were analyzed with an inductively coupled plasma spectrometer (ICP, Hitachi 300). The operating conditions of ICP were as follows: photomultiplier voltage, 610 V; anode current, 220 mA; anode voltage, 2.0 kV; sheath gas, argon 0.78 kg/cm^2, 3.5 l/min; plasma gas, argon 1.28 kg/cm^2, 3.5 l/min; slit width (μm) (entrance 30, exit 30). The detecting wavelengths were: P, 2553.2 Å; Mn, 2576.1 Å; Mg, 2795.5 Å; Fe, 3719.9 Å; Ca, 3933.6 Å; Al, 3961.5 Å; K, 4044.1 Å; and Sr, 4215.5 Å.

The peak heights of the eight elements were corrected against the blank solution. The concentrations of the eight elements in the samples were determined by the standard addition method using the regression equation obtained from the corrected values of three sample solutions and two solutions to which two concentrations of the standard solution had been added.

All chemicals were of an atomic absorption spectral grade and were purchased from Kanto Chemical Co., Inc.

Quantitative Analysis of Saikosaponins——The methanol extracts of the dried and powdered three tissues (ph1 and xy, 250 mg; ph2, 50 mg) obtained from five roots were treated with a Sep-Pak C_{18} cartridge (Waters) as reported previously.[1] Concentrations of saikosaponins a, c, and d in the samples were determined by high-performance liquid chromatography (HPLC) on a LC-3A apparatus (Shimadzu) equipped with a NQVA-PAK C_{18} stainless-steel column (15 cm $\times$ 3.9 mm, Waters), an ultraviolet (UV) detector (SPD-6A, Shimadzu) and a computing integrator (C-R1A, Shimadzu). The mobile phase, flow rate, monitoring wavelength, and other detection conditions were the same as reported previously.[1]

Elution times of the three saikosaponins in the samples were comparable to those of the authentic saikosaponins (kindly supplied by the Shionogi Research Laboratory[2]) under the same HPLC conditions.

Statistical Analysis——To compare the twelve mean values (x_i: $i = 1$—12), *i.e.*, concentrations of Ca, K, P, Mg, Mn, Al, Sr, Fe, saikosaponins a (ss-a), c (ss-c), d (ss-d) and total saikosaponins (total-ss), each variable was standardized according to the equation $z_i = (x_i - X_i)/SD$, where X_i represents the twelve grand means and SD is a standard deviation obtained for all three tissues. The twelve standardized values (z_i: $i = 1$—12) of each tissue were calculated on a computer (PC-9801Vm2, NEC) with a multivariate analysis program written by Nippon Maikon Gakuin. The data were analyzed by using a radar chart (Fig. 4) to determine the tissue-specific distribution patterns of elements and saikosaponins.

Results and Discussion

Direct point analysis by EXMA at five spots of various tissues in the cross-section of a main root of *B. falcatum* was first carried out to clarify the distribution of the elements. The nine elements Mg, Al, Si, P, S, Cl, Ca, K and Fe were detected simultaneously. Although copper (Cu), zinc (Zn) and Mn in Bupleuri Radix have been determined by quantitative X-ray fluorescence spectrometry after several pretreatments (ignition at 500 °C, digestion with 6 N HCl and complex formation with pyrrolidine dithiocarbamate[3]), these three elements were not measured in the present non-destructive EXMA examination. However, Mn in the samples was determined by the ICP method as described later.

A semi-quantitative comparison of the elements was made with the intensity along the vertical scale as shown on the right side of Fig. 1. The distribution of elements was not uniform throughout the root tissues as judged from the peak heights obtained at the five different points.

The two high peaks at 1.49 and 1.74 keV observed in the outer point (A) in the pericycle were identified as being due to Al and Si. This seems to be the first report of the occurrence of Al in *B. falcatum* root. The site of accumulation of Al was also identified by line analysis of EXMA along the electron scanning line. It starts in the cork layer, passes the pericycle, parenchyma, phloem, cambium and ends in the xylem of the cross-sectioned slice as shown in Fig. 1. The Al concentration along the line represents the intensity of the horizontal scale on the left side of Fig. 1. This is a direct proof of the high concentration of Al in the outermost layer of the root, and is in agreement with the finding[4] of Al in the epidermal cells of the root of *Zea mays*.

In the direct point analysis, sizable peaks of K at 3.31 keV and of Ca at 3.69 keV were also found in point (A), although K and Ca were present in all tissue types of the root. Crude

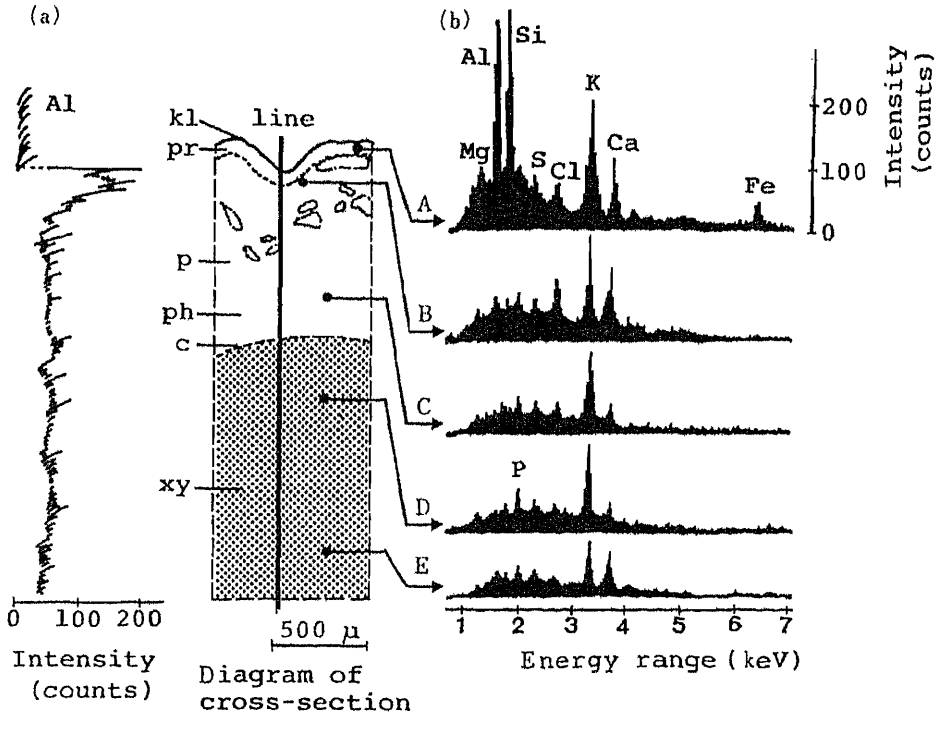

Fig. 1. Line (a) and Point (b) Analyses of *Bupleurum falcatum* Root (Cultivated in Ibaraki) with an Electron Microprobe X-Ray Microanalyzer (EXMA)

kl, cork layer; pr, pericycle; p, parenchyma; ph, phloem; c, cambium; xy, xylem.

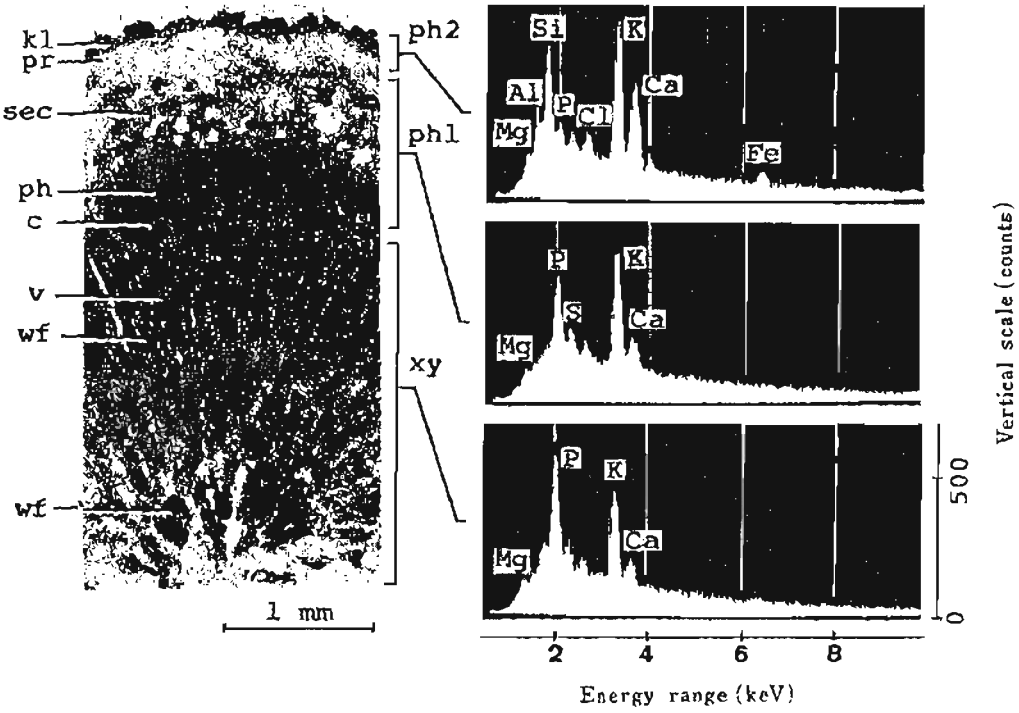

Fig. 2. Point Analysis Using Pellets of Three Tissues Divided from *Bupleurum falcatum* Root (Cultivated in Kumamoto) by EXMA

sec, secretory canal; v, vessel; wf, wood fiber; others are given in the legend to Fig. 1. Three tissues: ph2, ph1 and xy (see text).

drugs and their decoctions generally contain K and Ca.[5]

A small peak of Fe at 6.40 keV was detected only in point (A). Bupleuri Radix characteristically contains Fe in a high concentration, like Carthami Flos, Schizonepetae Herba and Asiasari Radix.[3,5,6]

All the above findings were supported by further EXMA analysis using the pellets prepared from the three tissues divided from the roots. Three electron beam scanning images (Fig. 2) showing the distributions of the nine elements in the three tissues were obtained. The principal feature was the high concentrations of Si and Al in the outermost layer (ph2), where Fe was also detected. A marked peak of P at 2.01 keV was detected in the two inner tissues (ph1 and xy).

The two EXMA scanning images of the pellets prepared by the same procedures using different samples harvested in Kumamoto and Ibaraki Prefectures were found to be similar. The EXMA methods described in this paper provide a qualitative and non-destructive means[7] for determining the tissue-specific distribution of elements in plants.

The patterns of elements in the three types of tissues examined by qualitative EXMA analyses were confirmed quantitatively by an ICP determination. The EXMA analyses showed that the outermost tissue (ph2) had higher contents of K, Al, Ca and Fe than the inner two tissues. Markedly low concentrations of K, Al and Ca were observed in the two inner tissues, as shown in Fig. 3. On the other hand, the main sites of concentration of P were the two inner tissues, and Mg was uniformly distributed within the three root tissues.

Mn and Sr were contained at μg/g concentrations, whereas the other seven elements were contained at mg/g levels, and they were also distributed mainly in the outermost tissues (ph2: Mn, 390.0 ± 10.8 μg/g; Sr, 134.1 ± 40.0 μg/g) and decreased toward the innermost xylem layer (xy: Mn, 21.0 ± 3.8 μg/g; Sr, 23.5 ± 5.2 μg/g).

Based on the weight ratios of tissues, element concentrations in the three tissues obtained by the ICP method were converted into the contents relative to the whole root weight: Ca, 2.10 mg/g; K, 5.85 mg/g; P, 4.13 mg/g; Mg, 2.00 mg/g; Al, 0.34 mg/g; Fe, 0.62 mg/g; Mn, 0.047 mg/g; Sr, 0.035 mg/g. Most of the quantitative data from the present ICP method are similar to the reported profiles[5,6] of mineral contents in crude drugs found by using atomic absorption spectrometry.

By combining the direct measurements using semi-quantitative EXMA and the quanti-

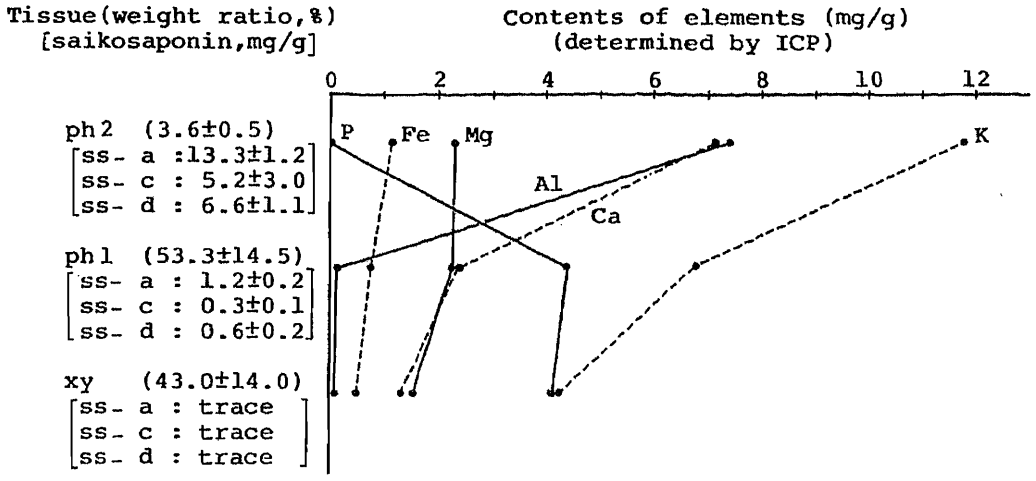

Fig. 3. Concentrations of Elements and Saikosaponins in Three Tissues Divided from *Bupleurum falcatum* Root (Cultivated in Kumamoto)

ph1, ph2, and xy are as shown in Fig. 2. ss-a, ss-c, and ss-d represent saikosaponins a, c, and d.

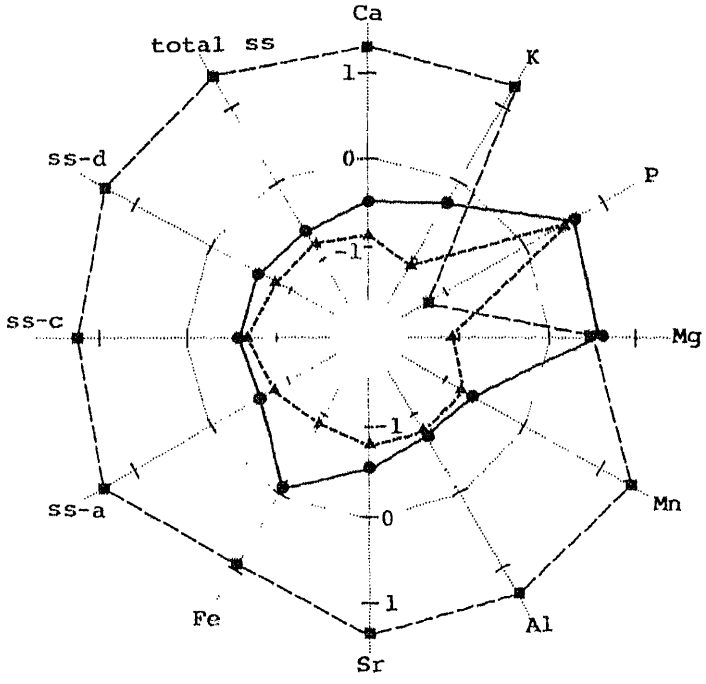

Fig. 4. Tissue-Specific Distribution Profiles of Elements and Saikosaponins of *Bupleurum falcatum* Root (Cultivated in Kumamoto)

●---●, ph1; ■---■, ph2; ▲---▲, xy. Each value of the twelve variables represents the standardized mean. ss-a, ss-c, ss-d and total ss are saikosaponins a, c, d and total saikosaponin.

tative ICP analyses, the tissue-specific occurrence of elements in crude drugs could be determined.

Previously,[1] we found that the saikosaponins in *B. falcatum* root were localized mainly in the outer peripheral tissues consisting of pericycle and parenchyma, containing many secretory canals. In the present sample, the outermost tissue (ph2), whose weight ratio on a whole root weight basis is 3.6%, was also found to contain the highest concentration ($2.51 \pm 0.29\%$) of saikosaponins a, c and d, amounting to 43% of the total saikosaponin content of the whole root. The inner phloem layer (ph1: weight ratio, 53.5%) had a lower concentration ($0.20 \pm 0.05\%$) of the three saikosaponins and only trace amounts of saikosaponins were found in the innermost xylem layer (xy: weight ratio, 43.0%) in the root. Thus, the outermost tissue (ph2) of the *B. falcatum* root contains a considerable part of the whole content of saikosaponins and elements such as Al, Si, Ca, K, Fe, Mn and Sr.

The radar graph summarizes the tissue-specific distribution patterns of saikosaponins and elements found by the HPLC and ICP analyses (Fig. 4). The comparison is simplified by the use of standardized mean values of the three tissues. It is noteworthy that the highest concentration of saikosaponins and elements except P and Mg are found in the outermost peripheral layer (ph2). Si, Al and Mn, along with the saikosaponins, occur exclusively in ph2, where minimal amounts of P are detected. A slightly higher content of P is observed in the innermost layer (xy), where the lowest concentrations of the three saikosaponins and other elements are found. Mg as well as P are present at the highest concentrations in the second phloem layer (ph1).

The physiological significance of the fact that saikosaponins and some elements coexist together in a specific tissue is a subject for future study. Further histochemical experiments on the effects of conditions of soil and manure and external root structures on the distribution of these components in *B. falcatum* root are necessary.

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Radioreceptor Assay for a New Antiallergic Agent, 1-(2-Ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole Difumarate (KB-2413), in Plasma[1)]

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A radioreceptor assay (RRA) for the quantitative determination of a new antiallergic agent, 1-(2-ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate (KB-2413), has been developed. This RRA method, based upon competitive binding of [^{3}H]mepyramine and KB-2413 to histamine H_1 receptors in guinea pig cerebellum, allows the simple, sensitive and reproducible determination of KB-2413 in plasma. The determination limit was 0.1 ng (as free base)/ml and the standard curve was linear over the range of 0.1 to 5.0 ng/ml. The intra- and inter-assay coefficients of variation for the determination of KB-2413 were 5.2—11.1% and 3.4—5.6%, respectively. The active metabolites, 1-(2-ethoxyethyl)-2-(1-homopiperazinyl)benzimidazole and 1-(2-ethoxyethyl)-5-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole, cross-reacted to the extents of 28.6 and 21.1%, respectively. However, plasma levels obtained by the RRA method and the gas chromatographic method for the intact drug were well correlated in guinea pigs after oral administration of KB-2413.

Keywords——antiallergic agent; 1-(2-ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate; KB-2413; determination; radioreceptor assay; [^{3}H]mepyramine; histamine H_1 receptor; plasma

Introduction

1-(2-Ethoxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole difumarate (KB-2413) has strong antiallergic activity and lower toxicity in animals as compared with other known antiallergic drugs such as ketotifen and chlorpheniramine.[2,3)] The chemical structure of KB-2413 is shown in Fig. 1.

A method for determination of the intact drug in plasma was established by capillary gas chromatography (GC) with a nitrogen-sensitive detector and the usefulness of the method was confirmed in animal experiments at relatively high doses.[4)] However, the dose in animal experiments was 50—100 times higher than the clinical dosage, so that a more sensitive determination method had to be developed to clarify the pharmacokinetics of KB-2413 in humans.

Radioreceptor assay (RRA) has proved to be valuable for measuring the plasma levels of a variety of drugs, for example, beta-blockers, benzodiazepines, neuroleptics, tricyclic antidepressants, calcium antagonists, *etc.* These RRAs are of great interest because of their

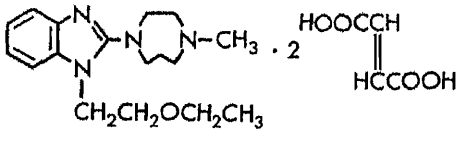

Fig. 1. Chemical Structure of KB-2413

ability to detect not only the parent drugs but also pharmacologically active metabolites by means of a simple technique.

Tran *et al.*[5] found that [^{3}H]mepyramine selectively bound to histamine H_1 receptors in mammalian brain membranes and examined in detail the properties of histamine H_1 receptors in mammalian brain labeled with [^{3}H]mepyramine.

We describe here an assay for a new antiallergic agent, KB-2413, based upon competitive binding of [^{3}H]mepyramine and KB-2413 to histamine H_1 receptors in guinea pig cerebellum. The present assay is simple, sensitive, reproducible and suitable for routine clinical application.

Experimental

Materials——KB-2413 was synthesized and supplied by Fuji Chemical Industry, Co., Ltd. (Toyama, Japan). [^{3}H]Mepyramine (specific activity; 26 Ci/mmol) used as a radioactive ligand was purchased from Amersham Japan Ltd. (Tokyo, Japan). 1-(2-Ethoxyethyl)-2-(1-homopiperazinyl)benzimidazole (metabolite A), 1-(2-hydroxyethyl)-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite B), 1-(2-ethoxyethyl)-2-(4-methyl-4-oxide-1-homopiperazinyl)benzimidazole (metabolite C), 1-(2-ethoxyethyl)-5-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite D), 1-(2-ethoxyethyl)-6-hydroxy-2-(4-methyl-1-homopiperazinyl)benzimidazole (metabolite E) and ketotifen fumarate were all synthesized at the Pharmaceuticals Research Center of Kanebo, Ltd. Antazoline phosphate, diphenhydramine hydrochloride, mepyramine maleate, methapyrilene fumarate, promethazine hydrochloride, triprolidine hydrochloride and tripelennamine hydrochloride were obtained from Sigma Chemical Company (St. Louis, U.S.A.). Cyproheptadine hydrochloride was obtained from Nippon Merck Banyu Co., Ltd. (Tokyo, Japan) and clemastine fumarate from Sankyo Co., Ltd. (Tokyo, Japan). Benzene for pesticide analysis (used as an extraction solvent) and other reagents of special grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Membrane Preparations——Male Hartley strain guinea pigs (250—400 g, Keali Co., Ltd., Osaka, Japan) were decapitated. The cerebellums collected from more than 20 animals were homogenized in 30 volumes of ice-cold 50 mM Na/K phosphate buffer (pH 7.5) with a Physcotron (Niti-On Medical and Physical Instruments Mfg. Co., Ltd., Chiba, Japan) for 3 periods of 10 s at setting 55, and the homogenate obtained was centrifuged for 20 min at 25000 × *g* at 4 °C. The pellet was resuspended in the same volume of fresh phosphate buffer with a Potter-Elvehjem Teflon-glass homogenizer, and centrifuged in the same manner as above. The resulting pellet was stored at −70 °C until use, and homogenized in 60 volumes of ice-cold phosphate buffer with a Potter-Elvehjem Teflon-glass homogenizer when required for the receptor assay. Protein in membrane preparations was measured by the method of Lowry *et al.*[6]

Receptor Assay——A 1 m aliquot of plasma was mixed with 1 ml of 0.2 N sodium hydroxide and 6 ml of benzene in a 10-ml glass-stoppered centrifuge tube. The mixture was vigorously shaken for 10 min and centrifuged for 10 min at 3000 rpm. Then 5 ml of the organic layer was transferred into another 10-ml glass tube, and evaporated to dryness under a stream of nitrogen at about 40 °C. The residue was redissolved in 0.5 ml of 50 mM Na/K phosphate buffer (pH 7.5) and 0.2 ml of [^{3}H]mepyramine solution in ice-cold phosphate buffer was added to give a final concentration of 0.9 nM. The mixture was pre-incubated for 1 min at 25 °C, then 0.3 ml of the membrane preparations containing approximately 0.34 mg of protein was added. Incubation was carried out at 25 °C for 30 min and was terminated by addition of 4 ml of ice-cold phosphate buffer. The mixture was immediately filtered onto a Whatman GF/C micro glass fiber filter under vacuum followed by washing three times with 4 ml of ice-cold phosphate buffer. A model VFM-1 cup-seal vacuum filtration manifold (Amicon Division, W. R. Grace & Co., Danvers, U.S.A.) and a model XX5500000 pump (Millipore Co., Bedford, U.S.A.) were used.

Radioactivity trapped on the filter was counted in 1 ml of methanol and 12 ml of Scintisol® EX-H (Dojindo Laboratories, Kumamoto, Japan) with a Tri-Carb liquid scintillation spectrometer (model B-2450, Packard Instrument Co., Downers Grove, Ill., U.S.A.). The counting efficiencies were determined automatically by the ^{226}Ra external standard ratio method and cpm was converted to dpm.

The assay was always done in triplicate and standard curves were routinely obtained for each experiment by adding known amounts of authentic KB-2413 directly to blank plasma. Specific binding was calculated by subtracting nonspecific binding determined according to a similar procedure in the presence of 2 μM triprolidine from total binding.

The cross-reactivities of the main metabolites relative to KB-2413 were calculated from their affinity constants (K_i values) which were determined from the equation $K_i = IC_{50}/(1 + C/K_d)$, where K_d is the dissociation constant (0.27 nM) derived from the Scatchard analysis of specific [^{3}H]mepyramine binding at 0.35 to 3.72 nM [^{3}H]mepyramine and C is the concentration of the labelled ligand in the binding assay. The IC_{50} values, the concentrations causing 50% inhibition of specific [^{3}H]mepyramine binding, were calculated after linearization of binding inhibition data by

using a logit-log conversion.

GC Method——The capillary gas chromatographic method with a nitrogen-sensitive detector described in the previous report[4] was used.

Animal Experiment——Male Hartley guinea pigs (240—320 g, Keali Co., Ltd.) fasted overnight were used. An isotonic saline solution of KB-2413 (0.6 mg/ml was administered orally at a dose of 2 mg/kg. At various times after dosing, the animals were anesthetized with ether, and blood samples were withdrawn from the abdominal vena cava with a heparinized plastic syringe. Plasma was promptly separated and stored at −20 °C until analysis by the GC and RRA methods.

Results

Binding of [^{3}H]Mepyramine to Receptors

Total [^{3}H]mepyramine binding with the membranes of guinea pig cerebellum was approximately 5500 dpm (95 fmol) with nonspecific binding levels assayed in the presence of 2 μM triprolidine being approximately 500 dpm (9 fmol). [^{3}H]Mepyramine binding was inhibited by the direct addition of plasma, for example, by 34.7 and 42.6% with 50 and 100 μl of plasma, respectively. On the other hand, only 5.8% of specific binding was inhibited by the benzene extracts of plasma, and the standard deviation of the inhibition was only 2.3% ($n=6$).

Stability of Brain Membranes

The pellet of brain membranes was stored for 3 months at 5, −20 and −70 °C, and the variations of specific [^{3}H]mepyramine binding were examined. The brain membranes have

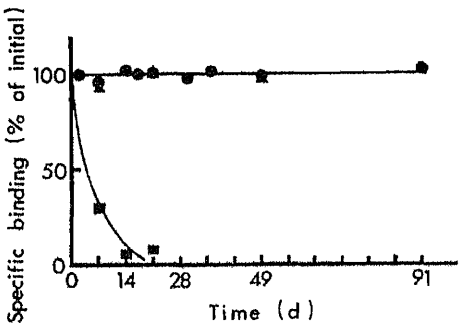

Fig. 2. Stability of Membrane Pellet on Storage at (■) 5 °C, (▲) −20 °C and (●) −70 °C

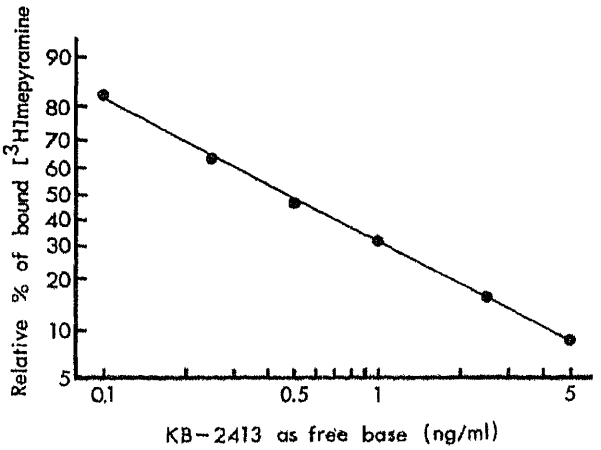

Fig. 3. Standard Curve for the Determination of KB-2413 in Plasma

Table I. Intra-assay Analytical Precision for the Determination of KB-2413 in Plasma

KB-2413 (as free base) (ng/ml)		Coefficients of variation (%)	n
Added	Found		
0.1	0.10	10.0	5
0.25	0.26	8.1	5
0.5	0.48	5.2	5
1.0	0.99	6.0	5
2.5	2.53	11.1	5
5.0	5.11	9.3	5

Table II. Inter-assay Analytical Precision for the Determination of KB-2413 in Plasma

KB-2413 (as free base) (ng/ml)		Coefficients of variation (%)	n
Added	Found		
0.1	0.09	5.6	6
0.25	0.25	4.8	6
0.5	0.53	4.9	6
1.0	1.06	3.4	6
2.5	2.51	3.7	6

proved to be stable for at least 3 months at less than −20 °C, as shown in Fig. 2. An extreme decrease of specific binding took place on storage at 5 °C.

Standard Curve

Various amounts (0.1 to 5.0 ng) of KB-2413 were added to 1 ml aliquots of the blank plasma obtained from normal subjects. A standard displacement curve for KB-2413 was converted to a straight line by means of a logit-log plot. A typical example of the standard curves is presented in Fig. 3. It showed a good linearity and allowed us to determine KB-2413 at a concentration as low as 0.1 ng (as free base)/ml.

Accuracy and Reproducibility

The accuracy and reproducibility of the assay were evaluated at concentrations of 0.1 to 5.0 ng of KB-2413/ml. The intra- and inter-assay data are summarized in Tables I and II, respectively.

In the intra-assay study, the overall recoveries of KB-2413 from plasma samples averaged 101 ± 8.3 (S.D.) % and the coefficients of variation were 5.2—11.1% in the concentration range of 0.1 to 5.0 ng/ml. On the other hand, in the inter-assay study, the overall recoveries of KB-2413 from plasma samples averaged 99.9 ± 5.83 (S.D.) % and the coefficients of variation were 3.4—5.6% in the concentration range of 0.1 to 2.5 ng/ml.

Specificity

As shown in Table III, metabolites A and D possessed about 30 and 20% cross-reactivities for KB-2413, respectively, while the other metabolites were less than 1.7% cross-reactive.

Correlation of Affinity Constant and Pharmacological Activity

Affinity constants of various antihistaminic drugs including KB-2413 to [^{3}H]mepyramine binding sites were determined from their IC_{50} values and compared with their potencies in blocking histamine-induced guinea pig ileal contraction (pA_2).[2,7-9] As shown in Fig. 4, there was a good correlation ($\gamma = 0.94$, $n = 10$) between them.

TABLE III. Cross-Reactivities of the Main Metabolites of KB-2413

Metabolites	Cross-reactivities (%)
A	28.6
B	0.8
C	0.6
D	21.1
E	1.7

A—E, see the text.

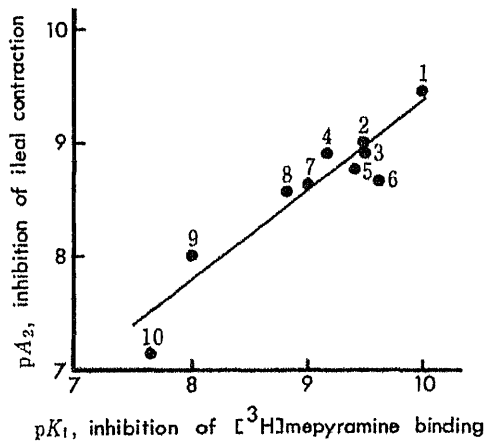

Fig. 4. Correlation of Affinities of Drugs for [^{3}H]Mepyramine Binding Sites (pK_i) with Their Potencies in Blocking Histamine-Induced Guinea Pig Ileal Constraction (pA_2)

pA_2 values were derived from published data.[2,7-9] Slope = 0.82, $\gamma = 0.94$.

1, clemastine; 2, mepyramine; 3, KB-2413; 4, promethazine; 5, cyproheptadine; 6, ketotifen; 7, methapyrilene; 8, tripelennamine; 9, diphenhydramine; 10, antazoline.

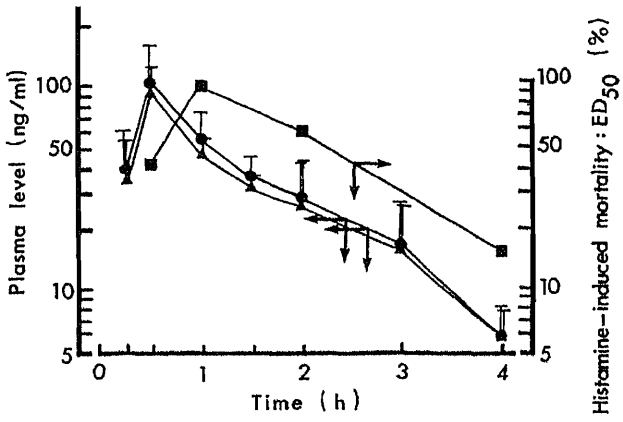

Fig. 5. Plasma Levels of KB-2413 Obtained by (▲) the GC Method and (●) RRA, and (■) Pharmacological Activity after Oral Administration of KB-2413 in Guinea Pigs

Plasma levels and pharmacological activities were obtained at doses of 2 and 0.00156—0.0125 mg/kg, respectively. Plasma levels are presented in terms of the free base of KB-2413.

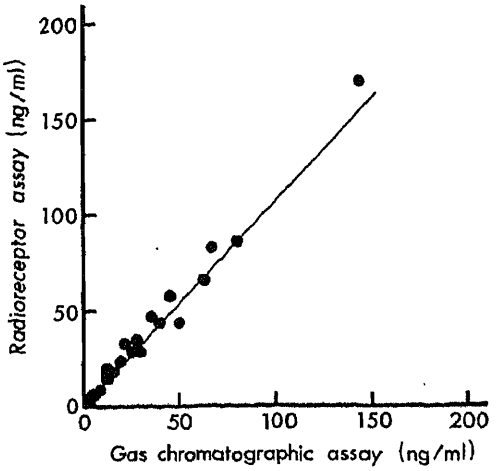

Fig. 6. Comparison of the Results of Radioreceptor Assay and Gas Chromatographic Assay of KB-2413 in Plasma Obtained from Guinea Pigs after Oral Administration of KB-2413 at a Dose of 2 mg/kg

Plasma levels are presented in terms of the free base of KB-2413.

Comparison of the RRA and GC Methods

Guinea pigs were orally given KB-2413 at a dose of 2 mg/kg. Plasma levels determined by the RRA method as well as by the GC method for the intact drug are shown in Fig. 5, which also shows pharmacological activity, that is, the inhibitory effect on histamine-induced mortality of guinea pigs, at doses of 0.00156 to 0.0125 mg/kg.[2] Plasma levels of the intact drug reached the maximum (about 90 ng/ml) at 30 min after dosing and then decreased with a half life of about 1 h. Plasma levels measured by the RRA method were similar to but slightly higher than those by the GC method.

The correlation between the values measured by the two methods, based on a total of 21 samples, is shown in Fig. 6. There was a good correlation with a slope of 1.15 and a correlation coefficient of 0.99. In addition, the pharmacological activity reached the maximum at 1 h after dosing and then decreased with almost the same half-life as that of the plasma levels.

Discussion

Chang *et al.*[10] demonstrated that considerable species differences existed in the affinity of [^{3}H]mepyramine to the brain membrane preparations of various animals. The affinities to guinea pig and human brain membrane preparations were 3—6 times greater than those to rat, mouse and rabbit preparations, and the greater affinity of [^{3}H]mepyramine in guinea pigs was attributable both to faster association rates and slower dissociation rates in guinea pigs than in rats. Moreover, Chang *et al.*,[10] Tran *et al.*[5] and Hill *et al.*[11] found pronounced differences between the regional localization of specific [^{3}H]mepyramine binding in various animals.

In our preliminary studies, species differences and regional distribution were also observed in specific [^{3}H]mepyramine binding to rat and guinea pig brains. Specific [^{3}H]mepyramine binding to guinea pig cerebellum was about 3 times higher than that to guinea pig whole brain excluding cerebellum and about 7 times higher than that to rat whole brain. In the present study, therefore, we used guinea pig cerebellum as histamine H_1

receptors. The membrane pellet of guinea pig cerebellum was easily prepared and was stable for at least 3 months at less than −20 °C (Fig. 2).

In the determination of KB-2413 in plasma by means of the RRA method, specific [^{3}H]mepyramine binding was inhibited by the direct addition of a small amount of plasma to the incubation mixture. Increasing volumes of plasma reduced the binding gradually, causing about 40% inhibition with 100 μl of plasma. This was presumably due to the binding of [^{3}H]mepyramine with plasma proteins. In order to decrease the inhibition of specific [^{3}H]mepyramine binding and raise the sensitivity of the determination of KB-2413 in plasma, the extraction of KB-2413 in plasma with benzene under basic conditions was carried out prior to the receptor assay. The inhibition of specific [^{3}H]mepyramine binding by benzene extracts was only 5.8%, and it had thus become feasible to determine 0.1 ng (as free base)/ml of KB-2413 in plasma by assaying the extracts of 1 ml of plasma (Fig. 3). Consequently, the sensitivity of the RRA method was about 20 times higher than that of the GC method,[4] and excellent reproducibility of the former method was indicated by the values in the intra- and inter-assay studies (Tables I and II).

The affinity constants (K_i value) of several antihistaminic drugs to histamine H_1 receptors correlated closely with their pharmacological activities, that is, potencies in blocking histamine-induced guinea pig ileal contraction (Fig. 4). It was suggested that KB-2413 was comparable in potency to the most active H_1 antihistaminics, ketotifen, cyproheptadine and mepyramine. Metabolites A (desmethylated compound) and D (5-hydroxylated compound) of the 5 main metabolites of KB-2413 possessed about 20—30% cross-reactivities for KB-2413 (Table III), so these metabolites might also have pharmacological activities.

The plasma levels of guinea pigs orally given 2 mg/kg of KB-2413 were determined by the RRA method and at the same time, the plasma levels of the intact drug were determined by the GC method. The levels obtained by the RRA method were similar to but slightly higher than those by the GC method (Figs. 5 and 6). It was considered that this slight difference was attributable to the presence of some active metabolites, for example, metabolites A and/or D. However, the substantial correspondence of the results of the RRA and GC methods suggested that presence of only small amounts of active metabolites in guinea pig plasma, if they are present. This will be examined in a future study.

The pharmacological activity (in terms of the inhibitory effect on histamine-induced mortality of guinea pigs) was compared with the concentration of KB-2413 in plasma after oral administration. The pharmacological activity reached the maximum about 30 min later than the peak of the plasma level, but their half-lives were comparable (Fig. 5). This time lag presumably represents the time required for KB-2413 to pass to the receptors from plasma, although the variations in experimental values need to be taken into consideration.

In conclusion, the present RRA method is simple, sensitive, reproducible and suitable for estimating the plasma levels, which well reflected the pharmacological activity. The usefulness of this RRA method in clinical studies was suggested in the preliminary report.[12] Further details will be reported elsewhere.

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Sensitivity of Steroid Enzyme Immunoassays. Comparison of Alkaline Phosphatase, β-Galactosidase and Horseradish Peroxidase as Labels in a Colorimetric Assay System[1)]

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The sensitivities of colorimetric testosterone enzyme immunoassays using alkaline phosphatase (AP), β-galactosidase (β-GAL) and horseradish peroxidase (HRP) as labels were compared. Enzyme labeling of testosterone was carried out by the *N*-succinimidyl ester method at an appropriate molar ratio of steroid to enzyme. In the competitive immunoassay, the bound and free enzyme-labeled antigens were separated by a double antibody method and the enzymic activity of the immune precipitate was determined by spectrophotometric methods. The AP activity was measured in four ways, using *p*-nitrophenyl phosphate, phenolphthalein monophosphate, phenyl phosphate, and nicotinamide adenine dinucleotide phosphate (NADP) as substrates. In the cases of β-GAL and HRP, *o*-nitrophenyl β-D-galactopyranoside and 3,3′,5,5′-tetramethylbenzidine were used, respectively. A dose-response curve with a satisfactory sensitivity was obtained in each testosterone assay system by the use of a minimum amount of the enzyme-labeled antigen at an appropriate dilution of anti-testosterone antiserum ($K_a = 2 \times 10^{10}\,M^{-1}$). The amount of testosterone needed to displace 50% of the bound label ranged from 9 to 90 pg. It was found that the highest sensitivity was obtained by the use of HRP, with AP next and β-GAL third; the former two sensitivities were each comparable to that of the corresponding fluorimetric assay.

Keywords——enzyme immunoassay; testosterone; steroid enzyme labeling; immunoassay sensitivity; alkaline phosphatase; β-galactosidase; horseradish peroxidase

In recent years, heterogeneous enzyme immunoassays of hormones and drugs have been developed using various enzymes as labels. The sensitivity of the assay depends on the enzyme employed. Enzymes currently used in the steroid assay systems are alkaline phosphatase (AP), horseradish peroxidase (HRP), β-galactosidase (β-GAL), glucose oxidase, glucose-6-phosphate dehydrogenase, glucose dehydrogenase, glucoamylase, penicillinase and urease; the former three enzymes are most commonly used. The choice of enzyme is based on various criteria such as activity and stability.[2)] It may also be possible to develop an enzyme immunoassay method using two enzyme systems.[3)] In order to obtain a practical basis for selecting the enzyme, it is necessary to perform comparative studies of assay sensitivity using the same anti-steroid antiserum and haptenic steroid derivative. Such a study requires excellent reproducibility of the enzyme labeling of steroids, since the number of steroid molecules incorporated per enzyme molecule (degree of hapten substitution) is a factor influencing the sensitivity. We have previously shown that the *N*-succinimidyl ester method is useful for the preparation of enzyme-labeled antigens.[4)] Further, AP,[4b)] β-GAL and HRP[4c)] have been compared, in a testosterone assay system, with regard to the effects of steroid/enzyme molar ratio in the labeling on the immunoreactivity of the labeled antigen with an anti-steroid antiserum and on the assay sensitivity, where the enzyme activities have been measured by fluorimetric methods. In view of the ready availability of instrumentation, however, colorimetric detection of the enzyme activity seems to be advantageous, although

the assay is usually less sensitive than fluorimetry.[5] This paper deals with the sensitivities of colorimetric testosterone enzyme immunoassays using the AP, β-GAL and HRP labels.

Materials and Methods

Materials——AP (EC 3.1.3.1) from calf intestine (enzyme label for enzyme immunoassay, 2500 U/mg) was obtained as a solution (1 mg/0.1 ml) from Boehringer–Mannheim Yamanouchi Co. (Tokyo); β-GAL (EC 3.2.1.23) from *Escherichia coli* (grade VI, 455 units/mg and grade VIII, 905 units/mg) and HRP (EC 1.11.1.7) (grade I-C, Reinheits–Zahl 3.41, 263 units/mg) were from Sigma Chemical Co. (St. Louis, MO) and Toyobo Co. (Osaka), respectively. The ^{17} succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was prepared by the method previously established in these laboratories.[6] Anti-testosterone antiserum used was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin.[7] Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo). Nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) and 3,3′,5,5′-tetramethylbenzidine were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Alcohol dehydrogenase (EC 1.1.1.1) from baker's yeast (catalogue number A3263, 185 units/mg), diaphorase (EC 1.6.4.3) from *Clostridium kluyveri* (type II-L, 10 units/mg), *p*-iodonitrotetrazolium violet, and phenolphthalein monophosphate (PMP, disodium salt) were obtained from Sigma Chemical Co.; phenyl phosphate (PP, disodium salt), 4-aminoantipyrine, *p*-nitrophenyl phosphate (*p*-NP, disodium salt), and *o*-nitrophenyl β-D-galactopyranoside were from Nakarai Chemicals, Ltd. (Kyoto).

Assay Buffer——A 0.05 M phosphate buffer, pH 7.3 (PB) containing 0.1% gelatin and 0.9% NaCl (gel-PBS) was used in the immunoassay with HRP as a label. In the systems with AP and β-GAL, gel-PBS containing 0.1% NaN_3 was used.

Preparation of Testosterone–Enzyme Conjugates——AP Labeling: This was carried out in the manner described previously.[4b] In short, a solution of the testosterone *N*-succinimidyl ester (13 μg) in dioxane (0.1 ml) was added to a solution of AP (100 μg, M.W. 116500) in PB (0.2 ml) at 0 °C, and the mixture was gently stirred at 4 °C for 4 h. After dialysis against cold PB, the solution was stored at 4 °C at a concentration of 100 μg/ml, adjusted with assay buffer.

β-GAL Labeling:[4c] Dioxane solutions (0.1 ml) containing calculated amounts of the activated ester corresponding to steroid/enzyme molar ratios of 5, 10, 30, and 60 (M.W. of β-GAL, 540000) were each added to a solution of β-GAL (1 mg) in PB (0.2 ml) at 0 °C. After dialysis against cold PB (2 l) for 2 d, the resulting solutions were stored at 4 °C at a concentration of 500 μg/ml, adjusted with assay buffer.

HRP Labeling:[4c] This was carried out in a manner similar to the β-GAL labeling described above. The activated ester was reacted with HRP (M.W. 40000) at a molar ratio of 30 to give an HRP-labeled antigen. For the immunoassay procedure, the label solutions were diluted with assay buffer containing 0.5% normal rabbit serum.

Enzyme Immunoassay Procedure——This was carried out in duplicate or triplicate in a glass test tube (10 ml). The standard procedure with the AP label is as follows: a solution of testosterone (0—500 pg) in assay buffer (0.1 ml) and AP-labeled testosterone (2—25 ng) in the buffer (0.1 ml) containing normal rabbit serum were added to diluted anti-testosterone antiserum (0.1 ml), and the mixture was incubated at 4 °C for 4 h. Goat anti-rabbit IgG antiserum (0.1 ml) diluted with assay buffer was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4 °C for 16 h. After addition of 0.05 M carbonate buffer (pH 10.0) containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3 (1.5 ml), the resulting solution was centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with the carbonate buffer (1.5 ml), and used for measurement of the enzymic activity (B_0 or B).

The enzyme immunoassays using β-GAL and HRP as labels were carried out in the manner described previously.[4c] In each system, the procedure without addition of the first antibody was also carried out to provide non-specific binding values; the background was estimated as a percentage of the absorbance for B_0, using distilled water as the zero reference.

Measurement of AP Activity—— NADP Method: Enzyme amplification was carried out according to the method of Stanley *et al.*[8a] with slight modifications. A solution of NADP (10—50 μM) in 0.05 M diethanolamine buffer, pH 10.0 (DEA, 1 ml), containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 25 °C for 30—60 min (first incubation). A PB solution (1 ml) containing alcohol dehydrogenase (100 μg), diaphorase (100 μg), 0.8 mM *p*-iodonitrotetrazolium violet, and 2% ethanol was added to the incubation mixture, and the whole was incubated at 25 °C for 10—30 min (second incubation). The reaction was terminated by addition of 0.1 M HCl (2 ml) and the absorbance was measured at 492 nm.

p-NP Method: A solution of *p*-NP (1 mM) in 1 M DEA or 0.05 M carbonate buffer, pH 10.0 (2 ml), each containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.1 M NaOH (2 ml) and the absorbance was measured at 405 nm.

PMP Method: A solution of PMP (0.25 mM) in 0.3 M DEA or 0.05 M carbonate buffer, pH 10.0 (2 ml), each containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.1 M phosphate buffer, pH 12.5 (2 ml) and the absorbance was measured at 550 nm.

PP Method: A solution of PP (5 mM) and 4-aminoantipyrine (5 mM) in 0.05M carbonate buffer, pH 10.0 (2 ml), containing 0.01% $MgCl_2$ was added to the assay tube, and the mixture was incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.2 M boric acid (2 ml) containing 1.2% $K_3Fe(CN)_6$ and the absorbance was measured at 500 nm.

Measurement of β-GAL Activity——The immune precipitate was diluted with assay buffer (1 ml) containing 0.1% $MgCl_2$ and 10% ethylene glycol, vortex-mixed, and preincubated at 37 °C for 3 min. *o*-Nitrophenyl β-D-galactopyranoside (0.06%, 1 ml) in assay buffer was added to the resulting solution and the mixture was incubated for 1 h. The reaction was terminated by addition of 1 M Na_2CO_3 (2 ml) and the absorbance was measured at 420 nm.

Measurement of HRP Activity——The immune precipitate was diluted with 0.05 M acetate–citric acid buffer, pH 5.5 (1.8 ml), containing 0.42 mM 3,3′,5,5′-tetramethylbenzidine and 3% dimethyl sulfoxide, vortex-mixed, and preincubated at 37 °C for 3 min. Hydrogen peroxide (0.02%, 0.2 ml) was added to the resulting solution, and the mixture was incubated for 1 h. The reaction was terminated by addition of 0.5 M H_2SO_4 (2 ml) and the absorbance was measured at 450 nm.

Results and Discussion

The purpose of this work was to compare the sensitivities obtainable with the testosterone enzyme immunoassay systems using AP, β-GAL and HRP as labels, when colorimetric methods were used for the measurement of the enzymic activity. Enzyme-labeled antigens were prepared by the active ester method. The *N*-succinimidyl ester of 4-hydroxytestosterone 4-hemiglutarate was treated with each enzyme in phosphate buffer (pH 7.3)–dioxane (Fig. 1). The activated ester should react readily with free amino groups of these enzymes. The steroid/enzyme molar ratios employed in the coupling reactions were 30 for AP and HRP, and 5—60 for β-GAL. Selection of the ratio in the former two labelings was based on both immunoreactivity of the resulting label and sensitivity of the assay, namely, on a balance between sensitivity and precision of each assay.[4b,c] The degrees of hapten substitution have been shown to be *ca.* 1 for AP,[4b] 2 for HRP, and 3 in the case of the β-GAL label prepared at a molar ratio of 10.[4c] The conjugates were dialyzed against the buffer to remove the unreacted steroid. No significant loss of enzymic activity was observed under the coupling conditions used.

For comparative purposes, anti-testosterone antiserum used must have a high binding affinity responsive to enzyme and substrate differences, since the affinity constant of the antibody limits the ultimate sensitivity of competitive enzyme immunoassay. The anti-steroid antiserum employed in this work was that elicited in a rabbit by immunization with the conjugate of 4-hydroxytestosterone 4-hemiglutarate with bovine serum albumin, that is, the combination of antibody and labeled antigen is homologous.[9] This antiserum showed a sufficient affinity for testosterone ($K_a = 2 \times 10^{10}\ M^{-1}$) in the radioimmunoassay procedure.[7] The bound and free enzyme-labeled antigens were separated by a double antibody method. The enzymic activity of the immune precipitate was determined by colorimetric methods. The assays were assessed in terms of the absorbance for B_0 and non-specific binding (background). The criteria that the optical density obtained upon 1 h incubation and the background should be at least 0.2 and less than 20%, respectively, were employed in this work.

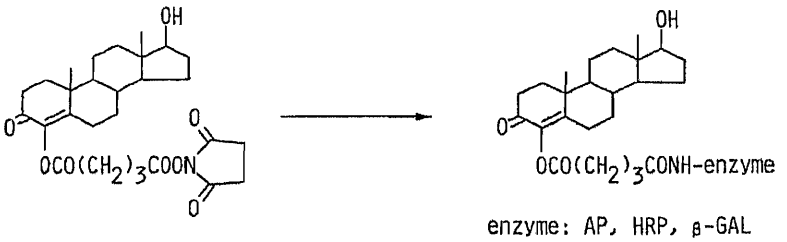

Fig. 1. Preparation of Enzyme-Labeled Antigens

TABLE I. Incubation Times and Absorbance for B_0 in the AP Amplification System[a]

Incubation time (min)		Absorbance	NSB[b] (%)
First	Second		
30	10	0.29	23
	20	0.41	22
	30	0.54	19
40	10	0.39	17
	20	0.57	16
	30	0.74	14
60	10	0.59	12
	20	0.90	11
	30	1.31	9

a) The concentration of NADP used in the first incubation was 20 μM. *b*) Non-specific binding (background).

TABLE II. Effects of Concentration of NADP and Amount of AP-Labeled Antigen on Absorbance for B_0 in the Amplification System[a]

NADP (μM)	AP-labeled antigen		
	2 ng	5 ng	10 ng
10	0.11 (52)	0.31 (17)	0.52 (11)
20	0.30 (31)	0.57 (16)[b]	0.94 (9)
50	0.50 (36)	1.03 (17)	1.39 (13)

a) The enzyme immunoassays were carried out with the given amounts of the AP label and a 1 : 40000 dilution of the anti-testosterone antiserum. In the AP activity measurement, the first and second incubation times were 40 and 20 min, respectively. Figures in parentheses indicate background (%). *b*) The inhibition data listed in Table III were obtained with this system.

First, the immunoassay systems using AP as a label were studied. The enzyme activity was measured in four ways. In the enzyme-amplified assay, the substrate NADP was dephosphorylated to nicotinamide adenine dinucleotide (NAD), which acts as a cofactor in the redox cycle system producing a formazan dye; this method has been reported to be highly sensitive.[8] In the other three cases, *p*-NP, PMP and PP were used as substrates.

In the NADP method, the effects of incubation times, concentration of NADP, and amount of AP label on absorbance were examined (Tables I and II). The results on the first reaction (NADP→NAD) time and the second cycling reaction time are listed in Table I. In the enzyme immunoassay procedure, 5 ng of the AP-labeled antigen at 1 : 40000 dilution of the anti-testosterone antiserum was employed on the basis of the previous findings.[4b] The amount of antigen corresponds to *ca.* 12 pg of testosterone, since the degree of hapten substitution was *ca.* 1. The data showed that satisfactory results can be obtained with a total incubation time of 60 min. Suitable concentrations of NADP were 10—50 μM, when 5 or 10 ng of the antigen was used (Table II). In the assay using 2 ng of the label, no satisfactory results were obtained with respect to the background.

Sensitivities obtainable with these four methods were then tested by examining the inhibition of enzymic activity caused by the addition of 200 pg of testosterone per tube, *i.e.* the extent of inhibition at the corresponding point of steroid amount in the dose–response curve, as shown in Fig. 2. The results are listed in Table III. With all the assay systems, satisfactory sensitivities were obtained. It is clear that the NADP method gives the highest sensitivity. In

TABLE III. Inhibition of Bound Enzymic Activity of the AP-Labeled Antigen by 200 pg of Testosterone

Method	Amount of label (ng)	Antiserum dilution	Buffer[a]	Inhibition (%)	Absorbance for B_0[b]
NADP	5	1:40000	0.05 M DEA	83	0.57 (16)[c]
p-NP	10	1:25000	1 M DEA	71	0.54 (8)
			0.05 M carbonate	70	0.23 (18)
	25	1:15000	0.05 M carbonate	68	0.45 (9)[c]
PMP	10	1:25000	0.3 M DEA	74	0.47 (20)
	25	1:15000	0.3 M DEA	63	1.04 (10)
			0.05 M carbonate	70	0.26 (25)
PP	10	1:25000	0.05 M carbonate	77	0.35 (10)

a) pH 10.0. *b*) Figures in parentheses indicate background (%). *c*) The dose–response curves shown in Fig. 2 were obtained with these systems.

the *p*-NP and PMP methods, the use of DEA rather than the carbonate buffer offered advantages according to our criteria, although the assays were somewhat less sensitive than the assay using PP as a substrate. Two typical dose–response curves with the AP systems are shown in Fig. 2. In the case of the NADP method, the amount of testosterone needed to displace 50% of the bound label was 30 pg: the sensitivity is 1.7-fold higher than that of the *p*-NP system and comparable to that of the fluorimetric assay using 4-methylumbelliferyl phosphate as a substrate.[4b]

β-GAL as a label was the next subject. *o*-Nitrophenyl β-D-galactopyranoside has mostly been used in the colorimetric detection. Using this enzyme, we have developed enzyme immunoassays of various steroids. In the present work, the effect of difference in specific activity (grade VI, 455 units *vs.* grade VIII, 905 units) on the assay sensitivity was examined. For this purpose, various molar ratios of the steroid to enzyme were employed in the β-GAL labeling reaction. The resulting labels (100 ng) gave essentially the same immunoreactivity patterns at 1 : 500 dilution of the anti-testosterone antiserum (the binding ability increased with increasing molar ratio for both enzyme grades), which are similar to that obtained in the previous study.[4a]

The results on sensitivity of the immunoassays with these labeled antigens are summarized in Table IV: 100 ng of the label corresponds to 53 pg of testosterone, if the degree of hapten substitution is 1. The data showed that the sensitivity was markedly influenced by the steroid/enzyme molar ratio, and that similar results were obtained with both β-GAL grades. It is clear that the β-GAL-labeled antigens prepared at a molar ratio of 10 gave higher sensitivities and the use of smaller amounts of the labels derived from grade VIII, which shows a higher specific activity, was not very effective in increasing sensitivity. Thus, no significant difference in sensitivity, according to our criteria, was observed between the two β-GAL grades. A typical dose–response curve obtained with the assay system using 100 ng of the β-GAL label at 1 : 10000 dilution of the antiserum is shown in Fig. 2. The sensitivity of the assay was lower than those of the assays using AP as a label; a fluorimetric system using 4-methylumbelliferyl β-D-galactopyranoside resulted in 3-fold increase in the sensitivity (data not shown).

HRP activity can be measured by using various substrates. In a previous paper,[10] we investigated the sensitivity of testosterone enzyme immunoassay systems with six chromogens; the assay using 3,3′,5,5′-tetramethylbenzidine, a non-mutagenic substrate, was found to give a high sensitivity comparable to that of the fluorimetric method using 3-(*p*-hydroxyphenyl)propionic acid. A dose-response curve obtained with the immunoassay using 2 ng of the HRP-labeled antigen and a 1 : 30000 dilution of the anti-testosterone antiserum is shown

TABLE IV. Inhibition of Bound Enzymic Activity of β-GAL-Labeled Antigens by 200 pg of Testosterone[a]

Molar ratio	Amount of label (ng)	Antiserum dilution	Inhibition (%)	
			Grade VI	Grade VIII
5	100	1:5000	57 (0.21)	58 (0.28)
10	100	1:10000	60 (0.27)	61 (0.44)[b]
		1:20000	63 (0.18)	62 (0.32)
	50	1:10000	—	63 (0.23)
30	100	1:20000	50 (0.21)	45 (0.49)
		1:40000	—	55 (0.29)
	25	1:10000	—	53 (0.25)
60	100	1:20000	39 (0.26)	34 (0.51)
		1:40000	41 (0.16)	40 (0.32)
	25	1:20000	—	43 (0.28)

a) Figures in parentheses indicate the absorbance for B_0 obtained after a 1 h enzyme reaction. In all cases, the background was less than 20%. —: not carried out. *b*) The dose-response curve shown in Fig. 2 was obtained with this system.

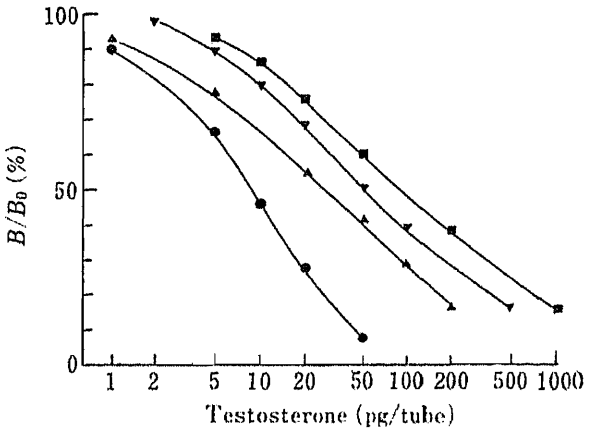

Fig. 2. Dose-Response Curves for Testosterone Enzyme Immunoassays Using AP (NADP, ▲; *p*-NP, ▼), β-GAL (■) and HRP (●) as Lebels

Assay conditions are given in Table III or IV, or in the text.

in Fig. 2: the antigen amount corresponds to *ca.* 14 pg of testosterone, since no significant effect of the degree of hapten substitution on the assay sensitivity has been observed.[4c] The absorbance value for B_0 obtained upon 1 h enzymic reaction was over 0.3 (the assay was carried out at pH 5.5, but the optimum pH value is 4.2).

In Fig. 2, it can be seen that the sensitivity of the assay using the HRP label is higher than those of the assays with the AP and β-GAL labels. The amounts of testosterone needed to displace 50% of the bound label ranged from 9 to 90 pg. The minimal detectable amounts of testosterone, that is, twice the standard deviation of the zero determination (B_0, $n=10$), were 1, 5 and 8 pg in the assays using the HRP, AP (*p*-NP) and β-GAL labels, respectively.

The present work showed that, in the colorimetric enzyme immunoassay, the highest sensitivity was obtained by the use of HRP, with AP next and β-GAL third. The order is the same as with the previous fluorimetric methods.[4b,c] In the cases of the former two enzymes, the sensitivities of the "best" assay systems were each found to be nearly equal to that of the corresponding fluorimetric assay. With β-GAL, chlorophenol red-β-D-galactopyranoside may be worthy of examination as a substrate. In the present study, the assay was carried out with the homologous system. We have previously found that the anti-testosterone antiserum used here has weak, if any, binding affinity for the bridge portion between enzyme and steroid in a

labeled antigen.[11] Therefore, the above result on the order of assay sensitivity seems to be free from the bridge binding phenomenon.[9,12] However, further studies are necessary to clarify this problem. In general, it is often found that such a homologous system does not provide satisfactory assay sensitivity. For the purpose of improving the sensitivity, a heterologous system has been designed. Previously, using β-GAL as a label in bridge heterologous assay systems for cortisol or 11-deoxycortisol, we showed that the bridge length is an important factor influencing the sensitivity, and that the use of a shorter bridge for enzyme labeling results in an increase in sensitivity.[12] There is the possibility that the bridge length effect depends on the label enzyme.

The information obtained here should be helpful in the further development of hapten enzyme immunoassays. It should be noted that the present high sensitivity was ascribable, in part, to the use of an appropriate molar ratio of steroid to enzyme in the enzyme labeling. We recommend molar ratios of 20—40, 10—20, and 10—60 in the AP, β-GAL, and HRP labelings, respectively.[4] In general, however, it is desirable to estimate the degree of hapten substitution, since the labeling rate is influenced by various factors, such as pH, solvent volume, and reactivity of steroid derivatives. Studies on the labeling of steroid with other enzymes currently used in enzyme immunoassays are in progress in these laboratories.

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Studies on Fe Complexes Produced by Yeast. II. Physicochemical Properties of an Fe(II)–Oligosaccharide Complex and Their Relation to Fe Absorption

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Oral administration to rats of Fe(II) complexes produced by yeast in $FeCl_3$-added wine resulted in effective intestinal absorption of Fe and incorporation of Fe into hemoglobin. The physicochemical properties of an Fe(II) complex showing the highest Fe absorption (designated B1-c) were studied. In paper electrophoresis, inorganic Fe salts migrated to the cathode, while B1-c was detected as a single spot consisting of Fe(II) and saccharides which moved to the anode in the pH range of 2—5.6. When methanol was added to an aqueous solution of B1-c, Fe was precipitated at alcohol contents over 50%. When incubated at 25 °C, Fe(II) in an aqueous solution of B1-c was more stable to oxidation than that in an inorganic Fe(II) salt solution. In the B1-c solution, more than 70% of Fe was maintained in the soluble Fe(II) form at pH 5.5, 7 and 10 even after 18 d of incubation. Just after adjustment of an aqueous B1-c solution to pH 11, 90% of Fe in the B1-c solution was soluble. Linear regression analysis of the data on B1-c showed a close correlation between the Fe solubility at the rat small intestinal pH (assumed to be 7.5) and the Fe absorption in the rat after oral administration. The results of infrared spectrometry, elementary analysis and colorimetry suggested that the ligand of B1-c was an oligosaccharide having a carboxyl group and with a molecular weight of around 1500.

Keywords——ferrous complex; oligosaccharide; yeast; physicochemical property; gastrointestinal ferrous absorption

Introduction

Gastrointestinal Fe absorption is affected by a variety of factors such as luminal and mucosal factors.[1-14] The luminal factors are, for example, the quantity, chemical forms, solubility, and interactions with ligands (endogenous and exogenous) of Fe compounds in the lumen of the intestine, and the mucosal factors are mostly related to the mechanism of Fe transport and kinetic parameters of the transport. In other words, the absorbability of Fe from Fe compounds depends on how the physicochemical properties of the Fe compounds are reflected in the above factors. For example, the difference in Fe absorption between Fe(II) and Fe(III) salts and the effects of ascorbic acid on Fe absorption have been explained mainly in terms of the solubility of Fe in the gastrointestinal tract.[1,2,4]

As reported previously,[15] we separated from wine Fe(II) complexes produced by yeast and found that the gastrointestinal Fe absorption from some of the complexes was excellent in rats. In this study, the physicochemical properties of the Fe(II) complexes showing such high Fe absorption were examined, especially in relation to luminal factors, using inorganic Fe salts as controls. In addition, the nature of the ligand of the Fe(II) complex having the highest Fe absorption was deduced from the results of instrumental analyses including infrared (IR), ultraviolet (UV), and visible spectrometries, elementary analysis and colorimetry.

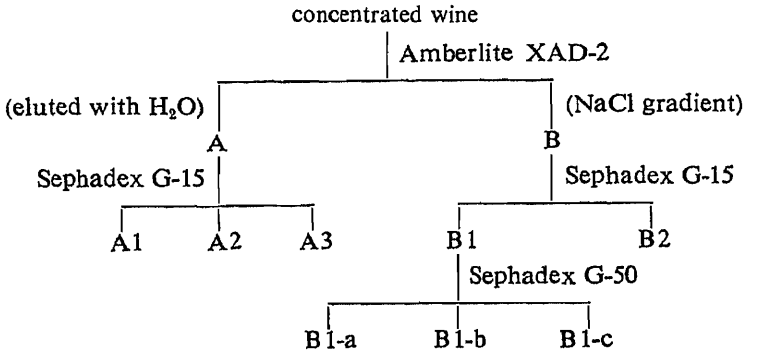

Fig. 1. Separation of Fe Complexes from Wine by Column Chromatography

The chromatographic conditions are detailed in our previous report.[15]

Materials and Methods

Preparation of Fe Complexes——The Fe(II) complexes were prepared by the method described in our previous report.[15] After fermentation by *Saccharomyces cerevisiae* OC-2, the wine containing ^{59}Fe-labeled Fe(II) complexes was concentrated and subjected to column chromatography as schematized in Fig. 1. Finally, seven fractions (designated A, A1, A2, A3, B, B1-c, B2) were obtained and subjected to examination. Of these fractions, A1, A2, B1-c, and B2 were previously found to be Fe(II) complexes, and B1-c has the highest activity for Fe absorption and incorporation into hemoglobin as reported in the previous paper.[15]

Paper Electrophoresis of Fe Complexes——The supporting medium used was filter paper No. 50 (Toyo Filter Paper Co.). Four unlabeled Fe fractions (A1, A2, B1-c, B2) concentrated under reduced pressure were spotted on the paper. Electrophoresis was performed with an Atto SJ-1060 apparatus at 50 V/cm for 30 min in the following three buffer solutions: (a) 0.3 M formic acid–acetic acid buffer (pH 2.0); (b) 0.2 M acetic acid–Na acetate buffer (pH 3.8); and (c) 0.2 M acetic acid–Na acetate buffer (pH 5.6). Fe was detected by spraying with 0.35% *o*-phenanthroline–ethanol solution, and saccharides, by spraying with an alkaline solution of 0.1 M silver nitrate. As control substances, two Fe salts ($FeCl_3$, $FeSO_4$) were tested in a similar manner.

Solubility of Fe Complexes in Organic Solvents——The solubilities of four Fe fractions (A1, A2, B1-c, B2) in methanol, ethyl ether and isopropanol were determined. An aqueous solution of each unlabeled Fe fraction (20 μg as Fe) was poured into a round-bottomed flask and evaporated to dryness under reduced pressure. After addition of 10 ml of the solvent, the flask was shaken at room temperature for 1 h. The mixture was then centrifuged at 10000 rpm for 10 min, and the supernatant was filtered. The total Fe and Fe(II) concentrations in the filtrate were determined by the bathophenanthroline method.[16]

Separately, methanol was added stepwise to an aqueous solution of B1-c (40 μg Fe/ml) in an ice bath, so as to increase the alcohol content in the medium. A sample was taken at each step and centrifuged at 10000 rpm for 10 min at 4 °C, and the Fe concentration in the supernatant was determined by atomic absorption spectrometry to examine the stepwise precipitation of B1-c.

Stability of Fe(II) in Fe Complexes to Oxidation in Aqueous Solution——The stability of Fe(II) in two Fe fractions (B1-c, B2) to oxidation at various pH values was examined. An aqueous solution of each unlabeled fraction (20 μg Fe/ml) was used without pH adjustment (pH 5.5 for B1-c, pH 4.0 for B2). Separately, Tris–HCl (pH 7.0) at a final concentration of 50 mM or borax buffer (pH 10.0) at 25 mM was added to the aqueous solution of each fraction, and the pH of the solution was adjusted to 7.0 or 10.0 by dropwise addition of dilute NaOH solution with an Iwaki pH meter (model 225). As control solutions, $FeSO_4$ solution was similarly adjusted to pH 5.5, 7.0 and 10.0 with phthalate (pH 5.5; final concentration, 25 mM), Tris–HCl (pH 7.0; 50 mM) and borax buffer (pH 10.0; 25 mM). Each test or control solution was adjusted to the ionic strength of 0.08 with NaCl and aerated with air (60 ml/min) for 10 min, and 2-ml portions were taken into 3-ml plastic syringes. After removal of the gas phase, the tip of each syringe was tightly sealed with a paraffin film, and the syringe was incubated at 25 °C. After 2, 9 and 18 d of incubation, 0.5-ml samples were taken from each syringe and centrifuged at 10000 rpm for 10 min to obtain portions of the supernatants. The concentration of Fe contained in the soluble Fe(II) form was determined by the bathophenanthroline method.[16]

pH Dependency of Fe Solubility in Aqueous Solutions of Fe Complexes——^{59}Fe-labeled Fe fractions A, A2, A3, B, B1-c and B2 were used. ^{59}Fe–Fe compound ($FeCl_3$, ferric citrate, $FeSO_4$, ferrous orothonate, ferrous ascorbate) solutions were prepared as reported previously[15] and used as the control solutions. Phthalate (pH 3—7), Tris–HCl (pH 7—9) or borax buffer (pH 9—11) was added to each test or control solution at a final concentration of 25 mM (phthalate, borax buffer) or 50 mM (Tris–HCl). The pH of the solution was adjusted to 3—11 by dropwise addition of

dilute NaOH solution with the Iwaki pH meter, and the ionic strength was adjusted to 0.08 with NaCl. The solution thus prepared was taken in a volume of 2 ml (1 μCi/40 μg Fe/ml) and centrifuged at 10000 rpm for 30 min. ^{59}Fe radioactivities in the supernatant and precipitate were determined with an Aloka auto-gamma-well scintillation system. The results were expressed as percentage of ^{59}Fe solubility.

Characterization of Bl-c——IR spectrometry was performed with untreated, methylated, and HCl-treated samples of Bl-c by the KBr disc method. The instrument used was a JASCO FTS-20 infrared spectrophotometer (Fourier transform type). The methylation of Bl-c was carried out by Hakomori's method,[17] and the methylated product was used after purification through a Merck silica gel 7734 column. The HCl-treated sample was prepared as follows. An aqueous solution of Bl-c was adjusted to pH 1 by the dropwise addition of 1 N HCl and allowed to stand at room temperature for 30 min. This solution was neutralized with silver carbonate, and the supernatant of the neutralized solution was concentrated under reduced pressure and used. The UV and visible spectra were determined with a Hitachi auto-recording spectrometer (model 200), and the elementary analysis (for C, H and N) was performed with a Perkin-Elmer 240 elementary analyzer. Colorimetry of oligosaccharide in Bl-c was carried out by the anthrone method,[18] and the amount of reducing sugars was determined, after hydrolysis of Bl-c in 1 N H_2SO_4 at 85 °C, by the Somogyi–Nelson method.[19]

Results

Physicochemical Properties of Fe Complexes

Paper Electrophoresis——Figure 2 shows the relative mobilities of four Fe fractions (A1, A2, Bl-c, B2) when the mobility of an inorganic Fe(II) salt ($FeSO_4$) was taken as -1.0. All Fe fractions differed in electrophoretic behavior from inorganic Fe(II) and Fe(III) salts, with relative mobilities varying from fraction to fraction. Of the four Fe fractions, A1 and A2 migrated to the anode at pH 3.8 and 5.6, in contrast to the inorganic Fe(II) and Fe(III) salts. These fractions moved to the cathode at pH 2.0, where the behavior of A1 was consistent with that of the Fe(II) salt. Bl-c migrated to the anode in the pH range of 2.0—5.6; the order of its

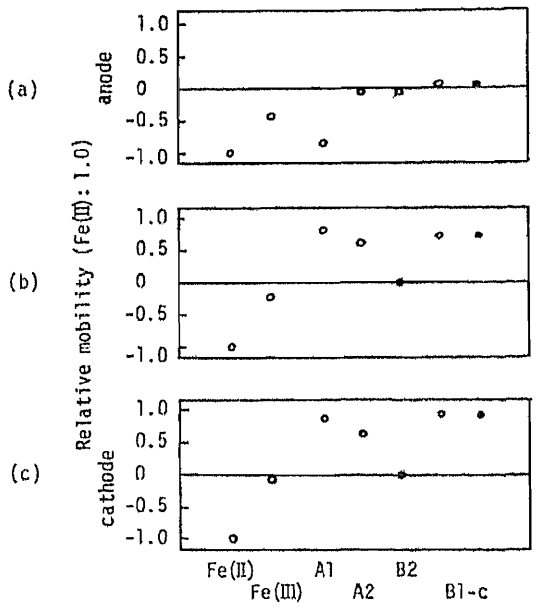

Fig. 2. Paper Electrophoresis of Fe Complexes Separated from Wine

Electrophoretic conditions: stationary phase, Toyo filter paper (No. 50); solvent, (a) 0.3 M formic acid-acetic acid buffer (pH 2.0), (b) 0.2 M acetic acid–Na acetate buffer (pH 3.8), and (c) 0.2 M acetic acid–Na acetate buffer (pH 5.6). Fe (open spot) was detected by spraying with *o*-phenanthroline–ethanol solution, and saccharides (closed spot), by spraying with an alkaline solution of silver nitrate.

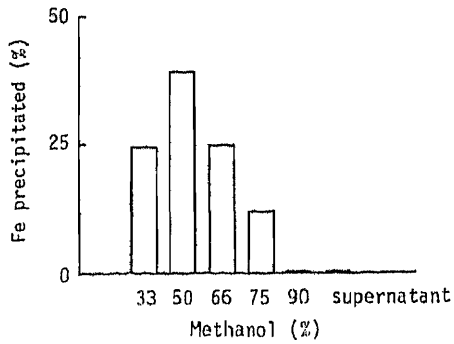

Fig. 3. Stepwise Precipitation of Fe in Methanol–H_2O Mixture of Bl-c

Methanol was added stepwise to an aqueous solution of Bl-c (40 μg Fe/ml). A portion of the mixture taken at each step was centrifuged at 10000 rpm for 10 min at 4 °C, and the Fe concentration in the supernatant was determined by atomic absorption spectrometry.

relative mobility was pH 5.6>3.8>2.0. At pH 2.0, the relative mobility of B1-c was less than 0.1. B1-c was further examined for saccharides, since the previous study had suggested that it might be a complex containing saccharides; a single spot was detected at pH 2.0—5.6, and this spot coincided with the Fe spot at all pH values. B2 moved slightly toward the cathode at pH 2.0, but it remained at the original position at pH 3.8 and 5.6.

Solubility in Organic Solvents——Attempts were made to extract Fe from the four Fe fractions (A1, A2, B1-c, B2) with three organic solvents. When diethyl ether and isopropanol were used as the solvents, Fe was not extracted from any fraction. In methanol, A1, A2, and B1-c were not dissolved. In contrast, 80.7% of Fe in B2 passed into methanol, and almost all the Fe in B2 was found as Fe(II) in the solvent.

Figure 3 presents the precipitation pattern of Fe in the aqueous solution of B1-c (40 μg Fe /ml) to which methanol was added stepwise. The amount of Fe precipitate was the largest in the 50% alcohol mixture, and the total amount of Fe precipitate in the 33, 50 and 60% alcohol mixtures accounted for 88% of the total Fe used. These precipitates were dissolved by the addition of water, and 90% or more of the Fe in this solution was present in the Fe(II) form.

Stability of Fe(II) in Fe Complexes to Oxidation——The pH values of fractions B1-c and B2 (20 μg Fe/ml) separated by column chromatography were 5.5 and 4.0, respectively. Table I presents the Fe(II) solubility in these fractions when they were incubated at 25 °C, without pH adjustment or after pH adjustment to 7.0 and 10.0.

In the B1-c sample in which the pH was not adjusted (pH 5.5), the Fe(II) concentration remained nearly unchanged from the initial value even after 18 d of incubation; thus, the sample was fairly stable to Fe oxidation. The B1-c samples adjusted to pH 7.0 and 10.0 were also stable, containing 85.2 and 72.8%, respectively, of Fe as Fe(II) after 18 d. In contrast, the B2 samples and the control ($FeSO_4$) solutions were relatively unstable. In the B2 sample of pH 4.0 (not adjusted), the Fe(II) concentration after 18 d of incubation was 58.8% of the initial value, and in the sample adjusted to pH 7.0, the value was 34.1%; both values were far lower than those in the B1-c samples. At pH 10.0, the value was decreased to 8.2% after 18 d. The Fe(II) concentrations in the control solution of pH 5.5 were 87.7 and 54.8%, respectively, after 2 and 18 d of incubation. When the pH was increased to 7.0 and 10.0, the concentrations after 2 d were as low as 25.2 and <0.5%, respectively.

The B1-c samples of pH 5.5 and 10.0 incubated for 18 d were applied to a Sephadex G-15 column, and their chromatographic patterns were compared with those of the unincu-

TABLE I. Stability of Fe(II) in Fe Complexes to Oxidation at 25 °C
(Percent Solubility of Fe(II))

Compound		Incubation time (d)		
		2	9	18
B1-c	5.5[a]	100.9±2.8	96.1±8.5	97.5±0.7
	7.0	90.1±1.4	92.6±0.7	85.2±1.4
	10.0	86.4±2.5	79.0±3.7	72.8±5.7
B2	4.0[a]	97.3±2.0	85.9±4.4	58.8±0.1
	7.0	66.1±3.4	38.8±5.4	34.1±0.7
	10.0	15.3±4.1	11.8±6.1	8.2±0.7
$FeSO_4$	5.5	87.7±3.0	71.0±8.2	54.8±0.3
	7.0	25.2±3.8	16.1±1.8	8.4±0.8
	10.0	<0.5	<0.5	<0.5

Each value is the mean±S.E. ($n=3$). The sample concentration used was 20 ppm as Fe(II). *a*) The pH was not adjusted.

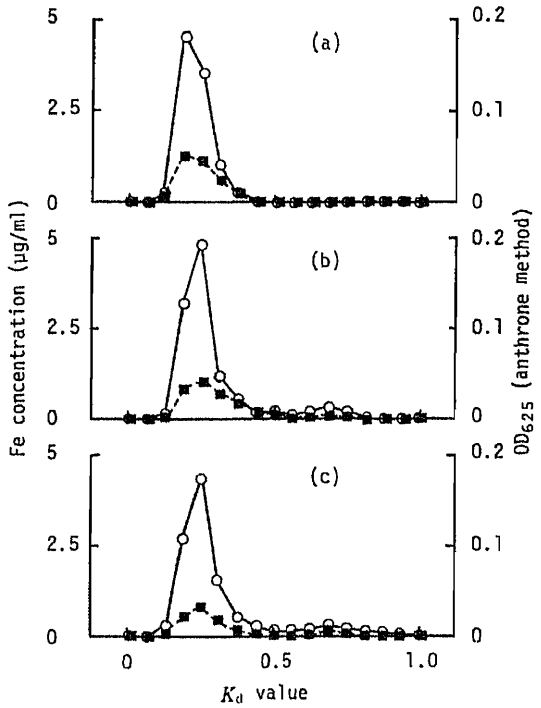

Fig. 4. Gel Filtration on Sephadex G-15 of Bl-c Incubated at 25 °C for 18 d

(a) Unincubated sample (pH 5.5); (b) incubated sample (pH 5.5); and (c) incubated sample (adjusted to pH 10.0).

○, Fe concn.; ■, OD_{625}/ml determined after reaction by the anthrone method with 1 ml of each fraction (the value represents a relative saccharide concentration).

Chromatographic conditions: column size, 1.5 cm i.d. × 52 cm; solvent, H_2O; flow rate, 20 ml/h.

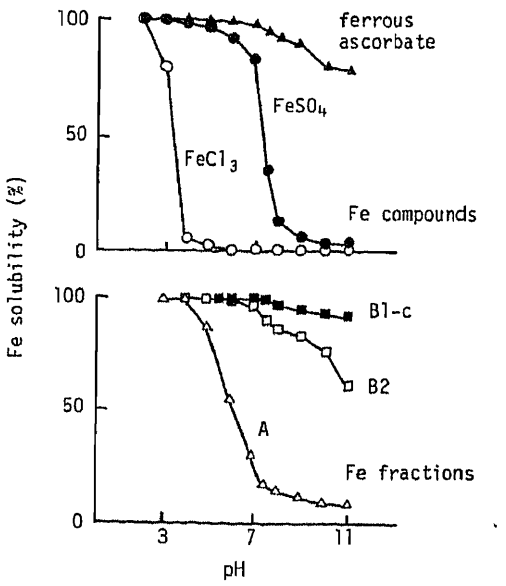

Fig. 5. Effects of pH on Fe Solubility in Aqueous Solutions of ^{59}Fe-Labeled Fe Fractions and Compounds (40 μg Fe/ml)

After pH adjustment, each solution was centrifuged at 10000 rpm for 30 min, and the Fe concentration in the supernatant and precipitate were determined.

bated sample (Fig. 4). Both incubated samples showed a small peak around the K_d value of 0.6 which did not appear on the chromatogram of the unincubated sample. However, the chromatograms of these incubated samples showed a main Fe peak at almost the same K_d (about 0.2) as in the case of the unincubated sample, showing that more than 90% of Fe was eluted.

pH Dependency of Fe Solubility in Aqueous Solution——Figure 5 shows the changes in Fe solubility in aqueous solutions of ^{59}Fe-labeled Fe fractions (A, Bl-c, B2) (40 μg Fe/ml) when the solutions were adjusted to pH 3—11; the results with the concentrated wine containing ^{59}Fe–Fe complexes and the ^{59}Fe–Fe compounds (ferrous ascorbate, $FeSO_4$, $FeCl_3$) are also shown in the figure.

In the inorganic Fe salt solutions, the Fe solubilities sharply decreased over pH 3 ($FeCl_3$) or pH 7 ($FeSO_4$). Ferrous ascorbate was soluble even in the alkaline range, and at pH 11, the solution contained 79% of ^{59}Fe in the soluble form. In the solution of fraction A separated from wine, the Fe solubility markedly decreased over pH 4, and the ^{59}Fe concentration at pH 11 was only 8% of the initial value. In contrast, Bl-c and B2 were stable in the alkaline range, and Bl-c showed Fe solubility as high as 90% even at pH 11.

The pH of the small intestine in the rat, a major Fe absorption site, is 6—8.[4,20] On the assumption that the small intestinal pH is 7.5, the correlation was examined between the Fe solubility in various Fe compound solutions at this pH and the gastrointestinal Fe absorption after oral administration of the compounds. The Fe solubility of the test compounds increased

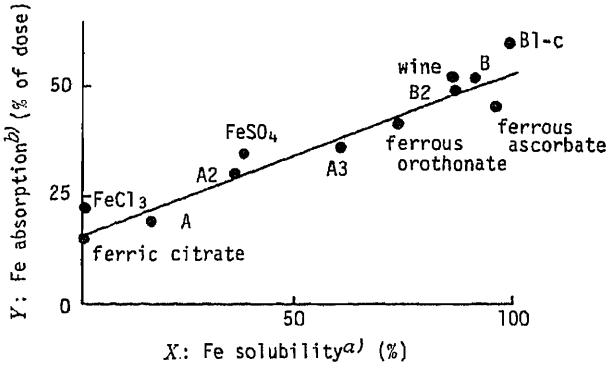

Fig. 6. Correlation between Fe Absorption and Solubility (pH 7.5)

a) Fe solubility in aqueous solutions of Fe compounds (40 μg Fe/ml) at the rat small intestinal pH (assumed to be 7.5); *b*) gastrointestinal Fe absorption in rats after oral administration of Fe compounds (250 μg Fe/kg) (the values are the means for 3—7 animals; data from the previous study[15]).
$Y=0.40X+15.7$, $r=0.924$.

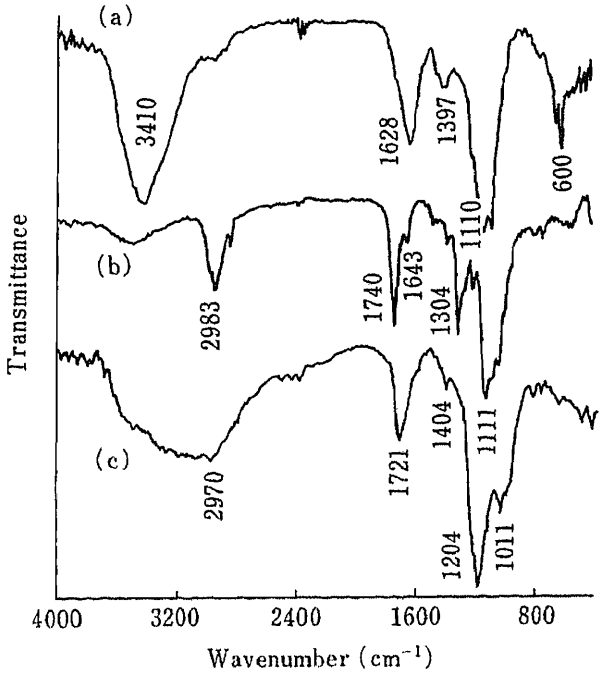

Fig. 7. Infrared Spectra of Bl-c (KBr Disc Method)

(a) Intact sample; (b) methylated sample; (c) HCl-treated sample.

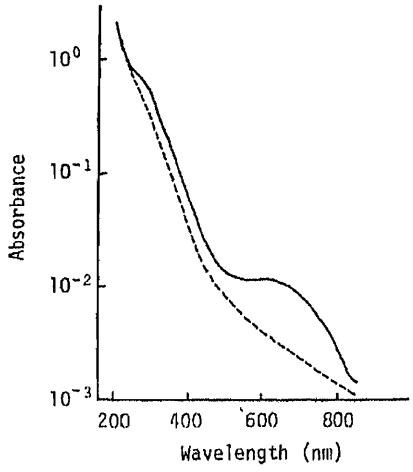

Fig. 8. Ultraviolet and Visible Spectra of Bl-c

Sample concentration in water: 8 μg Fe/ml. — —, Intact sample (pH 5.5); ------, sample adjusted to pH 1.0 with HCl.

in the following order: Fe(III) salts < fraction A < Fe(II) salts ($FeSO_4$, *etc.*) < ferrous ascorbate, concentrated wine and Fe(II) complexes (Bl-c, B2). When these ^{59}Fe-labeled Fe compound solutions (40 μg Fe/ml) were orally given to rats, the gastrointestinal Fe absorption was found to increase in almost the same order.[15] As shown in Fig. 6, a linear regression line was computed, and the correlation coefficient (r) was as high as 0.924.

Characterization of Bl-c

IR Spectrum——The IR spectra of Bl-c and its methylated and HCl-treated products were compared (Fig. 7). Bl-c had the following characteristic absorptions: an O–H stretching vibration around 3400 cm^{-1}, a C=O stretching vibration around 1630 cm^{-1}, a band at

1400 cm^{-1}, and a broad band at 1000—1200 cm^{-1}. In the spectrum of the methylated product, the absorption band due to the O–H stretching vibration nearly disappeared, and instead, the absorption due to the C–H stretching vibration around 2900 cm^{-1} was increased. The carbonyl absorption band was shifted from 1630 to 1740 cm^{-1} by methyl esterification of the carboxyl group. In addition, a strong absorption appeared at 1304 cm^{-1}, but the absorption bands at 1000—1200 cm^{-1} remained almost unchanged. Fe was cleaved from Bl-c by the methylation, so that no Fe was detected in the extract from the methylated Bl-c sample. The Fe cleavage from Bl-c also occurred on treatment with HCl, and the supernatant of the HCl-treated product after neutralization with silver carbonate contained no Fe. This was reflected in the spectrum by the shift of the carbonyl absorption band from 1630 to 1721 cm^{-1}.

UV and Visible Spectra——The UV and visible spectra of an aqueous solution of Bl-c adjusted to pH 1 with HCl were compared with those of the nonadjusted solution (Fig. 8). The spectrum of the intact Bl-c sample showed a weak shoulder around 270 nm and a maximum absorption at 610 nm, while the Bl-c solution adjusted to pH 1 had no such shoulder or maximum absorption.

Elementary Analysis——Table II shows the C, H and N contents of Bl-c together with the atomic ratios of the respective elements. The C, H and N were determined by the

Table II. Elementary Analysis of Bl-c

Element	% by weight	Atomic ratio[a)]
C	41.21	1.000
H	5.95	1.721
N	0.31	0.006
O	50.76	0.925
Fe	3.29	0.017

The C, H and N contents were determined with an elementary analyzer (Perkin-Elmer type 240), and the Fe content was obtained by atomic absorption spectrometry. The O content was obtained by subtracting the other elemental weights from the sample weight. *a)* Relative number of atoms when the number of C is taken as 1.000.

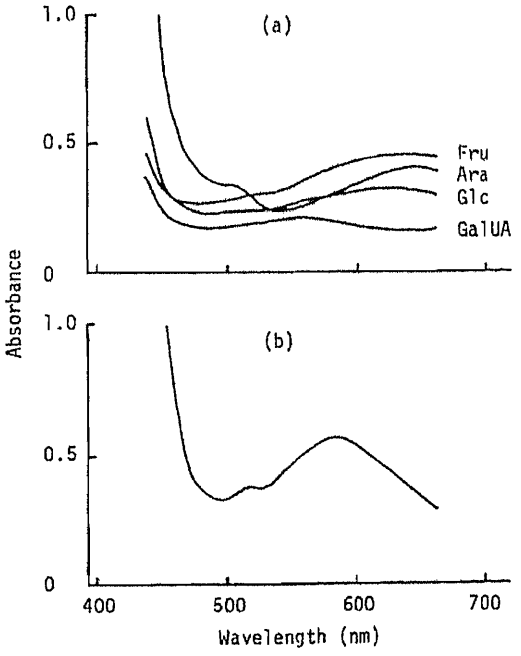

Fig. 9. Visible Spectra of Bl-c and Monosaccharides Determined at 1 h after Reaction by the Anthrone Method

(a) Monosaccharides, 0.2 μmol/sample Fru=fructose, Ara=arabinose, Glc=glucose, GalUA=galacturonic acid; (b) Bl-c, 50 μg Fe/sample.

TABLE III. Determination of the Increase in Reducing Power of B1-c in 1 N H_2SO_4 on Incubation at 85 °C

Item	Incubation time (h)			Blank value
	0	1	3	
Reducing power (A)[a]	0.149	0.468	0.580	0.032
Saccharide content (B)[b]	0.632	0.534	0.480	0.050
A/B[c]	0.201	0.901	1.274	—

Sample (B1-c) amount: 50 g Fe. *a*) Values are expressed as OD_{660} (Somogyi-Nelson method). *b*) Values are expressed as OD_{625} (anthrone method). *c*) Calculated from A and B, corrected by subtraction of the respective blank values.

combustion method, and the Fe, by atomic absorption spectrometry. B1-c contained neither S nor Cl when examined qualitatively by reaction with lead acetate and silver nitrate, respectively, and so its O content was obtained by subtraction of the other elemental weights from the sample weight. When the number of C atoms was taken as 1.000, the relative numbers of the other atoms were as follows: H, 1.721; O, 0.925; Fe, 0.017; and N, trace.

Determination of Saccharides by Colorimetric Method——Figure 9 shows the visible absorption spectra of B1-c and various monosaccharides allowed to stand at room temperature for 1 h after reaction by the anthrone method. The monosaccharides used showed the following characteristic maximum absorptions, as reported[21]: aldohexose (glucose) and hexose (fructose), at 620—630 nm (green); aldopentose (arabinose), at about 510 and 650 nm (brown); and uronic acid (galacturonic acid), at about 560 nm (purple). B1-c showed a spectrum combining the characteristics of several monosaccharides; that is, B1-c developed a brownish green color, with maximum absorptions at about 510 and 590 nm.

Table III shows the amount of reducing sugars of B1-c in 1 N H_2SO_4 incubated at 85 °C. The amount of reducing sugars (A) increased as B1-c was hydrolyzed by H_2SO_4, and reached about 5 times the initial value at 3 h after the start of incubation. The total saccharide content (B) in the mixture, which was concurrently determined by the anthrone method, showed a tendency to decrease with time. The amount of reducing sugars per unit of saccharides (A/B) at 3 h was about 6 times the initial value.

Discussion

It has been qualitatively shown that most Fe in wine is present as organic complexes.[22,23] Our previous[15] and present studies showed that the paper chromatographic and electrophoretic behaviors of the four main Fe fractions separated from wine (A1, A2, B1-c, B2) were different from those of inorganic Fe(II) and Fe(III) salts, suggesting that they may be Fe(II) complexes having different properties. When these Fe complexes were orally given to rats in the previous study,[15] Fe absorption and incorporation into hemoglobin varied greatly from complex to complex, and B1-c showed the highest activities. This variation was presumed to be due to the differences in physicochemical properties.

One of the most important factors affecting the gastrointestinal Fe absorption is the solubility of an Fe compound in the small intestine, a major Fe absorption site,[1-3] and many reports[4,8-12] have shown that a variety of ligands (such as sugars, amino acids and ascorbic acid) which form complexes with Fe increase Fe solubility at the small intestinal pH and promote Fe absorption. In the present study, in contrast to inorganic Fe salts and fractions A1 and A2, the Fe(II) complex obtained as fractions B1-c and B2 showed high Fe solubility at alkaline pH, and the Fe solubility of B1-c had a close correlation with the Fe absorption

therefrom. This suggests that the variation in Fe absorption among the Fe complexes and salts can be explained by the differences in Fe solubility at the physiological pH. Furthermore, B1-c was found, by electrophoresis and stability examination, to retain its structure as a complex even at acid and alkaline pH and to be stable to Fe oxidation. These findings suggest that when an Fe complex such as B1-c is given orally, the structure of complex may be relatively stable to the pH changes from the stomach to the small intestine and to contact with various oxidants in the intestine. In addition, the Fe in the complex is considered to be little affected by endogenous or exogenous ligands (phosphoric acid, phytic acid, proteins, *etc.*) that tend to inhibit Fe absorption.[1-3,13,14] Accordingly, these luminal factors seem to be important for the high Fe absorption from B1-c.

The present study showed that there were differences in Fe absorption among the Fe compounds having approximately 100% Fe solubilities at pH 7.5. This result suggests that mucosal factors such as the Fe transport mechanism in the intestine and the kinetic parameters of the transport also affect the Fe absorption from the Fe complexes. A further study on the process of Fe transport in the intestine will be necessary to elucidate the reasons for the high activity of the wine-derived Fe complex, B1-c, in Fe absorption.

When B1-c was examined by electrophoresis, Fe and saccharides were each detected as a single spot and coincided completely. These results indicate that B1-c might be a single complex of Fe(II) and saccharides. The ligand of this complex was considered to be an oligosaccharide having an alcoholic hydroxyl group and a carboxyl group, from the IR spectra of the intact B1-c sample and its methylated and HCl-treated products. In addition, the elementary analysis of B1-c showed that it had a C:H ratio of 1:1.7. When the value obtained by subtracting the total percent weight of C, H, N and Fe contents from 100 was taken as the O content (50.76%), the C:H:O ratio was about 1:1.7:1. This result also suggests that the ligand is an oligosaccharide. The colorimetric results and the increase in amount of reducing sugars after hydrolysis of B1-c suggest that this oligosaccharide may be composed of heterogenous monosaccharides and have a polymerization degree of over 6. Thus, the results support our previous estimate that the molecular weight of B1-c might be about 1500.[15]

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Study on the Immunomodulating Activities of Hot Water Extracts of Sclerotia of *Sclerotinia sclerotiorum* IFO 9395 Strain Cultured on Several Media

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Sclerotinia sclerotiorum IFO 9395 strain produces "sclerotia" on agar media. The immunomodulating activities of the non-dialyzable fraction of hot water extract of sclerotia (TSHW) grown on six kinds of agar media (*i.e.*, malt (M), potato-sucrose (PS), potato-dextrose (P), YpSs (Y), Leonian-yeast extract (L) and malt-yeast extract (MY)) were comparatively studied. The sclerotial formation was strongly affected by the composition of the media. The yield was the highest on PS agar (26.8 g as average dry weight of sclerotia per 100 agar plates), and the lowest on MY agar. However, the yield, composition (protein *ca.* 30%, carbohydrate *ca.* 60%, phosphate (<0.4%)), carbohydrate components (glucose and mannose in a molar ratio of 1.0: *ca.* 0.5) and amino acid composition of TSHW were similar on all the media. The results of methylation analysis indicated that all the TSHW preparations possessed quite similar branched β-1,3-glucan structures. These TSHW preparations showed various immunomodulating effects (mitogenic, polyclonal B-cell activating (PBA), reticuloendothelial system (RES)-activating, and antitumor activities). Antitumor and RES activation activities of TSHW were quite similar regardless of the difference of culture media, while mitogenic and PBA activities varied somewhat. These results suggest that the sclerotia contained immunomodulating material(s), the production of which was affected, in part, by the composition of the culture media.

Keywords——*Sclerotinia sclerotiorum*; immunomodulating material; sclerotia; mitogenic activity; β-1,3-glucan; antitumor activity

Introduction

Immunomodulators extracted from bacteria, such as lipopolysaccharide (LPS), peptidoglycans, and amphipathic polymer derived from gram-positive bacteria,[1] have been investigated in many laboratories. In the case of LPS, most of its activities originate from the lipid A moiety. Lipid A and various analogues have already been synthesized, and structure–activity relationships have been investigated.[2] Structure–activity relationships of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), which is the minimal structure of the bacterial cell-wall peptidoglycans required for immunoadjuvant activitiy, have also been extensively examined by using chemically modified analogues.[3] On the other hand, the antitumor glucans from several fungi, such as lentinan from *Lentinus edodes*,[4] schizophyllan (SPG) from *Schizophyllum commune*,[5] and Krestin (PS-K) from *Coriolus versicolor*,[6] have been applied clinically.

Previously, we have examined the immunomodulating activities of extracts from fungal fruit bodies, and found that they contained mitogens and polyclonal B cell activators, as well as antitumor polysaccharides.[7] From the results of purification and chemical modification studies of the mitogenic substances of the hot water extract from the fruit body of a fungus, *Peziza vesiculosa*, it was suggested that polypeptides having molecular weight and charge heterogeneities showed mitogenic activity.[8] Antitumor glucans have also been isolated from the alkali extract of this fungus.[9]

Some kinds of fungi form sclerotia, which is the endurance type of plectenchyma of some plant pathogenic fungi. Marukawa *et al.* have studied the chemical factors affecting the development of sclerotia of *Sclerotinia libertiana* FUCKEL, and suggested that both chemical and physical factors affected the formation of sclerotia.[10] *Sclerotinia sclerotiorum* IFO 9395, which belongs to Ascomycotina, Discomycetes, Helotiales, also produced sclerotia when it was cultured on agar media. We wish to know whether the extract of sclerotia showed immunomodulating activity comparable to that of the extracts of fungal fruit body. In this paper, we used six kinds of agar media to obtain sclerotia and compared the chemical properties and immunomodulating activities of their hot water extracts.

Materials and Methods

Mice——Six- to 12-week-old male ICR mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka.

Materials and Microorganisms——Six kinds of agar media (Table I) were used as production media of sclerotia. Soluble starch was purchased from Sigma Chemical Co., Ltd. Malt extract was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Agar was purchased from Wako Pure Chemical Industries, Ltd. Yeast extract and Peptone were purchased from Daigo Eiyo Kagaku Co., Ltd. Portions of about 20 ml of these media were plated into sterilized 9-cm diameter dishes. The mycelia of *Sclerotinia sclerotiorum* IFO 9395 was obtained from the Institute for Fermentation, Osaka, Japan (IFO), and cultured at 25 °C in our laboratory. The sclerotia produced on each agar medium were picked up after about 4 weeks of culture, and lyophilized. Grifolan NMF-5N was prepared from *Grifola frondosa* as described previously.[11] Heat-killed cells of *Propionibacterium acnes* C7 were kindly provided by Kowa Co., Tokyo. *Escherichia coli* 055 : B5 prepared by a phenol–water extraction method was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Sepharose CL-4B and diethylaminoethyl (DEAE)-Sephadex A-25 were purchased from Pharmacia Fine Chemicals.

Preparation of the Hot Water Extract of Sclerotia (TSHW)——Sclerotia (5 g each) grown on six kinds of agar media were disrupted to form a suspension in water (about 200 ml), and then extracted in an autoclave (1 h). The resulting suspension was separated into the extract and the residue by centrifugation. This extraction was repeated three times. The combined extracts were dialyzed against distilled water and the non-dialyzable fraction was recovered by lyophilization.

Immunomodulating Activities——Mitogenic activity,[12] polyclonal B-cell activating (PBA) activity[13] reticuloendothelial system (RES) activation activity,[14] and antitumor activity[15] were assessed by the methods described previously.

Chemical Analysis——Carbohydrate, protein, and phosphate contents were determined by the phenol–H_2SO_4 method,[16] Lowry–Folin method,[17] and Chen, Toribara and Warner method,[18] respectively. Component sugars were determined by gas-liquid chromatography (GLC) as alditol acetates. Methylation analysis was performed by the method of Hakomori.[19] Amino acid analysis was performed in a Hitachi L-8500 amino acid analyzer after hydrolysis in 6N HCl at 110 °C for 24 h.

Results

Chemical Properties of the TSHW Grown on Six Kinds of Media

We obtained TSHW (TSHW-M, TSHW-PS, TSHW-P, TSHW-Y, TSHW-MY, TSHW-L) from six kinds of agar media. First, several quantitative and qualitative analyses were performed (Table II-1). The yield of sclerotia per 100 agar plates varied markedly. The sclerotial production was the highest on the potato-sucrose (PS) agar (26.8 g), followed by potato-dextrose (P), YpSs (Y), malt (M), Leonian-yeast extract (L), and malt-yeast extract (MY) agar media. The yield (g) from PS was 50-fold higher than that from MY. These results indicated that the production of sclerotia depends on the composition of the culture medium, and potato extract is suggested to be effective for producing sclerotia.

Sclerotia suspended in distilled water were disrupted with a homogenizer and then extracted by autoclaving. The yield (%) of the TSHW was similar in each medium (Table II-1). Though the chemical properties (carbohydrate, protein and phosphate contents, and component sugars) of the TSHW preparations varied slightly depending on the culture media, all of them were composed of protein *ca.* 30%, carbohydrate *ca.* 60% and a trace amount of

phosphate (<0.4%). The major carbohydrate components of TSHW preparations were glucose (Glc) and mannose (Man) in all cases. However, the molar ratio of Man/Glc varied from 0.2 to 0.6 depending on the culture media. Methylation analysis showed three major peaks corresponding to the authentic alditol acetates of 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4,-di-*O*-methylglucose in a molar ratio of 1: *ca.* 1.1: *ca.* 0.8. Previously, Kitahara and Takeuchi demonstrated that the main carbohydrate constituent of sclerotia of *Sclerotinia libertiana* is a glucan mainly containing β-1,3 linkages.[20] From the ultrastructural aspects of sclerotia of *S. sclerotiorum*, Saito suggested the presence of β-1,3-glucans in medullary hyphae within sclerotia.[21] These results suggested that the main carbohydrate constituent of the TSHW is a β-1,3-glucan having a branch at C-6 of every other main chain glucosyl unit. Amino acid compositions of TSHW obtained from the six kinds of media were similar to each other, and all TSHW contained a large amount of aspartic acid and glutamic acid and/or their amides (Table II-2). Previously, we have demonstrated that the main amino acids of the mitogenic substances (VGM) in the hot water extract (vesiculogen) of *P. vesiculosa*, which also belongs to Ascomycotina, are Ser, Gly, Glu and Ala,[8] and we suggested that some anionic groups might be involved in the mitogenic activity of vesiculogen. Concerning these facts, it is interesting to know the participation of aspartic acid and glutamic acid in the mitogenic activity of TSHW.

The above results indicate that the production of the sclerotia is affected by the culture media, but the yield and chemical properties of the TSHW are only slightly affected.

TABLE 1. Agar Media Components

Malt (M) agar[a]	Malt extract	25 g
	Agar	20 g
	Distilled water	1 l
Potato-sucrose (PS) agar	Potato[b]	about 300 g
	Sucrose	20 g
	Agar	20 g
	Distilled water	1 l
Potato-dextrose (P) agar[c]	Potato extract	200 g
	Glucose	20 g
	Agar	15 g
	Distilled water	1 l
YpSs (Y) agar	Yeast extract	4 g
	Soluble starch	15 g
	K_2HPO_4	1 g
	$MgSO_4 \cdot 7H_2O$	0.5 g
	Agar	20 g
	Distilled water	1 l
Malt-yeast extract (MY) agar	Malt extract	20 g
	Yeast extract	2 g
	Agar	20 g
	Distilled water	1 l
Leonian-yeast extract (L) agar	KH_2PO_4	1.25 g
	$MgSO_4 \cdot 7H_2O$	0.625 g
	Peptone	0.625 g
	Maltose	6.25 g
	Malt extract	6.25 g
	Yeast extract	3.00 g
	Agar	20.00 g
	Distilled water	1 l

a) Malt agar was purchased from Nissui Seiyaku Co., Ltd. *b*) About 300 g of sliced potatoes was boiled in 1 l of distilled water for 30 min and the supernatant was obtained by filtration through gauze. *c*) Potato-dextrose agar was purchased from Eiken Chemical Co., Ltd.

TABLE II-1. Physicochemical Properties of TSHW Obtained from Some Agar Media

	Media[a]					
	M	PS	P	Y	MY	L
Sclerotia						
Dry weight (g)/ 100 agar plates	6.5	26.8	17.8	7.7	0.5	6.3
TSHW-						
Yield (%)	14±3	12±5	10±4	13± 5	15±3	10± 3
Protein (%)[b]	29±7	38±6	27±5	31±11	38±5	34±10
Carbohydrate (%)[c]	63±7	59±5	63±9	55±10	57±5	56± 9
Phosphate (%)[d]	<0.3	<0.4	<0.4	<0.4	<0.4	<0.3
Component sugars[e]						
Glucose	1.00	1.00	1.00	1.00	1.00	1.00
Mannose	0.6±0.3	0.4±0.1	0.4±0.2	0.2±0.1	0.5	0.6±0.2
Galactose	<0.1	<0.03	<0.04	<0.03	<0.03	<0.04
Methylpentose	<0.1	<0.03	<0.05	<0.02	<0.04	<0.03

a) See Table 1 and text. *b*) Lowry–Folin method[17] as bovine serum albumin. *c*) Phenol–H_2SO_4 method[16] as glucose. *d*) Chen, Toribara and Warner method[13] as phosphoric acid. *e*) Determined as alditol acetate derivatives by GLC.

TABLE II-2. Amino Acid Compositions[a] of TSHW Obtained from Some Agar Media

Amino acids	TSHW-					
	M	PS	P	Y	MY	L
Asp (Asn)	13.2[b]	15.1	14.8	14.4	14.1	14.6
Thr	9.3	7.8	9.1	8.5	8.8	6.2
Ser	7.7	5.9	7.9	7.1	7.4	5.0
Glu (Gln)	11.8	12.9	10.2	13.2	13.2	11.6
Gly	5.7	5.8	6.0	5.8	5.6	6.1
Ala	7.8	6.1	7.1	6.3	6.2	7.5
Cys	0.9	0.9	1.2	0.9	0.6	0.7
Val	5.1	5.0	4.8	4.9	4.9	4.4
Met	4.1	3.4	5.3	2.5	2.3	9.5
Ile	3.7	4.2	3.6	3.9	3.9	4.3
Leu	6.2	5.9	6.3	7.2	7.1	6.3
Tyr	3.4	4.9	3.1	4.2	4.3	2.9
Phe	3.9	4.7	4.7	4.4	4.6	4.4
Lys	5.9	6.9	4.1	6.4	6.4	5.9
His	2.6	2.4	3.0	2.5	2.6	2.2
Arg	3.6	3.0	3.4	3.5	3.6	3.7
Pro	5.1	5.0	5.4	4.0	4.3	4.8

a) Amino acid analysis was performed in a Hitachi L-8500 amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for 24 h. *b*) Each value is the percentage of each amino acid by weight with respect to total amino acids.

Immunomodulating Activities of TSHW Obtained by Cultivation on Six Kinds of Media

To compare the immunomodulating activities of each TSHW preparation, mitogenic activity, PBA activity, RES activation activity, and antitumor activity were measured. The results on mitogenic activity are shown in Table III. Spleen cells of ICR mice were cultured with TSHW preparations for 48 h at 37 °C and tritiated thymidine (^{3}H-TdR) was added during the last 20 h of the culture. Every TSHW preparation showed significant mitogenic

activity against ICR spleen cells in a dose-dependent manner and there was little difference among preparations. However, these preparations showed little or no mitogenic activity against thymus cells. Mitogenic activity of TSHW preparations against spleen cells was comparable to that of LPS, but the thymus cell-stimulating effect was negligible as compared to that of concanavalin A (ConA, about 50-fold less). No mitogenic activity was detected from the components of the culture media (data not shown). These results suggest that every TSHW preparation contained B-cell mitogenic substances.

To elucidate the polyclonal antibody synthesis by each TSHW preparation, plaque-forming cell (PFC) response to the sheep red blood cell (SRBC) was compared with or without 500 μg of TSHW preparation *in vivo*. Each TSHW preparation was injected intraperitoneally into ICR mice, and 3 d later, the number of direct PFC, which represents the number of immunoglobulin M (IgM)-producing lymphocytes, was measured (Table IV). All TSHW

TABLE III. Mitogenic Activity[a] of TSHW Obtained from Some Agar Media

Sample	Dose (μg/culture)	Spleen cells		Thymus cells	
		Mean cpm±S.D.	S.I.[b]	Mean cpm±S.D.	S.I.[b]
TSHW-M	0.4	23307± 376	3.66	4357± 963	1.77
	2	38298± 505	6.02	5088± 894	2.07
	10	71207±3086	11.18	5395± 30	2.20
	50	69630±7474	10.94	6047± 39	2.46
TSHW-PS	0.4	31145± 117	4.89	2950± 180	1.20
	2	50094±1464	7.87	5766± 472	2.35
	10	67912± 685	10.67	7728± 132	3.15
	50	77379±7592	12.15	8045± 89	3.28
TSHW-P	0.4	16186± 238	2.54	3706± 696	1.51
	2	32785± 636	5.15	4124± 861	1.68
	10	51143±2348	8.03	5718± 605	2.33
	50	60718±1141	9.54	6008± 223	2.45
TSHW-Y	0.4	19216± 534	3.02	4274± 787	1.74
	2	37556± 366	5.90	5298± 186	2.16
	10	55867±2015	8.77	8474± 402	3.45
	50	56635±2810	8.90	8268± 263	3.37
TSHW-MY	0.4	28499±1785	4.48	3015± 245	1.23
	2	45855±1454	7.20	3664± 272	1.49
	10	69975± 808	10.99	5301± 165	2.16
	50	65466±1291	10.28	6059± 415	2.47
TSHW-L	0.4	25109± 434	3.94	2548± 178	1.04
	2	41185±3975	6.47	4343± 64	1.77
	10	68082±4128	10.69	6594± 905	2.68
	50	82118±1401	12.90	4746± 458	1.93
LPS	0.4	61515± 373	9.66	—	
	2	73433± 396	11.53	—	
	10	72513±2636	11.39	—	
	50	65459± 955	10.28	—	
ConA	0.02	—		2145± 101	0.87
	0.1	—		2269± 33	0.92
	0.5	—		81386± 5330	33.14
	2.5	—		143792±15296	58.55
Control	—	6367± 59	—	2456± 412	—

a) Spleen or thymus cells (5×10^5/culture) from ICR mice were cultured for 48 h at 37 °C in a CO_2 incubator. Twenty hours before harvesting, 0.5 μCi of tritiated thymidine (^{3}H-TdR) was added to the culture medium in a volume of 20 μl. *b*) Stimulation index (S.I.) = mean cpm in each experimental group/mean cpm in control group.

TABLE IV. PBA Activity *in Vivo* and RES Activation Activity of TSHW Obtained from Some Agar Media

Sample	Dose (μg/mouse)	Day 3 direct PFC/spleen[a]		Phagocytic index[b]	S.I.[d]
		Mean±S.D.[c]	S.I.[d]	K (Mean±S.D.)[c]	
TSHW-M	500	130±102	1.67	0.1647±0.0543[e]	2.47
TSHW-PS	500	183±140[e]	2.35	0.1429±0.0578[e]	2.14
TSHW-P	500	308±269[e]	3.95	0.1084±0.0291[e]	1.62
TSHW-Y	500	303±250[e]	3.88	0.1350±0.0464[e]	2.02
TSHW-MY	500	155± 64[e]	1.99	0.1071±0.0280[e]	1.60
TSHW-L	500	138± 69	1.77	0.1047±0.0080[e]	1.57
LPS	10	536±205[f]	6.87	—	—
P. acnes	350	—	—	0.1633±0.0200[g]	2.44
Nil	—	78± 73	—	0.0668±0.0221	—

a) Arithmetic mean number of PFC±S.D. for 3 mice. *b*) Phagocytic index (K) was calculated by means of the following equation: $K=(\ln OD_1-\ln OD_2)/(t_2-t_1)$ where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. *c*) The significance (p value) of differences between the control and experimental groups was evaluated according to Student's t-test. Significant difference from the control. *d*) S.I. was calculated as the mean number of sample-injected group/mean number of saline-injected group. *e*) $p<0.05$. *f*) $p<0.01$. *g*) $p<0.001$.

TABLE V. Antitumor Effect of TSHW Obtained from Some Agar Media[a]

Sample	Dose (μg×5)	No. of mice	Tumor weight (g, mean±S.D.)	Inhibition ratio (%)[b]	Complete regression[b]
TSHW-M	100	10	0.30±0.66[c]	96	3/10
	500	10	0.30±0.63[c]	96	3/10
	1000	6	0.09±0.13[c]	99	3/ 6
TSHW-PS	100	10	0.16±0.12[c]	98	1/10
	500	9	0.10±0.09[c]	99	1/ 9
	1000	10	0.07±0.10[c]	99	5/10
TSHW-P	100	10	0.50±0.70[c]	94	4/10
	500	9	0.18±0.20[c]	98	2/ 9
	1000	7	0.24±0.40[c]	97	2/ 7
TSHW-Y	100	9	0.08±0.12[c]	99	3/ 9
	500	10	0.07±0.09[c]	99	3/10
	1000	7	0.18±0.18[c]	98	1/ 7
TSHW-MY	100	10	0.17±0.16[c]	98	1/10
	500	10	0.05±0.06[c]	99	5/10
	1000	10	0.05±0.07[c]	99	5/10
TSHW-L	100	10	0.32±0.56[c]	96	6/10
	500	9	0.15±0.11[c]	98	0/ 9
	1000	10	0.16±0.20[c]	98	1/10
Grifolan-NMF 5N	100	10	0.04[c]	>99	9/10
Nil	—	22	7.66±4.97	—	0/22

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered 5 times (days +7, +9, +11, +13, +15) as a saline solution by intraperitoneal injection. *b*) Inhibition and complete regression were determined at 35d after tumor inoculation. *c*) $p<0.001$.

preparations possessed PBA activity compared with saline-injected mice. TSHW-P and -Y showed higher PBA activity than the others. The reason for these difference is not yet clear, but it is assumed that the material(s) possessing mitogenic and PBA activities showed some qualitative and/or quantitative differences depending on the culture media used.

Next, we examined the RES-activating activity of each TSHW. RES activation was assessed by comparing the clearance time of carbon particles from the blood stream, and was expressed as the phagocytic index (K). Each TSHW preparation (500 μg) was injected i.p. at 48 h before assay. As shown in Table IV, phagocytic index was increased ($p<0.001$) by every TSHW preparation, suggesting that no significant difference was caused by the different media used.

The antitumor activity of each TSHW preparation is shown in Table V. Each TSHW preparation was injected i.p. (100, 500, and 1000 μg/mouse) into ICR mice bearing on solid form of Sarcoma 180. As a positive control, grifolan NMF-5N, which is a β-1,3-glucan branched at the C-6 of every third main chain glucosyl unit and possesses antitumor activity,[11] was used. As shown in Table V, every TSHW preparation showed strong antitumor activity (>90%), and no significant difference was seen.

These results suggested that the TSHW preparations showed marked immunomodulating activities and that there is a little difference arising from the use of different culture media, especially in the mitogenic and PBA activities.

Fractionation of TSHW–PS

As described above, the TSHW preparations showed various immunomodulating activities. However, it is not certain whether all of these activities result from only one active substance or not. Therefore, ammonium sulfate fractionation was performed at the first step. TSHW–PS was used in this experiment because it was obtained in the highest yield. TSHW–PS (1 mg/ml) dissolved in distilled water was precipitated with stepwise increases of 20% saturation of ammonium sulfate. Each precipitate was dissolved in distilled water and then dialyzed against distilled water. The recovery, chemical composition, and mitogenic activity of each fraction are shown in Table VI. Recoveries of ppt-60 (57%) and sup-100 (17%) were higher than those of other fractions (<2%). From the component sugar analysis, mannan was abundant in sup-100 and ppt-60 contained a large amount of glucan. Mitogenic activity was examined in each fraction at a dose of 2 μg/culture. The activities of fractions ppt-20 and ppt-60 were higher than that of TSHW–PS, and the activities of other fractions were weaker than that of TSHW–PS. It is suggested that the majority of the mitogenic substance(s)

Table VI. Some Chemical Properties and Mitogenic Activity of Ammonium Sulfate-Fractionated TSHW and Fractions of Ion-Exchange Chromatography-Fractionated ppt-60

	Fractions						DEAE-Sephadex A-25	
	ppt-20	ppt-40	ppt-60	ppt-80	ppt-100	sup-100	ppt-60 ads.	ppt-60 pass.
Yield (%)[a]	1	2	57	1	1	17	11	43
Protein (%)[b]	39	22	30	17	6	22	88	<1
Carbohydrate (%)[c]	26	25	59	40	49	67	18	86
Component sugars[d]								
Glucose	1.00	1.00	1.00	1.00	1.00	1.00	n.d.	n.d.
Mannose	0.53	0.22	0	0.27	0.25	9.56		
Galactose	0	0.01	0	0.08	0.11	0.13		
Methylpentose	0	0	0	0	0.03	0.10		
Mitogenic activity[e]								
S.I.	12.23	8.51	12.77	5.98	2.59	1.74	1.65	1.69

a) From 100 mg of TSHW. *b*—*d*) See Table II-1. *e*) Mitogenic activity was determined by 3H-TdR incorporation and expressed as S.I. at a dose of 2 μg/culture. In the case of ammonium sulfate-fractionated TSHW-PS, the number of mean cpm ± S.D. (S.I.) of TSHW-PS was 11618 ± 2185 (11.78) and the control value was 676 ± 28 (1.00), and in the case of ion-exchange chromatography-fractionated ppt-60, the number of mean cpm ± S.D. (S.I.) of fraction ppt-60 and the control were 16269 ± 1749 (3.83) and 4245 ± 613 (1.00), respectively.

Table VII. Antitumor Activity of Ammonium Sulfate-Fractionated TSHW Obtained from PS Agar Media[a]

Sample	Dose ($\mu g \times 5$)	No. of mice	Tumor weight (g, mean ± S.D.)	Inhibition ratio (%)[b]	Complete regression[b]
ppt-60	200	10	0.03 ± 0.08[c]	>99	5/10
sup-100	200	8	5.24 ± 4.62	32	1/ 8
Grifolan-NMF 5N	100	10	0.04[c]	>99	9/10
Nil	—	22	7.66 ± 4.97	—	0/22

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered 5 times (days +7, +9, +11, +13, +15) as a saline solution by intraperitoneal injection. *b*) Inhibition and complete regression were determined at 35 d after tumor inoculation. *c*) $p < 0.001$.

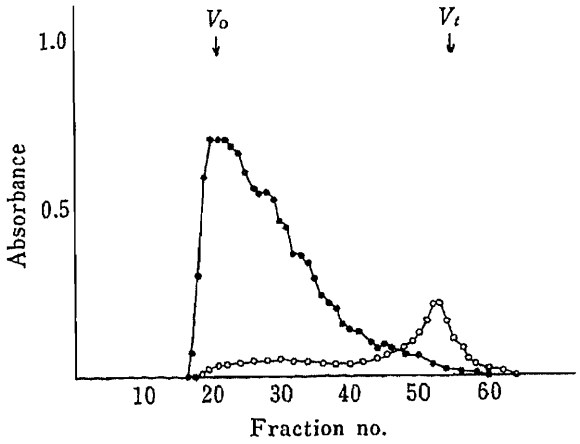

Fig. 1. Elution Profiles of Ammonium Sulfate-Fractionated ppt-60 from a Column of Sepharose CL-4B

The column (1.7 × 50 cm) was equilibrated with 8 M urea, and 14 mg of ppt-60 was applied. Fractions of 2.0 ml were collected and carbohydrate and protein were assayed by the phenol-sulfuric acid method (carbohydrate, 490 nm; —●—), and by ultraviolet absorption measurement (protein, 280 nm; —○—), respectively.

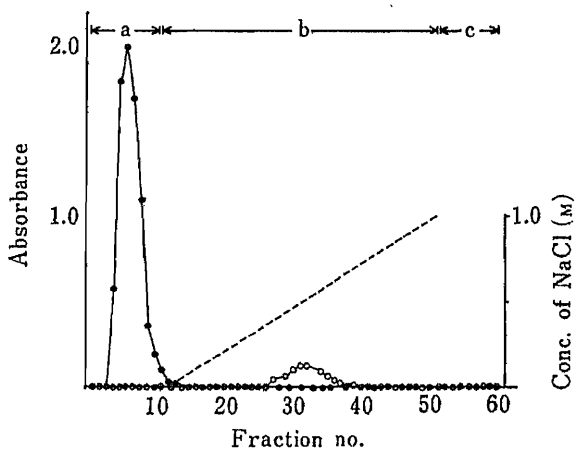

Fig. 2. Elution Profiles of Ammonium Sulfate-Fractionated ppt-60 from a Column of DEAE-Sephadex A-25 (Cl^-)

The column (25 ml) was equilibrated with 8 M urea, and 15 mg of fraction ppt-60 was applied. The column was initially eluted with the same buffer (a), then with a linear gradient (from 0 to 1 M NaCl containing 8 M urea) (b), and finally with 1 M NaCl containing 8 M urea (c). Fractions of 2.0 ml were collected and carbohydrate (490 nm; —●—) and protein (280 nm; —○—) were monitored by the phenol-sulfuric acid method and by ultraviolet absorption measurement, respectively.

was recovered from ppt-60, because the yield of this fraction is the greatest and the mitogenic activity of this fraction is the strongest. The antitumor activities of ppt-60 and sup-100 were measured, but only ppt-60 showed antitumor activity (Table VII).

To elucidate the molecular weight distribution and charge of the glucan moiety in ppt-60, gel filtration (Fig. 1) and ion-exchange chromatography (Fig. 2) were performed. Fraction ppt-60 was dissolved in 8 M urea and applied to a column of Sepharose CL-4B equilibrated with 8 M urea. As shown in Fig. 1, most of the glucan was eluted near the void volume and showed a uniform molecular weight distribution. Further, ppt-60 dissolved in 8 M urea was applied to a column of DEAE-Sephadex A-25 (Cl^-) equilibrated with 8 M urea. After being washed with the same buffer, the column was eluted with a linear gradient from 0 to 1 M NaCl containing 8 M urea. Finally, the column was eluted with 1 M NaCl containing 8 M urea. As shown in Fig. 2, almost all of the carbohydrate was recovered from the pass-through frac-

tion. These data suggest that the glucan moiety in ppt-60 has a uniform molecular weight distribution and has no charge.

In methylation analysis of ppt-60, 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose and 2,4-di-*O*-methylglucose were detected in a molar ratio of 1.0 : 1.4 : 1.1. The configuration of the glucan should be β for the reasons given in the above section. These facts suggested that the TSHW contained a β-1,3-glucan having a branch at C-6 of every other (or two in every five) main chain glucosyl unit.

Further, the mitogenic activities of the pass-through and adsorbed fractions on DEAE-Sephadex A-25 were measured. These fractions showed no mitogenic activity, but the reason for this is not clear. It is assumed that mitogenic substance(s) might bind firmly to the DEAE function and not be eluted with 1 M NaCl. These facts suggested that most of the glucan, which is neutral and has antitumor activity, did not show mitogenic activity.

Discussion

It would be interesting to know how many materials in fungi can modulate immune systems, and why these materials show such activities. Previously, we found that the hot water extract of fungal fruit bodies possesses several immunomodulating activities. In the previous paper, we showed that the hot water extract of the sclerotia contained B-cell mitogenic, polyclonal B-cell-activating, antitumor-active, and/or reticuloendothelial-activating materials.[22] However, it is not clear what factors affect the production of these materials. In this paper, we tried to clarify whether the immunomodulating materials were present in the extracts of the sclerotia in various conditions of culture. It was found that (1) the production of sclerotia was greatly influenced by the components of the media, (2) the molar ratio of Man/Glc varied from 0.2 to 0.6 and there was a high proportion of acidic amino acids, and (3) TSHW preparations showed various immunomodulating activities, and some of the activities depended on the culture media used.

The production of sclerotia was greatly influenced by the culture media, as mentioned above. Previously, Marukawa *et al.* precisely examined the factors affecting the sclerotia production and reported that the sclerotial formation of *S. libertiana* using Czapex-Dox agar medium was the highest at pH 4.0—6.0 and at 22—25 °C in darkness.[10] They also reported a correlation between the sclerotial formation and the production of sclerin, a hormone-like metabolite.[10] It would be thought that, in our experiments, the productivity of some differentiation factors, like as sclerin, is affected by the culturing media.

It has been reported that carbohydrate components in the ethanol-soluble fraction of sclerotia grown in media containing various carbon sources (*e.g.* D-glucose, D-fructose, D-mannose, sucrose, *etc.*) varied. However, the component sugars of the hydrolysates of the ethanol-insoluble portion of sclerotia were not influenced by the carbon sources, and glucose is the major carbohydrate component.[23] These facts agree, in part, with our finding that the major carbohydrate component of TSHW is glucose. However, the Man/Glc ratio varied depending on the culture media. The fact that the ammonium sulfate fractionation of TSHW-PS gave Man and Glc from different fractions suggests that the carbohydrate moiety in TSHW-PS is not composed of glucan–mannan complex but glucan and mannan individually. Thus, it is assumed that the mannan synthesis would be mainly affected by the culture media. On the other hand, methylation data indicated that branching points existed at every other main chain glucosyl unit, suggesting that the glucan structures were not affected by the culture media. Previously, Weete *et al.* reported the amino acid composition of the water-insoluble fraction of sclerotia of *S. sclerotiorum*, and suggested that Val, Asp, Glu, Ala, Leu and Ser were the major components.[24] While they did not examine the amino acid composition of the hot water extracts of sclerotia of *S. sclerotiorum*, the major amino acids were similar to those

of TSHW preparations. Our finding that the amino acid composition was not affected by the culture media also suggested similarities of protein moiety in the sclerotia grown in the different media.

The antitumor activity against Sarcoma-180 should be owing to the β-1,3-D-glucan moiety, because the purified glucan, SSG, obtained from the liquid culture filtrate of *S. sclerotiorum* showed antitumor activity.[25] As judged from the results of methylation analysis of TSHW preparations, the essential structures of the β-glucan moiety were quite similar. Further, the antitumor activities of the TSHW preparations were quite similar. These results suggested that the antitumor glucans in these preparations were both quantitatively and qualitatively similar to each other. β-1,3-D-Glucan is known to be a skeletal component of fungi, thus, diversity of this activity was not observed.

Previously, we examined the chemical and immunological properties of mitogen(s) obtained from the hot water extracts of *P. vesiculosa*, and suggested that the mitogenic activity was owing to the polypeptide (VGM).[8] The activity was degraded by alkaline treatment. VGM showed molecular weight and charge heterogeneities. Compared with the case of TSHW, the mitogenic activity and PBA activity were moderately dependent on the culture media. The mitogenic activity of TSHW preparations was reduced by alkaline treatment (data not shown). It may be speculated that the mitogenic activity of TSHW preparations is owing to polypeptides, such as vesiculogen, and it showed some quantitative and qualitative differences depending on the culture media. In ammonium sulfate fractionation of TSHW–PS, mitogenic substance(s) were mainly recovered in fraction ppt-60. No activity was recovered from the pass-through fraction, in which antitumor glucan would be recovered, or the absorbed fraction on DEAE–Sephadex A-25. These results suggest that the mitogenic substance(s) of ppt-60 may consist of acidic amino acids having a strong negative charge. It appears to bind firmly to the DEAE group. Further investigations are in progress.

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Reinvestigation of the Modification of Nucleic Acids with Malonaldehyde

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The reaction of yeast ribonucleic acid (RNA) with malonaldehyde, a product of lipid oxidation, at pH 4 and 37 °C produced modified RNA with absorption at 325 nm and fluorescence. The fluorescence intensity was extremely low as compared with that of bovine serum albumin modified similarly. *Torula* yeast transfer RNA (tRNA), calf thymus deoxyribonucleic acid (DNA), polycytidylic acid (polyC) and polyadenylic acid (polyA) underwent similar modification. The modification took place at cytosine, adenine and probably guanine residues. The absorption maximum at 325 nm may be due to the modified cytosine and adenine residues, which form 1 : 3 adducts with a methylene cyclopropane ring and a six-membered ring (Nair *et al.*, *J. Am. Chem. Soc.*, **106**, 3370 (1984)). These modifications did not produce any significant fluorescence. The present data are inconsistent with those of Reiss *et al.* (*Biochem. Biophys. Res. Commun.*, **48**, 921 (1972)), who reported that the modification of nucleic acids with malonaldehyde produced fluorescent cross-links due to conjugated Schiff bases.

Keywords——malonaldehyde; nucleic acid; RNA; DNA; cytidine; adenosine; fluorescence

Malonaldehyde is considered to be one of the secondary products formed during oxidation of polyunsaturated fatty acids.[1] Its formation has been regarded as significant because the aldehyde is toxic,[2] carcinogenic[3] and mutagenic.[4] Reactions of proteins with malonaldehyde have been investigated with respect to the formation of fluorescent components in lipofuscin. Tappel and his associates suggested the formation of fluorescent cross-links due to conjugated Schiff bases between the amino groups of proteins and malonaldehyde.[5] Our recent studies demonstrated that the fluorescence was due to the formation of 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde moieties and the cross-links to the formation of the less fluorescent conjugated Schiff bases.[6]

Malonaldehyde can react with deoxyribonucleic acid (DNA) to cause various biological and physicochemical changes.[7,8] While several reaction modes of nucleic acids have been postulated, they are not consistent. Formation of fluorescent cross-links due to conjugated Schiff bases between the amino groups of base moieties and malonaldehyde has been emphasized.[8] Several papers demonstrated that the reactions of guanosine,[9] cytidine[10] and adenosine[10] afforded 1 : 1, 1 : 2 and 1 : 3 adducts other than the conjugated Schiff bases. We reinvestigated the reaction of nucleic acids with malonaldehyde, in order to clarify the fluorescence characteristics and the reaction modes.

Materials and Methods

Adenosine, uridine-2′(3′)-monophosphate (2′,3′-UMP), adenosine-2′(3′)-monophosphate (2′,3′-AMP), guanosine-2′(3′)-monophosphate (2′,3′-GMP), polycytidylic acid sodium salt (polyC) and polyadenylic acid sodium salt (polyA) were obtained from Yamasa Shoyu Co., Ltd., Tokyo, Japan. Uridine, cytidine, arabinosylcytosine, guanosine, cytidine-2′(3′)-monophosphate (2′,3′-CMP), ribonucleic acid (RNA) from yeast, and transfer RNA (tRNA) from *Torula* yeast were obtained from Kohjin Company, Ltd., Tokyo, Japan. Calf thymus DNA and bovine serum albumin (BSA) were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Malonaldehyde solution at 1 M concentration was prepared by acid hydrolysis of malonaldehyde

bis(dimethylacetal) as described elsewhere.[6b] The solution was diluted into the appropriate buffer for use. Phosphorus was determined according to the method of Gerlach and Deuticke.[11] Paper chromatography was performed on Toyo Roshi No. 51A paper with a solvent system of 1-butanol–water (84 : 16). Spots were detected under ultraviolet (UV) light. Cellulose column chromatography was performed with Toyo Roshi cellulose powder (above 100 mesh) for chromatography. UV absorption spectra were taken with a Shimadzu UV-200S double-beam spectrophotometer. Fluorescence spectra were taken with a Hitachi 60—40 fluorescence spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken in dimethylsulfoxide-d_6 with Me_4Si as an internal standard using a JEOL PS-100 machine. High performance liquid chromatography (HPLC) was performed by the use of a Shimadzu LC-2 liquid chromatograph equipped with a column of Zorbax ODS (4.6 mm i.d. × 25 cm), and the peaks were detected at 260 nm with a Shimadzu SPD-1 spectrophotometric detector. The chromatograph was operated with a solvent system of methyl alcohol–0.05 M ammonium phosphate buffer (pH 6.0) (5 : 95) at a flow rate of 0.5 ml/min.

Nucleic Acids of Polynucleotides Modified with Malonaldehyde——A mixture of RNA (tRNA, DNA, polyC or polyA), and malonaldehyde or malonaldehyde/acetaldehyde in 0.1 M acetate buffer (pH 4.0) or 0.1 M phosphate buffer (pH 7.0) was incubated. Aliquots were periodically removed from the reaction mixture and dialyzed against 0.9% sodium chloride solution. The dialyzate was mixed with 2 volumes of ethyl alcohol, and the precipitate thus formed was collected by centrifugation at 3000 rpm for 10 min. The precipitate was redissolved in 1.0 ml of 0.9% sodium chloride solution and recovered by addition of ethyl alcohol. The precipitate was dried over calcium chloride. Under these conditions, no precipitate was obtained from the incubation mixture of malonaldehyde or malonaldehyde/acetaldehyde alone.

Reaction Products of Nucleosides with Malonaldehyde——A 100 ml reaction mixture of 40 mM cytidine and 100 mM malonaldehyde in 0.1 M acetate buffer (pH 4.0) was incubated at 37 °C for 6 d, then evaporated at below 40 °C. The residue was applied to a column (2 × 75 cm) of cellulose powder and eluted with 1-butanol–water (84 : 16). The fractions containing product CR′ were collected and evaporated. The residue was crystallized from ethyl alcohol–water to afford a pale yellow crystalline powder (41 mg). UV $\lambda_{max}^{0.1\ M\ phosphate,\ pH\ 7.0}$: 238, 322 nm; $\lambda_{max}^{0.1\ N\ HCl}$: 238, 322 nm. Fluorescence λ_{max} (excitation): 380 nm; λ_{max} (emission): 453 nm. Relative fluorescence intensity: 4×10^{-3} with respect to the intensity of quinine sulfate in 0.1 N sulfuric acid. ^{1}H-NMR (dimethylsulfoxide-d_6) ppm: 9.37 (1H, s, CHO), 9.20 (1H, s, CHO), 8.61 (1H, d, C^6H, J=7 Hz), 8.39 (1H, br. s), 7.71 (1H, s), 6.84 (1H, br s), 6.75 (1H, d, C^5H, J=7 Hz), 5.78 (1H, s, $C^{1'}$H), 2.00 (1H, d, *gem*-H, J=12 Hz), 1.81 (1H, d, *gem*-H, J=12 Hz).

Product AC′ from arabinosylcytosine was isolated as a pale yellow crystalline powder (73 mg). UV $\lambda_{max}^{0.1\ M\ phosphate,\ pH\ 7.0}$: 237, 324 nm; $\lambda_{max}^{0.1\ N\ HCl}$: 237, 324 nm. ^{1}H-NMR (dimethylsulfoxide-d_6) ppm: 9.38 (1H, s, CHO), 9.21 (1H, s, CHO), 8.39 (1H, br s), 8.18 (1H, d, C^6H, J=7 Hz), 7.72 (1H, s), 6.82 (1H, br s), 6.72 (1H, d, C^5H, J=7 Hz), 6.08 (1H, d, $C^{1'}$H), 2.03 (1H, d, *gem*-H, J=12 Hz), 1.80 (1H, d, *gem*-H, J=12 Hz).

Product AR′ from adenosine was isolated as a pale yellow crystalline powder. UV $\lambda_{max}^{0.1\ M\ phosphate,\ pH\ 7.0}$: 240, 270, 330 nm; $\lambda_{max}^{0.1\ N\ HCl}$: 240, 270, 330 nm.

Results and Discussion

The extents of fluorescence formation in the reactions of RNA and BSA with malonaldehyde were compared (Fig. 1). When 0.04 or 0.1% BSA was reacted with 10 mM malonaldehyde at pH 7.0 and 37 °C, fluorescence with an excitation maximum at 404 nm and an emission maximum at 467 nm increased. The fluorescence can be attributed to the formation of 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde moieties at the N and the ε-amino groups of BSA.[6a,c] In contrast, the reaction of 0.04 or 0.1% RNA with malonaldehyde at pH 7.0 or 4.0 at 37 °C produced much less fluorescence. The increase in fluorescence was only a little higher than that in the incubation mixture of malonaldehyde alone.

RNA, tRNA and DNA were reacted with malonaldehyde at 37 °C, and the modified nucleic acids were recovered. While the RNA modified at pH 7 did not show any change in the absorption spectrum, the RNA modified at pH 4 showed an absorption maximum at 325 nm. The extinction coefficients at 325 nm increased as the modification time was increased, and the ratio of $\varepsilon\ (p)^{325\ nm}/\varepsilon\ (p)^{260\ nm}$ increased to 0.26 after 90 h of modification (Table I). The fluorescence spectrum of the modified RNA revealed an excitation maximum at 325 nm and an emission maximum at 500 nm. Relative molar fluorescence intensity with respect to quinine sulfate increased with the modification time, but the relative intensity reached only less than 0.1% after 90 h of modification (Table I). The modified tRNA and DNA showed lower ratios of the extinction coefficients, indicating that tRNA and DNA were resistant to the modification. When the nucleic acids were reacted at 70 °C, a marked increase in absorbance

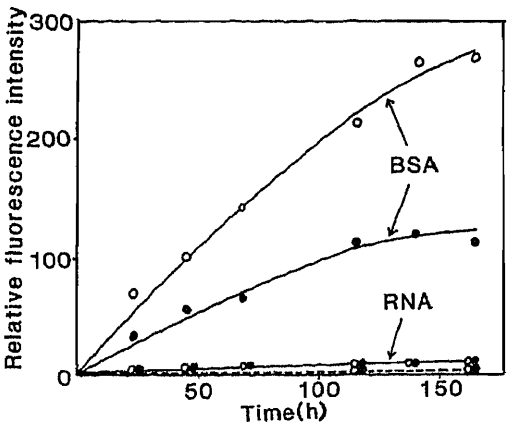

Fig. 1. Time Course of Fluorescence Formation in the Reaction Mixture of RNA and BSA with Malonaldehyde

A mixture of RNA or BSA, and 10 mM malonaldehyde in 0.1 M phosphate buffer (pH 7.0) (——) or 0.1 M acetate buffer (pH 4.0) (– – – –) was incubated at 37 °C. Relative fluorescence intensities of the reaction mixtures at the maximum wavelengths of excitation and emission were expressed with respect to 0.1 μM quinine sulfate in 0.1 N sulfuric acid (excitation at 350 nm and emission at 450 nm). ●, 0.04% RNA or BSA; ○, 0.1% RNA or BSA.

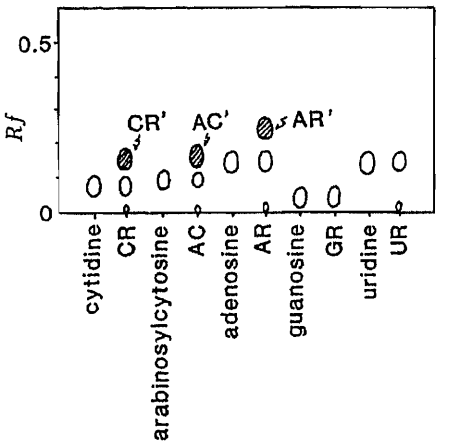

Fig. 2. Paper Chromatography of the Reaction Mixtures of Nucleosides with Malonaldehyde

A solution of 40 mM nucleoside (10 mM in case of guanosine) and 100 mM malonaldehyde in 0.1 M acetate buffer (pH 4.0) was incubated at 37 °C for 5 d. Chromatography was done with a solvent system of 1-butanol–water (84 : 16). Spots were visualized under UV light. CR, AC, AR, GR and UR indicate the reaction mixtures of cytidine, arabinosylcytosine, adenosine, guanosine and uridine, respectively.

Table I. Extinction Coefficients and Fluorescence Intensity of Nucleic Acids and Polynucleotides Modified with Malonaldehyde

Nucleic acids and polynucleotides	Extinction coefficient			Relative molar fluorescence intensity[f]
	$\varepsilon(p)^{260\,nm}$	$\varepsilon(p)^{325\,nm}$	Ratio[e]	
Unmodified RNA	9500	0	0	—
Malonaldehyde-modified				
RNA 37 °C, 24 h[a]	9400	860	0.09	2.7×10^{-4}
37 °C, 48 h[a]	9800	1400	0.14	3.8×10^{-4}
37 °C, 90 h[a]	10100	2600	0.26	8.1×10^{-4}
70 °C, 5 h[b]			0.27	
tRNA 37 °C, 90 h[a]			0.16	
70 °C, 5 h[b]			0.29	
DNA 37 °C, 90 h[a]			0.05	
70 °C, 5 h[b]			0.29	
PolyC 70 °C, 5 h[b]			0.26	
PolyA 70 °C, 5 h[b]			0.17	
Malonaldehyde/acetaldehyde-modified				
RNA 37 °C, 24 h[c]	9100	820	0.09	2.7×10^{-4}
37 °C, 48 h[c]	9800	1500	0.15	5.0×10^{-4}
37 °C, 90 h[c]	10000	2400	0.24	8.3×10^{-4}
Acetaldehyde-treated				
RNA 37 °C, 90 h[d]	8100	0	0	0.7×10^{-4}
Quinine sulfate				1.00

A solution of *a*) 1% nucleic acid and 60 mM malonaldehyde, *b*) 0.25% nucleic acid or polynucleotide and 250 mM malonaldehyde, *c*) 1% nucleic acid and 60 mM malonaldehyde/60 mM acetaldehyde, or *d*) 1% nucleic acid and 60 mM acetaldehyde, in 0.1 M acetate (pH 4.0) was incubated, and the modified nucleic acid or polynucleotide was recovered. *e*) $\varepsilon(p)^{325\,nm}/\varepsilon(p)^{260\,nm\ (or\ 280\,nm\ for\ polyC)}$. *f*) The intensities determined at the excitation and emission maxima are expressed relative to that of quinine sulfate.

TABLE II. Decrease in 2′,3′-Nucleotides in Malonaldehyde-Modified RNA Estimated by Alkaline Hydrolysis and Subsequent HPLC

Peak number	Retention time (min)	Identification	Relative peak height		% decrease in modification
			Unmodified RNA	Malonaldehyde-modified RNA[a]	
1	7.7	2′,3′-CMP	0.83	0.65	22
2	8.6	2′,3′-UMP	1.00	1.00	0
3	9.5		0.77	0.69	10
4	12.4	2′,3′-GMP	0.74	0.48	35
5	24.1		0.39	0.17	56
6	31	2′,3′-AMP	0.35	0.19	46
7	72		0.17	0.08	53

a) The RNA modified with malonaldehyde at pH 4.0 and 37 °C for 90 h (10 mg) was hydrolyzed in 1.0 ml of 0.3 N potassium hydroxide at 37 °C for 18 h. The mixture was neutralized with 5.8 N perchloric acid and centrifuged. The supernatant was subjected to HPLC.

at 325 nm was observed. PolyC and polyA produced modified polynucleotides with the same absorption maximum at 325 nm when treated at 70 °C. At elevated temperature, their secondary structures were disrupted and the molecules could be readily modified.

Malonaldehyde may coexist with other aliphatic aldehydes as secondary degradation products of polyunsaturated fatty acids, and the formation of fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde was enhanced by the presence of acetaldehyde in the reaction of amino acids and malonaldehyde.[12] RNA was reacted with malonaldehyde/acetaldehyde at pH 4 and 37 °C, but the modified RNA showed the same absorption spectrum as malonaldehyde-modified RNA with similar ratios of the extinction coefficients (Table I). Fluorescence spectra and relative fluorescence intensity (Table I) were essentially similar to those of the malonaldehyde-modified RNA. Thus, the presence of acetaldehyde did not affect the formation of fluorescence.

The malonaldehyde-modified RNA was hydrolyzed with alkali and the 2′,3′-nucleotides were fractionated by HPLC. While no modified nucleotides could be detected, probably owing to their instability to alkaline treatment, the contents of most of the nucleotides except for 2′,3′-UMP were decreased, and the lost nucleotide had presumably been modified (Table II).

The reaction mixtures of cytidine, arabinosylcytosine and adenosine showed new spots corresponding to CR′, AC′ and AR′, respectively (Fig. 2). The UV absorption spectra of the extracts of these spots had maxima at around 325 nm. All the spots corresponding to the parent nucleosides showed spectra similar to those of the parent nucleosides. Each reaction mixture was fluorescent, but the intensity was extremely low as compared to the standard quinine sulfate and did not exceed that of the control reaction mixture of malonaldehyde alone (Table III).

Product CR′ which was isolated by the use of a cellulose column exhibited absorption maxima at 238 and 322 nm. It is fluorescent but its intensity is much less than that of quinine sulfate. The ^{1}H-NMR spectrum of the product suggested that two signals at 9.37 and 9.20 ppm were assignable to two aldehyde protons, three signals at 8.39, 7.71 and 6.84 ppm to six-membered ring protons and two signals at 2.00 and 1.81 ppm to two geminal cyclopropane ring protons. This compound appears to be the hypermodified cytidine with a methylene cyclopropane-ring and a six-membered ring reported by Nair *et al.*[10] Product AC′ showed characteristics similar to those of CR′. Product AR′ showed an absorption spectrum which coincided with that reported by Nair *et al.*[10]

TABLE III. Fluorescence Spectra and Intensity of the Reaction Mixture of Nucleosides and Malonaldehyde

Reaction mixture[a]	Fluorescence maxima (nm)[b]		Relative fluorescence intensity[c]
	Excitation	Emission	
40 mM cytidine	470	562	130
40 mM adenosine	325	444	1530
10 mM guanosine	435	562	630
40 mM uridine	472	562	300
None	371	565	400

a) Reaction mixtures containing 100 mM malonaldehyde in 0.1 M acetate buffer (pH 4.0) were incubated at 37 °C for 7 d. *b*) Spectra of the mixture were measured after 10^4 dilution. *c*) Fluorescence intensity at the maxima is expressed relative to 0.1 μM quinine sulfate.

The present data demonstrated that malonaldehyde modifies nucleic acids without forming significant fluorescence at cytosine, adenine and probably guanine residues. Absorption maxima at around 325 nm of the modified nucleic acids may be due to the modified cytosine and adenine residues. The modified cytosine and adenine residues may be the 1 : 3 adducts of the bases and malonaldehyde with a methylene cyclopropane ring and a six-membered ring.[10] Brooks and Klamerth[7] suggested that guanine residues were more susceptible to the modification than the other bases. Seto *et al.*[9b,c] obtained the 1 : 1 adduct of guanosine with a cyclic structure showing an absorption maximum at 253 nm, and Marnett *et al.*[9d] obtained the 1 : 2 adduct with a bicyclic structure having an absorption maximum at 249 nm. While the former adduct has been reported to be fluorescent, its intensity was not elucidated.[9c] Since the content of guanine residues in the modified nucleic acids was decreased, the residues are presumably modified in either way. The reaction products of guanosine could not be detected on paper chromatography, and might not be separated under the conditions used.

Reiss *et al.*[8] demonstrated that the reaction of nucleic acids with malonaldehyde produced significant fluorescence with maxima at 390 nm (excitation) and 460 nm (emission) and characteristic absorption with a maximum at 325 nm, and suggested that the alterations were due to the formation of the conjugated Schiff bases of the amino groups of the base moieties and malonaldehyde. However, they did not measure the fluorescence intensities of the modified nucleic acids. Nevertheless, the formation of fluorescent cross-links due to conjugated Schiff base has been regarded as a clue to the biological actions of malonaldehyde.[4] Several attempts were made to detect the cross-links of DNA by fluorescence measurement or radiolabeling through borotritide reduction of the conjugated Schiff bases.[13] On the other hand, no cross-linked base pairs due to conjugated Schiff bases could be detected under a variety of conditions,[14] and it was suggested that cross-linking was not prerequisite for the biological activity.[15] The present data are consistent with those of Fujimoto *et al.*[16] who did not detect the formation of significant fluorescence in the reaction of DNA and malonaldehyde. It is suggested that fluorescent cross-links due to conjugated Schiff bases are hardly produced, but much less fluorescent and uncross-linked hypermodified bases are preferentially produced in the interaction of nucleic acids and malonaldehyde.

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In Vitro Drug Release from Macromolecule–Drug Conjugates of 3′-(7-Carboxyheptanoyl)-5-fluoro-2′-deoxyuridine with Decylenediamine-dextran T70 of Poly-L-lysine

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3′-(7-Carboxyheptanoyl)-5-fluoro-2′-deoxyuridine (C_6-FUdR) was conjugated to decylenediamine-dextran T70 (T70-C_{10}) or poly-L-lysine (PLL) by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). Drug release patterns from the conjugates between C_6-FUdR and T70-C_{10} (T70-C_{10}–C_6-FUdR) or PLL (PLL–C_6-FUdR) by enzymatic and nonenzymatic processes were investigated at neutral and weakly acidic pHs. Under the nonenzymatic conditions, 5-fluoro-2′-deoxyuridine (FUdR) was released slowly only at neutral pH. This property is similar to that of C_6-FUdR. Gradual drug release was observed enzymatically from T70-C_{10}–C_6-FUdR at both pHs, while PLL–C_6-FUdR did not show enzymatic drug release. However, after the treatment of PLL–C_6-FUdR with trypsin, FUdR was released enzymatically from the products; in this case, the release rate of FUdR was faster at neutral pH than at the weakly acidic pH. However, the release of FUdR by enzymatic processing was much slower from these conjugates than from C_6-FUdR.

Keywords——conjugation; T70-C_{10}–C_6-FUdR; PLL–C_6-FUdR; drug release; esterase; breakdown

Introduction

The method of binding antitumor agents to macromolecules has been developed recently as a chemical drug delivery system in attempts to improve the chemotherapeutic activities of the parent drugs. Such macromolecule–drug conjugates have the characteristics of remaining at the administered site for a long time, releasing the active agents gradually and accelerating penetration into the cells through endocytosis.[1–3] This approach might be effective for drugs with undesirable pharmacodynamics, or whose activity is dependent on the residence time in the diseased part.

5-Fluoro-2′-deoxyuridine (FUdR) is known to show more than 100 times higher activity than 5-fluorouracil *in vitro*,[4] to show time-dependent chemotherapeutic activity and to be effective against solid neoplasms.[5,6] Therefore, the derivation of FUdR with macromolecules could be fruitful. Brodano *et al.* have already reported the albumin conjugate of FUdR as a lysosomotropic agent.[7,8] The mechanism of its action seemed to be complicated but they suggested that the conjugate would exert toxic action within the cells after the digestion of the protein moiety and release of the free drug from the conjugate.[7,8] Trouet *et al.* reported that the release of the free drug was dependent on the kind of the spacers used in the case of albumin-succinyl-peptide-daunorubicin.[9] Thus, it is important to examine the drug release properties *in vitro* in order to clarify the drug release mechanism from macromolecule–drug conjugates. In this study, macromolecular FUdR esters, whose structures are well defined, were prepared and the release of FUdR was examined *in vitro* in the presence and absence of esterase.

Experimental

Materials——FUdR, poly-L-lysine (PLL) (M.W. 90000), esterase (2600 units/ml) from porcine liver and trypsin (10000 units/mg) from bovine pancreas were purchased from Sigma Chemicals Co. Dextran T70 (T70) (M.W. 70000) was obtained from Tokyo Kasei Industrial Co. 1,10-Diaminodecane (C_{10}) was purchased from Wako Pure Chemical Industries, Ltd. All other chemicals were commercial reagent-grade products.

Conjugates——Conjugation was carried out as follows; T70 was converted into decylenediamine-dextran T70 (T70-C_{10}) for use as a drug carrier. T70-C_{10} was synthesized by Schiff's base formation between C_{10} and periodate-oxidized T70, followed by borohydride reduction, as reported in detail in the previous paper.[10] T70-C_{10} (600 mg), 3'-(7-carboxyheptanoyl)-5-fluoro-2'-deoxyuridine (C_6-FUdR)[11] (10 mg) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (600 mg) were mixed in 20 ml of purified water and stirred for 24 h in the dark at room temperaure. PLL (200 mg), C_6-FUdR (20 mg) and EDC (400 mg) were mixed in 20 ml of purified water and treated similarly. After filtration of the reacted mixture through filter paper, the filtrate was applied to a Sephadex G50 column and eluted with 50 mM NaCl aqueous solution. The high-molecular-weight fractions were combined and dialyzed against water. After freeze-drying, decylenediamine-dextran T70 conjugate of C_6-FUdR (T70-C_{10}–C_6-FUdR) or PLL conjugate of C_6-FUdR (PLL-C_6-FUdR) was obtained. The content of FUdR was estimated from that of C_6-FUdR, which was calculated from the difference of absorbance at 270 nm between the conjugate and the polymer support at the same concentration (w/v) in pH 7.4 phosphate-buffered saline (PBS) (NaCl, 137 mM; KCl, 3 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM).

***In Vitro* Drug Release**——Drug release tests were done in PBS (pH 7.4) and in 0.1 M acetate buffer (pH 3.8). Esterase was used at the concentraion of 1000 units/ml, when present. The pH value of each sample solution was checked by using a pH-test paper, which was purchased from Toyo Roshi Co., Ltd. The samples were incubated at 37.5 °C, with shaking at 100 cycles/min. Aliquots were taken at appropriate times. The released FUdR was determined by high-performance liquid chromatography (HPLC) with a Shimadzu LC-3A apparatus equipped with a Nucleosil C-18 column (4 × 250 mm) and an ultraviolet detector set at 270 nm. The mobile phase used was a mixture of 0.02 M acetate buffer and methanol (19 : 1, v/v).

Trypsin-treated PLL–C_6-FUdR was prepared as follows: PLL–C_6-FUdR (30 mg) was dissolved in 5 ml of PBS, and trypsin (2.3 mg) was added. The mixture was incubated for 24 h at 37.5 °C. The products were obtained from the low-molecular fractions in gel-filtration (Sephadex G50). The freeze-dried powder was used for drug release tests. As a control, trypsin (2.3 mg) alone was treated similarly. The drug release experiment was performed in the same way as that on macromolecule–drug conjugates.

In all the experiments, the content of FUdR of each sample was checked by ultraviolet absorption measurement at 270 nm before incubation.

Results and Discussion

Conjugation

It was confirmed by gel-chromatography that C_6-FUdR was combined with the polymer support by using EDC. Since C_6-FUdR was considered to bind through its ω-carboxyl group

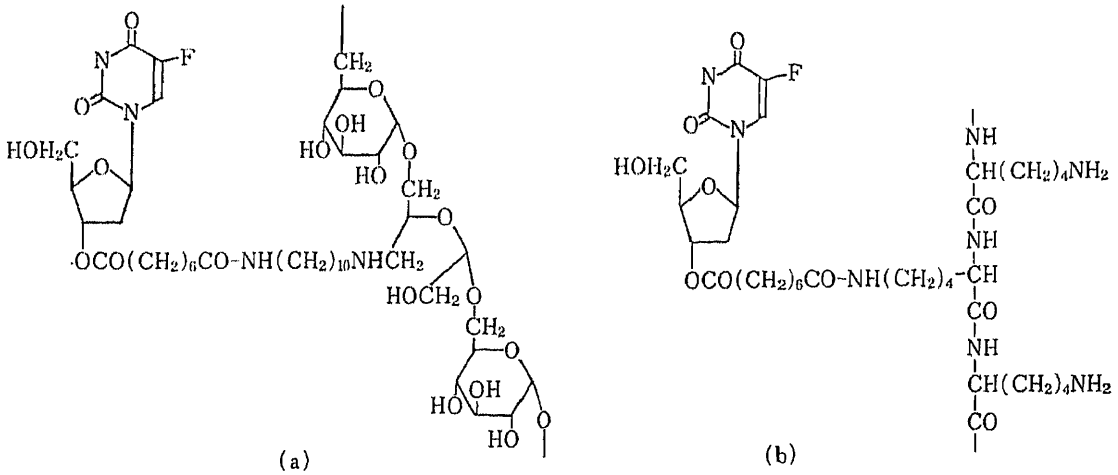

Fig. 1. Proposed Structures of T70-C_{10}–C_6-FUdR Conjugate (a) and PLL–C_6-FUdR Conjugate (b)

to the amino groups of the polymer support, the structures of the conjugates are assumed to be as shown in Fig. 1. However, C_6-FUdR was not combined with T70-C_{10} as effectively as methotrexate, which could be introduced onto T70-C_{10} quite efficiently, as had been reported in the previous paper.[10] In the case of PLL–C_6-FUdR, a similar tendency was noted. Namely, C_6-FUdR was not bound to PLL efficiently, in contrast to methotrexate.[3] The reproducibility of the binding of C_6-FUdR to both polymer supports was good, so it appears that C_6-FUdR is not easily introduced onto the polymer supports. There may be unfavorable steric or electrostatic interactions involved in the course of the reaction. Some chemical characteristics of the obtained conjugates are given in Table I.

Drug Release from C_6-FUdR

FUdR release from the native C_6-FUdR was complete within 2 h in the presence of esterase, both at pH 7.4 and at pH 3.8. Under the nonenzymatic conditions, 17% of FUdR was released at pH 7.4 and none was released at pH 3.8, after incubation for 48 h.

Drug Release from T70-C_{10}–C_6-FUdR

Gradual drug release by esterase was obtained both at neutral pH (pH 7.4) and in weak acid (pH 3.8). In the first 24 h, about 15% of the combined FUdR was released, as shown in Fig. 2. The subsequent release of FUdR was slower. As regards nonenzymatic hydrolysis, drug release was detected only at neutral pH, as was the case with C_6-FUdR. Thus, it can be

TABLE I. Properties of the Conjugates

Compound	FUdR content (μM/g)	λ_{max} (nm)[a]	λ_{min} (nm)[a]
C6-FUdR	—	266	236
T70-C_{10}–C_6-FUdR	6.4	275	262
PLL–C_6-FUdR	20.1	266	255

a) The spectra were measured in PBS, pH 7.4.

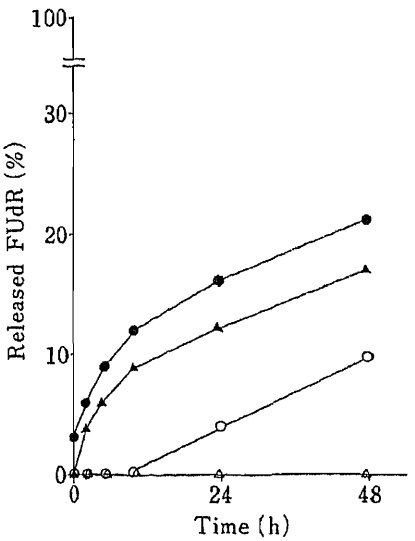

Fig. 2. *In Vitro* Release of FUdR from T70-C_{10}–C_6-FUdR Conjugate

●, with esterase at pH 7.4; ○, without esterase at pH 7.4; ▲, with esterase at pH 3.8; △, without esterase at pH 3.8. The concentration of esterase was 1000 units/ml.

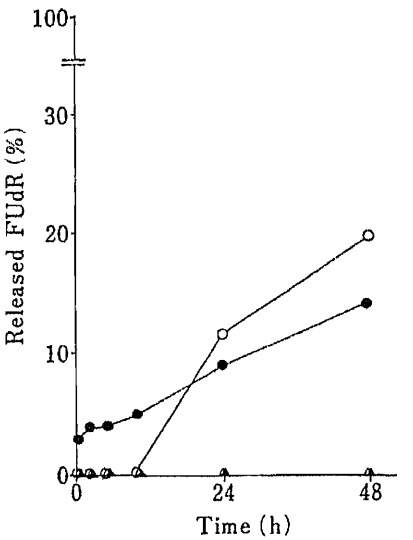

Fig. 3. *In Vitro* Release of FUdR from PLL–C_6-FUdR Conjugate

●, with esterase at pH 7.4; ○, without esterase at pH 7.4; ▲, with esterase at pH 3.8; △, without esterase at pH 3.8. The concentration of esterase was 1000 units/ml.

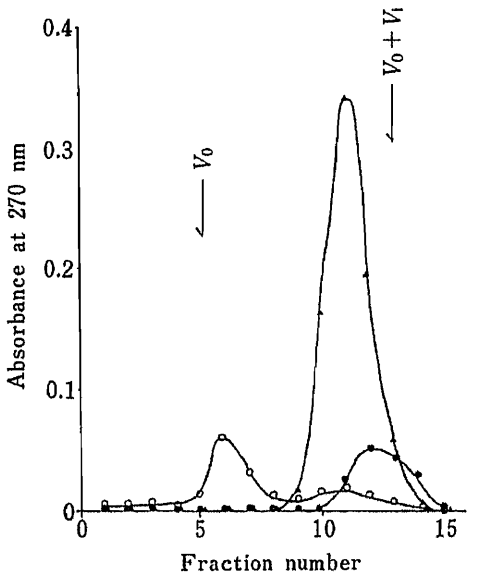

Fig. 4. Elution Patterns of the Products (—▲—) after the Treatment of PLL-C_6-FUdR with Trypsin for 24 h, Trypsin Alone (—○—) Immediately after Dissolution and Trypsin Alone (—●—) after Incubation for 24 h

The concentration of trypsin was 0.46 mg/ml.

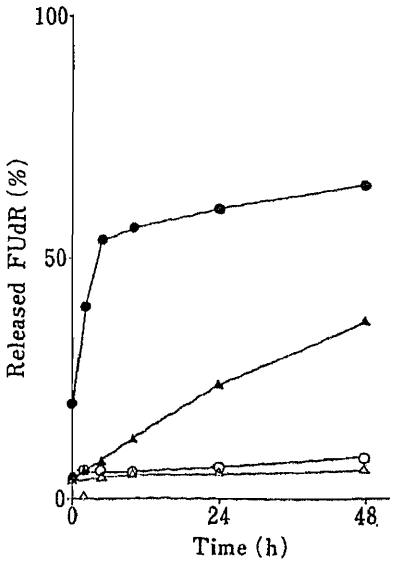

Fig. 5. *In Vitro* Release of FUdR from the Products Obtained after Incubation of PLL-C_6-FUdR with Trypsin

●, with esterase at pH 7.4; ○, without esterase at pH 7.4; ▲, with esterase at pH 3.8; △, without esterase at pH 3.8. The concentration of esterase was 1000 units/ml.

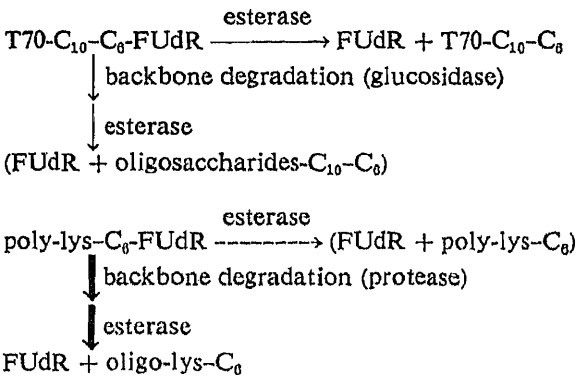

Fig. 6. Proposed Mechanisms of FUdR Release from T70-C_{10}-C_6-FUdR and PLL-C_6-FUdR

(⟶), minor or less important route; (⟶), main or more important route.

expected that FUdR will be released enzymatically both physiologically (pH 7.4) and lysosomally (pH 3.8) from the macromolecule.[12] Since the degradation of dextran is generally known to be slow,[12] the effect of the breakdown of the polymer support on the action of esterase was not checked in this study.

Drug Release of PLL-C_6-FUdR

The release of FUdR from PLL-C_6-FUdR by esterase was very little, and the drug release profile was different from that of T70-C_{10}-C_6-FUdR, as shown in Fig. 3. This may be related to the fact that the aminoalkyl side chains of PLL are shorter and more densely distributed than the spacers of T70-C_{10}, sterically hindering the esterase action. Marked release of FUdR in the absence of the esterase was found at pH 7.4. At pH 3.8, no release of FUdR was observed in the presence of absence of the esterase. The possibility should be

considered that the esterase interacts with PLL.

Figure 4 showed the gel-filtration pattern after the degradation of PLL–C_6-FUdR by using trypsin. The drug release profiles from the degraded products are shown in Fig. 5. Under the physiological conditions, several enzymes act specifically on peptide bonds involving basic amino acid residues.[13-15] Several studies on the enzymatic hydrolysis of PLL have been reported,[16-19] and PLL is considered to be biodegradable.[20-22] The results in Fig. 5 indicate that the release of FUdR, that is, the action of PLL–C_6-FUdR as a prodrug, can occur after the breakdown of the polymer support to low-molecular products such as lysine–C_6-FUdR and oligolysine–C_6-FUdR. Nearly 60% of the contained FUdR was released enzymatically after 10 h at pH 7.4, while the release was slower at pH 3.8. Nonenzymatic drug release was very small at both pHs. These results presumably reflect the release of steric hindrance upon destruction of the bulky polymer skeleton. However, the release rate of FUdR from the products was still slower than that from C_6-FUdR. The results are consistent with those on drug release from the albumin-FUdR conjugate reported by Brodano *et al.*[7,8]

Based on the *in vitro* drug release studies, the process of FUdR release could be as shown in Fig. 6. T70-C_{10}–C_6-FUdR was found to be useful for providing gradual drug release and PLL–C_6-FUdR showed the characteristics of an ester prodrug after breakdown of the PLL backbone.

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Preparation of and Drug Release from W/O/W Type Double Emulsions Containing Anticancer Agents Using an Oily Lymphographic Agent as an Oil Phase

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W/O/W type double emulsions stable for a long time were prepared by two-step emulsification procedures using an oily lymphographic agent, Lipiodol Ultra-Fluid, and a derivative of hydrogenated castor oil (HCO-10) as an oil phase. The yield of W/O/W type double emulsions was about 90%. Although the yield was not lowered by vigorous dispersion with a homogenizer, the size of oil droplets was decreased and the release rate of cytarabine was enhanced. Neither the concentration of cytarabine in, nor the volume of, the inner aqueous phase affected the release pattern of cytarabine from W/O/W type double emulsions. The release rate of 5-fluorouracil (5-FU) from the W/O/W type double emulsion was so fast that the release was completed within 1 h. The release of cytarabine and glucose was sustained and peplomycin was hardly released from the W/O/W type double emulsion. W/O type emulsions or W/O/W type double emulsions prepared in this study might be suitable as carriers of drugs such as peplomycin.

Keywords——W/O type emulsion; W/O/W type double emulsion; multiple emulsion; sustained release; anticancer drug; lipiodol; oily lymphographic agent; peplomycin; hydrogenated castor oil

Introduction

In the previous study,[1] we developed a quick preparative method for W/O/W type double emulsions and examined the release characteristics of cytarabine and 5-fluorouracil (5-FU) as representative drugs. The stability of the W/O/W type double emulsions, however, was not sufficient, so that coalescence among inner aqueous droplets occurred and the W/O/W type double emulsions ruptured in the absence of agitation during the drug release experiment. Thus, we have tried to prepare more stable W/O/W type double emulsions by examining suitable combinations of oils and HCO-10, which was found to be a good lipophilic surfactant in the previous investigation,[1] and we found that more stable W/O/W type double emulsions could be prepared by using an oily lymphographic agent, Lipiodol Ultra-Fluid (hereafter abbreviated to Lipiodol), as an oil phase. Lipiodol after injection into the body could be detected by X-ray examination, and W/O/W type double emulsions or W/O type emulsions prepared with Lipiodol were also detectable.[2] Recently it was reported that Lipiodol selectively remained at a hepatic cancer site when injected into the hepatic artery,[3,4] and W/O type emulsions prepared with Lipiodol were applied to the treatment of hepatic cancer.[2,5,6]

In the present investigation, we examined the stability and drug release properties of W/O type emulsions and W/O/W type double emulsions prepared with Lipiodol as an oil phase and HCO-10 as a lipophilic surfactant.

Experimental

Materials——Lipiodol was purchased from Kodama Co., Tokyo; it was a product of Laboratorie Guerbert,

Paris, France. Nonionic surfactants, derivatives of hydrogenated castor oil (HCO-10 as a lipophilic surfactant and HCO-60 as a hydrophilic surfactant) were generously supplied by Nikko Chemicals Co., Tokyo. 5-Fluorouracil, cytarabine and peplomycin were gifts from Kyowa Hakko Kogyo Co., Nihon Shinyaku Co., Kyoto, and Nihon Kayaku Co., Tokyo, respectively. Water was distilled and treated with a Milli-Q reagent-grade water system (Millipore Corp., Massachusetts). All other chemicals were of reagent grade.

Preparation of W/O/W Type Double Emulsions——An improved version of the two-step emulsification procedure reported previously[1] was employed. An aqueous solution of a drug (inner aqueous phase) was introduced into Lipiodol containing HCO-10 and emulsified with a sonicator (UR-20P, Tomy Seiko Co., Tokyo) in an ice-water bath to prepare W/O type emulsion (1st emulsification). An aqueous solution of HCO-60 (an outer aqueous phase) was then introduced into the W/O type emulsion and the whole was agitated with a vibrator mixer (MM-2, Kayagaki Rika Co., Tokyo) to prepare W/O/W type double emulsion (2nd emulsification). The standard conditions were as follows: the volume of inner aqueous phase was 0.4 ml, the volume of oily phase was 2.0 ml, the volume of the outer aqueous phase was 5.0 ml, the concentraion of HCO-10 was 1% (w/v), the concentration of HCO-60 was 0.1% (w/v), the time period of the 1st emulsification was over 2 min and the time period of the 2nd emulsification was 30 s. In an experiment to examine the effect of varying the conditions of the 2nd emulsification, more vigorous dispersion by a homogenizer (Physcotron NS-500, Niti-on, Co., Tokyo) was carried out after emulsification with the vibrator mixer.

Examination of Physical Stability of W/O Type Emulsions Prepared at the 1st Emulsification——Freshly prepared W/O type emulsions were kept at 4, 25 or 37 °C and the occurrence of phase separation was examined. When phase separation occurred, water separated on top, because the specific gravity of Lipiodol is larger than that of water.

Measurement of Yields of W/O/W Type Double Emulsions——Freshly prepared W/O/W type double emulsions were kept at 4 °C for a few minutes so that the oil droplets sank. Then a part of the outer aqueous phase was taken and centrifuged at 3000 rpm for 10 min. The supernatant solution was taken and the concentration of a marker (dissolved previously in the inner aqueous phase) was measured. Yields of W/O/W type double emulsions were calculated from the following equation:

$$\text{yield in percent} = \frac{C_o V_o}{V_i(C_i - C_o)} \times 100$$

where C_i, initial drug concentration in inner aqueous phase (mg/ml); C_o, drug concentraion in outer aqueous phase (mg/ml); V_i, inner aqueous phase volume (ml); V_o, outer aqueous phase volume (ml). The amount of drug that permeated through the oil phase of the prepared W/O/W type double emulsions was ignored in this calculation. Cytarabine and blue dextran were used as markers at the concentration of 1% in the inner aqueous phase.

Microscopic Observation——Emulsions were observed with an optical microscope (BH2, Olympus Optics Co., Tokyo) and photomicrographs were taken.

Release Study——Release patterns of a drug from W/O/W type double emulsions were investigated as previously reported.[1] The whole of the W/O/W type double emulsion was introduced into dialysis tubing and dialyzed in 200 ml of water at 37 °C. The dialysis solution was agitated with a magnetic stirring bar; 5 ml of the dialysis solution was withdrawn (and 5 ml of fresh water was added to maintain the original volume) at appropriate intervals. Percent of drug released was calculated as previously reported.[1] Points and bars in each figure represent mean values+S.E.M. When the S.E.M. is smaller than the size of the symbol, no bar is shown.

Measurement of Drug Concentrations——The concentrations of cytarabine, 5-FU and blue dextran were analyzed spectrophotometrically (UV 240 spectrophotometer, Shimadzu Seisakusho Co., Kyoto); cytarabine at 271.5 nm, 5-FU at 266.0 nm and blue dextran at 620 nm. Peplomycin was analyzed by high performance liquid chromatography (HPLC) (LC-3A, SPD-2A, C-R2AX, Shimadzu) under the following conditions: column, Zorbax ODS; mobile phase, CH_3COONH_4 (1%): MeOH = 6:4; flow rate, 0.9 ml/min; temperature, 40 °C; UV, 290.0 nm. Glucose was analyzed using Glucose C-Test Wako (Wako Junyaku Kogyo Co., Osaka).

Results and Discussion

Preparation of W/O/W Type Double Emulsions with or without HCO-10

Yields of, the release of cytarabine from, and structural change of W/O/W type double emulsions prepared with or without HCO-10 in Lipiodol are presented in Table I, Figs. 1 and 2, respectively.

Although W/O/W type double emulsions could be prepared without HCO-10 in Lipiodol, the yields were low (70.8% or 79.8%, Table I) and the release of cytarabine was almost complete within 6 h (Fig. 1). In these systems, W/O/W type double emulsions ruptured within 6 h during the release study (Fig. 2) and such instability lowered the emulsion yield and enhanced the release rate of cytarabine from the W/O/W type double emulsions.

Table I. Yields of W/O/W Type Double Emulsions

Emulsion	Inner aqueous phase	Oily phase	Outer aqueous phase	Yields, mean ± S.E.M. ($n=3$) Marker	
				Cytarabine	Blue dextran
A	Water	Lipiodol	0.1% HCO-60	70.8±0.6	
B	0.5% HCO-60	Lipiodol	0.1% HCO-60	79.8±0.8	
C	Water	1% HCO-10 Lipiodol	0.1% HCO-60	90.1±0.2	92.7±0.4
D	Water	1% HCO-10 Lipiodol	Water	91.4±0.2	89.5±0.6

Volumes of each phase and time periods of the 1st and 2nd emulsification were the standard conditions described in Experimental.

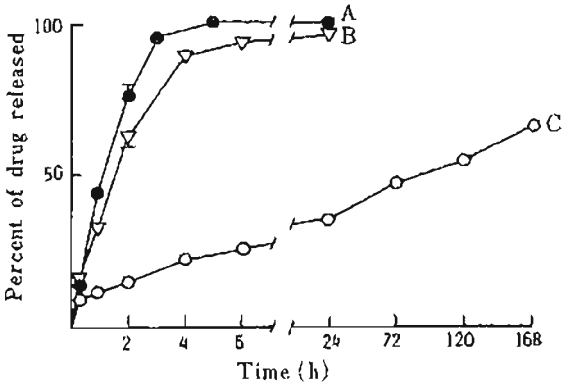

Fig. 1. Release Patterns of Cytarabine from W/O/W Type Double Emulsions Prepared with or without HCO-10 in Lipiodol

A, B and C in the figure correspond to emulsions in Table I.

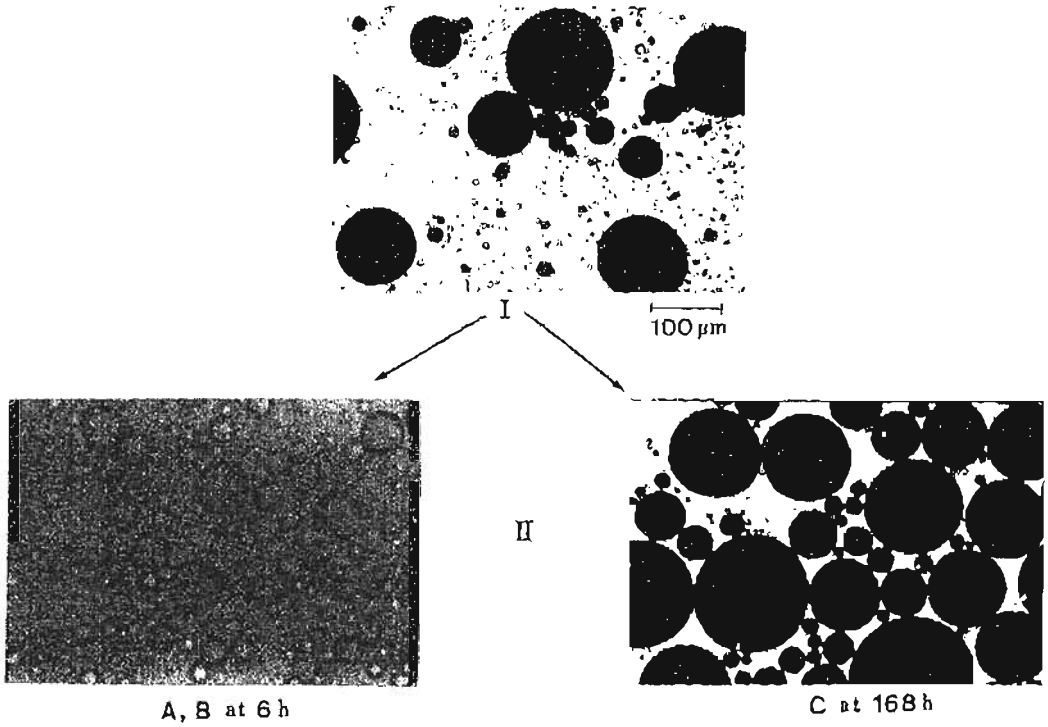

Fig. 2. Photomicrographs of W/O/W Type Double Emulsions Freshly Prepared (I) and after Release Study (II)

A, B and C in the figure correspond to emulsions in Table I.

When Lipiodol contained 1% HCO-10, yields were increased to over 90% (Table I) and the release of cytarabine was sustained; only 65.4% was released within 168 h (Fig. 1). In this system, the structure did not change during a 168 h period and obvious coalescence among inner aqueous droplets or rupture of W/O/W type double emulsions in the absence of agitation[1] was not observed (Fig. 2).

The presence of HCO-60 in the outer aqueous phase at the concentration on 0.1% did not affect the yield of W/O/W type double emulsions (Table I).

Physical Stability of W/O Type Emulsions Prepared with Lipiodol Containing 1% HCO-10

The stability of W/O type emulsions prepared at the 1st emulsification is an important factor determining the stability of W/O/W type double emulsions. Therefore, the physical stability of W/O type emulsions prepared with Lipiodol containing 1% HCO-10 was examined at 4, 25 and 37 °C.

Phase separation did not occur over 21 d at 4 °C. Although partial separation of water was observed on day 3 and day 5 at 25 °C and 37 °C, respectively, complete phase separation did not occur over 21 d.

A W/O type emulsion prepared with Lipiodol containing 1% HCO-10 was so stable that the W/O/W type double emulsions were stable for a long time.

Yields of and Release of Cytarabine from W/O/W Type Double Emulsions Dispersed with a Homogenizer after Emulsification with a Vibrator Mixer

The conditions at the 2nd emulsification are important for the yield of or release pattern

Table II. Yields of W/O/W Type Double Emulsions

Emulsion	Conditions at 2nd emulsification		Yield (%) mean ± S.E.M. ($n=3$)
C	Vibrator mixer (control)		96.9 ± 0.4
	Homogenizer scale setting	Time period	
E	55	1 min	98.1 ± 1.0
F	55	2 min	95.7 ± 2.7
G	55	4 min	81.9 ± 6.0
H	60	1 min	60.5 ± 2.7
I	65	1 min	45.6 ± 3.0

Conditions at preparation were the standard conditions described in Experimental except for dispersion with a homogenizer.

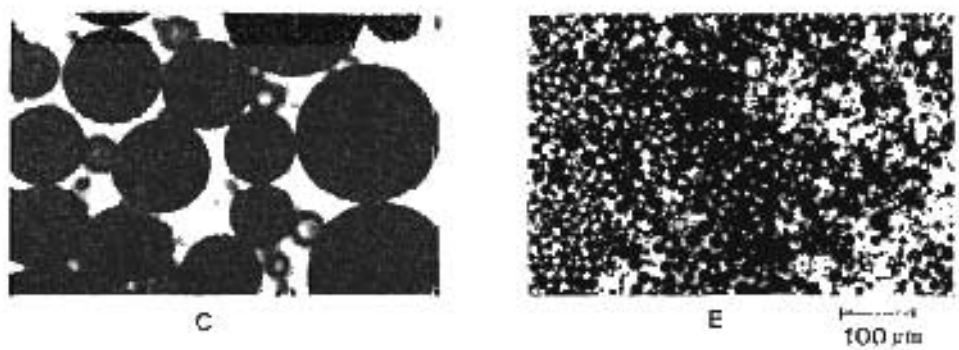

Fig. 3. Photomicrographs of Freshly Prepared W/O/W Type Double Emulsions Dispersed by the Vibrator Mixer Alone (C) and Followed by Dispersion with the Homogenizer (E)

C and E in the figure correspond to emulsions in Table II.

of a drug from W/O/W type double emulsions. Therefore, the effect of more vigorous dispersion by a homogenizer than a vibrator mixer on the yields of and the release of a drug from W/O/W type double emulsions were examined.

Table II shows the yields of W/O/W type double emulsions at the 2nd emulsification when dispersion was done with a vibrator mixer and a homogenizer. The marker was blue dextran. Since we could not measure accurately the rate of revolution of the homogenizer, the instrument scale was used as a measure of the intensity of dispersion (about 19000, 21800 and 23500 rpm at scale values of 55, 60 and 65, respectively, in distilled water). Although the yields hardly changed when the time period of dispersion was 1 or 2 min at scale setting of 55 compared to the control, the yield decreased as the time period of dispersion was increased to 4 min. Yields were also decreased as the scale setting was increased from 55 to 65 at the time period of 1 min.

Figure 3 shows the structures of W/O/W type double emulsions prepared under the

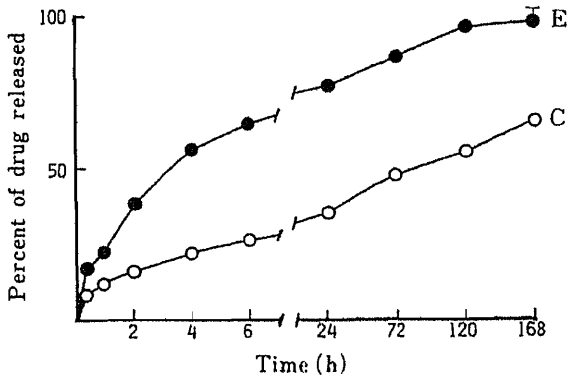

Fig. 4. Release Patterns of Cytarabine from W/O/W Type Double Emulsions Dispersed by the Vibrator Mixer Alone (C) and Followed by Dispersion with the Homogenizer (E) at the 2nd Emulsification ($n=3$)

C and E in the figure correspond to emulsions in Table II.

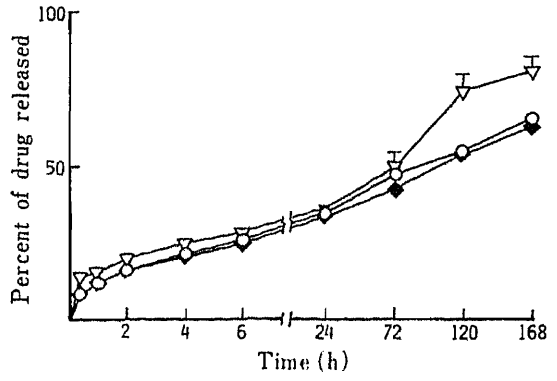

Fig. 5. Effect of Concentration of Cytarabine in the Inner Aqueous Phase on Release from W/O/W Type Double Emulsions ($n=3$)

○, 1%; ◆, 3%; ▽, 5%. Conditions at preparation were the standard conditions described in Experimental.

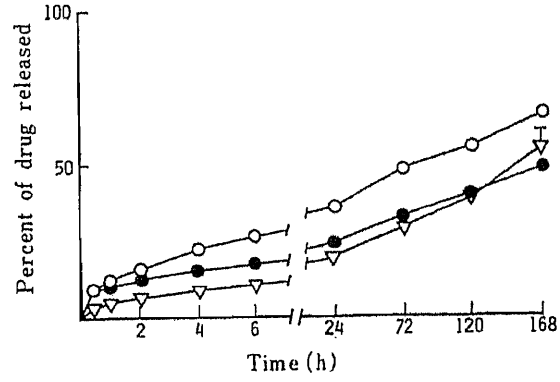

Fig. 6. Effect of the Inner Aqueous Phase Volume on Release of Cytarabine from W/O/W Type Double Emulsions ($n=3$)

○, 0.4 ml; ▽, 0.8 ml; ●, 1.2 ml. Conditions at preparation were the standard conditions described in Experimental except as otherwise described in the text.

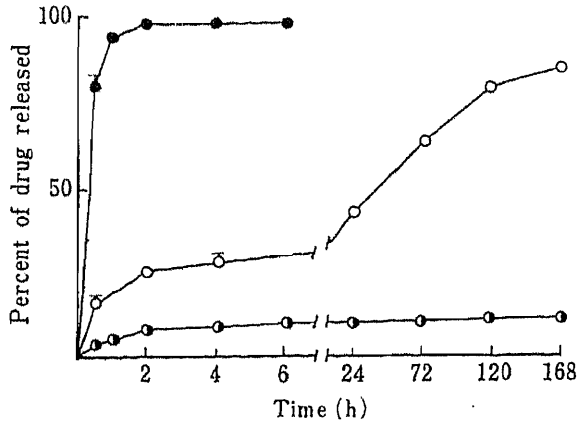

Fig. 7. Release Patterns of 5-FU, Glucose and Peplomycin from W/O/W Type Double Emulsions ($n=3$)

○, glucose; ●, 5-FU; ◑, peplomycin. Conditions at preparation were the standard conditions described in Experimental.

conditions of Table II (C, vibrator mixer; E, vibrator mixer and homogenizer; scale setting of 55, 1 min). The sizes of oil droplets containing the inner aqueous phase clearly became smaller on dispersion with the homogenizer.

Figure 4 shows the release of cytarabine from W/O/W type double emulsions prepared under the conditions of Table II. Although yields were similar in the two systems, the release of cytarabine from the W/O/W type double emulsion dispersed by the homogenizer was enhanced compared to the release from the W/O/W type double emulsion dispersed by the vibrator mixer alone. This enhancement might be due to the increase in surface areas of oil droplets resulting from the decrease in oil droplet sizes.

Effect of Concentration of Cytarabine in the Inner Aqueous Phase and Effect of the Inner Aqueous Phase Volume on the Release of Cytarabine from W/O/W Type Double Emulsions

Figure 5 shows the effect of the concentration of cytarabine in the inner aqueous phase on the release. Values of percent of the drug released were similar among the three systems within 72 h. Although percent of the drug released was larger at 120 and 168 h in the system containing 3% drug than in the other systems, the difference was not significant.

Figure 6 shows the release patterns of cytarabine from W/O/W type double emulsions when the volume of the inner aqueous phase was changed from 0.4 to 1.2 ml. Sonication at the 1st emulsification was applied for 2 min to every 0.4 ml volume of the inner aqueous phase before the next addition. The emulsion type prepared at the 1st emulsification was W/O type in all three systems. The outer aqueous phase volume was 5, 8 and 9 ml when the inner aqueous phase volume was 0.4, 0.8 and 1.2 ml, respectively, in order to keep the dispersion stable in the dialysis tubing during the release study. The percent of the drug released was larger in the 0.4 ml system than in the other systems. This difference might be due to the difference of the time period of sonication or of the outer aqueous phase volume, but the difference was not statistically significant at any point. Thus, the effect of the volume of the inner aqueous phase on the release of cytarabine from W/O/W type double emulsions seems to be slight.

These results indicate that the dose of drugs can be controlled by adjustment of the drug concentration in the inner aqueous phase and/or of the inner aqueous phase volume.

Release of 5-FU, Peplomycin and Glucose from W/O/W Type Double Emulsions

Figure 7 shows the release patterns of 5-FU, glucose and peplomycin from W/O/W type double emulsions. The concentrations of drugs in the inner aqueous phase were 1% for 5-FU, 2% for glucose and 0.25% for peplomycin.

The release of 5-FU was so fast that it was almost complete within 1 h. The release of glucose was sustained and was similar to the release of cytarabine. The release of peplomycin was most sustained; in other words, peplomycin was entrapped for a long time in the inner aqueous phase, and only 7.4% of the drug was released within 168 h. The structures of W/O/W type double emulsions in all systems in Fig. 7 were similar to the structures in Fig. 1C. It is therefore considered that the permeability of 5-FU is very large, while that of glucose or cytarabine is small and that of peplomycin is almost negligible through the oil phase.

General Discussion

The combination of Lipiodol and HCO-10 as an oil phase was suitable for the preparation of W/O/W type double emulsions stable for a long time. The concentration of HCO-10 in Lipiodol necessary for preparation of W/O/W type double emulsions was much lower (1%) than in other systems[1,7-9] and this low concentraion might be favorable for clinical application of W/O/W type double emulsions from the point of view of side effects or toxicity of surfactants. The effect of the concentration of HCO-10 was not examined in the

present study, because the effect might be complex and difficult to evaluate acculately. We only demonstrated that stable W/O/W type double emulsions could be prepared at a low concentraion of HCO-10, 1%, in Lipiodol, and further examination of the concentration or combinations of other oils and other derivatives of hydrogenated castor oil is still required.

High yields of W/O/W type double emulsions (over 90%) were obtained and the yield was not lowered by vigorous dispersion with the homogenizer, though the oil droplet sizes became smaller. The decrease of oil droplet sizes might affect the disposition of W/O/W type double emulsions after injection into the body. On the other hand, the release of cytarabine from W/O/W type double emulsions was enhanced with decrease in oil droplet size. Therefore, it may be expected that the release of drugs from W/O type emulsions or W/O/W type double emulsions injected into the body might be enhanced as emulsions are dispersed to smaller droplets.

Although it is advantageous for the preparation of W/O/W type double emulsions or for dose control that neither the concentration of drugs in the inner aqueous phase nor the inner aqueous phase volume affected the release pattern of cytarabine, this phenomenon must be examined individually with every drug entrapped in W/O/W type double emulsions because osmotic pressure or the interface effect might be different with every drug.

Permeability through the oil phase of W/O/W type double emulsions was different among drugs. 5-FU, which has both hydrophilicity and lipophilicity and small molecular weight, permeated so fast that the release was completed within 1 h. Permeation of cytarabine and glucose, which have high hydrophilicity was sustained for a long time. Peplomycin, which has high hydrophilicity and much higher molecular weight, hardly permeated through the oil phase. These results suggest that W/O/W type double emulsions are superior as carriers of such drugs as peplomycin. Formulations containing bleomycin were examined in clinical studies to treat or prevent metastasis to lymph nodes.[10,11] Peplomycin is a derivative of bleomycin, and W/O/W type double emulsions containing peplomycin might be clinically useful.

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Effects of Sodium or Glucose Exclusion from the Incubation Medium on Drug Transfer across Rat Jejunal Membrane *in Vitro*: An Electrophysiological Study

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The effects of buffer components, glucose and sodium, on the passive transfer of sulfanilic acid (SA) and sulfaguanidine (SG) across isolated rat jejunal membranes were investigated, together with their effects on the transmural potential difference (*PD*) and the membrane resistance (R_m). Glucose or sodium exclusion from bathing solutions significantly increased the mucosal-to-serosal transfer of both drugs. Glucose exclusion caused an irreversible reduction in *PD*, and the active transfer of L-phenylalanine (L-Phe) was also inhibited. However, effects of sodium exclusion on *PD* and the transfer of SA were reversible. The addition of phloridzin (Phl) to the mucosal solution induced the effect similar to that of glucose exclusion. In contrast, R_m was not significantly altered by the exclusion of glucose or the addition of Phl. It is clear that environmental conditions which reduce the active transport activity of the membrane enhance the membrane permeability to drugs.

Keywords——isolated rat jejunum; potential difference; membrane resistance; passive transfer; Na^+-glucose co-transport; paracellular route

Introduction

There are many papers concerning the effect of buffer composition on drug transfer across the gastrointestinal membrane *in vitro*. Mayersohn and Gibaldi[1,2] showed that the replacement of sodium ion by potassium ion reduced the passive transfer of various drugs across the everted rat intestine. In addition, they found that the presence of glucose in the buffer solution also reduced the passive transfer of drugs.[3] Benet *et al.*[4] examined the ability of various buffers to maintain the integrity of intestinal tissues by measuring the transfer rate of salicylate for 2 h. On the other hand, it was reported that the change in ionic composition of the buffer solution affected the active transport of sodium ion across the intestinal membrane.[5] Also, the addition of some sugars or amino acids to the buffer solution evoked an increase in transmural potential difference (*PD*) and short-circuit current (I_{sc}) due to the increase in electrogenic transfer of Na^+, which was co-transported with the sugar or amino acid.[6-8] These variations in the permeability or the activity of the membrane under various conditions *in vitro* would disturb the accurate study of drug absorption.

The present study was undertaken to investigate the effect of buffer components on the permeability of rat jejunal membrane in relation to the electrophysiological parameters.

Materials and Methods

Preparation of Jejunal Sheets——Wistar strain male rats, 200—250 g were used to prepare jejunal sheets. All studies were performed using sheets of rat jejunum mounted between two Lucite half chambers (Ussing-type) as

TABLE I. The Composition of Modified Ringer Solutions Used in This Study

Modified Ringer solution	NaH_2PO_4 (mM)	NaCl	KCl	$CaCl_2$	$NaHCO_3$	SA-Na	D-Glu (mg/100 ml)	D-Man (mg/100 ml)
Control	1.2	125	5	1.4	10	—	200	—
Cont-SA	1.2	115	5	1.4	10	10	200	—
Glu-free	1.2	125	5	1.4	10	—	—	202.2
Glu-free-SA	1.2	115	5	1.4	10	10	—	202.2

Modified Ringer solution	KH_2PO_4	Choline-Cl	KCl	$CaCl_2$	$KHCO_3$	SA-K	D-Glu
Na-free	1.2	125	5	1.4	10	—	200
Na-free-SA	1.2	120	—	1.4	10	10	200

described previously.[9]

Ringer Solutions——Compositions of Ringer solutions used in this study are listed in Table I. In glucose-free (Glu-free) Ringer solution, D-glucose was replaced with an equimolar amount of D-mannitol, and in sodium-free (Na-free) Ringer solution, NaCl, NaH_2PO_4 and $NaHCO_3$ were replaced with choline chloride, KH_2PO_4 and $KHCO_3$, respectively. Sulfanilic acid (SA) was dissolved in distilled water containing an equimolar amount of NaOH or KOH. This solution was added to each Ringer solution to prepare SA-containing (10 mM) Ringer solution. In Na-free SA-containing Ringer solution, KCl was not added because K^+ was supplied from the SA-KOH solution. Sulfaguanidine (SG; 4 mM), L-phenylalanine (L-Phe; 1 mM) and phloridzin (Phl; 1 mM) were added to each Ringer solution directly without a further modification of the composition. In these cases, the influence of osmolarity change may be negligibly small since the concentrations of these agents are very low.

Prior to the experiment, all solutions were adjusted to pH 7.4 at 37 °C and oxygenated with 95%O_2–5%CO_2 mixed gas.

Measurement of Electrical Parameters——*PD* and I_{sc} of the membrane were measured at 5—10 min intervals. The membrane resistance (R_m) was calculated according to Ohm's law, taking into account the resistance of the Ringer solution. The apparatuses for these measurements were the same as described in our previous report.[10]

Measurement of Drug Transfer across the Membrane——Mucosal-to-serosal transfer of SA, SG or L-Phe was measured under various conditions. After preincubation with a drug-free Ringer solution for 25 min, the drug-containing solution was introduced to the mucosal side of the membrane. Thereafter, a 1 ml sample was taken every 10 min from the serosal side for 1 h. The volume of bathing solution was kept constant by the addition of fresh Ringer solution. The mucosal-to-serosal transfer rate of drug was calculated from the rate of increase in the serosal concentration of each drug.[11]

Analytical Methods——SA and SG were estimated spectrophotometrically as described previously.[12] L-Phe was determined by high-performance liquid chromatography after derivatization with *o*-phthaldialdehyde, as described previously.[11]

Materials——All reagents used in these experiments were of reagent grade and were used without further purification.

Results

Effect of Glucose or Sodium Exclusion on the Transfer of SA or SG

Figure 1 shows a plot of the cumulative amount of SA or SG transferred from the mucosal side to the serosal side across the membrane as a function of time. An apparent increase in the amount transferred was observed for both drugs when glucose or sodium was excluded from the bathing solution. In all cases, the regression lines became linear from 20—30 min arter the addition of drug, and transfer rates calculated from the linear portion of each plot were as follows (nmol/cm^2·min): control (SA), 2.05±0.07; Glu-free (SA), 3.59±0.28; Na-free (SA), 3.02±0.28: control (SG), 0.597±0.042; Glu-free (SG), 1.09±0.04; Na-free

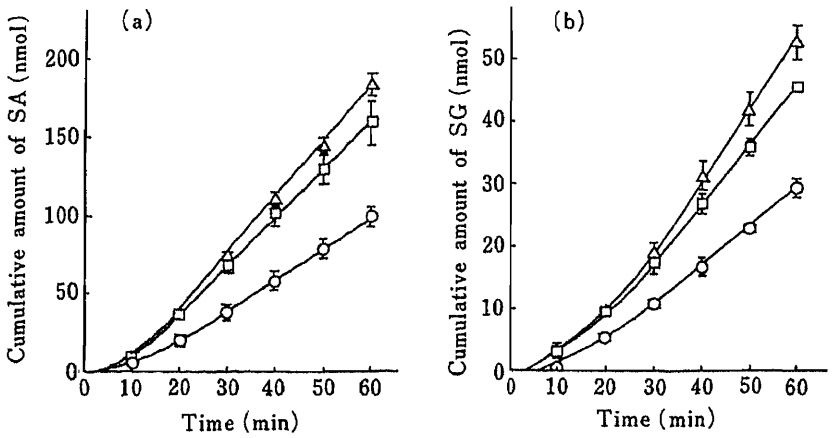

Fig. 1. Effects of Glucose or Sodium Exclusion from the Bathing Solution on the Mucosal-to-Serosal Transfers of SA (a) and SG (b)

○, control; △, Glu-free; □, Na-free conditions. Each point represents the mean of 4—7 experiments with the S.E.

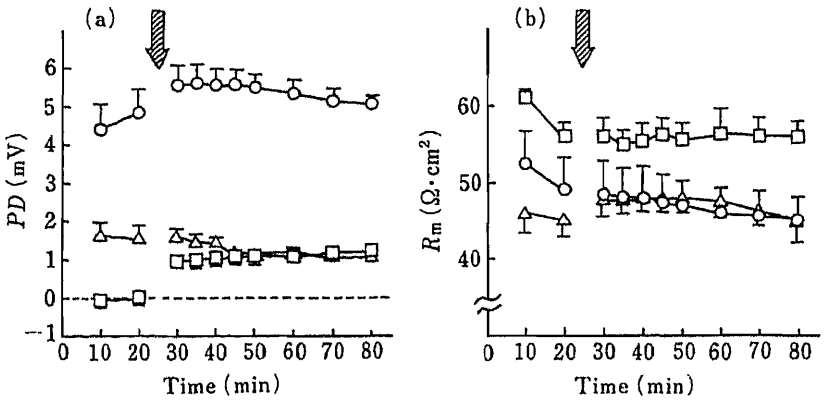

Fig. 2. Effects of Glucose or Sodium Exclusion from the Bathing Solution on *PD* (a) and R_m (b)

○, control; △, Glu-free; □, Na-free conditions. The arrows at 25 min indicate the time when SA containing Ringer solution was introduced to the mucosal side of the membrane. Each point represents the mean of 4—7 experiments with the S.E.

(SG), 0.938 ± 0.049. It is obvious that mucosal-to-serosal transfers of both drugs were influenced in a similar manner by altering the composition of the bathing solution.

Effect of Glucose or Sodium Exclusion on *PD* and R_m

The effects of glucose or sodium exclusion from the bathing solution on *PD* and R_m were examined concurrently with their effect on drug transfer. Figure 2 demonstrates the time-course of *PD* and R_m in control, Glu-free and Na-free conditions. In these experiments, Ringer solution containing 10 mM SA was introduced to the mucosal side after a 25-min preincubation. Similar results were obtained in the case of SG (data are not shown). *PD* was markedly reduced when glucose or sodium was excluded from the bathing solution. After SA-containing solution was introduced to the mucosal side, a rapid increase in *PD* was observed, especially under Na-free condition. This was probably due to the diffusion potential produced by the small differences of ionic compositions between the mucosal and serosal sides.

On the other hand, R_m was not influenced by the presence or absence of glucose, while R_m was significantly increased under Na-free condition.

Evaluation of the Viability of the Intestinal Membrane

In order to investigate the membrane viability under Glu-free or Na-free condition, we examined the recovery of *PD* after replacing the bathing solution from Glu-free to control or from Na-free to control solutions. The results are shown in Fig. 3. The solution replacement time and the side are indicated by arrows and letters (M or S) in Fig. 3. After preincubation with Glu-free Ringer solution for 25 min, replacement with control solution in the mucosal side alone or in both the mucosal and serosal sides caused only a temporary increase in *PD* of about 1—2 mV. When the preincubation period was shortened to 10 min, *PD* was rapidly increased to above 4 mV but thereafter declined gradually. However, the preincubation with Na-free Ringer solution did not affect the ability of *PD* to recover to the normal level after replacement with control solution. In this case, both mucosal and serosal solutions were replaced to exclude the influence of diffusion potentials.

Figure 4 shows the mucosal-to-serosal transfer of L-Phe in the presence or absence of glucose. It is known that L-Phe, an essential amino acid, is transported actively across the small intestinal membrane.[13] As is evident from the figure, the transfer of L-Phe was markedly inhibited under Glu-free condition; the rates of L-Phe transfer under control and Glu-free

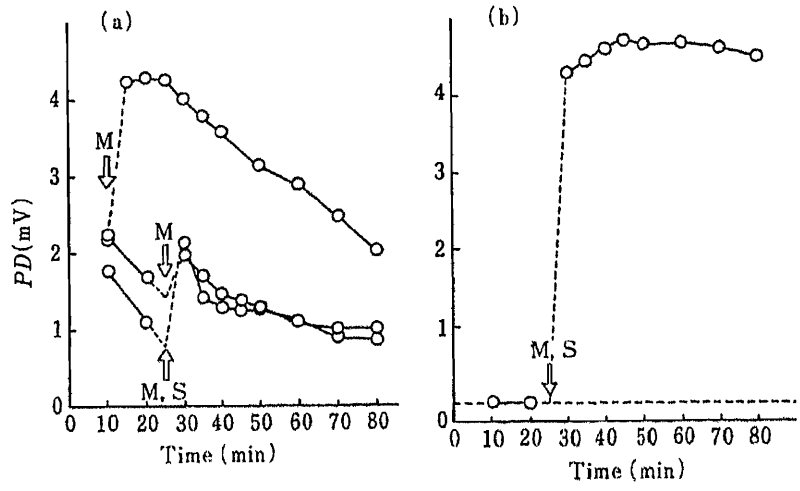

Fig. 3. Recovery of *PD* after Replacement of the Bathing Solution from Glu-Free to Control (a) and from Na-Free to Control Solutions (b)

The replacement of the solution was carried out at the time indicated by arrows. The letter M or S represents the side of replacement, mucosal or serosal, respectively. Each point represents the mean of at least 2 experiments.

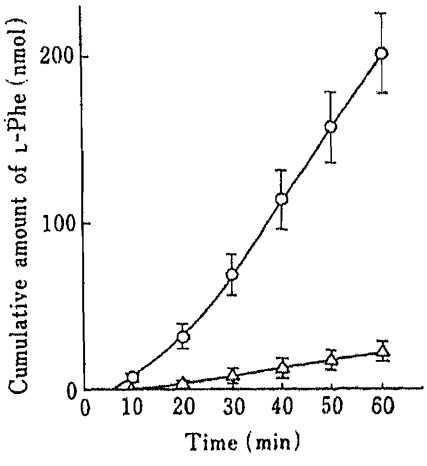

Fig. 4. Effect of Glucose Exclusion from the Bathing Solution on the Mucosal-to-Serosal Transfer of L-Phe

○, control; △, Glu-free conditions.
Each point represents the mean of 7 (control) or 3 (Glu-free) experiments with the S.E.

conditions were 4.41 ± 0.39 and 0.493 ± 0.084 (nmol/cm^2·min), respectively.

Effect of Sequential Replacement of Bathing Solution (Na-Free to Control) on *PD*, R_m and SA Transfer

As shown in Fig. 5, *PD* and R_m of the jejunal membrane and the transfer of SA under Na-free condition were similar to those in the previous experiments (Figs. 1 and 2). After the replacement with the control solution, *PD* recovered to the control level of around 5 mV in spite of the prolonged (70 min) incubation with Na-free solution. This replacement also reduced the transfer rate of SA gradually to the control level of 1.8—2.0 nmol/cm^2·min. In this figure, the transfer of SA is represented by the amount of SA transferred in each 10 min. R_m increased rapidly after the replacement and, thereafter, decreased gradually to the level which was slightly higher than that under the control condition.

Effect of Phl on *PD*, R_m and SA Transfer

Phl is a specific inhibitor of Na^+-glucose coupled transport due to its high binding activity to the carrier protein for glucose transport, which is located in the brush-border

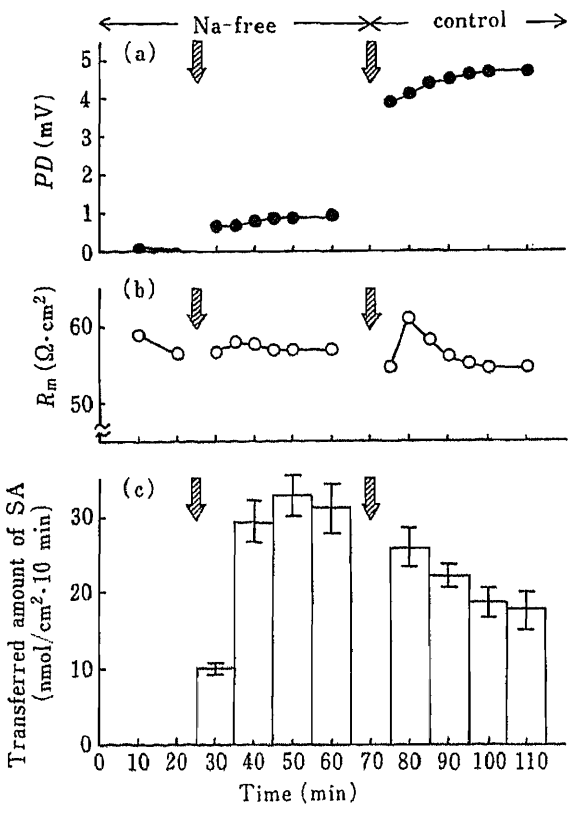

Fig. 5. Effects of Sequential Replacement of Bathing Solutions on *PD* (a), R_m (b) and the Mucosal-to-Serosal Transfer of SA (c)

The time course of the experiment was as follows: 0—25 min, preincubation with Na-free Ringer solution; 25—70 min, experiment under Na-free condition with SA in the mucosal side; 70—115 min, experiment under control condition with SA in the mucosal side. The solution replacement times are indicated by arrows. The mucosal-to-serosal transfer of SA was measured as the amount of SA transferred during each 10 min and expressed as the height of bars with the S.E. (c). Each point in (a) or (b) represents the mean of 4 experiments.

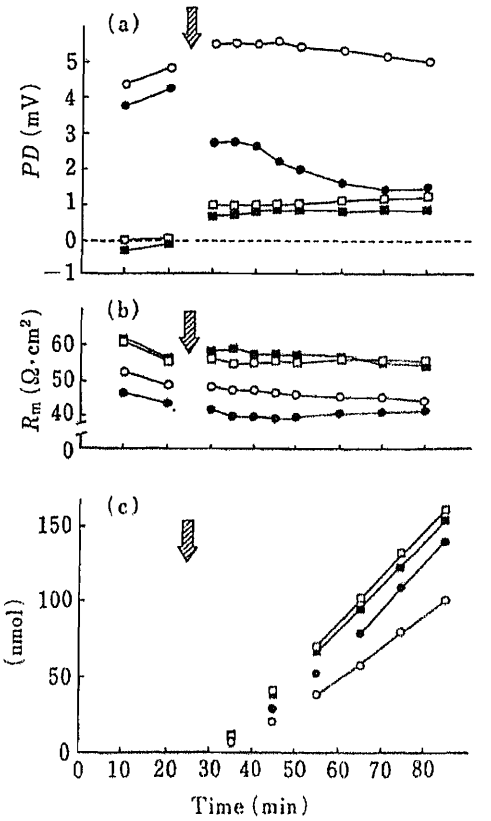

Fig. 6. Effects of Phloridzin on *PD* (a), R_m (b) and the Mucosal-to-Serosal Transfer of SA (c) under Control or Na-Free Condition

○, control; ●, control+Phl; □, Na-free; ■, Na-free+Phl conditions.

Phloridzin and SA containing Ringer solution was introduced to the mucosal side at 25 min (indicated by arrows). Each point represents the mean of 4—6 experiments.

membrane of epithelial cells. Phl at 1 mM was added to the mucosal side under control or Na-free condition to examine its effects on *PD*, R_m and the transfer of SA. The results are shown in Fig. 6. Under Na-free condition, the addition of Phl caused no significant change in *PD*, R_m or the transfer of SA, while under the control condition, *PD* decreased rapidly and SA transfer increased. At 40 min after the Phl addition, the rate of SA transfer became as high as that under Na-free condition. R_m under the control condition showed a decreasing tendency after the addition of Phl, but the difference was not statistically significant.

Discussion

The present study was undertaken with a view to establishing the experimental conditions which provide the best environment for maintaining the viability and the integrity of intestinal membranes *in vitro*. When examining drug absorption mechanisms or, further, the influences of pharmaceutical adjuvants on the membrane permeability or functions, one must take into account how long the membrane isolated from the body can maintain its physiological functions.

The marked reduction in *PD* in Glu-free or Na-free Ringer solution (Fig. 2) would be due to the disappearance of Na^+-glucose coupled transport, the electrogenic active transport system which supports a large part of *PD* across the intestinal membrane. Further, from *PD* recovery experiments, it was found that the incubation with Glu-free Ringer solution caused an irreversible reduction in the ability of the membrane to transport Na^+ actively. This damage to the viability of the membrane also reduced the active transfer of L-Phe. However, the effect of Na-free condition was reversible. The everted sac study on L-Phe transfer under control and Glu-free conditions gave the following result, which is similar to that in the case of the membrane sheet (Fig. 4): the transfer rates across the everted jejunal sac were 0.120 ± 0.007 and 0.0265 ± 0.0088 (μmol/min·sac) under control and Glu-free conditions, respectively. This indicates that exclusion of glucose from the bathing solution reduced the ability of the intestinal membrane to transport L-Phe actively, regardless of the experimental method employed, *in vitro*. Pritchard and Porteous[14] demonstrated that glucose is transported actively by rat small intestine *in vitro* and about 50% of the absorbed glucose is metabolized within the mucosal tissue, 90% to lactate and 10% to CO_2. This metabolism (glycolysis) would elevate the level of adenosine triphosphate in the cell and supply the energy required for active transport and to support the viability of the membrane. Even under Glu-free condition, sodium can be actively transported as Na^+ or NaCl[5] and, thus, the endogeneous energy is consumed. However, under Na-free condition, most of the active transport was blocked and the consumption of endogeneous energy would be small. These differences between Glu-free and Na-free conditions might cause the differences in the reversibility of their effect on *PD*.

The alteration in the mucosal-to-serosal transfer of SA and SG reflects the effect of buffer composition on the permeability, in other words, the barrier functions of the membrane. Both SA and SG are poorly absorbable drugs due to their low lipophilicity at the physiological pH region, where SA is completely ionized but SG is not at all. Thus, the similar increases in the transfer of the two drugs under Glu-free or Na-free conditions indicated that these effects are not due to the change in the electrical charge of the membrane by altering the ionic composition of bathing solutions. In addition, loss of integrity of the intestinal membrane with time, as shown by Benet *et al.*,[4] was negligible in our experiments since the rates of SA and SG transfers were constant after the lag time of 10—20 min (Fig. 1).

When considering the factors which alter the rate of drug transfer, one of the most important things to know is the route of movement of the drug molecule across the membrane. It is likely that leaky epithelium such as small intestine or gallbladder could

provide the extracellular route for low-molecular-weight and low lipophilic drugs.[15] We have demonstrated that SA penetrated the intestinal membrane not only *via* the intracellular route but also *via* the extracellular tight-junctional one.[16] Therefore, the effects of glucose or Na^+ exclusion on these two routes should be discussed separately.

Relating to the effect on the paracellular route, there have been a series of studies by Mayersohn and Gibaldi.[3,17] They found that there is a good correlation between the extent of tissue-fluid uptake under various experimental conditions and the mucosal-to-serosal transfer of riboflavin across the everted intestine. They concluded that the presence of materials causing tissue-fluid uptake (glucose, xylose or potassium) in bathing solutions would induce swelling of the epithelial cells to narrow the extracellular route. Under these conditions, transfer of drugs *via* the extracellular route would be suppressed. In the present study, however, there was no significant change in R_m between the control and Glu-free conditions, as shown in Fig. 2. Also, the addition of Phl to the mucosal solution caused only a small (not significant) decrease in R_m. Okada *et al.*[18] have reported that the presence of D-glucose in the mucosal solution did not cause any significant changes in R_m, by using rat duodenum, jejunum and ileum. Since the major ionic conductance pathway in leaky tissues is the extracellular route,[19] the structural change in the extracellular portion of the epithelium must cause changes in R_m values. Thus, if the exclusion of glucose or sodium from the incubation medium reduced the fluid uptake by tissue, resulting in a widening of the extracellular space, and enhancing the permeability of the extracellular route, the significant change in R_m should be detected. In this respect, the electrophysiological observations are in disagreement with the fluid uptake hypothesis.

One possible interpretation of this contradiction is as follows. The major barrier to ionic conductance in the extracellular route in leaky epithelia is tight-junctions, while the bulk of the extracellular volume consists of the lateral intercellular space.[19] Thus, the swelling of the epithelial cells induced by the increase in fluid uptake might narrow mainly the lateral intercellular space and probably would not influence R_m till the lateral space was closed to the level limiting the mobility of ions such as Na^+ or Cl^- in preference to the tight-junctions. Wiedner and Wright[20] demonstrated that mucosal hypertonicity caused the collapse of the lateral intercellular space and suppressed ion permeation through the extracellular route. Under this condition, the lateral intercellular space could govern drug movement *via* the paracellular route. Therefore, to clarify the factors which enhance the paracellular permeability to drugs, more detailed investigations, which take into account the role of the lateral intercellular space, would be necessary.

The increase in R_m observed when sodium was replaced with choline is almost certainly attributable to the fact that the extracellular pathway, which accounts for more than 80% of the membrane conductance, is relatively impermeable to choline.[5] Thus, this phenomenon should not be due to alteration of the membrane structures and, under Na-free condition, direct comparison of R_m values with those under the control condition would not be successful.

As to the influences on the permeability of the transcellular route, there is no evidence at the present stage to suggest that buffer compositions affect the permeability of the apical membrane of epithelial cells. However, it is likely that the low viability of epithelial cells under Glu-free condition is related to the loss of integrity of the cellular membrane, since the transfer rates of both SA and SG were increased more under Glu-free condition than under Na-free condition.

When deciding the composition of buffer solution to be used in *in vitro* transport experiment, one must make clear the purpose of the experiment and choose buffers which provide suitable conditions. Himukai *et al.*[21] noted that low Na^+ concentration (50 mM) and the replacement of Cl^- with SO_4^{2-} gave good reproducibility of the sugar- or amino acid-

induced change in *PD* of the intestinal membrane. This suggests that one must take care to confirm the viability of the membrane in examining the properties of drug transfer across the intestinal membrane, especially in the case of active transport, under *in vitro* conditions. In addition, when examining the effects of the pharmaceutical adjuvants on the membrane permeability to drugs, it would be necessary to check whether the adjuvants affect the active transport activity of the membrane or not, because the inhibition of the active transport itself could enhance the permeability of the membrane, as in the case of Phl.

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Enhancement of Percutaneous Absorption of Molsidomine

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Molsidomine, which is effective in treating angina pectoris, was poorly absorbed through the skin from a simple solution in oleic acid or propylene glycol, and its bioavailability was below 1% in rats. However, molsidomine was efficiently absorbed from a two-component system consisting of oleic acid and propylene glycol. Maximum absorption enhancement was observed in the two-component system containing 10% oleic acid; the bioavailability was about 95% within 6 h. Remarkable percutaneous absorption enhancement was also observed in the presence of linoleic acid when a series of unsaturated straight fatty acids with different carbon numbers were substituted for oleic acid. Lauric acid was the most effective in the series of saturated straight fatty acids. Effective percutaneous absorption enhancement also occurred with lauryl alcohol and oleyl alcohol, but little enhancement was observed with any fatty acid ester or sodium oleate. The two-component system is also effective for some water-soluble drugs and poorly water-soluble drugs, besides molsidomine.

Keywords——controlled-release transdermal dosage form; percutaneous absorption enhancer; molsidomine; angina pectoris; oleic acid; propylene glycol; lauric acid; linoleic acid; two-component system; bioavailability

Transdermal preparations have been used with drugs intended principally for local therapy. However, in recent years the development of transdermal dosage forms for systemic therapeutics has been actively pursued by many research groups. Beckett[1] maintained that the major advantages of a transdermal preparation are that it permits long-acting or controlled-release delivery to achieve constant plasma concentration of a drug, thus conveniently enhancing the drug's efficacy and minimizing its side-effects; it permits the use of pharmacologically active agents with short biological half-lives; and it avoids first-pass metabolism and allows drug input to be rapidly terminated by removing the system from the skin surface if side effects develop.

Drugs such as scopolamine,[2] nitroglycerin,[3] and isosorbide dinitrate,[4] which are commercially available as transdermal drug delivery systems, do not present any problem because they are relatively easily absorbed through the skin and their dose is small. Unlike these drugs, molsidomine[5] (*N*-ethoxycarbonyl-3-morpholinosydononimine) is poorly absorbed through the skin and consequently it is necessary to enhance its percutaneous absorption.

From the viewpoint of safety it is desirable that enhancers are materials conventionally used in pharmaceutical preparations. Various fatty acids and alcohols have been examined as effective enhancers[6] for percutaneous absorption and for rectal absorption. We attempted to find appropriate enhancers for molsidomine from among the fatty acids and alcohols, and found that the use of oleic acid and propylene glycol gave greatly improved percutaneous absorption of molsidomine in rats.

Experimental

Materials——Molsidomine was synthesized by Takeda Chemical Industries, Ltd., Japan. It is weakly basic with

a molecular weight of 242.23, a melting point of 139 to 142 °C, and a pK_a of 3.2. Protirelin tartrate (TRH-T), oxendolone, and TAI-908 (4-(4-methylbenzoyl)-1-indancarboxylic acid) were supplied by Takeda Chemical Industries, Ltd. and indomethacin, diazepam, and nifedipine were obtained commercially.

Propylene glycol, polyethylene glycol 400, glycerin, oleic acid, lauric acid, and all other chemicals were of analytical grade, obtained from Wako Pure Chemical Ind. Ltd., Osaka.

Animals——SD-JCL strain male rats, aged 7 weeks and weighing 240 to 280 g, were supplied by Clea Japan Inc., Tokyo.

Preparation of Test Solutions——The test solutions used for the percutaneous absorption of molsidomine were prepared by dissolving or suspending a constant amount of 10 mg of the drug and making the weight up to 200 mg by adding a vehicle alone or a vehicle containing an enhancer. The test solution of protirelin tartrate was prepared by mixing 10 μCi of ^{3}H-labeled protirelin (TRH) with a specific radioactivity of 100 Ci/mmol and 0.8 mg of TRH-T and adding the mixture to a vehicle to give a total weight of 200 mg.

Percutaneous Absorption——The rats were anesthetized with pentobarbital, and their abdominal hair was clipped with an electric hair clipper without damaging the skin. A test solution of 200 mg was directly administered to a part of the denuded area (20 cm^2; 5 × 4 cm) and blood samples of 0.5 ml were taken from the tail vein at 1, 2, 4, and 6 h afterward.

Determination of Molsidomine in Plasma——A venous blood sample (0.5 ml) was centrifuged (3000 g × 10 min), and 0.2 ml of the resulting plasma was taken into a test tube containing 1 ml of water and 5 ml of chloroform. This mixture was shaken for 10 min, and molsidomine was extracted into the chloroform layer; then 4 ml of the chloroform layer was pipetted out, and the solvent was evaporated off. The residue was taken up in 0.2 ml of a mixed solution of 0.05 M sodium acetate, acetonitrile, and tetrahydrofuran (a volume ratio of 70 : 30 : 0.2), and 50 μl of the resulting solution was injected into a liquid chromatograph (LC-4A: Shimadzu Seisakusho Ltd., Kyoto), equipped with a μ Bondapak C_{18} (i.d. 4 mm × 300 mm) column, and a ultraviolet (UV) detector (313 nm). The flow rate of the mobile phase was 0.8 ml/min. This method gave adequate separation of molsidomine and its metabolite cyanoethylene-aminomorpholine (retention time of molsidomine, 5.8 min; retention time of the metabolite, 6.7 min).

Molsidomine was extremely stable; 99.8% was recovered intact after incubation in rat plasma at 37 °C for 3 h.

Bioavailability of Molsidomine——An aliquot of saline solution (0.5 ml) containing 4 mg of molsidomine was administered intravenously to rats. At 10, 20, 30, 45, 60, 90, 120, and 180 min afterward, venous blood samples were taken to determine the plasma concentraion of molsidomine. The area under the plasma concentration–time curve (AUC) was calculated, and found to be 13.858 (μg·h/ml). The bioavailability (absolute bioavailability) through the percutaneous absorption of molsidomine was determined by applying Eq. 1.

$$\text{bioavailability } (\%) = [AUC^{6}_{0\,\text{transdermal}}\ (\mu\text{g}\cdot\text{h/ml}) / AUC^{3}_{0\,\text{i.v.}}\ (\mu\text{g}\cdot\text{h/ml})] \times 100 \qquad (1)$$

Solubility of Molsidomine——An excess of molsidomine was added to each vehicle and the mixture was shaken at 25 ± 1 °C for 2 h. The undissolved molsidomine was collected on a filter paper and the molsidomine concentration in the filtrate was determined by liquid chromatography.

Measurement of the Residual Amount of Molsidomine on the Skin——The residual amount of molsidomine remaining at the site of application on the skin was determined by washing the skin 10 to 15 times with absorbent cotton soaked with a solution of chloroform and ethanol (1 : 1); the amount of molsidomine in the recovered solution was measured by liquid chromatography. The recovery was investigated in a separate experiment. First, 200 mg of 10% oleic acid–propylene glycol solution containing 10 mg of molsidomine was applied to the denuded abdomen (20 cm^2) of a rat, then 5 min later, the area was washed and the amount of molsidomine recovered was measured. The average recovery for 5 rats was 97.2%. The residual percent (%) found when a test sample was applied was corrected by applying Eq. 2.

$$\text{corrected residual percent } (\%) = [\text{residual percent found } (\%) / 97.2\ (\%)] \times 100 \qquad (2)$$

Determination of TRH-T——At a fixed time after application of the test solution, a 0.12 ml blood sample was taken from the tail vein, and centrifuged. The resulting plasma (0.05 ml) was pipetted into a polyethylene vial containing 5 ml of a scintillator based on toluene, and the contents of the vial were mixed. The mixture was allowed to stand, then the whole radioactivity was measured with a β-ray scintillation counter to determine the protirelin equivalent concentration (μg·eq/ml) in the plasma.

Determination of Applied Drugs——The plasma concentrations of diazepam and TAI-908 were determined by liquid chromatography in accordance with the procedure for determining molsidomine; benzene was used as an extraction solvent for diazepam. Oxendolone, indomethacin and nifedipine were determined by the methods of Itakura,[7] Misaki *et al.*,[8] and Pietta *et al.*,[9] respectively.

Results and Discussion

Percutaneous Absorption of Molsidomine from Single Vehicles

The results of the percutaneous absorption of molsidomine from single lipid-soluble and

TABLE I. Percutaneous Absorption of Molsidomine from Various Vehicles in Rats

Vehicle	C_{max} (μg/ml)	AUC_0^6 (μg·h/ml)	Solubility at 25 °C (%)	Partition coefficient at 25 °C	Bioavailability[a] (%)	Percent[b] remaining on skin
Glycol salicylate	0.06	0.16	15.1	42.7[c]	0.46	—
Oleic acid	0.11	0.43	1.37	0.43	1.2	98.0
Octyl·decyl·oil	0.81	1.42	0.36	0.21	4.1	94.8
Isopropyl myristate	0.03	0.12	0.09	0.07	0.35	—
Propylene glycol	0.04	0.24	6.37	0.48[d]	0.69	98.9
Polyethylene glycol 400	0.03	0.09	5.23	—	0.26	—
Dimethyl sulfoxide	0.47	1.31	2.60	—	3.8	94.4
Glycerin	0.02	0.08	1.80	0.79	0.52	—

a) Bioavailability $=[AUC_{transdermal}/AUC_{i.v.}]\times 100$. *b*) Percent of molsidomine at 6 h after administration. Each value is the mean of three animals. *c*) Oily vehicle-water. *d*) Benzene/vehicle.

water-soluble vehicles are presented in Table I. Molsidomine was best absorbed percutaneously from octyl·decyl oil (medium chain triglyceride) among lipid-soluble vehicles and from dimethyl sulfoxide (DMSO) among water-soluble vehicles; the bioavailability in both instances was about 4%. With all other vehicles the bioavailability was not more than 1%. These values were consistent with the absorbed amounts of molsidomine as determined from the residual amounts remaining at the application sites. However, no vehicle was found that specifically enhanced the percutaneous absorption of molsidomine.

It is known that percutaneous absorption of a drug varies with its physico-chemical properties,[10] such as molecular weight, melting point, solubility, and partition coefficient between oil and water, and also factors associated with the pharmaceutical preparation, such as the type of vehicle or base employed. Higuchi[11] has shown that the absorption rate of a fairly water-soluble drug in the steady state can be expressed by Eq. 3.

$$\text{rate of percutaneous absorption} = [\text{partition coefficient (skin/vehicle)}]\,[\text{drug concentration in pharmaceutical preparation}]\,[\text{drug diffusibility in skin}][\text{applied area}]/[\text{thickness of skin}] \quad (3)$$

This equation indicates that to enhance percutaneous absorption, it is necessary to increase the drug concentration in the vehicle and the partition of the drug from the vehicle to the skin, *etc.*

No particular relation was found between the percutaneous absorption of molsidomine (AUC_0^6) and its solubility or partition coefficient in the vehicles shown in Table I.

Enhanced Percutaneous Absorption of Molsidomine

To enhance the percutaneous absorption of molsidomine, a screening test of possible absorption enhancers was carried out. Incorporation of either oleic acid (10%, w/w) or lauric acid (10%, w/w) into propylene glycol dramatically enhanced the percutaneous absorption of molsidomine (Fig. 1). In the case of oleic acid, the absorption, in terms of AUC_0^6, was enhanced 140 times over that of the control (propylene glycol). The plasma concentration of molsidomine rose sharply from 30 min after application of the drug, reached the maximum (C_{max}) in about 2 h, and thereafter decreased rapidly. The bioavailability was as high as 97%, and molsidomine was mostly absorbed within 6 h after it was administered. The residual amount of the drug at the site of application was found to be 3%, which was in good agreement with the bioavailability. When lauric acid was used, the absorption of molsidomine

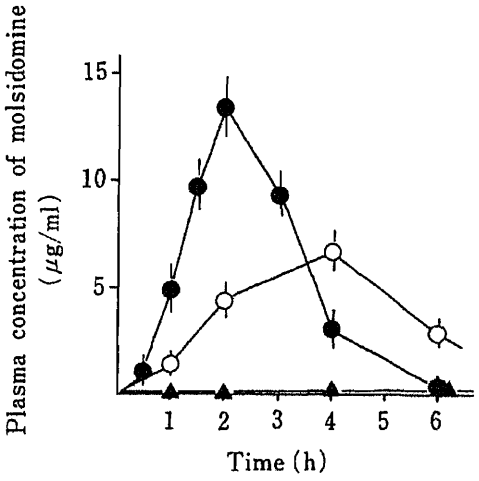

Fig. 1. Effect of Fatty Acids on the Percutaneous Absorption of Molsidomine in Rats

—●—, oleic acid (10%, w/w); —○—, lauric acid (10%, w/w); —▲—, control (propylene glycol alone). Each point represents the mean±S.E. of six rats.

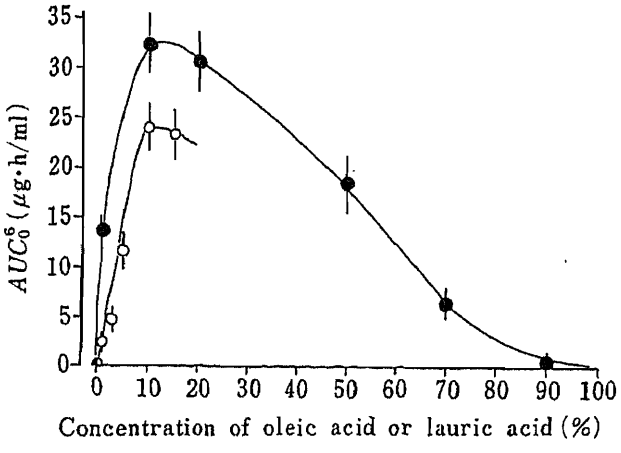

Fig. 2. Degree of Enhancement of Molsidomine Percutaneous Absorption as a Function of Enhancer Concentration from a Saturated Solution of Molsidomine in Rats

—●—, oleic acid/propylene glycol; —○—, lauric acid/propylene glycol. Each point represents the mean±S. E. of four rats.

was slower, but was still markedly enhanced over the control. The maximum absorption occurred at 4h after administration, and the bioavailability within 6h was 64%. If the bioavailability had been determined over a more prolonged period, however, a higher value would have been obtained. It is interesting that the percutaneous absorption of molsidomine from a single vehicle of oleic acid or propylene glycol is low, whereas the absorption from the two-component system is strikingly enhanced.

The percutaneous absorption of molsidomine in relation to the amount of oleic acid or lauric acid in propylene glycol was next investigated (Fig. 2). In the oleic acid–propylene glycol system, the absorption rose rapidly as the amount of oleic acid incorporated was increased. Maximum enhancement was observed with an incorporation of about 10%, and decreased gradually with further addition. When more than 50% oleic acid was added, the drug hardly dissolved in the two-component system; consequently, the decrease of absorption at higher proportions of oleic acid is at least partially attributable to a reduction in the amount of molsidomine dissolved. Lauric acid was found to behave similarly. In the two-component system, oleic acid or lauric acid is thought to act as an absorption enhancer, whereas propylene glycol is assumed to act as an auxiliary agent for absorption enhancement. Few cases are known where a specific ratio of two components provides the maximum percutaneous absorption-enhancing effect. In a study by Nelson Research and Development[12] maximum permeation through the skin of triamcinolone acetonide was attained with a 10% Azone®–ethanol solution. Such a specific two-component system is considered to be of use in designing percutaneous dosage forms; alteration in the ratio of the two components permits selection of systems giving arbitrary percutaneous absorption of molsidomine. Furthermore, the percutaneous absorption can be adjusted by diluting the system with any vehicle or base.

The effect of an enhancer on the percutaneous absorption of molsidomine was also examined using saturated or unsaturated fatty acids having different numbers of carbon atoms (Fig. 3). The saturated fatty acids, except lauric acid (having 12 carbon atoms), showed only minor enhancement. Although the reason why lauric acid enhances the absorption of molsidomine has not been clarified, it is assumed that, together with propylene glycol, it may

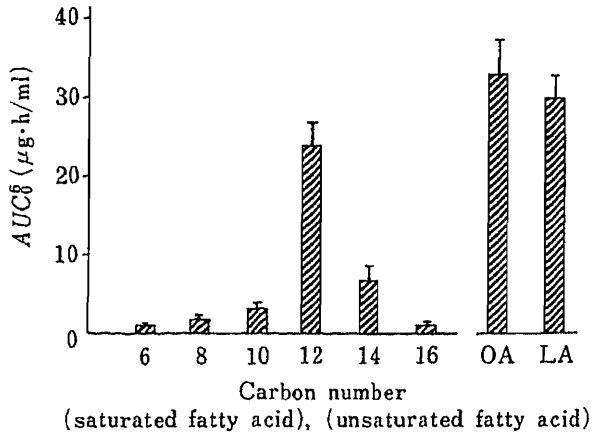

Fig. 3. Effect of Carbon Number of Saturated Fatty Acids and Unsaturated Fatty Acids on the Percutaneous Absorption of Molsidomine in Rats

OA, oleic acid; LA, linoleic acid. Each bar is the mean±S.E. of three rats.

TABLE II. Effect of Functional Groups on Percutaneous Absorption of Molsidomine in Rats

Enhancer	AUC_0^6 (μg·h/ml)	C_{max} (μg/ml)	Enhancer	AUC_0^6 (μg·h/ml)	C_{max} (μg/ml)
Lauric acid	20.51±1.50	5.20±0.41	Oleic acid	33.6 ±2.75	13.14±0.89
Lauryl alcohol	24.76±2.54	12.42±0.79	Oleyl alcohol	15.66±3.57	6.08±2.48
Methyl laurate	6.08±2.14	4.00±0.93	Methyl oleate	0.88±0.11	0.32±0.15
			Sodium oleate	5.69±1.41	1.80±0.53

Each value is the mean±S.E. of three rats.

act on the sebum in the stratum corneum to alter the permeability. Linoleic acid, an unsaturated fatty acid, resulted in a bioavailability of 78% and produced an absorption-enhancing effect nearly equal to that of oleic acid.

The effect of the type of functional groups of aliphatic compounds on the percutaneous absorption of molsidomine was also investigated (Table II). When the functional group was replaced by a hydroxyl group, the enhancing effect was still present. Lauryl alcohol was as effective as lauric acid, whereas oleyl alcohol was only half as effective as oleic acid. In contrast, replacement of the functional group with a methyl ester group brought about a marked decrease in the enhancing effect. A small enhancing effect was seen with sodium oleate. Okada *et al.*[13] reported that citric and succinic acids were exceptionally effective as vaginal absorption enhancers for leuprolide, whereas their sodium salts were not. Similar results were observed in the present percutaneous absorption system.

Effects of Vehicles on Absorption Enhancement

Propylene glycol, a basic vehicle component, was replaced by water-soluble polyhydric alcohols or oily vehicles in combination with oleic acid (Table III). The polyhydric alcohols and lipid-soluble vehicles, except ethylene glycol, all produced results similar to those obtained with the control and did not show enhancement. It was confirmed that oleic acid, combined with propylene glycol, gave the most striking enhancement. This combination is considered to be most effective to dissolve the sebum or to act on the whole stratum corneum to alter the permeability to molsidomine, but the precise mechanism is unknown.

Dose Dependence on the Plasma Concentraion of Molsidomine

Plasma concentrations of molsidomine were measured after application of 5 and 10 mg of molsidomine to rats (Fig. 4). The *AUC* from 0—6 h after the 5 mg administration was approximately half that after the 10 mg administration and the plasma concentration was

TABLE III. Effect of Vehicles on the Percutaneous Absorption of Molsidomine in Rats

	C_{max} (μg/ml)	AUC_0^6 (μg·h/ml)
Oily vehicle		
Glycol salicylate	0.14±0.01	0.65±0.05
Methyl salicylate	0.40±0.02	1.96±0.12
Olive oil	0.65±0.11	2.06±0.28
Liquid paraffin	ND[a)]	ND
Polyhydric alcohol		
Ethylene glycol	2.40±0.16	5.12±1.81
Polyethylene glycol 400	0.42±0.05	1.23±0.14
Glycerin	0.31±0.02	0.71±0.05

ND: Not detected. Each test solution contained 10% oleic acid. Each value is the mean±S.E. of three rats.

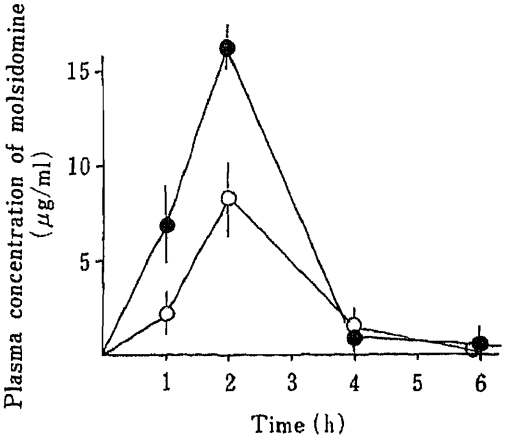

Fig. 4. Dose Dependence in the Percutaneous Absorption of Molsidomine in Rats

—●—, 10 mg of molsidomine/rat; —○—, 5 mg of molsidomine/rat. Each point represents the mean± S.E. of four to six rats.

found to be nearly proportional to the dose.

Pharmacokinetics of Percutaneous Absorption of Molsidomine

The rate of percutaneous absorption of molsidomine in rat was determined using the 10% oleci acid–propylene glycol system. To determine the rate of absorption, an analysis based on the following compartment theory was used. Assuming that the behavior of a drug *in vivo* follows the one-compartment model and that the process of absorption is zero- or first-order, the following two kinds of model equations can be derived:

zero-order model;

$$C_p=\frac{K_0}{K_e K_d}[1-e^{-K_e(t-t_0)}] \quad (t_0 \leqq t \leqq t_1) \tag{4}$$

$$C_p=\frac{K_0}{K_e \cdot V_d}[1-e^{-K_e(t-t_0)}]\cdot e^{-K_e(t-t_1)} \quad (t>t_1) \tag{5}$$

first-order model;

$$C_p=\frac{K_a \cdot F \cdot D}{V_d(K_a-K_e)}[e^{-K_e(t-t_0)}-e^{-K_a(t-t_0)}] \tag{6}$$

wherein;

K_0: zero-order absorption rate
K_a: first-order absorption rate constant
K_e: first-order elimination rate constant
V_d: distribution volume
t_1: completion time of absorption
t : time
t_0: lag time
D : dose
F : fraction absorbed
C_p: plasma concentration

Further, the plasma concentration of the drug after intravenous administration is given by the following equation:

$$C_p=\frac{D_{iv}}{V_d}e^{-K_e \cdot t} \tag{7}$$

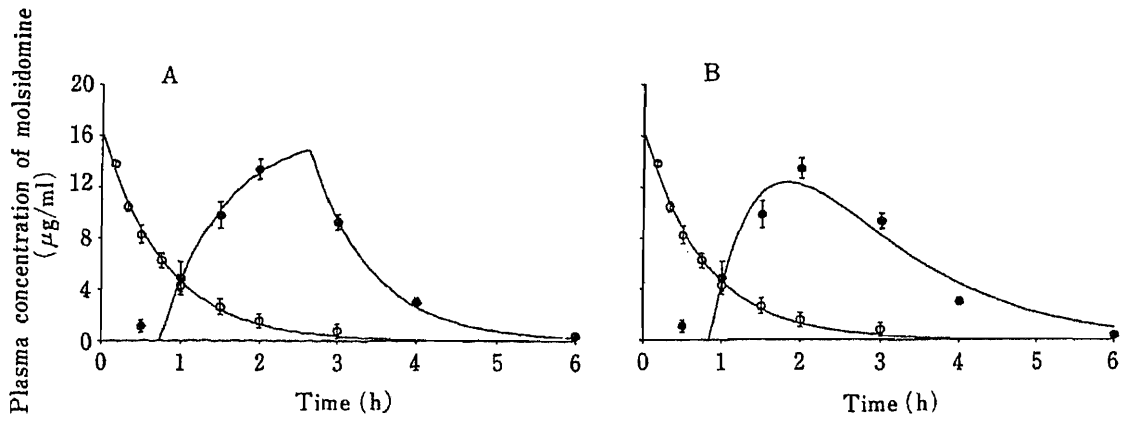

Fig. 5. Simultaneous Nonlinear Least-Squares Fitting of Intravenous and Percutaneous Administration Data for Molsidomine

A: Zero-order absorption model. B: First-order absorption model. —○—, intravenous injection data; —●—, percutaneous administration data. Each point represents the mean±S.E. of six rats.

TABLE IV. Results of Simultaneous Nonlinear Least-Squares Fitting by Means of the Program MULTI

	Zero-order model	First-order model
AIC	24.49	45.01
Sum of squares	2.628	10.320
Parameters:		
Absorption rate	K_0= 5.103 (mg/h)	K_a= 0.8238 (h^{-1})
Lag time (h)	0.7329	0.8538
Availability (%)	97.14	103.6
Elimination rate constant (h^{-1})	1.271	1.270
Distribution volume (ml)	244.1	244.1

where D_{iv} is the dose administered intravenously. By analyzing plasma concentraion data obtained after intravenous injection and percutaneous administration in terms of these model equations, the pharmacokinetic parameters for each can be determined. The calculations were performed using MULTI,[14] a program for a personal computer. The results of the calculations are shown in Fig. 5 and Table IV. A comparison of the zero-order model with the first-order model indicates that the former yields a smaller AIC[15] (Akaike's information criterion to select the optimum statistical model). Therefore, in this case the plasma concentration change of molsidomine after percutaneous administraion is consistent with the zero-order absorption model. As is evident from Fig. 5, the zero-order model fits the data better. In addition, it is convenient for investigating to what extent the data obtained *in vivo* and *in vitro* are consistent with each other, because the steady-state flux obtained from the permeation experiment *in vitro* has a zero-order permeation rate. The absorption rate of molsidomine from the 10% oleic acid–propylene glycol system is 5.103 (mg/h) (Table IV). The absorption rate per unit surface area J and the availability were calculated as 255 ($\mu g/cm^2/h$) and 97.14 (%), respectively.

Application of the Percutaneous Absorption Enhancer to Other Drugs

These studies were extended to determine whether the 10% oleic acid–propylene glycol system is effective for drugs other than molsidomine (Table V). The drugs selected were those that require sustained release for systemic administraion. The absorptions of indomethacin

TABLE V. Application of the Enhancer to Various Drugs

Drug	Component[a]	Dose (mg)	C_{max} (μg/ml)	AUC_0^6 (μg·h/ml)	E (AUC) / C (AUC)	Percent remaining on skin (%)
TAI-908[b]	C	5	1.27± 0.03	2.50± 0.09	49.8	97.4
	E	5	39.01± 0.14	124.50± 8.23		27.8
Diazepam	C	5	0.04± 0.01	0.25± 0.07	6.7	97.0
	E	5	0.61± 0.13	1.66± 0.17		54.2
Indomethacin	C	5	1.22± 0.28	2.73± 0.85	90	52.3
	E	5	97.6 ±16.91	246.1 ±38.09		9.3
Nifedipine	C	3	0.56± 0.03	2.16± 0.07	3.5	82.6
	E	3	2.05± 0.35	7.63± 0.39		62.8
Oxendolone	C	10	6.6 ± 0.8[c]	28.1[c]± 0.4	2.6	97.9
	E	10	13.4 ± 1.8	72.5 ± 2.3		—
Protirelin tartrate	C	0.8	0.04± 0.002	0.16± 0.03	6.8	93.7
	E	0.8	0.81± 0.10	1.09± 0.02		63.9

a) C, control (propylene glycol alone); E, enhancer (10% oleic acid/propylene glycol). *b*) 4-(4-Methylbenzoyl)-1-indancarboxylic acid. *c*) C_{max} in ng/ml, AUC in ng·h/ml. Each value is the mean±S.E. of three rats.

and its homologous compound, TAI-908, were enhanced 50 to 100 times over the control, with 70 to 90% availability. The absorptions of diazepam and nifedipine were enhanced 2- to 7-fold over the control, with about 40% availability. Oxendolone, a lipid-soluble steroid derivative, was absorbed least among the drugs investigated.

The stratum corneum consists of a dense layer of dead cells filled mainly with a polymerized keratin matrix and lipids, and is regarded as a lipid barrier to drug permeation. Many reports[16] have described the comparative advantage of lipid-soluble drugs for percutaneous absorption; this is the so-called lipid theory of percutaneous absorption.

However, the absorption of TRH-T, a water-soluble peptide compound, was enhanced 7-fold over the control, with about 30% availability. The two-component system was thus confirmed to exhibit absorption enhancing action for water-soluble drugs as well. On the other hand, insulin, with a molecular weight of about 6000, was not absorbed. Percutaneous absorption of drugs with a molecular weight of more than 1000 seems to be difficult and presumably insulin was not absorbed because of its high molecular weight.

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Mechanism of Enhancement of Percutaneous Absorption of Molsidomine by Oleic Acid

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The mechanism of percutaneous absorption of molsidomine, enhanced by a two-component system consisting of oleic acid and propylene glycol, was studied *in vitro* and *in vivo* in rats. About 10—20% of the oleic acid was absorbed from the skin independently of the oleic acid–propylene glycol ratio. In contrast, about 95% of molsidomine and propylene glycol were transdermally absorbed from the two-component system containing 10% oleic acid in 6 h. The permeability (percent of dose) of molsidomine through the excised rat skin was comparable to that of propylene glycol (both about 80% in 24 h). These results suggest that the molsidomine and propylene glycol permeated simultaneously through rat skin. Our proposed mechanism of percutaneous absorption of molsidomine assumes that oleic acid and propylene glycol penetrate into the stratum corneum and improve the permeability of the skin by dissolving hard lipoidal components, and then molsidomine, dissolved in the propylene glycol, passes through the modified stratum corneum.

Keywords——controlled-release transdermal dosage form; percutaneous absorption mechanism; percutaneous absorption enhancer; molsidomine; oleic acid; stratum corneum; propylene glycol

In designing controlled-release transdermal dosage forms of molsidomine containing percutaneous absorption enhancers, it is necessary to control the release not only of molsidomine but also of the absorption enhancers from the pharmaceutical preparation. Furthermore, it is important to know the mechanism by which such absorption enhancers modify the absorption of molsidomine.

The principal barrier to percutaneous absorption of drugs is said to be the stratum corneum,[2)] which is composed of dead cells consisting mainly of keratin and lipids. As a whole, it is considered to possess the nature of a lipid barrier.[3)] Mixed systems of fatty acids and alcohols have been examined as effective enhancers[4)] of percutaneous absorption, but the mechanism by which the epidermal barrier properties are altered is not known.

We have sought absorption enhancers acting on the lipid barrier to improve the percutaneous absorption of molsidomine, and reported in the previous paper[5)] that the two-component system of oleic acid and propylene glycol was remarkably effective; 10% oleic acid gave the maximum effect. Here we report our investigations of the mechanism of action of oleic acid and propylene glycol on the skin permeability to molsidomine.

Experimental

Materials——Molsidomine was produced by Takeda Chemical Industries, Ltd., Japan. Oleic acid, propylene glycol, polyethylene glycol 200, and glycerin were of reagent grade. Oleic acid labeled with ^{14}C, specific activity 59 mCi/mmol, was supplied by New England Nuclear Co., Boston. The reagents were of analytical grade.

Animals——SD-JCL strain male rats aged 7 weeks and weighing 240 to 280 g, were supplied by Clea Japan Inc., Tokyo.

Test Solutions for Absorption Studies *in Vivo*——All test solutions to be administered were prepared by

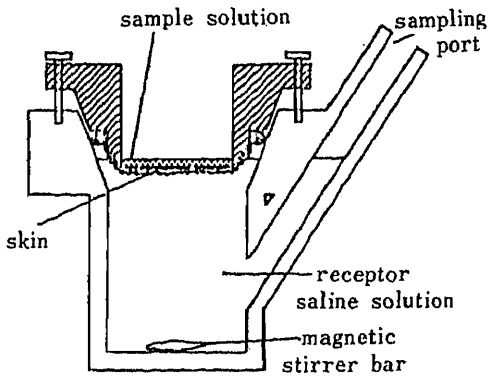

Fig. 1. Schematic Illustration of the Skin Diffusion Cell

dissolving or suspending 10 mg of molsidomine and making the weight up to 200 mg. Oleic acid labeled with ^{14}C was used after being appropriately diluted with the non-labeled substance. A single dose (200 mg) contained 10 μCi of the labeled compound. The test solution was administered by the procedure described in the previous paper.[5]

Determination of the Plasma Concentraion——The plasma concentration of molsidomine was determined in the manner reported previously.[5] The plasma concentration of oleic acid labeled with ^{14}C was measured by the same method as used for ^{3}H-labeled protirelin (TRH) in the previous study.[5]

Determination of the Residual Amount on the Skin——The residual amount of molsidomine or propylene glycol remaining at the site of application was measured by the procedure reported in the previous paper.[5] The residual amount of oleic acid was determined by measuring the ^{14}C-labeled compound in the recovered solution by the same procedure as employed for molsidomine.

Skin Permeation *in Vitro*——The diffusion cell used was similar to the apparatus of Hashida[6] (Fig. 1), on which a piece of excised skin was mounted. The rats were killed by exsanguination, and their denuded abdominal skin was excised, immediately immersed in saline in a refrigerator kept at about 5 °C, and allowed to stand for 8 to 18 h. No significant difference was observed in the permeation of molsidomine through excised skin allowed to stand for various periods of time up to 24 h.

The test solution, except for specific test samples, contained 50 mg of molsidomine (5%). Oleic acid, an absorption enhancer, was incorporated at a concentraion of 10%, unless otherwise stated. One gram of the test solution was applied to the skin. The lower chamber, filled with saline, was stirred vigorously. The temperature was 25±1 °C. Samples, 50 to 200 μl, were taken from the sampling port for assay of molsidomine.

Determination of Molsidomine and Solvent——Molsidomine was determined by liquid chromatography in the manner described in the previous paper.[5] Propylene glycol and glycerin were determined by the method of Lambert and Neish.[7] A 1 ml aliquot of a buffer (NH_3+NH_4Cl) at pH 6 and 0.1 ml of 0.05 M metaperiodic acid as an oxidizing agent were added to 50 μl of the sample, and the mixture was allowed to stand at 37 °C for 15 min. Then 2.0 ml of acetyl acetone was added as a coloring reagent. The resulting mixture was allowed to stand at 37 °C for 40 min and cooled with water; the absorbance was measured at λ_{max} 410 nm. The concentration of propylene glycol or glycerin in the sample was determined from a standard curve prepared previously. Polyethylene glycol 200 was determined by the phosphomolybdic acid method.[8] Three drops of HCl (1→5), two drops of $BaCl_2 \cdot HCl$, and two drops of phosphomolybdic acid were added to 5 ml of the sample solution. The resulting precipitate was separated by centrifugation, the supernatant solution was removed, and 5 ml of distilled water was added to the remaining precipitate. The mixture was shaken and centrifuged again, and the supernatant solution was eliminated. The precipitate was dissolved in 1.2 ml of concentrated sulfuric acid, then 5 ml of distilled water, 1.0 ml of ammonium thiocyanate, and 0.5 ml of $SnCl_2 \cdot HCl$ were added to the solution; the absorbance was measured at 470 nm. The amount of polyethylene glycol 200 was determined from a standard curve prepared separately.

Skin Treated with 10% Oleic Acid–Propylene Glycol——The treated skin was obtained as follows. A 10% oleic acid–propylene glycol solution (200 mg) free of molsidomine was applied to the denuded abdominal skin. The rats were killed by exsanguination 1 and 6 h later and the treated areas were excised, then cleaned 20 times with adsorbent cotton soaked with 50 ml of 50% aqueous ethanol without damaging the skin. The skin was mounted in the diffusion cell, and propylene glycol or glycerin solution (1 g) containing molsidomine (50 mg) was applied to it. The permeation proportions of molsidomine and vehicles were measured 10 h after the application. As a control, the same sample preparations were applied to non-treated skin, and the permeation proportions of molsidomine and vehicles were determined 24 h after the application.

Skin Treated with Oleic Acid——A 50 μl aliquot of ethanol containing 10 mg (corresponding to 1%) of oleic acid was applied to the excised skin (7 cm^2), which was allowed to stand for 1 h. Then the skin was mounted in the diffusion cell and 1 g of propylene glycol containing 50 mg of molsidomine was applied to it. The amounts of molsidomine that permeated were measured with time. In another experiment, 1 g of 1% oleic acid–propylene glycol solution containing 50 mg of molsidomine was applied to the skin coated with 50 μl of ethanol, and the amounts

of molsidomine that permeated were measured.

Stripping of the Stratum Corneum——The stratum corneum was stripped 15 times with a commercially available cellophane adhesive tape.

Histological Examination of the Stratum Corneum——Histological changes in the stratum corneum were examined by applying 10% oleic acid–propylene glycol to denuded abdominal skin, which was excised 1 h later, fixed in formalin by the conventional procedure, stained with hematoxylin-eosin, and examined under a microscope. Untreated skin served as a control.

Results and Discussion

Percutaneous Absorption of Oleic Acid

Figure 2 shows values (ml^{-1}) obtained by dividing the plasma concentrations of oleic acid by the dose of oleic acid when either 200 mg of oleic acid alone or a 10% oleic acid (20 mg)–propylene glycol solution was applied. In both cases, oleic acid was absorbed nearly proportionally to the dose. The residual amount of oleic acid remaining on the skin after 6 h was 77% for the former system and 90% for the latter system (Table I). These results indicate that about 10 to 20% of oleic acid was absorbed percutaneously in 6 h. It is interesting that only about 10% of the oleic acid was absorbed from the two-component system that allowed the absorption of nearly 95% of the molsidomine.

Percutaneous Absorption of Propylene Glycol

The residual amounts of propylene glycol on the skin were measured at 6 h after application of the two-component system containing various proportions of oleic acid. At the same time, the amounts of molsidomine were measured. These results are summarized in

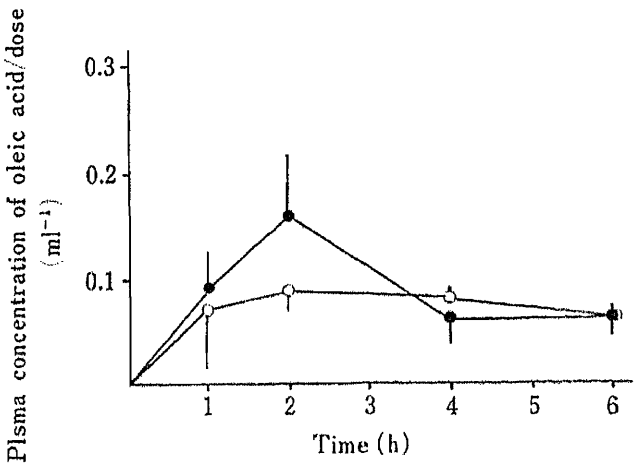

Fig. 2. Percutaneous Absorption of C^{14}-Oleic Acid through the Abdominal Skin of the Rat

—●—, oleic acid alone (200 mg); —○—, 10% oleic acid (20 mg)–propylene glycol. Each point represents the mean±S.E. of three rats.

TABLE I. Percutaneous Absorption of Vehicles in Rats

OA Proportion (%)	Percent remaining on the skin (%)			Bioavailability of molsidomine (%)
	PG	OA	Molsidomine	
0	95	—	97	1
5	—	—	—	35
10	3	90	5	97
20	—	—	—	87
50	45	—	50	53
95	—	77	—	2

OA, oleic acid; PG, propylene glycol. Bioavailability $=[AUC^{6}_{0\ \text{transdermal}}\ (\mu g \cdot h \cdot ml)/AUC^{3}_{0\ \text{i.v.}}\ (\mu g \cdot h/ml)] \times 100$.

Table I.

The greatest enhancement of absorption of propylene glycol occurred at about 10% oleic acid. The residual percent of propylene glycol on the skin was 3%, which is close to the residual percent for molsidomine. The same tendency was observed in the other systems tested. Therefore, the percutaneous absorptions of propylene glycol and molsidomine were assumed to be very similar.

Permeation of Molsidomine and Water-Soluble Polyhydric Alcohols

In the *in vivo* experiment described above, propylene glycol was percutaneously absorbed in larger amounts than expected from the system in which oleic acid was simultaneously used. A more detailed investigation was carried out *in vitro* on the permeation of molsidomine and propylene glycol. Similar investigations were performed with other water-soluble polyhydric alcohols, polyethylene glycol 200 and glycerin. An attempt was also made to clarify what roles these vehicles play in enhancing the percutaneous absorption of molsidomine.

Three rats were used to measure the permeation (percent permeated with respect to the dose) of molsidomine and of propylene glycol from the 10% oleic acid–propylene glycol

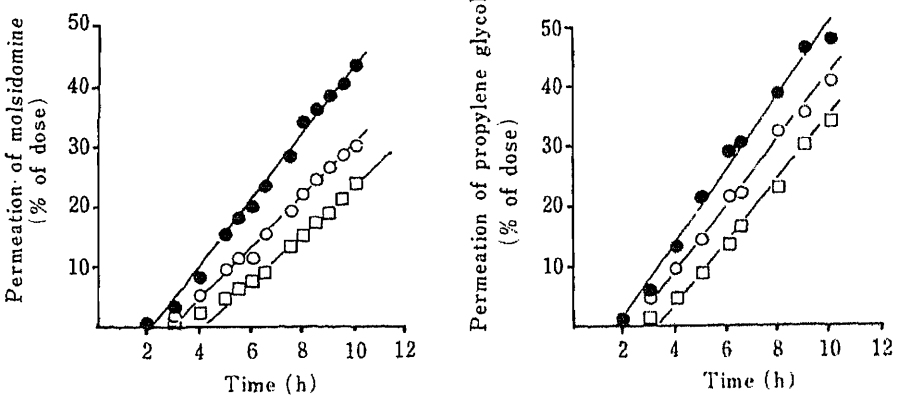

Fig. 3. Permeation of Molsidomine (Left) and Propylene Glycol (Right) through Excised Rat Skin (10% Oleic Acid–Propylene Glycol)

—●—, rat No. 1; —○—, rat No. 2; —□—, rat No. 3.

Table II. Permeation of Molsidomine and Polyethylene Glycol 200 through Excised Rat Skin

Time (h)	Molsidomine (%)	PEG 200[a] (%)
8	1.1±0.1	1.4±0.2
16	9.7±0.9	9.3±1.8
24	25.1±2.3	21.6±4.1

a) PEG 200, polyethylene glycol 200. All values represent mean±S.E. ($n=3$).

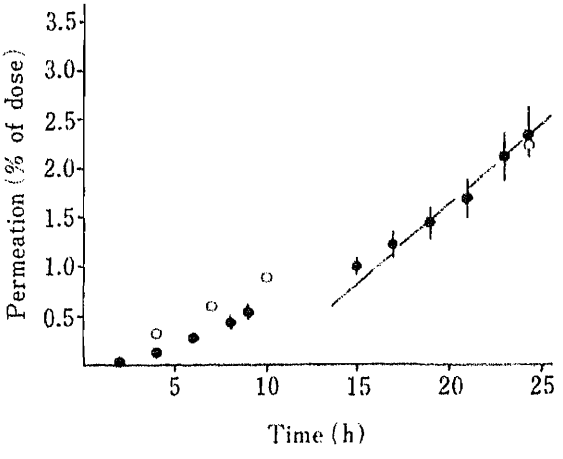

Fig. 4. Permeation of Molsidomine and Glycerin through Excised Rat Skin (10% Oleic Acid–Glycerin)

—●—, molsidomine; ○, glycerin. Each point represents the mean±S.E. of three rats.

TABLE III. Permeability of Molsidomine and Vehicles through Excised Rat Skin

	Vehicle		
	Propylene glycol	Polyethylene glycol 200	Glycerin
Molecular weight of vehicle	76.1	200	92.1
Partition coefficient (benzene/vehicle)	0.48	—	0.79
Solubility of molsidomine (%)	6.4	6.0	1.8
Percent of molsidomine permeated after 24 h (A)	78.3	25.1	2.3
Percent of vehicle permeated after 24 h (B)	80	21.6	2.2
(A)/(B)	0.98	1.2	1.0
Rate of permeation of molsidomine (steady-state flux: $\mu g/cm^2/h$)	299	136	4.1
Lag time of molsidomine (h)	3.4	10.6	11.3
Percent of vehicle permeated after 24 h (C)	1	0.3	0.2

(A), (B) 10% oleic acid/vehicles. (C) Vehicle alone (oleic acid free).

system (Fig. 3). In both cases, permeation began after a lag time of 2 h, and about 30 to 40% of each substance permeated over a 10 h period. There was adequate consistency in the permeation curves for both substances among the three samples.

Polyethylene glycol 200 showed a greatly lowered permeation (Table II) and molsidomine and polyethylene glycol 200 were found to permeate in equal proportions. As shown in Fig. 4, glycerin exhibited a slower rate of permeation than did polyethylene glycol 200, whereas molsidomine and glycerin also permeated in equal proportions.

These results are summarized in Table III. As was the case with the investigations[5] conducted *in vivo*, the ability of molsidomine to permeate the skin was greatly influenced by the type of water-soluble polyhydric alcohol used as a vehicle. Molsidomine and the polyhydric alcohols were found to permeate in equal proportions in each case; this suggests that molsidomine permeates through the skin dissolved in the polyhydric alcohol. The use of a vehicle that is capable of more readily dissolving molsidomine, and that permeates through the skin more effectively, results in better permeation of the drug.

Permeation of Molsidomine and Vehicles through Skin Treated with 10% Oleic Acid–Propylene Glycol

The permeation of molsidomine, propylene glycol, and glycerin through rat skin treated with 10% oleic acid–propylene glycol was investigated *in vitro* (Table IV). Molsidomine dissolved in propylene glycol showed the same permeation behavior irrespective of the duration of treatment with the absorption enhancer; at both 1 and 6 h the permeation was increased markedly over that of the control. Thus, treatment with the absorption enhancer for 1 h was sufficient. A glycerin solution of molsidomine did not affect the permeation. Although the reason for this is not clear, it is presumed that the stratum corneum adsorbs or absorbs oleic acid and permeation of glycerin is prevented. Actually, it was found that oleic acid is poorly compatible with glycerin. The reduced permeation of glycerin through the skin treated with the absorption enhancer suggested that the enhancer did not damage the skin.

Permeability of Molsidomine from Skin Treated with Oleic Acid

To evaluate the effect of oleic acid on the percutaneous absorption of molsidomine, an investigation was carried out *in vitro* on the permeation of molsidomine through excised skin

Table IV. Permeation of Molsidomine and Vehicles through Excised Rat Skin Treated with 10% Oleic Acid/Propylene Glycol

Drug and vehicle	Permeation (% of dose)		
	24 h	10 h	
	No treatment (Control)	Treatment time 1 h	6 h
Molsidomine in propylene glycol	0.1	18.5	17.4
Propylene glycol	1	28.6	28.1
Molsidomine in glycerin	0.1	1.4	1.9
Glycerin	0.2	0.8	0.8

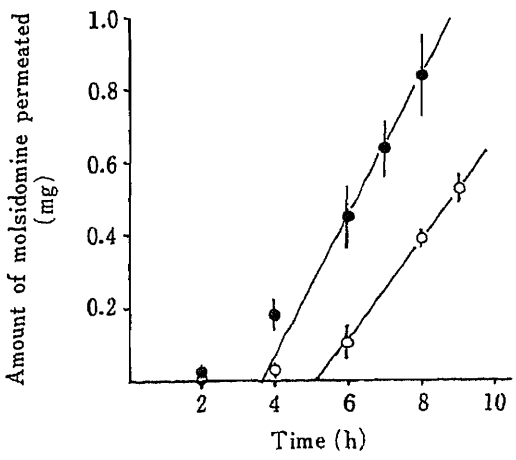

Fig. 5. Effect of Skin Treatment with Oleic Acid on the Permeation of Molsidomine through Excised Rat Skin

—●—, skin was treated with oleic acid, then molsidomine in propylene glycol was applied to the skin. —○—, molsidomine in 10% oleic acid–propylene glycol was applied to the skin without oleic acid treatment. Each point represents the mean±S.E. of three experiments.

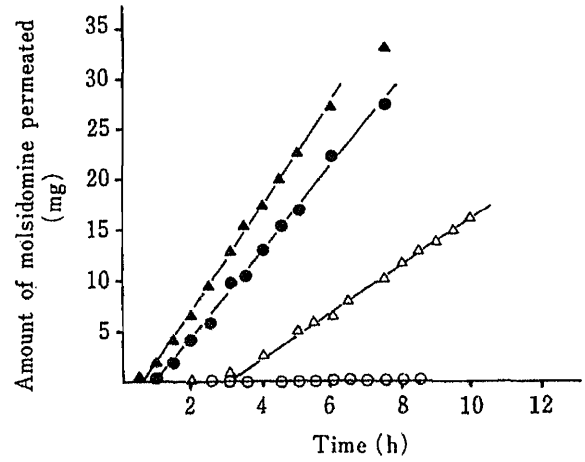

Fig. 6. Permeation of Molsidomine through Stripped Skin and Intact Skin of Rats

—▲—, stripped skin, 10% oleic acid–propylene glycol. —●—, stripped skin, propylene glycol alone. —△—, intact skin, 10% oleic acid–propylene glycol. —○—, intact skin, propylene glycol alone.

pretreated with oleic acid (Fig. 5). The rate of permeation of molsidomine was about twice that through the non treated skin. The lag time was also shortened by the pretreatment. Perhaps the oleic acid adsorbed on the surface or absorbed inside the stratum corneum permits the molsidomine together with propylene glycol to permeate comparatively easily.

Permeability of Molsidomine through Damaged Skin

The stratum corneum, which functions[9] to prevent the invasion of foreign material, naturally constitutes a major barrier to the permeation and absorption of drugs administered percutaneously. To evaluate the magnitude of this barrier to percutaneous absorption of molsidomine, an investigation was performed *in vitro* on the permeability of skin from which the stratum corneum had been stripped (Fig. 6). The rates of permeation of molsidomine from 10% oleic acid–propylene glycol and a simple vehicle consisting exclusively of propylene glycol were 714 (μg/cm^2/h) and 641 (μg/cm^2/h), respectively; this difference is not significant. Stripping of the stratum corneum resulted in a 900-fold increase in the rate of permeation; the rate of permeation from propylene glycol in the intact skin was 0.7 (μg/cm^2/h). The

Fig. 7. Stained Cross Section of Stratum Corneum of Rat Skin

Arrows indicate the stratum corneum. A: Intact skin. B, C: Skin treated with 10% oleic acid–propylene glycol.

apparent rate of permeation of molsidomine from the absorption-enhancing system in the intact skin was 399 ($\mu g/cm^2/h$). This striking difference from the control value demonstrates how effectively the absorption enhancer altered the barrier of the stratum corneum.

Histological Examination of the Stratum Corneum

Investigations were carried out to determine whether 10% oleic acid–propylene glycol brings about a change in the stratum corneum. The photomicrographs shown in Fig. 7 indicate that the treatment with the absorption enhancer does not cause changes as exfoliation or appreciable damage to the stratum corneum.

Mechanism of Percutaneous Absorption of Molsidomine

The rate-determining step in the percutaneous absorption of molsidomine is the stratum corneum, as is obvious from the finding that the rate of absorption from skin stripped of the stratum corneum is 900 times faster than that from the intact skin. Consequently, to enhance the percutaneous absorption of molsidomine, it is necessary to alter the permeability of the stratum corneum. The stratum corneum consists mainly of keratin and lipid, and as a whole, is considered to be a lipid membrane. In general there is great interest in how this lipid layer can be altered to allow the permeation and absorption of drugs. We have found that the 10% oleic acid–propylene glycol system caused remarkable enhancement of molsidomine absorption. The mechanism of this effect is discussed below on the basis of the results of *in vivo* and *in vitro* studies in rats.

It is presumed that oleic acid, alone or together with propylene glycol, might act to dissolve the hard sebum[10] on the surface of or within the stratum corneum to lower its viscosity and thus alter its permeability. This is supported by the results of the study carried out *in vitro* on the permeability of molsidomine through skin treated with oleic acid. Thus, it is further assumed that improving the permeability with oleic acid facilitates the permeation of molsidomine and propylene glycol. Over a 6 h period, the absorption of oleic acid was as low as 10 to 20% of the dose, whereas molsidomine and propylene glycol were almost entirely absorbed. As shown in the study carried out *in vitro*, molsidomine and propylene glycol permeated in the same proportions with respect to the dose. This indicates that molsidomine, dissolved in propylene glycol, permeates through the stratum corneum as if it were subject to solvent drag.[11] A number of reports[12] have appeared on drugs whose percutaneous absorption is consistent with the lipid theory, but it became clear that molsidomine was absorbed from the oleic acid–propylene glycol system by a different mechanism. The marked decrease in the absorption-enhancing effect exhibited when glycerin replaced propylene glycol is explained by the fact that since glycerin is less compatible with oleic acid in the stratum corneum, the segregated oleic acid constitutes a barrier which inhibits the permeation of glycerin. It is still not established whether the main pathway of the percutaneous permeation

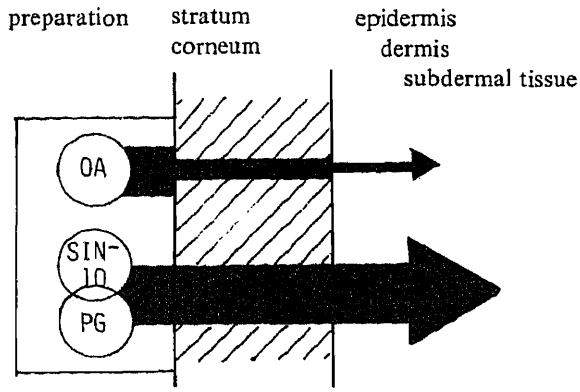

Fig. 8. Proposed Mechanism of Permeation Enhancement in the Percutaneous Absorption of Molsidomine

OA, oleic acid; PG, propylene glycol; SIN-10, molsidomine. Molsidomine and propylene glycol are absorbed together in the same properties. Oleic acid is poorly absorbed.

or absorption of molsidomine is intercellular or transcellular permeation through the stratum corneum. The absorption enhancement of molsidomine from the oleic acid–propylene glycol system is not due to damage to the stratum corneum. This is clear from the result of the experiment on drug administration with glycerin on skin treated with the absorption enhancer, and from photomicrographs of the stratum corneum.

The findings obtained in this study are expected to be of help in designing controlled-release transdermal dosage forms.

Acknowledgement The authors are grateful to Dr. T. Shimamoto, Dr. T. Yashiki, and Mr. H. Toguchi of our Central Research Division for encouragement throughout this work. Thanks are also due to Mrs. M. Satoh for her technical assistance.

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The Design of Membrane-Controlled Transdermal Therapeutic Systems Containing Molsidomine

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A basic investigation aimed at developing a membrane-controlled transdermal therapeutic system was carried out. The drug used was molsidomine with oleic acid and propylene glycol as the absorption enhancer and the vehicle, respectively. It was concluded that a microporous polyethylene membrane was most suitable for release control. A desirable pattern of the molsidomine plasma concentration *versus* time curve could be obtained by controlling the amounts of the three components, molsidomine, oleic acid, and propylene glycol in the device. Furthermore, the plasma concentration of molsidomine was well sustained by adding polyethyleneglycol 400 to the oleic acid–propylene glycol solution.

Keywords——molsidomine; transdermal; controlled release; microporous membrane; drug delivery system

Transdermal drug dosage forms are considered to be of two types. One is the skin-controlled form in which the rate of release of a drug from the preparation is always greater than the rate of percutaneous absorption; the rate of percutaneous absorption determines the overall rate of absorption of the drug. The other is the system-controlled form in which the rate of release of a drug from the preparation or device is slower than the rate of percutaneous absorption: the rate of release determines the overall drug absorption rate. The latter type is thought to be preferable for transdermal dosage forms aimed at systemic therapeutics.

A porous membrane of polyethylene has proved to be preferable for controlling the release from preparations of molsidomine, effective for the treatment of angina pectoris, and a percutaneous absorption enhancer. Trial transdermal dosage forms designed to sustain the plasma concentraion of molsidomine at a specified concentration for 24 h were produced and applied to rats for evaluation.

Experimental

Materials——Molsidomine[2)] was produced by Takeda Chemical Industries, Ltd., Japan. Oleic acid, propylene glycol, and polyethylene glycol 400 were of reagent grade, supplied by Wako Pure Chemical Industries, Ltd., Japan. Figure 1 is a sectional view of the device (transdermal therapeutic system) used. The drug solution was absorbed onto a piece of non-woven fabric, which was sealed in the device. The use of non-woven fabric prevented air-bubbles from being entrapped in the device, and hence gave the device a more agreeable "feel" and improved its adherence to the

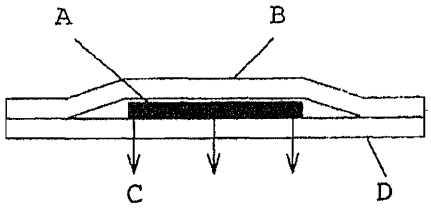

Fig. 1. Sectional View of the Transdermal Therapeutic System (Device)

A, non-woven fabric; B, backing; C, drug, absorption enhancer and vehicles; D, rate-controlling membrane.

TABLE I. Transdermal Preparations of Molsidomine

No.	Molsidomine		OA		PG		PEG400		Total (mg)	Membrane
	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)		
1	2.5	50	10	200	87.5	1750			2000	1200
2	5	75	2	30	93	1395			1500	1200
3	5	75	2	30	93	1395			1500	2200
4	5	75	5	75	45	675	45	675	1500	2200
5	2.5	37.5	5	75	45	675	47.5	712.5	1500	2200
6	5	75	3	45	92	1380			1500	2200
7	5	75	3	45	45	675	47	705	1500	2200
8	5	75	3	45	27	405	65	975	1500	2200
9	5	75	1	15	94	1410			1500	2200
10	5	75	1	15	46	690	48	720	1500	2200
11	5	75	1	15	9	135	85	1275	1500	2200

OA, oleic acid; PG, propylene glycol; PEG400, polyethylene glycol 400.

skin. The porous membranes used were Hipore 1200 (pore size: 0.1 μm) and 2200 (pore size: 0.4 μm) made of polyethylene (both are produced by Sekisui Chemical Co., Ltd., Japan); the device measured 4 by 5 cm (20 cm^2).

Animals——SD-JCL strain male rats aged 7 weeks and weighing 240 to 280 g were used as supplied by Clea Japan Inc., Japan. The device was applied to the denuded abdomen of a rat, and fixed with surgical tape. A jacket made of aluminum was mounted around the entire mid-region to prevent the rat from tearing off the device with its teeth; the jacket allowed the experiments to be undertaken without anesthesia. Three to 6 rats were employed in each test.

Determination of Molsidomine——Molsidomine was determined by the method reported in the previous paper.[3]

Formulation of the Drug Solutions——The formulations of the drug solutions used in the device and the type of controlled-release membranes employed are shown in Table I.

Analysis of the Data——The release of molsidomine from the device *in vivo* and the sustainability of the plasma concentration of molsidomine were evaluated by the moment analysis method.[4] Almost all reports dealing with evaluating the release of a drug from a given pharmaceutical preparation use simulation or curve-fitting by a mathematical model. The plasma concentration curves after percutaneous administration of drugs do not always yield an ideal pattern; analysis of the data is difficult in many cases. The moment analysis method permits the separation of drug release from a device, absorption and elimination processes, and is a very convenient method of analysis in designing controlled-release dosage forms.

The release time of molsidomine *in vivo* after percutaneous administration can be determined by subtracting the mean residence time (MRT) in the case of administration of a solution containing the drug from the MRT of the drug using the device. Thus,

$$\text{release time} = MRT_{\text{device}} - MRT_{\text{solution}} \quad (1)$$

where MRT was calculated in accordance with the trapezoidal rule. The rate of percutaneous absorption of molsidomine (K_r) was determined from the steady-state plasma concentration (C_{ss}) by applying the equation

$$K_r = C_{ss} \cdot CL \quad (2)$$

wherein CL is total body clearance. The value of CL was determined to be 289 (ml/h) by analyzing the plasma concentration curve when molsidomine was given i.v. to a rat.

Results and Discussion

Sustaining the Plasma Concentration of Molsidomine by Use of a Porous Polyethylene Membrane

A 10% of oleic acid–propylene glycol solution containing molsidomine was placed in the device utilizing a porous polyethylene membrane as a release control membrane. The time course of plasma concentration of molsidomine when the device was applied to a rat is shown in Fig. 2. The device provided a well sustained plasma concentration of molsidomine as

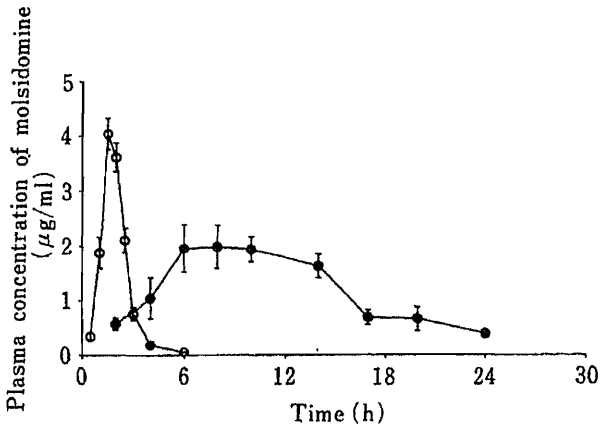

Fig. 2. Sustaining the Plasma Concentration of Molsidomine by Use of a Porous Polyethylene Membrane in Rats

—○—, control (drug solution: 5 mg of molsidomine–10% oleic acid–85% propylene glycol). —●—, Hipore 1200 device (50 mg of molsidomine–10% oleic acid–87.5% propylene glycol). Each point represents the mean ± S.E. of four rats.

Pharmacokinetic Variables

Dosage form	*AUC* (μg·h/ml)	*MRT* (h)	*F* (%)	K_r (μg/h)	Dose (mg)
Control	7.96	1.70	46.0	1167	5
TTS	30.10	10.11	17.4	573	50

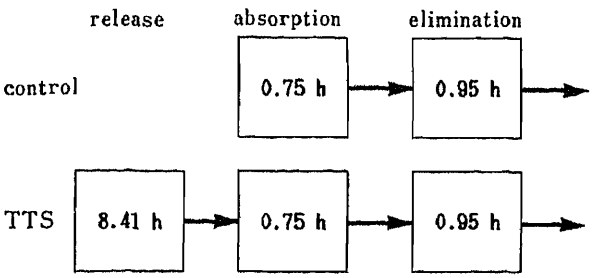

Fig. 3. Moment Analysis of Release *in Vivo* and Absorption Processes of Molsidomine and Pharmacokinetic Variables Following Percutaneous Application of the Device in Rats

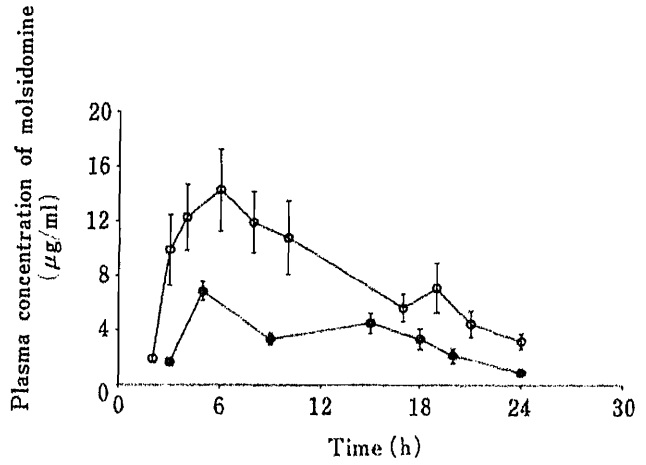

Fig. 4. Controlled-Releasing Properties of Polyethylene Microporous Membranes with Different Pore Sizes

—○—, Hipore 2200 device (75 mg of molsidomine–2% oleic acid–93% propylene glycol). —●—, Hipore 1200 device (75 mg of molsidomine–2% oleic acid–93% propylene glycol). Each point represents the mean ± S.E. of three rats.

compared with administration of a drug solution (5 mg of molsidomine). The results of separating the release, absorption, and elimination processes by moment analysis, together with the pharmacokinetic parameters are shown in Fig. 3. The release time, 8.41 h, was the longest, and it is evident that the release is the rate-determining step. Thus, the porous polyethylene membrane was effective for the controlled release of molsidomine. The device gave a bioavailability (*F*) of molsidomine of 17.4%.

Controlled-Releasing Properties of Hipore 1200 and Hipore 2200

A 2% oleic acid–propylene glycol solution was placed in the devices utilizing either Hipore 1200 or Hipore 2200, which have different pore sizes. The device utilizing Hipore 1200 (with the smaller pore size) sustained a plasma concentration of molsidomine of 1 to 2 μg/ml over 24 h, while the device utilizing Hipore 2200 sustained a concentration of over 4 μg/ml (Fig. 4). The higher plasma concentration of molsidomine was associated with the larger pore size of the membrane. The devices showed bioavailability values of 39.4% and 76.9%, respectively (Table II; Nos. 2 and 3). Figure 5 shows the results of separating the release,

TABLE II. Pharmacokinetic Parameters of Molsidomine Administered Transdermally

No.	AUC_0^{24} (μg·h/ml)	MRT (h)	F (%)	C_{max} (μg/ml)
1	30.1	10.11	17.4	1.98
2	102.3	13.83	39.4	6.85
3	199.5	10.05	76.9	14.26
4	73.9	11.70	28.5	5.78
5	33.2	11.99	25.6	2.20
6	279.9	7.11	107.8	21.31
7	83.3	8.88	32.1	7.24
8	30.8	23.79	11.9	1.04
9	39.3	11.40	15.1	3.99
10	23.6	13.69	9.1	1.51
11	14.3	26.57	5.5	0.47

Pharmacokinetic Variables

Membrane	AUC (μg·h/ml)	MRT (h)	F (%)	K_r (μg/h)	Dose (mg)
Hipore 2200	199.51	10.05	76.9	4120	75
Hipore 1200	102.26	13.83	39.4	1980	75

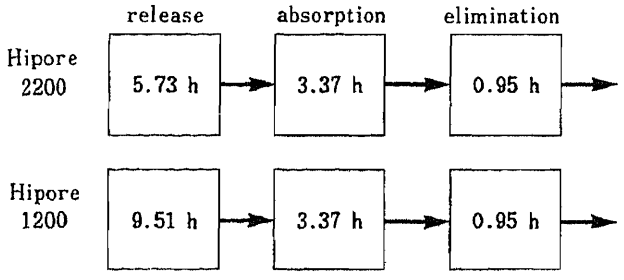

Fig. 5. Moment Analysis of Release *in Vivo* and Absorption Processes of Molsidomine and Pharmacokinetic Variables Following Percutaneous Application of Devices with Different Pore Sizes of Polyethylene Membrane in Rats

absorption, and elimination processes of molsidomine from the two devices: both provided better sustained plasma concentrations than the drug solution (control) and the release-controlling membranes functioned effectively. The Hipore 2200, which yielded higher plasma concentrations and greater bioavailability, was used for further investigations on the design of controlled-release dosage forms.

Proportion of Oleic Acid in Relation to Percutaneous Absorption

The device containing oleic acid–propylene glycol was used to examine the relations between the proportion of oleic acid incorporated and the percutaneous absorption and sustained plasma concentration of molsidomine. Devices Nos. 6, 3, and 9 (Table I) contained 3%, 2%, and 1% oleic acid, respectively; all used the same release-controlling membrane. The pharmacokinetic parameters of these three devices (Table II) revealed that as the proportion of oleic acid was increased, the C_{max}, the area under the plasma concentration–time curve (AUC), and F of molsidomine increased, whereas its MRT decreased. Thus, a larger proportion of oleic acid increased both the amount and rate of absorption of molsidomine.

A similar investigation was carried out on the oleic acid–propylene glycol–polyethylene glycol 400 system. Devices Nos. 4, 7, and 10 (Table I) contained 5%, 3%, and 1% oleic acid, respectively; their propylene glycol–polyethylene glycol 400 ratios are about 1 : 1. Comparison of the plasma concentration–time curves for molsidomine obtained when these devices were

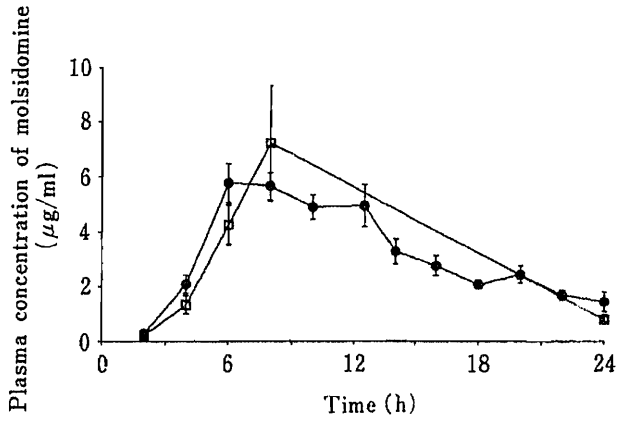

Fig. 6. Effect of the Proportion of Oleic Acid on the Plasma Concentration of Molsidomine in Rats

—□—, 3% oleic acid–45% propylene glycol–47% polyethylene glycol 400. —●—, 5% oleic acid–45% propylene glycol–45% polyethylene glycol 400. Each device contained 75 mg of molsidomine. Each point represents the mean ± S.E. of three rats.

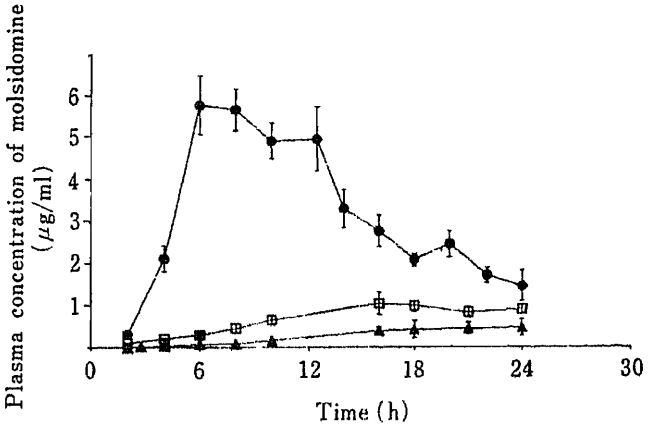

Fig. 7. Effect of Diluting 10% Oleic Acid–Propylene Glycol with Polyethylene Glycol 400 on the Percutaneous Absorption and Sustained Plasma Concentration of Molsidomine in Rats

—●—, 5% oleic acid–45% propylene glycol–45% polyethylene glycol 400. —□—, 3% oleic acid–27% propylene glycol–65% polyethylene glycol 400. —▲—, 1% oleic acid–9% propylene glycol–85% polyethylene glycol 400. Each device contained 75 mg of molsidomine. Each point represents the mean ± S.E. of three rats.

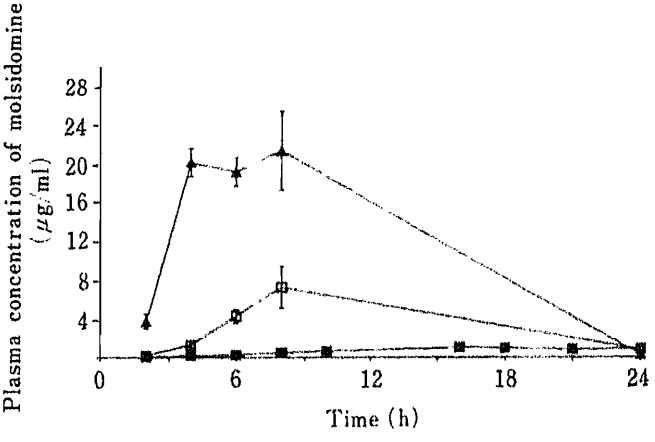

Fig. 8. Effect of the Proportions of Propylene Glycol and Polyethylene Glycol 400 with a Constant 3% Oleic Acid on the Percutaneous Absorption and Sustained Plasma Concentration of Molsidomine in Rats

—▲—, 3% oleic acid–92% propylene glycol. —□—, 3% oleic acid–45% propylene glycol–47% polyethylene glycol 400. —■—, 3% oleic acid–27% propylene glycol–65% polyethylene glycol 400. Each device contained 75 mg of molsidomine. Each point represents the mean ± S.E. of three rats.

applied percutaneously revealed no significant difference between the 5% and 3% preparations (Fig. 6). On the other hand, in the case of 1% oleic acid, the C_{max}, AUC, and F were far lower than in the cases of 5% and 3% (Table II; Nos. 4, 7, and 10). In the previous paper,[3] it was reported that as the proportion of oleic acid was decreased from 5% to 1%, the percutaneous absorption of molsidomine became lower; the system having polyethylene glycol 400 incorporated also displayed a similar tendency.

Effect of Polyethylene Glycol 400

Figure 7 shows a molsidomine plasma concentration–time curve obtained when the 10% oleic acid–propylene glycol system was diluted with polyethylene glycol 400 to various degrees

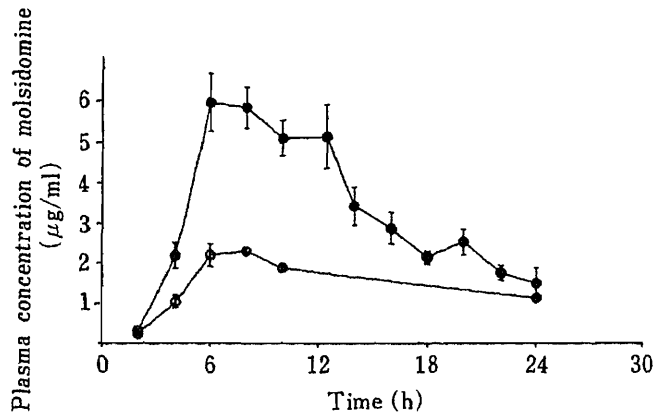

Fig. 9. Relation between the Dose and Percutaneous Absorption of Molsidomine in Rats

—●—, dose 75 mg/Hipore 2200 device (5% oleic acid–45% propylene glycol–45% polyethylene glycol 400). —○—, dose 47.5 mg/Hipore 2200 device (5% oleic acid–45% propylene glycol–47.5% polyethylene glycol 400). Each point represents the mean±S.E. of three rats.

(Table I; devices Nos. 4, 8, and 11). As the proportion of polyethylene glycol 400 was increased, the plasma concentration of molsidomine decreased steeply. Polyethylene glycol 400 contributes little to the absorption enhancement of molsidomine; it is a mere diluent vehicle.

Figure 8 shows molsidomine plasma concentration–time curves obtained when the proportions of propylene glycol and polyethylene glycol 400 were altered while oleic acid was maintained at 3%. As the proportion of propylene glycol was increased, the C_{max}, AUC, and F increased, whereas MRT decreased (Table II; Nos. 6, 7, and 8); that is, both the amount and rate of absorption of molsidomine increased in proportion to the content of propylene glycol. In the case of the device having the largest amount of propylene glycol added, a quick pulse rise in the plasma concentration occurred initially, and thereafter, it decreased rapidly. In contrast, the device having the smallest amount of propylene glycol added did not produce a steep rise in plasma concentration and sustained a lower concentration. Therefore, the plasma concentration and/or its pattern can be controlled by suitably changing the raio of propylene glycol to polyethylene glycol 400 in addition to the content of oleic acid.

Relation between the Dose and Absorption of Molsidomine

The relation between the dose and absorption of molsidomine is shown in Fig. 9. When the dose of molsidomine was doubled, C_{max} and AUC increased nearly in proportion, whereas MRT and F remained almost unchanged (Table II; Nos. 4 and 5). Thus, there is a linear relation between the dose and absorption of molsidomine.

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Inclusion Complexes of Poorly Water-Soluble Drugs with Glucosyl-cyclodextrins[1)]

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The inclusion complex-forming ability of three glucosyl-cyclodextrins (CDs), 6-*O*-α-D-glucosyl-α-CD (G-α-CD), 6-*O*-α-D-glucosyl-β-CD (G-β-CD), and 6^A,6^D-di-*O*-α-D-glucosyl-β-CD (2G-β-CD) with slightly soluble or insoluble drugs in aqueous solution and in the solid state was studied by the solubility method, ultraviolet spectroscopy, and differential scanning calorimetry. These glucosyl-CDs were capable of forming inclusion complexes with compounds which can form complexes with the parent CDs. The enhancement of solubility of poorly water-soluble drugs by glucosyl-β-CDs was much more marked than that by β-CD. The hemolytic activity of glucosyl-CD was lower than that of each parent CD. The estriol–glucosyl-β-CD complex may be practically applicable to injectable preparations because of its high water-solubility and low hemolytic activity.

Keywords——glucosyl-α-cyclodextrin; glucosyl-β-cyclodextrin; solubility; inclusion complex; estriol–glucosyl-β-cyclodextrin complex; solubility method; stability constant; UV spectroscopy; differential scanning calorimetry; hemolysis

In recent years, cyclodextrin (CD) research has made remarkable progress and the application of CDs has been extended to various fields. In particular, research on pharmaceutical application is very active at present, since the safety of CD has been confirmed.[2)] Hitherto β-CD, which is the most readily available CD and can easily form inclusion complexes with many kinds of compounds, has been most frequently employed for this purpose. However, the relatively low water-solubility of β-CD itself and β-CD complexes is a limiting factor.

Recently, we have obtained three branched CDs, 6-*O*-α-D-glucosyl-α-CD (G-α-CD), 6-*O*-α-D-glucosyl-β-CD (G-β-CD), and 6^A,6^D-di-*O*-α-D-glucosyl-β-CD (2G-β-CD), in a high state of purity, from the mother liquor of a large-scale preparation of β-CD with *Bacillus ohbensis* cyclomaltodextrin glucanotransferase, by high-performance liquid chromatography (HPLC) using a Hibar LiChroprep RP-18 column and an Asahipak GS-320 column.[3)] Their chromatographic behavior on C_{18}-bonded silica[4)] suggested that their solubilities in water would be much higher than that of β-CD.

This paper deals with the solubilities of the three glucosyl-CDs in water and their inclusion behavior, compared with those of the parent CDs.

Experimental

Materials——G-α-CD, G-β-CD, and 2G-β-CD were prepared and purified according to the reported method[3)]: G-α-CD, mp > 283 °C (dec.), $[\alpha]_D^{22}$ + 156.7°; G-β-CD mp > 283 °C (dec.), $[\alpha]_D^{22}$ + 164.3°; 2G-β-CD, mp 289—290 °C (dec.), $[\alpha]_D^{22}$ + 169.7°. α-CD and β-CD were used after recrystallization from water, $[\alpha]_D^{22}$ + 150.3° and + 163.0°, respectively. γ-CD, which was supplied by Sanraku Incorporated, was purified by HPLC on a Hibar LiChroprep RP-18 column, $[\alpha]_D^{25}$ + 174.8°. Nitrazepam, mp 224—226 °C (dec.), was kindly supplied by Shionogi & Co., Ltd. Phenobarbital (JP X grade) was purified by recrystallization, mp 174—178 °C. The other drugs used were of reagent

grade: digitoxigenin, mp 253 °C; digitoxin, mp 256—257 °C; estriol, mp 282 °C; griseofulvin, mp 220 °C; vitamin D_3, mp 84—85 °C; vitamin K_3, mp 105—107 °C; vitamin E and vitamin K_1, both oils. All other materials were of analytical-reagent grade. Water used in all experiments was deionized and distilled twice. Reagent-grade organic solvents used for HPLC were distilled and filtered through a 0.45-μm membrane filter.

General Methods——Optical rotations were determined with a DIP-360 digital polarimeter (JASCO, Tokyo, Japan). The pH measurements were carried out on an M-8 pH meter (Horiba, Kyoto, Japan). Melting points were measured with a micro melting point apparatus (Yanagimoto, Kyoto, Japan) and are uncorrected. Lyophilization was carried out with an FD-1 freeze dryer (Tokyo Rika, Tokyo, Japan). A UVIDEC-610C double-beam spectrophotometer (JASCO) was used for the determination of absorbances. HPLC analyses were performed using an 880-PU Intelligent HPLC pump (JASCO), a model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), and a UVIDEC-100V variable-wavelength ultraviolet (UV) detector (JASCO). Preparative HPLC was carried out using a Twincle pump and a VL-611 variable-loop injector (both from JASCO) with an SE-11 refractive index (RI) monitor (Showa Denko, Tokyo, Japan). The columns used were a Finepak SIL-C_{18} (250 × 4.6 mm i.d.) (JASCO), a YMC-Pack AL-312 ODS (150 × 6 mm i.d.) and a YMC-Pack A-212 C_8 (150 × 6 mm i.d.) (both from Yamamura Chemical, Kyoto, Japan). For preparative chromatography, a column packed with LiChroprep RP-18 (5—20 μm, 300 × 20 mm i.d.) (Yamazen, Osaka, Japan) and an Asahipak GS-320 (500 × 7.6 mm i.d.) (Asahi Kasei, Tokyo, Japan) were used.

Solubility Studies——Solubility of Glucosyl-CD: Water was carefully added in portions of 0.01—0.1 ml to a glass vessel containing 300 mg of lyophilized glucosyl-CD, and the volume of water required for complete dissolution of the CD within 30 min at 25 ± 1 °C, by vigorous shaking for 30 s periods at 5-min intervals was measured. For reference, the solubilities of lyophilized α-, β-, and γ-CDs were determined in the same way.

Estimation of Complex-Forming Ability of Glucosyl-CD by the Solubility Method[5]: Excess amounts of drug were added to aqueous solutions containing various concentrations of CD, and the mixtures were shaken for 24 h at 30 °C to allow equilibration to be attained. Then the aqueous solution was filtered through a 0.2-μm membrane filter and the drug concentraion in the filtrate was determined by HPLC under the conditions shown in Table I. An apparent stability constant, $K(M^{-1})$, was calculated from the initial straight line portion of the phase solubility diagrams according to the following equation.[5]

$$K = \frac{\text{slope}}{\text{intercept} \times (1 - \text{slope})} \quad (1)$$

Preparation of Complexes——The solid complexes were prepared by mixing equimolecular amounts of a CD and a drug in water. The mixture was shaken at 30 °C for 24 h, and filtered through 0.2-μm membrane filter to remove excess drug, then the filtrate was lyophilized. The freeze-dried samples obtained were used for thermal analysis and

TABLE I. Conditions of Drug Determination by HPLC

Drug	Column	Eluent $CH_3OH:H_2O$	Flow rate (ml/min)	Detection at (nm)	Retention time (min)
Digitoxigenin	YMC-Pack AL-312 ODS	65:35	1.0	220	8.5
Digitoxin	YMC-Pack AL-312 ODS	75:25	1.0	220	10.0
Estriol	YMC-Pack AL-312 ODS	55:45	1.0	280	9.5
Griseofulvin	YMC-Pack A-212 C_8	60:40	1.0	291	11.0
Nitrazepam	Finepak SIL-C_{18}	55:45	1.0	310	10.5
Phenobarbital	Finepak SIL-C_{18}	25:75	1.0	220	13.0
Vitamin D_3	Finepak SIL-C_{18}	90:10	1.5	265	12.3
Vitamin E	Finepak SIL-C_{18}	95: 5	1.0	292	10.5
Vitamin K_1	Finepak SIL-C_{18}	95: 5	1.5	249	11.0
Vitamin K_3	Finepak SIL-C_{18}	55:45	1.0	250	13.0

the study on hemolytic acitivity as solid inclusion complexes. The drug content in the complex was determined by HPLC.

Thermal Analysis——The DSC study was carried out by the use of a differential scanning calorimeter, model 1090 (Du Pont Company, Wilmington, DE, U.S.A.). The scanning temperature range was 30—300 °C and the scanning speed was 10 °C/min.

Determination of Hemolytic Activity——A 0.2% human erythrocytes suspension (1 ml) in 0.1 M isotonic phosphate buffer (pH 7.4, PBS) was added to 1 ml of PBS containing various concentrations of CDs. The mixture was incubated at 37 °C for 30 min and centrifuged at 1300 × *g* for 10 min. Percent hemolysis was expressed in terms of the ratio of the absorbance at 541 nm of hemoglobin released from erythrocytes with CDs to the absorbance after the complete hemolysis of erythrocytes in water. The hemolytic effects of estriol–G-β-CD and estriol–2G-β-CD complexes on human erythrocytes were examined in the same way.

Results and Discussion

Solubility of Glucosyl-CD

Solubilities of glucosyl-CDs in water at 25 °C are listed together with data on α-, β-, and γ-CDs (for reference) in Table II as the amount of CD dissolved in 1 ml of water. All three glucosyl-CDs are much more soluble than γ-CD, which has been recognized as the most soluble CD. The solubilities of G-β-CD and 2G-β-CD are about 50 times and 70 times greater than that of β-CD, respectively, while that of G-α-CD is higher than that of α-CD by a factor of about 5.

Inclusion Complex-Forming Ability of Glucosyl-CD

The complex-forming ability of three glucosyl-CDs with ten poorly water-soluble (slightly soluble and insoluble) drugs in aqueous solution was studied mainly by the solubility method[5] and was compared with that of the parent CDs, α-CD and β-CD. Since CDs are capable of forming inclusion complexes with compounds having a size compatible with the dimensions of the cavity,[6] the complexation abilities of glucosyl-CDs were almost the same as those of the parent CDs.

Table II. Solubilities of CDs in Water at 25 °C

CD	Glucose unit	Solubility (mg/ml)
α-CD	6	180
G-α-CD	7	890
β-CD	7	19
G-β-CD	8	970
2G-β-CD	9	1400
γ-CD	8	260

Table III. Apparent Stability Constant (M^{-1}) of Slightly Soluble Drug–CD Complexes Determined by the Solubility Method in Water at 30 °C

Guest molecule	Host molecule				
	α-CD	G-α-CD	β-CD	G-β-CD	2G-β-CD
Digitoxigenin	1700	2200	130000	130000	130000
Digitoxin	350	340	37000	38000	38000
Estriol	—	—	38000	39000	33000
Griseofulvin	—	—	30	20	20
Nitrazepam	30	30	130	140	130
Phenobarbital	30	30	1400	1400	1100
Vitamin K_3	40	40	190	190	180

TABLE IV. Enhancement of the Solubilities of Slightly Soluble or Insoluble Drugs in Water by Complexation with CDs at 30 °C

Drug	Solubility in H_2O (μg/ml)	Solubility in 1.5×10^{-2} M[a] CD soln. (μg/ml)				
		α-CD	G-α-CD	β-CD	G-β-CD	2G-β-CD
Digitoxigenin	10	260	330	730[b]	4400	4400
Digitoxin	17	100	100	1300[b]	5800	5300
Estriol	29	32	30	710[b]	2600	2500
Griseofulvin	15	16	16	21	19	20
Nitrazepam	43	61	60	120	130	130
Phenobarbital	1400	1900	1900	4500	4500	4400
Vitamin D_3	0	0	0	3[b]	520	520
Vitamin E	0	0	0	1[b]	1	1
Vitamin K_1	0	0	0	1[b]	0	0
Vitamin K_3	150	230	230	510	510	500

a) This concentration is near the saturated one in the case of β-CD. b) Precipitates appeared.

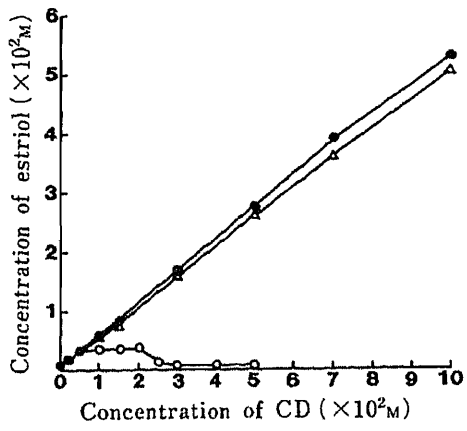

Fig. 1. Phase Solubility Diagrams of Estriol–CD Systems in Water at 30 °C
○, β-CD; ●, G-β-CD; △, 2G-β-CD.

The apparent stability constant (K), as a tentative measure of inclusion complexation, was estimated from Eq. 1 based on the assumption that a 1 : 1 complex was initially formed. The magnitudes of K values calculated from the initial rising portion of the solubility diagrams were found to be about the same in the two systems of α-CD and G-α-CD, and in the three systems of β-CD, G-β-CD, and 2G-β-CD, as shown in Table III.

However, the enhancement of solubility of poorly water-soluble drug by glucosyl-β-CDs was much more marked than that by β-CD, as shown in Table IV. The reason is that most β-CD systems show typical Bs type solubility curves[5] and precipitates of the complexes appear, whereas the solubilitites of drugs in G-β-CD and 2G-β-CD solutions increase linearly as a function of CD concentration and the solubility curve can be generally classified as being of type A.[5] Figure 1 shows the phase solubility diagrams obtained for estriol with the three β-CDs in water at 30 °C, for example. The estriol–β-CD complex began to precipitate at a β-CD concentration of 5×10^{-3} M and therefore the maximum concentration of estriol in β-CD solution was 3.2×10^{-3} M (=0.7 mg/ml). On the other hand, the solubility of estriol in G-β-CD or 2G-β-CD solution linearly increased and estriol concentrations of 5.2×10^{-2} M (=15.0 mg/ml) and 5.0×10^{-2} M (=14.4 mg/ml) were reached in 0.1 M G-β-CD and 2G-β-CD solutions, respectively. If necessary, estriol solution of higher concentration than these can be prepared by the use of G-β-CD or 2G-β-CD solution of higher concentration (up to 0.7 and 0.9 M, respectively).

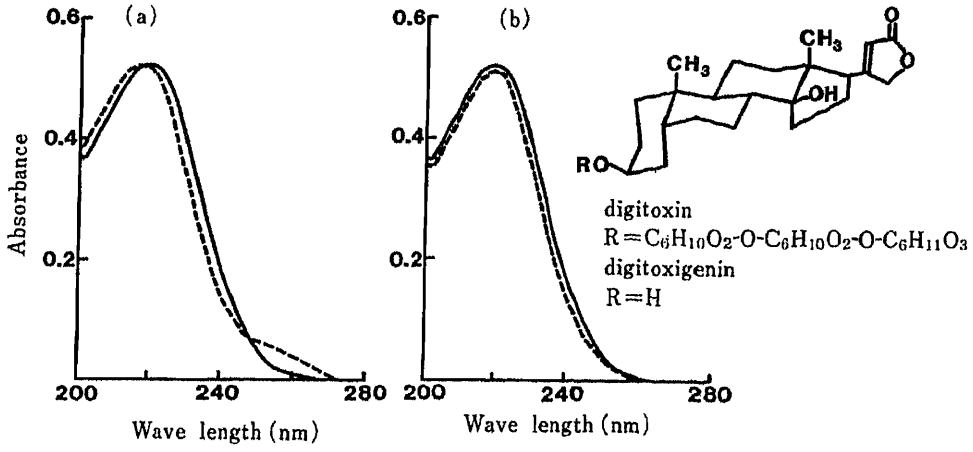

Fig. 2. UV Absorption Spectra of Digitoxin (a) and Digitoxigenin (b) in the Absence and in the Presence of G-β-CD in Water

(a) Concentration of digitoxin = 2.5×10^{-5} M, concentration of G-β-CD = 1.0×10^{-4} M. ----, digitoxin alone; ——, digitoxin + G-β-CD. (b) Concentration of digitoxigenin = 2.5×10^{-5} M, concentration of G-β-CD = 1.0×10^{-4} M. ----, digitoxigenin alone; ——, digitoxigenin + G-β-CD.

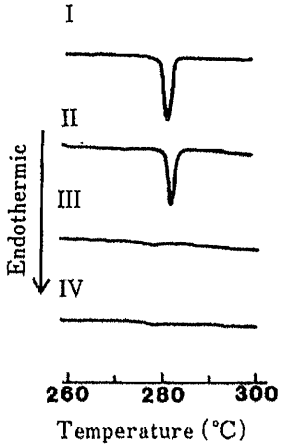

Fig. 3. DSC Thermograms of Estriol–G-β-CD Systems

(I), Estriol alone; (II), physical mixture of estriol and G-β-CD in 1 : 2 molar ratio; (III), 1 : 2 complex of estriol with G-β-CD; (IV), G-β-CD alone.

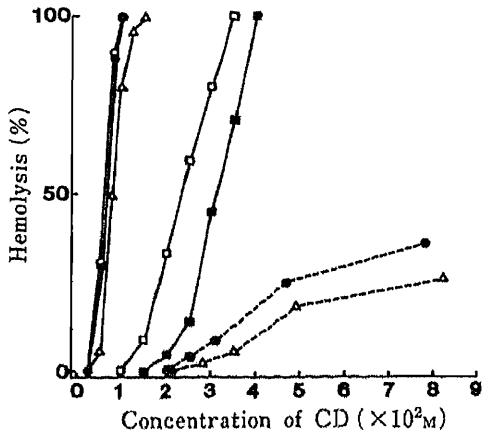

Fig. 4. Hemolytic Effects of CDs and Estriol–CDs Complexes on Human Erythrocytes in 0.1 M Isotonic Phosphate Buffer (pH 7.4) at 37 °C

□—□, α-CD; ■—■, G-α-CD; ○—○, β-CD; ●—●, G-β-CD; △—△, 2G-β-CD; ●---●, estriol–G-β-CD complex; △---△, estriol–2G-β-CD complex.

Interaction of glucosyl-CDs and slightly soluble drugs in aqueous solution was further examined by UV spectroscopy. Figure 2 shows the UV spectra of digitoxin and digitoxigenin in the absence and the presence of G-β-CD in water. In the absence of G-β-CD, digitoxin and digitoxigenin have an absorption maximum at 217 and 220 nm, respectively. On adding G-β-CD to a digitoxin solution, the absorption maximum is shifted to longer wavelength, while the absorption maximum of digitoxigenin in the presence of G-β-CD remains unchanged at 220 nm. This phenomenon may suggest that the digitoxigenin molecule penetrates into the G-β-CD cavity from the A-ring side and the lactone-ring (the chromophore) remains outside the cavity, whereas the digitoxin molecule, having a large and hydrophilic group on the A-ring, is included from the opposite side, that is, the lactone ring is incorporated inside the G-β-CD

cavity. This UV change of digitoxin was also observed in the presence of 2G-β-CD, β-CD, G-α-CD or α-CD. The UV absorption maximum of estriol was shifted similarly to that of digitoxin in the presence of G-β-CD, 2G-α-CD or β-CD.

Figure 3 shows the DSC thermograms of estriol–G-β-CD complex, the physical mixture and estriol. Estriol alone and the physical mixture showed an endothermic peak at around 282 °C. However, the endothermic peak disappeared with formation of the complex. This disappearance of the endothermic peak was also observed in the case of digitoxin–2G-β-CD complex and complexes of estriol, digitoxigenin and vitamin D_3 with G-β-CD or 2G-β-CD. These results indicate that these drugs interact with G-β-CD and 2G-β-CD in the solid state to form inclusion complexes.

Hemolytic Activity of Glucosyl-CD

The high solubilities of G-β-CD- and 2G-β-CD-complexes suggested the possibility of utilizing these complexes as injectable agents. However, CDs at higher concentrations have been found to cause hemolysis of human erythrocytes in the order of β->α->γ-CD in isotonic solution.[7] Thus, the hemolytic effects of glucosyl-CDs on human erythrocytes in isotonic solution were investigated (Fig. 4). The hemolytic activity of G-β-CD was about the same as that of β-CD, but those of 2G-β-CD and G-α-CD were lower than those of the parent CDs. Moreover, stable complexes such as estriol–G-β-CD and –2G-β-CD exhibited very low hemolytic activity; hemolysis was initiated at 20 mM G-β-CD and >20 mM 2G-β-CD containing >3 mg estriol.

Estriol is one of the follicle hormones and is on the market today in dosage forms of tablets and two kinds of injections (1 and 10 mg/ml aqueous suspension). In general, the injection (aqueous suspension) causes local irritation and tissue injury, and it would therefore be preferable to use a solution. The estriol–G-β-CD or –2G-β-CD complex may be applicable to injectable preparations because of its satisfactorily high water-solubility and low hemolytic activity.

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Specificity in the Pharmacological Actions of Optical Isomers of *cis*-2,3-Dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one (BTM)[1)]

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The pharmacological actions of the optical isomers of *cis*-2,3-dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one (BTM) were studied and compared. Antiacetylcholine activity determined by the Magnus method was higher but antihistamine activity was lower with the (−)-compound than with the (+)-compound; the (±)-compound showed intermediate activities. The antiulcer action and antisecretory action in pylorus-ligated rats as well as the anti-stress ulcer action in mice were more potent with the (−)-compound than the (+)-compound. The optical isomers showed similar local anesthetic action, when tested by using the corneal reflex in guinea pigs. Analgesic action, tested by the acetic acid-induced writhing method, was observed with the (+)-compound, but not the (±)- or (−)-compound. The (+)-compound showed the strongest acute toxicity in mice, followed by the (±)- and the (−)-compounds in that order. These results suggest the presence of multiple targets for the various pharmacological activities. The (−)-compound appears to be more promising as an antiulcer agent, since the antiulcer effect was greater and the toxicity was milder as compared with (+)-compound.

Keywords——(−)-*cis*-2,3-dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one dihydrochloride (BTM-1042); optical isomer; pharmacological activity; antiulcerogenic activity

(−)-*cis*-2,3-Dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one (dihydrochloride (BTM-1042) and monohydrochloride (BTM-1086); Fig. 1), a benzothiazepine derivative newly synthesized by Ohno *et al.*,[2,3)] shows potent antiulcer activity and a persistent inhibitory action on gastric acid secretion.[4−7)] Takayanagi *et al.* have shown[8)] that this compound has a parasympathicolytic action, inhibiting acetylcholine release and blocking the acetylcholine receptor. The potent action of this compound as an antimuscarinic drug with high selectivity for M_1-receptor was confirmed recently by means of a receptor binding assay.[9)] A metabolite[10)] of BTM-1086 found in blood (demethylated derivative of BTM-1086) was also shown to have antiulcer and antisecretory actions comparable to those of the parent compound.

In the present study, the pharmacological activities of the optical isomers of BTM-1042 were compared.

Materials and Methods

BTM-1042 has a molecular weight of 440.44 [the chemical structure is shown in Fig. 1: (±), BTM-1018 (2HCl); (+), BTM-1041 (2HCl); (−), BTM-1042 (2HCl)], and occurs as white crystals that are readily soluble in water. The optical isomers were resolved by recrystallizing the (−)- and (+)-tartaric acid salts of the enantiomers to constant rotation in order to determine the absolute configuration, and these salts were subsequently converted to the hydrochloride salts.[2,3)]

Reference compounds used were atropine sulfate (Wako Pure Chemical Ind., Ltd.), diphenhydramine

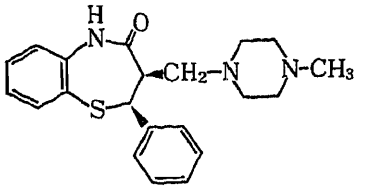

Fig. 1. Chemical Structure of *cis*-2,3-Dihydro-3-(4-methylpiperazinylmethyl)-2-phenyl-1,5-benzothiazepin-4(5*H*)-one

(±) Dihydrochloride, BTM-1018; (+) dihydrochloride, BTM-1041; (−) dihydrochloride, BTM-1042 (monohydrochloride: BTM-1086).

hydrochloride (Sigma), acetylcholine chloride (Tokyo Kasei Co.), histamine diphosphate (Wako), aminopyrine (Sigma), procaine hydrochloride (Merck), and lidocaine hydrochloride (Merck).

Gastric Secretion in Rats——Gastric Secretion in Pylorusligated Rats: Male Wistar rats weighing about 200 g, fasted for 24 h, were anesthetized with ethyl ether and the pylorus was ligated. Drugs were dissolved in saline and administered s.c. immediately after pyloric ligation. At 6 h after the pyloric ligation, the content of the stomach was collected and the volume and acidity of gastric juice were measured. The acidity was determined by titration with 0.02 N NaOH using phenolphthalein as the indicator.

Experimental Gastric Ulcers——Pyloric Ligation Ulcer (Shay's Ulcer): Male Wistar rats weighing about 200 g were fasted for 48 h but provided with water *ad libitum*. The pylorus was ligated according to the method of Shay *et al.*,[11] and 5 ml/kg of a test drug solution was given *p.o.* immediately after ligation. After 18 h, the rats were sacrificed and the stomach was removed. The severity of the gastric ulcers was scored according to an arbitrary scale and expressed in terms of an ulcer index: 0=no lesion, 1=one to three small ulcers (3 mm or smaller), 2=more than three small ulcers or one large ulcer, 3=one large and several small ulcers, 4=several large ulcers, and 5=perforated ulcers. The inhibition ratio was calculated as follows;

$$\text{inhibition ratio (\%)} = \frac{\text{ulcer index (control)} - \text{ulcer index (drug)}}{\text{ulcer index (control)}} \times 100$$

Water-Immersion Stress Ulcer in Mice: The stress ulcers were produced following the method described by Watanabe *et al.*[12] Male ddY mice weighing about 20 g were fasted for about 18 h before being immersed in water. Drugs were administered orally 30 min before the stress. These mice were placed in a stress cage and immersed in a water bath (15 °C) to the depth of the xiphoid process. At the end of the stress, the animal was sacrificed by dislocation of the cervix, then the stomach was removed, inflated with 1 ml of 1% formalin solution and placed in the same solution for 5 min. The stomach was cut open along the greater curvature and examined grossly for lesion in the glandular portion. As a measure of the degree of ulceration, the sum of the length (mm) of each lesion in the stomach was calculated.

Measurement of Ulcerous Lesions: The inner surface of damaged mucosa in pyloric ligation ulcers and the gastric erosions induced by water-immersion stress were examined with a stereoscopic microscope (10×). The person measuring the size of lesions had no knowledge of which treatment an animal had received.

Effect on Isolated Guinea Pig Ileum——Guinea pig (male Hartley, 300—400 g b.w.) ileum was isolated, and suspended in Tyrode's solution (37 °C) according to a standard method. The contraction of the ileum was recorded with the aid of an isotonic transducer (AP-620G, Nihon Kohden). Acetylcholine chloride (Ach; 2×10^{-7} g/ml) and histamine diphosphate (Hist; 2×10^{-7} g/ml) were used to induce contraction. Each test drug was added to the organ bath 5 min before the addition of the stimulants. ID_{50} values of the test drugs were determined from regression lines.

Analgesic Activity——Acetic Acid-Induced Writhing Test: Male ddY mice weighing about 20 g were injected i.p. with 0.1 ml of 0.7% acetic acid solution/10 g of body weight 30 min after *p.o.* administration of the test drugs. The number of writhes was counted for 20 min.

Local Anesthetic Action (Surface Anesthesia)——Male Hartley guinea pigs weighing 300 to 400 g were used. A drop of test drug solution was instilled into one side of the conjunctival sacs and saline into the other side. The cornea was touched 5 times with a mandrin and the loss of corneal reflex was taken as a measure of the surface anesthetic activity.

Acute Toxicity——Five-week-old ddY mice (Shizuoka Agr. Coop. Assn. for Laboratory Animals) were purchased and quarantined for about 1 week. Six healthy male mice weighing 20—28 g were selected for each group. The animals were fasted for 15—20 h, then a test drug dissolved in distilled water was administered i.v. or *p.o.* General signs and body weights of the mice were noted every day for 7 d. The LD_{50} and the 95% confidence limits were calculated by the method of Litchfield and Wilcoxon.[13]

Statistical Analysis——Results are each given as the mean±standard error. Statistical significance was determined by using Student's *t*-test. ED_{50} values were determined from plots of log dose against percentage inhibition compared with control groups.

Results

Gastric Secretion

Gastric Secretion in Pylorus-Ligated Rats——Subcutaneous injection of BTM-1042

TABLE I. Effects of BTM Compounds and Atropine Sulfate on Gastric Secretion in Pylorus-Ligated Rats

Compounds	Dose (mg/kg) s.c.	No. of rats	Volume (ml/rat) Mean±S.E.	Acid output (meq/6 h) Mean±S.E.
Control	—	6	12.1±0.8	1.01±0.04
BTM-1018 (±)	0.2	6	6.3±0.4[b]	0.58±0.06[b]
BTM-1041 (+)	0.2	6	12.9±0.8	1.27±0.06
BTM-1042 (−)	0.2	6	3.5±0.6[b]	0.32±0.04[b]
Atropine sulfate	0.2	6	6.3±1.8[a]	0.54±0.19[a]

Pyloric ligation was performed in rats fasted for 24 h. The animals were killed 6 h after pyloric ligation. The test drugs were administered s.c. immediately after the ligation. *a*) Significantly different from the control, $p<0.01$. *b*) Significantly different from the control, $p<0.001$.

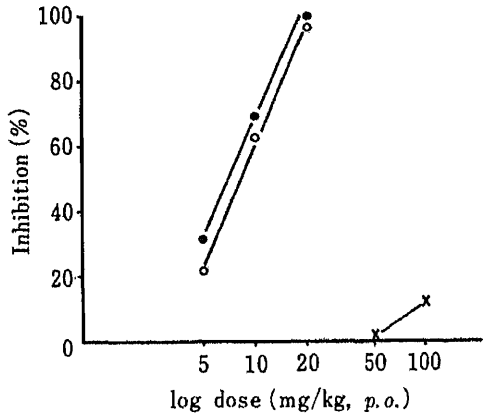

Fig. 2. Effects of BTM Compounds on Gastric Ulcers Induced by Pyloric Ligation in Rats

Ulcers were produced by pyloric ligation in rats fasted for 48 h. The animals were sacrificed 18 h after pyloric ligation. Drugs were administered *p.o.* immediately after the ligation.

Each point represents the mean of 6—7 animals. ●—●, BTM-1042 (−); ○—○, BTM-1018 (±); ×—×, BTM-1041 (+).

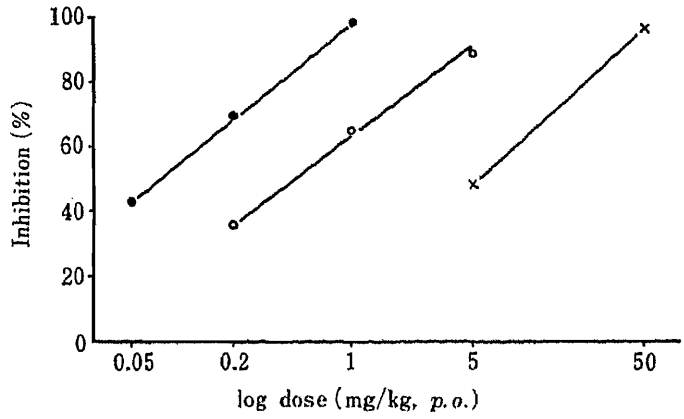

Fig. 3. Effects of BTM Compounds on Water-Immersion Stress Ulcers in Mice

Ulcers were produced in mice fasted for 18 h by immersing the animals in water (15°C) up to the xiphoid process. The animals were sacrificed after 5 h. A test drug was administered *p.o.* before the stress.

Each point represents the mean of 8—10 animals. ●—●, BTM-1042 (−); ○—○, BTM-1018 (±); ×—×, BTM-1041 (+).

significantly decreased the secretion of gastric juice (Table I); the secreted volumes were 29% ($p<0.001$) with the (−)-compound and 52% ($p<0.001$) with the (±)-compound as compared with the control. The (+)-compound was without effect. Gastric acid secretion showed a good correlation with the volume of gastric juice. After injection of the (−)-compound, gastric acid secretion was also decreased markedly to 32% ($p<0.001$) of the control value. Similar inhibition levels of about 45% ($p<0.05$) were noted with the (±)-compound and atropine sulfate (used as a reference drug). Administration of the (+)-compound (BTM-1041) scarcely influenced the gastric acid secretion.

Anti-ulcerogenic Activity

Pyloric Ligation Ulcer——Both the (−)- and (±)-compounds dose-dependently inhibited the development of ulcers produced by pyloric ligation (Fig. 2). The ED_{50} values were calculated to be 7 and 8 mg/kg for the (−)- and (±)-compounds, respectively, based on the dose–response curves. A slight inhibitory effect was observed with the (+)-compound at higher concentrations.

Water-Immersion Stress Ulcer in Mice——As shown in Fig. 3, the (−)-, (±)- and (+)-compounds exhibited dose-dependent antiulcer activities. The ED_{50} values calculated from the dose–response curves were 0.08, 0.5 and 6 mg/kg for the (−)-, (±)- and (+)-compounds, respectively. The antiulcer activity of the (−)-compound was 75 times more potent than that of the (±)-compound.

Antiacetylcholine and Antihistamine Actions

The antagonistic actions of BTM compounds against acetylcholine and histamine were studied by using guinea pig ileum (Table II). The most potent antiacetylcholine action was found with atropine sulfate, followed by the (−)-compound, the (±)-compound and diphenhydramine hydrochloride in that order. The (+)-compound showed antiacetylcholine effect only at higher concentrations. Antihistamine action was the most potent with diphenhydramine hydrochloride, followed by the (+)-compound, the (±)-compound and the (−)-compound in that order. Atropine sulfate showed very weak antiacetylcholine action.

BTM-1086 was found to be competitively antagonistic to acetylcholine. It was about one-fifth as active as atropine sulfate. Antagonistic actions of the compound to barium salts, histamine, serotonin and nicotine were also observed; all these effects were noncompetitive in nature (data not shown).

Analgesic Activity

Acetic Acid-Induced Writhing Test——Analgesic activity was tested by the acetic acid-

Table II. Inhibitory Effects of BTM Compounds on the Contraction of Isolated Guinea Pig Ileum Induced by Acetylcholine or Histamine

Compounds	ID_{50} (g/ml)	
	Ach (2×10^{-7} g/ml)	Hist (2×10^{-7} g/ml)
BTM-1018 (±)	4.8×10^{-7}	5.5×10^{-6}
BTM-1041 (+)	1.9×10^{-5}	1.7×10^{-6}
BTM-1042 (−)	2.3×10^{-7}	8.4×10^{-6}
Atropine sulfate	4.5×10^{-9}	1.8×10^{-5}
Diphenhydramine·HCl	3.7×10^{-6}	2.6×10^{-8}

The ID_{50} is the concentrations of a drug which produced 50% inhibition of the contraction as determined by linear regression analysis. The number of preparations was 5. The pA_2 values for Ach action were reported to be 8.26 and 9.43 with BTM-1042 (−) and atropine sulfate, respectively.[6]

Table III. Effects of BTM Compounds and Aminopyrine on Acetic Acid-Induced Writhing in Mice

Compounds	Dose (mg/kg) *p.o.*	Frequency Mean±S.E.	Inhibition (%)
Control	—	22.7±2.9	—
BTM-1018 (±)	100	21.8±7.8	4.0
	200	3.2±1.0[a]	85.9
BTM-1041 (+)	50	9.9±2.2[a]	56.4
	100	6.2±2.1[a]	72.7
BTM-1042 (−)	100	20.2±2.4	11.0
	200	16.0±2.6	29.5
Aminopyrine	100	5.5±4.2[a]	75.8

The number of mice at each dose: 10. *a*) Significantly different from the control, $p<0.01$.

Table IV. Local Anesthetic Activity of BTM Compounds in Guinea Pigs

Compounds	ED_{50} w/v %	Relative potency
Procaine·HCl	1.27	1.0
Lidocaine·HCl	0.22	5.8
BTM-1018 (±)	0.58	2.2
BTM-1041 (+)	0.51	2.5
BTM-1042 (−)	0.58	2.2

Table V. Acute Toxicity of BTM Compounds in Mice

Compounds	LD_{50} (95% C.L.) (mg/kg)	
	p.o.	i.v.
BTM-1018 (±)	610 (555— 671)	63
BTM-1041 (+)	280 (199— 395)	25
BTM-1042 (−)	870 (669—1131)	160

Each value was calculated by the Litchfield-Wilcoxon method.

TABLE VI. Relative Pharmacological Potencies

Activity	BTM-1018 (±)	BTM-1041 (+)	BTM-1042 (−)
Gastric secretion	1.0	0.0	1.8
Pyloric ligation ulcer	1.0	0.0	1.2
Stress ulcer	1.0	0.1	6.1
Antiacetylcholine	1.0	0.03	2.1
Antihistamine	1.0	3.2	0.7
Analgesic activity	1.0	3.3	0.4
Local anesthetic activity	1.0	1.1	1.0
Acute toxicity	1.0	2.6	0.4

induced writhing method in mice (Table III). Both the (+)- and the (±)-compounds showed dose-dependent analgesic effects. The ED_{50} values were calculated from the dose–response curves to be 40 and 150 mg/kg for the (+)- and the (±)-compounds, respectively. No significant inhibitory effect was noted for the (−)-compound. Aminopyrine (used as a control) exhibited a significant inhibitory effect.

Local Anesthetic Action (Surface Anesthesia)

As shown in Table IV, the effects of BTM compounds were less than that of lidocaine hydrochloride, but were 2.2—2.5 times greater than that of procaine hydrochloride.

Acute Toxicity

Compared to the (±)- and the (+)-compounds, the (−)-compound showed lower acute toxicity (Table V). The LD_{50} value of the (−)-compound was 870 mg/kg by oral administration or 160 mg/kg by intravenous administration.

Discussion

The pharmacological activities of the optical isomers of BTM are summarized in Table VI. The antisecretory, antiulcer and antiacetylcholine actions were greater with the (−)-compound than the (+)-compound. On the other hand, the (+)-compound showed highly selective antihistaminic, analgesic and toxic effects. These results suggest that BTM has multiple targets for its various effects. Although the molecular mechanisms of the actions of these compounds have not been clarified, the present experiments show that the (−)-compound, obtained by optical resolution from the (±)-compound, exhibits more potent antiulcer action with reduced side effects.

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Binding Characteristics of ^{125}I-Iodocyanopindolol to β-Adrenergic Receptors: Biphasic Scatchard Plots. II. Effects of Selective Antagonists

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The characteristics of the high- and low-affinity binding sites for ^{125}I-iodocyanopindolol (^{125}I-ICYP) in rat cerebral cortex membrane were examined. The Scatchard plots of ^{125}I-ICYP binding were biphasic in the absence of antagonists as well as in the presence of non-selective antagonists (*d*-, *dl*- and *l*-propranolol) and selective antagonists. Two of the latter (atenolol and practolol) are β_1-selective antagonists and two others (butoxamine and ICI-118551) are β_2-selective antagonists. The β_2-selective antagonists showed more potent action on the high-affinity sites than on the low-affinity sites, while the β_1-selective antagonists had a more potent effect on the low-affinity sites. These results were consistent with those obtained from pharmacological experiments, and suggest that the high- and low-affinity sites in the biphasic Scatchard plots correspond to β_2- and β_1-adrenoceptors, respectively.

By means of this approach, the relative percentages of the two receptor subtypes in rat brain (cerebral cortex), lung, heart, and spleen were calculated (ratios of β_1 : β_2-adrenoceptors) to be brain 89 : 11, lung 28 : 72, heart 92 : 8, and spleen 59 : 41. The absolute capacities of β-adrenoceptors per g wet weight in these tissues were 10—14 pmol/g tissue except for heart (approx. 2.4 pmol/g tissue).

Keywords——β-adrenoceptor; rat brain; two-site model; selective antagonist

In our previous reports, the Scatchard plots of ^{125}I-iodocyanopindolol (^{125}I-ICYP) binding to β-adrenoceptors in the rat brain (cerebral cortex) were shown to be biphasic in character.[1] The present study was designed to establish whether the high- and low-affinity sites thus identified correspond to β_2- and β_1-adrenoceptors, respectively, by examining ^{125}I-ICYP binding to rat cerebral cortex in the presence of selective antagonists. The findings were checked from a pharmacological point of view.

Experimental

(−)-^{125}I-ICYP (2200 Ci/mmol) was purchased from New England Nuclear Corp., and *l*-, *dl*-, and *d*-propranolol were kindly donated by ICI Pharmaceuticals. The following drugs were also used in this study: atenolol, ICI-118551, practolol, and butoxamine.

Membrane-enriched fractions were prepared as follows. After their removal from male Wistar rat (250—350 g), heart (ventricular muscle), lung, and spleen were each minced and homogenized twice in a Polytron homogenizer for 10 s at setting 8 in 250 mM sucrose and 10 mM Tris–HCl, pH 7.6. The homogenate from each tissue was filtered through 4 layers of gauze, and the filtrate was centrifuged at 40000 *g* for 30 min. The resultant pellet was rinsed at once with 75 mM Tris–HCl, 25 mM $MgCl_2$, pH 7.2 and homogenized twice in a Polytron homogenizer for 10 s at setting 8 in 20 ml of the same buffer. The membrane-enriched fraction from cerebral cortex was prepared as described previously.[1,2] It was frozen in liquid nitrogen and stored in −80 °C. Immediately before use, it was diluted to the desired concentrations as indicated in the text. Protein concentrations were determined by the method of Lowry *et al.*[3] using bovine serum albumin as the standard.

The β-adrenoceptor binding assay was carried out in duplicate with ^{125}I-ICYP in the presence (non-specific) or absence (total) of 10 μM *l*-propranolol by the method described previously.[1] In brief, 0.25 ml of membrane suspension (0.1 mg of protein) was incubated for 60 min at 23 °C with various concentrations (0.015—1.0 nM) of ^{125}I-ICYP in a

total volume of 0.5 ml containing 60 mM Tris–HCl and 20 mM $MgCl_2$ (pH 7.2). At the end of the incubation period, the incubation medium was immediately filtered through a GF/C glass fiber filter using an improved method.[4] The radioactivity was counted with an auto well gamma counter. The difference in mean values between total and non-specific binding was taken as the specific binding.

All kinetic analyses were carried out on an NEC PC-9801F computer system that performed iterative non-linear regression as described previously,[1] based on the theory of Munson and Rodbard.[5] The goodness of fit was evaluated for a model having only one receptor subtype and a model having two receptor subtypes, and the superior model was selected by application of Akaike's information criterion.[6] The programs were made available by Mr. S. Nagatsuka (Daiichi Pure Chem. Co., Ltd.) and partially modified in our laboratory. For the purpose of this report, the concentrations of the high- and low-affinity sites are expressed as percentages of the total concentration of the binding sites (high + low). In order to quantify the mode of saturation, Hill numbers of ^{125}I-ICYP binding to the membranes were determined from Hill plots.[7]

In order to determine the inhibition constant (K_i) of an added cold ligand, the biphasic Scatchard plot was separated into two affinity components (high- and low-affinity sites) and each component was compared with the control values. The K_i values were calculated by using the following equations:

(a) competitive inhibition

$$1/K_{d'} = 1/K_d \times K_i/(K_i + [I])$$

(b) non-competitive inhibition

$$B_{max'} = B_{max} \times K_i/(K_i + [I])$$

where K_d and $K_{d'}$ are the dissociation constants in the absence (control) and presence of the cold ligand, B_{max} and $B_{max'}$ are the capacities of the receptor for the radioligand in the absence (control) and presence of the cold ligand, respectively, and [I] is the concentration of the cold ligand.

β-Blocking actions were also studied pharmacologically by using the methods described previously.[8] In brief, the right and left atria of guinea pig were used for the assessment of the antagonistic potencies against the positive chronotropic and inotropic actions of *l*-isoproterenol (β_1-effect). The rate of the spontaneous contraction of the right atrium was recorded with an ink-writing oscillograph to test the positive chronotropic action. The left atrium was stimulated electrically by a square-wave stimulator (Nihon Koden SEN-3101) at the frequency of 1 Hz with voltages 30% above the threshold to evaluate the inotropic effect. The contractile tension was recorded on an ink-writing oscillograph with a strain-gauge transducer and a carrier amplifier. Isolated tracheal smooth muscle preparations of the guinea pig were used to assess the antagonistic effects towards the β_2-adrenoceptor. Seven tracheal rings removed from guinea pigs were sutured together and mounted vertically in a 50 ml organ bath. The contractile tension of the preparation was recorded on a potentiometric recorder (Medical Electronics Commercial Co., Ltd., E-4032). Relaxation of the preparation was induced by adding *l*-isoproterenol. Drugs were administered in a cumulative fashion, and the pA_2 values of the chemicals were calculated using the equation described previously.[8] The bathing solution was Krebs–Henseleit's solution: 118 mM NaCl, 4.7 mM KCl, 2.55 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 24.88 mM $NaHCO_3$, 11.1 mM glucose, 0.001% ascorbic acid and 0.001% disodium ethylenediaminetetraacetate (EDTA-2Na). The temperature of the solution was kept at 32 ± 0.5 °C. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2.

The significance of differences was analyzed by using Student's *t*-test.

Results

Figure 1 shows plots of the binding of ^{125}I-ICYP to β-adrenoceptors in the cerebral cortex and lung. The Scatchard plots in both tissues were biphasic in character.

In order to characterize the high- and low-affinity sites thus detected, an inhibition study with non-selective and selective antagonists was designed. When ^{125}I-ICYP binding to rat brain was examined in the presence of various concentrations of the antagonists, the only change observed was an increase in the K_d values of both high- and low-affinity sites, indicating that the inhibition was competitive in all cases. Therefore, the K_i values were calculated. Figures 2 and 3 depict the relation between the K_i values and the concentrations of the chemicals. The K_i values of *d*- and *l*-propranolol were concentration-independent, while the K_i values of *dl*-propranolol, racemic practolol, atenolol, butoxamine, and ICI-118551 were concentration-dependent. Furthermore, the K_i values of *dl*-propranolol varied between those of *l*- and *d*-propranolol. Table I lists the changes in K_d, B_{max}, high : low ratios and Hill

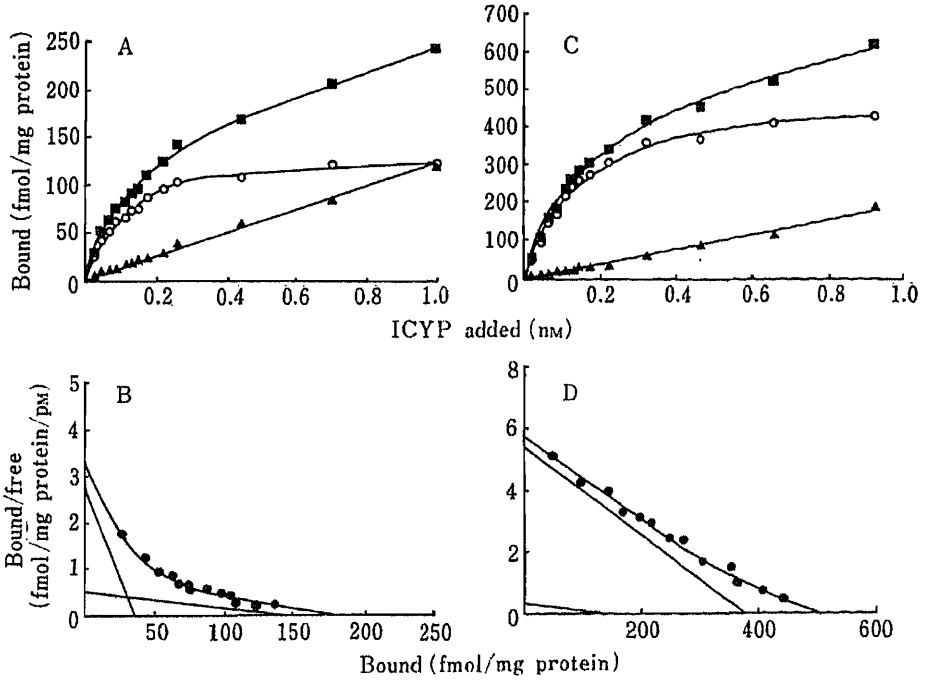

Fig. 1. Saturation Experiments and Scatchard Plots of ^{125}I-ICYP Binding to the Rat Brain (A, B) and Lung (C, D)

Specific binding is defined as the difference between total binding and the binding in the presence of 10 μM *l*-propranolol (non-specific binding) at ^{125}I-ICYP concentrations between 0.01 and 1.0 nM. The values of K_d and B_{max} for ^{125}I-ICYP binding were: brain, 13.13 pM and 36.29 fmol/mg protein (high-affinity site) and 301.89 pM and 147.73 fmol/mg protein (low-affinity site); lung, 69.43 pM and 376.64 fmol/mg protein (high-affinity site) and 426.73 pM and 127.83 fmol/mg protein (low-affinity site). The data shown are those from a single experiment performed in duplicate. The experiment shown is representative of twelve (brain) and three (lung) such experiments. The points show total (■), specific (● or ○), and non-specific (▲) binding.

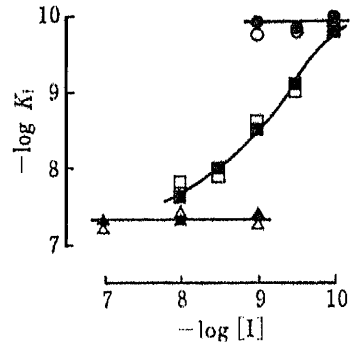

Fig. 2. Plots of $-\log K_i$ *vs.* $-\log[I]$ of *l*- (●, ○), *dl*- (■, □), and *d*-Propranolol (▲, △)

The K_i values for high- (solid symbols) or low-affinity sites (open symbols) were plotted. Each K_i value was calculated on the assumption of competitive inhibition. The data shown are those from a single experiment performed in duplicate.

coefficients produced by low concentrations of these chemicals in the rat cerebral cortex.

Table II summarizes the K_i values of these chemicals calculated from the data listed in Table I. The K_i values for high-affinity sites of non-subtype-selective antagonists, *d*-, *l*-, and *dl*-propranolol, were similar to those for low-affinity sites, while the K_i values for high-affinity sites of subtype-selective antagonists were different from those for low-affinity sites. Thus, β_1-selective antagonists, atenolol and practolol, were selective to low-affinity sites, and β_2-selective antagonists, butoxamine and ICI-118551 were selective to high-affinity sites. These results suggest that the high- and low-affinity sites in ^{125}I-ICYP correspond to β_2- and β_1-adrenoceptor subtypes, respectively.

Table III summarizes the K_d and B_{max} values of ^{125}I-ICYP binding to membranes from

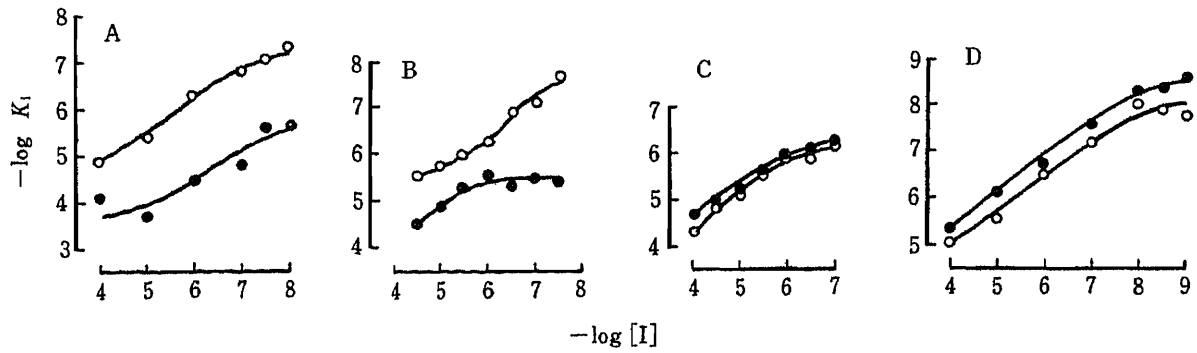

Fig. 3. Plots of $-\log K_i$ *vs.* $-\log[I]$ of Atenolol (A), Practolol (B), Butoxamine (C), and ICI-118551 (D)

The K_i values for high- (●) or low-affinity sites (○) were plotted. Each K_i value was calculated on the assumption of competitive inhibition. The data shown are those from a single experiment performed in duplicate.

TABLE I. Effects of β_1- or β_2-Selective Antagonists on β-Adrenoceptor Binding

	Drugs added (M)		Low-affinity (β_1) site	
			K_d (pM)	B_{max} (fmol/mg protein)
Control	—	(12)	387.70± 36.48	169.89± 3.64
l-Propranolol	1×10^{-10}	(3)	922.53±293.64	211.88±40.26
dl-Propranolol	1×10^{-10}	(3)	823.87±359.14	176.83±59.20
	1×10^{-8}	(4)	663.02± 11.02	133.17±14.75
d-Propranolol	1×10^{-8}	(3)	577.95±134.66	190.33±52.13
Practolol	1×10^{-7}	(3)	679.14±185.09	200.86±20.44
Atenolol	3×10^{-8}	(3)	536.10± 93.77	204.99±29.98
Butoxamine	3×10^{-7}	(3)	436.67± 60.05	182.87±19.20
ICI-118551	3×10^{-9}	(3)	397.95± 53.22	164.53±21.23

	High-affinity (β_2) site		High : low ratio	Hill coefficient
	K_d (pM)	B_{max} (fmol/mg protein)		
Control	18.63± 3.64	20.84±2.79	89.1 : 10.9	0.79±0.02
l-Propranolol	49.31±31.81	30.43±7.37	87.4 : 12.6	0.82±0.08
dl-Propranolol	44.35±33.92	30.14±6.22	85.4 : 14.6	0.79±0.05
	22.70± 2.50	12.58±1.70	91.4 : 8.6	0.82±0.05
d-Propranolol	20.95± 5.72	22.49±8.45	89.4 : 10.6	0.80±0.07
Practolol	28.52± 9.16	18.19±3.62	91.7 : 8.3	0.89±0.01
Atenolol	24.23± 7.14	24.52±2.76	89.3 : 10.7	0.82±0.01
Butoxamine	28.15± 0.46	15.67±4.70	92.1 : 7.9	0.88±0.05
ICI-118551	36.12±12.77	22.42±3.02	88.0 : 12.0	0.86±0.02

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

various tissues. Some of these results are also shown in Figs. 1 and 4. The calculated affinity (K_d) values of ^{125}I-ICYP for β_1- and β_2-receptors are quantitatively similar in each of the four tissues examined. The calculated ratios of β_1- and β_2-adrenoceptors are: brain 89.1:10.9, heart 92.0 : 8.0, lung 28.2 : 71.8, and spleen 58.8 : 41.2. The absolute capacities of β-adrenoceptors

TABLE II. Inhibition Constants of β_1- or β_2-Selective Antagonists

	Drugs added (M)	K_i (nM)		$\beta_1 K_i/\beta_2 K_i$ ratio
		β_1 (Low-affinity) site	β_2 (High-affinity) site	
l-Propranolol	1×10^{-10} (3)	0.194±0.086	0.560±0.476	1.1±0.4
dl-Propranolol	1×10^{-10} (3)	0.606±0.292	0.911±0.400	0.8±0.2
	1×10^{-8} (4)	11.3±3.5	14.5±5.8	1.2±0.5
d-Propranolol	1×10^{-8} (3)	31.4±8.9	56.9±44.6	1.8±1.1
Practolol	1×10^{-7} (3)	277±184	5250±2900	0.052±0.015
Atenolol	3×10^{-8} (3)	69.3±11.2	1370±676	0.097±0.053
Butoxamine	3×10^{-7} (3)	2010±474	686±347	3.8±1.0
ICI-118551	3×10^{-9} (4)	55.6±27.2	3.31±0.65	23±12

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

TABLE III. Affinity Constants (K_d) and Numbers of Receptors (B_{max}) in Various Tissues from Rat

	β_1 (Low-affinity) site		
	K_d (pM)	B_{max} (fmol/mg protein)	B_{max} (fmol/g tissue)
Brain (12)	387.70± 36.48	169.89±3.64	13883.16±1346.98
Heart (3)	421.33± 60.18	33.36±6.61	2264.07± 523.78
Lung (3)	644.43±206.09	139.98±6.57	3269.72± 224.99
Spleen (3)	683.13±136.56	88.54±8.23	6171.46± 448.03

	β_2 (High-affinity) site			$\beta_1:\beta_2$ ratio	Hill coefficient
	K_d (pM)	B_{max} (fmol/mg protein)	B_{max} (fmol/g tissue)		
Brain (12)	18.63± 3.64	20.84± 2.79	1703.01±206.54	89.1:10.9	0.79±0.02
Heart (3)	19.53± 4.71	2.89± 0.78	183.12± 22.49	92.0: 8.0	0.76±0.02
Lung (3)	71.99± 2.72	355.65±22.28	8357.64±893.13	28.2:71.8	0.84±0.03
Spleen (3)	76.56±14.18	62.02±11.49	4302.94±687.36	58.8:41.2	0.82±0.01

Each value in parenthesis is the number of experiments. Data are the mean values±S.E.

TABLE IV. pA_2 Values of β-Blockers

No.[a]	β-Blocker used	Positive chronotropic action (pA_2)	Positive inotropic action (pA_2)	Trachea (pA_2)
1	*l*-Propranolol	9.08±0.31 (6)	8.92±0.26 (6)	9.25±0.26 (5)
2	*d*-Propranolol	6.94±0.77 (4)	7.38±0.31 (4)	6.98±0.03 (2)
3	Practolol[b]	6.83±0.12 (6)	7.10±0.18 (6)	4.39±0.09 (6)
4	Atenolol[b]	7.14±0.03 (5)	7.44±0.04 (5)	5.61±0.06 (5)
5	Butoxamine	6.00±0.09 (5)	6.44±0.05 (4)	6.72±0.13 (5)
6	ICI-118551	7.26±0.17 (6)	7.40±0.16 (6)	9.08±0.14 (5)

Each value in parenthesis is the number of experiments. Data are the mean values±S.E. *a*) Numbers refer to the points in Fig. 5. *b*) The values were obtained from the report published by Nagatomo *et al.*[8]

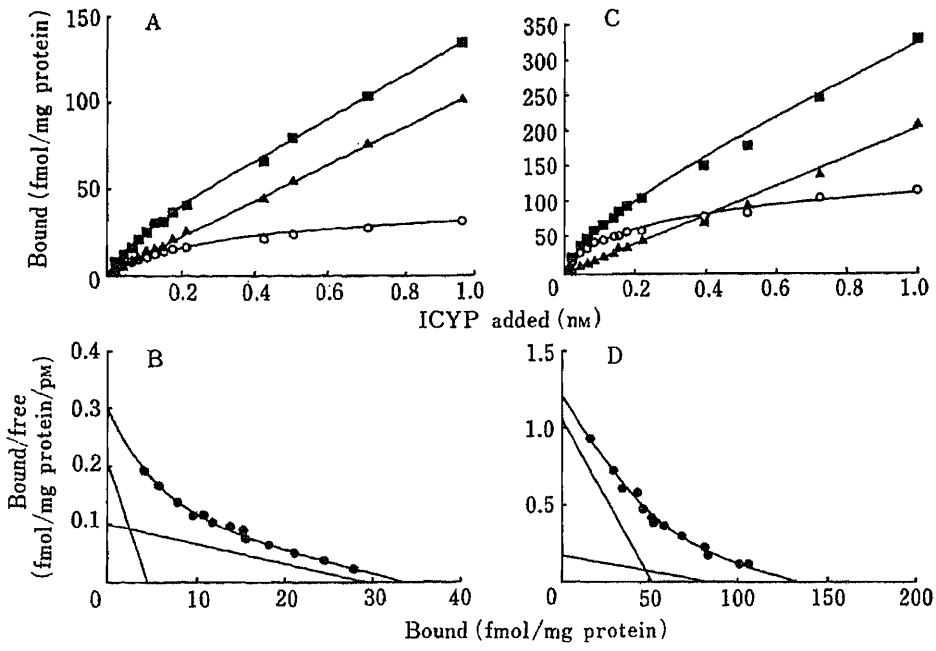

Fig. 4. Saturation Experiments and Scatchard Plots of ^{125}I-ICYP Binding to the Rat Heart (A, B) and Spleen (C, D)

The values of K_d and B_{max} with ^{125}I-ICYP using the rat heart and spleen were: heart, 21.70 pM and 4.41 fmol/mg protein (high-affinity site) and 301.54 pM and 29.52 fmol/mg protein (low-affinity site); spleen, 48.69 pM and 51.07 fmol/mg protein (high-affinity site) and 499.38 pM and 80.62 fmol/mg protein (low-affinity site). The data were obtained in a single experiment performed in duplicate. The experiment shown is representative of three such experiments. The points show total (■), specific (● or ○), and non-specific (▲) binding.

per g wet weight were 10—14 pmol/g tissues in brain, lung and spleen, but 2.4 pmol/g tissues in heart.

Table IV summarizes the pA_2 values of the β-blockers used. These results confirmed the selectivity of atenolol and practolol for β_1-adrenoceptor and of ICI-118551 and butoxamine for β_2-adrenoceptor. Figure 5 depicts the relationship between the K_i values of β-blockers for β_1- and β_2-adrenoceptor sites determined by the binding assay method and the pA_2 values obtained by the pharmacological method as regards the antagonistic effects towards the positive chronotropic action, the positive inotropic action and tracheal relaxing effect of isoproterenol. Good correlations were observed between the $-\log K_i$ values and pA_2 for β_1-sites *vs.* positive chronotropic action, for β_1-sites *vs.* positive inotropic action, and for β_2-sites *vs.* tracheal relaxation.

Discussion

The K_i values of *d*- and *l*-propranolol were concentration-independent in the range of concentrations used in this study, whereas those of *dl*-propranolol and selective β-adrenoceptor antagonists varied with concentration. These results may be explained by assuming that the β-adrenoceptor recognized the *l*-isomer and *d*-isomer separately. The antagonists used, *dl*-propranolol, atenolol, practolol, butoxamine, and ICI-118551 were racemic mixtures. As shown in Fig. 2 and Table II, the two K_i values of *dl*-propranolol may correspond to those of *l*- and *d*-propranolol. In fact, we found that the β-adrenoceptor in rat brain could discriminate the *l*-isomer and *d*-isomer of *dl*-propranolol.[9] Thus, in the radioligand binding assay with ^{3}H-dihydroalprenolol as the ligand, the displacement curves of *dl*-propranolol were biphasic and the two affinity sites were identical with those of the *d*- and *l*-isomers. On the other hand, the

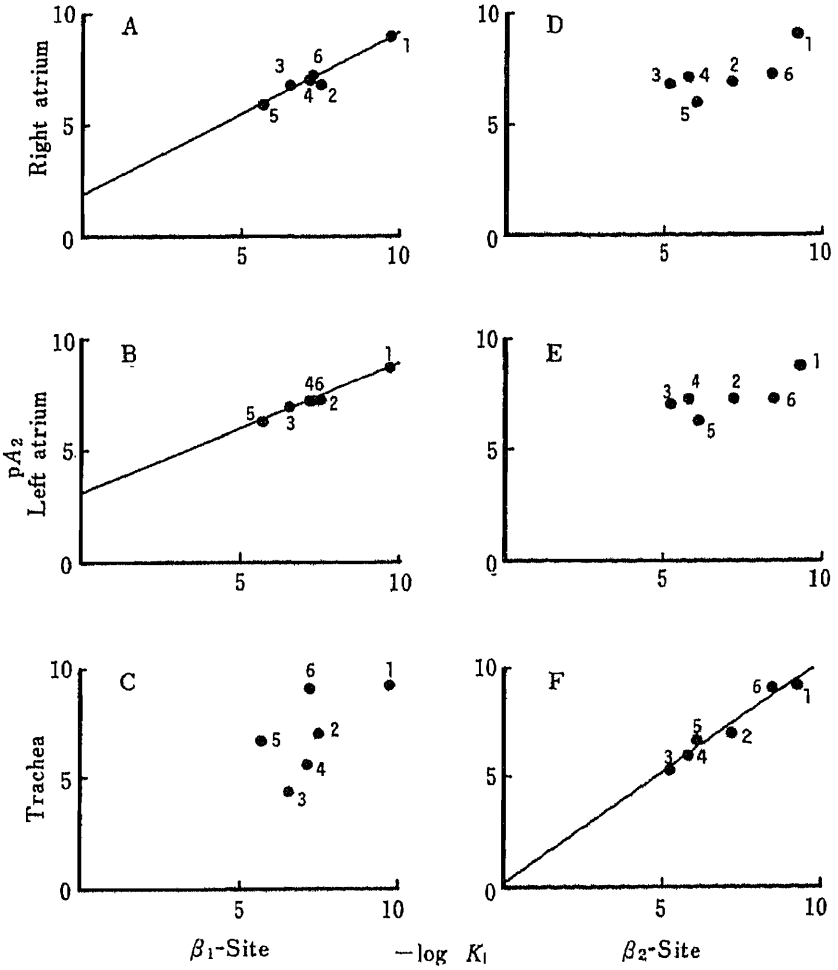

Fig. 5. Relationship between the Potencies of β-Adrenoceptor Antagonists for Inhibition of ^{125}I-ICYP Binding to Receptor Sites of the Rat Brain Membrane Preparation ($-\log K_i$) and the Potencies of Inhibition of the Positive Chronotropic (A, D), the Positive Inotropic (B, E) and the Tracheal Relaxation (C, F) Effects of *l*-Isoproterenol as Assessed by Pharmacological Methods in Guinea Pig (pA_2)

The K_i values used were those of β_1-affinity sites (A, B, C) and β_2-affinity sites (D, E, F) in Table III. Numbers refer to individual drugs in Table V.

A: $y=0.74x+1.78$, $r=0.98$ ($p<0.001$). B: $y=0.60x+3.05$, $r=0.99$ ($p<0.001$). C: $y=0.88x+0.57$, $r=0.62$. D: $y=0.49x+3.78$, $r=0.75$. E: $y=0.38x+4.76$, $r=0.73$. F: $y=1.01x+0.10$, $r=0.98$ ($p<0.001$).

displacement curve of either *d*- or *l*-isomer was uniphasic and the slope factor was unity. One possibility is that the *d*-isomer and *l*-isomer have different dissociation rate constants. Binding assays using (±)-carazolol and (±)-ICYP as the radioligands suggested that each of these racemic radioligands also possesses the two different dissociation rate constants due to the *d*- and *l*-isomers.[10]

The selectivity of ^{125}I-ICYP to β_2-adrenoceptor subtypes was shown by Scatchard plots of the binding in the presence and absence of subtype-selective antagonists. Further, from a pharmacological viewpoint, the subtype specificity of drugs was assessed by analyzing the relative potency of the agonists, isoproterenol (Iso), epinephrine (Epi), and norepinephrine (Nor).[11] Thus, the order of β_1-adrenoceptor potency was Iso > Epi = Nor, whereas that of the β_2-adrenoceptor potency was Iso > Epi > Nor. In the previous report,[1] we showed that the agonists competed for the high- and low-affinity binding sites of ^{125}I-ICYP in rank orders

corresponding to those of β_2- and β_1-adrenoceptor subtypes, respectively. This finding was further supported by the present results. (1) The low-affinity sites identified from the biphasic Scatchard plots were selectively inhibited by the β_1-selective antagonists, atenolol and practolol, whereas the high-affinity sites were selectively inhibited by the β_2-selective antagonists, butoxamine and ICI-118551 (Table II). (2) Good correlations were observed between the $-\log K_i$ values obtained from radioligand binding assays and pA_2 values obtained in pharmacological experiments (Fig. 5).

It has been found that the $\beta_1 : \beta_2$-receptor ratios in various tissues are: rat cerebral cortex 60—80 : 40—20,[12] lung 20—25 : 80—75,[12a,13] heart 83 : 17,[12a] and spleen 35 : 65.[14] These values are similar to those found in our experiments on the assumption that the high- and low-affinity sites correspond to β_2- and β_1-adrenoceptor subtypes, respectively: cerebral cortex 89.1 : 10.9, lung 28.2 : 71.8, heart 92.0 : 8.0, and spleen 58.8 : 41.2.

The present paper is the first to distinguish clearly β_1- and β_2-adrenoceptor recognition sites by means of radioligand binding studies using ^{125}I-ICYP as the ligand. This method can be used to assess directly the subtypes in various tissues and the inhibition type of an inhibitor from Scatchard plots in the presence and absence of the inhibitor.

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Adsorption Behavior of Ammonia and Trimethylamine in Pores of Oxygen Plasma Treated Activated Carbon

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The adsorption characteristics of ammonia and trimethylamine into pores of oxygen plasma treated activated carbon (PT-AC) were investigated in terms of adsorption isotherms, desorption isotherms, and isosteric differential heats of adsorption. The acid functional group concentration of PT-AC was increased 4 times as compared with that of raw activated carbon (R-AC). The surface area and pore volume of PT-AC were about the same as those of R-AC, but the adsorption capacity for ammonia was remarkably increased over that of R-AC. The adsorption capacity for trimethylamine was about the same as that of R-AC. The adsorption of ammonia and trimethylamine on PT-AC was found to be physical adsorption from the desorption isotherms. In the initial stage of adsorption, the isosteric differential heat of adsorption of ammonia on PT-AC was approximately twice that on R-AC. It seems that the amount of ammonia adsorbed on activated carbon is greatly increased by oxygen plasma treatment, probably because acid functional groups produced in the pores attract ammonia in the initial stage of adsorption. It is concluded that PT-AC is a characteristic adsorbent having a remarkably increased adsorption capacity for ammonia, which is a small and weakly basic molecule.

Keywords——ammonia; trimethylamine; oxygen plasma; acid functional group; plasma treated activated carbon; isosteric differential heat; adsorption

Recently, surface modification of substances by the use of low-temperature plasma has attracted attention in various fields of study. Low-temperature plasma has been applied in studies on the production of thin membranes,[1] catalytic reactions,[2] surface modification of metals,[3] and polymeric substances.[4] However, few reports have appeared on the adsorption characteristics of an adsorbent modified with plasma. Usually, wet treatment is carried out to introduce functional groups into pores of activated carbon, and the adsorption characteristics of such modified activated carbons have been reported.[5,6]

In this study, we examined the adsorption characteristics of oxygen plasma treated activated carbon (PT-AC) for ammonia and trimethylamine, which are basic malodorous which differ in molecular size. Moreover, selective adsorption by PT-AC is discussed in terms of adsorption isotherms and isosteric differential heat of adsorption of ammonia on raw activated carbon (R-AC) and PT-AC.

Experimental

Materials——Ammonia gas was of certified grade from Seitetsu Kagaku Co., and its purity was indicated to be 100.0%. Trimethylamine gas with a purity of better than 99.0% was obtained from Matheson Gas Products. R-AC (32—48 mesh) was obtained commercially from Wako Pure Chemical Ind., Ltd. PT-AC was prepared as follows. R-AC (10 g) was placed in a plasma container, degassed for 1 h at 0.1 Torr and then stirred for 3 h at 110 °C; the pressure was kept at 2.5 Torr by adjusting the oxygen flow rate. The R-AC was irradiated at 13.56 MHz for 3 h at 30 W (the maximum output of this plasma apparatus is 100 W). The specific surface area of activated carbon was measured with a BET apparatus by using nitrogen gas at liquid nitrogen temperature (−198 °C); the procedures for measurement

of the pore volume[7] and the concentration of surface functional groups on activated carbon[8] were described previously. The isosteric differential heat of adsorption was determined by applying the Clausius–Clapeyron equation.

Procedure for Adsorption——Adsorption isotherms of ammonia and trimethylamine on activated carbon were determined in an all-glass vacuum system similar to that described previously.[9] The equilibrium amounts adsorbed at pressures up to 600 Torr were measured at 30 °C by a gravimetric method by using a BET apparatus with a spring balance.

Results and Discussion

Adsorption Isotherms of Ammonia and Trimethylamine on PT-AC

Figure 1 shows a schematic diagram of the inductively coupled plasma reactor apparatus. Table I summarized the physicochemical properties of R-AC and PT-AC, and Table II gives the concentrations of surface functional groups on R-AC and PT-AC. In general, the amount adsorbed on an adsorbent is dominated by surface area, pore volume, and surface polarity.

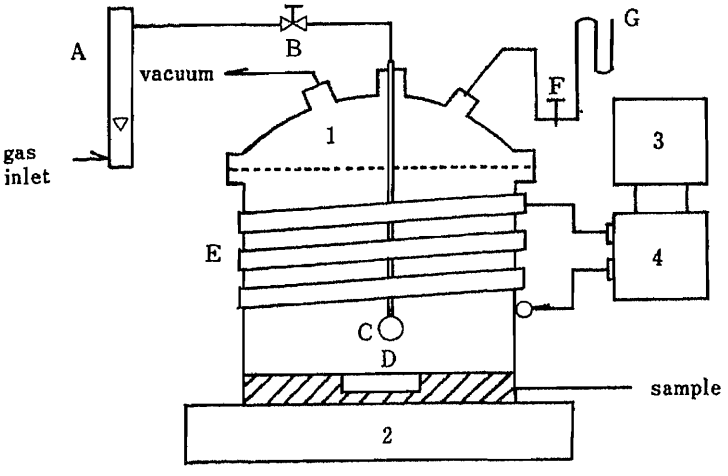

Fig. 1. Schematic Diagram of Plasma Apparatus

1, plasma reactor; 2, magnetic stirrer; 3, 13.56 MHz radio-frequency generator; 4, matching network; A, flowmeter; B, needle valve; C, ball filter; D, magnet; E, radio-frequency coil; F, stop valve; G, macleod gauge.

TABLE I. Physicochemical Properties of R-AC and PT-AC

Adsorbent	Specific surface area (m^2/g)	Pore volume (ml/g)	Surface pH
R-AC	849.9	0.5007	5.9
PT-AC	896.4	0.5020	4.8

TABLE II. Concentration of Surface Functional Groups on R-AC and PT-AC

Adsorbent	-COOH type (meq/g)	-OH type (meq/g)	=CO type (meq/g)	Total acidity (meq/g)
R-AC	0.1135	0.0000	0.0098	0.1233
PT-AC	0.4391	0.0338	0.0072	0.4791

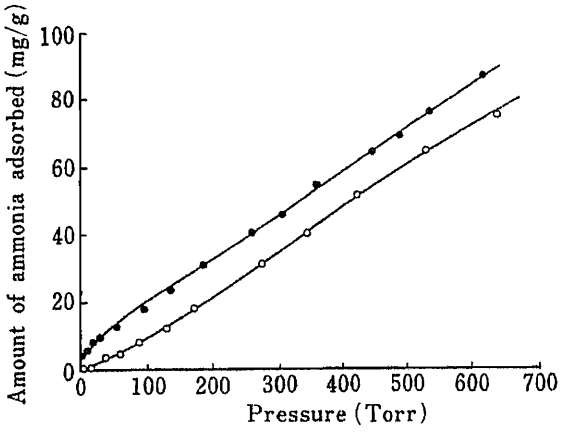

Fig. 2. Adsorption Isotherms of Ammonia on R-AC and PT-AC
○, R-AC; ●, PT-AC.

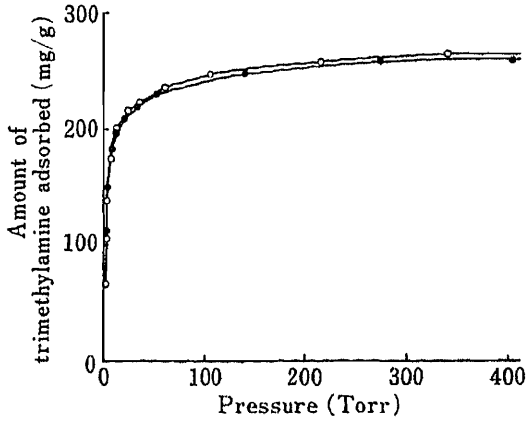

Fig. 3. Adsorption Isotherms of Trimethylamine on R-AC and PT-AC
○, R-AC; ●, PT-AC.

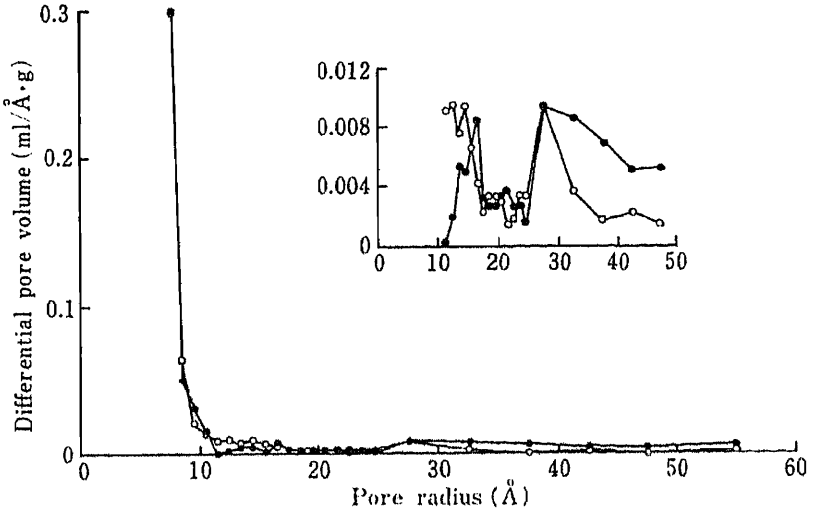

Fig. 4. Pore Size Distribution Curves of R-AC and PT-AC
○, R-AC; ●, PT-AC.

By oxygen plasma treatment, the pH of the surface appeared to lower and the total concentration of acid functional groups was increased 4 times; in particular, the concentration of carboxyl groups was remarkably increased over that of R-AC. Figures 2 and 3 show the adsorption isotherms of ammonia and trimethylamine on R-AC and PT-AC at 30 °C. The adsorption isotherms of ammonia and trimethylamine resembled type III and type I, respectively, according to the classification of Brunauer *et al.*[10] The adsorption capacity of PT-AC for ammonia was remarkably increased as compared with that on R-AC in the range of less than 600 Torr. The adsorption capacity of PT-AC for trimethylamine was about the same as that of R-AC over the whole experimental range. Figure 4 shows the pore size distribution curves of R-AC and PT-AC in the range of radii up to 55 Å. Dubinin[11] divided pore structure into three classes, *i.e.*, micropores (radius < 15—16 Å), transitional pores (15—16 < radius < 1000—2000 Å), and macropores (radius > 1000—2000 Å). In the range of micropores of 10—15 Å, the pore volumes of PT-AC was slightly decreased from that of R-AC and in the range of transitional pores of 30—60 Å, the pore volume of PT-AC was conversely increased. These results show that oxygen plasma treatment influences pore size

distribution. These findings indicate that the amount of ammonia adsorbed on activated carbon is greatly influenced by the content of acid functional groups (Table II) generated by oxygen plasma treatment while that of trimethylamine is little influenced, but surface area and pore volume are important (Table I).

Adsorption Behavior of Ammonia and Trimethylamine on PT-AC

Figure 5 shows the adsorption–desorption isotherms of ammonia and trimethylamine on PT-AC at 30 °C. The adsorption–desorption isotherms of ammonia and trimethylamine on PT-AC showed hysteresis loops. According to Sing *et al.*,[12] such hysteresis loops are often associated with narrow slit-like pores. Ammonia and trimethylamine adsorbed on PT-AC were degassed to the extent of more than 98% in 12 h at 30 °C, at 0.001 Torr. Thus, the adsorption mode of ammonia or trimethylamine on PT-AC is mainly physical adsorption. The forces involved in physical adsorption include both van der Waals forces and electrostatic interactions including polarization.[13] Thus, to clarify the extent of increase in the amount of ammonia adsorbed on PT-AC, the adsorption isotherms of ammonia on R-AC and PT-AC were obtained at 10 and 30 °C. The Clausius–Clapeyron equation was used to obtain the isosteric differential heats of adsorption of ammonia on R-AC and PT-AC. Figure 6 shows the relationship between the isosteric differential heat of adsorption and the amount of

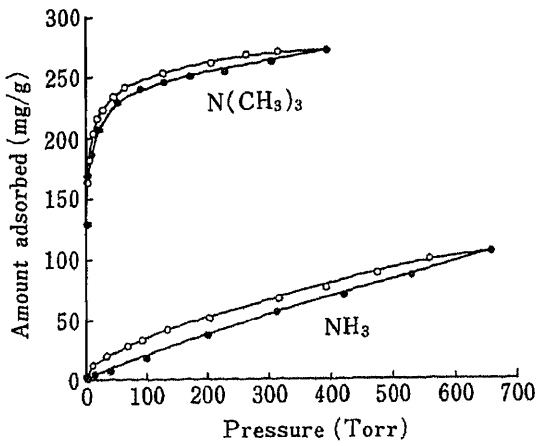

Fig. 5. Adsorption–Desorption Isotherms of Ammonia and Trimethylamine on PT-AC at 30 °C

●, adsorption; ○, desorption.

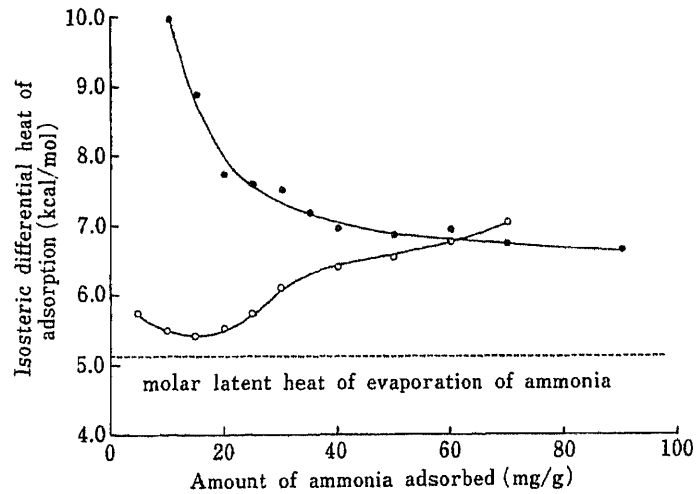

Fig. 6. Isosteric Differential Heat of Adsorption *vs.* Amount of Ammonia Adsorbed on R-AC and PT-AC

○, R-AC; ●, PT-AC.

ammonia adsorbed on R-AC and PT-AC. In the initial stage of adsorption, the isosteric differential heats of adsorption on R-AC and PT-AC were 5.5 and 10.0 kcal/mol, respectively, and that on PT-AC was approximately twice than that on R-AC. According to the increase in the amounts of ammonia adsorbed on R-AC and PT-AC, the difference in the isosteric differential heats of adsorption on R-AC and PT-AC became small. These results indicate that the characteristics of adsorption of ammonia on R-AC and PT-AC are very different in the initial stage of adsorption. According to Ruthven,[13] the adsorption is physical adsorption when the heat of adsorption is below 2 or 3 times the latent heat of evaporation. Therefore, the adsorption mode of ammonia on PT-AC obtained from the isosteric differential heat of adsorption is consistent with that obtained from the desorption isotherms of ammonia on PT-AC, because the latent heat of evaporation of ammonia (10—30 °C) is 5.2 kcal/mol.[14] It seems that the amount of ammonia adsorbed on activated carbon is remarkably increased by oxygen plasma treatment because of the acid functional groups produced in the pores. Trimethylamine is more basic than ammonia, but the molecular dimensions of trimethylamine and ammonia were found to be 5.9 and 3.7 Å,[15] respectively, so that trimethylamine is approximately 1.6 times larger than ammonia. It may be concluded that PT-AC characteristically has a remarkably increased adsorption capacity for ammonia, a small and weakly basic molecule.

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Notes

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Promoting Effect of Pentamethylbenzene on the Deprotection of *O*-Benzyltyrosine and N^{ε}-Benzyloxycarbonyllysine with Trifluoroacetic Acid[1)]

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O-Benzyltyrosine was rapidly deprotected without formation of 3-benzyltyrosine by treatment with trifluoroacetic acid containing pentamethylbenzene. This method was also found to be useful in the deprotection of N^{ε}-benzyloxycarbonyllysine [Lys(Z)] and N^{G}-4-methoxy-2,3,6-trimethylbenzenesulfonylarginine [Arg(Mtr)]. The new deprotecting method was successfully applied to the synthesis of kyotorphin (Tyr–Arg).

Keywords——deprotecting method; pentamethylbenzene–trifluoroacetic acid; *O*-benzyltyrosine; N^{α}-benzyloxycarbonyllysine; N^{G}-4-methoxy-2,3,6-trimethylbenzenesulfonylarginine; kyotorphin

A prevailing strategy in peptide synthesis involves a final acidolytic cleavage of all side chain protecting groups.[2)] Though trifluoroacetic acid (TFA) has many advantages such as high volatility, comparatively low toxicity and good solvency, it does not have sufficient acidity to remove various protecting groups.[3)] In 1980, Kiso *et al.*[4)] presented a novel method to improve the characteristics of TFA. They reported that though TFA alone deprotected *O*-benzyltyrosine [Tyr(Bzl)] slowly to yield tyrosine and 3-benzyltyrosine (by-product) in the ratio of 57 to 43,[5)] a combination of thioanisole with TFA could deprotect Tyr(Bzl) rapidly without formation of 3-benzyltyrosine.[6)] Moreover, this system could deprotect N^{ε}-benzyloxycarbonyllysine [Lys(Z)] quantitatively. The proposed mechanism for the deprotection of Tyr(Bzl) was addition of H^{+} to the oxygen atom of the ether bond and subsequent nucleophilic attack of the sulfur atom of thioanisole on the electron-deficient benzyl carbon atom.

On the other hand, it has been reported that in several deprotecting procedures the addition of compounds which consume a reactive species formed in the process increases the rate of the reaction or brings the reaction to completion.[7)] We have now examined whether potent cation scavengers can promote the removal of benzyl-type side chain protecting groups with TFA, in order to find another deprotecting procedure suitable for large-scale peptide synthesis. The present paper describes the efficient deprotection of Tyr(Bzl), Lys(Z) and N^{G}-4-methoxy-2,3,6-trimethylbenzenesulfonylarginine [Arg(Mtr)] with TFA containing pentamethylbenzene.

Anisole, the methoxy group of which activates the benzene ring for electrophilic attack, has been widely employed as a cation scavenger, so that the promoting effect of methoxybenzenes on the deprotection of Tyr(Bzl) with TFA at 30 °C was first examined. The progress of

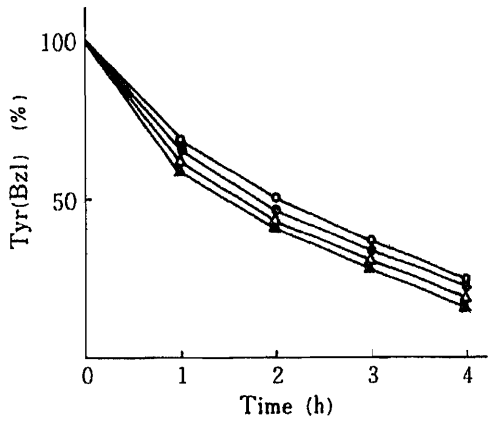

Fig. 1. Effect of Methoxybenzenes (2 mmol) on the Deprotection of Tyr(Bzl) (0.2 mmol) with TFA (4 ml) at 30 °C

○, no additive; ●, anisole; △, 1,3-dimethoxybenzene; ▲, 1,2,3-trimethoxybenzene.

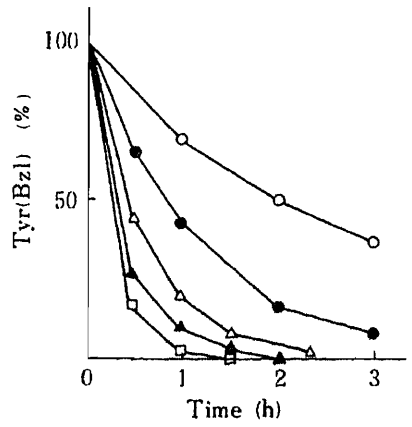

Fig. 2. Effect of Methylbenzenes (2 mmol) on the Deprotection of Tyr(Bzl) (0.2 mmol) with TFA (4 ml) at 30 °C

○, no additive; ●, 1,3-dimethylbenzene; △, 1,2,3-trimethylbenzene; ▲, 1,2,3,4-tetramethylbenzene; □, PMB.

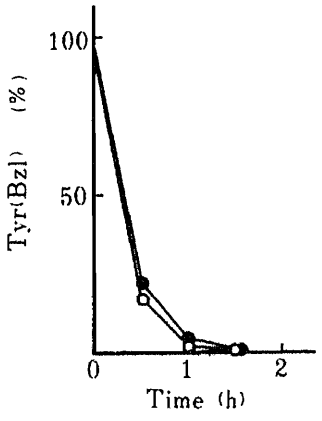

Fig. 3. Comparison of the Effects of Scavengers (2 mmol) on the Deprotection of Tyr(Bzl) (0.2 mmol) with TFA (4 ml) at 30 °C

○, PMB; ●, thioanisole.

the reaction was followed by high-performance liquid chromatography (HPLC). As can be seen in Fig. 1, these additives had a little ability to accelerate the cleavage of the benzyl ether. However, in the case of methylbenzenes the rate of the cleavage increased with increase of the number of methyl groups in the benzene ring, as shown in Fig. 2. Pentamethylbenzene (PMB) was the most effective among the agents tested. Complete deprotection of Tyr(Bzl) was achieved in PMB–TFA at 30 °C within 1.5 h and the rate of this cleavage was as fast as that in thioanisole–TFA, as shown in Fig. 3. Moreover, Tyr was found to be regenerated from Tyr(Bzl) almost quantitatively by the PMB–TFA method.

Though the mechanism of this cleavage reaction has not been clarified yet, except that 1-benzyl-2,3,4,5,6-pentamethylbenzene is produced, trapping of the benzyl cation by PMB presumably promotes the reaction as shown in Fig. 4. It appears to be strange that the methoxy group, which is a stronger electron donor than the methyl group,[8] did not contribute much to the promotion of the cleavage reaction. The reason may be that protonation of the methoxy group by TFA reduces its electron-releasing activity.

In addition, the deprotection of Lys(Z)[9] and Arg(Mtr)[10] was examined in PMB–TFA. In both cases the removal of the protecting group was accelerated, as shown in Figs. 5 and 6.

Furthermore, we examined the application of this new method to the synthesis of kyotorphin (Tyr–Arg).[11] Boc–Tyr(Bzl)–Arg(Mtr)–OH, which was prepared by coupling

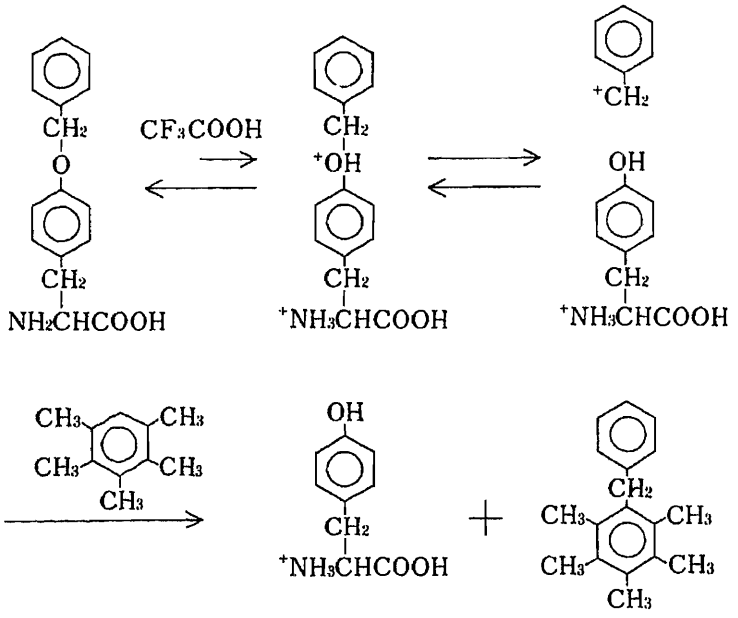

Fig. 4.

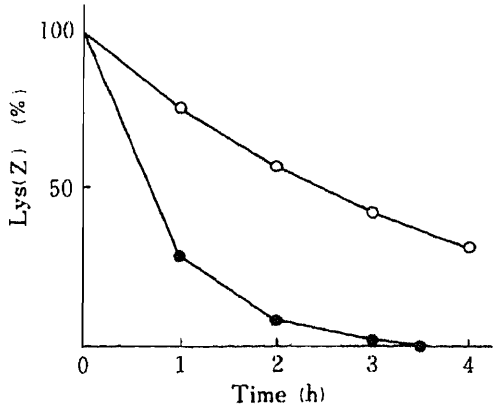

Fig. 5. Effect of PMB (2 mmol) on the Deprotection of Lys(Z) (0.2 mmol) with TFA (4 ml) at 30 °C

○, no additive; ●, PMB.

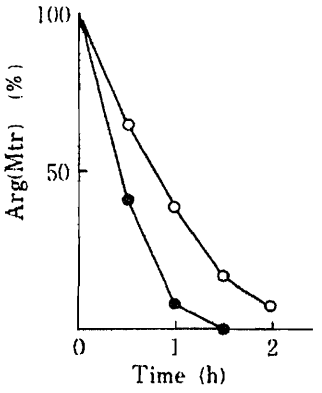

Fig. 6. Effect of PMB (2 mmol) on the Deprotection of Arg(Mtr) (0.2 mmol) with TFA (4 ml) at 30 °C

○, no additive; ●, PMB.

Boc–Tyr(Bzl)–OH[12] with H–Arg(Mtr)–OH by the HOSu active ester method, was deprotected by the PMB–TFA method at room temperature overnight. The product was purified by column chromatography on YMC gel (ODS) using 0.05% HCl as an eluent. Lyophilization of the main fraction yielded the hygroscopic pure peptide, which was converted to the acetate salt by treatment with Amberlite IRA-93 (AcOH form). The homogeneous peptide was obtained in 52% overall yield from the protected peptide.

These results show that this deprotecting method using PMB, which is commercially available, might be as useful as the thioanisole–TFA method in practical peptide synthesis. Further experiments are needed to determine the scope and limitations of this new method.

Experimental

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum was measured on a JEOL FX-90Q spectrometer using tetramethylsilane as an internal standard. Optical rotations were determined with a JASCO model DIP-140

polarimeter. Acid hydrolysis was carried out in 6 N HCl at 110 °C for 22 h. Amino acid analysis was performed in a Hitachi 835 amino acid analyzer. Thin-layer chromatograms (TLC) were run on silica gel plates (precoated silica gel plates $60F_{254}$, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–AcOH (7 : 1 : 0.1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (15 : 5 : 5 : 8). HPLC was carried out using an LC-5A pump (Shimadzu), an SDP-2A variable-wavelength UV detector (Shimadzu) and an AM-312 packed column (ODS, Yamamura Chemical Laboratory Co., Ltd.).

Measurement of Deprotection Rates of Tyr(Bzl), Lys(Z) and Arg(Mtr)——The protected amino acid (0.2 mmol), an additive (2 mmol) and benzoic acid (0.1 mmol, used as an internal standard) were dissolved in TFA (4 ml) and the solution was kept at 30 °C. The remaining amount of the protected amino acid in the solution was estimated by HPLC (detected at 210 nm) using 0.05% HCl (H_2O–CH_3CN, 78 : 22) as an eluent. The results are shown in Figs. 1, 2, 4 and 5.

Measurement of the Amount of Tyr Regenerated from Tyr(Bzl) by Treatment with PMB–TFA——Tyr(Bzl) (54.2 mg, 0.2 mmol) was dissolved in TFA (4 ml) containing PMB (296 mg, 2 mmol). The solution was left to stand at room temperature overnight, then 2-hydroxybenzamide (27.4 mg, 0.2 mmol, used as an internal standard) and D-10-camphorsulfonic acid (60 mg, 0.24 mmol) were added. The amount of Tyr in the solution was estimated by HPLC (detected at 280 nm) using 0.1% D-camphorsulfonic acid (H_2O–CH_3CN, 92 : 8) as an eluent; recovery 98%.

Isolation of 1-Benzyl-2,3,4,5,6-pentamethylbenzene——Tyr(Bzl) (2.71 g, 0.01 mol) and PMB (14.85 g, 0.1 mol) were dissolved in TFA (200 ml) and the solution was left to stand at room temperature for 7 h. After evaporation of the TFA, *n*-hexane and 1 N HCl were added. The *n*-hexane layer was washed with water and both *n*-hexane and PMB were removed *in vacuo*. The oily residue was triturated with *n*-hexane to give crystals; yield 2.17 g (91%), mp 111—112 °C (uncorrected). *Anal.* Calcd for $C_{18}H_{22}$: C, 90.70; H, 9.30. Found: C, 90.85; H, 9.31. ^{1}H-NMR ($CDCl_3$) δ: 2.16 (6H, s, $CH_3 \times 2$), 2.24 (9H, s, $CH_3 \times 3$), 4.10 (2H, s, $\underline{CH_2}Ph$), 6.90—7.30 (5H, m, $CH_2\underline{Ph}$).

Boc–Tyr(Bzl)–Arg(Mtr)–OH——Boc–Tyr(Bzl)–OH (2.60 g, 7.0 mmol) and HOSu (0.97 g, 8.4 mmol) were dissolved in DMF (15 ml) and *N,N'*-dicyclohexylcarbodiimide (1.45 g, 7.0 mmol) was added at 0 °C. The mixture was stirred at 4 °C overnight, then a solution of H–Arg(Mtr)–OH (2.70 g, 7.0 mmol) and *N*-methylmorpholine (0.77 ml, 7.0 mmol) in DMF (16 ml) was added. The mixture was stirred at room temperature for 1 d. The solvent was evaporated, the residue was dissolved in AcOEt and the precipitate was filtered off. The filtrate was washed with 0.1 N citric acid. After evaporation of the AcOEt, the residue was purified by column chromatography on silica gel ($CHCl_3$–MeOH, 30 : 1); yield 4.0 g (77%), $[\alpha]_D^{20}$ +5.6° (*c* = 2, MeOH), Rf_1 0.49. *Anal.* Calcd for $C_{37}H_{49}N_5O_9S \cdot 3/2\ H_2O$: C, 57.95; H, 6.83; N, 9.13. Found: C, 58.11; H, 6.48; N, 9.09.

H–Tyr–Arg–OH——Boc–Tyr(Bzl)–Arg(Mtr)–OH (1.481 g, 2 mmol) was dissolved in TFA (40 ml) containing PMB (2.96 g, 20 mmol) and the solution was left to stand overnight at room temperature. The solvent was evaporated off at 30 °C, ether and water were added to the residue and the water layer was lyophilized. The crude product was purified by column chromatography on YMC gel ODS (3 × 30 cm, 60/200, Yamamura Chemical Laboratory Co., Ltd.) using 0.05% HCl as an eluent. The main fraction was lyophilized, dissolved in water and treated with Amberlite IRA-93 (AcOH). The solvent was removed by lyophilization; yield 460 mg (52%), $[\alpha]_D^{20}$ +22.5° (*c* = 0.5, 0.2 M AcOH), Rf_2 0.48. Amino acid ratios (acid hydrolysate): Tyr 1.00, Arg 1.04 (recovery 93%). *Anal.* Calcd for $C_{15}H_{23}N_5O_4 \cdot CH_3COOH \cdot 5/2H_2O$: C, 46.15; H, 7.29; N, 15.83. Found: C, 46.42; H, 7.18; N, 16.02.

References and Notes

1) Amino acids and their derivatives in this paper are of the L-configuration. The following abbreviations are used: Bzl = benzyl, Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, HOSu = *N*-hydroxysuccinimide, DMF = dimethylformamide.
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Synthesis of Neurokinin B Analogs and Their Activities as Agonists and Antagonists

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Nine neurokinin B (NKB) related octapeptide analogs, substituted with Arg for His^3, or Gly, D-Trp, D-Ala or D-Phe for Phe^6, or Gly for Val^7, or D-Trp or D-Ala for Gly^8 in the original sequence, were synthesized by the solid-phase method. The biological activity of the synthetic analogs was assayed on isolated guinea-pig ileum and rat duodenum. None of the analogs possessed agonistic activity. In the guinea-pig ileum assay, [Arg^3, Gly^6, D-Ala^8]- and [Arg^3, Gly^6, D-Trp^8]-NKB (3—10) were found to act as fairly potent antagonists against NKB, but showed no antagonistic effect against substance P (SP) or neurokinin A (NKA). [Arg^3, D-Ala^6, D-Trp^8]- and [Arg^3, D-Trp^{6,8}, Gly^7]-NKB (3—10) showed antagonistic activities against NKB and SP, and NKB, SP and NKA, respectively, in the ileum assay, while the latter acted as an antagonist against NKB, but not against SP or NKA in the rat duodenum assay.

Keywords——neurokinin B; neurokinin B octapeptide analog; solid-phase synthesis; isolated guinea-pig ileum; isolated rat duodenum; agonistic activity; relative activity; antagonistic activity; nonspecific antagonist; specific antagonist

Neurokinin B[1,2] (NKB) is a decapeptide amide isolated from porcine spinal cord, and has close structural homologies with tachykinins such as neurokinin A (NKA), substance P (SP), kassinin and physalaemin (Fig. 1), particularly in the C-terminal regions.

In a study[3] of the relationship between chain length and activity, it was found that the contractile activity of NKB on the guinea-pig ileum, rat *vas* deferens and rat duodenum remains nearly intact after removal of the N-terminal tripeptide portion from the native peptide.

Our study[4] on the structure–activity relationship of NKB (3—10) provided a clear indication of the location of the active site, that is, the replacement of Phe^6 or Val^7 with Gly brings about a drastic decrease of the contractile activities on isolated guinea-pig ileum and rat *vas* deferens, while the substitution of Gly for Phe^5 enhances the activities. In other words, NKB related octapeptide analogs [Gly^6]-NKB (3—10) **1** and [Gly^7]-NKB (3—10) **2** have no intrinsic activity on isolated guinea-pig ileum and rat *vas* deferens. In a recent study[5] of NKB related peptides, it has been found that the substitution of an unusual amino acid such as Sar, D-Trp, D-Ala, D-Phe, D-Arg, D-Pro, D-α-phenylglycine, D-homoglutamine,[6] or D-homo-

	1	10
neurokinin B (NKB)	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2	
neurokinin A (NKA)	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2	
substance P (SP)	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	
kassinin	H-Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2	
physalaemin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2	

Fig. 1. Amino Acid Sequences of Tachykinin Peptides

glutamic acid[7] for Phe[6] significantly reduces the contractile activity on isolated guinea-pig ileum, and the replacement of Phe[6] with Gly or D-Ala changes the pharmacological spectrum of NKB from that of an agonist to that of an antagonist. Octapeptide analogs [Gly[6]]-NKB (3—10) **1** and [Arg[3], D-Ala[6]]-NKB (3—10) **3** have been found to act as fairly potent antagonists of NKB.

Based on these observations, a new series of octapeptide analogs of NKB (3—10) (Fig. 2) was designed in order to obtain a specific and potent antagonist of NKB. Analogs **1** and **2** were modified by replacements with Arg at position 3, Gly, D-Ala, D-Trp and D-Phe at position 6, Gly at position 7, and D-Ala and D-Trp at position 8 of NKB. It has been indicated that the antagonistic activity[8] of SP related peptide analogs against SP can be increased with double or triple substitutions of D-amino acids such as D-Trp and D-Ala for natural residues, mainly at the C-terminal region of SP. The N-terminal Arg[3] moiety was incorporated to improve the solubility of the peptides in water, as in SP antagonists[8] derived from the C-terminal hexapeptide sequence of SP.

The new NKB analogs were synthesized in the same way as described for the preparation of NKB analogs[4] on an automated peptide synthesizer by the standard solid-phase method[9] starting from *tert*-butoxycarbonyl(Boc)-Met-benzhydrylamine-resin. Boc protection was used

		3 6 7 8 10
1	[Gly[6]]-NKB (3—10)	H-His-Asp-Phe——Gly-Val——Gly-Leu-Met-NH_2
2	[Gly[7]]-NKB (3—10)	H-His-Asp-Phe——Phe-Gly——Gly-Leu-Met-NH_2
3	[Arg[3], D-Ala[6]]-NKB (3—10)	H-Arg-Asp-Phe-D-Ala-Val——Gly-Leu-Met-NH_2
4	[Arg[3], Gly[6], D-Ala[8]]-NKB (3—10)	H-Arg-Asp-Phe——Gly-Val-D-Ala-Leu-Met-NH_2
5	[Arg[3], Gly[6], D-Trp[8]]-NKB (3—10)	H-Arg-Asp-Phe——Gly-Val-D-Trp-Leu-Met-NH_2
6	[Arg[3], D-Ala[6], D-Trp[8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Ala-Val-D-Trp-Leu-Met-NH_2
7	[Arg[3], D-Trp[6,8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Trp-Val-D-Trp-Leu-Met-NH_2
8	[Arg[3], D-Phe[6], Gly[7], D-Ala[8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Phe-Gly-D-Ala-Leu-Met-NH_2
9	[Arg[3], D-Phe[6], Gly[7], D-Trp[8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Phe-Gly-D-Trp-Leu-Met-NH_2
10	[Arg[3], D-Ala[6], Gly[7], D-Trp[8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Ala-Gly-D-Trp-Leu-Met-NH_2
11	[Arg[3], D-Trp[6], Gly[7], D-Ala[8]]-NKB (3—10)	H-Arg-Asp-Phe-D-Trp-Gly-D-Ala-Leu-Met-NH_2
12	[Arg[3], D-Trp[6,8], Gly[7]]-NKB (3—10)	H-Arg-Asp-Phe-D-Trp-Gly-D-Trp-Leu-Met-NH_2

Fig. 2. NKB Related Octapeptide Analogs

TABLE I. Physical Properties and Yields of the Synthetic Analogs

Analog	$[\alpha]_D^{18}$ (°) (c=0.1, DMF)	Retention time[a] (min)	Rf^1	Rf^2	Yield (%)
4	−23.0	17.1	0.07	0.64	31.1
5	−36.0	21.6	0.09	0.67	18.4
6	−32.0	21.8	0.10	0.68	29.2
7	−20.0	26.3	0.16	0.70	15.8
8	−10.0[b]	17.4	0.07	0.65	14.9
9	+0.3[b]	23.5	0.13	0.68	14.2
10	−26.0[b]	18.3	0.06	0.64	11.5
11	+3.0[b]	18.4	0.07	0.64	29.1
12	−17.0	23.0	0.11	0.67	12.3

a) Conditions of analytical HPLC: column, Chemcosorb ODS (3.9×300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 14% to 35% MeCN (15 min) in 20 mM phosphate buffer (pH 3.0). *b*) $[\alpha]_D^{14}$ (°).

TABLE II. Amino Acid Analyses of the Synthetic Analogs

Analog	Found (Calcd)									
	Asp	Gly	Ala	Val	Met	Leu	Phe	Trp	Arg	NH_3
4	1.00 (1)	1.01 (1)	1.03 (1)	1.05 (1)	0.99 (1)	1.03 (1)	1.01 (1)	—	0.88 (1)	1.10 (1)
5	1.03 (1)	0.99 (1)	—	0.94 (1)	0.91 (1)	1.01 (1)	0.97 (1)	1.05 (1)	1.09 (1)	1.12 (1)
6	1.04 (1)	—	1.02 (1)	0.93 (1)	0.94 (1)	1.03 (1)	1.00 (1)	1.05 (1)	0.98 (1)	1.14 (1)
7	1.06 (1)	—	—	0.94 (1)	0.92 (1)	1.03 (1)	0.98 (1)	2.00 (2)	1.07 (1)	1.31 (1)
8	1.00 (1)	0.98 (1)	0.99 (1)	—	0.99 (1)	1.01 (1)	1.98 (2)	—	1.06 (1)	1.34 (1)
9	1.00 (1)	1.00 (1)	—	—	0.97 (1)	1.02 (1)	1.96 (2)	1.07 (1)	0.99 (1)	1.41 (1)
10	0.99 (1)	1.02 (1)	0.98 (1)	—	0.98 (1)	1.01 (1)	0.98 (1)	0.91 (1)	1.14 (1)	1.42 (1)
11	0.97 (1)	1.05 (1)	0.97 (1)	—	1.01 (1)	1.02 (1)	0.99 (1)	1.04 (1)	0.95 (1)	1.23 (1)
12	1.02 (1)	0.98 (1)	—	—	0.91 (1)	0.99 (1)	0.96 (1)	2.10 (2)	1.05 (1)	1.26 (1)

TABLE III. Biological Activities of the Synthetic Analogs on Isolated Guinea-Pig Ileum and Rat Duodenum

Analog	Guinea-pig ileum				Rat duodenum			
	RA[a]	pA_2/NKB	pA_2/SP	pA_2/NKA	RA	pA_2/NKB	pA_2/SP	pA_2/NKA
1	0[b]	5.82[c]	—	—	0	—	—	—
2	0[b]	—[c]	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
3	0[c]	5.69[c]	—	—	0	—	—	—
4	0	5.11	—	—	0	—	—	—
5	0	6.30	—	—	0	—	—	—
6	0	5.61	4.32	—	0	—	—	—
7	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
8	<0.01	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
9	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
10	<0.01	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
11	0	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
12	0	6.70	5.94	5.85	0	5.70	—	—

a) Relative activity based on NKB as 1. *b*) Data from ref 4). *c*) Data from ref 5). —, no antagonistic activity. N.T., not tested.

for the α-amino function. The side-chain protective groups used were cyclohexyl for Asp and *p*-toluenesulfonyl for Arg. The synthetic products were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) using 0.1% TFA in MeCN as the eluent. Highly purified peptides (Tables I and II) were obtained after gel filtration on a Sephadex LH-20 column. Homogeneity of the peptides was demonstrated by analytical HPLC and thin-layer chromatography (TLC). When a single peak and a single spot were observed for a peptide in all chromatographic systems, the peptide was considered to be sufficiently pure for bioassay. The chromatographic data were supported by the amino acid analytical data.

The biological properties of the synthetic peptides were examined on isolated guinea-pig ileum and rat duodenum. The agonistic effects of analogs **4**—**12** were compared with that of our synthetic NKB in the ileum assay. Analogs **4**—**12** were tested for their antagonistic effects against NKB, and analogs **1**, **3**—**6**, and **12** were examined against SP and NKA in the ileum assay. The agonistic and antagonistic activities of analogs **1**, **3**—**6**, and **12** were further assayed in the rat duodenum preparation. The antagonistic activities of the analogs were evaluated in terms of pA_2 (negative concentration of antagonist that reduces the contractile response to a double dose of agonist in the presence of the antagonist to that obtained with a

single dose without the antagonist), according to Schild.[10] In the presence of analogs at concentrations of 8×10^{-6} M for SP and 2×10^{-6} M for NKB and NKA, the maximal responses induced by NKB, SP and NKA were almost the same as those without analogs. The results are presented in Table III.

Analogs **4**—**12**, and **1**, **3**—**6**, and **12** showed no agonistic activities in the ileum and duodenum assays, respectively. Though NKB at the concentration of 10^{-7} M induced maximal response in both assays, the analogs did not induce a contractile response when applied at up to 10^{-6} M. Analogs **4**—**6** and **12** were found to act as antagonists of NKB, while analogs **7**—**11** exerted no antagonistic effect against NKB in the ileum assay. Analog **12** possessed the highest antagonistic activity against NKB among analogs **1**, **3**—**6**, and **12**. The six NKB antagonists were tested for their antagonistic effects against SP and NKA. Analog **6** was a weak antagonist against SP, but not against NKA. The most potent antagonist of NKB, analog **12**, also showed fairly potent antagonism to SP and NKA. None of analogs **1** and **3**—**5** was found to act as an antagonist of SP of NKA. The analogs **1**, **3**—**6**, and **12**, which possessed antagonistic activities in the ileum assay, were tested for their agonistic activities in the duodenum assay. Analogs **1** and **3**—**6** were found to exert no antagonistic effect against NKB, SP, or NKA, while analog **12**, a nonspecific antagonist in the ileum assay, showed antagonism to NKB, but not to SP or NKA. The data indicate that analogs **12**, and **1**, **3**—**5** are specific and fairly potent antagonists against NKB in the duodenum and ileum assays, respectively, under the conditions of this investigation. The results show that a double substitution in positions 6 and 8 of NKB did not increase the activity of the octapeptide analog as an antagonist. A triple substitution in positions 6, 7, and 8 appears to be unfavorable for a specific NKB antagonist.

Experimental

Amino acid derivatives were purchased from the Peptide Institute, Inc., Osaka, Japan, and benzhydrylamine (BHA) resin (available amine of the resin: 0.6 mmol/g of support) from Beckman Inc., Palo Alto, Calif. Optical rotations were measured in a Nippon Bunkoh DIP-4 polarimeter. Amino acid analyses on samples previously hydrolyzed with 6 N HCl (110 °C, 24 h) or on analogs **5**—**7** and **9**—**12** previously hydrolyzed with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (115 °C, 24 h) were performed on a Beckman System 7300 amino acid analyzer. TLC was carried out on silica-gel plates (Merck). The following solvent systems were used, and allowed to ascend for 10 cm: Rf^1, *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper phase); Rf^2, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24). Analytical HPLC was done with the following systems: column, Chemcosorb ODS (Chemco, 3.9 × 300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient for 15 min from 14% to 35% MeCN in 20 mM phosphate buffer (pH 3.0).

General Procedure for the Preparation of Analogs——The solid-phase synthesis was carried out using a Beckman System 990C peptide synthesizer as described previously.[4] BHA-resin hydrochloride (0.5 g for each analog) served as the solid support. A 2.5 fold excess of the amino acid derivative was used for all couplings. The coupling was effected with dicyclohexylcarbodiimide-1-hydroxybenzotriazole. The Boc group on Trp-peptide resin was deblocked by the use of 25% TFA in dichloromethane containing 5% 1,2-ethanedithiol. The protected peptide resin was treated with anhydrous liquid HF[11] containing 10% anisole. After evaporation of the HF *in vacuo* under ice-cooling, the residue was washed with AcOEt and the peptide was extracted with 50% AcOH.

Purification of the Peptides——The crude peptide was subjected to HPLC as reported previously.[4] The apparatus was composed of a model 590 pump and a U6K injector (Waters) connected to a column of Chemcosorb ODS (Chemco, 20 × 300 mm) or μ-Bondasphere C_{18} (Waters, 19 × 150 mm). The eluates were monitored with a UV detector (S-310A model-II, Soma) at 210 nm. The MeCN–0.1% TFA solvent system was used for elution at a flow rate of 10 or 7 ml/min. Each peptide emerged at 40—60 min on isocratic elution with 21—32% MeCN in the solvent system. The desired fraction was passed through a Sephadex LH-20 column (16 × 500 mm) eluted with aqueous DMF (90%) containing 0.02% 1,2-ethanedithiol. Homogeneity of the peptides was checked by analytical HPLC and TLC.

Bioassay——The agonistic activity of the synthetic analogs was measured on isolated guinea-pig ileum and rat duodenum, as described before.[4] The contraction was recorded by means of an isotonic transducer (Nippon Kohden, TD-111T) with a load of 2 g on a Servocorder (Watanabe Instruments, SR6204). Concentration–response curves were obtained by using a cumulative dose assay, and the time between measurements of two consecutive dose–response curves was longer than 10 min. Though NKB at the concentration of 10^{-7} M induced maximal response, all

the analogs were applied up to 10^{-6} M. In the tests for antagonistic activity in the guinea-pig ileum and rat duodenum systems, the analog was added 10 min before NKB, SP or NKA. The cumulative dose–response curve of NKB, SP or NKA in the presence or absence of the analog was obtained. In the presence of analogs at concentrations of 8×10^{-6} M for SP and 2×10^{-6} M for NKB and NKA, the maximal response induced by NKB, SP and NKA was almost the same as the response in the absence of the analogs.

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2) Amino acids, peptides and their derivatives mentioned in this paper are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature given in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations used: DMF, dimethylformamide; TFA, trifluoroacetic acid; AcOH, acetic acid; BuOH, butyl alcohol; MeCN, acetonitrile; AcOEt, ethyl acetate.
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Studies on Peptides. CLII.[1,2] Hard Acid Deprotecting Procedure for Peptide Synthesis

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The use of trimethylsilyl trifluoromethanesulfonate as a peptide deprotecting reagent was examined, together with the role of soft bases employed. A new deprotecting procedure based on the hard acid principle is presented.

Keywords——new peptide deprotecting procedure; hard acid principle; trimethylsilyl trifluoromethanesulfonate; trimethylsilyl methanesulfonate; trimethylsilyl trifluoroacetate; hard–soft acid–base concept; thioanisole-mediated deprotection; ^{29}Si-NMR chemical shift; human glucose-dependent insulinotropic polypeptide synthesis

The use of silyl compounds, such as Me_3SiI[3] and recently $MeSiCl_3$ or $SiCl_4$,[4] as peptide deblocking reagents has been examined. However, somewhat elevated temperature or prolonged treatment was required for complete removal of the Bzl group and even the Z group. We found that trimethylsilyl trifluoromethanesulfonate (TMSOTf), a powerful trimethylsilylating reagent,[5] which can be classified as a super hard acid,[6] has an ability to cleave, not only the Boc group,[7] but also readily various other protecting groups currently employed in peptide synthesis, including the Z and Bzl groups. The resulting trimethylsilylated compounds can be hydrolyzed easily with water or ammonium fluoride[8] to afford free peptides. The properties of several trimethylsilyl compounds were examined, together with the role of soft bases[9] employed in this hard acid deprotecting reaction.

Various benzyl-type (N^{ε}-Z, O-Bzl, O-Cl_2-Bzl[10]) and phenylsulfonyl-type (N^{G}-Mts,[11] N^{G}-MBS,[12] and N^{in}-Mts[13]) protecting groups, which are stable to TFA, were cleaved by treatment with 1 M TMSOTf/TFA in an ice-bath within 30 min. As a cation scavenger, thioanisole[14] (at a concentration of 1 M) was employed and amino acids regenerated were determined on an amino acid analyzer as shown in Table I. Several S-protecting groups of cysteine (MBzl,[15] Ad,[16] and *tert*-Bu[17]) were found to be cleaved similarly. The other protecting groups, N^{im}-Tos,[18] N^{im}-Bom,[19] and O-Chp,[20] were also cleaved smoothly from His, Trp and Asp, respectively, under the above conditions. Cys(Bzl)[21] and Cys(Acm)[22] resisted the action of this hard acid. Complete removal of two N^{G}-protecting groups of Arg, Tos[23] and NO_2,[24] could not be achieved under the above conditions. However, recovery of Arg from Arg(Tos) reached 86% after a 60 min treatment. From these data, the rate of this cleaving reaction was judged to be much faster than that of 1 M TFMSA/TFA.[25]

Thioanisole is a good scavenger to suppress side reactions in TFMSA/TFA deprotection, *i.e.*, Lys(Bzl) formation[14] and 3-Bzl-Tyr formation[26] and is known to play a role as a soft base[9,14] to accelerate the cleaving reaction.[27] This thioether was judged to be still a better soft base than diphenylsulfide (PhSPh)[28] and dimethylsulfide (MeSMe)[29] in the present

TABLE I. Removal of Various Protecting Groups by 1 M TMSOTf/TFA in the Presence of a Soft Base

Treated amino acid derivative (0 °C, 30 min)	Parent amino acid regenerated			
	Soft base added			
	None	Thioanisole	MeSMe	PhSPh
Z(OMe)-Lys(Z)-OH	48.4	98.7	87.0	73.1
Z(OMe)-Ser(Bzl)-OH	84.0	91.7	90.2	97.8
Boc-Thr(Bzl)-OH	91.8	98.0	94.6	100.0
Z(OMe)-Glu(OBzl)-OH	92.2	99.3	97.3	100.0
Z(OMe)-Asp(OBzl)-OH	100.0	99.2	97.7	100.0
Boc-Asp(OChp)-OH	91.4	100.0		100.0
Boc-Tyr(Bzl)-OH	63.3	100.0		87.5
Boc-Tyr(Cl_2-Bzl)-OH	81.5	100.0	88.3	86.6
Boc-His(Tos)-OH	88.5	94.5	96.3	100.0
Boc-His(Bom)-OH	88.5	88.9	50.5	90.2
Boc-Trp(Mts)-OH	74.7	100.0	25.6	100.0
Boc-Trp(For)-OH[a]		100.0		
Z(OMe)-Arg(Mts)-OH	93.3	97.6	67.2	100.0
Z(OMe)-Arg(MBS)-OH	80.6	93.5		84.3
Z-Arg(Tos)-OH	3.4	62.1	0	8.3
Z-Arg(NO_2)-OH		11.0		10.8
H-Cys(MBzl)-OH	100.0	100.0		100.0
Boc-Cys(*tert*-Bu)-OH	≒0	87.3	32.9	97.1
Z(OMe)-Cys(Ad)-OH	91.7	100.0	11.0	100.0
Boc-Cys(Acm)-OH	0	0		
H-Cys(Bzl)-OH	0	0		
Z(OMe)-Met(O)-OH	0	27.6	4.0	≒0
Boc-Ile-PAM-resin[b]	15.4	81.9	12.4	23.8

a) In the presence of ethanedithiol. *b*) 60 min treatment.

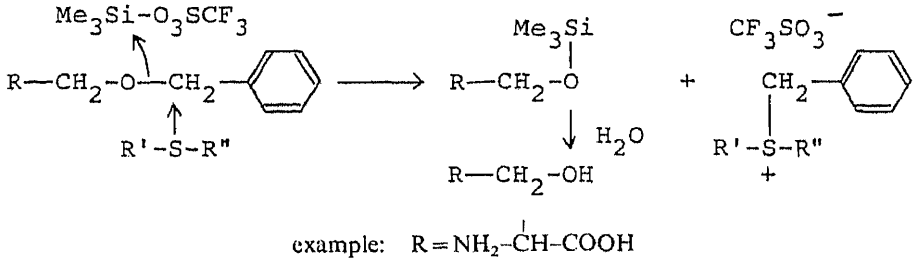


Fig. 1. Possible Mechanism of TMSOTf–Soft Base/TFA Deprotection

deprotecting reaction, and a possible mechanism of its action is shown in Fig. 1. When PhSPh (at a concentration of 0.5 M, due to its lower solubility in TFA) was employed, the recoveries of Lys and Tyr were somewhat low. MeSMe (at a concentration of 1 M) was not effective enough to cleave the Mts and MBS groups (Table I). The results suggested the existence of a subtle relationship between soft acids (protecting groups) and soft bases. Reduction of Met(O)[30] by 1 M TMSOTf/TFA treatment with the aid of these soft bases was unsuccessful. Trp(For)[31] was deprotected by this TMSOTf/TFA treatment in the presence of ethanedithiol, presumably through a thioacetal intermediate.[29b]

It seems noteworthy that Ile, possessing a bulky side chain, could be cleaved from Boc–Ile–O–CH_2–PAM-resin[32] by treatment with 1 M TMSOTf–thioanisole/TFA in fairly good

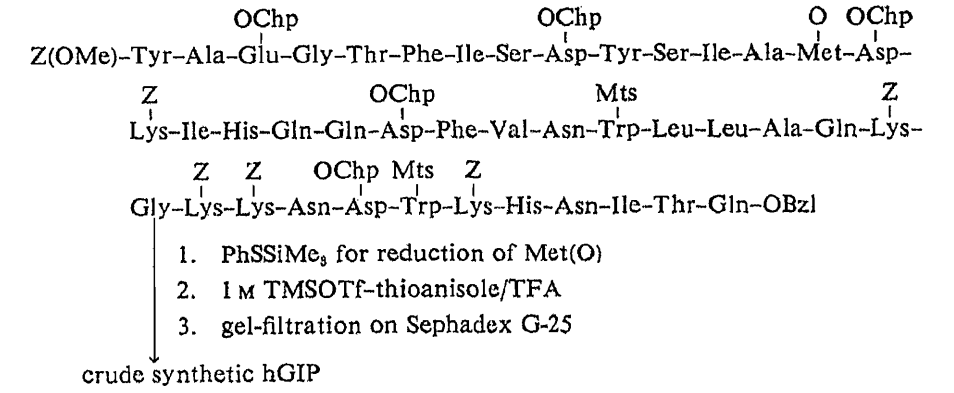
yield. The result seems to demonstrate the usefulness of this deprotecting procedure for solid-phase peptide synthesis.

When the ^{29}Si-NMR signal[33] of three trimethylsilyl compounds in TFA were measured, using Me_4Si as an internal standard, the signal of TMSOTf appeared at the lowest magnetic field, compared with those of trimethylsilyl methanesulfonate (TMSOMs) and trimethylsilyl trifluoroacetate (TMSOTa). These values seem to correlate well with cleaving efficiencies of these trimethylsilyl compounds tested as sources of a hard acid. For example, the protecting groups of four compounds, Ser(Bzl), Asp(OBzl), Lys(Z) and Arg(Mts), were cleaved by TMSOMs by increasing the concentration and elevating the temperature (22 °C), but complete

TABLE II. Removal of Protecting Groups by Trimethylsilyl Compounds in the Presence of Thioanisole

Hard acid (22 °C, 60 min)	^{29}Si ppm[a]	Ser(Bzl)	Lys(Z)	Arg(Mts)	Asp(OBzl)
1 M TMSOTf/TFA[b]	46.1[c]	91.7	98.7	97.6	99.2
2 M TMSOMs/TFA	37.9	93.5	92.4	80.0	99.5
3 M TMSOTa/TFA	35.6	7.1	16.9	0	1.6

a) Me_4Si was used as an internal standard. *b*) Data taken from Table I (conducted at 0 °C for 30 min). *c*) Lit.[33] 44.6 ppm in benzene.



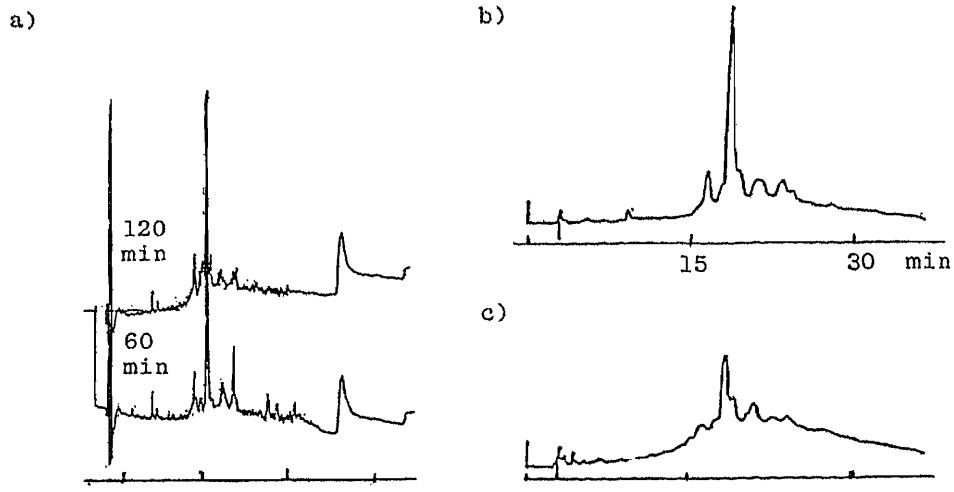


Fig. 2. HPLC of Crude Synthetic hGIP

a) TMSOTf-treated sample. b) TMSOTf-gel-filtered sample. c) TFMSA-gel-filtered sample.

removal of these protecting groups by treatment with 3 M TMSOTa–thioanisole/TFA was unsuccessful, as shown in Table II. Thus, of the hard acids tested, TMSOTf was judged to be the most attractive reagent for deprotection.

Next, we wished to demonstrate the usefulness of this new deprotecting procedure in practical peptide syntheses. As an example, protected human GIP (glucose-dependent insulinotropic polypeptide)[34] was treated with 1 M TMSOTf–thioanisole/TFA and periodically a part of the solution was examined by high-performance liquid chromatography (HPLC). As shown in Fig. 2a, the desired peak increased, with a decrease of other side peaks. After gel-filtration on Sephadex G-25, the crude deprotected peptide obtained at this stage exhibited much higher purity than the product obtained by 1 M TFMSA–thioanisole/TFA deprotection, when compared by HPLC (Fig. 2b, c). After subsequent HPLC purification, a 42-residue peptide corresponding to the entire amino acid sequence of hGIP[35] was obtained in a better yield (28%) than before (10%).

We have described here a new deprotecting procedure by the use of a hard acid, which differs from the Brönsted acid deprotecting procedures currently employed in peptide synthesis. This procedure, which seems to have several attractive features, may gain wide applicability in solution-phase peptide synthesis, as well as in solid-phase peptide synthesis, particularly for large-scale preparation.

Experimental

Amino acid analysis and HPLC were conducted with a Hitachi 835-02 model and a Waters 204 compact model, respectively. Rotation and ultraviolet absorption (UV) were determined with a Union PM-101 model and a Hitachi 100-20 model, respectively. *Rf* values in thin layer chromatography (TLC) conducted on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2) and Rf_2 *n*-BuOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1). Leucine-aminopeptidase was purchased from Sigma Chem. Co. (Lot. No. L-6007).

Treatment of Amino Acid Derivatives with Trimethylsilyl Compounds——(a) A mixture of an amino acid derivative (0.1 mmol each) and Gly (internal standard) was treated with 1 M TMSOTf/TFA in the presence or the absence of a soft base in an ice-bath for 30 min. As a soft base, three thioether compounds were tested: thioanisole (at a concentration of 1 M), MeSMe (at a concentration of 1 M) and PhSPh (at a concentration of 0.5 M; this concentration was judged to be practical, since PhSPh is not freely soluble in TFA). A part of the solution was subjected to quantitative amino acid analysis and the results are listed in Table I. (b) To examine the cleaving ability of other trimethylsilyl compounds, four derivatives, Ser(Bzl), Lys(Z), Arg(Mts) and Asp(OBzl), were selected. A mixture of each derivative (0.8 mmol) and Gly (internal standard) was treated with 2 M TMSOMs/TFA or 3 M TMSOTa/TFA in the presence of thioanisole (at a concentration of 1 M) at room temperature (22 °C) for 60 min. An aliquot was examined on an amino acid analyzer and the results are listed in Table II, together with the ^{29}Si chemical shift values.

Cleavage of Ile from Boc–Ile–O–CH_2–PAM-resin——TMSOTf was added at a concentration of 1 M to a suspension of Boc–Ile–O–CH_2–PAM-resin (25 mg, Ile content, 0.80 mmol/g) and Gly (internal standard) in 1 M thioanisole/TFA, and the mixture was stirred in an ice-bath for 60 min, then a part of the solution was subjected to quantitative amino acid analysis. The results are shown in Table I.

Deprotection of Protected GIP by Treatment with 1 M TMSOTf–Thioanisole/TFA——Protected GIP (50 mg, 74 μmol) was treated with 1 M TMSOTf–thioanisole/TFA (5 ml; the amount of TMSOTf was 45 eq/protecting group) in an ice-bath for 120 min to ensure complete deprotection. *m*-Cresol (15 μl, 20 eq) and ethanedithiol (12 μl, 20 eq) were added as additional scavengers to protect the Tyr and Trp residues. Periodically, an aliquot was examined by HPLC. The elution patterns obtained after 60 min and 120 min treatments are shown in Fig. 2a. Dry ether was added and the resulting powder was collected by centrifugation and washed with fresh ether. The deprotected peptide was dissolved in H_2O (*ca.* 5 ml) and the solution was adjusted to pH 8.0 with Et_3N. After addition of mercaptoethanol (200 μl) and 1 M NH_4F (400 μl), the solution was stirred in an ice-bath for 30 min, then the pH was adjusted to 5 with 1 N AcOH. Next, the solution was applied to a column of Sephadex G-25 (3.3 × 108 cm), which was eluted with 1 N AcOH. The desired fractions (8 ml each, tube Nos. 40—52; monitored by UV absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 35 mg (96%). Its HPLC elution pattern is shown in Fig. 2b in comparison with that of the gel-filtered sample obtained by 1 M TFMSA–thioanisole/TFA treatment.

The gel-filtered sample was next applied to a column of CM-Trisacryl M (2.0 × 5.0 cm), which was eluted first with pH 6.46, 0.01 M $AcONH_4$ buffer (120 ml), then with a gradient formed from 0.2 M NaCl in 0.01 M $AcONH_4$

(250 ml) through a mixing flask containing the starting buffer (250 ml). The desired fractions (5.8 ml each, tube Nos. 25—35; monitored by UV absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization. The residue was desalted by gel-filtration on Sephadex G-25 using 1 N AcOH under the conditions stated above. Lyophilization of the desired fraction gave a fluffy white powder; yield 13.6 mg (37%).

The product (6.8 mg) was next purified by HPLC using a Nucleosil 5C18 column (4.0 × 150 mm) with a gradient of MeCN (30—35%) in 0.1% TFA aq. The desired eluates (retention time, 20 min) were collected and the residue was lyophilized to give a fluffy white powder. The rest of the sample was similarly purified; yield 5.1 mg (28% from the protected GIP), Rf_1 0.26, Rf_2, 0.11. $[\alpha]_D^{18}$ −43.2° (c = 0.3, 1 N AcOH, lit.[34] −39.7° in 0.1 N AcOH). Amino acid ratios in a leucine-aminopeptidase digest (numbers in parentheses are theoretical values): Asp 3.68(4), Ser 1.75(2), Glu 1.09 (1), Gly 2.19(2), Ala 3.21(3), Val 1.17(1), Met 0.98(1), Ile 3.98(4), Leu 2.00(2), Tyr 1.95(2), Phe 2.19(2), Lys 4.76(5), His 1.98(2), Trp 2.11(2) (recovery of Leu 78%; Thr, Gln and Asn were not determined).

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An Efficient and Practical Synthesis of Bicyclo[3.3.1]nonane-2,4-diones[1)]

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Cyclohexane-1,3-dicarboxylic anhydrides (IVa—c), prepared from isophthalic acids in several steps, were treated with diethyl magnesiomalonate and triethylamine to give 3-di(ethoxycarbonyl)-acetylcyclohexanecarboxylic acids (Va—c) in good yields. Compounds Va—c were converted into methyl 3-acetylcyclohexanecarboxylates (VIa—c) by decarboxylation and esterification. Cyclization of VIa—c to bicyclo[3.3.1]nonane-2,4-diones (I) was performed by refluxing a mixture of VI and potassium hydride in xylene. In the case of VIb, two products (Id and Ie) were obtained. Compounds Ic and Ie were hydrolyzed to Ib and Ig, respectively, by treatment with *p*-toluenesulfonic acid in acetone and phosphorus tribromide. The 7α-hydroxy β-diketone (If) was obtained from Ib-triketal in three steps *via* reduction of Ib-2,4-diketal with lithium aluminum hydride. The keto-enol equilibrium of these β-diketones (Ia—f) and 9-substituted bicyclo[3.3.1]-nonane-2,4-diones (Xa—b) in deuteriochloroform is also described.

Keywords——bicyclo[3.3.1]nonane; β-diketone; bicyclo[3.3.1]nonane-2,4-dione; cyclization; hydrolysis; keto-enol equilibrium; 3-acetylcyclohexanecarboxylic acid

We have previously examined the chemical properties of derivatives of β-diketones,[2)] and bicyclo[3.3.1]nonane compounds.[3)] In the present work, we were interested in the reactivity of derivatives of bicyclo[3.3.1]nonane-2,4-dione (Ia). Musso *et al.*[4)] and Blechert *et al.*[5)] reported the synthesis of bicyclo[3.3.1]nonane-2,4,7-trione (Ib) and its derivatives as synthons for natural products. However, the synthetic method seems to be impractical in the former case, and insufficient experimental detail was given in the latter case. Recently we examined the scope and limitations of the synthetic method for β-diketone using dicarboxylic acid, acyl chloride, and aluminum chloride in nitromethane and showed that the method could also be applied to the synthesis of bicyclo[3.3.1]nonane-2,4-dione bearing an alkyl or acyl group at the C_3-position.[6)] We also developed a general and practical synthetic method for the system having no substituent at the C_3-position. The synthetic pathway adopted for Ia, Ib, Ib-7-ethyleneketal (Ic), 7α-methoxybicyclo[3.3.1]nonane-2,4-dione (Id), and 7β-methoxybicyclo[3.3.1]nonane-2,4-dione (Ie) is outlined in Chart 1.

Isophthalic acid (IIa) was led to 5-hydroxyisophthalic acid (IIb) *via* its 5-sulfonic acid and subsequently to 5-methoxyisophthalic acid (IIc) by treatment with dimethyl sulfate. *cis*-1,3-Cyclohexanedicarboxylic acid (IIIa) and its 5-methoxy analogue (IIIb) were prepared by catalytic reduction of IIa and IIc, respectively, over rhodium-alumina at a medium pressure.[4,7)] The nuclear magnetic resonance (NMR) spectra indicated that all these substituents in compound III were equatorial. The ethyleneketal of 5-oxo-*cis*-cyclohexanedicarboxylic acid (IIIc) was prepared in five steps from IIb.[4)] Anhydrides (IVa—c) were synthesized from the corresponding diacid (IIIa—c) by using dicyclohexylcarbodiimide (DCC) in methylene chloride or by using acetic anhydride. Compound IVb exhibited a quintet

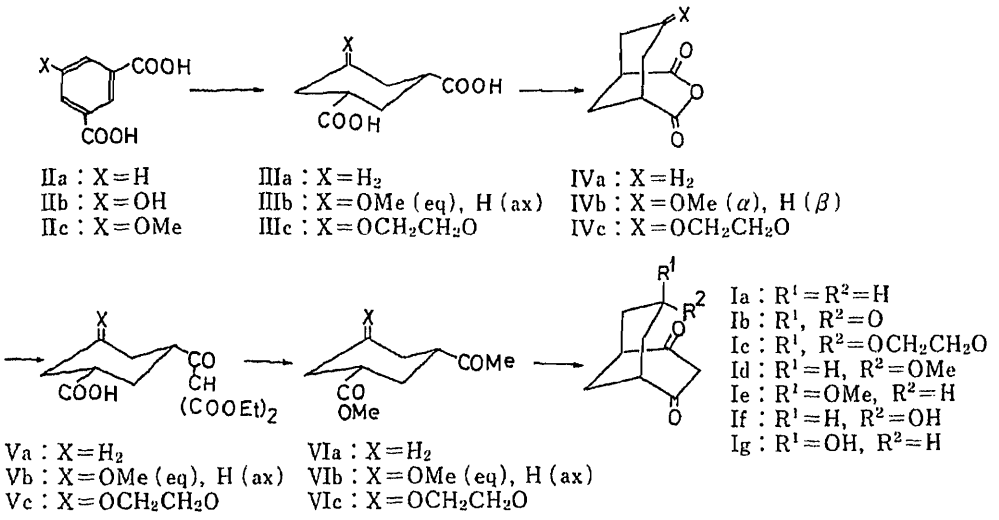

Chart 1

signal (J=3.0 Hz) at δ3.60 due to the proton at the carbon bearing the methoxy group. Blechert and coworkers[5] used methyl lithium in order to convert the anhydride (IV) to the 3-acetylcyclohexanecarboxylic acid. However, this method is difficult to scale and is not economical. We adopted in this step the method used for the synthesis of methyl ketones from acyl chlorides; treatment of the anhydride (IV) with diethyl magnesiomalonate and triethylamine in acetonitrile at room temperature.[8] Thus, 3-di(ethoxycarbonyl)acetyl-5-cyclohexanecarboxylic acid (Va), the 5-methoxy analogue (Vb), and an ethyleneketal of the 5-oxo analogue (Vc) were prepared in 89, 94, and 74% yields from IVa, IVb, and IVc, respectively. Compounds Va and Vb were esterified by using methanol in the presence of a catalytic amount of *p*-toluenesulfonic acid (*p*-TsOH), and then subjected to decarboxylation at the malonate moiety with sodium chloride in aqueous dimethyl sulfoxide (DMSO) at 160—170 °C to give methyl 3-acetylcyclohexanecarboxylate (VIa) and the 5-methoxy analogue (VIb) in overall yields of 90 and 73%, respectively. It was suggested from the NMR spectrum that VIb was a single product; it exhibited three singlet signals at δ2.21, 3.40, and 3.70 due to the methyl protons of one acetyl and two methoxy groups, respectively. Methyl 3-acetyl-5,5-ethylenedioxycyclohexanecarboxylate (VIc) was obtained in 82% yield from Vc by decarboxylation at the malonate moiety followed by esterification with diazomethane. The cyclization reaction of aceto esters (VI) to β-diketones (I) was carried out using potassium hydride (KH) with monitoring of the reaction mixture by means of gas chromatography (GC). Both compounds Ia and Ic were obtained in 87% yield by refluxing the mixture of KH and VIa or VIc in xylene for a relatively long time. Recently Ia was synthesized by Kojima's group[9] *via* a unique route, but the melting point reported was remarkably different from ours. When tetralin was used as a solvent in the reaction of VIc, the reaction time could be shortened but the yield fell to 76%. The cyclization reaction of VIb gave a mixture of two products, Id and Ie, in the ratio of *ca.* 3:1 in 83% yield. The determination of the configuration of the methoxy groups at C_7 was performed by comparing the coupling patterns of the C_7-proton in the NMR spectra of Id and Ie; a broad singlet [half-band width ($W_{1/2}$)=8.1 Hz] in Id and a triplet of triplets (J=9.5 and 4.8 Hz) in Ie. Compound Ib was prepared in 84% yield from Ic by treatment with a catalytic amount of *p*-TsOH in acetone at room temperature. 1-Hydroxy-6-oxobicyclo[3.2.1]nonane-3β-carboxylic acid (VIIa) was obtained when Ic was treated with 10% HCl at reflux temperature for 4 h. A possible mechanism for the formation of VIIa from Ic is shown in Chart 2.

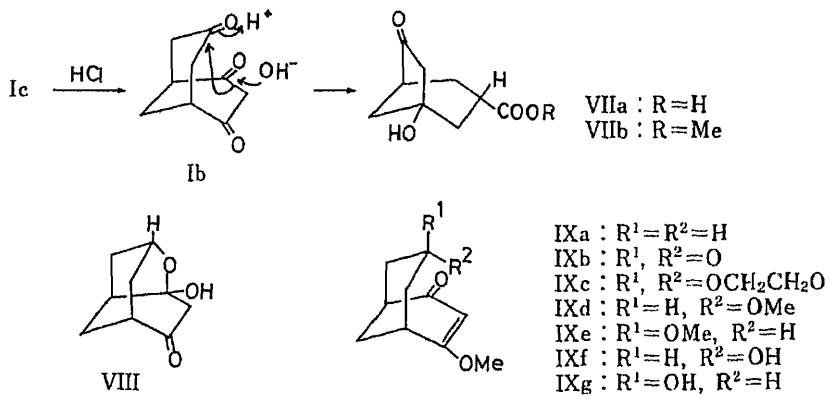

Chart 2

Although compound Id was treated with phosphorus tribromide in methylene chloride to give an inseparable mixture of products, its isomer (Ie) afforded 7α-hydroxybicyclo-[3.3.1]nonane-2,4-dione (Ig) under similar conditions. 7α-Hydroxybicyclo[3.3.1]nonane-2,4-dione (If), which exists in equilibrium with the intramolecular hemiketal (VIII) in the ratio of *ca.* 2:1, was obtained *via* lithium aluminum hydride (LAH) reduction of Ib-2,4-di(ethyleneketal), which was prepared by partial hydrolysis of Ib-2,4,7-tri(ethyleneketal) with *p*-TsOH in acetone–benzene. Compounds Ia—g were treated with an excess of diazomethane to give quantitatively the corresponding vinylogous esters (IXa—g). Compound VIII could also be converted to IXf with the same reagent. A remarkable difference was observed in the mass spectra (MS) of IXd and IXe; compound IXd exhibited a weak molecular ion peak and the base peak at *m/z* 111 due to a protonated resorcinol, whereas IXe showed the base peak at *m/z* 124 due to resorcinol monomethyl ether. Similar differences were also found in the spectra of IXf and IXg.

Finally the ratio of keto and enol forms in these β-diketones (I) was investigated by analysis of the NMR spectra measured in deuteriochloroform ($CDCl_3$). The NMR spectrum of Ig could not be measured because of its low solubility in this solvent.

The results, shown in Chart 3, may be summarized as follows.

(1) The substituents at C_7 show a similar effect on the equilibrium to those at C_9 (Ib—Xa and Ic—Xb).

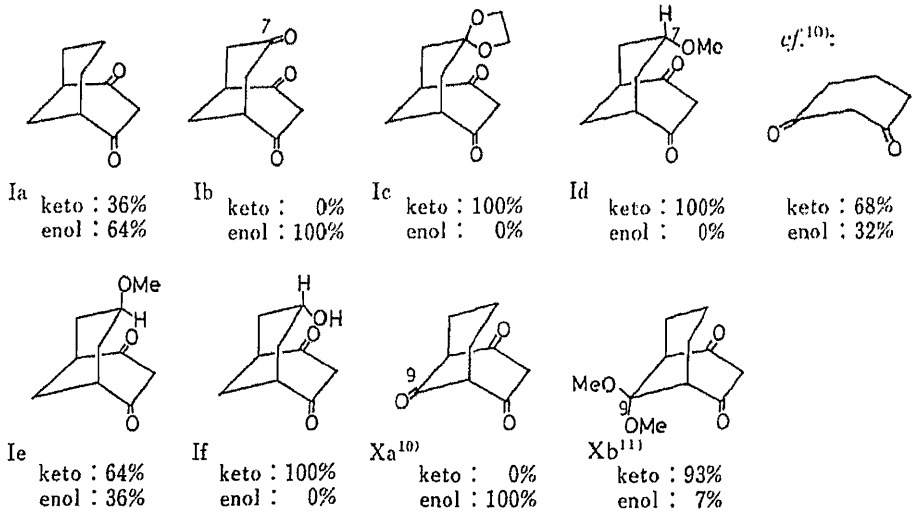

Chart 3. Keto–Enol Equilibrium in Bicyclo[3.3.1]nonane-2,4-dione in $CDCl_3$

(2) Ketalization dramatically changes the ratio (Ib—Ic and Xa—Xb).

(3) A carbonyl group at C_7 favors the enol form (Ib), while others favor the keto form (Ic—f).

(4) The *exo*-substituted compound behaves similarly to the *endo*-substituted one (Id and Ie).

These phenomena may be partially explained in terms of the streric repulsion between the substituents at the C_7-position and the plane of the enol moiety (Ic—f) or the overlap of their π-electrons (Ib). However, we can not explain the results in the X-series.

Experimental

All melting points taken on a Kofler block and the boiling points are uncorrected. Infrared (IR) spectra were determined by using a JASCO IR-A1 diffraction grating spectrophotometer; absorption data are given in cm^{-1}. NMR spectra were recorded in $CDCl_3$ on a JEOL PMX-60, XL-200, or FX-270 spectrometer with tetramethylsilane (TMS) as an internal standard. The chemical shifts and coupling constants (J) are given in δ and Hz, respectively. GC was carried out on a Shimadzu GC-6AM instrument with a stainless steel column (3 m/m × 3 m) packed with 5% SE-30. The N_2 gas flow was 40 ml/min. The MS were measured with a JEOL D-200 (70 eV, direct inlet system). All solvents were removed under reduced pressure after drying of the solution over anhydrous $MgSO_4$.

3-Oxabicyclo[3.3.1]nonane-2,4-dione (IVa)——A solution of DCC (6.36 g, 30.8 mmol) in CH_2Cl_2 (50 ml) was added dropwise to a suspension of 1,3-cyclohexanedicarboxylic acid[7] (5.30 g, 30.8 mmol) in CH_2Cl_2 (30 ml), and the mixture was stirred at room temperature for 2 h. The precipitated dicyclohexylurea was filtered off and washed several times with cold CH_2Cl_2. The combined organic layer was concentrated to give a white solid, which was recrystallized from benzene–petroleum ether to give IVa: mp 138—140 °C (colorless plates). Yield, 4.40 g (92.6%). NMR (60 MHz): 1.5—2.3 (8H, m), 3.08 (2H, brs, $W_{1/2}$=8.2, C_1- and C_5-H).

Methyl 3-Acetylcyclohexanecarboxylate (VIa)——Anhydrous Et_3N (2.47 ml, 35.6 mmol) was added during 15 min to a suspension of $MgCl_2$ (1.69 g, 17.8 mmol) and diethyl malonate (2.70 ml, 17.8 mmol) in CH_3CN (30 ml) at 0 °C with stirring. Stirring was continued for 15 min at 0 °C, then a solution of IVa (2.74 g, 17.8 mmol) in CH_3CN (20 ml) was added to the mixture at 0 °C in 30 min. The whole mixture was stirred for a further 4 h at room temperature and then acidified with 10% HCl. The aqueous layer was extracted with Et_2O (40 ml × 3), and the combined organic layer was washed with 10% HCl (20 ml × 1) and brine (20 ml × 2), and then concentrated to give a colorless viscous oil. 3-Di(ethoxycarbonyl)acetylcyclohexanecarboxylic acid (Va): IR (film): ν_{OH} 3600—2400, $\nu_{C=O}$ 1720, 1640, 1600, δ_{C-O} 1260. NMR (60 MHz): 1.07—2.90 (10H, m), 1.28 (6H, t, J=7, CH_3CH_2), 4.27 (4H, q, J=7, OCH_2), 4.65 (0.6H, s, $COCH_2$), 11.13 (1H, s, OH), 13.47 (0.4H, s, C(OH)=C<). A methanolic solution (150 ml) of the crude Va and *p*-TsOH (0.3 g) was refluxed for 3 h. The residue obtained after concentration of the methanolic mixture was dissolved in benzene (150 ml) and then the benzene layer was washed with H_2O (40 ml × 1) and brine (40 ml × 2). Removal of the solvent gave the methyl ester of Va. A mixture of the crude triester, DMSO (20 ml), NaCl (1.0 g, 17.8 mmol), and H_2O (0.6 g, 35.6 mmol) was warmed at 160—170 °C for 6 h with stirring. The reaction mixture was poured into ice-water (200 ml). The Et_2O extract (30 ml × 4) was washed with brine (20 ml × 2) and then concentrated to give a pale yellow oil, bp 123—125 °C (5 mmHg). Yield, 2.61 g (80% from IVa). IR (film): $\nu_{C=O}$ 1730, 1710, δ_{C-O} 1250, 1205, 1160. NMR (60 MHz): 1.0—2.8 (10H, m), 2.17 (3H, s, Ac), 3.68 (3H, s, OMe).

Bicyclo[3.3.1]nonane-2,4-dione (Ia)——KH (20% w/w in oil, 5.7 g, 28 mmol) was added to a solution of VIa (2.61 g, 14.2 mmol) in xylene (30 ml). The mixture was refluxed for 28 h, and the resulting brown solution was poured into ice-water. The xylene layer was extracted with water. The combined aqueous layer was washed with Et_2O (20 ml × 2), acidified with conc. HCl under ice-cooling, and then extracted with AcOEt (30 ml × 4). The brown solid obtained from the extract after removal of the solvent was recrystallized from AcOEt. mp 153—155 °C (Lit.[9] mp 136—138 °C). The yield was 1.87 g (87%). NMR (270 MHz): 1.1—1.3 (0.4H, m), 1.4—1.9, 1.9—2.3, 2.3—2.5 (each m), 2.50 (1.2H, brs, $W_{1/2}$=10.8, C_1- and C_5-H of enol form), 3.72 (0.8H, brs, $W_{1/2}$=10, C_1- and C_5-H of keto form), 3.24, 3.60 (each 0.4H, d, J=19.4, C_3-H of keto form), 5.64 (0.6H, s, C_3-H of enol form). MS *m/z* (%): 152 (M^+, 65), 124 ($M^+ - CO$, 5), 110 (resorcinol, 22), 97 (18), 82 (49), 81 (40), 68 (49), 67 (100). *Anal.* Calcd for $C_9H_{12}O_2$: C, 71.02; H, 7.95. Found: C, 70.74; H, 8.02.

4-Methoxy-3-bicyclo[3.3.1]nonen-2-one (IXa)——An excess of CH_2N_2 in Et_2O was added to a methanolic solution of Ia and the mixture was allowed to stand overnight at room temperature. The residue obtained after removal of the solvent was recrystallized from hexane–AcOEt. mp 65—65.5 °C. IR (KBr): $\nu_{C=O}$ 1645, $\nu_{C=C}$ 1600. NMR (270 MHz): 1.4—1.8 (7H, m), 2.20 (1H, d, like, J=12.5), 2.46, 2.55 (each 1H, brs, $W_{1/2}$=13.5, C_1- and C_5-H, respectively), 3.71 (3H, s, OMe), 5.52 (1H, s, C_3-H). MS *m/z* (%): 166 (M^+, 51), 151 ($M^+ - Me$, 6), 138 ($M^+ - CO$, 7), 137 (7), 125 (50), 124 (resorcinol monomethyl ether, 22), 111 (resorcinol+1, 100). *Anal.* Calcd for $C_{10}H_{14}O_2$: C, 72.26; H, 8.49. Found: C, 72.44; H, 8.61.

7α-Methoxy-3-oxabicyclo[3.3.1]nonane-2,4-dione(IVb)——5-Methoxy-1,3-cyclohexanedicarboxylic acid(IIIb)[12]

was prepared from 5-hydroxyisophthalic acid (IIb)[7] by methylation with Me_2SO_4 followed by catalytic reduction using Rh–Al_2O_3 in MeOH–AcOH at a medium pressure in the overall yield of 70.6%. IIIb was converted ino the anhydride (IVb) by refluxing in acetic anhydride overnight in 91% yield (Lit.[4] 58%).

Methyl 3-Acetyl-5-methoxycyclohexanecarboxylate (VIb)——Under conditions similar to those used in the preparation of VIa, VIb was obtained in the yield of 69% from IVb *via* 3-di(ethoxycarbonyl)acetyl-5-methoxycyclohexanecarboxylic acid (Vb). Vb: IR (film): ν_{OH} 3600—2400 (br), $\nu_{C=O}$ 1725, 1645, 1600. NMR (60 MHz): 1.1—2.8 (m), 1.27 (6H, t, J=7, CH_3CH_2–), 3.29 (1H, brs, C_5-H), 3.30 (3H, s, OMe), 4.30 (4H, q, J=7, OCH_2), 4.68 (0.2H, s, $COCH_2$), 8.42 (1H, brs, –COOH), 13.33 (0.8H, brs, enol H). VIb: IR (film): $\nu_{C=O}$ 1735, 1710. NMR (60 MHz): 0.8—1.7 (4H, m), 1.9—2.8 (m), 2.21 (3H, s, Ac), 3.10 (1H, brs, C_5-H), 3.40 (3H, s, OMe), 3.70 (3H, s, –COOMe).

7α- and 7β-Methoxybicyclo[3.3.1]nonane-2,4-dione (Id and Ie)——Under conditions similar to those used in the preparation of Ia, a mixture of Id and Ie (*ca.* 3 : 1) was obtained in the yield of 83%. They were separated by SiO_2 column chromatography; Id and Ie were successively eluted with $CHCl_3$. Id: IR (film): ν_{OH} 3600—2400 (br), $\nu_{C=O}$ 1710 (br), 1650—1550 (br). NMR (270 MHz): 1.7—1.9 (2H, m), 1.9—2.0 (1H, m), 2.3—2.5 (3H, m), 2.65 (2H, brs, $W_{1/2}$=10.8, C_1- and C_5-H), 3.04 (3H, s, OMe), 3.27, 3.40 (each 1H, d, J=18.0, C_3-H), 3.47 (1H, brs, $W_{1/2}$=8.1, C_7-H). MS *m/z* (%): 182 (M^+, 48), 152 (M^+-CH_2O, 27), 108 ($M^+-MeOH-CH_2CO$, 40), 85 (52), 81 (76), 80 (dihydrobenzene, 100). Ie: mp 187—189 °C (from benzene–AcOEt). IR (KBr): ν_{OH} 3400—2200 (br), ν 1650—1450 (br). NMR (270 MHz): keto form, 2.89 (2H, brs, $W_{1/2}$=10.8, C_1- and C_5-H), 3.06 (1H, tt, J=9.5, 4.8, C_7-H), 3.26 (3H, s, OMe), 3.25, 3.61 (each 1H, d, J=19.4, C_3-H); enol form, 2.69 (2H, brs, $W_{1/2}$=10.0, C_1- and C_5-H), 3.30 (3H, s, OMe), 3.38 (1H, tt, J=11.0, 5.5, C_7-H), 5.49 (1H, s, C_3-H). MS *m/z* (%): 182 (M^+, 16), 73 [CH_3–CH(OMe)–CH_2^+, 100].

4,7α- and 4,7β-Dimethoxy-3-bicyclo[3.3.1]nonen-2-one (IXd and IXe)——Compounds IXd and IXe were obtained in the usual manner. IXd: bp<150 °C (1.5 mmHg). IR ($CHCl_3$): $\nu_{C=O}$ 1655, $\nu_{C=C}$ 1605. NMR (200 MHz): 1.6—1.9 (3H, m), 2.1—2.4 (3H, m), 2.47 (2H, brs, $W_{1/2}$=12, C_1- and C_5-H), 3.12 (3H, s, C_7-OMe), 3.57 (1H, brs, $W_{1/2}$=12, C_7-H), 3.73 (3H, s, C_4-OMe), 5.31 (1H, s, C_3-H). MS *m/z* (%): 196 (M^+, 12), 166 ($M^+-CH_2=O$, 34), 125 (12), 124 (13), 111 (resorcinol+1, 100). *Anal.* Calcd for $C_{11}H_{16}O_3\cdot 1/8\ H_2O$: C, 66.56; H, 8.25. Found: C, 66.59; H, 8.08. IXe: bp<150 °C (1.5 mmHg). IR ($CHCl_3$): $\nu_{C=O}$ 1640, $\nu_{C=C}$ 1600. NMR (200 MHz): 1.2—1.6 (1H, m), 1.6—1.8 (2H, m), 2.1—2.4 (3H, m), 2.69 (2H, brs, $W_{1/2}$=12.8, C_1- and C_5-H), 3.30 (3H, s, C_7-OMe), 3.34 (1H, tt, J=11.0, 5.5, C_7-H), 3.74 (3H, s, C_4-OMe), 5.42 (1H, s, C_3-H). MS *m/z* (%): 196 (M^+, 5), 166 (0.3), 125 (15), 124 (resorcinol monomethyl ether, 100), 111 (34). *Anal.* Calcd for $C_{11}H_{16}O_3\cdot 1/5\ H_2O$: C, 66.11; H, 8.27. Found: C, 66.18; H, 8.08.

7β-Hydroxybicyclo[3.3.1]nonane-2,4-dione (Ig)——A solution of PBr_3 (0.22 ml, 2.4 mmol) in CH_2Cl_2 (2 ml) was added to a solution of Ie (94 mg, 0.52 mmol) with stirring at 0 °C. After being stirred for a further 1 h at room temperature, the mixture was poured into ice-water. The $CHCl_3$ extract (10 ml × 3) was washed with brine (5 ml × 1) and concentrated to give Ig (72 mg, 83%) as a white solid. mp 191—192 °C (from AcOEt). IR (KBr): ν_{OH} 3350 (sharp), 3600—2200, ν 1650—1470. The NMR spectrum could not be measured because of the low solubility of Ig in $CDCl_3$. MS *m/z* (%): 168 (M^+, 28), 111 (resorcinol +1, 100).

7β-Hydroxy-4-methoxy-3-bicyclo[3.3.1]nonen-2-one (IXg)——MS *m/z* (%): 182 (M^+, 6), 139 ($M^+-CH_2CH=O$, 14), 125 (31), 124 (resorcinol monomethyl ether, 100), 111 (resorcinol+1, 86). High-resolution MS, Calcd for $C_{10}H_{14}O_3$: 182.094. Found: 182.093.

7,7-Ethylenedioxy-3-oxabicyclo[3.3.1]nonane-2,4-dione (IVc)——Dimethyl 5-oxo-1,3-cyclohexanedicarboxylate was prepared from IIb in three steps *via* the improved Jones oxidation [94% (Lit.[4] 57%)] of the corresponding 5-hydroxy compound. It was ketalized and hydrolyzed with 10% NaOH to give 5,5-ethylenedioxy-1,3-cyclohexanedicarboxylic acid (IIIc), which was dehydrated to IVc by using DCC as a reagent in the overall yield of 94% (Lit[4] 57%).

Methyl 3-Acetyl-5,5-ethylenedioxycyclohexanecarboxylate (VIc)——Under conditions similar to those used in the preparation of VIa, VIc was obtained in the yield of 61% from IVc *via* 3-di(ethoxycarbonyl)acetyl-5,5-ethylenedioxycyclohexanecarboxylic acid (Vc). Vc: IR (film): ν_{OH} 3600—2400, $\nu_{C=O}$ 1720, 1640, 1600. NMR (60 MHz): 1.28 (6H, t, J=7, CH_3CH_2–), 4.00 (4H, s, OCH_2), 4.30 (4H, q, J=7, CH_3CH_2–), 4.66 (0.8H, s, $COCH_2$), 11.00 (1H, s, COOH), 13.50 (0.2H, s, enol H). VIc: bp 142—143 °C (1 mmHg). IR (film): $\nu_{C=O}$ 1730, 1710. NMR (60 MHz): 1.2—3.1 (11H, m), 2.20 (3H, s, Ac), 3.69 (3H, s, OMe), 3.95 (4H, s, CH_2O). MS *m/z* (%): 242 (M^+, 3.5), 139 ($M^+-C_2H_4O_2-Ac$, 100). *Anal.* Calcd for $C_{12}H_{18}O_5$: C, 59.49; H, 7.49. Found: C, 59.20; H, 7.47.

Bicyclo[3.3.1]nonane-2,4,7-trione 7-Ethyleneketal (Ic)——Under conditions similar to those used in the preparation of Ia, Ic was obtained after refluxing its xylene solution for 4 d or its tetralin solution for 12 h in yields of 87 and 73%, respectively. Ic: mp 158—160 °C (from AcOEt, Lit.[4] mp 162—163.5 °C). NMR (270 MHz): keto form, 1.9—2.0 (3H, m), 2.2—2.3 (2H, m), 2.4—2.5 (1H, m), 2.83 (2H, brs, $W_{1/2}$=10.8, C_1- and C_5-H), 3.32 (2H, s, $COCH_2CO$), 3.7—4.0 (4H, m, CH_2O).

4-Methoxy-3-bicyclo[3.3.1]nonene-2,7-dione 7-Ethyleneketal (IXc)——mp 121—123 °C (from Et_2O). NMR (60 MHz): 3.77 (3H, s, OMe), 3.87 (4H, brs, OCH_2). MS *m/z* (%): 224 (M^+, 94), 182 (M^+-CH_2CO, 20), 113 (100), 112 (methoxycyclohexene, 94). *Anal.* Calcd for $C_{12}H_{16}O_4$: C, 64.27; H, 7.19. Found: C, 64.20; H, 7.15.

Bicyclo[3.3.1]nonane-2,4,7-trione (Ib)——A mixture of Ic (527 mg, 2.51 mmol) and *p*-TsOH (100 mg) in acetone

(20 ml) was stirred for 19 h at room temperature. The white solid obtained after removal of the solvent was recrystallized from AcOEt. mp 158—160 °C (colorless crystals, Lit.[4] mp 154—155 °C). Yield: 407 mg (97%). NMR (270 MHz): enol from, 2.1—2.8 (6H, m), 2.95 (2H, brs, $W_{1/2}=13.5$, C_1- and C_5-H), 5.23 (1H, s, C_3-H). *Anal.* Calcd for $C_9H_{10}O_3$: C, 65.05; H, 6.07. Found: C, 65.14; H, 6.04.

4-Methoxy-3-bicyclo[3.3.1]nonene-2,7-dione (IXb)——mp 129—130 °C (from hexane). NMR (CCl_4, 60 MHz): 3.72 (3H, s, OMe), 5.16 (1H, s, C_3-H). MS *m/z* (%): 180 (M^+, 33), 149 (M^+-OMe, 7), 129 (10), 111 (resorcinol +1, 100). *Anal.* Calcd for $C_{10}H_{12}O_3$: C, 66.65; H, 6.71. Found: C, 66.84; H, 6.64.

1-Hydroxy-6-oxobicyclo[3.2.1]nonane-3-*exo*-carboxylic Acid (VIIa)——A mixture of Ic (200 mg, 0.95 mmol) and 10% HCl (15 ml) was refluxed for 4 h, and then concentrated *in vacuo* to give a white solid (170 mg, 97%), mp 219—222 °C (from AcOEt). IR (KBr): ν_{OH} 3420 (sharp), 3600—2200, $\nu_{C=O}$ 1740, 1700. MS *m/z* (%): 184 (M^+, 10), 164 (13), 138 ($M^+-HCOOH$, 24), 111 ($M^+-CO-HCO$, 14), 97 (cyclohexenone+1, 100), 93 (toluene+1, 37). VIIa-Methyl Ester: bp 140—150 °C (bath temperature, 1 mmHg). IR (film): ν_{OH} 3430, $\nu_{C=O}$ 1735. MS *m/z* (%): 198 (M^+, 22), 180 (M^+-H_2O, 3), 167 (M^+-MeO, 27), 166 (23), 155 (M^+-CH_2CO-1, 12), 152 (*m/z* 180−CO, 16), 138 ($M^+-HCOOMe$, 24), 111 (55), 97 (100), 93 (49). NMR (270 MHz): 1.77 (1H, dd, $J=11.0$, 3.3), 1.81 (1H, ddd, $J=13.6$, 8.4, 2.9, C_4-H), 1.90 (1H, s, OH), 2.0—2.2 (2H, m), 2.24 (1H, dt, $J=18.3$, 2.2), 2.4—2.8 (4H, m), 2.92 (1H, tm, $J=8.4$, C_3-H), 3.67 (3H, s, OMe).

Bicyclo[3.3.1]nonane-2,4,7-trione Tri(ethyleneketal)——A mixture of Ic (300 mg, 1.43 mmol), *p*-TsOH (50 mg) and ethyleneglycol (0.44 g, 7.2 mmol) in benzene was refluxed for 11 h in a flask equipped with a Dean-Stark trap. The reaction mixture was poured into 10% KOH (20 ml) and the separated organic layer was washed with water (10 ml × 3). The white solid obtained after removal of the solvent was recrystallized from hexane. Yield: 408 mg (96%). mp 81—85 °C. IR (KBr): δ_{C-O} 1200—1000. NMR (60 MHz): 1.3—2.2 (9H, m), 2.96 (1H, d, $J=14$), 3.6—4.2 (12H, m, CH_2O). MS *m/z* (%): 298 (M^+, 1), 212 [$M^+-CH_2=C(OCH_2)_2$, 25], 113 ($C_6H_{11}O^+=CH_2$, 100). *Anal.* Calcd for $C_{15}H_{22}O_6$: C, 60.39; H, 7.43. Found: C, 60.34; H, 7.44.

Bicyclo[3.3.1]nonane-2,4,7-trione 2,4-Di(ethyleneketal)——An acetone (20 ml) solution of the triketal (200 mg, 0.67 mmol) was stirred in the presence of *p*-TsOH (catalytic amount) at room temperature for 5 h. The residue obtained after evaporation of the solvent was dissolved in benzene (70 ml). The benzene solution was washed with sat. $NaHCO_3$ (20 ml × 1), H_2O (20 ml × 1), and brine (20 ml × 1), and then evaporated to give a white solid (164 mg), which was recrystallized from AcOEt–hexane. Yield: 155 mg (91%). mp 134—136 °C (colorless crystal). IR (KBr): $\nu_{C=O}$ 1700. NMR (270 MHz): 1.70 (1H, d, $J=15.4$), 1.8—2.0 (2H, m), 2.24 (2H, brs, $W_{1/2}=10.8$, C_1- and C_5-H), 2.29—2.40 (3H, dd, $J=16$, 1.1), 3.8—4.1 (8H, m, OCH_2). ^{13}C-NMR (50.1 MHz): 26.9 (t, C_9), 38.0 (d, C_1 and C_5), 39.0 (t, C_7), 42.9 (t, C_2 and C_4), 64.1, 65.1 (t, OCH_2), 109.9 (s, C_6 and C_8), 210.8 (s, C_3). *Anal.* Calcd for $C_{13}H_{18}O_5$: C, 61.40; H, 7.14. Found: C, 61.58; H, 7.22. Oxime: mp 241—242 °C (from benzene). *Anal.* Calcd for $C_{13}H_{19}NO_5$: C, 57.98; H, 7.11; N, 5.20. Found: C, 57.87; H, 7.22; N, 5.05.

7α-Hydroxybicyclo[3.3.1]nonane-2,4-dione Di(ethyleneketal)——A tetrahydrofuran (THF) solution (20 ml) of the keto-diketal (616 mg, 2.43 mmol) was added to a suspension of $LiAlH_4$ (369 mg, 9.72 mmol) in THF (20 ml) and the mixture was refluxed for 4 d. H_2O (1.0 ml) and then 10% NaOH (3 ml) were added to the cooled mixture. The resulting white solid was removed by decantation and washed several times with THF. The combined THF solution was concentrated to give a colorless waxy material, which was purified through an SiO_2 column. The title compound was eluted with $CHCl_3$. Yield: 520 mg (84%). IR (film): ν_{OH} 3480. NMR (270 MHz): 1.42 (1H, d, $J=3.7$), 1.54 (1H, brs), 1.7—1.9 (3H, m), 1.9—2.1 (5H, m), 3.06 (1H, d, $J=16.7$), 3.8—4.1 (8H, m, OCH_2), 4.1 (1H, m, C_7-H). MS *m/z* (%): 256 (M^+, 5), 81 (cyclohexene−1, 100). *Anal.* Calcd for $C_{13}H_{20}O_5 \cdot 1/5\ H_2O$: C, 60.08; H, 7.91. Found: C, 60.10; H, 7.96.

Hydrolysis of the Hydroxy-diketal——A mixture of the hydroxy-diketal (254 mg, 0.99 mmol) and 5% HCl (5 ml) in MeOH (1 ml) was stirred for 8 h at room temperature. The reaction mixture was diluted with brine (30 ml) and then extracted with AcOEt (20 ml × 4). The organic layer was washed with brine (10 ml × 1) and concentrated to give a colorless waxy material, which was purified through an SiO_2 column. A mixture of 7α-hydroxybicyclo[3.3.1]nonane-2,4-dione (If) and its intramolecular hemiketal (VIII) (*ca.* 2:1) was eluted with $CHCl_3$ as a white solid, which was trituated with AcOEt. mp 202—218 °C. Yield: 148 mg (89%). IR (KBr): ν_{OH} 3460, 3600—2200, $\nu_{C=O}$ 1700, ν 1620—1540. NMR (270 MHz): If, keto form; 2.75, 2.95 (each 1H, d, $J=6.1$, C_3-H), 4.15 (1H, brs, $W_{1/2}=8.1$, C_7-H), VIII, 4.48 (1H, t, $J=5.1$, >CH-O). *Anal.* Calcd for $C_9H_{12}O_3 \cdot 1/10\ H_2O$: C, 63.56; H, 7.24. Found: C, 63.62; H, 6.98.

7α-Hydroxy-4-methoxy-3-bicyclo[3.3.1]nonen-2-one (IXf)——CH_2N_2 in Et_2O was added to a methanolic mixture of If and VIII. After usual work-up, IXf was obtained in quantitative yield. IR (film): ν_{OH} 3400 (sharp), $\nu_{C=O}$ 1635, $\nu_{C=C}$ 1600. NMR (270 MHz): 1.7—2.1 (3H, m), 2.0—2.2 (2H, m), 2.2—2.4 (1H, m), 2.53 (2H, brs, $W_{1/2}=8.1$, C_1- and C_5-H), 3.74 (3H, s, -OMe), 4.13 (1H, brs, $W_{1/2}=10.8$, C_7-H), 5.35 (1H, s, C_3-H). MS *m/z* (%): 182 (M^+, 30), 125 (18), 124 (resorcinol monomethyl ether, 24), 111 (resorcinol+1, 100). High-resolution MS, Calcd for $C_{10}H_{14}O_3$: 182.094. Found: 182.090.

References and Notes

1) A part of this work was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba,

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12) In the literature,[4] IIIb was prepared from IIc-dimethyl ester by catalytic reduction with PtO_2 followed by saponification in an overall yield of 32%.

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Studies on Fungal Products. XIV.[1] Emestrin B, a New Epitrithiodioxopiperazine, from *Emericella striata*

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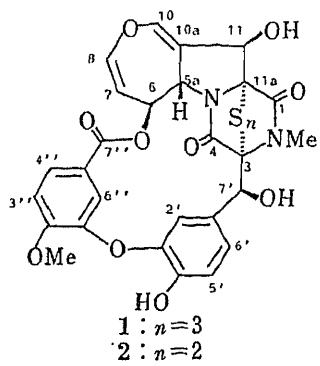
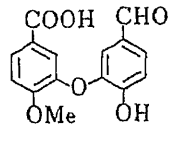
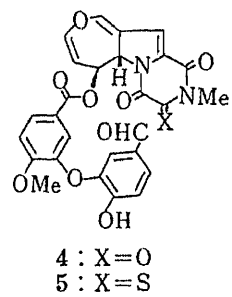
In the course of a search for metabolites related to emestrin (**2**) from *Emericells striata*, a new compound designated emestrin B (**1**) was isolated, together with shamixanthone, from the mycelial acetone extract. Shamixanthone is a compound related to emericellin, which had previously been isolated from the mycelium of the above fungus. The structure of emestrin B (**1**), including its absolute configuration, was established on the basis of the spectroscopic investigations. Emestrin B (**1**) is an epitrithio derivative corresponding to the antifungal macrocyclic epidithiodioxopiperazine, emestrin (**2**).

Keywords——*Emericella striata*; epitrithiodioxopiperazine; epidithiodioxopiperazine; emestrin B; emestrin; shamixanthone; antibacterial activity

Recently we reported[2,3] the isolation and structural elucidation of the characteristic macrocyclic epidithiodioxopiperazine, emestrin (**2**), from the mycelium and its degradation product, violaceic acid (**3**), from the culture filtrate of *Emericella striata* (RAI, TEWARI *et* MUKERJI) MALLOCH *et* CAIN (strain 80-NE-22). In the course of a search for metabolites related to **2**, dethiosecoemestrin (**4**)[4] and aurantioemestrin (**5**)[5,6] were isolated from the culture filtrate of the above fungus. From the structures and chemical reactions of **4** and **5**, we postulated[5,6] that **2** was biologically degraded to **5** at first, then to **4**, and finally to **3**. In order to find key intermediates in the transformation from **2** to **3**, **4**, and/or **5**, further investigation of the mycelial acetone extract was performed, and two compounds were isolated: a new compound designated as emestrin B (**1**), from the fraction slightly more polar than emerstrin (**2**), and shamixanthone, from the fraction slightly less polar than emericellin. Shamixanthone, accompanied with emericellin, was also obtained from *E. variecolor* BERK. *et* BR.[7] and *E.*







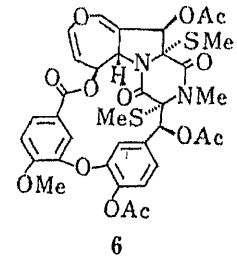


Chart 1

ruglosa (THOM *et* RAPER) C. R. BENJAMIN,[8] whose anamorphs are belonging to the section *Nidulantes* in the genus *Aspergillus* as same as *E. striata*. The structural elucidation of **1** is reported in this paper.

Emestrin B (**1**), mp 230—238 °C (dec.), gave a molecular ion at *m/z* 630 in field desorption (FD) mass spectrometry, and the elemental analysis, including sulfur, confirmed the molecular formula as $C_{27}H_{22}N_2O_{10}S_3$, which showed that **1** had only one more sulfur atom than **2**. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** is similar to that of **2** (Table I), and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of **1** is also closely similar to that of **2** (Table II). Positive silver nitrate test (dark brown)[9] and the ion at *m/z* 566 (M-S_2) and 534 (M-S_3 in the FD mass spectrum suggested the presence of a trithio bond in emestrin B (**1**). From the above results, the relative structure of emestrin B was determined as **1**. The ^{1}H-NMR chemical shifts of the signals assigned to the protons of the dihydrooxepine ring and to the aromatic protons in **1** are close to those of triacetyldidethiobis(methylthio)emestrin (**6**), which was derived from **2** by acetylation and reductive methylation,[3] rather than **1**, as shown in Table I. This may be because of a change of conformation in going from **2** to **1**.

The circular dichroism (CD) spectrum of **1**, whose absolute stereochemistry is known,[3]

TABLE I. ^{1}H-NMR Chemical Shifts of Emestrin B (**1**) and Related Compounds in $CDCl_3$

Proton No.	1	2[a]	6	Proton No.	1	2[a]	6
2-NMe	3.536	3.255	3.15	6′-H	6.933[d]	7.166	7.25[d]
5a-H	5.402	5.672	5.35	7′-H	4.809	4.967	5.93
6-H	5.328	4.672	5.35	2″-OMe	4.065	3.944	3.98
7-H	5.001	4.910	4.98	3″-H	7.026[c]	7.211	7.05[c]
8-H	6.358	6.412	6.33	4″-H	7.862[d]	7.576	7.76[d]
10-H	6.828	7.063	7.04	6″-H	8.351[b]	7.377	8.08[b]
11-H	5.226	5.466	6.51	OAc			2.18, 2.31, 2.34
2′-H	8.718[b]	7.767	8.80[b]	SMe			1.50, 1.98
5′-H	6.805[c]	6.883	6.96[c]				

a) The spectrum was measured in DMSO-d_6. *b*—*d*) Assignments may be reversed.

TABLE II. ^{13}C-NMR Chemical Shifts of Emestrin B (**1**) and Emestrin (**2**)

Carbon No.	1[a]	2[b]	Carbon No.	1[a]	2[b]
1	165.36 (Sq)[c]	164.28 (Sm)	1′	127.85 (Sdd)	127.08 (Sm)
2-NMe	28.32 (Q)	27.32 (Q)	2′	121.16 (Dbrd)	120.35 (Dbrd)
3	83.41 (Sd)	75.55 (Sd)	3′	146.87 (Sm)[e]	145.31 (Sdd)[d]
4	163.28 (Sd)	160.55 (Sd)	4′	154.11 (Sm)	153.26 (Sm)
5a	58.27 (Dm)	59.96 (Dm)	5′	114.80 (D)	115.42 (Dbrs)
6	74.61 (Dm)[d]	72.88 (Dm)	6′	126.34 (Dm)	122.66 (Dm)
7	109.73 (Ddd)	107.15 (Ddd)	7′	78.39 (Dd)	74.76 (Dd)
8	138.34 (Dm)	137.18 (Ddd)	1″	144.54 (Sm)[e]	143.44 (Sdd)[d]
10	141.10 (Dm)	141.68 (Dm)	2″-OMe	56.27 (Q)	55.84 (Q)
10a	110.38 (Sdd)	112.39 (Sdd)	3″	112.19 (D)	112.34 (D)
11	75.47 (Dm)[d]	74.76 (Dm)	4″	128.87 (Dm)	124.92 (Ddd)
11a	78.78 (Sm)	81.00 (Sm)	5″	122.51 (Sd)	122.15 (Sdd)
			6″	125.94 (Dd)	123.95 (Dd)
			7″	166.38 (Sdd)	164.43 (Sdd)

a) The spectrum was measured in a mixture of $CDCl_3$ and DMSO-d_6. *b*) The spectrum was measured in DMSO-d_6. *c*) Capital and small letters refer to $^1J_{C,H}$ and $^{>1}J_{C,H}$, respectively. *d*, *e*) Assignments may be reversed.

showed maxima at 228 (negative), 257 (positive), and 293 nm (positive), whereas that of **2** showed maxima at 233 (negative), 266 (positive), and 301 nm (positive). Thus, it is clear that **1** and **2** have 3*R*, 11a*R* configurations and consequently the absolute structure of emestrin B is as depicted in **1**.[10]

It is interesting that emestrin B (**1**), an epitrithiodioxopiperazine, was isolated along with emestrin (**2**), an epidithiodioxopiperazine, from the same fungus, *E. striata*, in relation to biosynthesis of dethiosecoemestrin (**4**) and aurantioemestrin (**5**). In the previous paper,[3,4] we reported that emestrin (**2**) had antifungal and antibacterial activity, and that the activities of **2** against bacteria seemed to be slightly less than those against fungi. Recently we reported[11] that **2** showed effective inhibition at 0.78—1.56 μg/ml against *Tricophyton* spp. and at 3.125—6.25 μg/ml against *Microsporum* spp. (*in vitro*). In a preliminary test,[4] emestrin B (**1**) also showed inhibitory activity at 25 μg per disc against *Bacillus subtilis* and *Escherichia coli*. Further studies on the antibacterial and antifungal activity are planned.

Experimental

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-181 spectrometer. Infrared (IR) and ultraviolet (UV) spectra were recorded on a Hitachi 215 spectrophotometer and a Hitachi 124 spectrophotometer, respectively. FD mass spectra (MS) were obtained on a JEOL JMS-D 300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX 400 spectrometer at 399.78 MHz for proton and at 100.43 MHz for carbon-13, using tetramethylsilane as an internal standard. The coupling patterns are indicated as follows: singlet=S or s, doublet=D or d, triplet=t, quartet=Q or q, multiplet=m, and broad=br. CD curves were determined on a JASCO J-40 spectrophotometer. Column chromatography was performed using Kieselgel 60 (Art. 7734; Merck). Low-pressure liquid chromatography (LPLC) was performed on a Chemco Low-Prep pump (81-M-2) and a glass column (200 × 10 mm) packed with silica gel CQ-3 (30—50 μ; Wako). Thin layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plates (Art. 5715; Merck). Spots on TLC were detected under UV light, and/or by spraying silver nitrate solution.

Isolation of Emestrin B (1)——*Emericella striata*, strain 80-NE-22, was cultivated at 30 °C for 3 weeks in Czapek-Dox medium (50 l). The dried mycelia (660 g) were pulverized and extracted with acetone at room temperature. After the chromatograhic separation of the acetone extract (26 g) with benzene–acetone (10 : 1, v/v), the fraction slightly more polar than emestrin (**2**) was purified by repeated LPLC [benzene–acetone (20 : 1, v/v)] to give emestrin B (**1**) (100 mg).

Emestrin B (**1**): Colorless crystalline powder, mp 230—238 °C (dec.) from MeOH. $[\alpha]_D^{20}$ +245° (c=0.19, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1715 (-COO-), 1690, 1680, 1670 (-CON-). UV λ_{max}^{MeOH} nm (log ε): 204 (4.66), 255 sh (4.32), 285 sh (3.92). FD-MS *m/z* (%): 630 (82, M$^+$), 629 (80, M-1), 566 (58, M-S_2), 534 (73, M-S_3), 533 (69), 532 (78), 288 (100). *Anal.* Calcd for $C_{27}H_{22}N_2O_{10}S_3$: C, 51.42; H, 3.52; N, 4.44; S, 15.25. Found: C, 51.16; H, 3.41; N, 4.43; S, 14.89. ^{1}H-NMR ($CDCl_3$) δ: 3.536 (3H, s, NMe), 4.065 (3H, s, OMe), 4.809 (1H, s, 7'-H), 5.001 (1H, dd, J=7.8, 2.4 Hz, 7-H), 5.226 (1H, br s, 11-H), 5.328 (1H, ddd, J=8.4, 2.4, 2.2 Hz, 6-H), 5.402 (1H, dd, J=8.4, 2.3 Hz, 5a-H), 6.358 (1H, dd, J=7.8, 2.2 Hz, 8-H), 6.805 (1H, d, J=8.3 Hz), 6.828 (1H, d, J=2.3 Hz, 10-H), 6.933 (1H, dd, J=8.3, 2.2 Hz), 7.016 (1H, s, 4'-OH), 7.026 (1H, d, J=8.5 Hz), 7.862 (1H, dd, J=8.5, 2.2 Hz), 8.351 (1H, d, J=2.2 Hz), 8.718 (1H, d, J=2.2 Hz). CD [θ] (nm): −5700 (228), +6900 (257), +20100 (293). The ^{13}C-NMR signals are summarized in Table II.

Isolation of Shamixanthone——The fraction slightly less polar than emericellin from the mycelial acetone extract was chromatographed on silica gel with benzene–ethyl acetate (98 : 2, v/v) to give a yellow crystalline powder (23 mg), mp 152—154 °C. This compound was identical with shamixanthone on the basis of IR, ^{1}H-NMR, and mass spectral comparisons.

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Synthesis of Nocardicins from Penicillins

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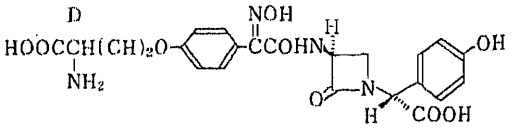
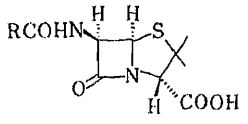
A nocardicin derivative was prepared from penicillin G, confirming that nocardicins have the same configuration as penicillins at C-3 of the β-lactam ring.

Keywords——stereochemistry; nocardicin; penicillin; unsubstituted monocyclic β-lactam; N-alkylation

The structure of nocardicin A (**1**),[1] a representative of the nocardicin group of antibiotics, was determined by analysis of its spectral data[2] and by total synthesis.[3] The stereochemistry at C-3 of the β-lactam ring of nocardicins was found, from those studies, to be the same as those at C-6 of penicillins and C-7 of cephalosporins. Prior to our total synthesis of nocardicin A, we had carried out investigations on the correlation between the C-3 stereochemistry of nocardicins and the C-6 stereochemistry of penicillins. We anticipated, at that time, that those investigations would also be of practical importance, because derivatization of monocyclic β-lactams from penicillins could provide much broader variability for studies on structure–activity relationships. Herein we describe a full account of the partial synthesis of nocardicin



1



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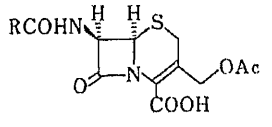

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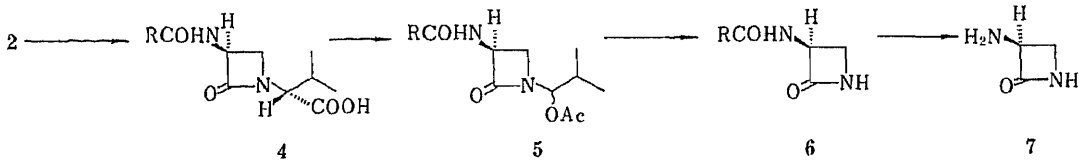


4 5 6 7

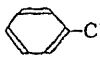
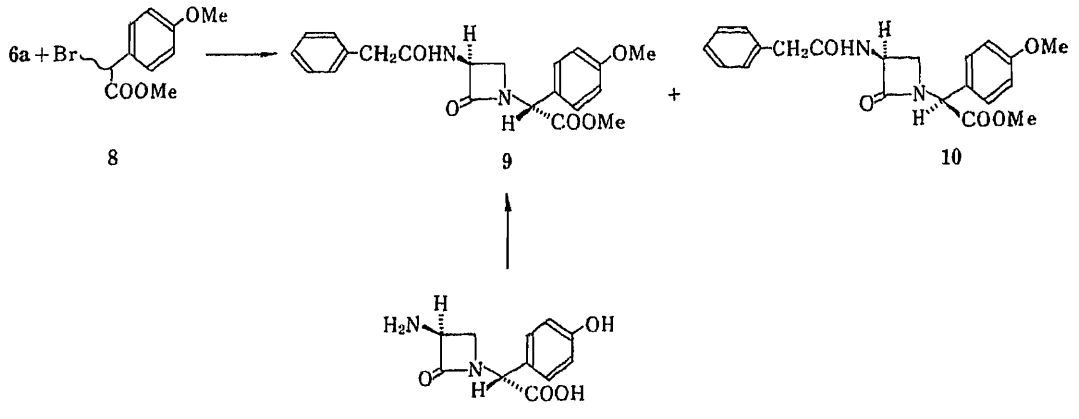
a : R=  b : R= 

Chart 1



8 9 10

11

Chart 2

from penicillin.

The approach which we adopted for conversion of penicillin to nocardicin was desulfurization of the thiazolidine sulfur of penicillin,[4] followed by oxidative removal of the N-1 side chain[5] of the resulting monocyclic β-lactam. Thus, desulfurization of penicillin G (**2a**) with Raney Ni gave the monocyclic β-lactam **4a** in 45% yield. Oxidation of **4a** with $Pb(OAc)_4/Cu(OAc)_2$, followed by treatment with $NaBH_4/K_2CO_3$ afforded, *via* **5a**, the N-unsubstituted monocyclic β-lactam **6a** in 30% yield. Similarly, **6b** was obtained from carbobenzyloxypenicillin **2b** in 24% total yield. Removal of the carbobenzyloxy group in **6b** by hydrogenolysis afforded 3-aminoazetidinone (**7**) in 56% yield.

Alkylation of **6a** with methyl α-bromo-(ρ-methoxyphenyl)-acetate (**8**) in the presence of NaH in dimethylformamide (DMF) provided a mixture of two diastereoisomers **9** and **10**. Separation by silica gel chromatography gave **9** and **10** in 12% and 5% yields, respectively. Compound **9** was identical with an authentic sample prepared from 3-aminonocardicinic acid (3-ANA, **11**)[6] by acylation with phenylacetyl chloride, followed by treatment with CH_2N_2. This result proved that the stereochemistry of the 3-acylamino group in the β-lactam ring of nocardicins is identical with that of the 6-acylamino group of penicillins.

This synthesis of compound **9** of the nocardicin type was found to be of limited practical importance because of the low yield of the N-alkylation step as described above. However, it does at least provide a variation in the synthesis of nocardicins from penicillins.

Experimental

3-Phenylacetamido-2-oxo-1-azetidinyl-2-(isopropyl)acetic Acid (4a)——A solution of **2a** (3.20 g) in H_2O (250 ml) was refluxed in the presence of Raney nickel W-2 type (32.0 g) for 15 min. After removal of the catalyst by filtration, the filtrate was adjusted to pH 8 (1 N HCl) and left at ambient temperature to give a precipitate, which was filtered off and washed with H_2O to give **4a** as needles (1.15 g, 44%). mp 108—110 °C. $[\alpha]_D^{24}$ −0.6° (c=0.5, EtOH). IR (Nujol): 3350, 1745, 1660 cm^{-1}. ^{1}H-NMR (D_2O+$NaHCO_3$) δ: 1.00 (6H, d, J=7 Hz), 1.98 (1H, m), 3.53 (1H, dd, J=3, 5 Hz), 3.63 (2H, s), 3.86 (1H, t, J=5 Hz), 3.89 (1H, d, J=7 Hz), 4.73 (1H, dd, J=3, 5 Hz), 7.20 (5H, s). *Anal.* Calcd for $C_{16}H_{20}N_2O_4 \cdot 1/2H_2O$: C, 61.33; H, 6.75; N, 8.94. Found: C, 61.24; H, 7.05; N, 8.90.

3-Benzyloxycarbonylamino-2-oxo-1-azetidinyl-2-(isopropyl)acetic Acid (4b)——This compound was prepared from **2b** as above. Yield 39%. mp 168—170 °C. $[\alpha]_D^{24}$ +9.5° (c=0.8, $CHCl_3$). IR (Nujol): 3400, 1745, 1680 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 0.95 (6H, t, J=6 Hz), 2.05 (1H, m), 3.36 (1H, dd, J=3, 5 Hz), 3.71 (1H, t, J=5 Hz), 3.88 (1H, d, J=7 Hz), 4.67 (1H, m), 5.08 (2H, s), 7.75 (5H, s), 8.04 (1H, d, J=8 Hz). *Anal.* Calcd for $C_{16}H_{20}N_2O_5$: C, 59.99; H, 6.29; N, 8.75. Found: C, 59.71; H, 6.35; N, 8.87.

3-Phenylacetamido-2-oxo-1-azetidine (6a)——A solution of **4a** (3.04 g) in AcOEt (100 ml) was refluxed in the presence of $Cu(OAc)_2 \cdot H_2O$ (0.1 g) and $Pb(OAc)_4$ (5.76 g) for 1 h. After cooling, the reaction mixture was filtered, and

the filtrate was washed with saturated aqueous $NaHCO_3$ and H_2O, dried over $MgSO_4$, and evaporated to give a crude oil, which was dissolved in MeOH (20 ml). K_2CO_3 (0.52 g) and $NaBH_4$ (0.15 g) were added, and the mixture was stirred for 1 h at 20 °C to give a precipitate, which was collected by filtration and washed with H_2O to give a crude powder. Recrystallization from MeOH gave **6a** as needles (0.7 g, 34%). mp 191—193 °C. $[\alpha]_D^{24}$ −22° (c=0.3, MeOH). IR (Nujol): 3325, 1780, 1660 cm^{-1}. ^{1}H-NMR (DMSO-d_6 + D_2O) δ: 3.05 (1H, dd, J=3, 5 Hz), 3.38 (1H, t, J=5 Hz), 3.45 (2H, s), 4.78 (1H, dd, J=3, 5 Hz), 7.23 (5H, s). *Anal.* Calcd for $C_{11}H_{12}N_2O_2$: C, 64.72; H, 5.82; N, 13.72. Found: C, 64.55; H, 5.97; N, 13.68.

3-Benzyloxycarbonylamino-2-oxo-1-azetidine (6b)——This compound was prepared from **4b** as above. Yield 77%. mp 164—165 °C. $[\alpha]_D^{24}$ −18.4° (c=0.4, MeOH). IR (Nujol): 3350, 1765, 1670 cm^{-1}. ^{1}H-NMR (DMSO-d_6 + D_2O) δ: 3.13 (1H, dd, J=3, 5 Hz), 3.48 (1H, t, J=5 Hz), 4.70 (1H, dd, J=3, 5 Hz), 5.03 (2H, s), 7.40 (5H, s). *Anal.* Calcd for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72. Found: C, 60.09; H, 5.33; N, 12.68.

3-Amino-2-oxo-1-azetidine Acetate (7)——A solution of **6b** (0.77 g) in a mixture of AcOH (270 ml) and EtOH (70 ml) was hydrogenated over 10% Pd–C (0.35 g) under atmospheric pressure of H_2. After removal of the catalyst by filtration, the filtrate was evaporated to give an oil, which was crystallized from AcOEt to give **7** as colorless needles (0.286 g, 56%). mp 130—131.5 °C (dec.). $[\alpha]_D^{25}$ −44.6° (c=0.2, H_2O). IR (Nujol): 3250, 1765 cm^{-1}. ^{1}H-NMR (D_2O) δ: 2.03 (3H, s), 3.64 (1H, dd, J=3, 5 Hz), 3.85 (1H, t, J=5 Hz). *Anal.* Calcd for $C_5H_{10}N_2O_3$: C, 41.09; H, 6.90; N, 19.17. Found: C, 40.94; H, 7.11; N, 19.03.

Methyl D-2-(3-Phenylacetamido-2-oxo-1-azetidinyl)-2-(4-methoxyphenyl)acetate (9)——A mixture of **6a** (0.612 g) and methyl α-bromo-(*p*-methoxyphenyl)acetate (**8**, 0.77 g) in DMF (7 ml) was treated with 50% NaH (0.144 g), and the whole was stirred for 30 min at ambient temperature. The reaction mixture was poured into ice-water (80 ml) and extracted with AcOEt. The organic layer was washed with 5% HCl and H_2O, dried over $MgSO_4$, and evaporated to give a mixture of **9** and **10**. Chromatography on silica gel with $CHCl_3$ gave **9**, which was crystallized from ether to give needles of **9** (0.134 g, 12%). mp 145—146 °C. $[\alpha]_D^{25}$ −209° (c=0.2, MeOH). IR ($CHCl_3$): 3340, 1760, 1745, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.03 (1H, dd, J=3, 5 Hz), 3.52 (1H, t, J=5 Hz), 3.75 (2H, s), 3.82 (3H, s), 3.84 (3H, s), 4.80 (1H, m), 5.52 (1H, s), 6.86 (2H, d, J=8 Hz), 7.20 (2H, d, J=8 Hz), 7.28 (5H, m). *Anal.* Calcd for $C_{21}H_{22}N_2O_5$: C, 65.95; H, 5.80; N, 7.33. Found: C, 65.83; H, 5.61; N, 7.11.

The isomer **10** was further purified by preparaive thin layer chromatography (TLC) on silica gel with a mixture of benzene and acetone (2:1) to give **10** as an oil (0.055 g, 5%). MS *m/z*: 382 (M^+). IR (film): 3430, 1760, 1750, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.40 (1H, dd, J=3, 5 Hz), 3.44 (1H, t, J=5 Hz), 3.52 (2H, s), 3.68 (3H, s), 3.77 (3H, s), 4.92 (1H, m), 5.48 (1H, s), 6.80 (1H, d, J=8 Hz), 6.88 (2H, d, J=8 Hz), 7.16 (2H, d, J=8 Hz), 7.27 (5H, s).

Preparation of 9 from 3-ANA (11)——A solution of phenylacetyl chloride (0.80 g) in acetone (5 ml) was added to a cooled (0 °C) solution of 3-ANA (0.94 g) and $NaHCO_3$ (0.80 g) in 50% aqueous acetone (20 ml), and the mixture was stirred for 2 h at the same temperature. After removal of the acetone by evaporation, the resulting aqueous layer was adjusted to pH 2 (10% HCl) and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$, and evaporated to give a powder (0.76 g), which was suspended in MeOH (20 ml). A solution of CH_2N_2 in ether was added dropwise to the suspension under stirring at 0 °C, during which time the precipitate gradually disappeared. The mixture was evaporated to give an oil, which was pulverized with ether to give a powder. Recrystallization from ether gave **9** as needles (0.48 g, 59%). mp 146 °C. $[\alpha]_D^{25}$ −206° (c=0.18, MeOH). IR (Nujol): 3340, 1760, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.03 (1H, dd, J=3, 5 Hz), 3.52 (2H, s), 3.74 (3H, s), 3.84 (1H, t, J=5 Hz), 4.90 (1H, m), 5.52 (1H, s), 6.25 (1H, d, J=8 Hz), 6.88 (2H, d, J=8 Hz), 7.22 (2H, d, J=8 Hz), 7.28 (5H, m). *Anal.* Calcd for $C_{21}H_{22}N_2O_5$: C, 65.95; H, 5.80; N, 7.33. Found: C, 65.72; H, 5.74; N, 7.18.

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Synthesis of 14α-Methylcholesterol

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14α-Methylcholesterol was synthesized from lanosterol *via* 19 steps in 1—2% overall yield.

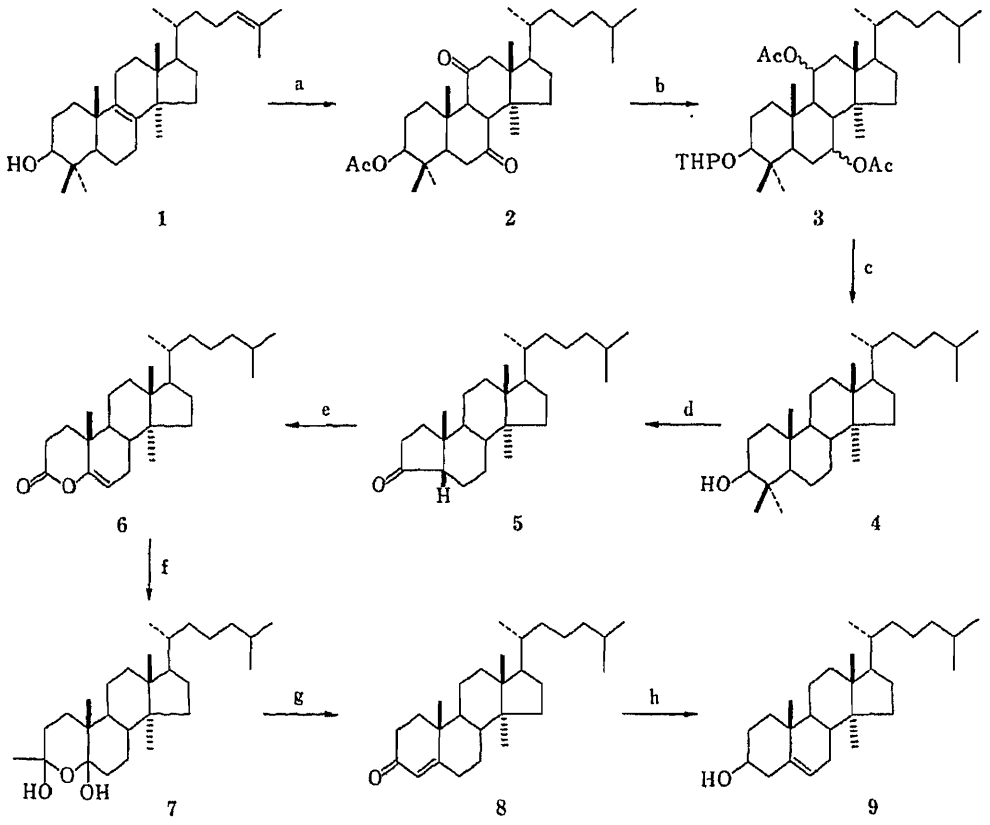
Keywords——14α-methylcholesterol; lanosterol; deacetoxylation; masked 1,5-diketone

For our investigation on sterol structure–function relationships,[1] we have required a sample of 14α-methylcholesterol. To our knowledge, this sterol (**9**) has never been prepared, although analogs such as 14α-methylcholestan-3β-ol,[2] 14α-methylcholest-4-en-3-one,[3] and 14α-methylcholest-7-en-3β-ol,[4] are known. Further, various 14α-methylated sterols were recently isolated from natural sources,[5] and have received considerable attention since they are potential intermediates in cholesterol biosynthesis. Several attempts to prepare 14α-methylestrone derivatives were also reported.[6] Here we describe the synthesis of 14α-methylcholesterol (**9**) from lanosterol (**1**) *via* 19 steps in 1—2% overall yield.

Lanosterol (**1**), through a conventional four step sequence[7] (catalytic hydrogenation, acetylation, chromic acid oxidation, reduction with zinc/acetic acid) was converted to the 7,11-diketone (**2**) in 59% yield. We avoided the hazardous Wolff–Kishner reduction to remove the 7,11-oxygen function of **2**[8] and instead, deacetoxylation of the 7,11-diacetate (**3**) was performed. Thus the latter, derived from **2** by successive saponification, tetrahydropyranyl ether formation, $LiAlH_4$ reduction and acetylation with acetic anhydride/pyridine in the presence of 4-dimethylaminopyridine, was subjected to reaction with sodium in hexamethylphosphoric triamide/*tert*-butanol.[9] Subsequent acid treatment afforded lanostanol (**4**) in 61% overall yield from **2**. Transformation of **4** into the enol lactone (**6**) *via* the A-nor-ketone (**5**), was effected essentially by the reported method (see Chart 1)[3a] in 18% yield. Grignard reaction of **6** with methyl magnesium iodide[10] gave the masked 1,5-diketone (**7**) in 75% yield. The latter had neither carbonyl absorption (infrared (IR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR)) nor an olefinic bond (^{1}H- and ^{13}C-NMR), and was much more polar than the starting enol lactone (**6**) on thin layer chromatography (TLC). Other NMR signals as well as the mass spectral peak at *m/z* 416 (M-18) strongly suggested the dihemiacetal structure (**7**), although its stereochemistry remained undetermined. Alkaline treatment of **7** gave the 3-oxo-4-ene (**8**).[3] Enol acetylation of **8** with isopropenyl acetate/*p*-toluenesulfonic acid,[11] followed by reduction with $NaBH_4$ furnished 14α-methylcholesterol (**9**, 42%), together with its 3-epimer (4%). The spectroscopic data of **9** unequivocally established the structure.

Experimental

Melting points were determined on a hot plate microscope and are uncorrected. Thin layer and column chromatography were carried out with Kieselgel $60F_{254}$ plates (Merck, 0.25 mm thick) and Kieselgel 60 (Merck, 70—230 mesh), respectively. ^{1}H-NMR and ^{13}C-NMR were recorded in $CDCl_3$ solution with a JEOL JNM-GX 270 at 270 MHz and at 67.8 MHz, respectively. Mass spectra (MS) were recorded on a JEOL JMS-DX303 with a direct-inlet



(a) i, H_2/Pd–C/AcOEt; ii, Ac_2O/pyr.; iii, CrO_3/AcOH; iv, Zn/AcOH.
(b) i, KOH/MeOH; ii, dihydropyran/ Amberlyst 15/CH_2Cl_2; iii, $LiAlH_4$/THF; iv) Ac_2O/DMAP/pyr.
(c) i, Na/HMPA/*tert*-BuOH; ii, d. HCl/MeOH–CH_2Cl_2.
(d) i, PCl_5/*n*-hexane; ii, O_3/CH_2Cl_2 and then Zn/AcOH; iii, KOH/MeOH.
(e) i, CF_3COOOH/CH_2Cl_2; ii, Jones oxid.; iii, $Ac_2O/HClO_4$. (f) CH_3MgI/Et_2O–C_6H_6. (g) NaOH/MeOH.
(h) i, $CH_2=C(OAc)Me$/*p*-TsOH; ii, $NaBH_4$/MeOH–THF.

Chart 1

system. Usual work-up refers to dilution with brine, extraction with CH_2Cl_2, drying ($MgSO_4$) and solvent evaporation under vacuum.

Lanostanol (4)——3β-Acetoxylanostane-7,11-dione (**2**, 230 mg)[7] was refluxed with 5% KOH–methanol (15 ml) for 20 min. Usual work-up gave the 3β-ol and this was stirred in a mixture of dihydropyran (150 mg), Amberlyst 15 (Rohm and Haas, 150 mg) and CH_2Cl_2 (8 ml) at room temperature for 5 h.[12] Filtration and solvent evaporation gave the tetrahydropyranyl (THP) ether as an oil, which was stirred with $LiAlH_4$ (150 mg) in dry tetrahydrofuran (10 ml) at room temperature overnight. The crude 7,11-diol obtained by usual work-up was allowed to stand in a mixture of acetic anhydride (1.5 ml), pyridine (6 ml) and 4-dimethylaminopyridine (150 mg) at room temperature for 5 h. Work-up as usual gave a yellow paste, which was chromatographed on silica gel with *n*-hexane–ethyl acetate (50 : 1) to give the 7,11-diacetate (**3**). This was dissolved in a mixture of dry *tert*-butanol (1.2 ml) and dry ethyl ether (1.5 ml), and added slowly (to maintain the deep blue color due to the radical anion) through a syringe to a stirred mixture of sodium (0.7 g), dry hexamethylphosphoric triamide (7 ml) and dry ethyl ether (5 ml) at room temperature under argon. Stirring was continued for 4 h and the mixture was worked up as usual. The crude product was allowed to stand in a mixture of conc. HCl (50 μl), methanol (2.5 ml) and CH_2Cl_2 (2.0 ml) at room temperature for 2 h. Usual work-up followed by column chromatography with *n*-hexane–ethyl acetate (40 : 1) gave lanostanol (**4**, 120 mg, 61% from **2**), mp 175—177 °C (needles from methanol, lit.[3b] 175—176 °C). MS *m/z*: 430 (M^+).

4-Oxa-3β,14α-dimethylcholestan-3ξ,5ξ-diol (7)——The enol lactone (**6**, 1.25 g) prepared from lanostanol (**4**) according to the literature,[3a] was dissolved in a 1 : 1 mixture (62 ml) of dry ethyl ether and dry benzene. To this stirred solution was added a methyl magnesium iodide solution (8.6 ml), which was prepared from magnesium (243 mg), methyl iodide (2.3 g) and dry ethyl ether (10 ml). Thirty min later, the mixture was worked up to give yellow amorphous material (1.3 g). A part (200 mg) of this material was chromatographed with *n*-hexane–ethyl acetate (30 : 1)—(5 : 1) to give the dihemiacetal (**7**, 150 mg), mp 163—165 °C (needles from methanol). ^{1}H-NMR δ: 0.73 (3H,

s, 13-Me), 0.84—0.89 (12H, m, 14-Me, 20-Me, 25-Me_2), 0.98 (3H, s, 10-Me), 1.28 (3H, s, 3-Me). MS *m/z*: 416 (M-18), 398, 380.

14α-Methylcholesterol (9)——The crude dihemiacetal (**7**, 1.15 g) was refluxed with a mixture of 10% NaOH (9.5 ml) and methanol (100 ml) for 2 h. Usual work-up gave the 3-oxo-4-ene (**8**),[3] which was then refluxed with a mixture of isopropenyl acetate (8.6 ml), *p*-toluenesulfonic acid (150 mg) and dry benzene (50 ml) under nitrogen for 5 h. Usual work-up gave the 3,5-dienol acetate. ^{1}H-NMR δ: 0.82 and 0.83 (each 3H, s, 13- and 14-Me), 0.86 (6H, d, J=6.5 Hz, 25-Me_2), 0.89 (3H, d, J=6.5 Hz, 20-Me), 1.03 (3H, s, 10-Me), 2.13 (3H, s, acetyl), 5.40 (1H, br s, 6-H), 5.68 (1H, s, 4-H), and this was stirred with $NaBH_4$ (2 g) in tetrahydrofuran (20 ml). Methanol (30 ml) was then added very slowly; a vigorous reaction occurred, resulting in solvent refluxing. After being stirred for 1 h, the mixture was worked up as usual and the crude product was chromatographed with *n*-hexane–ethyl acetate (50 : 1)—(10 : 1) to give the recovered enol acetate (384 mg), 14α-methylcholest-5-en-3α-ol (46 mg), mp 135—137 °C (needles from methanol). ^{1}H-NMR δ: 0.81 and 0.84 (each 3H, s, 13- and 14-Me), 0.87 (6H, d, J=6.5 Hz, 25-Me_2), 0.89 (3H, d, J=6.5 Hz, 20-Me), 4.0 (1H, m, 3-H), 5.4 (1H, m, 6-H), and 14α-methylcholest-5-en-3β-ol (**9**, 480 mg), mp 155—157 °C (needles from methanol). MS *m/z*: 400 (M^+), 395 (M-15), 392 (M-18). ^{1}H-NMR δ: 0.81 and 0.83 (each 3H, s, 13- and 14-Me), 0.87 (6H, d, J=6.5 Hz, 25-Me_2), 0.88 (3H, d, J=6.5 Hz, 20-Me), 1.05 (3H, s, 10-Me), 3.5 (1H, m, 3-H), 5.37 (1H, m, 6-H). ^{13}C-NMR δ: 37.3 (C1), 31.7 (C2), 71.7 (C3), 42.4 (C4), 140.2 (C5), 122.1 (C6), 32.0 (C7), 36.2 (C8), 43.1 (C9), 37.4 (C10), 20.1 (C11), 33.9 (C12), 45.2; 47.5 (C13; C14), 27.0 (C15), 28.0 (C16), 51.2 (C17), 14.2; 16.8 (C18; C32), 19.1 (C19), 35.5 (C20), 18.7 (C21), 36.6 (C22), 24.1 (C23), 39.5 (C24), 28.0 (C25), 22.6; 22.8 (C26; C27). Recycling of the recovered enol acetate afforded further 14α-methylcholesterol (**9**). Total yield of **9**: 445 mg (42% from **7**).

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Quinolizidines. XXI.[1] Syntheses of (±)- and (−)-Alancines

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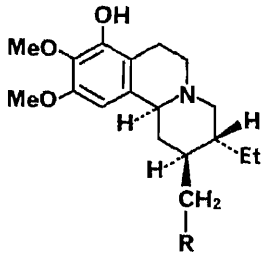
The first total synthesis of the *Alangium* alkaloid alancine (**10**) has been achieved in the form of a racemic modification by means of catalytic hydrogenolysis of the tricyclic amino acid (±)-**9**. Treatment of (±)-**10** with aqueous HCl afforded the hydrochloride salt (±)-**11**. A parallel synthesis starting with (−)-**9** gave (−)-alancine [(−)-**10**] as well as its hydrochloride [(−)-**11**] in good yields. The synthetic hydrochloride (−)-**11** was found to be identical with a sample isolated from *Alangium lamarckii* THW., indicating that the natural sample, previously considered to be in the free base form [(−)-**10**], was actually in the hydrochloride form [(−)-**11**].

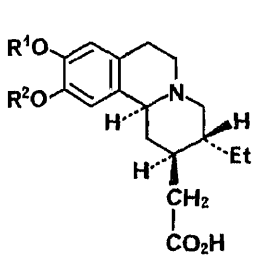
Keywords——*Alangium* alkaloid; alancine hydrochloride; racemic synthesis; chiral synthesis; benzyl ether hydrogenolysis; alancine ^{13}C-NMR; ankorine ^{13}C-NMR; alancine IR

In quite a recent communication,[2] Schiff and co-workers reported the isolation of alancine [(−)-**10**],[3] a phenolic benzo[*a*]quinolizidine alkaloid, from the stem bark of the Indian medicinal plant *Alangium lamarckii* THWAITES (Alangiaceae). The tricyclic amino acid structure of this alkaloid, unique among known benzo[*a*]quinolizidine-type alkaloids,[4] was elucidated[2] by them on the basis of spectral evidence and chemical correlation with ankorine (**1**),[5] another *Alangium* alkaloid with established absolute stereochemistry. They observed that the infrared (IR) absorption spectrum of their alancine sample exhibited an absorption band at 1725 cm^{-1} assignable to a carboxylic acid function.[2] This seemed to us discordant with our earlier finding that similar tricyclic amino acid structures such as **3**,[6] **4**,[6a,7] and **9**[8] show carbonyl absorptions below 1700 cm^{-1} together with N^+H absorptions in the 2200—2560 cm^{-1} region, reflecting their dipolar ion structures **5**—**7**. We thus undertook the synthesis of structure **10**, in both the racemic and (−) forms, in order to confirm the correctness of the structure assigned to (−)-alancine and to explain the appearance of the 1725 cm^{-1} band in the IR spectrum of the natural sample.[9]

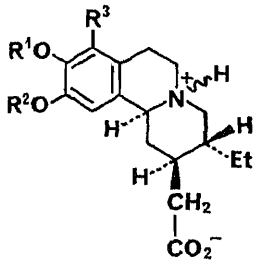
One of the shortest routes for the racemic and chiral syntheses of the target structure **10** would be that starting with the (±)- and (−)-tricyclic amino acids **9**. These starting materials have been available in our laboratory since their use[8] as key intermediates in the previous racemic and chiral syntheses of alangicine[8] and alangimarckine,[10] two other *Alangium* alkaloids of **2**-type. Thus, debenzylation of (±)-**9** was carried out in EtOH by using hydrogen and 10% Pd–C catalyst at 24 °C for 3 h, and racemic alancine [(±)-**10**] was obtained in 82% yield. Treatment of (±)-**10** with aqueous HCl produced the corresponding hydrochloride salt (±)-**11** in quantitative yield. A parallel sequence of reactions starting with (−)-**9** gave (−)-alancine [(−)-**10**] and its hydrochloride (−)-**11** in 82% and 85% yields, respectively. The spectral identity of (−)-**10** with (±)-**10** and that of (−)-**11** with (±)-**11** were confirmed by comparison of their ^{1}H nuclear magnetic resonance (NMR) (in CD_3OD), ^{13}C-NMR (in CD_3OD or CD_3OD–D_2O), ultraviolet (UV) (in MeOH, 0.1 N aq. HCl, and 0.1 N aq. NaOH), and mass spectra. Table I lists the chemical shifts for all carbons of (−)-**10**, (−)-**11**, and the



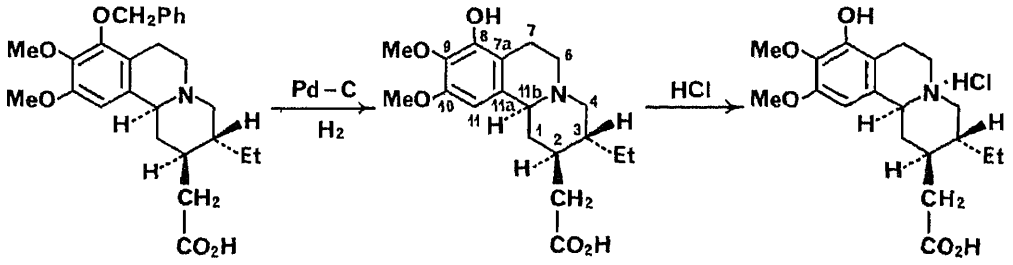
1: R = CH_2OH
2: R = a heterocyclic ring



3: $R^1 = CH_2Ph$; $R^2 = Me$
4: $R^1 = Me$; $R^2 = CH_2Ph$



5: $R^1 = CH_2Ph$; $R^2 = Me$; $R^3 = H$
6: $R^1 = Me$; $R^2 = CH_2Ph$; $R^3 = H$
7: $R^1 = R^2 = Me$; $R^3 = OCH_2Ph$
8: $R^1 = R^2 = Me$; $R^3 = OH$



(±)-9 (−)-9 (±)-10 (−)-10 (±)-11 (−)-11

Chart 1

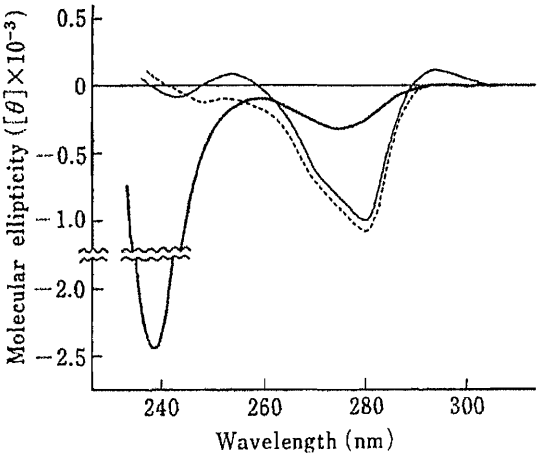


Fig. 1. CD Curves of Ankorine [(−)-**1**], Alancine [(−)-**10**], and Alancine·HCl [(−)-**11**]
——, (−)-**1** in EtOH ($c = 2.49 \times 10^{-4}$ M) at 20 °C.
– – –, (−)-**10** in MeOH ($c = 2.66 \times 10^{-4}$ M) at 18 °C.
– · – · –, (−)-**11** in MeOH ($c = 4.72 \times 10^{-4}$ M) at 22 °C.

structurally related alkaloid ankorine (**1**), which have been assigned as in the case[8] of alangicine (type **2**). The circular dichroism (CD) curves of these three compounds are shown in Fig. 1. As expected, the solid state IR spectrum of the synthetic (−)-**10** exhibited a few weak bands (N^+H) in the 2500 cm^{-1} region, a weak and broad band (CO_2H) at 1707 cm^{-1}, and a strong band (CO_2^-) at 1566 cm^{-1}, suggestive of the presence of the dipolar ion form (**8**) in a considerable proportion. It is reasonable that the hydrochloride (−)-**11** showed a strong absorption band (CO_2H) at 1725 cm^{-1} together with a few weak bands (N^+H) in the 2620—2710 cm^{-1} region.

To our surprise, the previously reported IR (KBr), ^{1}H-NMR (CD_3OD), and ^{13}C-NMR

TABLE I. ^{13}C Chemical Shifts of Ankorine [(−)-**1**], Alancine [(−)-**10**], and Alancine·HCl [(−)-**11**]

Carbon[b]	Chemical shift[a]				
	(−)-**1**		(−)-**10**	(−)-**11**	
	in $CDCl_3$	in CD_3OD[c]	in CD_3OD	in CD_3OD	in M–D[d]
C(1)	36.0[e]	36.7[i]	36.3	35.8	35.3
C(2)	37.8	38.7	38.2	36.9	36.3
C(3)	41.2	42.2	40.1	37.7	37.9
C(4)	61.7[f]	60.6[j]	59.8	59.0	59.2
C(6)	52.1	53.1	51.5	—[n]	52.4
C(7)	23.4[g]	24.2[k]	22.3[m]	21.9	21.3
C(7a)	115.0	116.1	113.7	113.0	113.5
C(8)	146.8	148.6	148.8	149.2	147.8
C(9)	133.9[h]	134.4[l]	136.5	137.1	136.6
C(10)	150.4	152.4	153.1	153.6	152.8
C(11)	100.5	101.2	101.0	101.1	101.8
C(11a)	134.3[h]	135.9[l]	129.9	128.4	128.6
C(11b)	63.0	64.2	63.8	—[n]	63.9
$\underline{C}H_2CH_3$	23.5[g]	24.4[k]	23.7[m]	23.6	23.0
$CH_2\underline{C}H_3$	11.1	11.3	10.7	10.4	10.1
$\underline{C}H_2CH_2OH$	37.3[e]	37.5[i]	—	—	—
$CH_2\underline{C}H_2OH$	60.5[f]	62.4[j]	—	—	—
$\underline{C}H_2CO_2H$	—	—	41.2	—[n]	39.9
$CH_2\underline{C}O_2H$	—	—	179.6	175.7	177.2
9-OCH_3	61.0	61.1	61.0	61.1	61.6
10-OCH_3	56.1	56.4	56.4	56.5	56.9

a) In ppm downfield from internal Me_4Si. *b*) See formula **10** in Chart 1 for the numbering system. The carbon marked with an underline in the partial structure is that to which the signal has been assigned. *c*) A sample of (±)-**1** was used. *d*) CD_3OD–D_2O (1:1, v/v). *e*—*m*) Assignments indicated by a given superscript may be reversed. *n*) An unmeasurably small peak resulting from the poor solubility of the synthetic salt.

(CD_3OD) spectra of "natural alancine"[2] did not match those of the synthetic (−)-**10**, but matched those of its hydrochloride salt (−)-**11** instead. It thus became evident that the physical, chemical, and spectral data reported by Schiff and co-workers[2] for "natural alancine" were in reality those for the hydrochloride salt [(−)-**11**] of alancine. Since the "natural alancine" had been isolated from the plant material by a procedure utilizing Mayer's complex formation and subsequent treatment with anion-exchange resin (Cl^-),[2] it is not unreasonable to consider that the alkaloid had actually been obtained in the form of the hydrochloride salt. Such a slip in characterization could have been avoided if elemental analysis data for "natural alancine" had been available then.

In summary, the above results not only represent the first total synthesis of the *Alangium* alkaloid alancine (**10**), in both the racemic and (−) forms, but also serve to characterize fully the free base of this alkaloid through the use of a synthetic sample in place of the natural sample that was isolated only in the form of the hydrochloride.

Experimental

General Notes——All melting points were determined by using a Yamato MP-1 capillary melting point apparatus and are corrected. Spectra reported herein were recorded on a Hitachi 320 UV spectrophotometer, a JASCO A-202 IR spectrophotometer, a JASCO J-500C spectropolarimeter, a Hitachi M-80 mass spectrometer, or a

JEOL JNM-FX-100 NMR spectrometer, equipped with a ^{13}C Fourier transform NMR system, at 24 °C with Me_4Si as an internal standard. Optical rotations were measured with a JASCO DIP-181 polarimeter using a 1-dm sample tube. Elemental analyses were performed by Mr. Y. Itatani and his associates at Kanazawa University. The following abbreviations are used: br = broad, s = singlet, sh = shoulder, t = triplet.

(±)-3α-Ethyl-1,3,4,6,7,11bα-hexahydro-8-hydroxy-9,10-dimethoxy-2*H*-benzo[*a*]quinolizine-2β-acetic Acid [(±)-Alancine] [(±)-10]——A solution of (±)-**9**[8] (419 mg, 0.953 mmol) in EtOH (50 ml) was hydrogenated over 10% Pd–C (150 mg) at atmospheric pressure and 24 °C for 3 h. Removal of the catalyst by filtration and concentration of the filtrate under reduced pressure gave a pale greenish solid. Recrystallization of the solid from EtOH furnished (±)-**10**·1/2H_2O (280 mg, 82%) as colorless prisms, mp 193—194 °C (dec.). Further recrystallizations from aqueous EtOH and drying over P_2O_5 at 2 mmHg and 40 °C for 13 h and then at 75 °C for 6 h yielded an analytical sample as colorless filaments, mp 217—218 °C (dec.); MS *m/z* (relative intensity): 349 (M^+) (88), 348 (100), 334 (36), 332 (44), 320 (12), 292 (14), 290 (34), 263 (17), 262 (75), 221 (51), 207 (57), 206 (17), 192 (24); IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2560 (br, N^+H), 1570 (CO_2^-). *Anal.* Calcd for $C_{19}H_{27}NO_5 \cdot 1/2H_2O$: C, 63.67; H, 7.87; N, 3.91. Found: C, 63.61; H, 7.87; N, 3.87. The UV (MeOH, 0.1 N aq. HCl, or 0.1 N aq. NaOH), ^1H-NMR (CD_3OD), and ^{13}C-NMR (CD_3OD) spectra of this sample were superimposable on those of the synthetic (−)-**10** described below.

(2*R*,3*R*,11b*S*)-3-Ethyl-1,3,4,6,7,11b-hexahydro-8-hydroxy-9,10-dimethoxy-2*H*-benzo[*a*]quinolizine-2-acetic Acid [(−)-Alancine] [(−)-10]——This was obtained in 82% yield from (−)-**9**[8] by catalytic hydrogenolysis similar to that described above for (±)-**10**. Purification by means of recrystallization from EtOH–H_2O (5 : 1, v/v) and drying over P_2O_5 at 2 mmHg and 40 °C for 15 h gave an analytical sample of (−)-**10**·1/2H_2O as colorless prisms, mp 216—220.5 °C (dec.); $[\alpha]_D^{20}$ −29° (*c* = 0.097, MeOH); MS *m/z* (relative intensity): 349 (M^+) (87), 348 (100), 334 (34), 332 (43), 320 (12), 292 (14), 290 (35), 263 (19), 262 (76), 221 (47), 207 (62), 206 (16), 192 (23); CD (Fig. 1)[11]; UV λ_{max} (MeOH) 230 nm (sh) (log ε 3.97), 273 (3.01), 280 (sh) (2.99); λ_{max} (0.1 N aq. HCl) 273 (3.02), 279 (sh) (3.01); λ_{max} (0.1 N aq. NaOH) 287 (3.43); IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2530 (br, N^+H), 1707 (weak, br, CO_2H), 1566 (CO_2^-); ^1H-NMR (CD_3OD) δ: 0.94 (3H, t, *J* = 7 Hz, $CCH_2\underline{Me}$), 3.76 and 3.79 (3H each, s, two OMe's), 6.41 (1H, s, $H_{(11)}$); ^{13}C-NMR (Table I). *Anal.* Calcd for $C_{19}H_{27}NO_5 \cdot 1/2H_2O$: C, 63.67; H, 7.87; N, 3.91. Found: C, 63.78; H, 7.74; N, 4.01.

(±)-3α-Ethyl-1,3,4,6,7,11bα-hexahydro-8-hydroxy-9,10-dimethoxy-2*H*-benzo[*a*]quinolizine-2β-acetic Acid Hydrochloride [(±)-Alancine Hydrochloride] [(±)-11]——To a suspension of (±)-**10**·1/2H_2O (232 mg, 0.647 mmol) in EtOH–H_2O (2 : 1, v/v) (15 ml) was added dropwise 1 N aqueous HCl (0.66 ml), and the mixture was concentrated *in vacuo* to leave (±)-**11** (251 mg, quantitative yield) as a colorless solid. Recrystallization of the solid from EtOH–H_2O (20 : 1, v/v) produced an analytical sample as colorless minute needles, mp 241.5—245 °C (dec.); MS *m/z*: 349 (M^+ − HCl); IR ν_{max}^{KBr} cm^{-1}: 3310 (OH), 2590—2710 (N^+H), 1727 (CO_2H). *Anal.* Calcd for $C_{19}H_{27}NO_5 \cdot HCl$: C, 59.14; H, 7.31; N, 3.63. Found: C, 59.12; H, 7.50; N, 3.42. The UV (MeOH, 0.1 N aq. HCl, or 0.1 N aq. NaOH), ^1H-NMR (CD_3OD), and ^{13}C-NMR [CD_3OD or CD_3OD–D_2O (1 : 1, v/v)] spectra of this sample were identical with those of the synthetic (−)-**11** described below.

(2*R*,3*R*,11b*S*)-3-Ethyl-1,3,4,6,7,11b-hexahydro-8-hydroxy-9,10-dimethoxy-2*H*-benzo[*a*]quinolizine-2-acetic Acid Hydrochloride [(−)-Alancine Hydrochloride] [(−)-11]——The tricyclic amino acid (−)-**10**·1/2H_2O (103 mg, 0.287 mmol) was dissolved in EtOH (16 ml), and 10% ethanolic HCl (3 ml) was added. After the solution had been kept in a refrigerator for 1 h, the crystals that resulted were filtered off and dried to give (−)-**11** (67 mg) as a first crop. The filtrate was concentrated *in vacuo* to leave a second crop (27 mg) of a slightly brownish solid. The total yield was 94 mg (85%). For analysis, the crude salt was recrystallized from EtOH, affording (−)-**11** as colorless minute prisms, mp 247.5—248.5 °C (dec.); $[\alpha]_D^{22}$ −28° (*c* = 0.101, MeOH); MS *m/z*: 349 (M^+ − HCl); CD (Fig. 1)[11]; UV λ_{max} (MeOH) 230 nm (sh) (log ε 3.98), 273 (3.04), 280 (sh) (3.01); λ_{max} (0.1 N aq. HCl) 273 (3.02), 279 (sh) (3.01); λ_{max} (0.1 N aq. NaOH) 287 (3.42); IR ν_{max}^{KBr} cm^{-1}: 3325 (OH), 2620—2710 (N^+H), 1725 (CO_2H); ^1H-NMR (CD_3OD) δ: 0.98 (3H, t, *J* = 7 Hz, $CCH_2\underline{Me}$), 3.77 and 3.83 (3H each, s, two OMe's), 6.41 (1H, s, $H_{(11)}$); ^{13}C-NMR (Table I). *Anal.* Calcd for $C_{19}H_{27}NO_5 \cdot HCl$: C, 59.14; H, 7.31; N, 3.63. Found: C, 58.91; H, 7.38; N, 3.64. The identity of this sample with "natural alancine"[2] was established by comparison of their UV, IR, ^1H-NMR, ^{13}C-NMR, CD, and mass spectra and optical rotations.

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Preparation of 1,6-Dihydro-3-benzazocines and Related Compounds by Base-Induced Intramolecular Cyclization of 1,2-Bis(2-isocyano-2-tosylethyl)benzene

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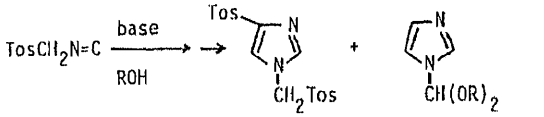
Treatment of 1,2-bis(2-isocyano-2-tosylethyl)benzene (**5a**) with potassium hydroxide in methanol, ethanol, or isopropyl alcohol afforded 2-substituted 1,6-dihydro-5-isocyano-3-benzazocines (**15a—c**) by intramolecular cyclization. When *tert*-butyl alcohol was used as a solvent under the same conditions, **5a** was led to 3,6-dihydro-5-isocyano-3-benzazocin-2(1*H*)-one (**18**) instead of the corresponding benzazocine of type **15d**. *N*-Alkylation of **18** with methyl, ethyl, and isopropyl iodides yielded 3-alkylated 3,6-dihydro-5-isocyano-3-benzazocin-2(1*H*)-ones (**20a—c**), which are structural isomers of **15a—c**.

Keywords——1,2-bis(2-isocyano-2-tosylethyl)benzene; intramolecular cyclization; 3-benzazocine; 3-benzazocinone; eight-membered heterocycle; tosylmethyl isocyanide

van Leusen *et al.* reported that the base-induced decomposition of tosylmethyl isocyanide (TosMIC, **1**), which is a useful synthetic tool for the preparation of five- or six-membered heterocycles,[1] afforded imidazoles (**2** and **3**) by the cyclodimerization of **1**.[2] In the course of our studies on the synthetic applications of TosMIC (**1**),[1f,3] we have previously reported the preparation of [3*n*]cyclophanes (**6**)[4] or dioxazolo[3²]cyclophanes (**8**)[5] by the reaction of bis(2-isocyano-2-tosylethyl)benzenes (**5a—c**), prepared by the alkylation of TosMIC (**1**) with α,α′-dibromoxylenes (**4a—c**), with **4a—c** or phthalaldehydes (**7a—c**), respectively. van Leusen's report on the cyclodimerization of **1** led us to investigate the intramolecular cyclization of **5a** possessing two accessible and reactive isocyanotosylmethyl groups under basic conditions.



1 2 3 : R=Me or Et

Chart 1

In this paper, we wish to report a facile preparation of 3-benzazocines (**15a—c**) and related compounds by the intramolecular cyclization of **5a** in the presence of base. Thus, treatment of **5a** with potassium hydroxide (KOH) in refluxing methanol for 3 h gave 1,6-dihydro-5-isocyano-2-methoxy-3-benzazocine (**15a**) as an intramolecular cyclization product of **5a** in 65% yield.

For ring closure of **5a** by the nucleophilic attack of the isocyanotosylmethyl carbanion (**9**), formed by loss of a proton, two routes as shown in Chart 3 can be considered. Thus, it might be possible for the carbanion (**9**) to attack either another isocyanotosylmethyl carbon

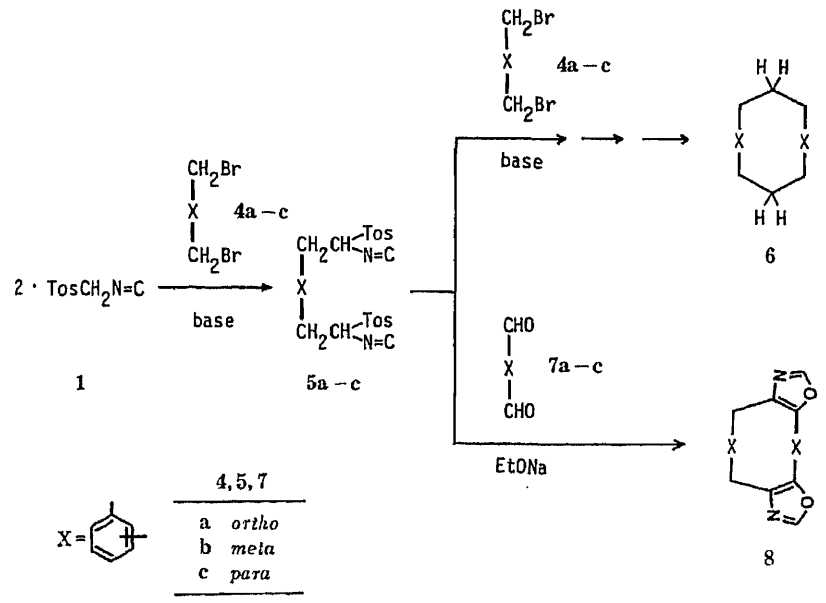


Chart 2

atom accompanied with loss of *p*-toluenesulfinate anion (Tos^-) to form six-membered carbocycles (**10**) (route A) or the electrophilic isocyano carbon atom to form eight-membered heterocycles (**11**) (route B). Since the structure of the cyclization product of **5a** was assigned as the 1,6-dihydro-3-benzazocine (**15a**) on the basis of the spectroscopic properties and analytical data (Table I), it was suggested that the intramolecular cyclization of **5a** proceeded along route B, as shown in Chart 3.

The formation of **15a** can be rationalized as follows: formation of the intermediate (**13**) occurs by the intramolecular nucleophilic attack of the carbanion (**9**) on the isocyano carbon atom to form **11**, followed by proton abstraction from methanol by **11** and 1,3-proton shift as indicated, and then the 3-benzazocine (**15a**) is formed by addition of methanol to **13**, followed by elimination of two molecules of toluenesulfinic acid (TosH) from the intermediate (**14a**).

In the same manner, treatments of **5a** with KOH in refluxing ethanol or in refluxing isopropyl alcohol gave the corresponding 3-benzazocines (**15b**; 74% or **15c**; 71%, respectively). Furthermore, the reactions of **5a** with potassium carbonate in methanol or with sodium ethoxide in ethanol similarly afforded 3-benzazocines (**15a**; 69% or **15b**; 74%, respectively).

When *tert*-butyl alcohol was used as a solvent, **5a** was led to 3,6-dihydro-5-isocyano-3-benzazocin-2(1*H*)-one (**18**) in 66% yield. With potassium *tert*-butoxide instead of KOH, analogous reaction of **5a** in *tert*-butyl alcohol afforded **18** in 69% yield. The structure of **18** was confirmed by the spectroscopic properties and analytical data (Table I). Namely, the infrared (IR) spectrum of **18** showed the characteristic absorptions of the vinylic isocyano group[6] and the amido N–H and carbonyl groups at 2110, 3225, and 1685 cm^{-1}, respectively, and the proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited a vinylic proton as a singlet at δ 6.60 and an amido proton as a broad singlet at δ 7.13.

These findings suggested that **16** was formed by addition of water, which was present as an azeotropic mixture with *tert*-butyl alcohol, to **13** instead of *tert*-butyl alcohol on account of the poor nucleophilicity of the *tert*-butoxy anion. The 3-benzazocinones (**18**) were then obtained by elimination of two molecules of TosH from **16**, followed by tautomerization of the imino alcohol of type **17**.

When dimethyl sulfoxide was used as an aprotic polar solvent, the reaction of **5a** with

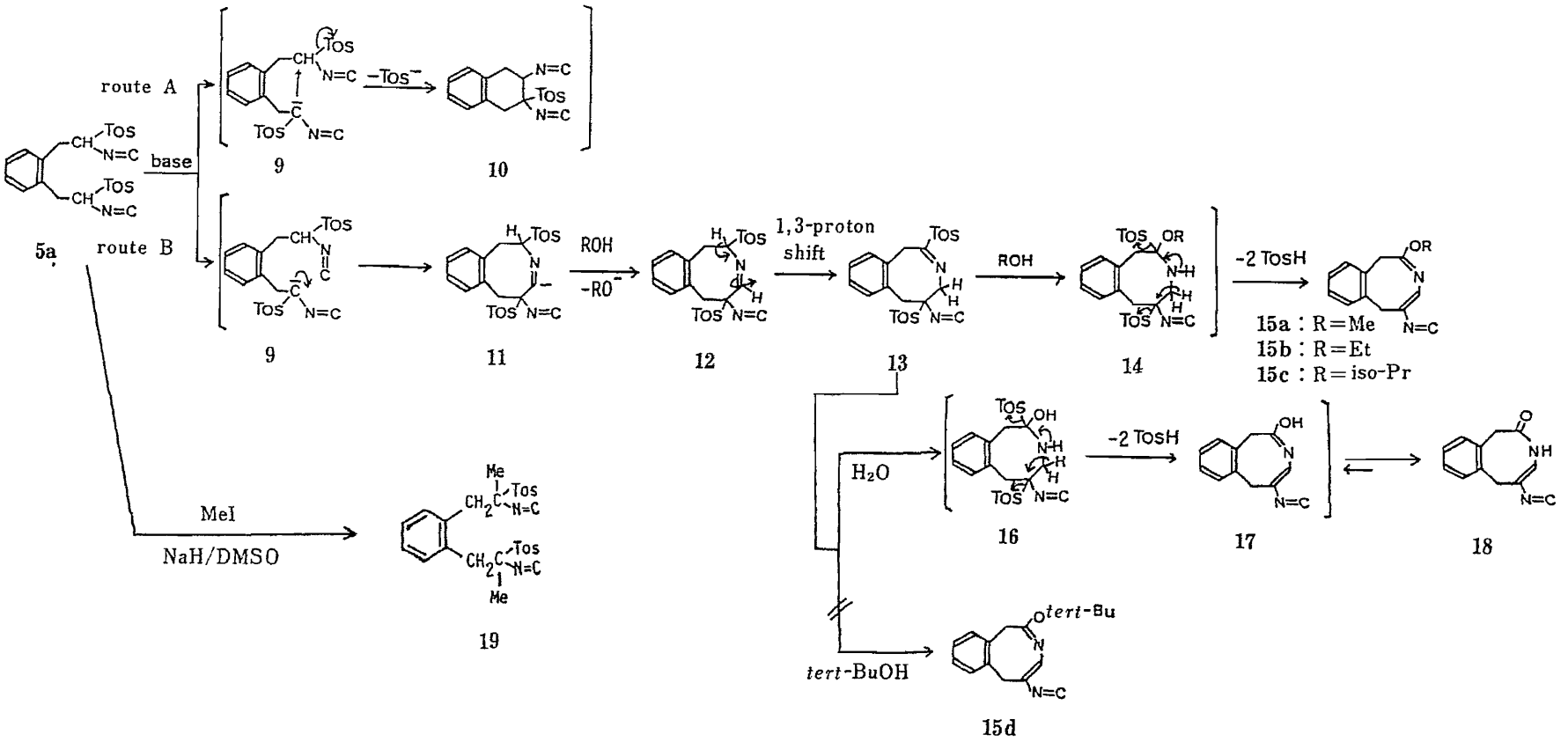

route A
base
5a
route B
9
10
11
12
13
14
ROH
-RO⁻
1,3-proton shift
ROH
-2 TosH
15a : R=Me
15b : R=Et
15c : R=iso-Pr
MeI
NaH/DMSO
19
H₂O
16
-2 TosH
17
18
tert-BuOH
15d

Chart 3

TABLE I. 2-Substituted 1,6-Dihydro-5-isocyano-3-benzazocines (**15a—c**) and 3,6-Dihydro-5-isocyano-3-benzazocin-2(1*H*)-one (**18**)

Compd. No.	Yield (%)	mp (°C)	IR (KBr, cm^{-1}) (N=C)	(C=C) or (C=N)	(C=O)	(NH)	^{1}H-NMR ($CDCl_3$, δ ppm) CH_3–	–CH_2–	–CH–	Benzyl-H	Vinyl-H	Aromatic-H	N–H	Formula Analysis (%) Calcd (Found) C	H	N	MS (*m/z*)	UV λ_{max}^{EtOH} (log ε)
15a	65,[a] 69[b]	127—128	2110	1630	—	—	3.74 s 3H	—	—	3.40 s 2H; 3.68 s 2H	7.13 s 1H	7.05—7.35 m 4H	—	$C_{13}H_{12}N_2O$ 73.56 (73.54)	5.70 (5.63)	13.20 (13.23)	212 (M^+) 197 (M^+-Me) 181 115 91	255.5 (3.87)
15b	74,[a] 74[c]	92—93	2110	1640	—	—	1.28 t 3H	4.22 q 2H	—	3.44 s 2H; 3.71 s 2H	7.11 s 1H	7.05—7.35 m 4H	—	$C_{14}H_{14}N_2O$ 74.31 (74.54)	6.24 (6.29)	12.38 (12.54)	226 (M^+) 197 (M^+-Et) 181 115 91	256.5 (3.90)
15c	71[a]	Oil[e]	2110	1630	—	—	1.18 d 6H	—	5.07 qu 1H	3.40 s 2H; 3.62 s 2H	7.13 s 1H	7.05—7.35 m 4H	—	$C_{15}H_{16}N_2O$ 74.97 (—)	6.71 (—)	11.66 (—)[f]	240 (M^+) 199 183 115 91	—
18	66,[a] 69[d]	146—148	2110	1645	1685	3225	—	—	—	3.62 s 2H; 3.74 s 2H	6.60 s 1H	7.10—7.30 m 4H	7.35 brs 1H	$C_{12}H_{10}N_2O$ 72.71 (72.62)	5.09 (4.92)	14.13 (14.14)	198 (M^+) 170 (M^+-H_2CN) 145 115	—

a) KOH. *b*) K_2CO_3. *c*) EtONa. *d*) *tert*-BuOK. *e*) IR (neat). *f*) Too unstable for analysis.

TABLE II. 3-Alkylated 3,6-Dihydro-5-isocyano-3-benzazocin-2(1*H*)-ones (**20a—c**)

Compd. No.	Yield (%)	mp (°C)	IR (KBr, cm^{-1}) (N=C) (C=O) (C=C)	^{1}H-NMR ($CDCl_3$, δ ppm) CH_3–	–CH_2–	–CH–	Benzyl-H		Vinyl-H	Aromatic-H	Formula Analysis (%) Calcd (Found)	MS (*m/z*)	UV λ_{max}^{EtOH} (log ε)
											C H N		
20a	43	170—171	2110	3.02	—	—	3.50	3.79	6.65	7.10—7.35	$C_{13}H_{12}N_2O$	212 (M^+)	253 (4.00)
			1665	s			s	s	s	m	73.56 5.70 1320	184 (M^+-H_2CN)	273 (sh) (3.56)
			1650	3H			2H	2H	1H	4H	(73.82 5.67 13.23)	158	
												115	
20b	48	110—111	2105	1.02	3.56	—	3.50	3.77	6.65	7.10—7.35	$C_{14}H_{14}N_2O$	226 (M^+)	253 (3.99)
			1655	t	q		s	s	s	m	74.31 6.24 12.38	198 (M^+-H_2CN)	273 (sh) (3.59)
			1640	3H	2H		2H	2H	1H	4H	(74.22 6.15 12.35)	172	
												115	
20c	28	129—130	2110	1.14	—	4.43	3.50	3.73	6.70	7.10—7.35	$C_{15}H_{16}N_2O$	240 (M^+)	254.5 (3.97)
			1655	d		qu	s	s	s	m	74.97 6.71 11.66	212 (M^+-H_2CN)	273 (sh) (3.58)
			1640	6H		1H	2H	2H	1H	4H	(75.00 6.74 11.55)	186	
												115	

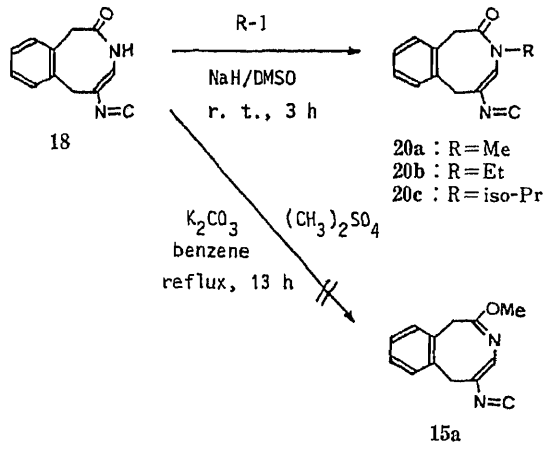


Chart 4

sodium hydride for 3 h at room temperature yielded tarry materials. Under the same conditions, addition of methyl iodide to this reaction system resulted in the formation of 1,2-bis(2-isocyano-2-tosylpropyl)benzene (**19**), the simple methylated product of **5a**. Judging from these findings, alcohol as a protic solvent seems to play an important role in the formation of 3-benzazocines (**15a—c**) and 3-benzazocinones (**18**).

O-Methylation of **18** to prepare **15a** by using dimethyl sulfate in the presence of potassium carbonate in refluxing benzene for 13 h was unsuccessful, and unchanged **18** was recovered from the reaction mixture. On the other hand, *N*-alkylation of **18** with alkyl iodides such as methyl, ethyl, and isopropyl iodides in the presence of sodium hydride in dimethyl sulfoxide at room temperature afforded the corresponding 3-alkylated 3,6-dihydro-5-isocyano-3-benzazocin-2(1*H*)-ones (**20a—c**) in the yields shown in Table II.

Thus, this report is the first to present a facile preparation of benzologs of eight-membered heterocycles with one nitrogen atom, such as 3-benzazocines (**15a—c**) and 3-benzazocinones (**18**), *via* 1,2-bis(2-isocyano-2-tosylethyl)benzene (**5a**).

Experimental

All melting points were taken on a Yanagimoto micro melting point determination apparatus and are uncorrected. IR spectra were recorded on a Hitachi model 260-30 infrared spectrophotometer. ^{1}H-NMR spectra were measured on a Hitachi R-22 spectrometer (90 MHz) using tetramethylsilane as an internal reference. Mass spectra (MS) were measured on a Hitachi mass spectrometer, model RMU-6MG. Ultraviolet (UV) spectra were measured on a Hitachi 323 spectrometer. The physical and analytical data for **15a—c** and **18** and for **20a—c** are listed in Tables I and II, unless otherwise noted.

1,6-Dihydro-5-isocyano-2-methoxy-3-benzazocine (15a)——Method 1: A suspension of 1,2-bis(2-isocyano-2-tosylethyl) benzene (**5a**) (2.46 g, 5 mmol) in methanol (100 ml) containing KOH (0.56 g, 10 mmol) was refluxed for 3 h with vigorous stirring. The alcohol was removed under reduced pressure, and a mixture of AcOEt (50 ml) and water (20 ml) was poured into the residue. The organic layer was separated, washed with two 20 ml portions of water, and then dried over anhydrous $MgSO_4$. The solvent was evaporated off, and the residue was chromatographed on silica gel with ether to give crude **15a**, which was recrystallized from ether to yield 0.69 g (65%) of **15a**; colorless prisms.

Method 2: A similar treatment of **5a** (2.46 g, 5 mmol) with K_2CO_3 (1.38 g, 10 mmol) in refluxing methanol (100 ml) gave a crude product, which was purified by the same procedure as mentioned above to yield 0.73 g (69%) of **15a**.

1,6-Dihydro-2-ethoxy-5-isocyano-3-benzazocine (15b)——Method 1: According to method 1 described above, treatment of **5a** (2.46 g, 5 mmol) with KOH (0.56 g, 10 mmol) in refluxing ethanol (100 ml) gave a crude product, which was purified by the same procedure as mentioned above to yield 0.84 g (74%) of **15b**; colorless prisms.

Method 2: Treatment of **5a** (2.46 g, 5 mmol) with EtONa (Na; 0.23 g, 10 mmol) in refluxing ethanol (100 ml) and the purification as described above yielded 0.84 g (74%) of **15b**.

1,6-Dihydro-5-isocyano-2-isopropoxy-3-benzazocine (15c)——According to method 1 for **15a**, treatment of **5a**

(2.46 g, 5 mmol) with KOH (0.56 g, 10 mmol) in refluxing isopropyl alcohol (100 ml) gave a crude product, which was chromatographed on silica gel with ether to yield 0.85 g (71%) of **15c**. However, an analytical sample of **15c** could not be obtained because of lability to heat.

3,6-Dihydro-5-isocyano-3-benzazocin-2(1*H*)-one (18)——Method 1: A suspension of **5a** (2.46 g, 5 mmol) in *tert*-butyl alcohol (100 ml) containing KOH (0.56 g, 10 mmol) was refluxed for 3 h with vigorous stirring. The alcohol was removed under reduced pressure, and a mixture of AcOEt (100 ml) and water (20 ml) was poured into the residue. The organic layer was separated, washed with two 20 ml portions of water, and dried over anhydrous $MgSO_4$. The solvent was evaporated off, and then the residue was chromatographed on silica gel with benzene–AcOEt (2:3) to afford crude solid, which was recrystallized from chloroform to yield 0.65 g (66%) of **18**; colorless needles.

Method 2: According to method 1 for **18**, reaction of **5a** (2.46 g, 5 mmol) with *tert*-BuOK (1.12 g, 10 mmol) in refluxing *tert*-butyl alcohol (100 ml) gave a crude product, which was purified by the same procedure as mentioned above to yield 0.68 g (69%) of **18**.

1,2-Bis(2-isocyano-2-tosylpropyl)benzene (19)——A solution of methyl iodide (1.42 g, 10 mmol) in dimethyl sulfoxide (DMSO) (20 ml) was added dropwise to a stirred solution of **5a** (2.46 g, 5 mmol) and NaH (0.48 g, 10 mmol) in DMSO (80 ml) at 5 °C. After being stirred for 3 h at room temperature, the resulting mixture was poured into water (500 ml), and the solution was extracted with three 50 ml portions of ether. The extracts were combined, washed with three 50 ml portions of water, and then dried over anhydrous $MgSO_4$. The organic solvent was evaporated off, and the residue was chromatographed on silica gel with benzene–AcOEt (20:1) to give a crude solid, which was recrystallized from methanol to yield 0.68 g (26%) of **19**; colorless prisms. mp 123—124 °C. *Anal.* Calcd for $C_{28}H_{28}N_2O_4S_2$: C, 64.59; H, 5.42; N, 5.38. Found: C, 64.32; H, 5.58; N, 4.98. IR (KBr): 2120 (N≡C), 1320, 1150 (SO_2) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.46 (6H, s, -Me), 2.45 (6H, s, aromatic-Me), 3.22 (2H, d, AB type, J=14 Hz, $-CH_2-$), 3.48 (2H, d, AB type, J=14 Hz, $-CH_2-$), 7.26 (4H, s, aromatic-H), 7.42 (4H, d, AB type, J=8 Hz, tosyl aromatic-H), 7.88 (4H, d, AB type, J=8 Hz, tosyl aromatic-H).

Preparations of 3-Alkylated 3,6-Dihydro-5-isocyano-3-benzazocin-2(1*H*)-ones (20a—c)——General Procedure: A solution of alkyl iodide (1 mmol) in DMSO (2 ml) was added dropwise to a stirred suspension of **18** (198 mg, 1 mmol) and NaH (24 mg, 1 mmol) in DMSO (8 ml) at room temperature. After being stirred for 3 h at room temperature, the resulting mixture was poured into ice-water (*ca.* 100 ml), and then the solution was extracted with AcOEt (40 ml). The extract was washed with two 20 ml portions of water, and dried over anhydrous $MgSO_4$. The organic solvent was evaporated off to give a crude product, which was purified by column chromatography (silica gel, ether) and recrystallization (ether) to provide an analytical sample.

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A New Amino Acid, (2*S*,3*R*)-(−)-3-Hydroxybaikiain from *Russula subnigricans* HONGO

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A new amino acid (I) was isolated from a toxic mushroom, *Russula subnigricans* HONGO, and characterized as (2*S*,3*R*)-(−)-3-hydroxybaikiain [(2*S*,3*R*)-(−)-1,2,3,6-tetrahydro-3-hydroxypyridine-2-carboxylic acid]. (*S*)-(−)-Baikiain (II), (*S*)-(−)-pipecolic acid (III), ergosterol (IV), ergosteryl peroxide (V) and cerevisterol (VI) were also isolated and identified.

Keywords——*Russula subnigricans*; *Russula nigricans*; *Russula adusta*; *Lactarius piperatus*; Basidiomycetes; amino acid; (2*S*,3*R*)-(−)-3-hydroxybaikiain; (*S*)-(−)-baikiain; (*S*)-(−)-pipecolic acid; ergosterol; ergosteryl peroxide; cerevisterol

A new amino acid (I), $C_6H_9O_3N$, (2*S*,3*R*)-(−)-3-hydroxybaikiain [(2*S*,3*R*)-(−)-1,2,3,6-tetrahydro-3-hydroxypyridine-2-carboxylic acid], was isolated from a toxic mushroom, *Russula subnigricans* HONGO (Russulaceae) (Japanese name: nisekurohatsu), along with (*S*)-(−)-baikiain (II), (*S*)-(−)-pipecolic acid (III), ergosterol (IV), ergosteryl peroxide (V) and cerevisterol (VI). The structure of I was elucidated on the basis of the spectroscopic data and the results of chemical transformation. Analysis of amino acids of this mushroom on an analyzer showed the presence of aspartic acid, glutamic acid, threonine, glycine, alanine, proline, tyrosine, phenylalanine, methionine, γ-aminobutyric acid, canavanine, cystine, valine, isoleucine, leucine, serine, lysine, histidine and arginine besides the above amino acids. Here we wish to report the isolation procedure and the structural elucidation of I and also the identification of other constituents.

The toxic mushroom, *Russula subnigricans* HONGO, is closely similar to an edible mushroom, *R. nigricans* (MERAT) FR. (Japanese name; kurohatsu). The latter mushroom shows blackening after reddening, while the former shows prolonged reddening. Intoxication of eleven persons, including three fatal cases, has been described.[1)]

Dried carpophores were extracted with methanol, and the extract was partitioned between ethyl acetate and water. The ethyl acetate layer was chromatographed on silica gel and the ergosterol, its peroxide and cerevisterol were isolated and identified. The water layer was applied to an Amberlite XAD-2 column and the passed fraction was chromatographed on Amberlite IR-120. The adsorbed partition was eluted with 2 N ammonia solution. The residue after evaporation of the solvent was subjected to chromatography on cellulose. The new amino acid (I) $C_6H_9O_3N$, mp 300—302 °C, was obtained as colorless needles after recrystallization of the fractions eluted with *n*-butanol saturated with water from a mixture of methanol and water.

The signals of the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of I were characterized by consideration of the chemical shifts and the splitting in the off-resonance spectrum. The signal (1C) at 171.49 ppm was attributed to a carbon of a carboxyl group. Signals (2C) at 126.81 and 123.70 ppm were assigned to two carbons of a 1,2-disubstituted

double bond. The signal (2C) at 60.82 ppm was assigned to two carbons having a hydroxy and an amino group. The signal (1C) at 42.39 ppm was assigned to a carbon of a methylene group attached to an amino group. The signals of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of I were characterized with the aid of the results of irradiation experiments as summarized in Fig. 1.

Because the $[\alpha]_D$ value of I in a neutral water solution was $-332.7°$ and that in 1 N HCl solution was $-324.0°$, this amino acid should be L-form.[2] The coupling constant ($J=3$ Hz) between the carbinyl hydrogen and the α-hydrogen of the amino carboxylic acid suggests *cis*-configuration. Therefore, the new amino acid (I) was concluded to be (2*S*,3*R*)-(−)-3-hydroxybaikiain [(2*S*,3*R*)-(−)-1,2,3,6-tetrahydro-3-hydroxypyridine-2-carboxylic acid]. This

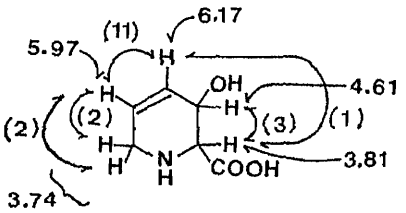

Fig. 1. ^{1}H-NMR Chemical Shifts and Coupling Constants (in Parenthesis) of I

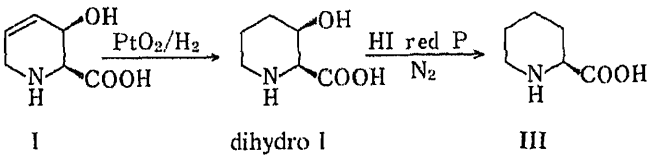

Fig. 2

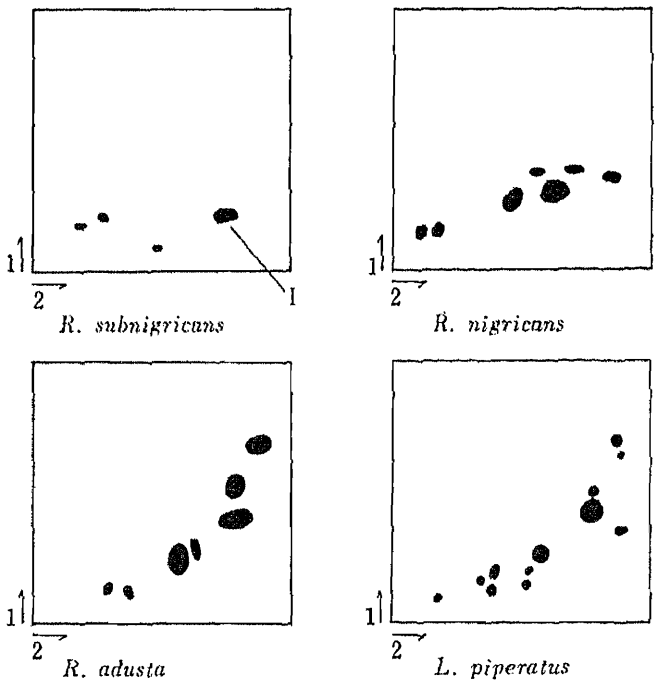

Fig. 3. Two-Dimensional Thin Layer Chromatograms of Amino Acids from 4 Mushrooms

1. BuOH : AcOH : H_2O = 4 : 1 : 1. 2. Phenol saturated with H_2O. 3. Kieselgel 60 F_{254}. 4. Ninhydrin, 120 °C.

conclusion was supported by the derivation into (*S*)-(−)-pipecolic acid (III) *via* the dihydro derivative, which was obtained through catalytic hydrogenation of the new amino acid (I), by treatment with red phosphorus and hydroiodic acid as shown in Fig. 2.

(*S*)-(−)-Baikiain (II) and (*S*)-(−)-pipecolic acid (III) were isolated from the mother solution of recrystallization of I by preparative high performance liquid chromatography (HPLC) and identified as described in the experimental section. The former amino acid, which is a deoxy derivative of I, was isolated from *Baikiaea plurijuna* (Leguminosae) and its structure was elucidated in 1950.[3] This amino acid was also isolated from *Caesalpinia tinctoria* and some allied species (Leguminosae),[4] and it was detected in leaves of *Derris elliptica*[5] and some marine algae.[6] Some biological activities of L-(−)-baikiain, especially an inhibitory activity towards glutamate as a neurotransmitter, were reported.[7] Because the new amino acid (I) showed no toxicity in mice given 1 g/kg perorally, work to identify the toxic principles of this mushroom is continuing.

On comparing the two-dimensional paper chromatograms of amino acids of *R. subnigricans*, *R. nigricans*, *R. adusta* (FR.) FR. (Japanese name: kogeirohatsutake) and *Lactarius piperatus* (FR.) S. F. GRAY, which all belong to the *Russula* family (the former three species are extremely similar to each other, and are difficult to differentiate), the new amino acid (I) was detected only in the case of *R. subnigricans* (Fig. 3). Therefore, the amino acid may be specific to this mushroom and may be useful for the identification of the mushroom, though more mushrooms of *Russula* genus and family are being investigated to confirm this.

Experimental[8]

Isolation of the New Amino Acid (I)——Dried fruiting bodies (250 g) of *Russula subnigricans* HONGO were extracted with methanol three times at room temperature. After evaporation of the solvent *in vacuo*, the residual extract was partitioned between ethyl acetate and water. The ethyl acetate solution was separated and the aqueous solution was subjected to Amberlite IRC-50 column chromatography after removal of dissolved ethyl acetate by evaporation under reduced pressure. The passed fraction was applied to an Amberlite IR-120 column. The adsorbed fraction was eluted with 2 N ammonia solution. The eluted fraction was concentrated under reduced pressure to remove ammonia and the residual solution was applied to an Amberlite IR-45 column after dilution with distilled water. The passed fraction was concentrated *in vacuo* and the residue was chromatographed on cellulose.

A new amino acid (I) was obtained as colorless needles (3.5 g) after recrystallization from aqueous methanol. The followig properties were observed: mp 300—302 °C. *Anal.* Calcd for $C_6H_9NO_3$: C, 50.34; H, 6.34; N, 9.79. Found: C, 50.07; H, 6.30; N, 9.96. $[\alpha]_D^{20}$ −332.7° (H_2O, $c=0.3$), −324.0° (1 N HCl, $c=0.3$). *Rf* on Avicel thin layer chromatography (TLC): 0.15 [BuOH–AcOH–H_2O (4 : 1 : 1)], as a yellow spot after usual treatment with ninhydrin. t_R on HPLC: 4.3 min (#2618, 8 mm i.d. × 50 cm, elution with pH 3.05 NH_3–HCOOH buffer, flow rate 3.0 ml/min, detected on radioisotope). IR (KBr) cm^{-1}: 3300 (OH), 3000, 2400 (NH), 1660, 1390 (carboxylate). MS (FD): *m/z* 144 ($M^+ + H$), 99.70. ^{13}C-NMR (D_2O) ppm: 171.49 (1C, s, $\underline{C}OOH$), 126.81 (1C, d, $\underline{C}H=CH$), 123.70 (1C, d, $CH=\underline{C}H$), 60.82 (2C, d, $\underline{C}H$–O and $\underline{C}H$–N), 42.39 (1C, t, $\underline{C}H_2$–N). ^{1}H-NMR (D_2O) ppm: 3.74 (2H, unresolved signal, C_6-2H), 3.81 (1H, splitted d, $J=3$ Hz, C_2-H), 4.61 (1H, unresolved, C_3-H), 5.97 and 6.17 (2H, m, C_5-H and C_4-H). ^{1}H-NMDR (D_2O): Irradiation at 4.61 ppm collapsed the multiplet at 6.17 and 5.97 ppm into a doublet (1H, $J=11$ Hz) and a sextet (1H, $J_1=11$, $J_2=J_3=2$ Hz), and transformed the unresolved signal at 3.74 ppm to a doublet (2H, $J=2$ Hz), and the split signal at 3.81 ppm to a doublet (1H, $J=1$ Hz). Irradiation at 6.0 ppm collapsed the signal at 4.61 ppm into a doublet (1H, $J=3$ Hz), the split doublet at 3.81 ppm to a doublet (1H, $J=3$ Hz), and the unresolved signal at 3.74 ppm into a singlet (2H).

Isolation of (*S*)-(−)-Baikiain (II)——The residue after recrystallization of I was subjected to HPLC for isolation. The fraction eluted at t_R 27 min with pH 3.05 NH_3–HCOOH buffer from a #2618 column (8 mm i.d. × 50 cm) at 3.0 ml/min flow rate was taken. The collected fraction was recrystallized from aqueous methanol and baikiain (27 mg) was obtained as colorless prisms: mp 265—270 °C. $[\alpha]_D^{20}$ −288° (H_2O, $c=0.1$). *Rf* 0.20 on Avicel TLC as a yellow spot after usual treatment with ninhydrin reagent [BuOH–AcOH–H_2O (4 : 1 : 1)]. IR (KBr) cm^{-1}: 3000, 2400 (NH), 1660 (COO^-). ^{13}C-NMR (D_2O) ppm: 173.95 (1C, s, COOH), 125.11 (1C, d, $\underline{C}H=CH$), 119.47 (1C, d, $CH=\underline{C}H$), 55.07 (1C, d, HN$\underline{C}$HCOOH), 41.74 (1C, t, $\underline{C}H_2$NH), 25.42 (1C, t, $\underline{C}H_2$). ^{1}H-NMR (D_2O) ppm: 2.44 (1H, octet, $J_1=18$, $J_2=12$, $J_3=3$ Hz, C_3-H), 2.70 (1H, unresolved d, $J=18$ Hz, C_3-H), 3.76 (2H, m, C_6-2H), 3.80 (1H, m, C_2-H), 5.9 (2H, m, C_4-H and C_5-H). ^{1}H-NMR (D_2O): Irradiation at 5.9 ppm collapsed the multiplet at 3.80 ppm into a singlet.

Irradiation at 2.44 ppm collapsed the multiplet at 5.9 ppm into a doublet (lower signal, 1H, $J=11$ Hz) and a sextet (higher signal, 1H, $J_1=10$ Hz, $J_2=J_3=3$ Hz). Irradiation at 3.8 ppm collapsed the multiplet at 5.9 ppm into an octet (lower signal, 1H, $J_1=11$, $J_2=4$, $J_3=2$ Hz) and a sextet (higher signal, 1H, $J_1=11$, $J_2=J_3=1$ Hz), the octet at 2.44 ppm into a doublet (1H, $J=18$ Hz) and the unresolved doublet into a double doublet (1H, $J_1=18$, $J_2=4$ Hz). The melting point, IR and NMR spectra were identical with those of reported L-(−)-baikiain.

Identification of (*S*)-(−)-Pipecolic Acid (III)——The residue after recrystallization of I was subjected to preparative HPLC (the same column, eluted with pH 3.4 NH_3–HCOOH buffer, at 0.5 ml/min flow rate). A peak at t_R 15 min was found to be identical with that of an authentic specimen of L-pipecolic acid. TLC: *Rf* 0.35 [BuOH–AcOH–H_2O (4:1:1)]. The collected fraction was recrystallized from MeOH to provide colorless needles (9 mg). $[\alpha]_D^{20}$ −32.5° (H_2O, $c=0.1$). The IR and NMR spectra were identical with those of an authentic specimen of L-pipecolic acid.

Hydrogenation of the New Amino Acid (I)——I (50 mg) was dissolved in a mixture of water (5 ml) and methanol (5 ml), and platinum dioxide (20 mg) was added. Hydrogen gas was introduced with stirring at room temperature for 4 h. The catalyst was filtered off and the filtrate was concentrated under reduced pressure to provide the dihydro derivative (45 mg) after recrystallization from MeOH. The product, mp >300 °C, $[\alpha]_D^{20}$ −93.3° ($c=0.3$, H_2O) was identified as (2*S*,3*R*)-(−)-3-hydroxypipecolic acid on the basis of the following data: MS (FD): *m/z* 146 (M^++H), 128 (M^++H−H_2O), 100 (M^+−CO_2−2H). ^{1}H-NMR (D_2O) ppm: 1.95 (4H, m, C_4-2H and C_5-2H), 3.02 (1H, t, $J=$ 10 Hz, C_6-H), 3.48 (1H, d, $J=10$ Hz), 3.72 (1H, s, C_2-H), 4.54 (1H, unresolved s, C_3-H). ^{1}H-NMDR (D_2O) ppm: Irradiation at 4.54 ppm increased the height of the singlet at 3.72 ppm, irradiation at 3.02 and 3.48 ppm changed the splitting pattern of the multiplet at 1.95 ppm, and irradiation at 1.95 ppm collapsed the doublet at 3.48 ppm into a sharpened doublet and the triplet at 3.02 ppm into a doublet.

Reduction of (2*S*,3*R*)-(−)-3-Hydroxypipecolic Acid——The dihydro derivative (30 mg) was heated with HI 0.5 ml and red phosphorus 10 mg in a shielded tube at 140 °C for 5 h. After cooling to room temperature, the tube was opened and the reaction solution was neutralized with 1 N NaOH. The diluted solution was introduced onto an Amberlite IR-120 column and the adsorbed fraction was eluted with 1 N NH_4OH. The eluate was evaporated under reduced pressure and the residue was recrystallized from MeOH. The product (10 mg) was identified as L-pipecolic acid on the basis of the following data. MS (FD): *m/z* 130 (M^++H), 75.61. $[\alpha]_D^{20}$ −32.7° (H_2O, $c=0.3$). ^{1}H-NMR (D_2O) ppm: 1.54—1.96 (5H, m, C_3-H, C_4, C_5-2H), 2.18 (1H, m, C_3-H), 3.05 (1H, m, C_6-H), 3.36 (1H, m, C_6-H), 3.70 (1H, m, C_2-H). These spectral data were identical with those of an authentic specimen.

Isolation of Ergosterol (IV), Its Peroxide (V) and Cerevisterol (VI)——From the fraction (16.5 g) soluble in ethyl acetate, mentioned briefly in connection with the isolation of the new amino acid (I), a part (14.5 g) was chromatographed on SiO_2 (70 g). After elution with *n*-hexane, in which higher fatty acids and their esters were eluted, elution with *n*-hexane–AcOEt (5:1) provided ergosterol (III), as colorless needles after recrystallization from AcOEt. *Rf* on TLC 0.3 (*n*-hexane:AcOEt=5:1). ^{1}H-NMR ($CDCl_3$) ppm: 0.63 (3H, s), 0.84 (6H, d, $J=8.0$ Hz), 0.92 (3H, d, $J=7.0$ Hz), 0.94 (3H, s), 1.04 (3H, d, $J=7.0$ Hz), 3.60 (1H, br s), 5.16—5.30 (2H, m), 5.41 (1H, m), 5.59 (1H, dd, $J=$ 1.5 Hz). The TLC behavior and ^{1}H-NMR spectrum were compared with those of an authentic specimen of ergosterol (IV) and the two were confirmed to be identical. Elution with *n*-hexane–AcOEt (4:1) provided ergosterol peroxide (V) as colorless needles after recrystallization from AcOEt. *Rf* on TLC 0.2 (*n*-hexane:AcOEt=2:1). ^{1}H-NMR ($CDCl_3$) ppm: 0.77 (3H, s), 0.85 (6H, d, $J=7.0$ Hz), 0.88 (6H, d, $J=7.0$ Hz), 0.96 (3H, s), 1.10 (3H, d, $J=6.0$ Hz), 5.18—5.35 (2H, m), 6.31 (1H, d, $J=14.9$ Hz), 6.60 (1H, d, $J=14.0$ Hz). After comparison of the TLC behavior and ^{1}H-NMR spectrum of the product with those of an authentic specimen, this isolated constituent was identified as ergosterol peroxide (V).

Elution with AcOEt, AcOEt–MeOH and MeOH provided gummy substances (2.5 g), which were chromatographed on Al_2O_3 (30 g). After elution with a mixture of *n*-hexane and AcOEt, elution with AcOEt–MeOH (4:1) provided cerevisterol (VI) as colorless needles after recrystallization from AcOEt. mp 251—253 °C. ^{1}H-NMR (pyridine-d_5 + CD_3OD) ppm: 0.65 (3H, s), 0.88 (6H, d, $J=7.0$ Hz), 0.90 (3H, s), 0.96 (3H, d, $J=7.0$ Hz), 1.01 (3H, d, $J=6.0$ Hz), 3.61 (1H, m), 4.92 (1H, br d, $J=5$ Hz), 5.20—5.36 (2H, m), 5.74 (1H, br d, $J=5.0$ Hz). After comparison of these data of an authentic specimen this compound was identified as cerevisterol (VI).

Amino Acids Analysis——Dried fruiting bodies (5.0 g) of *R. subnigricans* were extracted three times with water and the combined water solution was submitted to Amberlite IR-120 (50 ml) column chromatography. After washing of the column with distilled water, the amino acids fraction was eluted with 1 N NH_4OH. The residue after evaporation under reduced pressure was analyzed in a JEOL amino analyzer (an analysis sample was prepared as a 1 mg/ml solution in pH 2.2 0.2 N sodium citrate buffer). Column, JEOL AA pack Na; detection, fluorescence analysis with OPA treatment. The same fraction was also submitted to two-dimensional paper chromatography (solvent systems: 1) *n*-BuOH:AcOH:H_2O=4:1:1, 2) phenol saturated with water). Dried fruiting bodies (2.2 g) of *R. nigricans* were extracted with water, followed by the usual treatment for preparation of the amino acids fraction. The obtained fraction was subjected to two-dimensional chromatography (Fig. 3). The fresh fruiting bodies (5.7 kg) of *R. adusta* were extracted with water, followed by the usual treatment for preparation of the amino acids fraction. The obtained fraction was subjected to two-dimensional paper chromatography (Fig. 3) and amino acids analysis (Thr, Glu, Pro, Glu, Ala, Cys, Val, Ileu, Leu, Tyr, Phe, γ-aminobutyric acid (GABA), Arg). Dried fruiting bodies (5.0 g) of

L. piperatus were treated similarly, providing the two-dimensional paper chromatogram shown in Fig. 3 and amino acids (Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Met, Ileu, Leu, Phe, Asp).

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8) Melting points were measured on a Yanagimoto micro hot plate and are uncorrected. IR spectra were measured with a Shimadzu IR-27G infrared spectrometer. ^{1}H-NMR spectra were taken with JEOL JNM-PMX60 (at 60 MHz) and JEOL JMN-FX-100 (100 MHz) spectrometers using tetramethylsilane as an internal (for organic solvents) or an external (for D_2O solution) standard. ^{13}C-NMR spectra were measured with a JEOL JMN-FX-100 spectrometer operating at 25.05 MHz. The coupling patterns are indicated as follows: singlet=s, doublet=d, multiplet=m, and broad=br. Mass spectra were measured with Hitachi M-52 and JEOL JMS-01SG-2 mass spectrometers. $[\alpha]_D$ values were measured on a JACS DIP-340 polarimeter.

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Antimicrobial Activity of Pyrrolizidine Alkaloids from *Heliotropium ellipticum*

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Pyrrolizidine alkaloids, known for their significant antitumor activity, have been isolated from the aerial parts of *H. ellipticum* LEDEB. Europine (0.120%), heliotridine (0.152%), lasiocarpine (0.136%) and lasiocarpine-*N*-oxide (0.090%) have been identified. Antimicrobial activity of the isolated alkaloids against selected pathogenic bacteria and fungi has been investigated for the first time.

Keywords——*Heliotropium ellipticum*; Boraginaceae; europine; heliotridine; lasiocarpine; lasiocarpine-*N*-oxide; antibacterial activity; antifungal activity

Heliotropium ellipticum LEDEB., (Boraginaceae) is a herbaceous weed and widely distributed in the state of Rajasthan. Genus *Heliotropium* has been known to possess a number of medicinal properties and these are chiefly attributed to pyrrolizidine alkaloids.[1-3] These alkaloids besides hepatotoxic[4-6] also possess antitumor activity[7,8] against a number of tumor system. A large number of *Heliotropium* species have been investigated[9-21] for their pyrrolizidine alkaloids and different biological properties.[22] However, *H. ellipticum* remained untouched so far and hence, in the present study isolation of pyrrolizidine alkaloids and their antimicrobial testings have been reported.

Four pyrrolizidine alkaloids, europine (0.120%), heliotridine (0.152%), lasiocarpine (0.136%) and lasiocarpine-*N*-oxide (0.090%) have been isolated and identified on the basis of mp, infrared (IR) and mass spectral data (see Table I and Experimental).

Varied amounts of europine from *H. digynum*,[18] *H. europaeum*[17] and *H. marifolium*,[19] heliotridine from *H. eichwaldi*[20] and *H. lasiocarpum*, both in free and as its *N*-oxide, have been reported from different species of *Heliotropium*.[12,18,19,21]

While investigating the antimicrobial activities of the isolated alkaloids, although, the maximum inhibitory activity was demonstrated by europine (12 mm) against *Escherichia coli* and lasiocarpine against *Fusarium moniliforme* (11 mm) as compared to lasiocarpine-*N*-oxide and heliotridine (see Table II), but, unexpectedly it was not much exhibited as compared to the crude ethanolic extract against the test organisms.

TABLE I. Yields, Melting Points and Spectral Data of the Isolated Pyrrolizidine Alkaloids

Compounds	Yield (%)	mp (°C)	IR ($CHCl_3$) νcm^{-1}	MS *m/z* (M^+)
Europine	0.120	115	3500 (br), 1690, 1250	329
Heliotridine	0.152	116—118	3480 (br), 1710	223
Lasiocarpine	0.136	95—96	3480, 3460, 3400, 1735, 1710, 1280	396
Lasiocarpine-*N*-oxide	0.090	133 (dec.)	3300 (br), 1680, 1180	—

TABLE II. Antimicrobial Activity of Ethanolic Extract and the Isolated Pyrrolizidine Alkaloids from *Heliotropium ellipticum* LEDEB.

Test organisms	Zone of inhibition (mm±S.E.)[a]				
	Ethanolic extract	Europine	Heliotridine	Lasiocarpine	Lasiocarpine-*N*-oxide
Bacteria					
E. coli	8.00±0.09	10.00±0.22	—	12.00±0.34	9.00±0.89
E. cloacae	—	12.00±0.67	—	10.00±0.41	9.00±0.70
S. aureus	—	—	—	—	—
S. faecalis	—	—	—	—	—
Fungi					
A. flavus	9.00±0.83	7.00±0.76	—	—	—
C. lunata	10.00±0.59	—	—	—	8.00±0.47
D. tetramera	±	8.00±0.62	7.00±0.51	—	—
F. moniliforme	—	11.00±1.34	—	—	7.00±0.54
C. albicans	8.00±0.43	—	—	—	—

a) Mean of three replicates±S.E.; (±) trace activity; (—) not measurable activity.

From the results, it is evident that the efficacy of the crude extract against the test organisms is either due to the pyrrolizidine alkaloids present and/or the activity might be further enhanced by other metabolites, on identification, present in the whole extract.

So far, ethanolic extract of *H. bacciferum* and *H. subulatum* have been tested against one or the other microorganisms[22] but no active principles have been isolated. Therefore, in the present investigation the exhibited antimicrobial activity of the pyrrolizidine alkaloids isolated from the ethanolic extract of *H. ellipticum* is first of its kind and noteworthy.

A great deal of attention has been paid on the antitumor properties of pyrrolizidine alkaloids and heliotrine, lasiocarpine, monocrotaline, spectabilline and senecionine have been reported to be highly active against Walker-256 (intramuscular) tumor system.[8] Besides this, some of the alkaloids are responsible for malignant tumor of liver, skin and intestine in rats. The toxicity of the pyrrolizidine alkaloids containing plants to monogastric animals[23] increases by high ratio between the tertiary bases and their *N*-oxides. Therefore, the occurrence of europine, heliotridine, lasiocarpine and lasiocarpine-*N*-oxide might be responsible for the mild poisonous properties of *H. ellipticum*.

Experimental

All melting points are uncorrected. IR spectra were measured with Perkin-Elmer 337 spectrophotometer and mass spectra were run on a Hitachi RMC-7 spectrometer. Thin layer chromatography (TLC) and preparative TLC (pTLC) were performed with Silica gel G. Spots were located by spraying with Dragendorff's reagent followed by heating or by I_2 vapors.

Plant Material——Whole plants of *H. ellipticum* were collected from the fields in the month of August, 1986 and authenticated from the Herbarium, Department of Botany, University of Rajasthan, Jaipur, India.

Microorganisms——Pure cultures of *Escherichia coli, Enterobacter cloacae, Staphylococcus aureus, Streptococcus faecalis* and *Candida albicans* obtained from S.M.S. Medical College, Jaipur, India, and of *Aspergillus flavus, Curvularia lunata, Drechslera tetramera* and *Fusarium moniliforme* from the Laboratory of Microbiology, Department of Botany, University of Rajasthan, Jaipur, India, were used as test organisms.

Isolation of Alkaloids——The air-dried and powdered aerial parts (500 g) of the plant material were defatted with light petroleum ether and extracted with ethanol for 48 h in a Soxhlet apparatus. The viscous mass (9.7%) thus obtained was extracted with 500 ml of 5% H_2SO_4[17] and filtered. The filtrate was basified with ammonia and fractionated by extracting sequentially with ether (fr. E) and chloroform (fr. C). The aqueous layer was evaporated to

dryness and the dried residual mass was re-extracted with petroleum ether (fr. P). These fractions were then examined by co-chromatography ($CHCl_3$–MeOH–NH_4OH (85 : 14 : 1)). Seven spots (A→G; *Rf* 0.08, 0.20, 0.30, 0.50, 0.52, 0.80, 0.90) in fr. C and three spots (A→C; *Rf* 0.23, 0.61, 0.82) in fr. E were observed but fr. P did not yield any Dragendorff's positive spot. Out of the observed spots in the fr. C, spot C (*Rf* 0.30), D (*Rf* 0.50), E (*Rf* 0.52) and G (*Rf* 0.90) coincided with those of authentic europine, heliotridine, lasiocarpine and lasiocarpine-*N*-oxide used as markers. However, the remaining spots could not be identified due to their poor yields. These compounds were isolated by pTLC using the above solvent system (each developed two times) to obtain pure compounds, crystallized with methanol–acetone and then subjected for mps, IR and mass spectral studies and compared[24] with those of previously isolated samples, as recorded above, and by direct comparison by TLC, *etc.*

Media and Cultivation of Organisms——The selected bacteria were grown in Nutrient Broth medium and incubated at 37 °C for 48 h. Each bacterial culture was maintained by transferring to fresh medium every 48 h. However, fungi were grown on potato dextrose agar (PDA) medium by incubating at 27 °C for 48 h and maintained by periodic subculturings on fresh medium.

Antimicrobial Assay——For the antimicrobial assays, the *in vitro* paper disc diffusion method was adopted.[25] The different organisms were pre-seeded separately using a sterile swab over previously sterilized culture medium plates and the zones of growth inhibition were observed around sterilized dried discs of Whatman No. 1 paper (6 mm in diameter) which were containing 4 mg of plant extract (0.1 g/ml) or 2 mg of the isolated alkaloids (0.1 g/ml of the respective solvent) or streptomycin (10 mg/ml) or mycostatin (100 units/ml) as reference compounds separately. Such treated discs were air-dried at room temperature to remove any residual solvent which might interfere with the determination.

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Diterpenoid Constituents of *Rabdosia inflexa*: Structure Elucidation of Two New Diterpenoids, Inflexanins A and B

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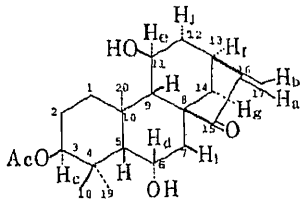
Two new diterpenoids, inflexanins A and B, have been isolated from the aerial part of *Rabdosia inflexa* (THUNB.) HARA together with the known compounds, inflexin (3) and inflexinol (4). The structures of the new diterpenoids have been elucidated as **1** and **2** on the basis of spectral and chemical evidence.

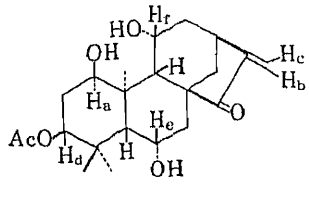
Keywords——*Rabdosia inflexa*; inflexanin A; inflexanin B; 15-oxo-*ent*-kaurenoid; inflexin; inflexinol; structure elucidation

Over one hundred diterpenoids have been isolated from plants belonging to the genus *Rabdosia* (Labiatae).[2)] These diterpenoids showed various biological activities,[3)] *i.e.*, antitumor and antimicrobial activities, inhibitory activity on the respiration of mitochondria and inhibitory activity on insect growth. From *Rabdosia inflexa* (THUNB.) HARA, two diterpenoids, named inflexin (**3**)[4)] and inflexinol (**4**)[5)] were isolated and characterized. In a continuation of our studies on biologically active substances from *Rabdosia* plants, we further examined the constituents of the title plant and isolated two new minor diterpenoids, named inflexanins A (**1**) and B (**2**), together with the known compounds, **3** and **4**. This paper deals with the structure elucidation of the new compounds.

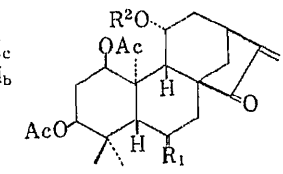
Inflexanin A (**1**) was isolated as an amorphous powder, $[\alpha]_D -108.3°$ (MeOH) and the molecular formula was determined as $C_{22}H_{32}O_5$ on the basis of the high-resolution mass spectrum (High-MS). Inflexanin A (**1**) showed an absorption maximum at 238 nm (ε 5990) in the ultraviolet (UV) spectrum, absorption bands at 1720 and 1650 cm^{-1} in the infrared (IR) ($CHCl_3$) spectrum, signals at δ 5.31 (H_b) and 5.91 (H_a) in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum ($CDCl_3$), and signals at δ 113.1 (t) and 149.8 (s) (*exo*-



1



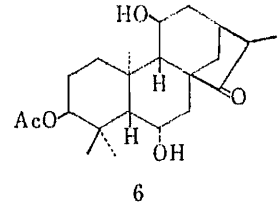
2



3 : R^1=O, R^2=H

4 : R^1=  , R^2=H

5 : R^1=, R^2=Ac



6

Chart 1

methylene), and 209.8 (s) (ketone) in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum ($CDCl_3$). From these spectral data, **1** was suggested to contain a five-membered ketone conjugated with an α-methylene group as a partial structure. The ^{1}H-NMR spectrum of inflexanin A (**1**) showed, besides the singlets at δ0.96, 1.28 and 1.42 due to three tertiary methyl groups and at δ2.07 assignable to an acetyl group, signals due to a proton attached to a carbon having an acetoxy group at δ4.62 (t, J=3 Hz, H_c) and signals due to protons attached to carbons having a hydroxy group at δ4.10 (br d, J=5 Hz, H_e) and 4.49 (dd, J=5 and 3 Hz after D_2O treatment, H_d). Besides the signals mentioned above and the signals due to an acetyl group (δ21.3 and 170.6), the ^{13}C-NMR spectrum of **1** showed signals due to three methyls, five methylenes, six methines and three quaternary carbons. These data, coupled with a consideration of the structures of diterpenoids isolated so far from the genus *Rabdosia*,[2] suggest that inflexanin A (**1**) has an 15-oxo-*ent*-kaurene structure as a basic skeleton. In fact, dihydroinflexanin A (**6**), obtained by catalytic hydrogenation of **1**, showed a negative Cotton effect in the optical rotatory dispersion (ORD) spectrum. The whole structure of inflexanin A was elucidated from the results of spin–spin decoupling and nuclear Overhauser effect (NOE)[6] experiments in the ^{1}H-NMR spectra. On irradiation at H_a or H_b, the signals due to H_b and H_f (δ3.09, 13-H) or those due to H_a and H_f were sharpened, respectively. On the other hand, the signals due to H_a and H_b were sharpened and the signal at δ2.27 (H_j, 12α-H) collapsed from a doublet of double doublets (J=14.5, 5 and 3 Hz) to a double doublet (J=14.5 and 5 Hz) on irradiation at the frequency of H_f. On irradiation at H_e, the signal due to H_j collapsed to a double doublet (J=14.5 and 3 Hz), and the signal due to H_e collapsed to a broad singlet on irradiation at H_j. These data suggested the structure around rings C and D, including the location of a β-axial secondary hydroxy group at C-11. This was further confirmed by the fact that NOE's (8 and 12%) were observed for H_e and H_g (δ2.78, 14α-H), respectively, on irradiation at δ1.42 (20-H_3). The proton H_c was presumed to be located between a methylene group and a quaternary carbon and to take an equatorial position. In fact, on irradiation at H_c, signals around δ1.5—1.6 changed. On irradiation at δ0.96 and 1.28 (19- and/or 18-H_3), NOE's for H_c (7 and 10%) were observed, leading to the assignment of H_c to the 3α-position. Consequently, the β-acetoxy group is located at C-3. On irradiation at H_d, a signal at δ2.31 (dd, J=3 and 14.5 Hz, H_j, 7β-H) collapsed to a doublet (J=14.5 Hz), suggesting that this equatorial proton might be located adjacent to a methylene group. The location was elucidated to be at the 6β position from the observation of NOE's for this proton (15 and 8.5%) on irradiation at δ0.96 and 1.28, respectively. On the basis of these data, the structure of inflexanin A was elucidated as *ent*-3α-acetoxy-6β,11α-dihydroxy-kaur-16-en-15-one (**1**).

Inflexanin B (**2**) was obtained as an amorphous powder, $[\alpha]_D$ −46.2° (MeOH), and the molecular formula was determined as $C_{22}H_{32}O_6$ on the basis of High-MS and fast atom bombardment mass spectrometry (FAB-MS). It also contained a five-membered ketone conjugated with an α-methylene group as judged from the following spectral data: UV 237.5 nm (ε 6618); IR ($CHCl_3$) 1725 and 1650 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 5.24 (H_c) and 6.04 (H_b, each 1H, br s); ^{13}C-NMR (C_5D_5N) δ 109.9 (t) and 152.2 (s) (*exo*-methylene), and 209.8 (ketone). Besides the signals due to three tertiary methyl groups (δ 1.09, 1.58 and 2.03) and an acetyl group (δ 1.95), the ^{1}H-NMR spectrum of **2** showed signals due to four secondary carbinyl protons [δ4.17 (dd, J=16 and 4 Hz, H_f), 4.72 (m, H_e), 4.95 (dd, J=3 and 3 Hz, H_d) and 6.18 (br d. like, J=3 Hz, H_a)], one of which is located on a carbon having an acetoxy group. The ^{13}C-NMR spectrum of **2** further showed the presence of three methyls, four methylenes, seven methines and three quaternary carbons in addition to an acetyl group and the above mentioned *exo*-methylene group conjugated with a ketone. These spectral data suggest that **2** also has the 15-oxo-*ent*-kaurene structure as a basic skeleton and are very similar to inflexinol (**4**) except for the number of acetyl groups. Acetylation of **2** gave the

diacetate (**5**) whcih was identical with inflexinol monoacetate, confirming the oxygenation pattern on the basic skeleton. The location of the acetyl group on the C-3β hydroxy group was determined by the comparison of the ^{1}H-NMR spectra of **2** and **4**. Namely, the signal (δ 4.87) assignable to the C-3α-equatorial proton in **4** was observed in almost the same region (δ 4.95) in **2**. On the other hand, the signal due to C-1-H in **4** was observed at δ 6.78 and the corresponding signal of **2** appeared at 0.6 ppm higher field (δ 6.18). On the basis of these data, the structure of inflexanin B should be represented as *ent*-3α-acetoxy-1α,6β,11β-trihydroxy-kaur-16-en-15-one (**2**).

Experimental

Melting points were determined with a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded with a Hitachi 215 spectrophotometer. ^{1}H-(200 MHz) and ^{13}C-(50 MHz)NMR spectra were obtained on a JEOL JNM FX-200 instrument. Chemical shifts are given in δ (ppm) values using tetramethylsilane as an internal standard. UV spectra were taken with a Hitachi 330 spectrophotometer. Optical rotation and ORD spectra were taken on a spectrometer, JASCO model ORD/UV-5. MS were determined with a JEOL JMS D-300 spectrometer. Kieselgel 60 (0.05—0.2 mm, Merck) was used for column chromatography and precoated silica gel plates F_{254} (0.25 and 0.5 mm in thickness) were used for thin layer chromatography (TLC). Extracts were dried over anhydrous $MgSO_4$.

Isolation of Inflexanin A (1) and Inflexanin B (2)——Dried aerial parts of *R. inflexa* (210 g), collected at Komagun, Yamanashi Prefecture, Japan in early October, 1983, were extracted with MeOH (4 l) for 2 weeks at room temperature. The plant material was then extracted again in the same manner. The combined methanolic extract was concentrated *in vacuo*. The residue was dissolved in 90% MeOH (300 ml) and the solution was washed with *n*-hexane (200 ml × 3). The 90% MeOH layer was concentrated *in vacuo*, the residue was suspended in H_2O (200 ml) and the mixture was extracted with AcOEt (250 ml × 3). After being washed with H_2O, the AcOEt extract was dried and evaporated *in vacuo* to give a residue (13 g), which was chromatographed on silica gel (200 g). The column was eluted first with $CHCl_3$ (2.2 l) and then successively with $CHCl_3$–Me_2CO (95 : 5, 1 l), $CHCl_3$–Me_2CO (9 : 1, 1.8 l), $CHCl_3$–Me_2CO (8 : 2, 0.8 l), and Me_2CO (1 l), collecting 100 ml fractions.

Fractions 16—23 were combined and evaporated *in vacuo* to give a residue (596 mg) which was chromatographed on a silica gel (60 g) column with Et_2O, collecting 7 ml fractions. Fractions 14—19 were combined and evaporated *in vacuo* to give a residue, which was separated by preparative TLC (solvent, Et_2O; developed three times) to furnish inflexinol (**4**) (193 mg) and inflexin (**3**) (107 mg).

Fractions 24—30 were combined and evaporaed *in vacuo* and the residue (69 mg) was purified by preparative TLC (solvent, Et_2O; developed twice) and by active charcoal treatment of an MeOH solution to give inflexanin A (**1**) (22 mg) as an amorphous powder.

Fractions 62—71 gave a residue (525 mg), which was purified by repeated column chromatography with $CHCl_3$–MeOH and Et_2O and preparative TLC ($CHCl_3$–Me_2CO (7 : 3), developed three times), to give inflexanin B (**2**) (54 mg) as an amorphous powder.

Inflexinol (**4**) and inflexin (**3**) were identified on the basis of comparisons of the spectral data with those of authentic samples. The data for the new diterpenoids are as follows.

Inflexanin A (**1**): An amorphous powder, $[\alpha]_D^{22}$ −108.3° (c = 0.12, MeOH). UV λ_{max}^{MeOH} nm (ε): 238 (5990). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3300, 1720, 1650, 1265. ^{1}H-NMR ($CDCl_3$) δ: 0.96, 1.28, 1.42 (each 3H, s, 3 × *tert*. Me), 2.07 (3H, s, OAc), 2.27 (1H, ddd, J = 14.5, 5, 3 Hz, 12α-H; H_j), 2.31 (1H, dd, J = 3, 14.5 Hz, 7β-H, H_i), 2.78 (1H, d, J = 12.5 Hz, 14α-H; H_g), 3.09 (1H, m, 13-H; H_f), 4.10 (1H, br d, J = 5 Hz, 11-H; H_e), 4.49 (1H, m, dd, J = 5, 3 Hz after D_2O treatment, 6-H; H_d), 4.62 (1H, t, J = 3 Hz, 3-H; H_c), 5.31 (H_b) and 5.91 (H_a) (each 1H, br s, 17-H_2). ^{13}C-NMR ($CDCl_3$) δ: 18.8 (q), 21.3 (q), 22.6 (t), 23.7 (q), 28.2 (q), 35.4 (t), 37.3 (d), 37.5 (s), 37.9 (t), 38.3 (s), 41.3 (t), 42.2 (t), 48.7 (s), 49.7 (d), 63.9 (d), 66.2 (d), 66.9 (d), 78.8 (d), 113.1 (t), 149.8 (s), 170.6 (s), 209.8 (s). MS *m/z*: Found 376.2238 (M^+). Calcd for $C_{22}H_{32}O_5$: 376.2250.

Inflexanin B (**2**): An amorphous powder, $[\alpha]_D^{26}$ −46.2° (c = 1.04, MeOH). UV λ_{max}^{MeOH} nm (ε): 237.5 (6618). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3575—3200, 1725, 1650, 1260. ^{1}H-NMR (C_5D_5N) δ: 1.09 and 1.58 (each 3H, s, 2 × *tert*-Me), 1.67 (1H, d, J = 1 Hz, 5-H), 1.73—1.91 (2H, 7α-H and 14β-H), 1.95 (3H, s, OAc), 2.03 (3H, s, *tert*-Me, 20-H_3), 2.12—2.42 (4H, 2-H_2 and 12-H_2), 2.52 (1H, br s, 9-H), 2.64 (1H, dd, J = 3, 14 Hz, 7β-H), 3.11 (1H, m, 13-H), 3.28 (1H, d, J = 12 Hz, 14α-H), 4.17 (1H, dd, J = 16, 4 Hz, 11-H; H_f), 4.72 (1H, m, 6-H; H_e), 4.95 (1H, dd, J = 3, 3 Hz, 3-H; H_d), 5.24 (1H, br s, 17-H_1; H_c), 5.90 (1H, d, J = 3 Hz, OH), 6.04 (1H, br s, 17-H_1; H_b), 6.18 (1H, br d-like, J = 3 Hz, 1-H; H_a). ^{13}C-NMR (C_5D_5N) δ: 14.9 (q), 21.0 (q), 23.9 (q), 28.4 (q), 34.1 (t), 37.8 (s), 38.7 (d), 39.7 (t), 41.7 (t), 43.7 (t), 44.9 (s), 49.4 (d), 49.7 (s), 65.4 (d), 66.4 (d), 67.2 (d), 77.3 (d), 80.1 (d), 109.9 (t), 152.2 (s), 170.3 (s), 209.8 (s). MS *m/z*: Found 374.2053 $(M-H_2O)^+$ and 332.2010 $(M-AcOH)^+$. Calcd for $C_{22}H_{30}O_5$ and $C_{20}H_{28}O_4$: 374.2093 and 332.1987. FAB-MS *m/z*: 415 $(M+Na)^+$ (+NaI) and 431 $(M+K)^+$ (+KI).

Dihydroinflexanin A (6)——Palladium carbon (5%, 10 mg) was added to a solution of inflexanin A (**1**) (9 mg) in MeOH (5 ml) and the mixture was stirred for 2 h in an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was evaporaed *in vacuo* to give a residue (8.3 mg), which was purified on a silica gel plate (solvent, $CHCl_3$–Me_2CO (9 : 1); developed twice) to give the dihydro derivative (**6**) (3.2 mg) as colorless needles, mp 243—246 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3450, 1730, 1380, 1255, 1042. ^{1}H-NMR ($CDCl_3$) δ: 0.95 and 1.27 (each 3H, s, 2 × *tert*-Me), 1.28 (3H, d, J=7 Hz, 17-H_3), 1.39 (3H, s, *tert*-Me), 2.06 (3H, s, OAc), 2.25 (1H, m, 16-H), 2.46 (1H, m, 13-H), 2.74 (1H, d, J=12 Hz, 14α-H), 3.97 (1H, dd, J=4, 2.5 Hz, 11-H), 4.44 (1H, dd, J=5, 3 Hz, 6-H), 4.61 (1H, dd, J=3, 3 Hz, 3-H). ORD λ_{max}^{MeOH} nm (ϕ): 332 (−1762), 292 (1453). MS *m/z*: Found 378.2407 (M^+). Calcd for $C_{22}H_{34}O_5$: 378.2407.

Inflexanin B Diacetate (5)——A solution of inflexanin B (**2**) (9.2 mg) in a mixture of acetic anhydride and pyridine (1 : 1, 0.4 ml) was stirred for 46 h at room temperature. Excess MeOH was added to the mixture to decompose the excess reagent and the solvent was evaporated off *in vacuo* to give a residue (13.8 mg), which was purified on a silica gel plate (solvent, $CHCl_3$–Me_2CO (9 : 1)) to give the diacetate (**5**) (4.0 mg) as colorless needles, mp 244—246 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3400, 1725, 1640, 1370, 1250, 1055. ^{1}H-NMR ($CDCl_3$) δ: 0.95, 1.32 and 1.62 (each 3H, s, 3 × *tert*-Me), 1.81, 2.00 and 2.11 (each 3H, s, 3 × OAc), 2.83 (1H, d, J=12.5 Hz, 14α-H), 3.07 (1H, m, 13-H), 4.48 (1H, m, 6-H), 4.68 (1H, dd, J=3, 3 Hz, 3-H), 4.90 (1H, dd, J=11, 5 Hz, 11-H), 5.24 (1H, br s, 17-H_1), 5.64 (1H, br d, J=3.5 Hz, 1-H), 5.88 (1H, br s, 17-H_1). This compound was identical with an authentic sample of inflexinol monoacetate[5] on the basis of mixed melting point determination and comparison of the IR spectra.

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6) Inflexanin A (**1**) (3.7 mg) was dissolved in $CDCl_3$ (0.5 ml), and an NOE experiment was performed using homogated decoupling conditions (flip angle 30°; irradiation time, 30 s; data points, 16K).

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Determination of Some Flavonoids in Scutellariae Radix by High-Performance Liquid Chromatography

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The determination of 7 kinds of flavonoids contained in Scutellariae Radix (ogon), *i.e.*, baicalin, baicalein, wogonin 7-*O*-glucuronide, wogonin, skullcapflavone II, oroxylin A, and oroxylin A 7-*O*-glucuronide, was performed by high-performance liquid chromatography. These substances could be determined within about 40 min on a C-18 reversed-phase column. Elution was carried out with mixtures of methanol, acetic acid and water in ratios of 5 : 5 : 90 and 90 : 5 : 5 in the form of a gradient from 6 : 4 to 2 : 8.

Keywords——flavonoid; baicalin; baicalein; Scutellariae Radix; ogon; high-performance liquid chromatography

Scutellariae Radix (ogon in Japanese) is the root of *Scutellaria baicalensis* GEORGI, with its bark removed. Ogon is usually mixed in dai-saiko-tou (a crude drug used for treatment of chronic diseases) and ouren-gedoku-tou (an antipyretic) to treat inflammation and fever.

Flavonoids are major components of ogon and about 40 kinds of flavonoids have been identified in it so far.[1] These flavonoids are known to have a broad range of physiological activities.[2] One of the authors morphopharmacologically studied the cholagogic effects of ogon's components (including extract) on bile canaliculi in rats.[3] Before beginning a study of the metabolism and distribution of these flavonoids in the body, we decided first to establish conditions for determination of the flavonoids of ogon by high-performance liquid chromatography (HPLC).

There are several methods with which to determine some of the flavonoids contained in ogon, such as thin-layer chromatography (TLC)[4,5] and HPLC.[5,6] Tomimori *et al.*[7] recently reported the determination of 11 flavonoids contained in ogon, including a microcomponent, using HPLC. Their method is effective for the separation and determination of a large number of components, but is too complex to be convenient, because each separation and determination must be performed individually. Therefore, we attempted to develop a method whereby we could simultaneously separate and determine as many components as possible. The present method for determination of the main flavonoids was consequently established.

Experimental

Preparations——Baicalin (I), baicalein (II), wogonin 7-*O*-glucuronide (III), wogonin (IV), oroxylin A 7-*O*-glucuronide (V), oroxylin A (VI), skullcapflavone II (VII), dihydrooroxylin A (VIII) and baicalein 7-*O*-glucoside (IX) were each separated from ogon.

Ogon——Several commercial preparations of ogon were obtained, as follows: ogons produced in Talien, Hopei

and Inner Mongolia; two types of ogon (sengon and rogon[8]) produced in Hopei and Tienchin. All of these ogons were obtained in the Osaka market.

Reagents——Extra-pure methanol and acetonitrile, acetic acid for microanalysis, and distilled water were used. To prepare an internal standard solution, 20 mg of 4-methylumbelliferone was precisely weighed and dissolved in 100 ml of 30% methanol.

Equipment——The apparatus consisted of pumps of the 510-type and 45J-type with a 680-type gradient controller (Nihon Waters, Ltd.), a column of NOVA PACK C-18 radial pack (8 mm i.d. × 100 mm), and a ultraviolet (UV) detector (KLC-2235, Kyowa Seimitsu, Ltd.), which was used at a wavelength of 275 nm and a sensitivity of 0.050 AU/FS.

Determination Method——Coarsely cut ogon was placed under decompression in a desiccator with silica gel in order to dry it. About 2.5 g of ogon was ground to below 30 mesh in size. The weighed ogon was extracted with 50 ml of 30% acetonitrile at 60—70 °C for 1 h; this procedure was repeated twice, then the three extracts were combined and the volume was adjusted to 250 ml by adding 30% acetonitrile. One milliliter of this solution and 2 ml of the internal standard solution were poured into a 10 ml measuring flask and the volume was adjusted to 10 ml by adding 15% acetonitrile. A 10 μl aliquot of this solution was injected into the liquid chromatograph. Linear gradient elution was performed for 40 min from a 6 : 4 ratio of solution A to solution B to a 2 : 8 ratio, at a flow rate of 1 ml/min; solution A consisted of methanol, acetic acid and water in the raio of 5 : 5 : 90, and solution B contained the same components in the ratio of 90 : 5 : 5.

Results and Discussion

A typical chromatogram is shown in Fig. 1. In this figure, because I and IX had almost the same retention times and could not be separated, they were taken to be I.

As the extraction solvent, 30% acetonitrile was used because the degree of turbidity was slight when the extract solution was cooled down. About 2.5 g of ogon was extracted with 50 ml of 30% acetonitrile at 60—70 °C for 1 h; this procedure was repeated three times, then the residue was again extracted, and this final extract solution was examined. The results revealed that residual I amounted to only 0.27—0.30% of total extracted I.

Calibration curves were obtained by the standard method. Every plot showed good linearity with correlation coefficient in the range of 0.997 to 0.999. The accuracy and precision of the proposed method were checked with I, II and IV. In five determinations, the recovery error of each flavonoid was less than 0.2 μg/ml and standard deviations were not more than 0.9 μg/ml for 48 μg/ml of I, 0.4 μg/ml for 8 μg/ml of II, and 0.3 μg/ml for 8 μg/ml of IV. In

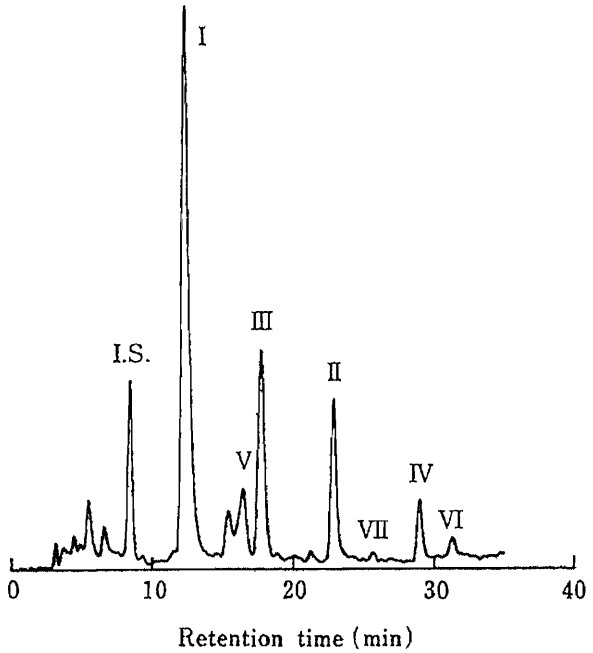

Fig. 1. Chromatogram of Flavonoids in Scutellariae Radix

I, baicalin; II, baicalein; III, wogonin 7-*O*-glucuronide; IV, wogonin; V, oroxylin A 7-*O*-glucuronide; VI, oroxylin A; VII, skullcapflavone II.

I.S., internal standard (4-methylumbelliferone); detector, 275 nm and 0.05 AU/FS; sample size, 10 μl; flow rate, 1.0 ml/min.

TABLE I. Determination of Flavonoids in Scutellariae Radix

	I	II	III	IV	V	VI	VII
					(Flavonoid mg/radix g)		
a) Produced in different place							
					(Mean±S.D. (n=3) of ground bulk)		
Talien	126.2± 5.4	13.8±4.6	37.7±1.9	7.9±3.9	12.0±0.6	3.3±1.8	nd
	152.2± 2.2	8.6±3.9	35.5±1.4	1.8±1.5	8.6±3.9	0.8±0.5	nd
	153.2± 4.4	24.3±0.9	35.6±3.2	6.2±1.2	15.0±2.1	4.9±0.8	nd
Hopei	189.9± 7.1	nd	43.5±1.8	nd	11.3±0.3	nd	nd
	188.0± 9.6	7.2±0.8	43.1±3.7	2.2±1.0	16.4±0.3	nd	nd
	206.5± 5.4	4.0±2.4	49.3±2.7	2.0±0.5	11.6±3.0	nd	nd
Inner Mongolia	109.7± 2.8	4.2±1.4	27.4±1.1	2.1±1.5	7.2±1.3	1.2±1.0	nd
	134.5± 3.0	2.7±0.5	37.5±0.5	1.5±0.4	10.6±0.9	1.1±1.0	nd
	149.5± 4.7	6.2±1.0	34.8±1.4	2.5±0.3	13.0±0.6	1.4±0.4	nd
b) Selected and not selected							
					(Mean±S.D. (n=6))		
Hopei (sengon)	141.2±15.5	5.1±1.7	37.3±2.1	1.8±0.8	7.7±2.3	1.2±0.4	0.6±0.5
Hopei (rogon)	119.7± 4.6	5.5±0.6	34.0±2.4	2.0±0.6	8.1±1.6	1.2±0.3	0.5±0.4
Hopei	191.8±14.3	4.9±0.8	44.6±9.8	1.2±0.2	12.8±2.8	1.1±0.3	0.2±0.2
Tienchin (sengon)	178.6± 7.1	6.5±1.3	46.0±5.4	1.7±0.5	10.8±7.0	1.1±0.3	nd
Tienchin (rogon)	121.6±15.4	5.5±0.5	32.6±2.4	1.3±0.3	12.0±0.3	0.5±0.5	nd
Tienchin	178.0± 8.3	14.3±6.3	49.1±7.0	4.6±1.7	10.1±0.6	2.3±0.6	nd

I, baicalin; II, baicalein; III, wogonin 7-*O*-glucuronide; IV, wogonin; V, oroxylin A 7-*O*-glucuronide; VI, oroxylin A; VII, skullcapflavone II; nd, less than 0.1 mg/g.

addition, the detection limit of IV was found to be 50 ng/ml at a S/N ratio of 2.

Table Ia shows the results for various samples of ogon without distinction between rogon and sengon, and Table Ib includes separate results for rogon and sengon. Compound VIII is not included in Table Ia or Ib because it was not detected in any sample. Compound I was the major component (56—60%); the glycosides, including I, III and V, accounted for 94.1—95.8% of the total. Measurement variations may be largely attributable to sampling errors, as the relative standard deviation of I in ogons produced in the same place was 18%.

Sengon contained slightly more glycosides than did rogon, and there was a significant difference in content of I between rogon and sengon.

Thus, 7 kinds of flavonoids contained in ogon at a content level of about 0.01% of the crude drug were simultaneously determined. The proposed method should be useful for investigation of the metabolism and distribution of ogon's flavonoids in the body.

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8) On the market, the form of ogon known as sengon consists of a thin section of the root to the tip with a substantial appearance and colored pale yellow-white, while ogon consisting of a thick part of the root and having a fragile and breakable central brown-colored part is called rogon.

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Effect of Methanol and Some Metal Compounds on the Frequency of Respiration-Deficient Mutation in Yeast Induced by Manganese Chloride

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The effect of methanol and KCl, NaCl, $MgCl_2$, $CaCl_2$ or $SrCl_2$ on the frequency of respiration-deficient (RD) mutation in yeast, *Saccharomyces cerevisiae*, induced by $MnCl_2$ was investigated. The frequency of RD mutation induced by 1×10^{-2} M $MnCl_2$ was increased from 9.5% to 59.6% by the addition of 4% methanol to the culture medium. However, the frequency of RD mutation induced by $MnCl_2$ was decreased by the addition of 1×10^{-2} M $MgCl_2$, $CaCl_2$ or $SrCl_2$ in the presence and absence of methanol. The growth-inhibitory effect of $MnCl_2$ was also partially reversed by the addition of these divalent metal compounds, whereas it was enhanced by methanol. Monovalent metal compounds, KCl and NaCl, had little effect on the RD mutation or growth inhibition by $MnCl_2$.

Uptake of Mn^{2+} by yeast cells was increased significantly by the addition of 4% methanol, and was decreased by $MgCl_2$, $CaCl_2$ or $SrCl_2$. Among these compounds, $CaCl_2$ decreased the uptake of Mn^{2+} most effectively.

Keywords——yeast; *Saccharomyces cerevisiae*; respiration-deficient mutation; growth inhibition; methanol-effect; metal compound; metal ion transport

In a previous paper,[1] we reported that the frequency of respiration-deficient (RD) mutation in yeast in the presence of 1×10^{-2} M $MnCl_2$ was increased by the addition of methanol to the culture medium, and that this increase was not observed when 1×10^{-2} M $MgCl_2$ was added with $MnCl_2$. It has been reported that Mg^{2+} is taken up preferentially over Mn^{2+} into yeast cells and that Mg^{2+} can totally inhibit Mn^{2+} uptake when the two cations are present at the same concentration.[2] These results suggested that the Mg^{2+}-dependent decrease of the frequency of RD mutation induced by Mn^{2+} resulted from the inhibition of Mn^{2+} uptake.[2] Though the uptake of Mn^{2+} was considered to be inhibited also by other divalent cations such as Co^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+} and Sr^{2+} transported by a relatively non-specific system,[2-4] the effect of these cations on the RD mutation by Mn^{2+} has not been tested.

In the present study, the relationship between the effect of these divalent cations on the frequency of RD mutaion induced by Mn^{2+} and the effect of these cations on the uptake of Mn^{2+} into yeast cells was examined. The effects of monovalent cations, K^+ and Na^+, were also tested because K^+ was reported to be slightly inhibitory to Mn^{2+} transport.

Experimental

Yeast Strain——*Saccharomyces cerevisiae* ATCC 26422 was used.

Chemicals——Methanol was of specially prepared grade (Nakarai Chemicals Co., Ltd.). Metal compounds were commercial preparations of guaranteed reagent grade.

Growth and Detection of RD Mutants——As described previously,[1] yeast cells were inoculated into Ogur's liquid

medium[5] at 1×10^5 cells/ml and incubated at 30 °C on a Monod shaker. $MnCl_2$ was added to the medium with or without methanol and each metal compound. After incubation for 24 h, cells were washed, diluted with water and spread onto Ogur's agar plates to give about 200 colonies per plate. Colonies of RD mutants were scored by the tetrazolium salt overlay method[6] after incubation at 30 °C for 2 d. Both completely white and sectored colonies were counted as RD mutants. The frequency of RD mutation was represented as the percentage of RD mutants in surviving cells. Results were expressed as averages of three independent experiments, each of which was done with 5 plates. Growth was given as the colony-forming cells per ml of culture incubated at 30 °C for 24 h.

Content of Mn^{2+} in Yeast——Cells were incubated at 30 °C for 24 h as described above, and washed three times with water. Dry weight was determined by filtering a cell suspension through a preweighed membrane filter and drying at 30 °C for 24 h. The dried cells were ashed in a muffle furnace. Mn^{2+} was oxidized to permanganate with ammonium peroxydisulfate in the presence of silver nitrate and determined spectrophotometrically at 525 nm.[7] The Mn^{2+} content in yeast cells was represented as μmoles of Mn^{2+} per 10 mg of dry cells.

Results

Isolation of RD Mutants Induced by $MnCl_2$

As shown in Table I, after incubation at 30 °C for 24 h in Ogur's liquid medium, the cell number reached 1.8×10^8/ml of culture. The frequency of RD mutants in the control was 1.4%. When 1×10^{-2} M $MnCl_2$ was added, the cell number in the 24-h culture was 4×10^7/ml and the frequency of RD mutation increased to 9.5%. At concentrations of less than 1×10^{-3} M $MnCl_2$, RD mutants were detected at the control level (data not shown).

Effect of Methanol on the Growth and Frequency of RD Mutation Induced by $MnCl_2$

The frequency of RD mutation induced by 1×10^{-2} M $MnCl_2$ was increased from 9.5% to

Table I. Effect of Metal Compounds on the Inhibition of Cell Growth and the Frequency of RD Mutation in Yeast Induced by Manganese Chloride

Compounds (1×10^{-2} M)		4% methanol	Growth ($\times 10^8$)	RD mutants (%)
—	—	−	1.8	1.4± 0.8
		+	2.0	0.9± 0.3
KCl	—	−	1.6	1.9± 0.4
		+	1.7	1.5± 0.1
NaCl	—	−	1.7	1.4± 0.8
		+	1.9	1.2± 0.4
$MgCl_2$	—	−	1.8	1.9± 0.8
		+	2.0	1.1± 0.5
$CaCl_2$	—	−	1.8	1.4± 0.5
		+	1.8	1.3± 0.5
$SrCl_2$	—	−	2.0	1.2± 0.6
		+	2.0	0.8± 0.6
—	$MnCl_2$	−	0.4	9.5± 2.6
		+	0.02	59.6± 8.2
KCl	$MnCl_2$	−	0.4	14.5± 7.3
		+	0.02	57.7±12.1
NaCl	$MnCl_2$	−	0.4	10.3± 6.9
		+	0.03	58.3±10.8
$MgCl_2$	$MnCl_2$	−	1.3	2.5± 1.5
		+	0.7	2.1± 1.9
$CaCl_2$	$MnCl_2$	−	1.4	2.3± 1.4
		+	1.5	2.1± 0.8
$SrCl_2$	$MnCl_2$	−	0.6	3.5± 0.2
		+	0.1	5.1± 0.6

Culture conditions are described in Experimental. Growth is given as the number of colony-forming cells per milliliter of culture incubated at 30 °C for 24 h. RD mutants were scored by the tetrazolium salt overlay method.[6] Results are the averages of at least three independent experiments ± S.D.

TABLE II. Effect of Metal Compounds on the Manganese Ion Content in Yeast Cells

Compounds (1×10^{-2} M)		4% methanol	Content of Mn^{2+} (μmol/10 mg of dry cells)
—	—	−	<0.01
		+	<0.01
—	$MnCl_2$	−	0.70±0.03
		+	1.19±0.23
KCl	$MnCl_2$	−	0.72±0.04
		+	1.02±0.13
NaCl	$MnCl_2$	−	0.67±0.05
		+	1.01±0.07
$MgCl_2$	$MnCl_2$	−	0.56±0.04
		+	0.63±0.06
$CaCl_2$	$MnCl_2$	−	0.08±0.03
		+	0.13±0.04
$SrCl_2$	$MnCl_2$	−	0.40±0.08
		+	0.45±0.14

Culture conditions are described in Experimental. Results are the averages of at least three independent experiments±S.D.

59.6% by the addition of 4% methanol. It should be stressed that 4% methanol alone did not induce any RD mutants. The final cell number was not decreased by the addition of 4% methanol alone. In the presence of both methanol and 1×10^{-2} M $MnCl_2$, the cell number of the 24-h culture was greatly decreased to only 2×10^6/ml (Table I), as compared with 4×10^7/ml in the presence of 1×10^{-2} M $MnCl_2$ alone, and 1.8×10^8/ml with neither.

Effect of Metal Compounds on the Growth and Frequency of RD Mutation Induced by $MnCl_2$

As shown in Table I, 1×10^{-2} M KCl, NaCl, $MgCl_2$, $CaCl_2$ and $SrCl_2$ showed little effect on final cell number or the frequency of RD mutation in yeast. The addition of $MgCl_2$, $CaCl_2$ and $SrCl_2$ to the culture medium decreased the frequency of RD mutation by $MnCl_2$ in the presence of methanol from 59.6% to almost the control level (2.1—5.1%). On the other hand, the frequency of RD mutation and growth inhibition by $MnCl_2$ were practically unaffected by KCl and NaCl in the presence or absence of methanol. The cell numbers in the 24-h culture with $MnCl_2$ recovered significantly when $MgCl_2$ or $CaCl_2$ was added.

Mn^{2+} Content in Yeast Cells

As shown in Table II, after the incubation at 30 °C for 24 h in Ogur's liquid medium containing 1×10^{-2} M $MnCl_2$, the Mn^{2+} content in yeast cells was 0.70 μmol per 10 mg of dry cells. The addition of 4% methanol to the medium caused an increase of Mn^{2+} content in yeast cells to about 1.7 times that in methanol-free medium. This Mn^{2+} content was practically not altered by the addition of 1×10^{-2} M KCl or NaCl to the culture medium containing $MnCl_2$. However, the addition of 1×10^{-2} M $MgCl_2$, $SrCl_2$ or $CaCl_2$ reduced the Mn^{2+} content in yeast cells to less than 0.70 μmol per 10 mg of dry cells. In particular, the Mn^{2+} content was only 0.08 μmol per 10 mg of dry cells in the presence of $CaCl_2$.

Discussion

Our results indicate that the frequency of RD mutation induced by Mn^{2+} was reduced by the addition of divalent cations, such as Mg^{2+}, Ca^{2+} and Sr^{2+}, but not by monovalent cations such as K^+ and Na^+. It has been reported that Mn^{2+} is transported into yeast cells by an energy-dependent uptake system for Mg^{2+},[2,4] which was found to be specific for divalent cations.[2] In the present study, it was confirmed that Mn^{2+} uptake by yeast cells was not

inhibited by K^+ or Na^+ but was by Mg^{2+}, Ca^{2+} and Sr^{2+}. This result supports the hypothesis that Mg^{2+} and other divalent cations decreased the induction of RD mutation by Mn^{2+} by inhibiting the uptake of Mn^{2+} due to competition for the divalent cation transport system. This inhibition of Mn^{2+} uptake is also in agreement with the finding in this paper that divalent cations antagonize Mn^{2+} in terms of the growth inhibition of yeast cells.

Sr^{2+} was less effective than Mg^{2+} or Ca^{2+} in decreasing the frequency of RD mutation by Mn^{2+} and in the restoration of cell growth (Table I). This result is consistent with the above conclusion, because divalent cations, such as Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+} and Sr^{2+}, can be taken up by the divalent cation transport system, and Sr^{2+} has the lowest affinity for the system.[4]

On the other hand, in spite of the fact that Mg^{2+} has the highest affinity for the divalent cation transport system,[4] the content of Mn^{2+} in yeast cells was higher in the presence of Mg^{2+} than in that of Ca^{2+} or Sr^{2+}. Therefore, the significant decrease in the frequency of Mn^{2+}-induced RD mutaion in the presence of Mg^{2+} could not be explained simply by the decrease of Mn^{2+} uptake by yeast cells. Putrament *et al.*[8] have suggested that the induction of RD mutants by Mn^{2+} resulted from a decrease in the fidelity of mitochondrial deoxyribonucleic acid (DNA) synthesis. They[9] have also reported that Mn^{2+} interacts with yeast mitochondrial DNA polymerase, which has a definite preference for Mg^{2+}.[10]

Recently Parkin and Ross[11] demonstrated that in another yeast, *Candida utilis*, Mn^{2+} uptake was highly specific and was unaffected by a 100-fold molar excess of Mg^{2+} or Ca^{2+}. This result contrasts with the relatively non-specific divalent cation transport observed in *Saccharomyces cerevisiae*.[2-4] The contrast may reflect the difference of Mn^{2+} concentration in the medium. Parkin and Ross[11] used a very low Mn^{2+} concentration (1×10^{-8} M) in their experiment. They assumed that at higher Mn^{2+} concentration, saturation of specific high-affinity transport systems of Mn^{2+} might occur and excess Mn^{2+} might be transported *via* the low-affinity Mg^{2+} transport system. Actually, competitive inhibition of Mn^{2+} transport by Mg^{2+}, Co^{2+} and Zn^{2+} was observed in *Candida utilis* at Mn^{2+} concentrations up to 5×10^{-5} M.[12]

The uptake of Mn^{2+} by yeast cells was significantly increased by the addition of methanol to the culture medium. Therefore, the primary site of action of methanol in enhancing the frequency of RD mutation by Mn^{2+} is the process of uptake or accumulation of Mn^{2+} in yeast cells rather than the process of expression of the RD phenotype[13] after the action of Mn^{2+}.

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Biological Activities of 3,3′-Dihydroxy-α,β-diethylstilbene and Its Isomer, Diethylstilbestrol

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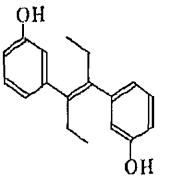
3,3′-Dihydroxy-α,β-diethylstilbene (I) and its isomer, diethylstilbestrol (II), showed antifungal activity against plant-pathogenic fungi, phytogrowth-inhibitory activity and a hypotensive effect on rats. First, I exhibited rather strong antifungal activity against plant-pathogenic fungi; the minimal inhibitory concentrations against *Fusarium oxysporum* f. sp. *lycopersici*. *Botryotinia fuckeliana* and *Pyrenophora graminea* were 4 μg/ml. On the other hand, the activity of II was weaker than that of I. Secondly, both compounds inhibited the growth of roots of two plants species even at low concentration of 50 ppm. Thirdly, I and II showed a hypotensive effect on rats (I, -23.33 ± 5.03 mmHg; II, -41.00 ± 2.40 mmHg; 10 mg/kg, i.v.). Unlike II, I has virtually no hormonal activity. It is noteworthy that in spite of the loss of hormonal character in going from II to I, the above-mentioned activities are fully retained.

Keywords——3,3′-dihydroxy-α,β-diethylstilbene; diethylstilbestrol; biological activity; antifungal activity; plant-pathogenic fungus; phytogrowth-inhibitory activity; hypotensive effect; hormonal side effect; phospholipidosis

Various oxystilbene compounds such as 3,3′,4,5′-tetrahydroxystilbene (III, Chart 1),[2,3] 3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene (IV, Chart 1)[4,5] and diethylstilbestrol (II, Chart 1)[6,7] have been reported to have the following biological activities by the authors; antifungal activity, phytogrowth-inhibitory activity, coronary vasodilator action on the isolated quinea-pig heart, hypotensive effect on rats and ichthyotoxicity. Among these



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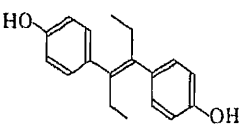
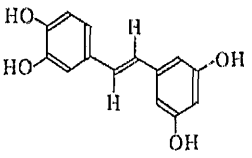
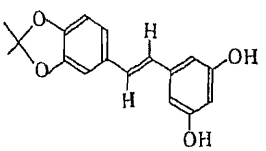


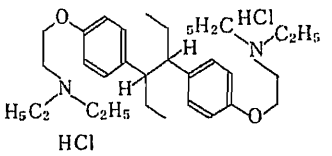
diethylstilbestrol (II)



3,3′,4,5′-tetrahydroxystilbene (III)



3,4-*O*-isopropylidene-3,3′,4,5′-tetrahydroxystilbene (IV)



Coralgil (V, hexestrol bis-(β-diethylaminoethyl ester) dihydrochloride)

Chart 1

compounds, II showed the strongest activities; but it had a hormonal side effect. Furthermore, Coralgil (hexestrol bis(β-diethylaminoethyl ether) dihydrochloride, V, Chart 1) a coronary vasodilator having the same basic skeleton as I and II, was found to have the severe side effects of phospholipidosis of the liver[8] and lung[9] cells, and foam cell syndrome.[10] Therefore, since 1970 its clinical use has been discontinued.

Subsequently, we synthesized various oxystilbene compounds in order to obtain more active substances. As a result, in the previous paper,[11] we reported that 3,3′-dihydroxy-α,β-diethylstilbene (I, Chart 1), an isomer of II, had rather strong ichthyotoxicity and coronary vasodilator action on the isolated guinea-pig heart. However, in contrast to II, I shows almost no side effect.[12] In this respect, the biological activities of I are noteworthy. Therefore, the antifungal activity on plant-pathogenic fungi, the phytogrowth-inhibitory activity and the hypotensive effect on rats of I were examined in detail in comparison with those of II. The results are presented here.

Materials and Methods

Chemicals——3,3′-Dihydroxy-α,β-diethylstilbene (I) and diethylstilbestrol (II, Aldrich Chemical Co., Ltd.) were used for the biological activity tests. Compound I was synthesized according to the method of Dodds *et al.*[13] I: mp 176—178 °C (reported value: mp 176 °C). ^{1}H-NMR ($CDCl_3$, δ ppm): 0.77 (6H, t, J=8 Hz, 2×-CH_3), 2.13 (4H, q, J=8 Hz, 2×-CH_2-), 6.65—7.25 (8H, m, arom H), 4.65 (2H, s, 2×-OH). Sodium 2,4-dichlorophenoxyacetate (Tokyo Kasei Co., Ltd.) was used as a standard for the phytogrowth-inhibitory activity test.

Organisms——The plant-pathogenic fungi were as follows: *Botryotinia fuckeliana* IFO-9760, *Pyrenophora graminea* IFO-6633, *Rhizoctonia solani* IFO-30464, *Cochliobolus miyabeanus* IFO-4870, *Ceratocystis fimbriata* IFO-4864, *Fusarium oxysporum* f. sp. *lycopersici* IFO-6531 and *Aureobasidium pullulans* IFO-4464. The plants used were *Brassica rapa* and *Raphanus sativus* L. var. *raphanistroides* MAKINO. The animals used for the experiments on blood pressure were male Wistar strain rats weighing 305—355 g (3 rats/group).

Biological Activity Tests——1) Antifungal Activity Test[5]: Antifungal activity was tested by the agar dilution method. The media used were as follows: potato sucrose agar in all cases except for *Fusarium oxysporum* f. sp. *lycoprsici* (potato dextrose agar: Eiken Chemical Co., Ltd.). The test fungi were applied to media containing various concentrations of I and II. The plates were incubated at 27 °C for 5 d and the growth was observed with the naked eye.

2) Phytogrowth-Inhibitory Activity Test[14]: Aliquots (1 ml) of acetone solutions of I, II and sodium 2,4-dichlorophenoxyacetate were each diluted in 100 ml of sterilized agar (0.8%, Difco Chemical Co., Ltd.) to the concentration of 50 ppm. The agar containing chemicals or acetone alone (control) was poured into a 500 ml sterilized beaker covered with aluminum foil. Then, 20 seeds of each plant sterilized with 70% EtOH and 1% NaClO were put on the agar and left for 7 d under 600 lux illumination. The lengths of the roots were measured and averaged. The phytogrowth-inhibitory activity was expressed as the ratio of the length of roots to that of the control (1.00).

3) Measurement of Blood Pressure[5]: Systemic blood pressure was measured with a pressure transducer (Nihon Kohden Kogyo Co., Ltd. P-23 ID, AP-601 G) following cannulation of the carotid artery in ras under anesthesia with sodium pentobarbital (40 mg/kg, i.p.). Compounds I and II were suspended in 5% acacia and administered *via* the femoral vein. It was shown that 5% acacia has no effect on blood pressure.

Results

Antifungal Activities of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II) on Plant-Pathogenic Fungi

The antifungal activities of 3,3′-dihydroxy-α,β-diethylstilbene (I) and diethylstilbestrol (II) were investigated by the agar dilution method. The results are summarized in Table I. Compounds I and II showed antifungal activities against all plant-pathogenic fungi tested except for *Ceratocystis fimbriata*. The activity of I was stronger than that of II. The minimal inhibitory concentrations (MIC) of I for *Fusarium oxysporum* f. sp. *lycopersici*, *Botryotiana fuckeliana* and *Pyrenophora graminea* were 4 μg/ml.

Phytogrowth-Inhibitory Activities of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II)

The inhibitory effects of 3,3′-dihydroxy-α,β-diethylstilbene (I) and diethylstilbestrol (II)

TABLE I. Antifungal Activities of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II) on Plant-Pathogenic Fungi

Fungi	MIC (μg/ml)	
	I	II
Fusarium oxysporum f. sp. *lycopersici* IFO-6531	4.0	7.0
Botryotinia fuckeliana IFO-9760	4.0	50.0
Pyrenophora graminea IFO-6633	4.0	10.0
Aureobasidium pullulans IFO-4464	25.0	25.0
Cochliobolus miyabeanus IFO-4870	25.0	50.0
Rhizoctonia solani IFO-30464	25.0	50.0
Ceratocystis fimbriata IFO-4864	1000.0	>1000.0

Culture conditions: 27 °C, 5 d. Media: potato sucrose agar (*Fusarium oxysporum* f. sp. *lycopersici* IFO-6531, potato dextrose agar). Method: agar dilution method.

TABLE II. Inhibitory Effects of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II) on Plant Growth

Plant	Growth (ratio)[a]		
	I	II	2,4-D[b]
Brassica rapa L.	0.46	0.43	0.06
Raphanus sativus L. var. *rahanistroides* MAKINO	0.83	0.65	0.10

a) Growth in control experiments after 7 d was taken as 1.00. Concentration: 50 ppm. Quantity of light: 600 lux. Experimental size: 20 seeds/group, 2 groups.
b) Sodium 2,4-dichlorophenoxyacetate.

TABLE III. Effects of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II) on Blood Pressure in Rats

Dose (mg/kg)	Mean arterial blood pressure (mmHg)	
	I	II
10	−23.33±5.03	−41.00±2.40
20	−45.67±2.52	−48.00±14.0
30	−52.66±0.58	−51.00±12.0

Each value represents the mean±S.D. of 3 rats. Route: Intravenous injection.

on two plant species were examined according to the previous paper.[14] As shown in Table II, compounds I and II inhibited the growth of roots both kinds of plants even at the low concentration of 50 ppm, but their activities were weaker than that of sodium 2,4-dichlorophenoxyacetate used as a standard.

Effect of 3,3′-Dihydroxy-α,β-diethylstilbene (I) and Diethylstilbestrol (II) on Blood Pressure in Rats

The effects of 3,3′-dihydroxy-α,β-diethylstilbene (I) and diethylstilbestrol (II) on blood pressure in rats were investigated. The results are summarized in Table III. Both compounds showed hypotensive action. In the group given 10 mg/kg, the action of II was stronger than that of I. However, at the doses of 20 and 30 mg/kg, I showed almost the same activity as II. The hypotensive actions of both compounds were transient and the blood pressure recovered to the original level within 5 min. The patterns of the hypotensive actions of both compounds were similar to those of III[3] and IV.[5]

Discussion

Like other oxystilbene compounds,[2-5] 3,3′-dihydroxy-α,β-diethylstilbene (I) and diethylstilbestrol (II) showed antifungal activity on plant-pathogenic fungi, as well as

phytogrowth-inhibitory activity and hypotensive action on rats.

First, compounds I and II showed rather strong antifungal activity against plant-pathogenic fungi (Table I). The antifungal activities of both compounds on plant-pathogenic fungi might be intrinsic to oxystilbene compounds. This view is supported by the findings that 3,4-*O*-isopropylidene-3,3′4,5′-tetrahydroxystilbene (IV),[5] which has the same skeleton as I, exhibited strong antifungal activity against plant-pathogenic fungi, and pinosylvine[15] and pterostilbene,[16] which also have the same skeleton as I, were reported to be phytoalexins. Further studies on the antifungal activities of many oxystilbene compounds against plant-pathogenic fungi should be fruitful.

Secondly, compounds I and II inhibited the growth of roots of two species of plants even at low concentration of 50 ppm (Table II). As regards phytogrowth-inhibitory activity of oxystilbene compounds, 3,3′,4,5′-tetrahydroxystilbene (III)[2] was reported to have rather strong activity. Plant growth regulators such as caffeic acid,[17] ferulic acid[17] and chlorogenic acid,[17] which have a partial structure in common with I, have been isolated from higher plants. Batatasin III[18] and lunularin,[19] having the same basic skeleton as I and II, were found to have phytogrowth-inhibitory activities. These findings suggest that oxystilbene compounds generally have phytogrowth-inhibitory activity. Further studies on the phytogrowth-inhibitory activity of many compounds having the same partial structures as I are in progress.

Thirdly, compounds I and II had a hypotensive effect on rats (Table III). The hypotensive effects of both compounds on rats were considered to be intrinsic to oxystilbene compounds, because II,[20] III[3] and IV[5] also show similar effects. In addition to the above-mentioned oxystilbene compounds, stilbamidine[21] and dimethylstilbamidine,[21] antiprotozal substances which are also stilbene compounds, show potent hypotensive activity. We are now investigating the hypotensive effects of many oxystilbene compounds.

It is very interesting that the above-mentioned activities of I were retained in spite of the loss of the hormonal side effect on shifting the phenolic hydroxyl group to the *m*-position (from II to I).[12] However, as stated above, Coralgil (V), in spite of its strong coronary vasodilator action, is no longer used clinically because of the severe side effects of phospholipidosis of the liver[8] and lung[9] cells, and foam cell syndrome.[10] Thus, I and other oxystilbene compounds (II—IV) need to be tested carefully for similar side effects. Such a study on I and other oxystilbene compounds (II—IV) is in progress.

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Effects of Long-Chain Fatty Acids and Fatty Alcohols on the Growth of *Streptococcus mutans*

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Minimal inhibitory concentrations (MICs) of a series of fatty acids and fatty alcohols against a cariogenic bacterium, *Streptococcus mutans*, were determined by a tube dilution technique. Among saturated fatty alcohols, tetradecanol and pentadecanol had the highest activity (MIC, 1.56 μg/ml), while among monounsaturated fatty alcohols, 10*Z*-pentadecenol had the strongest activity (MIC, 0.78 μg/ml). Saturated fatty acids showed relatively weak activity; tridecanoic acid had the highest activity among them (MIC, 12.5 μg/ml). Among unsaturated fatty acids, 10*Z*-heptadecenoic, 6*Z*-octadecenoic, 11*Z*-octadecenoic and 9*Z*,12*Z*-octadecadienoic acids had potent activity (MIC, 3.13 μg/ml). The antibacterial activities of methyl-branched and hydroxyl fatty acids as well as long chain dicarboxylic acids were also investigated.

Keywords——antibacterial action; dental caries; fatty acid; fatty alcohol; plaque control; *Streptococcus mutans*

The effects of fatty acids (FACs) and fatty alcohols (FALs) of various chain lengths on the growth of microbes have been reported by many workers.[1-7] The growth of a variety of gram-positive bacteria is inhibited by long-chain FACs and FALs in general but the sensitivity of the bacteria depends strongly on the chain length of these compounds.[1] A partial study on the antibacterial action of unsaturated FACs against a cariogenic bacterium, *Streptococcus mutans*, has been reported by Fukui *et al.*[7] This bacterium is the primary cause of dental caries; it adheres to the tooth surface to form dental plaque and then the enamel is dissolved by organic acids produced by the cellular carbohydrate metabolism.[8]

In the course of basic studies on dental caries prevention by naturally occurring substances[9-15] we have now examined the effects of a series of long-chain FACs and FALs, including saturated, unsaturated, methyl-branched and hydroxylated ones, on the growth of *S. mutans*. The results are presented here.

Materials and Methods

Chemicals——Saturated and unsaturated FACs/FALs were purchased from Funakoshi Yakuhin Co., Tokyo, Japan. Methyl-branched FACs and hydroxy-FACs were obtained from Larodan Fine Chemicals AB, Malmö, Sweden. All FACs and FALs were of the highest grade available (purity, above 98%).

Minimal Inhibitory Concentration (MIC)——Two-fold serial dilutions of FACs (free form) and FALs were prepared by diluting the original methanolic solutions (2 mg/ml) with the same solvent. The dilutions (0.1 ml each) were added to brain heart infusion (BHI; Difco Laboratories, Detroit, U.S.A.) broth containing precultured *S. mutans* cells (*ca.* 10^6 cells/ml). The mixtures were cultured for 48 h at 37 °C. MICs were determined by visually judging the bacterial growth in the series of test tubes.[9]

Results

Effect of FALs on the Growth of *S. mutans*

According to the tube dilution technique, MICs of various saturated FALs in BHI broth were investigated for *S. mutans*. Among the FALs examined (Table I), tetradecanol and pentadecanol showed the strongest inhibition of the growth of *S. mutans* (serotype c) which is the most frequent type detected in dental caries among Japanese,[16] the MICs being 1.56 μg/ml. Other saturated FALs with higher or lower carbon numbers had weaker inhibitory action.

Among the various monounsaturated FALs examined (Table II), 10*Z*-pentadecenol showed the strongest inhibition on the growth of *S. mutans*, its MIC being 0.78—1.56 μg/ml. As in the case of the FALs, a plot of the logarithm of the MIC *versus* the carbon number for the *Z*-monounsaturated FALs showed a parabolic curve centered around C_{15}. Among the unsaturated C_{18} FALs, none of the monoenols examined (6*Z*, 6*E*, 9*Z*, 9*E*, 11*Z* and 11*E*)

TABLE I. MICs of Saturated Fatty Alcohols and Saturated Fatty Acids against *S. mutans* MT 5091

Chain length: No. of double bonds	Fatty alcohol	MIC (μg/ml)	Fatty acid	MIC (μg/ml)
$C_{8:0}$	Octanol	100	Octanoic acid	>200
$C_{9:0}$	Nonanol	100	Nonanoic acid	>200
$C_{10:0}$	Decanol	25	Decanoic acid	>100
$C_{11:0}$	Undecanol	12.5	Undecanoic acid	>100
$C_{12:0}$	Dodecanol	6.25	Dodecanoic acid	100
$C_{13:0}$	Tridecanol	3.13	Tridecanoic acid	12.5
$C_{14:0}$	Tetradecanol	1.56	Tetradecanoic acid	25
$C_{15:0}$	Pentadecanol	1.56	Pentadecanoic acid	25
$C_{16:0}$	Hexadecanol	>100	Hexadecanoic acid	>100
$C_{17:0}$	Heptadecanol	>100		
$C_{18:0}$	Octadecanol	>100		

The experiments were done in triplicate. Under the same conditions, chlorhexidine gluconate showed an MIC of 0.78 μg/ml.

TABLE II. MICs of Unsaturated Fatty Alcohols against *S. mutans* MT5091

Chain length: No. of double bonds	MIC (μg/ml)			
	Z-Form		*E*-Form	
$C_{11:1}$	10*Z*-Undecenol	50.0		
$C_{12:1}$	11*Z*-Dodecenol	3.13		
$C_{13:1}$	12*Z*-Tridecenol	3.13		
$C_{14:1}$	9*Z*-Tetradecenol	6.25	9*E*-Tetradecenol	6.25
$C_{15:1}$	10*Z*-Pentadecenol	0.78—1.56		
$C_{16:1}$	9*Z*-Hexadecenol	1.56	9*E*-Hexadecenol	25.0
$C_{17:1}$	10*Z*-Heptadecenol	12.5		
$C_{18:1}$	6*Z*-Octadecenol	100	6*E*-Octadecenol	>100
$C_{18:1}$	9*Z*-Octadecenol	>100	9*E*-Octadecenol	>100
$C_{18:1}$	11*Z*-Octadecenol	>100	11*E*-Octadecenol	>100
$C_{18:2}$	9*Z*,12*Z*-Octadecadienol	12.5	9*E*,12*E*-Octadecadienol	100
$C_{18:3}$	6*Z*,9*Z*,12*Z*-Octadecatrienol	0.78		
$C_{18:3}$	9*Z*,12*Z*,15*Z*-Octadecatrienol	1.56		

showed antibacterial activity, but the trienols (6*Z*, 9*Z*, 12*Z* and 9*Z*, 12*Z*, 15*Z*) had potent antibacterial action (MICs, 0.78—1.58 μg/ml) and the dienols (9*Z*, 12*Z* and 9*E*, 12*E*) showed moderate activity.

Effect of FACs on the Growth of *S. mutans*

Saturated FACs did not show potent inhibitory action on the growth of *S. mutans* (Table I). Tridecanoic acid, however, had moderate antibacterial action with an MIC of 12.5 μg/ml. With higher or lower carbon numbers in the chain, the activity decreased significantly.

Among the monounsaturated FACs examined (Table III), 10*Z*-heptadecenoic and 6*Z*-octadecenoic acids strongly inhibited the growth of *S. mutans* with an MIC of 3.13 μg/ml. A comparison of the antibacterial effects of *Z*- and *E*-isomers of the unsaturated FACs showed that the *Z*- and *E*-forms of tetradecenoic and hexadecenoic acids had identical MIC, but the *Z*-forms of octadecenoic and octadecadienoic acids had stronger antibacterial action than the corresponding *E*-isomers. In contrast to the case of the unsaturated C_{18} FALs, the *Z*-monoenoic acids (6*Z* and 11*Z*) and *Z*,*Z*-dienoic acid (9*Z*,12*Z*) showed higher antibacterial activity than the *Z*,*Z*,*Z*-trienoic acids (6*Z*, 9*Z*, 12*Z* and 9*Z*, 12*Z*, 15*Z*), except for 9*Z*-octadecenoic acid.

Table IV shows the MICs of iso- and anteiso-FACs with a methyl branch at the ω and ω-1 positions, respectively, against *S. mutans*. 13-Methyltetradecanoic acid had the highest

TABLE III. MICs of Unsaturated Fatty Acids against *S. mutans* MT5091

Chain length: No. of double bonds	MIC (μg/ml)			
	Z-Form		*E*-Form	
$C_{12:1}$	11*Z*-Dodecenoic acid	>200		
$C_{13:1}$	12*Z*-Tridecenoic acid	>100		
$C_{14:1}$	9*Z*-Tetradecenoic acid	25.0	9*E*-Tetradecenoic acid	25.0
$C_{15:1}$	10*Z*-Pentadecenoic acid	12.5		
$C_{16:1}$	9*Z*-Hexadecenoic acid	6.25	9*E*-Hexadecenoic acid	6.25
$C_{17:1}$	10*Z*-Heptadecenoic acid	3.13		
$C_{18:1}$	6*Z*-Octadecenoic acid	3.13	6*E*-Octadecenoic acid	100
$C_{18:1}$	9*Z*-Octadecenoic acid	12.5	9*E*-Octadecenoic acid	100
$C_{18:1}$	11*Z*-Octadecenoic acid	3.13	11*E*-Octadecenoic acid	>100
$C_{18:2}$	9*Z*,12*Z*-Octadecadienoic acid	3.13	9*E*,12*E*-Octadecadienoic acid	25.0
$C_{18:3}$	6*Z*,9*Z*,12*Z*-Octadecatrienoic acid	6.25		
$C_{18:3}$	9*Z*,12*Z*,15*Z*-Octadecatrienoic acid	6.25		
$C_{19:1}$	7*Z*-Nonadecenoic acid	>100	7*E*-Nonadecenoic acid	>100

TABLE IV. MICs of Methyl-Branched Fatty Acids against *S. mutans* MT5091

Iso-fatty acid	MIC (μg/ml)	Anteiso-fatty acid	MIC (μg/ml)
10-Methylundecanoic acid	100	9-Methylundecanoic acid	100
11-Methyldodecanoic acid	100	10-Methyldodecanoic acid	25
12-Methyltridecanoic acid	12.5	11-Methyltridecanoic acid	12.5
13-Methyltetradecanoic acid	3.13	12-Methyltetradecanoic acid	3.13
14-Methylpentadecanoic acid	6.25	13-Methylpentadecanoic acid	1.56
15-Methylhexadecanoic acid	50	14-Methylhexadecanoic acid	3.13
16-Methylheptadecanoic acid	>200	15-Methylheptadecanoic acid	>200
17-Methyloctadecanoic acid	>200	16-Methyloctadecanoic acid	>200
18-Methylnonadecanoic acid	>200		

antibacterial activity in the iso series (MIC, 3.13 μg/ml), while 13-methylpentadecanoic acid was the most potent in the anteiso series (MIC, 1.56 μg/ml). Other methyl-branched FACs with higher and lower carbon numbers in both series were less inhibitory. The anteiso-FACs had stronger antibacterial action than the corresponding iso-FACs in general.

Saturated and unsaturated hydroxy-FACs had no significant inhibitory action on the growth of *S. mutans*, though only a limited number of hydroxy fatty acids were examined.[17]

Long-chain dicarboxylic acids such as dodecanedioic and tridecanedioic acids and an ω-amino carboxylic acid, such as 12-aminododecanoic acid, were not inhibitory to the growth of *S. mutans* at concentrations of less than 100 μg/ml.[17]

Discussion

The homologous series of FACs and FALs showed a characteristic pattern of inhibitory effect against *S. mutans* depending on their chain lengths. In the series of saturated and unsaturated FACs and FALs, the curves of ln(MIC) *versus* chain length were parabolic. A similar chain length-activity relationship of FACs and FALs was recently reported for larvicidal activity against *Toxocara canis* (dog round worm) by Kiuchi *et al.*[18] Long-chain dicarboxylic acids, dodecanedioic acid and tridecanedioic acid, had no inhibitory action against *S. mutans*. This finding is in agreement with the findings on *Neisseria gonorrhoeae*, which is less susceptible to *n*-hexanedioic acid and *n*-heptanedioic acid than to the corresponding monocarboxylic acids.[3] The presence of a polar group at both ends of the non-polar chain may prevent the binding of the molecule to the membrane of the cells. The mechanism of antibacterial action by long-chain FACs and FALs is not fully understood, but it may involve changes in bacterial cell membrane permeability caused by adsorption of FACs and FALs. Long-chain FACs and FALs occur in nature as components of fats and waxes, and are ingested in the normal diet. Our results show that some FACs and FALs have significant antibacterial action against *S. mutans*. It might be possible to develop these compounds as anti-cariogenic bacterial agents for dental caries prevention.

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Effect of the Molecular Weight of Polyethylene Glycol on the Bioavailability of Indomethacin Sustained-Release Suppositories Prepared with Solid Dispersions[1)]

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Conventional suppositories were prepared by using polyethylene glycol (PEG) alone, and sustained-release suppositories were prepared by using hydroxypropylmethylcellulose phthalate (HP55)–PEG 1000, 2000 and 4000 matrices. The effect of the molecular weight of PEG on the *in vitro* release characteristic and bioavailability in rabbits of indomethacin (IM) in those suppositories was investigated. The release rate of IM from the conventional and matrix suppositories decreased slightly with increase of the molecular weight of PEG. The bioavailability of the conventional suppositories was independent of the molecular weight of PEG, but the bioavailability of the matrix suppositories decreased with increase of the molecular weight of PEG.

Keywords——indomethacin; sustained-release suppository; solid dispersion; hydroxypropylmethylcellulose phthalate–polyethylene glycol matrix; molecular weight; X-ray diffraction; *in vitro* release rate; rectal administration; bioavailability

Solid dispersion techniques have been employed in order to increase the solubility, dissolution rate and bioavailability of poorly water-soluble and water-insoluble drugs.[2-4)] Polyethylene glycol (PEG) has often been used as the water-soluble carrier for solid dispersion systems,[5-7)] but comparisons of the relative effectiveness of different molecular weights of PEG have been inconclusive.[8)] Increase of the molecular weight of PEG decreased the dissolution of spironolactone[9)] and digoxin[10)] from solid dispersions, but enhanced the dissolution of papaverine[11)] and chlorothiazide.[12)]

In the previous paper,[13)] we reported that indomethacin (IM) suppositories, prepared by using a solid dispersion of PEG 2000 as a water-soluble carrier and hydroxypropylmethylcellulose phthalate 200731 (HP55) as a poorly water-soluble carrier, showed a desirable sustained-release characteristic with good bioavailability.

The purpose of the present study was to investigate the effect of the molecular weight of PEG on the release characteristics and bioavailability of IM in conventional suppositories prepared by using PEG alone and sustained-release suppositories prepared by using HP55–PEG matrices.

Experimental

Materials——IM was a gift from Sumitomo Pharmaceutical Co., Ltd., HP55 (kinematic viscosity: 40 cSt) was supplied by Shin-etsu Chemical Co., Ltd. and PEG 1000, 2000 and 4000 were purchased from Wako Pure Chemical Ind., Ltd. All other chemicals were reagent-grade commercial products.

Preparation of Suppositories——1) Conventional Suppositories (C-1000, C-2000 and C-4000): These sup-

positories were prepared by the fusion method with PEG 1000, 2000 and 4000 as a base, respectively.

2) Matrix Suppositories (M-1000, M-2000 and M-4000): These suppositories were prepared by the fusion method as follows. Physical mixtures of HP55 (2 g) and PEG of various molecular weights (7.75 g) were prepared. These mixtures were heated at 120 °C with occasional stirring until clear homogeneous fusions were formed. Then, IM (0.25 g) was melted in the HP55-PEG fusions, and the fusions were quickly poured into steel molds and allowed to solidify at room temperature. HP55–PEG 1000, HP55–PEG 2000 and HP55–PEG 4000 matrix suppositories were termed M-1000, M-2000 and M-4000, respectively.

The weight and IM content of all suppositories were 1 g and 25 mg, respectively. All suppositories were stored in a desiccator at room temperature, and were administered within 24 h after preparation.

X-Ray Diffractometry——For determinations of the crystallinity of IM in the matrices, parts of the fusions of each suppository prepared as described above were poured into aluminum holders, then solidified at room temperature. X-Ray diffraction spectra were determined with an X-ray diffractometer (Miniflex, Rigaku Denki, Ltd.; Cu-K_α radiation, 30 kV, 10 mA).

Release Test of Suppositories *in Vitro*——The release test was carried out by using a suppository release test apparatus (Toyama Ind., Ltd.) at 37 °C according to the method previously reported.[13] Five hundred milliliters of 0.1 M phosphate buffer solution (pH 7.2, $\mu=0.5$, NaCl) was used as the test solution.

Animal Experiments——White male rabbits weighing from 2.5 to 3.8 kg were fasted for 36 h prior to the experiments but were allowed free access to water. After rectal administration of a suppository, blood samples were taken from the ear vein at regular intervals. The plasma samples were frozen and stored at −5 °C until assay.

Measurements of IM in Plasma——The determination of IM concentration in plasma was performed by high-performance liquid chromatography as reported in the previous paper.[13]

Results and Discussion

Crystallinity of IM in Matrices

Figure 1 shows the X-ray diffraction spectra of IM powder alone and the IM–PEG matrices (IM: 2.5%). IM powder showed the characteristic crystal peaks at 11.5, 16.6 and 21.8 ° (2θ). On the other hand, IM–PEG 1000, IM–PEG 2000 and IM–PEG 4000 matrices did

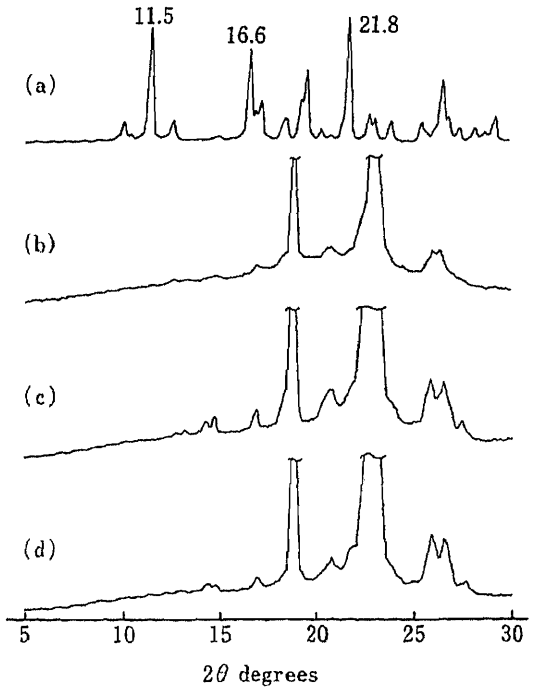

Fig. 1. X-Ray Diffraction Spectra of IM–PEG Matrices

(a) IM powder alone, (b) IM–PEG 1000, (c) IM–PEG 2000, (d) IM–PEG 4000, IM content 2.5%. Range: (a) 240000 cpm; (b—d) 120000 cpm.

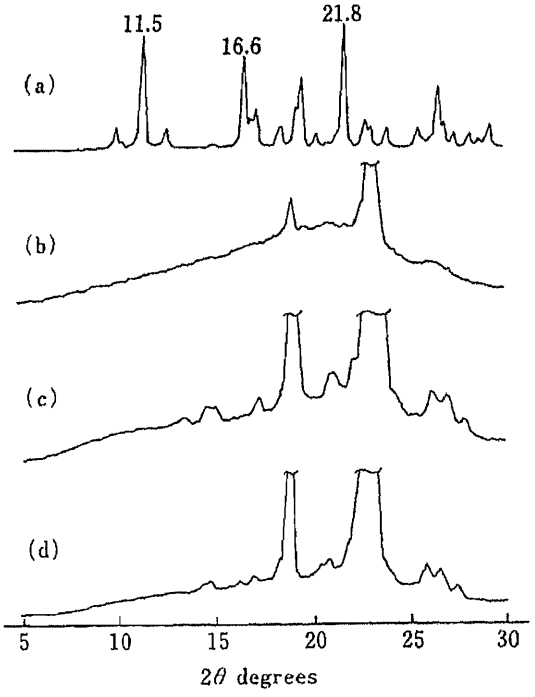

Fig. 2. X-Ray Diffraction Spectra of IM–HP55–PEG Matrices

(a) IM powder alone, (b) IM–HP55–PEG 1000, (c) IM–HP55–PEG 2000, (d) IM–HP55–PEG 4000, IM content 2.5%, HP55 content 20%. Range: (a) 240000 cpm; (b—d) 120000 cpm.

not show these peaks, though two major peaks at about 19 and 23° (2θ) were observed, which were identified as being due to PEG. The X-ray diffraction spectra of the IM–HP55–PEG matrices (IM, 2.5%; HP55, 20%) are shown in Fig. 2. IM–HP55–PEG 1000, IM–HP55–PEG 2000 and IM–HP55–PEG 4000 matrices did not show any peak attributable to IM crystals.

These results confirmed that IM was present in an amorphous form in both IM–PEG and IM–HP55–PEG matrices, and that the crystallinity of IM was unaffected by the molecular weight of PEG.

Release of IM from Suppositories *in Vitro*

Figure 3 shows the effect of the molecular weight of PEG on the release behavior of IM from the suppositories. All conventional suppositories gave complete drug release within 10—25 min (Fig. 3a). Increase of the molecular weight of PEG slightly decreased the release rate of IM from the conventional suppositories. On the other hand, all the matrix suppositories gave much slower release than the conventional suppositories. M-1000, M-2000 and M-4000 gave complete drug release at 90—100 min after the start of the test, and the release rates of IM from the matrix suppositories decreased slightly with increase of the molecular weight of PEG (Fig. 3b).

The mechanism of sustained release from these suppositories was discussed in our previous paper[13]; HP55 controlled the release of the PEG-entrapped IM by developing a network structure.

Plasma Levels and Bioavailability of IM after Rectal Administration in Rabbits

Figure 4 shows the plasma levels of IM after rectal administration of C-1000, C-2000 and

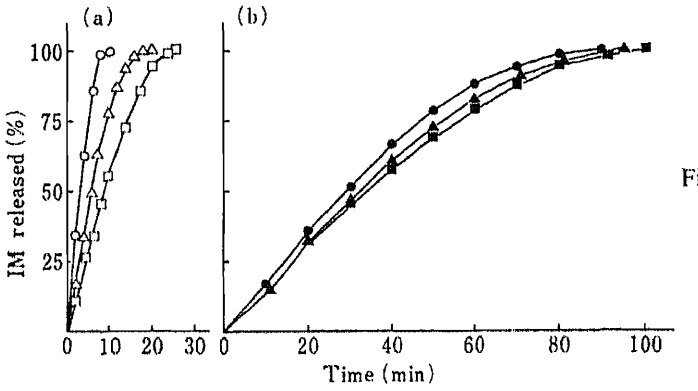

Fig. 3. Effect of Molecular Weight of PEG on the Release Behavior of IM from Suppositories *in Vitro*

(a) ○, C-1000; △, C-2000; □, C-4000. (b) ●, M-1000; ▲, M-2000; ■, M-4000. Each point represents the mean of three experiments.

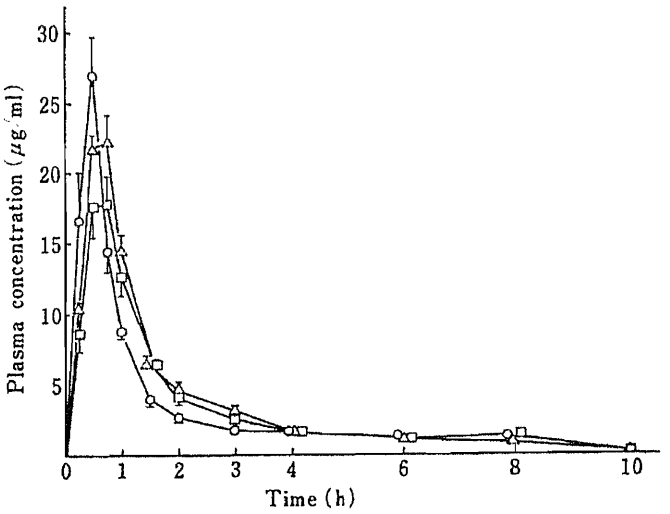

Fig. 4. Plasma Levels of IM after Rectal Administration of Conventional Suppositories

○, C-1000; △, C-2000; □, C-4000. Each point represents the mean ± S.E. of 4—6 rabbits.

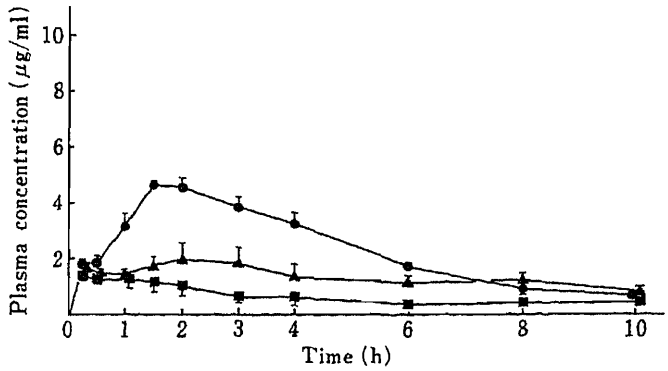

Fig. 5. Plasma Levels of IM after Rectal Administration of Matrix Suppositories

●, M-1000; ▲, M-2000; ■, M-4000. Each point represents the mean±S.E. of 4—7 rabbits.

TABLE I. Bioavailability Parameters[a] after Rectal Administration of Suppositories (IM: 25 mg) in Rabbits

	n[b]	Body weight (kg)	C_{max} (μg/ml)	AUC_0^{10}[c] (μg·h/ml)
C-1000	5	2.8±0.3	26.9±2.8	30.0±2.5
C-2000	4	3.5±0.1	22.2±2.0	32.1±2.1
C-4000	6	3.3±0.1	17.8±2.0	30.4±2.6
M-1000	4	3.4±0.1	4.6±0.2	22.9±1.6
M-2000	7	3.5±0.1	1.9±0.7	12.8±2.1
M-4000	4	3.3±0.1	1.4±0.2	6.2±1.2

a) Each value represents the mean±S.E. *b*) The number of rabbits used. *c*) Calcd by use of the trapezoidal rule from 0 to 10 h.

C-4000. These plasma level–time curves were similar; the plasma levels of IM reached a peak at 30—45 min and then declined rapidly. The values of the area under the plasma concentraion–time curve (*AUC*) were not significantly different among the conventional suppositories (Table I). It was concluded that the bioavailability of the conventional suppositories was not affected by the molecular weight of PEG. This may be because the dissolution of the conventional suppositories is rapid *in vivo* as well as *in vitro*.

On the other hand, the plasma levels of IM after rectal administration of M-1000, M-2000 and M-4000 are shown in Fig. 5. Each suppository showed a sustained-release characteristic. M-1000 gave a sustained-release pattern with a mild peak of 4.6 μg/ml at 90 min. Administrations of M-2000 and M-4000 resulted in plateau plasma levels for 15 min to 10 h, but the plasma level of IM from M-4000 was lower than that from M-2000. The *AUC* values of the matrix suppositories increased in the order of M-1000, M-2000 and M-4000 (Table I); *i.e.*, the *AUC* value increased with decreasing molecular weight of PEG. The *AUC* value of M-1000 was significantly higher than that of M-2000 ($p<0.05$) or M-4000 ($p<0.01$). Thus, the bioavailability was significantly different among the matrix suppositories. This may be because the *in vivo* dissolution of the matrix suppositories is much slower than the *in vitro* dissolution because of the very small amount of rectal fluid in rabbits, so that the bioavailability of the matrix suppositories decreases with increase of the molecular weight of PEG. In fact, about one-half of M-4000 remained in the rectum of a rabbit at 10 h after administration, though M-1000 was completely dissolved within 10 h. It was concluded that the use of low-molecular-weight PEG is preferable to that of high-molecular-weight PEG to ensure good bioavailability of IM sustained-release suppositories prepared by using the HP55–PEG matrix.

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Biopharmaceutical Studies of Thiazide Diuretics. III. *In Vivo* Formation of 2-Amino-4-chloro-*m*-benzenedisulfonamide as a Metabolite of Hydrochlorothiazide in a Patient

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Urine, plasma and erythrocytes from a patient receiving hydrochlorothiazide were examined by high-performance liquid chromatography. The urine and erythrocyte showed an unknown peak in the chromatograms. This substance was identified as 2-amino-4-chloro-*m*-benzenedisulfonamide and the identification was confirmed by comparison of the substance with an authentic sample obtained by synthesis. All data were analyzed with a PC 9801VM computer in conjunction with a JASCO MULTI-320 multi-wavelength ultraviolet detector.

Keywords——hydrochlorothiazide; hydrolysate; 2-amino-4-chloro-*m*-benzenedisulfonamide; plasma; urine; erythrocyte; nephrotic patient; metabolite

We have investigated the pharmacodynamics of hydrochlorothiazide (HCT), a widely used diuretic, in patients in order to clarify the relationship between the absorption behavior and the clinical responce.[1–4] Beermann *et al.*[5] have reported a bioavailability study with ^{14}C-HCT in 5 healthy volunteers. They found that over 95% of the absorbed or injected ^{14}C-HCT was excreted unchanged and that the ethyl acetate extracts of the urine samples collected from almost all volunteers showed only one radioactive spot on the thin-layer chromatogram (TLC). They also found that some labeled material accumulated in an area that did not correspond to HCT in a few samples obtained from only one volunteer, but the amount of the material was less than 0.5% of the excreted radioactivity. There is no other information on the metabolites of HCT. We have sometimes found an unknown peak which seemed to be a metabolite on the high-performance liquid chromatograms of a patient's urine, plasma and erythrocyte. The aim of the present study was to identify the metabolite of HCT.

Experimental

Materials——Powder and tablets (Esidrex®) of HCT were obtained from Ciba-Geigy (Japan) Ltd. 2-Amino-4-chloro-*m*-benzenedisulfonamide (ACBS) was prepared by heating HCT with 10 N NaOH for 1 h at 95 °C.[6] After the solution had cooled, it was acidified by adding HCl. The white precipitate was separated on a glass filter, washed with distilled water, and dried under reduced pressure. The product was isolated by column chromatography on silica gel (22 × 450 mm, Wako Gel C-200, Wako Pure Chemical Ind., Ltd., Osaka) using ethyl acetate–chloroform (30 : 1, v/v).

Apparatus and Conditions—The high-performance liquid chromatography (HPLC) system consisted of a TRIROTAR-II pump, a MULTI-320 multi-wavelength ultraviolet (UV) detector (Japan Spectroscopic Co., Japan) and a PC-9801 VM computer (NEC, Japan). The column (250 × 4.6 mm, i.d.) was packed with 5-μm diameter silica gel (Fine SIL-5, Japan Spectroscopic Co., Japan). A precolumn (23 × 3.8 mm, i.d.) of porous silica gel (Perisorb A, Merck) was fitted to protect the main column from plasma or urinary components. The mobile phase was ethanol–dichloroethane–hexane (14 : 10 : 74, v/v) for plasma or erythrocyte and ethanol–hexane (24 : 76, v/v) for urine. The

flow rate was 2 ml/min.

Determination of ACBS and HCT in Urine, Plasma and Erythrocyte——The extraction and clean-up procedures were described previously.[2]

Clinical Study——A single dose of 100 mg of HCT (as four tablets of Esidrex®, 25 mg) was administered to a nephrotic patient (female, 56 years old, body weight 55 kg) at 9 a.m. Informed consent was obtained from the patient beforehand. Blood samples (2 ml) were taken from the forearm vein into a heparinized syringe, and centrifuged immediately to separate the plasma and erythrocyte. Urine was collected up to 24 h postdosing. All samples were stored frozen at −20 °C until assayed.

Results and Discussion

The chromatograms obtained from the patient's plasma, erythrocyte and urine are shown in Fig. 1. These chromatograms were monitored by measuring the absorbance at 270 nm. The extracts from the urine or plasma sample showed a small unknown peak, which seemed to be a metabolite, just behind hydroflumethiazide as an internal standard. In the chromatogram obtained from the erythrocyte sample the unknown peak was much higher than in the cases of the other samples and showed a peak height comparable to that of HCT. The three-dimensional chromatogram of the extract from the erythrocyte sample is shown in

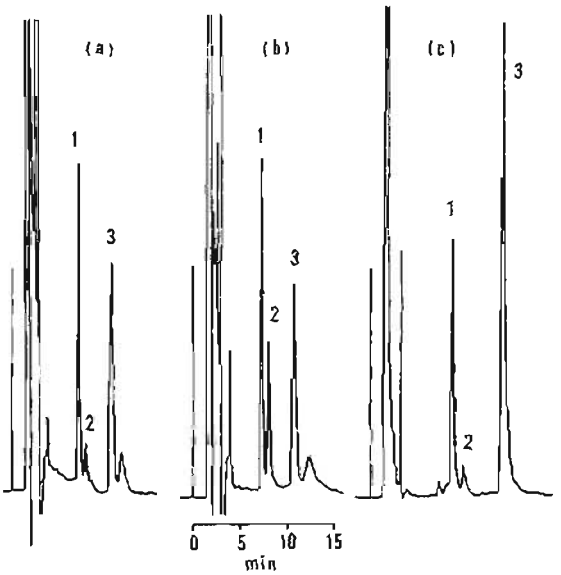

Fig. 1. Typical Chromatograms Obtained from Plasma (a), Erythrocyte (b) and Urine (c) Samples of a Patient

1, hydroflumethiazide; 2, unknown; 3, hydrochlorothiazide. The plasma and erythrocyte samples were obtained 24 h after administration and the urine sample was collected between 12 and 24 h postdosing.

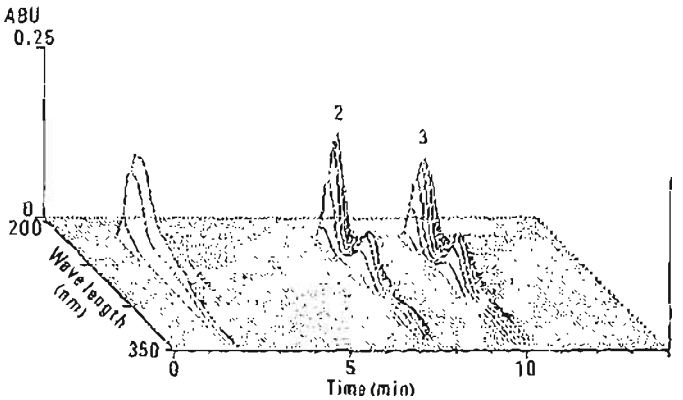

Fig. 2. Three-Dimensional Chromatogram Obtained from an Erythrocyte Sample of the Patient 24 h after Administration

Key: see Fig. 1.

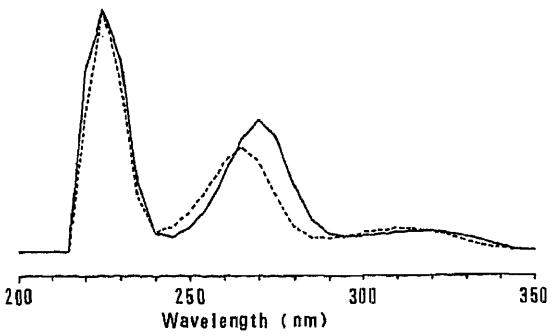

Fig. 3. UV Spectra of Hydrochlorothiazide Peak and Unknown Peak

——, hydrochlorothiazide; ------, unknown.

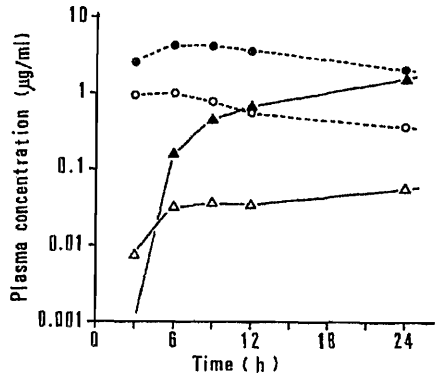

Fig. 4. Concentrations of Hydrochlorothiazide and Its Hydrolysate in Plasma and Erythrocyte from the Patient

---●---, HCT in erythrocyte; ---○---, HCT in plasma; —▲—, ACBS in erythrocyte; —△—, ACBS in plasma.

Fig. 2. The UV spectra of the HCT peak and the unknown peak are shown in Fig. 3. The unknown substance was identified by comparison of its HPLC elution profile and UV spectrum with those of authentic compounds; it was found to be a hydrolysis product of HCT, ACBS. It has been reported that commercial HCT tablets and bulk powder contain small amounts of ACBS as a degradation product of HCT.[6] Therefore, the amount of ACBS in tablets of the same lot as those administered to the patient was determined. The amount of ACBS was less than 0.4% of HCT. On the other hand, the amount of ACBS in the patient's urine collected up to 24 h was about 4.3% of HCT excreted in the urine.

As shown in Fig. 4, the HCT concentration in erythrocyte and plasma rose to a peak at 6 h postdosing and then declined slowly, although the ACBS concentrations in erythrocyte and plasma were still increasing at the end of the experimental period. The results indicate that ACBS is mainly formed *in vivo* by hydrolysis of HCT after administration and that the excretion rate of ACBS is slower than the rate of HCT hydrolysis to ACBS. The plasma concentration of HCT was about 10 times higher than that of ACBS. However, in erythrocyte, the concentration of ACBS was about equivalent to that of HCT. This fact shows that the affinity of ACBS to erythrocyte is much stronger than that of HCT.

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Further Metabolism of 4-Acetylaminoantipyrine, the Major Metabolite of Aminopyrine, in Rats

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A significant reduction of urinary excretion of 4-acetylaminoantipyrine (AcAA) after administration of aminopyrine was observed in rats treated with phenobarbital or with 3-methylcholanthrene. Urinary excretion data and results obtained in the isolated rat hepatocyte system clearly demonstrated that both an alternative route of metabolism of 4-aminoantipyrine, an AcAA precursor, and further metabolism of AcAA itself accounted for the considerable reduction of AcAA recovery. The further metabolism of AcAA, which had been regarded as one of the final metabolic products of aminopyrine, was especially enhanced by 3-methylcholanthrene pretreatment.

Keywords——aminopyrine; 4-acetylaminoantipyrine; metabolism; enzyme induction; phenobarbital; 3-methylcholanthrene

Aminopyrine (AM) has been widely used as a model substrate for investigation of drug metabolism.[1-3] The main metabolic pathway of AM is demethylation, giving 4-monomethylaminoantipyrine (MAA), which is subsequently demethylated to 4-aminoantipyrine (AA). AA undergoes acetylation to form 4-acetylaminoantipyrine (AcAA). 4-Formylaminoantipyrine (FAA) is also produced from AM *via* MAA in man and animals.[4]

The urinary recovery of the metabolites amounts to 15—50% of the dose in man.[5] Our previous paper[6] demonstrated the significance of the formation of 4-dimethylamino-3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one (AM-3-CH_2OH) in the metabolism of AM. The purpose of the present study was to gain a better understanding of the fate of AM in the body by elucidating in more detail the pathways of biotransformation. In addition, we attempted to investigate the effect of enzyme inducers on AM metabolism.

Experimental

Chemicals——AM, MAA, AA, FAA, AcAA and deuterium-labeled AM, MAA and AA were prepared as described previously.[7-9] Rubazonic acid (RA) was synthesized according to the method of Knorr.[10,11] mp 181—182 °C. *Anal.* Calcd for $C_{20}H_{17}N_5O_2$: C, 66.84; H, 4.77; N, 19.49. Found: C, 66.55; H, 4.84; N, 19.49.

Animals and Treatment——Male Wistar rats (11 weeks old) weighing 250—300 g were pretreated with phenobarbital (PB; 50 mg/kg, i.p.) or 3-methylcholanthrene (3-MC; 20 mg/kg, i.p.) once daily for 3 d before experimentation. The last dose of the inducers was given approximately 24 h prior to the urinary excretion study or prior to sacrifice to obtain the isolated hepatocytes. AM, AA or AcAA was administered intraperitoneally at a dose of 0.13 mmol/kg in the urinary excretion study. The urine was collected over a 24-h period from the time of drug administration.

Isolated Rat Hepatocytes——The hepatocytes were prepared by the method of Moldéus *et al.*[12] The substrate (0.2 mM AA or 0.1 mM AcAA) was incubated with the hepatocytes (4.0×10^6 cells/ml) in a rotating round-bottomed flask at 37 °C under a stream of 95%O_2–5%CO_2 in a total volume of 5 ml. The incubation medium used was Krebs–Henseleit buffer, pH 7.4, containing 0.3% albumin, 12.6 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) and 2 U/ml penicillin G. The viability of the fresh cells was 94—98% according to the lactic dehydrogenase

latency test[12] and was slightly decreased (89—93%) by the 60-min incubation with the substrates.

Chemical Analysis——AM, MAA, AA, FAA and AcAA were determined by mass fragmentography using a gas chromatography-mass spectrometer equipped with a multiple ion detector-peak matcher,[7] and also partially by high performance liquid chromatography (HPLC).[13]

RA in the urine was determined as follows. The 24-h urine was adjusted to pH 2 with 4 N HCl then extracted with 20 ml of ethyl acetate. A portion of the organic layer (15 ml) was evaporated to dryness. The extract was dissolved in 200 μl of MeOH and separated quantitatively by thin-layer chromatography (TLC) on silica gel with EtOH–$CHCl_3$(1 : 9). The spot of RA was scraped off and extracted with 5 ml of $CHCl_3$. A portion of the organic layer (4 ml) was evaporated to dryness. The residue was dissolved in the mobile phase of 60% MeOH in 10 mM phosphate buffer, adjusted to pH 6.0, and a portion (80 μl) was injected into the HPLC apparatus. A 250 × 4.6-mm i.d., 5-μm particle-size, C_{18} reversed-phase column (TSKgel ODS-120T, Toso) was used at ambient temperature with a mobile phase flow rate of 1.0 ml/min, and the UV detector was set at 251 nm.

Results

The urinary excretion of AM and its metabolites during 24 h following the i.p. administration of AM (0.13 mmol/kg) is summarized in Table I. AcAA accounted for the greater part (80%) of the total recovery, amounting to 42% of the dose in the control experiments. The excretion of FAA was relatively high (6.6% of the dose), and only trace amounts of AM, MAA, AA and RA, were found.

The percentage of the dose excreted as AcAA was one-half the control value in the rats treated with phenobarbital. In the case of 3-MC pretreatment, the AcAA excretion was reduced significantly to one-fourth of the control value. The decrease in the urinary excretion of AcAA resulted in a significant reduction of the total recovery of AM and its metabolites. The amount excreted as RA was significantly increased by these enzyme inducers, though the contribution to the total recovery was minimal. The percentages of the dose excreted as unchanged AM and metabolic products other than AcAA and RA were not significantly altered by the pretreatments with PB and 3-MC.

When AA (0.13 mmol/kg) was administered to rats, urinary AcAA accounted for 68% of the dose (Table II). The total recovery of AA was 71% in the control experiment. A significant decrease in the percentage of the dose excreted as AcAA was observed in the rats pretreated with 3-MC.

Table I. Rat Urinary Excretion of AM and Its Metabolites during 24 h Following the Intraperitoneal Administration of AM (0.13 mmol/kg)

Metabolite	% of dose[a]		
	Control	PB-treated	3-MC-treated
AM	0.29±0.14	0.22±0.11	0.16±0.09
MAA	0.99±0.24	0.72±0.26	0.16±0.11
AA	1.78±0.42	2.53±0.69	1.07±0.55
FAA	6.60±5.27	3.25±3.27	4.68±1.44
AcAA	41.9±9.1	21.2±3.4[b]	11.0±4.0[b]
RA	0.02±0.01	0.13±0.05[b]	0.22±0.02[b]
Total recovery	51.6±10.5	28.0±4.8	17.3±4.3

a) Mean±standard deviations (S.D.) of 4 experiments. *b*) $p<0.01$, when compared with the control value. AM, aminopyrine; MAA, 4-monomethylaminoantipyrine; AA, 4-aminoantipyrine; FAA, 4-formylaminoantipyrine; AcAA, 4-acetylaminoantipyrine; RA, rubazonic acid.

Table II. Rat Urinary Excretion of AA and Its Metabolites during 24 h Following the Intraperitoneal Administration of AA (0.13 mmol/kg)

Metabolite	% of dose[a]		
	Control	PB-treated	3-MC-treated
AA	2.92±1.01	2.79±1.09	1.84±0.74
AcAA	67.8±8.5	50.9±13.3	13.4±7.5[b]
RA	0.02±0.01	0.04±0.01[b]	0.32±0.11[b]
Total recovery	70.7±8.6	53.7±13.3	15.6±7.5

a) Mean±S.D. of 4 experiments. *b*) $p<0.01$, when compared with the control value.

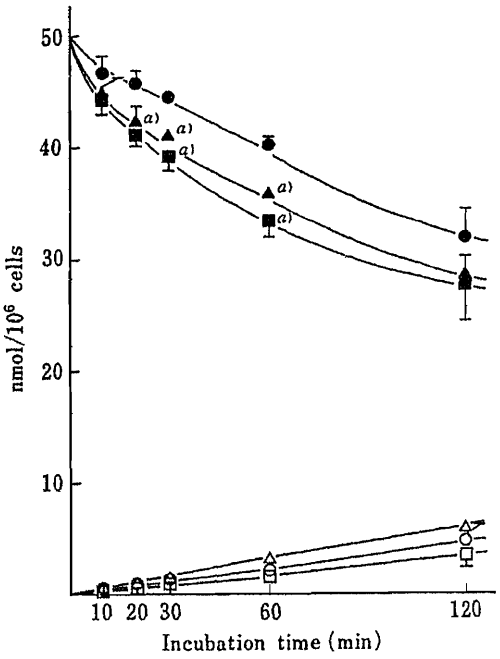

Fig. 1. Time Course of AA Metabolism in Isolated Hepatocytes

The initial concentration of AA was 0.2 mM. Each value is the mean of 3 experiments, and the vertical bar represents the standard error. AA: ●, control; ▲, PB-pretreated; ■, 3-MC-pretreated. AcAA: ○, control; △, PB-pretreated; □, 3-MC-pretreated. *a*) $p<0.05$, when compared with the control value.

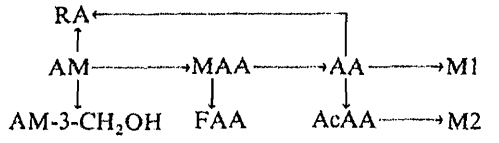

Chart 1. Main Metabolic Pathways of Aminopyrine

TABLE III. Rat Urinary Excretion of AcAA during 24 h Following the Intraperitoneal Administration of AcAA (0.13 mmol/kg)

% of dose[a)]		
Control	PB-treated	3-MC-treated
90.2±5.6	74.9±6.9[b)]	44.3±9.8[b)]

a) Mean±S.D. of 4 experiments. *b*) $p<0.05$, when compared with the control value.

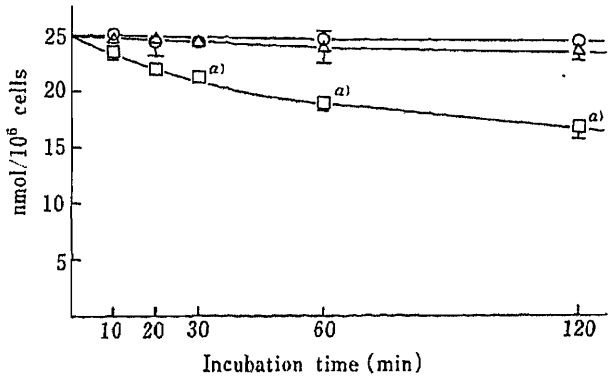

Fig. 2. Elimination of AcAA in Isolated Hepatocytes

The initial concentration of AcAA was 0.1 mM. Each value is the mean of 3 experiments, and the vertical bar represents the standard error. ○, control; △, PB-pretreated; □, 3-MC-pretreated. *a*) $p<0.05$, when compared with the control value.

The urinary recovery of AcAA during 24 h following AcAA administration (0.13 mmol/kg, i.p.) is shown in Table III. Although AcAA has been regarded as one of the final metabolic products of AM, the results suggest that further metabolic transformation occurs, and is greatly facilitated by the 3-MC pretreatment.

Figure 1 shows the time courses of disappearance of AA and formation of AcAA in the isolated hepatocyte system. The PB and 3-MC pretreatments facilitated the elimination of AA to the same extent. The effect of the enzyme inducers on the formation of AcAA could not be clearly observed in this system because AcAA formation was slow, amounting to only 10% in 120 min.

The disappearance of AcAA was then examined in the same system (Fig. 2). The elimination of AcAA was very slow in both the control group and the PB-pretreatment group. The percentages of AcAA remaining at 120 min were 97.6±1.7% and 95.3±4.0%, respectively. On the other hand, the AcAA elimination was significantly increased in the isolated hepatocyte system prepared from the rats treated with 3-MC. The percentage of AcAA remaining at 120 min was 67.4±4.2%.

Discussion

A significant reduction of the urinary excretion of AcAA was observed in rats treated with PB or 3-MC. This finding suggests that an alternative route of metabolism of AA exists, or subsequent metabolism of AcAA occurs, or both, as shown in Chart 1.

As a next step, we employed the rat isolated hepatocyte system in order to elucidate the contribution of the unknown biotransformation to the metabolic mass balance of AA and AcAA.

Figure 1 shows that the total recovery of AA was decreased by the treatment with the inducers, while formation of AcAA was not affected. Therefore, it was suggested that an alternative route of metabolism of AA exists (M1 in Chart 1). This idea was confirmed by the finding that formaldehyde production in this system was enhanced significantly by pretreatment with PB or 3-MC (unpublished data). It was considered that *N*-demethylation might be the first step of the alternative metabolic route of AA, as reported in the study on the metabolism of antipyrine.[14)]

As indicated by the urinary excretion data (Table III) and the metabolic mass balance study *in vitro* (Fig. 2), the further metabolic breakdown of AcAA was drastically enhanced by the pretreatment with 3-MC. This process was not a simple deacetylation of AcAA, because no trace of AA was detected in the urine or in the medium.

From the results obtained in this study, it may be concluded that both an alternative route of metabolism of AA and further metabolism of AcAA are involved in the considerable reduction in the urinary excretion of AcAA. In addition, it was found that the further metabolism of AcAA is especially enhanced by 3-MC pretreatment.

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Communications to the Editor

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SYNTHESIS OF BREDININ 5'-ALKYLPHOSPHATES INVOLVING PHOTOCHEMICAL MANIPULATION OF THE IMIDAZOLE MOIETY, AND THEIR ANTITUMOR ACTIVITIES

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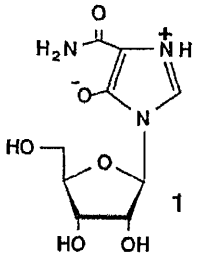
Bredinin 5'-oleyl and 5'-cetyl phosphates (2a and 2b), prepared from 1-β-D-ribofuranosyl-5-aminoimidazole-4-carboxamide via a photochemical ring-opening reaction of the imidazole moiety, showed remarkable antitumor effects against various transplantable mouse tumors, and were clearly superior to bredinin (1).

KEYWORDS——bredinin; nucleoside antibiotic; photochemical ring-opening reaction; phosphorylation; antitumor activity; bredinin 5'-alkylphosphate; AICA-riboside

The nucleoside antibiotic bredinin (1) has been used clinically as a potent immunosuppressive agent.[1] In some experimental tumor systems, bredinin also had only a weak antitumor effect due to the inhibition of cellular guanine nucleotide synthesis.[2] Some recent investigations of the cytotoxic mechanism of bredinin[3] suggest that it may be metabolized by cellular adenosine kinase to its 5'-phosphate (3), the actual inhibitor of cellular IMP dehydrogenase, however the metabolic rate is often quite low, so that bredinin exhibits hardly any antitumor effect in vivo. Bredinin 5'-monophosphate (3) will not surpass bredinin itself in antitumor potency, since nucleoside monophosphates do not penetrate cells.[4]

In order to improve the potency of bredinin as an antitumor agent, we planned to synthesize and evaluate 5'-higher-alkylphosphoryl derivatives of bredinin (2). We hypothesized that these compounds might have a high affinity for cell membranes and thus might penetrate the cells, then release bredinin 5'-phosphate (3), the potent inhibitor of cellular IMP dehydrogenase which is one of the most favorable target enzymes in cancer chemotherapy.[5] These derivatives may also be highly bioavailable because of their lipophilicities.



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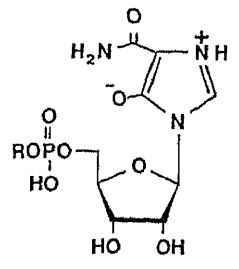
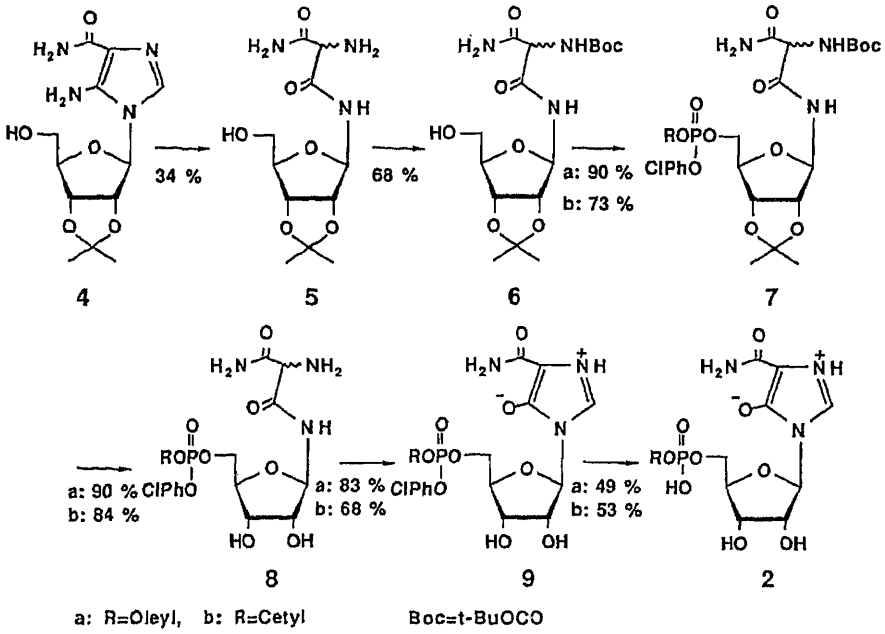


2a: R=Oleyl
2b: R=Cetyl
3 : R=H



The initial attempt to prepare the desired compounds (2) from 2',3'-O-isopropylidenebredinin by the usual phosphodiester or triester method were unsuccessful due to the unusual zwitterionic structure of the base moiety of bredinin.[6]

On the other hand, we have recently reported a novel synthesis of bredinin from 1-β-D-ribofuranosyl-5-aminoimidazole-4-carboxamide (AICA-riboside) via a photochemical imidazole ring-opening reaction.[7] This method was found to be applicable to the preparation of the 5'-alkylphosphates of bredinin (2). 2',3'-O-Isopropylidene-AICA-riboside (4)[8] in 0.1 M aqueous formic acid was photo-irradiated with a low-pressure mercury lamp to afford the desired ring-opened product (5) in 34% yield. Compound 5 was treated with di-tert-butyl pyrocarbonate in dimethylformamide (DMF) to give the N-Boc derivative (6), which was phosphorylated with p-chlorophenylphosphoroditriazolide[9] in dioxane, then treated with an excess of oleyl alcohol in the presence of N,N-dimethylaminopyridine (DMAP) to give the phosphotriester (7a). The isopropylidene and N-Boc groups of 7a were removed simultaneously by treatment with 90% aqueous trifluoroacetic acid to furnish 8a, which was heated at 90°C with triethyl orthoformate in DMF to afford the ring-closure product (9a). Deprotection at the phosphate moiety of 9a was carried out by treatment with N,N,N',N'-tetramethylguanidinium pyridine-2-aldoximate in aqueous dioxane[10] to furnish the desired bredinin 5'-oleylphosphate (2a). The structure of 2a was confirmed by instrumental analysis.[11] Similarly, 5'-cetylphosphoryl derivative (2b)[12] was obtained from 6 in 22% over-all yield.

The antitumor effects of bredinin and the synthesized 5'-alkylphosphoryl derivatives of bredinin, given ip, against mouse tumor systems inoculated ip are shown in Table I. Under our experimental conditions, the optimum dose found for bredinin was 50 mg/kg per day. So, in this study, the same dose was given in treatments with the alkylphosphoryl derivatives. All compounds were inactive against P388 leukemia. However, 2a and 2b had marked effects against all other tumor systems examined. It is noteworthy that 2a and 2b had significant antitumor effects against both Meth A fibrosarcoma and Sarcoma180 tumor systems, while its parent nucleoside, bredinin (1) was inactive against them.

Table I. Antitumor Activity against ip-Implanted Tumors in Mice

	Dose (mg/kg/day)	% ILS[a] P388[b]	Meth A[b]	Ehrlich carcinoma[c]	Sarcoma180[c]
Bredinin (1)	50	4 (0)[d]	13 (0)	> 57 (1)	21 (0)
2a	50	12 (0)	71 (0)	>128 (3)	> 89 (1)
2b	50	15 (0)	58 (0)	> 87 (1)	>190 (1)

a) Percent increase in life span: (T/C - 1) x 100. b) On day 0, each group of five BDF_1 mice (P388) or BALB/C mice (Meth A) received ip-inoculations of tumor cells (1×10^6). The compounds were given ip once daily on days 1 to 5. c) On day 0, each group of five ICR mice received ip-inoculations of tumor cells (2×10^6). The compounds were given ip once daily on day 2 to 8. d) Numbers in parentheses are 45-day survivors out of 5 mice.

Table II. Antitumor Activity against sc-Implanted Tumors in Mice

	Dose (mg/kg/day)	T/C (%) Meth A[a]	Ehrlich carcinoma[b]	Sarcoma180[b]
Bredinin (1)	50	95	27	124
2a	50	42[c]	12[d]	53

a) On day 0, Meth A fibrosarcoma cells (1×10^6) were implanted sc in BALB/C mice (5 mice per group). The compound was given ip once daily on days 1 to 5. Tumor weights were measured on day 10. b) On Day 0, tumor cells (2×10^6) were implanted sc in ICR mice (5 mice per group). The compounds were given ip once daily on days 2 to 8. Tumor weights were measured on day 14. c) Significant difference ($p<0.01$) from the controls. d) Significant difference ($p<0.05$) from the controls.

The antitumor effects of bredinin (**1**) and **2a** against tumors implanted sc in mice were also investigated (Table II). Bredinin did not have an antitumor effect against these solid tumors, but **2a** inhibited the growth of the solid tumors (Meth A fibrosarcoma and Ehrlich carcinoma), the effects being statistically significant.

These results may clearly support the above hypothesis. Although similar 5'-alkylphosphoryl derivatizations of antitumor nucleoside analogues have been performed by several investigators (e.g., arabinofuranosylcytosine[13a], 5-fluorouridine[13b]), none of them has shown such evident improvement in the antitumor effect as in the present case, when compared with the corresponding parent nucleosides. This may be due to the fact that these parent antitumor nucleosides are, in the first phase, good substrates of cellular nucleoside kinases.

Further evaluation of **2a** and **2b** is under investigation.

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11) Physical data of 2a: mp 160-163°C (dec.). Anal. Calcd for $C_{27}H_{48}N_3O_9P \cdot H_2O$; C,53.37; H,8.29; N,6.91. Found; C,53.25; H,8.36; N,6.68. MS (FAB); m/e 590 (MH). ^{1}H-NMR (DMSO-d_6·D_2O, 100 MHz); δ, 8.23(s,1H,H-2), 5.56(d,1H,H-1',$J_{1',2'}$=4.9 Hz), 5.32(t,2H,oleyl CH=CH), 4.31(dd,1H,H-2'), 4.02-3.72(m,6H, H-3',4',5',and oleyl CH_2O), 1.95(broad,4H,oleyl $\underline{CH_2}CH=$), 1.50-1.24(m,24H,oleyl CH_2), 0.89(t,3H,oleyl CH_3).

12) Physical data of 2b: mp 157-160°C (dec.). Anal.Calcd for $C_{25}H_{46}N_3O_9P \cdot 2/3H_2O$; C,52.16; H,8.29; N,7.30. Found; C,52.11; H,8.32; N,7.14. MS (FAB); m/e 564 (MH). ^{1}H-NMR (DMSO-d_6·D_2O, 100 MHz); δ, 8.23(s,1H,H-2), 5.57(d,1H,H-1',$J_{1',2'}$=5.1 Hz), 4.32(dd,1H,H-2'), 4.03-3.73(m,6H,H-3',4',5', and cetyl CH_2O), 1.55-1.24(m,28H, cetyl CH_2), 0.85(t,3H,cetyl CH_3).

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DIKETOPIPERAZINE DERIVATIVES, A NEW SERIES OF PLATELET-ACTIVATING FACTOR INHIBITORS

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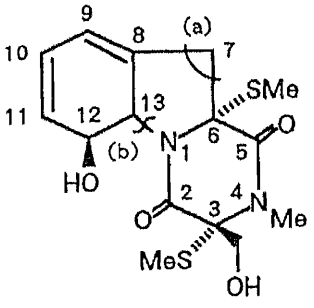
Some diketopiperazine derivatives (3a-f, 4a-f, and 5a-c) related to natural products (1 and 2) have been prepared and evaluated for their inhibition of platelet aggregation induced by platelet-activating factor (PAF).

KEYWORDS——diketopiperazine; platelet-activating factor; platelet aggregation; structure-activity relationship

Since the characterization of platelet-activating factor (PAF), an endogeneous mediator in allergic and inflammatory responses, research on PAF antagonists has increased to exploit a new type of agent for treating these diseases.[1] As part of a research program for novel PAF inhibitors in our laboratories, the potent inhibitors bisdethiobis(methylthio)gliotoxin (1) and 3,6-bismethylthio-3-hydroxymethyl-6-phenylmethylpiperazine-2,5-dione (2) were isolated from Penicillium terlikowskii No. 5348[2] and Penicillium citrinum No. 2973,[3] respectively. In studying these natural products, we were interested in clarifying their structure-activity relationships to find more active compounds in these series. Both of these products have a diketopiperazine framework and a bis(methylthio) function as common structural units. To design molecules of compounds related to 1 and 2, we removed the C7 methylene (a) and disconnected the



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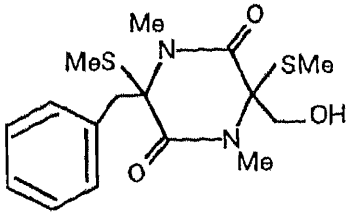

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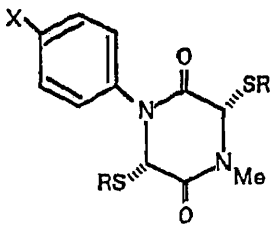
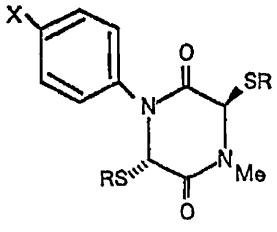
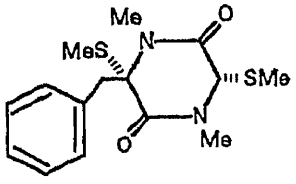
Fig. 1



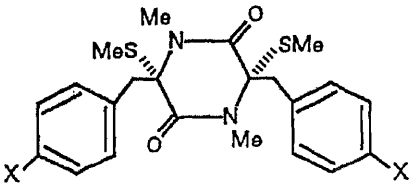
3a ; X=H , R=Me
3b ; X=Cl , R=Me
3c ; X=OMe, R=Me
3d ; X=H , R=Ph
3e ; X=Cl , R=Ph
3f ; X=OMe, R=Ph



4a ; X=H , R=Me
4b ; X=Cl , R=Me
4c ; X=OMe, R=Me
4d ; X=H , R=Ph
4e ; X=Cl , R=Ph
4f ; X=OMe, R=Ph



5a



5b ; X=H
5c ; X=Cl

Fig. 2

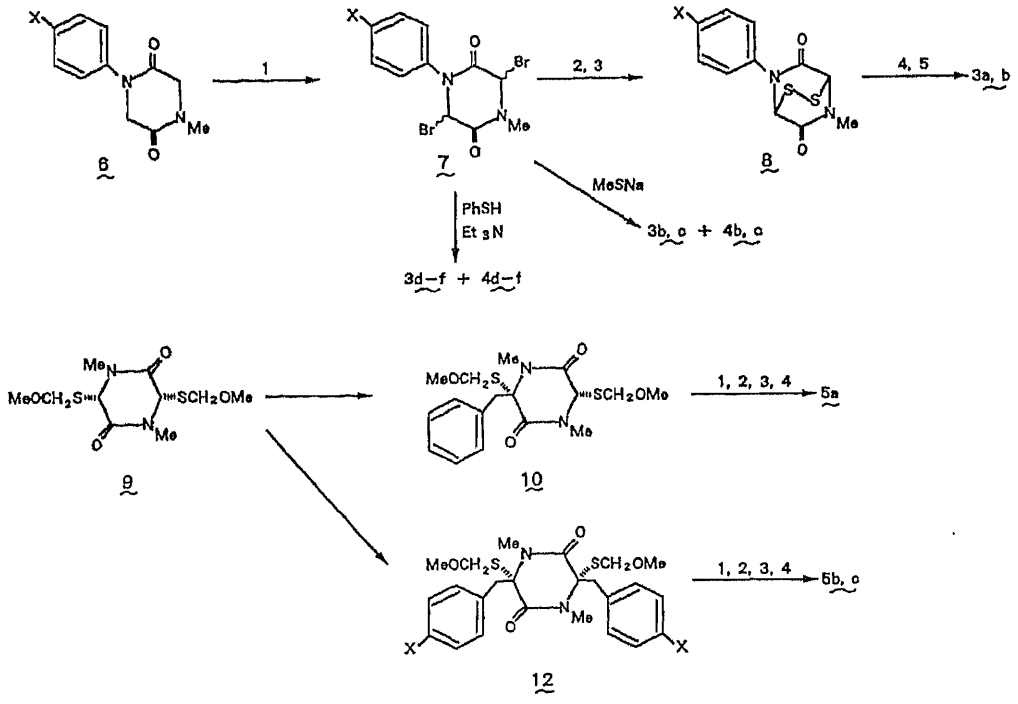


Chart 1

C13-N1 bond (b) in the structure of 1. These chemical variations lead to N-phenyl diketopiperazines (3, type A) and benzyldiketopiperazines (5, type B). The latter precisely corresponds to the structure of 2. Here we report the synthesis of these two compounds and their biological activities.

Compounds 3a,b[4] of the A type with cis configuration were prepared by a sequence of reactions (1. NBS/mCPBA, 2. AcSK, 3. I_2/HCl, 4. $NaBH_4$, 5. MeI)[5] starting from diketopiperazines 6 (X=H, Cl).[6] Alternatively, compound 3b[4] was prepared, together with the corresponding trans isomer 4b,[4] by a direct substitution of bromide 7 (X=Cl) with MeSNa. In the ^1H NMR spectrum of 3b, the diketopiperazine ring protons were by ca 0.25 ppm upfield from the protons of 4b (3b, 4.69, 4.90; 4b, 4.93, 5.14). This phenomenon was observed in general in this series of compounds and utilized in the structural assignment of these compounds. The related cis and trans compounds (3c-f and 4c-f)[4] were prepared using the direct substitution method.

Compounds of the B type were prepared as follows. Alkylation of diketopiperazine 9[5] with benzyl bromide (1 equiv/$LiNPr^i_2$) gave the monobenzyl derivative 10, which was converted, via disulfide 11, to compound 5a[4] (1. BCl_3, 2. I_2, 3. $NaBH_4$, 4. MeI). Compounds 5b,c[4] were obtained from 9 by similar treatment after alkylation using 2 equivalent benzyl bromide and p-chlorobenzyl bromide (2 equiv/$LiNPr^i_2$, respectively) of 9.

The compounds were evaluated in vitro by inhibition of a PAF-induced rabbit platelet aggregation and the results are summarized in comparison with those of 1 and 2 in Table I. In the compounds of the A type, without exception the cis compounds were more active than the corresponding trans isomers, particularly so in the S-phenyl series of compounds. The S-phenyl derivatives were more active than the corresponding S-methyl derivatives in the cis series, while the activities of the compounds in the trans series were entirely in reverse. Compound 3f was thus the most active in the A type of compounds. The three compounds prepared as the B type were all moderately active, but considerably less so than 3f.

Table I. Inhibitory Activity of Diketopiperazine Derivatives[a]

Compound	IC_{50} (uM)	Compound	IC_{50} (uM)
1	4.0	4b	15.4
2	16.9	4c	8.0
3a	16.9	4d	54.8
3b	7.3	4e	28.8
3c	5.2	4f	42.2
3d	5.2	5a	12.3
3e	1.7	5b	9.7
3f	0.69	5c	8.3

a) IC_{50} is the concentration of a compound required for 50% inhibition of rabbit platelet aggregation induced by PAF (20 nM).

In conclusion, the most active PAF inhibitor was 3f. It had no effect on platelet aggregations induced by collagen, arachidonic acid, or ADP,[7] thus proving to be a PAF-specific inhibitor.

ACKNOWLEGEMENT We are grateful to Dr. K. Yoshida and his colleagues for the biological assays.

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4) Physical data of the compounds prepared in the present study: 3a; mp 98-101°C, NMR(4.70 and 4.96). 3b; mp 113-115°C, NMR(2.26(3H,s, S-Me), 2.33(3H,s, S-Me), 3.15(3H,s, N-Me), 4.69(1H,s, H_2 or H_5), 4.70(1H,s, H_5 or H_2), and 7.38(4H,s). 3c; mp 80-83°C, NMR(4.69 and 4.90). 3d; mp 185-186°C, NMR(4.41 and 4.80). 3e; mp 194-196°C, NMR(4.39 and 4.71). 3f; mp 197-198°C, NMR(4.42 and 4.79). 4b; mp 153-155°C, NMR(2.10(3H,s, S-Me), 2.18(3H,s, S-Me), 3.17(3H,s, N-Me), 4.93(1H,s, H_2 or H_5), 5.14(1H,s, H_5 or H_2), 7.25(2H,d,J=8Hz), and 7.43(2H,d,J=8). 4c; mp 167-170°C, NMR(4.96 and 5.14). 4d; mp 147-149°C, NMR(5.10 and 5.33). 4e; mp 147-149°C, NMR(5.04 and 5.26). 4f; mp 172-173°C, NMR(5.06 and 5.25). 5a; mp 118-119°C. 5b; mp 98-99°C, NMR(2.20(6H,s, S-Me), 2.92(2H,d,J=15, benzyl-CH_2), 3.14(6H,s, N-Me), 3.36(2H,d,J=15, benzyl-CH_2), 6.7-7.3(10H,m). 5c; mp 148-150°C, NMR(2.25(6H,s), 2.93(2H,d,J=15), 3.12(6H,s), 3.60(2H,d,J=15), 6.76(4H,d,J=8), and 7.07(4H,d,J=8).

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6) The compounds of 6 were synthesized by Ullmann-type reaction of substituted iodobenzenes and glysine-sarcosine anhydride.

7) Details will be reported elsewhere.

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SPIROTUBIPOLIDE, A NEW MARINE SESQUITERPENOID FROM THE STOLONIFER TUBIPORA MUSICA LINNAEUS

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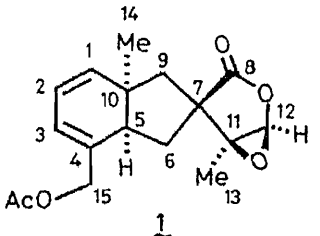
The structure of spirotubipolide (1), a new type of marine sesquiterpenoid isolated from the Japanese stolonifer Tubipora musica Linnaeus, was characterized on the basis of spectroscopic data.

KEYWORDS —— spirotubipolide; stolonifer; Tubipora musica Linnaeus; sesquiterpenoid; β,γ-epoxy-γ-lactone; spirolactone; NOESY

Previously, we reported the structures of new bioactive furanosesquiterpenoids[1] from the Japanese stolonifer Tubipora musica Linnaeus. In our continuing study of the chemical constituents of T. musica, a new sesquiterpenoid, spirotubipolide (1), has been isolated. It has what appears to be a heretofore unknown type of carbon skeleton. This paper describes the isolation and structure of 1.

The hexane extract (36 g) of the freeze-dried organisms (3.7 kg) of T. musica, collected at the coral reef of Ishigaki Island (Okinawa, Japan), was subjected to silica gel column chromatography (hexane-ethyl acetate as the eluent). The eluates, obtained after elution of tubipofuran[1] and 15-acetoxytubipofuran,[1] were purified by HPLC (silica gel, hexane-ethyl acetate 5:1) to give spirotubipolide (1)[2] as colorless crystals [10 mg, $C_{17}H_{20}O_5$, $[\alpha]_D$ -194° (c 0.34, $CHCl_3$)].

The presence of a conjugated homoannular diene system in 1 was indicated by UV absorption at 262 nm (ε 4520), ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) [δ_{ppm} 5.52 (1H, br d, J = 9.5 Hz, H-1), 5.85 (1H, dd, J = 5.5, 9.5 Hz, H-2), 5.95 (1H, br d, J = 5.5 Hz, H-3)], and ^{13}C-NMR spectrum (100 MHz, $CDCl_3$) [δ_{ppm} 120.1 (d), 120.7 (d), 136.3 (d), 134.0 (s)]. The ^{1}H- and ^{13}C-NMR spectra also showed signals due to an acetoxymethyl [δ_H 2.12 (3H, s), 4.61 (1H, d, J = 13 Hz), 4.63 (1H, d, J = 13 Hz), δ_C 21.0 (q), 67.2 (t), 170.8 (s)], a methyl on a quaternary carbon [δ_H 1.09 (3H, s), δ_C 14.2 (q), 44.8 (s)], two methylenes [δ_H 1.98 (1H, d, J = 14.4 Hz, H-9α), 2.26 (1H, d, J = 14.4 Hz, H-9β), 2.19 (1H, t, J = 11.7 Hz, H-6β), 2.42 (1H, dd, J = 5.4,



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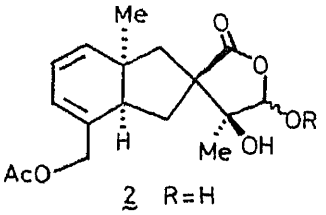
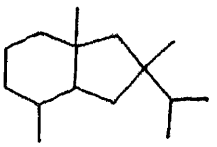


2 R=H
3 R=Ac



(4)

11.7 Hz, H-6α), δ_C 40.6 (t), 47.8 (t)], and a methine [δ_H 2.25 (1H, ddd, J = 0.8, 5.4, 11.7 Hz), δ_C 47.7 (d)]. The ^{1}H-NMR decoupling experiments[3] confirmed the relationships of the protons on the A and B rings. The presence of a β-methyl-β,γ-epoxy-γ-lactone moiety was indicated by the following spectral data; IR ($CHCl_3$) 1795 cm^{-1}, ^{1}H-NMR 1.58 (3H, s, H-13), 5.41 (1H, s, H-12), ^{13}C-NMR 25.4 (q, C-13), 52.1 (s, C-7), 64.6 (s, C-11), 82.7 (d, C-12), 179.7 (s, C-8). These spectral data closely resemble those of the known β-methyl-β,γ-epoxy-γ-lactone,[4] and the formation of 2[5] and 3[6] in the reaction of 1 with 80% aqueous acetic acid at 50°C supported the presence of the β,γ-epoxy-γ-lactone moiety. The mass spectrum of 1 showed a strong peak at m/z 105 (52%) due to the fragment ion (a) as shown in Chart 1. On the basis of these findings, the structure of spirotubipolide was determined as represented by 1.

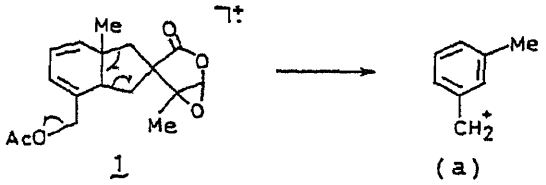


Chart 1

The relative stereochemistry of 1 was established by measuring its nuclear Overhauser effect correlation two-dimensional NMR spectrum (NOESY) as shown in Fig. 1. The correlation between the angular methyl signal (H-14) and the H-5 signal indicates the cis A/B ring junction. The correlation between the methyl signal on the epoxide and the H-9α signal, which is also correlated with the angular methyl signal (H-14), reveals the stereochemistry of the β,γ-epoxy-γ-lactone moiety. Consideration of the other NOE correlations and the observation of the W-shape long-range coupling (J = 0.8 Hz) between H-1 and H-5 show the nonsteroidal conformation of 1 as shown in Fig. 1. The absolute configurations at C-5 and C-10 appear to be the same as those of 15-acetoxytubipofuran (5) which coexisted with 1 in T. musica by the biogenetic consideration; 1 could be derived from 5 in a stereocontrolled manner involving oxidation of the furan moiety followed by rearrangement as shown in Chart 2.

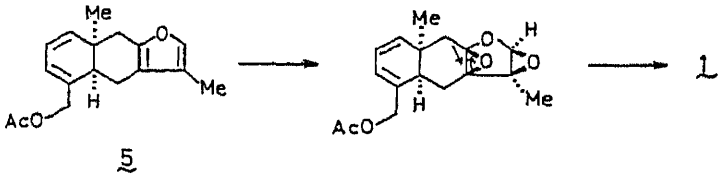


Chart 2

The structure of 1 is characterized by the new carbon skeleton represented by (4). The β,γ-epoxy-γ-lactone moiety in 1 is a very rare functional array in natural products and 1 is the first compound with this moiety from stolonifer: ptychanolide[7a] from the liverwort and dysetherin[7b] from the marine sponge are natural products known to have this moiety.

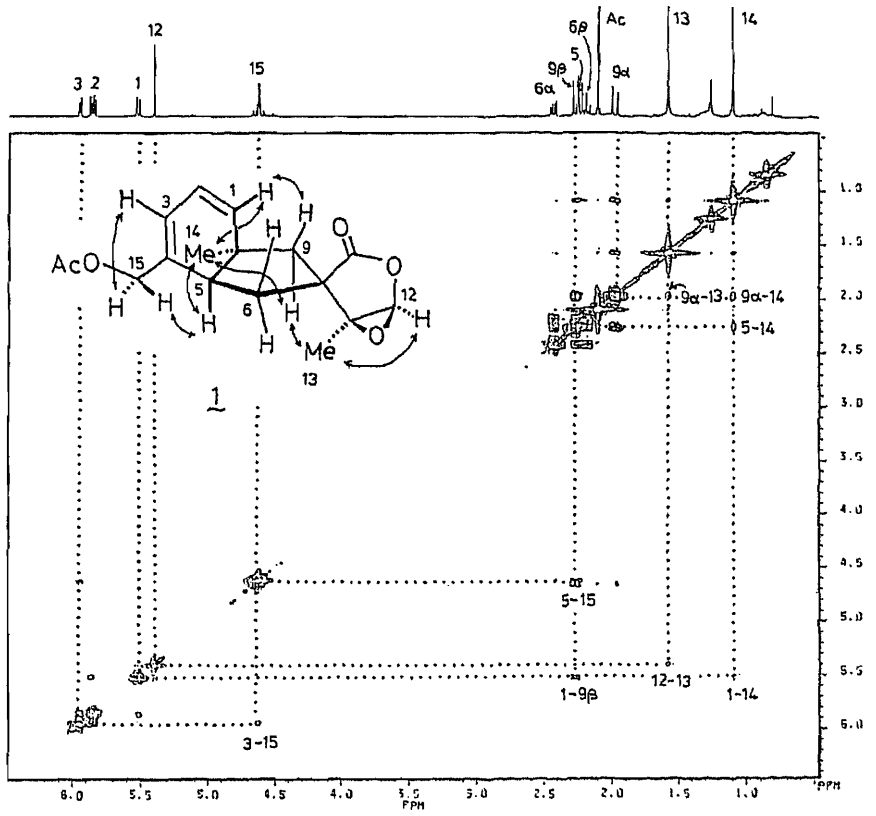


Fig. 1. Nuclear Overhauser Effect Correlation 2D NMR Spectrum (400 MHz, $CDCl_3$) and Conformation of 1

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2) Satisfactory high resolution mass data were given for all new compounds.

3) The results of the ^{1}H-NMR decoupling experiments of 1 are summarized as follows;

irradiated proton (δ_{ppm})	observed protons and changes (J in Hz)
H-1 (5.52)	H-2 (5.85, dd) ⟶ d J = 5.5
	H-5 (2.25, ddd) ⟶ dd J = 5.4, 11.7 (W-shape long-range coupling)
H-3 (5.95)	H-2 (5.85, dd) ⟶ changed H-5 (2.25, ddd) ⟶ sharpened
	H-15 (4.61, d) ⟶ sharpened H-15 (4.63, d) ⟶ sharpened
H-9α (1.98)	H-9β (2.26, d) ⟶ s
H-6α (2.42)	H-5 (2.25, ddd) ⟶ br d J = 11.7 H-6β (2.19, t) ⟶ d J = 11.7

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5) 2: $[\alpha]_D$ -241° (c 0.044, $CHCl_3$); IR ($CHCl_3$) 3300, 1770, 1720 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) 1.04 (3H, s, H-14), 1.33 (3H, s, H-13), 2.09 (3H, s), 4.57 (1H, d, J = 13 Hz, H-15), 4.66 (1H, d, J = 13 Hz, H-15), 5.37 (1H, s, H-12). The compound 2 and 3 were obtained as a single isomer, respectively, and acetylation of 2 gave 3.

6) 3: $[\alpha]_D$ -252° (c 0.054, $CHCl_3$); IR ($CHCl_3$) 3300, 1785, 1725 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) 1.04 (3H, s, H-14), 1.34 (3H, s, H-13), 2.10 (3H, s), 2.18 (3H, s), 4.62 (2H, br s, H-15), 6.22 (1H, s, H-12).

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Communications to the Editor

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STRUCTURES OF TRIPTOFORDININE A-1 AND A-2 DETERMINED BY TWO-DIMENSIONAL NMR SPECTROSCOPY. HIGHLY ESTERIFIED SESQUITERPENE ALKALOIDS FROM TRIPTERYGIUM WILFORDII HOOK FIL. VAR. REGELII MAKINO

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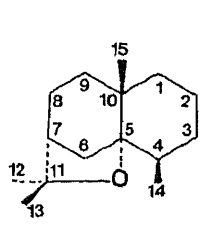
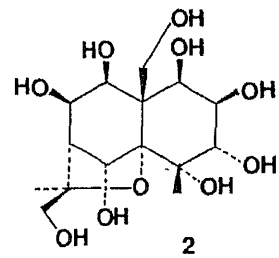
The structures of triptofordinine A-1 and A-2, highly esterified sesquiterpene alkaloids isolated from *Tripterygium wilfordii* Hook fil. var. *regelii* Makino, were established by the application of two-dimensional NMR.

KEYWORDS—— sesquiterpene; alkaloid; triptofordinine; two-dimensional NMR; celastraceae; tripterygium wilfordii

The sesquiterpene dihydroagarofuran (1) occurs in nature as euonymiol (2) in various oxygenated forms, bearing as many as nine hydroxy groups.[1] These polyols occur mostly in members of the *Celastraceae* plants as polyesters, in which the constituent acids are aliphatic (acetic, 2-methylbutyric, hydroxyisobutyric, etc.) and/or aromatic (benzoic, cinnamic, furoic, nicotinic, etc.).[2] In the structural elucidation of these sesquiterpenes, the difficulty of determining the linking sites of respective ester groupings when more than three kinds of acids are involved as esters in the molecule necessitates the use of the X-ray crystallographic method[3,4] or selective hydrolyses.[5] In fact, determination of the estersites in such compounds has been unsuccessful in many cases.[3,6] This problem can be solved conveniently by using 2-D NMR spectroscopy including ^{1}H-^{13}C long-range COSY.[7] Here we describe the structural elucidation of two new sesquiterpene alkaloids, triptofordinine A-1 (3) and A-2 (4), from *Tripterygium wilfordii* Hook fil. var. *regelii* Makino,[8] which is used in China as a drug for the treatment of cancer and as an insecticide.[9]

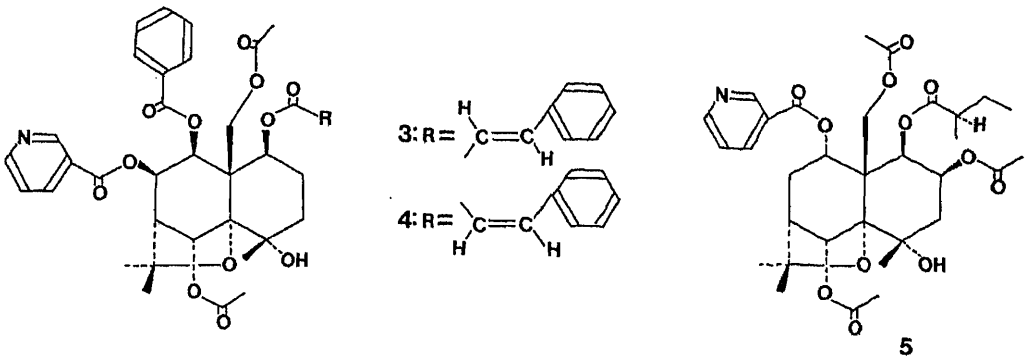


1



2

The methanol extract from the leaves of *T. wilfordii* var. *regelii* was separated by a combination of silica gel and Sephadex LH-20 column chromatography and HPLC to give five new sesquiterpene alkaloids: triptofordinine A-1, A-2, A-3, B, and C.



5

In its MS, triptofordinine A-1 (3), $C_{41}H_{43}NO_{12}$, mp 193-194°C, $[\alpha]_D$ -81.4°(MeOH), UV λ_{max} nm (ε): 220(36000), 275(17700), 282(17500), had M^+ peaks at *m/z* 741(exact mass: 741.2787; Calcd. 741.2785) along with significant fragment ion peaks at *m/z* 131(C_6H_5CH=CHCO), 106(C_5H_4NCO), 105 (C_6H_5CO), and 43(CH_3CO). The ^1H and ^{13}C NMR(in $CDCl_3$ and pyridine-d_5) and ^1H-^1H and ^1H-^{13}C COSY spectra (in pyridine-d_5) of 3 indicated the presence of partial structures A, B, and C (Fig. 1) in addition to a benzoyl, a *trans*-cinnamoyl, a nicotinoyl, two acetyl (δ_H and δ_C for CH_3: 2.17 and 21.4; 2.31 and 21.5), three *tert*-methyl groups (δ_H and δ_C: 1.66 and 24.2; 1.63 and 23.5; 1.63 and 29.2), and four *quaternary* carbons (δ_C: 53.1, 70.8, 83.8, and 92.5). These spectral data suggest that this compound is a dihydroagarofuran derivative (3), having a structure analogous to ever-1 (5).[3]

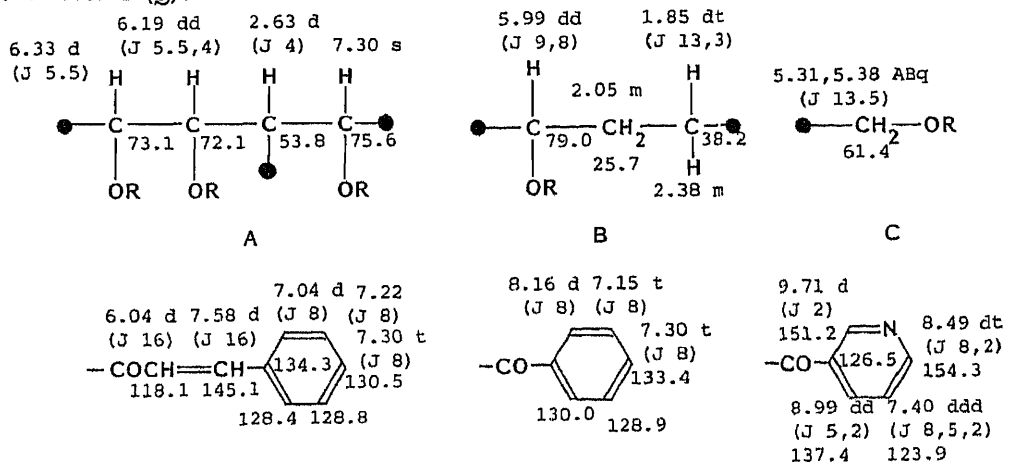


Fig. 1. Partial Structures in 3 (δ-Values in Pyridine-d_5)

As this stage, we measured the ^1H-^{13}C long-range COSY of 3 in order to confirm the assumed structure (3). As expected, the ^{13}C-signals at δ 92.5(C-5) and at δ 53.1(C-10) showed long-range correlations with the ^1H-signals at δ 7.30(6-H), 5.31, 5.38(15-H_2), 2.63(7-H), 1.85(3-H), and 1.63(14-H_3) and at δ 7.30(6-H), 6.19(8-H), 6.33(9-H), 5.99(1-H), 5.31, 5.38(15-H_2), and 2.05(2-H). respectively (Fig. 2). In turn, the ^{13}C signal at δ 70.9(C-4) was correlated with the ^1H-signals at δ 2.38, 1.85(3-H_2), 2.05(2-H), and 1.63(14-H_3), and the signal at δ 53.8 (C-7) with the ^1H-signals at δ 1.66(12-H_3) and 1.63(13-H_3). Also some other significant long-range correlations are shown by arrows in formula in Fig. 2.

Turning now to the location of the ester groupings, the carbonyl ^{13}C signal at δ 165.25

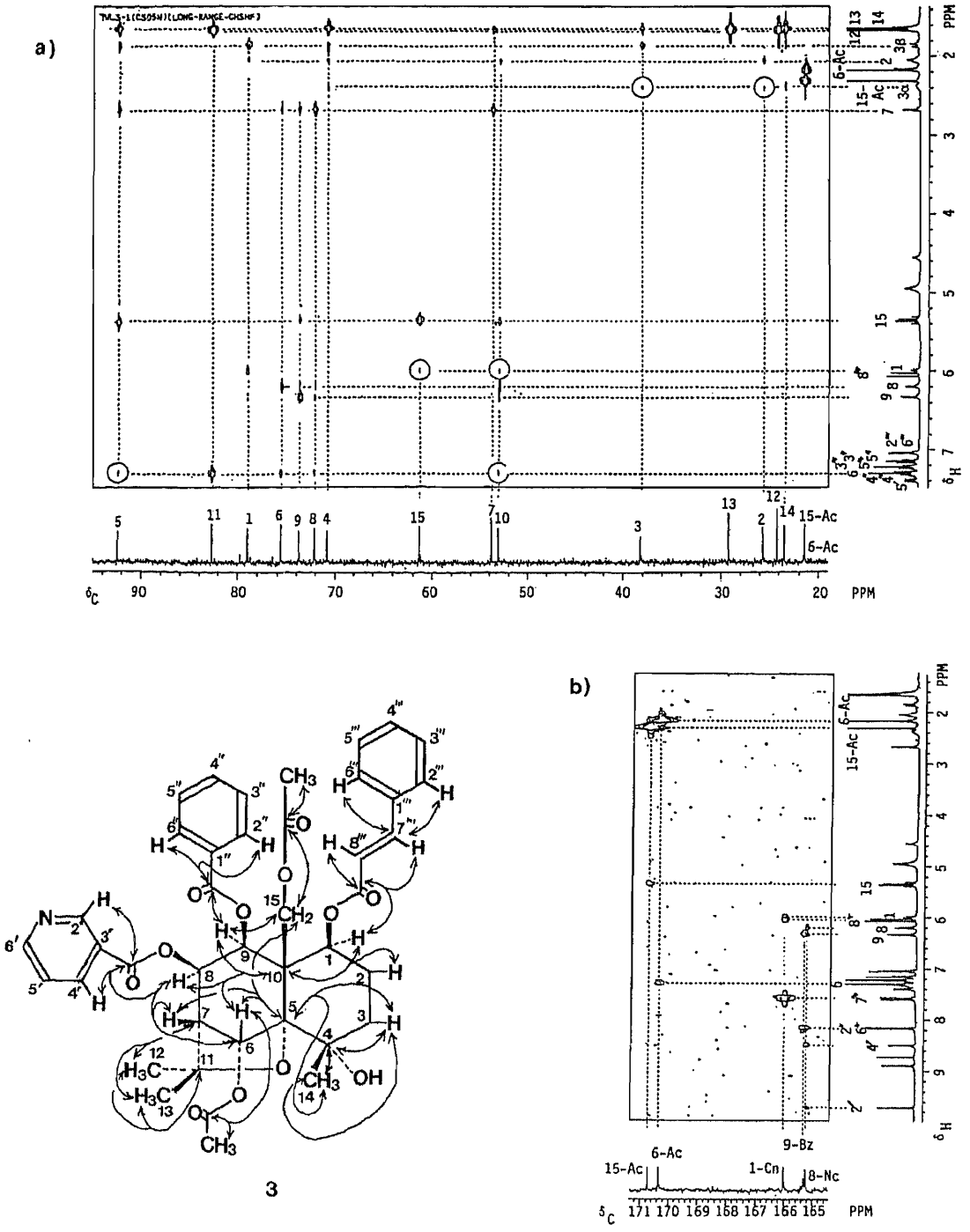


Fig. 2. ^{1}H-^{13}C Long-Range COSY Spectra of 3 in Pyridine-d_5
a) High field region, b) Low field region, (sample: 15 mg, 20°C, 12 h run, J_{CH}=10 Hz).
Open circle indicate significant but weak peaks at this threshold level.
Abbreviation; Ac: acetyl, Bz: benzoyl, Cn: cinnamoyl, Nc: nicotinoyl.

showed long range correlations with the ^{1}H-signals at δ 6.19(8-H), 8.99 and 9.71(nicotinoyl 2′- and 4′-H), and the signals at δ 165.32 and δ 166.00 were correlated with the ^{1}H-signals at δ 6.33(9-H) and 8.16(benzoyl 2″- and 6″-H) and at δ 5.99(1-H), 6.04 and 7.58(cinnamoyl 7‴- and 8‴-H), respectively. On the other hand, the carbonyl ^{13}C-signals at δ 170.35 and at δ 170.74 were correlated with the ^{1}H-signals at δ 7.36(6-H) and 2.13($COCH_3$) and at δ 5.31, 5.38(15-H_2) and 2.31($COCH_3$), respectively. Thus the plannar structure of 3 was proved.

The relative stereochemistry of 3 was determined on the basis of the coupling constants of each proton and the results of NOE experiments (200 MHz, $CDCl_3$). Irradiation at the 14-methyl group enhanced the signal intensity of 6- and 15 protons (8 and 6%, respectively) and irradiation at the 1-methine proton enhanced the signal intensity of the 9-proton (10%). Also, irradiation at the 12- and 13-methyl groups gave NOE enhancement of the 8- and 9-protons and the 7-proton, respectively. Therefore the structure of triptofordinine A-1 was proved to be 3.

Triptofordinine A-2 (4), $C_{41}H_{43}NO_{12}$, mp 94-95°C, $[\alpha]_D$ -101.0°(MeOH), contains a benzoyl, a *cis*-cinnamoyl, a nicotinoyl, and two acetyl residues as indicated by the MS, ^{1}H and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra are almost identical with those of 3 except for the signals due to the olefinic protons in the cinnamoyl residue [3: δ_H 5.72, 7.28(each d, J=16 Hz), 4: δ_H 5.21, 6.43(each d, J=12.5 Hz), in $CDCl_3$, 200 MHz]. From this comparison of ^{1}H NMR, it was concluded that in the structure of 4, the *trans*- cinnamoyl residue of 3 was replaced by the *cis*-cinnamoyl residue at C-1.

The structure elucidation of triptofordinine A-3, B, and C is in progress.

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