Binding Position of Azathioprine with Bovine Serum Albumin Determined by Measuring Nuclear Magnetic Resonance Relaxation Time[1)]

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The interaction between azathioprine (AZ) and bovine serum albumin (BSA) is mainly due to hydrophobic binding according to the dependence of the binding constant on the ionic strength obtained by equilibrium dialysis. The binding constant and partition coefficient of AZ were smaller than those of warfarin, phenylbutazone and ibuprofen. Little variation in the proton chemical shift of AZ was observed whether there was an absence or presence of BSA (7.25×10^{-5} M). The spin–lattice relaxation time (T_1) of AZ decreased in the presence of BSA to 6—22%. The spin–spin relaxation rate ($1/T_2$) of AZ increased 16—24 times for the methyl group and the imidazole ring and 8—13 times for the purine ring in the presence of BSA. The ratio of the spin–spin relaxation rate of the free AZ to the bound AZ ($(1/T_2)_b/(1/T_2)_f$) of the methyl group and the imidazole ring was 2—3 times larger than that of the purine ring. The binding of AZ to BSA was concluded to be mainly at the methyl group on the imidazole ring of AZ.

Keywords hydrophobic interaction; azathioprine; bovine serum albumin: nuclear magnetic resonance; spin–lattice relaxation time; spin–spin relaxation rate; binding position; partition coefficient; equilibrium dialysis

In part I[2)] of this series concerning the interaction between drugs and water-soluble polymers, the interaction between warfarin and water-soluble polymers (polyvinylpyrrolidone and vinylpyrrolidone-acrylamide copolymer) was ascribed to hydrophobic binding. In parts II[3)] and III,[4)] it was concluded that the hydrophobic binding predominantly played a role in the interaction between bovine serum albumin (BSA) and two kinds of drugs, phenylbutazone (PB) and ibuprofen (IB), which were classified by Sudlow *et al.*[5)] into site I drug and site II drug, respectively, according to specific sites on human serum albumin. Furthermore, the binding positions of both drugs to BSA were elucidated to be at the phenyl group by means of the nuclear magnetic resonance (NMR) spin–spin relaxation rate ($1/T_2$). Therefore, it is predicted that the microscopic investigation of binding state, specially the binding position of the drug, offers useful information about the interaction between other drugs and polymers.

In the present paper, the interaction between an immunosuppressive, azathioprine (AZ) and BSA was elucidated to be predominantly hydrophobic and weak by means of equilibrium dialysis, partition coefficient and dissociation constant. Furthermore, the binding position of AZ to BSA was determined to be at the methyl group on the imidazole ring on the basis of an increase of the relaxation rate $(1/T_2)_b$ of AZ bound to BSA.

Experimental

Materials AZ was of special reagent grade from Sigma, and was used without further purification. BSA was from Wako and its average molecular weight was 6.9×10^4. Other reagents were from commercial sources and were used without further purification.

Equilibrium Dialysis The equilibrium dialysis method was the same as described previously.[4)] Temperatures were regulated within 0.2 °C during all experiments. The drug concentration was determined by absorbance on a Shimadzu UV-190 spectrometer. UV λ_{max}^{pH7} nm (ε): AZ, 278 (14700).

NMR Spectroscopy The NMR spectra were measured in deuterium oxide (D_2O, phosphate buffer, 0.1 M, pH = 7) on a JEOL GX-400 spectrometer (radio frequency, 400 MHz; $\pi/2$ pulse, 11.1 μs) at 40.0 ± 0.5 °C. The spin–lattice relaxation time (T_1) was obtained by the inversion recovery method[6)] according to Eq. 1:

$$\ln(M_0 - M_t) = -\frac{t}{T_1} + \ln(2M_0) \quad (1)$$

where t is the interval between the π pulse and $\pi/2$ pulse, M_0 is the equilibrium magnetization at $t=0$, and M_t is the macroscopic magnetization at t. The spin–spin relaxation time (T_2) was obtained by the Carr-Purcell-Meiboom-Gill (CPMG)[7)] method according to Eq. 2:

$$\ln(M_{t_2}) = \ln(M_{t_1}) - \frac{\tau}{T_2} \quad (2)$$

where t_1 and t_2 are the times when a free induction decay (FID) is observed after the $\pi/2$ pulse, M_{t_1} and M_{t_2} are the macroscopic magnetizations at t_1 and t_2, respectively, and τ is the interval between t_1 and t_2. The spin–spin relaxation rate ($1/T_2$) and the standard deviation (S.D.) were calculated by the nonlinear least-squares method.[8)] The pulse delay time (60 s), when the next pulse was irradiated after the observation of FID, was selected to be more than five times T_1. The homo-gated irradiation was carried out in order to depress the HDO peak. An nuclear Overhauser effect (NOE) was observed by the double homo-gated irradiation method.

Measurement of pK_a Measurement of pK_a by ultraviolet (UV) spectrum was carried out by the method proposed by Flexser *et al.*[9)] pK_a, 8.0 (λ = 295 nm). Measurement of pK_a by titration was carried out as follows; 0.025 M AZ solutions involving 0.03 N HCl and 30—50% *N,N*-dimethylformamide (DMF) were titrated by 0.01 N NaOH at 25.0 ± 0.2 °C. The pK_a in the aqueous solution was estimated from pH at half equivalence after extrapolating the curve of apparent pK_a' *vs.* DMF concentration. pK_{a1}, <3.2; pK_{a2}, 7.5.

Results and Discussion

The Binding Constant of AZ to BSA Determined by Equilibrium Dialysis The binding of the drug (AZ) to the polymer (BSA) was examined by equilibrium dialysis at 20—40 °C. The free drug concentration (Df) was determined from the residual drug concentration, and the number of mol of the drug binding to one mol of the polymer (r) was estimated from the decrease in drug concentration. The plot of r *vs.* r/Df produced a linear relationship, as shown in Fig. 1, and satisfied Eq. 3 proposed by Scatchard[10)]:

$$\frac{r}{Df} = Kn - Kr \quad (3)$$

where n is the number of binding sites per mol of the polymer and K is the binding constant between the drug and the polymer. The n- and K-values were calculated from the intercept on the abscissa and the slope of the line. From the linear relationship in Fig. 1, it was considered that the

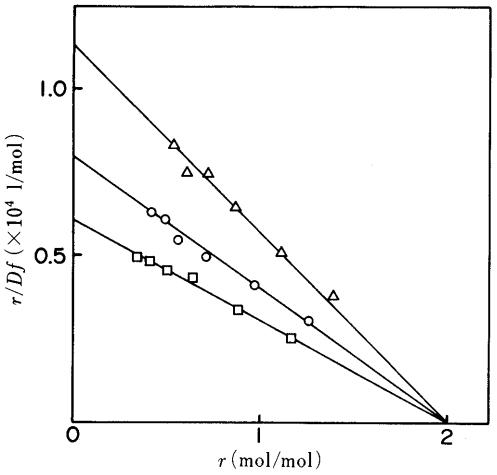


Fig. 1. Scatchard Plots for the Binding of AZ to BSA (7.25×10^{-5} M) in 0.1 M Phosphate Buffer (pH 7) at 20 °C (△), 30 °C (○) and 40 °C (□)

Table I. Dependence of the Binding Constant (K) on the Concentration of Phosphate Buffer (C)

C (M)	K ($\times 10^3$ M^{-1})
0.05	1.29
0.1	3.03
0.2	4.06

[BSA] = 7.25×10^{-5} M, pH = 7, 40 °C.

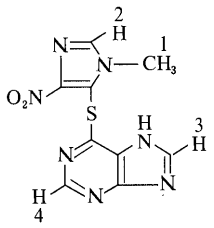
Table II. Thermodynamic Parameters for the Binding of Drugs to BSA

Drug	Temp. (°C)	K ($\times 10^3$ M^{-1})	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (Jmol^{-1} K^{-1})	n	$P^{a)}$	pK_a
AZ	20	5.63	−21.05	−23.43	−8.16	2	1.61	<3.2[b]
	30	3.92	−20.84		−8.58			8.0[c]
	40	3.03	−20.88		−8.16			
					(Av.) −8.28			
WF[2]	30	13.9	−24.03	−21.76	7.49	2	36.03	4.8
PB[3]	30	13.8	−24.02	−20.80	10.63	3	6.82	4.2
IB[4]	30	12.8	−23.80	−9.20	48.18	3	13.71	4.35

[BSA] = 7.25×10^{-5} M, pH = 7, [phos. buf.] = 0.1 M. *a*) Partition coefficient, octanol/water. *b*) pK_{a1} of the imidazolium. *c*) pK_{a2} of the purine at 25 °C.

binding site in this work was only of one class. Since the binding constant increased with ionic strength (Table I), it was considered that the interaction between AZ and BSA was not electrostatic. The binding constant of AZ to BSA was smaller than the constants of the site I drug (WF, PB) or the site II drug (IB) (Table II). As reported previously, the bindings of these three drugs to BSA were predominantly hydrophobic. Therefore, the partitions of these drugs between octanol and water were measured (Table II). The small binding constant of AZ was explained based on its small hydrophobic property. As the dissociation constants of AZ were estimated to be <3.2 (pK_{a1}) and 8.0 (pK_{a2}), the imidazole ring was present in the undissociated form, and *ca.* 10% of the purine ring existed in the ionic form at pH 7. Therefore, the hydrophobic binding of AZ to BSA was due to the methylimidazole moiety. Although WF, PB and IB were weak acids (pK_a 4—5) and present in the ionic forms at pH 7, they exhibited a hydrophobic tendency because of the strong hydrophobic property of the phenyl group. The reason why the binding constant of AZ was smaller than

Table III. Chemical Shifts ($\delta^{a)}$) and NOE of AZ



	Concentration	1-CH_3	2-CH	3-CH	4-CH
δ	1 mM AZ[b]	3.717	8.030	8.432	8.499
	1 mM AZ/7.25×10^{-5} M BSA[b]	3.708	8.024	8.424	8.492
	10 mM AZ/0.05 N NaOH	3.654	7.974	8.218	8.320
NOE[c]	1 mM AZ[b]		18.3	0	3.8

a) From TMS (external reference). *b*) pH = 7, [phos. buf.] = 0.1 M, 40 °C. *c*) Increment of peak area at irradiation to 1-CH_3, (%).

Table IV. Spin–Lattice Relaxation Time (T_1, s) of AZ

Concentration	1-CH_3	2-CH	3-CH	4-CH
1 mM AZ	2.429	6.776	3.157	7.823
1 mM AZ/7.25×10^{-5} M BSA	0.528	0.540	0.474	0.463

pH = 7, [phos. buf.] = 0.1 M, 40 °C.

the constants of WF, PB and IB was attributed to the smaller hydrophobic ability of the methylimidazole group than that of the phenyl group.

The number of binding sites of AZ on BSA was independent of temperature (20—40 °C), similarly to WF, PB and IB. The thermodynamic parameters were calculated from the linear relationship between ln K and the reciprocal absolute temperature ($1/T$) (Table II). The values of the standard increases of enthalpy (ΔH°) and free energy (ΔG°) were negative and large, similarly to WF, PB and IB. Therefore, it was considered that the binding was advantageous for enthalpy. The value of the standard increase of entropy (ΔS°) for the binding of AZ to BSA was negative and small, while those values for WF, PB and IB were positive and small. As reported previously, the binding positions of PB and IB to BSA were at the phenyl group and the hydrophobic binding may cause destruction of the iceberg structure and increase the entropy (ΔS°). However, since ΔS° was negative in the case of AZ, the weak hydrophobic binding may result in little destruction of the iceberg structure.

The Chemical Shift of AZ The chemical shifts of AZ under typical conditions are shown in Table III. The peak at 3.7 was obviously assigned to the methyl group on the imidazole ring. The 2-CH signal was assigned to the methine at the 2-position on the imidazole ring since the NOE was observed at 2-CH irradiating to 1-CH_3 (Table III). The 3-CH peak was assigned to the methine at the 8-position on the purine ring because of the largest shift by dissociation of NH on the purine ring in an alkaline solution. Bullock and Jardetzky[11] reported similar assignment of a purine derivative based on deuterium exchange. The variation of chemical shifts between AZ in the absence and presence of BSA (7.25×10^{-5} M) was less than 0.009 ppm. Therefore, it was difficult to determine the binding position from the

TABLE V. Spin–Spin Relaxation Rates ($1/T_2$) of AZ

Peak	$1/T_2 \pm$S.D. (s^{-1})	$(1/T_2)_f \pm$S.D. (s^{-1})	$n=2$ (Dialysis)		$n=100$ (Arbitrary)	
			$(1/T_2)_b$ ($\times 10^2 s^{-1}$)	$(1/T_2)_b/(1/T_2)_f$ ($\times 10$)	$(1/T_2)_b$ ($\times 10 s^{-1}$)	$(1/T_2)_b/(1/T_2)_f$ ($\times 10$)
1-CH_3	17.12±1.08	0.719±0.038	1.556	21.65	6.17	8.58
2-CH	11.46±0.79	0.727±0.031	1.021	14.05	4.06	5.59
3-CH	12.00±0.78	1.526±0.047	1.004	6.58	4.05	2.65
4-CH	14.93±0.74	1.192±0.117	1.310	10.99	5.23	4.39

$1/T_2$, overall observed (1 mM AZ/7.25×10^{-5} M BSA); $(1/T_2)_f$, observed for free AZ (1 mM); $(1/T_2)_b$, calculated for AZ bound to BSA; n, number of binding sites on BSA; S.D., standard deviation.

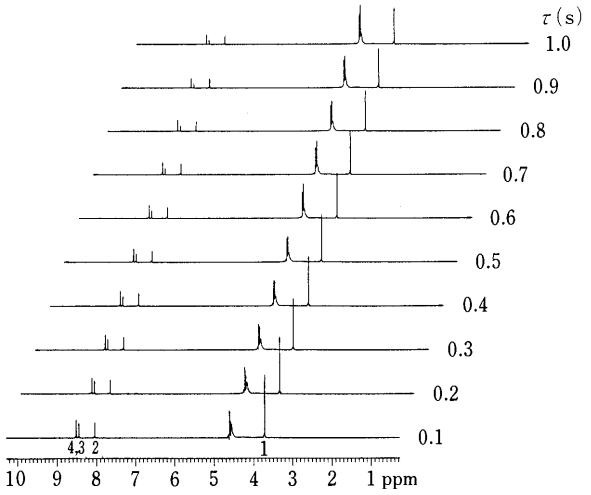


Fig. 2. Spin–Spin Relaxation Traces Obtained by the Carr-Purcell-Meiboom-Gill Method for Protons of AZ at 40 °C

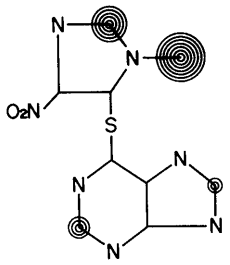


Chart 1. Contour Plot of the Ratio of the Spin–Spin Relaxation Rate of Free AZ to Bound AZ

TABLE VI. Binding Constants in the Absence and Presence of Competitor

Drug	Competitor	K ($\times 10^3 M^{-1}$)
WF		10.46
WF	AZ	4.18[a]
AZ		3.03
AZ	IB	1.30[a]

[BSA] = 7.25×10^{-5} M, [competitor] = 1 mM, pH = 7, 40 °C. *a*) Apparent binding constant.

change of chemical shift of AZ.

Spin–Lattice Relaxation Time The spin–lattice relaxation time (T_1) of AZ under typical conditions was measured by the inversion recovery method (Table IV). The T_1-values of each proton in the presence of BSA (7.25×10^{-5} M) decreased to 21.74% (1-CH_3), 7.97 (2-CH), 15.01% (3-CH), 5.92% (4-CH) respectively, compared with its absence. Though it was evident from these results that the motion of the imidazole and purine rings slowed down, it might be difficult to determine clearly the binding position of AZ to BSA.

Spin–Spin Relaxation Rate The spin–spin relaxation time (T_2) of AZ was measured by the CPMG method and a series of specta are shown in Fig. 2. Ueda *et al.*[12] described that the spin–spin relaxation rate ($1/T_2$) was a sensitive parameter for analyzing small variations in molecular environment. Therefore, $1/T_2$ was used instead of T_2 (Table V). In the presence of BSA (7.25×10^{-5} M), the $1/T_2$-values of the *N*-methylimidazole group and the purine ring increased *ca.* 16—24 and 8—13 times, respectively, compared with its absence. Therefore, it was concluded that the binding position of AZ to BSA was the *N*-methylimidazole group. Furthermore, in order to consider the binding position quantitatively, the spin–spin relaxation rate of the bound drug $(1/T_2)_b$ was calculated according to Eq. 4 proposed by Jardetzky[13]:

$$\frac{1}{T_2}=\left(\frac{1}{T_2}\right)_f+B\left[\left(\frac{1}{T_2}\right)_b-\left(\frac{1}{T_2}\right)_f\right] \quad (4)$$

where $(1/T_2)_f$ is the spin–spin relaxation rate of the free drug and B is the proportion of the drug (AZ) bound to the protein (BSA). The B-value could be calculated from the binding constant (K) and the number of binding sites (n) obtained by equilibrium dialysis. When the calculated value of B and the values of $1/T_2$ and $(1/T_2)_f$ were substituted into Eq. 4, $(1/T_2)_b$ could be evaluated (Table V). It was impossible to determine the binding position based on the value of $(1/T_2)_b$ because of different spin–spin relaxation rates of the free drug $(1/T_2)_f$. Therefore, the ratio between the $1/T_2$ values of the bound drug and the free ($(1/T_2)_b/(1/T_2)_f$) was used. The $(1/T_2)_b/(1/T_2)_f$ values of the methyl group and the imidazole ring were 2—3 times as large as that of the purine ring. Thus, it was quantitatively confirmed that the binding position of AZ to BSA was at the methyl group on the imidazole ring. The contour plot of the ratio $(1/T_2)_b/(1/T_2)_f$ is clearly illustrated in Chart 1.

As mentioned above, the imidazole ring existed in the undissociated form at pH 7 (in other words, it was hydrophobic), while *ca.* 10% of the purine ring existed in the ionic form (in other words, hydrophilic). Since the hydrophobic nature of each proton of AZ agreed with the order of the $(1/T_2)_b/(1/T_2)_f$-values (1-CH_3 > 2-CH > 4-CH > 3-CH), the conclusion mentioned above was supported again. The drug/protein ratio examined by NMR was larger than that of equilibrium dialysis or the other

methods. Therefore, it was considered that the binding site examined by NMR ([AZ] = 1×10^{-3} M, [BSA] = 7.25×10^{-5} M) was secondary (in other words, nonspecific). Even if the nonspecific site (n) was assumed arbitrarily to be 100, which was sufficiently larger than that of the primary site ($n=2$), the relative values of $(1/T_2)_b/(1/T_2)_f$ did not vary, although the absolute values decreased as shown in Table V. Consequently, the conclusion mentioned above stands even if the binding is nonspecific.

AZ competed with WF or IB binding to BSA in the equilibrium dialysis method (Table VI). From these results, it was understood that the binding site of AZ on BSA competed with those of WF and IB, and the site of AZ might be similar to the site I and site II classified by Sudlow *et al.*[5]

References and Notes

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Revised Substituent Entropy Constants σ_{s° for Di- and Tri-Substituted Benzene Derivatives, and Their Applications for Substrates Having Two Substituted Phenyl Rings

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The revised substituent entropy constants σ_{s° representing both dispersion and repulsion interactions are expressed by the next equations; $\sigma_{s^\circ}(12)=0.859\Sigma\sigma_{s^\circ}(\text{mono})-0.011$; $\sigma_{s^\circ}(13)=0.894\Sigma\sigma_{s^\circ}(\text{mono})-0.013$; $\sigma_{s^\circ}(14)=0.905\Sigma\sigma_{s^\circ}(\text{mono})-0.022$; $\sigma_{s^\circ}(123)=0.779\Sigma\sigma_{s^\circ}(\text{mono})-0.021$; $\sigma_{s^\circ}(124)=0.765\Sigma\sigma_{s^\circ}(\text{mono})-0.007$; $\sigma_{s^\circ}(135)=0.817\Sigma\sigma_{s^\circ}(\text{mono})-0.033$; where the correction due to the symmetry number, $n\times R\ln 2$, should be introduced optionally. The descriptors σ_{s° of the substrates having two substituted phenyl rings—namely, biphenyls, diphenyl ethers, -amines, -methanes, sulfides and benzophenones—could be determined by the joint use of the next equations; $\sigma_{s^\circ}(1234)=0.704\Sigma\sigma_{s^\circ}(\text{mono})-0.024$; $\sigma_{s^\circ}(1235)=0.702\Sigma\sigma_{s^\circ}(\text{mono})-0.018$; $\sigma_{s^\circ}(1245)=0.706\Sigma\sigma_{s^\circ}(\text{mono})-0.023$; $\sigma_{s^\circ}(\text{penta})=0.618\Sigma\sigma_{s^\circ}(\text{mono})-0.012$; $\sigma_{s^\circ}(\text{hexa})=0.570\Sigma\sigma_{s^\circ}(\text{mono})-0.012$.

Observed results suggest that the concept of *isosterism* could probably be related to a similar level of dispersion and repulsion interactions between receptor and substrate.

Keywords entropy constant; absolute entropy; quantitative structure–activity relationship; regression analyses; polychlorinated biphenyls; substituted diphenyl ether herbicide; substituted benzophenone antiinflammatory agent

Introduction

In our previous report,[1] the authors proposed the procedure for the estimation of the descriptor σ_{s° for tetra-, penta-, and hexa-substituted benzene derivatives, taking the number of symmetry into account, where the observed and calculated values of σ_{s° as well as $S^\circ_{298}(g)$ agreed well with each other.

On the other hand, Kawaki *et al.*[2] previously proposed the empirical method for the estimation of unknown descriptor by means of statistical treatment, referencing those of monosubstituted benzene derivatives. Afterwards, their treatments were also extended to tri-substituted benzene series,[3] but it is impossible for us to extend their procedures for polysubstituted benzene derivatives.

In this work, in view of the successful result of polysubstituted benzene series in our previous work,[1] we have reexamined the di- and tri-substituted benzene series by means of regression analysis, using $\Sigma\sigma_{s^\circ}(\text{mono})$ as an independent variable, and agreements between observed and calculated values are established after the correction of the symmetry. Next, we tried to extend our procedures to the estimation of the descriptor of substituted aromatics having two kinds of phenyl rings—namely, polychlorinated biphenyls, substituted diphenyl ether herbicides and substituted benzophenone antiinflammatory agents, and referred to the substances of "*isosterism.*"

Experimental

Absolute Entropy $S^\circ_{298}(g)$ Observed absolute entropies cited in this work are all referred from the data sources.[4]

Substituent Entropy Constant σ_{s°[5] The substituent entropy constant σ_{s° is defined as below;

$$\sigma_{s^\circ}=\log\{S^\circ_{298}(g)_A/S^\circ_{298}(g)_B\}$$

where subscript A denotes substituted benzene or methane, and B means unsubstituted benzene or methane.

Descriptors σ_{s° for $C_6H_{6-n}R_n$ ($n=2$ and 3) Descriptors σ_{s° for 1,2-, 1,3-, 1,4-, 1,2,3-, 1,2,4-, and 1,3,5-, substituted benzene series are estimated according to the next equations 1—6, using the data arranged in Tables I and II;

$$\sigma_{s^\circ}(12)=0.859\Sigma\sigma_{s^\circ}(\text{mono})-0.011 \quad (1)$$

$$\sigma_{s^\circ}(13)=0.894\Sigma\sigma_{s^\circ}(\text{mono})-0.013 \quad (2)$$

$$\sigma_{s^\circ}(14)=0.905\Sigma\sigma_{s^\circ}(\text{mono})-0.022 \quad (3)$$

$$\sigma_{s^\circ}(123)=0.779\Sigma\sigma_{s^\circ}(\text{mono})-0.021 \quad (4)$$

$$\sigma_{s^\circ}(124)=0.765\Sigma\sigma_{s^\circ}(\text{mono})-0.007 \quad (5)$$

$$\sigma_{s^\circ}(135)=0.817\Sigma\sigma_{s^\circ}(\text{mono})-0.033 \quad (6)$$

Equations 7—11 determined for tetra-, penta-, and hexa-substituted benzene series have been shown in our previous report.[1]

$$\sigma_{s^\circ}(1234)=0.704\Sigma\sigma_{s^\circ}(\text{mono})-0.024 \quad (7)$$

$$\sigma_{s^\circ}(1235)=0.702\Sigma\sigma_{s^\circ}(\text{mono})-0.018 \quad (8)$$

$$\sigma_{s^\circ}(1245)=0.706\Sigma\sigma_{s^\circ}(\text{mono})-0.023 \quad (9)$$

$$\sigma_{s^\circ}(\text{penta})=0.618\Sigma\sigma_{s^\circ}(\text{mono})-0.012 \quad (10)$$

$$\sigma_{s^\circ}(\text{hexa})=0.570\Sigma\sigma_{s^\circ}(\text{mono})-0.012 \quad (11)$$

Regression Analyses Regression analyses were carried out on NEC PC-9801/M/VX and Epson PC-286V personal computers using a program package for multi-variate analyses MVA developed by Takagi *et al.*[6]

Results and Discussion

Disubstituted Benzene Derivatives The descriptors σ_{s° obtained from the observed $S^\circ_{298}(g)$ of $C_6H_4R_2$ (R=Me, Et, F, Cl, Br) cited from the data sources[4] afford linear relations with $\Sigma\sigma_{s^\circ}(\text{mono})$ after regression analyses as below;

$$\sigma_{s^\circ}(12)=0.859(0.005)\Sigma\sigma_{s^\circ}(\text{mono})-0.011(0.008) \quad (1)$$
$$n=5,\ r=1.000,\ F=3020,\ \text{S.D.}=0.002$$

$$\sigma_{s^\circ}(13)=0.894(0.055)\Sigma\sigma_{s^\circ}(\text{mono})-0.013(0.009) \quad (2)$$
$$n=5,\ r=0.999,\ F=2685,\ \text{S.D.}=0.002$$

$$\sigma_{s^\circ}(14)=0.905(0.063)\Sigma\sigma_{s^\circ}(\text{mono})-0.022(0.011) \quad (3)$$
$$n=5,\ r=0.999,\ F=2098,\ \text{S.D.}=0.002$$

The calculated descriptors given by Eqs. 1—3 agreed well with those of the observed ones as arranged in Table I, and the unknown values were also estimated as shown in Table II.

Furthermore, as estimated in Table III, the calculated descriptors for $C_6H_4R_1R_2$ ($R_1\neq R_2$), after correcting the symmetry element, agreed well with the observed values, but some of their ΔS° exceed over ± 2.0 e.u.

Trisubstituted Benzen Derivatives The descriptors σ_{s°

TABLE I. Observed and Calculated S°_{298}(g)/e.u. of Disubstituted Benzene Derivatives

	Obs.		Calcd.		ΔS°
	S°_{298}(g)	$\sigma_{s^{\circ}}$	S°_{298}(g)	$\sigma_{s^{\circ}}$	
1. *o*-F_2	76.94	0.078	76.75	0.077	+0.19
2. *m*-F_2	76.50	0.075	77.03	0.078	−0.53
3. *p*-F_2	75.30	0.068	75.65	0.070	−0.35
4. *o*-Cl_2	81.66	0.104	81.45	0.102	+0.21
5. *m*-Cl_2	82.07	0.106	81.94	0.105	+0.13
6. *p*-Cl_2	80.46	0.097	80.53	0.097	−0.07
7. *o*-Br_2	86.43	0.128	86.42	0.128	+0.01
8. *m*-Br_2	87.69	0.134	87.16	0.132	+0.53
9. *p*-Br_2	86.33	0.128	85.72	0.125	−0.61
10. *o*-Me_2	84.31	0.117	84.73	0.120	−0.42
11. *m*-Me_2	85.49	0.123	85.38	0.123	+0.11
12. *p*-Me_2	84.23	0.117	83.95	0.115	+0.28
13. *o*-Et_2	103.81	0.208	103.67	0.207	+0.14
14. *m*-Et_2	104.99	0.213	105.33	0.214	−0.34
15. *p*-Et_2	103.73	0.207	103.84	0.208	−0.11

TABLE II. Calculated Descriptors $\sigma_{s^{\circ}}$ of Disubstituted Benzene $C_6H_4R_1R_2$ ($R_1=R_2$) Derivatives

	$\Sigma\sigma_{s^{\circ}}$ (mono)	12		13		14
NO_2	0.208		0.168		0.173	0.166
CN	0.152[a]		0.120		0.123	0.116
CF_3	0.282[a]		0.231		0.239	0.233
COMe	0.282[a]		0.231		0.239	0.233
CO_2Me	0.370[a]		0.307		0.318	0.313
F	0.102[a]	0.078[a]	0.077	0.076[a]	0.078	0.069[a] 0.070
Cl	0.132[a]	0.103[a]	0.102	0.106[a]	0.105	0.097[a] 0.097
Br	0.162[a]	0.128[a]	0.128	0.134[a]	0.132	0.128[a] 0.125
I	0.188[a]		0.150		0.155	0.148
Me	0.152[a]	0.117[a]	0.120	0.123[a]	0.123	0.117[a] 0.166
Et	0.254[a]	0.208[a]	0.207	0.213[a]	0.214	0.207[a] 0.208
OMe	0.254[a]		0.207		0.214	0.208
OH	0.138[a]		0.108		0.110	0.103
NH_2	0.148[a]		0.116		0.119	0.112
NMe_2	0.268[a]		0.219		0.227	0.221

a) Observed values.

calculated from the observed S°_{298}(g)[4] for $C_6H_3R_3$ (R = F, Cl, Me, Et) and 1,3,5-Br_3-C_6H_3 gave Eqs. 4—6, using $\Sigma\sigma_{s^{\circ}}$(mono) as an independent variable.

$$\sigma_{s^{\circ}}(123)=0.779\Sigma\sigma_{s^{\circ}}(\text{mono})-0.021 \quad (4)$$
$$n=4,\ r=0.999,\ F=992.6$$

$$\sigma_{s^{\circ}}(124)=0.765\Sigma\sigma_{s^{\circ}}(\text{mono})-0.007 \quad (5)$$
$$n=4,\ r=0.999,\ F=27006$$

$$\sigma_{s^{\circ}}(135)=0.817\Sigma\sigma_{s^{\circ}}(\text{mono})-0.033 \quad (6)$$
$$n=5,\ r=0.999,\ F=1511$$

The agreements between observed and calculated S°_{298}(g), as well as those of $\sigma_{s^{\circ}}$, are summarized in Table IV, and the unknown values are also arranged in Table V.

But, unfortunately, because of a shortage of observed S°_{298}(g) data for $C_6H_3R_1R_2R_3$ ($R_1=R_2\neq R_3$ or $R_1\neq R_2\neq R_3$), we are unable to confirm visible proof on the validity of Eqs. 4—6.

After all, the procedures shown above are more logical than that of the statistical one given in our previous reports,[2,3] and Eqs. 1—6, together with Eqs. 7—11, could be expected to display their merits in the estimation of the

TABLE III. Observed and Calculated $\sigma_{s^{\circ}}$ as well as S°_{298}(g)/e.u. of Disubstituted Benzene $C_6H_4R_1R_2$ ($R_1=R_2$) Derivatives

	Obs.		Calcd.		ΔS°
	S°_{298}(g)	$\sigma_{s^{\circ}}$	S°_{298}(g)	$\sigma_{s^{\circ}}$	
1. *o*-F–NO_2	85.18	0.122	88.49	0.138	−3.31
2. *m*-F–NO_2	85.85	0.125	89.26	0.142	−3.41
3. *p*-F–NO_2	84.55	0.119	86.44	0.128	−1.89
4. *o*-Cl–NO_2	88.60	0.139	91.11	0.151	−2.51
5. *m*-Cl–NO_2	89.15	0.142	92.01	0.155	−2.86
6. *p*-Cl–NO_2	87.76	0.135	89.18	0.142	−1.42
7. *o*-Br–NO_2	91.97	0.135	93.81	0.164	−1.84
8. *m*-Br–NO_2	92.91	0.159	94.85	0.169	−1.94
9. *p*-Br–NO_2	90.53	0.148	92.02	0.155	−1.19
10. *o*-I–NO_2	94.24	0.166	96.22	0.175	−1.98
11. *m*-I–NO_2	95.01	0.169	97.39	0.180	−2.38
12. *p*-I–NO_2	92.52	0.158	94.54	0.167	−2.02
13. *o*-F–Cl	80.74	0.099	80.44	0.097	+0.30
14. *o*-F–Br	83.59	0.114	82.82	0.110	+0.77
15. *o*-F–I	85.60	0.124	84.94	0.121	+0.66
16. *m*-F–Cl	81.02	0.100	80.82	0.099	+0.20
17. *p*-F–Cl	76.90	0.077	78.05	0.084	−1.15
18. *o*-Cl–Br	86.58	0.129	85.28	0.122	+1.30
19. *o*-Me–Et	95.42	0.171	95.10	0.170	+0.32
20. *m*-Me–Et	96.60	0.176	96.21	0.175	+0.39
21. *p*-Me–Et	95.34	0.171	93.37	0.162	+1.97
22. *o*-Me–OH	85.47	0.123	84.94	0.121	+0.57
	84.80	0.120	84.94	0.121	−0.14
23. *m*-Me–OH	85.27	0.122	85.54	0.124	−0.27
	83.20	0.112	85.54	0.124	−2.34
24. *p*-Me–OH	83.09	0.111	82.74	0.109	+0.35
	81.82	0.104	82.74	0.109	−0.92
25. *p*-Me–CN	84.60	0.119	83.95	0.116	+0.65
26. *o*-F–NH_2	82.91	0.110	81.70	0.104	+1.21
27. *m*-F–NH_2	83.54	0.113	82.14	0.106	+1.40
28. *p*-F–NH_2	81.84	0.104	79.36	0.091	+2.48
29. *p*-F–CHO	89.43	0.143	88.44	0.138	+0.99
30. *p*-Cl–CHO	92.09	0.156	91.25	0.152	+0.84
31. *p*-Br–CHO	94.90	0.169	94.15	0.165	+0.75
32. *p*-F–Me	81.19	0.101	79.69	0.093	+1.50

TABLE IV. Observed and Calculated S°_{298}(g)/e.u. of Trisubstituted Benzene Derivatives

	Obs.		Calcd.		ΔS°
	S°_{298}(g)	$\sigma_{s^{\circ}}$	S°_{298}(g)	$\sigma_{s^{\circ}}$	
1. 123-F_3	80.07	0.095	80.66	0.098	−0.59
2. 124-F_3	82.61	0.109	82.89	0.110	−0.28
3. 135-F_3	78.75	0.088	79.52	0.092	−0.77
4. 123-Cl_3	88.47	0.138	87.44	0.133	+1.03
5. 124-Cl_3	89.94	0.145	89.73	0.144	+0.21
6. 135-Cl_3	86.64	0.129	86.55	0.129	+0.09
7. 135-Br_3	95.30	0.171	94.19	0.165	+1.11
8. 123-Me_3	91.98	0.155	92.28	0.157	−0.30
9. 124-Me_3	94.59	0.167	94.60	0.167	−0.01
10. 135-Me_3	92.09	0.156	91.57	0.153	+0.52
11. 123-Et_3	121.23	0.275	121.42	0.276	−0.19
12. 124-Et_3	123.84	0.284	123.86	0.284	−0.02
13. 135-Et_3	121.34	0.276	122.11	0.278	−0.77

descriptor $\sigma_{s^{\circ}}$ for substituted benzenes having an optional set of substituent groups, by correcting the symmetry factor.

In the next section, using Eqs. 1—11, the authors tried to estimate the descriptor $\sigma_{s^{\circ}}$ of the substrate having two kinds of aryl rings in the molecular framework.

Estimation of the Descriptor $\sigma_{s^{\circ}}$ for Substrate Having Two

TABLE V. Calculated Descriptors σ_{s° of Trisubstituted Benzene C_6H_3-$R_1R_2R_3$ ($R_1=R_2=R_3$) Derivatives

	$\Sigma\sigma_{s^\circ}$ (mono)	123-		124-		135-	
NO_2	0.312		0.222		0.232		0.222
CN	0.228[a]		0.157		0.167		0.153
COMe	0.423[a]		0.309		0.317		0.313
COEt	0.540		0.400		0.406		0.408
CF_3	0.423[a]		0.309		0.317		0.312
CO_2Me	0.555		0.411		0.418		0.420
CO_2Et	0.645		0.481		0.486		0.494
F	0.153[a]	0.095[a]	0.098	0.109[a]	0.110	0.088	0.092
Cl	0.198[a]	0.138[a]	0.133	0.145[a]	0.144	0.129[a]	0.129
Br	0.243[a]		0.168		0.179	0.171[a]	0.166
I	0.282[a]		0.199		0.211		0.197
Me	0.228[a]	0.155[a]	0.157	0.167[a]	0.167	0.156[a]	0.153
Et	0.381[a]	0.275[a]	0.276	0.284[a]	0.284	0.276[a]	0.278
OMe	0.381[a]		0.276		0.284		0.278
OEt	0.510		0.376		0.383		0.384
OH	0.207[a]		0.140		0.151		0.136
NH_2	0.222[a]		0.152		0.163		0.148
NMe_2	0.402[a]		0.292		0.301		0.295

a) Observed values.

TABLE VIa. Chlorinated Biphenyls C_6H_5–$C_6H_{5-n}Cl_n$ ($n=1,\cdots,5$) and Their Descriptor σ_{s°

Position	σ_{s°	Position	σ_{s°
1. H	0.164	11. 2,3,4-Cl_3	0.236
2. 2-Cl	0.193	12. 2,4,5-Cl_3	0.243
3. 3-Cl	0.199	13. 2,3,6-Cl_3	0.236
4. 4-Cl	0.192	14. 2,4,6-Cl_3	0.236
5. 2,4-Cl_2	0.219	15. 3,4,5-Cl_3	0.241
6. 3,4-Cl_2	0.219	16. 2,3,5-Cl_3	0.241
7. 3,5-Cl_2	0.220	17. 2,3,4,5-Cl_4	0.258
8. 2,6-Cl_2	0.215	18. 2,3,5,6-Cl_4	0.253
9. 2,5-Cl_2	0.219	19. 2,3,4,6-Cl_4	0.258
10. 2,3-Cl_2	0.215	20. Cl_5	0.283

TABLE VIb. Chlorinated Biphenyls $Cl_nC_6H_{5-n}$–$C_6H_{5-n'}Cl_{n'}$ ($n, n'=1,\cdots,5$) and Their Descriptors σ_{s°

Position n	Position n'	σ_{s°	Position n	Position n'	σ_{s°
1. 4	4′	0.217	18. 2,4	2′,4′	0.262
2. 3,4	4′	0.241	19. 2,5	2′,5′	0.262
3. 3,4	3′,4′	0.262	20. 2,3,4,5	2′	0.274
4. 3,4,5	3′,4′,5′	0.295	21. 2,3,4	2′,5′	0.275
5. 2	4′	0.217	22. 2,4,5	2′,5′	0.281
6. 3,4	2′	0.241	23. 2,3,4	2′,3′	0.286
7. 2,4	3′,4′	0.262	24. 2,3,4,5	2′,3′	0.289
8. 2,5	3′,4′	0.262	25. 2,4,5	2′,4′,5′	0.293
9. 2,4	3′	0.247	26. 2,3,4,5	2′,3′,4′,5′	0.315
10. 2,5	4′	0.241	27. 2,3,4,6	2′	0.274
11. 2,5	3′	0.244	28. 2,3,5,6	2′,3′	0.287
12. 2,4	4′	0.241	29. 2,3,5,6	2′,4′,5′	0.306
13. 2,3,4,5	3′,4′,5′	0.307	30. 2,3,4,5,6	2′,3′,4′,5′	0.335
14. 2	2′	0.217	31. 2,6	2′,6′	0.249
15. 2,5	2′	0.241	32. 2,4,6	2′,4′,6′	0.287
16. 2,3	2′,3′	0.255	33. 2,3,5,6	2′,3′,5′,6′	0.308
17. 2,3,5	2′	0.260	34. 2,3,4,5,6	2′,3′,4′,5′	0.248

Substituted Phenyl Rings. Polychlorinated Biphenyls The estimation of the descriptor σ_{s° for polychlorinated biphenyl has important significance at present to the evaluation of aquatic toxicity[7] in the field of echotoxicology. In this work, the authors determined the descriptor by the step shown in Chart 1, where two examples are taken as exercise.

1. 2,3′,5-Cl_3-biphenyl

Using $\sigma_{s^\circ}(Ph)=0.164$ and $\sigma_{s^\circ}(Cl)=0.066$, we get the descriptor for 2,5-Cl_2-biphenyl as $\sigma_{s^\circ}(124)=0.219$. Similarly, 3-Cl-biphenyl gives $\sigma_{s^\circ}(13)=0.193$. The sum of converted $S^\circ_{298}(g)=100.25$ e.u. from $\sigma_{s^\circ}(13)=0.193$ and the correction of symmetry $R\ln 2=1.38$ e.u. afford $S^\circ_{298}(g)=101.63$ e.u. and $\sigma_{s^\circ}=0.199$. Under the prerequisite given above, we get the descriptor of the substrate by two ways 1) and 2) as below;

1) $\sigma_{s^\circ}(124)=0.246$.

2) $\sigma_{s^\circ}(13)=0.241$. Sum of $S^\circ_{298}(g)=112.27$ e.u. converted from $\sigma_{s^\circ}(13)=0.241$ and the correction of symmetry $R\ln 2=1.38$ e.u. afford $S^\circ_{298}(g)=113.65$ e.u. and $\sigma_{s^\circ}=0.247$.

The mathematical mean value of 1) and 2) becomes $\sigma_{s^\circ}=0.247$.

2. 2,2′,3,4′,5,5′,6-Cl_7-biphenyl

Using $\sigma_{s^\circ}(Ph)=0.164$ and $\sigma_{s^\circ}(Cl)=0.066$, we get the descriptor for 2,3,5,6-Cl_4-biphenyl, having one axis of symmetry, as below;

$\sigma_{s^\circ}(penta)=0.253$.

Similarly 2,4,5-Cl_3-biphenyl gives $\sigma_{s^\circ}(1245)=0.233$. The sum of the converted $S^\circ_{298}(g)=109.91$ e.u. from $\sigma_{s^\circ}(1245)=0.233$ and the correction of $2\times R\ln 2=2.75$ e.u. afford $S^\circ_{298}(g)=112.66$ e.u. and $\sigma_{s^\circ}=0.243$. Here we get the descriptor of the substrate by two ways 1) and 2) as below;

1) $\sigma_{s^\circ}(penta)=0.301$.

The sum of $S^\circ_{298}(g)=128.77$ e.u. converted from $\sigma_{s^\circ}(penta)=0.301$ and the correction of symmetry $R\ln 2=1.38$ e.u. afford $S^\circ_{298}(g)=130.15$ e.u. and $\sigma_{s^\circ}=0.305$.

2) $\sigma_{s^\circ}(1245)=0.295$.

The sum of $S^\circ_{298}(g)$ converted from $\sigma_{s^\circ}(1245)=0.295$ and the correction of symmetry $2\times R\ln 2=2.75$ e.u. afford $S^\circ_{298}(g)=129.78$ e.u. and $\sigma_{s^\circ}=0.305$. The mathematical average of 1) and 2) gives $\sigma_{s^\circ}=0.306$.

Chart 1. Estimation of σ_{s° for 2,3′,5-Cl_3- and 2,2′,3,4′,5,5′,6-Cl_7-Biphenyls

	MeR			PhR		
	$S^\circ_{298}(g)$	σ_{s°	$R\ln 3$	$S^\circ_{298}(g)$	σ_{s°	$R\ln 3$
COMe	70.49	0.200	×2	89.12	0.141	×1
COEt	80.81	0.259	2		0.180	1
CO–*n*-Pr	89.91	0.305	2		0.215	1
COPh	89.12	0.301	1			
	86.94[a]	0.290[a]	2	102.68[a]	0.203[a]	1
				+2.18		
				−1.38[b]		
				103.48[a]	0.206[a,b]	0
Me	54.85	0.091	2	76.64	0.076	1
Et	64.51	0.161	2	86.15	0.127	1
n-Pr	74.12	0.221	2	95.76	0.173	1
n-Bu	83.40	0.273	2	105.04	0.213	1
CH_2Ph	86.15	0.284	1	105.56[a]	0.215[a]	1
	83.97[a]	0.276[a]	2	107.74[a]	0.224[a,b]	0
OMe	63.83	0.156	2	86.2	0.127	1
OEt	74.24	0.222	2		0.175	1
O–*n*-Pr	84.13	0.276	2		0.215	1
OPh	86.2	0.287	1	105.56[a]	0.215[a]	1
	−2.18[b]			+2.18[b]		
	84.02[a]	0.276[a]	2	107.74	0.224[a,b]	0
SMe	68.32	0.186	2	89.84[a]	0.145[a]	1
SEt	79.62	0.252	2		0.195	1
S–*n*-Pr	88.84	0.300	2		0.230	1
SPh	89.84[a]	0.305[a]	1	+109.26[a]	0.230[a]	
	−2.18[b]			+2.18[b]		
	87.66	0.294	2	111.44[a]	0.239[a,b]	0

Chart 2. Estimations of Descriptors σ_{s° for Ph–X–Ph (X=CO, CH_2, O, S)[c]

a) Estimated values. *b*) Final values. *c*) $S^\circ_{298}(g)$ and σ_{s° for four series having same set of $R\ln 3$ are mutually linear.

Namely, in conclusion, it is common practice for us to determine the descriptor as a whole molecule after estimating the descriptor of the substrate having a Cl atom on one side phenyl ring. The results are summarized in Tables VIa and VIb, respectively.

Table VIIa. Substituted Diphenyl Ethers and Their Descriptors σ_{s°

Substituent	σ_{s°	Substituent	σ_{s°
1. H	0.224	5. 2,4-Cl_2	0.265
2. 4-NO_2	0.294	6. 2,4,6-Cl_3	0.278
3. 2,4-Cl_2-6-F	0.273	7. 3-OEt-4-NO_2	0.382
4. 2-Cl-4-CF_3	0.322		

Table VIIb. Substituted Diphenyl Ethers and Their Descriptors σ_{s°

Substituent					σ_{s°
2	4	6	3′	4′	
F	Cl	Cl		NO_2	0.330
NO_2	CF_3			NO_2	0.415
Cl	CF_3			NO_2	0.379
Cl	Cl			NO_2	0.325
Cl	Cl	Cl		NO_2	0.333
Cl	CF_3		OEt	NO_2	0.451

Substituted Ph–X–Ph (X= C=O, CH_2, NH, O, S) and Their Descriptors σ_{s° Ph–X–Ph type molecules, having X= C=O, CH_2, O, and S, are well known, because some of them are important as herbicides in antiinflammatory agents. In view of the successful estimation of σ_{s° for polychlorinated biphenyls, we have tried to extend our procedure to the entitled substrate.

In the first place, the descriptors of unsubstituted Ph–X–Ph (X= C=O, CH_2, O, S) are determined stepwise as given in Chart 2.

1. Substituted Diphenyl Ether as Herbicide[8] As summarized in Table VIIa, the descriptor σ_{s° for substrate having a substituent on one side of the phenyl ring is established by the usual way. And, using the descriptors of Table VIIa, we are able to estimate the descriptor of substrate having a substituent on both sides, and the results are given in Table VIIb. In this section, the estimation of the descriptor for 2-Cl–4-CF_3–3′-OEt–4′-NO_2–diphenyl ether is taken as exercise.

2-Cl–4-CF_3–3′-OEt–4′-NO_2: In the first step, the descriptors for 2-Cl–4-CF_3– and 3-OEt–4-NO_2–diphenyl ether are necessary, and are determined as 1) and 2); 1) 2-Cl–4-CF_3–C_6H_3–O–C_6H_5: Using σ_{s°(OPh)=0.224, σ_{s°(Cl)=0.066, and σ_{s°(CF_3)=0.141, the descriptor of the substrate is given by σ_{s°(124). σ_{s°(124)=0.322. 2) 3-OEt–4-NO_2–C_6H_3–O–C_6H_5: Using σ_{s°(OPh)=0.224, σ_{s°(OEt)=0.170, and σ_{s°(NO_2)=0.115, the descriptor of substrate is given as σ_{s°(124). σ_{s°(124)=0.382.

Using the two conditions mentioned above, we are able to determine the descriptor σ_{s° for 2-Cl–4-CF_3–3′-OEt–4′-NO_2–diphenyl ether as σ_{s°(124) by two ways 3) and 4); 3) Using σ_{s°(2-Cl–4-CF_3–C_6H_3–O–)=0.322, σ_{s°(NO_2)=0.115, and σ_{s°(OEt)=0.170, we get σ_{s°(124)=0.457. 4) Using σ_{s°(3-OEt–4-NO_2–C_6H_3–O–)=0.382, σ_{s°(CF_3)=0.141, and σ_{s°(Cl)=0.066, we get σ_{s°(124)=0.444. The mathematical mean value gives σ_{s°=0.451.

2. Substituted 2-Amino-3-benzoyl Phenylacetic Acid as Antiinflammatory Agent[9] At first, we determined the descriptor σ_{s° for $PhCH_2COOH$ by the step given in Table VIII where the descriptors of $PhCH_2R$ became linear with those of $MeCH_2R$ and MeR, and after the correction of symmetry $n\times R\ln 3$ given by Me group, σ_{s°(CH_2COOH)=0.185 was finally obtained.

Table VIII. Estimation of S°_{298}(g)/e.u. and Descriptor σ_{s° for $PhCH_2R$, $MeCH_2R$, and MeR[a]

	$PhCH_2R$			$MeCH_2R$			MeR		
	S°_{298}(g)	σ_{s°	$R\ln 3$	S°_{298}(g)	σ_{s°	$R\ln 3$	S°_{298}(g)	σ_{s°	$R\ln 3$
Me	86.15	0.127	1	64.51	0.161	2	54.85	0.091	2
Et	95.76	0.173	1	74.12	0.221	2	64.51	0.161	2
n-Pr	105.04	0.213	1	83.40	0.273	2	74.12	0.221	2
CO_2H	96.27[b]	0.175[b]	1	74.40[b]	0.223[b]	2	65.32[b]	0.166[b]	2
	98.51[b,c]	0.185[b,c]	0	76.66[b,c]	0.236[b,c]	1	67.52	0.181	1

a) S°_{298}(g) and σ_{s° for three series having same set of $R\ln 3$ are mutually linear. b) Estimated values. c) Final values.

In view of the result given above, we are able to determine the descriptors for several kinds of substituted 2-amino-3-benzoyl phenylacetic acid homologs as given below.

2-Amino-3-benzoyl Phenylacetic Acid: Using σ_{s°(CO-Ph)=0.206, σ_{s°(NH_2)=0.074, and σ_{s°(CH_2COOH)=0.185, we get descriptor of substrate as σ_{s°(123)=0.341. The conversion of σ_{s°=0.341 to S°_{298}(g)=141.16 e.u., and the correlation by $R\ln 2$=1.38 e.u. afford S°_{298}(g)=142.54 e.u. and σ_{s°=0.345. This result means σ_{s°(3-CO–2-NH_2–C_6H_3–CH_2COOH)=0.345.

4-Cl-Benzophenone: Using σ_{s°(COPh)=0.206 and σ_{s°(Cl)=0.066, we get the descriptor as σ_{s°(14)=0.224. The conversion of σ_{s°(14)=0.224 to S°_{298}(g)=107.81 e.u., and the correction by $R\ln 2$=1.38 e.u. afford S°_{298}(g)=109.19 e.u. and σ_{s°=0.230.

2-Amino-3-(4′-Cl-benzoyl)phenylacetic Acid: Using σ_{s°(CH_2COOH)=0.185, σ_{s°(NH_2)=0.074 and σ_{s°(4-Cl–C_6H_4–CO–)=0.230, we get the descriptor as σ_{s°(123)=0.360. The conversion of σ_{s°(123) to S°_{298}(g)=147.37 e.u., and the correction by $R\ln 2$=1.38 e.u. afford S°_{298}(g)=148.75 e.u. and σ_{s°=0.364.

Another way, we also get the descriptor of the same substrate as follows; Using σ_{s°(Cl)=0.066 and σ_{s°(3-CO–2-NH_2–C_6H_3–CH_2COOH)=0.345, we get the descriptor of the substrate as σ_{s°(14)=0.350. The conversion of σ_{s°(14)=0.350 to S°_{298}(g)=144.02 e.u. and the correction of $R\ln 2$=1.38 e.u. afford S°_{298}(g)=145.40 e.u. and σ_{s°=0.354. The mathematical mean value gives σ_{s°=0.359.

2-Amino-3-benzoyl-5-Cl-phenylacetic Acid: Using σ_{s°(Cl)=0.066, σ_{s°(NH_2)=0.074, σ_{s°(CH_2COOH)=0.185, and σ_{s°(COPh)=0.206, we get the descriptor of the substrate as σ_{s°(1235)=0.355. The conversion of σ_{s°(1235)=0.355 to S°_{298}(g)=145.63 and the correction of $R\ln 2$=1.38 e.u. afford S°_{298}(g)=147.01 e.u. and σ_{s°=0.359.

2-Amino-3-(4′-Cl-benzoyl)-5-Cl-phenylacetic Acid: The descriptor of the substrate can be given by two ways[1,2]; 1) Using σ_{s°(Cl)=0.066 and σ_{s°(3-CO–2-NH_2–5-Cl–C_6H_2–CH_2COOH)=0.359, we get the descriptor as σ_{s°(14)=0.363. The conversion of σ_{s°(14)=0.363 to S°_{298}(g)=148.29 e.u. and the correction of $R\ln 2$=1.38 e.u. afford S°_{298}(g)=149.67 e.u. and σ_{s°=0.367. 2) Using σ_{s°(4-Cl–C_6H_4–CO–)=0.230, σ_{s°(Cl)=0.066, σ_{s°(NH_2)=0.074, and σ_{s°(CH_2COOH)=0.185, we get the descriptor as σ_{s°(1235)=0.372. The conversion of σ_{s°(1235)=0.372 to S°_{298}(g)=151.39 e.u. and the correction of symmetry $R\ln 2$=1.38 e.u. afford σ_{s°=0.376. The mathematical mean value gives σ_{s°=0.372.

The descriptors determined for the above five examples

TABLE IX. Substituted Benzophenones and Their Descriptors σ_{s°

A-ring			B-ring	
1	2	5	4′	σ_{s°
CH_2CO_2H	NH_2			0.345
			Cl	0.230
CH_2CO_2H	NH_2		Cl	0.359
CH_2CO_2H	NH_2	Cl		0.359
CH_2CO_2H	NH_2	Cl	Cl	0.372

are summarized in Table IX.

Conclusion

Logical estimation of the QSAR descriptor σ_{s° for $C_6H_{6-n}R_n$ ($n=1,2,3,\cdots,6$) could be given by means of the regression analyses using $\Sigma\sigma_{s^\circ}$(mono) as an independent variable. And, when different kinds of substituents are introduced, correction of the symmetry is necessary. This procedure could be extended for the substrates having two kinds of substituted benzene rings, and successful determinations are carried out for polychlorinated biphenyls, diphenyl ether herbicides, and benzophenone antiinflammatory agents. The results suggest that the concept of "*isosterism*" could be ascribed to the similarity of the descriptor σ_{s°, representing both dispersion and repulsion interactions between receptor and substrate.

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Interaction of Bleomycin with Deoxyribonucleic Acid Oligomer: Proton Nuclear Magnetic Resonance Titration Study Using Novel Bleomycin Complexes with Ni^{2+} and VO^{3+}

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Two metal complexes of bleomycin (BLM), BLM–Ni^{2+} and BLM–VO^{3+} are used for studying interactions between BLM and deoxyribonucleic acid (DNA) by nuclear magnetic resonance. Although these BLMs do not mediate DNA strand scission under the usual conditions, they bind to DNA in the same manner as the active metal complexes of bleomycin (BLM–Fe^{2+} and BLM–Co^{3+}). A self-complementary dodecanucleotide, d(CCCCAGCTGGGG), having a single site for cleavage was synthesized. d(CCCCAATTGGGG), which contains no –GpC– sequence, was also synthesized. The BLM–metal complexes were shown to bind specifically to the GpC site by circular dichroism and fluorescence titration studies. We assigned all the resonances for imino protons and phosphorus, and most of the nonexchangeable proton resonances of d(CCCCAGCTGGGG). No substantial change in the chemical shifts of these signals was observed upon titration with either BLM–Ni^{2+} or BLM–VO^{3+}. This result is not consistent with a model of the strong intercalation of the BLMs between the base-pairs. The BLMs bind to DNA in a different manner, and DNA does not change its conformation upon binding with BLMs.

Keywords bleomycin; DNA; ^{1}H-NMR; binding; metal; oligonucleotide; sequence specificity; association constant; CD; fluorescence

Introduction

The bleomycins (BLMs, Fig. 1A) are antitumor antibiotics used clinically for the treatment of certain carcinomas.[2)] The therapeutic effects of BLMs are believed to be due to their activity in deoxyribonucleic acid (DNA) degradation; this process has been shown to proceed in the presence of any of several redox-active metal ions and oxygen or ultraviolet light.[3)]

The BLMs preferentially cleave double-stranded DNA at the sequences GpC and GpT, and the most preferred site is Py–G–C–Pu.[4)] The mechanism of these chemical reactions has been well studied using synthetic oligonucleotides as well as polymer DNA.[5,6)] In contrast, the mode of DNA–BLM interactions, how BLMs interact with DNA strands and recognize the base sequences, is not known. There are two major hypotheses for the interaction between BLMs and DNA: [1] BLM intercalates into the DNA duplex with its bithiazole moiety,[7)] and [2] BLM binds along the minor groove of the DNA duplex with its tetrapeptide and terminal cationic groups.[8,9)] Many experiments for each hypothesis have been tried, but the problem has not been definitely solved because of lack of direct evidence provided by X-ray crystallographic or nuclear magnetic resonance (NMR) solution studies. Moreover, the structure of the metallobleomycin itself has not been analyzed by X-ray crystallography. Only the crystal structure of P-3A, a biosynthetic intermediate of BLM, coordinating Cu^{2+} is known.[10)]

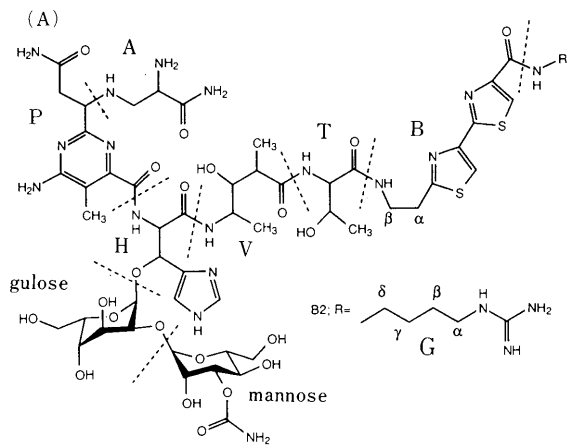

Fig. 1. Chemical Structure of Bleomycin-B2 (A) and Structure and Numbering System of the Dodecanucleotides GC-12 and AT-12 (B)

Abbreviations for the structural units in BLM are also indicated in accordance with ref. 12 (A).

Fluorescence studies show that the coordinating metal is necessary for BLM to bind to DNA.[11)] Therefore we should prepare a certain metal complex for observing the interaction between BLM and DNA. For this purpose, an inactive BLM complex; BLM–Zn^{2+} has been studied by Haasnoot *et al.* because zinc ions are diamagnetic.[12-14)] Interactions between BLM–Zn^{2+} and a DNA polymer or d(CGCGCG) were studied by NMR,[15,16)] but these DNA's contain multiple binding sites. Moreover, it is known that Zn^{2+} forms not only octahedral but also tetrahedral coordination complexes with small molecules.[17)]

To elucidate the mode of metal BLM–DNA interactions by NMR, we can adopt two simple strategies: [1] using an oligonucleotide designed suitably for observing the "specific interaction," and [2] using an octahedral-coordinating metal for the BLM reaction center. For the first purpose, we synthesized a dodecanucleotide d(CCCCAGCTGGGG) that has a single cleavage site for BLMs.[18)] For the second purpose, we used two BLM–metal complexes: BLM–Ni^{2+} and BLM–VO^{3+}. Ni^{2+} and VO^{3+} form an octahedral coordination as assumed for the coordination metals of

most of the "active" BLMs.

In this paper, we report studies on the site-specific interactions of BLM–Ni^{2+} and BLM–VO^{3+} complexes with the d(CCCCAGCTGGGG) duplex. Although a specific interaction occurs, the oligonucleotide duplex shows only small changes in its NMR spectra. The results are not consistent with a model of the strong intercalation.

Materials and Methods

Oligonucleotides Dodecanucleotides, GC-12: d(CCCCAGCTGGGG) and AT-12: d(CCCCAATTGGGG), were synthesized by the phosphoramidite method.[19] Both oligomers were purified, desalted and the counter cations were converted to sodium ions using a Dowex 50 (Na^+ form) column. The molar absorption coefficients, ε_{260}, were determined by enzymatic digestion experiments (GC-12, 107400; AT-12, 106700).

Bleomycin–Metal Complexes Bleomycin B2·2HCl (BLM-B2; metal-free, highly purified) was a generous gift from Dr. T. Takita (Nippon Kayaku, Co., Ltd.).

Metal ion solution (10 mM) were prepared using chloride derivatives: VO^{3+} from $VOCl_3$ and Ni^{2+} from $NiCl_2$–$6H_2O$. The metal–BLM complexes were prepared by mixing equal volumes of a 10 mM BLM-B2 stock solution and a solution of either metal ion (10 mM).

Cleavage Reaction To a mixture of 5′-^{32}P-labeled GC-12 or AT-12 (50 μM, final concentration) and BLM–Fe^{2+} (50 μM) in phosphate buffer (10 mM, pH 7.1), H_2O_2 (0.2 mM) was added at 0 °C and left for 15 min. For BLM–Ni^{2+} and BLM–VO^{3+}, the same conditions were used but H_2O_2 was not added and the mixture was left for 1 h. Then the mixture was immediately lyophilized and electrophoresed on 20% polyacrylamide/7 M urea gel.

Titration Experiments Circular dichroism (CD) spectra were recorded by a JASCO J500A spectrometer. Each oligomer (20 nmol) was dissolved in 3 ml of 100 mM NaCl, 10 mM sodium phosphate buffer (pH 7.5) and the solution was kept at 90 °C for 1 h, cooled slowly and kept at room temperature overnight. Then it was titrated with small volumes of 4 mM BLM–metal complex solution at 20 °C. The light path length was 10 mm.

Fluorescence was observed using a Hitachi MPF-4A fluorescence spectrometer. Excitation was done at 300 nm and emission at 350 nm was measured. BLM–metal complex (6.7 nmol) was dissolved in the same buffer (2 ml) and put into a 10 mm cuvette. A solution containing GC-12 or AT-12 (4 mM) in the same buffer was annealed and then added to the BLM–metal solution.

NMR Experiments NMR spectra were recorded by a JEOL GX-500 spectrometer (500 MHz for proton and 203 MHz for phosphorus). The NMR sample was prepared in the following manner: for exchangeable protons, 1 μmol of GC-12 (107 A_{260} units) was dissolved in 400 μl of H_2O–D_2O (4:1) containing 0.1 M NaCl and 10 mM sodium phosphate buffer (pH 6.8); for non-exchangeable protons and phosphorus, GC-12 (1.5 μmol) was dissolved in 400 μl of 99.98% D_2O containing 0.1 M NaCl, 10 mM phosphate buffer (pH 6.8). For titration, GC-12 (1 μmol) was dissolved in 400 μl of H_2O–D_2O (4:1) containing 0.1 M NaCl without the buffer, then the pH was adjusted to 6.8 using NaOH.

An NMR titration experiment was done observing exchangeable proton and phosphorus resonances with an optional ϕ5 mm ^{31}P probe. Small volumes of a BLM–metal solution (50 mM) were added, and then the pH was adjusted. Exchangeable protons were observed using a solvent-suppression 1-1 pulse sequence,[20] and nuclear Overhauser effect (NOE) experiments were done at 5 °C for spectral assignment. The ^{1}H chemical shifts were measured relative to internal 2-methyl-2-propanol (1.23 ppm). The ^{31}P chemical shifts were measured relative to external trimethyl phosphate (10% in ethanol).

Pure absorption double quantum filter-correlated spectroscopy (DQF-COSY) and nuclear Overhauser effect correlation spectroscopy (NOESY) spectra of GC-12 were recorded with 2048 × 256 data points at 30 °C. Mixing times of 150 ms and 250 ms were used for NOESY. Before Fourier transformation, t_1 data were zero-filled four times (2048 × 1024 data points) and a slightly shifted Gaussian function window was applied. For each spectrum at least 80 scans of free induction decays (FIDs) were accumulated. Also for GC-12: BLM–Ni^{2+} (1:1) complex, DQF-COSY and NOESY (mixing time 250 ms) spectra were measured.

^{31}P-Detected two dimensional-insensitive nuclei enhanced by polarization transfer (2D-INEPT) spectra (pure absorption mode) were recorded with 2048 × 256 data points at 30 °C. The frequency width was 1600 Hz for ^{31}P and 5000 Hz for ^{1}H. J_{P-H} was estimated to be 12.5 Hz, which is rather larger than the standard value, in the pulse sequence. Eighty scans of FIDs for each column were accumulated. The data were processed similarly to those from the ^{1}H–^{1}H 2D NMR experiments.

Results

DNA Cleavage Reaction The results for the cleavage reactions of GC-12 and AT-12 are shown in Fig. 2. GC-12 was cleaved specifically at the position of the G6pC7 site by BLM–Fe^{2+} in the presence of H_2O_2 (Fig. 2, lane 2). Several non-specific cleavages with lower efficiency than that for GC-12 were observed for AT-12, probably because AT-12 contains no strongly preferred binding site for BLM (Fig. 2, lane 6). GC-12 was not cleaved at all by either BLM–Ni^{2+} or BLM–VO^{3+} in the absence of H_2O_2, confirming that GC-12 complexed with these metal–BLMs is stable under the usual conditions of NMR measurement.

Specific Binding as Studied by CD and Fluorescence Spectroscopy Figure 3 shows the results of CD titration experiments of GC-12 and AT-12 with BLM–Ni^{2+}. GC-12 shows a marked change in $[\theta]$ values upon addition of BLM–Ni^{2+} (Fig. 3A). On the other hand, AT-12 does not show such changes. The CD titration curve for GC-12 is shown in Fig. 3C. The change reaches a plateau at about 8 eq of the metal–BLM. Considering the size of BLM molecule, we assumed that one molecule of BLM–Ni^{2+} binds to the duplex at a time. Though GC-12 duplex has two GpC sites, they are located symmetrically at the center of the duplex molecule and share the same minor-groove. Thus the association constant (K_a) between GC-12 and BLM–Ni^{2+} is described by

$$K_a = \frac{[\text{complex}]}{[\text{free duplex}][\text{free BLM}]} = \frac{f}{[\text{duplex}](1-f)(R-f)} \quad (1)$$

where [duplex] is a total duplex concentration, R is a ratio of total BLM to total duplex concentrations and f is a fraction of complex formation for the duplex ([complex]/

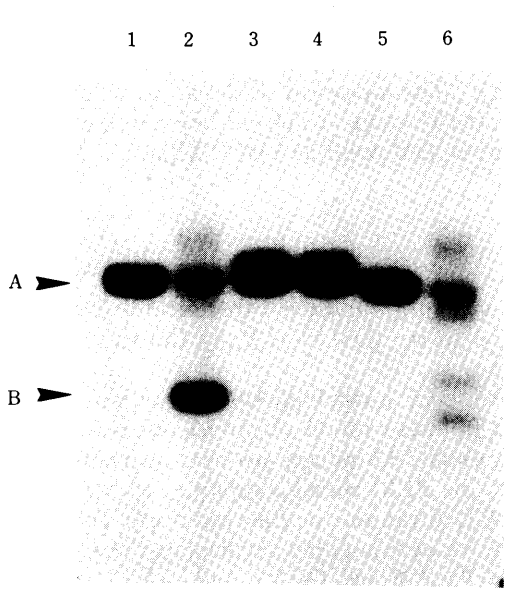

Fig. 2. 20% Polyacrylamide/7 M Urea Gel Electrophoresis for the Cleavage Reactions

Lanes 1—4: For the reactions of GC-12, without drug (lane 1), with BLM–Fe^{2+} and H_2O_2 (lane 2), with BLM–Ni^{2+} (lane 3) and BLM–VO^{3+} (lane 4). Lanes 5,6: For the reactions of AT-12, without drug (lane 5) and with BLM–Fe^{2+} and H_2O_2 (lane 6). A and B show the positions of the 5′-labeled DNA and the cleaved product, respectively.

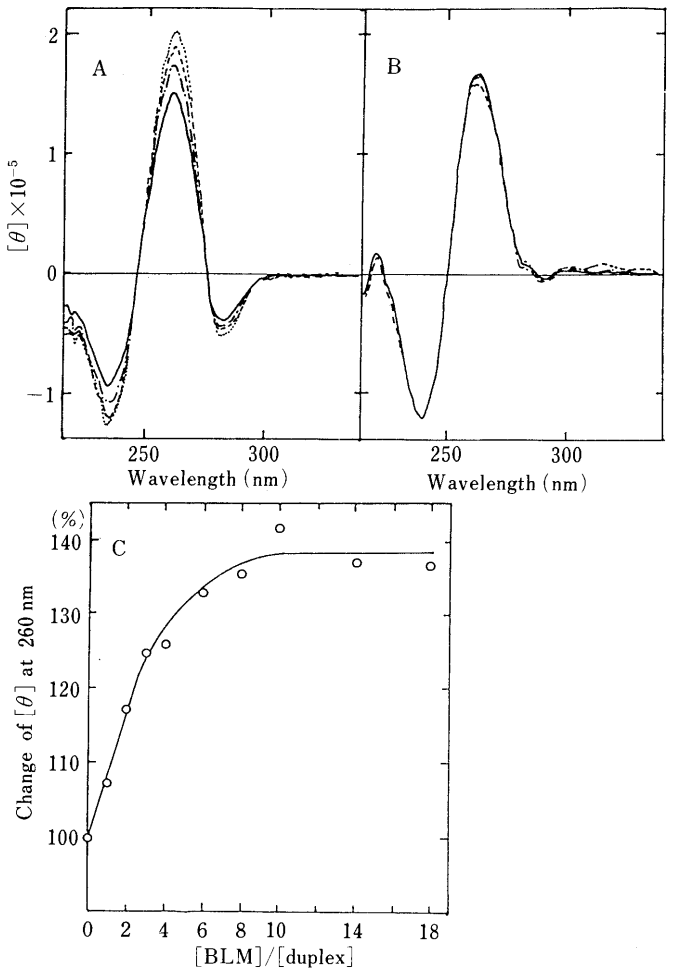

Fig. 3. CD Spectra of GC-12 (A) and AT-12 (B) upon Titration with BLM–Ni^{2+}

A: ——, 0 eq; —·—, 2 eq; – – –, 4 eq; ------, 8 eq. B: ——, 0 eq; —·—, 2 eq; – – –; 4 eq. DNA (6.7 μM) in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.5) at 20 °C. The [BLM]/[duplex] ratios are shown. The changes of $[\theta]$ values of GC-12 at 260 nm are plotted against the [BLM]/[duplex] ratio (C).

[duplex]). The observed fraction is calculated by the change of $[\theta]$ value at 260 nm; we estimated the value of the titration end point as 138%. Equation 1 can be solved for f as

$$f=\frac{1}{2}\left\{\left(R+1+\frac{1}{[\text{duplex}]K_a}\right)-\sqrt{\left(R+1+\frac{1}{[\text{duplex}]K_a}\right)^2-4R}\right\} \quad (2)$$

with a given K_a for each experimental data set. K_a was estimated to be $4.0\times10^5\ \text{M}^{-1}$ by the least squares fitting method.

Fluorescence quenching upon titration of the BLM–metal complexes with the oligonucleotides was also measured (Fig. 4). GC-12 induces stronger quenching than AT-12 for both the BLM–metal complexes. In contrast to the result of CD titration, the fluorescence quenching does not level off; we were not able to calculate the K_a from the fluorescence data. Since GC-12 and AT-12 still have some absorption at 300 nm which is the excitation wavelength, the effect of light loss cannot be neglected when a large amount of DNA is added to BLM. Nevertheless, this result again suggests that GC-12 binds to the BLM–metal more strongly than AT-12.

Effects of Coordinating Metals for BLM on ^{1}H-NMR Spectra Although Ni^{2+} is paramagnetic, its paramagnetic effect on ^{1}H-NMR spectra of BLM was rather small, and there was no extreme shift; only two broad peaks at around 10 ppm and 0 ppm were observed (data not shown). This result is distinct from the case of BLM–Fe^{2+}. ^{1}H-NMR spectral dispersion for BLM–Fe^{2+} is from −18 ppm to 48 ppm: 7 signals at the field lower than 12 ppm and 9 signals at the field higher than 0 ppm were observed.[21] In the case of BLM–Ni^{2+}, the paramagnetic effect of Ni^{2+} mainly causes broadening of the signals, and the broadening is smaller than that of some paramagnetic metallobleomycins (*cf.* BLM–Cu^{2+}, see Fig. 3 in ref. 22). This is the reason we chose Ni^{2+} as an "inactive" BLM complex for this study. VO^{3+} is diamagnetic. None of the proton signals for BLM–VO^{3+} either greatly shifted or broadened.

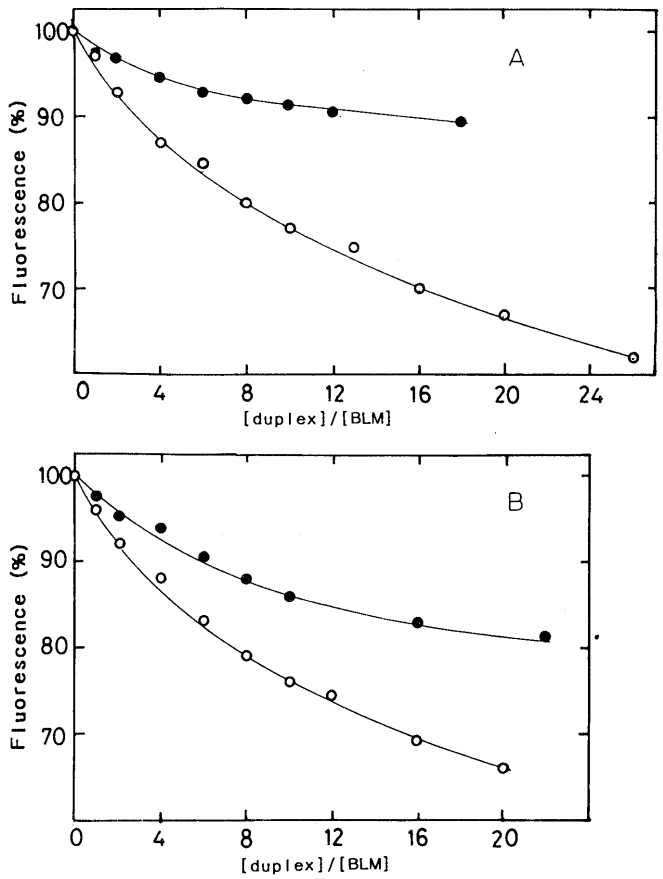

Fig. 4. Quenching Effects of DNA on the Fluorescence of BLM–Ni^{2+} (A) and BLM–VO^{3+} (B)

BLM (3.3 μM) in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.5) was titrated with AT-12 (closed circle) and GC-12 (open circles) at room temperature.

We assigned most of the non-exchangeable proton signals of BLM–Ni^{2+} and BLM–VO^{3+} using DQF-COSY and 2D-HOHAHA spectra (H. Hiroaki, unpublished result). The preliminary results suggest that the coordinating nitrogen atoms are the same as those expected for BLM–Fe^{2+} (see Fig. 1 in ref. 3).

Assignment of Proton Resonances by GC-12 Using standard methods, all the imino proton and most of the cytosine amino proton signals were assigned. NOE experiments with a 1-1 pulse for GC-12 were done and the assignments are presented in Table I. Since G12 is at the end of the duplex, the imino proton resonance was not observed at this temperature.

TABLE I. ^{1}H-NMR Chemical Shifts for d(CCCCAGCTGGGG) at 30 °C (ppm)

Residue	NH[a]	H8/H6	H2/H5/CH_3	H1′	H2′	H2″	H3′
C1		7.79 (−0.02)	5.94 (−0.03)	5.96 (−0.01)	2.18 (−0.03)	2.53 (0.00)	4.64 (0.01)
C2		7.66 (−0.02)	5.71 (−0.04)	6.00 (0.00)	2.23 (−0.02)	2.48 (−0.01)	4.85 (−0.02)
C3		7.57 (−0.07)	5.64 (0.00)	5.94 (−0.01)	2.12 (−0.04)	2.41 (0.03)	4.83 (−0.02)
C4		7.48 (−0.03)	5.66 (−0.03)	5.27 (0.04)	2.05 (−0.05)	2.30 (−0.07)	4.81 (−0.02)
A5		8.17 (0.00)	7.64[c]	6.04 (0.01)	2.75 (−0.02)	2.90 (0.00)	5.04 (0.00)
G6	12.80 (0.00)	7.64 (−0.01)		5.73 (0.00)	2.50 (0.00)	2.61 (0.00)	4.97 (−0.04)
C7		7.30 (0.00)	5.18 (0.00)	5.84 (0.02)	1.93 (−0.01)	2.42 (−0.02)	4.70 (−0.01)
T8	14.07 (0.06)	7.27 (−0.01)	1.90 (0.03)	5.65 (0.02)	1.96 (−0.01)	2.29 (−0.02)	4.81 (0.00)
G9	13.03 (0.02)	7.80 (−0.01)		5.49 (0.01)	2.61 (−0.01)	2.62 (0.01)	4.93 (0.00)
G10	13.11 (0.00)	7.67 (0.00)		5.57 (0.01)	2.53 (−0.03)	2.64 (−0.04)	4.94 (−0.01)
G11	13.18 (0.02)	7.63 (0.00)		5.76 (−0.03)	2.51 (−0.01)	2.68 (−0.13)	4.92 (0.01)
G12	13.27[b]	7.69 (−0.02)		6.11 (−0.03)	2.43 (−0.12)	2.33 (0.10)	4.60 (0.01)

a) The spectra were recorded at 10 °C. *b*) The signal for the complex was not observed due to broadening. *c*) The signal for the complex was not identified due to overlapping. The chemical shift differences between free GC-12 and GC-12 complexed with BLM–Ni^{2+}, ($\delta_{free} - \delta_{complex}$), are shown in parentheses.

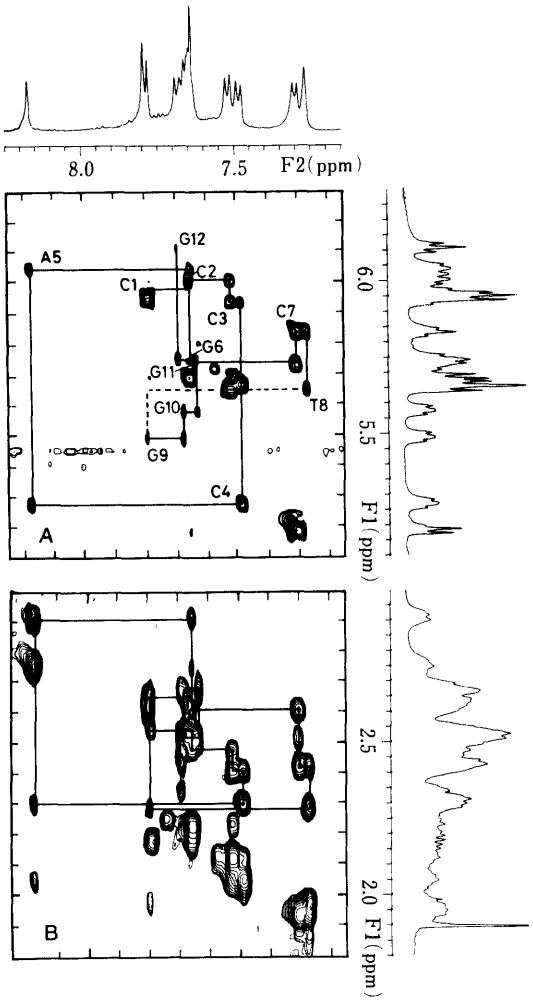

Fig. 5. Expanded NOESY Spectra of GC-12

Mixing time, 250 ms; 30 °C. (A) The base proton-H1′ region and (B) the base proton-H2′/H2″ regions. The lines indicate the sequential assignments of the signals. Intraresidue cross-peaks are labelled (A).

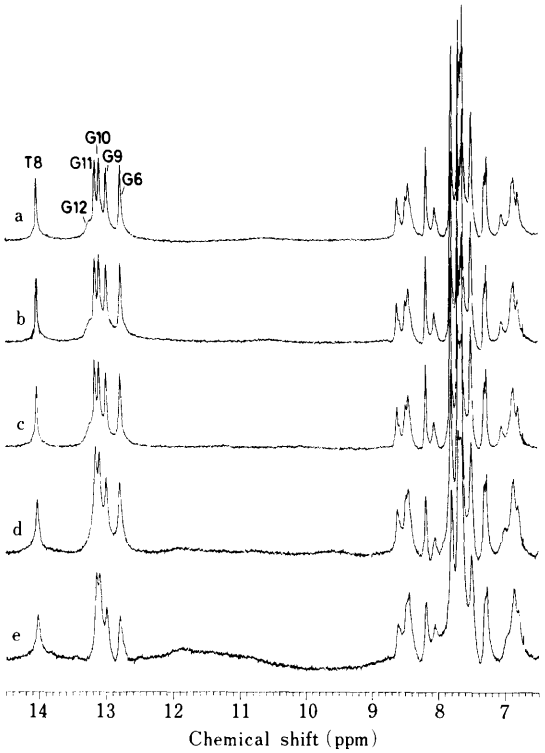

Fig. 6. ^{1}H-NMR Spectra of GC-12 (2.5 mM) upon Titration with BLM–Ni^{2+} in H_2O–D_2O (4 : 1) at 10 °C

The imino, amino and aromatic proton regions are shown. Assignments of the imino proton signals are shown above the spectrum (a). The [BLM]/[duplex] ratios are (a) 0.0, (b) 0.1, (c) 0.2, (d) 0.6, and (e) 1.0.

For non-exchangeable protons, sequential NOEs between base protons (Pu-H8 and Py-H6) and sugar 1′, 2′, or 2″ protons can be traced, as shown in Fig. 5 (A: H8/H6-H1′ and B: H8/H6-H2′/H2″).[23] Since a B-DNA-type structure was assumed, it is reasonable to apply the standard sequential assignment method. No cross peak between T8H1′ and G9H8 is observed, but one is seen between T8H2′ and G9H8.

All the H3′ resonances were assigned using DQF-COSY spectrum (data not shown). The result confirms the assignment made by the NOESY analysis. All the deoxyriboses except for the terminal C1 and G12 residues show a pattern of a C2′-*endo* sugar puckering in DQF-COSY, confirming the B-form structure.[24] All the H4′ resonances and 10 of the H5′ and H5″ resonances were also assigned using the sugar–sugar region of the NOESY spectra (data not shown).

Titration Study of GC-12 with Metal–BLM on ^{1}H-NMR Observing the exchangeable protons, GC-12 was titrated with BLM–Ni^{2+} and BLM–VO^{3+} (Fig. 6 and Fig. 7, respectively). At each drug addition, the pH of the sample

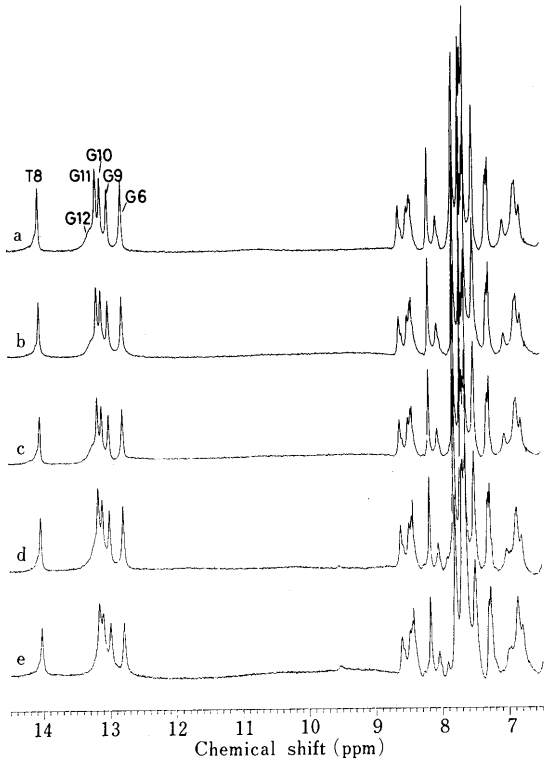

Fig. 7. ^{1}H-NMR Spectra of GC-12 (2.5 mM) upon Titration with BLM–VO^{3+} in H_2O–D_2O (4 : 1) at 10 °C

The imino, amino, and aromatic proton regions are shown. Assignments of the imino proton signals are shown above the spectrum (a). The [BLM]/[duplex] ratios are (a) 0.0, (b) 0.1, (c) 0.2, (d) 0.6, and (e) 1.0.

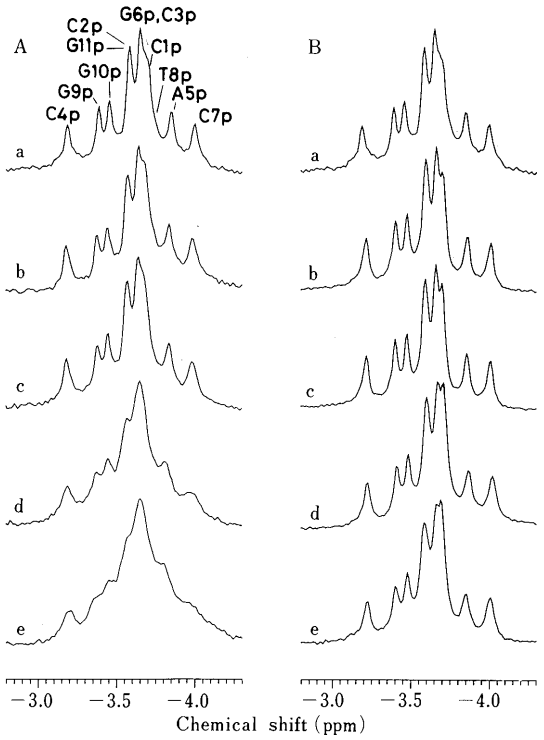

Fig. 8. ^{31}P-NMR Spectra of GC-12 (2.5 mM) upon Titration with BLM–Ni^{2+} (A) and BLM–VO^{3+} (B) in D_2O at 10 °C

The assignments of the signals are shown above the spectrum (a). The [BLM]/[duplex] ratios are (a) 0.0, (b) 0.1, (c) 0.2, (d) 0.6, and (e) 1.0.

was adjusted by diluted NaOH.

Considering the K_a of the BLMs (about $4 \times 10^5\ M^{-1}$), an equimolar mixture (1.25 mM) of GC-12 duplex (2.5 mM strand) and BLM should form a BLM–DNA complex by more than 90%. We stopped titration at this point. Ni^{2+} is paramagnetic, so that the imino proton signals become slightly broader (Fig. 6). VO^{3+} is diamagnetic, so that no broadening was observed (Fig. 7). In both cases, no change in signal positions occurs. The G12N1H signal vanishes at the end of titration. Throughout the titration, no additional resonance for either imino proton or phosphorus was observed, suggesting a fast interconversion between free GC-12 and bound GC-12.

Titration Study of GC-12 with Metal–BLM by ^{31}P-NMR A 2D-INEPT pulse sequence originally used for ^{1}H–^{13}C 2D NMR was used for assignment of the ^{31}P signals. Under these conditions (overestimating J_{P-H} as 12.5 Hz), only two correlations for each phosphorus were observed: one between H3′(n) and P(n) and the other between P(n) and H4′($n+1$) in the system of H3′(n)–C3′–O3′–P(n)–O5′–C5′–C4′–H4′($n+1$) (data not shown). Correlations for P–H5′/H5″ were not observed due to their relaxation rates under these conditions. On the chart, we could easily assign all the 11 phosphorus signals (Fig. 8, top).

Observing the phosphorus signals, GC-12 was titrated with BLM–Ni^{2+} and BLM–VO^{3+} (Fig. 8A and 8B, respectively). In the case of BLM–Ni^{2+}, the signal broadening is more profound than that for the protons. There is no change in the signal positions in either case.

Assignment for GC-12: BLM–Ni^{2+} Complex Because of the paramagneticity of Ni^{2+}, signal broadening occurs,

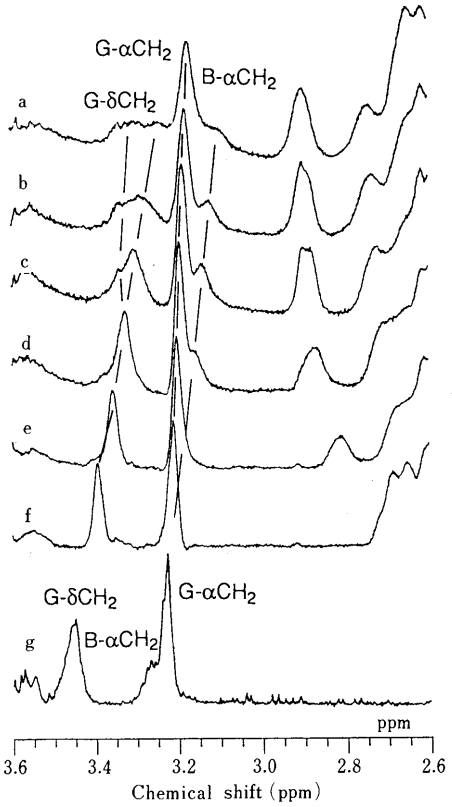

Fig. 9. ^{1}H-NMR Spectra of GC-12 (2.5 mM): BLM–Ni^{2+} (1.25 mM) (a—f) and BLM–Ni^{2+} Alone (g)

(a) 30 °C, (b) 40 °C, (c) 50 °C, (d) 60 °C, (e) 70 °C, (f) 80 °C, and (g) 30 °C.

especially in the NOESY spectrum, making sequential assignment difficult. Nevertheless, most of the cross-peaks involving the base protons correspond to those of free

GC-12. We assigned each base proton signal except for A5H2. The sugar region of the DQF-COSY spectrum also corresponded well to those of free DNA. We assigned the non-exchangeable proton signals except for the 4′ and 5′ protons of the DNA component of the complex. The chemical shift differences with respect to the free DNA are presented in Table I.

Since GC-12 is self-complementary, the ratio of GC-12 strand to BLM–Ni^{2+} is 2 : 1 in the complex. Low intensities and severe overlapping of the signals from the BLM–Ni^{2+} component make assignment difficult. Three methylene proton resonances in the region from 2.6 to 3.6 ppm (Fig. 9) were assigned to δCH_2 of the δ-aminobutylguanidinium residue (denoted G in Fig. 1), αCH_2 of the G-residue, and αCH_2 of the bithiazole residue (denoted B in Fig. 1). Two separate resonances are observed for G-δCH_2 at 30 °C (Fig. 9a) whereas no separation is observed in the free BLM–Ni^{2+} (Fig. 9g). Upon raising temperature, those resonances sharpen and shift downfield. The chemical shift differences with respect to those of free BLM–Ni^{2+} for G-δCH_2 and B-αCH_2 are both about 0.15 ppm at 30 °C. This result strongly suggests that a complex between GC-12 and BLM–Ni^{2+} is indeed formed under such stoichiometric conditions. The interaction is specific for the DNA duplex, because BLM–Ni^{2+} dissociates from GC-12 at higher temperature where the duplex dissociates.

Discussion

Site-Specific Binding of BLM–Ni^{2+} and BLM–VO^{3+} For study by NMR or other spectroscopic methods, it is essential that the sample be stable under the measurement conditions. An ideal "inactive" BLM should bind to DNA without cleaving it. We were able to satisfy the criteria by using BLM–Ni^{2+} and BLM–VO^{3+}. We confirmed that BLM–Ni^{2+} and BLM–VO^{3+} induce no DNA strand scission under the conditions of NMR measurement (Fig. 2) but actually bind to DNA.

Only GC-12 shows great changes upon titration with the metal–BLM. The magnitudes of the CD bands increase without significant changes in the positive and negative maximum wavelength, suggesting that the base stacking mode in GC-12 does not change upon complex formation. The difference between the two oligomers is in the central two bases, A6pT7 and G6pC7, therefore the sequence-specific interaction actually occurs at this site. This is confirmed by the observation that GC-12 is specifically cleaved at the GpC site and is also supported by fluorescence quenching experiments. Relatively small fluorescence quenching and the result of cleavage experiment for AT-12 suggest that some non-specific interactions also occur between AT-12 and metal–BLM. The NMR spectral changes for the BLM terminal residues strongly suggest the complex formation through the residues under the stoichiometric conditions.

Binding Mode of BLMs to the Dodecanucleotide GC-12 Although site-specific binding was confirmed, the changes of NMR spectra of the DNA component were much smaller than expected. No chemical shift change of the imino proton or the phosphorus resonances around the interacting site was observed. The base protons and sugar 1′, 2′, 2″, and 3′ protons did not show any drastic change in their chemical shifts either, upon complex formation with BLM–Ni^{2+}. Since no additional signal was observed throughout the titration, fast rate of exchange between free GC-12 and bound GC-12 is assumed, and the chemical shifts of DNA protons should be numerically averaged ones between those of free and bound DNA. In contrast, small but obvious change of signal position was observed for BLM terminal residues. Our results for DNA proton and phosphorus signals appear completely negative, but we assume that they actually reflect the nature of the interaction between BLM and GC-12. None of these results is consistent with BLM intercalation between the base pairs.

The effects of intercalation on imino proton resonances are well defined. Feigon *et al.* have shown by NMR studies that most DNA-binding drugs acting as intercalators cause an upfield shift of imino proton resonances of the DNA polymers.[25] This can be explained by a shielding effect of the aromatic ring moiety of the intercalators on the imino protons at the center of the DNA helix and very close to the rings.

The effects of intercalators on chemical shifts of phosphorus atoms were also studied.[26,27] Chemical shifts for the phosphate groups that form a backbone of DNA strands are affected by torsion angles about the 3′O–P and P–5′O bonds. If a drug intercalates between the base pairs, the backbone conformation will be distorted and produce certain changes in the ^{31}P-NMR spcetrum. Our result suggests that the conformation about the P–O bonds of GC-12 is not affected by interaction with either of the metal–BLM complexes.

Studies on the activity of modified or semi-synthetic BLMs show that the positive charge at the end of the BLM main chain is necessary, suggesting some interaction between the phosphate negative charges and the guanidinium group of BLM-B2.[22,28] Though no change in the phosphorus chemical shifts is observed, the methylene proton signals near the bithiazole moiety (G-δCH_2, B-αCH_2) show a marked upfield shift. The two hydrogens of G-δCH_2 show different chemical shifts suggesting reduced mobility.

No intermolecular NOE cross-peaks for the BLM–Ni^{2+}: GC-12 complex were observed. Intermolecular NOEs are observed for some minor-groove binding drugs,[29,30] but their association constants are much greater than that of BLM.

A couple of research groups proposed a model in which BLM binds to DNA along the minor groove.[8,9] The peptide moiety of BLM contains two possible hydrogen acceptor sites, the nitrogens of the bithiazole, and two hydrogen donor sites, the amide protons near the bithiazole. Our results may be better explained by the minor-groove binding model. We observed considerable changes upon complex formation only for the 3′-terminal residues (G11, G12) but not for the 5′-terminal residues (Table I, Fig. 6 and Fig. 7). This result is rather consistent with the minor-groove binding model since the distance between G12 and G6 of the opposite strand across the minor groove is relatively small.

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Synthesis and Application of Imidazole Derivatives. Synthesis of Pyrrolo[1,2-*a*]benzimidazoles and Azepino[1,2-*a*]benzimidazoles

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4-Methyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one derivatives (11a—d) were synthesized by intramolecular acylation of 1-carboxymethyl-2,3-dimethylbenzimidazolium halides (9a and 8b—d) in good yields. Treatment of the iodide (8a) with an excess of refluxing thionyl chloride gave 1,1,3-trichloro-4-methyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (14). Introduction of electrophiles into the 1-position of 11d and 6-position of 5-methyl-9,10-dihydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (2a) was achieved by successive treatment with lithium diisopropylamide and electrophiles such as methyl iodide and ketones. The azepinone 2a was reacted with various electrophiles to give 6-substituted products in good yields.

Keywords benzimidazolium salt; 2-methylbenzimidazole; 1-acyl-1*H*-imidazole; intramolecular cyclization; pyrrolo[1,2-*a*]benzimidazole; azepino[1,2-*a*]benzimidazole; thionyl chloride; enaminone; electrophilic substitution

Imidazole and benzimidazole are interesting heterocycles because they are not only presented in many naturally occurring products and various useful drugs[1] but also show characteristic chemical behavior.[2] In this series of studies, our aims have been to prepare azepino-, pyrido- and pyrrolo[1,2-*a*]benzimidazole derivatives (**2a**, **2b**, **2c**, respectively) and their derivatives such as **3** and **4**, and we also wish to examine their chemical properties and biological activities. In the previous paper, we reported the synthesis and structure determination of tricyclic 5-alkyl-9,10-tetrahydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (**2a**), which was prepared starting from 2-methylbenzimidazole (**1a**).[3]

This paper deals with several synthetic procedures for a novel tricyclic system, 4-methyl-1,2-dihydro-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one derivatives (**11**), and electrophilic substitution at the α- and α′-position of the carbonyl group in **11** and **2a**.

The 1-position of 2-methylbenzimidazole (**1a**) was alkylated in 92% yield with ethyl bromoacetate in the presence of sodium hydride, and the resultant ester (**6a**) was treated with methyl iodide to give **7a**, which was hydrolyzed with an equimolar amount of aqueous sodium hydroxide. Neutralization of the hydrolyzate with hydrochloric acid gave the crystalline acid (**8a**) in 85.4% yield from **6a**. Attempts to prepare **11a** by the activation of **8a** by treating with *N*,*N*′-carbonyldiimidazole (CDI) in *N*,*N*′-dimethylformamide (DMF) according to the procedure used for the preparation of **2a**[3] were unsuccessful because of the poor solubility of **8a** in DMF. Therefore, in an attempt to increase the solubility of the benzimidazolium halide, an exchange of the iodide ion to chloride ion was conducted by passing a methanolic solution of **8a** through a column of anion exchanging resin (Cl^- form of Amberlyst A-27 resin). The solubility of the chloride (**9**) in DMF was enhanced, as expected and the reaction of **9** with CDI proceeded smoothly to give the desired cyclized product (**11a**) in 35.3% yield from **9a**.

Spectral and analytical data of **11a** supported the proposed structure (Chart 2). Positive color reaction with methanolic ferric(III) chloride solution was observed with **11a** as well as **2a**, probably because in solution **11a** existed as an enol form (**12a**) to a considerable degree. The proposed structure of **11a** or **12a** in Chart 2 was also supported by the similarity to **2a** in ultraviolet (UV), infrared (IR) and other properties such as appearance, solubility and thin layer chromatography (TLC) behavior.

The analogues **11b**—**d** were also prepared in fair yields from the corresponding starting materials **1** and **5** in a similar manner.

Next, we tried alkylation of the 1-position of **11d**. Thus, treatment of **11d** with lithium diisopropylamide (LDA) followed by addition of methyl iodide gave the tetramethylpyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (**11e**) in 73.2% yield. The structure of **11e** was confirmed by spectral and analytical data (IR, mass spectrum (MS), ^{1}H-nuclear magnetic resonance (^{1}H-NMR)), and a positive color reaction in the ferric(III) chloride test.

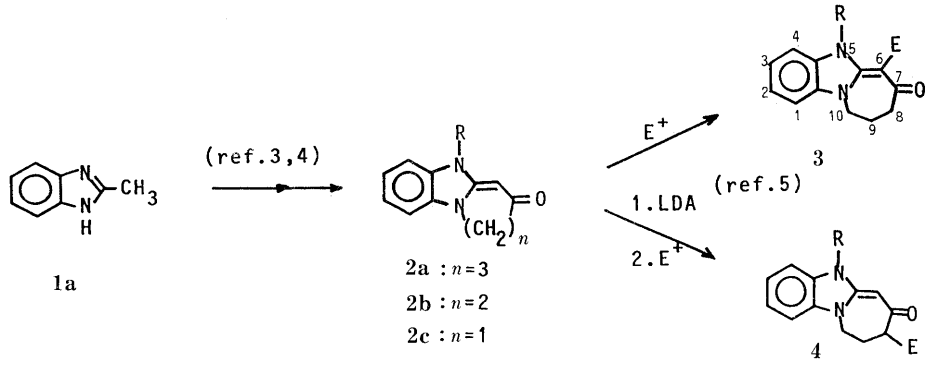

Chart 1

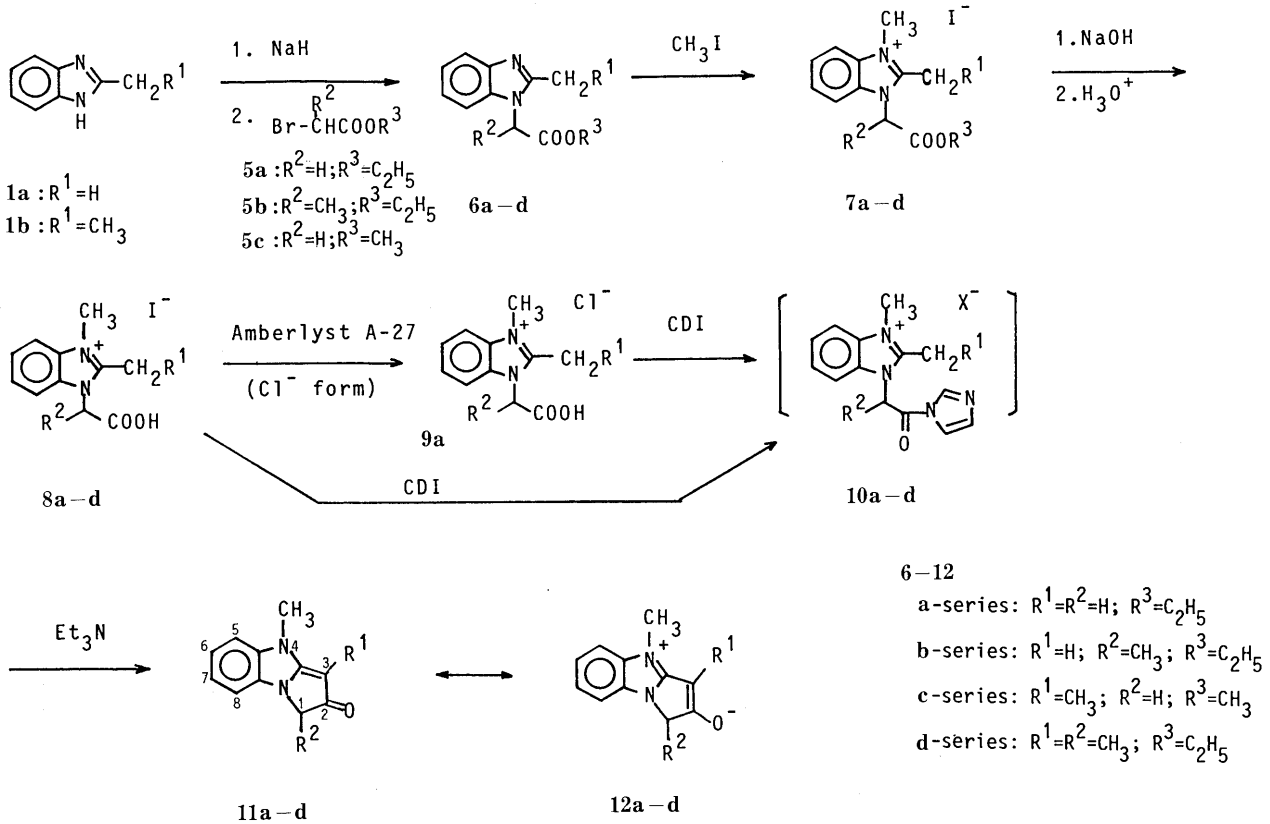

Chart 2

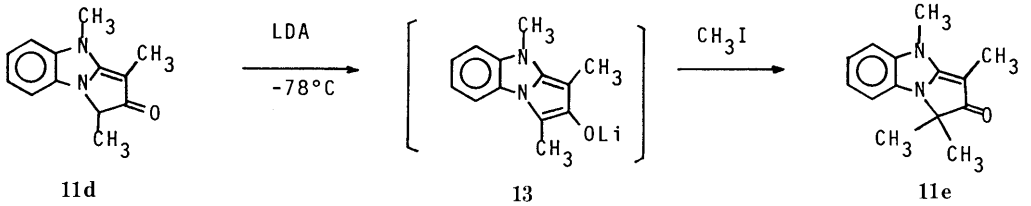

Chart 3

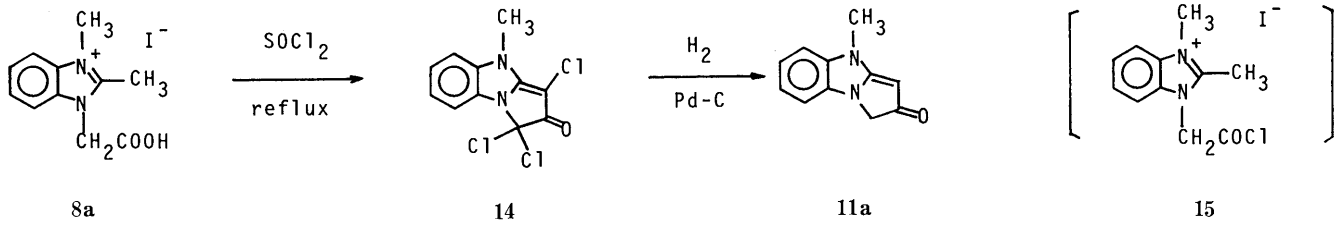

Chart 4

In the initial stage of the present work, we had planned an intramolecular cyclization of the acid chloride **15**. Thus, we heated the carboxylic acid **8a** in an excess of thionyl chloride at 80 °C for 2 h, but the corresponding acid chloride **15** was not obtained. From the reaction mixture, yellow prisms were isolated in 54.8% yield and the structure was, interestingly, estimated as 1,1,3-trichloro-4-methyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (**14**) based on the positive reaction in the ferric(III) chloride test, MS ($M^+ = 288$ *m/z*), analytical data, and absence of any aliphatic proton signal other than that of $NC\underline{H}_3$ (3.88 ppm, s, 3H). The structure of the trichloride (**14**) was finally confirmed by catalytic hydrogenation in the presence of Pd–charcoal, resulting in the formation of **11a** in quantitative yield.

It is presumed that the trichloride (**14**) is probably produced through a radical reaction process, and in the literature, some abnormal reactions involving thionyl chloride have been described.[4,5] For example, Castle *et al.* reported that 3-(2-naphthyl)-2-propenoic acid was converted in 75% yield to 1-chloronaphtho[2,1-*b*]thiophene-2-carbonyl chloride by treatment with a refluxing mixture of thionyl chloride, chlorobenzene and a catalytic amount of pyridine.[5] The reaction of **8a** to **14** is also presumed to proceed *via* a similar abnormal process.

It is noteworthy that $\nu_{C=O}$ of the trichloride (**14**) is

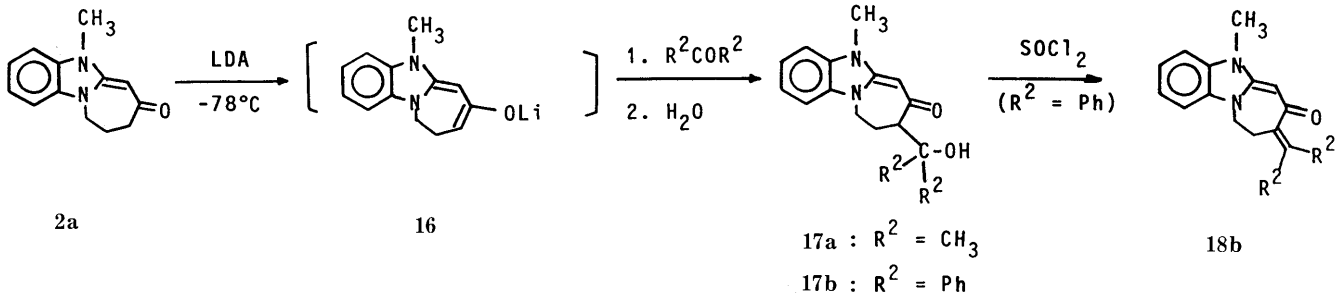

Chart 5

TABLE I. 6-Substituted 5-Methyl-7,8,9,10-tetrahydro-6*H*-azepino[1,2-*a*]benzimidazol-7-ones Prepared

Electrophile	Product (E)	Yield (%)	mp (°C) (Solvent)	Molecular formula	IR (KBr) $\nu_{C=O}$ or $\nu_{C=C}$ (cm^{-1})	^{1}H-NMR (TMS) δ, J (Hz)
$(CH_3CO)_2O$	**19** (CH_3CO)	68	230—232 (AcOEt)	$C_{15}H_{16}N_2O_2$ (256.3)	1615, 1580	2.23—2.53 (m, 4H, $-CH_2CH_2CO$), 2.63 (s, 3H, $COCH_3$), 3.66 (s, 3H, NCH_3), 4.27 (t, 2H, NCH_2, J=7 Hz), 7.43 (m, $4H_{arom}$)
$(C_6H_5CO)_2O$	**20** (C_6H_5CO)	57	267—269 (C_6H_6–*n*-hexane)	$C_{20}H_{18}N_2O_2$ (318.4)	1615, 1550	2.25—2.65 (m, 4H, CH_2CH_2CO), 3.64 (s, 3H, NCH_3), 4.21—4.38 (m, 2H, NCH_2), 7.26—7.72 (m, $9H_{arom}$)
$CH_3COCH_2=CH$	**21** ($CH_3COCH_2CH_2$)	46	152—154 (C_6H_6–*n*-hexane)	$C_{17}H_{20}N_2O_2$ (284.4)	1695, 1520	2.16 (s, 3H, $COCH_3$), 2.50 (m, 4H, CH_2CH_2-CH_2CO), 2.80 (m, 4H, $CH_2CH_2COCH_3$), 3.60 (s, 3H, NCH_3), 3.90 (t, 2H, NCH_2, J=7 Hz), 7.20 (m, $4H_{arom}$)
PhNCO	**22** (PhNHCO)	82	202—204 (AcOEt–*n*-hexane)	$C_{20}H_{19}N_3O_2$ (333.4)	1640	2.30—2.55 (m, 4H, CH_2CH_2CO), 3.84 (s, 3H, NCH_3), 4.18—4.39 (m, 2H, NCH_2), 7.04—7.74 (m, $9H_{arom}$), 12.17 (s, 1H, NH)
n-BuNCO	**23** (*n*-BuNHCO)	35	132—135 (AcOEt–*n*-hexane)	$C_{18}H_{23}N_3O_2$ (313.4)	1580	0.95 (t, 3H, CH_2CH_3, J=6 Hz), 1.20—1.80 (m, 4H, $CH_2CH_2CH_3$), 2.15—2.70 (m, 4H, CH_2-CH_2CO), 3.35 (q, 2H, $NHCH_3$), 4.23 (br, 2H, NCH_2, J=6 Hz), 7.45 (m, $4H_{arom}$), 9.86 (br, 1H, NH)
PhNCS	**24** (PhNHC=S)	31	211—213 (AcOEt–*n*-hexane)	$C_{20}H_{19}N_3OS$ (349.5)	1650	2.03—2.46 (m, 4H, CH_2CH_2CO), 3.98 (s, 3H, NCH_3), 4.35—4.55 (m, 2H, NCH_2), 7.12—7.92 (m, $9H_{arom}$), 13.96 (br, 1H, NH)
PhN_2Cl	**25**·HCl[a] (PhN=N–)	72	222—224 (EtOH–Et_2O)	$C_{19}H_{19}ClN_4O$ (354.8)	1660	2.32—2.54 (m, 2H, $CH_2CH_2CH_2$), 2.72—2.80 (t, 2H, CH_2CO, J=6 Hz), 4.18 (s, 3H, NCH_3), 4.74 (t, 2H, NCH_2, J=7 Hz), 7.27—8.22 (m, $9H_{arom}$), 14.40 (br, 1H, N^+H)
$4\text{-}MeOC_6H_4N_2Cl$	**26**·HCl[a]	36	247—249 (MeOH–Et_2O)	$C_{20}H_{21}ClN_4O_2$ (384.9)	1500	2.47—2.52 (m, 2H, $CH_2CH_2CH_2$), 2.70—2.79 (m, 2H, CH_2CO), 3.80 (s, 3H, OCH_3), 4.16 (s, 3H, NCH_3), 4.67 (m, 2H, NCH_2), 7.00—8.18 (m, $8H_{arom}$), 14.69 (N^+H)
PhSeCl	**27**	75	174—176 (AcOEt–*n*-hexane)	$C_{19}H_{18}N_2OSe$ (369.0)	1495	2.65 (m, 4H, CH_2CH_2CO), 3.63 (s, 3H, NCH_3), 4.13 (t, 2H, NCH_2, J=6 Hz), 7.00—7.50 (m, $9H_{arom}$)

a) ^{1}H-NMR of the compound was measured in DMSO-d_6.

observed at 1710 cm^{-1} while **11a—d** as well as **2a** do not show any $\nu_{C=O}$ absorption near 1700 cm^{-1} in their IR spectra because of the contribution of the dipole structure (**12a**).[3] It can be considered that in **14** the three electronegative chlorine atoms at the 1- and 3-positions electrostatically supress the enolization of the carbonyl group at the 2-position.

Next, we applied the above-described enolate formation procedure to the azepinone (**2a**). The azepinone (**2a**) was treated with an equimolar amount of LDA at −78 °C followed by addition of acetone to give the aldol-type product (**17a**) in 58% yield. The structure of **17a** was supported by its ^{1}H-NMR spectrum. When the diphenyl analogue (**17b**) was treated with thionyl chloride at room temperature, the corresponding dehydration product (**18b**) was obtained in 67% yield.

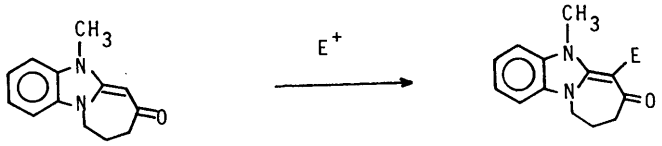

Chart 6

Finally we examined reactivity to electrophiles at the 6-position of the enaminone (**2a**), because the position probably holds key to the reactivity of enamines and ketone enolates, like the 4-position of antipyrine.[6] It was found that treatment of **2a** with acetic anhydride gave the 6-

acetylated product (**19**) in 68% yield as expected. The structure of **19** was supported by its ^{1}H-NMR spectrum. Thus, the azepinone (**2a**) was treated with various electrophilic reagents to give smoothly the corresponding 6-substituted products (**20**—**27**) in various yields.

Biological activities of the newly prepared compounds described in this paper are under examination.

Experimental

All melting points are uncorrected. IR spectra were taken with a Shimadzu IR-410 spectrometer. ^{1}H-NMR were obtained at 80 MHz on a Varian CFT-20 spectrometer and the chemical shifts are expressed in δ (ppm) values with tetramethylsilane as an internal standard. Abbreviations of ^{1}H-NMR signal patterns are as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); br (broad). UV spectra were obtained on a Shimadzu UV-200S spectrometer. Low-resolution MS (LRMS) and high-resolution MS (HRMS) were obtained on a Hitachi M-80 spectrometer. All solvents were removed under reduced pressure in the usual work-up procedure. Unless otherwise stated, anhydrous sodium sulfate was used as a drying agent. A Kugelrohr apparatus was used for vacuum distillations of oily crude products. Silica gel (Merck Art. 7734) was used in column chromatography.

1-(3-Ethoxycarbonylmethyl)-2-methylbenzimidazole (6a) 2-Methylbenzimidazole (**1a**, 2.64 g, 20 mmol) was added under an N_2 atmosphere to an ice-cooled suspension of 97% NaH (0.55 g, 22 mmol) in dry tetrahydrofuran (THF, 20 ml) and the mixture was stirred for 30 min. Ethyl bromoacetate (**5a**, 3.67 g, 22 mmol) was added to the cooled mixture and the whole was stirred for 2 h at room temperature. Water (20 ml) was added to the reaction mixture, and the product was extracted with AcOEt (50 ml × 3). The organic phase was washed with brine (10 ml) and dried. A pale yellow crystalline residue was obtained after evaporation. The product was purified by recrystallization from CCl_4 to give colorless needles, mp 105—106 °C. Yield, 3.99 g (91.5%). IR ν_{max} cm^{-1}: 1750 (C=O). ^{1}H-NMR (in $CDCl_3$) ppm: 1.25 (t, 3H, $CH_2C\underline{H}_3$, J=7 Hz), 2.57 (s, 3H, $C\underline{H}_3C$=), 4.17 (q, 2H, $OC\underline{H}_2CH_3$, J=7 Hz), 4.78 (s, 2H, $NC\underline{H}_2$), 7.18—7.31 (m, 3H, Ar-H), 7.64—7.73 (m, 1H, Ar-H). *Anal.* Calcd for $C_{12}H_{14}N_2O_2$: C, 66.04; H, 6.47; N, 12.84. Found: C, 65.63; H, 6.76; N, 12.75.

1-(1-Ethoxycarbonylethyl)-2-methylbenzimidazole (6b) **6b** was prepared in a similar manner starting from **1a** (5.0 g, 38 mmol), ethyl 2-bromopropionate (7.20 g, 40 mmol) and NaH (1.03 g, 42 mmol). The crude product was purified by vacuum distillation, bp 150—152 °C (3 mmHg). Yield, quantitative. IR ν_{max} cm^{-1}: 1740 (C=O). ^{1}H-NMR (in $CDCl_3$) ppm: 1.15 (t, 3H, $OCH_2C\underline{H}_3$, J=7 Hz), 1.80 (d, 3H, $>CHC\underline{H}_3$, J=7 Hz), 2.62 (s, 3H, $=CC\underline{H}_3$), 4.19 (q, 2H, $OC\underline{H}_2CH_3$, J=7 Hz), 5.10 (q, 1H, $>C\underline{H}CH_3$, J=7 Hz), 7.12—7.38 (m, 3H, Ar-H), 7.60—7.75 (m, 1H, Ar-H). *Anal.* Calcd for $C_{13}H_{16}N_2O_2$: C, 67.22; H, 6.94; N, 12.06. Found: C, 67.03; H, 7.03; N, 11.91.

2-Ethyl-1-methoxycarbonylmethylbenzimidazole (6c) **6c** was prepared in a similar manner starting from **1b** (0.60 g, 4.1 mmol), methyl bromoacetate (0.69 g, 4.5 mmol) and NaH (0.11 g, 4.5 mmol). The crystalline crude product was recrystallized from C_6H_6–*n*-hexane to give colorless needles, mp 124—125 °C. Yield, 0.77 g (85.7%). IR ν_{max} cm^{-1}:1740 (C=O). ^{1}H-NMR (in $CDCl_3$) ppm: 1.46 (t, 3H, $CH_2C\underline{H}_3$, J=7 Hz), 2.85 (q, 2H, $=CC\underline{H}_2CH_3$, J=7 Hz), 3.75 (s, 3H, $COOC\underline{H}_3$), 4.81 (s, 2H, $C\underline{H}_2COO$), 7.13—7.33 (m, 3H, Ar-H), 7.66—7.81 (m, 1H, Ar-H). *Anal.* Calcd for $C_{12}H_{14}N_2O_2$: C, 66.04; H, 6.47; N, 12.84. Found: C, 66.38; H, 6.36; N, 13.04.

1-(1-Ethoxycarbonylethyl)-2-ethylbenzimidazole (6d) **6d** was prepared in a similar manner starting from **1b** (1.00 g, 6.9 mmol), ethyl 2-bromopropionate (1.30 g, 7.2 mmol) and NaH (0.19 g, 7.5 mmol). The crude oily product was purified by vacuum distillation, bp 153—155 °C (3 mmHg). Yield, quantitative. IR ν_{max} cm^{-1}:1740 (C=O). ^{1}H-NMR (in $CDCl_3$) ppm: 1.14 (t, 3H, $OCH_2C\underline{H}_3$, J=7 Hz), 1.47 (t, 3H, $=CCH_2C\underline{H}_3$, J=7 Hz), 1.80 (d, 3H, $CHC\underline{H}_3$, J=7 Hz), 2.91 (q, 2H, $=CC\underline{H}_2CH_3$, J=7 Hz), 4.18 (q, 2H, $OC\underline{H}_2CH_3$, J=7 Hz), 5.12 (q, 1H, $C\underline{H}CH_3$, J=7 Hz), 7.13—7.38 (m, 3H, Ar-H), 7.64—7.80 (m, 1H, Ar-H). HRMS *m/z*: Calcd for $C_{14}H_{18}N_2O_2$: 246.1368. Found: 246.1356 (M^+).

1-Ethoxycarbonylmethyl-2,3-dimethylbenzimidazolium Iodide (7a) A mixture of **6a** (1.15 g, 5.3 mmol), CH_3I (3.3 ml, 53 mmol) and AcOEt (20 ml) was refluxed at 80 °C for 2 h. The reaction mixture was cooled in an ice-water bath, then the precipitated crystals were filtered off under suction. Recrystallization of the crude product from 2-propanol gave pale yellow prisms, mp 197—199 °C. Yield, 1.77 g (92.9%). IR ν_{max} cm^{-1}: 1740 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.26 (t, 3H, $OCH_2C\underline{H}_3$, J=7 Hz), 2.89 (s, 3H, $=CC\underline{H}_3$), 4.06 (s, 3H, $NC\underline{H}_3$), 4.24 (q, 2H, $OC\underline{H}_2CH_3$, J=7 Hz), 5.62 (s, 2H, $NC\underline{H}_2$), 7.59—7.71 (m, 2H, Ar-H), 7.93—8.05 (m, 2H, Ar-H). *Anal.* Calcd for $C_{13}H_{17}IN_2O_2$: C, 43.35; H, 4.76; N, 7.78. Found: C, 43.25; H, 4.84; N, 8.10.

1-(1-Ethoxycarbonylethyl)-2,3-dimethylbenzimidazolium Iodide (7b) **7b** (yield, 12.97 g, 91.6%) was obtained in a similar manner starting from **6b** (8.70 g, 37.5 mmol), MeI (12.3 ml, 190 mmol) and AcOEt (40 ml). The crude product was recrystallized from 2-propanol to give pale yellow needles, mp 150—151 °C. IR ν_{max} cm^{-1}:1750 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.19 (t, 3H, $OCH_2C\underline{H}_3$, J=7 Hz), 1.83 (d, 3H, $>CHC\underline{H}_3$, J=7 Hz), 2.93 (s, 3H, $=CC\underline{H}_3$), 4.03 (s, 3H, $NC\underline{H}_3$), 4.23 (q, 2H, $OC\underline{H}_2CH_3$, J=7 Hz), 6.04 (q, 1H, $>C\underline{H}CH_3$, J=7 Hz), 7.50—7.84 (m, 2H, Ar-H), 7.87—8.11 (m, 2H, Ar-H). *Anal.* Calcd for $C_{14}H_{19}IN_2O_2$: C, 44.93; H, 5.12; N, 7.49. Found: C, 45.08; H, 5.07; N, 7.53.

2-Ethyl-1-methoxycarbonylmethyl-3-methylbenzimidazolium Iodide (7c) **7c** (yield, 0.517 g, 95.2%) was obtained in a similar manner starting from **6c** (329 mg, 1.5 mmol), CH_3I (0.5 ml, 7.6 mmol) and AcOEt (3 ml). The crude product was recrystallized from MeOH–AcOEt to give pale yellow prisms, mp 190—192 °C. IR ν_{max} cm^{-1}:1745 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.25 (t, 3H, $CH_2C\underline{H}_3$, J=7 Hz), 3.35 (q, 2H, $C\underline{H}_2CH_3$, J=7 Hz), 3.78 (s, 3H, $COOC\underline{H}_3$), 4.11 (s, 3H, $NC\underline{H}_3$), 5.67 (s, 2H, $C\underline{H}_2COOCH_3$), 7.60—7.78 (m, 2H, Ar-H), 7.93—8.10 (m, 2H, Ar-H). *Anal.* Calcd for $C_{13}H_{17}IN_2O_2$: C, 43.35; H, 4.75; N, 7.78. Found: C, 43.33; H, 4.81; N, 7.63.

1-(1-Ethoxycarbonylethyl)-2-ethyl-3-methylbenzimidazolium Iodide (7d) **7d** was obtained in a similar manner starting from **6d** (1.68 g, 6.8 mmol), MeI (2.2 ml, 34 mmol) and AcOEt (15 ml). The viscous crude product was used in the next step without further purification.

1-Carboxymethyl-2,3-dimethylbenzimidazolium Iodide (8a) A solution of **7a** (2.53 g, 7 mmol) in 4 N NaOH (2.0 ml, 8 mmol) was stirred for 1 h at room temperature. The solution was evaporated to dryness after neutralization with 2 N HCl (4.0 ml, 8 mmol), and the residual solid was recrystallized from MeOH–AcOEt to give colorless needles, mp 220—223 °C (dec.). Yield, 2.14 g (91.9%). IR ν_{max} cm^{-1}:1740 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 2.88 (s, 3H, $=CC\underline{H}_3$), 4.05 (s, 3H, $NC\underline{H}_3$), 5.50 (s, 2H, $NC\underline{H}_2$), 7.58—7.70 (m, 2H, Ar-H), 7.95—8.08 (m, 2H, Ar-H). *Anal.* Calcd for $C_{11}H_{13}IN_2O_2$: C, 39.78; H, 3.94; N, 8.43. Found: C, 39.78; H, 4.05; N, 8.42.

1-(1-Carboxyethyl)-2,3-dimethylbenzimidazolium Iodide (8b) **8b** (yield, 9.75 g, 92.4%) was obtained in a similar manner starting from **7b** (11.40 g, 30.5 mmol) and 4 N NaOH (8.0 ml, 32 mmol). Colorless needles, mp 249—250 °C (dec.). IR ν_{max} cm^{-1}:1760 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.80 (d, 3H, $CHC\underline{H}_3$, J=7 Hz), 2.93 (s, 3H, $=CC\underline{H}_3$), 4.03 (s, 3H, $NC\underline{H}_3$), 5.94 (q, 1H, $C\underline{H}CH_3$, J=7 Hz), 7.56—7.84 (m, 2H, Ar-H), 7.88—8.10 (m, 2H, Ar-H). *Anal.* Calcd for $C_{12}H_{15}IN_2O_2$: C, 41.64; H, 4.37; N, 8.09. Found: C, 41.80; H, 4.29; N, 8.18.

1-Carboxymethyl-2-ethyl-3-methylbenzimidazolium Iodide (8c) **8c** (yield, 458 mg, 92.2%) was obtained in a similar manner starting from **7c** (517 mg, 1.4 mmol) and 4 N NaOH (0.5 ml, 2 mmol). The crude product was recrystallized from 2-propanol to give pale yellow needles, mp 189—191 °C (dec.). IR ν_{max} cm^{-1}:1740 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.26 (t, 3H, $CH_2C\underline{H}_3$, J=7 Hz), 3.35 (q, 2H, $C\underline{H}_2CH_3$, J=7 Hz), 4.10 (s, 3H, $NC\underline{H}_3$), 5.54 (s, 2H, $C\underline{H}_2COOH$), 7.53—7.77 (m, 2H, Ar-H), 7.88—8.10 (m, 2H, Ar-H). *Anal.* Calcd for $C_{12}H_{15}IN_2O_2$: C, 41.64; H, 4.37; N, 8.09. Found: C, 42.09; H, 4.43; N, 8.30.

1-(1-Carboxyethyl)-2-ethylbenzimidazolium Iodide (8d) **8d** (yield, 2.33 g, 94.5%, calculated from **6d**) was obtained in a similar manner starting from **7d** [prepared from 1.68 g (6.8 mmol) of **6d**] and 2 N NaOH (4.0 ml, 8 mmol). Recrystallization from MeOH–AcOEt gave colorless prisms, mp 255—257 °C (dec.). IR ν_{max} cm^{-1}:1750 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 1.28 (t, 3H, $CH_2C\underline{H}_3$, J=7 Hz), 1.82 (d, 3H, $CHC\underline{H}_3$, J=7 Hz), 3.38 (q, 2H, $C\underline{H}_2CH_3$, J=7 Hz), 4.07 (s, 3H, $NC\underline{H}_3$), 5.96 (q, 1H, $C\underline{H}CH_3$, J=7 Hz), 7.57—7.88 (m, 3H, Ar-H), 7.95—8.11 (m, 1H, Ar-H). *Anal.* Calcd for $C_{13}H_{17}IN_2O_2$: C, 43.35; H, 4.76; N, 7.78. Found: C, 43.36; H, 4.67; N, 7.79.

1-Carboxymethyl-2,3-dimethylbenzimidazolium Chloride (9a) A solution of **8a** (14.54 g, 43.8 mmol) in MeOH (50 ml) was passed through a column (i.d. 3.5 cm) of Amberlyst A-21 (Cl^- form; 75 g) and the column was washed with 250 ml of MeOH. The combined MeOH solution was evaporated and the residue was recrystallized from MeOH–AcOEt to give colorless needles, mp 240—242 °C (dec.). Yield, quantitative. IR ν_{max} cm^{-1}: 1740 (C=O). ^{1}H-NMR (in DMSO-d_6) ppm: 2.89 (s, 3H, $=CC\underline{H}_3$), 4.06 (s, 3H, $NC\underline{H}_3$), 5.54 (s, 2H, $NC\underline{H}_2$), 7.57—7.69 (m, 2H,

Ar-H), 7.96—8.07 (m, 2H, Ar-H). *Anal.* Calcd for $C_{11}H_{13}ClN_2O_2$: C, 54.89; H, 5.44; N, 11.64. Found: C, 54.64; H, 5.55; N, 11.40.

4-Methyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (11a) a) CDI (1.49 g, 9.2 mmol) was added to a solution of **9a** (2.00 g, 8.3 mmol) in DMF (16 ml) at room temperature under an N_2 atmosphere. The mixture was stirred for 1 h, then Et_3N (3.5 ml, 25 mmol) was added and the whole was heated at 70 °C for 5 h. The reaction mixture was concentrated *in vacuo* and the residue was purified by chromatography on silica gel (solvent: $CHCl_3$–MeOH, 5:1). Crystals obtained from the main fraction were recrystallized from C_6H_6 to give pale yellow needles, mp 199—201 °C. Yield, 574 mg (35.3%). IR ν_{max} cm^{-1}:1570 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 3.55 (s, 3H, NCH_3), 4.14 (s, 2H, NCH_2), 4.80 (s, 1H, >C=CH–), 7.11 (br s, 4H, Ar-H). UV λ_{max}^{EtOH} nm (log ε): 241 (4.31), 322 (4.30), 332 (4.40). LRMS *m/z*: 186 (M^+). *Anal.* Calcd for $C_{11}H_{10}N_2O$: C, 70.95; H, 5.41; N, 15.04. Found: C, 70.94; H, 5.35; N, 15.04.

b) A mixture of **14** (183 mg, 0.64 mmol), 5% Pd–C (100 mg) and 2-propanol (2.0 ml) was stirred for 1 h at room temperature under an H_2 atmosphere (1 atm). The catalyst was removed by filtration and the filtrate was concentrated. The residue was diluted with water (1 ml), basified with solid K_2CO_3, and extracted with AcOEt (10 ml). Removal of the solvent of the extract after drying gave a solid mass, which was recrystallized from C_6H_6 to give pale yellow needles, mp 199—201 °C. Yield, quantitative. The product was identical (IR, TLC, mp, and mixed-melting-point test) with **11a** obtained by method a.

1,4-Dimethyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (11b) 11b (yield, 581 mg, 56.2%) was obtained in a similar manner to method a as used for **11a** starting from **8b** (1.79 g, 5.2 mmol), CDI (924 mg), DMF (10 ml) and Et_3N (2.15 ml, 15.5 mmol). The crude product was recrystallized from C_6H_6 to give pale yellow needles, mp 159—162 °C (dec.). IR ν_{max} cm^{-1}:1550, 1570 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 1.62 (d, 3H, $CHCH_3$, J=6 Hz), 3.55 (s, 3H, NCH_3), 4.20 (q, 1H, $CHCH_3$, J=6 Hz), 4.75 (s, 1H, >C=CH–), 7.12 (br s, 4H, Ar-H). HRMS *m/z*: Calcd for $C_{12}H_{12}N_2O$: 200.0949. Found: 200.0932 (M^+).

3,4-Dimethyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (11c) 11c (yield, 187 mg, 70.6%) was obtained in a similar manner to that described for **11a** starting from **8c** (458 mg, 1.3 mmol), CDI (236 mg, 1.5 mmol), DMF (3 ml) and Et_3N (0.55 ml, 4 mmol). The crude product was recrystallized from C_6H_6 to give pale yellow needles, mp 216—218 °C. IR ν_{max} cm^{-1}:1540, 1560 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 2.00 (s, 3H, $=CCH_3$), 3.71 (s, 3H, NCH_3), 4.05 (s, 2H, NCH_2), 7.08 (br s, 4H, Ar-H). LRMS *m/z*: 200 (M^+). *Anal.* Calcd for $C_{12}H_{12}N_2O$: C, 71.98; H, 6.04; N, 13.99. Found: C, 71.70; H, 6.08; N, 13.84.

1,3,4-Trimethyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (11d) 11d (yield, 468 mg, 81.3%) was obtained in a similar manner to that used for **11a** starting from **8d** (969 mg, 2.7 mmol), CDI (480 mg, 3.0 mmol), DMF (6 ml) and Et_3N (1.1 ml, 8.1 mmol). The crude product was recrystallized from C_6H_6 to give pale yellow needles, mp 166—167.5 °C. IR ν_{max} cm^{-1}: 1550, 1570 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 1.58 (d, 3H, $CHCH_3$, J=7 Hz), 2.00 (s, 3H, $=CCH_3$), 3.73 (s, 3H, NCH_3), 4.11 (q, NCH, J=7 Hz), 7.08 (s, 4H, Ar-H). LRMS *m/z*: 214 (M^+). *Anal.* Calcd for $C_{13}H_{14}N_2O$: C, 72.87; H, 6.59; N, 13.08. Found: C, 72.55; H, 6.56; N, 13.06.

1,1,3,4-Tetramethyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (11e) A LDA solution (0.98 mmol, prepared in a usual manner) was added dropwise at −78 °C under an N_2 atmosphere to a solution of **11d** (200 mg, 0.93 mmol), and the mixture was stirred for 1 h at −78 °C. CH_3I (63 μl, 0.93 mmol) was added to the mixture, followed by stirring for 1 h. The reaction was quenched by addition of water (5 ml), and the product was extracted with AcOEt (5 ml × 3). The combined organic phase was evaporated to give a solid residue, which was recrystallized from C_6H_6 to give pale yellow prisms, mp 163—164.5 °C. Yield, 156 mg (73.2%). IR ν_{max} cm^{-1}: 1550, 1570 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 1.51 (s, 6H, $>C(CH_3)_2$), 2.01 (s, 3H, $=CCH_3$), 3.74 (s, 3H, NCH_3), 7.08 (br s, 4H, Ar-H). LRMS *m/z*: 228 (M^+). *Anal.* Calcd for $C_{14}H_{16}N_2O$: C, 73.65; H, 7.06; N, 12.27. Found: C, 73.70; H, 7.10; N, 12.30.

1,1,3-Trichloro-4-methyl-4*H*-pyrrolo[1,2-*a*]benzimidazol-2(1*H*)-one (14) A mixture of **8a** (1.00 g, 3 mmol) and $SOCl_2$ (10 ml) was refluxed at 80 °C for 2 h in a usual manner. Excess reagent was removed by evaporation under reduced pressure and the residue was washed with dry C_6H_6 (10 ml). The resulting residue was basified with 5% $NaHCO_3$ (5 ml) and the product was extracted with AcOEt (5 ml × 3). The organic phase was evaporated after drying and the residue was purified by column chromatography on silica gel (solvent, AcOEt). Removal of the solvent of the main fraction gave a solid residue, which was recrystallized from MeOH to give pale yellow needles, mp 219—220.5 °C (dec.). Yield, 475 mg (54.8%). IR ν_{max} cm^{-1}: 1580, 1620, 1710 (C=O or C=C). ^{1}H-NMR (in $CDCl_3$) ppm: 3.88 (s, 3H, NCH_3), 7.21—7.46 (m, 4H, Ar-H). UV λ_{max}^{EtOH} nm (log ε): 247 (4.12), 293 (3.54), 379 (3.86). LRMS *m/z*: 288 (M^+). *Anal.* Calcd for $C_{11}H_7Cl_3N_2O \cdot 1/2H_2O$: C, 44.25; H, 2.70; N, 9.38. Found: C, 44.75; H, 2.77; N, 9.47.

6-Acetyl-5-methyl-9,10-dihydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (19) A mixture of **2a** (R = CH_3, 2.14 g, 10 mmol) and acetic anhydride (10 ml) was stirred for 4 h at 100 °C under an N_2 atmosphere. The volatile portion of the mixture was removed by evaporation under reduced pressure. Water (10 ml) was added to the residue and the mixture was stirred for 30 min then basified with powdered K_2CO_3. The mixture was extracted with $CHCl_3$ (100 ml), and the extract was dried. Evaporation of the solvent gave a crystalline residue, which was purified by column chromatography on silica gel (solvent, AcOEt–MeOH, 5:1) to give a crystalline mass. The product was recrystallized from AcOEt to give slightly brown prisms; yield, 1.74 g (68%). *Anal.* Calcd for $C_{15}H_{16}N_2O_2$: C, 70.29; H, 6.29; N, 10.93. Found: C, 70.21; H, 6.36; N, 10.91.

6-Benzoyl-5-methyl-9,10-dihydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (20) A mixture of **2a** (R = CH_3, 2.14 g, 10 mmol) and benzoic anhydride (11.30 g, 50 mmol) was stirred for 18 h at 80 °C under an N_2 atmosphere. After the mixture was cooled, water (50 ml) was added, the whole was basified with K_2CO_3, and the product was extracted with $CHCl_3$ (100 ml × 3). The extract was concentrated after drying to give a crystalline residue. Product: pale yellow needles; yield, 1.76 g (57%). *Anal.* Calcd for $C_{20}H_{18}N_2O_2$: C, 75.45; H, 5.70; N, 8.80. Found: C, 75.18; H, 5.69; N, 8.85.

5-Methyl-6-(3-oxobutyl)-9,10-dihydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (21) Methyl vinyl ketone (1.25 ml, 15 mmol) was added to a solution of **2a** (R = CH_3, 2.14 g, 10 mmol) in EtOH (20 ml), and the mixture was stirred for 5 h at room temperature. Removal of the solvent gave a viscous residue, which was purified by column chromatography on silica gel (solvent, $CHCl_3$–MeOH, 10:1). The main fraction gave a crystalline residue. Product: slightly brown needles; yield, 1.30 g (46%). *Anal.* Calcd for $C_{17}H_{20}N_2O_2$: C, 71.80; H, 7.09; N, 9.85. Found: C, 71.86; H, 7.14; N, 9.87.

5-Methyl-6-(*N*-phenylcarbamoyl)-9,10-tetrahydro-5*H*-azepino[1,2-*a*]-benzimidazol-7(8*H*)-one (22) Phenyl isocyanate (1.63 ml, 10 mmol) was added to a solution of **2a** (R = CH_3, 2.14 g, 10 mmol) in $CHCl_3$ (8 ml), and the mixture was stirred for 3 h at room temperature. The solvent was removed by evaporation to give a viscous material, which was purified by column chromatography on silica gel (solvent: AcOEt–MeOH, 10:1). Removal of the solvent gave a crystalline residue, which was recrystallized. Product: colorless needles; yield, 1.75 g (82%). *Anal.* Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.61. Found: C, 71.87; H, 5.64; N, 12.84.

6-(*N*-Butylcarbamoyl)-5-methyl-9,10-tetrahydro-5*H*-azepino[1,2-*a*]-benzimidazol-7(8*H*)-one (23) **23** (yield, 0.66 g, 35%) was obtained in a similar manner starting from **2a** (R = CH_3, 1.28 g, 6 mmol), *n*-butyl isocyanate (1.06 ml, 9 mmol) and $CHCl_3$ (5 ml). Product: colorless needles. *Anal.* Calcd for $C_{18}H_{23}N_3O_2$: C, 67.81; H, 7.46; N, 13.20. Found: C, 67.80; H, 7.76; N, 12.80.

5-Methyl-6-(*N*-phenylthiocarbamoyl)-7,8,9,10-tetrahydro-6*H*-azepino-[1,2-*a*]benzimidazol-7-one (24) **24** (yield, 0.57 g, 31%) was obtained in a similar manner starting from **2a** (R = CH_3, 1.07 g, 5 mmol), phenyl isothiocyanate (0.66 ml, 5.5 mmol) and $CHCl_3$ (5 ml). *Anal.* Calcd for $C_{20}H_{19}N_3OS$: C, 68.74; H, 5.48; N, 12.03. Found: C, 68.61; H, 5.31; N, 11.91.

5-Methyl-6-phenylazo-9,10-tetrahydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one Hydrochloride (25·HCl) A mixture of aniline (0.92 ml, 10 mmol), 2 N HCl (15 ml, 30 mmol), EtOH (22.5 ml) and isoamyl nitrite (1.35 ml, 10 mmol) was stirred at 0 °C for 15 min. The reaction mixture was added dropwise to an ice-cooled solution of **2a** (R = CH_3, 2.14 g, 10 mmol) in EtOH (5 ml) under stirring, then the resulting mixture was stirred at room temperature for 1 h. The volatile portion of the reaction mixture was evaporated off. A solution of the residue in EtOH (30 ml) was passed through a column of Amberlyst A-21 -HCl form (30 g; Rohm & Haas Ltd.). The resin was washed with ethanol (50 ml), and the combined ethanolic solution was evaporated to give a crystalline residue. Product: yellow needles. Yield, 2.56 g (72%). *Anal.* Calcd for $C_{19}H_{19}ClN_4O$: C, 58.38; H, 5.92; N, 14.33. Found: C, 58.41; H, 5.72; N, 14.22.

6-(4-Methoxyphenylazo)-5-methyl-9,10-dihydro-5*H*-azepino[1,2-*a*]-benzimidazol-7(8*H*)-one Hydrochloride (26·HCl) **26**·HCl (yield, 1.64 g, 36%) was obtained in a similar manner starting from **2a** (R = CH_3, 2.14 g, 10 mmol) in EtOH (5 ml), *p*-anisidine (1.23 g, 10 mmol) in EtOH (22.5 ml), isoamyl nitrite (1.35 ml, 10 mmol) and 2 N HCl (15 ml, 30 mmol). Product:

yellow needles. *Anal.* Calcd for $C_{20}H_{21}ClN_4O_2$: C, 52.47; H, 5.06; N, 12.23. Found: C, 52.64; H, 4.69; N, 12.28.

5-Methyl-6-phenylselenenyl-9,10-dihydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (27) A mixture of **2a** (R=CH_3, 1.07 g, 5 mmol), phenylselenenyl chloride (0.96 g, 5 mmol) and $CHCl_3$ (10 ml) was stirred for 2.5 h at room temperature, and the solvent was evaporated under reduced pressure. The viscous residue was purified by chromatography on silica gel (solvent: AcOEt–MeOH, 5:1). The solvent was evaporated to give a crystalline mass, which was recrystallized from AcOEt–*n*-hexane to give pale yellow powder; yield, 1.38 g (75%). *Anal.* Calcd for $C_{19}H_{18}N_2OSe$: C, 61.79; H, 4.91; N, 7.59. Found: C, 61.64; H, 5.12; N, 7.27.

8-(1-Hydroxy-1-methylethyl)-5-methyl-9,10-tetrahydro-5*H*-azepino-[1,2-*a*]benzimidazol-7(8*H*)-one (17a) Powdered **2a** (R=CH_3, 1.07 g, 5 mmol) was added at −78 °C under an N_2 atmosphere to a solution of LDA (5.1 mmol), which was prepared in a usual manner, and the mixture was stirred for 15 min. Acetone (0.39 ml, 5.25 mmol) was added to the mixture, and the reaction was quenched after 1 h by addition of water (10 ml). The resulting mixture was extracted with AcOEt (100 ml) followed by washing of the organic phase with brine. Evaporation of the solution after drying with Na_2SO_4 gave a crystalline residue, which was recrystallized from AcOEt to give colorless needles; yield, 0.78 g (58%). *Anal.* Calcd for $C_{16}H_{20}N_2O_2$: C, 82.51; H, 5.86; N, 7.40. Found: C, 82.27; H, 6.10; N, 7.18.

5-Methyl-8-(diphenylhydroxymethyl)-9,10-tetrahydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (17b) **17b** (yield, 0.80 g, 50%) was obtained in a similar manner starting from **2a** (R=CH_3, 0.86 g, 4.0 mmol), LDA (4.2 mmol) and benzophenone (0.74 g, 4.2 mmol). *Anal.* Calcd for $C_{26}H_{24}N_2O_2$: C, 78.76; H, 6.10; N, 7.07. Found: C, 78.53; H, 6.10; N, 7.14.

5-Methyl-8-diphenylmethylene-9,10-tetrahydro-5*H*-azepino[1,2-*a*]benzimidazol-7(8*H*)-one (18b) The hydroxy ketone (**17b**, 0.60 g, 1.5 mmol) was stirred in $SOCl_2$ (0.58 ml, 8 mmol) for 5 min at room temperature. Excess reagent was removed by evaporation under reduced pressure. The residue was treated with water (5 ml) and $CHCl_3$ (25 ml), and the aqueous phase was basified with powdered K_2CO_3 under shaking. The $CHCl_3$ phase was separated and dried with Na_2SO_4, followed by evaporation under reduced pressure to give a crystalline residue. Recrystallization of the crude product gave pale yellow needles; yield, 0.39 g (67%), mp 255—257 °C. IR (KBr) cm^{-1}: 1560 (C=O). ^{1}H-NMR (80 MHz in $CDCl_3$) ppm: 2.99 (t, 2H, $-CH_2C\underline{H}_2C-$, J=6 Hz), 3.46 (s, 3H, $-NC\underline{H}_3$), 3.85 (t, 2H, $-NC\underline{H}_2-$, J=6 Hz), 5.15 (s, 1H, $C=C\underline{H}CO-$), 6.96—7.41 (m, 14H, aromatic protons). *Anal.* Calcd for $C_{26}H_{22}N_2O$: C, 70.56; H, 7.40; N, 10.29. Found: C, 70.53; H, 7.46; N, 10.21.

References and Notes

1) Examples of the imidazole-contained drug: cimetidine, clotrimazole, miconazole, ozagrel, nizofenone, histidine, histamine; examples of the benzimidazole-contained drug: omeprazole, cremizole, mebendazole, cyanocobalamine.
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Triazolo[4,5-*d*]pyrimidines. X.[1] Halogen–Metal Exchange Reaction of 7-Halo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidines with Butyllithium

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The amino group at the 7-position on the 3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine ring was converted into halogen atoms by treatment with isopentyl nitrite in halomethanes, in satisfactory yields. The halogen–metal exchange reaction between 7-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (2) and butyllithium in the presence of *N,N,N′,N′*-tetramethylethylenediamine proceeded, giving the 7-lithio compound (9). The lithio compound (9) reacted smoothly with electrophiles to give the corresponding 7-substituted compounds (15—18). On the other hand, the reaction of 7-chloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (1) with butyllithium gave the ring fission product, 5-amino-1-phenyl-1*H*-1,2,3-triazole-4-carbonitrile (14).

Keywords 7-halo-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine; halogen–metal exchange reaction; butyllithium; electrophile; ring fission

We reported that the substitution of a methylsulfonyl group at the 5-[1] and 7-positions[2] on the 3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (triazolopyrimidine) ring with Grignard reagents as carbanions gave the corresponding 5- and 7-alkylated derivatives, while the addition[1] of Grignard reagents across the C^7,N^6-double bond on the triazolopyrimidine ring proceeded when 5-chloro-3-pyenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine reacted with Grignard reagents. It is well known[3] that lithio compounds of heteroarenes react smoothly with electrophiles, and this is a useful method for introduction of functionalized carbon chains into heteroarenes. However, only a few studies[4] have been reported on the reaction of fused pyrimidine rings with organo lithium compounds. In this paper, we describe the reaction of the 7-halotriazolopyrimidines (**1**—**5**) with butyllithium (BuLi).

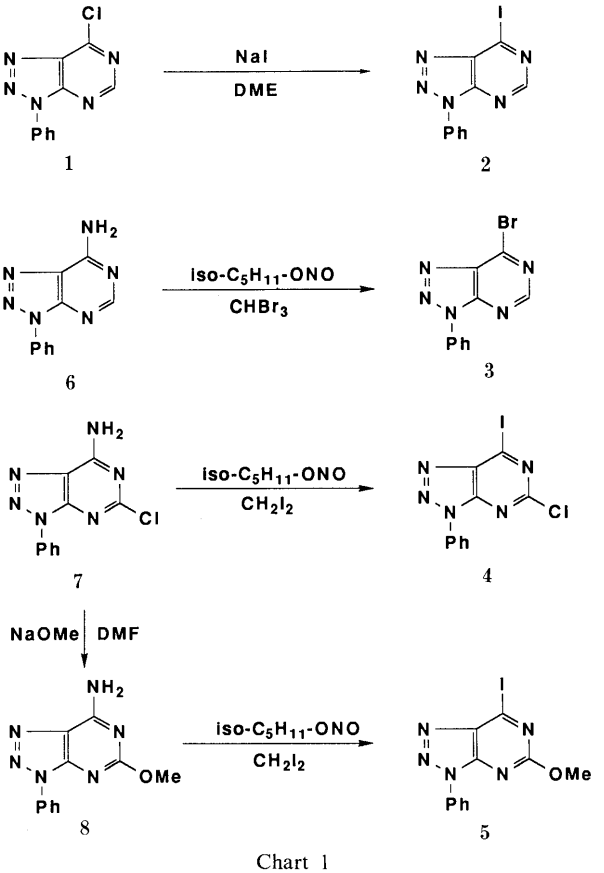

Chart 1

Two methods, A and B, have been reported for the preparation of iodonated condensed pyrimidines. Method A[5] is substitution of a chlorine atom on the condensed pyrimidine ring with sodium iodide in dimethoxyethane (DME), and method B[6] is conversion of the amino group into a halogen atom by treatment with pentyl nitrite in halomethanes, such as tetrachloromethane, tribromomethane and diiodomethane, in good yields.

Firstly, we examined the preparation of 7-iodo- (**2**, **4** and **5**) and 7-bromo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidines (**3**) by methods A and B. 7-Chloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**1**) reacted with sodium iodide to give 7-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**2**) in 87% yield by method A. The conversion of the amino group at the 7-position on the triazolopyrimidine ring into a bromo or an iodo atom by method B proceeded and the corresponding 7-halo derivatives (**3**—**5**) were obtained in satisfactory yields, as shown in Table I.

Next, we investigated the reaction of the 7-halotriazolopyrimidines (**1**—**3**) with BuLi. When a solution of **2** and BuLi in the presence of *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) in tetrahydrofuran (THF) was stirred at −100 °C for 1 min and quenched with aqueous ammonium chloride, 3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**10**) was obtained in 41% yield, together with 7,7′-bis[3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine] (**11**). This result indicates that the lithio compound (**9**) was initially formed as

TABLE I. Yields, Melting Points and Elemental Analysis Data for **2**—**5**

Compd.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
2	87	143—144	$C_{10}H_6IN_5$	37.17 (37.45)	1.87 (1.87)	21.68 (21.59)
3	69	134—136	$C_{10}H_6BrN_5$	43.50 (43.67)	2.19 (2.22)	25.37 (25.35)
4	66	186—188	$C_{10}H_5ClIN_5$	33.59 (33.70)	1.41 (1.47)	19.59 (19.59)
5	41	165—167	$C_{11}H_8IN_5O$	37.42 (37.40)	2.28 (2.26)	19.83 (19.85)

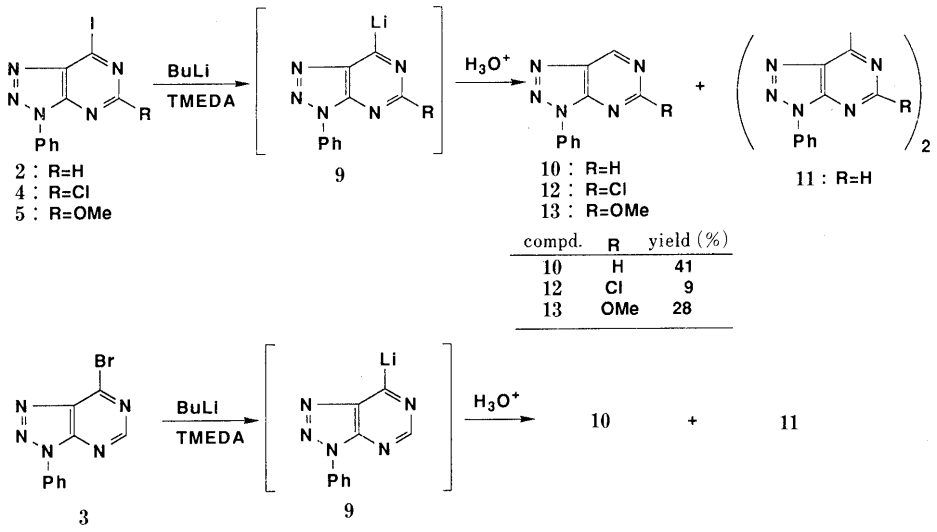

Chart 2

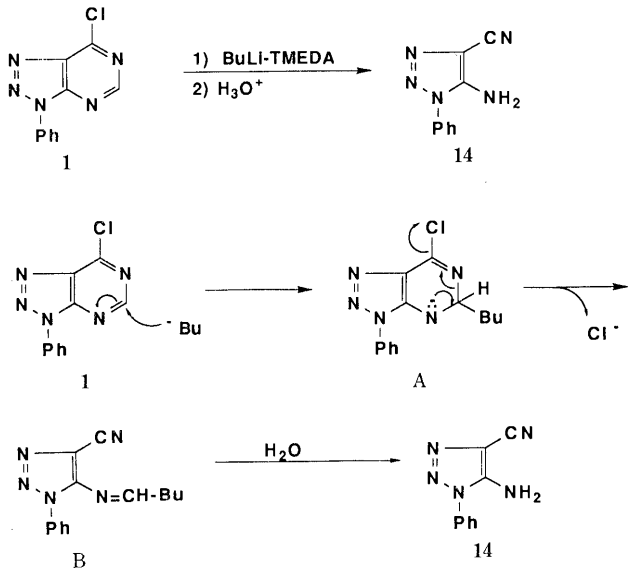

Chart 3

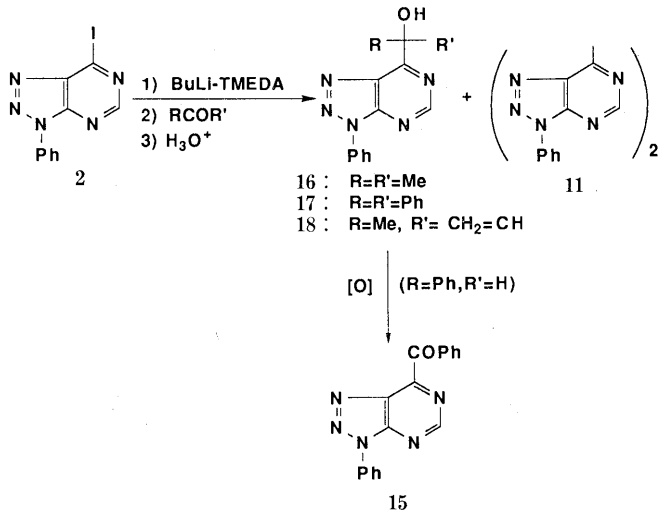

Chart 4

an intermediate. Similarly, the halogen–metal exchange reaction of **3** with BuLi under the same conditions proceeded to give **10**, though the yield was low. On the other hand, in the case of the 7-chloro compound (**1**), ring fission between the C^5 and N^6-positions occurred, resulting in the formation of 5-amino-1-phenyl-1*H*-1,2,3-triazole-4-carbonitrile (**14**) in 62% yield.

The formation of **14** may be explained by the process shown in Chart 3. The first step is nucleophilic addition of BuLi across the C^5,N^4-double bond to form the intermediate A, followed by ring fission between the C^5 and N^6-positions to give the intermediate B, which then gives **14** with loss of valeraldehyde. Moreover, the halogen–metal exchange reaction of the 5-substituted 7-iodo-3-phenyltriazolopyrimidines (**4** and **5**) proceeded under the same conditions to give the desired products (**12** and **13**), but in low yields, as shown in Chart 2.

Finally, we investigated the reaction of the lithio compound (**9**) with electrophiles such as benzaldehyde and ketones. When a solution of **9**, prepared from **2** with BuLi and TMEDA, and benzaldehyde in THF was stirred at −100——10 °C for 1 h, 7-benzoyl-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**15**) was obtained in 36% yield, together with **11**. Similarly, the lithio compound (**9**) reacted smoothly with ketones such as acetone and benzophenone under the same conditions, giving 7-(2-hydroxy-2-propyl)- (**16**) and 7-(hydroxydiphenylmethyl)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidines (**17**), together with **11**. In the case of methyl vinyl ketone, the nucleophilic addition of the

TABLE II. Yields, Melting Points and Elemental Analysis Data for **15**—**18**

Compd.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
15	36	141—142[a]				
16	39	115—116	$C_{13}H_{13}N_5O$	61.16	5.13	27.44
				(61.37	5.12	27.44)
17	25	209—210	$C_{23}H_{17}N_5O$	72.81	4.52	18.46
				(73.07	4.54	18.23)
18	18	86—88	$C_{14}H_{13}N_5O$	62.91	4.90	26.20
				(62.93	4.94	26.17)

a) Lit.[10] mp 141 °C.

lithio compound (**9**) to the carbonyl group proceeded, giving 7-(2-hydroxy-3-buten-2-yl)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**18**).

The experimental results may be summarized as follows. i) The 7-iodo- and 7-bromotriazolopyrimidines were easily prepared from the 7-aminotriazolopyrimidines by treatment with isopentyl nitrite in halomethanes. ii) The halogen–metal exchange reaction between the 7-iodo derivative and BuLi proceeded and the 7-lithio compound reacted smoothly with electrophiles. iii) The reaction of the lithio compound with electrophiles provides a method for the introduction of functionalized carbon chains into the 7-position of the triazolopyrimidine ring.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were measured with a JASCO IR-700 diffraction grating IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz and 23 °C with a Hitachi R-24B high resolution ^{1}H-NMR spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, dd = double doublet, m = multiplet, br = broad.

7-Iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (2) A solution of **1**[7] (2.3 g, 10 mmol) and NaI (10 g, 66 mmol) in DME (200 ml) was refluxed for 3 d. The generated NaCl was filtered off, and the filtrate was evaporated under reduced pressure. The residue was diluted with H_2O and extracted with benzene. The crude product was purified by SiO_2 column chromatography with benzene and recrystallized from petroleum benzin–benzene to give colorless needles (**2**), mp 143—144 °C. Yield 2.8 g (87%).

7-Bromo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (3) Isopentyl nitrite (15 ml, 112 mmol) was added to a suspension of **6**[2] (2 g, 9.4 mmol) in tribromomethane (45 ml) under a nitrogen atmosphere and the mixture was stirred at 80 °C for 30 min. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless prisms (**3**), mp 134—136 °C. Yield 1.8 g (69%).

7-Amino-5-chloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (7) A solution of 5,7-dichloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine[8] (4 g, 15 mmol) and 28% aqueous NH_4OH (7 ml) in dimethylformamide (DMF) (55 ml) was stirred at room temperature for 5 min. The reaction mixture was poured onto ice-water. The generated solids were filtered off to give colorless solids (**7**), mp > 300 °C (lit.[8] mp > 300 °C). Yield 3.4 g (93%).

5-Chloro-7-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (4) Isopentyl nitrite (30 ml, 233 mmol) was added to a suspension of **7**[8] (2 g, 8.1 mmol) in diiodomethane (60 ml) under a nitrogen atmosphere and the mixture was stirred at 85 °C for 4 d. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from MeOH to give colorless needles (**4**), mp 186—188 °C. Yield 1.9 g (66%).

7-Amino-5-methoxy-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (8) A solution of NaOMe (4.4 g, 83 mmol) in MeOH (40 ml) was added to a solution of **7** (1 g, 4.1 mmol) in DMF (20 ml). The mixture was stirred at 60 °C for 1 h. The generated NaCl was filtered off and the filtrate was evaporated under reduced pressure. The residue was diluted with H_2O and neutralized with concentrated HCl. The generated solids were filtered off and recrystallized from MeOH to give colorless powders (**8**), mp 233—236 °C (dec.). Yield 0.85 g (85%). MS *m/z*: 242 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3176, 3320 (NH_2). ^{1}H-NMR (DMSO-d_6): 3.94 (3H, s, OCH_3), 7.25—7.79 (3H, m, N^3-Ph), 7.80—8.81 (4H, m, N^3-Ph, NH_2).

7-Iodo-5-methoxy-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (5) Isopentyl nitrite (30 ml, 224 mmol) was added to a suspension of **8** (2 g, 8.3 mmol) in diiodomethane (60 ml) under a nitrogen atmosphere. The mixture was stirred at 85 °C for 2.5 h. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless needles (**5**), mp 165—167 °C. Yield 1.2 g (41%).

Reaction of 2 with BuLi A solution of TMEDA (235 mg, 2 mmol) in THF (15 ml) was cooled to −100 °C under a nitrogen atmosphere and treated with BuLi (1.62 M in hexane, 1.4 ml, 2.2 mmol). After 5 min, a solution of **2** (323 mg, 1 mmol) in THF (10 ml) was added dropwise to the solution for 10 min at −100 °C. After 30 s, aqueous NH_4Cl was added to the mixture, and the whole was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography. The fraction eluted with petroleum benzin–benzene (1 : 1) gave 3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**10**) as pale yellow needles, mp 112—114 °C (lit.[9] mp 114—115 °C). Yield 80 mg (41%). ^{1}H-NMR ($CDCl_3$): 7.39—7.82 (3H, m, N^3-Ph), 8.00—8.39 (2H, m, N^3-Ph), 9.20 (1H, s, C^5-H), 9.55 (1H, s, C^7-H). The fraction eluted with benzene–$CHCl_3$ (1 : 1) gave 7,7′-bis[3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine] (**11**) as pale yellow needles, mp 264—268 °C (dec.) (lit.[9] mp 266—270 °C). Yield 54 mg (28%). ^{1}H-NMR ($CDCl_3$): 7.40—8.60 (10H, m, N^3-Ph), 9.70 (2H, s, C^5-H).

Reaction of 3 with BuLi A solution of **3** (276 mg, 1 mmol) in THF (10 ml) was treated with BuLi and TMEDA as described for the reaction of **2** with BuLi. Work-up as described above gave **10** (40 mg) in 20% yield and **11** (25 mg) in 13% yield.

Reaction of 1 with BuLi A solution of **1** (232 mg, 1 mmol) in THF (10 ml) was treated with BuLi and TMEDA as described for the reaction of **2** with BuLi, and the reaction mixture was worked up in the same way. The eluate from benzene was recrystallized from petroleum benzin–benzene to give 5-amino-1-phenyl-1*H*-1,2,3-triazole-4-carbonitrile (**14**) as colorless needles, mp 126—129 °C (lit.[10] mp 127 °C). IR ν_{max}^{KBr} cm^{-1}: 3320, 3196 (NH_2), 2238 (CN). ^{1}H-NMR ($CDCl_3$): 4.42—4.95 (2H, br, NH_2), 7.41 (5H, s, N^1-Ph).

Reaction of 4 with BuLi A soluton of TMEDA (120 mg, 1 mmol) in THF (15 ml) was cooled to −100 °C under a nitrogen atmosphere and treated with BuLi (1.62 M in hexane, 0.7 ml, 1.1 mmol). After 5 min, a solution of **4** (358 mg, 1 mmol) in THF (10 ml) was added dropwise to the solution for 10 min at −100 °C. After 30 s, aqueous NH_4Cl was added to the mixture, and the whole was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography with benzene and recrystallized from petroleum benzin–benzene to give 5-chloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**12**) as colorless needles mp 137—138 °C (lit.[8] mp 137—138 °C). Yield 20 mg (9%). ^{1}H-NMR ($CDCl_3$): 7.27—7.83 (3H, m, N^3-Ph), 7.85—8.32 (2H, m, N^3-Ph), 8.88 (1H, s, C^7-H).

Reaction of 5 with BuLi A solution of **5** (353 mg, 1 mmol) in THF (10 ml) was treated with BuLi and TMEDA as described for the reaction of **2** with BuLi. A similar work-up gave 5-methoxy-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**13**) as colorless needles, mp 93—94 °C (lit.[8] mp 94—95 °C). Yield 36 mg (28%). ^{1}H-NMR ($CDCl_3$): 4.14 (3H, s, OCH_3), 7.38—7.65 (3H, m, N^3-Ph), 8.10—8.28 (2H, m, N^3-Ph), 9.41 (1H, s, C^7-H).

7-Benzoyl-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (15) A solution of TMEDA (235 mg, 2 mmol) in THF (15 ml) was cooled to −100 °C under a nitrogen atmosphere and treated with BuLi (1.62 M in hexane, 1.4 ml, 2.2 mmol). After 5 min, a solution of **2** (323 mg, 1 mmol) in THF (10 ml) was added dropwise to the solution for 10 min at −100 °C. After 30 s, a solution of benzaldehyde (1.1 g, 10 mmol) in THF (10 ml) was dropped into the mixture at −100 °C and the whole was stirred at −100—−10 °C for 1.5 h. Aqueous NH_4Cl was added, and the mixture was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography. The fraction eluted with petroleum benzin–benzene (1 : 1) gave pale yellow needles (**15**) from petroleum benzin–benzene, mp 141—142 °C (lit.[11] mp 141 °C). Yield 109 mg (36%). The fraction eluted with benzene–$CHCl_3$ (1 : 1) gave **11**. Yield 42 mg (22%).

7-(2-Hydroxy-2-propyl)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (16) A solution of **2** (323 mg, 1 mmol) in THF (15 ml) and acetone (580 mg, 10 mmol) was treated with BuLi and TMEDA as described for **15**. A similar work-up gave **16** (100 mg) as colorless needles from petroleum benzin–benzene, mp 115—116 °C, in 39% yield and **11** (40 mg) in 21% yield.

7-(Hydroxydiphenylmethyl)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (17) A solution of **2** (323 mg, 1 mmol) in THF (15 ml) and benzophenone (1.8 g, 10 mmol) in THF (10 ml) was treated with BuLi and

TABLE III. ^{1}H-NMR Spectral Data for **2**—**5**

Compd.	^{1}H-NMR ($CDCl_3$) δ
2	7.31—7.80 (3H, m, N^3-Ph), 7.89—8.30 (2H, m, N^3-Ph), 8.75 (1H, s, C^5-H)
3	7.21—7.65 (3H, m, N^3-Ph), 7.89—8.20 (2H, m, N^3-Ph), 8.71 (1H, s, C^5-H)
4	7.25—7.78 (3H, m, N^3-Ph), 7.85—8.20 (2H, m, N^3-Ph)
5	4.10 (3H, s, OCH_3), 7.29—7.71 (3H, m, N^3-Ph), 7.85—8.22 (2H, m, N^3-Ph)

TABLE IV. IR and ^{1}H-NMR Spectral Data for **15**—**18**

Compd.	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR ($CDCl_3$) δ
15	1665 (C=O)	7.32—7.79 (6H, m, N^3-Ph, COPh), 7.80—8.36 (4H, m, N^3-Ph, COPh), 9.23 (1H, s, C^5-H)
16	3442 (OH)	1.91 (6H, s, $C(CH_3)_2$), 4.95 (1H, br, OH), 7.31—7.78 (3H, m, N^3-Ph), 8.04—8.32 (2H, m, N^3-Ph), 9.08 (1H, s, C^5-H)
17	3396 (OH)	6.26 (1H, s, OH), 7.15—7.96 (13H, m, N^3-Ph, $C(Ph)_2$), 8.05—8.46 (2H, m, N^3-Ph)
18	3410 (OH)	2.01 (3H, s, CH_3), 5.13 (1H, s, OH), 5.14 (1H, dd, J_{AB}=2.0 Hz, J_{AX}=18.0 Hz, $CH_X=C\underline{H}_AH_B$), 5.52 (1H, dd, J_{AB}=2.0 Hz, J_{BX}=12.0 Hz, $CH_X=CH_A\underline{H}_B$), 6.49 (1H, dd, J_{AX}=18.0 Hz, J_{BX}=12.0 Hz, $C\underline{H}_X=CH_AH_B$), 7.24—7.71 (3H, m, N^3-Ph), 7.90—8.27 (2H, m, N^3-Ph), 8.98 (1H, s, C^5-H)

TMEDA as described for **15**. A similar work-up gave **17** (93 mg) as colorless prisms from benzene–MeOH, mp 209—210 °C, in 25% yield and **11** (30 mg) in 15% yield.

7-(2-Hydroxy-3-buten-2-yl)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (18) A solution of **2** (323 mg, 1 mmol) in THF (15 ml) and methyl vinyl ketone was treated with BuLi and TMEDA as described for **15**. A similar work-up gave **18** (47 mg) as pale yellow needles from petroleum benzin, mp 86—88 °C, in 20% yield and **11** (45 mg) in 23% yield.

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Studies toward Total Synthesis of Non-aromatic *Erythrina* Alkaloids. (1).[1] Synthesis and Isomerization of Unsaturated Bicyclic δ-Lactones

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As a model of the C/D ring system of erythroidines, bicyclic unsaturated δ-lactones were synthesized in a regio-selective manner, and their isomerization reactions in the presence of acid, base (DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene), and NaOH were studied. In the lactone form, the 6-ene (3) was the most unstable and isomerized to the 5-ene (1) then to the $1^{(6)}$-ene (2). The latter two lactones equilibrate to give *ca.* 3:2 mixture of 1 and 2 in the presence of DBU. On the contrary, in the opened form (NaOH), the lactone 1 was the most unstable and isomerized to 2 and 3. The $1^{(10)}$-ene (4) was inert under all of the conditions examined.

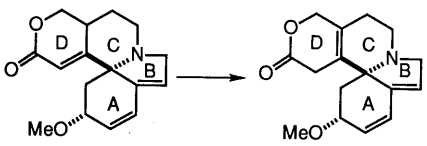
Keywords *Erythrina* alkaloid; erythroidine; intramolecular Horner–Emmons reaction; decarbomathoxylation; unsaturated δ-lactone; 3-oxabicyclo[4.4.0]dec-5-en-4-one; 3-oxabicyclo[4.4.0]dec-$1^{(6)}$-en-4-one; 3-oxabicyclo[4.4.0]dec-6-en-4-one; 3-oxabicyclo[4.4.0]dec-$1^{(10)}$-en-4-one; isomerization

Erythrina alkaloids are remarkable for their strong curare-like action on oral administration. There are more than 60 alkaloids of this group. Total syntheses of those which have an aromatic group at ring D (aromatic *Erythrina* alkaloids) have been accomplished,[2] but synthesis of so-called non-aromatic *Erythrina* alkaloids, all of which have an unsaturated lactone ring at ring D instead of an aromatic group, is still elusive.

In 1958, Boekelheide and Morrison[3] reported that, on treatment with 10% NaOH solution, α-erythroidine exclusively isomerized into β-erythroidine. The reaction includes an interesting migration of a conjugated double bond to an unconjugated position.

In the connection with synthetic studies of erythroidines, we are interested in the above migration and wondered whether it is due to an inherent property of the erythroidine skeleton or not. Therefore, as a model experiment, we have examined isomerization reactions of a simple bicyclic system corresponding to rings C and D of erythroidines, because the results obtained should provide useful information for the synthesis of these alkaloids. For this purpose, we have synthesized the four unsaturated lactones **1**—**4** in a selective manner and examined their isomerization reactions under various conditions.

Synthesis of the Unsaturated Lactones 1—4 **A. The Lactones 1 and 2** The syntheses of lactones **1** and **2** were previously reported by Lythgoe's group.[4] They obtained **1** in 48% yield from 2-acetoxymethylcyclohexanone by Reformatsky reaction with methyl bromoacetate followed by dehydration with acetic anhydride. They also prepared **2** in 44% yield from the enol benzoate **5** (prepared from 2-hydroxymethylenecyclohexanone in two steps) by acid hydrolysis, hydride reduction, and Jones oxidation. Our synthesis of these lactones is more straightforward and selective.



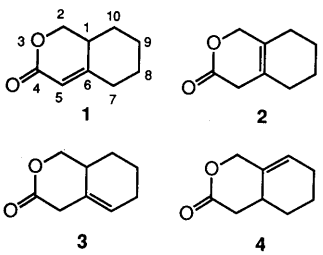


Chart 1

2-Hydroxymethylcyclohexanone **6** was phosphonoacetylated with diethylphosphonoacetyl chloride[5] to give **7** (70%). Intramolecular Horner–Emmons reaction of **7** in dimethoxyethane (DME) on treatment with KH gave the lactone **1**, mp 59—60 °C, in 76% yield.

Treatment of 2-methoxycarbonylcyclohexanone **8a** with 35% formaldehyde and a catalytic amount of triethylamine in methanol gave the 2-hydroxymethyl derivative **9a** in almost quantitative yield.[6] This was phosphonoacetylated as above and the resulting **10a** was subjected to an intramolecular Horner–Emmons reaction with NaH to yield the lactone **11a**. The corresponding ethyl ester **11b** was similarly synthesized from **8b** *via* the phosphonoacetate **10b**. When the cyclization of **10b** was performed with EtONa–EtOH, **11b** was obtained in low yield accompanied with a solvolytic ring opening product **12**.

The methoxycarbonyl group of **11a** was removed by application of Tsuda's kinetically controlled decarbomethoxylation method of vinylogous β-keto methyl esters.[7] Thus, heating of **11a** with $CaCl_2 \cdot 2H_2O$ in dimethyl sulfoxide (DMSO) containing *tert*-heptylmercaptan at 145 °C for 2 h gave the lactone **2** in 68% yield as a kinetically controlled product. Prolonged heating of the reaction mixture promoted the isomerization of the double bond, thus decreasing the amount of the lactone **2**. The ethyl ester **11b** resisted decarbethoxylation as expected.

B. The Lactone 3 The lactone **3** was synthesized by a modification of Belleau's procedure.[8] Prins reaction of the homoallylic acid **13** with trioxane in trifluoroacetic acid at 10 °C for 18 h gave a mixture of **1**, **2**, and **3** in a ratio of *ca.* 2:1:7 in 45% yield. From this mixture, the lactone **3** was purified by preparative high performance liquid chromatography (HPLC) on an octadecyl silica (ODS)

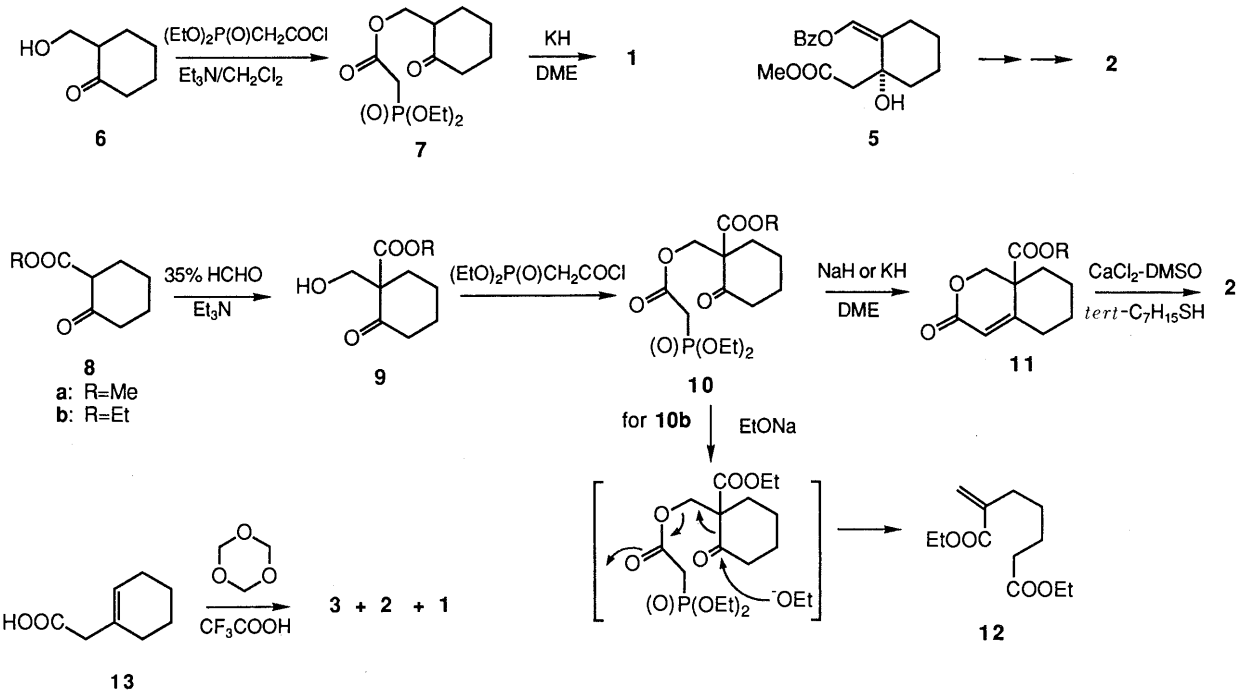


Chart 2

column (see Experimental).

C. The Lactone 4 The lactone **4** is not known, and was synthesized as follows. Methylenation of ethyl 2-oxocyclohexaneacetate **14** with dimethyl oxosulfonium methylide gave a single oxirane derivative **15a** in 30% yield. Dimethylsulfonium methylide gave the same oxirane **15a** in 13% yield.

The stereochemistry of the oxirane ring in **15a** was clarified by the following experiments. Compound **14** was transformed to ethyl 2-methylenecyclohexaneacetate **16** by Nozaki's methylenation.[9] Epoxidation of **16** with *m*-chloroperbenzoic acid (*m*CPBA) gave a mixture of two stereoisomeric oxiranes, **15a** and **15b**, in a ratio of 2:1 (determined by HPLC on an ODS column). On the other hand, iodolactonization of the acid **17** derived from **16** gave a single iodolactone **18** quantitatively. The stereochemistry of **18** is clear from a mechanistic consideration of the iodolactonization: the I^+ should attack from the opposite face of the side chain and the carboxylate anion should attack from the back side of the intermediary iodonium ion. Treatment of **18** with EtONa in EtOH gave a single oxirane which was identical with **15a**. Thus, **15a** and **15b** have *syn* and *anti* configurations for O to CH_2COOR, respectively. Predominant formation of **15a** over **15b** in *m*CPBA oxidation may be explained by the Henbest effect.[10]

Treatment of **15a** with 2% methanolic hydrochloric acid[11] did not give any unsaturated lactones, but instead gave four products, **19**—**22**. Compounds **19** and **20** were γ-lactones (1772 and 1767 cm^{-1}, respectively). The former had the formula $C_9H_{13}O_2Cl$ and contained Cl (MS: M^+ at *m/z* 190 and 188 were 1:3), and the latter ($C_9H_{14}O_3$) contained a hydroxy group (3440 cm^{-1}) instead of Cl, thus indicating the structures. Compounds **21** and **22** were δ-lactones (1733 and 1725 cm^{-1}, respectively). Compound **21** was isomeric to **19** and contained a Cl atom, and compound **22** contained an OMe group (δ 3.26) instead of

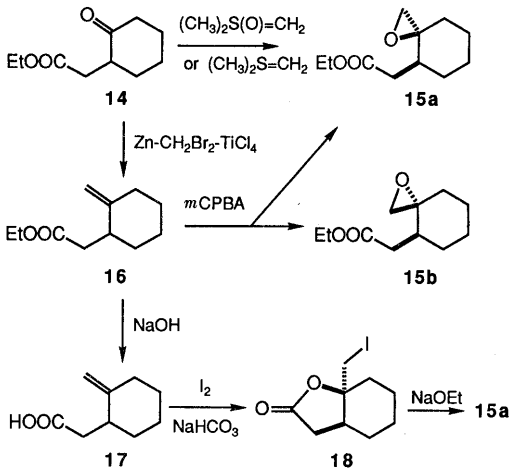


Chart 3

Cl. These and the other spectral data supported the assigned structures. The stereochemistries were assigned from mechanistic considerations, as shown in Chart 4.

On the other hand, heating of **15a** in benzene with pyridinium *p*-toluenesulfonate (PPTS) afforded the lactones **2** and **4** together with a mixture of the aldehyde **23** and a tosylate **24** (**2**:**4**:**23**+**24**=4:6:17). A similar treatment of **15b** gave **2**, **4**, and the hydroxy-lactone **20** in a ratio of 4:9:21. The aldehyde **23** and the lactone **20** are the stereospecific products from the oxiranes **15a** and **15b**, respectively, and the tosylate **24** is also a specific product from **15a** (see Chart 5). The formation of the same unsaturated lactones **2** and **4** from both isomers **15a** and **15b** suggests that these lactones were produced mainly through the carbocation **25**. However, a contribution of the concerted mechanism can not be ruled out, because the ratios of **4**/**2** from **15a** and **15b** were different (6/4 *vs.* 9/4).

D. Characterization of the Lactones 1—4 Each of the above unsaturated lactones **1**—**4** was readily purified by

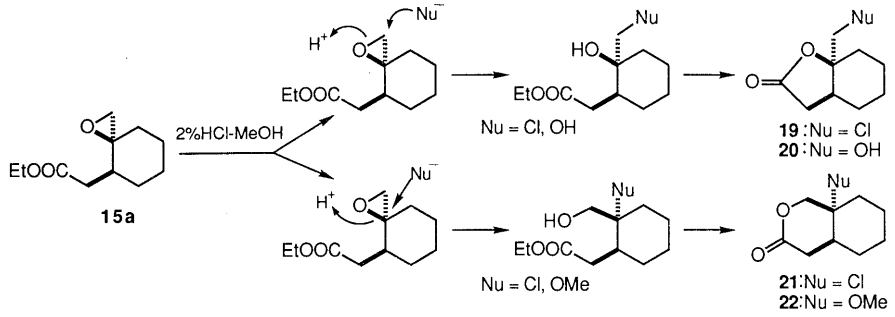


Chart 4

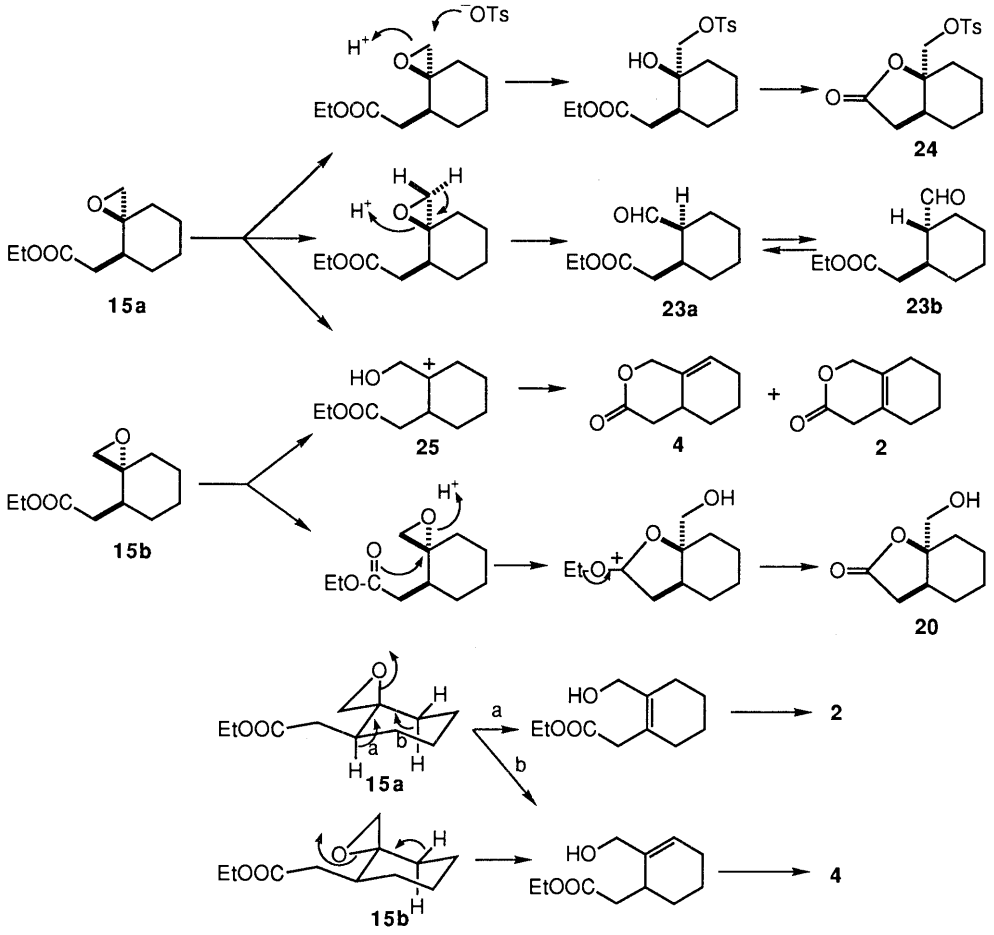


Chart 5

preparative HPLC on an ODS column and characterized spectroscopically as shown in Tables I and II.

Isomerization of Unsaturated Lactones **A. Acid-Catalyzed Isomerization** Each of the lactones **1**—**4** was heated with 2% PPTS in benzene at 120 °C in a sealed tube for 5 h and the products were analyzed by HPLC. The results are shown in Table III.

The lactone **3** isomerized to **1**, giving rise to a 9 : 1 mixture of **1** and **3**, while the other lactones were unchanged under these conditions.

B. Base-Catalyzed Isomerization The lactones were heated with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene at 120 °C in a sealed tube and the products were analyzed by HPLC. The results are shown in Table IV.

The lactone **3** was very labile: it irreversibly isomerized to **1** giving rise to an 8 : 2 mixture of **1** and **2** after 0.5 h. The lactones **1** and **2** slowly isomerized to give an equilibrium mixture of **1** and **2** (*ca.* 3 : 2) from either compound. The lactone **4** was stable under these conditions.

Lithgoe's group[4] isolated the lactone **1** in 30% yield on isomerization of **2** with piperidine in ethanol (reflux, 17 h). This agrees with our observation.

C. Isomerization with NaOH The lactones were heated with 10% NaOH at 120 °C for 5 h and the product was analyzed by HPLC after acidification and extraction. The results are shown in Table V.

The lactones **2**—**4** were unchanged under these conditions. However, the lactone **1** was unstable and gave a mixture of **1**, **2**, and **3** in a ratio of 1 : 8 : 6. The result corresponds to the exclusive isomerization of α-erythroidine to β-erythroidine.[3]

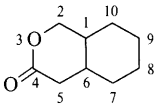
The above results indicate that for the hydroxy-acid

TABLE I. Characterization of the δ-Lactones **1**—**4**

	1	**2**	**3**	**4**
State	mp 59—60 °C	Oil	Oil	Oil
IR (cm^{-1})[a]	1721, 1700	1730	1736	1731
^{1}H-NMR[b] δ, (*J*)				
Olefin H	5.69 br s	—	5.58 br s	5.75 br s
OCH_2	4.37 dd (11, 6)	4.68 br s	4.33 dd (11, 5)	4.80 dd (13, 1.5)
	3.93 t (11)		3.89 t (11)	4.66 dd (13, 1.2)
$COCH_2$	—	2.93 br s	3.30 s	2.78 dd (16.5, 6)
				2.27 dd (16.5, 11)
HPLC t_R (min)[c]	12.3	16.8	15.4	14.4

a) $CHCl_3$ solution. *b*) $CDCl_3$ solution. *c*) Column, TSK gel 120T (ODS, 4.6 × 250 mm); mobile phase: 50% MeOH (0.6 ml/min).

TABLE II. ^{13}C-NMR Data (100 MHz) for Unsaturated δ-Lactones (in $CDCl_3$)



Carbon	5-Ene **1**	$1^{(6)}$-Ene **2**	6-Ene **3**	$1^{(10)}$-Ene **4**
1	35.2 d	124.3 s	34.6 d	131.4 s
2	70.6 t	71.7 t	72.6 t	72.0 t
4	164.6 s	169.7 s	170.7 s	171.7 s
5	114.0 d	34.4 t	36.9 t	37.2 t
6	163.3 s	124.3 s	129.0 s	31.5 d
7	33.1 t	24.7[a] t	123.6 d	24.8[a] t
8	28.8[a] t	22.1 t	24.7 t	20.8[a] t
9	25.9[a] t	22.1 t	24.4[a] t	29.3 t
10	24.3[a] t	28.3[a] t	21.1[a] t	124.3 d

a) Assignments may be interchanged in each column.

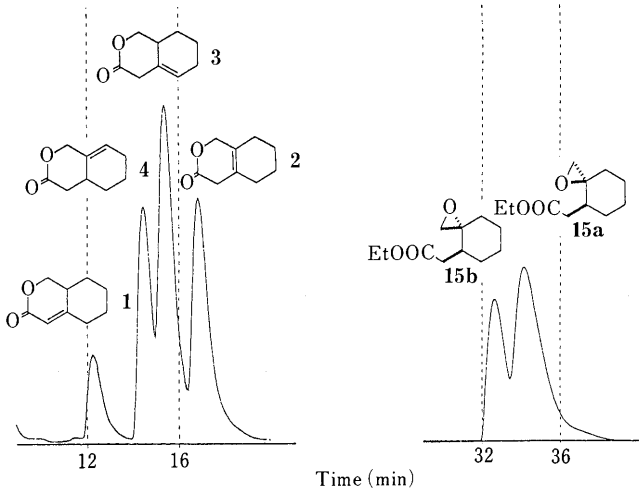


Fig. 1. HPLC of δ-Lactones **1**—**4** and Oxiranes **15a** and **15b**

Mobile phase: 50% MeOH (0.6 ml/min). Column: TSK gel 120T (ODS, 4.6 × 250 mm). Detector: refractive index.

forms, in contrast to the lactone forms, the *exo*-cyclic unsaturated structure **26** is unstable and rapidly isomerizes to the *endo*-cyclic unsaturated structures **27** and **28**. Such a migration of the double bond from an *exo*-cyclic (conjugated) to an *endo*-cyclic (unconjugated) position was supported by the isomerization reaction of the conjugated acid **29** with KOH. On treatment with 10 N KOH at 140 °C for 10 h, **29** gave a 8 : 1 mixture of **13** and **29** with preference for the unconjugated *endo*-cyclic isomer **13**.[12] The stability

TABLE III. Isomerization of Unsaturated δ-Lactones under Acidic Conditions (PPTS)[a]

Compound	Product (%)[b]			
	1	**2**	**3**	**4**
1	100	—	—	—
2	—	100	—	—
3	90	—	10	—
4	—	—	—	100

a) Reaction conditions: 2% PPTS in benzene at 120 °C for 5 h. *b*) Products were analyzed by HPLC on an ODS column.

TABLE IV. Isomerization of Unsaturated δ-Lactones under Basic Conditions (DBU)[a]

Compound	Product (%)[b]			
	1	**2**	**3**	**4**
1	60	40	—	—
2	58	42	—	—
3[c]	77	22	1	—
4	—	—	—	100

a) Reaction conditions: 2% DBU in benzene at 120 °C for 5 h. *b*) Products were analyzed by HPLC on an ODS column. *c*) Reaction time 0.5 h.

TABLE V. Isomerization of Unsaturated δ-Lactones under Basic Conditions (NaOH)[a]

Compound	Product (%)[b]			
	1	**2**	**3**	**4**
1	7	53	40	—
2	—	100	—	—
3	2	—	98	—
4	—	—	—	100

a) Reaction conditions: 10% NaOH at 120 °C for 5 h. *b*) Products were analyzed by HPLC on an ODS column.

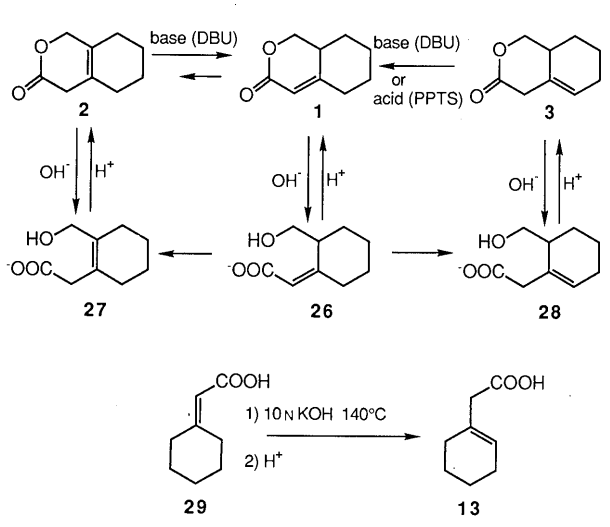


Chart 6

and isomerization of these lactones and the corresponding hydroxy acid are summarized in Chart 6.

Lythgoe's group[4] isolated only the lactone **2** (98%) after treatment of the lactone **1** with 10 N KOH at 130 °C for

18 h. Reinvestigation of their isomerization reaction (at 140 °C) revealed that the products were a mixture of **2** and **3** in a ratio of 1 : 1 after 15 h and in a ratio of 2 : 1 after 30 h.

The findings revealed in this investigation will be very useful for the synthesis not only of natural products but also of unnatural compounds containing an unsaturated lactone ring.

Experimental

General Unless otherwise stated, the following procedures were adopted. Melting points were determined on a Yanaco micro hot stage melting point apparatus and are uncorrected. Infrared (IR) spectra were taken in chloroform solutions and the data are given in cm^{-1}. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL FX-100 (100 MHz) or a JEOL GX-400 (400 MHz) spectrometer and carbon-13 NMR (^{13}C-NMR) spectra on a JEOL GX-400 (100 MHz), both in chloroform-*d* solutions with tetramethylsilane as an internal standard, and the chemical shifts are given in δ values. Mass spectra (MS) and high-resolution mass spectra (HRMS) were taken with a Hitachi M-80 machine and M^+ and/or major peaks are indicated as *m/z* (%). Column chromatography was performed on silica gel (Fuji-Devison BW-820MH). For thin layer chromatography (TLC), Merck precoated plates GF_{254} were used and spots were monitored under ultraviolet (UV) (254 nm), then developed by spraying 10% phosphomolybdic acid in EtOH and heating the plates at 100 °C until coloration took place. All organic extracts were washed with brine and dried over anhydrous sodium sulfate before concentration. Identities were confirmed by mixed melting-point determination (for crystalline compounds) and also by comparison of TLC behavior and ^{1}H-NMR and IR spectra.

HPLC Conditions Analytical HPLC was performed on a Toso TSK-gel 120T column (ODS, 4.6 × 250 mm). Mobile phase: 50% MeOH. Flow rate: 0.6 ml/min. Detection: rafractive index. Preparative HPLC was carried out on an ODS column (20 × 250 mm) with a flow rate of 5 ml/min.

3-Oxabicyclo[4.4.0]dec-5-en-4-one (1) Triethylamine (2.0 g) was added to a mixture of 2-hydroxymethylcyclohexanone (**6**) (1.28 g) and diethylphosphonoacetyl chloride (3.2 g) in CH_2Cl_2 (25 ml) at −78 °C under an Ar atmosphere. After being stirred for 30 min at the same temperature, the mixture was poured into ice-water and extracted with CH_2Cl_2. Concentration of the extract *in vacuo* below 30 °C and chromatography of the residue with AcOEt gave the phosphonate **7** (2.14 g, 70%) as a colorless oil. IR (neat): 1739, 1710, 1260, 1010. ^{1}H-NMR: 4.6—3.9 (6H, m, $OC\underline{H}_2CH_3 \times 2$ and OCH_2), 2.96 (2H, d, J=22 Hz, CH_2PO), 1.35 (6H, t, J=7 Hz, $OCH_2C\underline{H}_3 \times 2$).

Potassium hydride (23.6% in oil, 221 mg) was added to a stirred solution of the phosphonoacetate **7** (400 mg) in DME (10 ml) at 0 °C and the mixture was stirred at room temperature for 30 min. The reaction was quenched with aqueous NH_4Cl, then the solution was acidified with 5% HCl and extracted with ether. Chromatography of the product gave, from the AcOEt–hexane (1 : 3) eluate, the lactone **1** (150 mg, 76%), mp 59—60 °C (lit. 59—60 °C).[8] *Anal.* Calcd for $C_9H_{12}O_2$: C, 71.03; H, 7.95. Found: C, 70.81; H, 8.25.

Methyl 1-Hydroxymethyl-2-oxocyclohexanecarboxylate (9a) Triethylamine (0.5 ml) was added to a mixture of methyl 2-oxocyclohexanecarboxylate (**8a**) (456 mg) and 35% formaldehyde (2 ml) in MeOH (10 ml) at room temperature. After being stirred for 2 h at the same temperature, the mixture was poured into saturated aqueous $NaHCO_3$ solution and extracted with CH_2Cl_2. Concentration of the extract and chromatography of the residue gave, from the hexane–AcOEt (2 : 1) eluate, **9a** (538 mg, 98%) as a colorless oil. IR: 3400, 1724, 1696. ^{1}H-NMR: 3.80 (3H, s, OMe), 3.96—3.58 (3H, m, $C\underline{H}_2O\underline{H}$). MS: 168 ($M^+$ − 18, 3), 167 (7), 156 (58), 128 (31), 124 (100).

Ethyl 1-Hydroxymethyl-2-oxocyclohexanecarboxylate (9b) The ethyl ester **8b** (8.5 g) in MeOH (10 ml) was similarly reacted with 35% formaldehyde (13 ml) under triethylamine (0.5 ml) catalysis to give the corresponding ethyl ester **9b**[6] (9.1 g, 91%) as a colorless liquid, bp 104 °C/3 mmHg. IR: 3570, 1730, 1705. ^{1}H-NMR ($CDCl_3$ + a drop of D_2O): 4.27 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 3.78 (2H, br s, CH_2O), 1.30 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$).

Methyl 4-Oxo-3-oxabicyclo[4.4.0]dec-5-ene-1-carboxylate (11a) Diethylphosphonoacetyl chloride (prepared from 3.2 g of the acid)[5] in CH_2Cl_2 (20 ml) was added to a mixture of **9a** (1.2 g) and triethylamine (3.3 g) in CH_2Cl_2 (40 ml). After being stirred for 30 min at room temperature, the mixture was washed with saturated aqueous $NaHCO_3$ solution and concentrated. Flash chromatography of the residue with benzene–acetone (4 : 1) gave crude **10a** (0.78 g) as an oil. ^{1}H-NMR: 4.44 (2H, ABq, $\Delta\delta$=0.22 ppm, J=11 Hz, OCH_2), 4.16 (4H, dq, J=8.3, 7 Hz, $POOC\underline{H}_2CH_3 \times 2$), 3.77 (3H, s, OMe), 2.95 (2H, d, J=21.5 Hz, CH_2PO), 1.35 (6H, t, J=7 Hz, $OCH_2C\underline{H}_3 \times 2$).

Crude **10a** (780 mg) in DME (10 ml) was added slowly to a stirred solution of NaH (130 mg) in DME (20 ml) at 0 °C, and the mixture was stirred for 30 min at 0 °C and 30 min at room temperature. After decomposition of the reagent with AcOH, the mixture was poured into 1 N HCl and extracted with ether. Chromatography of the product gave, from the benzene–acetone (10 : 1) eluate, **11a** (346 mg, 26% from **9a**) as colorless plates from ether, mp 83—84 °C. IR (KBr): 1727, 1701. ^{1}H-NMR: 5.89 (1H, br s, =CH), 4.59, 4.04 (each 1H, d, J=11.2 Hz, OCH_2), 3.77 (3H, s, OMe). MS: 210 (M^+, 48), 178 (22), 150 (100). *Anal.* Calcd for $C_{11}H_{14}O_4$: C, 62.85; H, 6.71. Found: C, 62.60; H, 6.97.

Ethyl 4-Oxo-3-oxabicyclo[4.4.0]dec-5-ene-1-carboxylate (11b) The ethyl ester **9b** (2.4 g) in CH_2Cl_2 (50 ml) was phosphonoacetylated with diethylphosphonoacetyl chloride (3.88 g) and triethylamine (2.4 g) at 0 °C for 30 min to give the phosphonoacetate **10b** (3.9 g, 97%) as a colorless liquid. IR (neat): 1742, 1739, 1719. ^{1}H-NMR: 4.5—3.9 (8H, m, OCH_2 and $OC\underline{H}_2CH_3 \times 3$), 2.94 (2H, d, J=22 Hz, CH_2PO), 1.36 (9H, t, J=7 Hz, $OCH_2C\underline{H}_3 \times 3$).

Compound **10b** (1 g) in DME (30 ml) was treated with KH (23.6% in oil, 120 mg) at 0 °C for 30 min and at room temperature for 30 min. Chromatography of the product gave, from the AcOEt–hexane (1 : 5) eluate, **11b** (550 mg, 82%) as colorless prisms, mp 58—60 °C. IR (Nujol): 1725. ^{1}H-NMR: 5.87 (1H, br s, =CH), 4.58, 4.06 (each 1H, d, J=11 Hz, OCH_2), 4.22 (2H, q, J=7 Hz, $OC\underline{H}_2CH_3$), 1.27 (3H, t, J=7 Hz, $OCH_2C\underline{H}_3$). *Anal.* Calcd for $C_{12}H_{16}O_4$: C, 64.24; H, 7.19. Found: C, 64.18; H, 7.23.

Treatment of 10b with EtONa–EtOH The phosphonoacetate **10b** (800 mg) in 2.5% EtONa–EtOH (10 ml) was stirred for 1.5 h at room temperature. The mixture was brought to pH 5 with 10% HCl and extracted with CH_2Cl_2. Chromatography of the product gave, from the AcOEt–hexane (1 : 4) eluate, **11b** (52 mg, 11%) and **12** (127 mg, 21%). Compound **12** was an oil. IR (neat): 1735, 1720, 1660, 1630. ^{1}H-NMR: 6.16, 5.54 (each 1H, br s, $=CH_2$), 4.22, 4.14 (each 2H, q, J=7.2 Hz, $OC\underline{H}_2CH_3 \times 2$), 1.31, 1.26 (each 3H, t, J=7.2 Hz, $OCH_2C\underline{H}_3 \times 2$). HRMS: Calcd for $C_{12}H_{20}O_4$: 228.1361. Found: 228.1385.

3-Oxabicyclo[4.4.0]dec-1$^{(6)}$-en-4-one (2) Compound **11a** (100 mg) and $CaCl_2 \cdot 2H_2O$ (559 mg) in DMSO (5 ml) containing *tert*-heptylmercaptan (0.4 ml) were heated in a sealed tube at 145 °C for 2 h under an Ar atmosphere. The mixture was poured into 1 N HCl and extracted with ether. Concentration of the extract and chromatography of the residue gave, from the benzene–acetone (20 : 1) eluate, the lactone **2** (49 mg, 68%). MS: 152 (M^+, 91), 124 (25), 108 (38), 95 (100). HRMS: Calcd for $C_9H_{12}O_2$: 152.0837. Found: 152.0812.

3-Oxabicyclo[4.4.0]dec-6-en-4-one (3) 1-Cyclohexene-1-acetic acid **13** (1.685 g) and trioxane (0.38 g) in trifluoroacetic acid (5 ml) were stirred at 10 °C for 18 h. The mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ and brine, dried, and concentrated. Chromatography of the residue gave, from the hexane–AcOEt eluate, a mixture of the lactones (0.9 g, 58%) **3**, **2**, and **1** in a ratio of *ca.* 7 : 1 : 2. A portion of this mixture was separated by preparative HPLC to give the pure lactone **3** as a colorless oil. MS: 152 (M^+, 15), 122 (12), 93 (73), 79 (100). HRMS: Calcd for $C_9H_{12}O_2$: 152.0837. Found: 152.0853.

Methylenation of Ethyl 2-Oxocyclohexaneacetate (14) with Sulfonium Ylides (1) Trimethyloxosulfonium iodide (616 mg) in DMSO (4 ml) was converted to the ylide on treatment with NaH (114 mg, 60% oil dispersion) for 30 min at room temperature under an Ar atmosphere. Compound **14** (500 mg) in DMSO (4 ml) was added to this solution, and the mixture was heated at 50 °C for 2 h with stirring, then poured into ice-water and extracted with ether. Chromatography of the extract gave, from the benzene–hexane (9 : 1) eluate, the oxirane **15a** (162 mg, 30%) as a colorless oil. Its homogeneity was confirmed by HPLC. IR: 1715. ^{1}H-NMR: 4.12 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 2.61 (2H, ABq, $\Delta\delta$=0.17 ppm, J=4.4 Hz, oxirane), 1.25 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 173.0, 60.3, 60.0, 53.2, 36.8, 34.1, 32.7, 30.2, 24.4, 23.4, 14.2. MS: 198 (M^+, 5), 137 (100). HRMS: Calcd for $C_{11}H_{18}O_3$: 198.1255. Found: 198.1277.

(2) Trimethylsulfonium iodide (651 mg) was converted to the ylide with NaH (60% oil dispersion, 130 mg) in DMSO (4 ml) as described above. Compound **14** (500 mg) in tetrahydrofuran (THF) (5 ml) was added to this solution and the mixture was stirred at 0 °C for 1 h. Work-up of the mixture gave **15a** (71 mg, 13%). Its homogeneity was confirmed by HPLC.

Ethyl 2-Methylenecyclohexaneacetate (16) Zn–CH_2Br_2–$TiCl_4$ re-

agent[9] was prepared by the method described in Organic Synthesis.[13] Compound **14** (200 mg) in CH_2Cl_2 (10 ml) was added dropwise to a stirred slurry of the above reagent (10 ml) at 0 °C and the mixture was stirred at room temperature. After 1.5 h, ether and saturated $NaHCO_3$ solution were added, and after further stirring for 1 h, the mixture was filtered. The separated organic layer was dried and concentrated. Chromatography of the residue in benzene gave **16**[14] (159 mg, 80%) as an oil. IR: 1720. ^{1}H-NMR: 4.66, 4.53 (each 1H, s, $=CH_2$), 4.12 (2H, q, J=7.3 Hz, $COOC\underline{H}_2CH_3$), 1.25 (3H, t, J=7.3 Hz, $COOCH_2C\underline{H}_3$). MS: 168 (M^+-14, 52), 137 (M^+-OEt, 100).

Epoxidation of 16 Compound **16** (470 mg) and *m*CPBA (555 mg) in CH_2Cl_2 (10 ml) were stirred for 3 h at 0 °C and for 2 h at room temperature. After addition of 10% Na_2SO_3 solution (5 ml), the mixture was poured into saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2 to give a mixture of **15a** and **15b** (496 mg, 98%). The ratio of **15a** to **15b** was 2 : 1 as evidenced from HPLC. These products were separated by preparative HPLC. Compound **15b** was a colorless oil. IR: 1720. ^{1}H-NMR: 2.57 (2H, ABq, $\Delta\delta$=0.18 ppm, J=4.4 Hz, oxirane), 4.12 (2H, q, J=7 Hz, $COOC\underline{H}_2CH_3$), 1.25 (3H, t, J=7 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 172.6, 60.4, 60.2, 50.7, 37.9, 34.7, 33.4, 31.2, 25.3, 23.7, 14.2. MS: 198 (M^+). HRMS: Calcd for $C_{11}H_{18}O_3$: 198.1255. Found: 198.1253.

The Iodolactone 18 Compound **16** (165 mg) was hydrolyzed with 5% NaOH (3 ml) and EtOH (1 ml) on heating at 50 °C for 3 h to give the acid **17** (151 mg), after acidification and extraction with AcOEt. This was dissolved in 0.5 M $NaHCO_3$ (7 ml) and KI_3 solution [prepared from iodine (465 mg), KI (908 mg), and water (3 ml)] was added, then the mixture was stirred overnight at room temperature, and extracted with CH_2Cl_2. The organic layer was washed with aqueous $Na_2S_2O_3$ and brine, and concentrated to give the iodolactone **18** (255 mg, 100%) as colorless prisms from ether, mp 60—61.5 °C. IR: 1781, 1762. ^{1}H-NMR: 3.45 (2H, s, CH_2I), 2.32—2.82 (3H, m, $COCH_2$, CH). MS: 280 (M^+, 5), 153 (26), 139 (100). *Anal.* Calcd for $C_9H_{13}IO_2$: C, 38.59; H, 4.86. Found: C, 38.45; H, 4.75.

Treatment of 18 with NaOEt A mixture of the iodolactone **18** (162 mg) and 1 M NaOEt–EtOH (3.6 ml) in EtOH (5 ml) was stirred for 4.5 h at room temperature and 30 min at 30 °C, then poured into ice-water and extracted with CH_2Cl_2 to give the oxirane **15a** (80 mg, 80%). Its homogeneity was confirmed by HPLC.

Treatment of the Oxirane 15a with 2% Methanolic Hydrochloric Acid The oxirane **15a** (50 mg) in MeOH (10 ml) containing concentrated hydrochloric acid (0.6 ml) was stirred for 40 h at room temperature. The mixture was diluted with water and extracted with $CHCl_3$. HPLC of the product gave **19** (8 mg, 17%), **20** (13 mg, 30%), **21** (3 mg, 6%), and **22** (6 mg, 13%).

Compound **19**: Colorless oil. IR: 1772. ^{1}H-NMR (400 MHz): 3.65 (2H, ABq, $\Delta\delta$=0.06 ppm, J=12 Hz, CH_2Cl), 2.72—2.64 (2H, m, $COCH_2$), 2.42 (1H, m, CH). MS: 190 (M^+ for ^{37}Cl, 0.2), 188 (M^+ for ^{35}Cl, 0.6), 139 (100).

Compound **20**: Colorless oil. IR: 3440, 1767. ^{1}H-NMR (400 MHz): 3.68 (2H, ABq, $\Delta\delta$=0.07 ppm, J=12.5 Hz, CH_2O), 2.7—2.6 (2H, m, $COCH_2$), 2.52—2.45 (1H, m, CH). MS: 170 (M^+, 0.14), 139 (100). HRMS: Calcd for $C_9H_{14}O_3$: 170.0942. Found: 170.0958.

Compound **21**: Colorless oil. IR: 1733. ^{1}H-NMR (400 MHz): 4.66, 4.36 (each 1H, d, J=12.5 Hz, OCH_2), 3.05 (1H, dd, J=19.1, 7.3 Hz), 2.44 (1H, dd, J=19.1, 4.4 Hz) ($COCH_2$). MS: 190 (M^+ for ^{37}Cl, 1.5), 188 (M^+ for ^{35}Cl, 4.3), 116 (100).

Compound **22**: Colorless oil. IR: 1725. ^{1}H-NMR (400 MHz): 4.46 (1H, d, J=12.5 Hz), 4.29 (1H, dd, J=12.5, 1.8 Hz) (OCH_2), 3.26 (3H, s, OMe), 2.97 (1H, dd, J=18.3, 7.3 Hz), 2.21 (1H, dd, J=18.3, 2 Hz) ($COCH_2$). MS: 184 (M^+, 26), 169 (28), 111 (100). HRMS: Calcd for $C_{10}H_{16}O_3$: 184.1099. Found: 184.1102.

Treatment of the Oxiranes 15 with PPTS (1) A mixture of the oxirane **15a** (50 mg), PPTS (12.7 mg), and molecular sieves 4A (excess) in benzene (10 ml) was heated under reflux for 5 h under an Ar atmosphere. The cooled mixture was washed with aqueous $NaHCO_3$ and concentrated. Chromatography of the residue gave, from the hexane–AcOEt (3 : 1) eluate, a mixture of **2** and **4** (19 mg, 50%). The ratio was 1 : 2 (HPLC). These products were separated by preparative HPLC.

The Lactone **4**: Colorless oil. MS: 152 (M^+, 12), 108 (50), 94 (39), 93 (67), 79 (100). HRMS: Calcd for $C_9H_{12}O_2$: 152.0837. Found: 152.0809.

(2) Each of the oxiranes **15a** and **15b** (7.1 mg and 9.5 mg) and PPTS (20 mg) in benzene (1.0 ml) were heated in a sealed tube at 110 °C for 5 h. Each mixture was washed with saturated $NaHCO_3$ and concentrated, and the residue (5 mg from **15a** and 3 mg from **15b**) was analyzed by HPLC. A mixture of **2**, **4**, and **23**+**24** (**23** and **24** gave an inseparable peak) in a ratio of 4 : 6 : 17 was obtained from **15a**, and a mixture of **2**, **4**, and **20** in a ratio of 4 : 9 : 21 from **15b**.

(3) A mixture of oxiranes **15a** and **15b** (2 : 1, 478 mg) and PPTS (100 mg) in benzene (5 ml) was heated in a sealed tube at 120 °C for 3 h and worked up as described above. Chromatography of the product eluting with benzene–acetone gave **23** (54 mg, 11%) from the 40 : 1 eluate, a mixture of **2**, **4**, and **24** (174 mg) from the 20 : 1 eluate, and **20** (74 mg, 18%) from the 2 : 1 eluate. The mixture of **2**, **4**, and **24** was separated by preparative HPLC to give **2** (30 mg, 8%), **4** (37 mg, 10%) and **24** (68 mg, 9%).

The Aldehyde **23**: Colorless oil, which was a mixture of **23a** and **23b** on the basis of the ^{1}H- and ^{13}C-NMR spectra. IR: 1724. ^{1}H-NMR (400 MHz): 9.75 (br s), 9.56 (d, J=3 Hz) (CHO), 4.15—4.09 (2H, seven peaks, $COOC\underline{H}_2CH_3$), 1.25 (3H, br t, J=7.3 Hz, $COOCH_2C\underline{H}_3$). ^{13}C-NMR: 204.1, 172.3, 60.3, 54.8, 39.3, 33.3, 31.0, 25.9, 25.0, 24.5, 14.2 and 204.5, 172.8, 60.3, 51.2, 36.4, 33.7, 29.8, 25.2, 23.8, 23.3, 14.2. MS: 198 (M^+, 19), 197 (100), 169 ($M-CHO$, 35), 168 (28), 153 (30), 152 (18), 151 (37), 139 (95), 123 (30), 95 (40), 88 (40).

The *p*-Toluenesulfonate **24**: Colorless oil. IR: 1784. ^{1}H-NMR: 7.75, 7.33 (each 2H, d, J=8 Hz, ArH), 4.00 (2H, s, CH_2O), 2.8—2.1 (3H, m, $COCH_2$, CH), 2.46 (3H, s, Me). MS: 324 (M^+, 3), 153 (48), 140 (51), 139 (100). HRMS: Calcd for $C_{16}H_{20}O_5S$: 324.1030. Found: 324.1039.

Isomerization of Lactones (1) With PPTS: The lactone (each 2—10 mg) was heated in a sealed tube with 2% PPTS in benzene (1 ml) at 120 °C for 5 h. The mixture was diluted with benzene, washed with saturated aqueous $NaHCO_3$, and concentrated. The residue was dissolved in 50% MeOH or MeOH and analyzed by HPLC (see Table III).

(2) With DBU: The lactone (each 2—10 mg) was heated in a sealed tube with 2% DBU in benzene (1 ml) at 120 °C for 5 h (for **1**, **2**, and **4**) or for 0.5 h (for **3**). The cooled mixture was diluted with ether, washed with 1 N HCl, and concentrated. The residue was analyzed by HPLC as described above (see Table IV).

(3) With NaOH: The lactone (each 2—10 mg) was heated in a sealed tube with 10% NaOH (1 ml) at 120 °C for 5 h. The cooled mixture was acidified with dilute H_2SO_4, stirred for 30 min at room temperature, then extracted with ether. The residue obtained from the ether extract was analyzed by HPLC as described above (see Table V).

Isomerization of the Conjugated Acid 29 The acid **29** (100 mg) was heated in a sealed tube with 10 N KOH at 140 °C for 10 h. The cooled mixture was acidified with HCl and extracted with CH_2Cl_2. The product was a mixture of **29** and **13** (1 : 8) on the basis of HPLC analysis.

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Studies on *Aconitum* Species. XV. Deoxygenation Reaction of Aconitine Type Alkaloids

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Mesaconitine (1) in tetrahydrofuran reacted with sodium hydride, a catalytic amount of imidazole, carbon disulfide and methyl iodide at room temperature to give the di-*O*-(*S*-methyl)thiocarbonate (5). The reductive cleavage of 5 with tri-*n*-butyltin hydride gave isodelphinine (3) in a high yield of 83%. The exact same reactions of aconitine (2) and jesaconitine (6) gave penduline (4) and 3,13-dideoxyjesaconitine (7) in 85 and 86% yields, respectively. The same reactions in diethylether, in place of tetrahydrofuran, gave the 3-deoxy compounds, hypaconitine (9), deoxyaconitine (10) and deoxyjesaconitine (11), in yields of 87, 88 and 85%, respectively. When the same reaction as used for the syntheses of the 3,13-dideoxy compounds was done at refluxing temperature, 3,13,15-trideoxy compounds, that is, 3,13,15-trideoxymesaconitine, 3,13,15-trideoxyaconitine and 3,13,15-trideoxyjesaconitine, were obtained in yields of 83, 83 and 88%, respectively.

Keywords aconitine; deoxygenation; mesaconitine; jesaconitine; hypaconitine

Aconitine type alkaloids are highly oxygenated compounds and studies on the reactivity of their oxygen functions, as well as chemical correlations and structure–activity relationships, are important in order to understand their potent biological activities.

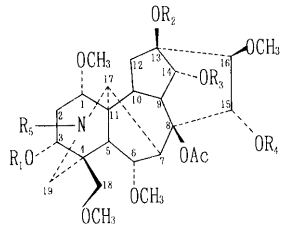
In 1988, Kulanthaivel and Pelletier[1] reported on the deoxygenation of several C19 diterpenoid alkaloids by using reductive cleavage of imidazolylthiocarbonylester with tri-*n*-butyltin hydride (*n*-Bu_3SnH). However, there have been few reports of deoxygenation reactions of aconitine-type alkaloids. We reported previously on the transformation of mesaconitine (**1**) and aconitine (**2**) into isodelphinine (**3**) and penduline (**4**), respectively, by deoxygenation of a C13-*O*-triflate by means of photochemical cleavage.[2–4] But the yield of the photochemical deoxygenation was low. Further, synthesis of compounds deoxygenated at C3, C13 and C15 is necessary to investigate the structure–activity relationships.

In this paper, we present high-yield deoxygenations at C3, C13 and C15 of aconitine-type alkaloids according to Barton and McCombic.[5]

Treatment of **1** with sodium hydride (NaH) in the presence of a catalytic amount of imidazole in tetrahydrofuran (THF), followed by the addition of carbon disulfide (CS_2) and methyl iodide (CH_3I) at room temperature, gave 3,13-di-*O*-(*S*-methyl)thiocarbonate (**5**) in 96% yield. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **5** revealed the presence of two *S*-methyl groups, at δ 2.57 and 2.46. The signal at δ 5.70 (1H, J = 12.7, 5.5 Hz) assignable to C3-β-H suggested the presence of a thiocarbonyloxy group at C3, and the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum suggested the presence of another thiocarbonyloxy group at C13. The other signals and the mass (MS) spectrum were in agreement with the assigned structure. The reductive cleavage of **5** with *n*-Bu_3SnH gave **3** in a yield of 83%. This product was identical with an authentic sample[2] in terms of melting point and (NMR), infrared (IR), and MS spectra.

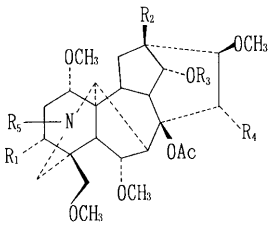
Application of this methodology to **2** gave **4** in a yield of 85%. This product was identical with an authentic sample[3] in terms of melting point and NMR, IR and MS spectra.

Jesaconitine (**6**) gave 3,13-dideoxyjesaconitine (**7**) by the above method in an 86% overall yield. Synthesis of the 3,13-dideoxy compound has not previously been reported,



1: R_1=H, R_2=H, R_3=Bz, R_4=H, R_5=CH_3
2: R_1=H, R_2=H, R_3=Bz, R_4=H, R_5=CH_2CH_3
5: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=Bz, R_4=H, R_5=CH_3
6: R_1=H, R_2=H, R_3=As, R_4=H, R_5=CH_2CH_3
8: R_1=$CS(SCH_3)$, R_2=H, R_3=Bz, R_4=H, R_5=CH_3
12: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=As, R_4=$CS(SCH_3)$, R_5=CH_2CH_3
14: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=Bz, R_4=H, R_5=CH_2CH_3
15: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=As, R_4=H, R_5=CH_2CH_3
16: R_1=$CS(SCH_3)$, R_2=H, R_3=Bz, R_4=H, R_5=CH_2CH_3
17: R_1=$CS(SCH_3)$, R_2=H, R_3=As, R_4=H, R_5=CH_2CH_3
18: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=Bz, R_4=$CS(SCH_3)$, R_5=CH_2CH_3
20: R_1=$CS(SCH_3)$, R_2=$CS(SCH_3)$, R_3=Bz, R_4=$CS(SCH_3)$, R_5=CH_3

Chart 1



3: $R_1=H$, $R_2=H$, $R_3=Bz$, $R_4=OH$, $R_5=CH_3$
4: $R_1=H$, $R_2=H$, $R_3=Bz$, $R_4=OH$, $R_5=CH_2CH_3$
7: $R_1=H$, $R_2=H$, $R_3=As$, $R_4=OH$, $R_5=CH_2CH_3$
9: $R_1=H$, $R_2=OH$, $R_3=Bz$, $R_4=OH$, $R_5=CH_3$
10: $R_1=H$, $R_2=OH$, $R_3=Bz$, $R_4=OH$, $R_5=CH_2CH_3$
11: $R_1=H$, $R_2=OH$, $R_3=As$, $R_4=OH$, $R_5=CH_2CH_3$
13: $R_1=H$, $R_2=H$, $R_3=As$, $R_4=H$, $R_5=CH_2CH_3$
19: $R_1=H$, $R_2=H$, $R_3=Bz$, $R_4=H$, $R_5=CH_2CH_3$
21: $R_1=H$, $R_2=H$, $R_3=Bz$, $R_4=H$, $R_5=CH_3$

Chart 2

and its structure was determined by analysis of the spectral data (see Experimental).

These results indicate that the deoxygenation procedure leads to the production of 3,13-dideoxygenated derivatives in considerable yield. Meanwhile, deoxygenation at C3 alone could also be performed by using diethyl ether instead of THF as a reaction solvent to give the 3-*O*-(*S*-methyl)thiocarbonate (**8**) of **1** in a yield of 93%. The ^{1}H-NMR spectrum of **8** showed a signal at δ 5.72 (dd, $J=12.7$, 5.4 Hz) assignable to C3-β-H. Other NMR and MS signals of **8** were in agreement with the assigned structure. Reductive cleavage of **8** with *n*-Bu_3SnH gave hypaconitine (**9**) in a yield of 87%. This product was identical with an authentic sample in terms of melting point and NMR, IR and MS spectra.

Application of the deoxygenation sequence to **2** and **6** gave deoxyaconitine (**10**) and deoxyjesaconitine (**11**) in overall yields of 88 and 85%, respectively. These transformation processes occur in considerably superior yields to those previously obtained.[7]

Lastly, deoxygenation at C3, C13 and C15 was achieved as follows.

A hydroxy group at C15 in aconitine-type alkaloids generally resists acylation, since the hydroxy group is shielded by the aroyl group at C14 and the acetyl group at C8. A mixture of **6** with NaH and a catalytic amount of imidazole in THF was stirred for 0.5 h, followed by the addition of CS_2 and CH_3I at room temperature. The reaction mixture was refluxed for 1.5 h to give the 3,13,15-tri-*O*-(*S*-methyl)thiocarbonate (**12**) in a yield of 93%. In the ^{1}H-NMR spectrum of **12**, three *S*-methyl signals were apparent, at δ 2.46, 2.57 and 2.66. Three (*S*-methyl)thiocarbonyloxy groups were concluded to be present at C3, C13 and C15 on the basis of the other ^{1}H-NMR signals and ^{13}C-NMR chemical shifts (see Experimental).

Reductive cleavage of **12** with *n*-Bu_3SnH furnished 3,13,15-trideoxyjesaconitine (**13**) in a yield of 88%. Its melting point and spectral data were identical with those of foresaconitine isolated from *Aconitum forrestii*.[10,11] Application of the same deoxygenation sequence to **1** and **2** also gave the 3,13,15-trideoxycompounds (**21**, **19**).

Experimental

All melting points are uncorrected. IR spectra were taken in KBr disks with a JASCO FT/7000 spectrometer, and ultraviolet (UV) spectra were measured in EtOH solution with a Shimadzu UV 240 spectrophotometer. NMR spectra were measured in $CDCl_3$ solution with a JEOL GX-270, using tetramethylsilane as an internal standard. MS and high resolution mass spectra (HR-MS) were measured with a Hitachi M-2000 spectrometer. Column chromatography was performed on silica gel (0.06—0.200 mm, Merck). Preparative thin layer chromatography (TLC) was performed on Silica gel F_{254} (thickness 1 mm, Merck).

3,13-Di-*O*-(*S*-methyl)thiocarbonylmesaconitine (5) A mixture of **1** (50 mg), dry THF (6 ml), imidazole (1.3 mg) and NaH (116 mg) was stirred at 0 °C for 30 min, then CS_2 (1.2 ml) and CH_3I (0.9 ml) were added dropwise. The mixture was stirred for 1.5 h at room temperature, then the reaction was quenched with ice-water and the whole was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water, then dried over anhydrous sodium sulfate. The solvent was evaporated off and the residue was purified by column chromatography on silica gel with Et_2O saturated with 28% ammonia water–hexane (3:2) as an eluting solvent to yield **5** (60 mg, 96%, amorphous). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1715 (C=O). ^{1}H-NMR δ: 1.41 (3H, s, $OCOCH_3$), 2.38 (3H, s, NCH_3), 2.46 (3H, s, SCH_3), 2.57 (3H, s, SCH_3), 3.18 (3H, s, OCH_3), 3.21 (3H, s, OCH_3), 3.27 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 5.24 (1H, d, $J=5.1$ Hz, C14-β-H), 5.70 (1H, dd, $J=12.7$, 5.5 Hz, C3-β-H), 7.43—8.09 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.8 and 19.4 (SCH_3 ×2), 81.9 (C3), 88.4 (C13), 213.9 and 214.9 (C=S ×2). MS *m/z*: 811 (M^+), 780 (M^+-OCH_3, base peak), 720 ($M^+-OCH_3-CH_3COOH$).

Isodelphinine (3) A solution of **5** (20 mg) in 1 ml of dry benzene was refluxed with *n*-Bu_3SnH (0.15 ml) in dry benzene (0.5 ml) for 2 h. The reaction mixture was concentrated *in vacuo*, then the residue was chromatographed on silica gel with Et_2O saturated with 28% ammonia water–hexane (4:1) as an eluting solvent and crystallized from Et_2O–hexane to give **3** (12.3 mg, 83% yield): mp 159—161 °C (lit.[4] 158—160 °C). IR ν_{max}^{KBr} cm^{-1}: 3500, 1720 and 1705 (C=O). ^{1}H-NMR δ: 1.43 (3H, s, $OCOCH_3$), 2.35 (3H, s, NCH_3), 3.18 (3H, s, OCH_3), 3.28 (6H, s, OCH_3), 3.52 (3H, s, OCH_3), 5.05 (1H, t, $J=4.3$ Hz, C14-β-H), 7.40—8.05 (5H, m, benzoyl group). MS *m/z*: 599 (M^+), 568 (M^+-OCH_3), 508 ($M^+-OCH_3-CH_3COOH$, base peak). The NMR and MS spectra of **3** were identical to those of an authentic sample.

3,13-Di-*O*-(*S*-methyl)thiocarbonylaconitine (14) Compound **14** was prepared from **2** in 93% yield in the same manner as used for the synthesis of compound **5**: amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 1717 (C=O). ^{1}H-NMR δ: 1.12 (3H, t, $J=7.1$ Hz, NCH_2CH_3), 1.41 (3H, s, $OCOCH_3$), 2.45 (3H, s, SCH_3), 2.57 (3H, s, SCH_3), 3.18 (3H, s, OCH_3), 3.20 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 5.24 (1H, d, $J=5.1$ Hz, C14-β-H), 5.71 (1H, dd, $J=12.3$, 5.5 Hz, C3-β-H), 7.45—8.07 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.9 and 19.3 (SCH_3 ×2), 81.6 (C3), 88.3 (C13), 213.9 and 215.0 (C=S ×2). MS *m/z*: 825 (M^+), 794 (M^+-OCH_3, base peak), 734 ($M^+-OCH_3-CH_3COOH$).

Penduline (4) Compound **4** was prepared from **14** in 91% yield in the same manner as used for the synthesis of compound **3**: mp 165—166 °C (acetone–hexane) (lit.[4] 166—167 °C). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1715 (C=O). ^{1}H-NMR δ: 1.06 (3H, t, $J=7.0$ Hz, NCH_2CH_3), 1.43 (3H, s, $OCOCH_3$), 3.18 (3H, s, OCH_3), 3.27 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 3.52 (3H, s, OCH_3), 5.05 (1H, t, $J=4.5$ Hz, C14-β-H), 7.38—8.14 (5H, m, benzoyl group). MS *m/z*: 613 (M^+), 582 (M^+-OCH_3), 522 ($M^+-OCH_3-CH_3COOH$, base peak). The NMR and MS spectra of **4** were identical to those of an authentic sample.

3,13-Di-*O*-(*S*-methyl)thiocarbonyljesaconitine (15) Compound **15** was prepared from **6** in 96% yield in the same manner as used for the synthesis of compound **5**: amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1717 (C=O). ^{1}H-NMR δ: 1.12 (3H, t, $J=7.1$ Hz, NCH_2CH_3), 1.42 (3H, s, $OCOCH_3$), 2.46 (3H, s, SCH_3), 2.56 (3H, s, SCH_3), 3.18 (3H, s, OCH_3), 3.21 (3H, s, OCH_3), 3.25 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 3.87 (3H, s, OCH_3 of anisoyl group), 5.21 (1H, d, $J=5.1$ Hz, C14-β-H), 5.71 (1H, dd, $J=12.7$, 7.8 Hz, C3-β-H), 6.94 and 8.03 (each 2H, d, $J=9.1$ Hz, anisoyl group). ^{13}C-NMR δ: 18.8 and 19.4 (SCH_3 ×2), 81.6 (C3), 88.4 (C13), 213.9 and 214.7 (C=S ×2). MS *m/z*: 855 (M^+), 824 (M^+-OCH_3, base peak), 764 ($M^+-OCH_3-CH_3COOH$).

3,13-Dideoxyjesaconitine (7) Compound **7** was prepared from **15** in 89% yield in the same manner as used for the synthesis of compound **3**: amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 1711 (C=O). ^{1}H-NMR δ: 1.11 (3H, t, $J=7.1$ Hz, NCH_2CH_3), 1.42 (3H, s, $OCOCH_3$), 3.19 (3H, s, OCH_3), 3.21 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.58 (3H, s, OCH_3), 3.87 (3H, s, OCH_3 of anisoyl group), 5.06 (1H, t, $J=4.5$ Hz, C14-β-H), 6.96 and 8.05 (each 2H, d, $J=9.0$ Hz, anisoyl group). ^{13}C-NMR δ: 85.1 (C1), 26.4 (C2), 34.8 (C3), 39.0 (C4), 49.0 (C5), 83.5 (C6), 44.3 (C7),

92.0 (C8), 45.3 (C9), 38.6 (C10), 50.0 (C11), 28.8 (C12), 44.5 (C13), 76.1 (C14), 75.4 (C15), 89.3 (C16), 61.5 (C17), 80.3 (C18), 53.2 (C19), 49.1 (NCH_2CH_3), 13.5 (NCH_2CH_3), 56.1 (C1′), 57.7 (C6′), 57.9 (C16′), 59.0 (C18′), 55.3 ($COC_6H_4OCH_3$), 172.2 ($COCH_3$), 21.3 ($COCH_3$), 166.0 ($COC_6H_4OCH_3$), 122.4, 131.6, 113.5 and 163.2 ($COC_6H_4OCH_3$). MS *m/z*: 643 (M^+), 612 (M^+-OCH_3, base peak), 552 ($M^+-OCH_3-CH_3COOH$). *Anal.* Calcd for $C_{35}H_{49}NO_{10}$: C, 65.30; H, 7.67; N, 2.18. Found: C, 65.57; H, 7.84; N, 2.23.

3-*O*-(*S*-Methyl)thiocarbonylmesaconitine (8) A mixture of **1** (50 mg), dry Et_2O (6 ml), imidazole (3 mg) and NaH (116 mg) was stirred at 0 °C for 30 min, then CS_2 (1.2 ml) and CH_3I (0.9 ml) were added dropwise. The mixture was stirred for 2 h at room temperature, then the reaction was quenched with ice-water and the whole was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water, then dried over anhydrous sodium sulfate. The solvent was evaporated off and the residue was purified by column chromatography on silica gel with Et_2O saturated with 28% ammonia water–hexane (3:2) as an eluting solvent to yield **8** (53 mg, 93%): amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 1710 (C=O). ^{1}H-NMR δ: 1.40 (3H, s, $OCOCH_3$), 2.44 (3H, s, NCH_3), 2.57 (3H, s, SCH_3), 3.18 (3H, s, OCH_3), 3.20 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 4.88 (1H, d, J=5.3 Hz, C14-β-H), 5.72 (1H, dd, J=12.7, 5.4 Hz, C3-β-H), 8.05—7.43 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.5 (SCH_3), 81.7 (C3), 214.9 (C=S). MS *m/z*: 721 (M^+), 690 (M^+-OCH_3, base peak), 630 ($M^+-OCH_3-CH_3COOH$).

Hypaconitine (9) A solution of **8** (20 mg) in 1 ml of dry benzene was refluxed with n-Bu_3SnH (0.15 ml) in dry benzene (0.5 ml) for 2 h. The reaction mixture was concentrated *in vacuo*, then the residue was chromatographed on silica gel with Et_2O saturated with 28% ammonia water–hexane (4:1) as an eluting solvent and crystallized from MeOH to give **9** (16 mg, 94% yield): mp 189—190 °C. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1730 (C=O). ^{1}H-NMR δ: 1.39 (3H, s, $OCOCH_3$), 2.35 (3H, s, NCH_3), 3.18 (3H, s, OCH_3), 3.33 (6H, s, $OCH_3\times2$), 3.75 (3H, s, OCH_3), 4.87 (1H, d, J=5.0 Hz, C14-β-H), 8.05—8.12 (5H, m, benzoyl group). MS *m/z*: 615 (M^+), 584 (M^+-OCH_3, base peak), 524 ($M^+-OCH_3-CH_3COOH$). *Anal.* Calcd for $C_{33}H_{45}NO_{10}$: C, 64.37; H, 7.37; N, 2.27. Found: C, 64.49; H, 7.42; N, 2.26. The NMR and MS spectra of **9** were identical to those of an authentic sample.

3-*O*-(*S*-Methyl)thiocarbonylaconitine (16) Compound **16** was prepared from **2** in 92% yield in the same manner as used for the synthesis of compound **8**: amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1725 (C=O). ^{1}H-NMR δ: 1.16 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.39 (3H, s, $OCOCH_3$), 2.56 (3H, s, SCH_3), 3.19 (3H, s, OCH_3), 3.20 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 4.88 (1H, d, J=4.9 Hz, C14-β-H), 5.71 (1H, dd, J=12.1, 6.8 Hz, C3-β-H), 7.4—8.02 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.7 (SCH_3), 81.6 (C3), 214.7 (C=S). MS *m/z*: 735 (M^+), 704 (M^+-OCH_3, base peak), 644 ($M^+-OCH_3-CH_3COOH$).

Deoxyaconitine (10) Compound **10** was prepared from **16** in 96% yield in the same manner as used for the synthesis of compound **9**: colorless needles (MeOH), mp 169.5—170.5 °C (lit.[6] 168—170 °C). IR ν_{max}^{KBr} cm^{-1}: 3550 (OH), 1730 and 1715 (C=O). ^{1}H-NMR δ: 1.07 (3H, t, J=7.0 Hz, NCH_2CH_3), 1.37 (3H, s, $OCOCH_3$), 3.15 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 4.87 (1H, d, J=5.0 Hz, C14-β-H), 7.32—8.12 (5H, m, benzoyl group). MS *m/z*: 629 (M^+), 598 (M^+-OCH_3), 538 ($M^+-OCH_3-CH_3COOH$, base peak). *Anal.* Calcd for $C_{34}H_{47}NO_{10}$: C, 64.84; H, 7.52; N, 2.22. Found: C, 64.69; H, 7.69; N, 2.23. NMR and MS spectra of **10** were identical with those of an authentic sample reported by Takayama *et al.*[6]

3-*O*-(*S*-Methyl)thiocarbonyljesaconitine (17) Compound **17** was prepared from **6** in 92% yield in the same manner as used for the synthesis of compound **8**: amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1725 (C=O). ^{1}H-NMR δ: 1.11 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.39 (3H, s, $OCOCH_3$), 2.56 (3H, s, SCH_3), 3.18 (3H, s, OCH_3), 3.20 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.87 (3H, s, OCH_3 of anisoyl group), 4.84 (1H, d, J=4.9 Hz, C14-β-H), 5.71 (1H, dd, J=12.7, 7.7 Hz, C3-β-H), 6.93 and 7.98 (each 2H, d, J=8.7 Hz, anisoyl group). ^{13}C-NMR δ: 18.8 (SCH_3), 81.7 (C3), 214.7 (C=S). MS *m/z*: 765 (M^+), 734 (M^+-OCH_3, base peak), 674 ($M^+-OCH_3-CH_3COOH$).

Deoxyjesaconitine (11) Compound **11** was prepared from **17** in 93% yield in the same manner as used for the synthesis of compound **9**: colorless needles (acetone), mp: 175—176 °C. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1715 and 1710 (C=O). ^{1}H-NMR δ: 1.08 (3H, t, J=7.0 Hz, NCH_2CH_3), 1.43 (3H, s, $OCOCH_3$), 3.17 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 3.30 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 3.88 (3H, s, OCH_3 of anisoyl group), 4.83 (1H, d, J=5.0 Hz, C14-β-H), 6.92 and 7.97 (each 2H, d, J=8.0 Hz, anisoyl group). MS *m/z*: 659 (M^+), 628 (M^+-OCH_3, base peak), 568 ($M^+-OCH_3-CH_3COOH$). NMR and MS spectra of **11** were identical with those of an authentic sample.[7]

3,13,15-Tri-*O*-(*S*-methyl)thiocarbonyljesaconitine (12) A mixture of **6** (50 mg), dry THF (6 ml), imidazole (3 mg) and NaH (116 mg) was stirred at 0 °C for 30 min, then CS_2 (1.2 ml) and CH_3I (0.9 ml) were added dropwise. The mixture was refluxed for 1.5 h, then the reaction was quenched with ice-water and the whole was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water, then dried over anhydrous sodium sulfate. The solvent was evaporated off and the residue was purified by column chromatography on silica gel with Et_2O saturated with 28% ammonia water–hexane (3:2) as an eluting solvent to yield **12** (65 mg, 93% yield): amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1715 (C=O). ^{1}H-NMR δ: 1.18 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.28 (3H, s, $OCOCH_3$), 2.46 (3H, s, SCH_3), 2.57 (3H, s, SCH_3), 2.66 (3H, s, SCH_3), 3.18, 3.19, 3.29, 3.38 (3H, s, OCH_3), 3.83 (3H, s, OCH_3 of anisoyl group), 4.18 (1H, d, J=5.8 Hz, C15-β-H), 5.30 (1H, d, J=5.4 Hz, C14-β-H), 5.73 (1H, dd, J=11.1, 7.4 Hz, C3-β-H), 6.95 and 8.21 (each 2H, d, J=9.1 Hz, anisoyl group). ^{13}C-NMR δ: 18.9, 19.4 and 19.7 ($SCH_3\times3$), 81.3 (C3), 88.2 (C13), 87.6 (C15), 214.0, 214.8 and 217.1 (C=S). MS *m/z*: 945 (M^+), 914 (M^+-OCH_3, base peak), 854 ($M^+-OCH_3-CH_3COOH$).

3,13,15-Trideoxyjesaconitine (13) A solution of **12** (30 mg) in dry benzene (1 ml) was refluxed with n-Bu_3SnH (0.2 ml) in dry benzene (0.5 ml) for 4 h. The reaction mixture was concentrated *in vacuo*, then the residue was chromatographed on silica gel with Et_2O saturated with 28% ammonia water–hexane (4:1) as an eluting solvent and crystallized from Et_2O–hexane to give **13** (18 mg, 88% yield), mp 151—152 °C (lit.[8] 153—154 °C). IR ν_{max}^{KBr} cm^{-1}: 1717 (C=O). $[\alpha]_D^{19}$ +29.8° (c=0.98, $CHCl_3$). ^{1}H-NMR δ: 1.08 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.39 (3H, s, $OCOCH_3$), 3.17 (3H, s, OCH_3), 3.26 (3H, s, OCH_3), 3.28 (3H, s, OCH_3), 3.38 (3H, s, OCH_3), 3.83 (3H, s, OCH_3 of anisoyl group), 5.03 (1H, t, J=4.8 Hz, C14-β-H), 6.91 and 8.01 (each 2H, d, J=8.9 Hz, anisoyl group). ^{13}C-NMR δ: 85.0 (C1), 26.3 (C2), 34.8 (C3), 39.2 (C4), 49.1 (C5), 82.8 (C6), 44.9 (C7), 85.8 (C8), 49.2 (C9), 43.9 (C10), 50.3 (C11), 28.9 (C12), 39.0 (C13), 75.8 (C14), 37.8 (C15), 83.3 (C16), 63.6 (C17), 80.4 (C18), 53.7 (C19), 49.1 (NCH_2CH_3), 13.4 (NCH_2CH_3), 56.5 (C1′), 57.8 (C6′), 56.0 (C16′), 59.0 (C18′), 55.3 ($COC_6H_4OCH_3$), 169.7 ($COCH_3$), 21.8 ($COCH_3$), 166.0 ($COC_6H_4OCH_3$), 163.3, 131.6, 122.8 and 113.6 ($COC_6H_4OCH_3$). MS *m/z*: 627 (M^+), 596 (M^+-OCH_3, base peak), 536 ($M^+-OCH_3-CH_3COOH$). HR-MS: Calcd for $C_{35}H_{49}NO_9$: 627.3407. Found: 627.3394. *Anal.* Calcd for $C_{35}H_{49}NO_9\cdot H_2O$: C, 65.10; H, 7.96; N, 2.17. Found: C, 65.11; H, 7.71; N, 2.16. NMR and MS spectra of **13** were identical with those of foresaconitine as reported by Chen and Breitmaier[8] and Yang *et al.*[9]

3,13,15-Tri-*O*-(*S*-methyl)thiocarbonylaconitine (18) Compound **18** was prepared from **2** in 83% yield in the same manner as used for the synthesis of compound **12**: colorless prism (Et_2O–hexane), mp 239—241 °C. IR ν_{max}^{KBr} cm^{-1}: 1717 (C=O). ^{1}H-NMR δ: 1.15 (3H, t, J=7.0 Hz, NCH_2CH_3), 1.27 (3H, s, $OCOCH_3$), 2.45 (3H, s, SCH_3), 2.56 (3H, s, SCH_3), 2.66 (3H, s, SCH_3), 3.16 (3H, s, OCH_3), 3.18 (3H, s, OCH_3), 3.29 (3H, s, OCH_3), 3.45 (3H, s, OCH_3), 5.31 (1H, d, J=5.3 Hz, C14-β-H), 5.73 (1H, dd, J=12.7, 5.4 Hz, C3-β-H), 7.45—8.20 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.9, 19.5 and 19.8 ($SCH_3\times3$), 81.3 (C13), 87.7 (C15), 214.1, 214.9 and 217.0 (C=S). MS *m/z*: 915 (M^+), 884 (M^+-OCH_3, base peak), 824 ($M^+-OCH_3-CH_3COOH$).

3,13,15-Trideoxyaconitine (19) Compound **19** was prepared from **18** in 83% yield in the same manner as used for the synthesis of compound **13**: colorless needles (Et_2O–hexane). mp 150—151 °C (lit.[10] 148—150 °C). IR ν_{max}^{KBr} cm^{-1}: 1717 (C=O). ^{1}H-NMR δ: 1.10 (3H, t, J=7.0 Hz, NCH_2CH_3), 1.38 (3H, s, $OCOCH_3$), 3.17 (3H, s, OCH_3), 3.28 (6H, s, $OCH_3\times2$), 3.37 (3H, s, OCH_3), 5.05 (1H, d, J=5.0 Hz, C14-β-H), 7.39—8.02 (5H, m, benzoyl group). ^{13}C-NMR δ: 85.1 (C1), 26.5 (C2), 35.1 (C3), 39.1 (C4), 49.0 (C5), 82.9 (C6), 47.9 (C7), 85.8 (C8), 49.2 (C9), 38.8 (C10), 50.5 (C11), 28.9 (C12), 34.7 (C13), 75.5 (C14), 38.1 (C15), 85.3 (C16), 63.0 (C17), 80.2 (C18), 53.8 (C19), 49.0 (NCH_2CH_3), 13.3 (NCH_2CH_3), 56.6 (C1′), 57.8 (C6′), 56.2 (C16′), 59.1 (C18′), 169.8 ($COCH_3$), 21.6 ($COCH_3$), 166.0 (COC_6H_5), 128.3, 129.3 and 132.9 (COC_6H_5). MS *m/z*: 597 (M^+), 566 (M^+-OCH_3, base peak), 506 ($M^+-OCH_3-CH_3COOH$). *Anal.* Calcd for $C_{34}H_{47}NO_8$: C, 68.32; H, 7.92; N, 2.34. Found: C, 67.97; H, 8.03; N, 2.23. These spectral data was identical to those of crassicaudine.[10,11]

3,13,15-Tri-*O*-(*S*-methyl)thiocarbonylmesaconitine (20) Compound **20** was prepared from **1** in 83% yield in the same manner as used for the synthesis of compound **12**: colorless prisms (Et_2O–hexane), mp 233—234 °C. IR ν_{max}^{KBr} cm^{-1}: 1715 (C=O). ^{1}H-NMR δ: 1.23 (3H, s,

$OCOCH_3$), 2.47 (3H, s, NCH_3), 2.57 (3H, s, SCH_3), 2.58 (3H, s, SCH_3), 2.65 (3H, s, SCH_3), 3.17 (3H, s, OCH_3), 3.19 (3H, s, OCH_3), 3.30 (3H, s, OCH_3), 3.44 (3H, s, OCH_3), 5.35 (1H, d, J=5.3 Hz, C14-β-H), 5.72 (1H, dd, J=12.7, 5.4 Hz, C3-β-H), 7.45—8.28 (5H, m, benzoyl group). ^{13}C-NMR δ: 18.9, 19.5 and 19.8 ($SCH_3 \times 3$), 81.2 (C3), 88.2 (C13), 87.7 (C15), 214.1, 214.9 and 217.0 (C=S). MS m/z: 901 (M^+), 870 ($M^+ - OCH_3$, base peak), 810 ($M^+ - OCH_3 - CH_3COOH$).

3,13,15-Trideoxymesaconitine (21) Compound **21** was prepared from **20** in 83% yield in the same manner as used for the synthesis of compound **13**: colorless needles (Et_2O–hexane). mp 156—157 °C. IR ν_{max}^{KBr} cm^{-1}: 1715 (C=O). ^{1}H-NMR δ: 1.37 (3H, s, $OCOCH_3$), 2.31 (3H, s, NCH_3), 3.17 (3H, s, OCH_3), 3.28 (6H, s, $OCH_3 \times 2$), 3.38 (3H, s, OCH_3), 5.07 (1H, d, J=5.0 Hz, C14-β-H), 7.40—8.08 (5H, m, benzoyl group). ^{13}C-NMR δ: 85.0 (C1), 26.4 (C2), 37.8 (C3), 39.2 (C4), 48.5 (C5), 82.7 (C6), 48.1 (C7), 85.8 (C8), 44.9 (C9), 38.6 (C10), 50.4 (C11), 28.7 (C12), 34.6 (C13), 75.6 (C14), 38.9 (C15), 83.3 (C16), 63.2 (C17), 80.3 (C18), 56.1 (C19), 42.5 (NCH_3), 56.4 (C1′), 58.1 (C6′), 56.5 (C16′), 59.1 (C18′), 169.7 ($\underline{C}OCH_3$), 21.6 ($CO\underline{C}H_3$), 166.0 ($\underline{C}OC_6H_5$), 128.5, 129.5 and 133.0 ($CO\underline{C}_6H_5$). MS m/z: 583 (M^+), 552 ($M^+ - OCH_3$, base peak), 462 ($M^+ - OCH_3 - CH_3COOH$). *Anal.* Calcd for $C_{33}H_{45}NO_8$: C, 67.90; H, 7.77; N, 2.40. Found: C, 67.87; H, 7.82; N, 2.40.

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Structure and Synthesis of Nectrisine, a New Immunomodulator Isolated from a Fungus[1)]

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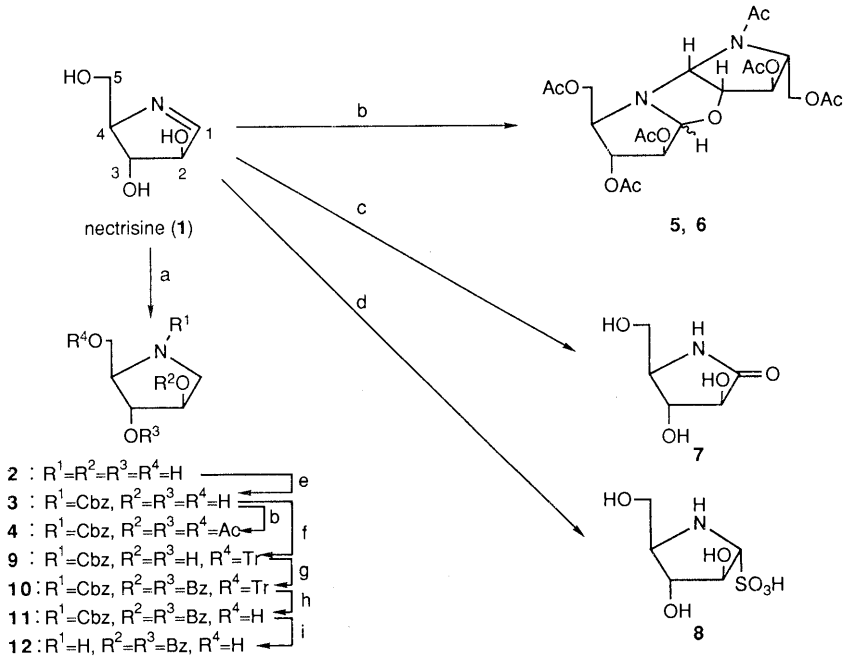
The structure of a novel immunomodulator, nectrisine (1), has been elucidated on the basis of chemical and spectroscopic evidence. Its absolute stereochemistry was predicted on the basis of the dibenzoate chirality rule and finally confirmed by a synthesis from D-glucose.

Keywords nectrisine; *Nectria lucida*; D-glucose; D-arabinose; enantiospecific synthesis; immunomodulator; α-glucosidase inhibitor; α-mannosidase inhibitor; Ia antigen

In our continuing screening program for immunologically active compounds from microorganisms, nectrisine (**1**), which was tentatively designated as WF4490, was isolated as a new type of immunomodulator from a fungus, *Nectria lucida* F-4490.[2)] This natural product induces the expression of Ia antigen[3)] and restores the immune response depressed by immunosuppressive factors of tumors.[2)] It also possesses potent α-glucosidase-inhibitory activity and α-mannosidase-inhibitory activity.[2)] In the previous communication,[1)] we reported the structural elucidation and synthesis of nectrisine. This paper is devoted to a full account of that work.

Nectrisine was isolated as a colorless powder: $[\alpha]_D$ +21.8° (c=0.6, H_2O). The molecular formula ($C_5H_9NO_3$) was established by elemental analysis and fast atom bombardment mass spectrometry (FAB-MS). The infrared (IR) spectrum showed absorption bands ascribed to hydroxyl groups (3330 cm^{-1}) and an imino function (1640 cm^{-1}). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (Table I) showed five signals consisting of one methylene (δ 61.8 (t)), three methines (δ 83.9 (d), 78.8 (d), 77.4 (d)), and one sp^2-carbon (δ 171.0 (d)) assignable to the C-1 imino carbon. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table II), the corresponding imino proton was observed at δ 7.71 (1H, d, J=2 Hz), together with five protons of methylene and methine moieties (δ 4.12—3.14, 5H, m). The chemical shifts of ^{1}H- and ^{13}C-NMR suggested that all carbons bear oxygen or nitrogen atoms.

Catalytic hydrogenation of **1** (H_2 (5 atm), 10% Pd–C, H_2O) provided the dihydro derivative **2** whose ^{13}C-NMR spectrum (Table I) showed a new methylene signal (δ 51.0 (t)), instead of the imino carbon signal of **1**, along with other carbons consisting of one methylene (δ 61.8) and three methines (δ 78.8 (d), 77.2 (d), 66.1 (d)). In the ^{1}H-NMR spectrum of **2** (Table II), the corresponding two methylene protons appeared at δ 3.20 (1H, dd, J=12, 6 Hz) and δ 2.92 (1H, dd, J=12, 4 Hz). A spin-decoupling experiment on **2** clarified the ^{1}H–^{1}H relationships as shown in Fig. 1 to reveal the structure of 3,4-dihydroxy-2-(hydroxymethyl)pyrrolidine for **2**. This structure was corroborated by the fact that treatment of **2** with carbobenzyloxy chloride followed by



(a) H_2, 10% Pd-C, H_2O (b) Ac_2O, Py (c) I_2, NaOH, H_2O (d) SO_2, H_2O (e)CbzCl, NaOH, H_2O
(f) TrCl, Et_3N, DMAP, CH_2Cl_2 (g)BzCl, Py, CH_2Cl_2 (h)TsOH, MeOH (i)H_2, 10% Pd-C, MeOH

Chart 1

TABLE I. ^{13}C-NMR (67.8 MHz, D_2O) Chemical Shifts (in ppm) for **1** and **2**[a]

C	1	2
1	171.0 (d)	51.0 (t)
2	83.9 (d)[b]	77.2 (d)[c]
3	78.8 (d)[b]	78.8 (d)[c]
4	77.4 (d)[b]	66.1 (d)[c]
5	61.8 (t)	61.8 (t)

a) Abbreviations given in parentheses denote signals observed in the off-resonance experiments. *b, c*) Assignments may be interchangeable in each column.

TABLE II. ^{1}H-NMR Chemical Shifts (D_2O, in ppm), Multiplicities, and Coupling Constants (in Hz, in parentheses) for **1**, **2**, and **7**

H	1[a]	2[a]	7[b]
1-H	7.71 d (2)	3.20 dd (12, 6) 2.92 dd (12, 4)	
2-H	4.12—3.14, 5H m	4.18 dt (6, 4)	4.08 d (8)
3-H		3.87 dd (5, 4)	3.77 t (8)
4-H		3.09 dt (6, 5)	3.21 ddd (8, 5, 4)
5-H		3.78 dd (12, 5) 3.69 dd (12, 6)	3.56 dd (12, 4) 3.48 dd (12, 5)

a) 270 MHz. *b*) 200 MHz.

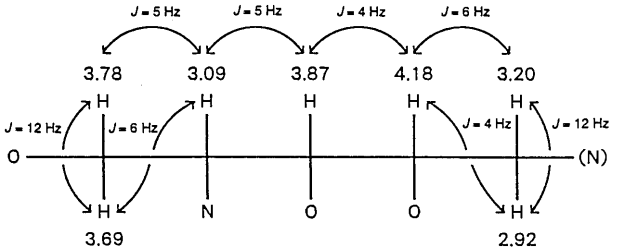


Fig. 1

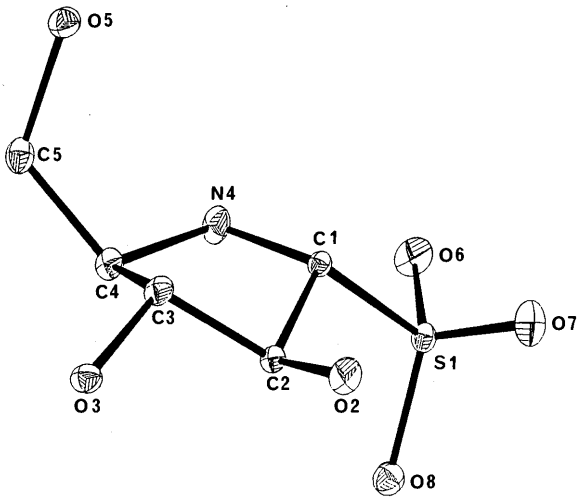


Fig. 2

TABLE III. Atomic Coordinates with e.s.d.'s in Parentheses and Thermal Parameters (Å^2)

Atom	x	y	z	B_{eq}
C1	0.399 (1)	0.842 (1)	0.703 (2)	1.2
C2	0.364 (1)	0.880 (1)	0.949 (2)	1.1
C3	0.179 (1)	0.803 (1)	0.908 (2)	1.3
C4	0.217 (1)	0.665 (1)	0.821 (2)	1.3
C5	0.048 (2)	0.580 (1)	0.674 (2)	1.7
N4	0.336 (1)	0.697 (1)	0.654 (2)	1.7
S1	0.6420 (3)	0.8544 (4)	0.7223 (4)	1.3
O2	0.358 (1)	1.0178 (9)	0.991 (1)	1.9
O3	0.142 (1)	0.7955 (9)	1.137 (1)	1.6
O5	−0.064 (1)	0.6405 (9)	0.432 (1)	1.7
O6	0.651 (1)	0.7589 (9)	0.526 (2)	2.2
O7	0.668 (1)	0.992 (1)	0.670 (2)	2.4
O8	0.757 (1)	0.8127 (9)	0.980 (1)	2.0

TABLE IV. Bond Lengths (Å) and Angles (°) with Their e.s.d.'s in Parentheses

Bond lengths (Å)			
C1–C2	1.55 (2)	C1–N4	1.52 (2)
C1–S1	1.80 (1)	C2–C3	1.53 (2)
C2–O2	1.41 (1)	C3–C4	1.53 (2)
C3–O3	1.42 (1)	C4–N4	1.55 (2)
C4–C5	1.51 (2)	S1–O6	1.48 (1)
S1–O7	1.44 (1)	S1–O8	1.46 (1)
C5–O5	1.45 (2)		
Bond angles (°)			
C2–C1–N4	104.8 (9)	C2–C1–S1	115.5 (9)
N4–C1–S1	108.9 (8)	C1–C2–C3	100.4 (9)
C1–C2–O2	114.7 (9)	C3–C2–O2	115.8 (9)
C2–C3–C4	103.8 (9)	C2–C3–O3	111.0 (9)
C4–C3–O3	111.0 (10)	C3–C4–N4	102.6 (9)
C3–C4–C5	118.1 (10)	N4–C4–C5	109.9 (10)
C1–N4–C4	107.5 (9)	C1–S1–O6	103.4 (6)
C1–S1–O7	105.5 (6)	C1–S1–O8	105.4 (6)
O6–S1–O7	115.0 (6)	O6–S1–O8	112.6 (6)
O7–S1–O8	113.7 (6)	C4–C5–O5	111.6 (10)

acetylation with acetic anhydride gave the *N*-benzyloxycarbonyl-tri-*O*-acetyl derivative **4**, *via* **3**. Hence the structure of nectrisine was deduced to be **1**, without the stereochemistry.

Chemical evidence supporting this presumed structure was obtained as follows. Acetylation of **1** with acetic anhydride in pyridine afforded the two epimeric dimeric hexaacetates **5** (18%) and **6** (30%).[4] The C-1′ configurations of the dimers were could not be clarified because the two epimers showed similar $J_{1',2'}$ values, 5 Hz for **5** and 4 Hz for **6**, in their ^{1}H-NMR spectra. The dimers were isomerized by treatment with 0.5% TsOH in $CHCl_3$ at room temperature to give a mixture of **5** and **6** (*ca.* 1 : 1). Further evidence in support of the structure **1** was provided by oxidation of **1** with iodine (NaOH, H_2O) to give the lactam **7**, whose IR spectrum showed an absorption band ascribed to an amide function at 1658 cm^{-1}. The ^{1}H-NMR spectrum of **7** (Table II) showed five signals at δ 4.08 (1H, d, J=8 Hz), 3.77 (1H, t, J=8 Hz), 3.56 (1H, dd, J=12, 4 Hz) and 3.48 (1H, dd, J=12, 5 Hz), and 3.21 (1H, ddd, J=8, 5, 4 Hz), which were assigned to 2-H, 3-H, 5-H$_2$ and 4-H, respectively. Thse data indicate that nectrisine has the structure **1**.

In order to gain information on the stereochemistry of nectrisine, we examined several reactions for obtaining the acetonide of **2** (*e.g.*, 2,2-dimethoxypropane, TsOH). These attempts were all unsuccessful, and accordingly the two hydroxyl groups and the hydroxymethyl function are presumed to be all *trans*. This presumed structure was confirmed by X-ray crystallographic analysis of the bisulfite adduct **8**, which was prepared by treatment of **1** with SO_2 in H_2O (Fig. 2).

The absolute stereochemistry was deduced to be the D-form by applying the dibenzoate chirality rule[5] to the 2,3-di-*O*-benzoyl derivative **12**, which was prepared as follows. The *N*-benzyloxycarbonyl derivative **3** was transformed into **10** *via* **9** by selective protection of the primary alcohol with trityl chloride and subsequent acylation of the two hydroxyl groups with benzoyl chloride. Treatment of **10** with TsOH and subsequent hydrogenolysis of the resulting **11** over 10% Pd–C afforded the dibenzoate **12**. A negative sign of the first Cotton effect ($[\theta]_{234}$ −67000) was observed in the circular dichroism (CD) spectrum of **12**, indicating that the configurations of C-2 and C-3 are both *R* and hence, that of C-4 is *R*. Since nectrisine showed potent α-glucosidase inhibitory activity, this deduction on the stereochemistry of C-2, C-3, and C-4 is reasonable by analogy with that of the corresponding C-3, C-4, and C-5 in nojirimycin (**13**), a representative α-glucosidase inhibitor[6] (Fig. 3).

Finally the presumed structure was confirmed by a synthesis from D-glucose, whose three asymmetric carbons, C-3, C-4, and C-5, correspond to C-2, C-3, and C-4 of nectrisine, respectively. We devised a synthetic route for **1** that includes oxidative cleavage between C-1 and C-2 of the appropriately protected 5-amino-5-deoxy-D-glucose (**14**) (Chart 2). The requisite intermediate **14** could be obtained from 5-amino-5-deoxy-3-*O*-benzyl-1,2-*O*-isopropylidene-6-*O*-trityl-α-D-glucofuranose (**15**), prepared by Niida *et al.* for their synthesis of nojirimycin.[6] We chose a trifluoroacetyl group for protection of the amino function in **15** because we expected that it would be stable under acidic or oxidative conditions and against hydrogenolysis, and it is easily removed by mild alkaline hydrolysis in the last step. *N*-Acylation of **15** with trifluoroacetic anhydride and subsequent acidic hydrolysis of the resulting **16** with 75% aqueous trifluoroacetic acid (TFA) afforded the triol **17**. The vicinal diol function was oxidatively cleaved with $NaIO_4$ to give the pyranose **18**. In this reaction the C-1 carbon of **17** remained as the *O*-formyl group at the 3-position of **18**. Although the 2-*O*-benzyl group resisted the usual hydrogenolysis (H_2 (5 atm), 10% Pd–C), deprotection was achieved by hydrogenolysis using Pd black in 4.4% HCOOH–MeOH to afford **19** in 98% yield. The two acyl groups in **19** were finally hydrolyzed with a slight excess of 0.5 N aqueous NaOH to furnish **1** in 96% yield. This product was identical with the natural product. The total yield from **15** was 56%. The structure of nectrisine was thus established to be **1**.

It is notable that nectrisine exists as the imino form, as judged from the ^1H- and ^{13}C-NMR spectral data, while the corresponding L-xylo (**21**)[4a] and L-lyxo (**22**)[4b] stereoisomers were reported to adopt mainly the dimeric forms **21c** and **22c**, respectively (Fig. 4). This might be explained by steric factors. It seems that **1** is much more stable than **21b** and **22b** because the three substituents on the pyrroline ring of

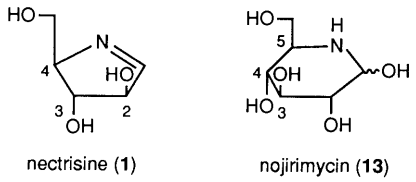


Fig. 3

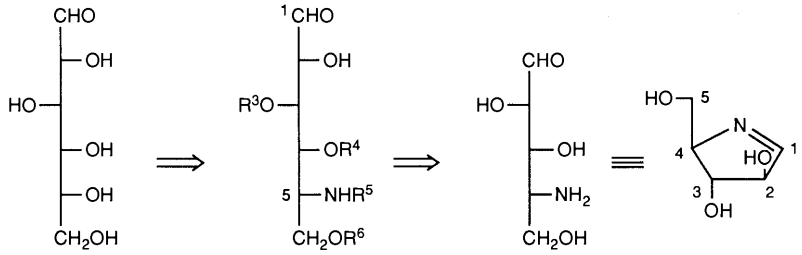
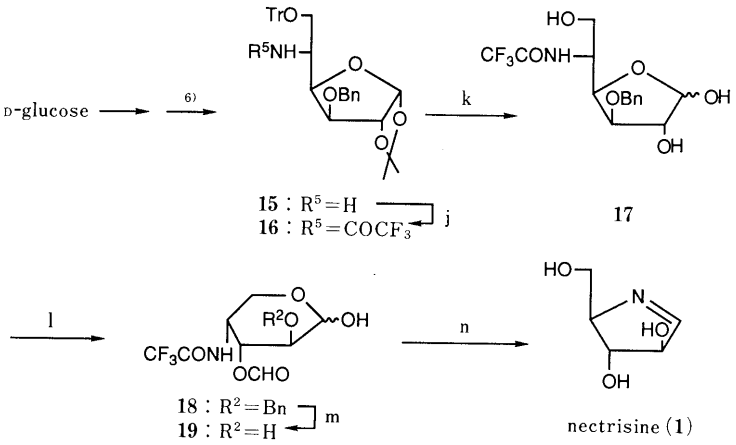
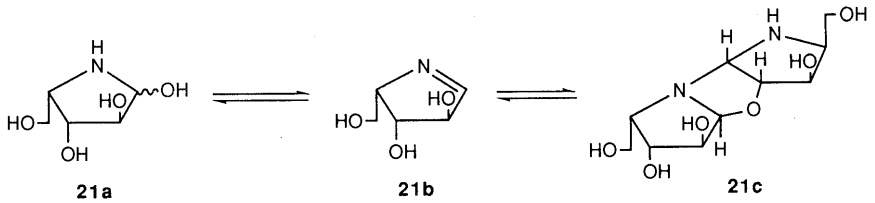


Chart 2



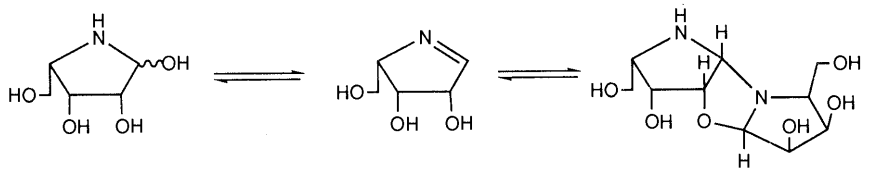
(j) $(CF_3CO)_2O$, Et_3N, CH_2Cl_2 (k) 75% aq. TFA (l) $NaIO_4$, aq. THF (m) Pd-black, 4.4% HCOOH–MeOH (n) 0.5 N aq. NaOH

Chart 3



21a 21b 21c

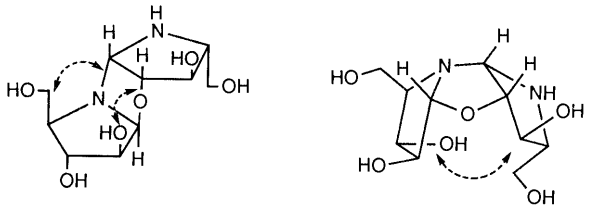
21 (L-xylo)



22a 22b 22c

22 (L-lyxo)

Fig. 4



23 24

Fig. 5

TABLE V. Biological Activities of Nectrisine Derivatives

Compound	α-Glucosidase IC_{50} (μg/ml)	α-Mannosidase IC_{50} (μg/ml)	Ia induction EC_{70} (μg/ml)	Restoration of immune response MEC (μg/ml)
Nectrisine	0.05	6.5	0.04	0.08
2	0.2	310	1.6	2.0
3	>33	>33	>12.5	>500
7	>33	>33	>12.5	N.T.
1-Deoxynojirimycin	0.03	>33	>25	N.T.
Swainsonine	>33	0.12	0.02	0.02

1 are in all *trans*, and not eclipsed, relationships, while **21b** has one pair and **22b** has two pairs of substituents which are in *cis*, and eclipsed, relationships. On the other hand, **23** and **24**, dimeric forms of **1**, are likely to be less stable than **21c** and **22c** because of steric hindrance (Fig. 5).

Biological activities[7] of nectrisine (**1**), **2**, **3** and **7** are shown in Table V. A representative α-glucosidase inhibitor, 1-deoxynojirimycin,[8] and a representative α-mannosidase inhibitor, swainsonine,[9] were also tested. It appears that the imino function and the basic nitrogen atom of nectrisine (**1**) are important for its biological activities. These results also seem to suggest that the immunomodulating activities, *i.e.*, induction of Ia antigen and restoration of immune response, are correlated with each other and that α-mannosidase-inhibitory activity, but not α-glucosidase-inhibitory activity, might contribute to the immunomodulating activities.

In conclusion, we established the structure of nectrisine to be 4-amino-4-deoxy-D-arabinose (**1**), and developed an efficient synthetic route from D-glucose which is capable of providing sufficient amounts for detailed biological evaluation.

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our preceding paper[10] except for the following. A JEOL FX-270 spectrometer was also used to take ^1H (270 MHz) and ^{13}C (67.8 MHz)-NMR spectra. CD spectra were measured with a JASCO J-20 automatic recording spectropolarimeter.

Nectrisine (1) A colorless amorphous powder, $[\alpha]_D$ +21.8° (c=0.6, H_2O). *Anal.* Calcd for $C_5H_9NO_3$: C, 45.80; H, 6.92; N, 10.68. Found: C, 45.08; H, 6.57; N, 10.16. IR (KBr): 3330, 2900, 1640, 1500, 1400, 1240, 1200, 1040 cm^{-1}. ^1H- and ^{13}C-NMR: see Tables I and II. FAB-MS m/z: 132 $(M+H)^+$.

1,4-Dideoxy-1,4-imino-D-arabinitol (2) A solution of **1** (120 mg) in H_2O (5.0 ml) was treated with 10% Pd–C (30 mg) under hydrogen (4 atm) at room temperature for 4 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to give a brownish oil (125 mg), which was purified by carbon treatment in H_2O to afford **2** (116 mg, 95%). **2**: A viscous colorless oil, $[\alpha]_D$ +15.7° (c=0.4, H_2O). *Anal.* Calcd for $C_5H_{11}NO_3$: C, 45.10; H, 8.33; N, 10.52. Found: C, 44.82; H, 8.60; N, 10.37. IR (neat): 3350, 2940, 1540, 1420, 1052 cm^{-1}. ^1H- and ^{13}C-NMR: see Tables I and II. FAB-MS m/z: 134 $(M+H)^+$.

***N*-Benzyloxycarbonyl-1,4-dideoxy-1,4-imino-D-arabinitol (3)** Carbobenzyloxy chloride (1.0 ml) and 1 N aqueous NaOH (0.7 ml) were added to a stirred ice-cold solution of **2** (665 mg) in H_2O (10 ml) at room temperature over a period of 10 min and the mixture was stirred for 1 h. After removal of the solvent under reduced pressure, the residue was extracted with CH_2Cl_2–MeOH (4:1, 20 ml). The extract was combined, evaporated *in vacuo*, and purified by column chromatography (SiO_2 20 g, CH_2Cl_2–MeOH = 20:1) to afford **3** (1.18 g, 88%). **3**: Colorless fine crystals, mp 126—128 °C (Et_2O), $[\alpha]_D$ −28.9° (c=1.9, MeOH). *Anal.* Calcd for $C_{13}H_{17}NO_5$: C, 58.42; H, 6.41; N, 5.24. Found: C, 58.10; H, 6.41; N, 5.15. IR (Nujol): 3340, 1664, 1420, 1352, 1190, 1118, 1072, 1050, 1010 cm^{-1}. ^1H-NMR (D_2O) δ: 7.49 (5H, s), 5.23 (2H, s), 4.30—4.18 (2H, m), 4.01—3.73 (4H, m), 3.39 (1H, m). FAB-MS m/z: 268 $(M+H)^+$.

2,3,5-Tri-*O*-acetyl-*N*-benzyloxycarbonyl-1,4-dideoxy-1,4-imino-D-arabinitol (4) Compound **3** (100 mg) was treated with acetic anhydride (1.0 ml) and pyridine (2.0 ml) at room temperature for 5 h. After concentration *in vacuo*, the residue was dissolved in Et_2O (5 ml) and washed with 1 N aqueous HCl, brine, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo* to afford **4** (140 mg, 95%). **4**: A colorless viscous oil, $[\alpha]_D$ −22.7° (c=0.6, MeOH). *Anal.* Calcd for $C_{19}H_{23}NO_8$: C, 58.01; H, 5.89; N, 3.56. Found: C, 57.78;

H, 5.84; N, 3.52. IR ($CHCl_3$): 1736, 1696, 1408, 1350, 1202 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.38 (5H, br s), 5.25—5.10 (4H, m), 4.43—3.84 (4H, m), 3.53 (1H, m), 2.11 (3H, s), 2.08 (6H, s). FAB-MS *m/z*: 394 $(M+H)^+$.

Acetylation of Nectrisine (1) A solution of **1** (1.2 g) in pyridine (20 ml) was treated with acetic anhydride (10 ml) at room temperature for 12 h. After concentration *in vacuo*, the residue was purified by column chromatography (SiO_2 100 g, *n*-hexane : AcOEt = 1 : 1—1 : 2) to afford two epimers of dimeric hexaacetates **5**, the faster-eluted one, (424 mg, 18%) and **6**, the later-eluted one, (716 mg, 30%). **5**: A colorless viscous oil, $[\alpha]_D$ +37.3° (*c* = 0.7, MeOH). IR ($CHCl_3$): 2990, 2950, 1738, 1660, 1366, 1224, 1204, 1040 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 5:33 (1H, d, *J* = 5 Hz), 5.24 (1H, t, *J* = 5 Hz), 5.13 (1H, br s), 5.03 (1H, m), 4.78 (1H, d, *J* = 2 Hz), 4.57 (1H, d, *J* = 5 Hz), 4.37—4.28 (3H, m), 4.12—4.00 (2H, m), 3.37 (1H, m). FAB-MS *m/z*: 515 $(M+H)^+$. High-resolution FAB-MS Calcd for $C_{22}H_{31}N_2O_{12}$ $(M+H)^+$: 515.188. Found: 515.188. **6**: A colorless viscous oil, $[\alpha]_D$ −39.2° (*c* = 0.6, MeOH). IR ($CHCl_3$): 2990, 2940, 1738, 1654, 1364, 1220, 1202, 1042 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 5.77 (1H, d, *J* = 4 Hz), 5.20 (1H, dd, *J* = 6, 4 Hz), 5.18—5.11 (2H, m), 5.04 (1H, br s), 4.40 (1H, d, *J* = 4 Hz), 4.30—4.22 (2H, m), 4.19 (1H, dd, *J* = 10, 5 Hz), 4.06 (1H, m), 3.98 (1H, dd, *J* = 10, 8 Hz), 3.66 (1H, m). FAB-MS *m/z*: 515 $(M+H)^+$. High-resolution FAB-MS Calcd for $C_{22}H_{31}N_2O_{12}$ $(M+H)^+$: 515.188. Found: 515.189.

Isomerization of 5 and 6 Compound **5** (50 mg) was treated with a 0.5% solution of TsOH·H_2O in $CHCl_3$ (5.0 ml) at room temperature for 30 min. After being washed with saturated aqueous $NaHCO_3$ and brine, the organic layer was dried over $MgSO_4$, evaporated *in vacuo*, and subjected to column chromatography (SiO_2 5.0 g, *n*-hexane : AcOEt = 1 : 1) to give **5** (19 mg, 38%) and **6** (20 mg, 40%) which were found to be identical with authentic samples by direct comparison: TLC (AcOEt, *Rf* = 0.50 for **5** and 0.40 for **6**; *n*-hexane : acetone = 1 : 1, *Rf* = 0.46 for **5** and 0.39 for **6**; CH_2Cl_2 : MeOH = 19 : 1, *Rf* = 0.47 for **5** and 0.45 for **6**) and ^{1}H-NMR (DMSO-d_6). The same treatment of **6** (50 mg) afforded **5** (21 mg, 42%) and **6** (23 mg, 46%).

(3*S*,4*R*,5*R*)-3,4-Dihydroxy-5-hydroxymethyl-2-pyrrolidone (7) Aqueous I_2 (0.1 N, 35 ml) and NaOH (52.5 ml) were added to a stirred solution of **1** (183 mg) in H_2O (7.5 ml) simultaneously over 12 min. After being stirred for 3 h, the reaction mixture was neutralized with 1 N HCl under ice-cooling and evaporated *in vacuo* to give a residue, which was extracted with $CHCl_3$: MeOH : H_2O = 6 : 4 : 1 (30 ml). The extract was evaporated *in vacuo*, and this residue was purified by cation exchange resin column chromatography (Dowex 50W × 8 (H^+ form) 15 ml, H_2O) and subsequent anion exchange resin column chromatography (Amberlite IRA-45 (OH^- form) 15 ml, H_2O) to afford **7** (55 mg, 27%). **7**: Colorless fine crystals, mp 136—137 °C (EtOH), $[\alpha]_D$ +15.6° (*c* = 0.5, H_2O). *Anal.* Calcd for $C_5H_9NO_4$: C, 40.82; H, 6.17; N, 9.52. Found: C, 40.52; H, 6.09; N, 9.22. IR (KBr): 3200, 2910, 2850, 1658, 1340, 1314, 1278, 1090, 1058 cm^{-1}. ^{1}H-NMR (D_2O) δ: 4.08 (1H, d, *J* = 8 Hz), 3.77 (1H, t, *J* = 8 Hz), 3.56 (1H, dd, *J* = 12, 4 Hz), 3.48 (1H, dd, *J* = 12, 5 Hz), 3.21 (1H, ddd, *J* = 8, 5, 4 Hz). FAB-MS *m/z*: 148 $(M+H)^+$.

Bisulfite Adduct of 1 (8) SO_2 gas was introduced into a stirred ice-cold solution of **1** (730 mg) in H_2O (0.73 ml) for 30 min. The reaction mixture was left to stand at room temperature for 2 d. MeOH (7.3 ml) was added thereto under ice-cooling and the mixture was stirred at the same temperature for 30 min. The precipitate was collected by vacuum filtration and washed with MeOH to afford **8** (836 mg, 72%). **8**: Colorless needles, mp > 250 °C (6% aqueous SO_2–EtOH), $[\alpha]_D$ +42.7° (*c* = 1.0, H_2O). *Anal.* Calcd for $C_5H_{11}NO_6S$: C, 28.17; H, 5.20; N, 6.57; S, 15.04. Found: C, 28.14; H, 5.03; N, 6.39; S, 14.72. IR (Nujol): 3370, 3300, 3150, 1568, 1247, 1234, 1213, 1200, 1160 cm^{-1}. ^{1}H-NMR (400 MHz, D_2O) δ: 4.46 (1H, dd, *J* = 7, 7 Hz), 4.38 (1H, d, *J* = 7 Hz), 4.14 (1H, dd, *J* = 10, 7 Hz), 3.96 (1H, dd, *J* = 12, 5 Hz), 3.91 (1H, dd, *J* = 12, 4 Hz), 3.69 (1H, ddd, *J* = 10, 5, 4 Hz). FAB-MS *m/z*: 425 $(2M-H)^+$, 212 $(M-H)^+$.

X-Ray Analysis of 8 The crystals were obtained by recrystallization from 6% aqueous SO_2–EtOH: $C_5H_{11}NO_6S$, monoclinic, space group $P2_1$, *a* = 7.545(1), *b* = 10.040(1), *c* = 5.627(1) Å, β = 111.28(1)°, *V* = 397.1(1) Å^3, *Z* = 2, D_x = 1.783 g/cm^3, μ = 36.4 cm^{-1}. The X-ray intensity data from a selected crystal (0.20 × 0.10 × 0.05 mm) were obtained on a Rigaku AFC-5 diffractometer equipped with a rotating anode X-ray generator (40 kV-100 mA), using graphite-monochromated CuK_α radiation (λ = 1.54178 Å). A total of 715 independent reflections $2\theta < 130°$ were collected with the $2\theta/\omega$ scan mode. The structure was solved by the direct method using MULTAN 84 (Main *et al.*, 1984). The refinement was carried out by the block-diagonal least-squares method with anisotropic thermal parameters for non H atoms. The *R* factor was reduced to 0.068 using 713 reflections with $F_o > 3\sigma(F_o)$. The atomic parameters, bond lengths and bond angles are given in Tables III and IV.

***N*-Benzyloxycarbonyl-1,4-dideoxy-1,4-imino-5-*O*-triphenylmethyl-D-arabinitol (9)** Triphenylmethyl chloride (463 mg) and 4-dimethylaminopyridine (10 mg) were added to a stirred anhydrous solution of **3** (423 mg) and Et_3N (0.24 ml) in CH_2Cl_2 (8.5 ml) at room temperature. After being stirred for 12 h, the reaction mixture was washed twice with water, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 30 g, *n*-hexane : AcOEt = 1 : 1) to afford **9** (644 mg, 84%). **9**: Amorphous, $[\alpha]_D$ −42.8° (*c* = 0.5, MeOH). *Anal.* Calcd for $C_{32}H_{31}NO_5$: C, 75.42; H, 6.13; N, 2.75. Found: C, 75.14; H, 6.28; N, 2.72. IR ($CHCl_3$): 3400, 1688, 1410, 1348, 1074 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.50—7.10 (20H, m), 5.20—4.90 (2H, m), 4.18—3.30 (7H, m). FAB-MS *m/z*: 267 $(M-Tr+H)^+$, 251 $(M-TrO+H)^+$.

2,3-Di-*O*-benzoyl-*N*-benzyloxycarbonyl-1,4-dideoxy-1,4-imino-5-*O*-triphenylmethyl-D-arabinitol (10) Benzoyl chloride (0.26 ml) was added dropwise to a stirred anhydrous solution of **9** (387 mg) and pyridine (0.2 ml) in CH_2Cl_2 (4 ml) at room temperature. After being stirred for 12 h, the reaction mixture was washed with 1 N aqueous HCl, brine, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 30 g, *n*-hexane : AcOEt = 9 : 1—5 : 1) to afford **10** (489 mg, 90%). **10**: Amorphous, $[\alpha]_D$ −32.8° (*c* = 0.6, MeOH). *Anal.* Calcd for $C_{46}H_{39}NO_7$: C, 76.97; H, 5.48; N, 1.95. Found: C, 76.65; H, 5.55; N, 1.88. IR ($CHCl_3$): 1716, 1700, 1444, 1410, 1258, 1100, 1088 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.07 (2H, dd, *J* = 7, 1 Hz), 7.96—7.05 (28H, m), 5.95 (1H, br s), 5.53 (1H, d like), 5.13—4.98 (2H, m), 4.40—4.13 (2H, m), 3.85—3.60 (2H, m), 3.32 (1H, m). FAB-MS *m/z*: 458 $(M-TrO)^+$.

2,3-Di-*O*-benzoyl-*N*-benzyloxycarbonyl-1,4-dideoxy-1,4-imino-D-arabinitol (11) A solution of **10** (421 mg) and TsOH·H_2O (100 mg) in MeOH (10 ml) was stirred at room temperature for 20 h. The reaction mixture was diluted with CH_2Cl_2 (40 ml), washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 100 g, *n*-hexane : AcOEt = 3 : 1) to afford **11** (269 mg, 96%). **11**: Amorphous, $[\alpha]_D$ −47.0° (*c* = 0.8, MeOH). *Anal.* Calcd for $C_{27}H_{25}NO_7$: C, 68.20; H, 5.30; N, 2.95. Found: C, 67.95; H, 5.32; N, 2.87. IR ($CHCl_3$): 3400, 1716, 1700, 1680, 1412, 1258, 1102 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.08—8.00 (4H, m), 7.68—7.30 (11H, m), 5.70—5.48 (2H, m), 5.17 (2H, s), 4.35—3.70 (5H, m). FAB-MS *m/z*: 476 $(M+H)^+$.

2,3-Di-*O*-benzoyl-1,4-dideoxy-1,4-imino-D-arabinitol (12) Compound **11** (179 mg) was treated with 10% Pd–C (36 mg) in MeOH (4 ml) under hydrogen (4 atm) at room temperature for 24 h. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 5 g, CH_2Cl_2 : MeOH = 50 : 1—100 : 3) to afford **12** (107 mg, 83%). **12**: Colorless oil, $[\alpha]_D$ −121.0° (*c* = 0.6, MeOH). *Anal.* Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.58; H, 5.68; N, 4.06. IR ($CHCl_3$): 3520, 3350, 1712, 1262, 1106 cm^{-1}. CD ($c = 1.75 \times 10^{-2}$, MeOH) $[\theta]^{25}$ (nm): −67000 (234) (negative maximum), +25000 (220) (positive maximum). ^{1}H-NMR (CD_3OD) δ: 8.18—8.08 (4H, m), 7.72—7.46 (6H, m). 5.77 (1H, m), 5.56 (1H, br s), 4.18—3.92 (4H, m), 3.80 (1H, d, *J* = 12 Hz). FAB-MS *m/z*: 342 $(M+H)^+$.

3-*O*-Benzyl-5-deoxy-1,2-*O*-isopropylidene-5-trifluoroacetamido-6-*O*-triphenylmethyl-α-D-glucofuranose (16) A solution of trifluoroacetic anhydride (2.25 ml) in CH_2Cl_2 (80 ml) was added dropwise to a stirred anhydrous solution of 5-amino-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-6-*O*-triphenylmethyl-α-D-glucofuranose (**15**,[6] 8.0 g) and Et_3N (2.5 ml) in CH_2Cl_2 (240 ml) in an ice–H_2O bath under an N_2 atmosphere over 40 min. After being stirred for 20 min, the reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2 250 g, *n*-hexane : AcOEt = 8 : 1) to afford **16** (9.35 g, quant.). **16**: Colorless fine crystals, mp 72—74 °C (*n*-heptane), $[\alpha]_D$ −51.3° (*c* = 0.5, $CHCl_3$). *Anal.* Calcd for $C_{37}H_{36}F_3NO_6$: C, 68.61; H, 5.60; N, 2.16. Found: C, 68.40; H, 5.65; N, 2.18. IR ($CHCl_3$): 3400, 2995, 2940, 1722, 1534, 1452, 1378, 1282, 1162, 1074 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 7.55—7.20 (21H, m), 7.10—7.00 (2H, m), 5.93 (1H, d, *J* = 4 Hz), 4.82 (1H, m), 4.58 (1H, dd, *J* = 6, 4 Hz), 4.56 (1H, d, *J* = 4 Hz), 4.42 (1H, d, *J* = 11 Hz), 3.97 (1H, d, *J* = 11 Hz), 3.78 (1H, d, *J* = 4 Hz), 3.52 (1H, dd, *J* = 10, 5 Hz), 2.94 (1H, t, *J* = 10 Hz), 1.53, 1.33 (each, 3H, s). FAB-MS *m/z*: 686 $(M+K)^+$, 670 $(M+Na)^+$.

3-*O*-Benzyl-5-deoxy-5-trifluoroacetamido-D-glucose (17) Compound **16** (585 mg) was treated with 75% aqueous TFA (2 ml) at room temperature for 40 min. After removal of the solvent under reduced pressure, the residue was purified by column chromatography (SiO_2 30 g, *n*-hexane : AcOEt = 1 : 1—AcOEt only) to afford **17** (270 mg, 82%). **17**: A hygroscopic amorphous powder, $[\alpha]_D$ −14.4° (*c* = 0.5, MeOH). IR (Nujol): 3495,

3330, 3180, 1728, 1662, 1552, 1220, 1200 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 9.10 (1H, d, J=9.5 Hz, D_2O-exchangeable), 6.23 (0.2H, d, J=8 Hz, D_2O-exchangeable), 6.18 (0.8H, d, J=9 Hz, D_2O-exchangeable), 5.42 (0.2H, d, J=5 Hz, D_2O-exchangeable), 5.24 (0.8H, dd, J=9, 4 Hz), 5.22 (0.8H, d, J=5 Hz, D_2O-exchangeable), 4.78 (1H, t, J=5 Hz, D_2O-exchangeable), 4.57 (1H, d, J=11 Hz), 4.35 (1H, d, J=11 Hz), 4.32—3.20 (6H, m). FAB-MS m/z: 404 $(M+K)^+$, 388 $(M+Na)^+$. High-resolution FAB-MS Calcd for $C_{15}H_{18}F_3NNaO_6$ $(M+Na)^+$: 388.098. Found: 388.095.

2-*O*-Benzyl-4-deoxy-3-*O*-formyl-4-trifluoroacetamido-D-arabinose (18) A solution of **17** (731 mg) in tetrahydrofuran containing 2.5% H_2O (24 ml) was added to a stirred solution of sodium metaperiodate (856 mg) in H_2O (24 ml) at 5—6 °C over a period of 35 min. Stirring was continued for 25 min under ice-cooling, then the insoluble material was removed by filtration and the filtrate was extracted with AcOEt. The extract was dried over $MgSO_4$, evaporated *in vacuo* and purified by column chromatography (SiO_2 10 g, *n*-hexane : AcOEt = 2 : 3) to afford **18** (531 mg, 73%). **18**: A colorless viscous oil, $[\alpha]_D$ −13.1° (c=0.5, MeOH). IR (Nujol): 3430, 3300, 1730, 1714, 1702, 1539 cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 8.09 (1H, s), 7.48—7.22 (5H, m), 5.10 (0.5H, d, J=2 Hz), 4.85—4.58 (1.5H, m), 4.37—4.26 (1H, m), 4.16—3.85 (2H, m), 3.72—3.43 (3H, m). FAB-MS m/z: 386 $(M+Na)^+$. High-resolution FAB-MS Calcd for $C_{15}H_{16}F_3NNaO_6$ $(M+Na)^+$: 386.083. Found: 386.086.

4-Deoxy-3-*O*-formyl-4-trifluoroacetamido-D-arabinose (19) A mixture of **18** (225 mg), Pd black (300 mg) and 4.4% HCOOH–MeOH (40 ml) was stirred at room temperature for 1.5 h under an N_2 atmosphere. After removal of the catalyst by filtration, the filtrate was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2 10 g, AcOEt) to afford **19** (188 mg, 98%). **19**: An amorphous solid, $[\alpha]_D$ −64.3° (c=0.5, MeOH). *Anal.* Calcd for $C_8H_{10}F_3NO_6$: C, 35.18; H, 3.69; N, 5.13. Found: C, 35.27; H, 3.95; N, 5.05. IR (Nujol): 3420, 3300, 1716, 1700, 1558, 1160 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 9.58 (0.5H, d, J=9 Hz, D_2O-exchangeable), 9.51 (0.5H, d, J=9 Hz, D_2O-exchangeable), 8.23 (1H, s), 6.92 (0.5H, d, J=5 Hz, D_2O-exchangeable), 6.63 (0.5H, d, J=5 Hz, D_2O-exchangeable), 5.47 (0.5H, d, J=5 Hz, D_2O-exchangeable), 5.11 (0.5H, d, J=5 Hz, D_2O-exchangeable), 5.09—4.96 (1H, m), 4.89 (0.5H, dd, J=9, 5 Hz, 4.52—4.28 (1.5H, m), 4.02 (0.5H, dd, J=13, 3 Hz), 3.92—3.54 (2H, m), 3.48 (0.5H, dd, J=13, 4 Hz). FAB-MS m/z: 296 $(M+Na)^+$.

Synthesis of Nectrisine (1) Compound **19** (134 mg) was treated with 0.5 N aqueous NaOH (3.0 ml) at room temperature for 30 min. The mixture was acidified to pH 4 with acetic acid under ice-cooling, diluted with H_2O (100 ml) and subjected to column chromatography (CM-Sephadex (NH_4^+ form) 100 ml, eluted with H_2O 400 ml, then 2% aqueous NH_3 200 ml). The aqueous NH_3 fractions containing the objective compound were collected and evaporated *in vacuo*. The residue was taken up in H_2O (5 ml) and lyophilized to afford **1** (62 mg, 96%), which was identical with an authentic sample by direct comparison: TLC ($CHCl_3$: MeOH : 28% aqueous NH_3 = 5 : 3 : 1, Rf=0.35; *n*-BuOH : AcOH : H_2O = 4 : 1 : 2, Rf=0.21; isopropyl alcohol : H_2O = 7 : 3, Rf=0.27), $[\alpha]_D$ +21.0° (c=0.6, H_2O), IR (KBr), ^{1}H-NMR (D_2O), and ^{13}C-NMR (D_2O) (Tables I and II).

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β-Lactam Antifungals. II.[1] Enantiocontrolled Synthesis of (2*R*,5*S*)-2-Hydroxymethyl-1-carbapenam, the Carba-Analog of a Clavam Antifungal

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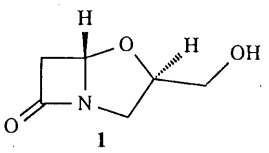
(2*R*,5*S*)-2-Hydroxymethyl-1-carbapenam (3), the carba-analog of an antifungal β-lactam (2*R*,5*S*)-2-(hydroxymethyl)clavam (1), was synthesized in an enantiocontrolled manner, starting from the coupling reaction of an optically active phthalimido-acetate (3*S*,4*S*)-4 and an allylsilane 7, followed by removal of the phthalimido group that was crucial for asymmetric induction. Hydroboration, protecting-group interconversion, and cyclization gave 3 stereoselectively.

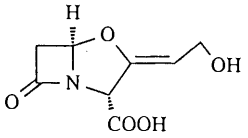
Keywords carbapenam; synthesis; enantiocontrol; antifungal; oxapenam; allylsilane; phthalimide; isonitrile; hydroboration

(3*R*,5*S*)-3-Hydroxymethyl-4-oxa-1-azabicyclo[3.2.0]-heptan-7-one [(2*R*,5*S*)-2-(hydroxymethyl)clavam] (**1**) is an antifungal β-lactam that was isolated by Brown and Evans[2] from culture fluides of *Streptomyces clavuligerus*. It is reported to exhibit activity against a number of species of fungi. From a stereochemical point of view, it is interesting that this and related clavam antifungals possess the 5*S* absolute configuration (hereafter in this paper, the clavam numbering shown in A will be used), which is opposite to that of the corresponding positions of the natural β-lactam antibacterials, such as penicillin and cephalosporin, and the β-lactamase inhibitor clavulanic acid (**2**).[3]

The clavam **1** is structurally rather simple and therefore deserves attention as a new lead for a novel type of antifungal agent. As a part of a program directed toward the development of novel antifungal agents, we first synthesized **1** in an enantiocontrolled manner.[1] The clavam **1** synthesized, however, showed only moderate antifungal potency and its chemical instability required improvement. Consequently, structural modification of **1** was started. We reasoned, by analogy with clavulanic acid **2**, that the chemical instability of the clavam **1** was brought about by protonation at O1, which activates the β-lactam system, followed by the attack of a nucleophile on the C7 carbonyl to cleave the β-lactam ring, as shown in B. Consequently, we designed the 1-carba-analog of the natural product **1**, the title compound, (2*R*,5*S*)-2-hydroxymethyl-1-carbapenam (**3**), in the expectation that **3** might be chemically more stable than **1**, and exhibit higher biological activty. In the present paper, we report an enantiocontrolled synthesis of **3**.



1



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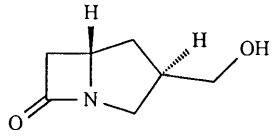

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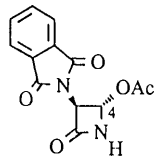

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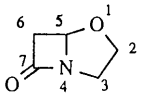
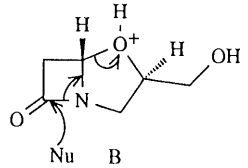


A clavam skeleton



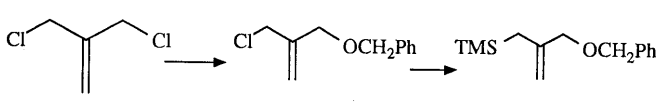
B

Chart 1

We recognized the importance of controlling the stereochemistry at C5 in the synthesis, because the absolute configuration at this position generally seems to determine whether bicyclic β-lactams were antibacterial or antifungal.[3] Toward this end, we selected the known, optically active phthalimido-acetate (3*S*,4*S*)-**4**,[1,4] which is readily available from 6-aminopenicillanic acid (6-APA), as a starting material for the synthesis. The bulky, subsequently removable phthalimido group in (3*S*,4*S*)-**4** was expected to control the stereochemistry upon C–C bond formation at the neighboring C4 position. For the remaining four-carbon segment, we selected the allylsilane **7**.

This allylsilane **7** was prepared in two steps from commercially available allylic dichloride **5**, as shown in Chart 2. Thus, the dichloride **5** was treated with sodium benzyloxide in tetrahydrofuran (THF) to afford a half-ether **6** in 66% yield, and **6** was converted to the allylsilane **7** in 77% yield *via* the Grignard reagent.

On the other hand, the optically active phthalimido-acetate (3*S*,4*S*)-**4** was converted by treatment with chlorotrimethylsilane and triethylamine to an *N*-silyl-β-lactam (3*S*,4*S*)-**8**. This lactam (3*S*,4*S*)-**8** reacted with the allylsilane **7** in 1,2-dichloroethane at 50 °C in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate[5] (TMSOTf) to afford, after aqueous work-up, the *trans* lactam (3*S*,4*S*)-**9** in 79% overall yield from (3*S*,4*S*)-**4** (Chart 3). The coupling constant between the C3 and C4 protons in the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of (3*S*,4*S*)-**9** was 2 Hz. This result shows that the relative configuration of the C3 and C4



5 **6** **7**

Chart 2

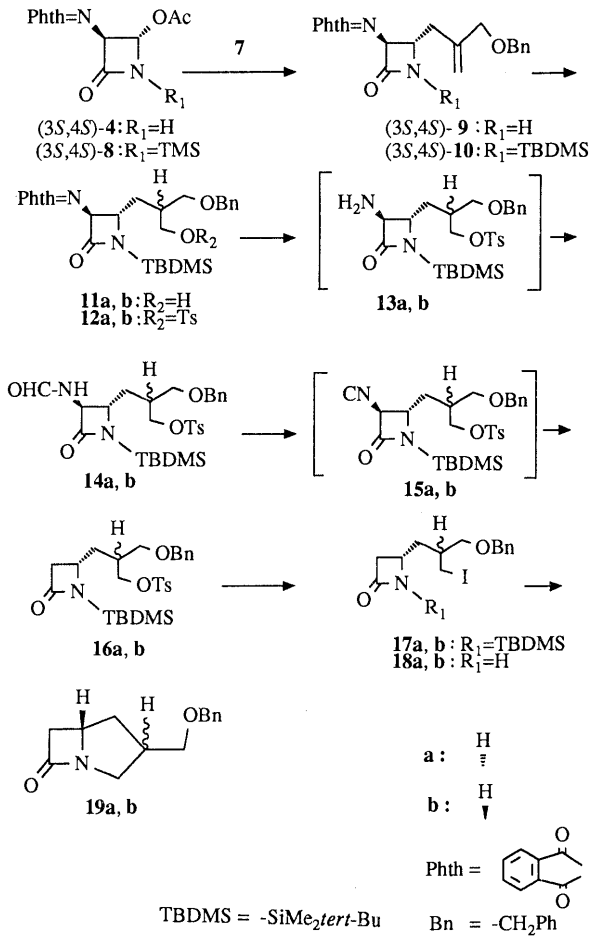


Chart 3

substituents is *trans*, and accordingly, the absolute configuration at C4 is *S*. The lactam nitrogen was silylated again using *tert*-butylchlorodimethylsilane and triethylamine in *N*,*N*-dimethylformamide (DMF), giving (3*S*,4*S*)-**10** in 94% yield. The olefin moiety in (3*S*,4*S*)-**10** was hydroborated using borane dimethylsulfide in THF, to afford, after oxidative work-up, a diastereomeric mixture of alcohols, which was separated by flash column chromatography to give the more polar isomer **11a** in 24% yield, and the less polar one **11b** in 43% yield. The absolute configuration of the chiral centers that were generated in **11a** and **11b** could not be determined at this stage, but it was later assigned to be as depicted in Chart 3.

In order to investigate the subsequent steps, the diastereomer **11a** was subjected to the following reaction first. This alcohol was tosylated by treatment with *p*-toluenesulfonyl chloride in pyridine to afford a tosylate **12a** in 99% yield. The phthaloyl group in **12a** was removed by reaction with methylhydrazine in dichloromethane to liberate an unstable amine **13a**, which, without isolation, was formylated to give a formamide **14a** in 73% overall yield from **12a**. The formamide **14a** was dehydrated using trichloromethyl chloroformate ("diphosgene") and triethylamine in dichloromethane to give an isocyanide **15a**, which was decyanated[6] by heating with tributylstannane and a catalytic amount of α,α′-azobisisobutyronitrile in benzene to give **16a** in 77% overall yield from **14a**. The tosyloxy group in **16a** was converted into the iodine atom in the conventional manner, quantitatively giving an iodide

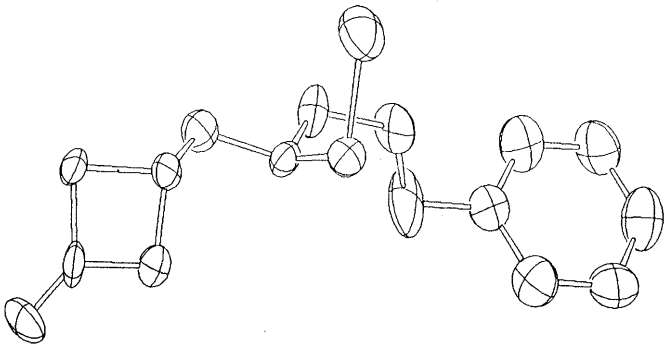


Fig. 1. Projection of the Structure of the Iodide **18a**

17a, which was desilylated by treatment with tetrabutylammonium fluoride to give a crystalline, *N*-unsubstituted β-lactam **18a** in quantitative yield.

Following exactly the same reaction sequence as described above, the diastereomeric alcohol **11b** was converted into the corresponding iodide **18b**.

At this stage, the diastereomer **18a** was subjected to X-ray diffraction analysis, and the relative configuration of the two chiral centers that exist in this diastereomer was determined (Fig. 1) to be *lk*. Taking into account that the absolute configuration at C4 in the azetidinone ring in **18a** is *S*, the configuration at the chiral center in the side chain was found to be *S*. Hence, the absolute configuration of **11a**, **b** through **18a**, **b** was found to be as depicted in Chart 3.

The iodides **18a**, **b** were separately treated with powdered potassium carbonate in DMF at room temperature to give the carbapenams **19a**, **b** in 64 and 82% yields, respectively. Then, the hydrogenolysis of the diastereomer **19a** to **3** was investigated. However, **19a** remained almost unchanged after being treated with 10% palladium–carbon catalyst under H_2 atmosphere even at 150 atm for 4 h; only a trace amount of the desired alcohol **3** was detected in the reaction mixture.[7]

Therefore, we next turned our attention to the preparation of the corresponding *tert*-butyldimethylsilyl ether **33**. This was achieved as follows (Chart 4).

The phthalimide (3*S*,4*S*)-**10** was converted, in a manner similar to that described for the preparation of **14a** from **12a**, into a formamide (3*S*,4*S*)-**21** in 64% yield. The formamido moiety was then removed, in the same manner as described above, *via* the isocyanide (3*S*,4*S*)-**22**, to give (*S*)-**23** in 82% yield. The olefin moiety in (*S*)-**23** was hydroborated as above, giving a diastereomeric mixture of alcohols, which was separated by flash column chromatography to give the more polar alcohol **24a** in 27% yield, and the less polar one **24b** in 34% yield. The configuration of the two alcohols **24a** and **24b** was determined to be as depicted, by leading these alcohols to the aforementioned tosylates **16a** and **16b**, respectively. Although the stereoselectivity in this hydroboration reaction was low, both diastereomers **24a** and **24b** could be converted into the sole, desired intermediate **30**.

Thus, the diastereomer **24a** was treated with isobutyryl chloride in pyridine to give an ester **25** in 90% yield, and this was hydrogenolyzed in ethanol using palladium black at 1 atm to give a half-alcohol **26**. Since this material was prone to suffer acyl migration upon standing for a long

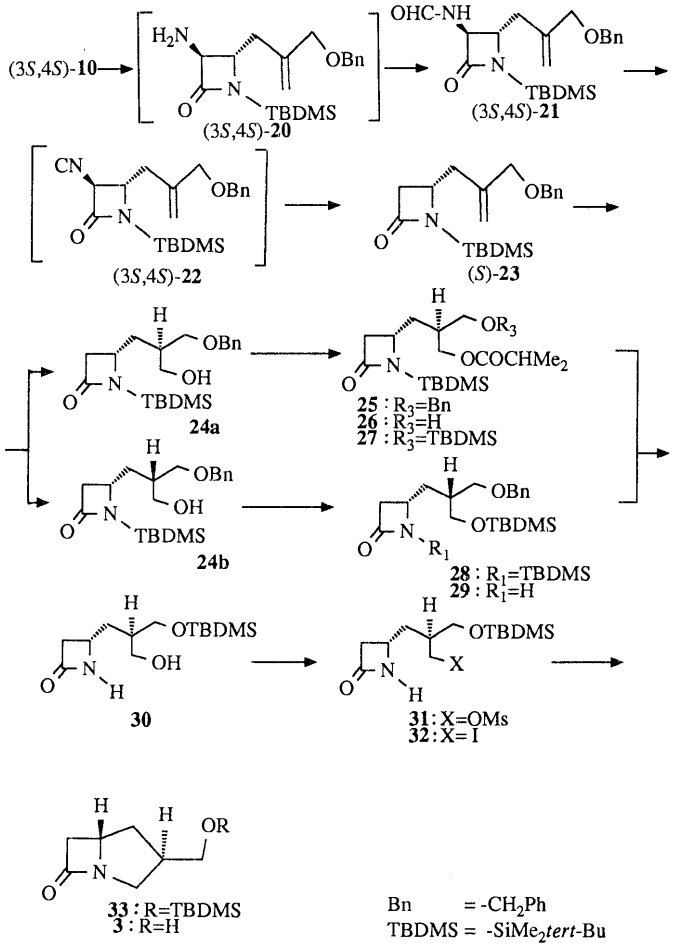


Chart 4

time, it was crucial to use palladium black instead of palladium–carbon, in order to shorten the reaction time. The product **26** was silylated immediately to give the silyl ether **27** in 71% overall yield from **25**. Upon treatment with sodium hydroxide in methanol–water, **27** was selectively *N*-desilylated and was converted into an alcohol **30** in 99% yield.

On the other hand, the other diastereomer **24b** was converted into a silyl ether **28** in 95% yield, and **28** was *N*-desilylated by sodium hydroxide into **29** in quantitative yield. Hydrogenolysis of **29** using 10% palladium–carbon catalyst gave the above-mentioned alcohol **30** in 97% yield.

The alcohol **30** thus obtained was mesylated to give **31** in 90% yield, and the latter was converted into an iodide **32** in 88% yield. The iodide **32** was cyclized, in a way similar to that described for **18a**, giving the desired carbapenam **33** in 72% yield.

Finally, the silyl protecting group was removed by treatment with tetrabutylammonium fluoride in THF which was buffered with acetic acid, liberating the title compound **3**, $[\alpha]_D^{25}$ −191° (c=1.20, $CHCl_3$), as a colorless oil in 57% yield after chromatographic purification.

Contrary to our expectation, the carbapenam **3** turned out to be much more unstable than the oxapenam **1**. When left to stand in chloroform overnight, **3** decomposed almost completely to give insoluble, gummy precipitates. The difference of "ring strain" that is inherent in oxa- and carbapenam systems presumably overwhelmed the effect of protonation shown in B. The carbapenam derivative **3** exhibited no antifungal activity *in vitro*.

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer, ^{1}H-NMR spectra on a Varian EM-360L spectrometer (60 MHz), or a JEOL GX-270 spectrometer (270 MHz) using tetramethylsilane as the internal standard, and mass spectra (MS) and high-resolution mass spectra (HRMS) on a JEOL JMS D300 spectrometer. Thin-layer chromatography (TLC) was performed on TLC plates, Silica gel $60F_{254}$ precoated, layer thickness 0.25 mm (E. Merck), and spots were made visible by ultraviolet (UV) irradiation or by spraying with phosphomolybdic acid or with vanadic acid–sulfuric acid followed by heating. Preparative TLC was performed on TLC plates, Silica gel $60F_{254}$ precoated, layer thickness 2 mm (E. Merck). Chromatography columns were prepared with silica gel (60—110 mesh, Kanto Chemical Co., Inc.), and flash chromatography columns were prepared with silica gel (230—400 mesh, E. Merck). The amount of silica gel used and the developing solvents are shown in parentheses. The abbreviations used are as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; dddd, doublet of doublets of doublets of doublets; ddddd, doublet of doublets of doublets of doublets of doublets; dt, doublet of triplets; m, multiplet; br, broad; sh, shoulder.

3-Benzyloxy-2-chloromethyl-1-propene (6) Benzyl alcohol (9.80 g, 90.7 mmol) was slowly added to a stirred suspension of NaH (55% mineral oil suspension, 3.60 g, 82.5 mmol, washed with hexane) in THF (100 ml) at 0 °C. When hydrogen gas ceased to evolve, 3-chloro-2-chloromethyl-1-propene (**5**, 21.0 g, 16.8 mmol) was added. The mixture was refluxed for 6 h. After cooling, it was partitioned between hexane and water. The organic layer was dried and concentrated under reduced pressure giving an oily residue, which was purified by distillation to give **6** (11.8 g, 66%, bp 110—116 °C (3 mmHg)) as an oil. *Anal.* Calcd for $C_{11}H_{13}ClO$: C, 67.18; H, 6.66. Found: C, 67.40; H, 6.63. IR ($CHCl_3$): 1725, 1705, 1090, 1070 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 4.08 (4H, s-like), 4.47 (2H, s), 5.06 (2H, br s), 7.30 (5H, s). MS *m/z*: 197, 195 (M^+-1), 166, 131, 122, 107, 91 (100%).

(2-Benzyloxymethyl-2-propenyl)trimethylsilane (7) A solution of **6** (13.0 g, 66 mmol) and chlorotrimethylsilane (14.3 g, 132 mmol) in THF (80 ml) was added to a suspension of Mg (3.20 g, 132 mmol) in THF (50 ml) with stirring under an N_2 atmosphere at 80 °C (bath temperature) over a period of 15 min. The mixture was refluxed for an additional 1 h. After cooling, the mixture was partitioned between hexane and a diluted aqueous solution of $NaHCO_3$. The resulting precipitates were dissolved by adding NH_4Cl (solid). The organic layer was collected, washed with brine, and dried. Evaporation of the solvent under reduced pressure and distillation of the oily residue gave **7** (11.9 g, 77%, bp 125—126 °C (8 mmHg)) as an oil. *Anal.* Calcd for $C_{14}H_{22}OSi$: C, 71.73; H, 9.46. Found: C, 71.51; H, 9.42. IR ($CHCl_3$): 2950, 1455, 1250 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: −0.02 (9H, s), 1.53 (2H, s), 3.81 (2H, br s), 4.44 (2H, s), 4.66 (1H, br s), 4.86 (1H, br s), 7.30 (5H, s). MS *m/z*: 235 (M^++1), 221, 181, 179, 143, 131, 115, 105, 91 (100%).

(3*S*,4*S*)-4-Acetoxy-3-phthalimido-1-trimethylsilyl-2-azetidinone [(3*S*,4*S*)-8] A mixture of (3*S*,4*S*)-4-acetoxy-3-phthalimido-2-azetidinone[1,4] [(3*S*,4*S*)-**4**, mp 184—188 °C (dec.), $[\alpha]_D^{25}$ −56.0° (c=0.48, acetone), 4.00 g, 14.6 mmol], triethylamine (1.60 g, 15.8 mmol), chlorotrimethylsilane (1.70 g, 15.6 mmol), and CH_2Cl_2 (130 ml) was stirred at 0 °C for 50 min. Then it was diluted with dry ether (400 ml) and allowed to warm to room temperature. The resulting precipitates were filtered off and the solvent was evaporated off to give (3*S*,4*S*)-**8** (4.98 g, 99%) as a solid, which was used for the next step without further purification.

(3*S*,4*S*)-4-(2-Benzyloxymethyl-2-propenyl)-3-phthalimido-2-azetidinone [(3*S*,4*S*)-9] A solution of (3*S*,4*S*)-**8** (4.98 g, 14.4 mmol), **7** (6.70 g, 30.0 mmol), and TMSOTf (0.5 g, 2.3 mmol) in 1,2-dichloroethane (30 ml) was heated at 45—50 °C for 5 h. After the mixture had been cooled to 0 °C, a saturated aqueous solution of $NaHCO_3$ was added, and the whole was stirred for 10 min. Then it was extracted with $CHCl_3$ (×3), and the organic layer was dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by column chromatography (150 g, AcOEt : hexane = 1 : 6, v/v) to afford (3*S*,4*S*)-**9** [4.32 g, 79% overall yield from (3*S*,4*S*)-**4**] as an oil. *Anal.* Calcd for $C_{22}H_{20}N_2O_4$: C, 70.20; H, 5.36; N, 7.44. Found: C, 69.98; H, 5.39; N, 7.29. IR ($CHCl_3$): 3420, 1780, 1760, 1720, 1390 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.3—2.7 (2H, m), 3.95 (2H, br s), 4.23 (1H, ddd, J=7, 6, 2 Hz), 4.46 (2H, s), 5.00 (1H, d, J=2 Hz), 5.02 (1H, br s), 5.15 (1H, br s), 6.4 (1H, br), 7.3 (5H, br s), 7.6—8.0 (4H,

m). MS *m/z*: 377 ($M^+ + 1$), 315, 286, 225, 160, 148, 95, 91, 80 (100%).

(3*S*,4*S*)-4-(2-Benzyloxymethyl-2-propenyl)-1-*tert*-butyldimethylsilyl-3-phthalimido-2-azetidinone [(3*S*,4*S*)-10] A solution of (3*S*,4*S*)-**9** (16.38 g, 43.6 mmol), triethylamine (6.91 g, 70.0 mmol), and *tert*-butylchlorodimethylsilane (10.30 g, 70.0 mmol) in DMF (220 ml) was stirred at room temperature for 10 h. The mixture was partitioned between PhH and a diluted aqueous solution of $NaHCO_3$. The organic layer was washed with water (×3) and brine (×1), successively. The extract was dried over Na_2SO_4, and the solvent was evaporated off to leave an oily residue, which was chromatographed (150 g, AcOEt : PhH = 1 : 12, v/v) to afford (3*S*,4*S*)-**10** (20.1 g, 94%) as an oil. *Anal.* Calcd for $C_{28}H_{34}N_2O_4Si$: C, 68.54; H, 6.98; N, 5.71. Found: C, 68.38; H, 7.05; N, 5.69. IR ($CHCl_3$): 2930, 1780, 1745, 1720, 1390, 1320 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.34 (6H, s), 1.06 (9H, s), 2.38 (1H, d, J = 14 Hz), 2.83 (1H, dd, J = 14, 5 Hz), 3.90 (2H, br s), 4.1—4.5 (1H, m), 4.39 (2H, s), 4.9—5.2 (3H, m), 7.2—7.5 (5H, m), 7.6—8.1 (4H, m). MS *m/z*: 491 ($M^+ + 1$), 475, 433, 331, 231, 189 (100%).

(3*S*,4*S*)-4-[(*R*)-3-Benzyloxy-2-(hydroxymethyl)propyl]-1-*tert*-butyldimethylsilyl-3-phthalimido-2-azetidinone (11a) and Its Side-Chain Epimer (11b) A solution of $BH_3 \cdot SMe_2$ (2 M solution in THF, 0.88 ml, 1.76 mmol) was added to a solution of (3*S*,4*S*)-**10** (865 mg, 1.76 mmol) in THF (8 ml) at 0 °C with stirring. The mixture was stirred at 0 °C for 50 min, and then at room temperature for 1.5 h. At the end of this period, the mixture was diluted with THF (30 ml). A solution of H_2O_2 (35%, w/v, 1 ml, 0.01 mol) and NaOH (0.1 g, 2.5 mmol) in water (1 ml) was added to this at 0 °C with stirring. After being stirred at 0 °C for 50 min, the mixture was partitioned between AcOEt and water. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to leave an oily residue, which was purified by flash column chromatography (15 g, AcOEt : PhH = 1 : 9—2 : 8, v/v) to afford the less polar isomer **11b** (387 mg, 43%) and the more polar one **11a** (210 mg, 24%) as oils. The spectral data of these alcohols are as follows.

11a: IR ($CHCl_3$): 3500, 1780, 1745, 1720, 1392 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.33 (6H, s), 1.03 (9H, s), 1.5—2.2 (3H, m), 3.3—3.7 (3H, m), 3.9—4.3 (2H, m), 4.30 (2H, s), 4.96 (1H, d, J = 3 Hz), 7.1—7.3 (5H, m), 7.6—8.0 (4H, m). MS *m/z*: 509 ($M^+ + 1$), 493, 451, 212, 204, 186, 113, 91 (100%). HRMS Calcd for $C_{28}H_{37}N_2O_5Si$ ($M^+ + 1$): 509.2470. Found: 509.2473.

11b: IR ($CHCl_3$): 3500, 1780, 1748, 1720, 1392 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.31 (3H, s), 0.34 (3H, s), 1.03 (9H, s), 1.5—2.3 (3H, m), 3.3—3.7 (3H, m), 3.9—4.4 (2H, m), 4.50 (2H, s), 4.93 (1H, d, J = 3 Hz), 7.32 (5H, br s), 7.6—8.0 (4H, m). MS *m/z*: 509 ($M^+ + 1$), 493, 451, 292, 212, 204, 113, 91 (100%). HRMS Calcd for $C_{28}H_{37}N_2O_5Si$ ($M^+ + 1$): 509.2470. Found: 509.2475.

(3*S*,4*S*)-4-[(*S*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-3-phthalimido-2-azetidinone (12a) *p*-Toluenesulfonyl chloride (1.00 g, 5.3 mmol) was added to a stirred solution of **11a** (1.00 g, 1.96 mmol) in pyridine (10 ml) at 0 °C, and stirring was continued for 5 h at room temperature. Then the solvent was evaporated off *in vacuo*, and the residue was partitioned between PhH and water. The organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by flash column chromatography (20 g, AcOEt : PhH = 1 : 9, v/v) to give **12a** (1.29 g, 99%) as an oil. IR ($CHCl_3$): 1780, 1740, 1720, 1390, 1172 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.30 (6H, s), 1.00 (9H, s), 1.2—2.2 (3H, m), 2.40 (3H, s), 3.0—3.5 (2H, m), 3.8—4.2 (3H, m), 4.16 (2H, s), 4.79 (1H, d, J = 3 Hz), 6.9—7.5 (7H, m), 7.5—8.0 (6H, m). MS *m/z*: 605 ($M^+ - C_4H_9$), 499, 469, 278, 222, 148, 131, 91 (100%). HRMS Calcd for $C_{31}H_{33}N_2O_7SSi$ ($M^+ - C_4H_9$): 605.1776. Found: 605.1765.

(3*S*,4*S*)-4-[(*R*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-3-phthalimido-2-azetidinone (12b) Following a procedure similar to that described for the preparation of **12a**, **11b** (1.80 g, 3.54 mmol) was tosylated to give **12b** (2.10 g, 89%) as a foam. IR ($CHCl_3$): 1780, 1750, 1720, 1390, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.26 (3H, s), 0.31 (3H, s), 1.02 (9H, s), 1.5—2.5 (3H, m), 2.39 (3H, s), 2.38 (2H, d, J = 5 Hz), 3.8—4.3 (3H, m), 4.40 (2H, s), 4.90 (1H, d, J = 3 Hz), 7.23 (2H, d, J = 9 Hz), 7.30 (5H, s), 7.67 (2H, d, J = 9 Hz), 7.6—8.0 (4H, m). MS *m/z*: 605 ($M^+ - C_4H_9$), 499, 469, 451, 229, 212, 186, 91 (100%). HRMS Calcd for $C_{31}H_{33}N_2O_7SSi$ ($M^+ - C_4H_9$): 605.1776. Found: 605.1775.

(3*S*,4*S*)-4-[(*S*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-3-formylamino-2-azetidinone (14a) Methylhydrazine (0.5 g, 11 mmol) was added to a solution of **12a** (1.00 g, 1.51 mmol) in CH_2Cl_2 (20 ml) at 0 °C with stirring. The mixture was stirred and allowed to warm to room temperature over a period of 3 h. Then it was diluted with CCl_4 (*ca.* 100 ml), and the solvent was evaporated off *in vacuo*, in order to remove excess methylhydrazine. The residue was dissolved in a mixture of CCl_4 (*ca.* 100 ml) and CH_2Cl_2 (*ca.* 20 ml) and the solvent was again evaporated off. After repeating this procedure two more times, the residue was dissolved again in CH_2Cl_2 (50 ml), and the mixture was stirred at room temperature for 12 h, during which time precipitates gradually emerged. To this slurry was added, at 0 °C, a solution of 1-formylimidazole, which had been obtained by mixing formic acid (206 mg, 4.5 mmol) and 1,1′-carbonyldiimidazole (726 mg, 4.5 mmol) in CH_2Cl_2 (1 ml). After 1 h, the mixture was partitioned between CH_2Cl_2 and a diluted aqueous solution of Na_2CO_3. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to give an oily residue, which was purified by flash column chromatography (15 g, AcOEt : PhH = 1 : 3, v/v) to give **14a** (0.62 g, 73%) as an oil. IR ($CHCl_3$): 3420, 1740, 1685, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.30 (6H, s), 0.94 (9H, s), 1.2—2.2 (3H, m), 2.41 (3H, s), 3.38 (1H, d, J = 5 Hz), 3.4—4.5 (2H, m), 4.08 (1H, d, J = 6 Hz), 4.38 (2H, s), 6.3 (1H, br), 7.2—7.5 (7H, m), 7.82 (2H, d, J = 9 Hz), 8.15 (1H, br s). MS *m/z*: 561 ($M^+ + 1$), 503, 418, 358, 267, 229, 91 (100%). HRMS Calcd for $C_{28}H_{41}N_2O_6SSi$ ($M^+ + 1$): 561.2452. Found: 561.2454.

(3*S*,4*S*)-4-[(*R*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-3-formylamino-2-azetidinone (14b) Following a procedure similar to that described for the preparation of **14a**, **12b** (2.10 g, 3.17 mmol) was dephthaloylated and then formylated, to give **14b** (1.00 g, 56%) as an oil. IR ($CHCl_3$): 3430, 1740, 1690, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.25 (3H, s), 0.26 (3H, s), 0.96 (9H, s), 1.53 (1H, ddd, J = 14, 12, 4 Hz), 1.96 (1H, ddd, J = 14, 9, 3 Hz), 2.3—2.5 (1H, m), 2.43 (3H, s), 3.37 (1H, d, J = 6 Hz), 3.67 (1H, dt, J = 12, 3 Hz), 4.04 (1H, dd, J = 10, 5 Hz), 4.08 (1H, dd, J = 10, 4 Hz), 4.38 (1H, d, J = 12 Hz), 4.41 (1H, d, J = 12 Hz), 4.43 (1H, dd, J = 7, 3 Hz), 6.0 (1H, br d, J = 7 Hz), 7.2—7.4 (7H, m), 7.76 (2H, d, J = 8 Hz), 8.13 (1H, d, J = 0.7 Hz). MS *m/z*: 560 ($M^+ + 1$), 503, 418, 358, 267, 229 (100%). HRMS Calcd for $C_{28}H_{40}N_2O_6SSi$: 560.2374. Found: 560.2369.

(*S*)-4-[(*S*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (16a) Trichloromethyl chloroformate (230 mg, 1.16 mmol) was added to a solution of **14a** (620 mg, 1.11 mmol) and triethylamine (570 mg, 5.6 mmol) in CH_2Cl_2 (32 ml) at −50 °C, and stirring was continued at −50 °C for 10 min. At the end of this period, a saturated aqueous solution of $NaHCO_3$ was added, and the mixture was stirred under ice-cooling for 5 min. The mixture was extracted with CH_2Cl_2, and the extract was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave the crude isocyanide **15a** (0.6 g) as a brown oil. This product was mixed with tributylstannane (650 mg, 2.23 mmol) and α,α′-azobisisobutyronitrile (23 mg, 0.14 mmol) in PhH (50 ml) under an atmosphere of argon, and the mixture was refluxed for 30 min. After cooling, the concentrated mixture was purified by flash column chromatography (15 g, AcOEt : hexane = 1 : 19, v/v) to give **16a** (443 mg, 77%) as a solid. Recrystallization from Et_2O–hexane gave an analytical sample as colorless needles, mp 79—80 °C. *Anal.* Calcd for $C_{27}H_{39}NO_5SSi$: C, 62.63; H, 7.59; N, 2.71; S, 6.19. Found: C, 62.58; H, 7.54; N, 2.63; S, 6.36. IR ($CHCl_3$): 1720, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.19 (6H, s), 0.95 (9H, s), 1.2—2.1 (3H, m), 2.41 (3H, s), 2.43 (1H, dd, J = 15, 3 Hz), 3.07 (1H, dd, J = 15, 5 Hz), 3.2—3.7 (1H, m), 3.40 (1H, d, J = 5 Hz), 4.03 (1H, d, J = 5 Hz), 4.40 (2H, s), 7.1—7.5 (7H, m), 7.81 (2H, d, J = 9 Hz). MS *m/z*: 517 (M^+), 460, 418, 229, 105 (100%).

(*S*)-4-[(*R*)-2-Benzyloxymethyl-3-(*p*-toluenesulfonyloxy)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (16b) Following a procedure similar to that described for the preparation of **16a**, **14b** (1.03 g, 1.84 mmol) was dehydrated and then decyanated to give **16b** (0.81 g, 85%) as an oil. IR ($CHCl_3$): 1725, 1360, 1175 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.20 (3H, s), 0.21 (3H, s), 0.94 (9H, s), 1.5—1.8 (1H, s), 1.8—2.0 (2H, m), 2.43 (3H, s), 2.54 (1H, dd, J = 15, 3 Hz), 3.07 (1H, dd, J = 15, 5 Hz), 3.33 (1H, dd, J = 10, 6 Hz), 3.36 (1H, dd, J = 10, 4 Hz), 3.56 (1H, dddd, J = 12, 5, 3, 3 Hz), 4.05 (1H, dd, J = 10, 5 Hz), 4.07 (1H, dd, J = 10, 5 Hz), 4.38 (2H, s), 7.2—7.4 (7H, m), 7.77 (2H, d, J = 9 Hz). MS *m/z*: 460 ($M^+ - C_4H_9$), 418, 229, 91 (100%). HRMS Calcd for $C_{23}H_{30}NO_5SSi$ ($M^+ - C_4H_9$): 460.1612. Found: 460.1631.

(*S*)-4-[(*S*)-3-Benzyloxy-2-(iodomethyl)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (17a) A mixture of **16a** (430 mg, 0.83 mmol), NaI (1.7 g, 11 mmol), and acetone (17 ml) was refluxed for 2 h. The mixture was partitioned between AcOEt and water, and the organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by flash column chromatography (6 g, AcOEt : hexane = 1 : 9, v/v) to afford **17a** (398 mg, 100%) as an oil. IR ($CHCl_3$): 2950, 1725, 1315, 1192, 1090, 1000, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.22 (6H, s), 0.95 (9H, s), 1.2—2.2 (3H, m), 2.60 (1H, dd,

$J=15$, 3 Hz), 3.15 (1H, dd, $J=15$, 5 Hz), 3.0—3.7 (1H, m), 3.36 (2H, d, $J=5$ Hz), 3.46 (2H, d, $J=5$ Hz), 4.50 (2H, s), 7.33 (5H, s). MS *m/z*: 458 (M^+-CH_3), 416 ($M^+-C_4H_9$), 374, 203, 91 (100%). HRMS Calcd for $C_{19}H_{29}INO_2Si$ (M^+-CH_3): 458.1011. Found: 458.1020.

(*S*)-4-[(*R*)-3-Benzyloxy-2-(iodomethyl)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (17b) Following a procedure similar to that described for the preparation of **17a**, **16b** (90 mg, 0.174 mmol) was treated with NaI in acetone to give **17b** (70 mg, 85%) as an oil. IR ($CHCl_3$): 2950, 1725, 1310, 1195, 1100, 1000, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.25 (3H, s), 0.26 (3H, s), 0.96 (9H, s), 1.2—1.8 (2H, m), 1.8—2.0 (1H, m), 2.61 (1H, dd, $J=15$, 3 Hz), 3.13 (1H, dd, $J=15$, 5 Hz), 3.24 (1H, dd, $J=10$, 4 Hz), 3.31 (1H, dd, $J=10$, 7 Hz), 3.37 (1H, dd, $J=10$, 5 Hz), 3.47 (1H, dd, $J=10$, 4 Hz), 3.57 (1H, dddd, $J=11$, 6, 3, 3 Hz), 4.51 (2H, s), 7.2—7.5 (5H, m). MS *m/z*: 474 ($M^+ + 1$), 458, 416, 374, 91 (100%). HRMS Calcd for $C_{20}H_{33}INO_2Si$ ($M^+ + 1$): 474.1324. Found: 474.1319.

(*S*)-4-[(*S*)-3-Benzyloxy-2-(iodomethyl)propyl]-2-azetidinone (18a) A solution of **17a** (398 mg, 0.84 mmol), Bu_4NF (1.0 M solution in THF, 0.90 ml, 0.90 mmol), and AcOH (160 mg, 2.7 mmol) in THF (7 ml) was stirred at 0 °C for 1 h. The mixture was partitioned between AcOEt and a diluted aqueous solution of $NaHCO_3$, and the organic layer was washed with brine. The extract was dried over Na_2SO_4, and the solvent was evaporated off to leave an oily residue, which was purified by flash column chromatography (6 g, AcOEt : PhH = 3 : 1, v/v) to afford **18a** (302 mg, 100%) as a solid. Recrystallization from Et_2O–hexane gave an analytical sample as colorless needles, mp 76—77 °C. *Anal.* Calcd for $C_{14}H_{18}INO_2$: C, 46.81; H, 5.05; N, 3.90; I, 35.33. Found: C, 47.10; H, 5.22; N, 3.94; I, 35.60. IR ($CHCl_3$): 3400, 1745, 1090 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.6—1.8 (3H, m), 2.63 (1H, ddd, $J=15$, 3, 1 Hz), 3.09 (1H, ddd, $J=15$, 5, 2 Hz), 3.25 (1H, dd, $J=10$, 5 Hz), 3.36 (1H, dd, $J=10$, 5 Hz), 3.40 (1H, dd, $J=9$, 7 Hz), 3.45 (1H, dd, $J=9$, 4 Hz), 3.69 (1H, dddd, $J=11$, 6, 6, 3 Hz), 4.52 (2H, s), 5.9 (1H, br), 7.3—7.4 (5H, m). MS *m/z*: 360 ($M^+ + 1$), 331, 314, 226, 204, 190, 126, 91 (100%).

(*S*)-4-[(*R*)-3-Benzyloxy-2-(iodomethyl)propyl]-2-azetidinone (18b) Following a procedure similar to that described for the preparation of **18a**, **17b** (64 mg, 0.13 mmol) was desilylated to give **18b** (47 mg, 97%) as an oil. IR ($CHCl_3$): 3420, 1750, 1090 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.6—1.8 (3H, m), 2.58 (1H, ddd, $J=15$, 2, 1 Hz), 3.08 (1H, ddd, $J=15$, 5, 2 Hz), 3.27 (1H, dd, $J=10$, 5 Hz), 3.36 (1H, dd, $J=10$, 5 Hz), 3.41 (1H, dd, $J=10$, 5 Hz), 3.48 (1H, dd, $J=10$, 5 Hz), 3.70 (1H, dddd, $J=7$, 7, 5, 2 Hz), 4.51 (2H, s), 5.9 (1H, br), 7.3—7.4 (5H, m). MS *m/z*: 360 ($M^+ + 1$), 314, 190, 126, 91 (100%). HRMS Calcd for $C_{14}H_{19}INO_2$ ($M^+ + 1$): 360.0460. Found: 360.0459.

(3*R*,5*S*)-3-Benzyloxymethyl-1-azabicyclo[3.2.0]heptan-7-one (19a) A mixture of **18a** (52 mg, 0.14 mmol), powdered K_2CO_3 (35 mg, 0.25 mmol), and DMF (0.9 ml) was vigorously stirred at room temperature for 18 h. The mixture was partitioned between PhH and a phosphate buffer solution (pH 7.0), and the organic layer was washed with water and brine. The extract was dried over Na_2SO_4 and the solvent was evaporated off to leave an oily residue, which was purified by preparative TLC (AcOEt : hexane = 2 : 3, v/v) to give **19a** (22.9 mg, 68%) as an oil. IR ($CHCl_3$): 1745, 1340 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.67 (1H, ddd, $J=13$, 9, 7 Hz), 2.05 (1H, ddd, $J=13$, 7, 4 Hz), 2.58 (1H, dd, $J=16$, 2 Hz), 2.69 (1H, ddd, $J=12$, 5, 1 Hz), 2.82 (1H, m), 3.23 (1H, ddd, $J=16$, 5, 1 Hz), 3.40 (1H, dd, $J=9$, 6 Hz), 3.44 (1H, dd, $J=9$, 6 Hz), 3.74 (1H, dddd, $J=7$, 7, 5, 2 Hz), 3.79 (1H, dd, $J=12$, 7 Hz), 4.51 (2H, s), 7.2—7.4 (5H, m). MS *m/z*: 231 (M^+), 203, 189, 91 (100%). HRMS Calcd for $C_{14}H_{17}NO_2$: 231.1258. Found: 231.1266.

(3*S*,5*S*)-3-Benzyloxymethyl-1-azabicyclo[3.2.0]heptan-7-one (19b) Following a procedure similar to that described for the preparation of **19a**, **18b** (43 mg, 0.12 mmol) was cyclized to give **19b** (22.7 mg, 82%) as an oil. IR ($CHCl_3$): 1740, 1346 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.24 (1H, ddd, $J=12$, 11, 9 Hz), 2.28 (1H, ddd, $J=12$, 6, 6 Hz), 2.60 (1H, dd, $J=15$, 2 Hz), 2.85 (1H, ddddd, $J=14$, 10, 8, 6, 6 Hz), 3.06 (1H, ddd, $J=11$, 8, 1 Hz), 3.20 (1H, ddd, $J=15$, 5, 1 Hz), 3.35 (1H, dd, $J=11$, 8 Hz), 3.46 (2H, d, $J=6$ Hz), 3.68 (1H, dddd, $J=9$, 6, 5, 2 Hz), 4.51 (2H, s), 7.2—7.5 (5H, m). MS *m/z*: 231 (M^+), 125, 107, 91 (100%). HRMS Calcd for $C_{14}H_{17}NO_2$: 231.1258. Found: 231.1263.

(3*S*,4*S*)-4-(2-Benzyloxymethyl-2-propenyl)-1-*tert*-butyldimethylsilyl-3-formylamino-2-azetidinone [(3*S*,4*S*)-21] Following a procedure similar to that described for the preparation of **14a**, (3*S*,4*S*)-**10** (20.1 g, 41 mmol) was dephthaloylated and then formylated to give (3*S*,4*S*)-**21** (7.62 g, 64%) as an oil. IR ($CHCl_3$): 3440, 1740, 1690 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.33 (6H, s), 1.05 (9H, s), 2.2—3.0 (2H, m), 3.93 (2H, br s), 4.0—4.5 (1H, m), 4.40 (2H, s), 4.7—5.2 (3H, m), 6.3 (1H, br), 7.1—7.5 (5H, m), 8.15 (1H, br s). MS *m/z*: 389 ($M^+ + 1$), 331, 303, 246, 227, 186, 140, 123, 91 (100%). HRMS Calcd for $C_{21}H_{33}N_2O_3Si$ ($M^+ + 1$): 389.2259. Found: 389.2263.

(*S*)-4-(2-Benzyloxymethyl-2-propenyl)-1-*tert*-butyldimethylsilyl-2-azetidinone [(*S*)-23] Following a procedure similar to that described for the preparation of **16a**, (3*S*,4*S*)-**21** (2.31 g, 6.0 mmol) was dehydrated and then decyanated to give (*S*)-**23** (1.66 g, 81%) as an oil. *Anal.* Calcd for $C_{20}H_{31}NO_2Si$: C, 69.52; H, 9.04, N, 4.05. Found: C, 69.29; H, 9.27; N, 4.09. IR ($CHCl_3$): 1720, 1460, 1330, 1255, 1188, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.23 (3H, s), 0.24 (3H, s), 0.96 (9H, s), 2.12 (1H, br dd, $J=14$, 11 Hz), 2.65 (1H, dd, $J=15$, 3 Hz), 2.73 (1H, br dd, $J=14$, 3 Hz), 3.11 (1H, dd, $J=15$, 5 Hz), 3.72 (1H, dddd, $J=11$, 5, 3, 3 Hz), 3.94 (2H, br s), 4.49 (2H, br s), 4.93 (1H, br s), 5.12 (1H, br s), 7.2—7.5 (5H, m). MS *m/z*: 346 ($M^+ + 1$), 288, 246, 181, 142, 100, 91 (100%), 73.

(*S*)-4-[(*R*)-3-Benzyloxy-2-(hydroxymethyl)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (24a) and Its Side-Chain Epimer (24b) Following a procedure similar to that described for the preparation of **11a** and **11b**, (*S*)-**23** (119 mg, 0.34 mmol) was hydroborated to give a diastereomeric mixture of alcohols, which was separated by flash column chromatography (2 g, AcOEt : PhH = 1 : 4—2 : 3, v/v) to afford the less polar isomer **24b** (42 mg, 34%) and the more polar one **24a** (34 mg, 27%) as oils. The spectral data of these alcohols are as follows.

24a: IR ($CHCl_3$): 3400, 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.21 (6H, s), 0.95 (9H, s), 1.46 (1H, ddd, $J=13$, 11, 4 Hz), 1.7—1.9 (1H, m), 1.96 (1H, ddd, $J=13$, 9, 3 Hz), 2.63 (1H, dd, $J=15$, 3 Hz), 3.13 (1H, dd, $J=15$, 5 Hz), 3.51 (1H, dd, $J=9$, 6 Hz), 3.5—3.8 (4H, m), 4.50 (1H, d, $J=11$ Hz), 4.53 (1H, d, $J=11$ Hz), 7.3—7.4 (5H, m). MS *m/z*: 364 ($M^+ + 1$), 348, 322, 306, 264, 91 (100%). HRMS Calcd for $C_{20}H_{34}NO_3Si$ ($M^+ + 1$): 346.2306. Found: 364.2303.

24b: IR ($CHCl_3$): 3400, 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.22 (6H, s), 0.96 (9H, s), 1.37 (1H, ddd, $J=13$, 11, 4 Hz), 1.7—1.9 (1H, m), 1.99 (1H, ddd, $J=13$, 9, 4 Hz), 2.59 (1H, dd, $J=15$, 3 Hz), 3.13 (1H, dd, $J=15$, 5 Hz), 3.46 (1H, dd, $J=9$, 6 Hz), 3.5—3.7 (2H, m), 3.57 (1H, dd, $J=9$, 4 Hz), 3.72 (1H, dd, $J=7$, 3 Hz), 4.50 (1H, d, $J=12$ Hz), 4.53 (1H, d, $J=12$ Hz), 7.3—7.4 (5H, m). MS *m/z*: 364 ($M^+ + 1$), 348, 306, 264, 91 (100%). HRMS Calcd for $C_{20}H_{34}NO_3Si$ ($M^+ + 1$): 364.2306. Found: 364.2298.

Conversion of the Alcohols 24a, b to the Tosylates 16a, b Following a procedure similar to that described for the conversion of **11a** to **12a**, the alcohols **24a** and **24b** were tosylated separately to give the corresponding tosylates **16a** and **16b**, respectively. The products (yields: 85 and 87%) were identical with **16a** and **16b** obtained from **14a** and **14b**, respectively.

(*S*)-4-[(*S*)-2-Benzyloxymethyl-3-(isobutyryloxy)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (25) Isobutyryl chloride (87 mg, 0.82 mmol) was added to a stirred solution of **24a** (240 mg, 0.66 mmol) in pyridine (2.1 ml) at 0 °C, and stirring was continued at 0 °C for 1 h. Then a saturated aqueous solution of $NaHCO_3$ was added, and the mixture was stirred for 10 min. The mixture was partitioned between Et_2O and water, and the organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by column chromatography (2.5 g, AcOEt : PhH = 1 : 19, v/v) to afford **25** (257 mg, 90%) as an oil. IR ($CHCl_3$): 1730 (sh), 1720 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.21 (6H, s), 0.95 (9H, s), 1.15 (6H, d, $J=7$ Hz), 1.3—1.6 (1H, m), 1.9—2.1 (2H, m), 2.53 (1H, septet, $J=7$ Hz), 2.58 (1H, dd, $J=15$, 2 Hz), 3.13 (1H, dd, $J=15$, 5 Hz), 3.42 (2H, d, $J=4$ Hz), 3.5—3.7 (1H, m), 4.05 (1H, dd, $J=11$, 6 Hz), 4.07 (1H, dd, $J=11$, 6 Hz), 4.49 (2H, s), 7.2—7.4 (5H, m). MS *m/z*: 434 ($M^+ + 1$), 418, 376, 334, 306, 264, 145, 91 (100%). HRMS Calcd for $C_{24}H_{40}NO_4Si$ ($M^+ + 1$): 434.2724. Found: 434.2721.

(*S*)-1-*tert*-Butyldimethylsilyl-4-[(*R*)-2-(*tert*-butyldimethylsilyl)oxymethyl-3-(isobutyryloxy)propyl]-2-zaetidinone (27) A suspension of palladium black in water (*ca.* 0.2 ml) was added to a solution of **25** (26 mg, 0.060 mmol) in EtOH (2 ml), and the mixture was stirred at room temperature under an atmosphere of H_2 (1 atm) for 1 h. Then the mixture was filtered and the solvent was evaporated off to give an oily residue, which was immediately mixed with *tert*-butylchlorodimethylsilane (30 mg, 0.19 mmol) and imidazole (30 mg, 0.44 mmol) in DMF (1 ml) at 0 °C. The mixture was stirred at room temperature for 10 min, then a saturated aqueous solution of $NaHCO_3$ was added, and the mixture was extracted with PhH. The extract was successively washed with water and brine, and then dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by column chromatography (0.5 g, AcOEt : PhH = 1 : 19, v/v) to afford **27** (17.5 mg, 64%) as an oil. IR ($CHCl_3$): 1720, 1470, 1255, 1190, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.04 (6H, s), 0.23 (6H, s), 0.89 (9H, s), 0.95 (9H, s), 1.17 (6H, d, $J=7$ Hz), 1.37 (1H, ddd, $J=13$, 11, 4 Hz), 1.7—1.9 (1H, m), 2.03 (1H, ddd, $J=12$, 9, 3 Hz), 2.55 (1H,

septet, $J=7$ Hz), 2.59 (1H, dd, $J=15$, 3 Hz), 3.14 (1H, dd, $J=15$, 5 Hz), 3.59 (2H, d, $J=5$ Hz), 3.66 (1H, dddd, $J=11$, 5, 3, 3 Hz), 3.99 (1H, dd, $J=11$, 6 Hz), 4.01 (1H, dd, $J=11$, 7 Hz). MS *m/z*: 458 ($M^+ + 1$), 442, 400, 358 (100%). HRMS Calcd for $C_{23}H_{48}NO_4Si_2$ ($M^+ + 1$): 458.3119. Found: 458.3123.

(*S*)-4-[(*R*)-2-Benzyloxymethyl-3-(*tert*-butyldimethylsilyloxy)propyl]-1-*tert*-butyldimethylsilyl-2-azetidinone (28) A solution of **24b** (0.48 g, 1.3 mmol), *tert*-butylchlorodimethylsilane (0.42 g, 2.8 mmol) and imidazole (0.196 g, 2.8 mmol) in DMF (12 ml) was stirred at 0 °C for 40 min. Then a saturated aqueous solution of $NaHCO_3$ was added, and the mixture was extracted with PhH. The extract was successively washed with water and brine, and then dried over Na_2SO_4. Evaporation of solvent gave an oily residue, which was purified by column chromatography (10 g, AcOEt : PhH = 1 : 19, v/v) to give **28** (0.60 g, 95%) as an oil. IR ($CHCl_3$): 1725, 1255, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.04 (6H, s), 0.22 (6H, s), 0.88 (9H, s), 0.95 (9H, s), 1.40 (1H, ddd, $J=13$, 11, 5 Hz), 1.7—1.8 (1H, m), 1.99 (1H, ddd, $J=14$, 9, 4 Hz), 2.61 (1H, dd, $J=15$, 3 Hz), 3.10 (1H, dd, $J=15$, 5 Hz), 3.34 (1H, dd, $J=9$, 7 Hz), 3.42 (1H, dd, $J=9$, 7 Hz), 3.60 (2H, d, $J=5$ Hz), 3.65 (1H, dddd, $J=14$, 5, 3, 3 Hz), 4.47 (2H, s), 7.2—7.4 (5H, m). MS *m/z*: 462 ($M^+ - CH_3$), 420 ($M^+ - C_4H_9$), 378, 91 (100%). HRMS Calcd for $C_{25}H_{44}NO_3Si_2$ ($M^+ - CH_3$): 462.2857. Found: 462.2858.

(*S*)-4-[(*R*)-2-Benzyloxymethyl-3-(*tert*-butyldimethylsilyloxy)propyl]-2-azetidinone (29) A solution of NaOH (0.1 g, 2.5 mmol) in water (1 ml) was added to a solution of **28** (0.60 g, 1.26 mmol) in MeOH (20 ml) at −15 °C with stirring. After being stirred at −15 °C for 13 h, the mixture was partitioned between Et_2O and water, and the organic layer was washed with a saturated aqueous solution of NH_4Cl and brine, successively. The extract was dried over Na_2SO_4, and the solvent was evaporated off, to leave an oily residue, which was purified by column chromatography to afford **29** (0.46 g, 100%) as an oil. IR ($CHCl_3$): 3425, 1750, 1190, 840 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.04 (6H, s), 0.88 (9H, s), 1.5—2.0 (3H, m), 2.54 (1H, ddd, $J=15$, 3.5, 1.5 Hz), 3.05 (1H, ddd, $J=15$, 5, 2 Hz), 3.40 (1H, dd, $J=9$, 6 Hz), 3.45 (1H, dd, $J=9$, 5 Hz), 3.5—3.7 (2H, m), 3.6—3.8 (1H, m), 4.48 (2H, s), 5.9 (1H, br), 7.2—7.4 (5H, m). MS *m/z*: 348 ($M^+ - CH_3$), 335, 321, 306 ($M^+ - C_4H_9$), 264, 91 (100%). HRMS Calcd for $C_{19}H_{30}NO_3Si$ ($M^+ - CH_3$): 348.1993. Found: 348.1984.

(*S*)-4-[(*R*)-3-(*tert*-Butyldimethylsilyloxy)-2-(hydroxymethyl)propyl]-2-azetidinone (30) (a) A solution of **27** (22 mg, 0.048 mmol) and NaOH (10% in water, w/v, 0.1 ml, 0.25 mmol) in MeOH (1 ml) was stirred at 0 °C for 7 h. The mixture was partitioned between Et_2O and water, and the organic layer was washed with brine, and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by column chromatography (0.5 g, AcOEt) to give **30** (13 mg, 99%) as an oil. IR ($CHCl_3$): 3400, 1750, 835 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.90 (9H, s), 1.5—1.9 (3H, m), 2.61 (1H, dd, $J=15$, 2 Hz), 3.11 (1H, dd, $J=15$, 5 Hz), 3.6—3.8 (5H, m), 5.9 (1H, br). MS *m/z*: 274 ($M^+ + 1$), 216, 174 (100%). HRMS Calcd for $C_{13}H_{28}NO_3Si$ ($M^+ + 1$): 274.1837. Found: 274.1819. (b) A mixture of **29** (59 mg, 0.16 mmol), 10% Pd/C (60 mg), and MeOH (0.8 ml) was stirred at room temperature under an atmosphere of H_2 (1 atm) for 17 h. Then it was filtered and the solvent was evaporated off to leave an oily residue, which was purified as described in (a) to give **30** (43 mg, 97%) as an oil.

(*S*)-4-[(*S*)-2-(*tert*-Butyldimethylsilyloxy)methyl-3-(methanesulfonyloxy)-propyl]-2-azetidinone (31) Methanesulfonyl chloride (50 mg, 0.44 mmol) was added to a solution of **30** (69 mg, 0.25 mmol) and triethylamine (78 mg, 0.77 mmol) in CH_2Cl_2 (1 ml) at 0 °C with stirring. After 10 min, the reaction was quenched by adding a saturated aqueous solution of $NaHCO_3$, and the mixture was partitioned between Et_2O and water. The organic layer was washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was purified by column chromatography to afford **31** (80 mg, 90%) as an oil. IR ($CHCl_3$): 3400, 1750, 1355, 1170, 830 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s), 0.90 (9H, s), 1.71 (1H, dt, $J=14$, 7 Hz), 1.76 (1H, ddd, $J=14$, 7, 6 Hz), 1.97 (1H, triplet of quintets, $J=7$, 5 Hz), 2.60 (1H, ddd, $J=15$, 2, 1 Hz), 3.02 (3H, s), 3.13 (1H, ddd, $J=15$, 5, 2 Hz), 3.59 (1H, dd, $J=15$, 5 Hz), 3.66 (1H, dd, $J=10$, 5 Hz), 3.77 (1H, dddd, $J=7$, 6, 5, 2 Hz), 4.21 (1H, dd, $J=10$, 5 Hz), 4.25 (1H, dd, $J=10$, 5 Hz), 5.95 (1H, br). MS *m/z*: 310, 294 ($M^+ - C_4H_9$), 252 (100%). HRMS Calcd for $C_{10}H_{20}NO_5SSi$ ($M^+ - C_4H_9$): 294.0830. Found: 294.0835.

(*S*)-4-[(*S*)-3-(*tert*-Butyldimethylsilyloxy)-2-(iodomethyl)propyl]-2-azetidinone (32) Following a procedure similar to that described for the preparation of **17a**, **31** (78 mg, 0.22 mmol) was treated with NaI in acetone to give **32** (75 mg, 88%) as an oil. IR ($CHCl_3$): 3420, 1755, 1100, 840 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 0.08 (6H, s), 0.90 (9H, s), 1.4—1.8 (3H, m), 2.65 (1H, ddd, $J=15$, 2, 1 Hz), 3.12 (1H, ddd, $J=15$, 5, 2 Hz), 3.21 (1H, dd, $J=10$, 5 Hz), 3.33 (1H, dd, $J=10$, 5 Hz), 3.52 (1H, dd, $J=10$, 6 Hz), 3.59 (1H, dd, $J=10$, 5 Hz), 3.72 (1H, dddd, $J=7$, 6, 5, 2 Hz), 5.9 (1H, br). MS *m/z*: 384 ($M^+ + 1$). 326, 284 (100%). HRMS Calcd for $C_{13}H_{27}INO_2Si$ ($M^+ + 1$): 384.0855. Found: 384.0851.

(3*R*,5*S*)-3-(*tert*-Butyldimethylsilyl)oxymethyl-1-azabicyclo[3.2.0]heptan-7-one (33) Following a procedure similar to that described for the preparation of **19a**, **32** (261 mg, 0.68 mmol) was cyclized to give **33** (126 mg, 72%) as an oil. IR ($CHCl_3$): 1740, 835 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.05 (6H, s), 0.89 (9H, s), 1.63 (1H, ddd, $J=13$, 8, 7 Hz), 2.04 (1H, ddd, $J=13$, 7, 4 Hz), 2.57 (1H, dd, $J=15$, 2 Hz), 2.6—2.8 (1H, m), 2.68 (1H, dd, $J=8$, 4 Hz), 3.23 (1H, ddd, $J=15$, 5, 1 Hz), 3.56 (1H, dd, $J=10$, 6 Hz), 3.57 (1H, dd, $J=10$, 6 Hz), 3.7—3.8 (2H, m). MS *m/z*: 256 ($M^+ + 1$), 240, 237, 212, 193, 156 (100%). HRMS Calcd for $C_{13}H_{26}NO_2Si$ ($M^+ + 1$): 256.1731. Found: 256.1735.

(3*R*,5*S*)-3-Hydroxymethyl-1-azabicyclo[3.2.0]heptan-7-one (3) A mixture of **33** (121 mg, 0.47 mmol), Bu_4NF (1.0 M solution in THF, 1.14 ml, 1.14 mmol), AcOH (41 mg, 0.68 mmol), and THF (0.7 ml) was stirred at room temperature for 3 h. Then, the mixture was charged on a column of silica gel (2 g, in PhH) and eluted with AcOEt (150 ml). The eluates were combined and the solvent was evaporated to leave an oily residue, which was chromatographed again on silica gel (2 g, AcOEt : PhH = 2 : 3, v/v) to afford **3** (58 mg, 57%) as an oil, $[\alpha]_D^{25}$ −191° ($c=1.20$, $CHCl_3$). IR ($CHCl_3$): 3450, 1740, 1340, 1300, 1040 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.68 (1H, ddd, $J=13$, 8, 7 Hz), 2.07 (1H, ddd, $J=13$, 7, 4 Hz), 2.6—2.8 (2H, m), 2.61 (1H, dd, $J=16$, 2 Hz), 3.26 (1H, ddd, $J=16$, 5, 1 Hz), 3.5—3.75 (1H, m), 3.75—3.9 (2H, m). MS *m/z*: 141 (M^+), 124, 113, 99, 82, 80, 68, 67 (100%). HRMS Calcd for $C_7H_{11}NO_2$: 141.0789. Found: 141.0792.

References and Notes

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7) When a more active catalyst such as palladium black was used, we observed an abnormal ring cleavage reaction. The details will be reported elsewhere.

Stereoselective Total Synthesis of 16-Membered Macrolide Aglycons, Leuconolides and Maridonolides. Macrocyclic Stereocontrol Based on Conformational Analysis of the 16-Membered Macrolide Ring[1)]

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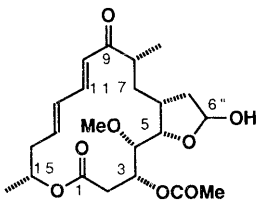
Sixteen-membered macrolide aglycons with different oxidation levels, leuconolide A_1 (3a), leuconolide A_3 (3b), midecanolide A_1 (3c), maridonolide II (4a), and maridonolide I (4b), were synthesized from two carbonolide type compounds (1, 2) by stereoselective reduction and epoxidation on the 16-membered ring system. The conformational analysis of macrolide rings based on nuclear magnetic resonance measurements and MMP2 calculations is also discussed in relation to the stereoselective synthesis of the five macrolide aglycons (3a—4b).

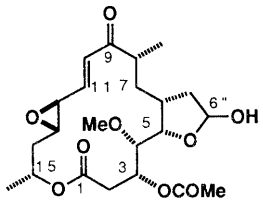
Keywords macrolide aglycon; total synthesis; leuconolide; maridonolide; conformation; conformational analysis; stereoselective reduction; NOE; MMP2 calculation

Macrolides and polyether antibiotics are interesting synthetic targets, and new synthetic methodologies have been developed to achieve their total synthesis, *i.e.* acyclic and macro ring stereocontrol, use of protecting groups, macrolactonization, *etc.*

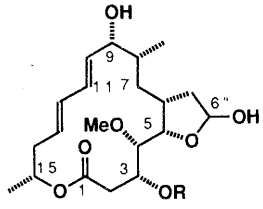
Recently[1,2)] we reported the total synthesis of 16-membered macrolide aglycons,[3)] carbonolide B (**1**) and carbonolide A (**2**), by virtue of the MPM (4-methoxyphenylmethyl) protection of hydroxy functions[4)] and some stereocontrolled reactions in an acyclic system. During the synthesis of **2**, we have been able to analyze the conformation of a 16-membered dienone compound using the combination of nuclear Overhauser enhancement (NOE) data and X-ray crystallography. The methodology was extended to the total synthesis of five 16-membered macrolide aglycons, leuconolide A_1 (**3a**),[5)] leuconolide A_3 (**3b**),[6)] midecanolide A_1 (**3c**),[7)] maridonolide I (**4b**), and maridonolide II (**4a**).[8,9)] Since these aglycons (**1**—**4**) have the same skeleton and only differ in their oxidation levels, the aglycons (**3**, **4**) were expected to be synthesized from the carbonolides (**1**, **2**) by stereoselective reduction and epoxidation. The conformational analysis of the 16-membered macrolide rings allowed us to achieve stereoselective synthesis of the five macrolide aglycons (**3a**—**4c**). The conformation of the 16-membered lactone ring plays a very important role in the stereoselectivity in such a large ring system.[10)] We focused on how to control the conformation by variation of the protection pattern of C3, C5, and C6″ hydroxy groups to obtain the desired stereoselection in reduction and epoxidation.



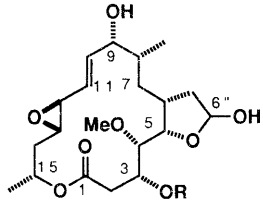
1: carbonolide B



2: carbonolide *A*



3a: R=H leuconolide A_1
3b: R=COMe leuconolide A_3
3c: R=COEt midecanolide A_1



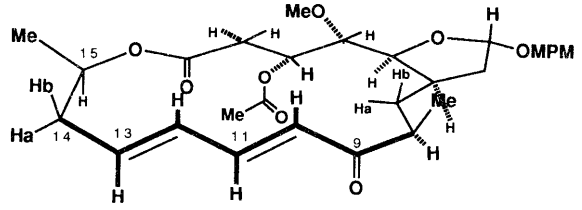
4a: R=COMe maridonolide II
4b: R=COEt maridonolide I

Fig. 1

Results and Discussion

Conformational Analysis of 16-Membered Macrolide Rings Combined with C9 Carbonyl Reduction[11)] The synthesis of leuconolides (**3a**—**c**) and maridonolides (**4a**, **b**) by the reduction of carbonolide B 4-methoxybenzylacetal (**5**) and carbonolide A 4-methoxybenzylacetal (**8**) was first examined.[12)] When **5** was treated with tetrabutylammonium borohydride in MeOH at 0 °C, a 1 : 1.8 mixture of the desired 9*R* alcohol (**6**)[13)] and its 9*S* isomer (**7**) was obtained. Reduction of **8** under the same conditions gave mainly the undesired 9*S* alcohol (**10**) with 22 : 1 selectivity. The stereochemistry of C9-alcohols was confirmed by comparing their *J*9,10 values with those in the reports by Grieco *et al.*[14)] and Freiberg *et al.*[15)]

On the basis of NOE and NOE spectroscopy (NOESY) measurements,[16)] this disappointing selectivity can be explained in terms of the unfavorable 9,10-*s-cis*, 11,12-*s-trans* (A) conformation. As can be seen from Fig. 2, the dienone group is almost at right angles to the 16-membered ring plane, so the inside of the ring (*si* face) is completely blocked. The reduction of the C9 ketone occurred from the less hindered peripheral (*re*) face to give mainly the undesired 9*S* alcohol (**7**). The hydride attack on the C9



5

Fig. 2

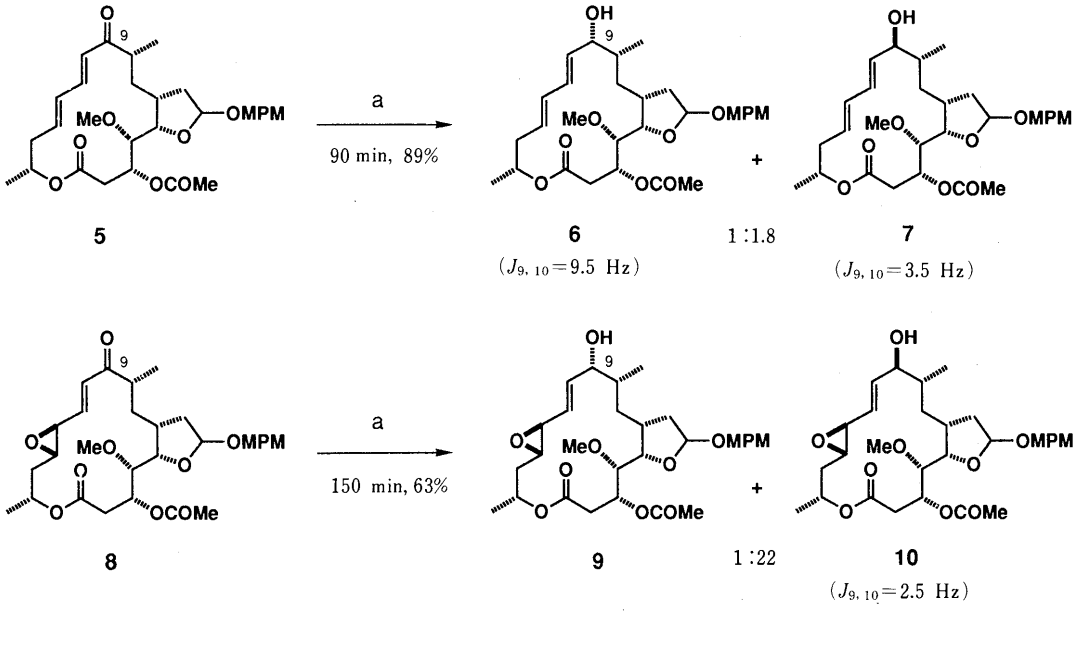


Chart 1

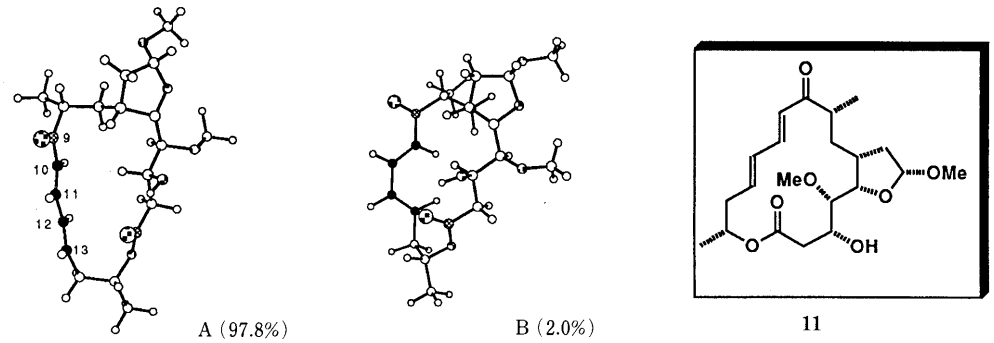


Fig. 3. MMP2–CONFLEX 2 Calculation of **11**

carbonyl was, however, hindered by the C8-methyl group and required a long reaction time. The MMP2 calculation combined with a systematic structure generation algorithm (CONFLEX)[17] supported this conclusion. Niddanolide methylacetal (**11**), with the same system, was calculated[18] to exist mainly in the A conformation (97.8%) together with the B conformer (9,10-*s*-*cis*, 11,12-*s*-*cis*; 2.0%) (Fig. 3).

We should completely reverse the stereoselectivity of the reduction to obtain the desired 9*R*-alcohol. In the 3,5-acetonide compound (**12**), A, B, C (9,10-*s*-*trans*, 11,12-*s*-*trans*), and A′ (the dienone portion inside out) conformers were observed by NOE and NOESY measurements (Fig. 4, 5). This result was supported by the MMP2–CONFLEX2 calculation for the 6-methyl 3,5-acetonide compound (**13**).[19] Compound **13** has interconvertible A (67.9%), B (10.5%), C (10.5%) and A′ (13H, 14Hb: *syn*, 8.8%) conformers. Typical computer drawings of conformers A, B, C, and A′ are shown in Fig. 6. In contrast to the A conformer giving the undesired 9*S* alcohol, the A′, B, and C conformers should be reduced much faster than the A conformer from the back side (*si* face) without steric hindrance to afford the desired 9*R* alcohol (Fig. 7). More than 30% of the 3,5-acetonide was found to exist as the desired conformers.

Synthesis of Leuconolides A_1 and A_3, and Midecanolide A_1 We sought to synthesize leuconolides A_1 (**3a**) and A_3 (**3b**), and midecanolide A_1 (**3c**) using reduction of the 6″-*O*-MPM-protected C3,C5-acetonide compound (**12**), which was synthesized from D-glucose *via* Yamaguchi's esterification of two fragments, **14** (C1—C10) and **15** (C11—C16), followed by Wittig–Horner cyclization as described in the previous paper.[1] When **12** was treated with sodium borohydride ($NaBH_4$) in methanol at 0 °C, a rapid reduction occurred stereoselectively to give the desired 9*R* alcohol (**16**) in 97% yield, and none of the stereoisomer was detected in the crude product. Chloroacetyl protection of **16** followed by 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) oxidation gave the primary alcohol (**18**) in 91% yield. The Swern oxidation and acid treatment with 1 N HCl–tetrahydrofuran (THF) (1 : 3) gave the hemiacetal compound (**20**), as a 1 : 1 mixture of anomers. The chloroacetyl group was hydrolyzed to afford leuconolide A_1 (**3a**) in 98% yield. Acetylation of the hemiacetal (**20**) gave the diacetates (**21**α, β), which were converted to leuconolide A_3 (**3b**) in 83% yield by careful alkaline treatment. Similarly, midecanolide A_1 (**3c**) was synthesized

NOE observed (%)

Signal irradiated

	1	2a	2b	3	4	5	6	7a	7b	8	8Me	9	10	11	12	13	14a	14b	15	16
1																				
2a			25.4																	
2b		19.5		3.0												3.2			2.2	
3			2.7		5.4	2.7														
4				6.5		5.4							2.7	1.1						
5				7.2	6.5		7.0			5.9										
6						NOE				NOE										
7a					NOE				NOE				NOE							
7b																				
8						NOE			NOE				NOE	NOE						
8Me										3.2			1.4							
9																				
10					2.4			3.2		2.4	0.9					7.0	2.4			
11										4.9						1.6	7.8			
12														NOE						
13																				
14a																NOE		NOE	NOE	
14b															8.9	3.5				2.0
15															1.9	3.2	3.2			1.6
16																			2.8	

Fig. 4. The Matrix of ^{1}H-NOE Obtained for **12** in $CDCl_3$ (NOE: Exact Measurement of NOE % Is Not Possible for Unresolved Peaks)

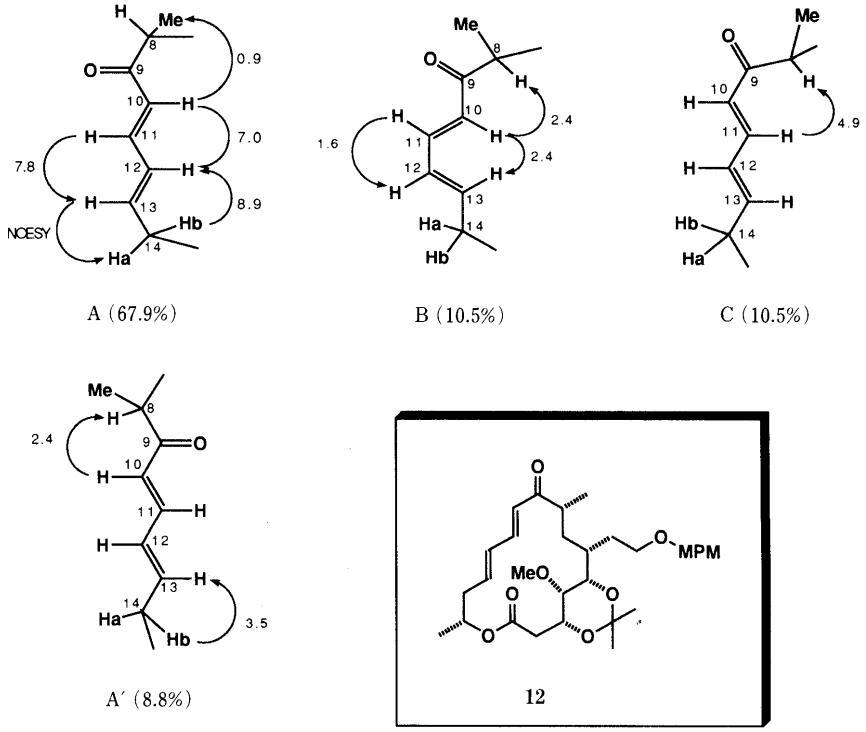


Fig. 5. NOE Correlation for the Conformational Isomers of **12** in $CDCl_3$

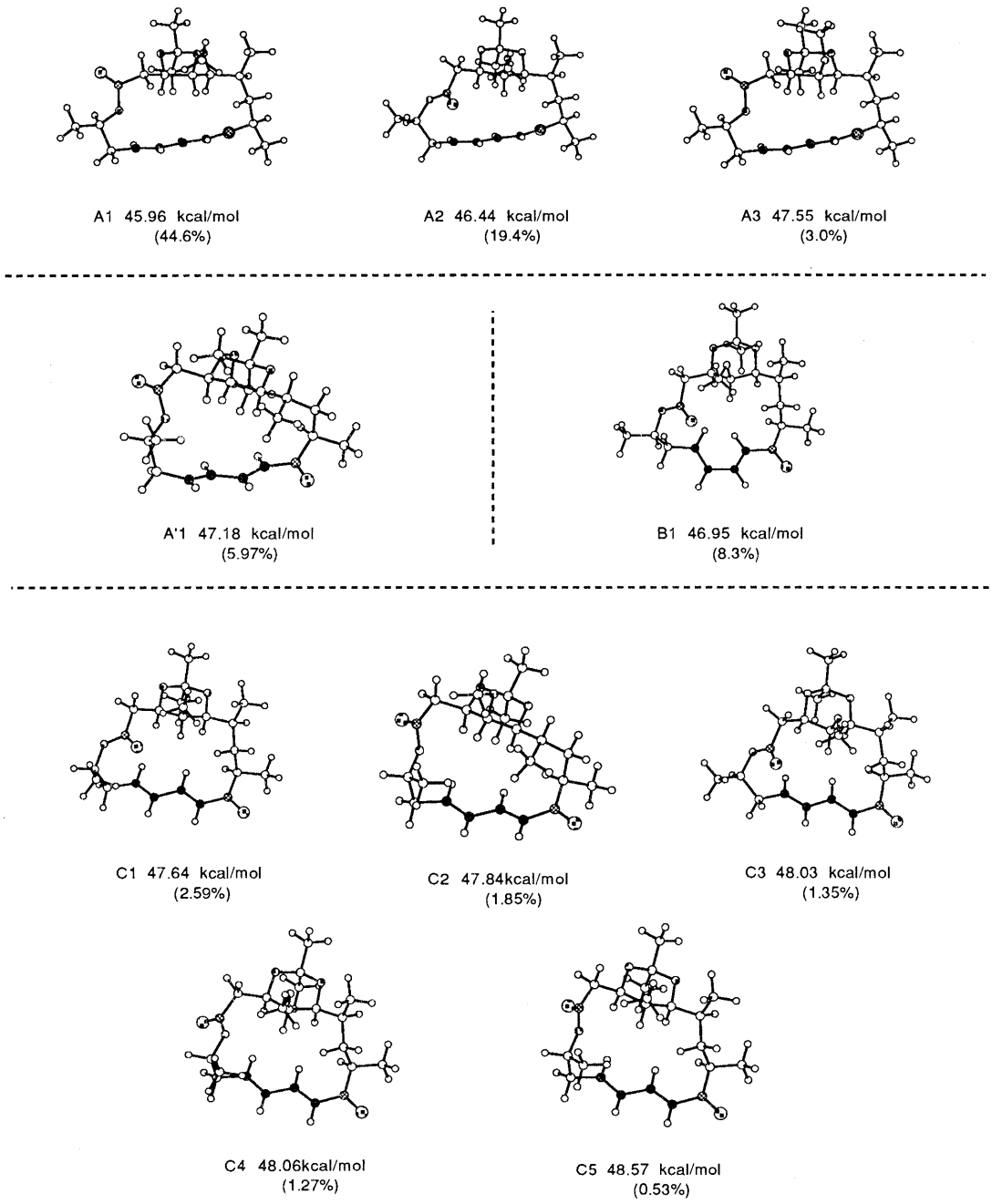


Fig. 6. Structures and Populations of Conformers by MMP2–CONFLEX 2 Calculation

from the dipropionates (**22**α, β).[20]

Synthesis of Maridonolides I and II We have succeeded in the total synthesis of carbonolide A (**2**), leuconolides (**3a**, **b**), and midecanolide A_1 (**3c**) based on the conformation-controlled reactions of 16-membered macrolide rings. This methodology has now been extended to the synthesis of maridonolide II (**4a**) and maridonolide I (**4b**), which are the aglycons of maridomycins II and I isolated from *Streptomyces hygroscopicus* No. B-5050.[8]

In order to synthesize maridonolides, two different routes, reduction of the epoxyenone (**23**) and epoxidation of the diene alcohols (**21**, **22**), were examined. Epoxidation of **12** with *meta*-chloroperbenzoic acid (MCPBA) gave the epoxide (**23**) in 22% yield, and the C10,11–C12,13-di-epoxide was concomitantly formed. A better result was obtained by using three conventional reactions; hydrolysis of the C3,C5-acetonide of **12** with 10-camphorsulfonic acid (CSA) in MeOH, MCPBA oxidation giving the desired β-epoxide in 56% yield, and acetonide formation with 2-methoxypropene and 0.1 eq of CSA to afford **23**.[21] When **23** was reduced with $NaBH_4$ in MeOH at 0 °C, the expected 9*R* alcohol (**24**) was obtained in quantitative yield. The alcohol was protected with a chloroacetyl group, and converted to the aldehyde (**27**) by DDQ deprotection of the MPM group and Swern oxidation. Careful hydrolysis of **27** to avoid opening of the epoxide ring with 2 N sulfuric acid–THF (1 : 20) at 0 °C for 10 h gave the hemiacetal (**28**), as a 1 : 1 mixture of anomers. The hemiacetal (**28**) was converted to the diacetate (**29**α, β) in the usual way, and then easily converted to maridonolide II (**4a**) by selective deprotection of the C9 chloroacetyl and C6″ acetyl groups under alkaline conditions. Maridonolide I (**4b**) was similarly

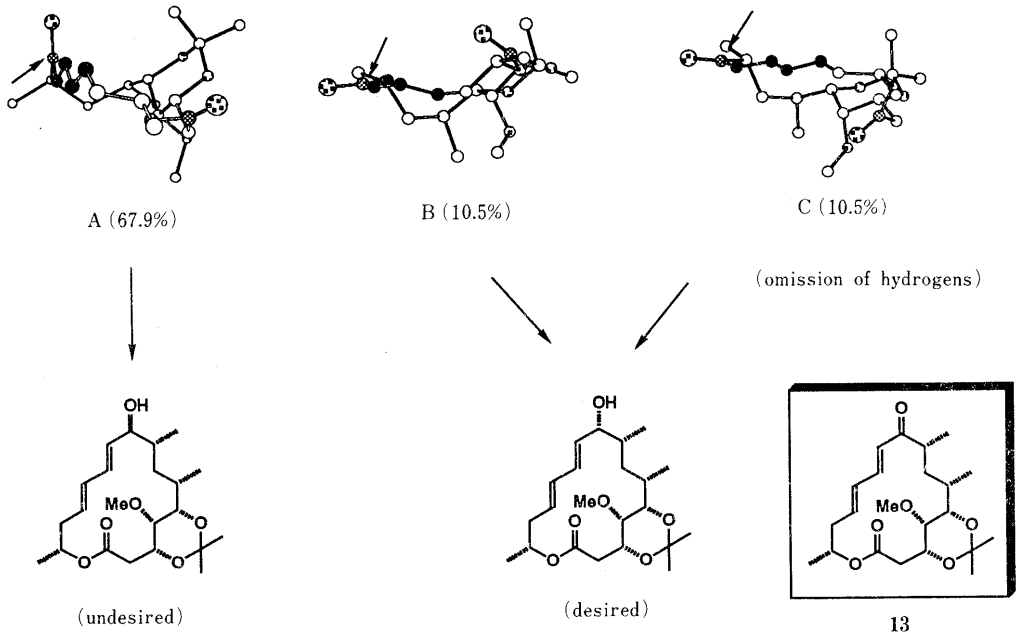
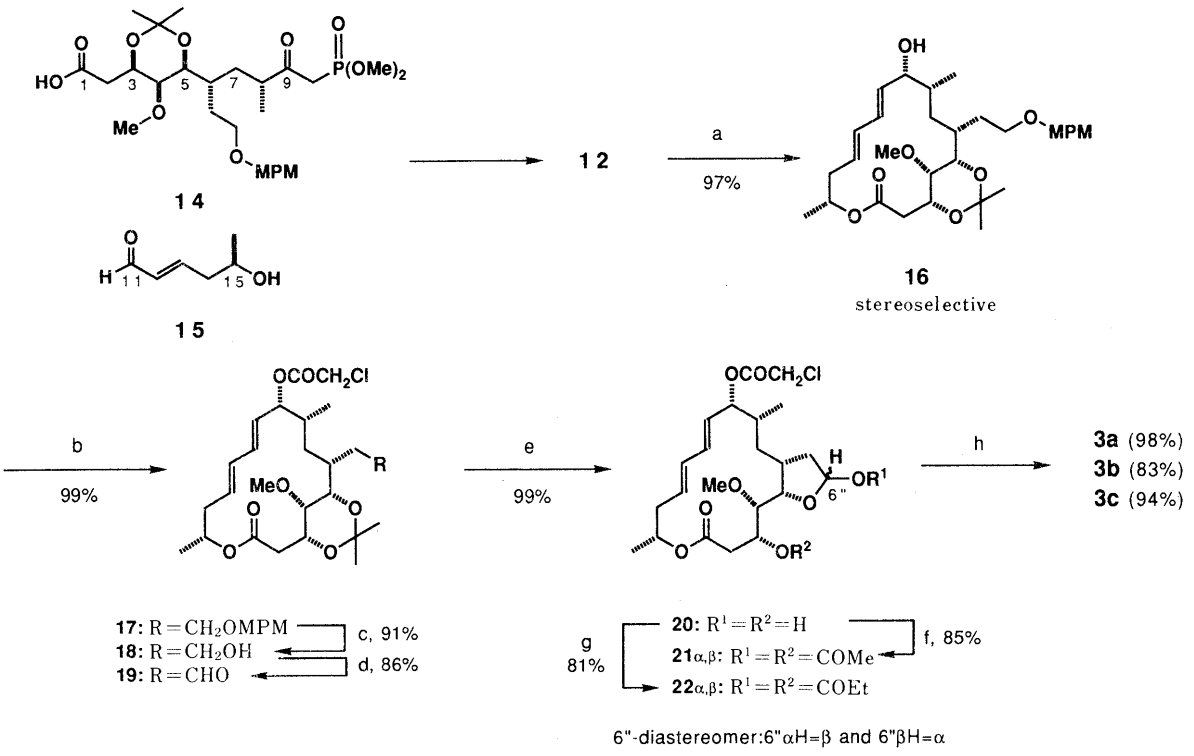


Fig. 7. Reduction of **13**

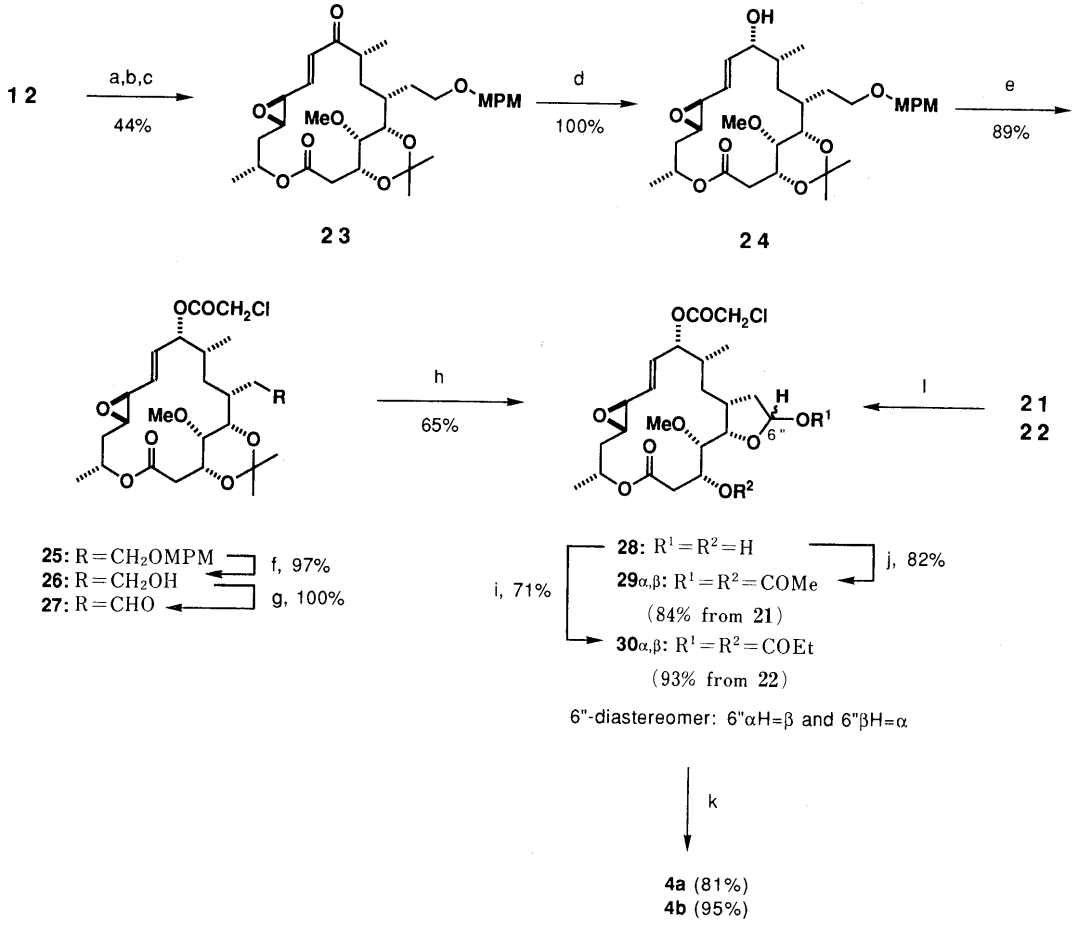


a) $NaBH_4$, MeOH, 0°C; b) $(ClCH_2CO)_2O$, py. DMAP; c) DDQ, CH_2Cl_2-H_2O (20:1); d) $(COCl)_2$, DMSO, Et_3N
e) 1 N HCl-THF (3:1); f) Ac_2O, Et_3N, DMAP, CH_2Cl_2; g) $(EtCO)_2O$, Et_3N, DMAP, CH_2Cl_2;
h) K_2CO_3 (3 eq), MeOH, 0°C

Chart 2

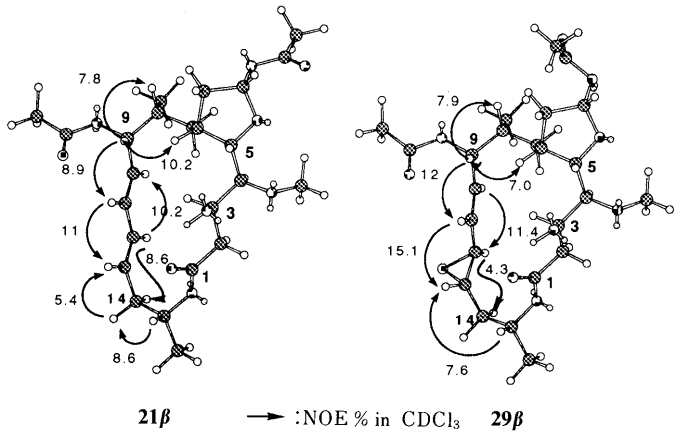
synthesized from **30α, β**.[22]

Epoxidation on the diene part to the 9*R*(α) alcohol compounds was next examined. From the nuclear magnetic resonance (NMR) spectra (coupling constants and NOE) of **21** and **29**, the most probable conformations of **21** and **29** were estimated to be as shown in Fig. 8. The diene is approximately perpendicular to the plane of the 16-membered ring and the H-9 lies nearly in the plane of the diene system. Therefore, epoxidation of the C10,11-double bond would be strongly hindered by the protective group of the C-9 hydroxy group, and the C12,13-β-epoxide was expected to be formed selectively. When the MPM-leuconolide A_3 (**6**) was treated with MCPBA in dichloromethane, a 1 : 2.3 regioisomeric mixture of the C10,11-β-



a) CSA, MeOH; b) MCPBA, $NaHCO_3$, CH_2Cl_2; c) $Me(MeO)C=CH_2$, PPTS, CH_2Cl_2; d) $NaBH_4$, MeOH, 0°C
e) $(ClCH_2CO)_2O$, py. DMAP; f) DDQ, CH_2Cl_2-H_2O (10:1); g) $(COCl)_2$, DMSO, Et_3N; h) 2 N H_2SO_4-dioxane (1:10)
i) Ac_2O, Et_3N, DMAP, CH_2Cl_2; j) $(EtCO)_2O$, Et_3N, DMAP, CH_2Cl_2; k) K_2CO_3 (3 eq), MeOH, 0°C
l) MCPBA (1.5eq), CH_2Cl_2

Chart 3



21β → :NOE % in $CDCl_3$ **29β**

Fig. 8. Estimated Conformations of **21β** and **29β**

and C12,13-β-epoxides was obtained. Also 3,5-acetonide compound (**17**) gave a 1 : 1 regioisomeric mixture of the β-epoxides. On the other hand, the diacyl compounds, **21** and **22**, were selectively epoxidized to give the C12,13-β-epoxides, **29** and **30**, in 84% and 93% yields, respectively.

Leuconolide A_3, midecanolide A_1, and maridonolide II were identical [NMR, infrared (IR) mass, $[\alpha]_D$] with the degradation products of natural leucomycin A_3 (josamycin), midecamycin A_1 and maridomycin IV, respectively.

Experimental

All melting points were measured with Yamato melting point apparatus model MP-21 and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. IR spectra were recorded in $CHCl_3$ or neat on a JASCO IRA-2-spectrometer. ^{1}H-NMR spectra were recorded in $CDCl_3$ on a JEOL FX-100, JEOL JNM GX-270, or JEOL JNM GX-500 instrument. Low- and high-resolution mass spectra (MS) were taken on a JEOL JMS HX-110 or JEOL JMS DX-303 spectrometer. Ultraviolet (UV) spectra were obtained on a Varian Cary 219 spectrophotometer using ethanol as a solvent.

Reduction of Carbonolide B 4-Methoxybenzylacetal (5) and Carbonolide A 4-Methoxybenzylacetal (8) (1) A stirred ethanol (1 ml) solution of **5** (20.0 mg, 37 μmol) was treated with *n*-Bu_4NBH_4 (9.5 mg, 37 μmol) at 0 °C for 60 min. *n*-Bu_4NBH_4 (9.5 mg, 37 μmol) was added to the reaction mixture and stirring was continued for an additional 30 min. Solid NH_4Cl was added to the reaction mixture to quench the reaction, and the solvent was removed. The residue was extracted with CH_2Cl_2, and the whole was washed with saturated aqueous NaCl, and dried over $MgSO_4$. The solvent was removed *in vacuo*, the residue was purified on a silica gel preparative thin layer chromatography (TLC) plate with hexane–AcOEt (3 : 2) to afford leuconolide A_1 4-methoxybenzylacetal (**6**) (6.4 mg, 32%) and 9*S* leuconolide A_1 4-methoxybenzylacetal (**7**) (11.4 mg, 57%). The ratio of **6** and **7** (1 : 1.8) was determined from the isolation yields.

Leuconolide A_1 4-Methoxybenzylacetal (**6**): $[\alpha]_D^{13}$ +67° (c=0.624, $CHCl_3$). ^{1}H-NMR δ: 0.99 (3H, d, J=7.0 Hz), 1.01—1.10 (2H, m), 1.26 (3H, d, J=6.0 Hz), 1.50—1.60 (1H, m), 1.82—1.92 (1H, m), 2.07—2.15

(2H, m), 2.12 (3H, s), 2.15—2.25 (2H, m), 2.22 (1H, dd, J=14.2, 1.5 Hz), 2.47 (1H, dt, J=13.0, 3.5 Hz), 2.93 (1H, dd, J=14.2, 11.5 Hz), 3.17 (1H, d, J=9.2 Hz), 3.99 (1H, dd, J=9.5, 4.0 Hz), 4.24 (1H, dd, J=9.5, 4.0 Hz), 4.42 (1H, d, J=11.5 Hz), 4.69 (1H, d, J=11.5 Hz), 5.07 (1H, m), 5.10 (1H, d, J=11.5 Hz), 5.22 (1H, dd, J=5.5, 4.2 Hz), 5.61 (1H, dd, J=15.5, 9.5 Hz), 5.63 (1H, ddd, J=15.5, 11.0, 4.0 Hz), 6.03 (1H, dd, J=15.5, 10.5 Hz), 6.50 (1H, dd, J=15.5, 10.5 Hz), 6.86—6.89 (2H, m), 7.26—7.29 (2H, m). MS m/z (relative intensity): 546 (M^+, 0.2%), 528 (M^+-18, 0.25%), 409 (1.7), 175 (5.2), 138 (6.3), 121 (100), 111 (7.7), 95 (9.6). Exact MS Calcd for $C_{30}H_{40}O_8$: 528.2723. Found: 528.2701. IR ν (neat) cm^{-1}: 3450, 1735, 1725. TLC Rf=0.30 (30% EtOAc/hexane).

9*S*-Leuconolide A_1 4-Methoxybenzylacetal (**7**): ^{1}H-NMR δ: 0.91 (3H, d, J=7.0 Hz), 1.24 (1H, d, J=6.2 Hz), 1.73—1.86 (1H, m), 1.92—1.98 (1H, m), 1.97—2.03 (1H, m), 2.06 (1H, ddd, J=13.8, 10.6, 9.50 Hz), 2.16 (1H, dd, J=15.5, 1.8 Hz), 2.30—2.40 (1H, m), 2.45 (1H, ddd, J=13.8, 4.5, 3.0 Hz), 2.73 (1H, dd, J=15.5, 10.5 Hz), 2.96 (1H, dd, J=9.2, 0.5 Hz), 3.49 (3H, s), 3.73 (3H, s), 3.79 (1H, br d, J=10.5 Hz), 4.08—4.11 (1H, m), 4.11 (1H, dd, J=9.0, 4.3 Hz), 4.65 (1H, d, J=11.5 Hz), 5.10 (1H, dd, J=5.5, 1.5 Hz), 5.05—5.14 (1H, m), 5.52 (1H, ddd, J=15.0, 10.5, 5.0 Hz), 5.68 (1H, dd, J=15.0, 4.0 Hz), 6.01 (1H, dd, J=15.0, 10.5 Hz), 6.26 (1H, ddd, J=15.0, 10.5, 1.5 Hz). TLC Rf=0.37 (30% EtOAc/hexane).

(2) A stirred ethanol (1 ml) solution of **8** (2.8 mg, 5.5 μmol) was treated with n-Bu_4NBH_4 (3.0 mg, 11 μmol) at 0 °C for 150 min. Solid NH_4Cl was added to the reaction mixture to quench the reaction, and the solvent was removed. The residue was extracted with CH_2Cl_2, and the whole was washed with saturated aqueous NaCl, dried over $MgSO_4$. After removal of the solvent, the residue was passed through a short silica gel column with hexane–AcOEt (2 : 1) to afford maridonolide II 4-methoxybenzylacetal (**9** and **10**) (1.9 mg, 63%, **9** : **10** = 1 : 22). The ratio of alcohols was determined from the NMR signals (methoxy peaks of δ 3.62 *vs.* δ 3.66 and δ 3.80 *vs.* δ 3.90).

9*S*-Maridonolide II 4-Methoxybenzylacetal (**10**): ^{1}H-NMR δ: 0.98 (3H, d, J=7.3 Hz), 1.28 (3H, d, J=6.2 Hz), 1.87—1.96 (2H, m), 1.96—2.07 (1H, m), 2.08—2.17 (2H, m), 2.09 (3H, s), 2.33 (1H, d, J=14.0 Hz), 2.35 (1H, dd, J=14.3, 1.8 Hz), 2.45—2.57 (1H, m), 2.99 (1H, dd, J=14.3, 11.4 Hz), 3.17 (2H, q, J=7.0 Hz), 3.62 (3H, s), 3.80 (3H, s), 4.02 (1H, dd, J=9.1, 4.0 Hz), 4.24 (1H, m), 4.43 (1H, d, J=11.3 Hz), 4.68 (1H, d, J=11.3 Hz), 5.07 (1H, ddd, J=11.0, 6.2, 2.8 Hz), 5.22 (1H, dd, J=5.5, 4.0 Hz), 5.27 (1H, d, J=10.4 Hz), 5.70 (1H, ddd, J=15.2, 9.0, 2.0 Hz), 6.17 (1H, dd, J=15.4, 3.9 Hz).

6″-Dihydro-3,5-isopropylidene-6″-*O*-(4-methoxybenzyl) Leuconolide A_1 (16) $NaBH_4$ (2.1 mg, 0.054 mmol) was added to a stirred solution of **12** (29.6 mg, 0.054 mmol) in MeOH (2 ml) at 0 °C and the reaction mixture was stirred for 5 min. Powdered NH_4Cl was added to the reaction mixture to quench the reaction and the solvent was evaporated off *in vacuo*. The residue was extracted with AcOEt, and the extract was washed with saturated aqueous NH_4Cl and dried. After removal of the solvent, the residue was chromatographed on a silica gel column with AcOEt–hexane (2 : 1) as the eluant to give the 9*R* alcohol **16** (28.6 mg, 97%) as a colorless oil. $[\alpha]_D^{13}$ +32° (c=0.7, $CHCl_3$). ^{1}H-NMR δ: 1.07 (3H, d, J=6.5 Hz), 1.28 (3H, d, J=6.5 Hz), 1.40 (3H, s), 1.44 (3H, s), 1.54 (1H, t, J=13.0 Hz), 2.20 (1H, ddd, J=15.5, 10.5, 9.0 Hz), 2.34 (1H, dd, J=15.0, 2.2 Hz), 2.41—2.54 (1H, m), 2.48 (1H, dd, J=14.0, 3.0 Hz), 2.81 (1H, dd, J=15.0, 10.5 Hz), 2.82 (1H, s), 3.41—3.55 (2H, m), 3.45 (3H, s), 3.76 (1H, d, J=5.0 Hz), 3.79 (3H, s), 4.23 (1H, d, J=11.0 Hz), 4.25 (1H, s), 4.38 (1H, d, J=11.3 Hz), 4.45 (1H, d, J=11.3 Hz), 5.35 (1H, ddq, J=8.5, 2.5, 6.5 Hz), 5.65 (1H, ddd, J=15.0, 10.0, 3.5 Hz), 5.66 (1H, dd, J=15.0, 5.0 Hz), 5.99 (1H, dd, J=15.5, 10.5 Hz), 6.12 (1H, ddd, J=15.5, 10.5, 0.5 Hz), 6.84—6.87 (2H, m), 7.24—7.27 (2H, m) MS m/z (relative intensity): 546 (M^+, 0.3%) 531 (0.4), 488 (0.8), 456 (1.3), 438 (1.0), 398 (1.0), 367 (1.7), 317 (1.5), 233 (4.2), 135 (4.6), 121 (100). Exact MS m/z Calcd for $C_{31}H_{46}O_8$ (M^+): 546.3192. Found: 546.3181. IR ν (neat) cm^{-1}: 3500, 1735, 1620.

9-*O*-Chloroacetyl-6″-dihydro-3,5-isopropylidene-6″-*O*-(4-methoxybenzyl) Leuconolide A_1 (17) Chloroacetic anhydride (23.4 mg, 0.14 mmol) was added to a stirred solution of **23** (25.0 mg, 0.046 mmol) and 4-dimethylaminopyridine (DMAP) (16.7 mg, 0.046 mmol) in pyridine (1 ml). Stirring was continued for 20 min at 5 °C, then the reaction mixture was diluted with CH_2Cl_2 and washed with 1 N HCl–brine (3 : 1) and brine. The organic layer was dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (3 : 1) as the eluant to give **17** as a colorless oil (27.7 mg, 99%). $[\alpha]_D^{13}$ +39.4° (c=0.72, $CHCl_3$). ^{1}H-NMR δ: 1.0 (3H, d, J=6.5 Hz), 1.28 (3H, d, J=6.5 Hz), 1.3—1.5 (2H, m), 1.41 (3H, s), 1.44 (3H, s), 1.64 (1H, t, J=13.0), 1.8—1.9 (1H, m), 2.18 (1H, dt, J=15.5, 10.5 Hz), 2.34 (1H, dd, J=15.5, 2.0 Hz), 2.42—2.55 (2H, m), 2.78 (1H, s), 2.80 (1H, dd, J=15.5, 10.5 Hz), 3.40—3.57 (2H, m), 3.47 (3H, s), 3.75 (1H, d, J=5.0 Hz), 3.80 (3H, s), 4.04 (2H, s), 4.12 (1H, br d, J=11.0 Hz), 4.39 (1H, d, J=12.5 Hz), 4.45 (1H, d, J=12.5 Hz), 5.30 (1H, ddq, J=8.8, 2.2, 6.5 Hz, 5.38 (1H, d, J=6.2 Hz), 5.59 (1H, dd, J=15.5, 6.0 Hz), 5.69 (1H, ddd, J=15.5, 9.5, 3.2 Hz), 5.92 (1H, ddd, J=15.5, 10.0, 0.5 Hz), 6.10 (1H, dd, J=10.5 Hz), 6.84—6.87 (2H, m), 7.24—7.27 (2H, m). MS m/z (relative intensity): 622 (M^+, 0.3%) 564 (0.8), 533 (3.0), 514 (0.5), 470 (0.9), 438 (1.0), 421 (0.75), 407 (1.36), 349 (2.0), 334 (1.3), 317 (2.0), 303 (2.5), 233 (10.4), 121 (100), 71 (100). Exact MS m/z Calcd for $C_{33}H_{47}ClO_9$ (M^+): 622.2908. Found: 622.2908. IR ν (neat) cm^{-1}: 1760, 1720 (CO), 1610 (C=C).

9-*O*-Chloroacetyl-6″-dihydro-3,5-isopropylideneleuconolide A_1 (18) DDQ (30.0 mg, 0.13 mmol) was added to a stirred solution of **17** (27.5 mg, 0.044 mmol) in CH_2Cl_2 (1 ml) and H_2O (0.05 ml). After being stirred for 40 min, the reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$, and brine. The extract was dried over $MgSO_4$ and concentrated to leave an oil, which was chromatographed on a silica gel column with hexane–AcOEt (2 : 1) as the eluant to give **18** as a colorless oil (20.2 mg, 91%). $[\alpha]_D^{13.5}$ +29.2° (c=0.85, $CHCl_3$). ^{1}H-NMR δ: 1.04 (3H, d, J=6.5 Hz). 1.29 (3H, d, J=6.5 Hz), 1.35—1.48 (2H, m), 1.44 (3H, s), 1.47 (3H, s), 1.58—1.60 (1H, m), 1.77 (1H, t, J=13.5 Hz), 1.86—1.96 (1H, m), 2.19 (1H, dt, J=15.2, 10.0 Hz), 2.28 (1H, ddd, J=10.5, 8.0, 6.5 Hz), 2.29—2.36 (1H, m), 2.36 (1H, dd, J=15.2, 2.0 Hz), 2.50 (1H, d, J=15.2 Hz), 2.80 (1H, s), 2.81 (1H, dd, J=15.2 Hz, 11.0 Hz), 3.48 (3H, s), 3.55 (1H, ddd, J=10.2, 8.0, 5.2 Hz), 3.74 (1H, t, J=5.5 Hz), 3.77 (2H, q, J=5.5 Hz), 4.09 (2H, s), 4.26 (1H, dt, J=10.0, 1.5 Hz), 5.32 (1H, ddq, J=11.0, 2.2, 6.5 Hz), 5.39 (1H, d, J=6.0 Hz), 5.60 (1H, dd, J=15.5, 5.5 Hz), 5.70 (1H, ddd, J=15.0, 9.5, 3.5 Hz), 5.94 (1H, ddd, J=15.0, 10.0, 0.5 Hz), 6.12 (1H, dd, J=15.5, 10.0 Hz). MS m/z (relative intensity): 504 (M^++2, 0.5%), 502 (M^+, 1.4), 489 (3.0), 487 (8.2), 446 (3.8), 444 (9.8), 426 (6.5), 393 (6.0), 350 (12), 310 (26), 301 (42), 234 (100), 148 (50), 123 (52), 94 (84). Exact MS m/z Calcd for $C_{25}H_{39}ClO_8$ (M^+): 502.2333. Found: 502.2335. IR ν (neat) cm^{-1}: 3600, 1755, 1720.

9-*O*-Chloroacetyl-3,5-isopropylideneleuconolide A_1 (19) Dry dimethyl sulfoxide (DMSO) (24 μl, 0.34 mmol) in dry CH_2Cl_2 (0.5 ml) was added dropwise during 15 min to an efficiently stirred solution of oxalyl chloride (15 μl, 0.15 mmol) in dry CH_2Cl_2 (0.5 ml) at −78 °C under an argon atmosphere. After 15 min at −78 °C, a solution of **18** (42.5 mg, 0.085 mmol) in CH_2Cl_2 (1 ml) was added to the mixture during 5 min. Stirring was continued at −78 °C for 15 min, then Et_3N (70 μl, 0.51 mmol) was added dropwise, and after removal of the cooling bath, the reaction mixture was allowed to warm to room temperature (over *ca.* 1 h). Then H_2O was added, the organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (5 ml × 2). The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (2 : 1) as the eluant to give the aldehyde (**19**) as a colorless oil (36.2 mg, 86%). $[\alpha]_D^{17}$ +33.8° (c=1.45, $CHCl_3$). ^{1}H-NMR δ: 1.09 (3H, d, J=6.5 Hz), 1.29 (3H, d, J=6.2 Hz), 1.39 (3H, s), 1.45 (3H, s), 1.72 (1H, t, J=13.0 Hz), 2.14 (1H, ddd, J=16.0, 10.5, 9.5 Hz), 2.26 (1H, ddd, J=10.5, 8.5, 2.5 Hz), 2.37 (1H, dd, J=16.0, 2.5 Hz), 2.45—2.55 (2H, m), 2.80 (1H, s), 2.80 (1H, dd, J=16.0, 11.0 Hz), 3.19 (1H, dd, J=4.5, 1.0 Hz), 3.26 (1H, dd, J=4.5, 1.0 Hz), 3.51 (3H, s), 3.84 (1H, dd, J=5.5, 0.5 Hz), 4.07 (2H, s), 4.27 (1H, d, J=10.0 Hz), 5.32 (1H, ddq, J=11.0, 2.2, 6.2 Hz), 5.38 (1H, d, J=6.0 Hz), 5.59 (1H, dd, J=15.5, 6.0 Hz), 5.71 (1H, ddd, J=15.5, 9.5, 3.5 Hz), 5.92 (1H, ddd, J=16.0, 10.0, 1.5 Hz), 6.12 (1H, dd, J=16.0, 10.0 Hz), 9.73 (1H, d, J=2.5 Hz), MS m/z (relative intensity); 500 (M^+, 1.4%) 485 (M^+−Me, 1.2), 442 (1.6), 424 (1.0), 406 (2.4), 391 (4.7), 348 (12.5), 326 (12), 299 (8.3), 232 (32.3), 148 (26), 123 (51.3), 71 (100), 43 (57). Exact MS m/z Calcd for $C_{25}H_{37}ClO_8$ (M^+): 500.2177. Found: 502.2190. IR ν (neat) cm^{-1}: 1750, 1720 (CO).

9-*O*-Chloroacetylleuconolide A_1 Hemiacetal (20) A solution of **19** (17 mg, 0.034 mmol) in 1 N HCl (0.3 ml) and THF (1 ml) was stirred at room temperature for 40 min. After neutralization with solid $NaHCO_3$, the reaction mixture was evaporated to dryness. CH_2Cl_2 and H_2O were added to the residue, and the CH_2Cl_2 layer was separated. The aqueous layer was extracted again with CH_2Cl_2, and the organic layers were combined, and dried over $MgSO_4$. After evaporation of the solvent, purification of the residue on a silica gel column with hexane–AcOEt (1 : 2) as the eluant afforded **20** as a colorless oil (15.5 mg, 99%). $[\alpha]_D^{17}$ +35.4° (c=1.08, $CHCl_3$). ^{1}H-NMR δ: 1.04 (3H, d, J=6.5 Hz), 1.13 (1H, t, J=7.0 Hz), 1.30 (1.5H, d, J=6.5 Hz), 1.31 (1.5H, d, J=6.5 Hz), 1.48—1.55 (1H, m), 1.65—1.83 (1H, m), 1.85—2.04 (1H, m), 2.09 (0.5H, dd, J=11.0, 2.9 Hz), 2.14—2.16 (0.5H, m), 2.19 (0.5H, dd, J=11.0, 2.9 Hz), 2.25 (0.5 H, dd, J=15.0, 3.0 Hz), 2.25—2.35 (0.5H, m), 2.39 (0.5H, dd, J=14.0, 8.0 Hz),

2.45—2.57 (1.5H, m), 2.55 (0.5H, dd, J=17.0, 8.0 Hz), 2.68 (0.5H, dd, J=6.2, 6.0 Hz), 2.77 (0.5H, dd, J=15.0, 10.5 Hz), 2.97 (0.5H, d, J=8.0 Hz), 3.07 (0.5H, d, J=4.0 Hz), 3.53 (1H, s), 3.62 (1H, s), 3.75 (0.5H, dd, J=2.2, 0.5 Hz), 4.04 (1.5H, s), 4.07 (1.5H, s), 4.47 (0.5H, dd, J=8.5, 4.0 Hz), 5.14—5.29 (1H, m), 5.36 (1H, dd, J=9.4, 4.2 Hz), 5.36—5.39 (0.5H, m), 5.43—5.46 (0.5H, m), 5.57 (0.5H, dd, J=15.5, 9.5 Hz), 5.58 (0.5H, dd, J=15.5, 9.5 Hz), 5.65 (0.5H, ddd, J=11.5, 8.5, 2.0 Hz), 5.72 (0.5H, ddd, J=15.5, 10.5, 4.0 Hz), 5.96 (0.5H, dd, J=15.0, 11.5 Hz), 6.00 (0.5H, dd, J=15.0, 11.5 Hz), 6.21 (0.5H, dd, J=15.0, 10.5 Hz), 6.35 (0.5H, dd, J=15.0, 10.5 Hz). MS m/z (relative intensity): 442 (M^+, 5.8%), 366 (3.8), 348 (23), 326 (18), 308 (11), 232 (47), 148 (44), 124 (100), 94 (97), 71 (95). Exact MS m/z Calcd for $C_{22}H_{31}ClO_7$ (M^+): 442.1759. Found: 442.1735. IR ν (neat) cm^{-1}: 3400, 1750, 1720, 1700.

Leuconolide A_1 Hemiacetal (3a) A solution of **20** (12.2 mg, 0.0265 mmol) in 1 ml of MeOH was treated with K_2CO_3 (5.5 mg, 0.04 mmol) at 0 °C for 10 min. After evaporation of the solvent, CH_2Cl_2 was added to the reaction mixture and precipitate was filtered off. The filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–AcOEt (1 : 3) as the eluant to give **3a** as a colorless solid (10 mg, 98%). $[\alpha]_D^{19}$ +43.6° (c=0.5, $CHCl_3$). ^{1}H-NMR δ: 1.02 (1.5H, d, J=7.0 Hz), 1.09 (1.5H, d, J=7.0 Hz), 1.29 (1.5H, d, J=7.0 Hz), 1.30 (1.5H, d, J=7.0 Hz), 1.36—1.45 (0.5H, m), 1.45—1.55 (0.5H, m), 1.55—1.7 (1H, m), 1.81—1.91 (1H, m), 2.13 (0.5H, dt, J=10.5, 3.0 Hz), 2.19 (0.5H, dt, J=10.5, 3.0 Hz), 2.25—2.35 (1H, m), 2.27 (0.5H, dd, J=15.0, 3.2 Hz), 2.37 (0.5H, dd, J=8.0, 6.5 Hz), 2.48 (1H, t, J=14.5 Hz), 2.64 (1H, d, J=7.7 Hz), 2.76 (0.5H, dd, J=15.0, 10.0 Hz), 2.97 (0.5H, d, J=8.2 Hz), 3.08 (0.5H, d, J=3.0 Hz), 3.53 (1.5H, s), 3.62 (1.5H, s), 3.79 (0.5H, dd, J=10.0, 2.5 Hz), 3.90—4.02 (0.5H, m), 4.02—4.12 (0.5H, m), 4.15 (0.5H, dd, J=9.5, 3.3 Hz), 4.19 (0.5H, dd, J=9.5, 7.2 Hz), 4.22 (0.5H, dd, 8.5, 4.0 Hz), 4.20—4.32 (0.5H, m), 4.42 (0.5H, dd, J=8.2, 4.5 Hz), 5.18—5.30 (1H, m), 5.42 (0.5H, s), 5.60 (0.5H, ddd, J=15.5, 6.0, 4.0 Hz), 5.67 (1H, dd, J=15.5, 9.5 Hz), 5.71 (0.5H, ddd, J=15.5, 9.5, 7.0 Hz), 5.98 (0.5H, dd, J=15.5, 9.5 Hz), 6.01 (0.5H, dd, J=15.5, 9.5 Hz), 6.14 (0.5H, dd, J=15.5, 9.5 Hz), 6.18 (0.5H, dd, J=15.5, 9.5 Hz). MS m/z (relative intensity): 384 (M^+, 0.1%), 366 (5.6), 349 (1.9), 232 (4.8), 211 (8.6), 155 (14), 123 (100), 95 (56), 43, (49). Exact MS m/z Calcd for $C_{20}H_{32}O_7$ (M^+): 384.2148. Found: 384.2127. IR ν (neat) cm^{-1}: 3400, 1730, 1700.

9-*O*-Chloroacetylleuconolide A_3 Acetoxyacetal (21) Acetic anhydride (50 μl) was added to a stirred solution of **20** (20 mg, 45 μmol), Et_3N (150 μl), and DMAP (1 mg) in CH_2Cl_2 (0.5 ml) at room temperature, and the solution was stirred for 10 h. The reaction mixture was evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (3 : 1) as the eluant to give the diacetate **21β** as a colorless oil (12 mg, 51%). $[\alpha]_D^{20}$ +32.5° (c=1.43, $CHCl_3$). ^{1}H-NMR δ: 1.02 (3H, d, J=6.5 Hz), 1.09—1.13 (1H, m), 1.27 (3H, d, J=6.2 Hz), 1.95—2.16 (4H, m), 2.05 (3H, s), 2.14 (3H, s), 2.22 (1H, dd, J=14.5, 1.7 Hz), 2.30 (1H, dd, J=14.5, 6.0 Hz), 2.30—2.40 (1H, m), 2.47 (1H, ddd, J=13.0, 3.0, 2.5 Hz), 2.93 (1H, dd, J=14.5, 11.5 Hz), 3.13 (1H, d, J=9.5 Hz), 3.54 (3H, s), 4.00 (1H, dd, J=9.5, 3.8 Hz), 4.04 (2H, s), 5.01 (1H, m), 5.04 (1H, dd, J=11.0, 0.5 Hz), 5.36 (1H, dd, J=9.5, 4.0 Hz), 5.55 (1H, dd, J=15.0, 9.5 Hz), 5.72 (1H, ddd, J=15.0, 11.0, 3.2 Hz), 6.03 (1H, ddd, J=15.0, 10.5, 0.5 Hz), 6.28 (1H, dd, J=5.5, 4.5 Hz), 6.63 (1H, dd, J=15.0, 10.5 Hz). MS m/z (relative intensity): 544 (M^+, 0.2%), 509 (0.26), 485 (6.3), 390 (8.3), 350 (4.6), 308 (6.3), 232 (12), 215 (13.5), 199 (8.8), 175 (13), 147 (12.5), 135 (29), 124 (29), 105 (30), 95 (34), 81 (34), 71 (34), 55 (50), 43 (100). Exact MS m/z Calcd for $C_{26}H_{37}ClO_{10}$: 544.2075. Found: 544.2057. IR ν (neat) cm^{-1}: 1750, 1740, 1730, 1720.

Continued elution provided **21α** as a colorless oil (8.0 mg, 34%). $[\alpha]_D^{19}$ +18.7° (c=0.6, $CHCl_3$). ^{1}H-NMR δ: 1.02 (3H, d, J=7.0 Hz), 1.27 (3H, d, J=6.2 Hz), 1.54 (1H, ddd, J=14.0, 13.0, 3.2 Hz), 2.03 (3H, s), 2.05—2.35 (5H, m), 2.10 (3H, s), 2.22 (1H, dd, J=14.0, 2.0 Hz), 2.48 (1H, dd, J=14.0, 4.0, 3.0 Hz), 2.92 (1H, dd, J=14.0, 11.5 Hz), 3.19 (1H, d, J=10.0 Hz), 3.55 (3H, s), 3.84 (1H, dd, J=10.0, 4.2 Hz), 4.05 (2H, s), 4.98 (1H, ddq, J=11.0, 3.0, 6.0 Hz), 5.05 (1H, d, J=11.2 Hz), 5.36 (1H, dd, J=10.0, 4.0 Hz), 5.56 (1H, dd, J=15.0, 10.0 Hz), 5.73 (1H, ddd, J=15.0, 11.5, 3.6 Hz), 6.05 (1H, ddd, J=15.0, 10.5, 0.5 Hz), 6.20 (1H, d, J=4.5 Hz), 6.61 (1H, dd, J=15.0, 10.5 Hz). MS m/z (relative intensity): 546 (M^++2, 0.15%), 544 (M^+, 0.3), 484 (6.3), 390 (7.5), 308 (8.3), 232 (12.5), 215 (12.5), 199 (10), 188 (8.7), 175 (13.5), 148 (11.5), 135 (25), 124 (30), 105 (29), 95 (29), 81 (29), 71 (29). Exact MS m/z Calcd for $C_{24}H_{33}ClO_8$ (M^+−60): 484.1864. Found: 484.1854. IR ν (neat) cm^{-1}: 1750, 1735, 1725, 1720.

9-*O*-Chloroacetylmidecanolide A_1 Propionyloxy Acetal (22) Propionic anhydride (25 μl) was added to a stirred solution of **20** (10.0 mg, 0.022 mmol), Et_3N (83 μl), and DMAP (4 mg) in CH_2Cl_2 (1 ml) at room temperature, and the solution was stirred for 13 h, then evaporated *in vacuo*. The residue was chromatographed on a silica gel columnn with hexane–AcOEt (3 : 1) as the eluant to give the dipropionate **22β** as a colorless oil (6.9 mg, 58%). $[\alpha]_D^{19}$ −19.3° (c=0.46, $CHCl_3$). ^{1}H-NMR δ: 1.02 (3H, d, J=7.0 Hz), 1.12 (3H, t, J=7.0 Hz), 1.16 (3H, t, J=7.0 Hz), 1.26 (3H, d, J=6.2 Hz), 1.98 (1H, ddd, J=14.2, 7.0, 4.8 Hz), 2.02—2.09 (1H, m), 2.14 (1H, dt, J=13.5, 11.5 Hz), 2.30 (1H, dd, J=14, 2.0 Hz), 2.315 (2H, q, J=7.0 Hz), 2.320 (1H, q, J=7.0 Hz), 2.41 (1H, d, J=7.0 Hz), 2.415 (1H, q, J=7.0 Hz), 2.47 (1H, ddd, J=13.5, 5.0, 3.0 Hz), 2.47—2.50 (1H, m), 2.89 (1H, ddd, J=14.0, 11.5 Hz), 3.15 (1H, dd, J=9.6, 1.0 Hz), 3.53 (3H, s), 3.98 (1H, dd, J=9.0, 6.0 Hz), 4.04 (2H, s), 4.99 (1H, ddd, J=11.6, 2.7, 6.2 Hz), 5.06 (1H, ddd, J=11.5, 2.0, 1.0 Hz), 5.37 (1H, dd, J=9.5, 3.5 Hz), 5.54 (1H, dd, J=15.5, 9.5 Hz), 5.73 (1H, ddd, J=15.0, 11.0, 3.5 Hz), 6.03 (1H, ddd, J=15.5, 10.5 Hz). MS m/z (relative intensity): 572 (M^+, 0.02%), 516 (0.015), 499 (14.2), 422 (1.3), 404 (9.5), 382 (2.5), 350 (4.0), 326 (5.1), 308 (7.6), 232 (18.3), 215 (15.6), 189 (15), 171 (9.6), 148 (12.9), 124 (20.8), 93 (22.9), 57 (108). Exact MS m/z Calcd for $C_{28}H_{41}ClO_{10}$ (M^+): 572.2388. Found: 572.2391.

Continued elution provided **22α** as a colorless oil (2.8 mg, 23%). ^{1}H-NMR δ: 1.02 (3H, d, J=7.0 Hz), 1.11 (3H, t, J=7.5 Hz), 1.12 (3H, t, J=7.5 Hz), 1.27 (3H, d, J=7.5 Hz), 2.05—2.20 (2H, m), 2.17 (1H, dt, J=14.0, 1.0 Hz), 2.22 (1H, br d, J=13.5, 1.5 Hz), 2.24—2.33 (1H, m), 2.30 (2H, q, J=7.5 Hz), 2.38 (1H, q, J=7.5 Hz), 2.40—2.49 (1H, m), 2.48 (1H, ddd, J=14.0, 4.0, 3.0 Hz), 2.92 (1H, dd, J=13.5, 1.5 Hz), 3.19 (1H, d, J=10.0 Hz), 3.53 (3H, s), 3.83 (1H, dd, J=9.6, 4.2 Hz), 4.05 (2H, s), 4.94 (1H, ddq, J=11.0, 3.5, 4.2 Hz), 5.06 (1H, dd, J=9.0, 4.2 Hz), 5.38 (1H, dd, J=9.5, 4.0 Hz), 5.56 (1H, dd, J=15.0, 9.8 Hz), 5.73 (1H, ddd, J=15.0, 11.0, 3.8 Hz), 6.05 (1H, ddd, J=15.2, 10.5, 1.0 Hz), 6.23 (1H, d, J=4.5 Hz), 6.64 (1H, dd, J=15.2, 10.5 Hz). MS m/z (relative intensity): 572 (M^+, 0.014%), 516, (0.04), 509 (0.14), 499 (5.5), 404 (4.4), 392 (2.5), 350 (9.4), 326 (6.3), 308 (6.3), 232 (16.5), 215 (9.6), 189 (15), 171 (7.5), 148 (13.0), 135 (41.7), 124 (26.0), 105 (28.1), 93 (29.2), 71 (37), 57 (108). Exact MS m/z Calcd for $C_{28}H_{41}ClO_{10}$ (M^+): 572.2388. Found: 572.2365. IR ν (neat) cm^{-1}: 1750, 1735, 1725 (CO), 1460, 1420, 1300, 1280, 1180, 1125, 1080, 1050.

Leuconolide A_3 Hemiacetal (3b) A solution of **21α,β** (10.0 mg, 0.0184 mmol) in 1 ml of MeOH was treated with K_2CO_3 (7.6 mg, 0.05 mmol) at 0 °C for 25 min. Solid NH_4Cl was added to the solvent, and the precipitate was filtered off. The filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with hexane–AcOEt (2 : 1) as the eluant to give **3b** as a colorless solid (6.5 mg, 83%). mp 95—96.6 °C (amorphous solid). $[\alpha]_D^{19}$ +35.3° (c=0.81, $CHCl_3$). ^{1}H-NMR δ: 1.00 (2H, d, J=7.0 Hz), 1.04 (1H, d, J=7.0 Hz), 1.27 (3H, d, J=6.5 Hz), 1.75—1.96 (2H, m), 2.10 (1H, s), 2.13 (2H, s), 2.19 (1H, dt, J=8.0, 2.8 Hz), 2.30—2.37 (1H, m), 2.42—2.75 (1H, m), 2.48—2.33 (1H, m), 2.77 (0.3H, dd, J=15.5, 8.0 Hz), 2.92 (0.7H, dd, J=14.0, 10.0 Hz), 3.14 (0.7H, d, J=9.2 Hz), 3.18 (0.3H, dd, J=6.2, 1.5 Hz), 3.55 (0.7H, s), 3.60 (0.3H, s), 4.06 (1H, dd, J=9.0, 4.0 Hz), 4.25 (1H, dd, J=9.5, 4.0 Hz), 4.97—5.13 (1H, m), 5.08 (0.3H, ddd, J=11.0, 2.0, 1.0 Hz), 5.28 (0.3H, dd, J=8.2, 4.5 Hz), 5.39—5.45 (0.3H, s), 5.61 (0.7H, ddd, J=16.0, 10.0, 4.5 Hz), 5.62 (1H, dd, J=16.0, 10.0 Hz), 5.69 (0.3H, ddd, J=16.0, 10.0, 4.5 Hz), 6.03 (1H, dd, J=15.0, 10.5 Hz), 6.35 (0.3H, dd, J=15.0, 10.5 Hz), 6.49 (0.7 H, dd, J=15.0, 10.5 Hz). MS m/z (relative intensity): 426 (M^+, 0.3%), 408 (8.9), 368 (3.2), 253 (6.4), 232 (1.7), 211 (11), 155 (15), 123 (77), 43 (100). Exact MS m/z Calcd for $C_{22}H_{34}O_8$ (M^+): 426.2254. Found: 426.2227. Calcd for $C_{22}H_{32}O_7$ (M^+−18): 408.2148. Found: 408.2123. IR ν (neat) cm^{-1}: 3400, 1740, 1730.

Midecanolide A_1 Hemiacetal (3c) A solution of **21α,β** (4.3 mg, 0.0075 mmol) in 1 ml of MeOH was treated with K_2CO_3 (3.1 mg, 0.022 mmol) at 0 °C for 60 min. Solid NH_4Cl was added to the reaction mixture and the solvent was removed *in vacuo*. The residue was taken up in CH_2Cl_2 and the precipitate was filtered off. The filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–MeOH (20 : 1) as the eluant to give **3c** as a colorless solid (3.1 mg, 94%). mp 80—81 °C (amorphous solid). $[\alpha]_D^{19}$ +31.6° (c=0.58, $CHCl_3$). ^{1}H-NMR δ: 1.00 (2.1H, d, J=7.0 Hz), 1.04 (0.9H, d, J=7.0 Hz), 1.14 (0.9H, t, J=7.0 Hz), 1.16 (2.1H, t, J=7.0 Hz), 1.26 (3H, d, J=6.2 Hz), 1.75—1.97 (2H, m), 2.12 (0.3H, dd, J=11.5, 2.5 Hz), 2.21 (0.7H, dd, J=14.0, 6.5 Hz), 2.23 (0.7H, dd, J=14.5, 2.0 Hz), 2.34 (0.3H, q, J=7.0 Hz), 2.35 (0.3H, q, J=7.0 Hz), 2.38 (0.7H, q, J=7.0 Hz), 2.39 (0.7H, q, J=7.0 Hz), 2.50 (0.3H, dd, J=10.5, 5.5 Hz), 2.75 (0.3H, dd, J=15.5, 8.0 Hz), 2.91 (0.7H, dd, J=14.0, 11.2 Hz), 3.14 (0.3H, dd, J=9.5, 0.5 Hz), 3.19 (0.3H, dd, J=9.0, 2.0 Hz), 3.54 (2.1H, s), 3.59 (0.9H, s), 3.84 (0.3H, t, J=5.5 Hz), 4.04 (0.3H, dd, J=9.2, 4.2 Hz), 4.28 (0.7H, dd, J=9.2, 4.2 Hz), 4.97—5.10 (1H, m), 5.08 (0.7H, ddd, J=11.0, 2.3, 1.5 Hz),

5.25—5.30 (0.3H, m), 5.29—5.32 (0.3H, m), 5.62 (0.7H, dd, J=15.5, 10.5 Hz), 5.64 (0.3H, dd, J=15.5, 11.5 Hz), 5.63 (0.7H, ddd, J=15.5, 10.5, 4.2 Hz), 5.79 (0.3H, ddd, J=15.5, 10.5, 4.2 Hz), 6.03 (0.7H, ddd, J=9.5, 4.0, 0.5 Hz), 6.22 (0.3H, ddd, J=9.5, 4.0, 0.5 Hz), 6.35 (0.3H, dd, J=15.5, 10.0 Hz), 6.52 (0.7 H, dd, J=15.5, 10.0 Hz). MS m/z (relative intensity): 440 (M^+, 0.4%), 422 (M^+-18, 7.2), 348 (2.1), 252 (4.3), 232 (5.7), 211 (11), 155 (13), 123 (72), 95 (51), 57 (100). Exact MS m/z Calcd for $C_{23}H_{34}O_7$ (M^+-18): 422.2305. Found: 422.2289. IR ν (neat) cm^{-1}: 3400, 1740, 1730, 1720, 1460, 1360, 1310, 1280, 1180, 920, 865, 740.

6″-Dihydro-12*S*,13*R*-epoxy-3,5-isopropylidene-6″-*O*-(4-methoxybenzyl)-niddanolide (23) CSA (0.7 mg) was added to a solution of **12** (17.0 mg, 0.031 mmol) in MeOH (0.5 ml) at room temperature. After 30 min, Et_3N (50 μl) was added, and the reaction mixture was evaporated *in vacuo*. The residue was chromatographed on a preparative TLC plate with hexane–AcOEt (1 : 3) as the developer to give 6″-dihydro-6″-*O*-(4-methoxybenzyl)niddanolide as a colorless oil (13.0 mg, 83%). $[\alpha]_D^{24}$ +14.5° (c=0.64, $CHCl_3$). ^{1}H-NMR δ: 1.18 (3H, d, J=7.0 Hz), 1.31 (3H, s), 1.32 (3H, d, J=6.2 Hz), 1.33 (3H, s), 1.37—1.45 (2H, m), 1.49 (1H, dd, J=7.0, 2.0 Hz), 1.62 (1H, d, J=4.0 Hz), 1.75 (1H, dd, J=14.5, 8.8 Hz), 1.65—1.95 (2H, m), 2.22 (1H, d, J=15.0 Hz), 2.35—2.62 (2H, m), 2.78 (1H, dd, J=16.1, 11.0 Hz), 2.94 (1H, dd, J=9.5, 1.5 Hz), 3.46—3.54 (2H, m), 3.72 (1H, dd, J=11.0, 1.5 Hz), 3.80 (3H, s), 3.99 (1H, d, J=8.4 Hz), 4.43 (2H, dd, J=16.5, 11.7 Hz), 5.20—5.30 (1H, m), 6.08—6.19 (2H, m), 6.31 (1H, d, J=15.0 Hz), 6.80—6.95 (2H, m), 7.20 (1H, d, J=15.0 Hz), 7.26—7.30 (2H, m). MS m/z (relative intensity): 504 (M^+, 0.3%), 399 (0.1), 384 (0.6), 366 (5.2), 351 (0.8), 334 (0.8), 261 (0.6), 249 (1.3), 234 (2.7), 150 (4.2), 137 (8.3), 121 (100). Exact MS m/z Calcd for $C_{28}H_{40}O_8$ (M^+): 504.2723. Found: 504.2709.

MCPBA (81.4 mg, 0.40 mmol; 85% activity) was added to a stirred solution of 6″-dihydro-6″-*O*-(4-methoxybenzyl)niddanolide (65.6 mg, 0.13 mmol), $NaHCO_3$ (40 mg) in CH_2Cl_2 (3 ml) at room temperature. After 20.5 h, the solution was diluted with Et_2O, washed with saturated aqueous $NaHCO_3$ and brine, and dried ($MgSO_4$). After removal of the solvent, the crude epoxide was chromatographed on a silica gel column with hexane–AcOEt (1 : 3) as the eluant to give 6″-dihydro-12*S*,13*R*-epoxy-6″-*O*-(4-methoxybenzyl)niddanolide as a colorless oil (37.8 mg, 56%). $[\alpha]_D^{24}$ +9.45° (c=0.74, $CHCl_3$). ^{1}H-NMR δ: 1.17 (3H, d, J=7.0 Hz), 1.24—1.26 (1H, m), 1.31 (3H, s), 1.39—1.92 (5H, m), 2.31 (1H, dd, J=16.1, 1.1 Hz), 2.39 (1H, dd, J=12.1, 2.2 Hz), 2.61—2.63 (1H, m), 2.82 (1H, dd, J=16.1, 11.0 Hz), 3.00—3.15 (2H, m), 3.08 (1H, dd, J=10.3, 2.2 Hz), 3.14 (1H, dd, J=9.2, 1.8 Hz), 3.42—3.56, (2H, m), 3.60, (3H, s), 3.74—3.76 (1H, m), 3.80 (3H, s), 3.98 (1H, d, J=9.8 Hz), 4.43 (1H, dd, J=16.5, 11.4 Hz), 5.28—5.30 (1H, m), 6.49 (1H, dd, J=16.5, 11.4 Hz), 6.84—6.87 (2H, m), 7.20—7.23 (2H, m). MS m/z (relative intensity): 520 (M^+, 0.4%), 383 (1.0), 383 (12.5), 350 (1.7), 336 (0.9), 318 (1.7), 249 (1.3), 234 (3.8), 223 (2.1), 195 (1.7), 181 (3.4), 150 (98.3), 121 (100). Exact MS m/z Calcd for $C_{28}H_{40}O_9$ (M^+): 520.2673. Found: 520.2701. IR ν (neat) cm^{-1}: 3450, 1720, 1685, 1620, 1510, 1450, 1350, 1300, 1180, 1100, 1040, 1010, 980.

2-Methoxypropene (20 μl, 0.21 mmol) and pyridinium *p*-toluenesulfonate (PPTS) (1 mg) were added to a solution of the above epoxide (9.1 mg, 17.5 mmol) in CH_2Cl_2 (1 ml) at room temperature under an argon atmosphere. After 5 min, Et_3N (50 μl) was added, and the reaction mixture was evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (1 : 1) as the eluant to give **23** as a colorless oil (9.2 mg, 94%). $[\alpha]_D^{22}$ −7.0° (c=0.83, $CHCl_3$). ^{1}H-MMR δ: 0.87—0.88 (0.5H, m), 0.95 (1.8H, d, J=6.6 Hz), 1.02—1.09 (0.5H, m), 1.12 (1.2H, d, J=6.6 Hz), 1.24 (1.8H, s), 1.26 (1.2H, s), 1.28 (1.8H, s), 1.30 (1.2H, s), 1.33 (1.2H, d, J=6.2 Hz), 1.40 (1.8H, d, J=7.0 Hz), 1.76—1.90 (2.5H, m), 1.94 (0.5H, dd, J=6.0, 3.5 Hz), 1.99 (0.5H, dd, J=6.0 Hz, 3.5 Hz), 2.24—2.40 (3H, m), 2.36 (0.5H, dd, J=14.7, 2.9 Hz), 2.55 (0.5H, dd, J=14.7, 4.5 Hz), 2.72 (0.5H, dd, J=14.7, 4.4 Hz), 2.85 (0.5H, dd, J=14.7, 9.2 Hz) 2.82—2.92 (5H, m), 3.045 (0.5H, s), 3.05 (0.5H, s), 3.30—3.36 (1.5H, m), 3.40—3.60 (5H, m), 3.46 (2H, s), 3.52 (1H, s), 3.72 (1H, d, J=9.2 Hz), 3.80 (2H, s), 3.81 (1H, s), 3.98—3.99 (0.5H, m), 4.22 (1H, ddd, J=9.5, 4.2, 1.0 Hz), 4.40 (0.5H, d, J=11.7 Hz), 4.49 (1H, d, J=11.7 Hz), 5.08 (0.5H, m), 5.25 (0.5H, m), 6.42 (0.5H, d, J=15.5 Hz), 6.49 (0.5H, d, J=15.5 Hz), 6.64 (0.5H, dd, J=15.5, 6.0 Hz), 6.85—7.23 (4H, m), 6.90 (0.5H, dd, J=15.4, 2.9 Hz). MS m/z (relative intensity): 560 (M^+, 0.07%), 502 (0.2), 471 (0.5), 421 (1.0), 406 (0.3), 366 (2), 348 (0.75), 334 (1.1), 150 (4.8), 137 (8.8), 121 (100). Exact MS m/z Calcd for $C_{31}H_{44}O_9$ (M^+): 560.2985. Found: 560.2963. IR ν (neat) cm^{-1}: 1725, 1695, 1635, 1620, 1300.

6″-Dihydro-12*S*,13*R*-epoxy-3,5-isopropylidene-6″-*O*-(4-methoxybenzyl)-leuconolide A_1 (24) A stirred MeOH solution of **23** (31.5 mg, 0.056 mmol in 1.5 ml of MeOH) was treated with $NaBH_4$ (5.2 mg, 0.138 mmol) at 0 °C for 10 min. Solid NH_4Cl was added to the reaction mixture to quench the reaction, and the solvent was removed to dryness. The residue was extracted with CH_2Cl_2, and the extract was washed with saturated aqueous NH_4Cl and dried over $MgSO_4$. After removal of the solvent, the residue was chromatographed on a silica gel column with hexane–AcOEt (1 : 1) to afford **24** as a colorless oil (31.6 mg, 100%). $[\alpha]_D^{23}$ +14.9° (c=0.66, $CHCl_3$). ^{1}H-NMR δ: 0.80—0.90 (2H, m), 1.08 (3H, d, J=5.5 Hz), 1.32 (3H, d, J=6.6 Hz), 1.42 (3H, s), 1.44 (3H, s), 1.23—1.81 (6H, m), 1.89 (1H, m), 2.07—2.09 (1H, m), 2.34 (1H, d, J=7.7 Hz), 2.41—2.51 (1H, m), 2.44 (1H, dd, J=16.5, 2.6 Hz), 2.90 (1H, dd, J=16.1, 11.0 Hz), 2.92 (1H, s), 2.98—3.08 (1H, m), 3.05 (1H, dd, J=6.6, 1.8 Hz), 3.51 (3H, s), 3.80 (1H, dd, J=6.6, 1.8 Hz), 4.21—4.28 (2H, m), 4.39 (1H, d, J=11.7 Hz), 4.46 (1H, d, J=11.7 Hz), 5.24—5.26 (1H, m), 5.53 (1H, dd, J=16.0, 6.6 Hz), 6.03 (1H, dd, J=16.0, 6.2 Hz), 6.86—6.88 (2H, m), 7.24—7.27 (2H, m). MS m/z (relative intensity): 562 (M^+, 0.03%), 544 (0.14), 486 (0.36), 472 (1.5), 365 (0.42), 249 (2.6), 234 (2.3), 202 (2.5), 121 (100), 98 (0.46). Exact MS m/z Calcd for $C_{31}H_{46}O_9$ (M^+): 562.3142. Found: 562.3172. IR ν (neat) cm^{-1}: 3450, 1740, 1625.

9*R*-*O*-Chloroacetyl-6″-dihydro-12*S*,13*R*-epoxy-3,5-isopropylidene-6″-*O*-(4-methoxybenzyl)leuconolide A_1 (25) Chloroacetic anhydride (26.1 mg, 0.153 mmol) was added to a stirred solution of **24** (28.4 mg, 0.051 mmol) and DMAP (6.0 mg, 0.049 mmol) in a 1 : 1 mixture of pyridine and CH_2Cl_2 (1 ml). Stirring was continued for 30 min at room temperature, then the reaction mixture was diluted with CH_2Cl_2 and washed with 1 N HCl–brine (1 : 1) and brine. The organic layer was dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (1 : 1) as the eluant to give **25** as a colorless oil (28.6 mg, 88.6%). $[\alpha]_D^{23}$ +18.9° (c=0.72, $CHCl_3$). ^{1}H-NMR δ: 1.00 (3H, d, J=6.6 Hz), 1.32 (3H, d, J=6.6 Hz), 1.35—1.45 (1H, m), 1.89—1.95 (1H, m), 2.07 (1H, d, J=15.2 Hz), 2.44 (1H, dd, J=5.5, 2.9 Hz), 2.40—2.55 (1H, m), 2.88 (1H, dd, J=16.1, 11.0 Hz), 2.90—3.05 (3H, m), 3.51 (3H, s), 3.44—3.57 (3H, m), 3.78 (1H, d, J=5.9 Hz), 3.80 (3H, s), 4.02 (2H, s), 4.26 (1H, dd, J=11.3, 2.2 Hz), 4.45 (1H, d, J=11.5 Hz), 5.21—5.27 (1H, m), 5.41 (1H, d, J=6.2 Hz), 5.52 (1H, dd, J=15.4, 6.6 Hz), 5.93 (1H, dd, J=15.4, 6.6 Hz), 6.87—6.89 (2H, m), 7.25—7.27 (2H, m). MS m/z (relative intensity): 638 (M^+, 0.07%), 620 (M^+-18, 0.07), 580 (0.3), 564 (0.2), 549 (0.9), 487 (0.4), 459 (0.3), 444 (0.4), 319 (0.6), 301 (0.4), 249 (1.5), 234 (1.0), 217 (1.9), 189 (2.3), 175 (2.7), 137 (4.2), 121 (100), 109 (4.2). Exact MS m/z Calcd for $C_{33}H_{47}ClO_{10}$ (M^+): 638.2857. Found: 638.2819. IR ν (neat) cm^{-1}: 1755, 1725, 1610, 1580, 1455, 1380, 1360, 1300, 1250, 1200, 1180, 1120, 1080, 1035, 975.

9*R*-*O*-Chloroacetyl-6″-dihydro-12*S*,13*R*-epoxy-3,5-isopropylideneleuconolide A_1 (26) DDQ (22.2 mg, 0.094 mmol) was added to a stirred solution of **25** (22.2 mg, 0.035 mmol) in CH_2Cl_2 (1 ml) and H_2O (0.05 ml). After being stirred for 40 min, the reaction mixture was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$, and brine. The extract was dried over Na_2SO_4 and concentrated to leave an oil, which was purified by silica gel column chromatography with hexane : AcOEt = 1 : 1 as an eluant to give **26** as a colorless oil (17.4 mg, 97%). $[\alpha]_D^{23}$ +10.5° (c=0.56, $CHCl_3$). ^{1}H-NMR δ: 1.03 (3H, d, J=6.6 Hz), 1.32 (3H, d, J=6.2 Hz), 1.20—1.80 (11H, m), 1.93—1.99 (1H, m), 2.10 (1H, ddd, J=13.2, 1.8, 1.5 Hz), 2.31—2.33 (1H, m), 2.48 (1H, dd, J=16.1, 2.9 Hz), 2.89 (1H, dd, J=16.1, 11.0 Hz), 2.95—3.05 (2H, m), 3.03 (1H, d, J=6.2 Hz), 3.50—3.62 (2H, m), 3.53 (3H, s), 3.75 (1H, t, J=5.5 Hz), 3.80 (1H, d, J=5.1 Hz), 4.09 (2H, s), 4.27 (1H, dd, J=11.0, 1.8 Hz), 5.25 (1H, m), 5.44 (1H, d, J=6.6 Hz), 5.55 (1H, dd, J=15.8, 6.6 Hz), 5.95 (1H, dd, J=15.8, 6.6 Hz). MS m/z (relative intensity): 503 (M^+-15, 0.1%), 460 (0.09), 442 (1), 366 (0.08), 349 (0.08), 317 (2), 251 (5.2), 233 (12.5), 163 (9.4), 121 (12.5), 109 (28), 98 (100). Exact MS m/z Calcd for $C_{24}H_{36}ClO_9$ (M^+-15): 503.2048. Found: 503.2075. IR ν (neat) cm^{-1}: 3400, 1750, 1720, 1450, 1375, 1360, 1300, 1280, 1260, 1200, 1175, 1120, 1080, 1040, 970.

9*R*-*O*-Chloroacetyl-12*S*,13*R*-epoxy-3,5-isopropylideneleuconolide A_1 (27) Dry DMSO (17 μl, 0.239 mmol) in dry CH_2Cl_2 (0.5 ml) was added dropwise during 15 min to a well stirred solution of oxalyl chloride (10 μl, 0.115 mmol) in dry CH_2Cl_2 (0.5 ml) cooled to below −73 °C under an argon atmosphere. After 15 min at −73 °C, a solution of **26** (12.7 mg, 0.024 mmol) was added to the mixture during 5 min. Stirring was continued at −73 °C for 15 min, then Et_3N (0.048 ml, 0.345 mmol) was added dropwise, and after removal of the cooling bath, the reaction mixture was allowed to warm to room temperature (over *ca.* 1 h). Then saturated aqueous NH_4Cl (5 ml) was added, the organic layer was separated, and the aqueous layer was re-extracted with ether (5 ml × 2). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was chromatographed on a silica gel column with hexane–AcOEt (1 : 1) as the eluant to give the aldehyde as a colorless oil (12.7 mg, 100%). $[\alpha]_D^{24}$ +3.05° (c=0.75, $CHCl_3$). ^{1}H-NMR δ: 1.09 (3H,

d, J=6.0 Hz), 1.32 (3H, d, J=6.6 Hz), 1.40 (3H, s), 1.44 (3H, s), 1.52—1.71 (5H, m), 2.11 (1H, d, J=16.5 Hz), 2.28 (1H, dq, J=8.2, 2.2 Hz), 2.48 (1H, dd, J=16.5, 2.9 Hz), 2.96 (1H, d, J=16.5 Hz), 3.01 (1H, d, J=7.3 Hz), 3.03—3.06 (1H, m), 3.22 (1H, ddd, J=17.2, 3.7, 1.5 Hz), 3.56 (3H, s), 3.86 (1H, d, J=6.2 Hz), 4.07 (2H, s), 4.27 (1H, dd, J=11.0, 2.2 Hz), 5.21—5.30 (1H, m), 5.42 (1H, d, J=6.0 Hz), 5.52 (1H, dd, J=16.1, 7.0 Hz), 5.95 (1H, dd, J=16.1, 7.0 Hz), 9.74 (1H, s). MS m/z (relative intensity): 501 (M^+-15, 0.07%), 458 (0.98), 442 (0.2), 431 (0.27), 407 (0.48), 364 (4.2), 249 (9.2), 175 (6.3), 163 (6.8), 147 (10), 121 (17), 98 (100). Exact MS m/z Calcd for $C_{24}H_{34}ClO_9$ (M^+-15): 501.1891. Found 501.1892. IR ν (neat) cm^{-1}: 1750, 1720, 1710, 1450, 1410, 1370, 1300, 1280, 1260, 1200, 1170, 1075, 970, 910.

9*R*-*O*-Chloroacetylmaridonolide II Acetoxyacetal (29) (a) A solution of **27** (9.3 mg, 0.018 mmol) in 1 ml of 2 N H_2SO_4–THF (1 : 10) was stirred at room temperature for 30 min. After dilution of the solvent with CH_2Cl_2, the reaction mixture was washed with saturated aqueous $NaHCO_3$ and brine, then dried over Na_2SO_4, and evaporated. Purification of the residue on a silica gel column with hexane–AcOEt (1 : 3) as the eluant gave recovered **27** (3.6 mg, 39%) and 9*R*-*O*-Chloroacetyl-12*S*,13*R*-epoxyleuconolide A_1 hemiacetal (**28**) as a colorless oil (3.4 mg, 40%). $[\alpha]_D^{24}$ +5.94° (c=0.42, $CHCl_3$). IR ν (neat) cm^{-1}: 3400, 1750, 1725, 1720, 1460, 1380, 1260, 1200, 1080, 1040. A solution of **28** (4.8 mg, 0.01 mmol) in pyridine (0.5 ml) and CH_2Cl_2 (0.5 ml) was treated with acetic anhydride (10 μl, 0.106 mmol) and DMAP (1 mg). Stirring was continued for 40 min, then the reaction mixture was diluted with CH_2Cl_2 and washed with 1 N HCl and brine. The organic layer was dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified on a silica gel column with hexane–AcOEt (1 : 1) as the eluant, affordeding **29α**, **β** as a colorless oil (4.6 mg, 82%).

(b) MCPBA (4.4 mg, 0.021 mmol; 85% activity) was added to a stirred solution of **21** (7.8 mg, 0.0143 mmol) and K_2CO_3 (2 mg) in CH_2Cl_2 (0.5 ml) at room temperature. After 16.5 h, the solution was diluted with CH_2Cl_2, washed with aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave the crude epoxide, which was chromatographed on a silica gel column with hexane–AcOEt (2 : 1) as the eluant to give the epoxide **29α**, **β** as a colorless oil (6.7 mg, 84%). ^{1}H-NMR δ: 1.07 (3H, d, J=7.0 Hz), 1.28 (3H, d, J=6.0 Hz), 1.97—2.17 (2H, m), 2.06 (1H, dd, J=11.5, 8.5 Hz), 2.06 (3H, s), 2.10 (3H, s), 2.20—2.37 (3H, m), 2.34 (1H, dd, J=12.5, 1.0 Hz), 2.93 (1H, dd, J=12.5, 11.5 Hz), 3.11 (1H, dd, J=9.2, 2.0 Hz), 3.15 (1H, dt, J=10.5, 1.0 Hz), 3.16 (1H, dd, J=9.0, 0.5 Hz), 3.56 (3H, s), 3.59 (1H, t, J=6.9 Hz), 4.04—4.08 (1H, m), 4.05 (2H, s), 4.99 (1H, ddq, J=8.5, 3.0, 6.0 Hz), 5.06 (1H, dd, J=11.0, 1.5 Hz), 5.39 (1H, dd, J=9.5, 3.5 Hz), 5.78 (1H, dd, J=15.5, 8.5 Hz), 5.99 (1H, dd, J=15.5, 9.5 Hz), 6.29 (1H, dd, J=6.0, 4.5 Hz). MS m/z (relative intensity): 560 (M^+, 0.36%), 501 (4.8), 472 (0.9), 440 (0.7), 407 (1.2), 249 (4.2), 231 (6.3), 175 (17), 98 (84), 43 (100). Exact MS m/z Calcd for $C_{26}H_{37}ClO_{11}$ (M^+): 560.2024. Found: 560.2012. IR ν (neat) cm^{-1}: 1750, 1740, 1730.

9*R*-*O*-Chloroacetylmaridonolide I (30) (a) A solution of **28** (4.2 mg, 9 μmol), Et_3N (32 μl), propionic anhydride (15 μl, 0.117 mmol) and DMAP (1 mg) in CH_2Cl_2 was stirred at room temperature for 90 min, then diluted with CH_2Cl_2 and washed with 1 N HCl and brine. The organic layer was dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified on a silica gel column with hexane–AcOEt (1 : 1) as the eluant to give **30** as a colorless oil (3.7 mg, 71%).

(b) MCPBA (5 mg, 0.029 mmol; 85% activity) was added to a stirred solution of **22** (10 mg, 0.0174 mmol) and K_2CO_3 (4 mg) in CH_2Cl_2 (1 ml) at room temperature. After 8.5 h, the solution was diluted with CH_2Cl_2, washed with aqueous $NaHCO_3$ and brine, dried ($MgSO_4$), and evaporated *in vacuo* to leave the crude epoxide, which was chromatographed on a silica gel column with hexane–AcOEt (2 : 1) as the eluant to give the epoxide **30** as a colorless oil (9.5 mg, 93%). $[\alpha]_D^{22.5}$ +22.4° (c=0.81, $CHCl_3$). ^{1}H-NMR δ: 1.07 (3H, d, J=6.5 Hz), 1.13 (6H, t, J=7.3 Hz), 1.28 (3H, d, J=6.2 Hz), 1.46 (1H, ddd, J=14.2, 12.0, 9.8 Hz), 2.01 (1H, ddd, J=14.0, 6.8, 4.8 Hz), 2.02—2.12 (1H, m), 2.20—2.42 (4H, m), 2.33 (2H, q, J=7.3 Hz), 2.37 (2H, q, J=7.3 Hz), 2.38 (2H, q, J=7.3 Hz), 2.93 (1H, ddd, J=14.0, 11.6 Hz), 3.12 (1H, dd, J=10.5, 5.5 Hz), 3.18 (1H, dd, J=9.6, 1.0 Hz), 3.55 (3H, s), 4.03 (1H, dd, J=9.3, 4.2 Hz), 4.05 (2H, s), 4.96 (1H, ddq, J=12.2, 3.4, 6.2 Hz), 5.11 (1H, d, J=10.5 Hz), 5.39 (1H, dd, J=9.5, 3.5 Hz), 5.80 (1H, dd, J=15.5, 9.0 Hz), 6.00 (1H, dd, J=15.5, 9.6 Hz), 6.31 (1H, dd, J=5.8, 5.0 Hz). MS m/z (relative intensity): 590 (M^++2, 0.1%), 588 (M^+, 0.2), 515 (4.7), 495 (0.3), 421 (0.7), 231 (2.7), 189 (6.3), 147 (3.5), 121 (6.5), 109 (11.3), 98 (27.9), 81 (12.0), 71 (22.6), 57 (100), 41 (9.2). Exact MS m/z Calcd for $C_{28}H_{41}ClO_{11}$ (M^+): 588.2337. Found: 588.2344. IR ν (neat) cm^{-1}: 1750, 1730, 1720, 1460, 1280, 1180, 1130, 1080, 970.

Maridonolide II Hemiacetal (4a) A solution of **29α**, **β** (13 mg, 0.023 mmol) in 0.5 ml of MeOH was treated with K_2CO_3 (10 mg, 0.072 mmol) at 0 °C for 20 min. After evaporation of the solvent, the residue was taken up in CH_2Cl_2, and the precipitate was filtered off. The filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–MeOH (20 : 1) as the eluant to give **4a** as a colorless solid (8.3 mg, 81%). mp 94—95 °C (amorphous solid). $[\alpha]_D^{19}$ −4.6° (c=0.56, $CHCl_3$). ^{1}H-NMR δ: 1.05 (2.1H, d, J=7.0 Hz), 1.07 (0.9H, d, J=7.0 Hz), 1.28 (2.1H, d, J=6.2 Hz), 1.30 (0.9H, d, J=6.2 Hz), 1.40 (1H, dt, J=11.5, 2.5 Hz), 1.81 (1H, dd, J=7.5, 3.5 Hz), 1.86 (1H, dd, J=7.0, 4.5 Hz), 1.92—2.05 (1H, m), 2.076 (0.9H, s), 2.084 (2.1H, s), 2.11 (1H, dd, J=12.0, 8.0 Hz), 2.19 (0.7H, ddd, J=11.5, 3.5, 1.5 Hz), 2.20 (0.7H, dd, J=8.2, 5.0 Hz), 2.28 (0.3H, ddd, J=7.5, 3.5, 1.5 Hz), 2.34 (0.7H, dd, J=13.0, 2.8 Hz), 2.53 (0.3H, dd, J=14.2, 4.2 Hz), 2.62 (0.7H, br d, J=2.5 Hz), 2.86 (0.3H, dd, J=14.0, 9.5 Hz), 2.94 (0.7 H, dd, J=13.5, 11.5 Hz), 3.12 (1H, d, J=9.2 Hz), 3.19 (0.7H, dd, J=9.2, 0.5 Hz), 3.30 (0.3H, dd, J=7.5, 2.0 Hz), 3.57 (2.1H, s), 3.62 (0.9H, s), 3.89 (0.3H, dd, J=7.2, 5.0 Hz), 4.12 (1H, dd, J=9.5, 4.0 Hz), 4.24 (1H, dd, J=9.0, 3.5 Hz), 4.95—5.17 (1H, m), 5.15 (0.7H, ddd, J=11.0, 2.2, 1.5 Hz), 5.23 (0.3H, ddd, J=9.8, 4.2, 2.0 Hz), 5.46 (0.3H, t, J=4.0 Hz), 5.55—5.65 (0.7H, m), 5.62 (0.7H, dd, J=15.5, 8.5 Hz), 6.07 (0.7H, dd, J=15.5, 8.5 Hz), 6.08 (0.3 H, dd, J=15.5, 8.5 Hz). MS m/z (relative intensity): 442 (M^+, 0.07%), 424 (M^+-18, 0.9), 382 (1.2), 364 (2.2), 350 (2.6), 249 (8.0), 179 (17), 117 (36), 98 (99), 71 (66), 43 (100). Exact MS m/z Calcd for $C_{22}H_{32}O_8$ (M^+): 424.2097. Found: 424.2095. IR ν (neat) cm^{-1}: 3400, 1730.

Maridonolide I Hemiacetal (4b) A solution of **30α**, **β** (6.8 mg, 0.012 mmol) in 1 ml of MeOH was treated with K_2CO_3 (6 mg, 0.043 mmol) at 0 °C for 60 min. After evaporation of the solvent, CH_2Cl_2 was added to the reaction mixture and precipitate was filtered off. The filtrate was evaporated *in vacuo* to leave an oil, which was chromatographed on a silica gel column with CH_2Cl_2–MeOH (20 : 1) as the eluant to give **4b** as a colorless solid (5.0 mg, 95%). mp 91—92.5 °C (amorphous solid). $[\alpha]_D^{19}$ = −6.4° (c=1.90). ^{1}H-NMR δ: 1.05 (2.1H, d, J=7.0 Hz), 1.07 (0.9H, d, J=7.0 Hz), 1.12 (3H, t, J=7.0 Hz), 1.28 (0.9H, d, J=6.2 Hz), 1.30 (2.1H, d, J=6.2 Hz), 1.39—1.49 (1H, m), 1.79—1.85 (1H, m), 1.82—1.90 (1H, m), 1.91—2.01 (1H, m), 2.10—2.20 (1H, m), 2.17 (0.3H, s), 2.21 (1H, dd, J=7.8, 5.0 Hz), 2.28 (0.3H, dd, J=6.0, 3.2 Hz), 2.36 (2H, dt, J=2.0, 6.2 Hz), 2.53 (0.3H, dd, J=14.5, 4.2 Hz), 2.63 (0.7H, d, J=3.5 Hz), 2.85 (0.3H, dd, J=14.0, 9.5 Hz), 2.93 (0.7H, dd, J=12.5, 11.5 Hz), 3.08—3.17 (0.3H, m), 3.13 (1H, d, J=9.0 Hz), 3.17 (0.7H, dd, J=9.2, 0.5Hz), 3.31 (0.3H, dd, J=7.0, 2.0 Hz), 3.56 (2.1H, s), 3.62 (0.9H, s), 3.89 (0.3H, dd, J=7.0, 5.0 Hz), 4.11 (1H, dd, J=9.0, 4.0 Hz), 4.24 (1H, dd, J=9.0, 3.5 Hz), 4.93—5.74 (1H, m), 5.12 (0.7H, ddd, J=11.8, 2.5, 1.0 Hz), 5.24 (0.3H, ddd, J=8.5, 4.0, 1.8 Hz), 5.44 (0.3H, t, J=5.0 Hz), 5.58 (0.7H, dd, J=8.0, 6.0 Hz), 5.62 (0.3H, dd, J=15.5, 8.5 Hz), 5.65 (0.7H, dd, J=15.5, 8.5 Hz), 6.06 (0.3H, dd, J=15.5, 9.5 Hz), 6.08 (0.7H, dd, J=15.5, 9.5 Hz). MS m/z (relative intensity): 456 (M^+, 0.04%), 438 (M^+-18, 0.48), 249 (4.3), 189 (8.9), 98 (60), 71 (57), 57 (100). Exact MS m/z Calcd for $C_{23}H_{34}O_8$ (M^+-18): 438.2101. Found: 438.2271. IR ν (neat) cm^{-1}: 3400, 1730.

References and Notes

1) Chiral Synthesis of Polyketide-Derived Natural Products. 33. For part 32, see: N. Nakajima, K. Uoto, and O. Yonemitsu, *Chem., Pharm., Bull.*, **39**, 64 (1991).
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11) Unless otherwise noted, the numberings are based on those of **1**—**4**.
12) Compound **6** was converted to leuconolide A_3 by treatment with 80% trifluoroacetic acid in 61% yield.
13) There are two precedents for the reduction of such a 16-membered macrolide. Grieco *et al.*[14] reported the reduction of tylonolide *O*-methylacetal to the 9*S*-alcohol selectively and Freiberg *et al.*[15] obtained the 9*R*-alcohol (josamycin) from carbomycin B in moderale selectivity.
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18) There were four conformers having Boltzmann distributions lagrer than 0.1%.
19) There were 32 conformers having Boltzmann distributions larger than 0.1%.
20) Leuconolide A_1 (**3a**), leuconolide A_3 (**3b**), and midecanolide A_1 (**3c**) are 1 : 1, 2.3 : 1, and 2.3 : 1 isomeric mixtures, respectively, with respect to the hemiacetal position.
21) NMR studies suggested that **23** was a mixture of multiple conformers of a single compound, as indicated by doubling of nearly every signal in the spectra. Details of the behavior and spectrum were reported in: T. Matsushima, N. Nakajima, and O. Yonemitsu, *Tetrahedron Lett.*, **32**, 5133 (1991).
22) Both maridonolide II (**4a**) and maridonolide I (**4b**) are 2.3 : 1 isomeric mixtures with respect to the hemiacetal position.

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Synthetic Studies of Indoles and Related Compounds. XXVIII.[1] Intramolecular Vinylation of Pyrrole Derivatives Using Palladium: a New Synthetic Method for Substituted Indoles

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The cyclization of methyl (*E*)-6-oxo-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate (8a) using an equimolar amount of palladium chloride gave methyl 4-hydroxy-1-(phenylsulfonyl)-1*H*-indole-7-acetate (11a) in 33% yield. The similar cyclization of methyl (*E*)-6-acetoxy-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate (8c) proceeded smoothly to give methyl 1-(phenylsulfonyl)-1*H*-indole-7-acetate (11c) and 4-acetoxy-7-methoxycarbonylmethylidene-1-phenylsulfonyl-4,5,6,7-tetrahydro-1*H*-indole (10c) in 41% and 22% yields, respectively. Conversion of the tetrahydroindole (10c) to the indole (11c) was accomplished in 44% yield by treatment with *p*-toluenesulfonic acid in benzene. Methyl (*E*)-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate (8d) gave 11c and 7-methoxycarbonylmethylidene-1-phenylsulfonyl-4,5,6,7-tetrahydro-1*H*-indole (10d) in 27% and 40% yields, respectively. The cyclization of ethyl (*E*)-4-(5-methoxycarbonyl-4-pentenyl)-1*H*-pyrrole-2-carboxylate (18) also gave the corresponding indole-7-acetate (20) and 7-methoxycarbonylmethylidene-4,5,6,7-tetrahydro-1*H*-indole (21) in 16% and 49% yields, respectively.

Keywords intramolecular vinylation; palladation; synthesis; 4,7-disubstituted indole; 1-(phenylsulfonyl)pyrrole; Friedel–Crafts acylation

Recently, new synthetic methods for the indole nucleus starting from pyrrole derivatives have been reported.[2,3] An important characteristic of those routes is that indole derivatives which have variously functionalized alkyl side chains and/or hetero atoms on the benzene ring can be prepared regiospecifically.[2] Several indole alkaloids, such as teleocidin analogues,[3a,d] ergot alkaloids,[3b] and trikentrins,[3c] have been synthesized by utilizing those methods. We have reported the regioselective C_4-acylation of ethyl pyrrole-2-carboxylate by Friedel–Crafts (F–C) reaction,[4] and the direct regioselective vinylation of indoles at the C_3-position with α,β-unsaturated carbonyl compounds using a stoichiometric amount of palladium.[5] On the basis of those results, we report here a new methodology for the synthesis of 4,7-disubstituted or 7-substituted indole derivatives starting from pyrroles (**5**, **6**).

Chart 1 shows the synthetic plan, which consists of the following steps; i) regioselective synthesis of a β-substituted pyrrole (**2**) which has a side chain with a double bond at the appropriate position (step 1), ii) novel intramolecular vinylation of **2** at the α-position using an equimolar amount of palladium (step 2), and iii) aromatization of the cyclized product (**3**) to give the 4,7-disubstituted indole (**4**) (step 3).

Results and Discussion

Introduction of the Side Chain Regioselective introduction of the side chain is essential for accomplishment of the new synthesis. For this purpose, 1-(phenylsulfonyl)-1*H*-pyrrole (**5**) and ethyl 1*H*-pyrrole-2-carboxylate (**6**) were selected as starting pyrroles, since these compounds undergo regioselective F–C acylation at the 3- (or β-) position of **5**

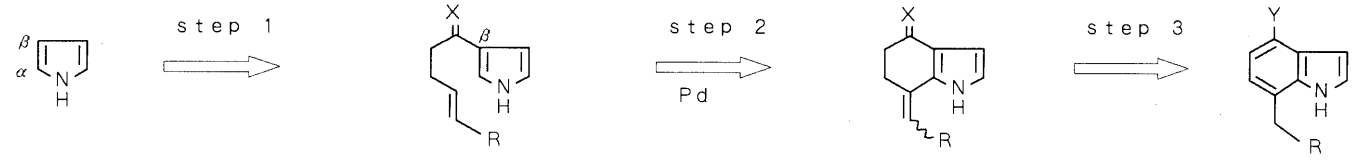

Chart 1

Chart 2

TABLE I. Cyclization of the *N*-Phenylsulfonylpyrrole Series (**8a—d**)

Run	Pyrrole (**8a—d**)	Conditions				Product (%)	SM[a] recovery (%)
		Solvent	Additive (4 eq)	Temperature	Time (h)		
1	**8a**	CH_3CN	$(CH_3COO)_2Cu$	Reflux	4	—	—
2	**8a**	CH_3COOH	CH_3COONa	Reflux	10	**11a** (13)	35
3	**8a**	CH_3COOH[b]	CH_3COONa	Reflux	4	**11a** (33)	40
4	**8b**	CH_3CN	CH_3COONa	60 °C	6	**10b** (20 and 5)	19
5	**8c**	CH_3COOH[b]	CH_3COONa	Reflux	1	**10c** (22), **11c** (41)	25
6	**8c**	CH_3CN	$(CH_3COO)_2Cu$	60 °C	4.5	**10c** (26), **11c** (9)	10
7	**8d**	CH_3COOH[b]	CH_3COONa[c]	Reflux	2.5	**10b** (40), **11c** (27)	—

a) Starting material. *b*) High dilution condition (**8**: 0.01 mol/l). *c*) Nine eq of AcONa was used.

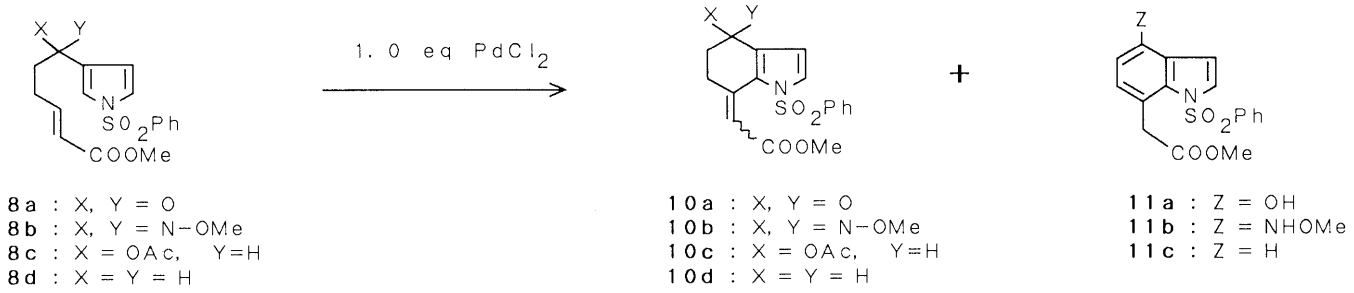

Chart 3

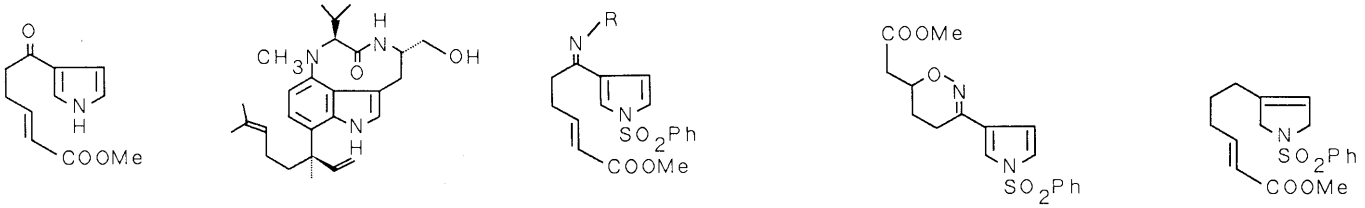

Chart 4

and the 4- (or β-) position of **6** as shown by Kakushima *et al.*[6] and ourselves.[4] Furthermore, those compounds should be stable under oxidative conditions (palladation) owing to the electron-withdrawing phenylsulfonyl or 2-ethoxycarbonyl group on the pyrrole ring.[7] The F–C acylation of the pyrroles (**5** and **6**) with the acid chloride (**7**) derived from (*E*)-5-methoxycarbonyl-4-pentenoic acid,[8] regioselectively gave the β-acylated products in good yields (73% for **8a**, 62% for **9a**) under the reported conditions.[4,6] If the ketone group in **8a** and **9a** is changed to other groups, various 4,7- or 7-substituted indoles should be obtained after cyclization.

Cyclization of the 1-(Phenylsulfonyl)-1*H*-pyrrole Series (8a—d) The cyclizations of the 1-(phenylsulfonyl)-1*H*-pyrrole series (**8a—d**) were examined in detail, and the results are summarized in Table I.

The cyclization of the ketone (**8a**) with 1.0 eq of $PdCl_2$ in the presence of 4.0 eq of $Cu(OAc)_2$ in CH_3CN gave no isolable product (run 1). So we changed the solvent and additive to AcOH and AcONa (run 2). The 4-hydroxy-7-alkylindole (**11a**) was obtained in low yield accompanied with the starting pyrrole (**8a**). The product (**11a**) should be formed by spontaneous isomerization of the double bond of the expected cyclized product (**10a**). This reaction was affected by the amount of solvent, since the yield was increased to 33% (with 40% recovery of starting material) by dilution about 10-fold with solvent (run 3). As the yield was still unsatisfactory, we tried to change the reactivity of the pyrrole ring towards palladation by deprotection of the phenylsulfonyl group. The deprotection of **8a** by Mg/MeOH[9] smoothly proceeded to give the NH-ketone (**12**) in good yield, but the cyclization of **12** was unsuccessful, giving only a tarry product accompanied with the starting material (49%) under the same conditions as those of run 2. The expected indole might be unstable under these conditions.

Next, we tried to synthesize 4-amino-7-alkylindole, which is potentially useful for the synthesis of teleocidines (**13** is a representative example). As the Schiff's base (**14**) could not be obtained by the reaction of the ketone (**8a**) with primary amines such as valine methyl ester (H–Val–OMe) or *n*-butylamine, preparation of the oxime (**15**) was examined. The reaction of **8a** with hydroxylamine gave the desired oxime (**15**) in only 35% yield as an unstable oil,

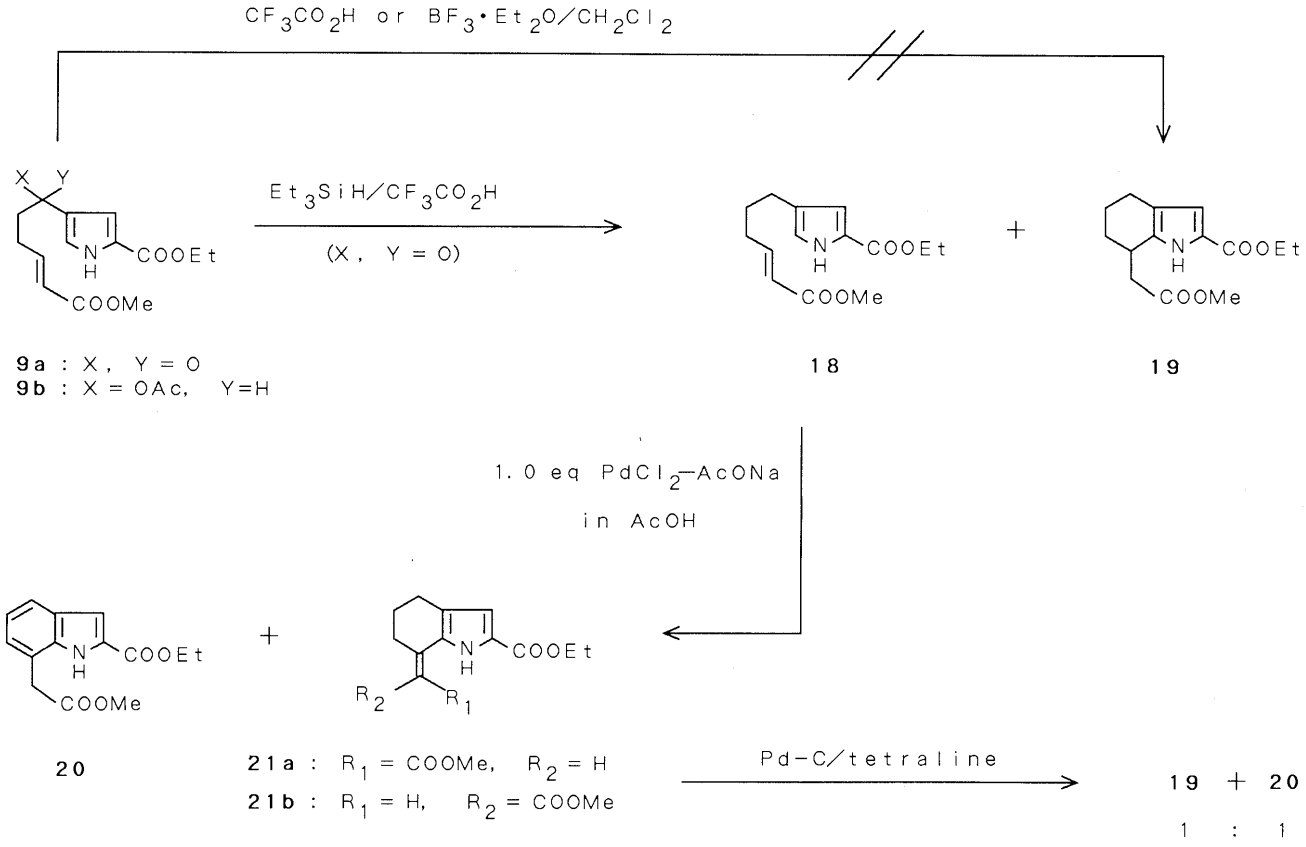

Chart 5

and the oxazole (**16**) in 62% yield as the major product. The oxazole (**16**) should be formed as a result of an intramolecular Michael addition of the hydroxyl group of the oxime (**15**). Thus, the methoxylamine was used in place of hydroxylamine to give the desired oxime (**8b**) in good yield as a mixture of *E*- and *Z*-isomers (91%). Both isomers were separable by silica gel chromatography, but the isomerization occurred very rapidly even in $CDCl_3$ during nuclear magnetic resonance (NMR) measurement. So a mixture of *E*- and *Z*-isomers was subjected directly to the cyclization. The cyclized product (**10b**) was obtained by using a combination of CH_3CN and AcONa in 25% yield as a mixture of two isomers out of four possible isomers (run 4). Each isomer was isolated as a pure form, but their structures could not be determined from the spectral data. Curiously, the desired indole (**11b**), which should be formed from **10b** by spontaneous isomerization of the double bond, was neither detected in the reaction mixture nor formed by heating **10b** with Pd–C or $RhCl(PPh_3)_3$.

Although the 4,7-disubstituted indoles were regiospecifically obtained in only a few steps from *N*-phenylsulfonylpyrrole (**5**), the yields of the cyclization products were unsatisfactory for the practical use. The reactivity of the ketone (**8a**) was presumed to be insufficient for palladation owing to the presence of electron-withdrawing substituents (1-phenylsulfonyl and C_3-carbonyl groups), since it is known[10] that electron-rich aromatic compounds show higher reactivity for palladation. As deprotection of the phenylsulfonyl group of **8a** led to unsatisfactory results in the cyclization as described before, the electron-withdrawing carbonyl group was reduced to alcohol or methylene. At first, the cyclization of the acetate (**8c**), which was obtained by reduction of the ketone (**8a**) with $NaBH_4$ followed by acetylation, was carried out, expecting the formation of the 7-monosubstituted indole (**11c**) in one step by spontaneous elimination of AcOH from the cyclization product (**10c**). In practice, however, the cyclic acetate (**10c**) was also formed as a minor product in addition to the expected indole (**11c**) (run 5). The combination of CH_3CN and $Cu(OAc)_2$ gave a much worse result (run 6). Treatment of the cyclic acetate (**10c**) with *p*-toluenesulfonic acid (*p*-TsOH) in benzene gave the corresponding indole (**11c**) in 46% yield.

Next we tried cyclization of the methylene compound (**8d**), although spontaneous aromatization could not be expected in the cyclized product (**10d**). When the ketone (**8a**) was reduced with triethylsilane (Et_3SiH) in CF_3COOH, not only the carbonyl group but also the pyrrole ring was reduced to give the dihydropyrrole derivative (**17**). The position of the double bond was tentatively determined to be between the 3- and 4-position based on the NMR spectrum, which showed one broad singlet at 4.02 ppm derived from two sets of methylene protons in the pyrrole ring. The crude product (**17**) was directly dehydrogenated with MnO_2 to give the aromatized α,β-unsaturated ester (**8d**). The reaction of **8d** under palladation conditions gave the cyclized product (**10d**) and aromatized indole (**11c**) in 40 and 27% yields, respectively, contrary to our prediction (run 7). Aromatization of **10d**[11] was accomplished in a stepwise manner according to our reported method.[4] Thus, **10d** was treated with $CuBr_2$ in AcOEt followed by heating with $LiBr/Li_2CO_3$ in *N*,*N*-dimethylformamide (DMF) to give the indole (**11c**) in 48% overall yield from **10d**.

Cyclization of the Ethyl Pyrrole-2-carboxylate Series (9a, b and 18) Next we turned our attention to the cyclization

of the ethyl pyrrole-2-carboxylate series (**9a, b** and **18**). The steric effect of the 2-ethoxycarbonyl group on the cyclization should be much less than that of the 1-phenylsulfonyl group, while the pyrrole ring would be stabilized by a 2-ethoxycarbonyl group to about the same extent as by the 1-phenylsulfonyl group. Contrary to our prediction, the ketone (**9a**) did not give the cyclized product at all, and the acetate (**9b**), a relatively unstable oil, afforded a complex mixture. But the cyclization of the methylene compound (**18**), which was obtained by the reduction of the ketone (**9a**) with Et_3SiH in CF_3COOH (*vide infra*), proceeded smoothly to give the indole (**20**), and the *Z*- and *E*-form of the tetrahydroindole (**21a** and **21b**) in 16%, 32%, and 17% yields, respectively. The geometries of the double bond of **21a** and **21b** were estimated from the chemical shifts of hydrogen at the pyrrole nitrogen [12.66 ppm for the major product (**21a**) and 9.63 ppm for the minor product (**21b**)], since hydrogen bonding of NH with the ester carbonyl was possible in the *Z*-form. Disappointingly, conversion of the cyclized olefin (**21a** or **21b**) to the indole (**20**) by Pd–C in tetraline gave a 1 : 1 mixture of the desired indole (**20**) and hexahydro derivative (**19**) as a result of disproportionation. It is not clear why disproportionation occurred instead of simple dehydrogenation.

Reduction of the ketone (**9a**) with Et_3SiH in CF_3COOH (room temperature, overnight) afforded an unexpected cyclized product (**19**) as the major product (59% yield) accompanied with the desired methylene compound (**18**) as a minor product (26% yield). The cyclized product (**19**) should be formed by an intramolecular Michael addition of the aromatic ring to the conjugated double bond. The shorter reaction time (4.5 h) increased the yield of the desired product (**18**, 56%), which was accompanied with **19** in 11% yield. On the basis of those results, we attempted the acid catalyzed cyclization of the ketone (**9a**), but the cyclized product was not obtained on treatment with CF_3COOH or $BF_3 \cdot OEt_2$ in CH_2Cl_2.

Conclusion

The present investigation, which involves palladium-mediated cyclization from the α- to the β-position of the pyrrole ring, provides a new methodology of indole synthesis, although it does not represent a practical approach so far, owing to the unsatisfactory yield and the requirement for stoichiometric use of expensive palladium salt.

Experimental

All melting points were determined on a Yanagimoto micromelting hot-stage apparatus and are uncorrected. Infrared (IR) spectra were recorded in Nujol mulls (unless otherwise stated) on a Shimadzu IR-400 spectrometer. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded in $CDCl_3$ (unless otherwise stated) on a Hitachi R-24B spectrometer (60 MHz) (unless otherwise stated) or a JEOL GX-400 (400 MHz) spectrometer with tetramethylsilane as an internal reference. Mass spectra (MS) were measured with JEOL JMS-D-300 and JMS-DX-303 spectrometers using a direct inlet system. For column chromatography, Kiesel gel 60 (70—230 mesh, Merck), and for thin layer chromatography (TLC), Kieselgel GF_{254}, were used. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; dt, double triplet; ddd, double double doublet; dddd, double double double doublet; ddt, double double triplet; ttt, triple triple triplet; q, quartet; m, multiplet; dif, diffused; br, broad; arom, aromatic.

Preparation of Methyl (*E*)-6-Oxo-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate (8a) Oxalyl chloride (12.8 ml, 0.15 mol) was added to a solution of (*E*)-5-methoxycarbonyl-4-pentenoic acid (15.82 g, 0.10 mol) in CH_2Cl_2 (50 ml) at room temperature under an Ar atmosphere. After refluxing of the mixture for 20 min, excess oxalyl chloride and solvent were removed under reduced pressure to give the crude acid chloride (**7**) as an oil. A solution of this crude oil in CH_2ClCH_2Cl (100 ml) was added to powdered $AlCl_3$ (27 g, 0.20 mol) at 0 °C under an Ar atmosphere. After 15 min, a solution of 1-(phenylsulfonyl)pyrrole[12] (10.36 g, 0.05 mol) in CH_2Cl_2 (50 ml) was slowly added to the above mixture under ice cooling and the whole was stirred for 30 min at room temperature. Then the reaction mixture was poured into ice water and extracted with CH_2Cl_2. The organic layer was washed successively with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave a brown oil (25.0 g), which was purified by silica gel column chromatography (AcOEt : *n*-hexane = 1 : 2) to give **8a** as a pale yellow solid (12.63 g, 73%). This solid was recrystallized from AcOEt–hexane to give colorless prisms, mp 74.5—75.5 °C. *Anal.* Calcd for $C_{17}H_{17}NO_5S$: C, 58.78; H, 4.93; N, 4.03. Found: C, 58.65; H, 4.86; N, 4.05. IR ν_{max} cm^{-1}: 1702, 1675 (C=O). ^{1}H-NMR (400 MHz) δ: 2.59 (2H, dq, *J*=7.0, 1.6 Hz, C$\underline{H}_2$CH=CH), 2.91 (2H, t, *J*=7.0 Hz, COC$\underline{H}_2$CH$_2$), 3.72 (3H, s, OCH_3), 5.87 (1H, dt, *J*=15.5, 1.6 Hz, =C$\underline{H}$CO), 6.69 (1H, dd, *J*=3.4, 1.8 Hz, 4-H), 6.98 (1H, dt, *J*=15.5, 7.0 Hz, C$\underline{H}$=CHCO), 7.17 (1H, dd, *J*=3.4, 2.0 Hz, 5-H), 7.56 (2H, t, *J*=7.5 Hz, 2×3′-H), 7.67 (1H, dt, *J*=7.5, 1.3 Hz, 4′-H), 7.74 (1H, dd, *J*=2.0, 1.8 Hz, 2-H), 7.92 (2H, dd, *J*=7.5, 1.3 Hz, 2×2′-H). MS *m/z* (%): 234 (100), 347 (M^+, 3.9).

Cyclization of 8a A mixture of **8a** (100 mg, 0.29 mmol), $PdCl_2$ (51 mg, 0.29 mmol), and AcONa (95 mg, 1.2 mmol) in AcOH (30 ml) was refluxed for 4 h under an Ar atmosphere. Then the reaction mixture was poured into H_2O and extracted with benzene. The organic layer was washed with H_2O and saturated aqueous NaCl and dried over $MgSO_4$. After evaporation of the solvent, the resultant brown oil (94 mg) was chromatographed on silica gel with AcOEt–hexane (1 : 2) to give methyl 4-hydroxy-1-(phenylsulfonyl)-1*H*-indole-7-acetate (**11a**) as a brown solid (33 mg, 33%), accompanied with 40 mg of starting material (40% recovery). **11a** was recrystallized from AcOEt–hexane to give pale brown prisms, mp 152.5—153.5 °C. *Anal.* Calcd for $C_{17}H_{15}NO_5S$: C, 59.12; H, 4.38; N, 4.06. Found: C, 59.11; H, 4.38; N, 4.06. IR ν_{max} cm^{-1}: 3365 (OH), 1718 (C=O). ^{1}H-NMR (400 MHz) δ: 3.71 (3H, s, OCH_3), 4.01 (2H, s, arom-C$\underline{H}_2$), 5.76 (1H, s, OH), 6.43 (1H, d, *J*=8.0 Hz, 5- or 6-H), 6.71 (1H, d, *J*=4.0 Hz, 2- or 3-H), 6.83 (2H, d, *J*=8.0 Hz, 5- or 6-H), 7.41 (2H, t, *J*=8.0 Hz, 2×3′-H), 7.52 (1H, dif t, *J*=8.0 Hz, 4′-H), 7.52 (1H, d, *J*=4.0 Hz, 2- or 3-H), 7.63 (2H, dd, *J*=8.0, 1.5 Hz, 2×2′-H). MS *m/z* (%): 149 (100), 345 (M^+, 40.4).

Preparation of Methyl 6-Oxo-6-(1*H*-pyrrol-3-yl)-2-hexenoate (12) A mixture of **8a** (2.00 g, 5.8 mmol) and Mg powder (210 mg, 8.6 mmol), and 2 drops of AcOH in absolute MeOH (30 ml) was refluxed for 7.5 h. Since the reaction was not completed, further Mg powder (140 mg, 5.8 mmol) and 1 drop of AcOH were added to the reaction mixture. Refluxing was continued for 2 h, then the precipitates were removed by filtration through Celite and washed thoroughly with MeOH. The combined filtrate was concentrated by evaporation and H_2O was poured into the residue. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed successively with H_2O and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant yellow oil (1.22 g) was chromatographed on silica gel with AcOEt–hexane (1 : 2) to give **12** as a pale yellow solid (734 mg, 62%), which was recrystallized from AcOEt–Et_2O to give colorless prisms, mp 64.0—66.0 °C. *Anal.* Calcd for $C_{11}H_{13}NO_3$: C, 63.76; H, 6.32; N, 6.76. Found: C, 63.62; H, 6.30; N, 6.80. IR ν_{max} cm^{-1}: 3340—3260 (NH), 1725 (C=O). ^{1}H-NMR δ: 2.22—3.10 (4H, m, CH_2CH_2), 3.67 (3H, s, $COOCH_3$), 5.82 (1H, d, *J*=16.0 Hz, =C$\underline{H}$CO$_2$CH$_3$), 6.5—7.1 (3H, m, C$\underline{H}$=CHCO and arom-H), 7.3—7.5 (1H, m, arom-H), 9.1—10.1 (1H, br s, NH). MS *m/z* (%): 94 (100), 207 (M^+, 6.5).

Preparation of Methyl 6-Hydroxyimino-6-[1-(phenylsulfonyl)-1*H*-pyrrol-2-yl]-2-hexenoate (15) A solution of hydroxylamine hydrochloride (63 mg, 0.91 mmol) and sodium acetate (123 mg, 1.50 mmol) in H_2O (0.8 ml) was added to a solution of **8a** (300 mg, 0.86 mmol) in MeOH (3 ml) at 0 °C. The mixture was stirred for 4 h at room temperature, then MeOH was removed by evaporation and the residue was extracted with benzene. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant colorless oil (310 mg) was separated into two fractions by silica gel column chromatography using AcOEt–hexane as an eluent. The first fraction was an isomeric mixture of oximes (**15**), which was obtained as an unstable oil (111 mg, 35%). IR ν_{max} cm^{-1}: 3140 (OH), 1720 (C=O). ^{1}H-NMR δ: 2.1—3.1 (4H, m, –C$\underline{H}_2$C$\underline{H}_2$–), 3.69 (3H, s, CO_2CH_3),

5.80 (1H, dif d, J=15 Hz, =CHCO), 6.51 (1H, dd, J=3, 1 Hz, 2- or 3-H), 6.6—8.0 (8H, m, arom-H and CH=CHCO), 8.2—8.8 (1H, br, O–H). MS m/z (%): 77 (100), 362 (M^+, 22). The second fraction was the oxazole (**16**), which was obtained as a colorless oil (188 mg, 60%). IR ν_{max} cm^{-1}: 1735 (C=O). ^{1}H-NMR δ: 1.7—3.5 (6H, m, aliphatic-H), 3.65 (3H, s, CO_2CH_3), 4.1—4.8 (1H, m, O–CH), 6.4—6.6 (1H, m, 2- or 3-H), 7.0—8.0 and 8.4—8.6 (7H, m, arom-H). MS m/z (%): 362 (M^+, 100). High-resolution MS m/z: Calcd for $C_{17}H_{18}N_2O_5S$: 362.0932. Found: 362.0923.

Preparation of Methyl 6-Methoxyimino-6-[1-(phenylsulfonyl)-1*H*-pyrrol-2-yl)-2-hexenoate (8b) A solution of methoxylamine hydrochloride (264 mg, 3.2 mmol) and AcONa (425 mg, 5.2 mmol) in H_2O (2.5 ml) was added to a solution of **8a** (1.00 g, 2.9 mmol) in MeOH (20 ml) at room temperature. After refluxing of the mixture for 15.5 h, MeOH was removed by evaporation and the residue was extracted with benzene. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant colorless oil (1.098 g) was chromatographed on silica gel with AcOEt–hexane (1 : 3) to give an isomeric mixture of **8b** as a colorless solid (985 mg, 91%). Each isomer was separated by careful silica gel column chromatography to give the pure isomers. The first fraction, the major isomer, was a colorless solid, mp 66.5—71.0 °C. IR ν_{max} cm^{-1}: 1710 (C=O), 1648 (C=N). ^{1}H-NMR (400 MHz) δ: 2.41 (2H, ddt, J=6.8, 1.6, 7.8 Hz, CH_2CH=), 2.67 (2H, t, J=7.8 Hz, N=CCH_2), 3.73 and 3.89 (2×3H, 2×s, 2×OCH_3), 5.83 (1H, dt, J=15.6, 1.6 Hz, =CHCO), 6.60 (1H, dd, J=3.3, 1.6 Hz, 4- or 5-H), 6.96 (1H, dt, J=15.6, 6.8 Hz, CH_2CH=CH), 7.13 (1H, dd, J=3.3, 2.1 Hz, 4- or 5-H), 7.30 (1H, dd, J=2.1, 1.6 Hz, 2-H), 7.52 (2H, t, J=7.7 Hz, 2×3′-H), 7.62 (1H, tt, J=7.7, 1.5 Hz, 4′-H), 7.87 (2H, dd, J=7.7, 1.5 Hz, 2×2′-H). MS m/z (%): 77 (100), 376 (M^+, 2.9). High-resolution MS m/z: Calcd for $C_{18}H_{20}N_2O_5S$: 376.1088. Found: 376.1090. The second fraction, the minor isomer, was a colorless oil. IR (film) ν_{max} cm^{-1}: 1710 (C=O), 1648 (C=N). ^{1}H-NMR (400 MHz) δ: 2.46—2.53 (2H, m, CH_2CH=), 2.63 (2H, t, J=7.9 Hz, N=CCH_2), 3.72 and 3.96 (2×3H, 2×s, 2×OCH_3), 5.84 (1H, dt, J=15.8, 1.7 Hz, =CHCO), 6.54 (1H, dd, J=3.4, 1.7 Hz, 4- or 5-H), 6.99 (1H, dt, J=15.8, 6.8 Hz), CH_2CH=CH), 7.15 (1H, dd, J=3.4, 2.2 Hz, 4- or 5-H), 7.53 (2H, t, J=7.5 Hz, 2×3′-H), 7.63 (1H, tt, J=7.5, 1.5 Hz, 4′-H), 7.90 (2H, dd, J=7.5, 1.5 Hz, 2×2′-H), 7.92 (1H, dd, J=2.2, 1.7 Hz, 2-H), MS m/z (%): 77 (100), 376 (M^+, 1.8). High-resolution MS m/z: Calcd for $C_{18}H_{20}N_2O_5S$: 376.1088. Found: 376.1091. These isomers were easily isomerized to each other during recrystallization or NMR measurement.

Cyclization of 8b A mixture of **8b** (100 mg, 0.27 mmol), $PdCl_2$ (47 mg, 0.27 mmol), and AcONa (87 mg, 1.1 mmol) in CH_3CN (3 ml) was heated at 60 °C for 25 h under an Ar atmosphere. Then the reaction mixture was poured into H_2O and extracted with AcOEt–benzene. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant dark brown oil (71 mg) was chromatographed on silica gel with benzene–AcOEt (40 : 1) to give the two pure isomers of 7-methoxycarbonylmethylidene-4-methoxyimino-1-phenylsulfonyl-4,5,6,7-tetrahydro-1*H*-indole (**10b**). The first fraction was a pale yellow solid (20 mg, 20%), which was recrystallized from AcOEt–hexane to give colorless prisms, mp 131.5—133.0 °C. *Anal.* Calcd for $C_{18}H_{18}N_2O_5S$: C, 57.74; H, 4.85; N, 7.48. Found: C, 57.63; H, 4.83; N, 7.36. IR ν_{max} cm^{-1}: 1695 (C=O), 1615 (C=N). ^{1}H-NMR (400 MHz) δ: 2.67 (2H, t, J=6.9 Hz, N=CCH_2), 2.98 (2H, dt, J=6.9, 0.9 Hz, CH=CCH_2), 3.77 and 3.91 (2×3H, 2×s, 2×OCH_3), 6.58 (1H, br s, =$CHCO_2CH_3$), 6.65 (1H, d, J=3.5 Hz, 2- or 3-H), 7.44 (2H, t, J=8.0 Hz, 2×3′-H), 7.48 (1H, d, J=3.5 Hz, 2- or 3-H), 7.59 (1H, tt, J=8.0, 1.5 Hz, 4′-H), 7.65 (2H, dd, J=8.0, 1.5 Hz, 2×2′-H). MS m/z (%): 201 (100), 374 (M^+, 86.3). The second fraction was a pale yellow solid (5.3 mg, 5.3%), which was recrystallized from benzene to give colorless prisms, mp 160.5—161.5 °C. *Anal.* Calcd for $C_{18}H_{18}N_2O_5S$: C, 57.74; H, 4.85; N, 7.48. Found: C, 57.62; H, 4.82; N, 7.46. IR ν_{max} cm^{-1}: 1700 (C=O), 1615 (C=N). ^{1}H-NMR (400 MHz) δ: 2.73 (2H, t, J=6.9 Hz, N=CCH_2), 3.18 (2H, dt, J=6.9, 1.6 Hz, CH=CCH_2), 3.73, 3.92 (2×3H, 2×s, 2×OCH_3), 6.14 (1H, t, J=1.6 Hz, =$CHCO_2CH_3$), 7.41 (1H, d, J=2.2 Hz, 2- or 3-H), 7.52 (1H, d, J=2.2 Hz, 2- or 3-H), 7.54 (2H, t, J=7.5 Hz, 2×3′-H), 7.66 (1H, tt, J=7.5, 1.9 Hz, 4′-H), 7.93 (2H, dd, J=7.5, 1.9 Hz, 2×2′-H). MS m/z (%): 77 (100), 374 (M^+, 75.9). The third fraction was recovered starting material as a mixture of isomers (**8b**, 19 mg, 19%).

Preparation of Methyl 6-Acetoxy-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl)-2-hexanoate (8c) A) Reduction of the ketone (**8a**) with $NaBH_4$: $NaBH_4$ (109 mg, 2.88 mmol) was added to a solution of **8a** (1.00 g, 2.88 mmol) in MeOH (20 ml) at room temperature and the mixture was stirred for 30 min. Since the reaction was not completed, further $NaBH_4$ (15 mg, 0.40 mmol) was added. After 30 min, the reaction was quenched with H_2O and the mixture was extracted with Et_2O. The organic layer was washed successively with 10% aqueous HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave a colorless oil (1.076 g), which was chromatographed on silica gel with AcOEt–benzene (1 : 4) to give methyl 6-hydroxy-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate as a a colorless oil (916 mg, 91%). IR (film) ν_{max} cm^{-1}: 1720 (C=O). ^{1}H-NMR δ: 1.5—2.5 (4H, m, CH_2CH_2), 2.30 (1H, br s, OH), 3.64 (3H, s, OCH_3), 4.53 (1H, t, J=6 Hz, CHOH), 5.71 (1H, d, J=16 Hz, CH=CHCO), 6.20 (1H, dd, J=4, 2 Hz, 4-H), 6.6—7.9 (8H, m, Ar-H and CH=CHCO). MS m/z (%): 236 (100), 349 (M^+, 1.6). High-resolution MS m/z: Calcd for $C_{17}H_{19}NO_5S$: 349.0979. Found: 349.0989. B) Acetylation of methyl 6-hydroxy-6-[1-(phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate: Ac_2O (0.451 ml, 4.78 mmol) was added to a solution of the above alcohol (835 mg, 2.39 mmol) in pyridine (8 ml) and the mixture was stirred for 17 h. The reaction mixture was poured into ice water, stirred for 1 h, and extracted with Et_2O. The organic layer was washed successively with 10% aqueous HCl, saturated $NaHCO_3$, and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave a pale yellow oil (0.924 g), which was chromatographed on silica gel with AcOEt–hexane (1 : 2) to give the title compound (**8c**) as a pale yellow oil (906 mg, 97%). *Anal.* Calcd for $C_{19}H_{21}NO_6S$: C, 58.30; H, 5.41; N, 3.58. Found: C, 58.26; H, 5.52; N, 3.64. IR (film) ν_{max} cm^{-1}: 1735, 1720 (C=O). ^{1}H-NMR δ: 1.6—2.5 (4H, m, CH_2CH_2), 2.00 (3H, s, $COCH_3$), 3.67 (3H, s, $COOCH_3$), 5.68 (1H, dif t, J=6 Hz, $CHOCOCH_3$), 5.71 (1H, dif d, J=16 Hz, =$CHCO_2CH_3$), 6.20 (1H, t, J=3 Hz, arom-H), 6.6—7.9 (8H, m, CH=CHCO and arom-H).

Cyclization of 8c A mixture of **8c** (100 mg, 0.26 mmol), $PdCl_2$ (46 mg, 0.26 mmol), and AcONa (84 mg, 1.0 mmol) in AcOH (30 ml) was refluxed for 1 h under an Ar atmosphere. Then most of the AcOH was removed by evaporation. The residue was diluted with H_2O and extracted with AcOEt. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant dark brown tar (96 mg) was chromatographed on silica gel with benzene–AcOEt (30 : 1) to give the two cyclized products. The first fraction gave methyl 1-(phenylsulfonyl)-1*H*-indole-7-acetate (**11c**) as a colorless solid (34 mg, 41%), which was recrystallized from benzene–hexane to give colorless prisms, mp 104.0—105.5 °C. *Anal.* Calcd for $C_{17}H_{15}NO_4S$: C, 61.99; H, 4.59; N, 4.25. Found: C, 62.09; H, 4.55; N, 4.25. IR (film) ν_{max} cm^{-1}: 1735 (C=O). ^{1}H-NMR δ: 3.60 (3H, s, OCH_3), 4.10 (2H, s, arom-CH_2CO), 6.58 (1H, d, J=4 Hz, 2- or 3-H), 7.0—7.7 (9H, m, arom-H). MS m/z (%): 374 (M^+, 100). The second fraction gave 4-acetoxy-7-methoxycarbonylmethylidene-4,5,6,7-tetrahydro-1*H*-indole (**10c**) as a colorless oil (22 mg, 22%). IR ν_{max} cm^{-1}: 1733 and 1700 (C=O). ^{1}H-NMR (400 MHz) δ: 1.8—1.9, 2.0—2.1, 2.9—3.0, 3.1—3.2 (each 1H, 4×m, CH_2CH_2), 2.04 (3H, s, CH_3CO), 3.74 (3H, s, OCH_3), 5.78 (1H, t, J=5.5 Hz, $CHOCOCH_3$), 6.34 (1H, d, J=3.5 Hz, 2- or 3-H), 6.59 (1H, br s, =$CHCO_2CH_3$), 7.47 (2H, t, J=8.5 Hz, 2×3′-H), 7.53 (1H, d, J=3.5 Hz, 2- or 3-H), 7.61 (1H, tt, J=8.5, 1.2 Hz, 4′-H), 7.67 (2H, dd, J=8.5, 1.2 Hz, 2×2′-H). MS m/z (%): 188 (100), 389 (M^+, 12.7). High-resolution MS m/z: Calcd for $C_{19}H_{19}NO_6S$: 389.0934. Found: 389.0926. The third fraction gave recovered starting material (**8c**, 24.8 mg, 25% recovery).

Conversion of 10c to 11c A mixture of 4-acetoxy-7-carbomethoxymethylidene-4,5,6,7-tetrahydro-1*H*-indole (**10c**) (46.7 mg, 0.12 mmol) and *p*-toluenesulfonic acid (20 mg, 0.11 mmol) in benzene was heated at 60 °C for 10 min under an Ar atmosphere. Then the reaction mixture was poured into H_2O and extracted with benzene. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant brown oil (39.2 mg) was chromatographed on silica gel with AcOEt–benzene (1 : 30) to give methyl 1-(phenylsulfonyl)-1*H*-indole-7-acetate (**11c**) as a solid (18.0 mg, 46%), whose IR and NMR spectra were identical with those of the product obtained from the cyclization of **8c** or **8d**.

Preparation of Methyl 6-[1-(Phenylsulfonyl)-1*H*-pyrrol-3-yl]-2-hexenoate (8d) A mixture of **8a** (1.39 g, 4.0 mmol) and Et_3SiH (2.1 ml, 13.2 mmol) in CF_3COOH (24 ml) was heated at 60 °C for 1 h. Then CF_3COOH was removed under reduced pressure below 40 °C and the residue was diluted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant oil (2.48 g) was chromatographed on silica gel with AcOEt–hexane (1 : 3) to give methyl 6-(1-phenylsulfonyl-2,5-dihydro-1*H*-pyrrol-3-yl)-2-hexenoate (**17**) as an unstable pale brown oil (0.992 g). IR (film) ν_{max} cm^{-1}: 1720 (C=O). ^{1}H-NMR δ: 1.3—1.8 (2H,

m, $CH_2C\underline{H}_2CH_2CH=$), 1.8—2.6 (4H, m, $C\underline{H}_2CH_2C\underline{H}_2CH=$), 3.70 (3H, s, OCH_3), 4.02 (4H, br s, $C\underline{H}_2NC\underline{H}_2$), 5.28 (1H, br s, $NCH_2C\underline{H}=$), 5.75 (1H, br d, $J=17$ Hz, $CH=C\underline{H}CO$), 6.5—7.2 (1H, m, $C\underline{H}=CHCO$), 7.3—7.6 (3H, m, $2\times3'$-H and 4′-H), 7.6—7.8 (2H, m, $2\times2'$-H).

A mixture of the above oil (**17**, 0.992 g) and MnO_2 (9.92 g) in benzene (99 ml) was refluxed for 1 h. MnO_2 was filtered off and washed thoroughly with hot benzene. After evaporation of the solvent, the title compound (**8d**) was obtained as a pale brown oil (0.607 g, 46% yield from **8a**). IR (film) ν_{max} cm^{-1}: 1730 (C=O). ^{1}H-NMR δ: 1.4—1.9 (2H, m, $CH_2C\underline{H}_2CH_2CH=$), 1.9—2.6 (4H, m, $C\underline{H}_2CH_2C\underline{H}_2CH=$), 3.67 (3H, s, OCH_3), 5.72 (1H, br d, $J=17$ Hz, $CH=C\underline{H}CO$), 6.0—6.2 (1H, m, 4-H), 6.6—7.2 (3H, m, $C\underline{H}=CHCO$, 2-H, and 5-H), 7.3—7.6 (3H, m, $2\times3'$-H and 4′-H), 7.6—8.1 (2H, m, $2\times2'$-H). MS *m/z* (%): 77 (100), 333 (M^+, 2.1). High-resolution MS *m/z*: Calcd for $C_{17}H_{19}NO_4S$: 333.1035. Found: 333.1020.

Cyclization of 8d A mixture of **8d** (100 mg, 0.30 mmol), $PdCl_2$ (53 mg, 0.30 mmol), and AcONa (218 mg, 2.7 mmol) in AcOH (30 ml) was refluxed for 3 h under an Ar atmosphere. Then most of the AcOH was distilled off under reduced pressure. The residue was diluted with H_2O and extracted with AcOEt. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the resultant dark brown tar (97 mg) was chromatographed on silica gel with AcOEt–hexane (1 : 5) to give the two cyclized products. The first fraction gave 7-methoxycarbonylmethylidene-1-phenylsulfonyl-4,5,6,7-tetrahydro-1*H*-indole (**10d**) as a colorless solid (39 mg, 40%), which was recrystallized from benzene–hexane to give colorless prisms, mp 91.0—92.5 °C. *Anal.* Calcd for $C_{17}H_{17}NO_4S$: C, 61.62; H, 5.17; N, 4.23. Found: C, 61.57; H, 5.17; N, 4.22. IR ν_{max} cm^{-1}: 1700 (C=O). ^{1}H-NMR (400 MHz) δ: 1.72 (2H, quintet, $J=6.2$ Hz, $CH_2C\underline{H}_2CH_2$), 2.50 (2H, t, $J=6.2$ Hz, $CH_2C\underline{H}_2$-arom), 2.96 (2H, dt, $J=6.2$, 1.3 Hz, $CH_2C\underline{H}_2C=$), 3.72 (3H, s, OCH_3), 6.17 (1H, d, $J=3.3$ Hz, 2- or 3-H), 6.51 (1H, br s, $CH=C\underline{H}CO$), 7.43 (2H, t, $J=7.5$ Hz, 3′-H), 7.47 (1H, d, $J=3.3$ Hz, 2- or 3-H), 7.57 (1H, dif tt, $J=7.5$, 1.5 Hz, 4′-H), 7.64 (2H, dif dd, $J=7.5$, 1.5 Hz, 2′-H). MS *m/z* (%): 77 (100), 331 (M^+, 11.9). The second fraction gave methyl 1-(phenylsulfonyl)-1*H*-indole-7-acetate (**11c**, 27 mg, 27%), of which the IR and NMR spectra were identical with those of the product obtained by the cyclization of the acetate (**8c**).

Conversion of 10d to 11c A) Bromination: A mixture of **10d** (23.7 mg, 0.07 mmol) and $CuBr_2$ (31.9 mg, 0.14 mmol) in AcOEt (1 ml) was refluxed for 20 min under an Ar atmosphere. Then the precipitates were removed by filtration and washed with AcOEt. The combined organic layer was washed with H_2O and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave dark a brown oil (30 mg), which was chromatographed on silica gel with benzene to give 6-bromo-7-methoxycarbonylmethylidene-1-phenylsulfonyl-4,5,6,7-tetrahydro-1*H*-indole as a colorless solid (18.1 mg, 62%), which was recrystallized from benzene–hexane to give a colorless prisms, mp 115—134 °C. IR ν_{max} cm^{-1}: 1700 (C=O). ^{1}H-NMR δ: 2.08 (1H, dddd, $J=14.5$, 11.0, 6.0, 3.1 Hz, 5-H_A), 2.21 (1H, dddd, $J=14.5$, 6.0, 3.1, 1.5 Hz, 5-H_B), 2.63 (1H, ddd, $J=17.0$, 6.0, 1.5 Hz, 4-H_A), 2.85 (1H, ddd, $J=17.0$, 11.0, 6.0 Hz, 4-H_B), 3.79 (3H, s, OCH_3), 6.22 (1H, d, $J=3.5$ Hz, 2- or 3-H), 6.60 (1H, t, $J=3.1$ Hz, 6-H), 6.66 (1H, s, C=CHCO), 7.42 (2H, dif t, $J=8.0$ Hz, 3′-H), 7.55 (1H, dif tt, $J=8.0$, 1.5 Hz 4′-H), 7.55 (1H, d, $J=3.5$ Hz, 2- or 3-H), 7.66 (2H, dif dd, $J=8.0$, 1.5 Hz, 2′-H). MS *m/z* (%): 188 (100), 409 (M^+, 18.7), 411 (M^++2, 18.7%). High-resolution MS *m/z*: Calcd for $C_{17}H_{16}BrNO_4S$: 408.9979. Found: 408.9979.

B) Aromatization: A mixture of the above bromide (11.0 mg, 0.027 mmol), LiBr (2.6 mg, 0.03 mmol), and $LiCO_3$ (2.2 mg, 0.03 mmol) in DMF (0.2 ml) was heated at 120 °C for 2 h under an Ar atmosphere. Then the reaction mixture was poured into H_2O and extracted with Et_2O. The organic layer was washed with H_2O and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave a residue (9.6 mg), which was chromatographed on silica gel to give methyl 1-(phenylsulfonyl)-1*H*-indole-7-acetate (**11c**, 6.8 mg, 77%) as a colorless solid. The IR and NMR spectra of this compound were identical with those of the cyclization product obtained from **8c** or **8d**.

Preparation of Ethyl 4-(5-Methoxycarbonyl-1-oxo-4-pentenyl)-1*H*-pyrrole-2-carboxylate (9a) Oxalyl chloride (12.6 ml, 144 mmol) was added to a solution of (*E*)-5-methoxycarbonyl-4-pentenoic acid (15.37 g, 97 mmol) in CH_2Cl_2 (30 ml) at room temperature under an Ar atmosphere and the mixture was heated at 40 °C for 1.5 h. Then the solvent and excess oxalyl chloride were removed by evaporation to give the crude acid chloride (**7**) as an oil. A solution of this crude oil in $CHCl_3$ (100 ml) was added to $AlCl_3$ (25.72 g, 193 mmol) under Ar, then a solution of ethyl 1*H*-pyrrole-2-carboxylate (6.78 g, 49 mmol) in $CHCl_3$ (30 ml) was added to the above solution. The whole mixture was refluxed for 3 h, poured into ice water, and then extracted with AcOEt. The organic layer was washed successively with saturated $NaHCO_3$ and saturated NaCl and dried over $MgSO_4$. Evaporation of the solvent gave a brown oil (24.0 g), which was purified by silica gel chromatography (AcOEt–benzene) to give the desired compound (**9a**) as a pale yellow solid (8.47 g, 62%). This solid was recrystallized from benzene–hexane to give colorless prisms, mp 76—79 °C. *Anal.* Calcd for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 59.95; H, 6.09; N, 5.02. IR ν_{max} cm^{-1}: 1705, 1645 (C=O). ^{1}H-NMR δ: 1.37 (3H, t, $J=7.0$ Hz, $OCH_2C\underline{H}_3$), 2.4—3.2 [4H, m, $CO(C\underline{H}_2)_2CH=$], 3.69 (3H, s, OCH_3), 4.33 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 5.83 (1H, d, $J=16$ Hz, $=C\underline{H}CO_2CH_3$), 6.7—7.2 (1H, m, $C\underline{H}=CHCO$), 7.21 (1H, t, $J=2$ Hz, 3- or 5-H), 7.50 (1H, dd, $J=3$, 2 Hz, 3- or 5-H), 10.1 (1H, br s, NH). MS *m/z* (%): 120 (100), 279 (M^+, 11.7).

Reduction of 9a Et_3SiH (0.31 ml, 1.9 mmol) was added to a solution of **9a** (140 mg, 0.50 mmol) in CF_3COOH (3.0 ml) at room temperature under an Ar atmosphere, and the mixture was stirred for 4.5 h, then poured into ice-water and extracted with AcOEt. The organic layer was washed with saturated aqueous $NaHCO_3$ and saturated aqueous NaCl, and dried over $MgSO_4$. Evaporation of the solvent gave a residue (184 mg), which was chromatographed on silica gel with benzene–hexane to provide two products. The first fraction gave ethyl 7-methoxycarbonylmethyl-4,5,6,7-tetrahydro-1*H*-indole-2-carboxylate (**19**) (15 mg, 11% yield), which was recrystallized from benzene–hexane to give colorless needles, mp 89—94 °C. *Anal.* Calcd for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.31; H, 7.32; N, 5.27. IR ν_{max} cm^{-1}: 3300 (NH), 1735, 1670 (C=O). ^{1}H-NMR δ: 1.32 (3H, t, $J=7$ Hz, $OCH_2C\underline{H}_3$), 1.6—2.3 (4H, m, arom-$CH_2C\underline{H}_2C\underline{H}_2$), 2.2—2.8 (4H, m, arom-$CH_2$ and $C\underline{H}_2COOCH_3$), 3.0—3.4 (1H, m, $C\underline{H}CH_2CO$), 3.72 (3H, s, OCH_3), 4.27 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 6.62 (1H, d, $J=2$ Hz, 3-H), 9.62 (1H, br s, NH). MS *m/z* (%): 192 (100), 265 (M^+, 50.3). The second fraction gave ethyl 4-(5-methoxycarbonyl-4-pentenyl)-1*H*-pyrrole-2-carboxylate (**18**) as an yellow oil (75 mg, 56% yield). IR (film) ν_{max} cm^{-1}: 3300 (NH), 1705 (C=O). ^{1}H-NMR δ: 1.25 (3H, t, $J=7$ Hz, $OCH_2C\underline{H}_3$), 1.5—1.9 (2H, m, arom-$CH_2C\underline{H}_2CH_2$), 1.9—2.7 (4H, m, arom-$C\underline{H}_2$ and $C\underline{H}_2CH=CH$), 4.22 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 3.62 (3H, s, OCH_3), 5.71 (1H, d, $J=15$ Hz, $CH=C\underline{H}CO$), 6.5—6.8 (2H, m, 3-H and 5-H), 6.7—7.2 (1H, m, $C\underline{H}=CHCO$), 9.38 (1H, br s, NH). MS *m/z* (%): 106 (100), 265 (M^+, 27.0). High-resolution MS *m/z*: Calcd for $C_{14}H_{19}NO_4$: 265.1314. Found: 265.1306.

Cyclization of 18 A mixture of **18** (162 mg, 0.61 mmol), $PdCl_2$ (108 mg, 0.61 mmol), and AcONa (200 mg, 2.44 mmol) in AcOH (6 ml) was heated at 60 °C for 3 h under an Ar atmosphere. The reaction mixture was filtered through Celite and the residue was washed with hot AcOEt and water. The aqueous layer was extracted with AcOEt. The combined organic layer was washed with saturated aqueous NaCl, and dried over $MgSO_4$. After evaporation of the solvent, the residue (171 mg) was chromatographed on silica gel with AcOEt–hexane to give four pure compounds as colorless solids. The first fraction gave ethyl (*Z*)-7-methoxycarbonylmethylidene-4,5,6,7-tetrahydro-1*H*-indole-2-carboxylate (**21a**, 51 mg, 32% yield), which was recrystallized from AcOEt–hexane to give colorless needles, mp 68—70 °C. *Anal.* Calcd for $C_{14}H_{17}NO_4$: C, 63.56; H, 6.51; N, 5.32. Found: C, 63.88; H, 6.59; N, 5.29. IR ν_{max} cm^{-1}: 3200 (NH), 1710, 1690, (C=O). ^{1}H-NMR δ: 1.37 (3H, t, $J=7$ Hz, $OCH_2C\underline{H}_3$), 1.89 (2H, q, $J=6$ Hz, arom-$CH_2C\underline{H}_2CH_2$), 2.56 and 2.65 (2×2H, $2\times$t, $J=6$ Hz, arom-$C\underline{H}_2$ and $C\underline{H}_2C=CH$), 3.73 (3H, s, OCH_3), 4.34 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 5.56 (1H, br s, $C=C\underline{H}CO$), 6.66 (1H, d, $J=3$ Hz, 3-H), 12.66 (1H, br s, NH). MS *m/z* (%): 263 (M^+, 100). The second fraction gave ethyl (*E*)-7-methoxycarbonylmethylidene-4,5,6,7-tetrahydro-1*H*-indole-2-carboxylate (**21b**, 28 mg, 17%), which was recrystallized from AcOEt–hexane to give colorless needles, mp 158—161 °C. *Anal.* Calcd for $C_{14}H_{17}NO_4$: C, 63.56; H, 6.51; N, 5.32. Found: C, 63.57; H, 6.57; N, 5.27. IR ν_{max} cm^{-1}: 3280 (NH), 1685 (C=O). ^{1}H-NMR δ: 1.33 (3H, t, $J=7$ Hz, $OCH_2C\underline{H}_3$), 1.6—2.2 (2H, m, arom-$CH_2C\underline{H}_2CH_2$), 2.61 (2H, t, $J=6$ Hz, Ar$C\underline{H}_2$), 3.10 (2H, br t, $J=6$ Hz, $C\underline{H}_2C=CH$), 3.69 (3H, s, OCH_3), 4.34 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 6.03 (1H, br s, $C=C\underline{H}CO$), 6.66 (1H, d, $J=2$ Hz, 3-H), 9.63 (1H, br s, NH). MS *m/z* (%): 263 (M^+, 100). The third fraction gave ethyl 7-(methoxycarbonylmethyl)-1*H*-indole-2-carboxylate (**20**, 26 mg, 16%), which was recrystallized from AcOEt–hexane to give colorless needles, mp 100—101 °C. *Anal.* Calcd for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.37; H, 6.01; N, 5.09. IR ν_{max} cm^{-1}: 3340 (NH), 1725, 1685 (C=O). ^{1}H-NMR δ: 1.40 (3H, t, $J=7$ Hz, $OCH_2C\underline{H}_3$), 3.67 (3H, s, CO_2CH_3), 3.87 (2H, s, arom-CH_2CO), 4.41 (2H, q, $J=7$ Hz, $OC\underline{H}_2CH_3$), 7.0—7.4 (3H, m, arom-H), 7.59 (1H, dd, $J=6$, 3 Hz, 4-H), 9.50 (1H, br s, NH). MS *m/z* (%): 261 (M^+, 100). Fourth fraction gave unchanged starting material (20 mg, 12% recovery).

Dehydrogenation of 21 with Pd–C A mixture of **21** (a mixture of *E*- and *Z*-isomer, 76 mg, 0.29 mmol) and 10% Pd–C (20 mg) in tetralin (2 ml) was heated at 220 °C for 11.5 h. Then Pd–C was filtered off and washed thoroughly with AcOEt. The combined organic layer was evaporated and the residue was chromatographed on silica gel with AcOEt–benzene to give a colorless solid (62 mg), which was a mixture of **19** and **20** (1 : 1). The ratio of **19** and **20** was determined by NMR.

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Synthesis of 2-Substituted 1,3,4-Thiadiazol-5-ylacetic, Propionic, and Glutaric Acids

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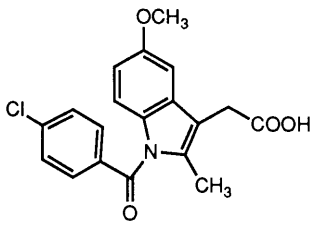
1,3,4-Thiadiazol-5-ylacetic, propionic, and glutaric acids (3—5) were synthesized *via* ethyl 2-substituted 1,3,4-thiadiazol-5-ylacetates (11 and 17) as key intermediates.

Keywords 2,3-dihydro-1,3,4-thiadiazole; 2-substituted 1,3,4-thiadiazol-5-ylacetate; 2-benzoyl-1,3,4-thiadiazol-5-ylacetic acid; ethyl bromoacetate; alkylation; hydrolysis; 1,3,4-thiadiazol-5-ylpropionic acid; 1,3,4-thiadiazol-5-ylglutaric acid

Previously, we have reported the synthesis of 5-substituted 3-acetyl-2-phenyl-2,3-dihydro-1,3,4-thiadiazoles by nucleophilic substitution of the methylsulfinyl group of 3-acetyl-5-methylsulfinyl-2-phenyl-2,3-dihydro-1,3,4-thiadiazole and the synthesis of 2-methylsulfinyl-5-phenyl-1,3,4-thiadiazole by the reaction of 3-acetyl-5-methylsulfinyl-2-phenyl-2,3-dihydro-1,3,4-thiadiazole 1-oxides with base.[1] We have also reported that a sulfonyl group at the 2 position of 1,3,4-thiadiazoles can be substituted with nucleophiles.[2]

We now report the application of these methods to the synthesis of novel 1,3,4-thiadiazole derivatives, which are expected to have antiinflammatory activity, based on the structures of indomethacin (**1**) and oxaprozin (**2**) (Chart 1). This paper describes the synthesis of 2-benzoyl-1,3,4-thiadiazol-5-ylacetic acid (**3a**), 3-(2-substituted 1,3,4-thiadiazol-5-yl)propionic acid derivatives (**4**) and 3-(2-substituted 1,3,4-thiadiazol-5-yl)glutaric acid derivatives (**5**) (Chart 2).

The synthetic route to the acetic acid derivative **3a** is shown in Chart 3. Benzoylation of phenylacetaldehyde



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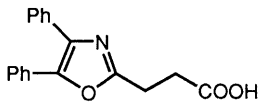
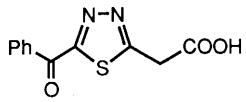
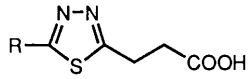


2

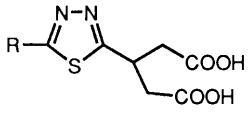
Chart 1



3a



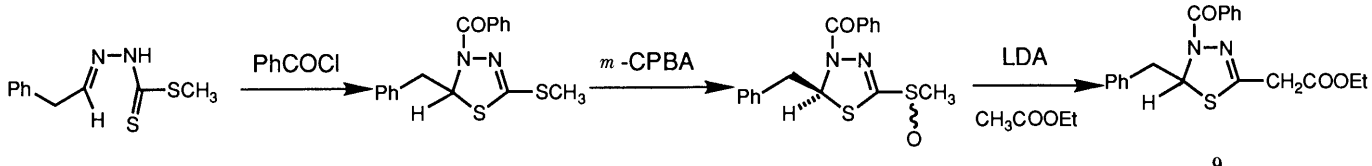
4a—e



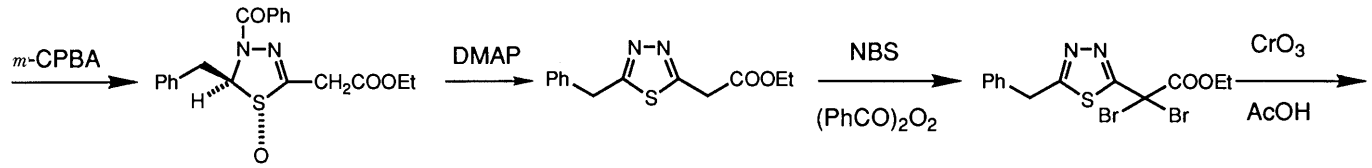
5a—e

a : R=PhCO
b : R=Ph
c : R=4-ClPh
d : R=4-BrPh
e : R=$PhCH_2$

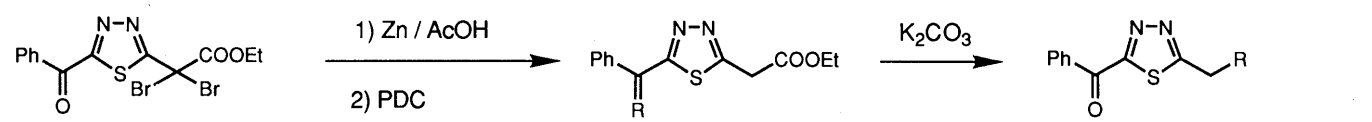
Chart 2



6 7 8 9



10 11 12



13

14 : R=C(OH)H

15 : R=O

3a : R=COOH

3b : R=H

Chart 3

methylthio(thiocarbonyl)hydrazone (**6**) prepared by the condensation of methylthio(thiocarbonyl)hydrazide with phenylacetaldehyde gave 3-benzoyl-2-benzyl-5-methylthio-2,3-dihydro-1,3,4-thiadiazole (**7**) in 98% yield. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of compound **7** showed a 2-H absorption at δ 6.55 with an upfield shift of 0.91 ppm from that of the methine proton of **6**, in good agreement with reported chemical shifts of other 2-protons of 2,3-dihydro-1,3,4-thiadiazole derivatives.[3] Oxidation of **7** with *m*-chloroperbenzoic acid (*m*-

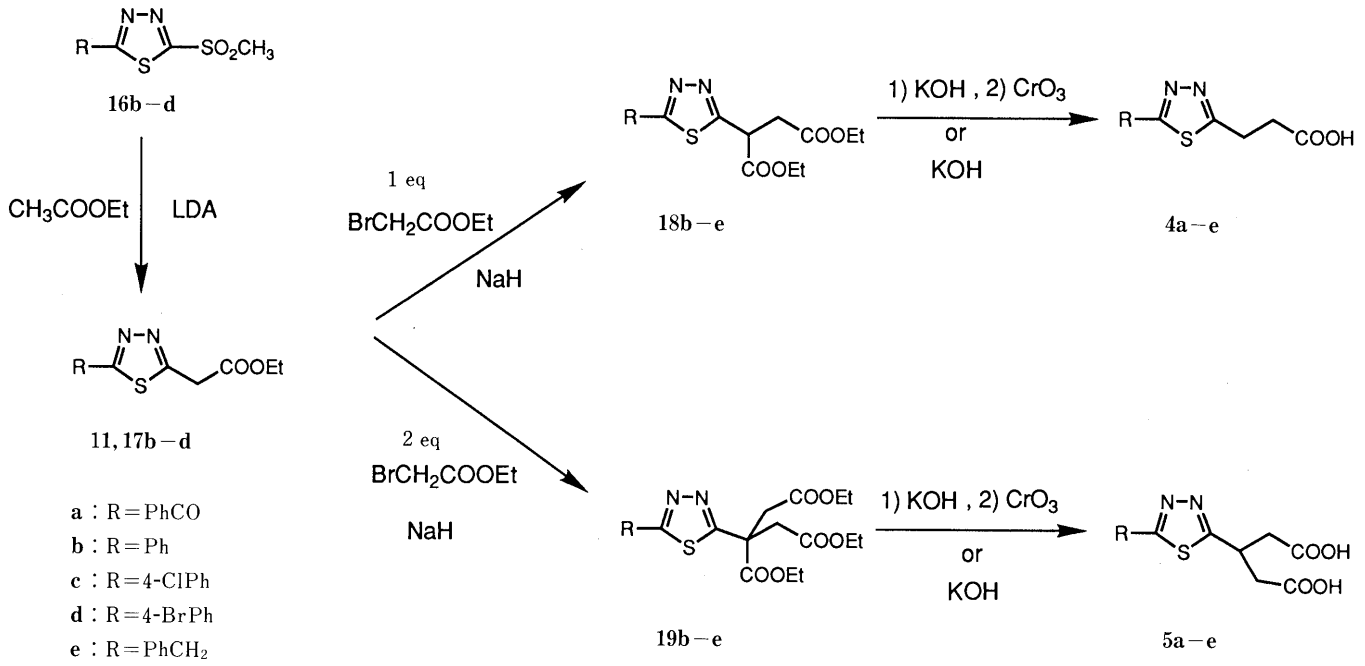


Chart 4

TABLE I. Spectral Data for Diethyl 2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-ylsuccinates (**18b—d**) and Diethyl 3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]-3-ethoxycarbonylglutarates (**19b—d**)

Compd. No.	Yield (%)	mp (°C) (Recrystn. solvent)	IR (KBr) cm^{-1} CO	^{1}H-NMR ($CDCl_3$) δ (J=Hz)	Formula	Analysis Calcd (Found) C	H	N	MS m/z (M^+)
18b	47		1745[a] 1730	1.25 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.27 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.29 (2H, d, J=7, CH_2), 4.17 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.30 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.70 (1H, t, J=7, CH), 7.40—7.65 (3H, m, ArH), 7.75—8.10 (2H, m, ArH)	$C_{16}H_{18}N_2O_4S$	334.0987[b] (334.0985)			334
18c	43	76—77 (*n*-Hexane)	1745 1720	1.24 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.27 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.47 (2H, d, J=7, CH_2), 4.14 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.24 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.61 (1H, t, J=7, CH), 7.45 (2H, dd, J=2, 9, ArH), 7.90 (2H, dd, J=2, 9, ArH)	$C_{16}H_{17}ClN_2O_4S$	52.10 (52.01	4.65 4.56	7.60 7.50)	368 370
18d	37	77—78 (*n*-Hexane–benzene)	1745 1720	1.23 (3H, t, J=7, $CH_2C\underline{H}_3$), 1.27 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.27 (2H, d, J=7, CH_2), 4.14 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.24 (2H, q, J=7, $C\underline{H}_2CH_3$), 4.61 (1H, t, J=7, CH), 7.60 (2H, dd, J=2, 9, ArH), 7.83 (2H, dd, J=2, 9, ArH)	$C_{16}H_{17}BrN_2O_4S$	46.50 (46.52	4.15 4.05	6.78 6.59)	412 414
19b	94		1740[a] 1735	1.21 (6H, t, J=7, $CH_2C\underline{H}_3$), 1.32 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.46 (4H, s, CH_2), 4.09 (4H, q, J=7, $C\underline{H}_2CH_3$), 4.32 (2H, q, J=7, $C\underline{H}_2CH_3$), 7.30—7.60 (3H, m, ArH), 7.80—8.15 (2H, m, ArH)	$C_{20}H_{24}N_2O_6S$	420.1354[b] (420.1389)			420
19c	95		1745[a] 1735	1.18 (6H, t, J=7, $CH_2C\underline{H}_3$), 1.29 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.41 (4H, s, CH_2), 4.05 (4H, q, J=7, $C\underline{H}_2CH_3$), 4.28 (2H, q, J=7, $C\underline{H}_2CH_3$), 7.46 (2H, dd, J=3, 7, ArH), 7.93 (2H, dd, J=3, 7, ArH)	$C_{20}H_{23}ClN_2O_6S$	454.0965[b] (454.0971)			454 456
19d	97		1745[a] 1735	1.17 (6H, t, J=7, $CH_2C\underline{H}_3$), 1.29 (3H, t, J=7, $CH_2C\underline{H}_3$), 3.38 (4H, d, J=2, CH_2), 4.04 (4H, q, J=7, $C\underline{H}_2CH_3$), 4.27 (2H, q, J=7, $C\underline{H}_2CH_3$), 7.59 (2H, dd, J=3, 7, ArH), 7.84 (2H, dd, J=3, 7, ArH)	$C_{20}H_{23}BrN_2O_6S$	498.0460[b] (498.0435)			498 500

a) Measured neat. *b*) Determined by high-resolution mass spectrometry (HR-MS). Upper figure, calcd for M^+; lower figure found.

TABLE II. Spectral Data for 3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]propionic Acids (**4b—d**) and 3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]glutaric Acids (**5b—d**)

Compd. No.	Yield (%)	mp (°C) (Recrystn. solvent)	IR (KBr) cm^{-1}		^{1}H-NMR (DMSO-d_6) δ (J=Hz)	Formula	Analysis Calcd (Found)			MS m/z (M^+)
			OH	CO			C	H	N	
4b	35	152—154 (Benzene–$CHCl_3$)	3200—2500	1700	2.82 (2H, t, J=7, CH_2), 3.36 (2H, t, J=7, CH_2), 7.48—7.65 (3H, m, ArH), 7.88—8.04 (2H, m, ArH), 8.0—11.0 (1H, br, CO_2H)	$C_{11}H_{10}N_2O_2S$	56.40 (56.48	4.30 4.18	11.96 11.84)	234
4c	94	181—182 (Benzene–$CHCl_3$)	3200—2500	1700	2.80 (2H, t, J=7, CH_2), 3.34 (2H, t, J=7, CH_2), 7.62 (2H, dd, J=2, 9, ArH), 7.98 (2H, dd, J=2, 9, ArH), 12.30 (1H, br s, CO_2H)	$C_{11}H_9ClN_2O_2S$	49.17 (48.97	3.38 3.17	10.42 10.16)	268 270
4d	81	192—193 (Benzene–$CHCl_3$)	3200—2500	1710	2.80 (2H, t, J=7, CH_2), 3.35 (2H, t, J=7, CH_2), 7.75 (2H, dd, J=2, 9, ArH), 7.91 (2H, dd, J=2, 9, ArH), 12.30 (1H, br s, CO_2H)	$C_{11}H_9BrN_2O_2S$	42.19 (42.39	2.90 2.84	8.95 8.66)	312 314
5b	59	179—181 (Acetone)	3200—2500	1720	2.86 (4H, d, J=7, CH_2), 4.00 (1H, t, J=7, CH), 7.45—7.65 (3H, m, ArH), 7.85—8.04 (2H, m, ArH), 9.0—12.5 (2H, br, CO_2H)	$C_{13}H_{12}N_2O_4S$	53.42 (53.25	4.14 4.11	9.58 9.49)	292
5c	82	186—188 ($CHCl_3$–acetone)	3150—2500	1710	2.84 (4H, d, J=7, CH_2), 3.96 (1H, t, J=7, CH), 7.60 (2H, dd, J=3, 7, ArH), 7.95 (2H, dd, J=3, 7, ArH), 12.0—12.6 (2H, br, CO_2H)	$C_{13}H_{11}ClN_2O_4S$	47.79 (47.82	3.39 3.32	8.57 8.29)	326 328
5d	65	193—195 (Benzene–acetone)	3150—2500	1705	2.84 (4H, d, J=7, CH_2), 3.97 (1H, t, J=7, CH), 7.73 (2H, dd, J=3, 7, ArH), 7.89 (2H, dd, J=3, 7, ArH), 12.0—12.6 (2H, br, CO_2H)	$C_{13}H_{11}BrN_2O_4S$	42.06 (42.17	2.99 2.87	7.55 7.29)	370 372

DMSO: dimethylsulfoxide.

CPBA) at room temperature gave the sulfoxide **8** as a diastereoisomeric mixture[1] in 92% yield. Nucleophilic substitution of the methylsulfinyl group in **8** with ethyl acetate in the presence of lithium diisopropylamide (LDA) at −78 °C afforded the acetate derivative **9** in 97% yield. Oxidation of **9** with *m*-CPBA at room temperature gave the 2,3-dihydro-1,3,4-thiadiazole 1-oxide (**10**) in 93% yield. The oxygen atom of the sulfoxide group of **10** was assigned as being *trans* to the benzyl group.[4—6] Treatment of **10** with 4-dimethylaminopyridine (DMAP) in ethanol under reflux gave the 1,3,4-thiadiazole derivative **11** in 82% yield.[1,3g]

An attempt to obtain the 2-benzoyl-1,3,4-thiadiazole derivative **15** directly from **11** by oxidation with selenium dioxide was unsuccessful. Therefore, an alternative approach to **15** from **11** was developed, as shown in Chart 3. The reaction of **11** with N-bromosuccinimide (NBS) (2 mol eq) catalyzed by benzoyl peroxide afforded the dibromo derivative **12**, which was oxidized with chromium(VI) oxide (CrO_3) in acetic acid to give the 5-benzoyl derivative **13**. Reduction of **13** with zinc in acetic acid gave the 2-(α-hydroxybenzyl)-1,3,4-thiadiazole derivative **14**, which was oxidized with pyridinium dichromate (PDC) to furnish **15**. Treatment of **15** with potassium carbonate in aqueous ethanol gave the corresponding carboxylic acid **3a**. Compound **3a** was found to be decarboxylated on standing at room temperature,[7] probably *via* the intramolecular participation of the N-4 atom, affording the 5-methyl derivative **3b** as evidenced by ^{1}H-NMR analysis. Thus, its biological activity could not be tested.

We then directed our attention toward the synthesis of 3-(2-benzoyl-1,3,4-thiadiazol-5-yl)propionic acid (**4a**) and the 3-glutaric acid derivative **5a**. Compound **11** was used as the starting material for the synthesis of these compounds. The synthetic routes are shown in Chart 4.

Alkylation of **11** with 1 mol eq of ethyl bromoacetate in the presence of sodium hydride (NaH) at 0 °C provided the diethyl succinate derivative **18e**, whose hydrolysis with potassium hydroxide gave the carboxylic acid **4e**. This was oxidized with CrO_3 to furnish **4a**. On the other hand, alkylation of **11** with 2 mol eq of ethyl bromoacetate provided the diethyl glutarate derivative **19e**. Similarly, hydrolysis of **19e** with potassium hydroxide followed by oxidation with CrO_3 gave **5a**.

The 2-phenyl substituted compounds **4b—d** and **5b—d** were prepared by using the sulfones **16b—d**. These were obtained by the oxidation of 5-(4-substituted phenyl)-2-methylthio-1,3,4-thiadiazoles with potassium permanganate ($KMnO_4$) in acetic acid according to Fujii's method.[8] Displacement of **16—d** with ethyl acetate in the presence of LDA at −78 °C gave **17b—d**, whose conversions to the target molecules were achieved through procedures similar to those described earlier for **4a** and **5a**.

The analytical and spectral data for compounds **18b—d**, **19b—d**, **4b—d** and **5b—d** are shown in Tables I and II. Compounds **4a—d** and **5a—d** did not exhibit any significant inhibitory effect on cyclooxygenase from bovine seminal vesicles.

Experimental

Melting points were determined by the capillary method and are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 215 spectrometer. ^{1}H-NMR spectra were recorded on a JEOL PS-100 or a JEOL JNM-PMX 60_{SI} spectrometer using tetramethylsilane as an internal standard. Mass spectra (MS) were measured with a JEOL D-300 instrument. For column chromatography, Silica gel 60 (230—400 mesh, Nacalai Tesque) was employed.

Phenylacetaldehyde Methylthio(thiocarbonyl)hydrazone (6) Compound

6 was prepared by the literature method.[9]

3-Benzoyl-2-benzyl-5-methylthio-2,3-dihydro-1,3,4-thiadiazole (7) A mixture of **6** (5.04 g, 22.5 mmol) and benzoyl chloride (6.2 ml, 53.41 mmol) in $CHCl_3$ was refluxed for 30 min. The mixture was neutralized with 5% aqueous sodium hydrogen carbonate and extracted with $CHCl_3$ (3×100 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was recrystallized from MeOH to give **7** (7.23 g, 98%), mp 86—88 °C. IR (KBr): 1630 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.38 (3H, s, SCH_3), 3.08 (1H, dd, J=8, 14 Hz, PhCH), 3.43 (1H, dd, J=4, 14 Hz, PhCH), 6.55 (1H, dd, J=4, 8 Hz, C_2-H), 7.28 (5H, s, ArH), 7.29—7.95 (5H, m, ArH). MS m/z: 328 (M^+). *Anal.* Calcd for $C_{17}H_{16}N_2OS_2$: C, 62.17; H, 4.91; N, 8.53. Found: C, 62.07; H, 4.79; N, 8.34.

3-Benzoyl-2-benzyl-5-methylsulfinyl-2,3-dihydro-1,3,4-thiadiazole (8) A solution of 80% *m*-CPBA (95 mg, 0.44 mmol) in $CHCl_3$ (3 ml) was added dropwise to a stirred solution of **7** (145 mg, 0.44 mmol) in $CHCl_3$ (1 ml) at 0 °C. After being stirred at room temperature for 30 min, the mixture was neutralized with 5% aqueous sodium hydrogen carbonate and extracted with $CHCl_3$ (3×50 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 20 : 1, v/v) to give **8** (140 mg, 92%) as an inseparable diastereomeric mixture. IR (neat): 1650 (CO), 1070 (SO) cm^{-1}. Major isomer ^{1}H-NMR ($CDCl_3$) δ: 2.35 (3H, s, $SOCH_3$), 2.88—3.65 (2H, m, $PhCH_2$), 6.60—6.79 (1H, m, C_2-H), 7.23 (5H, s, ArH), 7.31—7.80 (5H, m, ArH). Minor isomer ^{1}H-NMR ($CDCl_3$) δ: 2.74 (3H, s, $SOCH_3$), 2.88—3.65 (2H, m, $PhCH_2$), 6.60—6.79 (1H, m, C_2-H), 7.25 (5H, s, ArH), 7.31—7.80 (5H, m, ArH). MS m/z: 344 (M^+).

Ethyl 3-Benzoyl-2-benzyl-2,3-dihydro-1,3,4-thiadiazol-5-ylacetate (9) A solution of ethyl acetate (0.95 ml, 9.73 mmol) in anhydrous tetrahydrofuran (THF) (3 ml) was added to a solution of LDA (9.41 mmol; prepared from a 1.65 M solution of *n*-BuLi in hexane, 5.7 ml, 9.41 mmol, and diisopropylamine, 1.4 ml, 9.99 mmol) in anhydrous THF (3 ml) at −78 °C under argon. After the mixture had been stirred at −78 °C for 30 min, a solution of **8** (1.61 g, 4.68 mmol) in anhydrous THF (10 ml) was added dropwise. After 1 min at −78 °C, the mixture was quenched with aqueous acetic acid and extracted with $CHCl_3$ (3×100 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 30 : 1, v/v) to give **9** (1.67 g, 97%) as an oil. IR (neat): 1745 (CO), 1650 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.12 (1H, dd, J=8, 14 Hz, PhCH), 3.42 (1H, dd, J=4, 14 Hz, PhCH), 3.39 (2H, s, C_5-CH_2), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.49 (1H, dd, J=4, 8 Hz, C_2-H), 7.25 (5H, s, ArH), 7.26—7.90 (5H, m, ArH). MS m/z: 368 (M^+).

Ethyl 3-Benzoyl-2-benzyl-2,3-dihydro-1,3,4-thiadiazol-1-oxide-5-ylacetate (10) A solution of 80% *m*-CPBA (937 mg, 4.34 mmol) in $CHCl_3$ (20 ml) was added dropwise to a stirred solution of **9** (1.6 g, 4.35 mmol) in $CHCl_3$ (10 ml) at 0 °C. After the mixture had been stirred at room temperature for 30 min, work-up as described for the preparation of compound **8** gave **10** (1.55 g, 93%), mp 102—103 °C. IR (KBr): 1735 (CO), 1670 (CO), 1055 (SO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.84 (1H, dd, J=10, 14 Hz, PhCH), 3.46 (1H, dd, J=4, 14 Hz, PhCH), 3.79 (2H, d, J=7 Hz, C_5-CH_2), 4.24 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 5.89 (1H, dd, J=4, 10 Hz, C_2-H), 7.32 (5H, s, ArH), 7.35—7.90 (5H, m, ArH). MS m/z: 384 (M^+). *Anal.* Calcd for $C_{20}H_{20}N_2O_4S$: C, 62.48; H, 5.24; N, 7.29. Found: C, 62.18; H, 5.02; N, 6.99.

Ethyl 2-Benzyl-1,3,4-thiadiazol-5-ylacetate (11) A mixture of **10** (5.16 g, 13.44 mmol) and DMAP (2 g, 16.37 mmol) in EtOH (10 ml) was refluxed for 30 min. The solvent was evaporated off under reduced pressure, and the residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 15 : 1, v/v) to give **11** (2.9 g, 82%) as an oil. IR (neat): 1735 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.27 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.09 (2H, s, CH_2), 4.19 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.40 (2H, s, CH_2), 7.29 (5H, s, ArH). MS m/z: 262 (M^+). HR-MS m/z: Calcd for $C_{13}H_{14}N_2O_2S$: 262.0776. Found: 262.0783.

Ethyl 2-Benzyl-1,3,4-thiadiazol-5-yldibromoacetate (12) A mixture of **11** (1.39 g, 5.31 mmol), NBS (1.88 g, 10.56 mmol), and benzoyl peroxide (5 mg) in CCl_4 (10 ml) was refluxed for 1 min. The mixture was filtered and the filtrate was neutralized with 5% aqueous sodium hydrogen carbonate and extracted with $CHCl_3$ (3×100 ml). The combined extracts were washed with 5% aqueous $Na_2S_2O_3$ and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 30 : 1, v/v) to give **12** (1.95 g, 88%) as an oil. IR (neat): 1760 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.33 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.38 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.40 (2H, s, CH_2), 7.33 (5H, s, ArH). MS m/z: 418, 420, 422 (M^+).

Ethyl 2-Benzoyl-1,3,4-thiadiazol-5-yldibromoacetate (13) A mixture of **12** (100 mg, 0.24 mmol) and CrO_3 (48 mg, 0.48 mmol) in acetic acid (3 ml) was heated at 60 °C for 30 min. The mixture was poured into ice-water, neutralized with 5% aqueous sodium hydrogen carbonate, and extracted with $CHCl_3$ (3×50 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$) to give a solid, which was recrystallized from EtOH to give **13** (95 mg, 92%), mp 105—106 °C. IR (KBr): 1735 (CO), 1645 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.37 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.43 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.40—7.75 (3H, m, ArH), 8.44—8.55 (2H, m, ArH). MS m/z: 432, 434, 436 (M^+). *Anal.* Calcd for $C_{13}H_{10}Br_2N_2O_3S$: C, 35.97; H, 2.32; N, 6.45. Found: C, 35.97; H, 2.23; N, 6.65.

Ethyl 2-(α-Hydroxybenzyl)-1,3,4-thiadiazol-5-ylacetate (14) A suspension of **13** (200 mg, 0.46 mmol) and activated zinc (120 mg, 1.84 mmol) in acetic acid (3 ml) was stirred at room temperature for 30 min. The mixture was filtered and the filtrate was neutralized with 5% aqueous sodium hydrogen carbonate, then extracted with $CHCl_3$ (3×50 ml). Work-up as described for the preparation of **13** gave the residue, which was chromatographed on a silica gel column ($CHCl_3$–MeOH, 10 : 1, v/v) to give **14** (120 mg, 94%) as an oil. IR (neat): 3300 (OH), 1740 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.01 (2H, s, CH_2), 4.17 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.52—5.20 (1H, br, OH), 6.16 (1H, s, CH), 7.20—7.50 (5H, m, ArH). MS m/z: 278 (M^+).

Ethyl 2-Benzoyl-1,3,4-thiadiazol-5-ylacetate (15) A mixture of **14** (1.1 g, 3.99 mmol) and PDC (2.97 g, 7.89 mmol) in CH_2Cl_2 (15 ml) was stirred at room temperature for 4 h, then mixed with ether, and filtered. The filtrate was concentrated under reduced pressure, and the residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 20 : 1, v/v) to give a solid, which was recrystallized from EtOH to give **15** (756 mg, 69%), mp 66—68 °C. IR (KBr): 1735 (CO), 1650 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.26 (2H, s, CH_2), 4.27 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.40—7.75 (3H, m, ArH), 8.44—8.53 (2H, m, ArH). MS m/z: 276 (M^+). *Anal.* Calcd for $C_{13}H_{12}N_2O_3S$: C, 56.51; H, 4.38; N, 10.14. Found: C, 56.41; H, 4.20; N, 9.92.

2-Benzoyl-1,3,4-thiadiazol-5-ylacetic Acid (3a) A suspension of **15** (125 mg, 0.45 mmol) and potassium carbonate (125 mg, 0.9 mmol) in 70% aqueous EtOH (6 ml) was heated at 60 °C for 2 h. After cooling, the mixture was acidified with 5% HCl and extracted with ethyl acetate (3×50 ml). The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated to give a solid, which was washed with $CHCl_3$ to provide **3a** (95 mg, 85%), mp 100—101 °C. IR (KBr): 2500—3100 (OH), 1720 (CO), 1665 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$–DMSO-d_6) δ: 4.27 (2H, s, CH_2), 7.35—7.75 (3H, m, ArH), 8.40—8.65 (2H, m, ArH). MS m/z: 204 ($M^+ - CO_2$). *Anal.* Calcd for $C_{11}H_8N_2O_3S$: C, 53.22; H, 3.25; N, 11.28. Found: C, 53.00; H, 3.01; N, 10.92. Decarboxylation of **3a** occurred gradually on standing at room temperature.

2-Benzoyl-5-methyl-1,3,4-thiadiazole (3b) The structure of compound **3b** was confirmed by examination of the ^{1}H-NMR spectrum. ^{1}H-NMR ($CDCl_3$) δ: 2.90 (3H, s, CH_3), 7.50—7.72 (3H, m, ArH), 8.49—8.54 (2H, m, ArH).

Diethyl 2-Benzyl-1,3,4-thiadiazol-5-ylsuccinate (18e) A suspension of sodium hydride (76 mg, 1.91 mmol, 60% dispersion in oil, washed twice with ether) in anhydrous THF (5 ml) was added dropwise to a stirred solution of **11** (500 mg, 1.91 mmol) in THF (15 ml) at 0 °C. After being stirred at 0 °C for 1 h, the mixture was treated dropwise with a solution of ethyl bromoacetate (0.21 ml, 1.91 mmol) in THF (3 ml). After 30 min, the mixture was neutralized with aqueous acetic acid and extracted with $CHCl_3$ (3×100 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 100 : 1, v/v) to give two fractions. Evaporation of the first fraction gave **18e** (443 mg, 67%). IR (KBr): 1730 (CO), 1740 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.19 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.22 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.16 (2H, dd, J=3, 7 Hz, CH_2), 4.10 (2H, q, J=7 Hz, CH_2CH_3), 4.17 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.35 (2H, s, C_2-CH_2), 4.51 (1H, t, J=7 Hz, CH), 7.29 (5H, s, ArH). MS m/z: 348 (M^+). HR-MS m/z: Calcd for $C_{17}H_{20}N_2O_4S$: 348.1143. Found: 348.1108. The starting material **11** (128 mg) was recovered from the second fraction.

3-(2-Benzyl-1,3,4-thiadiazol-5-yl)propionic Acid (4e) A solution of 85% KOH (444 mg, 6.73 mmol) in H_2O (5 ml) was added to a stirred solution of **18e** (395 mg, 1.13 mmol) in EtOH (5 ml) at room temperature. After being stirred at 50 °C for 1 h, the mixture was cooled on ice, acidified with

10% aqueous HCl, and extracted with $CHCl_3$ (3 × 100 ml). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–MeOH, 20 : 1, v/v) to give a solid, which was recrystallized from isopropyl ether–acetone to give **4e** (127 mg, 45%), mp 89—90 °C. IR (KBr): 3100—2500 (OH), 1715 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.71 (2H, t, J=7 Hz, CH_2), 3.22 (2H, t, J=7 Hz, CH_2), 4.23 (2H, s, C_2-CH_2), 7.34 (5H, s, ArH), 11.2—12.8 (1H, br, OH). MS m/z: 248 (M^+). *Anal.* Calcd for $C_{12}H_{12}N_2O_2S$: C, 58.05; H, 4.87; N, 11.28. Found: C, 58.20; H, 4.80; N, 11.32.

3-(2-Benzoyl-1,3,4-thiadiazol-5-yl)propionic Acid (4a) A suspension of CrO_3 (40 mg, 0.4 mmol) in acetic acid (2 ml) was added to a stirred solution of **4e** (50 mg, 0.2 mmol) in acetic acid (2 ml) at room temperature. After being stirred at 60 °C for 1 h, the mixture was cooled on ice, treated with water (5 ml), and extracted with $CHCl_3$. Work-up as described for the preparation of **4e** gave **4a** (38 mg, 72%), mp 117—118 °C (acetone). IR (KBr): 3200—2500 (OH), 1720 (CO), 1690 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.85 (2H, t, J=7 Hz, CH_2), 3.43 (2H, t, J=7 Hz, CH_2), 7.50—7.85 (3H, m, ArH), 8.25—8.45 (2H, m, ArH), 12.42 (1H, br s, OH). MS m/z: 262 (M^+). *Anal.* Calcd for $C_{12}H_{10}N_2O_3S$: C, 54.95; H, 3.84; N, 10.68. Found: C, 55.09; H, 3.70; H, 10.68.

Diethyl 3-(2-Benzyl-1,3,4-thiadiazol-5-yl)-3-ethoxycarbonylglutarate (19e) Compound **19e** was obtained from **11** (3 g, 11.44 mmol) and ethyl bromoacetate (2.52 ml, 22.78 mmol) in a similar manner to that described for compound **18e**. Yield 3.26 g (66%). IR (KBr): 1750 (CO), 1740 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.13 (6H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.23 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 3.32 (4H, s, CH_2), 3.99 (4H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.19 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.34 (2H, s, C_2-CH_2), 7.27 (5H, s, ArH). MS m/z: 434 (M^+). HR-MS m/z: Calcd for $C_{21}H_{26}N_2O_6S$: 434.1511. Found: 434.1501.

3-(2-Benzyl-1,3,4-thiadiazol-5-yl)glutaric Acid (5e) Compound **5e** was obtained from **19e** (559 mg, 1.29 mmol) and 85% KOH (434 mg, 6.57 mmol) in a similar manner to that described for compound **4e**. Yield 275 mg (70%), mp 96—98 °C (acetone). IR (KBr): 3100—2500 (OH), 1730 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.77 (4H, d, J=7 Hz, CH_2), 3.88 (1H, t, J=7 Hz, CH), 4.40 (2H, s, C_2-CH_2), 7.35 (5H, s, ArH), 9.0—12.0 (2H, br s, OH). MS m/z: 306 (M^+). *Anal.* Calcd for $C_{14}H_{14}N_2O_4S$: C, 54.89; H, 4.61; N, 9.14. Found: C, 54.99; H, 4.55; N, 9.11.

3-(2-Benzoyl-1,3,4-thiadiazol-5-yl)glutaric Acid (5a) Compound **5a** was obtained from **5e** (150 mg, 0.49 mmol) and CrO_3 (98 mg, 0.98 mmol) in a similar manner to that described for compound **4a**. Yield 119 mg (76%), mp 157—158 °C (acetone). IR (KBr): 3200—2500 (OH), 1720 (CO), 1705 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.91 (4H, d, J=7 Hz, CH_2), 4.07 (1H, t, J=7 Hz, CH), 7.50—7.90 (3H, m, ArH), 8.28—8.46 (2H, m, ArH), 9.0—12.0 (2H, br s, OH). MS m/z: 320 (M^+). *Anal.* Calcd for $C_{14}H_{12}N_2O_5S$: C, 52.50; H, 3.78; N, 8.75. Found: C, 52.58; H, 3.76; N, 8.51.

2-Methylsulfonyl-5-phenyl-1,3,4-thiadiazole (16b) Compound **16b**[8] was prepared by the literature method.

5-(4-Chlorophenyl)-2-methylsulfonyl-1,3,4-thiadiazole (16c) Compound **16c** was prepared by the literature method.[8] mp 189—190 °C (lit.,[3g] mp 189—190 °C). IR (KBr): 1325, 1150 (SO_2) cm^{-1}.

5-(4-Bromophenyl)-2-methylsulfonyl-1,3,4-thiadiazole (16d) Compound **16d** was prepared by the literature method.[8] mp 186—187 °C. IR (KBr): 1330, 1160 (SO_2) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.52 (3H, s, CH_3), 7.69 (2H, dd, J=2, 9 Hz, ArH), 7.93 (2H, dd, J=2, 9 Hz, ArH). MS m/z: 318, 320 (M^+). *Anal.* Calcd for $C_9H_7BrN_2O_2S_2$: C, 33.87; H, 2.21; N, 8.78. Found: C, 33.89; H, 2.13; N, 8.82.

Ethyl 2-Phenyl-1,3,4-thiadiazol-5-ylacetate (17b) A solution of ethyl acetate (1.64 ml, 16.8 mmol) in anhydrous THF (3 ml) was added to a solution of LDA (16.8 mmol; prepared from a 1.68 M solution of *n*-BuLi in hexane, 10 ml, 16.8 mmol, and diisopropylamine, 2.36 ml, 16.8 mmol) in anhydrous THF (10 ml) at −78 °C under argon. The mixture was stirred at −78 °C for 30 min, then a solution of **16b** (2.02 g, 8.42 mmol) in anhydrous THF (20 ml) was added dropwise. After 1 h at −78 °C, the reaction was quenched with aqueous acetic acid and the mixture was extracted with ethyl acetate (2 × 100 ml). The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column ($CHCl_3$–acetone, 50 : 1, v/v) to give a solid, which was recrystallized from EtOH to give **17b** (1.96 g, 94%), mp 87—88 °C (lit.,[7] 88—89 °C). IR (KBr): 1730 (CO) cm^{-1}.

Ethyl 2-(4-Chlorophenyl)-1,3,4-thiadiazol-5-ylacetate (17c) Compound **17c** was obtained from **16c** (450 mg, 1.64 mmol) and ethyl acetate (0.48 ml, 4.91 mmol) in a similar manner to that described for compound **17b**. Yield 454 mg (98%), mp 105—106 °C (EtOH). IR (KBr): 1730 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.29 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.18 (2H, s, CH_2), 4.23 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.43 (2H, dd, J=2, 9 Hz, ArH), 7.90 (2H, dd, J=2, 9 Hz, ArH). MS m/z: 282, 284 (M^+). *Anal.* Calcd for $C_{12}H_{11}ClN_2O_2S$: C, 50.98; H, 3.92; N, 9.91. Found: C, 50.97; H, 3.85; N, 9.98.

Ethyl 2-(4-Bromophenyl)-1,3,4-thiadiazol-5-ylacetate (17d) Compound **17d** was obtained from **16d** (523 mg, 1.64 mmol) and ethyl acetate (0.48 ml, 4.91 mmol) in a similar manner to that described for compound **17b**. Yield 481 mg (90%), mp 105—106 °C (EtOH). IR (KBr): 1725 (CO) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.31 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 4.19 (2H, s, CH_2), 4.24 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.60 (2H, dd, J=2, 9 Hz, ArH), 7.83 (2H, dd, J=2, 9 Hz, ArH). MS m/z: 326, 328 (M^+). *Anal.* Calcd for $C_{12}H_{11}BrN_2O_2S$: C, 44.05; H, 3.39; N, 8.56. Found: C, 44.09; H, 3.21; N, 8.49.

Diethyl 2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-ylsuccinates (18b—d) Compounds **18b—d** were obtained from **17b—d** and ethyl bromoacetate in a similar manner to that described for compound **18e**. Yields, melting points, recrystalization solvents, and analytical and spectral data for compounds **18b—d** are given in Table I.

3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]propionic Acids (4b—d) Compounds **4b—d** were obtained from **18b—d** and potassium hydroxide in a similar manner to that described for compound **4e**. Yields, melting points, recrystallization solvents, and analytical and spectral data for compounds **4b—d** are given in Table II.

Diethyl 3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]-3-ethoxycarbonylglutarates (19b—d) Compounds **19b—d** were obtained from **17b—d** and ethyl bromoacetate in a similar manner to that described for compound **19e**. Yields, and analytical and spectral data for compounds **19b—d** are given in Table I.

3-[2-(4-Substituted phenyl)-1,3,4-thiadiazol-5-yl]glutaric Acids (5b—d) Compounds **5b—d** were obtained from **19b—d** and potassium hydroxide in a similar manner to that described for compound **5e**. Yields, melting points, recrystallization solvents, and analytical and spectral data for compounds **5b—d** are given in Table II.

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Synthesis of Metabolites of S-1452, an Orally Active Thromboxane A_2 Receptor Antagonist

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The synthesis of 16 metabolites of S-1452, an orally active thromboxane A_2 (TXA_2) receptor antagonist, is described. Regioselective hydroxylation at C-5 or C-6 of the bicyclo[2.2.1]heptane skeleton of the optically active intermediate 16 was attempted by using 9-borabicyclo[3.3.1]nonane followed by H_2O_2 or *m*-chloroperbenzoic acid (*m*-CPBA) and then $LiAlH_4$, to obtain the hydroxylated product 17a or 17b, respectively. Modification of the C-2 substituent of 17a and 17b afforded eight metabolites of S-1452. Eight non-hydroxylated metabolites were synthesized by using a similar reaction sequence.

Keywords thromboxane; TXA_2 receptor antagonist; S-1452 metabolite; enantioselective synthesis; regioselective hydroxylation

Thromboxane A_2 (TXA_2), which has potent platelet aggregation, vasoconstriction, and bronchoconstriction activities, is one of the major mediators causing problems in circulatory disorders and asthmatic conditions.[1] S-1452, calcium (1*R*,2*S*,3*S*,4*S*)-(5′*Z*)-7-(phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)hept-5′-enoate, has been established as a chemically stable and orally active TXA_2 receptor antagonist.[2] The metabolism of S-145, the racemic acid form of S-1452,[3] in isolated rat hepatocytes was preliminarily examined by using high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS).[4] Extensive studies have been done on the metabolism of S-1452 in rats in these laboratories, and 16 metabolites were isolated[5] (Fig. 1). The structures of these metabolites were first proposed on the basis of HPLC, proton nuclear magnetic resonance (^{1}H-NMR) and GC/MS findings, and then confirmed by direct comparison with authentic synthetic samples. Described herein is the enantio- and regioselective synthesis of these compounds.

Results and Discussion

The possibility that a hydroxy group is introduced at the C-5 or C-6 position of the bicyclo[2.2.1]heptane skeleton in living tissues was suggested on the basis of GC/MS spectroscopy[6] of eight metabolites. This led us to try regioselective hydroxylation of a common synthetic intermediate **16**, which could be converted to metabolites of S-1452, by employing two different types of reactions. Although the stereochemistry of the hydroxy group of the metabolites, *i.e.*, *exo* or *endo*, could not be ascertained from the physicochemical data, the production of *exo* hydroxy metabolites was presumed from the steric preference in bicyclo[2.2.1]heptane chemistry. Moreover, as the conversion from "*exo*" to "*endo*" was well established by the oxidation–reduction procedure, the synthesis of C-5 or C-6 *exo* hydroxy compounds could be designed as required.

For the present purpose, (1*S*,2*S*,3*S*,4*R*)-3-carboxy-2-methoxycarbonylbicyclo[2.2.1]hept-5-ene (**13**) was considered to be a very suitable starting material. It was prepared by the highly enantioselective synthesis of half-esters of bicyclo[2.2.1]heptane-2,3-dicarboxylic acid from bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride in 50% overall yield (99.8% ee).[7] Transformation of **13** into the sulfonamide **15** was carried out by the reported procedure[3,8] in 59% yield. Reduction of **15** with lithium aluminum hydride ($LiAlH_4$) followed by protection of the resulting alcohol using dihydropyran gave the tetrahydropyranyl (THP) ether **16** in 74% yield. As described above, **16** was a key intermediate for the introduction of a hydroxy group in the bicyclo[2.2.1]heptane skeleton, and

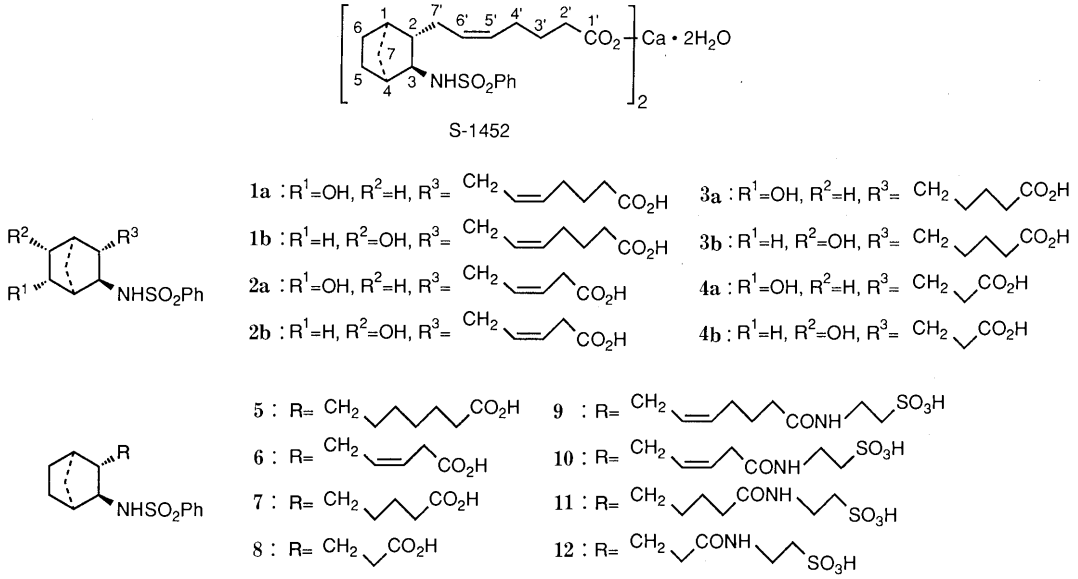


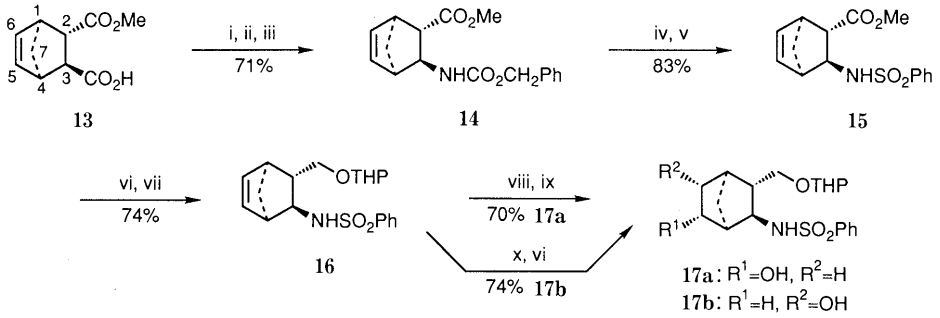
Fig. 1. Metabolites of S-1452 in Rat

regioselective hydroxylation at C-5 or C-6 was attempted.

Treatment of **16** with 9-borabicyclo[3.3.1]nonane (9-BBN) in tetrahydrofuran (THF) at 20 °C followed by oxidation with aqueous NaOH–H_2O_2 at 60 °C afforded a mixture of the hydroxy products in 85% yield in a ratio of 82:18 (determined by HPLC analysis). Chromatographic separation gave the major product **17a** as crystals and the minor one **17b** as an oil. Each compound, **17a** or **17b**, was a mixture of diastereomers concerning the THP protecting group and showed two peaks in HPLC identical with those of the major component or the minor one of the crude product, respectively. Epoxidation of **16** with *m*-chloroperbenzoic acid (*m*-CPBA) in CH_2Cl_2 at 0 °C gave the *exo* epoxide exclusively, and subsequent reduction with $LiAlH_4$ produced a mixture of **17a** and **17b** (2:98) in 86% yield (Chart 1). The major product, in this case, was identical with **17b** formed as the minor product in the 9-BBN procedure.

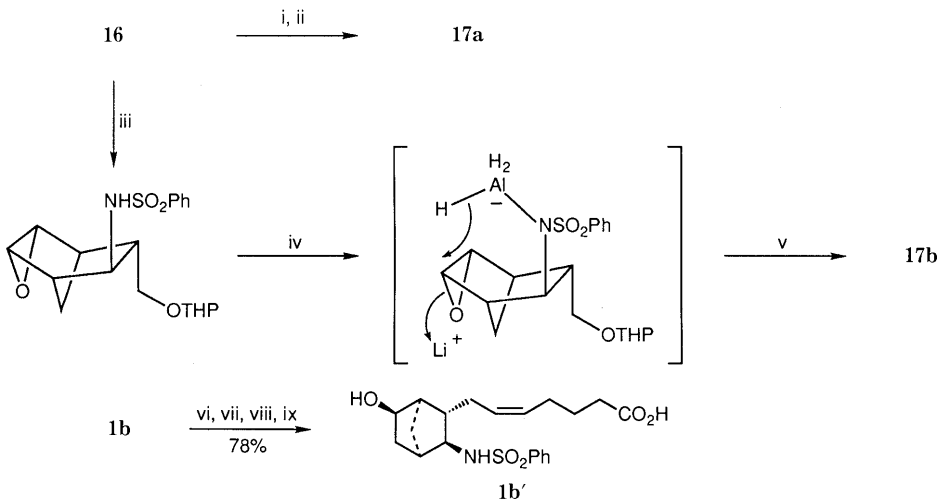
The position and stereochemistry of hydroxy groups of these compounds were determined by ^{1}H-NMR spectroscopy using the decoupling method because the values of the chemical shift (ppm) and the proton-proton coupling constant had already been well studied for bicyclo[2.2.1]-heptane derivatives.[9] ^{1}H-NMR spectroscopy of the hydroxymethyl derivatives **18a** and **18b** derived from **17a** and **17b**, respectively, as described in the next step, revealed a coupling constant corresponding to *endo-endo* coupling (J=6—7 Hz) between the C-5 and C-6 proton at δ4.13 (J=6.2 Hz, 5-H) and δ3.71 (J=6.5 Hz, 6-H), respectively. Thus, the *exo* configuration of these compounds was determined and the structure of the major product in the 9-BBN procedure was found to be **17a**, and that of the minor one to be **17b**. Epoxidation of **16** also occurred from the less-hindered *exo* side, while $LiAlH_4$ reduction of the *exo* epoxide occurred from the *endo* side by intramolecular hydride transfer *via* formation of a complex between $LiAlH_4$ and the nitrogen atom[10] of the C-3 sulfonamide as depicted in Chart 2, giving the 6-hydroxy isomer **17b** with high regioselectivity.

To obtain chemical evidence of the *exo* configuration of the C-6 hydroxy compounds, **1b**, derived from **17b** as described later, was converted to the *endo* isomer **1b′** by a four-step procedure (esterification, pyridinium chlorochromate (PCC) oxidation, $NaBH_4$ reduction and hydrolysis) in 78% yield. The stereochemistry of **1b′** could easily be predicted from the course of the hydride reduction of the C-6 keto derivative prepared by PCC oxidation of **1b**. Clearly, the hydride reduction occurs from the *exo* face and gives the *endo* alcohol **1b′**. ^{1}H-NMR spectroscopy of **1b′** also supported the stereochemistry, based on a comparison of the coupling constant[9] of the C-6 proton ($J_{C5exo\text{-}C6exo}$ 10.4 Hz) with that of the C-6 *exo* hydroxy



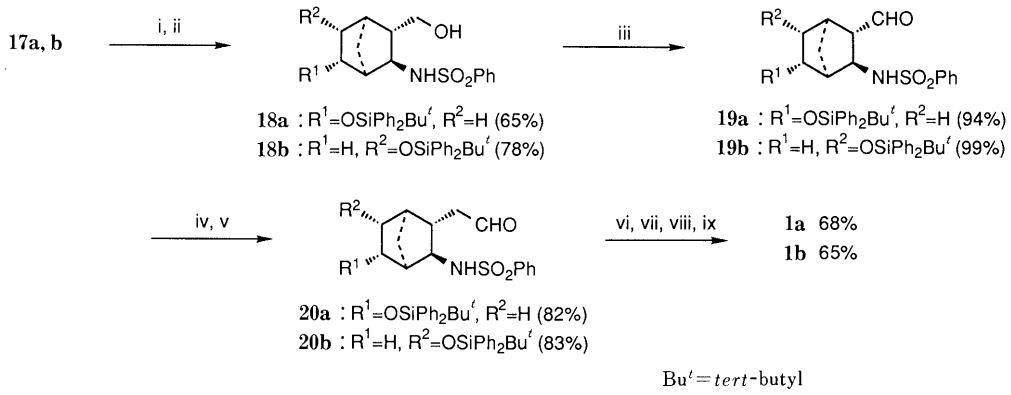
i, $ClCO_2Et$, Et_3N, NaN_3; ii, benzene, 80°C; iii, $PhCH_2OH$, Et_3N, benzene, 80°C; iv, TFA, anisole; v, $PhSO_2Cl$, Et_3N; vi, $LiAlH_4$; vii, dihydropyran, *p*-Ts-OH; viii, 9-BBN; ix, H_2O_2-NaOH; x, *m*-CPBA

Chart 1



i, 9-BBN; ii, H_2O_2-NaOH; iii, *m*-CPBA; iv, $LiAlH_4$; v, H^+; vi, CH_2N_2; vii, PCC; viii, $NaBH_4$; ix, 1 N NaOH

Chart 2. Presumed Transition State of Regioselective Hydroxylation



i, Bu^tPh_2SiCl, DMAP; ii, AcOH-H_2O-THF; iii, PCC; iv, Ph_3P=CHOMe; v, 90%HCO_2H; vi, Ph_3P=$CH(CH_2)_3CO_2K$; vii, CH_2N_2; viii, n-Bu_4NF; ix, 1 N NaOH

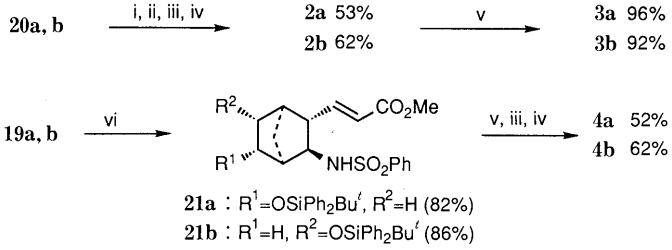
Chart 3

isomer **1b** ($J_{C5endo\text{-}C6endo}$ 6.2 Hz).

For the differentiation of the secondary alcohol of **17a** or **17b** from the C-1 primary alcohol, *tert*-butyldiphenylsilyl ether was used as a protecting group. It was stable to mild acid hydrolysis, which gave rise to effective deprotection of the C-1 THP ether. Treatment of **17a** with *tert*-butyldiphenylsilyl chloride and dimethylaminopyridine in dimethylformamide (DMF) and deprotection of the primary alcohol with aqueous AcOH at 50 °C gave the hydroxymethyl derivative **18a**, which, when oxidized with PCC, afforded the secondary aldehyde **19a** in 61% yield from **17a**.

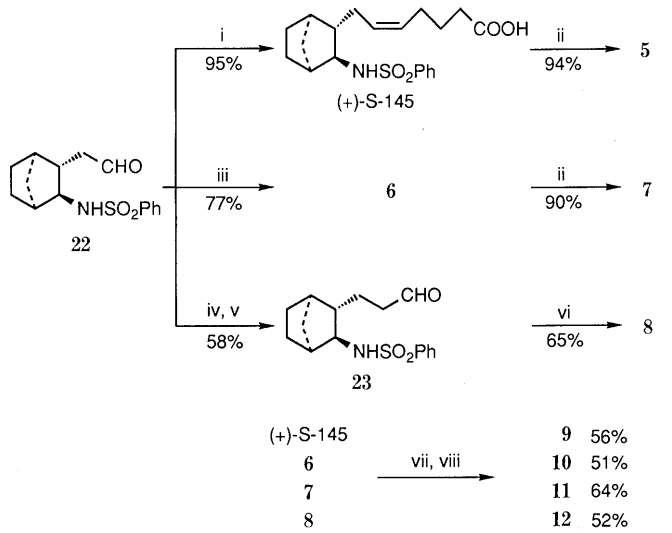
Methylene homologation of the aldehyde **19a** was accomplished by the Wittig reaction using methoxymethylenetriphenylphosphorane followed by acid treatment, to obtain the primary aldehyde **20a** in 82% yield. Compound **20a** was converted to the 5-hexenoic acid **1a** as follows. The Wittig reaction using (4-carboxybutyl)-triphenylphosphorane and the usual esterification with diazomethane afforded the methyl 5-hexenoate derivative. Deprotection of the silyl ether at C-5 with n-Bu_4NF followed by hydrolysis with 1 N KOH gave the objective compound **1a** in 68% yield from **20a**. The 6-hydroxy isomer **1b** was also derived from **17b** in the same manner as **1a** in 45% overall yield.

3-Pentenoic acid derivatives **2a** and **2b** were obtained from **20a** and **20b** in a similar manner to those of **1a** and **1b**, except that the Wittig reaction was performed using 2-carboxyethyltriphenylphosphorane, in 53 and 62% yields, respectively. In these cases, the 3-pentenoic acid derivatives were unstable under the Wittig reaction conditions, and double bond migration occurred, causing a decrease in the yield.[11] Catalytic hydrogenation of **2a** and **2b** using 10% Pd–C in methanol gave the saturated pentanoic acids **3a** and **3b** in 96 and 92% yields, respectively. The propionic acid derivatives **4a** and **4b** were obtained by four-step conversion as depicted in Chart 4. Transformation of **19a** into the α,β-unsaturated ester **21a** by application of the Wittig–Horner reaction using methyl diethylphosphonoacetate followed by catalytic reduction gave the saturated ester. Deprotection of the silyl ether and subsequent hydrolysis afforded **4a** in 43% overall yield. The 6-hydroxy isomer **4b** was also derived from **19b** by the same procedure.



i, Ph_3P=$CHCH_2CO_2K$; ii, CH_2N_2; iii, n-Bu_4NF; iv, 1 N NaOH; v, H_2, Pd/C; vi, $(MeO)_2P(O)CH_2CO_2Me$, NaH

Chart 4



i, Ph_3P=$CH(CH_2)_3CO_2K$; ii, H_2, Pd/C; iii, Ph_3P=$CHCH_2CO_2K$; iv, Ph_3P=CHOMe; v, 90%HCO_2H; vi, Jones oxidation; vii, DCC, HOSu; viii, $H_2N(CH_2)_2SO_3H$, Et_3N

Chart 5

Among the four non-hydroxylated metabolites (**5**—**7** and **8**), **5** could be prepared easily by catalytic reduction of (+)-S-145 and the other three compounds were synthesized from a common intermediate, (1*R*,2*S*,3*S*,4*S*)-(3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)acetaldehyde (**22**),[2] which was derived from the saturated analog of **13**.

Two kinds of Wittig reaction of **22** afforded the β,γ-

unsaturated carboxylic acid **6** and the methoxymethylene derivative. Hydrogenation of **6** gave **7** in 69% yield from **22**. Acid treatment of the methoxymethylene derivative gave the propionyl aldehyde **23**, and subsequent Jones oxidation afforded the propionic acid derivative **8** in 38% yield from **22**. Taurine derivatives of non-hydroxylated compounds, **9**—**11** and **12** were prepared by the coupling reaction of (+)-S-145, **6**, **7** and **8** with taurine, respectively, using the active ester method (DCC–HOSu; dicyclohexylcarbodiimide–*N*-hydroxysuccinimide).

Sixteen metabolites of S-1452 were identified by comparison with authentic synthetic samples based on HPLC, MS and ^{1}H-NMR findings, and the structures of the metabolites were definitely established.

All the synthesized compounds were tested for their biological activities as TXA_2 receptor antagonists. Among them, the C-5 hydroxy derivative **1a** and the C-6 hydroxy derivative **1b** inhibited platelet aggregation in guinea pigs induced by U-46619,[12] exhibiting higher IC_{50} values (**1a**, 1.80 μM; **1b**, 0.68 μM) than that of S-1452 (0.07 μM).[13] These values correspond to 1/25 and 1/10 of the TXA_2 antagonistic activity of S-1452, respectively. Details of the biological activity will be reported elsewhere.[14]

Experimental

Reactions using anhydrous solvents that had been dried over type 4A molecular sieves were carried out in a nitrogen atmosphere. Melting points were determined on a Yanagimoto apparatus and were not corrected. Infrared (IR) spectra were recorded on a JASCO A702 spectrometer. The value of $[\alpha]_D$ was determined with a Perkin–Elmer 241 polarimeter. Unless otherwise stated, ^{1}H-NMR spectra were obtained in $CDCl_3$ with a Varian VXR-200 spectrometer using tetramethylsilane as an internal reference. HPLC analysis was carried out on a Shimadzu LC-6A chromatograph equipped with a C-6A integrator and an SPD-6A variable-wavelength ultraviolet monitor. Mass spectrometry (MS) was conducted on a Hitachi M-68 spectrometer. To dry organic solutions, anhydrous magnesium sulfate was used. For column chromatography, silica gel (Merck Silica gel 60) or a Merck Lobar column was used.

The hydroxylated products, **1a**, **1b**, **2a**, **2b**, **4a** and **4b**, were highly purified by preparative HPLC using Develosil (Nomura Chemical, Japan) ODS-15/30; column size, 50/500 (mm); mobile phase, CH_3CN–MeOH–H_2O–AcOH (200 : 200 : 450 : 1); flow rate, 80 ml/min; detection, 225 nm.

(1*S*,2*S*,3*S*,4*R*)-3-Carbobenzoxyamino-2-carbomethoxybicyclo[2.2.1]-hept-5-ene (14) Triethylamine (7.74 ml, 42.7 mmol × 1.3) and $ClCO_2Et$ (4.08 ml, 42.7 mmol) were added to a solution of **13** (7.70 g, 42.7 mmol) in acetone (120 ml) at 0 °C, and the mixture was stirred for 30 min. Next, a solution of NaN_3 (8.33 g, 42.7 mmol × 3) in H_2O (46 ml) was added and the mixture was stirred for 1 h, then poured into cold H_2O and extracted with ethyl acetate. The extract was washed with 5% aqueous $NaHCO_3$, dried, and concentrated *in vacuo* to obtain an oily residue, which was dissolved in benzene (90 ml) and heated to reflux for 1 h. After the gas evolution ceased, benzyl alcohol (8.70 ml, 42.7 mmol × 2) and triethylamine (7.7 ml, 42.7 mmol × 1.3) were added, and the mixture was stirred for 1 h under reflux and then poured into cold 2 N HCl. The organic solution was washed with aqueous 5% $NaHCO_3$ and H_2O, dried, and concentrated *in vacuo*. Chromatography on silica gel with toluene–ethyl acetate (10 : 1) as the eluent gave the carbobenzoxy derivative **14** (9.0 g, 71%), mp 81—82 °C. *Anal.* Calcd for $C_{17}H_{19}NO_4$: C, 67.76; H, 6.36; N, 4.65. Found: C, 67.78; H, 6.42; N, 4.62. $[\alpha]_D^{24}$ +138.3 ± 0.9° (c = 2.013, $CHCl_3$). IR ($CHCl_3$): 3442 (NH), 1730 (CO) cm^{-1}. ^{1}H-NMR δ: 1.52 (1H, ABq, A part, J = 8.0 Hz, 7-H), 1.83 (1H, ABq, B part, J = 8.0 Hz, 7-H), 1.93 (1H, dd, J = 1.4, 3.6 Hz, 2-H), 3.02 (2H, brs, 1-H, 4-H), 3.74 (3H, s, Me), 4.55 (2H, brs, 3-H, NH), 5.10 (2H, s, CH_2), 6.17 (1H, dABq, A part, J = 3.0, 5.4 Hz, olefinic H), 6.43 (1H, dABq, B part, J = 2.6, 5.4 Hz, olefinic H), 7.35 (5H, s, aromatic H).

(1*S*,2*S*,3*S*,4*R*)-2-Carbomethoxy-3-phenylsulfonylaminobicyclo[2.2.1]-hept-5-ene (15) A solution of **14** (8.0 g, 26.5 mmol) in a mixture of anisole (25 ml) and trifluoroacetic acid (100 ml) was stirred for 6 h at 45 °C, then concentrated *in vacuo*, and the residue was rinsed with *n*-hexane. To a solution of the residue in CH_2Cl_2 (50 ml), triethylamine (14.8 ml, 26.5 mmol × 4) and phenylsulfonyl chloride (4 ml, 26.5 mmol × 1.2) were added at 0 °C, and the mixture was stirred for 30 min at the same temperature. The reaction mixture was washed with 2 N HCl, aqueous 5% $NaHCO_3$ and H_2O, and then dried and concentrated *in vacuo*. Chromatography on silica gel with *n*-hexane–ethyl acetate (2 : 1) as the eluent gave the sulfonamide **15** (6.75 g, 83%), mp 128—129 °C. *Anal.* Calcd for $C_{15}H_{17}NO_4S$: C, 58.61; H, 5.58; N, 4.56; S, 10.43. Found: C, 58.60; H, 5.57; N, 4.52; S, 10.23. $[\alpha]_D^{25}$ +100.9 ± 0.5° (c = 3.005, $CHCl_3$), IR ($CHCl_3$): 3275 (NH), 1730 (CO) cm^{-1}. ^{1}H-NMR δ: 1.38—1.47 (1H, m, 7-H), 1.59—1.68 (1H, m, 7-H), 1.87 (1H, dd, J = 3.0, 2.8 Hz, 2-H), 2.88 (1H, s, 4-H), 2.91 (1H, s, 1-H), 3.58 (3H, s, Me), 4.23 (1H, ddd, J = 9.6, 3.7, 3.6 Hz, 3-H), 4.35 (1H, d, J = 9.6 Hz, NH), 6.10 (1H, dd, J = 2.6, 5.6 Hz, olefinic H), 6.41 (1H, dd, J = 2.6, 5.6 Hz, olefinic H), 7.46—7.65 (3H, m, aromatic H), 7.84—7.92 (2H, m, aromatic H).

(1*S*,2*S*,3*S*,4*R*)-3-Phenylsulfonylamino-(2-tetrahydropyranyloxymethyl)-bicyclo[2.2.1]hept-5-ene (16) A solution of **15** (6.54 g, 21.3 mmol) in a mixture of THF (30 ml) and ether (90 ml) was added to a suspension of $LiAlH_4$ (3.88 g, 21.3 mmol × 4.8) in ether (120 ml) over 40 min at 0 °C, and the reaction mixture was stirred for 30 min at the same temperature. Next, 2 N HCl was added carefully, and the mixture was partitioned between ethyl acetate and H_2O. The organic solution was washed with aqueous 5% $NaHCO_3$ and H_2O, and then dried and concentrated *in vacuo*. Dihydropyran (3.35 ml) and *p*-toluenesulfonic acid hydrate (100 mg) were added to a solution of the residue in CH_2Cl_2 (50 ml), and the mixture was stirred for 1.5 h at room temperature, then washed with aqueous 5% $NaHCO_3$ and H_2O, dried, and concentrated *in vacuo*. Chromatography of the residue on silica gel with *n*-hexane–ethyl acetate (4 : 1) as the eluent yielded the THP ether **16** (5.73 g, 74%), which was dissolved in benzene, and the solution was concentrated *in vacuo*. By repeating the procedures, contaminating solvents, except benzene, were removed, giving a colorless oil. *Anal.* Calcd for $C_{19}H_{25}NO_4S \cdot 0.3C_6H_6$: C, 64.57; H, 6.98; N, 3.62; S, 8.29. Found: C, 64.59; H, 6.99; N, 3.69; S, 8.03. $[\alpha]_D^{24}$ +61.8 ± 1.0° (c = 1.003, $CHCl_3$). IR ($CHCl_3$): 3380 (NH), 1345 (SO_2), 1158 (SO_2) cm^{-1}. ^{1}H-NMR δ: 1.30—1.88 (9H, m, 2-H, 7-H, methylene H of THP), 2.62—2.84 (2H, m, 1-H, 4-H), 3.13—3.87 (5H, m, 3-H, methylene H of THP), 4.30—4.60 (2H, m, NH, O–CH(C)–O), 5.90—6.00 (1H, m, olefinic H), 6.35—6.45 (1H, m, olefinic H), 7.37 (s, contaminated benzene), 7.46—7.65 (3H, m, aromatic H), 7.84—7.95 (2H, m, aromatic H). MS m/z: 364 $(M+H)^+$ (PILSIMS).

(1*S*,2*S*,3*S*,4*S*,5*R*)-5-Hydroxy-3-phenylsulfonylamino-(2-tetrahydropyranyloxymethyl)bicyclo[2.2.1]heptane (17a) A solution of 9-BBN (0.5 N, 50.6 ml, 25.3 mmol) was added to a solution of **16** (4.6 g, 12.7 mmol) in THF (23 ml) at room temperature, and the mixture was stirred for 1.5 h. To this solution, 6 N NaOH (6.3 ml, 38.0 mmol) and 30% H_2O_2 (5.0 ml, 44.3 mmol) were added, and the mixture was stirred for 1 h at 60 °C. The reaction mixture was poured into cold H_2O and extracted with ethyl acetate. The extract was washed with 2 N HCl and aqueous 5% $NaHCO_3$, dried, and evaporated to dryness to give 4.83 g of the crude product (**17a** : **17b** = 82 : 18). The ratio of the regioisomer was determined by HPLC analysis using Nucleosil 100-5: Mobile phase, *n*-hexane–THF (4 : 1); flow rate, 7 ml/min; detection, 225 nm. Chromatography on silica gel with *n*-hexane–ethyl acetate (2 : 1) as the eluent gave **17a** (3.4 g, 70%), mp 155—160 °C. *Anal.* Calcd for $C_{19}H_{27}NO_5S$: C, 59.82; H, 7.13; N, 3.67; S, 8.41. Found: C, 59.56; H, 7.00; N, 3.69; S, 8.45. ^{1}H-NMR δ: 1.17—1.90 (12H, m, 2-H, 6-H, 7-H, methylene H of THP, OH), 1.94—2.10 (1H, m, 4-H), 2.35—2.49 (1H, m, 1-H), 2.83—3.19 (2H, m, methylene H of THP), 3.29—3.62 (2.5H, m, 3-H, methylene H of THP), 3.79—3.95 (0.5H, m, 3-H), 4.27—4.48 (2H, m, 5-H, O–CH(C)–O), 4.87 (0.5H, d, J = 5 Hz, NH), 5.40 (0.5H, d, J = 3 Hz, NH), 7.47—7.67 (3H, m, aromatic H), 7.85—7.97 (2H, m, aromatic H). Polar fractions gave **17b** (0.65 g, 14%) as an oil, which was identical with the major product described in the next reaction.

(1*R*,2*S*,3*S*,4*R*,6*S*)-6-Hydroxy-3-phenylsulfonylamino-(2-tetrahydropyranyloxymethyl)bicyclo[2.2.1]heptane (17b) A solution of **16** (3.75 g, 10.3 mmol) in CH_2Cl_2 (80 ml) was treated with *m*-CPBA (80% purity, 3.33 g, 15.5 mmol) at 0 °C, and the mixture was stirred for 2.5 h at room temperature. The resulting crystals were removed by filtration and the filtrate was washed with aqueous 5% $Na_2S_2O_3$ solution, 5% $NaHCO_3$, and H_2O, and then dried and concentrated *in vacuo*. Chromatography on silica gel with *n*-hexane–ethyl acetate (1 : 1) as the eluent gave 3.55 g of the *exo* epoxide, which was dissolved in ether (20 ml) and reduced with $LiAlH_4$ (1.4 g) suspended in a mixture of ether (40 ml) and THF (20 ml) at room temperature. After the reaction mixture had been stirred for 30 min, H_2O was added carefully, and the mixture was partitioned

between ethyl acetate and 2 N HCl. The organic solution was washed with H_2O and aqueous 5% $NaHCO_3$, dried, and concentrated to dryness. Chromatography on silica gel with *n*-hexane–ethyl acetate (1 : 1) as the eluent gave **17b** (3.0 g, 74%), colorless oil. *Anal.* Calcd for $C_{19}H_{27}NO_5S \cdot 0.3H_2O$ (hygroscopic): C, 58.99; H, 7.19; N, 3.62; S, 8.29. Found: C, 58.84; H, 6.96; N, 3.68; S, 8.00. ^{1}H-NMR δ: 1.02—2.46 (14H, m, 1-H, 2-H, 4-H, 5-H, 7-H, methylene H of THP, OH), 2.82—3.20 (2H, m, methylene H of THP), 3.34—3.69 (2.5H, 3-H, methylene H of THP), 3.75—3.93 (1.5H, m, 3-H, 6-H), 4.33—4.46 (1H, m, O–CH(C)–O), 4.83 (0.5H, d, J=5 Hz, NH), 5.25 (0.5H, d, J=4.8 Hz, NH), 7.25—7.66 (3H, m, aromatic H), 7.84—7.95 (2H, m, aromatic H). MS m/z: 382 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*R*,6*R*)-(5′*Z*)-7-(6-Hydroxy-3-phenylsulfonylaminobicyclo-[2.2.1]hept-2-yl)hept-5′-enoic Acid (1b′) Compound **1b** was esterified with diazomethane and the methyl ester was oxidized with PCC, in the same manner as **19a**, reduced with $NaBH_4$ in the usual way, and hydrolyzed with 1 N NaOH, giving **1b′** (78%), a colorless gum. *Anal.* Calcd for $C_{20}H_{27}NO_5S$: C, 61.65; H, 6.93; N, 3.49. Found: C, 61.90; H, 7.05, N, 3.35. IR ($CHCl_3$): 3600—2400 (COOH), 3270 (NH), 1710 (COOH), 1322 (SO_2), 1157 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 1.23—2.17 (13H, m, 1-H, 2-H, 4-H, 5-H, 7-H, 3′-H, 4′-H, 7′-H), 2.23 (2H, t, J=5.5 Hz, 2′-H), 2.96—3.08 (1H, m, 3-H), 4.13 (1H, td, J=4.2, 10.4 Hz, 6-H), 5.10—5.29 (2H, m, olefinic H), 7.49—7.69 (3H, m, aromatic H), 7.80—7.94 (2H, m, aromatic H).

(1*S*,2*S*,3*R*,4*S*,5*R*)-5-*tert*-Butyldiphenylsilyloxy-2-hydroxymethyl-3-phenylsulfonylaminobicyclo[2.2.1]heptane (18a) and (1*R*,2*S*,3*S*,4*R*,6*S*)-6-*tert*-Butyldiphenylsilyloxy-2-hydroxymethyl-3-phenylsulfonylaminobicyclo-[2.2.1]heptane (18b) Dimethylaminopyridine (865 mg, 3.5 mmol × 2) and *tert*-butyldiphenylchlorosilane (1.4 ml, 3.5 mmol × 1.5) were added to a solution of **17a** (1.35 g, 3.5 mmol) in DMF (17 ml), and the mixture was stirred overnight at 50 °C, then poured into cold water and extracted with ethyl acetate. The extract was washed with aqueous 5% $NaHCO_3$ and H_2O, dried, and evaporated to dryness. Chromatography of the residue on silica gel with *n*-hexane–ethyl acetate (9 : 1)—(4 : 1) as the eluent gave 2.15 g of silylated product, which was dissolved in a mixture of THF (5 ml), acetic acid (15 ml), and H_2O (5 ml). The mixture was warmed overnight at 50 °C, then poured into H_2O and extracted with CH_2Cl_2. The extract was washed with aqueous 5% $NaHCO_3$ and H_2O, dried, and evaporated to dryness. Chromatography on silica gel with *n*-hexane–ethyl acetate (2 : 1) as the eluent gave the primary alcohol **18a** (1.18 g, 65%), mp 131—133 °C. *Anal.* Calcd for $C_{30}H_{37}NO_4SSi$: C, 67.25; H, 6.96; N, 2.61. Found: C, 67.22; H, 6.98; N, 2.75. $[\alpha]_D^{24}$ −6.9±0.5° (c=1.011, $CHCl_3$). IR ($CHCl_3$): 3510 (OH), 1324 (SO_2), 1162 (SO_2) cm^{-1}. ^{1}H-NMR δ: 1.04 (9H, s, *tert*-Bu), 1.15—1.30 (2H, m, 2-H, 7-H), 1.54—1.80 (6H, m, 6-H, 7-H, OH), 1.98—2.10 (2H, m, 1-H, 4-H), 2.80 (1H, q, J=8.0 Hz, 3-H), 3.29 (1H, dABq, A part, J=7.6, 11.0 Hz, CH_2O), 3.35 (1H, dABq, B part, J=7.6, 11.0 Hz, CH_2O), 4.03 (1H, d, J=5.2 Hz, NH), 4.08—4.18 (1H, m, 5-H), 7.34—7.77 (15H, m, aromatic H). ^{1}H-NMR (400 MHz; $CDCl_3$) δ: 4.13 (1H, d, J=6.2 Hz, 5-H).

18b: 78% yield; colorless oil. *Anal.* Calcd for $C_{30}H_{37}NO_4SSi$: C, 67.25; H, 6.96; N, 2.61. Found: C, 67.39; H, 7.11; N, 2.71. $[\alpha]_D^{24}$ +21.7±0.6° (c=1.014, $CHCl_3$). IR ($CHCl_3$): 3620 (OH), 3380 (NH), 1322 (SO_2), 1160 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.71—1.96 (7H, m, 1-H, 2-H, 5-H, 7-H, OH), 1.03 (9H, s, *tert*-Bu), 2.16 (1H, br s, 4-H), 2.90—3.02 (1H, m, 3-H), 3.22 (1H, dABq, A part, J=4.0, 10.0 Hz, CH_2O), 3.29 (1H, dABq, B part, J=4.0, 10.0 Hz, CH_2O), 3.71 (1H, d, J=6.5 Hz, 6-H), 4.62 (1H, d, J=6.0 Hz, NH), 7.30—7.70 (13H, m, aromatic H), 7.78—7.90 (2H, m, aromatic H).

(1*S*,2*S*,3*S*,4*S*,5*R*)-5-*tert*-Butyldiphenylsilyloxy-2-formyl-3-phenylsulfonylaminobicyclo[2.2.1]heptane (19a) and (1*R*,2*S*,3*S*,4*R*,6*S*)-6-*tert*-Butyldiphenylsilyloxy-2-formyl-3-phenylsulfonylaminobicyclo[2.2.1]heptane (19b) PCC (724 mg, 1.1 mmol × 3) and molecular sieves (type 4A, powder, 1.0 g) were added to a solution of **18a** (600 mg, 1.1 mmol) in CH_2Cl_2 (45 ml), and the mixture was stirred for 1 h at room temperature. The reaction mixture was passed through silica gel with CH_2Cl_2 and then *n*-hexane–ethyl acetate (4 : 1) as the eluents, and the secondary aldehyde **19a** (564 mg, 94%), a colorless oil was obtained. ^{1}H-NMR δ: 1.05 (9H, s, *tert*-Bu), 1.10 (1H, m, 2-H), 1.60—2.00 (4H, m, 6-H, 7-H), 2.27 (1H, br s, 4-H), 2.41—2.49 (1H, m, 2-H), 3.35—3.46 (2H, m, 3-H, NH), 4.00—4.11 (1H, m, 5-H), 7.37—7.73 (15H, m, aromatic H), 9.20 (1H, s, CHO).

19b: 99% yield, colorless oil. ^{1}H-NMR δ: 1.04 (9H, s, *tert*-Bu), 1.07—2.37 (7H, m, 1-H, 2-H, 4-H, 5-H, 7-H), 3.65—3.74 (1H, m, 3-H), 3.80—3.90 (1H, m, 6-H), 4.42 (1H, d, J=5.4 Hz, NH), 7.34—7.86 (15H, m, aromatic H), 9.20 (1H, s, CHO). These aldehydes **19a** and **19b** were found to be unstable and were used for the next reaction without further purification.

(1*S*,2*S*,3*R*,4*S*,5*R*)-5-*tert*-Butyldiphenylsilyloxy-2-formylmethyl-3-phenylsulfonylaminobicyclo[2.2.1]heptane (20a) and (1*R*,2*S*,3*S*,4*R*,6*S*)-6-*tert*-Butyldiphenylsilyloxy-2-formylmethyl-3-phenylsulfonylaminobicyclo[2.2.1]-heptane (20b) A suspension of methoxymethyltriphenylphosphonium chloride (19.27 g, 18.7 mmol × 3) in THF (190 ml) was treated with *tert*-BuOK (6.0 g, 18.7 mmol × 2.9) at 0 °C, and the mixture was stirred for 1 h at the same temperature. Then a solution of **19a** (10.0 g, 18.7 mmol) in THF (60 ml) was added. The reaction mixture was stirred for 20 min at 0 °C and poured into a mixture of toluene and H_2O. The organic solution was washed with H_2O, dried, and concentrated *in vacuo*. The residue was dissolved in a mixture of 90% formic acid (20 ml) and THF (2 ml), and the mixture was stirred for 1.5 h at room temperature, then poured into a mixture of aqueous 5% $NaHCO_3$ and CH_2Cl_2. The organic solution was washed with H_2O, dried, and concentrated *in vacuo*. Chromatography of the residue on silica gel with *n*-hexane–ethyl acetate (2 : 1) as the eluent gave the primary aldehyde **20a** (8.43 g, 82%), mp 137—139 °C. *Anal.* Calcd for $C_{31}H_{37}NO_4SSi$: C, 67.97; H, 6.81; N, 2.56. Found: C, 68.07; H, 6.77; N, 2.65. $[\alpha]_D^{24}$ +7.9±0.5° (c=1.004, $CHCl_3$). IR ($CHCl_3$): 3380 (NH), 1722 (CHO) cm^{-1}. ^{1}H-NMR δ: 1.05 (9H, s, *tert*-Bu), 1.14—1.82 (5H, m, 2-H, 6-H, 7-H), 1.88 (1H, br s, 1-H), 2.04 (1H, br s, 4-H), 2.26 (1H, dd ABq, A part, J=1.6, 18.0, 6.2 Hz, $CH_2(O)$, 2.42 (1H, dABq, B part, J=18.0, 6.2 Hz, CH_2CO), 2.61 (1H, q, J=4.0 Hz, 3-H), 4.15—4.30 (1H, m, 5-H), 4.34 (1H, d, J=4.0 Hz, NH), 7.34—7.80 (15H, m, aromatic H), 9.50 (1H, s, CHO).

20b: 83% yield, colorless foam. *Anal.* Calcd for $C_{31}H_{37}NO_4SSi \cdot 0.1H_2O$: C, 67.75; H, 6.82; N, 2.55. Found: C, 67.55; H, 6.76; N, 2.56. $[\alpha]_D^{24}$ +42.2±0.8° (c=1.004, $CHCl_3$). IR ($CHCl_3$): 3310 (NH), 1722 (CO), 1335 (SO_2), 1168 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.93—1.16 (1H, m, 2-H), 1.02 (9H, s, *tert*-Bu), 1.19 (1H, d, J=10.7 Hz, 7-H), 1.42 (1H, dd, J=4.8, 14.0 Hz, 5-H), 1.64 (1H, br s, 1-H), 1.80 (1H, dd, J=1.7, 10.7 Hz, 7-H), 2.05 (1H, ddd, J=2.2, 6.6, 14.0 Hz, 5-H), 2.29 (2H, d, J=7.2 Hz, CH_2CO), 2.33—2.40 (1H, m, 4-H), 2.50—2.61 (1H, m, 3-H), 3.74 (1H, d, J=6.6 Hz, 6-H), 5.07 (1H, d, J=3.9 Hz, NH), 7.28—7.67 (13H, m, aromatic H), 7.75—7.86 (2H, m, aromatic H), 9.40 (1H, s, CHO). MS m/z: 548 $(M+H)^+$ (PILSIMS).

(1*S*,2*S*,3*R*,4*S*,5*R*)-(5′*Z*)-7′-(5-Hydroxy-3-phenylsulfonylaminobicyclo-[2.2.1]hept-2-yl)hept-5′-enoic Acid (1a) General Procedure for the Preparation of **1b**, **2a**, **2b** and **6**: A suspension of 4-carboxybutyltriphenylphosphonium bromide (1.43 g, 1.1 mmol × 3) in THF (60 ml) was treated with *tert*-BuOK (654 mg, 1.1 mmol × 5.4) at 0 °C, and the mixture was stirred for 1 h at room temperature. To this solution of the phosphorane, a solution of **20a** (590 mg, 1.1 mmol) in THF (5 ml) was added at −15 °C, and the mixture was stirred for 1 h (−15 °C→0 °C), then poured into a mixture of 2 N HCl and ethyl acetate. The organic solution was washed with H_2O, dried, and concentrated *in vacuo*. The residue was treated with diazomethane in ether as usual, giving the crude product. Chromatography on silica gel with *n*-hexane–ethyl acetate (9 : 1)—(2 : 1) as the eluent gave the methyl 5-pentenoate derivative (647 mg), which was treated with *n*-Bu_4NF (1 mol solution in THF, 30 ml) in THF (5 ml) overnight at 60 °C. The reaction mixture was poured into aqueous saturated NH_4Cl solution and extracted with ethyl acetate. The extract was washed with H_2O, dried and evaporated to dryness. Chromatography of the residue on silica gel with *n*-hexane–ethyl acetate (9 : 1)—(1 : 1) as the eluent gave 360 mg of the desilylated product. Usual hydrolysis of the product with 1 N KOH (2.6 ml) in MeOH (3 ml) for 2 h at 40 °C gave the desired product **1a** (281 mg, 68%), colorless foam. *Anal.* Calcd for $C_{20}H_{27}NO_5S \cdot 0.25H_2O$ (hygroscopic): C, 60.36; H, 6.96; N, 3.52; S, 8.06. Found: C, 60.26; H, 6.92; N, 3.57; S, 7.99. $[\alpha]_D^{24}$ +46.0±0.8° (c=1.039, MeOH). IR (KBr): 3680—2400 (COOH), 3260 (OH), 1705 (COOH), 1315 (SO_2), 1155 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.93—1.07 (1H, m, 2-H), 1.27—1.98 (11H, m, 1-H, 6-H, 7-H, 3′-H, 4′-H, 7′-H), 2.15—2.30 (3H, m, 4-H, 2′-H), 2.80—2.91 (1H, m, 3-H), 4.06—4.17 (1H, m, 5-H), 5.00—5.24 (2H, m, olefinic H), 7.51—7.69 (3H, m, aromatic H), 7.84—7.95 (2H, aromatic H). MS m/z: 394 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*R*,6*S*)-(5′*Z*)-7′-(6-Hydroxy-3-phenylsulfonylaminobicyclo-[2.2.1]hept-2-yl)hept-5′-enoic Acid (1b) 65% yield, mp 127—128 °C. *Anal.* Calcd for $C_{20}H_{27}NO_5S$: C, 61.05; H, 6.92; N, 3.56; S, 8.15. Found: C, 61.02; H, 6.95; N, 3.57; S, 8.04. $[\alpha]_D^{24}$ +28.5±0.7° (c=1.013, MeOH). IR (KBr): 3650—2400 (COOH), 3430 (OH), 3210 (NH), 1710 (COOH), 1308 (SO_2), 1145 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.80—0.96 (1H, m, 2-H), 1.01—2.17 (12H, m, 1-H, 4-H, 5-H, 7-H, 3′-H, 4′-H, 7′-H), 2.23 (2H, t, J=7.4 Hz, 2′-H), 2.82—2.91 (1H, m, 3-H), 3.63

(1H, d, J=6.2 Hz, 6-H), 5.09—5.31 (2H, m, olefinic H), 7.50—7.69 (3H, m, aromatic H), 7.83—7.92 (2H, m, aromatic H).

The pentenoic derivatives **2a**, **2b** and **6** were obtained by using 2-carboxyethyltriphenylphosphonium bromide, in place of 4-carboxybutyltriphenylphosphonium bromide, in the same manner.

(1*S*,2*S*,3*R*,4*S*,5*R*)-(3′*Z*)-5′-(5-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)pent-3′-enoic Acid (2a) 53% yield, colorless foam. *Anal.* Calcd for $C_{18}H_{23}NO_5S$: C, 59.16; H, 6.34; N, 3.83; S, 8.77. Found: C, 58.95; H, 6.52; N, 3.84; S, 8.59. $[\alpha]_D^{24}$ +44.2±0.8° (c=1.008, MeOH). IR (KBr): 3760—2280 (COOH), 3260 (NH), 1710 (COOH), 1320 (SO_2), 1158 (SO_2) cm^{-1}. ^{1}H-NMR δ: (CD_3OD) 0.93—1.07 (1H, m, 2-H), 1.26—1.98 (7H, m, 1-H, 6-H, 7-H, 5′-H), 2.27 (1H, d, J=5.0 Hz, 4-H), 2.71—2.98 (3H, m, 3-H, 2′-H), 4.16—4.26 (1H, m, 5-H), 5.12—5.43 (2H, m, olefinic H), 7.52—7.68 (3H, m, aromatic H), 7.84—7.94 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*R*,6*S*)-(3′*Z*)-5′-(6-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)pent-3′-enoic Acid (2b) 62% yield, colorless foam. *Anal.* Calcd for $C_{18}H_{23}NO_5S \cdot 0.2H_2O$ (hygroscopic): C, 58.84; H, 6.42; N, 3.81; S, 8.73. Found: C, 58.63; H, 6.42; N, 3.88; S, 8.80. $[\alpha]_D^{24}$ +28.5±0.7° (c=1.017, MeOH). IR (KBr): 3700—2320 (COOH), 3280 (NH), 1710 (COOH), 1320 (SO_2), 1157 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.84—2.19 (9H, m, 1-H, 2-H, 4-H, 5-H, 7-H, 5′-H), 2.77—3.03 (3H, m, 3-H, 2′-H), 3.64 (1H, d, J=6.0 Hz, 6-H), 5.23—5.50 (2H, m, olefinic H), 7.50—7.67 (3H, m, aromatic H), 7.82—7.91 (2H, m, aromatic H). MS m/z: 366 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*S*)-(3′*Z*)-5′-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)-pent-3′-enoic Acid (6) 77% yield, mp 68—69 °C. *Anal.* Calcd for $C_{18}H_{23}NO_4S$: C, 61.87; H, 6.63; N, 4.01; S, 9.17. Found: C, 61.65; H, 6.72; N, 4.22; S, 8.93. $[\alpha]_D^{24}$ +11.2±0.5° (c=1.004, $CHCl_3$). IR (KBr): 3720—2200 (COOH), 3290 (NH), 1710 (COOH), 1325 (SO_2), 1165 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.80—1.65 (7H, m, 2-H, 5-H, 6-H, 7-H), 1.75—2.04 (3H, m, 1-H, 5′-H), 2.17 (1H, br s, 4-H), 2.85—3.14 (3H, m, 3-H, 2′-H), 5.09 (1H, d, J=6.8 Hz, NH), 5.27—5.56 (2H, m, olefinic H), 7.43—7.64 (3H, m, aromatic H), 7.83—7.97 (2H, m, aromatic H).

(1*S*,2*S*,3*R*,4*S*,5*R*)-5′-(5-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]-hept-2-yl)pentanoic Acid (3a) General Procedure for the Preparation of **3b**, **5** and **7**: A solution of **2a** (288 mg) in MeOH (5 ml) containing 10% Pd–C (100 mg) was stirred for 20 min under a hydrogen atmosphere at room temperature. The solid was removed by filtration, and concentration of the filtrate *in vacuo* gave the saturated product **3a** (279 mg, 96%), a colorless oil. *Anal.* Calcd for $C_{18}H_{25}NO_5S$: C, 58.83; H, 6.85; N, 3.81. Found: C, 58.95; H, 6.93; N, 3.47. IR ($CHCl_3$): 3600—2400 (COOH), 3270 (NH), 1710 (COOH), 1322 (SO_2), 1161 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.74—1.80 (11H, m, 2-H, 6-H, 7-H, 3′-H, 4′-H, 5′-H), 1.84—1.92 (1H, m, 1-H), 2.07 (2H, dd, J=7.8, 16.0 Hz, 2′-H), 2.20 (1H, d, J=5.0 Hz, 4-H), 2.84 (1H, t, J=4.0 Hz, 3-H), 4.14—4.26 (1H, m, 5-H), 7.52—7.70 (3H, m, aromatic H), 7.85—7.95 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*R*,6*S*)-5′-(6-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]-hept-2-yl)pentanoic Acid (3b) 92% yield, colorless oil. *Anal.* Calcd for $C_{18}H_{25}NO_5S$: C, 58.83; H, 6.86; N, 3.81. Found: C, 59.01; H, 6.87; N, 3.54. IR ($CHCl_3$): 3600—2270 (COOH), 3260 (NH), 1710 (COOH), 1324 (SO_2), 1159 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.76—1.57 (10H, m, 2-H, 5-H, 7-H, 3′-H, 4′-H, 5′-H), 1.79 (1H, s, 1-H), 1.96—2.20 (4H, m, 4-H, 5-H, 2′-H), 2.81—2.90 (1H, m, 3-H), 3.64 (1H, d, J=6.0 Hz, 6-H), 7.51—7.70 (3H, m, aromatic H), 7.82—7.92 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*S*)-7′-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)heptanoic Acid (5) 94% yield, mp 89—90 °C. *Anal.* Calcd for $C_{20}H_{29}NO_4S$: C, 63.30; H, 7.70; N, 3.69; S, 8.45. Found: C, 63.09; H, 7.62; N, 3.63; S, 8.22. $[\alpha]_D^{24}$ +16.3±0.6° (c=1.013, $CHCl_3$). IR (KBr): 3720—2280 (COOH), 3280 (NH), 1705 (CO), 1323 (SO_2), 1159 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.73—1.73 (17H, m, 2-H, 5-H, 6-H, 7-H, 3′-H, 4′-H, 5′-H, 6′-H, 7′-H), 1.84 (1H, br s, 1-H), 2.07 (1H, br s, 4-H), 2.35 (2H, t, J=7.2 Hz, 2′-H), 2.94—3.10 (1H, m, 3-H), 5.21 (1H, d, J=7.4 Hz, NH), 7.40—7.70 (3H, m, aromatic H), 7.80—8.06 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*S*)-5′-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)pentanoic Acid (7) 90% yield, mp 84—86 °C. *Anal.* Calcd for $C_{18}H_{25}NO_4S$: C, 61.51; H, 7.17; N, 3.99; S, 9.12. Found: C, 61.71; H, 7.53; N, 4.19; S, 8.84. $[\alpha]_D^{24}$ +13.2±0.5° (c=1.002, $CHCl_3$). IR (KBr): 3720—2300 (COOH), 3275 (NH), 1706 (COOH), 1325 (SO_2), 1162 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.80—1.66 (13H, m, 2-H, 5-H, 6-H, 7-H, 3′-H, 4′-H, 5′-H), 1.84 (1H, br s, 1-H), 2.06 (1H, br s, 4-H), 2.26 (2H, t, J=7.1 Hz, 2′-H), 2.95—3.08 (1H, m, 3-H), 5.25 (1H, d, J=7.0 Hz, NH), 7.45—7.64 (3H, m, aromatic H), 7.84—7.98 (2H, m, aromatic H).

(1*S*,2*R*,3*R*,4*S*,5*R*)-Methyl (5-hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propenylate (21a) and (1*R*,2*S*,3*S*,4*R*,6*S*)-Methyl (6-hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propenylate (21b) A solution of methyl diethylphosphonoacetate (706 mg, 1.1 mmol×3) in THF (10 ml) was treated with NaH (60% in mineral oil, 130 mg, 1.1 mmol×2.9) at −10 °C and the mixture was stirred for 35 min at −5 °C. Next, a solution of **19a** (598 mg, 1.1 mmol) in THF (3 ml) was added at the same temperature. The mixture was stirred for 1 h at room temperature, then poured into a mixture of 2 N HCl and ethyl acetate. The organic solution was washed with aqueous 5% $NaHCO_3$ and H_2O, dried, and concentrated *in vacuo*. Chromatography on silica gel with *n*-hexane–ethyl acetate (2:1) as the eluent gave **21a** (540 mg, 82%), mp 178—184 °C. *Anal.* Calcd for $C_{33}H_{39}NO_5SSi$: C, 67.20; H, 6.67; N, 2.38. Found: C, 67.10; H, 6.90; N, 2.31. IR ($CHCl_3$): 1720 (COOMe) cm^{-1}. ^{1}H-NMR δ: 1.05 (9H, s, *tert*-Bu), 1.24—1.39 (2H, m, 2-H, 7-H), 1.63—1.84 (3H, m, 6-H, 7-H), 2.04—2.17 (2H, m, 1-H, 4-H), 2.94 (1H, q, J=5.0 Hz, 3-H), 3.69 (3H, s, Me), 3.90 (1H, d, J=5.4 Hz, NH), 4.05—4.16 (1H, m, 5-H), 5.47 (1H, dd, J=2.0, 15.6 Hz, olefinic H), 6.56 (1H, dd, J=15.6, 8.4 Hz, olefinic H), 7.34—7.74 (15H, m, aromatic H).

21b: 86% yield, mp 64—66 °C. *Anal.* Calcd for $C_{33}H_{39}NO_5SSi$: C, 67.20; H, 6.67; N, 2.38. Found: C, 67.46; H, 6.82; N, 2.58. IR ($CHCl_3$): 1721 (COOMe) cm^{-1}. ^{1}H-NMR δ: 0.84—0.95 (1H, m, 2-H), 1.02 (9H, s, *tert*-Bu), 1.20—1.48 (3H, m, 5-H, 7-H), 1.75—1.90 (2H, m, 1-H, 5-H), 2.20—2.30 (1H, m, 4-H), 3.10—3.21 (1H, m, 3H), 3.64—3.73 (1H, m, 6-H), 3.69 (3H, s, Me), 4.48 (1H, d, J=7.0 Hz, NH), 5.32 (1H, dd, J=1.2, 15.6 Hz, olefinic H), 6.48 (1H, dd, J=15.6, 8.2 Hz, olefinic H), 7.30—7.68 (13H, m, aromatic H), 7.72—7.83 (2H, m, aromatic H).

(1*S*,2*S*,3*R*,4*S*,5*R*)-(5-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propionic Acid (4a) and (1*R*,2*S*,3*S*,4*R*,6*S*)-(6-Hydroxy-3-phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propionic Acid (4b) Catalytic reduction of **21a** using 10% Pd–C gave the saturated product, which was subjected to silyl group deprotection and hydrolysis, in the same manner as **1a**, to obtain **4a** in 52% yield, colorless pillars, mp 187—189 °C. *Anal.* Calcd for $C_{16}H_{21}NO_5S$ (hygroscopic): C, 56.62; H, 6.24; N, 4.13; S, 9.45. Found: C, 56.40; H, 6.33; N, 4.06; S, 9.17. $[\alpha]_D^{26}$ +36.3±0.8° (c=1.011, MeOH). IR (Nujol): 3560—2200 (COOH), 3290 (NH), 1732 (COOH) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.86—1.06 (1H, m, 2-H), 1.10—2.25 (10H, m, 1-H, 4-H, 6-H, 7-H, 2′-H, 3′-H), 2.86 (1H, t, J=5.0 Hz, 3-H), 4.10—4.24 (1H, m, 5-H), 7.50—7.73 (3H, m, aromatic H), 7.84—8.03 (2H, m, aromatic H).

4b: 62% yield, mp 182—185 °C. *Anal.* Calcd for $C_{16}H_{21}NO_5S$: C, 56.62; H, 6.24; N, 4.13; S, 9.45. Found: C, 56.31; H, 6.22; N, 4.24; S, 9.13. $[\alpha]_D^{23}$ +30.9±0.7° (c=1.008, MeOH). IR (Nujol): 3500—2360 (COOH), 3435 (OH), 3265 (NH), 1714 (COOH), 1328 (SO_2), 1160 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.77—1.01 (2H, m, 2-H, 7-H), 1.21—1.61 (4H, m, 5-H, 7-H, 3′-H), 1.80 (1H, s, 1-H), 1.92—2.25 (4H, m, 4-H, 5-H, 2′-H), 2.82—2.94 (1H, m, 3-H), 3.64 (1H, d, J=6.0 Hz, 6-H), 7.49—7.68 (3H, m, aromatic H), 7.80—7.94 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*S*)-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propionic Acid (8) Wittig reaction of **22** with methoxymethylenetriphenylphosphorane, in the same manner as **20a**, gave the enol ether (100%). A solution of the enol ether (6.55 g) in 90% formic acid (6.5 ml) was allowed to stand for 1.5 h at room temperature, and the mixture was poured into aqueous 10% $NaHCO_3$, and extracted with ethyl acetate. The extract was washed with H_2O, dried and evaporated to dryness. Chromatography on silica gel with toluene–ethyl acetate (9:1) as the eluent gave **23** (3.6 g, 58%), mp 99—101 °C. *Anal.* Calcd for $C_{16}H_{21}NO_3S$: C, 62.52; H, 6.89; N, 4.56; S, 10.43. Found: C, 62.32; H, 6.79; N, 4.60; S, 10.14. $[\alpha]_D^{24}$ +6.3±0.5° (c=1.010, $CHCl_3$). IR (KBr): 3290 (NH), 1725 (CHO), 1312 (SO_2), 1162 (SO_2) cm^{-1}. ^{1}H-NMR δ: 0.85—1.00 (1H, m, 2-H), 1.06—1.68 (6H, m, 5-H, 6-H, 7-H), 1.83—1.91 (1H, br s, 1-H), 1.98—2.08 (1H, br s, 4-H), 2.33 (2H, d, J=7.3 Hz, CH_2CO), 3.00—3.12 (1H, m, 3-H), 4.81 (1H, d, J=7.6 Hz, NH), 7.45—7.65 (3H, m, aromatic H), 7.83—7.97 (2H, m, aromatic H), 9.66 (1H, s, CHO). A solution of **23** (4.46 g, 14.5 mmol) in acetone (44 ml) was oxidized with Jones' reagent (14.4 mmol) at room temperature for 1.5 h. The mixture was poured into H_2O and extracted with ethyl acetate. The extract was washed with brine, dried, and evaporated to dryness. The residue was partitioned between toluene and 1 N NaOH, and the aqueous solution was acidified with 2 N HCl, and extracted with ethyl acetate. The extract was washed with H_2O, dried, and evaporated to dryness. Recrystallization of the crude product from a mixture of MeOH–ethyl acetate gave the title compound **8** (3.03 g, 65%), mp 174—177 °C. *Anal.* Calcd for $C_{16}H_{21}NO_4S$: C, 59.42; H, 6.54; N, 4.33; S, 9.91. Found: C, 59.20; H, 6.54; N, 4.32; S, 9.73. $[\alpha]_D^{25}$ +19.6±0.6° (c=1.008, MeOH). IR (KBr): 3680—2600 (COOH), 3240 (NH), 1728 (COOH), 1318 (SO_2),

1156 (SO_2) cm^{-1}. ^{1}H-NMR (CD_3OD) δ: 0.94—1.70 (9H, m, 2-H, 5-H, 6-H, 7-H, 3′-H), 1.82—1.92 (1H, br s, 1-H), 1.92—2.24 (3H, m, 4-H, 2′-H), 2.87—2.99 (1H, m, 3-H), 7.50—7.68 (3H, m, aromatic H), 7.83—7.96 (2H, m, aromatic H).

(1*R*,2*S*,3*S*,4*S*)-(5′*Z*)-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)-hept-5′-enamidoethylsulfonic Acid (9) General Procedure for Preparation of the Taurine Derivatives (**10**, **11** and **12**): DCC (274 mg, 1.3 mmol) and HOSu (153 mg, 1.3 mmol) were added to a solution of (+)-S-145 (500 mg, 1.3 mmol) in DMF (7.5 ml) at 0 °C, and the mixture was stirred for 1.5 h at room temperature. Then taurine (168 mg, 1.3 mmol) and triethylamine (370 μl, 2.7 mmol) were added, and the mixture was stirred overnight at the same temperature. The resulting crystals were removed by filtration and the filtrate was partitioned between ethyl acetate and 2 N HCl. The aqueous solution was washed with ethyl acetate and concentrated *in vacuo* (<40 °C). Purification with HP-20 resin using H_2O, and then H_2O–MeOH (1 : 1) as the eluent gave **9** (359 mg, 56%), colorless powder. *Anal.* Calcd for $C_{22}H_{32}N_2O_6S_2 \cdot 0.5H_2O$ (hygroscopic): C, 53.53; H, 6.74; N, 5.67; S, 12.99. Found: C, 53.86; H, 6.74; N, 5.97; S, 12.64. $[\alpha]_D^{23}$ +37.3±0.8° (c=1.002, H_2O). IR (KBr): 1650 (CO), 1320 (SO_2), 1155 (SO_2) cm^{-1}; ^{1}H-NMR (D_2O–DSS) δ: 1.00—1.96 (13H, m, 2-H, 5-H, 6-H, 7-H, 3′-H, 4′-H, 7′-H), 2.06—2.23 (3H, m, 1-H, 2′-H), 2.84—2.96 (1H, br s, 4-H), 3.05 (2H, t, J=6.8 Hz, CH_2S), 3.55 (2H, t, J=6.5 Hz, CH_2N), 4.97—5.27 (2H, br s, olefinic H), 7.55—7.78 (3H, m, aromatic H), 7.80—7.93 (2H, m, aromatic H). MS m/z: 483 $(M-H)^-$ (NILSIMS), 485 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*S*)-(3′*Z*)-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)-pent-3′-enamidoethylsulfonic Acid (10) 51% yield (a mixture of double bond isomers), purified by preparative HPLC, colorless powder. ^{1}H-NMR (D_2O–DSS) δ: 1.02—1.97 (9H, m, 2-H, 5-H, 6-H, 7-H, 5′-H), 1.84—1.92 (1H, br s, 1-H), 2.10—2.21 (1H, br s, 4-H), 2.65—2.88 (2H, m, 2′-H), 2.88—2.97 (1H, m, 3-H), 3.04 (2H, t, J=6.7 Hz, CH_2S), 3.54 (2H, t, J=6.8 Hz, CH_2N), 5.28 (1H, td, J=7.2, 10.8 Hz, olefinic H), 5.41 (1H, td, J=7.2, 10.8 Hz, olefinic H), 7.55—7.76 (3H, m, aromatic H), 7.83—7.95 (2H, m, aromatic H). MS m/z: 455 $(M-H)^-$ (NILSIMS), 457 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*S*)-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)pentamidoethylsulfonic Acid (11) 64% yield, colorless powder. *Anal.* Calcd for $C_{20}H_{30}N_2O_6S_2 \cdot 0.1H_2O$ (hygroscopic): C,52.18; H, 6.58; N, 6.08; S, 13.93. Found: C, 51.92; H, 6.67; N, 6.47; S, 13.59. $[\alpha]_D^{23}$ +17.9±0.6° (c=1.003, H_2O). IR (KBr): 1670 (CO), 1315 (SO_2), 1155 (SO_2) cm^{-1}. ^{1}H-NMR (D_2O–DSS) δ: 0.70—1.63 (13H, m, 2-H, 5-H, 6-H, 7-H, 3′-H, 4′-H, 5′-H), 1.82—1.91 (1H, br s, 1-H), 1.97—2.14 (1H, br s, 4-H), 2.07 (2H, t, J=7.2 Hz, 2′-H), 2.89—3.00 (1H, m, 3-H), 3.07 (2H, t, J=6.9 Hz, CH_2S), 3.56 (2H, t, J=6.8 Hz, CH_2N), 7.57—7.78 (3H, m, aromatic H), 7.85—7.94 (2H, m, aromatic H). MS m/z: 457 $(M-H)^-$ (NILSIMS), 459 $(M+H)^+$ (PILSIMS).

(1*R*,2*S*,3*S*,4*S*)-(3-Phenylsulfonylaminobicyclo[2.2.1]hept-2-yl)propionamidoethylsulfonic Acid (12) 52% yield, colorless powder. *Anal.* Calcd for $C_{18}H_{26}N_2O_6S_2 \cdot 0.2H_2O$ (hygroscopic): C, 49.80; H, 6.13; N, 6.45; S, 14.77. Found: C, 49.68; H, 6.15; N, 6.70; S, 14.49. $[\alpha]_D^{23}$ +12.3±0.5° (c=1.005, H_2O). IR (Nujol): 1675 (CO), 1322 (SO_2), 1155 (SO_2) cm^{-1}. ^{1}H-NMR (D_2O–DSS) δ: 0.89—1.63 (9H, m, 2-H, 5-H, 6-H, 7-H, 3′-H), 1.70—1.95 (2H, m, 1-H, 2′-H), 1.95—2.17 (2H, m, 4-H, 2′-H), 2.94—3.04 (1H, m, 3-H), 3.05 (2H, t, J=6.8 Hz, CH_2S), 3.52 (2H, t, J=6.8 Hz, CH_2N), 7.57—7.77 (3H, m, aromatic H), 7.87—7.95 (2H, m, aromatic H). MS m/z: 429 $(M-H)^-$ (NILSIMS), 431 $(M+H)^+$ (PILSIMS).

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Tannins of Tamaricaceous Plants. II.[1] New Monomeric and Dimeric Hydrolyzable Tannins from *Reaumuria hirtella* and *Tamarix pakistanica*

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Two new monomeric hydrolyzable tannins [remurins A (12) and B (13)] and a new dimer [hirtellin A (7)], have been isolated from *Reaumuria hirtella* JAUB. *et* SP. (Tamaricaceae). Hirtellin A and an additional new dimeric hydrolyzable tannin, tamarixinin A (6), along with three known tannins including hirtellin B (5), have also been isolated from *Tamarix pakistanica* QAISER. The structures of the new tannins have been elucidated based on chemical method and two-dimensional nuclear magnetic resonance analyses including ^1H–^{13}C long-range shift correlation spectroscopy. Tamarixinin A (6) exhibited a host-mediated antitumor activity comparable to that of hirtellin B.

Keywords *Reaumuria hirtella*; *Tamarix pakistanica*; Tamaricaceae; tannin; dimeric ellagitannin; remurin A; remurin B; hirtellin A; tamarixinin A; antitumor activity

The oligomeric hydrolyzable tannins are biogenetically the products of intermolecular oxidative C–O or C–C coupling among two or more monomeric hydrolyzable tannins, and diversity of their structures and wide distribution in various plant families have been revealed by extensive studies during the last decade.[3] The plant families from which such oligomers have been hitherto isolated include Rosaceae, Lythraceae, Cornaceae, Coriariaceae, Euphorbiaceae, Melastomataceae, Onagraceae, Nyssaceae, Betulaceae, Fagaceae and Theaceae.[3] Several oligomeric hydrolyzable tannins have remarkable biological activities, such as host-mediated anti-tumor activity,[4] inhibition of reverse transcriptase of retro-virus[5] and promotion of iodination in leucocytes.[6] We previously found the occurrence of some such oligomers in *Reaumuria hirtella* JAUB. *et* SP. (Tamaricaceae), and isolated a new antitumor-active dimeric hydrolyzable tannin, hirtellin B (5),[1] which has a unique linking unit (hellinoyl group) between the monomers. In a continuing study on the tannins in the family Tamaricaceae, we have isolated additional new tannins from the same plant, and named them remurins A (**12**) and B (**13**), and hirtellin A (**7**). We have also found that *Tamarix pakistanica* QAISER, an erect shrub endemic to Pakistan,[7] whose twigs and flowers are used as a remedy for diarrhoea, produces hydrolyzable tannins similar to those of *R. hirtella*. The tannins isolated from the flower of this plant include hirtellins A (**7**) and B (**5**), and a new dimer, tamarixinin A (**6**), which exhibited host-mediated antitumor activity comparable to that of hirtellin B (**5**). This paper deals with the isolation of the new tannins and several known compounds from the above two plant species, and their structural elucidation.

Repeated column chromatography of the ethyl acetate extract, which was obtained from the homogenate of the dried leaves of *R. hirtella* in 70% aqueous acetone, yielded remurins A (**12**) and B (**13**), and hirtellin A (**7**). Four known tannins, gemin D (**1**),[8] 1,3-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-*β*-D-glucose (**2**),[9] tellimagrandin I (**3**),[10] and hirtellin B (**5**),[1] were also isolated.

The ethyl acetate-soluble portion of the 70% aqueous acetone homogenate of the dried flowers of *T. pakistanica* was similarly subjected to chromatographic separation over Toyopearl HW-40 and MCI-gel CHP-20P, to yield **1**, tellimagrandin II (**4**), **5**, and two new tannins, tamarixinin A (**6**) and hirtellin A (**7**).

The new compounds isolated are off-white amorphous powders, and the colorations with the $FeCl_3$ and with $NaNO_2$–AcOH reagents showed that they are ellagitannins.[11]

Tamarixinin A (**6**) was established to be a degalloyl congener of hirtellin B (**5**) as follows. Its fast-atom bombardment mass spectrum (FAB-MS) showed the $(M+Na)^+$ ion peak at m/z 1743, which is 152 mass unit lower than that of **5**. It was shown to form an equilibrium mixture of two anomers, by the duplication of signals in the proton nuclear magnetic resonance (^1H-NMR) spectrum. The presence of two galloyl groups was indicated by two pairs of galloyl proton signals [δ 6.94, 6.93 (each s, 2H in total) and 6.89, 6.88 (each s, 2H in total)]. The six pairs of 1H singlet (see Experimental), and *meta*-coupled signals [δ 6.02 (1H, d, $J=2$ Hz) and 7.04, 7.06 (each d, $J=2$ Hz, 1H in total)] in the aromatic region, are consistent with the presence of two hexahydroxydiphenoyl (HHDP) groups and a hellinoyl (dehydrotrigalloyl) group in the same way as in hirtellin B (**5**). Methylation of **6** with diazomethane followed by methanolysis gave methyl tri-*O*-methylgallate (**8a**), dimethyl hexamethoxydiphenate (**9**) and trimethyl hepta-*O*-methylhellinate (**10**),[1] which substantiated the presence of the aroyl units assigned above in **6**. The ^1H–^1H shift correlation (COSY) spectrum (assignments of the signals are summarized in Table I) shows that the sugars in **6** are two glucopyranoses with the $^4C_1$ conformation.

The signals of the fully acylated glucose core (glucose-II) were analogous to those of hirtellin B (**5**). The substitution pattern of the acyl groups, including the hellinoyl group on the glucose-II, should be similar to that of **5**.

Conclusive evidence for the structure (**6**) of tamarixinin A, including the atropisomerism of the HHDP groups, was provided by selective degalloylation of **5** with tannase,[12] yielding **6**.

Hirtellin A (**7**), $[\alpha]_D$ +82° (MeOH), showed an $[M+Na]^+$ ion peak at m/z 1897 in the FAB-MS, which is in accord with its dimeric nature. Acid hydrolysis of **7** yielded gallic acid (**8**), ellagic acid, dehydrodigallic acid (**11**) and glucose. The presence of these constructing units in **7** was further confirmed by methanolysis of the nonacosamethyl derivative (**7a**) [FAB-MS m/z 2303 $(M+Na)^+$], which gave methyl tri-*O*-methylgallate (**8a**), dimethyl hexamethoxydiphenate (**9**) and dimethyl penta-*O*-methyldehydrodigallate (**11a**).[13] The presence of four galloyl, two HHDP

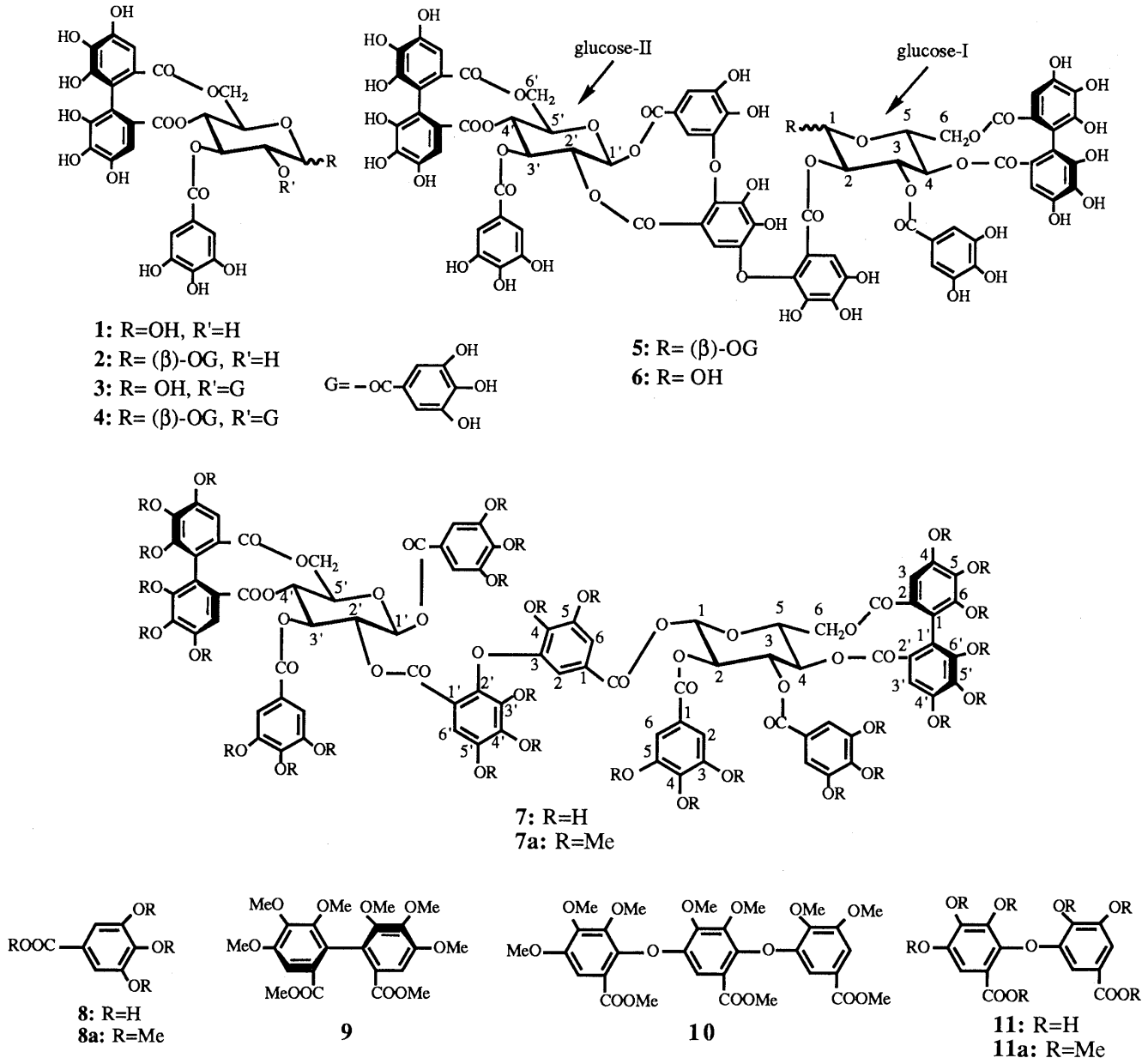


Chart 1

and a dehydrodigalloyl (DHDG) groups in **7** was indicated by the ^{1}H-NMR spectrum, which exhibited four 2H-singlets, five 1H-singlets and two *meta*-coupled doublets in the aromatic region (see Experimental). Two sets of glucose proton signals (Table I) were unambiguously distinguished from each other by the ^{1}H–^{1}H COSY. All-axial orientation of H-1(1′)—H-5 (5′) in the two $^4C_1$ glucopyranose cores is evident from the coupling constants of the signals. The chemical shifts of the C-6 (6′) methylene protons of each glucose core [δ 5.33 (5.29), 3.98 (3.82)] are characteristic of an ellagitannin having an HHDP group at 0-4/0-6 of the $^4C_1$ glucopyranose core.[14] Since the two glucose cores in **7** are fully acylated as evidenced by the chemical shifts of their protons, four galloyl groups and a DHDG group should be placed at 0-1—0-3 and 0-1′—0-3′. The glucose carbon resonances in the ^{13}C-NMR spectrum of **7** are analogous to those of tellimagrandin II (**4**)[15] (Table II). Therefore, hertellin A is regarded as a dimer, which has two tellimagrandin II moieties linked through a dehydrodigalloyl group.

The binding sites of the dehydrodigalloyl group including the other acyl groups on the glucose cores were determined from the ^{1}H–^{13}C long-range COSY spectrum, which gives cross peaks for nuclei separated by two or three bonds. The measurement of the COSY with an average J value of 8 Hz for two- or three-bond coupling revealed all connectivities among sugar proton–ester carbonyl carbon–aromatic proton(s) through three-bond coupling (Fig. 1), except for that of the glucose H-6 (H-6′) and HHDP proton. The *meta*-coupled doublets at δ 7.27 and 6.45 were correlated with H-1 (δ 6.07) of glucose-I through the common ester carbonyl carbon resonance at δ 164.4. On the other hand, a cross peak between the H-2′ signal (δ 5.55) of glucose-II and the carboxyl carbon signal at δ 164.1, which is correlated with the H-6′ signal (δ 7.05) of the DHDG group, was also observed. These facts unequivocally indicated that the DHDG moiety connects with 0-1 and 0-2′ in the glucopyranoses.

TABLE I. ^{1}H-NMR Spectral Data for the Glucose Moieties of **5**, **6**, **7** and **11**

	5	6[a] α-Anomer	6[a] β-Anomer	7	11
H-1	6.16 d (J=8)	5.51 d (J=4)	5.16 d (J=8)	6.07 d (J=8)	
H-2	5.66 dd (J=8, 10)	5.25 dd (J=4, 10)	5.24 dd (J=8, 10)	5.58 dd (J=8, 10)	
H-3	5.74 t (J=10)	5.57 t (J=10)	5.81 t (J=10)	5.77 t (J=10)	
H-4	5.14 t (J=10)	5.06 t (J=10)	5.04 t (J=10)	5.22 t (J=10)	
H-5	4.51 dd (J=6, 10)	4.27 dd (J=6, 10)	4.67 dd (J=6, 10)	4.53 dd (J=6, 10)	
H-6	5.35 dd (J=6, 13)	5.26 dd (J=6, 13)		5.33 dd (J=6, 13)	
	3.86 d (J=13)	3.75 d (J=13)		3.98 d (J=13)	
H-1′	5.60 d (J=8.5)	5.77 d (J=8)	5.78 d (J=8)	5.35 d (J=8)	
H-2′	5.36 dd (J=8.5, 10)	5.39 dd (J=8, 10)	5.42 dd (J=8, 10)	5.55 dd (J=8, 10)	5.49—5.54 Overlapped
H-3′	5.69 t (J=10)	5.74 t (J=10)		5.64 t (J=10)	
H-4′	5.19 t (J=10)	5.08 t (J=10)	5.09 t (J=10)	5.13 t (J=10)	5.14 t (J=10)
H-5′	4.35 ddd (J=1.5, 7, 10)	4.42 br dd (J=6, 10)		4.22 dd (J=6.5, 10)	4.22 dd (J=6.5, 10)
H-6′	5.31 dd (J=7, 13)	5.29 dd (J=6, 13)		5.29 dd (J=6.5, 13)	5.31 dd (J=6.5, 13.5)
	4.13 dd (J=1.5, 13)	3.85 br d (J=13)		3.82 d (J=13)	3.81 d (J=13)

500 MHz, acetone-d_6 + D_2O, J in Hz. *a*) Ratio of α- and β-anomers = 3 : 2.

TABLE II. ^{13}C-NMR Data for the Glucose Moieties of **7** and **4**

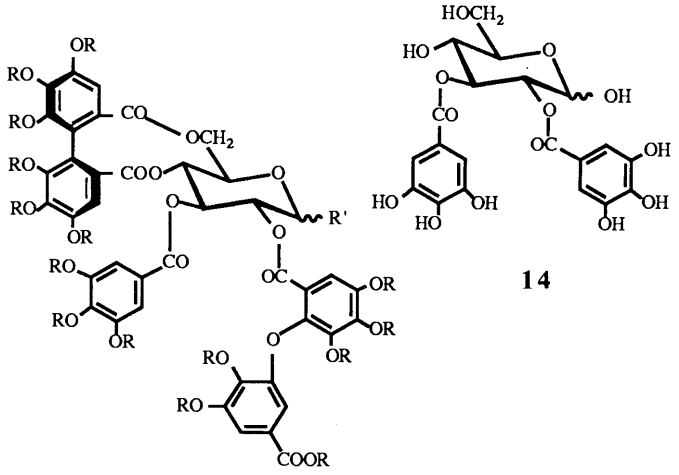
	C-1 (C-1′)	C-2 (C-2′)	C-3 (C-3′)	C-4 (C-4′)	C-5 (C-5′)	C-6 (C-6′)
7	93.5	71.5	73.4	70.3	72.9	62.9
	(93.5)	(71.8)	(73.2)	(70.6)	(72.9)	(63.1)
4	93.8	71.8	73.3	70.8	73.1	63.1

126 MHz, acetone-d_6 + D_2O.

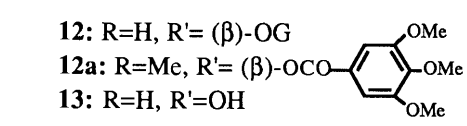
The circular dichroism (CD) spectrum of **7** exhibited a strong postitive Cotton effect at 233 nm, $[\theta]$ $+21 \times 10^4$, with a pattern similar to that of **4**[16] but having an amplitude twice that of the latter, indicating the (*S*)-configuration of both HHDP groups in **7**. Based on these data, hirtellin A was assigned the structure **7**.

The anomeric proton (H-1′) of the glucose-II in **7** resonates at a higher field (δ 5.35) than that of **4** (δ 6.20).[17] This unusual upfield shift is analogous to that of H-1′ of **5** and **6** (see Table I), and can be explained by the anisotropic effect of the aromatic ring of the DHDG and hellinoyl groups attached to 0-2′ in these dimers. It is known that hydrolyzable tannins which have the galloyl part of a valoneoyl group at 0-2 of the $^4C_1$ glucopyranose show a similar upfield shift of the anomeric proton.[18,19] This anomaly may thus be a characteristic feature of hydrolyzable tannins possessing the DHDG group at 0-2 of the $^4C_1$ glucopyranose, as well as those having the galloyl part of the valoneoyl group at 0-2.

Remurin A (**12**), $[\alpha]_D$ +60° (MeOH), showed the $[M+Na]^+$ ion peak at m/z 1129 in the FAB-MS. Its ^{1}H-NMR spectrum indicated the presence of an HHDP [δ 6.48, 6.61 (each 1H, s)], a DHDG [δ 7.00 (1H, s), 6.51, 7.25 (each 1H, d, J=2 Hz)] and two galloyl groups [δ 6.94, 6.99 (each 2H, s)]. These component units were confirmed by methanolysis of the octadecamethyl derivative (**12a**), which produced **8a**, **9** and **11a**. The sugar liberated upon acid hydrolysis of **12** was identified as glucose. The ^{1}H–^{1}H COSY indicated that the glucose proton signals are closely similar to those of glucose-II of **7**, including the characteristic upfield shift of the anomeric proton signal (Table I). The structure of remurin A was thus assumed to be as shown in the formula **12**, and was confirmed by demonstrating its identity with the main product from hydrolysis of hirtellin A (**7**) in hot water, which was accompanied with **2** and 2,3-di-*O*-galloyl-D-glucose (**14**).



14



12: R=H, R'= (β)-OG
12a: R=Me, R'= (β)-OCO-
13: R=H, R'=OH

Chart 2

Remurin B (**13**) exhibited an $(M+Na)^+$ peak at m/z 977 in the FAB-MS, which is 152 mass (galloyl) unit lower than that of **12**. Upon methylation with dimethyl sulfate and potassium carbonate, **13** afforded a mixture of α- and β-anomers of the hexadecamethyl derivative (**13a**), which gave **8a**, **9** and **11a** upon methanolysis. The anomeric proton signals of the sugar moiety in the ^{1}H-NMR spectrum of **13**, at δ 5.37 (d, J=4 Hz) and 4.48 (d, J=8 Hz), indicated its existence as an equilibrium mixture of α- and β-anomers. The large coupling constants (*ca.* 10 Hz) among the other sugar proton signals (H-2—H-5) of each anomer are characteristic of those of a glucopyranose residue adopting the $^4C_1$ conformation. This spectrum also showed the presence of a galloyl group [δ 6.92, 6.97 (each, s, 2H in total), an HHDP group [δ 6.46, 6.60 (each 1H, s)] and a DHDG group [δ 6.64, 6.65 (each d, J=2 Hz, 1H in total), 7.17, 7.22 (each d, J=2 Hz, 1H in total)]. Therefore, remurin B (**13**) is a degalloyl congener of remurin A (**12**), and this structure was substantiated by enzymatic hydrolysis of **12**, yielding **13**.

Tamarixinin A (**6**) and hirtellin A (**7**) exhibited a significant host-mediated antitumor activity against sarcoma 180 in mice.[4] These dimers (10 mg/kg) were administered intraperitoneally to mice on the 4 th day before inoculation of tumor cells (1×10^5). Although the average lifespan of the control group (6 mice) was 15 d, three the one mice in the group treated with **6** and **7**, respectively, sur-

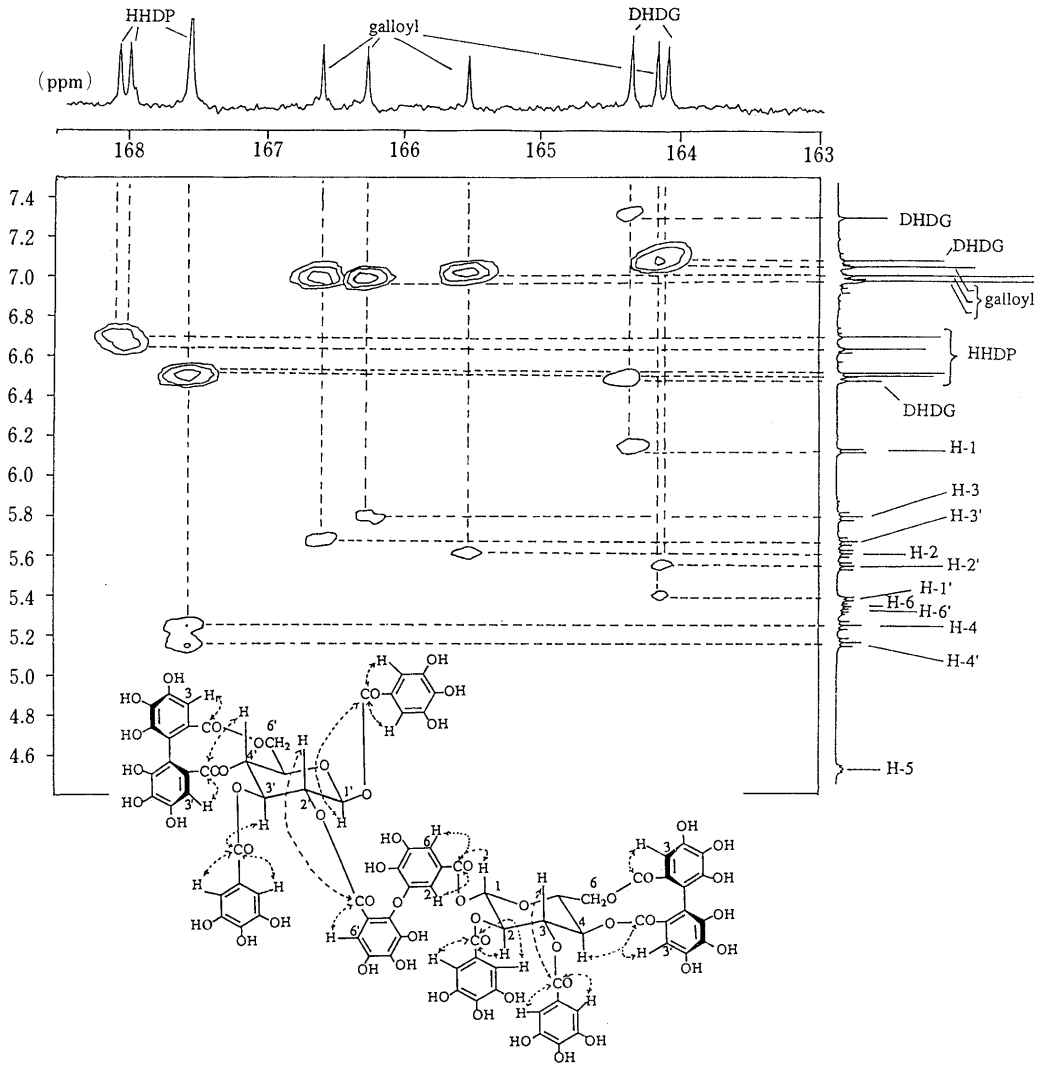


Fig. 1. ^{1}H–^{13}C Long-Range Shift Correlation Spectrum of **7** in Acetone-d_6–D_2O (J_{CH}=8 Hz)
^{13}C-NMR: δ_C 163—168.5.

vived over 60 d and the activity of **6** was comparable to those of hirtellin B (**5**),[1] oenothein B and coriariin A.[4,20]

Experimental

General The material (*R. hirtella*), instruments and chromatographic methods in this work are the same as those described in the preceding paper.[1] The flowers of *T. pakistanica* were collected at the campus of Sindh University, Pakistan, in August 1990, and identified by Prof. M. Qaiser, Department of Botany, University of Karachi. A voucher specimen is deposited at Sindh Uviversity.

Isolation of Tannins a) From *Reaumuria hirtella*: A part (28 g) of the EtOAc extract (65.7 g) previously obtained from 70% aqueous acetone homogenate of the dried leaves of *R. hirtella* (3.3 kg) was suspended in 70% MeOH (150 ml). Insoluble material was collected by centrifugation and washed thoroughly with 70% MeOH to give ellagic acid (0.9 g). The supernatant and washing were combined, and applied to a column of Toyopearl HW-40 (coarse) (7 cm i.d. × 45 cm) and developed with 70% MeOH→MeOH–H_2O–acetone (7:2:1 → 6:2:2 → 5:2:3) in a stepwise gradient mode. The 70% MeOH eluate gave gallic acid (336 mg) and gemin D (**1**) (169 mg). The MeOH–H_2O–acetone (7:2:1) eluate afforded tellimagrandin I (**3**) (654 mg), 1,3-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (**2**) (559 mg), and crude remurin A, which was purified by rechromatography over MCI-gel CHP-20P with H_2O containing increasing amounts of MeOH to yield remurin A (**12**) (202 mg). The MeOH–H_2O–acetone (6:2:2) eluate gave hirtellin A (**7**) (2.38 g). A part (35 g) of the *n*-BuOH extract (130 g)[1] was subjected to column chromatography over Toyopearl HW-40 (coarse, 7 cm i.d. × 50 cm) developing with the same solvent system (aqueous MeOH and MeOH–H_2O–acetone) as that used for the chromatography of the EtOAc extract. The MeOH–H_2O–acetone (7:2:1) eluate afforded remurin B (**13**) (133 mg).

b) From *Tamarix pakistanica*: The dried flowers (3 kg) of *T. pakistanica* were homogenized in 70% aqueous acetone (9 l × 3). The homogenate was filtered and the filtrate was concentrated *in vacuo* to *ca.* 2.5 l. After removal of insoluble viscous materials by decantation, the aqueous solution was extracted with Et_2O, EtOAc and *n*-BuOH (presaturated with H_2O), successively. A part (6 g) of the EtOAc extract (39.8 g) was subjected to column chromatography over Toyopearl HW-40 (coarse, 2.2 cm I.D. × 52 cm), developing with MeOH–H_2O (50% MeOH→60% MeOH→70% MeOH)→MeOH–H_2O–acetone (7:2:1→6:2:2) in a stepwise gradient mode. The 60% MeOH eluate gave **2** (52 mg) and tellimagrandin II (**4**) (30 mg). Rechromatography of the 70% MeOH eluate over MCI-gel CHP-20P with 20% MeOH afforded tamarixinin A (**6**) (58 mg). The eluate with MeOH–H_2O–acetone (7:2:1) gave hirtellins B (**5**) (114 mg) and A (**7**) (147 mg).

1,3-Di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (2) An off-white amorphous powder, $[\alpha]_D$ +24° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{34}H_{25}O_{22}\cdot 4H_2O$: C, 47.62; H, 3.80. Found: C, 47.63; H, 3.83. ^{1}H-NMR (acetone-d_6+D_2O): 7.19, 7.03 [each 2H, s, galloyl (Gal)], 6.44, 6.62 (each 1H, s, HHDP), 5.89 [1H, d, *J*=8 Hz, glucose (Gluc) H-1], 5.48 (1H, dd, *J*=9, 10 Hz, Gluc H-3), 5.08 (1H, t, *J*=10 Hz, Gluc H-4), 5.30 (1H, dd, *J*=6.5, 13 Hz, Gluc H-6), 4.34 (1H, dd, *J*=6.5, 10 Hz, Gluc H-5), 3.98 (1H, dd, *J*=8, 9 Hz, Gluc G-2), 3.81 (1H, d, *J*=13 Hz, Gluc H-6).

Tamarixinin A (6) An off-white amorphous powder, $[\alpha]_D$ +98° (*c*=1,1, MeOH). Ultraviolet (UV) λ_{max} (MeOH) nm (log ε): 218 (5.23), 272 (4.84). FAB-MS: *m/z* 1743 $(M+Na)^+$. CD (MeOH) [θ] (nm): $+41.0\times10^4$ (233), -8.3×10^4 (263), $+4.3\times10^4$ (290), -2.6×10^4 (324). ^{1}H-NMR (acetone-d_6+D_2O): δ 7.62, 7.59 [each s, 1H in total, hellinoyl (Hel)], 7.06,

7.04 (each d, $J=2$ Hz, 1H in total, Hel), 6.72, 6.71 (each s, 1H in total, Hel), 6.02 (1H, d, $J=2$ Hz, Hel), 6.94, 6.93 (each s, 2H in total, Gal), 6.89, 6.88 (each s, 2H in total, Gal), 6.62, 6.61, 6.60, 6.51, 6.50, 6.46, 6.47 (each s, 4H in total, HHDP), and glucose protons, see Table I.

Methylation of Tamarixinin A (6) Followed by Methanolysis A solution of **6** (6.8 mg) in MeOH (1 ml) was methylated with ethereal CH_2N_2 at room temperature for 5 h. The residue obtained after removal of the solvent was directly methanolyzed with 1% NaOMe (0.2 ml) in MeOH (1 ml) at room temperature for 10 h to give **8a**, **9** and **10**, which were identified by co-chromatography with authentic samples on thin-layer chromatography (TLC) (Kieselgel PF_{254}, ligroin–CH_2Cl_2–acetone 6 : 4 : 1).

Degalloylation of Hirtellin B (5) to Tamarixinin A (6) A solution of hirtellin B (**5**) (50 mg) in H_2O (5 ml) was incubated with tannase (10 drops) prepared according to the literature[12] at 37 °C for a week. The reaction mixture was acidified with 0.1 N HCl and subjected to column chromatography over MCI-gel CHP-20P with H_2O containing increasing amounts of MeOH. The 30% MeOH eluate yielded a degalloylated derivative (9 mg), identified as tamarixinin A (**6**), by co-chromatography on high performance liquid chromatography (HPLC) and ^{1}H-NMR spectral comparison with an authentic sample.

Hirtellin A (7) An off-white amorphous powder, $[\alpha]_D$ +82° ($c=1.0$, MeOH). *Anal.* Calcd for $C_{82}H_{58}O_{52}\cdot 13H_2O$: C, 46.69; H, 4.01. Found: C, 46.78; H, 3.67. UV λ_{max} (MeOH) nm (log ε): 219 (5.17), 277 (4.84). FAB-MS: m/z 1897 $(M+Na)^+$. CD (MeOH) [θ] (nm): $+2.1\times10^5$ (233), -4.5×10^4 (261), $+4.5\times10^4$ (285). ^{1}H-NMR (acetone-d_6+D_2O): δ 6.95 (s, 4H, Gal ×2), 6.98, 7.02 (each 2H, s, Gal), 6.47, 6.49, 6.61, 6.67 (each 1H, s, HHDP), 6.45, 7.27 [each 1H, d, $J=2$ Hz, DHDG], 7.05 (1H, s, DHDG), and glucose protons, see Table I. ^{13}C-NMR (acetone-d_6+D_2O): δ 107.1 (DHDG C-2), 107.7 (2C), 107.9, 108.1 (HHDP C-3, C-3′), 109.9 (2C), 110.0 (3C), 110.2 (2C), 110.4 (2C) (Gal C-2, C-6, DHDG C-6′), 112.9 (DHDG C-6), 113.2 (DHDG C-1), 115.5 (2C), 115.7 (2C) (HHDP C-1, C-1′), 119.5 (DHDG C-1′), 119.8, 119.9, 120.0, 120.2 (Gal C-1), 125.5, 125.7, 126.2, 126.3 (HHDP C-2, C-2′), 136.3 (2C), 136.5 (2C), 136.6 (HHDP C-5, C-5′, DHDG C-2′), 139.1, 139.3 (2C), 139.6 (Gal C-4), 139.8, 140.3, 140.6 (DHDG C-4, C-3′, C-4′), 143.4 (DHDG C-5′), 144.3 (3C), 144.4 (HHDP C-6, C-6′), 145.1 (2C), 145.2 (2C) (HHDP C-4, C-4′), 145.7 (4C), 145.8 (4C) (Gal C-3), 146.0 (DHDG C-5), 147.8 (DHDG C-3), 164.1, 164.2, 164.4, 165.0, 166.3, 166.6, 167.6 (2C), 168.0, 168.1 (ester carbonyl), and glucose carbons, see Table II.

Acid Hydrolysis of Hirtellin A (7) and Remurin A (12) A solution of **7** (2 mg) in 1 N H_2SO_4 (1 ml) in a sealed ampule was heated on a boiling-water bath for 5 h. After cooling, the solution was extracted with EtOAc. HPLC analysis of the EtOAc extract showed the presence of gallic acid (**8**), dehydrodigallic acid (**11**) and ellagic acid. The aqueous layer was neutralized with Amberlite IR-410 (OH form) and evaporated. The gas liquid chromatography (GLC) analysis after trimethylsilylation of the syrup showed the presence of glucose. GLC conditions: G-250 capillary column, column temperature, 170 °C, flow rate of He, 30 ml/min.

Similar acid hydrolysis of **12** gave the same products as those from **7**.

Methylation of Hirtellin A (7) A mixture of **7** (50 mg), anhydrous K_2CO_3 (350 mg) and dimethyl sulfate (0.5 ml) in dry acetone (20 ml), was stirred overnight at room temperature, and refluxed for 6 h. After removal of the inorganic material by filtration, the filtrate was concentrated and submitted to preparative TLC (silica gel, benzene–acetone (5 : 1)) to give a nonacosamethyl derivaitve (**7a**) (35 mg) as a white amorphous powder. FAB-MS: m/z: 2280 (M^+), 2303 $(M+Na)^+$. ^{1}H-NMR (acetone-d_6): δ 6.24 (1H, d, $J=8$ Hz, Gluc H-1), 5.62 (1H, dd, $J=8$, 10 Hz, Gluc H-2), 5.87 (1H, t, $J=10$ Hz, Gluc H-3), 5.28 (1H, t, $J=10$ Hz, Gluc H-4), 4.61 (1H, ddd, $J=1.5$, 6.5, 10 Hz, Gluc H-5), 5.33 (1H, dd, $J=6.5$, 13.5 Hz, Gluc H-6), the other H-6 is overlapped by OMe signals, 6.17 (1H, d, $J=8$ Hz, Gluc H-1′), 5.64 (1H, t, $J=8$ Hz, Gluc H-2′), 5.76 (1H, dd, $J=8$, 10 Hz, Gluc H-3′), 5.37 (1H, t, $J=10$ Hz, Gluc H-4′), 4.39 (1H, dd, $J=6$, 10 Hz, Gluc H-5′), 5.19 (1H, br dd, $J=6$, 13.5 Hz, Gluc H-6′), the other H-6′ is overlapped by OMe signals, 3.91, 3.90, 3.87, 3.86, 3.80, 3.75, 3.72, 3.69, 3.68, 3.65, 3.64, 3.63, 3.62 (each 3H, s, OMe ×13), 3.85 (21H, s, OMe ×7), 3.84, 3.79, 3.76 (each 6H, s, OMe ×6), 3.73 (9H, s, OMe ×3), 7.19, 7.23 (each 2H, s, Gal), 7.21, 7.20 (each 3H, s, Gal and DHDG), 6.95, 6.89, 6.82 (each 1H, s, HHDP), 6.77 (2H, s, HHDP and DHDG).

Methanolysis of the Nonacosamethyl Derivative (7a) A solution of **7a** (10 mg) in 1% NaOMe (0.1 ml) was left standing overnight at room temperature. After acidification with AcOH, the solvent was evaporated under an N_2 stream, and the residue was treated with an excess of ethereal CH_2N_2 for 6 h. The residue obtained after removal of the solvent was subjected to preparative TLC (Kieselgel PF_{254}, benzene–acetone (15 : 1)) to yield methyl tri-*O*-methylgallate (**8a**) (1.8 mg), dimethyl penta-*O*-methyldehydrodigallate (**11a**) (1.2 mg) and dimethyl hexamethoxydiphenate (**9**) (1.4 mg).

Remurin A (12) An off-white amorphous powder, $[\alpha]_D$ +60° ($c=1.0$, MeOH). *Anal.* Calcd for $C_{48}H_{34}O_{31}\cdot 5H_2O$: C, 48.17; H, 3.71. Found: C, 48.83; H, 4.01. FAB-MS: m/z 1129 $(M+Na)^+$. UV λ_{max} (MeOH) nm (log ε): 225 (4.87), 275 (4.59). CD (MeOH) [θ] (nm): $+1.2\times10^5$ (234), -2.1×10^4 (261), $+2.2\times10^4$ (282). ^{1}H-NMR (acetone-d_6+D_2O): δ 7.25, 6.51 (each 1H, d, $J=2$ Hz, DHDG), 7.00 (1H, s, DHDG), 6.61, 6.48 (each 1H, s, HHDP), 6.94, 6.99 (each 2H, s, Gal), and glucose protons, see Table I.

Remurin B (13) An off-white amorphous powder, $[\alpha]_D$ +53° ($c=1.0$, MeOH), FAB-MS: m/z 977 $(M+N)^+$. UV λ_{max} (MeOH) nm (log ε): 212 (4.91), 218 (sh, 4.88), 273 (4.46). CD (MeOH) [θ] (nm): $+9\times10^4$ (234), -2.9×10^4 (261), $+2.7\times10^4$ (281). ^{1}H-NMR (acetone-d_6+D_2O): α-anomer; δ 5.37 (d, $J=4$ Hz, Gluc H-1), 5.00 (dd, $J=4$, 10 Hz, Gluc H-2), 5.79 (t, $J=10$ Hz, Gluc H-3), 5.05 (t, $J=10$ Hz, Gluc H-4), 4.59 (ddd, $J=1.5$, 6.5, 10 Hz, Gluc H-5), 5.21 (dd, $J=6.5$, 13 Hz, Gluc H-6), 3.72 (dd, $J=1.5$, 13 Hz, Gluc H-6), β-anomer; 4.48 (d, $J=8$ Hz, Gluc H-1), 5.15 (dd, $J=8$, 10 Hz, Gluc H-2), 5.37 (t, $J=10$ Hz, Gluc H-3), 5.02 (t, $J=10$ Hz, Gluc H-4), 4.01 (dd, $J=6.5$, 10 Hz, Gluc H-5), 5.22 (dd, $J=6.5$, 13 Hz, Gluc H-6), 3.78 (d, $J=13$ Hz, Gluc H-6), and aromatic protons, see text.

Methylation of Remurin A (12) Followed by Methanolysis A mixture of remurin A (**12**) (10 mg), anhydrous K_2CO_3 (70 mg) and dimethyl sulfate (0.1 ml) in dry acetone (5 ml) was stirred overnight at room temperature, and then refluxed for 4 h. The reaction mixture was worked up in a similar way to that described for the methylation of **7**, to give octadeca-*O*-methylremurin A (**12a**) (5.5 mg) as a white amorphous solid, FAB-MS: m/z 1381 $(M+Na)^+$ and 1397 $(M+K)^+$. *Anal.* Calcd for $C_{66}H_{70}O_{31}\cdot 3H_2O$: C, 56.09; H, 5.42. Found: C, 56.34; H, 5.71. ^{1}H-NMR (acetone-d_6+D_2O) δ: 6.12 (1H, d, $J=7$ Hz, Gluc H-1), 5.75 (2H, Gluc H-2, H-3), 5.31 (1H, t, $J=10$ Hz, Gluc H-4), 4.43 (1H, dd, $J=6$, 10 Hz, Gluc H-5), 5.24 (1H, dd, $J=6$, 13.5 Hz, Gluc H-6), the other H-6 signal was overlapped by OMe signals, 7.21, 7.18 (each 2H, s, Gal), 7.26 (1H, s), 7.11, 6.57 (each 1H, d, $J=2$ Hz) (DHDG), 6.89, 6.80 (each 1H, s, HHDP), 3.60, 3.62, 3.64, 3.65, 3.72, 3.80, 3.88, 3.90 (each 3H, s, OMe), 3.75, 3.77 (each 6H, s, OMe), 3.85, 3.86 (each 9H, s, OMe).

Methanolysis of **12a** (1 mg) with 1% NaOMe (50 μl) gave **8a**, **9** and **11a**, which were identified by co-chromatography on TLC with authentic specimens.

Partial Hydrolysis of Hirtellin A (7) A solution of **7** (100 mg) in H_2O (30 ml) was heated at 95 °C for 10 h. After cooling, the precipitate obtained was removed by centrifugation, and the conentrated supernatant was subjected to column chromatography over MCI-gel CHP-20P (1.1 cm i.d. ×23 cm) developing with 10% MeOH→20% MeOH→30% MeOH→40% MeOH in a stepwise gradient mode. The 10% MeOH and 20% MeOH eluates gave gallic acid (2.6 mg) and 2,3-di-*O*-galloyl-D-glucose (**14**) (2.7 mg), respectively. The 30% MeOH eluate afforded remurin A (**12**) (5.5 mg) and 1,3-di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose (**2**) (3.9 mg). Their identities were confirmed by co-chromatography with authentic samples on HPLC and by ^{1}H-NMR spectral comparison. Unreacted **7** (5.7 mg) was also recovered from the 40% MeOH eluate.

Methylation of Remurin B (13) Remurin B (**13**) (5 mg) was methylated with anhydrous K_2CO_3 (35 mg) and dimethyl sulfate (0.1 ml) in dry acetone (3 ml), in a way similar to that described for methylation of **7** to yield hexadecamethylremurin B (**13a**) (mixture of α- and β-anomers) (2.8 mg) as a white amorphous solid. ^{1}H-NMR (acetone-d_6): δ 7.22, 7.14 (2H in total, Gal), 7.34, 7.25 (each s, 1H in total), 7.29, 7.24, 6.70, 6.65 (each d, $J=2$ Hz, 2H in total) (DHDG), 6.91 (1H, s), 6.77, 6.74 (each s, 1H in total) (HHDP), 5.09 (2/3H, d, $J=4$ Hz, Gluc H-1 of α-anomer), 4.54 (1/3H, d, $J=7.5$ Hz, Gluc H-1 of β-anomer). Methanolysis of **13a** (2.8 mg) with 1% NaOMe (1.0 ml) in MeOH (0.2 ml) gave **8a**, **9** and **11a**, which were identified by co-chromatography with authentic samples on TLC (Kieselgel PF_{254}, benzene–acetone (16 : 1)).

Partial Hydrolysis of Remurin A (12) with Tannase A mixture of remurin A (**12**) (25 mg) and tannase (10 drops) in H_2O (4 ml) was kept standing at 37 °C for 6 h. After removal of the solvent, the residue was dissolved in 10% MeOH and chromatographed over MCI-gel CHP-20P with 10% MeOH→20% MeOH to yield gallic acid (1 mg), and a monodegalloylated derivative (15 mg) which was shown to be identical with remurin B (**13**) by co-chromatography on HPLC and ^{1}H-NMR spectral comparison.

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Purines. XLIX.[1] Synthesis and Proton Nuclear Magnetic Resonance Study of 3,7-Dialkylxanthines and 1,3,7-Trialkylxanthines

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A general synthetic route to 3,7-dialkylxanthines (type 9) from 3,7-dialkyladenines (6) [hence from 3- or 7-alkyladenines (11 or 10)] has been established. The route started with ethoxycarbonylation of 1-alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (7), readily obtainable from 6 by alkaline hydrolysis, and proceeded through cyclization of the resulting carbamates (8) under alkaline conditions. Alkylation of 9 with alkyl halide in *N*,*N*-dimethylformamide in the presence of anhydrous K_2CO_3 extended the above synthetic route to the 1,3,7-trialkylxanthine level (type 14). Hydrogenolytic debenzylation of 3-benzyl-1,7-dimethylxanthine (16), prepared by following this general synthetic route, furnished paraxanthine (26) in fair yield. Conversion of 26 into 3-(4-hydroxy-3-nitrobenzyl)-1,7-dimethylxanthine (24), isomeric with the bryozoan purine phidolopin (2), was effected through aralkylation with 4-(methoxymethoxy)-3-nitrobenzyl bromide (28) followed by *O*-deprotection.

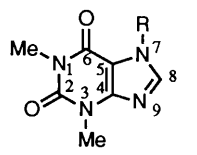
On the basis of proton nuclear magnetic resonance data for the 3,7-dialkylxanthines (3 and 9b—i) and 1,3,7-trialkylxanthines (5 and 14—22) thus prepared, reliable criteria for distinguishing signals of *N*-alkyl substituents at various positions are put forward.

Keywords dialkylxanthine; trialkylxanthine; dialkyladenine; ring fission–reclosure synthesis; ethoxycarbonylation; amide carbamate cyclization; xanthine *N*-alkylation; amide *N*-debenzylation; phidolopin regioisomer; *N*-alkylxanthine ^{1}H-NMR

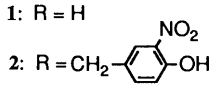
N-Alkylxanthines constitute a small but important group of pharmacologically and biologically active compounds, of which theophylline (**1**), theobromine (**3**), caffeine (**5**), pentoxifylline (**4**), and the bryozoan purine phidolopin (**2**)[2] are representative,[3] and many derivatives of this type have been synthesized by a variety of conventional methods.[4,5] In a previous paper from this laboratory,[6] we have reported that 3,7-dialkyladenine salts (**6**), readily accessible from either 3-alkyladenines (**11**) or 7-alkyladenines (**10**) by alkylation,[7] undergo hydrolytic ring fission under alkaline conditions, producing mainly 1-alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (**7**). Recyclization of **7** with the C(2)-precursor ethyl chloroformate,[8] as exemplified by that of the dimethyl analogue **7a** to theobromine (**3**) in the presence of Et_3N,[6] may provide a novel, general synthetic route to 3,7-dialkylxanthines (type **9**) from 3- or 7-alkyladenines (**11** or **10**) and hence from adenine (**12**)[7,9] through **6**, **7**, and the carbamates **8**. In this paper, we present the details of our further study on such recyclization, establishing the generality of the above synthetic route. We also report an extension of this route to the synthesis of 1,3,7-trialkylxanthines (type **14**), including the 3-(arylmethyl)-type isomer (**24**) of phidolopin (**2**), and proton nuclear magnetic resonance (^{1}H-NMR) spectral criteria for differentiation of *N*-methyl or *N*-methylene groups in the 3,7-dialkyl- and 1,3,7-trialkylxanthine systems.

The substrates selected for the cyclization study were all nine 1-alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (**7a**—**i**), in which each of the two alkyl groups is any one of the methyl, ethyl, and benzyl groups, and they were prepared according to the procedures reported previously.[6] Treatment of **7c** with an excess of ethyl chloroformate in MeCN in the presence of Et_3N below 38 °C for 1.5 h, the procedure being patterned after that described before for the cyclization of **7a** to theobromine (**3**),[6] gave the cyclized product **9c** and a product presumed to be its N(1)-ethoxycarbonyl derivative (**13**) in 37% and 3.4% yields, respectively, together with a 28% recovery of the starting material (**7c**). This unsatisfactory result led us to apply the acetate buffer method of Itaya and Harada,[8h] with some modification. On treatment with an excess of ethyl chloroformate in a heterogeneous mixture of acetate buffer (pH 5) and AcOEt at 2—5 °C for 1 h, **7c** produced the carbamate **8c** in 71% yield. Cyclization of **8c** was then effected in boiling 1 N aqueous NaOH for 10 min to give 7-benzyl-3-methylxanthine (**9c**) in 94% yield. The omission of the process for purifying **8c** from this two-step sequence raised the overall yield of **9c** to 100%. Under similar reaction conditions, the other eight aminoimidazolecarboxamides (**7a**,**b**,**d**—**i**) also cyclized smoothly to afford the corresponding 3,7-dialkylxanthines (**3** and **9b**,**d**—**i**), as shown in Table I. The structures of **3** and **9c** were confirmed by direct comparisons with authentic theobromine and 7-benzyl-3-methylxanthine,[10] respectively, and it may be seen from Table II that all dialkylxanthines thus obtained had similar ultraviolet (UV) spectra, indicative of identical positional disubstitution.

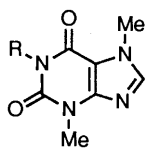
The above synthetic route to 3,7-dialkylxanthines (type **3** or **9**) was then extended beyond them by one step to secure some 1,3,7-trialkylxanthines (**5** and **14**—**22**), required as reference compounds for the present ^{1}H-NMR spectroscopic study. Treatment of **9h** with an excess of MeI in *N*,*N*-dimethylformamide (DMF) in the presence of an equimolar amount of anhydrous K_2CO_3, an application of the known procedure for *N*-alkylation of xanthine derivatives,[3d,f,5b,j] at room temperature for 3 h furnished 3-benzyl-7-ethyl-1-methylxanthine (**17**) in 95% yield. Several of the remaining 3,7-dialkylxanthines were similarly



1: R = H

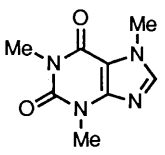


2: R = CH_2-(3-nitro-4-hydroxyphenyl)

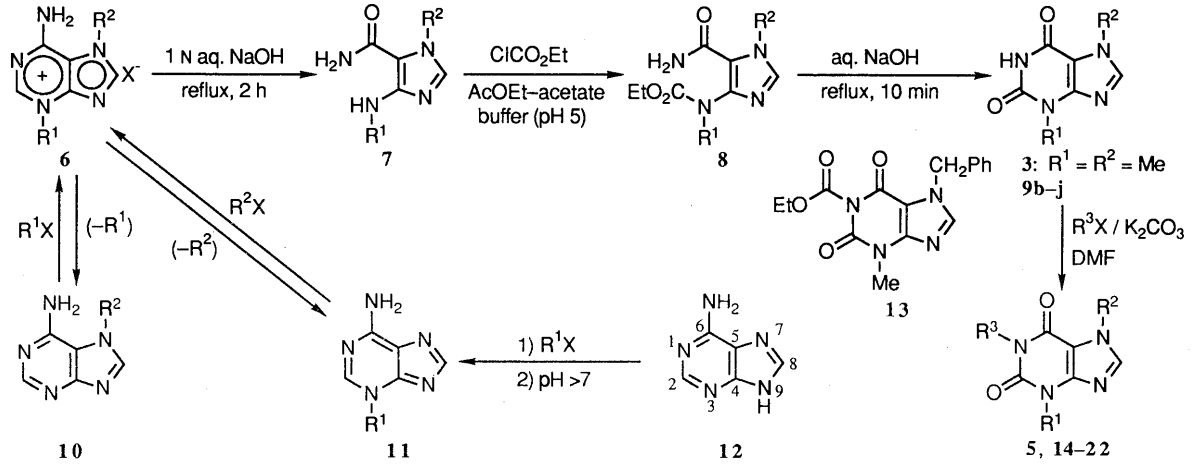


3: R = H

4: R = $(CH_2)_4COMe$



5



a: R^1 = Me; R^2 = Me b: R^1 = Me; R^2 = Et c: R^1 = Me; R^2 = CH_2Ph

d: R^1 = Et; R^2 = Me e: R^1 = Et; R^2 = Et f: R^1 = Et; R^2 = CH_2Ph

g: R^1 = CH_2Ph; R^2 = Me h: R^1 = CH_2Ph; R^2 = Et i: R^1 = CH_2Ph; R^2 = CH_2Ph

j: R^1 = CH_2–(2-NO_2-5-OMe)-Ph; R^2 = Me k: R^1 = CH_2–(2-NO_2-5-OCH_2OMe)-Ph

compd.	R^3	R^1	R^2
5	Me	Me	Me
14	Me	Me	CH_2Ph
15	Me	Et	CH_2Ph
16	Me	CH_2Ph	Me
17	Me	CH_2Ph	Et
18	Et	Me	CH_2Ph
19	Et	CH_2Ph	Me
20	CH_2Ph	Me	Me
21	CH_2Ph	Me	Et
22	CH_2Ph	Et	Me

Chart 1

TABLE I. Synthesis of 3,7-Dialkylxanthines (**3** and **9b—i**) from 1-Alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (**7a—i**) through the Ethoxycarbonyl Derivatives (**8a—i**)

Product			Reaction step: Acylation Temp.[a] (°C)	Acylation Time (h)	Cyclization[b]	Overall yield (%)	Appearance and recrystn. solvent[c]	mp (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
No.	N(3)–R^1	N(7)–R^2										
3	Me	Me	r.t.	2	A	85	Colorless solid	>300[d]	$C_7H_8N_4O_2$	46.67	4.48	31.10
										(—	—	—)
9b	Me	Et	r.t.	3	B	87	Colorless needles (E)	289.5—290	$C_8H_{10}N_4O_2$	49.48	5.19	28.85
										(49.47	5.22	28.78)
9c	Me	CH_2Ph	r.t.	2	A	100	Colorless needles (F)	278.5—280[d,e]	$C_{13}H_{12}N_4O_2$	60.93	4.72	21.86
										(—	—	—)
9d	Et	Me	r.t.	0.5	C	77	Colorless needles (E)	264—264.5	$C_8H_{10}N_4O_2$	49.48	5.19	28.85
										(49.47	5.23	29.04)
9e	Et	Et	r.t.	3	A	70	Colorless prisms (G)	187.5—188[f]	$C_9H_{12}N_4O_2$	51.92	5.81	26.91
										(51.96	5.88	26.82)
9f	Et	CH_2Ph	r.t.	2	D	89	Colorless needles (G)	222.5—223.5	$C_{14}H_{14}N_4O_2$	62.21	5.22	20.73
										(62.28	5.16	20.64)
9g	CH_2Ph	Me	r.t.	3.5	D	70	Colorless prisms (G)	236—236.5[g]	$C_{13}H_{12}N_4O_2$	60.93	4.72	21.86
										(60.72	4.71	21.80)
9h	CH_2Ph	Et	r.t.	3	D	63	Colorless prisms (G)	185—185.5	$C_{14}H_{14}N_4O_2$	62.21	5.22	20.73
										(62.46	5.15	20.62)
9i	CH_2Ph	CH_2Ph	r.t.	4	A	73	Colorless needles (H)	186.5—188	$C_{19}H_{16}N_4O_2$	68.66	4.85	16.86
										(68.49	4.75	16.65)

a) r.t., room temperature. *b*) The letter A stands for 10-min reflux with 1 N aqueous NaOH; B, 20-min reflux with 1 N aqueous NaOH; C, 2-h reflux with 1 N aqueous NaOH; D, 10-min reflux with 0.5 N aqueous NaOH. *c*) The letter in parentheses refers to the recrystallization solvent: E, EtOH; F, AcOH; G, AcOEt; H, MeCN. *d*) This sample was identical (by comparison of the IR spectrum) with an authentic specimen. *e*) Lit.[10] mp 275—277 °C. *f*) Lit.[11] mp 183 °C. *g*) Lit. mp 232—233 °C[5a]; 232—234 °C.[12]

alkylated with or without slight modification, giving the corresponding 1,3,7-trialkylxanthines in good yields. Table III summarizes the results of these alkylations. The identity of the synthetic trimethyl derivative **5** with caffeine was confirmed by a direct comparison with an authentic sample, and the correctness of the structures of the other

TABLE II. UV Spectra of 3,7-Dialkylxanthines (**3** and **9b—i**) and 1,3,7-Trialkylxanthines (**5** and **14—22**)

Compound				UV spectra							
				95% (v/v) aq. EtOH		H_2O (pH 1)[a]		H_2O (pH 7)[b]		H_2O (pH 13)[c]	
No.	N(1)–R^3	N(3)–R^1	N(7)–R^2	λ_{max} (nm)	$\varepsilon \times 10^{-3}$	λ_{max} (nm)	$\varepsilon \times 10^{-3}$	λ_{max} (nm)	$\varepsilon \times 10^{-3}$	λ_{max} (nm)	$\varepsilon \times 10^{-3}$
3	—	Me	Me	273	9.0	273	9.8	273	10.1	275	10.1
9b	—	Me	Et	273	9.6	273	9.9	273	10.5	274	10.3
9c	—	Me	CH_2Ph	274	9.3	274	9.5	274	9.7	275	9.5
9d	—	Et	Me	273	10.0	273	10.2	273	10.5	275	10.4
9e	—	Et	Et	273	10.0	273	10.1	273	10.4	274	10.2
9f	—	Et	CH_2Ph	275	10.0	274	9.2	274	9.4	276	9.0
9g	—	CH_2Ph	Me	273	10.5	273	11.7	273	11.8	275	11.4
9h	—	CH_2Ph	Et	273	11.1	273	11.1	273	11.2	275	10.7
9i	—	CH_2Ph	CH_2Ph	274	9.7	273	10.7	273	10.7	275	10.0
5	Me	Me	Me	273	9.6	273	9.6	273	9.9	273	9.6
14	Me	Me	CH_2Ph	274	8.3	273	8.8	273	9.1	273	9.1
15	Me	Et	CH_2Ph	274	8.7	274	9.2	274	9.5	274	9.2
16	Me	CH_2Ph	Me	273	10.2	272	10.6	272	10.7	272	10.0
17	Me	CH_2Ph	Et	273	10.1	273	10.3	273	10.7	273	10.5
18	Et	Me	CH_2Ph	275	8.4	275	9.0	275	9.2	275	9.1
19	Et	CH_2Ph	Me	274	10.5	273	10.6	273	10.7	273	10.3
20	CH_2Ph	Me	Me	274	9.7	274	10.2	274	10.3	274	10.0
21	CH_2Ph	Me	Et	274	9.7	274	10.0	274	10.3	274	10.2
22	CH_2Ph	Et	Me	275	9.9	275	10.2	275	10.3	275	10.1

a) Measured in 0.1 N aqueous HCl. *b*) Measured in 0.005 M phosphate buffer (pH 7). *c*) Measured in 0.1 N aqueous NaOH.

TABLE III. Synthesis of 1,3,7-Trialkylxanthines (**5** and **14—21**) from 3,7-Dialkylxanthines by Alkylation

3,7-Dialkylxanthine			Alkylation in DMF				1,3,7-Trialkylxanthine					Analysis (%) Calcd (Found)		
No.	N(3)–R^1	N(7)–R^2	K_2CO_3 (mol. eq)[a]	R^3X[b]	Temp.[c] (°C)	Time (h)	No.	Yield (%)	Appearance and recrystn. solvent[d]	mp (°C)	Formula	C	H	N
3	Me	Me	1.2[e]	MeI (2.5)	r.t.	7	**5**	99	Colorless solid	235.5—237[f]	$C_8H_{10}N_4O_2$	49.48 (—	5.19 —	28.85 —)
9c	Me	CH_2Ph	1.0	MeI (1.5)	r.t.	2	**14**	81	Colorless needles (A)	158—159[g]	$C_{14}H_{14}N_4O_2$	62.21 (62.38	5.22 5.04	20.73 20.88)
9f	Et	CH_2Ph	1.0	MeI (5.0)	r.t.	6	**15**	89	Colorless needles (B)	103.5—105.5	$C_{15}H_{16}N_4O_2$	63.37 (63.63	5.67 5.73	19.71 19.62)
9g	CH_2Ph	Me	1.1	MeI (1.5)	r.t.	3	**16**	93	Colorless prisms (B)	163.5—164.5[h]	$C_{14}H_{14}N_4O_2$	62.21 (62.43	5.22 5.31	20.73 20.60)
9h	CH_2Ph	Et	1.0	MeI (5.0)	r.t.	3	**17**	95	Colorless prisms (B)	141—141.5	$C_{15}H_{16}N_4O_2$	63.37 (63.61	5.67 5.71	19.71 19.49)
9c	Me	CH_2Ph	1.0	EtI (1.5)	r.t.—60	8[i]	**18**	85	Colorless needles (B)	107.5—108.5	$C_{15}H_{16}N_4O_2$	63.37 (63.12	5.67 5.69	19.71 19.73)
9g	CH_2Ph	Me	1.0	EtI (1.5)	r.t.	10	**19**	86	Colorless prisms (B)	140—140.5	$C_{15}H_{16}N_4O_2$	63.37 (63.19	5.67 5.63	19.71 19.62)
3	Me	Me	2.0	$PhCH_2Br$ (3.1)	r.t.—95	13[j]	**20**	84	Colorless needles (C)	141—141.5[k]	$C_{14}H_{14}N_4O_2$	62.21 (62.17	5.22 5.22	20.73 20.72)
9b	Me	Et	2.0	$PhCH_2Br$ (5.0)	r.t.	6.5	**21**	73	Colorless prisms (D)	103.5—104.5	$C_{15}H_{16}N_4O_2$	63.37 (63.52	5.67 5.72	19.71 19.76)
9d	Et	Me	2.0	$PhCH_2Br$ (5.0)	r.t.	3	**22**	88	Colorless prisms (C)	111.5—113.5	$C_{15}H_{16}N_4O_2$	63.37 (63.44	5.67 5.69	19.71 19.55)

a) Relative to the amount of a 3,7-dialkylxanthine used. *b*) The figures in parentheses indicate the amounts of alkylating agents in molar eq relative to 3,7-dialkylxanthines used. *c*) Unless otherwise stated, bath temperature is recorded; r.t., room temperature. *d*) The letter in parentheses refers to the recrystallization solvent: A, AcOEt; B, MeOH; C, EtOH; D, benzene–hexane (1 : 10, v/v). *e*) The reaction was carried out in the presence of 1.3 molar eq of 18-crown-6. *f*) This sample was identical (by comparison of the IR spectrum) with an authentic specimen. *g*) Lit.[3d,13] mp 157 °C. *h*) Lit. mp 163—164 °C[12]; 158—159 °C.[5a] *i*) At r.t. for 6 h and then at 60 °C for 2 h. *j*) At r.t. for 6 h and then at 95 °C for 7 h. *k*) Lit.[14] mp 140 °C.

trialkylxanthines was supported by the similarity of their UV spectra with that of **5**, as may be seen from Table II. Thus, we were able to corroborate the generality of the conversion of 3,7-dialkyladenines (**6**) into 3,7-dialkylxanthines (type **9**) and 1,3,7-trialkylxanthines (type **14**) through the monocycle **7**.

With the completion of characterization of the above two series of *N*-alkylxanthines, it was possible to catalog their ^{1}H-NMR spectral data obtained from Me_2SO-d_6 solutions. It may be seen from Table IV that *N*-methyl groups at various positions in both the 3,7-dialkyl- and 1,3,7-trialkylxanthine systems can be distinguished by their ^{1}H-NMR signals: their δ values may be aligned in the order of N(1)-Me < N(3)-Me < N(7)-Me, being in agreement with the order observed[15] in other solvents and interpreted by Bergmann and co-workers.[15b] As anticipated, the same

TABLE IV. ^{1}H-NMR Data for 3,7-Dialkylxanthines (**3** and **9b—i**) and 1,3,7-Trialkylxanthines (**5** and **14—22**)

Compd.	Chemical shift (δ)[a] in Me_2SO-d_6																
	N(1)-R^3					N(3)-R^1					N(7)-R^2					N(1)-H	C(8)-H
	Me	Et		CH_2Ph		Me	Et		CH_2Ph		Me	Et		CH_2Ph			
		CH_2[b]	Me[c]	CH_2	Ph		CH_2[b]	Me[c]	CH_2	Ph[d]		CH_2[b]	Me[c]	CH_2	Ph[d]		
3	—	—	—	—	—	3.33	—	—	—	—	3.84	—	—	—	—	11.05	7.94
9b	—	—	—	—	—	3.35	—	—	—	—	—	4.23	1.38	—	—	11.08	8.03
9c	—	—	—	—	—	3.34	—	—	—	—	—	—	—	5.45	7.33[e]	11.14	8.22
9d	—	—	—	—	—	—	3.95	1.19	—	—	3.86	—	—	—	—	11.06	7.97
9e	—	—	—	—	—	—	3.95	1.20	—	—	—	4.23	1.39	—	—	11.07	8.04
9f	—	—	—	—	—	—	3.95	1.20	—	—	—	—	—	5.45	7.04—7.44	11.12	8.22
9g	—	—	—	—	—	—	—	—	5.09	7.30[e]	3.85	—	—	—	—	11.19	7.96
9h	—	—	—	—	—	—	—	—	5.10	7.10—7.45	—	4.24	1.39	—	—	11.21	8.04
9i	—	—	—	—	—	—	—	—	5.08	7.24—7.40	—	—	—	5.44	7.24—7.40	11.23	8.21
5	3.21	—	—	—	—	3.40	—	—	—	—	3.87[f]	—	—	—	—	—	7.98[g]
14	3.21	—	—	—	—	3.42	—	—	—	—	—	—	—	5.49	7.33[e]	—	8.26
15	3.21	—	—	—	—	—	4.02	1.21	—	—	—	—	—	5.48	7.26—7.40	—	8.27
16	3.24	—	—	—	—	—	—	—	5.15	7.22—7.40	3.89	—	—	—	—	—	8.00
17	3.25	—	—	—	—	—	—	—	5.13	7.10—7.40	—	4.28	1.40	—	—	—	8.07
18	—	3.89	1.10	—	—	3.41	—	—	—	—	—	—	—	5.48	7.33[e]	—	8.25
19	—	3.91	1.12	—	—	—	—	—	5.15	7.10—7.40	3.89	—	—	—	—	—	8.00
20	—	—	—	5.05	7.10—7.40	3.43	—	—	—	—	3.89	—	—	—	—	—	8.02
21	—	—	—	5.06	7.05—7.40	3.43	—	—	—	—	—	4.28	1.39	—	—	—	8.11
22	—	—	—	5.06	7.28[e]	—	4.03	1.22	—	—	3.89	—	—	—	—	—	8.03

a) Measured at 18—139 mM concentration and expressed in ppm downfield from internal Me_4Si. *b*) Quartet with $J=7$ Hz. *c*) Triplet with $J=7$ Hz. *d*) Appeared as a multiplet, unless otherwise noted. *e*) Appeared as a single peak. *f*) Doublet with $J=0.5$ Hz. *g*) Appeared as an unresolved multiplet.

order also holds for the methylene protons of *N*-Et and *N*-CH_2Ph groups and even for the methyl protons of *N*-Et groups. The C(8)-H signals for the N(7)-Me and N(7)-Et analogues fall in the range of δ 7.94—8.11, whereas those for the N(7)-CH_2Ph analogues (**9c**, **9f**, **9i**, **14**, **15**, and **18**) fall in the δ 8.21—8.27 region. Such a downfield shift observed for the N(7)-CH_2Ph analogues may be interpretable in terms of the effect of the electron-withdrawing (relative to a simple alkyl group) benzyl group,[16] as we have experienced in the adenine ring system.[1b,17] In addition, the N(1)-H signals for 3,7-dialkylxanthines (**3** and **9b—i**)[18] fall in the range of δ 11.05—11.23, but those for the N(3)- and N(7)-CH_2Ph analogues (**9c** and **9f—i**) have a tendency to lie to the downfield side. On the basis of the above NMR data, the δ values listed in Table V may be employed as criteria for distinguishing *N*-alkyl or *N*-(arylmethyl) substituents at various positions in the 3,7-dialkyl- and 1,3,7-trialkylxanthine systems in Me_2SO-d_6 solution. These criteria should be reliable and useful since the assigned δ-value ranges for the three kinds of regioisomeric protons in each substituent series have no crossovers and since the solvent Me_2SO-d_6 is suitable for NMR measurements of almost all derivatives of xanthine.

We next planned to extend the scope of the above synthetic strategy to include the synthesis of functionalized, 1,3,7-trisubstituted xanthines. Phidolopin (**2**), a recent addition to the xanthine group from the bryozoan *Phidolopora pacifica*[2] or *Diaperoecia californica*,[2b] and its two unnatural, positional isomers (**24** and **25**) would be fascinating targets along this line. However, **2** and its 1-(arylmethyl)-type isomer (**25**) had already been synthesized from theophylline (**1**) and theobromine (**3**), respectively, by Hirota *et al.*[5b] while our present work was in progress. This led us to choose the remaining positional isomer (**24**) as the target structure for synthesis. Thus, adenine (**12**) was first treated with 4-(methoxymethoxy)-3-nitrobenzyl bromide (**28**)[2b,5b] in $AcNMe_2$ according to the previously reported, general 3-alkylation procedure[7a,b] (Chart 1). The reaction mixture became brown and failed to give the desired product (**11k**), most probably owing to *O*-deprotection by HBr generated as arylmethylation proceeded. On the other hand, replacement of the acid-labile bromide **28** by the 4-methoxy analogue **29** in the above arylmethylation afforded, after basification, the 3-substituted adenine **11j** in 43% yield. Subsequent methylation of **11j** with MeI in $AcNMe_2$ at 28 °C for 3.5 h furnished the 3,7-disubstituted adenine salt **6j** (X=I) (87% yield), which was then hydrolyzed in boiling 1 N aqueous NaOH for 2 h to provide the monocycle **7j** in 56% yield. Cyclization of **7j** through the carbamate **8j** was effected according to the above general ethoxycarbonylation procedure, giving the 3,7-disubstituted xanthine **9j** in 54% overall yield (from **7j**). Methylation of **9j** with MeI in DMF in the presence of anhydrous K_2CO_3 at room temperature for 6 h produced the penultimate compound **23** (85% yield), from which the ultimate compound **24** should be reached by *O*-demethylation. However, *O*-demethylation experiments with **23** or the model compound **30** using HBr/AcOH,[19]

TABLE V. ^{1}H-NMR Spectroscopic Criteria for Distinguishing *N*-Alkyl Substituents at Various Positions in the 3,7-Dialkyl- and 1,3,7-Trialkylxanthine Systems

Substituent[a]	Chemical shift (δ)[b] in Me_2SO-d_6		
	at N(1)	at N(3)	at N(7)
Me	3.21—3.25	3.33—3.43	3.84—3.89
$C\underline{H}_2Me$	3.89—3.91	3.95—4.03	4.23—4.28
$CH_2\underline{Me}$	1.10—1.12	1.19—1.22	1.38—1.40
$C\underline{H}_2Ph$	5.05—5.06	5.08—5.15	5.44—5.49

a) The underscored portion in the partial structure indicates the protons for which the δ values are listed. *b*) In ppm downfield from internal Me_4Si.

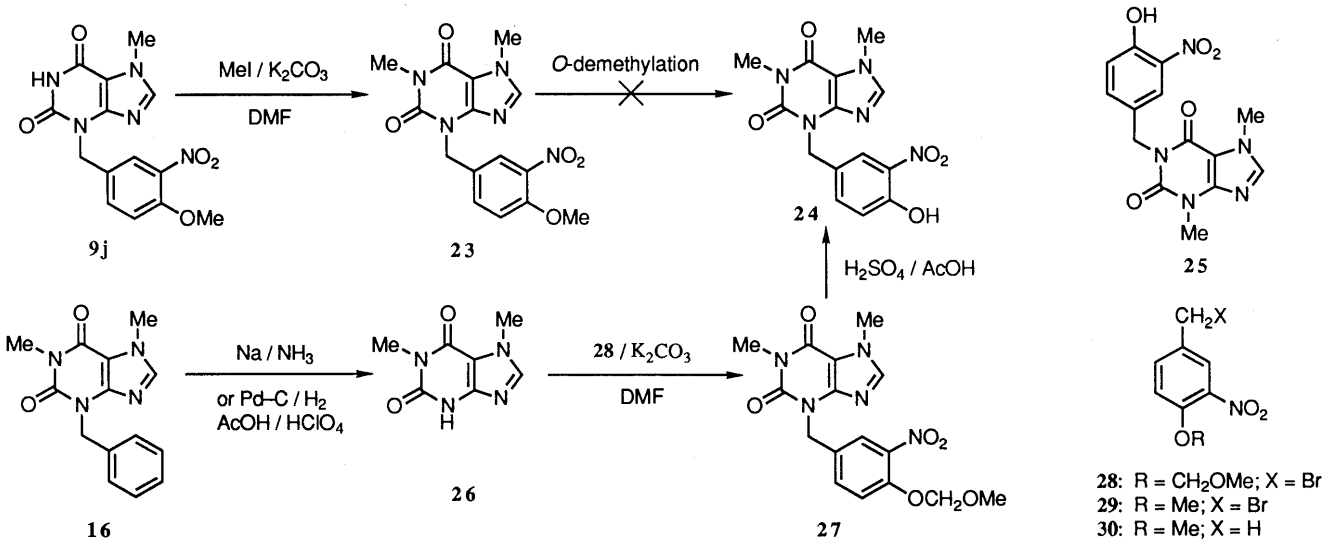


Chart 2

$AlBr_3$,[20] BBr_3,[21] Me_3SiI,[22] $NaCN/Me_2SO$,[23] and sodium *p*-thiocresolate[24] were all unsuccessful. Thus, we switched to an alternative synthetic route starting with 3-benzyl-1,7-dimethylxanthine (**16**). Treatment of **16** with sodium in liquid NH_3, according to a general debenzylation procedure for *N*-benzylated lactams and amides,[25] furnished paraxanthine (**26**) in 48% yield. It is known that *N*-debenzylation at the amide- or lactam-type nitrogen by catalytic hydrogenolysis is extremely difficult.[25a,26] Indeed, catalytic hydrogenolysis of **16** in AcOH in the presence of perchloric acid over 10% Pd–C at atmospheric pressure and 40—60 °C was very slow, but after 17 h it was possible to obtain **26** in 70% yield. Aralkylation of **26** was then effected with **28** in DMF in the presence of anhydrous K_2CO_3 at room temperature for 3 h, giving the 3-(arylmethy) derivative **27** in 84% yield.[27] Finally, *O*-deprotection of **27** with a small amount of 2 N aqueous H_2SO_4 in boiling AcOH for 1 h[28] provided the target compound **24**, isomeric with phidolopin (**2**), in 81% yield. In view of the antibacterial, antifungal, and antialgal activities exhibited by **2**,[2,29] it would be of interest to test **24** for biological activity. It should also be noted that the criteria proposed in Table V proved to be very useful for interpretation of the ^{1}H-NMR spectra of the above functionalized xanthines (**9j**, **23**, **24**, **26**, and **27**).

In conclusion, the present work has established a general synthetic route to 3,7-dialkyl- (type **9**) and 1,3,7-trialkylxanthines (type **14**) from 3- or 7-alkyladenines (**11** or **10**) *via* 3,7-dialkyladenines (**6**) and 1-alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (**7**). It represents an application of the "fission and reclosure" technology,[30,31] developed in our laboratory for modification of the adenine ring, to synthesis of dioxopurines. In this connection, the above hydrogenolytic debenzylation of 3-benzyl-1,7-dimethylxanthine (**16**) leading to paraxanthine (**26**) may exemplify a novel synthetic route to 1,7-dialkylxanthines, and the 3-(arylmethyl)-type isomer (**24**) of phidolopin (**2**) has been synthesized from **26**. As a result of the syntheses of some twenty *N*-alkylxanthines, it has now become possible to lay down ^{1}H-NMR spectroscopic criteria (Table V) for distinguishing *N*-alkyl or *N*-(arylmethyl) substituents at various positions in the 3,7-dialkyl- and 1,3,7-trialkylxanthine systems.

Experimental

General Notes All melting points were determined by using a Yamato MP-1 capillary melting point apparatus and are corrected. See ref. 6 for details of instrumentation and measurements. Elemental analyses were performed by Mr. Y. Itatani and his associates at Kanazawa University. The following abbreviations are used: br = broad, d = doublet, dd = doublet-of-doublets, m = multiplet, q = quartet, s = singlet, sh = shoulder, t = triplet.

Cyclization of 1-Alkyl-4-(alkylamino)-1*H*-imidazole-5-carboxamides (7a—i) Leading to 3,7-Dialkylxanthines (3 and 9b—i) through 1-Alkyl-4-[(ethoxycarbonyl)alkylamino]-1*H*-imidazole-5-carboxamides (8a—i) The cyclization of 1-benzyl-4-(ethylamino)-1*H*-imidazole-5-carboxamide (**7f**) will be described below in detail as a typical example.

A suspension of **7f**[6] (977 mg, 4 mmol) in a mixture of AcOEt (28 ml) and 1 M aqueous AcOH–AcONa buffer (pH 5) (40 ml) was stirred at room temperature, and a 2 M solution of ethyl chloroformate in AcOEt (10 ml, 20 mmol) was added dropwise over a period of 60 min at such a rate that the inner temperature did not exceed 26 °C. Stirring was continued at room temperature for a further 2 h. During the reaction, the pH of the medium had been maintained at 5.2—5.3 by occasional addition of 10% aqueous NaOH. The reaction mixture was then adjusted to pH 7.0 with 10% aqueous NaOH, and the AcOEt layer was separated from the aqueous layer, which was further extracted with AcOEt. The AcOEt extracts were combined with the above AcOEt layer, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to leave crude 1-benzyl-4-[(ethoxycarbonyl)-ethylamino]-1*H*-imidazole-5-carboxamide (**8f**) as a pale brown oil. The oil was then heated with boiling 0.5 N aqueous NaOH (40 ml) for 10 min, and the reaction mixture was kept in a refrigerator overnight after neutralization with 1 N aqueous HCl. The crystals that resulted were filtered off, washed with a little H_2O, and dried to give crude 7-benzyl-3-ethylxanthine (**9f**) (979 mg). Recrystallization from AcOEt (*ca.* 100 ml) afforded a first crop (910 mg) of **9f** as colorless needles, mp 220.5—222 °C. The usual work-up of the mother liquor of this recrystallization yielded a second crop (50 mg), mp 218.5—220.5 °C. The total yield of **9f** was 960 mg (89% from **7f**). Further recrystallization from AcOEt produced an analytical sample of **9f** as colorless needles, mp 222.5—223.5 °C; MS *m/z*: 270 (M^+); UV (Table II); ^{1}H-NMR (Table IV); *Anal.* (Table I).

Cyclizations of the other aminoimidazolecarboxamides (**7a—e** and **7g—i**)[6] were conducted in a similar manner. The results are shown in Tables I, II, and IV.

1-Benzyl-4-[(ethoxycarbonyl)methylamino]-1*H*-imidazole-5-carboxamide (8c) A suspension of 1-benzyl-4-(methylamino)-1*H*-imidazole-5-carboxamide (**7c**)[6] (464 mg, 2.02 mmol) in a mixture of AcOEt (10 ml) and 1 M aqueous AcOH–AcONa buffer (pH 5) (15 ml) was stirred under ice-cooling, and a solution of ethyl chloroformate (1.10 g, 10 mmol) in

AcOEt (10 ml) was added dropwise at 2—5 °C over a period of 20 min. After having been stirred at the same temperature for 1 h, the reaction mixture was brought to pH 7 by addition of 10% aqueous NaOH. The AcOEt layer was separated from the aqueous layer, which was then extracted with $CHCl_3$. The $CHCl_3$ extracts were combined with the above AcOEt layer, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to leave a colorless solid. Recrystallization of the solid from AcOEt gave **8c** (430 mg, 71%) as colorless prisms, mp 126—129 °C. Further recrystallization from AcOEt furnished an analytical sample as colorless prisms, mp 127.5—129.5 °C; MS *m/z*: 302 (M^+); UV $\lambda_{max}^{95\%\ aq.\ EtOH}$ 241 nm (ε 7000); IR ν_{max}^{Nujol} cm^{-1}: 3425 and 3180 ($CONH_2$), 1704 (carbamate CO), 1672 (amide CO); ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3H, t, J=7 Hz, $OCH_2\underline{Me}$), 3.28 (3H, s, NMe), 4.21 (2H, q, J=7 Hz, $OC\underline{H}_2Me$), 5.50 [2H, s, N(1)-$C\underline{H}_2Ph$], 5.67 (br, $CONH_2$), 7.08—7.48 [5H, m, N(1)-$CH_2\underline{Ph}$], 7.45 [1H, s, C(2)-H]. *Anal.* Calcd for $C_{15}H_{18}N_4O_3$: C, 59.59; H, 6.00; N, 18.53. Found: C, 59.63; H, 6.04; N, 18.58.

Cyclization of 8c Leading to 7-Benzyl-3-methylxanthine (9c) A mixture of **8c** (302 mg, 1 mmol) and 1 N aqueous NaOH (5 ml) was heated under reflux for 10 min. After cooling, the reaction mixture was brought to pH 7 with 1 N aqueous HCl and then kept in a refrigerator overnight. The colorless solid that deposited was filtered off, washed with a little H_2O, and dried to give crude **9c** (258 mg). Recrystallization from AcOH produced a pure sample (242 mg, 94%) as colorless needles, mp 278.5—280 °C. This sample was identical (by comparison of the IR spectrum) with authentic **9c**.[10]

Reaction of 7c with Ethyl Chloroformate in MeCN in the Presence of Et_3N A suspension of **7c**[6] (1.15 g, 5 mmol) in MeCN (35 ml) containing Et_3N (2.53 g, 25 mmol) was stirred at room temperature, and a solution of ethyl chloroformate (3.81 g, 35 mmol) in MeCN (15 ml) was added dropwise over a period of 15 min at such a rate that the inner temperature did not exceed 38 °C. After having been stirred at room temperature for 75 min, the reaction mixture was filtered to collect the colorless solid that resulted. The solid was washed with MeCN and recrystallized from AcOEt to yield a first crop (429 mg) of 7-benzyl-3-methylxanthine (**9c**) as colorless needles, mp 278—279.5 °C. This sample was identical (by comparison of the IR spectrum) with authentic **9c**.[10] The filtrate and washings, obtained when the crude **9c** was isolated, were combined and concentrated *in vacuo*, and the residue was chromatographed on a column of silica gel (80 g) using AcOEt–MeOH (20:1, v/v) as the eluent. Earlier fractions gave a yellow oil (55.2 mg, 3.4%), presumed to be 7-benzyl-1-ethoxycarbonyl-3-methylxanthine (**13**), MS *m/z*: 328 (M^+); ^{1}H-NMR ($CDCl_3$) δ: 1.43 (3H, t, J=7.5 Hz, $OCH_2\underline{Me}$), 3.54 [3H, s, N(3)-Me], 4.51 (2H, q, J=7.5 Hz, $OC\underline{H}_2Me$), 5.45 [2H, s, N(7)-$C\underline{H}_2Ph$], 7.35 [5H, s, N(7)-$CH_2\underline{Ph}$], 7.57 [1H, s, C(8)-H].

Middle fractions eluted from the above silica gel column furnished a colorless solid, which was recrystallized from AcOEt to give a second crop (48 mg) of **9c** as colorless needles, mp 278.5—279.5 °C. The total yield of **9c** was 477 mg (37%).

Later fractions of the chromatoraphy yielded unaltered **7c** (321 mg, 28%), mp 159.5—160.5 °C, which was identical (by comparison of the IR spectrum) with an authentic sample.[6]

Alkylation of 3,7-Dialkylxanthines (3 and 9b—d,f—h) Leading to 1,3,7-Trialkylxanthines (5 and 14—22) The procedure employed for the methylation of 3-benzyl-7-ethylxanthine (**9h**) will be described below in detail as a typical example.

A stirred suspension of **9h** (540 mg, 2 mmol) and anhydrous K_2CO_3 (276 mg, 2 mmol) in DMF (17 ml) was heated at 80 °C for 1 h. After cooling, MeI (1.42 g, 10 mmol) was added, and the resulting mixture was stirred at room temperature for 3 h and then concentrated *in vacuo*. The residue was washed successively with 10% aqueous NaOH (2×3 ml) and H_2O (15 ml) to leave, after drying, 3-benzyl-7-ethyl-1-methylxanthine (**17**) (541 mg, 95%) as colorless prisms, mp 139.5—141 °C. Recrystallization from MeOH produced an analytical sample as colorless prisms, mp 141—141.5 °C; MS *m/z*: 284 (M^+); UV (Table II); ^{1}H-NMR (Table IV); *Anal.* (Table III).

The other 3,7-dialkylxanthines (**3** and **9b—d, f, g**) were similarly alkylated under the reaction conditions specified in Table III, affording the results shown in Tables II—IV.

3-(4-Methoxy-3-nitrobenzyl)adenine (11j) A stirred mixture of adenine (**12**) (2.70 g, 20 mmol) and 4-methoxy-3-nitrobenzyl bromide (**29**)[32] (5.91 g, 24 mmol) in $AcNMe_2$ (200 ml) was heated at 61—65 °C for 5 h. The reaction mixture was concentrated *in vacuo* to leave a slightly yellow solid. The solid was washed with ether (4×20 ml) and then recrystallized from 70% (v/v) aqueous EtOH to yield **11j**·HBr as pale yellowish needles. The crude salt was dissolved in warm H_2O (150 ml), and the resulting solution was made alkaline with 28% aqueous NH_3 and then kept in a refrigerator for 2 d. The crystals that deposited were filtered off, washed with H_2O, and recrystallized from EtOH to furnish **11j**·1/7EtOH (2.62 g, 43%) as pale yellowish needles, mp 256—257 °C (dec.). Further recrystallization from EtOH and drying over P_2O_5 at 2 mmHg and 100 °C for 33 h gave an analytical sample, mp 262—262.5 °C (dec.); MS *m/z*: 300 (M^+); UV $\lambda_{max}^{95\%\ aq.\ EtOH}$ 273 nm (ε 14100), 330 (sh) (2200); $\lambda_{max}^{H_2O}$ (pH 1) 274 (22000), 335 (2600); $\lambda_{max}^{H_2O}$ (pH 7) 272 (17100), 339 (2500); $\lambda_{max}^{H_2O}$ (pH 13) 272 (16500), 339 (2500); $\lambda_{min}^{H_2O}$ (pH 1) − $\lambda_{min}^{H_2O}$ (pH 7) = 241 nm − 247 nm = −6 nm[7b,33]; IR ν_{max}^{Nujol} cm^{-1}: 1540, 1337 ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 1.06 (3/7H, t, J=7 Hz, $HOCH_2\underline{Me}$), 3.45 (2/7H, q, J=7 Hz, $HOC\underline{H}_2Me$), 3.89 (3H, s, OMe), 5.50 [2H, s, N(3)-$C\underline{H}_2Ar$], 7.35 [1H, d, J=9 Hz, C(5′)-H], 7.76 and 8.59 (1H each, s, purine H's),[34] 7.84 [1H, dd, J=9, 2 Hz, C(6′)-H], 7.94 (2H, br, NH_2), 8.11 [1H, d, J=2 Hz, C(2′)-H].[35] *Anal.* Calcd for $C_{13}H_{12}N_6O_3 \cdot 1/7EtOH$: C, 52.00; H, 4.32; N, 27.39. Found: C, 52.05; H, 4.19; N, 27.69.

3-(4-Methoxy-3-nitrobenzyl)-7-methyladenine Hydriodide [6j (X=I)] A mixture of **11j**·1/7EtOH (9.21 g, 30 mmol) and MeI (12.78 g, 90 mmol) in $AcNMe_2$ (150 ml) was stirred at 28 °C for 3.5 h. The reaction mixture was concentrated *in vacuo*, leaving a yellow solid. The solid was washed with ether (3×20 ml) and recrystallized from 50% (v/v) aqueous EtOH to give **6j**·1/7EtOH (X=I) (11.74 g, 87%) as yellowish prisms, mp 244.5 °C (dec.). Further recrystallization in the same manner and drying over P_2O_5 at 2 mmHg and 50 °C for 24 h yielded an analytical sample, mp 245.5 °C (dec.); UV $\lambda_{max}^{95\%\ aq.\ EtOH}$ 281 nm (ε 18700), 320 (2200); $\lambda_{max}^{H_2O}$ (pH 1) 277 (20400), 336 (2700); $\lambda_{max}^{H_2O}$ (pH 7) 277 (20300), 336 (2700); $\lambda_{max}^{H_2O}$ (pH 13) 279 (17300), 336 (sh) (2800); IR ν_{max}^{Nujol} 1534 cm^{-1} ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 1.06 (3/7H, t, J=7 Hz, $HOCH_2\underline{Me}$), 3.45 (2/7H, q, J=7 Hz, $HOC\underline{H}_2Me$), 3.90 (3H, s, OMe), 4.07 [3H, s, N(7)-Me], 5.59 [2H, s, N(3)-$C\underline{H}_2Ar$], 7.37 [1H, d, J=9 Hz, C(5′)-H], 7.83 [1H, dd, J=9, 2 Hz, C(6′)-H], 8.07 [1H, d, J=2 Hz, C(2′)-H], 8.3—8.6 (1H, br, NH), 8.58 (1H, s, purine H), 9.02 (1H, s, purine H), 9.40 (1H, br, NH).[35] *Anal.* Calcd for $C_{14}H_{14}N_6O_3 \cdot HI \cdot 1/7EtOH$: C, 38.23; H, 3.56; N, 18.73. Found: C, 37.96; H, 3.54; N, 18.93.

4-[(4-Methoxy-3-nitrobenzyl)amino]-1-methyl-1*H*-imidazole-5-carboxamide (7j) A stirred suspension of **6j**·1/7EtOH (X=I) (898 mg, 2 mmol) in 1 N aqueous NaOH (10 ml) was heated under reflux for 2 h. After cooling, the reaction mixture was neutralized with 10% aqueous HCl and kept in a refrigerator for 3 h. The precipitate that deposited was filtered off, washed with H_2O, and dried to give a dark brown solid (538 mg), mp 178 °C (dec.). Recrystallization of the solid from H_2O afforded **7j** (344 mg, 56%) as a brown solid, mp 183—185 °C (dec.). Further recrystallization from H_2O yielded an analytical sample as brownish needles, mp 189.5—190.5 °C (dec.); MS *m/z*: 305 (M^+); UV $\lambda_{max}^{95\%\ aq.\ EtOH}$ 292 nm (ε 12100); $\lambda_{max}^{H_2O}$ (pH 1) 245 (11900), 277 (13000), 339 (2800); $\lambda_{max}^{H_2O}$ (pH 7) 286 (12400), 339 (2800); $\lambda_{max}^{H_2O}$ (pH 13) 286 (12500), 339 (2800); IR ν_{max}^{Nujol} 1535 cm^{-1} ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 3.70 [3H, s, N(1)-Me], 3.88 (3H, s, OMe), 4.41 (2H, d, J=6 Hz, $NHC\underline{H}_2Ar$), 6.29 (1H, t, J=6 Hz, $N\underline{H}CH_2Ar$), 6.75 (2H, s, $CONH_2$), 7.28 [1H, d, J=9 Hz, C(5′)-H], 7.31 [1H, s, C(2)-H], 7.60 [1H, dd, J=9, 2 Hz, C(6′)-H], 7.80 [1H, d, J=2 Hz, C(2′)-H].[35] *Anal.* Calcd for $C_{13}H_{15}N_5O_4$: C, 51.15; H, 4.95; N, 22.94. Found: C, 51.18; H, 4.97; N, 22.67.

3-(4-Methoxy-3-nitrobenzyl)-7-methylxanthine (9j) A suspension of **7j** (1.53 g, 5 mmol) in a mixture of AcOEt (30 ml) and 1 M aqueous AcOH–AcONa buffer (pH 5) (50 ml) was stirred at room temperature, and a 2 M solution of ethyl chloroformate in AcOEt (25 ml, 50 mmol) was added dropwise over a period of 50 min at such a rate that the inner temperature did not exceed 30 °C. Stirring was continued at room temperature for 8 h, and for a further 6 h after another addition of a 2 M solution of ethyl chloroformate in AcOEt (12.5 ml, 25 mmol) in a similar manner. During the reaction, the pH of the medium had been kept at 5.1—5.3 by occasional addition of 10% aqueous NaOH. Isolation of crude **8j** from the reaction mixture and subsequent treatment of **8j** with 1 N aqueous NaOH (25 ml) were performed in a manner similar to that described above for the cyclization of **7f** to **9f** *via* **8f**, producing **9j** (891 mg, 54%) as yellowish needles, mp 279—280.5 °C (dec.). Recrystallization from AcOH and drying over KOH at 2 mmHg and room temperature for 20 h gave an analytical sample as yellowish needles, mp 280.5—281.5 °C (dec.); MS *m/z*: 331 (M^+); UV $\lambda_{max}^{95\%\ aq.\ EtOH}$ 271 nm (ε 13000), 332 (2300); $\lambda_{max}^{H_2O}$ (pH 1) 271 (15000), 340 (2600); $\lambda_{max}^{H_2O}$ (pH 7) 271 (15100), 340 (2700); $\lambda_{max}^{H_2O}$ (pH 13) 275 (14600), 343 (2700); IR ν_{max}^{Nujol} 1540 cm^{-1} ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 3.85 [3H, s, N(7)-Me], 3.89 (3H, s, OMe), 5.08 [2H, s, N(3)-$C\underline{H}_2Ar$], 7.31 [1H, d, J=9 Hz, C(5′)-H], 7.64 [1H, dd, J=9, 2 Hz, C(6′)-H], 7.86 [1H, d, J=2 Hz, C(2′)-H], 7.98 [1H, s, C(8)-H], 11.22 [1H, s, N(1)-H].[35] *Anal.* Calcd for $C_{14}H_{13}N_5O_5$: C, 50.76; H, 3.96; N, 21.14.

Found: C, 50.58; H, 3.77; N, 21.23.

3-(4-Methoxy-3-nitrobenzyl)-1,7-dimethylxanthine (23) A mixture of **9j** (662 mg, 2 mmol) and anhydrous K_2CO_3 (553 mg, 4 mmol) in DMF (20 ml) was stirred at 90—92 °C for 1 h. After cooling, MeI (1.42 g, 10 mmol) was added, and the resulting mixture was stirred at room temperature for 6 h. The yellowish precipitate that resulted was filtered off, washed successively with EtOH, H_2O, and EtOH (in that order), and recrystallized from AcOH to afford **23** (585 mg, 85%) as yellowish needles, mp 283—284 °C (dec.). Further recrystallization from AcOH and drying over KOH at 2 mmHg and 100 °C for 6 h yielded an analytical sample, mp 285—286 °C (dec.); MS *m/z*: 345 (M^+); UV $\lambda_{max}^{95\%\,aq.\,EtOH}$ 272 nm (ε 12600), 328 (2200); $\lambda_{max}^{95\%\,aq.\,EtOH}$ (H^+)[36] 270 (12500), 326 (2300); $\lambda_{max}^{95\%\,aq.\,EtOH}$ (OH^-)[37] 270 (12400), 326 (2200); IR ν_{max}^{Nujol} 1536 cm^{-1} ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 3.23 [3H, s, N(1)-Me], 3.89 [6H, s, N(7)-Me and OMe], 5.15 [2H, s, N(3)-C$\underline{H}_2$Ar], 7.29 [1H, d, *J*=9 Hz, C(5′)-H], 7.65 [1H, dd, *J*=9, 2 Hz, C(6′)-H], 7.86 [1H, d, *J*=2 Hz, C(2′)-H], 8.03 [1H, s, C(8)-H].[35] *Anal.* Calcd for $C_{15}H_{15}N_5O_5$: C, 52.17; H, 4.38; N, 20.28. Found: C, 52.32; H, 4.28; N, 20.01.

1,7-Dimethylxanthine (Paraxanthine) (26) i) By Dissolving Metal Reduction of **16**: To a stirred solution of **16** (542 mg, 2 mmol) in liquid NH_3 (*ca.* 120 ml) was added metallic Na (370 mg, 16 mg.-atom) in small pieces over a period of 50 min at the boiling point of liquid NH_3. The reaction mixture was slowly concentrated at room temperature to leave a pale brown solid, to which EtOH (5 ml) and H_2O (20 ml) were added in that order under ice-cooling. Concentration of the resulting solution under reduced pressure left a pale brown solid. The solid was quickly washed with H_2O (6.5 ml) and then dissolved in H_2O (20 ml). The resulting aqueous solution was neutralized with 10% aqueous HCl and kept in a refrigerator overnight. The slightly yellowish crystals that deposited were filtered off, washed with a little H_2O, and dried to give a first crop (124 mg) of **26**, mp 295.5—297.5 °C (dec.). The filtrate and washings obtained at this stage were combined with the above, first aqueous washings, and the resulting mixture was concentrated, after neutralization with 10% aqueous HCl, under reduced pressure. The pale brownish residue was dried and extracted with hot MeOH, and the methanolic extracts were subjected to flash chromatography[38] [silica gel, CH_2Cl_2–EtOH (10:1, v/v)], yielding a second crop (51 mg) of **26**, mp 293.5—295.5 °C (dec.). The total yield of **26** was 175 mg (48%). Recrystallization from H_2O furnished an analytical sample as colorless needles, mp 297.5—298 °C (dec.) (lit.[8b] mp 297—298 °C); MS *m/z*: 180 (M^+); UV $\lambda_{max}^{95\%\,aq.EtOH}$ 270 nm (ε 9400); $\lambda_{max}^{H_2O}$ (pH 1) 267 (9000); $\lambda_{max}^{H_2O}$ (pH 7) 269 (9600); $\lambda_{max}^{H_2O}$ (pH 13) 289 (8300); ^{1}H-NMR (Me_2SO-d_6) δ: 3.17 [3H, s, N(1)-Me], 3.85 [3H, s, N(7)-Me], 7.90 [1H, s, C(8)-H], 11.81 [1H, s, N(3)-H]. *Anal.* Calcd for $C_7H_8N_4O_2$: C, 46.67; H, 4.48; N, 31.10. Found: C, 46.60; H, 4.39; N, 31.15.

ii) By Catalytic Hydrogenolysis of **16**: A solution of **16** (196 mg, 0.73 mmol) in AcOH (15 ml) containing 70% aqueous $HClO_4$ (0.2 ml) was hydrogenated over 10% Pd–C (200 mg) at atmospheric pressure and 40 °C for 4 h. The reaction was so slow that a suspension of more catalyst (100 mg) in AcOH (1 ml) was added at this stage, and hydrogenation was continued at 60 °C for a further 13 h. After cooling, the catalyst was removed by filtration and washed with MeOH. The filtrate and methanolic washings were combined and concentrated *in vacuo* to leave a brown oil, which was dissolved in H_2O (2 ml). The resulting aqueous solution was neutralized with 10% aqueous NaOH and kept in a refrigerator overnight. The colorless needles that resulted were filtered off, washed with a little H_2O, and dried to give **26** (92 mg, 70%), mp 295.5—296 °C (dec.). This sample was identical (by comparison of the IR spectrum and thin-layer chromatographic mobility) with the one obtained by method (i).

3-[(4-Methoxymethoxy)-3-nitrobenzyl]-1,7-dimethylxanthine (27) A suspension of **26** (292 mg, 1.6 mmol) and anhydrous K_2CO_3 (246 mg, 1.8 mmol) in DMF (15 ml) was stirred at 88 °C for 1 h. After cooling, 4-(methoxymethoxy)-3-nitrobenzyl bromide (**28**)[2b,5b] (671 mg, 2.43 mmol) was added, and the mixture was stirred at room temperature for 3 h. The reaction mixture was then filtered to remove an insoluble solid, which was washed with EtOH. The filtrate and washings were combined and concentrated *in vacuo*, and the residual yellow solid was recrystallized from EtOH to give **27** (511 mg, 84%) as colorless needles, mp 160.5—164 °C. Further recrystallization from MeOH and drying over P_2O_5 at 2 mmHg and 75 °C for 12 h yielded an analytical sample as colorless needles, mp 168.5—169.5 °C; MS *m/z*: 375 (M^+); UV $\lambda_{max}^{95\%\,aq.\,EtOH}$ 271 nm (ε 12300), 317 (1800); $\lambda_{max}^{95\%\,aq.\,EtOH}$ (H^+)[36] 271 (12400), 317 (1900); $\lambda_{max}^{95\%\,aq.\,EtOH}$ (OH^-)[37] 271 (12400), 317 (1900); IR ν_{max}^{Nujol} 1541 cm^{-1} ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 3.23 [3H, s, N(1)-Me], 3.38 (3H, s, OMe), 3.89 [3H, s, N(7)-Me], 5.15 [2H, s, N(3)-C$\underline{H}_2$Ar], 5.32 (2H, s, OCH_2O), 7.36 [1H, d, *J*=9 Hz, C(5′)-H], 7.61 [1H, dd, *J*=9, 2 Hz, C(6′)-H], 7.85 [1H, d, *J*=2 Hz, C(2′)-H], 8.02 [1H, s, C(8)-H].[35] *Anal.* Calcd for $C_{16}H_{17}N_5O_6$: C, 51.20; H, 4.57; N, 18.66. Found: C, 51.13; H, 4.55; N, 18.45.

3-(4-Hydroxy-3-nitrobenzyl)-1,7-dimethylxanthine (24) A solution of **27** (375 mg, 1 mmol) in AcOH (15 ml) containing 2 N aqueous H_2SO_4 (6 drops) was heated under reflux for 1 h. The reaction mixture was concentrated *in vacuo* to leave a yellow solid, which was dissolved in CH_2Cl_2 (80 ml). The CH_2Cl_2 solution was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residual solid obtained was recrystallized from MeCN to give **24** (269 mg, 81%) as yellow needles, mp 238—239 °C. Further recrystallization from MeCN produced an analytical sample, mp 239.5—240.5 °C; MS *m/z*: 331 (M^+); UV $\lambda_{max}^{95\%\,aq.\,EtOH}$ 273 nm (ε 16100), 351 (2900); $\lambda_{max}^{H_2O}$ (pH 1) 273 (14400), 355 (2900); $\lambda_{max}^{H_2O}$ (pH 7) 273 (13600), 405 (2500); $\lambda_{max}^{H_2O}$ (pH 13) 233 (23100), 273 (11700), 420 (4400); IR ν_{max}^{Nujol} cm^{-1}: 3320 (OH), 1541 ($ArNO_2$); ^{1}H-NMR (Me_2SO-d_6) δ: 3.22 [3H, s, N(1)-Me], 3.89 [3H, s, N(7)-Me], 5.10 [2H, s, N(3)-C$\underline{H}_2$Ar], 7.07 [1H, d, *J*=8.5 Hz, C(5′)-H], 7.54 [1H, dd, *J*=8.5, 2 Hz, C(6′)-H], 7.91 [1H, d, *J*=2 Hz, C(2′)-H], 8.02 [1H, s, C(8)-H], 10.91 (1H, s, OH).[35] *Anal.* Calcd for $C_{14}H_{13}N_5O_5$: C, 50.76; H, 3.96; N, 21.14. Found: C, 50.81; H, 3.91; N, 21.06.

References and Notes

1) *a*) This article is dedicated to the memory of Emeritus Professor Dr. Shigehiko Sugasawa (University of Tokyo), who ended a most distinguished, constantly inspiring, and exemplary chemist's life at the age of 92 on March 1, 1991; *b*) Paper XLVIII in this series, T. Fujii, T. Saito, K. Kizu, H. Hayashibara, Y. Kumazawa, S. Nakajima, and T. Fujisawa, *Chem. Pharm. Bull.*, **39**, 301 (1991).
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35) For convenience, each position of the benzene ring is indicated by a primed number.
36) Since the sample was not sufficiently soluble in H_2O, 95% (v/v) aqueous EtOH containing HCl at 0.1 M concentration was used.
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Erythro-Selective Aldol Reaction of Tricarbonyl(η^6-*o*-trialkylsilylbenzaldehyde)chromium(0) Complexes with Cyclic Ketene Silyl Acetals

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The aldol reaction of tricarbonyl(*o*-trimethylsilyl (TMS)-benzaldehyde)chromium(0) complex (1a) with cyclic ketene silyl acetals (2—4) afforded the corresponding *erythro* products selectively. Changing the *ortho* TMS group to a triisopropylsilyl (TIPS) group in the complex brought about an improvement of the *erythro* selectivity in the case of the five-membered acetal (4).

Keywords stereoselective aldol reaction; *erythro* isomer; *threo* isomer; *o*-trimethylsilylbenzaldehyde–chromium complex; *o*-triisopropylsilylbenzaldehyde–chromium complex; cyclic ketene silyl acetal; boron trifluoride etherate; cerium(IV) ammonium nitrate

The stereoselective aldol reaction[1] has been well recognized as one of the most useful tools in organic synthesis. Recently we developed a highly *erythro*-selective aldol reaction[2,3] of tricarbonyl(η^6-*o*-trimethylsilylbenzaldehyde)chromium(0) complex (**1a**)[4] with cyclic silyl enol ethers. This stereoselective aldol reaction was successfully extended to an asymmetric situation[2] where a high enantiomeric excess was attained. In order to evaluate the inherent selectivity of the *o*-trimethylsilyl (TMS)-benzaldehyde–chromium complex (**1a**) in the aldol reaction, we investigated the reaction between **1a** and cyclic ketene silyl acetals (**2**—**4**). This paper describes the *erythro*-selective aldol reaction of the *o*-trialkylsilylbenzaldehyde–chromium(0) complexes (**1a**, **f**) with several cyclic ketene silyl acetals.

At the outset we chose the seven-membered ketene silyl acetal (**2**) as a nucleophile because the seven-membered silyl enol ether had previously been shown to give the highest *erythro* selectivity in our aldol reaction.[2,3] The aldol reaction was carried out as follows. A mixture of the complex (**1a**) and the acetal (**2**) in dry methylene chloride was treated with boron trifluoride etherate ($BF_3 \cdot OEt_2$) under a nitrogen atmosphere at $-78\,^{\circ}C$. After usual work-up, the residue was exposed to cerium(IV) ammonium nitrate (CAN)[5] in methanol at $0\,^{\circ}C$ to give the aldol condensation products in 72% yield. This adduct was found to be made up of the *erythro* and *threo* isomers (**6a** and **7a**) in a ratio of 90 : 10 by careful examination of its proton magnetic resonance (^{1}H-NMR) spectrum. Stereochemical assignment of the *erythro* and *threo* isomers was also made by consideration of the chemical shifts as well as coupling constants of benzylic protons on the basis of the literature precedents.[1] The signal of the benzylic proton of the *erythro* isomer (**6a**) appeared at δ 5.48 ppm as a doublet ($J = 1.5$ Hz), whereas that of the *threo* isomer (**7a**) appeared at δ 5.21 ppm as a doublet of doublets ($J = 9.3$, 3.4 Hz). Thus, the *erythro* isomer (**6a**) was obtained in a highly selective manner. This result was in line with the expectation based on our earlier results[2,3] with cyclic silyl enol ethers.

The *o*-ethylbenzaldehyde–chromium(0) complex (**1b**), however, showed only a good *erythro* selectivity (*erythro* : *threo* = 76 : 24) under identical reaction conditions. This observation was in sharp contrast to the case of the reaction[2,3] between **1b** and 1-trimethylsilyloxycyclohe-

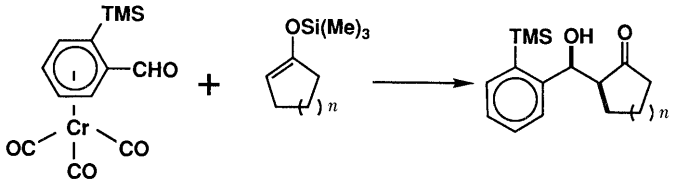
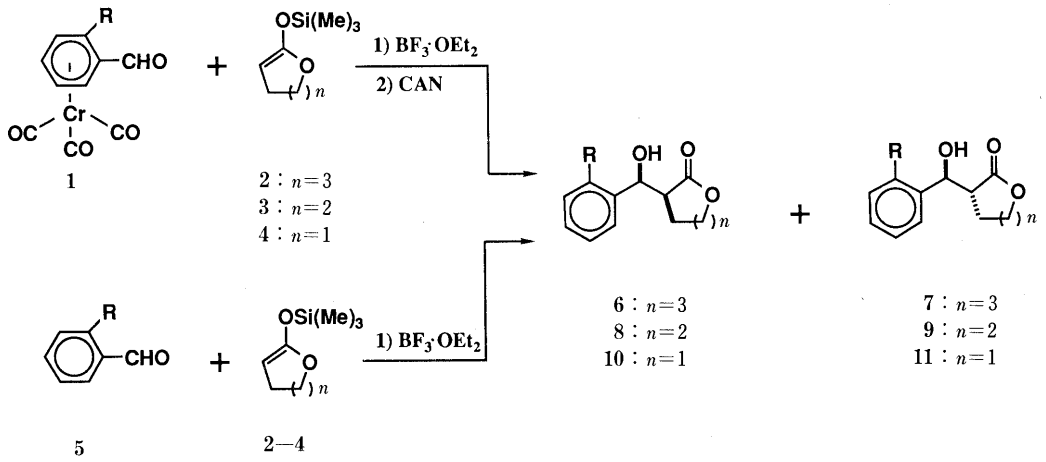


Chart 1



a : R=TMS b : R=Et c : R=Me d : R=OMe e : R=H f : R=TIPS

Chart 2

ptene, where the corresponding *erythro* isomer was exclusively formed. Other *ortho* substituents such as methyl and methoxy groups in the chromium complex exhibited much less selectivity, as anticipated.[2] Interestingly, the benzaldehyde–chromium complex (**1e**) provided the *threo* isomer predominantly, although the selectivity is rather low. The results obtained are summarized in Table I. Control experiments using the *ortho* substituted benzaldehydes (**5**) afforded the aldol products nonselectively (Table II) except for **5e**, which showed a moderate *threo* selectivity, as the complex (**1e**) did. This *threo*-selective reaction of **5e** is understandable based on the moderate *threo* selectivity observed in the reaction of **5e**[1,6] with trimethylsilyloxycyclohexene.

Since the *o*-TMS-benzaldehyde–chromium complex (**1a**) was found to provide the highest selectivity, we next examined the aldol reaction of **1a** with the six- and five-membered ketene silyl acetals (**3** and **4**). Upon treatment with **3** under conditions similar to those described for **2**, **1a** yielded a mixture of **8a** and **9a** in a ratio of 87 : 13, the *erythro* selectivity of which is in good agreement with that observed for **2**. However, it disappointingly became manifest that contracting the ring size from six to five enormously decreased the *erythro* selectivity. Indeed **1a** furnished a mixture of **10a** and **11a** in a ratio of 66 : 34 when exposed to **4** (Table I). The uncomplexed aldehyde (**5a**) did not exhibit any selectivity in the aldol reaction (Table II). To sum up these results, the aldol reaction of **1a** with cyclic ketene silyl acetals afforded the *erythro* isomer in a stereoselective manner, but the selectivity is generally lower than that obtained from the reaction between **1a** and cyclic silyl enol ethers.

We previously explained a high *erythro* selectivity obtained from the reaction of **1a** with cyclic silyl enol ethers in terms of an acyclic transition state[2] where the *ortho* TMS group in the complex plays an important role in producing the high selectivity. In the favorable transition states (A_1 and B_1), the smallest substituent, the hydrogen on the double bond of the silyl enol ether was placed in the most sterically demanding position to minimize the nonbonding interaction with the bulky *ortho* TMS moiety. When cyclic ketene silyl acetals (Chart 3, X = O: **2**—**4**) were employed instead of cyclic silyl enol ethers (X = CH_2), there was another significant interaction to be considered. Namely unfavorable dipole–dipole interaction between the ethereal oxygen of the acetals and the aldehyde oxygen might exist in the transition states (A_1 and B_1). This interaction seems not to be serious in other staggered transition states (A_2, A_3, B_2, and B_3). Therefore, the reaction might be able to proceed *via* other transition states besides A_1, resulting in a decrease of the *erythro* selectivity. The above analysis can also be applied to interpret the low *erythro* selectivity of **4** in comparison with that of **2** and **3**. Molecular model examination indicated that the dipole–dipole interaction is much severer in the case of the five-membered acetal (**4**) than in seven- and six-membered ones (**2** and **3**). If the *ortho* silyl group in the complex is sterically large enough to prevent an approach of substituents other than the smallest one, hydrogen, on the double bond of the cyclic acetal, steric factors will override electronic factors and again favor the transition state (A_1) leading to the *erythro* isomer. On the basis of the above consideration, we pursued the reaction of the chromium complex having the triisopropylsilyl (TIPS)

Table I. Aldol Reaction of Chromium-Complexes (**1**) with Cyclic Ketene Silyl Acetals (**2**—**4**)

Entry	Aldehyde	R	Ketene silyl acetal	Product[a]	Yield[b] (%)
1	**1a**	TMS	**2**	**6a** : **7a** = 90 : 10	72
2	**1b**	Et	**2**	**6b** : **7b** = 76 : 24	87
3	**1c**	Me	**2**	**6c** : **7c** = 66 : 34	98
4	**1d**	OMe	**2**	**6d** : **7d** = 53 : 47	62
5	**1e**	H	**2**	**6e** : **7e** = 33 : 67	63
6	**1a**	TMS	**3**	**8a** : **9a** = 87 : 13	61
7	**1a**	TMS	**4**	**10a** : **11a** = 66 : 34	91
8	**1f**	TIPS	**4**	**10f** : **11f** = 85 : 15	98
9	**1f**	TIPS	**3**	**8f** : **9f** = 83 : 17	85
10	**1f**	TIPS	**2**	**6f** : **7f** = 67 : 33	100

a) Ratios were determined from the 400-MHz ^{1}H-NMR spectra. *b*) Yield of a mixture of *erythro* and *threo* isomers.

Table II. Aldol Reaction of *o*-Substituted Benzaldehydes (**5**) with Cyclic Ketene Silyl Acetals (**2**—**4**)

Entry	Aldehyde	R	Ketene silyl acetal	Product[a]	Yield[b] (%)
1	**5a**	TMS	**2**	**6a** : **7a** = 47 : 53	99
2	**5b**	Et	**2**	**6b** : **7b** = 55 : 45	70
3	**5c**	Me	**2**	**6c** : **7c** = 47 : 53	94
4	**5d**	OMe	**2**	**6d** : **7d** = 54 : 46	88
5	**5e**	H	**2**	**6e** : **7e** = 22 : 78	99
6	**5a**	TMS	**3**	**8a** : **9a** = 42 : 58	66
7	**5a**	TMS	**4**	**10a** : **11a** = 47 : 53	85
8	**5f**	TIPS	**4**	**10f** : **11f** = 70 : 30	99
9	**5f**	TIPS	**3**	**8f** : **9f** = 84 : 16	98
10	**5f**	TIPS	**2**	**6f** : **7f** = 76 : 24	98

a) Ratios were determined from the 400-MHz ^{1}H-NMR spectra. *b*) Yield of a mixture of *erythro* and *threo* isomers.

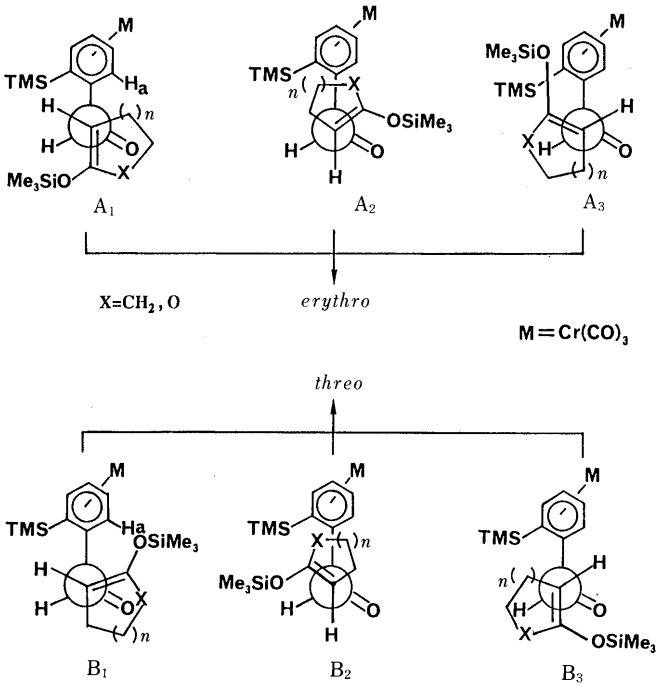


Chart 3

group at the *ortho* position.

The required *o*-TIPS-benzaldehyde–chromium complex (**1f**) was prepared according to the literature.[7] We first performed the aldol reaction of **1f** with the five-membered acetal (**4**) under the standard conditions to give the aldol adducts in 98% yield. The ratio of the *erythro* and *threo* isomers was determined to be 85 : 15. The *erythro* selectivity was greatly improved in comparison with the *o*-TMS-benzaldehyde derivative (**1a**) (*erythro* : *threo* = 66 : 34). A six-membered acetal (**3**) also showed a high *erythro* selectivity (*erythro* : *threo* = 83 : 17). However, the seven-membered acetal (**2**), the best nucleophile for *erythro* selectivity in the reaction of **1a**, produced a mixture of the *erythro* and *threo* isomers, the ratio of which was found to be only 67 : 33 (Table I). The *erythro* selectivity observed here was much lower than that of the *o*-TMS derivative (**1a**). In addition, control experiments using *o*-TIPS-benzaldehyde (**5f**) consistently provided a good *erythro* selectivity as shown in Table II.

We could not interpret all of these results in terms of only the aforementioned mechanism. However, we found that a high *erythro* selectivity could be achieved by appropriate choice of the *ortho* trialkylsilyl group (TMS or TIPS) in the chromium complex.

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were measured with a JASCO A-102 spectrometer in $CHCl_3$, mass spectra (MS) with a Hitachi M-80 mass spectrometer, and ^{1}H-NMR spectra with a JEOL JNM-GX 400 spectrometer in $CDCl_3$. Silica gel (Silica gel 60, 230—400 mesh, Nacalai Tesque) was used for chromatography. All reactions were carried out under a nitrogen atmosphere. Organic extracts were dried over anhydrous Na_2SO_4. The starting chromium complexes (**1a**,[2] **b**,[2] **c**,[8] **d**,[8] **e**,[8] **f**[7]) were prepared according to the literature.

General Procedure for the Aldol Reaction of the Chromium Complexes (1) with Cyclic Ketene Silyl Acetals (2—4) A solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (1 M solution, 1.2—2.0 eq) was slowly added to a solution of one of the complexes (**1**, 1.0 eq) and a ketene silyl acetal (**2**—**4**, 1.1—2.0 eq) in dry CH_2Cl_2 (5 ml) at −78 °C. The reaction mixture was stirred at the same temperature for 30 min—3 h. The reaction was monitored by thin-layer chromatography (TLC) and quenched by addition of saturated NH_4Cl solution (0.5 ml). The reaction mixture was washed with H_2O and brine, dried, and concentrated. The residue was then dissolved in MeOH (5 ml). CAN (3.0 eq) was added portionwise to the stirred MeOH solution at −20 °C. Stirring was continued until the decomplexation was completed (monitored by TLC, 10—20 min). MeOH was evaporated off and the residue was taken up in CH_2Cl_2. The CH_2Cl_2 solution was washed with H_2O and brine, dried, and concentrated to dryness. Chromatography of the residue with hexane–AcOEt (10 : 1—4 : 1) afforded the corresponding aldol product as a mixture of the *erythro* (**6**, **8**, **10**) and *threo* (**7**, **9**, **11**) isomers.

(*R,*R**)- and (*R**,*S**)-2-[Hydroxy(2-trimethylsilylphenyl)methyl]-6-hexanolide (6a and 7a)** The aldehyde (**1a**) (53 mg, 0.17 mmol) and **2** (40 mg, 0.21 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.25 ml) and then with CAN (283 mg, 0.52 mmol) to afford a mixture of **6a** and **7a** (36 mg, 90%, **6a** : **7a** = 90 : 10). Careful chromatography was repeated several times to provide **6a** and **7a** in pure form. **6a**: A colorless solid, mp 97—97.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3550 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.61—7.28 (4H, m, aromatic H), 5.48 (1H, d, J = 1.5 Hz, benzylic H), 4.35—4.10 (m, 2H), 3.44 (1H, br s, OH), 2.76—2.74 (1H, m), 2.09—1.28 (6H, m), 0.32 (9H, s, TMS). MS m/z (%): 292 (M^+, 0.38), 163 (100). *Anal.* Calcd for $C_{16}H_{24}O_3Si$: C, 65.71; H, 8.27. Found: C, 65.67; H, 8.49. **7a**: A colorless solid, mp 142—143 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3550 (OH), 1715 (C=O). ^{1}H-NMR δ: 7.54—7.29 (4H, m, aromatic H), 5.21 (1H, dd, J = 9.3, 3.4 Hz, benzylic H), 4.38—4.36 (2H, m), 3.54 (1H, d, J = 3.4 Hz, OH), 3.17—3.14 (1H, m), 1.94—1.31 (6H, m), 0.37 (9H, s, TMS). MS m/z (%): 292 (M^+, 0.16), 163 (100). *Anal.* Calcd for $C_{16}H_{24}O_3Si$: C, 65.71; H, 8.27. Found: C, 65.46; H, 8.51.

(*R,*R**)- and (*R**,*S**)-2-[(2-Ethylphenyl)hydroxymethyl]-6-hexanolide (6b and 7b)** The aldehyde (**1b**) (57 mg, 0.21 mmol) and **2** (79 mg, 0.43 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.42 ml) and then with CAN (346 mg, 0.63 mmol) to afford a mixture of **6b** and **7b** (46 mg, 87%, **6b** : **7b** = 76 : 24). Careful chromatography was repeated several times to provide **6b** and **7b** in pure form. **6b**: Colorless plates, mp 84—84.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.71—7.11 (4H, m, aromatic H), 5.49 (1H, br s, benzylic H), 4.25—3.96 (2H, m), 2.86—2.20 (4H, m), 1.97—1.04 (6H, m), 1.24 (3H, t, J = 7.6 Hz, $CH_2C\underline{H}_3$). MS m/z (%): 248 (M^+, 0.46), 114 (100). *Anal.* Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.53; H, 8.49. **7b**: A colorless solid, mp 97.5—99 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.54—7.06 (4H, m, aromatic H), 5.24 (1H, d, J = 9.3 Hz, benzylic H), 4.31—4.18 (2H, m), 3.17—2.20 (4H, m), 2.01—1.04 (6H, m), 1.24 (3H, t, J = 7.6 Hz, $CH_2C\underline{H}_3$). MS m/z (%): 248 (M^+, 0.27), 114 (100). *Anal.* Calcd for $C_{15}H_{20}O_3$: C, 72.55; H, 8.12. Found: C, 72.24; H, 8.46.

(*R,*R**)- and (*R**,*S**)-2-[Hydroxy(2-methylphenyl)methyl]-6-hexanolide (6c and 7c)** The aldehyde (**1c**) (149 mg, 0.58 mmol) and **2** (164 mg, 0.88 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (1.2 ml) and then with CAN (958 mg, 1.75 mmol) to afford a mixture of **6c** and **7c** (133 mg, 98%, **6c** : **7c** = 66 : 34). Careful chromatography was repeated several times to provide **6c** and **7c** in pure form. **6c**: Colorless pillars, mp 92.5—93 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3550 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.58—7.14 (4H, m, aromatic H), 5.43 (1H, br s, benzylic H), 4.37—4.15 (2H, m), 3.58 (1H, d, J = 2.8 Hz, OH), 2.76—2.74 (1H, m), 2.29 (3H, s, CH_3), 1.99—1.24 (6H, m). MS m/z (%): 234 (M^+, 6.9), 114 (100). *Anal.* Calcd for $C_{14}H_{18}O_3$: C, 71.77; H, 7.74. Found: C, 71.85; H, 8.00. **7c**: A colorless solid, mp 131.5—132.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1715 (C=O). ^{1}H-NMR δ: 7.41—7.18 (4H, m, aromatic H), 5.19 (1H, d, J = 10.5 Hz, benzylic H), 4.36—3.33 (2H, m), 3.60 (1H, br s, OH), 3.03—3.02 (1H, m), 2.39 (3H, s, CH_3), 1.90—1.44 (6H, m). MS m/z (%): 234 (M^+, 1.3), 114 (100). *Anal.* Calcd for $C_{14}H_{18}O_3$: C, 71.77; H, 7.74. Found: C, 71.50; H, 7.88.

(*R,*R**)- and (*R**,*S**)-2-[Hydroxy(2-methoxyphenyl)methyl]-6-hexanolide (6d and 7d)** The aldehyde (**1d**) (138 mg, 0.51 mmol) and **2** (113 mg, 0.61 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.76 ml) and then with CAN (834 mg, 1.52 mmol) to afford a mixture of **6d** and **7d** (78 mg, 62%, **6d** : **7d** = 53 : 47). Careful chromatography was repeated several times to provide **6d** and **7d** in pure form. **6d**: Colorless pillars, mp 125—125.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3550 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.57—6.86 (4H, m, aromatic H), 5.46 (1H, br s, benzylic H), 4.34—4.18 (2H, m), 3.83 (3H, s, OMe), 3.78 (1H, d, J = 3.1 Hz, OH), 3.04—3.01 (1H, m), 1.89—1.23 (6H, m). MS m/z (%): 250 (M^+, 13.3), 137 (100). *Anal.* Calcd for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25. Found: C, 67.00; H, 7.38. **7d**: A pale yellow solid, mp 79.5—80 °C (AcOEt–hexane). IR ν_{max} cm^{-1} 3400 (OH), 1715 (C=O). ^{1}H-NMR: δ: 7.52—6.83 (4H, m, aromatic H), 5.26 (1H, d, J = 7.1 Hz, benzylic H), 4.41—4.01 (3H, m), 3.84 (3H, s, OMe), 3.21—2.89 (1H, m), 2.15—1.10 (6H, m). MS m/z (%): 250 (M^+, 9.1), 137 (100). *Anal.* Calcd for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25. Found: C, 66.97; H, 7.43.

(*R,*R**)- and (*R**,*S**)-2-(1-Hydroxy-1-phenylmethyl)-6-hexanolide (6e and 7e)** The aldehyde (**1e**) (169 mg, 0.70 mmol) and **2** (158 mg, 0.85 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (1.05 ml) and then with CAN (1.15 g, 2.10 mmol) to afford a mixture of **6e** and **7e** (96 mg, 63%, **6e** : **7e** = 33 : 67). Careful chromatography was repeated several times to provide **6e** and **7e** in pure form. **6e**: Colorless needles, mp 100—101.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3550 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.38—7.27 (5H, m, aromatic H), 5.29 (1H, br s, benzylic H), 4.38—4.21 (2H, m), 3.70 (1H, d, J = 2.7 Hz, OH), 2.86—2.83 (1H, m), 1.92—1.25 (6H, m). MS m/z (%): 220 (M^+, 7.3), 114 (100). *Anal.* Calcd for $C_{13}H_{16}O_3$: C, 70.89; H, 7.32. Found: C, 70.82; H, 7.40. **7e**: Colorless plates, mp 166.5—167 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3500 (OH), 1710 (C=O). ^{1}H-NMR δ: 7.38—7.30 (5H, m, aromatic H), 4.84 (1H, dd, J = 8.6, 4.0 Hz, benzylic H), 4.37—4.29 (2H, m), 3.76 (1H, d, J = 4.0 Hz, OH), 2.96 (1H, dt, J = 8.6, 1.8 Hz), 1.94—1.26 (6H, m). MS m/z (%): 220 (M^+, 9.3), 114 (100). *Anal.* Calcd for $C_{13}H_{16}O_3$: C, 70.89; H, 7.32. Found: C, 70.75; H, 7.36.

(*R,*R**)- and (*R**,*S**)-2-[Hydroxy(2-trimethylsilylphenyl)methyl]-5-pentanolide (8a and 9a)** The aldehyde (**1a**) (50 mg, 0.16 mmol) and **3** (56 mg, 0.32 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.32 ml) and then with CAN (270 mg, 0.49 mmol) to afford a mixture of **8a** and **9a** (27 mg, 61%, **8a** : **9a** = 87 : 13), a colorless solid, mp 120.5—122 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1730 (C=O). ^{1}H-NMR δ: 7.59—7.26 (4H, m, aromatic H), 5.82 (87/100 × 1H, t, J = 2.8 Hz, benzylic H), 5.09 (13/100 × 1H, dd, J = 9.5, 1.5 Hz), 4.61—4.27 (2H, m),

3.07—2.63 (2H, m), 2.08—1.53 (4H, m), 0.39 (9H, s, TMS). *Anal.* Calcd for $C_{15}H_{22}O_3Si$: C, 64.71; H, 7.96. Found: C, 64.54; H, 8.16.

(*R*,R)- and (*R*,S**)-2-[Hydroxy(2-trimethylsilylphenyl)methyl]-4-butanolide (10a and 11a)** The aldehyde (**1a**) (63 mg, 0.20 mmol) and **4** (63 mg, 0.40 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.40 ml) and then with CAN (340 mg, 0.62 mmol) to afford a mixture of **10a** and **11a** (48 mg, 91%, **10a**:**11a**=66:34), colorless plates, mp 134.5—135.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1770, 1760 (C=O). ^{1}H-NMR δ: 7.58—7.26 (4H, m, aromatic H), 5.64 (66/100×1H, d, J=1.8 Hz, benzylic H), 5.00 (34/100×1H, d, J=9.8 Hz, benzylic H), 4.44—4.11 (2H, m), 3.19—2.66 (2H, m), 2.13—1.74 (2H, m), 0.39 (66/100×9H, s, TMS), 0.37 (34/100×9H, s, TMS). *Anal.* Calcd for $C_{14}H_{20}O_3Si$: C, 63.59; H, 7.62. Found: C, 63.52; H, 7.81.

(*R*,R)- and (*R*,S**)-2-[Hydroxy(2-triisopropylsilylphenyl)methyl]-6-hexanolide (6f and 7f)** The aldehyde (**1f**) (106 mg, 0.27 mmol) and **2** (74 mg, 0.40 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.40 ml) and then with CAN (447 mg, 0.81 mmol) to afford a mixture of **6f** and **7f** (100 mg, quantitative, **6f**:**7f**=67:33). Careful chromatography was repeated several times to provide **6f** and **7f** in pure form. **6f**: A colorless solid, mp 83.5—84.5 °C (AcOEt—hexane). IR ν_{max} cm^{-1}: 3380 (OH), 1715 (C=O). ^{1}H-NMR δ: 7.69—7.26 (4H, m, aromatic H), 5.41 (1H, br s, benzylic H), 4.34—4.05 (2H, m), 3.49 (1H, d, J=2.7 Hz, OH), 2.73—2.70 (1H, m), 2.25—1.24 (9H, m), 1.14 (9H, d, J=7.5 Hz, CHC$\underline{H}_3$), 1.08 (9H, d, J=7.5 Hz, CHC$\underline{H}_3$). Chemical ionization MS m/z (%): 377 (M^++1, 0.1), 41 (100). *Anal.* Calcd for $C_{22}H_{36}O_3Si$: C, 70.16; H, 9.63. Found: C, 69.77; H, 10.01. **7f**: A colorless solid, mp 138.5—139 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3400 (OH), 1715 (C=O). ^{1}H-NMR δ: 7.60—7.26 (4H, m, aromatic H), 5.16 (1H, dd, J=9.2, 3.6 Hz, benzylic H), 4.42—4.33 (2H, m), 3.37 (1H, d, J=3.6 Hz, OH), 3.20—3.15 (1H, m), 1.94—1.27 (9H, m), 1.16 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$), 1.14 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$). Chemical ionization MS m/z (%): 377 (M^++1, 0.2), 41 (100). *Anal.* Calcd for $C_{22}H_{36}O_3Si \cdot H_2O$: C, 69.79; H, 10.12. Found: C, 69.65; H, 10.26.

(*R*,R)- and (*R*,S**)-2-[Hydroxy(2-triisopropylsilylphenyl)methyl]-5-pentanolide (8f and 9f)** The aldehyde (**1f**) (65 mg, 0.16 mmol) and **3** (42 mg, 0.24 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.24 ml) and then with CAN (274 mg, 0.50 mmol) to afford a mixture of **8f** and **9f** (50 mg, 85%, **8f**:**9f**=83:17), a colorless solid, mp 124—125 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3430 (OH), 1725 (C=O). ^{1}H-NMR δ: 7.62—7.25 (4H, m, aromatic H), 5.71 (83/100×1H, br s, benzylic H), 5.00 (17/100×1H, d, J=9.1 Hz, benzylic H), 4.43—4.19 (2H, m), 2.99—2.62 (2H, m), 2.16—1.23 (7H, m), 1.19—1.11 (18H, m, CHC$\underline{H}_3$). Chemical ionization MS m/z (%): 363 (M^++1, 0.5), 41 (100).

(*R*,R)- and (*R*,S**)-2-[Hydroxy(2-triisopropylsilylphenyl)methyl]-4-butanolide (10f and 11f)** The aldehyde (**1f**) (60 mg, 0.15 mmol) and **4** (35 mg, 0.22 mmol) were treated with a solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (0.23 mmol) and then with CAN (250 mg, 0.46 mmol) to afford a mixture of **10f** and **11f** (52 mg, 98%, **10f**:**11f**=85:15). Careful chromatography was repeated several times to provide **10f** and **11f** in pure form. **10f**: A colorless solid, mp 124—124.5 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3375 (OH), 1770 (C=O). ^{1}H-NMR δ: 7.58—7.27 (4H, m, aromatic H), 5.54 (1H, br s, benzylic H), 4.45—4.11 (2H, m), 2.86—2.12 (3H, m), 2.21 (1H, d, J=3.7 Hz, OH), 1.68—1.48 (3H, m, C$\underline{H}$CH$_3$), 1.17 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$), 1.12 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$). Chemical ionization MS m/z (%): 349 (M^++1, 4.3), 87 (100). *Anal.* Calcd for $C_{20}H_{32}O_3Si$: C, 68.92; H, 9.25. Found: C, 68.45; H, 9.60. **11f**: A colorless solid, mp 99—101 °C (AcOEt–hexane). IR ν_{max} cm^{-1}: 3500 (OH), 1755 (C=O). ^{1}H-NMR δ: 7.58—7.29 (4H, m, aromatic H), 4.92 (1H, dd, J=9.2, 1.5 Hz, benzylic H), 4.36—4.16 (2H, m), 4.33 (1H, d, J=1.5 Hz, OH), 3.24—3.17 (1H, dt, J=9.2, 11.6 Hz), 2.03—1.75 (2H, m), 1.50—1.40 (3H, m, C$\underline{H}$CH$_3$), 1.14 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$), 1.12 (9H, d, J=7.3 Hz, CHC$\underline{H}_3$). Chemical ionization MS m/z (%): 349 (M^++1, 1.3), 87 (100). *Anal.* Calcd for $C_{20}H_{32}O_3Si$: C, 68.92; H, 9.25. Found: C, 68.68; H, 9.60.

General Procedure for the Aldol Reaction of *o*-Substituted Benzaldehydes (5) with Cyclic Ketene Silyl Acetals (2—4) A solution of $BF_3 \cdot OEt_2$ in dry CH_2Cl_2 (1 M solution, 1.2—2.0 eq) was slowly added to a solution of **5** (1.0 eq) and a ketene silyl acetal (**2**—**4**, 1.2—2.0 eq) in dry CH_2Cl_2 (5 ml) at −78 °C. The reaction mixture was stirred for 30 min—3 h at the same temperature. The reaction was monitored by TLC and quenched by addition of saturated NH_4Cl solution (0.5 ml). The reaction mixture was washed with H_2O and brine, dried, and concentrated to dryness. Chromatography of the residue gave the aldol products. The results are summarized in Table II.

References and Notes

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Marine Natural Products. XXVIII.[1] The Structures of Sarasinosides A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, and C_3, Nine New Norlanostane-Triterpenoidal Oligoglycosides from the Palauan Marine Sponge *Asteropus sarasinosum*

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The chemical structures of sarasinosides A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, and C_3, nine 30-norlanostane-triterpenoidal oligoglycosides isolated from the Palauan marine sponge *Asteropus sarasinosum* have been elucidated on the basis of chemical and physicochemical evidence. Sarasinosides A_2 (2) and A_3 (3) were shown to be the 7,9(11)-diene and 8,14-diene analogs of sarasinoside A_1 (1), whereas sarasinosides B_2 (7) and B_3 (9) were the 7,9(11)-diene and 8,14-diene analogs of sarasinoside B_1 (5), respectively. Similar structural correlations of sarasinosides C_2 (6) and C_3 (8) with sarasinoside C_1 (4) were demonstrated. These sarasinosides characteristically contain one mole each of *N*-acetylglucosamine and *N*-acetylgalactosamine in their oligosaccharide moieties.

Keywords sarasinoside A_1; sarasinoside A_2; sarasinoside A_3; sarasinoside B_1; sarasinoside C_1; marine saponin; norlanostane triterpene oligoglycoside; marine sponge; *Asteropus sarasinosum*; *N*-acetylglucosamine

Saponins occur very widely in the plant kingdom,[2,3] and among marine organisms, sea cucumber and starfish characteristically produce these saponins. Namely, sea cucumber produces lanostane-triterpenoidal oligoglycosides, while starfish metabolites steroidal oligoglycosides.

During the course of our studies in search of new biologically active marine natural products,[3] we have investigated the chemical constituents of the Palauan marine sponge *Asteropus sarasinosum* and isolated nine new ichthyotoxic norlanostane-triterpenoidal oligoglycosides named sarasinosides A_1 (**1**), A_2 (**2**), A_3 (**3**), B_1 (**5**), B_2 (**7**), B_3 (**9**), C_1 (**4**), C_2 (**6**), and C_3 (**8**).[4] This was the first isolation of saponins from a marine organism other than sea cucumber and starfish (echinoderm). These sarasinosides character-

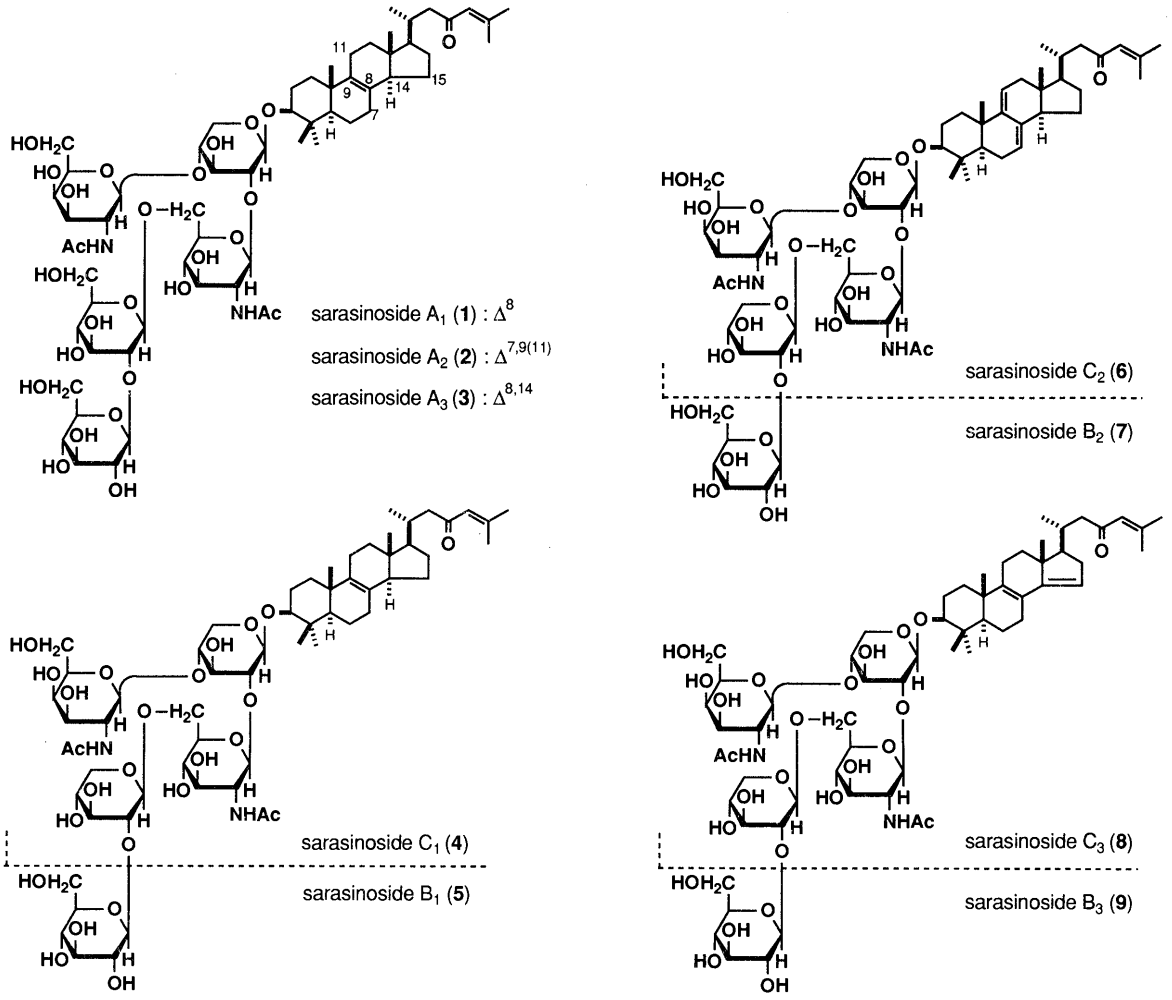


Chart 1

istically contain one mole each of *N*-acetylglucosamine and *N*-acetylgalactosamine in their oligosaccharide moieties. In a previous paper,[4] we reported the isolation of these nine oligoglycosides and the chemical structures of three major oligoglycosides: sarasinosides A_1 (**1**), B_1 (**5**), and C_1 (**4**).[5] This paper presents a full account of the chemical evidence supporting the proposed structures of sarasinosides A_1 (**1**), B_1 (**5**), and C_1 (**4**) and also deals with the structure elucidation of six congeneric oligoglycosides: sarasinosides A_2 (**2**), A_3 (**3**), B_2 (**7**), B_3 (**9**), C_2 (**6**), and C_3 (**8**).

The freeze-dried sponge, which was collected at −5 m in the Palau Islands, was extracted with methanol (MeOH) and the extract was partitioned into a mixture of ethyl acetate and water. The water-soluble portion was then partitioned into a mixture of 1-butanol and water. Chromatographic separation of the 1-butanol soluble portion provided three saponin fractions containing sarasinosides A, B, and C, which each gave a single spot on a thin-layer chromatogram (TLC). Each fraction was further separated by high-performance liquid chromatography (HPLC) finally to afford sarasinosides A_1 (**1**), A_2 (**2**), A_3 (**3**), B_1 (**5**), B_2 (**7**), B_3 (**9**), C_1 (**4**), C_2 (**6**), and C_3 (**8**) in 0.68, 0.25, 0.40, 0.50, 0.17, 0.33, 0.13, 0.04, and 0.10% yields, respectively, from the 1-butanol soluble portion.

Sarasinosides A_1 (1), B_1 (5), and C_1 (4) Sarasinoside A_1 (**1**), the major oligoglycoside, was obtained as fine crystals of mp 207—210 °C. In the infrared (IR) spectrum, it showed strong absorption bands at 3370 (br) and 1067 (br) cm^{-1}, indicative of glycosidic structure. Sarasinoside A_1 (**1**) showed an ultraviolet (UV) absorption maximum at 237 nm ($\varepsilon = 13500$) which was ascribable to a conjugated enone chromophore. The presence of this conjugated enone moiety was also suggested by the carbon-13 signals observed at δ_C 200.5 (s, C-23), 124.8 (d, C-24), and 153.7 (s, C-25), together with the signals of two olefinic carbons observed at δ_C 127.5 (s, C-8) and 136.4 (s, C-9) in the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum.

Acidic hydrolysis of sarasinoside A_1 (**1**) with 20% aqueous H_2SO_4–MeOH (1 : 1) yielded two secondarily formed sapogenols **10** and **11**. The proton nuclear magnetic resonance (^{1}H-NMR) decoupling experiments and the ^{13}C-NMR analysis of **10** and **11** led us to presume that **10** and **11** were double bond isomers having a norlanostane skeleton.[6] The relative configuration of **11** having a Δ^{14} double bond was established by X-ray crystallographic analysis.[4] The ^{1}H- and ^{13}C-NMR spectra of **11** showed the signals due to the Δ^{14} double bond at δ 5.13 (m, 15-H) and δ_C 155.8 (s, C-14), 117.2 (d, C-15) together with the signals assignable to the conjugated enone moiety in the side chain at δ 6.07 (s, 24-H) and δ_C 200.6 (s, C-23), 125.0 (d, C-24), and 153.9 (s, C-25). The ^{13}C-NMR spectrum of the other sapogenol **10** showed the signals of two olefinic carbons, which were assigned to the $\Delta^{8(14)}$ double bond, at δ_C 127.1 (s, C-8) and 141.8 (s, C-14), and the signals of the conjugated enone moiety in the side chain at δ_C 200.6 (s, C-23), 125.0 (d, C-24), and 153.9 (s, C-25). On the other hand, the ^{13}C-NMR signals assignable to the tetrasubstituted olefinic carbons in the aglycone part of sarasinoside A_1 (**1**) were observed at δ_C 127.5 (s) and 136.4 (s) as mentioned above. Interestingly, acidic hydrolysis of **1** under milder conditions, *e.g.* 10% H_2SO_4–MeOH (1 : 1) provided the sapogenol **10** as a single product. Comparison in detail

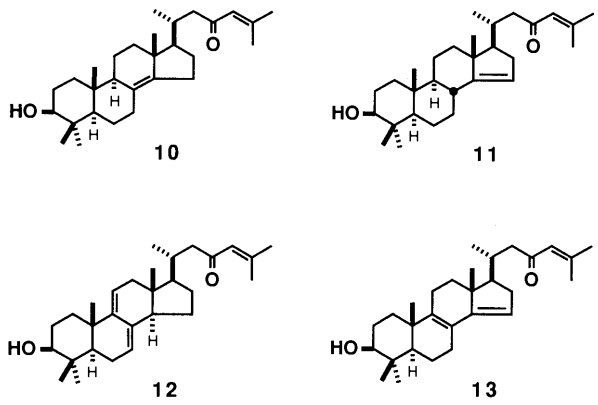


Chart 2

of the ^{13}C-NMR data for **1**, **10**, and **11** with those for known compounds[6] led us to conclude that the genuine aglycone of **1** has a Δ^8 double bond as depicted in **14**, and the acidic hydrolysis of **1** gave rise to double bond migration in the aglycone part to liberate the artifactual sapogenols **10** and **11** having a thermodynamically more favored double bond.[7]

Sarasinoside A_1 (**1**) is a pentaglycoside comprising 2 mol of D-glucose and 1 mol each of D-xylose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine in its oligosaccharide portion. The ^{1}H- and ^{13}C-NMR spectra of **1** showed signals at δ 5.58 (d, $J = 8.6$ Hz), 5.56 (d, $J = 8.5$ Hz), 5.20 (d, $J = 7.6$ Hz), 5.15 (d, $J = 8.6$ Hz), and 4.53 (d, $J = 8.2$ Hz) and at δ_C 106.7, 105.5, 102.6, 102.2, and 101.7 (each d) which suggested the β-anomeric configurations of these five monosaccharide constituents. In order to determine the sequence in the oligosaccharide portion, **1** was subjected to enzymatic hydrolysis with crude hesperidinase to afford two prosapogenols, A_1-pro-1 (**15**) and A_1-pro-2 (**16**).

A_1-pro-1 (**15**) was a diglycoside having 1 mol each of xylose and *N*-acetylglucosamine. Methanolysis of fully methylated A_1-pro-1, which was prepared by complete methylation of **15**, liberated methyl 3,4-di-*O*-methylxylopyranoside. Thus, the structure of A_1-pro-1 has been determined as **15**.

A_1-pro-2 (**16**) was a tetraglycoside having 1 mol each of xylose, glucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine in its carbohydrate portion. Methanolysis of fully methylated A_1-pro-2, which was also obtained by complete methylation of **16**, liberated methyl 2,3,4,6-tetra-*O*-methylglucopyranoside and methyl 3-*O*-methylxylopyranoside. Another enzymatic hydrolysis of **1** using β-glucosidase (type II from almonds) gave another tetraglycoside A_1-pro-3 (**17**) which comprised 2 mol of glucose and 1 mol each of xylose and *N*-acetylglucosamine in its carbohydrate portion. Complete methylation of **17** followed by methanolysis liberated methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 3,4,6-tri-*O*-methylglucopyranoside, and methyl 3,4-di-*O*-methylxylopyranoside. In the ^{13}C-NMR spectrum of **16** and **17**, the signals of C-6″ in the *N*-acetylglucosamine moieties were observed at a lower field (δ_C 69.8 in **16** and δ_C 69.6 in **17**) than the C-6″ signal of **15** (δ_C 62.5) due to the glycosidation shift.[8,9a] Based on the foregoing evidence, the structures of A_1-pro-2 and A_1-pro-3 have been determined as **16** and **17**, respectively.

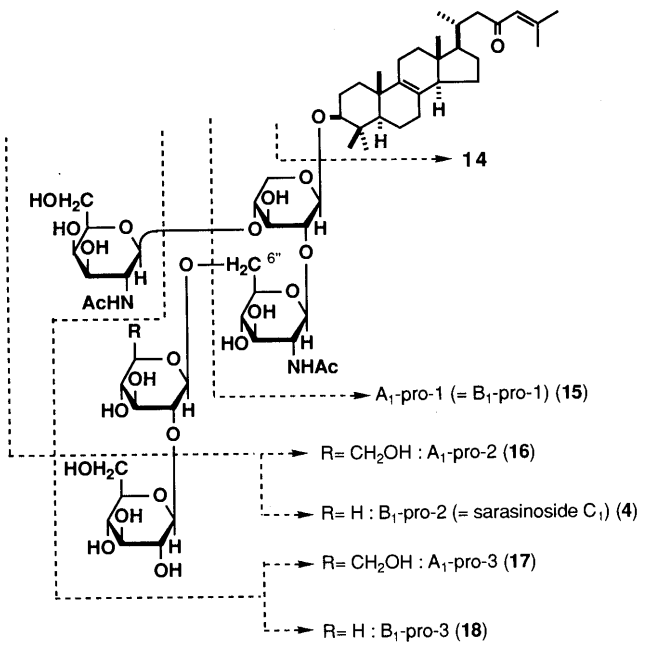


Chart 3

In a similar manner, complete methylation followed by methanolysis of sarasinoside A_1 (**1**) liberated methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 3,4,6-tri-*O*-methylglucopyranoside, and methyl 3-*O*-methylxylopyranoside. Consequently, the structure of sarasinoside A_1 (**1**) has been determined to be as shown. In the ordinary methylation analysis of **1** and its prosapogenols (**15**, **16**, **17**) mentioned above, unambiguous results in regard to the branching at the *N*-acetylamino sugar moieties were not obtained. A similar result was also experienced in the following structure elucidation of sarasinoside B_1 (**5**). Direct chemical evidence on the branching at the *N*-acetylamino sugar moiety has been obtained by other means as described later.

Sarasinoside B_1 (**5**) was obtained as fine crystals of mp 197—199 °C. It showed a UV absorption maximum at 239 nm ($\varepsilon = 13600$) similarly to sarasinoside A_1 (**1**). Sarasinoside B_1 (**5**) is a pentaglycoside containing 2 mol of D-xylose and 1 mol each of D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine. Acidic hydrolysis of **5** yielded two artifactual sapogenols **10** and **11** as in the case of sarasinoside A_1 (**1**). The ^{13}C-NMR spectrum of **5** showed the Δ^8 olefinic carbon signals at δ_C 127.7 (s, C-8) and 136.4 (s, C-9) together with many other signals closely resembling those of **1**, except for some signals due to the oligosaccharide moiety. Thus, the genuine aglycone of sarasinoside B_1 (**5**) has been shown to be identical with that of sarasinoside A_1 (**1**).

The ^{1}H- and ^{13}C-NMR spectra of sarasinoside B_1 (**5**) showed signals at δ 5.56 (d, $J = 8.5$ Hz), 5.43 (d, $J = 7.3$ Hz), 5.21 (d, $J = 7.3$ Hz), 5.20 (d, $J = 8.2$ Hz), and 4.57 (d, $J = 7.6$ Hz) and at δ_C 106.7, 105.5, 103.2, 102.3, and 102.0 (each d), which suggested the β-anomeric configurations of the five monosaccharide moieties in **5**. Enzymatic hydrolysis of **5** using crude hesperidinase furnished B_1-pro-1 ($= A_1$-pro-1) (**15**) and B_1-pro-2 (**4**), whereas another enzymatic hydrolysis of **5** using β-glucosidase afforded B_1-pro-3 (**18**). Among these partial hydrolysates, B_1-pro-1 was shown to be identical with A_1-pro-1 (**15**).

Acidic hydrolysis of the prosapogenol B_1-pro-2 (**4**), a tetraglycoside, furnished 2 mol of xylose and 1 mol each of *N*-acetylglucosamine and *N*-acetylgalactosamine. Methanolysis of fully methylated B_1-pro-2, prepared by complete methylation of **4**, liberated methyl 2,3,4-tri-*O*-methylxylopyranoside and methyl 3-*O*-methylxylopyranoside. On the other hand, acidic hydrolysis of B_1-pro-3 (**18**) furnished 2 mol of xylose and 1 mol each of glucose and *N*-acetylglucosamine. Complete methylation of **18** followed by methanolysis liberated methyl 2,3,4,6-tetra-*O*-methylglucopyranoside and methyl 3,4-di-*O*-methylxylopyranoside. Based on the above-mentioned evidence, the structures of B_1-pro-2 and B_1-pro-3 have been determined as **4** and **18**, respectively.

Furthermore, complete methylation of sarasinoside B_1 (**5**) followed by methanolysis liberated methyl 2,3,4,6-tetra-*O*-methylglucopyranoside, methyl 3,4-di-*O*-methylxylopyranoside, and methyl 3-*O*-methylxylopyranoside. Consequently, the structure of sarasinoside B_1 (**5**) has been concluded to be as shown.

Sarasinoside C_1 was a tetraglycoside containing 2 mol of xylose and 1 mol each of *N*-acetylglucosamine and *N*-acetylgalactosamine in its carbohydrate portion. Acidic hydrolysis of sarasinoside C_1 (**4**) provided two artifactual sapogenols **10** and **11**, as were obtained above on the acidic hydrolysis of sarasinosides A_1 (**1**) and B_1 (**5**). Eventually, sarasinoside C_1 was shown to be identical with B_1-pro-2 (**4**).

As mentioned above in connection with the methylation analysis of sarasinosides A_1 (**1**), B_1 (**5**), and C_1 (**4**), rather complicated products were obtained from the *N*-acetylamino sugar moieties. Thus, in the initial complete methylation, *N*-methylation of *N*-acetylglucosamine and *N*-acetylgalactosamine moieties occurred, so that subsequent methanolysis of the fully methylated derivatives of **1**, **5**, and **4** gave rise to ready cleavage of the *N*-acetamide linkage to yield HCl salts of *N*-methylglucosamine and *N*-methylgalactosamine derivatives. These co-occurring reactions presumably resulted in the failure of the gas-liquid chromatographic (GLC) and TLC analyses of the methylated *N*-acetylglucosamine and *N*-acetylgalactosamine moieties.

In order to get more direct proof of the carbohydrate sequence of the *N*-acetylamino sugar moieties, fully methylated derivatives of sarasinosides A_1 (**1**) and B_1 (**5**) were subjected to Hakomori's degradation method.[10] Thus, the fully methylated derivatives of **1** and **5** were each heated with H_2SO_4–AcOH. Then, the reaction mixture was diluted with water and the whole mixture was heated again. The hydrolysates thus obtained were treated with sodium borohydride and subsequently acetylated with acetic anhydride. After purification, partially methylated hexitol acetates were obtained as follows: 3-*O*-methyl-1,2,4,5-tetra-*O*-acetylxylitol (**19**), 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol (**20**), 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetylglucitol (**21**) from **1**; **19**, **20**, 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetylxylitol (**22**) from **5**; and 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol (**23**) and 3,4-di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol (**24**) from both **1** and **5**. The latter *N*-methylacetamidohexitols, **23** and **24**, were identified by direct comparisons with authentic samples which were synthesized from *N*-acetyl-D-glucosamine and *N*-acetyl-D-galac

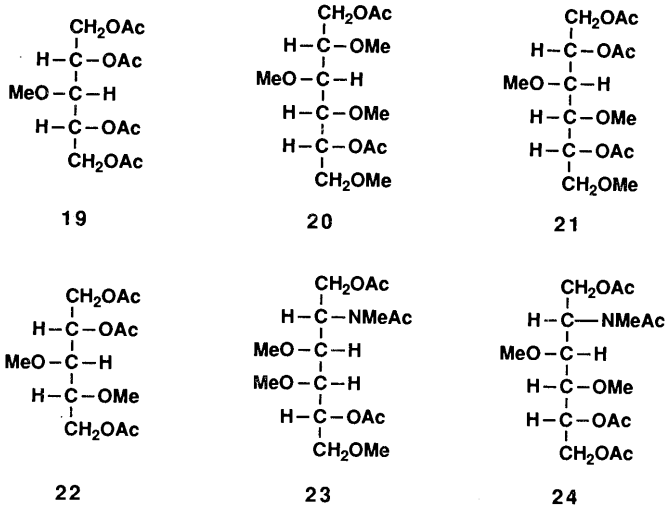
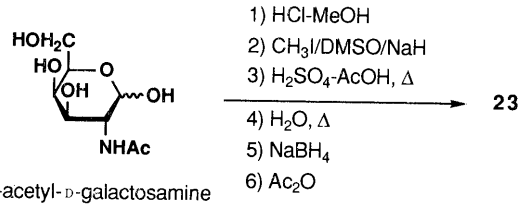


Chart 4



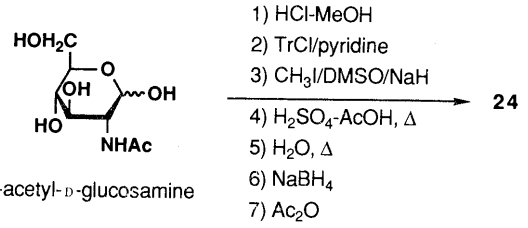


Chart 5

tosamine through the procedures shown in Chart 5. The ^{1}H-NMR spectra of **23** and **24** showed that **23** and **24** were each present as a mixture of two conformers in solution.

Sarasinosides A_2 (2) and A_3 (3) Sarasinoside A_2 (**2**) and sarasinoside A_3 (**3**), which moved with the same *Rf* value as sarasinoside A_1 (**1**) on ordinary TLC, were obtained as fine crystals having the same melting point, 205—208 °C. Sarasinoside A_2 (**2**) showed UV absorption maxima at 239 (shoulder) nm (ε=22000), 243 (23500), 251 (sh) (16500) and an IR absorption band at 1630 (br) cm^{-1}, which were attributable to a conjugated enone and *N*-acetylamino moieties. The IR spectrum of **2** also showed strong, broad absorption bands at 3360 and 1050 cm^{-1} due to the glycosidic structure (Table I). The circular dichroism (CD) spectrum of **2** showed complex maxima ($[\theta]_{234}$ +6200, $[\theta]_{248}$ −3500, $[\theta]_{256}$ −3200) ascribable to the conjugated diene and conjugated enone moieties in **2**.

Acidic hydrolysis of sarasinoside A_2 (**2**) with 3% aqueous H_2SO_4 provided another sapogenol **12**. The sapogenol (**12**) showed UV absorption maxima at 239 (sh) nm (ε=22900),

Table I. UV, CD, and ^{13}C-NMR Data for Sarasinosides A_1 (**1**), A_2 (**2**), A_3 (**3**), B_1 (**5**), B_2 (**7**), B_3 (**9**), C_1 (**4**), C_2 (**6**), C_3 (**8**) and Sapogenols (**12**, **13**)

Compound	UV λ_{max} nm (ε)	CD $[\theta]_{nm}$ (peak or trough)	^{13}C-NMR δ_C (Olef. C of sapogenol)
Sarasinoside A_1 (**1**)	237 (13500)	222 (−6600)	127.5 (s), 136.4 (s)
Sarasinoside A_2 (**2**)	239sh(22000) 243 (23500) 251sh(16500)	234 (+6200) 248 (−3500) 256 (−3200)	117.2 (d), 122.0 (d) 135.8 (s), 147.3 (s)
Sarasinoside A_3 (**3**)	245 (22000)	231 (−15900)	116.9 (d), 122.8 (s) 142.3 (s), 151.4 (s)
Sarasinoside B_1 (**5**)	239 (13600)	222 (−7200)	127.7 (s), 136.4 (s)
Sarasinoside B_2 (**7**)	239sh(21700) 243 (22800) 251sh(16200)	233 (+8000) 248 (−3500) 256 (−4000)	117.2 (d), 121.9 (d) 135.8 (s), 147.2 (s)
Sarasinoside B_3 (**9**)	243 (21600)	231 (−14800)	116.9 (d), 122.8 (s) 142.1 (s), 151.2 (s)
Sarasinoside C_1 (**4**)	237 (13600)	222 (−6500)	127.3 (s), 135.9 (s)
Sarasinoside C_2 (**6**)	239sh(22500) 243 (23800) 251sh(16800)	234 (+4700) 248 (−5000) 255 (−4400)	117.0 (d), 121.7 (d) 135.8 (s), 147.0 (s)
Sarasinoside C_3 (**8**)	244 (25900)	231 (−15900)	116.7 (d), 122.7 (s) 141.9 (s), 151.1 (s)
12	239sh(22900) 243 (24000) 250sh(17800)	229 (+1200) 247 (−2700) 254 (−2200)	117.4 (d), 121.9 (d) 136.1 (s), 147.3 (s)
13	243 (33300)	231 (−5500)	117.1 (d), 123.1 (s) 142.5 (s), 151.6 (s)

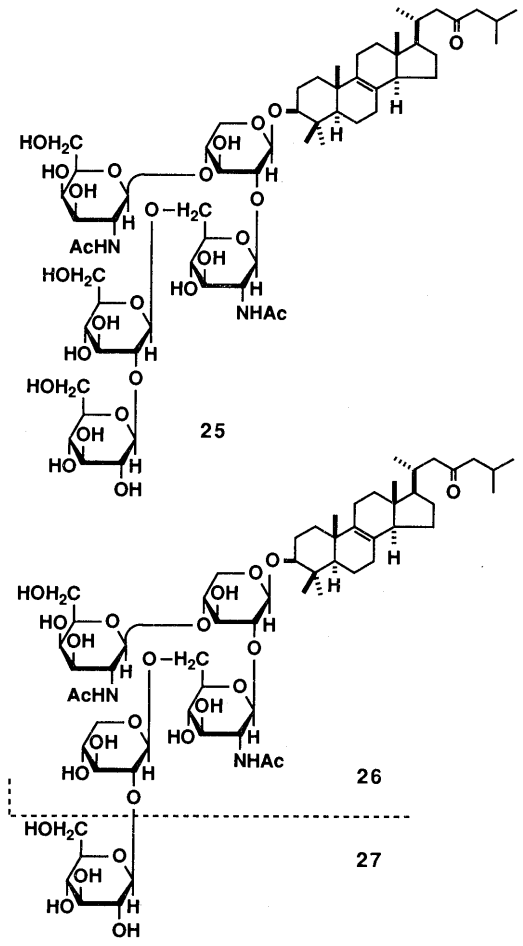


Chart 6

243 (24000), 250 (sh) (17800) and complex CD maxima ($[\theta]_{229}$ +1200, $[\theta]_{247}$ −2700, $[\theta]_{254}$ −2200) which were similar to those observed for **2**. The ^{1}H-NMR spectrum of **12** showed four singlet methyl signals at δ 0.55, 0.90, 0.98, and 1.01, a doublet methyl signal at δ 0.95 (J=6.4 Hz), and two olefinic proton signals at δ 5.40 and 5.45 (both m) together with the signals of an olefinic proton [δ 6.06 (s)] and two olefinic methyls [δ 1.89, 2.15 (both, s)] ascribable to the 24-en-23-one structure in the side chain. The ^{1}H- and ^{13}C-NMR analysis[6] in detail of **12** disclosed that **12** was a 7,9(11)-diene analog of the above-described norlanostane-type sapogenol **11**, which was obtained by the acidic hydrolysis of sarasinoside A_1 (**1**) and the structure of which had been determined by X-ray crystallographic analysis.[4]

The carbon signals assignable to the sugar moiety in the ^{13}C-NMR spectrum of sarasinoside A_2 (**2**) were shown to be superimposable on those of sarasinoside A_1 (**1**). So, **2** was presumed to be a 7,9(11)-diene analog of **1**. In order to verify this presumption, sarasinoside A_2 (**2**) was subjected to catalytic hydrogenation over 10% palladium–carbon to afford a tetrahydro derivative **25**, which was shown to be identical with a reduction product prepared from sarasinoside A_1 (**1**) under the same reaction conditions. Based on the combined evidence, the structure of sarasinoside A_2 (**2**) has been determined to be as shown.

Sarasinoside A_3 (**3**) showed a UV absorption maximum at 245 nm (ε=22000) and a negative CD maximum at $[\theta]_{231}$ −15900 due to its conjugated diene and conjugated enone chromophores. Acidic hydrolysis of sarasinoside A_3 (**3**) with 5% aqueous H_2SO_4–MeOH (1 : 1) furnished another sapogenol **13**. The sapogenol (**13**) showed a UV absorption maximum at 243 nm (ε=33300) and a negative CD maximum ($[\theta]_{231}$ −5500) similar to those of the parent glycoside sarasinoside A_3 (**3**). The ^{1}H-NMR spectrum of **13** showed four singlet methyl signals at δ 0.84, 0.86, 1.02, and 1.04, a doublet methyl signal at δ 0.97 (J=6.4 Hz) and one olefinic proton signal at δ 5.34 (m) together with the signals of an olefinic proton [δ 6.08 (s)] and two olefinic methyls [δ 1.89, 2.15 (both s)], which were ascribable to the 24-en-23-one structure as seen in the aglycone parts of sarasinoside A_1 (**1**) and A_2 (**2**). The ^{1}H-NMR decoupling experiments and the ^{13}C-NMR analysis in detail of **13** led us to presume that **13** was an 8,14-diene isomer of the sapogenol **12**. The carbon signals assignable to the aglycone part in the ^{13}C-NMR spectrum of sarasinoside A_3 (**3**) were very similar to those of **13**, whereas the carbon signals assignable to the sugar moiety were shown to be superimposable on those of sarasinoside A_1 (**1**). These findings indicated that **3** was an 8,14-diene analog of **1**. In order to verify this, sarasinoside A_3 (**3**) was subjected to catalytic hydrogenation over 10% palladium–carbon to afford the tetrahydro derivative **25**, which was identical with the reduction product obtained above from sarasinoside A_1 (**1**). Consequently, the chemical structure of sarasinoside A_3 has been determined to be **3**.

Sarasinosides B_2 (7) and C_2 (6) Sarasinoside B_2 (**7**) and sarasinoside C_2 (**6**) were obtained as fine crystals of mp 192—195 °C and mp 188—191 °C, respectively. The ^{13}C-NMR spectra of **7** and **6** closely resembled the spectrum of sarasinoside A_2 (**2**) except for some signals due to the oligosaccharide moieties. Sarasinosides B_2 (**7**) and C_2 (**6**) showed characteristic UV absorption maxima: 239 (sh) nm (ε=21700), 243 (22800), 251 (sh) (16200) for **7**; 239 (sh) (22500), 243 (23800), 251 (sh) (16800) for **6**, and CD maxima: $[\theta]_{233}$ +8000, $[\theta]_{248}$ −3500, $[\theta]_{256}$ −4000 for **7**; $[\theta]_{234}$ +4700, $[\theta]_{248}$ −5000, $[\theta]_{255}$ −4400 for **6**, which were very similar to the maxima observed for sarasinoside A_2 (**2**) (Table I). Acidic hydrolysis of sarasinosides B_2 (**7**) and C_2 (**6**) with 3% aqueous H_2SO_4 respectively provided the same sapogenol **12**. So, it was shown that sarasinosides B_2 (**7**) and C_2 (**6**) possessed the same aglycone **12** as sarasinoside A_2 (**2**).

Sarasinoside B_2 (**7**) and sarasinoside C_2 (**6**) moved with the same *Rf* values as sarasinoside B_1 (**5**) and C_1 (**4**), respectively, on ordinary TLC. The carbon signals due to the sugar moiety in the ^{13}C-NMR spectrum of **7** were superimposable on those of **5**, while the carbon signals due to the sugar moiety of **6** were superimposable on those of **4**. Thus, **7** and **6** were presumed to be the 7,9(11)-diene analogs of **5** and **4**, respectively. In order to verify this, **7** and **6** were each subjected to catalytic hydrogenation over 10% palladium–carbon to afford their tetrahydro derivatives **27** and **26**. The reduction products, **27** and **26**, thus obtained were shown to be identical with the reduction products prepared from sarasinosides B_1 (**5**) and C_1 (**4**) under the same reaction conditions. Consequently, the chemical structures of sarasinosides B_2 and C_2 have been determined to be **7** and **6**, respectively.

Sarasinosides B_3 (9) and C_3 (8) Sarasinoside B_3 (**9**) and sarasinoside C_3 (**8**) were obtained as fine crystals of mp 190—193 °C and 187—190 °C, respectively. Sarasinosides B_3 (**9**) and C_3 (**8**) showed UV absorption maxima at 243 nm (ε=21600) (for **9**) and at 244 nm (ε=25900) (for **8**) and negative CD maxima of $[\theta]_{231}$ −14800 (for **9**) and $[\theta]_{231}$ −15900 (for **8**), which were characteristically similar to those observed for sarasinoside A_3 (**3**). Acidic hydrolysis of **9** and **8** with 5% aqueous H_2SO_4–MeOH (1 : 1) furnished the same sapogenol **13**. Therefore, it was shown that the aglycone of sarasinosides B_3 (**9**) and C_3 (**8**) had the same structure as that of sarasinoside A_3 (**3**).

The mobilities of sarasinoside B_3 (**9**) and sarasinoside C_3 (**8**) on ordinary TLC were the same as those of sarasinosides B_1 (**5**) and C_1 (**4**), as observed among the foregoing sarasinoside congenors, *e.g.* A_1 (**1**), A_2 (**2**), and A_3 (**3**). The carbon signals assignable to the sugar moiety in the ^{13}C-NMR spectrum of sarasinoside B_3 (**9**) closely resembled those observed for sarasinoside B_1 (**5**), while the carbon signals due to the sugar moiety of sarasinoside C_3 (**8**) were shown to be superimposable on those of sarasinoside C_1 (**4**). These findings indicated that **9** and **8** were the 8,14-diene analogs of **5** and **4**, respectively. In order to substantiate this presumption, sarasinosides B_3 (**9**) and C_3 (**8**) were subjected to catalytic hydrogenation over 10% palladium–carbon to afford the tetrahydro derivatives **27** and **26**. The products, **27** and **26**, thus obtained were shown to be identical with the reduction products which were synthesized from sarasinoside B_1 (**5**) and C_1 (**4**), respectively, through the same reaction procedures. Based on the accumulated evidence, the structures of sarasinoside B_3 and C_3 have been determined to be **9** and **8**, respectively.

The structures of sarasinosides elucidated in this paper are characterized by a norlanostane-triterpenoid aglycone. As far as we know, this is the first example of naturally occurring norlanostane-triterpenoidal saponins. The nor-

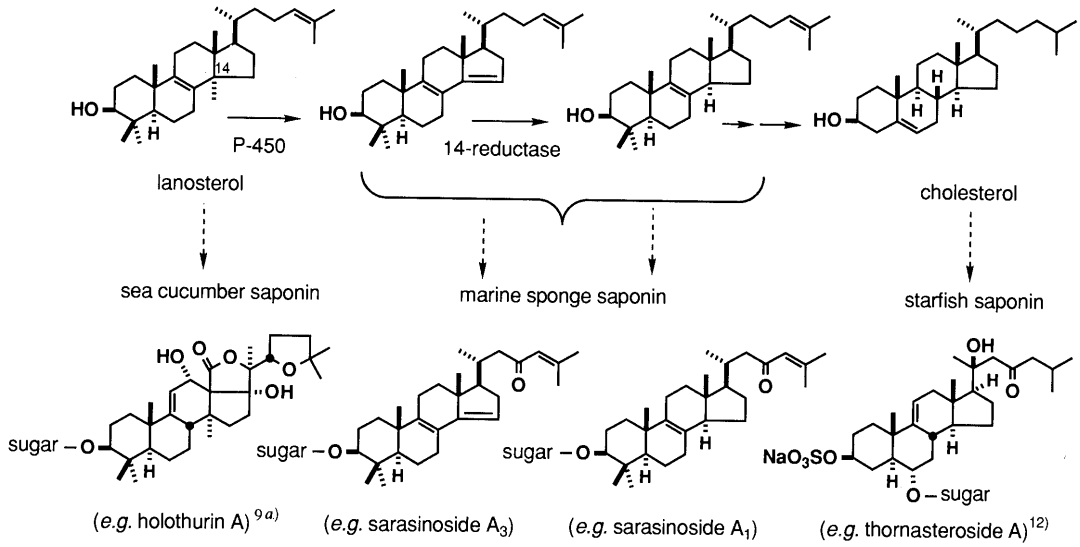


Fig. 1. Conversion of Lanosterol to 4,4-Dimethylzymosterol by Yeast Microsomes

lanostane-triterpenoid, the carbon skeleton of the aglycone of sarasinosides, has been placed as an intermediate in the biogenetic pathway from lanosterol to cholesterol in yeast and animal organs, where oxidative elimination of the 14α-methyl group has been demonstrated.[11] It is noteworthy from the viewpoint of chemosystematics that saponins from sea cucumbers possess lanostane-triterpenoidal aglycones[2,3,9] and those from starfish comprise steroidal aglycones,[12] while the saponins isolated in this paper from the marine sponge *Asteropus sarasinosum* have 30-norlanostane triterpenoids as their aglycones, although it remains to be proved that they are really biosynthesized by the respective parent organisms (Fig. 1).

Among the nine sarasinosides, the major saponins sarasinosides A_1 (**1**) and B_1 (**5**) have been subjected to some biological activity tests. It has been found so far that **1** and **5** exhibit piscicidal activity (against *Pocilia reticulata*), with LD_{50} (48 h) = 0.39 μg/ml for **1** and 0.71 μg/ml for **5**, and inhibit the cell division of fertilized eggs of the starfish *Asterina pectinifera* with LD_{100} = 10 μg/ml for **1** and **5**.

Experimental

The instruments used to obtain physical data and experimental conditions for chromatography were the same as described in our preceding paper.[13]

Isolation of Sarasinosides A_1 (1), A_2 (2), A_3 (3), B_1 (5), B_2 (7), B_3 (9), C_1 (4), C_2 (6), and C_3 (8) Freeze-dried whole marine sponge (1.2 kg) *Asteropus sarasinosum* (collected in the Palau Islands), was extracted with 95% aqueous MeOH (20 l) at room temperature 3 times for 8 h each. The MeOH extract obtained after evaporation of the solvent *in vacuo* was partitioned into an ethyl acetate–H_2O mixture and then the H_2O phase was further partitioned with 1-butanol to furnish the 1-butanol-soluble portion (140 g after evaporation of the solvent). The 1-butanol-soluble portion (20 g) was then purified by column chromatography [Silica gel 60, 60—230 mesh (Merck), 2 kg, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)→6 : 4 : 1] to give three oligoglycoside fractions [sarasinoside A (1.4 g), sarasinoside B (1.1 g), and sarasinoside C (0.3 g)]. The oligoglycoside fractions were further subjected to HPLC (Zorbax ODS, MeOH : H_2O = 5 : 1) to isolate nine oligoglycosides: sarasinosides A_1 (**1**) (136 mg), A_2 (**2**) (49 mg), and A_3 (**3**) (80 mg) from the sarasinoside A fraction, sarasinosides B_1 (**5**) (100 mg), B_2 (**7**) (33 mg), and B_3 (**9**) (67 mg) from the sarasinoside B fraction, and sarasinosides C_1 (**4**) (26 mg), C_2 (**6**) (7 mg), C_3 (**8**) (20 mg) from the sarasinoside C fraction, respectively.

Sarasinoside A_1 (**1**): mp 207—210 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −14° (c = 1, MeOH, 25 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3370 (br), 2929, 2870, 1640 (br), 1550, 1067 (br). CD ($c = 1.0 \times 10^{-1}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.60 (3H, s), 1.00 (3H, d, J = 5.8 Hz, 20-CH_3), 1.01, 1.16, 1.29 (each 3H, s), 1.75 (3H, s, 25-CH_3), 2.04, 2.09 (both 3H, s, NHCOCH_3), 2.15 (3H, s, 25-CH_3), 3.12 (1H, dd, J = 4.4, 11.1 Hz, 3α-H), 4.53 (1H, d, J = 8.2 Hz), 5.15 (1H, d, J = 8.6 Hz), 5.20 (1H, d, J = 7.6 Hz), 5.56 (1H, d, J = 8.5 Hz), 5.58 (1H, d, J = 8.6 Hz), 6.14 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 172.0, 171.5 (both s, NHCOCH_3), 153.7 (s, C-25), 136.4 (s, C-9), 127.5 (s, C-8), 124.8 (d, C-24), 106.7 (d), 105.5 (d), 102.6 (d), 102.2 (d), 101.7 (d), 90.1 (d, C-3). *Anal.* Calcd for $C_{62}H_{100}N_2O_{26} \cdot 2H_2O$: C, 56.18; H, 7.91; N, 2.11. Found: C, 56.36; H, 7.79; N, 2.16.

Sarasinoside A_2 (**2**): mp 205—208 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −5.5° (c = 0.5, MeOH, 23 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3360 (br), 2915, 2873, 1630 (br), 1548, 1050 (br). CD ($c = 5.8 \times 10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.54 (3H, s), 0.99 (3H, d, J = 4.6 Hz, 20-CH_3), 1.03, 1.25, 1.29 (each 3H, s), 1.74 (3H, s, 25-CH_3), 2.03, 2.09 (both 3H, s, NHCOCH_3), 2.16 (3H, s, 25-CH_3), 3.15 (1H, dd, J = 3.7, 11.3 Hz, 3α-H), 4.55 (1H, d, J = 7.6 Hz), 5.18 (1H, d, J = 7.9 Hz), 5.20 (1H, d, J = 9.2 Hz), 5.53 (1H, d, J = 7.9 Hz), 5.55 (1H, d, J = 8.5 Hz), 5.33 (1H, m), 5.44 (1H, m), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.6 (s, C-23), 172.0, 171.5 (both s, NHCOCH_3), 153.9 (s, C-25), 147.3 (s, C-9), 135.8 (s, C-8), 124.9 (d, C-24), 122.0 (d, C-7), 117.2 (d, C-11), 106.8 (d), 105.6 (d), 102.8 (d), 102.4 (d), 101.9 (d), 90.1 (d, C-3). *Anal.* Calcd for $C_{62}H_{98}N_2O_{26} \cdot 2H_2O$: C, 56.27; H, 7.77; N, 2.12. Found: C, 56.20; H, 8.00; N, 1.94.

Sarasinoside A_3 (**3**): mp 205—208 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −22° (c = 1.2, MeOH, 23 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3290 (br), 2902, 2869, 1630 (br), 1549, 1050 (br). CD ($c = 5.5 \times 10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.85 (3H, s), 1.02 (3H, d, J = 6.4 Hz, 20-CH_3), 1.07, 1.17, 1.30 (each 3H, s), 1.75 (3H, s, 25-CH_3), 2.04, 2.09 (both 3H, s, NHCOCH_3), 2.16 (3H, s, 25-CH_3), 3.13 (1H, dd, J = 4.4, 11.5 Hz, 3α-H), 4.57 (1H, d, J = 7.6 Hz), 5.19 (1H, d, J = 8.6 Hz), 5.22 (1H, d, J = 7.6 Hz), 5.59 (1H, d, J = 8.9 Hz), 5.61 (1H, d, J = 8.9 Hz), 5.37 (1H, br s, 15-H), 6.16 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 171.9, 171.5 (both s, NHCOCH_3), 153.9 (s, C-25), 151.4 (s, C-14), 142.3 (s, C-9), 124.9 (d, C-24), 122.8 (s, C-8), 116.9 (d, C-15), 106.7 (d), 105.6 (d), 102.7 (d), 102.2 (d), 101.8 (d), 89.9 (d, C-3). *Anal.* Calcd for $C_{62}H_{98}N_2O_{26} \cdot 2H_2O$: C, 56.27; H, 7.77; N, 2.12. Found: C, 56.13; H, 7.93; N, 2.10.

Sarasinoside B_1 (**5**): mp 197—199 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −16° (c = 0.99, MeOH, 20 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3340 (br), 2933, 2871, 1640 (br), 1554, 1063 (br). CD ($c = 5.6 \times 10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.62, 0.98 (both 3H, s), 1.01 (3H, d, J = 5.5 Hz, 20-CH_3), 1.16, 1.32 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.04, 2.08 (both 3H, s, NHCOCH_3), 2.16 (3H, s, 25-CH_3), 3.15 (1H, dd, J = 4.3, 11.9 Hz, 3α-H), 4.57 (1H, d, J = 7.6 Hz), 5.20 (1H, d, J = 8.2 Hz), 5.21 (1H, d, J = 7.3 Hz), 5.43 (1H, d, J = 7.3 Hz), 5.56 (1H, d, J = 8.5 Hz), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.7 (s, C-23), 172.0, 171.5 (both s, NHCOCH_3), 153.8 (s, C-25), 136.4 (s, C-9), 127.7 (s, C-8), 124.9 (d, C-24),

106.7 (d), 105.5 (d), 103.2 (d), 102.3 (d), 102.0 (d), 90.0 (d, C-3). *Anal.* Calcd for $C_{61}H_{98}N_2O_{25}\cdot 2H_2O$: C, 56.56; H, 7.94; N, 2.16. Found: C, 56.61; H, 7.88; N, 2.32.

Sarasinoside B_2 (**7**): mp 192—195 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −10° ($c=0.3$, MeOH). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3370 (br), 2912, 2866, 1640 (br), 1542, 1065 (br). CD ($c=1.3\times10^{-1}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine−D_2O) δ: 0.54 (3H, s), 1.00 (6H, br s), 1.23, 1.31 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.03, 2.08 (both 3H, s, NHCOCH_3), 2.17 (3H, s, 25-CH_3), 3.16 (1H, dd, $J=4.1$, 11.8 Hz, 3α-H), 4.58 (1H, d, $J=7.3$ Hz), 5.20 (1H, d, $J=7.3$ Hz), 5.23 (1H, d, $J=8.5$ Hz), 5.38 (1H, d, $J=7.3$ Hz), 5.54 (1H, d, $J=8.5$ Hz), 5.35 (1H, m), 5.44 (1H, m), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 172.0, 171.4 (both s, NHCOCH_3), 153.8 (s, C-25), 147.2 (s, C-9), 135.8 (s, C-8), 124.9 (d, C-24), 121.9 (d, C-7), 117.2 (d, C-11), 106.7 (d), 105.3 (d), 103.2 (d), 102.3 (d), 102.0 (d), 89.8 (d, C-3). *Anal.* Calcd for $C_{61}H_{96}N_2O_{25}$: C, 58.27; H, 7.70; N, 2.23. Found: C, 58.12; H, 8.00; N, 2.28.

Sarasinoside B_3 (**9**): mp 190—193 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −23° ($c=0.4$, MeOH, 25 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3360 (br), 2919, 2875, 1640 (br), 1547, 1071 (br). CD ($c=8.3\times10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.87, 1.03 (both 3H, s), 1.03 (3H, d, $J=5.8$ Hz, 20-CH_3), 1.16, 1.31 (both 3H, s), 1.75 (3H, s, 25-CH_3), 2.04, 2.08 (both 3H, s, NHCOCH_3), 2.18 (3H, s, 25-CH_3), 3.15 (1H, dd, $J=4.3$, 11.9 Hz, 3α-H), 4.58 (1H, d, $J=7.3$ Hz), 5.22 (2H, d, $J=7.9$ Hz), 5.49 (1H, d, $J=7.6$ Hz), 5.57 (1H, d, $J=8.2$ Hz), 5.37 (1H, br s, 15-H), 6.16 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 171.9, 171.5 (both s, NHCOCH_3), 153.8 (s, C-25), 151.2 (s, C-14), 142.1 (s, C-9), 124.8 (d, C-24), 122.8 (s, C-8), 116.9 (d, C-15), 106.4 (d), 105.3 (d), 103.0 (d), 102.1 (d), 101.8 (d), 89.6 (d, C-3). *Anal.* Calcd for $C_{61}H_{96}N_2O_{25}$: C, 58.27; H, 7.70; N, 2.23. Found: C, 58.29; H, 8.01; N, 2.34.

Sarasinoside C_1 (**4**): mp 194—197 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −22° ($c=2.65$, MeOH, 25 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3320 (br), 2933, 2860, 1637 (br), 1546, 1043 (br). CD ($c=6.7\times10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.60, 0.94 (both 3H, s), 1.01 (3H, d, $J=5.5$ Hz, 20-CH_3), 1.12, 1.27 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.09, 2.10 (both 3H, s, NHCOCH_3), 2.15 (3H, s, 25-CH_3), 3.18 (1H, dd, $J=4.3$, 11.9 Hz, 3α-H), 4.62 (1H, d, $J=7.3$ Hz), 4.94 (1H, d, $J=7.6$ Hz), 5.09 (1H, d, $J=8.6$ Hz), 5.43 (1H, d, $J=8.5$ Hz), 6.14 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 172.1, 171.7 (both s, NHCOCH_3), 153.7 (s, C-25), 135.9 (s, C-9), 127.3 (s, C-8), 124.5 (d, C-24), 104.6 (d), 104.3 (d), 101.6 (2C, d), 89.0 (d, C-3). *Anal.* Calcd for $C_{55}H_{88}N_2O_{20}\cdot 2H_2O$: C, 58.29; H, 8.18; N, 2.47. Found: C, 58.02, H, 7.88; N, 2.44.

Sarasinoside C_2 (**6**): mp 188—191 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −11° ($c=0.3$, MeOH, 25 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3370 (br), 2930, 2873, 1640 (br), 1547, 1050 (br). CD ($c=7.5\times10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.53, 0.95 (both 3H, s), 0.98 (3H, d, $J=6.1$ Hz, 20-CH_3), 1.19, 1.26 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.08, 2.10 (both 3H, s, NHCOCH_3), 2.15 (3H, s, 25-CH_3), 3.18 (1H, dd, $J=3.8$, 12.1 Hz, 3α-H), 4.60 (1H, d, $J=7.3$ Hz), 4.94 (1H, d, $J=7.3$ Hz), 5.10 (1H, d, $J=8.2$ Hz), 5.39 (1H, d, $J=8.5$ Hz), 5.35 (1H, m), 5.41 (1H, m), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.5 (s, C-23), 172.3, 171.6 (both s, NHCOCH_3), 153.8 (s, C-25), 147.0 (s, C-9), 135.8 (s, C-8), 124.7 (d, C-24), 121.7 (d, C-7), 117.0 (d, C-11), 105.0 (d), 104.5 (d), 102.0 (2C, d), 89.1 (d, C-3). *Anal.* Calcd for $C_{55}H_{86}N_2O_{20}\cdot 2H_2O$: C, 58.39; H, 8.02; N, 2.48. Found: C, 58.65; H, 8.16; N, 2.44.

Sarasinoside C_3 (**8**): mp 187—190 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −31° ($c=0.9$, MeOH, 25 °C). UV (MeOH) λ_{max}: Table I. IR (KBr) ν_{max} cm^{-1}: 3380 (br), 2913, 2865, 1630 (br), 1543, 1040 (br). ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.85, 0.98 (both 3H, s), 1.03 (3H, d, $J=6.1$ Hz, 20-CH_3), 1.12, 1.27 (both 3H, s), 1.75 (3H, s, 25-CH_3), 2.08, 2.09 (both 3H, s, NHCOCH_3), 2.16 (3H, s, 25-CH_3), 3.17 (1H, dd, $J=4.0$, 11.6 Hz, 3α-H), 4.63 (1H, d, $J=7.0$ Hz), 4.95 (1H, d, $J=7.3$ Hz), 5.11 (1H, d, $J=8.2$ Hz), 5.43 (1H, d, $J=8.2$ Hz), 5.37 (1H, br s, 15-H), 6.16 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.4 (s, C-23), 172.7, 171.8 (both s, NHCOCH_3), 153.8 (s, C-25), 151.1 (s, C-14), 141.9 (s, C-9), 124.7 (d, C-24), 122.7 (s, C-8), 116.7 (d, C-15), 104.8 (d), 104.4 (d), 101.8 (2C, d), 88.8 (d, C-3). *Anal.* Calcd for $C_{55}H_{86}N_2O_{20}\cdot H_2O$: C, 59.34; H, 7.97; N, 2.52. Found: C, 59.16; H, 8.16; N, 2.51.

Acidic Hydrolysis of Sarasinosides A_1 (1), B_1 (5), and C_1 (4) A mixture of **1** (60 mg) and 20% aqueous H_2SO_4–MeOH (1 : 1) (2 ml) was heated under reflux for 3 h on a water-bath. After dilution with H_2O, the reaction mixture was extracted with AcOEt. The AcOEt extract was washed with aqueous saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure from the AcOEt extract gave a product (20 mg), which was purified by column chromatography (SiO_2 treated with $AgNO_3$, benzene : acetone = 30 : 1) to furnish the sapogenols, **10** (9 mg) and **11** (5 mg). **10**: mp 124—127 °C (MeOH–AcOEt), colorless needles. $[\alpha]_D$ +5.2° ($c=0.5$, $CHCl_3$, 20 °C). UV (MeOH) λ_{max} nm (ε): 236 (12200). IR (CCl_4) ν_{max} cm^{-1}: 3628, 2938, 2872, 1690, 1621, 1448, 1029. CD ($c=1.2\times10^{-2}$, MeOH): $[\theta]_{223}$ −7600 (neg. max.), $[\theta]_{203}$ +5500 (pos. max.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.75, 0.81, 0.89 (each 3H, s), 0.95 (3H, d, $J=6.1$ Hz, 20-CH_3), 1.01 (3H, s), 1.88, 2.14 (both 3H, s, 25-CH_3), 3.26 (1H, dd, $J=4.1$, 11.8 Hz, 3α-H), 6.06 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.6 (s, C-23), 153.9 (s, C-25), 141.8 (s, C-14), 127.1 (s, C-8), 125.0 (d, C-24), 78.4 (d, C-3). Mass *m/z* (%): 426 (8, M^+), 408 (3), 83 (100). High-resolution Mass Calcd for $C_{29}H_{46}O_2$: 426.350. Found: 426.349. **11**: mp 153—155 °C (MeOH–AcOEt), colorless needles. $[\alpha]_D$ +12.2° ($c=0.18$, $CHCl_3$, 20 °C). UV (MeOH) λ_{max} nm (ε): 239 (13300). IR (CCl_4) ν_{max} cm^{-1}: 3622, 2930, 2850, 1688, 1618, 1446, 1031. CD ($c=1.77\times10^{-2}$, MeOH): $[\theta]_{242}$ −1400 (neg. max.), $[\theta]_{210}$ −1900 (neg. max.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.81, 0.89 (both 3H, s), 0.94 (3H, d, $J=6.4$ Hz, 20-CH_3), 0.95, 0.98 (both 3H, s), 1.89, 2.15 (both 3H, s, 25-CH_3), 3.21 (1H, dd, $J=4.6$, 11.6 Hz, 3α-H), 5.13 (1H, m, 15-H), 6.07 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.6 (s, C-23), 155.8 (s, C-14), 153.9 (s, C-25), 125.0 (d, C-24), 117.2 (d, C-15), 78.3 (d, C-3). Mass *m/z* (%): 426 (4, M^+), 408 (3), 125 (100). High-resolution Mass Calcd for $C_{29}H_{46}O_2$: 426.350. Found : 426.349.

Sarasinosides B_1 (**5**) (20 mg) and C_1 (**4**) (15 mg) were hydrolyzed as described above to give the sapogenols, **10** and **11**, respectively. Compounds **10** and **11** thus obtained were shown to be identical with authentic samples obtained above from **1** by TLC and mass spectral comparisons. Furthermore, a mixture of **1** (23 mg) and 10% aqueous H_2SO_4–MeOH (1 : 1) (2 ml) was heated under reflux for 2 h on a water-bath. The reaction mixture was worked up as described above to give **10** (3 mg) as a single product.

Enzymatic Hydrolysis of Sarasinoside A_1 (1) a) A suspension of **1** (350 mg) in H_2O (5 ml) was treated with crude hesperidinase (500 mg, lot No. 680930 provided by Tanabe Pharm. Co.) and the whole mixture was stirred at 40 °C for 4 d. The reaction mixture was then treated with 1-butanol (5 ml), heated at 60 °C for 10 min and filtered. The filtrate was extracted with 1-butanol and the 1-butanol extract was washed with H_2O and concentrated under reduced pressure to give a product (280 mg). Purification of the product by column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] furnished A_1-pro-1 (**15**) (30 mg) and A_1-pro-2 (**16**) (62 mg). A_1-pro-1 (**15**): mp 171—174 °C (80% MeOH), fine crystals. $[\alpha]_D$ −20° ($c=0.3$, MeOH, 20 °C). UV (MeOH) λ_{max} nm (ε): 238 (12200). IR (KBr) ν_{max} cm^{-1}: 3310 (br), 2935, 2865, 1640 (br), 1544, 1071 (br). CD ($c=2.74\times10^{-2}$, MeOH): $[\theta]_{222}$ −5600 (neg. max). ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.59, 0.89 (both 3H, s), 1.00 (3H, d, $J=5.8$ Hz, 20-CH_3), 1.06, 1.27 (both 3H, s), 1.75 (3H, s, 25-CH_3), 2.09 (3H, s, NHCOCH_3), 2.14 (3H, s, 25-CH_3), 3.22 (1H, dd, $J=4.1$, 11.9 Hz, 3α-H), 4.76 (1H, d, $J=7.0$ Hz), 5.62 (1H, d, $J=8.5$ Hz), 6.14 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.9 (s, C-23), 172.0 (s, NHCOCH_3), 154.0 (s, C-25), 135.9 (s, C-9), 127.3 (s, C-8), 124.5 (d, C-24), 104.6 (d), 101.7 (d), 89.2 (d, C-3), 62.5 (t, C-6″). *Anal.* Calcd for $C_{42}H_{67}NO_{11}\cdot 2H_2O$: C, 63.21; H, 8.46; N, 1.76. Found: C, 63.55; H, 8.73; N, 1.75. A_1-pro-2 (**16**): mp 196—198 °C (80% MeOH), fine crystals. $[\alpha]_D$ −13° ($c=0.26$, MeOH, 20 °C). UV (MeOH) λ_{max} nm (ε): 239 (13800). IR (KBr) ν_{max} cm^{-1}: 3360 (br), 2941, 2875, 1655 (br), 1549, 1069 (br). CD ($c=4.05\times10^{-2}$, MeOH): $[\theta]_{222}$ −7200 (neg. max.). ^{1}H-NMR (500 MHz, d_5-pyridine+D_2O) δ: 0.60, 0.94 (both 3H, s), 1.01 (3H, d, $J=5.8$ Hz, 20-CH_3), 1.13, 1.27 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.09, 2.10 (both 3H, s, NHCOCH_3), 2.15 (3H, s, 25-CH_3), 3.17 (1H, dd, $J=4.3$, 11.9 Hz, 3α-H), 4.61 (1H, d, $J=7.3$ Hz), 5.05 (1H, d, $J=7.6$ Hz), 5.10 (1H, d, $J=8.2$ Hz), 5.46 (1H, d, $J=8.5$ Hz), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.7 (s, C-23), 172.2, 171.7 (both s, NHCOCH_3), 153.8 (s, C-25), 135.8 (s, C-9), 127.5 (s, C-8), 124.7 (d, C-24), 104.6 (d), 104.5 (d), 101.9 (d), 101.6 (d), 89.3 (d, C-3), 69.8 (t, C-6″). *Anal.* Calcd for $C_{56}H_{90}N_2O_{21}\cdot 3H_2O$: C, 56.93; H, 8.19; N, 2.37. Found: C, 56.68; H, 7.87; N, 2.43.

b) A suspension of **1** (200 mg) in H_2O (5 ml) was treated with β-glucosidase (type II, from almonds) (400 mg) and the whole was kept stirring at 40 °C for 6 d. The 1-butanol extractive (170 mg), which was obtained by working up of the reaction mixture as for the above-described hydrolysis with crude hesperidinase, was subjected to column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to furnish A_1-pro-3 (**17**) (20 mg) and unchanged **1** (80 mg). A_1-pro-3 (**17**): mp

189—192 °C (80% MeOH), fine crystals. $[\alpha]_D$ −25° (c = 0.2, MeOH, 20 °C). UV (MeOH) λ_{max} nm (ε): 237 (13400). IR (KBr) ν_{max} cm^{-1}: 3390 (br), 2936, 2847, 1629 (br), 1555, 1077 (br). CD ($c = 4.3 \times 10^{-2}$, MeOH): $[\theta]_{223}$ −5500 (neg. max.). ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.65 (3H, s), 1.05 (3H, d, J = 6.3 Hz, 20-CH_3), 1.07, 1.24, 1.34 (each 3H, s), 1.75 (3H, s, 25-CH_3), 1.95 (3H, s, NHCOCH_3), 2.21 (3H, s, 25-CH_3), 3.25 (1H, dd, J = 4.3, 11.9 Hz, 3α-H), 4.78 (1H, d, J = 7.3 Hz), 5.28 (1H, d, J = 7.0 Hz), 5.53 (1H, d, J = 7.3 Hz), 5.79 (1H, d, J = 6.7 Hz), 6.16 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.8 (s, C-23), 171.8 (s, NHCOCH_3), 153.8 (s, C-25), 135.8 (s, C-9), 127.5 (s, C-8), 124.8 (d, C-24), 106.3 (d), 105.6 (d), 102.8 (d), 101.5 (d), 90.0 (d, C-3), 69.6 (t, C-6″). *Anal.* Calcd for $C_{54}H_{87}NO_{21} \cdot 2H_2O$: C, 57.79; H, 7.99; N, 1.25. Found: C, 57.94; H, 8.18; N, 1.26.

Carbohydrate Composition of Sarasinoside A_1 (1) and Prosapogenols (15, 16, 17) A solution of **1**, **15**, **16**, or **17** (3 mg each) in anhydrous 9% HCl–MeOH (0.5 ml) was heated under reflux for 1 h. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated under reduced pressure to give the product, which was trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (0.2 ml) in pyridine (0.1 ml), and the resulting trimethylsilyl derivatives were quantitatively analyzed by GLC 2% silicone SE-30 on Uniport B 80—100 mesh; 3 mm × 2 m; column temperature, 180 °C; N_2 flow rate, 35 ml/min) to identify methyl xylopyranoside (a) (t_R = 2 min 57 s, 3 min 11 s), methyl glucopyranoside (b) (t_R = 7 min 07 s, 7 min 47 s), methyl 2-acetamide-2-deoxy-galactopyranoside (c) (t_R = 16 min 02 s), and methyl 2-acetamide-2-deoxy-glucopyranoside (d) (t_R = 17 min 54 s). As standards, 10 mg each of xylose, glucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine were treated in a similar manner. The relative integrated areas (given in parentheses) of the GLC peaks were as follows: **1**: a (1), b (2), c (1), d (1); **15**: a (1), d (1); **16**: a (1), b (1), c (1), d (1); **17**: a (1), b (2), d (1). A solution of sarasinosides mixture (1.2 g) in anhydrous 9% HCl–MeOH (10 ml) was heated under reflux for 1 h. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The filtrate was evaporated under reduced pressure to give the product (1.4 g), which was separated by column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] to furnish methyl glucopyranoside (213 mg), methyl xylopyranoside (199 mg) and a mixture of methyl aminoglycosides (286 mg). The mixture of methyl aminoglycosides (286 mg) was further separated by HPLC (Zorbax ODS, MeOH : H_2O = 1 : 10) to give methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (78 mg) and methyl 2-acetamido-2-deoxy-α-D-galactopyranoside (45 mg), which were identical with authentic samples by ^{1}H-NMR, HPLC, TLC, and $[\alpha]_D$ comparisons. Both methyl glucopyranoside (90 mg) and methyl xylopyranoside (126 mg) obtained above were hydrolyzed with 5% aqueous HCl (3 ml) under reflux for 1 h, respectively. The reaction mixtures were neutralized with Dowex 1 × 2 OH^- form and filtered. After removal of the solvent, both products were purified by column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to give D-glucose (20 mg) and D-xylose (30 mg), which were identical with the respective authentic samples by ^{1}H-NMR, TLC, and $[\alpha]_D$ comparisons.

Methylation of A_1-pro-1 (15) Followed by Methanolysis 1) A solution of **15** (18 mg) in dimethyl sulfoxide (DMSO) (1 ml) was treated with a dimsyl carbanion solution (1 ml) [prepared from NaH (2 g), which was washed with dry *n*-hexane before use, and DMSO (35 ml) by stirring at 60 °C for 1 h under an N_2 atmosphere] and the whole solution was sirred at room temperature (25 °C) for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (1 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. The reaction mixture was then poured into ice-H_2O and the whole was extracted with AcOEt. The AcOEt extract was washed with H_2O, then dried over $MgSO_4$. Removal of the solvent from the AcOEt extract under reduced pressure gave a product, which was purified by column chromatography (SiO_2, benzene : acetone = 3 : 1) to furnish the fully methylated derivative (10 mg); IR (CCl_4): no OH.

2) A solution of the fully methylated derivative (4 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1 h and neutralized with Ag_2CO_3. The whole mixture was filtered and the filtrate was subjected to GLC analysis [1) 15% polyethylene glycol succinate (PEGS) on Chromosorb WAW (80—100 mesh), 3 mm × 2 m; column temperature, 190 °C; N_2 flow rate, 35 ml/min, and 2) 5% butane disuccinate (BDS) on Uniport B (80—100 mesh); 3 mm × 2 m; column temperature, 165 °C; N_2 flow rare, 35 ml/min] and TLC (benzene–acetone = 3 : 1). Methyl 3,4-di-*O*-methylxylopyranoside [I] was identified [1) PEGS t_R = 4 min 55 s, 5 min 59 s; 2) BDS t_R = 4 min 50 s, 5 min 53 s; Rf = 0.32].

Methylation of A_1-pro-2 (16) Followed by Methanolysis 1) A solution of **16** (20 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (2 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (1 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work up of the reaction mixture as described above gave a product, which was purified by column chromatography (SiO_2, benzene : acetone = 3 : 1) to furnish the fully methylated derivative (13 mg); IR (CCl_4): no OH.

2) A solution of the fully methylated derivative (5 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was examined by GLC and TLC (benzene : acetone = 1 : 1). The following methyl glycosides were identified: methyl 3-*O*-methylxylopyranoside [II] [1) PEGS t_R = 12 min 52 s, 20 min 02 s; 2) BDS t_R = 11 min 49 s; Rf = 0.34] and methyl 2,3,4,6-tetra-*O*-methylglucopyranoside [III] [1) PEGS t_R = 3 min 15 s, 4 min 27 s; 2) BDS t_R = 3 min 41 s, 5 min 11 s; Rf = 0.75, 0.80].

Methylation of A_1-pro-3 (17) Followed by Methanolysis 1) A solution of **17** (15 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (2 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (2 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work-up of the reaction mixture as described above gave a product, which was purified by column chromatography (SiO_2, benzene : acetone = 3 : 1) to furnish the fully methylated derivative (7 mg); IR (CCl_4): no OH.

2) A solution of the fully methylated derivative (4 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1.5 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was analyzed by GLC and TLC (benzene : acetone = 3 : 1) to identify [I] [1) PEGS t_R = 4 min 58 s, 6 min 03 s; 2) BDS t_R = 4 min 37 s, 5 min 32 s; Rf = 0.34], [III] [1) PEGS t_R = 3 min 22 s, 4 min 36 s; 2) BDS t_R = 3 min 45 s, 5 min 18 s; Rf = 0.50, 0.60], and methyl 3,4,6-tri-*O*-methylglucopyranoside [IV] [1) PEGS t_R = 9 min 50 s, 11 min 54 s; 2) BDS t_R = 10 min 47 s, 13 min 07 s; Rf = 0.26].

Methylation of Sarasinoside A_1 (1) Followed by Methanolysis 1) A solution of **1** (20 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (2 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (2 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work-up of the reaction mixture as described above gave a product, which was purified by column chromatography (SiO_2, benzene : acetone = 1 : 1) to furnish the fully methylated derivative (10 mg); IR (CCl_4) ν_{max} cm^{-1}: no OH, 2926, 1707, 1648, 1057 (br).

2) A solution of the fully methylated derivative (4 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1.5 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was analyzed by GLC [3) 15% neopentyl glycol succinate (NPGS) on Chromosorb WAW (80—100 mesh), 3 mm × 2 m; column temperature, 190 °C; N_2 flow rate 35 ml/min] and TLC (benzene : acetone = 1 : 1) to identify [II] [1) PEGS t_R = 13 min 04 s, 20 min 08 s; 3) NPGS t_R = 13 min 20 s, 19 min 22 s; Rf = 0.35], [III] [1) PEGS t_R = 3 min 15 s, 4 min 26 s; 3) NPGS t_R = 5 min 20 s, 7 min 06 s; Rf = 0.72, 0.79], [IV] [1) PEGS t_R = 9 min 23 s, 11 min 26 s; 3) NPGS t_R = 12 min 48 s, 14 min 46 s; Rf = 0.50].

Enzymatic Hydrolysis of Sarasinoside B_1 (5) a) A suspension of **5** (170 mg) in H_2O (4 ml) was treated with crude hesperidinase (500 mg, lot NO. 680930 provided by Tanabe Pharm. Co.) and the whole mixture was stirred at 40 °C for 12 d. The reaction mixture was then treated with 1-butanol (5 ml), heated at 60 °C for 10 min, and filtered. The filtrate was extracted with 1-butanol and the 1-butanol extract was washed with H_2O and concentrated under reduced pressure to give a product (140 mg). Purification of the product by column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] furnished B_1-pro-1 (**15**) (10 mg) and B_1-pro-2 (**4**) (37 mg). B_1-pro-1 and B_1-pro-2 were shown to be identical with A_1-pro-1 (**15**) and sarasinoside C_1 (**4**), respectively, by mixed melting-point determination and UV, IR, ^{13}C-NMR, HPLC, and TLC comparisons.

b) A suspension of **5** (50 mg) in H_2O (2 ml) was treated with β-glucosidase (type II, from almonds) (200 mg) and the whole was kept stirring at 38 °C for 12 d. The 1-butanol extractive (40 mg), which was obtained by work-up of the reaction mixture as for the above-described hydrolysis with crude hesperidinase, was subjected to column chromatography [SiO_2, $CHCl_3$: MeOH : H_2O = 7 : 3 : 1 (lower phase)] to furnish B_1-pro-3 (**18**) (19 mg) and unchanged **5** (12 mg). B_1-pro-3 (**18**): mp 188—191 °C (80% MeOH), fine crystals. $[\alpha]_D$ −20° (c = 0.24, MeOH, 20 °C). UV (MeOH) λ_{max} nm (ε): 238 (13700). IR (KBr) ν_{max} cm^{-1}: 3410 (br), 2920, 2850, 1628 (br), 1561, 1076 (br). CD ($c = 6.2 \times 10^{-2}$, MeOH): $[\theta]_{223}$ −5800 (neg. max.). ^{1}H-NMR

(500 MHz, d_5-pyridine + D_2O) δ: 0.61, 0.97 (both 3H, s), 1.01 (3H, d, J=5.8 Hz, 20-CH_3), 1.16, 1.34 (both 3H, s), 1.74 (3H, s, 25-CH_3), 2.01 (3H, s, NHCOCH_3), 2.17 (3H, s, 25-CH_3), 3.19 (1H, dd, J=4.3, 11.9 Hz, 3α-H), 4.70 (1H, d, J=7.3 Hz), 5.23 (1H, d, J=6.7 Hz), 5.27 (1H, d, J=7.3 Hz), 5.70 (1H, d, J=8.2 Hz), 6.15 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.9 (s, C-23), 171.9 (s, NHCOCH_3), 153.9 (s, C-25), 135.8 (s, C-9), 127.5 (s, C-8), 124.7 (d, C-24), 106.0 (d), 105.3 (d), 103.0 (d), 101.5 (d), 89.7 (d, C-3), 69.2 (t, C-6″). *Anal.* Calcd for $C_{53}H_{85}NO_{20}\cdot 2H_2O$: C, 58.28; H, 8.21; N, 1.28. Found: C, 58.22; H, 8.12; N, 1.27.

Carbohydrate Composition of Sarasinoside B_1 (5) and Prosapogenols (4, 18) A solution of **5**, **4**, or **18** (4 mg each) in anhydrous 9% HCl–MeOH (1 ml) was heated under reflux for 1 h. The reaction mixture was worked up and analyzed by GLC as described above to obtain the following results. Sarasinoside B_1 (**5**): a (2), b (1), c (1), d (1); **4**: a (2), c (1), d (1); **18**: a (2), b (1), d (1).

Methylation of B_1-pro-2 (4) Followed by Methanolysis 1) A solution of **4** (35 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (1 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (1 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work up of the reaction mixture as described above gave an AcOEt extract, which was purified by column chromatography (SiO_2, benzene : acetone = 2 : 1) to furnish the fully methylated derivative (19 mg); IR (CCl_4): no OH.

2) A solution of the fully methylated derivative (5 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was examined by GLC and TLC (benzene : acetone = 1 : 1). The following methyl glycosides were identified: [II] [1) PEGS t_R = 12 min 43 s, 19 min 34 s; 2) BDS t_R = 11 min 38 s; Rf = 0.34] and methyl 2,3,4-tri-*O*-methylxylopyranoside [V] [1) PEGS t_R = 1 min 45 s, 2 min 11 s; 2) BDS t_R = 1 min 46 s, 2 min 10 s; Rf = 0.76, 0.84].

Methylation of B_1-pro-3 (18) Followed by Methanolysis 1) A solution of **18** (15 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (2 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (2 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work-up of the reaction mixture as described above gave an AcOEt extract, which was purified by column chromatography (SiO_2, benzene : acetone = 3 : 1) to furnish the fully methylated derivative (7 mg); IR (CCl_4): no OH.

2) A solution of the fully methylated derivative (4 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was examined by GLC and TLC (benzene : acetone = 3 : 1). The following methyl glycosides were identified: [I] [1) PEGS t_R = 4 min 55 s, 5 min 58 s; 2) BDS t_R = 4 min 49 s, 5 min 47 s; Rf = 0.32] and [III] [1) PEGS t_R = 3 min 20 s, 4 min 32 s; 2) BDS t_R = 3 min 52 s, 5 min 31 s; Rf = 0.52, 0.61].

Methylation of Sarasinoside B_1 (5) Followed by Methanolysis 1) A solution of **5** (24 mg) in DMSO (1 ml) was treated with a dimsyl carbanion solution (3 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (3 ml) under ice-cooling and stirred at room temperature for a further 1 h in the dark. Work-up of the reaction mixture as described above gave an AcOEt extract, which was purified by column chromatography (SiO_2, benzene : acetone = 1 : 1) to furnish the fully methylated derivative (10 mg), IR (CCl_4) ν_{max} cm^{-1}: no OH, 2926, 1705, 1649, 1097 (br).

2) A solution of the fully methylated derivative (4 mg) in 9% HCl–MeOH (1 ml) was heated under reflux for 1 h. After neutralization with Ag_2CO_3, the reaction mixture was filtered and the filtrate was examined by GLC and TLC (benzene : acetone = 1 : 1). The following methyl glycosides were identified: [I] [1) PEGS t_R = 4 min 45 s, 5 min 44 s; 3) NPGS t_R = 6 min 07 s, 7 min 05 s; Rf = 0.59] [II] [1) PEGS t_R = 13 min 05 s, 20 min 08 s; 3) NPGS t_R = 13 min 25 s, 19 min 23 s; Rf = 0.34], and [III] [1) PEGS t_R = 3 min 18 s, 4 min 23 s; 3) NPGS t_R = 5 min 16 s, 7 min 05 s; Rf = 0.72, 0.80].

Complete Methylation of Sarasinoside A_1(1) Followed by Acetolysis, Hydrolysis and Derivation to Give Partially Methylated Hexitol Acetates 1) A solution of **1** (50 mg) in DMSO (2 ml) was treated with a dimsyl carbanion solution (4 ml) and the whole mixture was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (4 ml) under ice-cooling and stirred at room temperature for 1 h in the dark. The reaction mixture was then poured into ice-H_2O and the whole was extracted with AcOEt. The AcOEt extract was washed with H_2O, then dried over $MgSO_4$. Removal of the solvent from the AcOEt extract under reduced pressure gave a product, which was purified by SiO_2 column chromatography (benzene : acetone = 1 : 1) to furnish the fully methylated derivative (27 mg).

2) The fully methylated derivative (27 mg) was treated with 0.5 N H_2SO_4 in 95% acetic acid (1 ml) [prepared from 10 N H_2SO_4 (5 ml) and glacial acetic acid (95 ml)], and the whole was heated at 80 °C for 8 h. The reaction mixture was then treated with H_2O (1 ml) and heated at 80 °C for an additional 5 h.

3) The reaction mixture was neutralized with Dowex 1 × 2 OH^- form and the filtrate was evaporated to dryness under reduced pressure. A solution of the residue in MeOH–H_2O (1 : 1) (1 ml) was treated with sodium borohydride ($NaBH_4$) (40 mg) and was then stirred at room temperature for 6 h. The reaction mixture was treated with glacial acetic acid (0.5 ml) and the whole was evaporated under reduced pressure. The residue was treated with acetic anhydride (3 ml) and heated at 100 °C for 3 h. The reaction mixture was evaporated under reduced pressure and the residue was partitioned into $CHCl_3$–H_2O. The $CHCl_3$-soluble portion was evaporated under reduced pressure to give the residue (35 mg), which was purifed by SiO_2 column chromatography (benzene : AcOEt = 3 : 1) to give 3-*O*-methyl-1,2,4,5-tetra-*O*-acetyl-xylitol (**19**, 3 mg), 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-glucitol (**20**, 3 mg), 3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl-glucitol (**21**, 2 mg), 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol (**23**, 3 mg), and 3,4-di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol (**24**, 3 mg). 3-*O*-Methyl-1,2,4,5-tetra-*O*-acetyl-xylitol (**19**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.05, 2.11 (both 6H, s), 3.52 (3H, s), 3.58 (1H, dd, J=5.0, 5.0 Hz, 3-H), 4.08 (2H, dd, J=6.6, 12.0 Hz, 1-H, 5-H), 4.39 (2H, dd, J=4.0, 12.0 Hz, 1-H, 5-H), 5.25 (2H, ddd, J=4.0, 5.0, 6.6 Hz, 2-H, 4-H). Mass m/z (%): 275 (1, M^+ − OAc), 189 (54), 129 (100). High-resolution Mass Calcd for $C_{14}H_{22}O_9$ − OAc (M^+ − OAc): 275.113. Found: 275.112. 2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl-glucitol (**20**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.10, 2.12 (both 3H, s), 3.37, 3.50, 3.50, 3.51 (each 3H, s), 3.38 (1H, dd, J=4.0, 5.5 Hz, 3-H), 3.60 (1H, dd, J=4.0, 5.5 Hz, 4-H), 3.61 (1H, dd, J=5.5, 11.0 Hz, 6-H), 3.65 (1H, ddd, J=3.5, 5.5, 6.4 Hz, 2-H), 3.69 (1H, dd, J=3.4, 11.0 Hz, 6-H), 4.17 (1H, dd, J=6.4, 12.0 Hz, 1-H), 4.37 (1H, dd, J=3.5, 12.0 Hz, 1-H), 5.15 (1H, ddd, J=3.4, 5.5, 5.5 Hz, 5-H). Mass m/z (%): 291 (0.1, M^+ − OCH_3), 263 (M^+ − OAc, 1.3), 101 (100). High-resolution Mass Calcd for $C_{14}H_{26}O_8$ − OAc: 263.149. Found: 263.149. 3,4,6-Tri-*O*-methyl-1,2,5,-tri-*O*-acetyl-glucitol (**21**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.07, 2.10, 2.14, 3.37, 3.50, 3.52 (each 3H, s), 3.40 (1H, dd, J=5.2, 5.2 Hz, 3-H), 3.58 (1H, dd, J=4.9, 10.7 Hz, 6-H), 3.59 (1H, m, 4-H), 3.69 (1H, dd, J=4.0, 10.7 Hz, 6-H), 4.20 (1H, dd, J=7.5, 12.1 Hz, 1-H), 4.45 (1H, dd, J=3.2, 12.1 Hz, 1-H), 5.09 (1H, ddd, J=4.0, 4.9, 5.2 Hz, 5-H), 5.37 (1H, ddd, J=3.2, 5.2, 7.5 Hz, 2-H). Mass m/z (%): 291 (0.6, M^+ − OAc), 233 (1.4), 205 (2.6), 129 (100). High-resolution Mass Calcd for $C_{15}H_{26}O_9$ − OAc: 291.144. Found: 291.142. 3,4,6-Tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol (**23**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.04[a], 2.06[b], 2.11[a] × 2, 2.12[b], 2.14[b] (total 9H, each s), 2.92[b], 3.02[a] (total 3H, both s), 3.30—3.48 (total 2H, 3-H, 4-H), 3.37[a] × 3, 3.40[b], 3.41[b], 3.43[b] (total 9H, each s), 3.49—3.60 (total 2H, 6-H), 4.17—4.38 (total 3H, 1-H, 2-H), 5.22 (total 1H, 5-H) (peak area a : b = 10 : 7). Mass m/z (%): 364 (2.8, M^+ + 1), 304 (1.1, M^+ − OAc), 116 (100). High-resolution Mass Calcd for $C_{16}H_{29}NO_8$: 363.189. Found: 363.189. 3,4-Di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol (**24**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.05[a], 2.07, 2.08[a], 2.10[b], 2.12[a], 2.15[b] (total 12H, each s), 2.88[b], 3.07[a] (total 3H, both s), 3.37—3.64 (total 2H, 3-H, 4-H), 3.41[b], 3.46[a], 3.50[a], 3.53[b] (total 6H, each s), 4.14—4.62 (total 5H, 1-H, 2-H, 6-H), 5.07[a], 5.22[b] (total 1H, 5-H) (peak area a : b = 5 : 2).

Synthesis of 23 A solution of *N*-acetyl-D-galactosamine (280 mg) in 9% HCl–MeOH (5 ml) was heated under reflux for 3 h and neutralized with 5% KOH–MeOH. The filtrate was evaporated under reduced pressure to give the residue, which was purified by SiO_2 column chromatography [$CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] to give methyl *N*-acetyl-D-galactosamide (136 mg). A solution of mtehyl *N*-acetyl-D-galactosamide (136 mg) in DMSO (2 ml) was treated with a dimsyl carbanion solution (6 ml) and the whole solution was stirred at room temperature for 1 h under an N_2 atmosphere. The reaction mixture was treated with CH_3I (6 ml) under ice-cooling and then stirred at room temperature for a further 1 h in the dark. Work up of the reaction mixture as described for methylation of sarasinoside A_1 (**1**) gave a product, which was purified by SiO_2 column chromatography (benzene : acetone = 5 : 2) to furnish the fully methylated derivative (102 mg). The fully methylated derivative was treated with 0.5 N H_2SO_4 in 95% acetic acid (2.5 ml) and the whole was heated at 100 °C for 3 h. The reaction mixture was then treated with H_2O (2.5 ml),

heated at 100 °C for an additional 3 h, neutralized with Dowex 1 × 2 OH^- form and filtered. The filtrate was evaporated to dryness under reduced pressure. A solution of the residue in MeOH–H_2O (1 : 1) (3 ml) was treated with $NaBH_4$ (250 mg) and the mixture was stirred at room temperature for 2 h, then treated with glacial acetic acid (0.6 ml). The whole was evaporated under reduced pressure. The residue was treated with acetic anhydride (5 ml) and heated at 100 °C for 2 h. The reaction mixture was evaporated under reduced pressure and the residue was partitioned into $CHCl_3$–H_2O. The $CHCl_3$-soluble portion was evaporated under reduced pressure to give the residue, which was purified by column chromatography (SiO_2, AcOEt) to give 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamido-D-galactitol (**23**) (25 mg).

Synthesis of 24 A solution of *N*-acetyl-D-glucosamine (295 mg) in 9% HCl–MeOH (6 ml) was heated under reflux for 6 h and neutralized with 5% KOH–MeOH. The filtrate was evaporated under reduced pressure to give the residue, which was purified by SiO_2 column chromatography [$CHCl_3$: MeOH : H_2O = 10 : 3 : 1 (lower phase)] to give methyl *N*-acetyl-D-glucosamide (165 mg). A solution of methyl *N*-acetyl-D-glucosamide in pyridine (5 ml) was treated with trityl chloride (215 mg) and the whole was stirred at 60 °C for 6 h. After dilution with H_2O, the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ phase was taken and washed with brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a product (450 mg), which was purified by column chromatography (SiO_2, $CHCl_3$: MeOH = 15 : 1) to give methyl 6-*O*-trityl-*N*-acetyl-D-glucosamide (274 mg). The methyl 6-*O*-trityl-*N*-acetyl-D-glucosamide (274 mg) was treated as described above for methyl *N*-acetyl-D-galactosamide to give 3,4,-di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamido-D-glucitol (**24**) (16 mg).

Complete Methylation of Sarasinoside B_1 (5) Followed by Acetolysis, Hydrolysis and Derivation into Partially Methylated Hexitol Acetates 1) A solution of **5** (60 mg) in DMSO (2 ml) was treated with a dimsyl carbanion solution (4 ml) and the whole mixture was stirred at room temperature for 1 h under an N_2 atmosphere, then treated with CH_3I (4 ml) and stirred at room temperature for an additional 1 h in the dark. Work-up of the reaction mixture as described above for methylation of sarasinoside A_1 (**1**) gave the fully methylated derivative (49 mg).

2) The fully methylated derivative (49 mg) was treated with 0.5 N H_2SO_4 in 95% acetic acid (1 ml) and the whole mixture was heated at 80 °C for 5 h. The reaction mixture was then treated with water (1 ml) and heated at 80 °C for an additional 5 h.

3) The reaction mixture was neutralized with Dowex 1 × 2 OH^- form and the filtrate was evaporated to dryness under reduced pressure. A solution of the residue in MeOH–H_2O (1 : 1) (2 ml) was treated with $NaBH_4$ (40 mg) and then stirred at room temperature for 5 h. The reaction mixture was treated with glacial acetic acid (0.5 ml) and the whole was evaporated under reduced pressure. The residue was treated with acetic anhydride (3 ml) and heated at 100 °C for 3 h. Work-up of the reaction mixture as described above for sarasinoside A_1 (**1**) gave 3-*O*-methyl-1,2,4,5-tetra-*O*-acetylxylitol (**19**, 3 mg), 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylglucitol (**20**, 7 mg), 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetylxylitol (**22**, 2 mg), 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidogalactitol (**23**, 4 mg), and 3,4-di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol (**24**, 4 mg). 3,4-Di-*O*-methyl-1,2,5-tri-*O*-acetylxylitol (**22**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.07, 2.09, 2.12, 3.48, 3.54 (each 3H, s), 3.49 (1H, m, 3-H), 3.55 (1H, m, 4-H), 4.17 (1H, dd, J = 5.5, 11.8 Hz, 5-H), 4.19 (1H, dd, J = 7.0, 12.2 Hz, 1-H), 4.31 (1H, dd, J = 4.7, 11.8 Hz 5-H), 4.42 (1H, dd, J = 3.7, 12.2 Hz, 1-H), 5.33 (1H, ddd, J = 3.7, 4.9, 7.0 Hz, 2-H).

Acidic Hydrolysis of Sarasinosides A_2 (2), B_2 (7), and C_2 (6) A mixture of **2** (43 mg) and 3% aqueous H_2SO_4 (1 ml) was heated for 2 h under reflux on a water-bath. After dilution with H_2O, the reaction mixture was extracted with AcOEt. The AcOEt extract was washed with aqueous saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure from the AcOEt extract gave a product (22 mg), which was purified by column chromatography (SiO_2 treated with $AgNO_3$, *n*-hexane : AcOEt = 5 : 2) to furnish a sapogenol **12**. **12**: mp 136—139 °C (MeOH–AcOEt), colorless needles. $[\alpha]_D$ +8.7° (c = 0.2, $CHCl_3$, 23 °C). UV (MeOH) λ_{max}: Table I. IR (CCl_4) ν_{max} cm^{-1}: 3620, 2923, 2851, 1688, 1445, 1026. CD ($c = 5.7 \times 10^{-2}$, MeOH). Table I. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.55, 0.90 (both 3H, s), 0.95 (3H, d, J = 6.4 Hz, 20-CH_3), 0.98, 1.01 (both 3H, s), 1.89, 2.15 (both 3H, s, 25-CH_3), 3.25 (1H, dd, J = 4.6, 11.6 Hz, 3α-H), 5.40 (1H, m), 5.45 (1H, m), 6.06 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.6 (s, C-23), 153.9 (s, C-25), 147.3 (s, C-9), 136.1 (s, C-8), 124.9 (d, C-24), 121.9 (d, C-7), 117.4 (d, C-11), 78.1 (d, C-3). Mass m/z (%): 424 (54, M^+), 326 (100), 297 (34). High-resolution Mass Calcd for $C_{29}H_{44}O_3$: 424.334. Found: 424.336. Sarasinosides B_2 (**7**) (20 mg) and C_2 (**6**) (18 mg) were hydrolyzed as described above to give the same sapogenol **12**, respectively. Compound **12** thus obtained was shown to be identical with an authentic sample obtained from **2** by TLC and mass spectral comparisons.

Acidic Hydrolysis of Sarasinosides A_3 (3), B_3 (9), C_3 (8) A mixture of **3** (32 mg) and 5% aqueous H_2SO_4–MeOH (1 : 1) (2 ml) was heated for 1 h under reflux. After dilution with H_2O, the reaction mixture was extracted with AcOEt. The AcOEt soluble portion was washed with aqueous saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Removal of the solvent under reduced pressure from the AcOEt extract yielded a product (17 mg), which was purified by SiO_2 column chromatography (benzene : acetone = 45 : 1) to give a sapogenol **13**. **13**: mp 135—138 °C (MeOH–AcOEt), colorless needles. $[\alpha]_D$ −10° (c = 0.2, $CHCl_3$, 23 °C). UV (MeOH) λ_{max}: Table I. IR (CCl_4) ν_{max} cm^{-1}: 3628, 2954, 2933, 2853, 1687, 1618, 1032. CD ($c = 6.5 \times 10^{-2}$, MeOH): Table I. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.84, 0.86 (both 3H, s), 0.97 (3H, d, J = 6.4 Hz, 20-CH_3), 1.02, 1.04 (both 3H, s), 1.89, 2.15 (both 3H, s, 25-CH_3), 3.25 (1H, dd, J = 4.6, 11.6 Hz, 3α-H), 5.34 (1H, m, 15-H), 6.08 (1H, s, 24-H). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 200.6 (s, C-23), 153.9 (s, C-25), 151.6 (s, C-14), 142.5 (s, C-9), 125.0 (d, C-24), 123.1 (s, C-8), 117.1 (d, C-15), 77.9 (d, C-3). Mass m/z (%): 424 (75, M^+), 406 (15, $M^+ - H_2O$), 326 (63), 83 (100). High-resolution Mass Calcd for $C_{29}H_{44}O_2$: 424.334. Found: 424.334. Sarasinosides B_3 (**9**) (20 mg) and C_3 (**8**) (15 mg) were hydrolyzed as described above to give the same sapogenol **13**. Compound **13** thus obtained was shown to be identical with an authentic sample obtained from **3** by TLC and mass spectral comparisons.

Catalytic Hydrogenation of Sarasinosides A_1 (1), A_2 (2), and A_3 (3) A suspension of 10% Pd–C (70 mg) in MeOH (4 ml) was stirred under a hydrogen atmosphere for 15 min and **1** (58 mg) was then added to this suspension. The whole mixture was stirred for a further 24 h, then filtered. Removal of the solvent from the filtrate under reduced pressure gave a product (56 mg). HPLC purification (Zorbax ODS, MeOH : H_2O = 5 : 1) of the product furnished **25** (45 mg). **25**: mp 212—215 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −11° (c = 0.3, MeOH, 25 °C). UV (MeOH) λ_{max} nm: transparent above 210 nm. IR (KBr) ν_{max} cm^{-1}: 3360 (br), 2925, 2869, 1640, 1547, 1054 (br). CD ($c = 7.5 \times 10^{-2}$, MeOH): $[\theta]_{221}$ −6600 (neg. max.). ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.61 (3H, s), 0.87 [6H, d, J = 6.7 Hz, 25-$(CH_3)_2$], 0.99 (3H, d, J = 5.8 Hz, 20-CH_3), 1.03, 1.19, 1.31 (each 3H, s), 2.04, 2.09 (both 3H, s, $NHCOCH_3$), 3.15 (1H, dd, J = 4.7, 11.4 Hz, 3α-H), 4.57 (1H, d, J = 7.9 Hz), 5.20 (1H, d, J = 8.2 Hz), 5.21 (1H, d, J = 7.6 Hz), 5.59 (1H, d, J = 7.9 Hz), 5.61 (1H, d, J = 7.3 Hz). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 210.1 (s, C-23), 171.9, 171.5 (both s, $NHCOCH_3$), 136.4 (s, C-9), 127.6 (s, C-8), 106.8 (d), 105.6 (d), 102.7 (d), 102.3 (d), 101.9 (d), 90.2 (d, C-3). *Anal.* Calcd for $C_{62}H_{102}N_2O_{26} \cdot 2H_2O$: C, 56.09; H, 8.05; N, 2.11. Found: C, 56.12; H, 7.85; N, 2.19. Sarasinosides A_2 (**2**) (17 mg) and A_3 (**3**) (21 mg) were hydrogenated over 10% Pd–C under similar conditions to give **25** (13 mg from **2**, 16 mg from **3**). Compound **25** thus obtained was shown to be identical with an authentic sample obtained from **1** by mixed melting-point determination, HPLC, and ^{13}C-NMR comparisons.

Catalytic Hydrogenation of Sarasinosides B_1 (5), B_2 (7), and B_3 (9) A suspension of 10% Pd–C (17 mg) in MeOH (4 ml) was stirred under a hydrogen atmosphere for 15 min. Compound **5** (12 mg) was added to this suspension and the reaction mixture was stirred for a further 24 h, then filtered. Removal of the solvent from the filtrate under reduced pressure gave a product (11 mg). HPLC purification (Zorbax ODS, MeOH : H_2O = 5 : 1) of the product furnished **27** (8 mg). **27**: mp 200—203 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −19° (c = 0.2, MeOH, 25 °C). UV (MeOH) λ_{max} nm: transparent above 210 nm. IR (KBr) ν_{max} cm^{-1}: 3350 (br), 2923, 2860, 1646, 1548, 1039 (br). CD ($c = 8.1 \times 10^{-2}$, MeOH): $[\theta]_{220}$ −6900 (neg. max). ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.61 (3H, s), 0.86, 0.87 [both 3H, d, J = 6.7 Hz, 25-$(CH_3)_2$], 0.98 (6H, br s), 1.16, 1.32 (both 3H, s), 2.05, 2.10 (both 3H, s, $NHCOCH_3$), 3.15 (1H, dd, J = 4.6, 11.0 Hz), 4.56 (1H, d, J = 7.9 Hz), 5.20 (1H, d, J = 8.2 Hz), 5.21 (1H, d, J = 7.3 Hz), 5.38 (1H, d, J = 7.3 Hz), 5.56 (1H, d, J = 8.6 Hz). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 210.1 (s, C-23), 172.3, 171.9 (both s, $NHCOCH_3$), 136.3 (s, C-9), 127.6 (s, C-8), 106.6 (d), 105.3 (d), 103.2 (d), 102.6 (d), 101.8 (d), 90.0 (d, C-3). *Anal.* Calcd for $C_{61}H_{100}N_2O_{25} \cdot H_2O$: C, 57.26; H, 8.03; N, 2.19. Found: C, 57.42; H, 7.99, N, 2.35. Sarasinosides B_2 (**7**) (14 mg) and B_3 (**9**) (20 mg) were hydrogenated over 10% Pd–C under similar conditions to give **27** (10 mg from **7**, 16 mg from **9**). Compound **27** thus obtained was shown to be identical with an authentic sample obtained from **5** by mixed melting-point determination, HPLC, and ^{13}C-NMR comparisons.

Catalytic Hydrogenation of Sarasinosides C_1 (4), C_2 (6), and C_3 (8) A

suspension of 10% Pd–C (100 mg) in MeOH (7 ml) was stirred under a hydrogen atmosphere for 15 min. Compound **4** (150 mg) was added to this suspension and the reaction mixture was stirred for a further 24 h, then filtered. Removal of the solvent from the filtrate under reduced pressure gave a product (120 mg). HPLC purification (Zorbax ODS, MeOH: $H_2O = 5:1$) of the product furnished **26** (100 mg): mp 197—200 °C (MeOH–H_2O), fine crystals. $[\alpha]_D$ −20° ($c = 1.2$, MeOH, 25 °C). UV (MeOH) λ_{max} nm: transparent above 210 nm. IR (KBr) ν_{max} cm^{-1}: 3360 (br), 2927, 2865, 1638, 1548, 1037 (br). CD ($c = 6.0 \times 10^{-2}$, MeOH): $[\theta]_{222}$ −6200 (neg. max.). ^{1}H-NMR (500 MHz, d_5-pyridine + D_2O) δ: 0.61 (3H, s), 0.86 (3H, d, $J = 6.7$ Hz, 25-CH_3), 0.87 (3H, d, $J = 6.4$ Hz, 25-CH_3), 0.95 (3H, s), 0.99 (3H, d, $J = 5.5$ Hz, 20-CH_3), 1.13, 1.28 (both 3H, s), 2.09, 2.10 (both 3H, s, $NHCOCH_3$), 3.18 (1H, dd, $J = 4.1$, 11.8 Hz, 3α-H), 4.64 (1H, d, $J = 7.0$ Hz), 4.96 (1H, d, $J = 7.3$ Hz), 5.12 (1H, d, $J = 8.6$ Hz), 5.44 (1H, d, $J = 8.5$ Hz). ^{13}C-NMR (125 MHz, d_5-pyridine) δ_C: 210.2 (s, C-23), 172.3, 171.6 (both s, $NHCOCH_3$), 136.4 (s, C-9), 127.7 (s, C-8), 105.3 (d), 104.6 (d), 102.3 × 2 (d), 89.4 (d, C-3). *Anal.* Calcd for $C_{55}H_{90}N_2O_{20} \cdot H_2O$: C, 59.23; H, 8.13; N, 2.51. Found: C, 59.11; H, 8.20; N, 2.38. Sarasinoside C_2 (**6**) (50 mg) and C_3 (**8**) (20 mg) were hydrogenated over 10% Pd–C under similar conditions to give **26** (30 mg from **6**, 14 mg from **8**). Compound **26** thus obtained was shown to be identical with an authentic sample obtained from **4** by mixed melting-point determination, HPLC, and ^{13}C-NMR comparisons.

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Synthesis of *ortho*-Substituted Arylacetic Esters and Related Compounds by Means of Sommelet–Hauser Rearrangement of Sulfur Ylides

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The rearrangement of a series of dimethylsulfonium α-substituted benzylides, *e.g.*, 8, in ethanol has been examined. The ylides 8a,b generated *in situ* by treatment of the sulfonium salts 7a,b with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in ethanol at room temperature, afforded the *o*-(methylthiomethyl)phenylacetic esters 10a,b as a result of the Sommelet–Hauser rearrangement of the tautomeric ylides 9a,b. By contrast, the ylide 14 possessing a furan ring was stable at room temperature, but, on heating in ethanol, gave the rearranged product 15. The ylide 22 stabilized by an acetyl group provided three rearranged products, 23, 24, and 25, in boiling ethanol. Treatment of the sulfonium salts 30a,b with DBU at room temperature afforded the corresponding rearranged products 31a,b. The sulfonium salt 34a prepared from 33a, on treatment with sodium ethoxide, gave the rearranged product 36a, which was then *S*-methylated and treated with DBU to give the 1,2,3-trisubstituted benzene 37a. This method was applied to the synthesis of the fenoprofen analog 39 from 39 from 33b,c.

Keywords Sommelet–Hauser rearrangement; sulfur ylide; arylacetic ester; Friedel–Crafts reaction; α-chlorosulfide; sulfonium salt; tautomerization; Favorskii rearrangement; [2,3] sigmatropic rearrangement; desulfurization

The Sommelet–Hauser rearrangement of benzylsulfonium methylides and related species has been widely used as a selective method for the *ortho* substitution of aromatic substrates.[1] Robert and his co-workers[2] reported that the crystalline stable ylide **2**, prepared from the *gem*-dicyano epoxide **1** and dimethyl sulfide in 3 steps (see Chart 1), on heating in methanol, gave the *ortho*-substituted arylacetic ester **4**. Formation of **4** can be rationalized in terms of the Sommelet–Hauser rearrangement of the tautomeric ylide **3** formed from **2** under the reaction conditions. The ylide **2** was stable in an aprotic solvent such as tetrahydrofuran (THF) even under reflux, indicating that the tautomerization between **2** and **3** occurred only in a protic solvent such as methanol. We have now investigated this rearrangement in more detail by variation of the aromatic ring and the stabilizing group of the ylide **2**. The present paper describes the results of our work in this area, including a new preparation of the requisite ylides and their rearrangement to give various *ortho*-substituted arylacetic esters and related compounds.

We began our investigation by examining the rearrangement of the sulfur ylide **8a**. The ylide **8a** was prepared as follows. Treatment of the Friedel–Crafts reaction product **6a**[3] derived from benzene and α-chlorosulfide **5**, with a stoichiometric amount of silver tetrafluoroborate ($AgBF_4$) in a large excess of methyl iodide at room temperature gave the sulfonium salt **7a**, which was then treated with sodium hydride in THF to give the ylide **8a** quantitatively.

In contrast to the ylide **2**, which requires refluxing conditions for the rearrangement, the ylide **8a** was found to undergo the rearrangement even at room temperature in ethanol to give ethyl 2-(methylthiomethyl)phenylacetate (**10a**) quantitatively. This result indicates that the tautomerization between **8a** and the reactive ylide **9a** readily occurred at room temperature. The relatively high stability

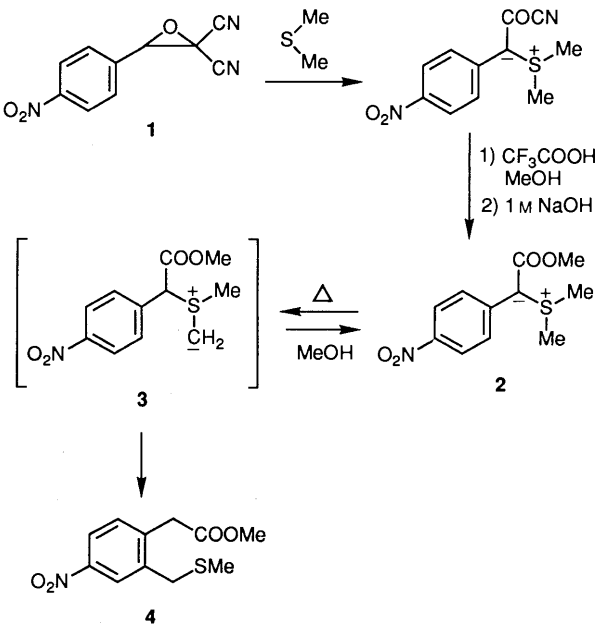

Chart 1

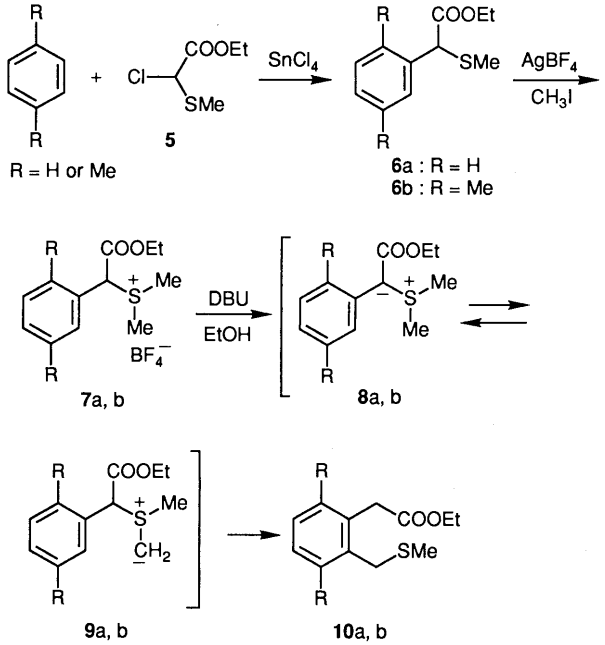

Chart 2

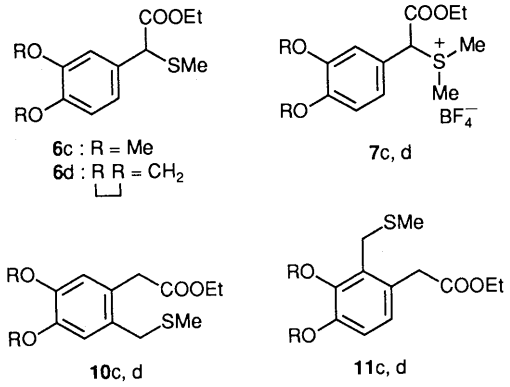

Chart 3

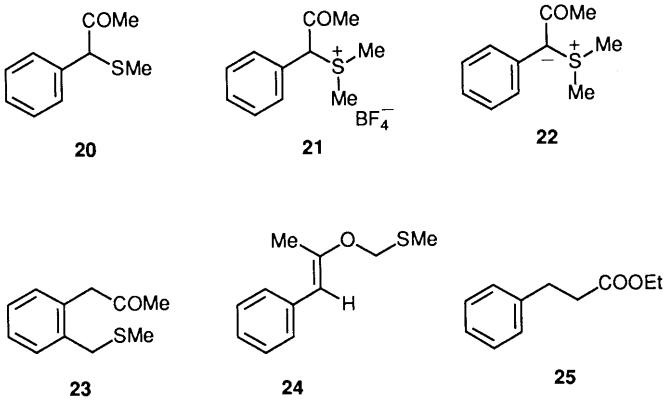

Chart 5

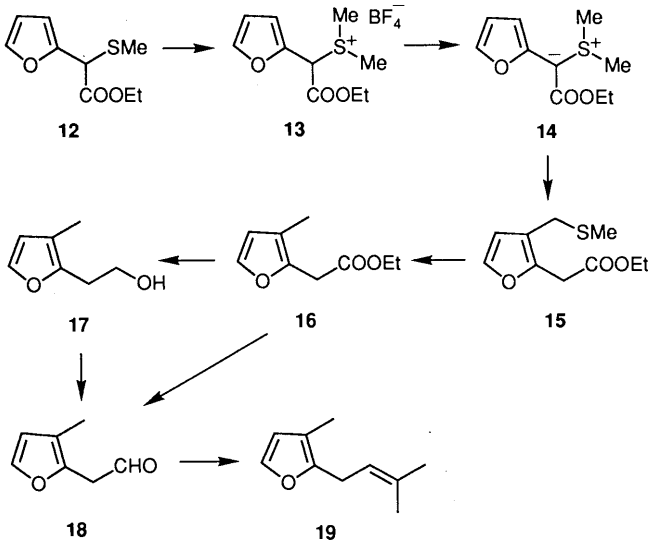

Chart 4

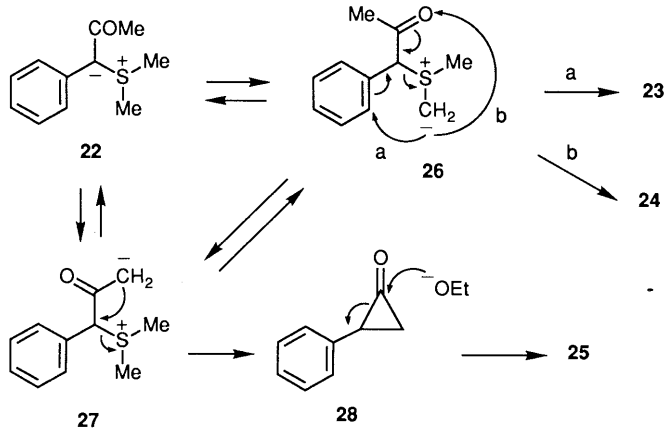

Chart 6

of the reported ylide **2** might be ascribed to the presence of an electron-withdrawing *p*-nitro group on the aromatic ring.

The rearranged product **10a** was obtained more conveniently by treatment of the sulfonium salt **7a** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in ethanol at room temperature in 98% yield. The use of triethylamine in place of DBU afforded a 70% yield of the product **10a**. Similar treatment of the sulfonium salt **7b** with DBU gave **10b** in 82% yield.

S-Methylation of **6c** followed by treatment of the resultant sulfonium salt **7c** with DBU afforded the 2,4,5- and 2,3,4-trisubstituted arylacetic esters **10c** and **11c** in 41 and 14% yields (based on **6c**), respectively. The structures of **10c** and **11c** were confirmed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy (see Experimental). The sulfonium salt **7d** gave an inseparable mixture of the rearranged products **10d** and **11d** (*ca.* 4 : 1).

The transformation of the furan derivative **13** into the rearranged product **15** was somewhat troublesome. Thus, treatment of **13** with DBU in ethanol at room temperature or under refluxing conditions gave a complex mixture of products. However, when the ylide **14** isolated from **13** was heated in ethanol, the expected rearranged product **15** was obtained in 67% yield (based on **12**). Direct heating of the salt **13** in boiling ethanol in the presence of sodium ethoxide again provided an unsatisfactory result.

We next examined transformation of the product **15** into the aldehyde **18**, which has been shown to be convertible into rose furan (**19**) *via* the Wittig reaction with isopropylidenetriphenylphosphorane.[4] Desulfurization of **15** with tributyltin hydride in the presence of azobisisobutyronitrile (AIBN) in boiling benzene afforded, in 83% yield, the ester **16**, which was then reduced with lithium aluminum hydride to give the alcohol **17** in 85% yield. The ester **16** and the alcohol **17** were subjected to reduction with diisobutylaluminum hydride (DIBAH) and to oxidation with Collins' reagent, respectively. The ^{1}H-NMR spectra of both reaction mixtures clearly showed the presence of the desired aldehyde **18**, but all attempts to purify the product by conventional means were unsuccessful.

The acetyl-stabilized ylide **22**, prepared from the sulfide **20**, was stable at room temperature. However, when the sulfonium salt **21** was heated in boiling ethanol in the presence of DBU, three rearranged products **23**, **24**, and **25** were obtained in 22, 8, and 14% yields (based on **20**), respectively. The *E*-configuration of **24** was determined by comparison of the chemical shift (δ 4.49) of the olefinic proton with those of (*E*)- (δ 4.50) and (*Z*)- (δ 5.23) 2-methoxy-1-propenylbenzenes.[5]

Formation of **23** and **24** can be explained in terms of the common intermediate **26** generated by tautomerization of the ylide **22**. The attack of the anionic center of **26** on the *ortho*-position of the aromatic ring gives the normal Sommelet–Hauser rearrangement product **23** (path a). On the other hand, when carbanion of **26** attacks the carbonyl

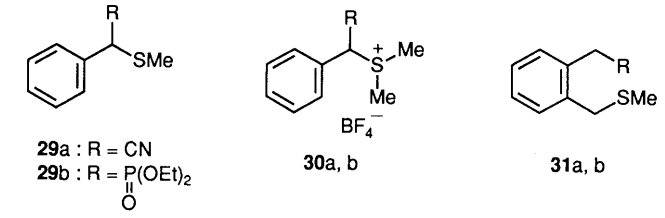

Chart 7

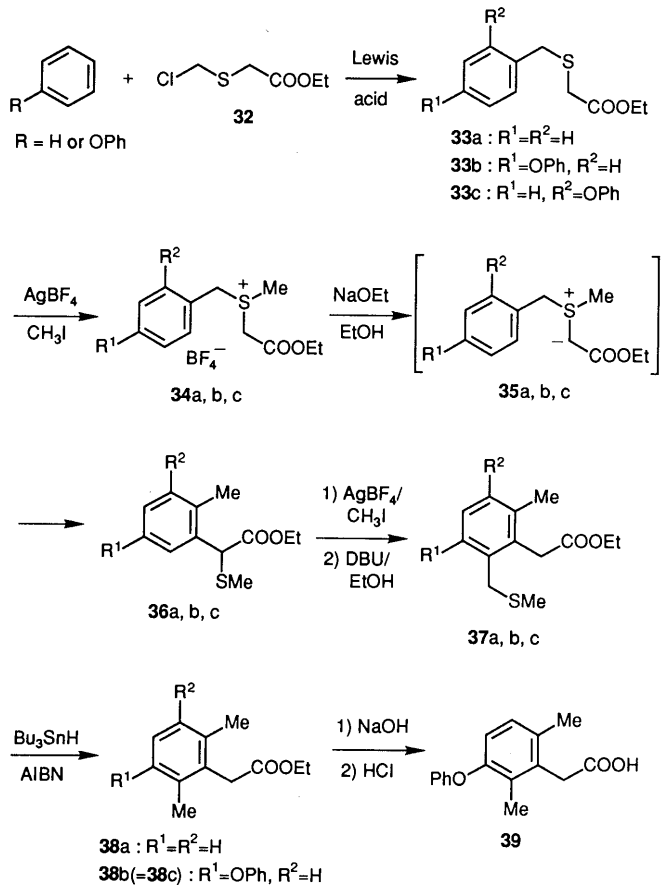

Chart 8

oxygen in a [2,3] sigmatropic manner (path b), the vinyl ether **24** might result.[6] Formation of **25** is of particular interest. This reaction might proceed *via* the Favorskii-type rearrangement of the third tautomer **27**, in which dimethyl sulfide acts as a leaving group, to give the cyclopropanone **28**, as shown in Chart 6.

Treatment of the sulfonium salts **30a** (R=CN) and **30b** [R=P(O)(OEt)$_2$] with DBU in ethanol gave the expected rearranged products **31a** and **31b** at room temperature in 62 and 93% yields, respectively.

Finally, we examined the reaction of the sulfonium salts **34** which were prepared by the Friedel–Crafts reaction of arenes with the α-chlorosulfide **32**[7] followed by *S*-methylation of the resultant products **33**. When **34a** was treated with a stoichiometric amount of sodium ethoxide in ethanol at room temperature, the rearrangement product **36a** was obtained in 60% yield. The use of DBU in place of NaOEt afforded a complex mixture of products containing **36a**. The formation of **36a** from **34a** can be easily rationalized in terms of the Sommelet–Hauser rearrangement of the initially formed stabilized ylide **35a**.

The product **36a** was next subjected to the same rearrangement as described above for **6**, giving the ester **37a** in 76% yield. Desulfurization of **37a** with Bu_3SnH–AIBN afforded the 2,6-dimethylphenylacetic ester **38a** in 60% yield.

The Friedel–Crafts reaction of diphenyl ether with **32** gave an inseparable mixture of the *p*- and *o*-substituted products **33b** and **33c** in a ratio of *ca.* 4:1 and 68% total yield. This mixture was then subjected to a similar sequence of reactions to that described for the preparation of **38a** from **33a** to give the sole product **38b** (=**38c**). A subsequent alkaline hydrolysis of **38b** gave the phenylacetic acid **39**. The carboxylic acid **39** thus obtained can be regarded as an analog of a potent anti-inflammatory agent fenoprofen, but showed no remarkable activity when examined by the carrageenin-induced rat paw edema method.

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO A-1 spectrophotometer. ^{1}H-NMR spectra were determined with a JEOL JNM-PMX 60 (60 MHz) or a Varian XL-300 (300 MHz) spectrometer using tetramethylsilane as an internal standard. High-resolution mass spectra (MS) were obtained with a Hitachi M-80 instrument at 20 eV. Column chromatography was performed under pressure on Silica gel 60 PF_{254} (Merck).

[Ethoxycarbonyl(phenyl)methyl]dimethylsulfonium Tetrafluoroborate (7a): A Typical Procedure for the Preparation of Sulfonium Salts Silver tetrafluoroborate (90%) (344 mg, 1.5 mmol) was added in one portion to a stirred solution of **6a**[3] (300 mg, 1.4 mmol) in methyl iodide (10 ml) at room temperature and stirring was continued for 2 h, during which time the sulfonium salt **7a** and silver iodide separated out. The supernatant was removed by decantation, the residue was extracted thoroughly with dichloromethane, and the extract was concentrated *in vacuo* to give the curde sulfonium salt **7a** (487 mg, >100%) as an oil. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.67 (3H, s, SMe), 3.09 (3H, s, SMe), 4.31 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 5.73 (1H, s, CH), 7.48 (5H, s, aromatic protons). This salt was used immediately in the next stage.

Ethyl 2-(Methylthiomethyl)phenylacetate (10a) Method A: A solution of the above salt **7a** (487 mg) in dry THF (2 ml) was added to a suspension of sodium hydride (60% dispersion in mineral oil) (75 mg, 1.9 mmol) in dry THF (8 ml) at −78 °C and the mixture was stirred at the same temperature for 1.5 h. The precipitated salts were removed by filtration and the filtrate was concentrated *in vacuo* to give the crude ylide **8a** (383 mg, >100%) as an oil. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.26 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.87 (6H, s, SMe_2), 4.20 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.7—7.5 (5H, m, aromatic protons). This ylide **8a** was dissolved in dry ethanol (5 ml) and the solution was allowed to stand at room temperature for 1 h. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene) to give **10a** (312 mg, 99% based on **6a**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.99 (3H, s, SMe), 3.73 (2H, s, $ArCH_2$), 3.77 (2H, s, $ArCH_2$), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.22 (4H, s, aromatic protons). *Anal.* Calcd for $C_{12}H_{16}O_2S$: C, 64.26; H, 7.19. Found: C, 64.44; H, 7.24.

Method B: DBU (380 mg, 2.5 mmol) was added to a solution of the crude sulfonium salt **7a** (594 mg), prepared from **6a** (325 mg, 1.55 mmol), in dry ethanol (10 ml) and the mixture was stirred at room temperature for 1 h. After removal of the solvent, dichloromethane (20 ml) was added to the residue and the whole was washed with water, then dried over $MgSO_4$. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene) to give **10a** (340 mg, 98% based on **6a**).

Ethyl 3,6-Dimethyl-1-(methylthiomethyl)phenylacetate (10b) According to a procedure similar to that described for the preparation of **10a** (method B), the sulfonium salt **7b** (207 mg), prepared from **6b**[3] (150 mg, 0.63 mmol) and MeI–$AgBF_4$, was treated with DBU (111 mg, 0.73 mmol) in ethanol to give **10b** (126 mg, 79% based on **6b**), mp 47—48 °C (from hexane). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.23 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.12 (3H, s, SMe), 2.27 (3H, s, ArMe), 2.38 (3H, s, ArMe), 3.80 (2H, s, $ArCH_2$), 3.83 (2H, s, $ArCH_2$), 4.12 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.93 (2H, s, aromatic protons). *Anal.* Calcd for $C_{14}H_{20}O_2S$: C, 66.63; H,

7.99. Found: C, 66.46; H, 8.12.

Ethyl 3,4-Dimethoxyphenyl(methylthio)acetate (6c) $SnCl_4$ (0.49 ml, 4.2 mmol) was added to a solution of **5** (700 mg, 4.2 mmol) and veratrole (580 mg, 4.2 mmol) in dichloromethane (20 ml) at 0 °C and the mixture was stirred at room temperature for 1 h. The reaction was quenched by the addition of water (10 ml) and the mixture was extracted with dichloromethane. The solvent was evaporated off and the residue was chromatographed on silica gel (hexane–ethyl acetate, 4 : 1) to give **6c** (1.0 g, 93%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.25 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.07 (3H, s, SMe), 3.83 (3H, s, OMe), 3.86 (3H, s, OMe), 4.18 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.45 (1H, s, SCH), 6.7—7.2 (3H, m, aromatic protons). *Anal.* Calcd for $C_{12}H_{18}O_4S$: C, 57.76; H, 6.71. Found: C, 58.03; H, 6.91.

Ethyl 1,3-Benzodioxol-5-yl(methylthio)acetate (6d) According to a procedure similar to that described for the preparation of **6c**, except for the use of $TiCl_4$ instead of $SnCl_4$, 1,3-benzodioxole (500 mg, 4.1 mmol) was allowed to react with **5** (691 mg, 4.1 mmol) to give **6d** (799 mg, 80%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.26 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.03 (3H, s, SMe), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.37 (1H, s, SCH), 5.88 (2H, s, OCH_2O), 6.7—7.1 (3H, m, aromatic protons). *Anal.* Calcd for $C_{12}H_{14}OS$: C, 56.71; H, 5.55. Found: C, 56.78, H, 5.68.

Ethyl 4,5-Dimethoxy-2-(methylthiomethyl)phenylacetate (10c) and Ethyl 3,4-Dimethoxy-2-(methylthiomethyl)phenylacetate (11c) Using a procedure similar to that described for the preparation of **10a** (method B), the sulfonium salt **7c** (177 mg), prepared from **6c** (131 mg, 0.51 mmol) and MeI–$AgBF_4$, was treated with DBU (87 mg, 0.57 mmol) in ethanol and the reaction mixture was chromatographed on silica gel (hexane–ethyl acetate, 10 : 1). The first eluate gave **11c** (20 mg, 14% based on **6c**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.07 (3H, s, SMe), 3.72 (2H, s, $ArCH_2$), 3.84 (8H, s, OMe×2 and $ArCH_2$), 4.14 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.77, 6.95 (1H each, AB q, J=8 Hz, aromatic protons). Exact MS m/z: Calcd for $C_{14}H_{20}O_4S$: 284.1080. Found: 284.1077.

The second eluate gave **10c** (60 mg, 41% based on **6c**), mp 32—33 °C (from light petroleum). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.26 (3H, t, J=7.2 Hz, $CH_2C\underline{H}_3$), 2.03 (3H, s, SMe), 3.70 (2H, s, $ArCH_2$), 3.71 (2H, s, $ArCH_2$), 3.87 (3H, s, OMe), 3.88 (3H, s, OMe), 4.15 (2H, q, J=7.2 Hz, $C\underline{H}_2CH_3$), 6.77 (1H, s, aromatic proton), 6.80 (1H, s, aromatic proton). *Anal.* Calcd for $C_{14}H_{20}O_4S$: C, 59.13; H, 7.09. Found: C, 59.11; H, 7.09.

Ethyl 6-Methylthiomethyl-1,3-benzodioxol-5-ylacetate (10d) and Ethyl 4-Methylthiomethyl-1,3-benzodioxol-5-ylacetate (11d) Using a procedure similar to that described for the preparation of **10a** (method B), the sulfonium salt **7d** (186 mg), prepared from **6d** (150 mg, 0.59 mmol), was treated with DBU (121 mg, 0.79 mmol) to give a mixture of **10d** and **11d** (118 mg, 75% based on **6d**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 1.25 (3H, t, J=7.2 Hz, $CH_2C\underline{H}_3$), 2.02 (12/5H, s, SMe for **10d**), 2.06 (3/5H, s, SMe for **11d**), 3.658 (8/5H, s, $ArCH_2$ for **10d**), 3.664 (8/5H, s, $ArCH_2$ for **10d**), 3.70 (2/5H, s, $ArCH_2$ for **11d**), 3.75 (2/5H, s, $ArCH_2$ for **11d**), 4.14 (2H, q, J=7.2 Hz, $C\underline{H}_2CH_3$), 5.93 (8/5H, s, OCH_2O for **10d**), 5.95 (2/5H, s, OCH_2O for **11d**), 6.68, 6.72 (1/5H each, AB q, J=7.9 Hz, aromatic protons for **11d**), 6.73 (4/5H, s, aromatic proton for **10d**), 6.75 (4/5H, s, aromatic proton for **10d**). *Anal.* Calcd for $C_{13}H_{16}O_4S$: C, 58.19; H, 6.01. Found: C, 57.72; H, 6.07.

Ethyl (3-Methylthiomethyl-2-furyl)acetate (15) A solution of sodium ethoxide (48 mg, 0.71 mmol) in ethanol (10 ml) was added to a solution of the sulfonium salt **13** (233 mg, 0.71 mmol), prepared from **12**[3] (146 mg, 0.73 mmol) and MeI–$AgBF_4$, in ethanol (5 ml) at 0 °C and the mixture was stirred at room temperature for 10 min. After removal of the solvent, chloroform was added to the residue, the precipitated salts were filtered off, and the filtrate was concentrated *in vacuo* to give the crude ylide **14** (156 mg) as an oil. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.74 (6H, s, SMe_2), 4.11 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.0—6.4 (2H, m, H-3 and H-4), 7.25 (1H, d, J=2 Hz, H-5). The ylide **14** thus obtained was dissolved in ethanol (10 ml) and the solution was heated under reflux for 3 h. The solvent was evaporated off and the residue was chromatographed on silica gel (benzene) to give **15** (105 mg, 67% based on **12**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.25 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.00 (3H, s, SMe), 3.47 (2H, s, $ArCH_2$), 3.63 (2H, s, $ArCH_2$), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.33 (1H, d, J=2 Hz, H-4), 7.27 (1H, d, J=2 Hz, H-5). Exact MS m/z: Calcd for $C_{10}H_{14}O_3S$: 214.0662. Found: 214.0639.

Ethyl (3-Methyl-2-furyl)acetate (16) A mixture of Bu_3SnH (0.28 ml, 1.06 mmol) and AIBN (18 mg, 0.11 mmol) in benzene (10 ml) was added dropwide to a solution of **15** (161 mg, 0.75 mmol) in boiling benzene (8 ml) over a period of 3 h and the mixture was further heated under reflux for 3 h. After removal of the solvent, ethyl ether (10 ml) and a solution of potassium fluoride (500 mg) in water (5 ml) were added to the residue and the mixture was stirred at room temperature overnight. The organic layer was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel (benzene) to give **16** (104 mg, 83%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1740. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.23 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 1.97 (3H, s, ArMe), 3.55 (2H, s, $ArCH_2$), 4.13 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.16 (1H, d, J=2 Hz, H-4), 7.19 (1H, d, J=2 Hz, H-5). Exact MS m/z: Calcd for $C_9H_{12}O_3$: 168.0785. Found: 168.0758.

2-(3-Methyl-2-furyl)ethanol (17) A solution of **16** (90 mg, 0.54 mmol) in dry ethyl ether (5 ml) was added to a suspension of $LiAlH_4$ (42 mg, 1.1 mmol) in dry ethyl ether (5 ml) at 0 °C and the mixture was stirred at room temperature for 2 h. Usual work-up gave **17** (57 mg, 84%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3600, 3420. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.98 (3H, s, ArMe), 2.10 (1H, br s, OH), 2.80 (2H, t, J=6.5 Hz, $ArCH_2$), 3.80 (2H, br t, J=6.5 Hz, $C\underline{H}_2OH$), 6.14 (1H, d, J=2 Hz, H-4), 7.20 (1H, d, J=2 Hz, H-5). Exact MS m/z: Calcd for $C_7H_{10}O_2$: 126.0680. Found: 126.0680.

Attempted Synthesis of (3-Methyl-2-furyl)acetaldehyde (18) Method A: A 1 M solution of DIBAH in hexane (0.48 ml, 0.48 mmol) was added to a solution of the ester **16** (53 mg, 0.32 mmol) in dry toluene (3 ml) at −78 °C and the mixture was stirred at the same temperature for 1.5 h. A saturated ammonium chloride solution was added to the reaction mixture and the whole was extracted with ethyl ether. The extract was dried over $MgSO_4$ and the solvent was evaporated off to give the crude aldehyde **18** (15 mg). ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.98 (3H, s, ArMe), 3.65 (2H, d, J=2 Hz, $ArCH_2$), 6.25 (1H, d, J=2 Hz, H-4), 7.30 (1H, d, J=2 Hz, H-5), 9.66 (1H, t, J=2 Hz, CHO).

Method B: A solution of the alcohol **17** (85 mg, 0.66 mmol) in dry dichloromethane (1 ml) was added dropwise to a solution of Collins' reagent, prepared from CrO_3 (400 mg, 4 mmol) and pyridine (632 mg, 8 mmol), in dry dichloromethane (15 ml) at room temperature and the mixture was stirred at the same temperature for 15 min. The resultant precipitates were removed by decantation and the organic layer was washed with 1% hydrochloric acid, then dried over $MgSO_4$. The solvent was evaporated off to give the crude aldehyde **18** (58 mg). Attempts to purify the aldehyde **18** by either distillation or chromatography on silica gel were unsuccessful.

Dimethylsulfonium Acetyl(phenyl)methylide (22) A solution of the crude sulfonium salt **21** (218 mg), prepared from **20**[8] (146 mg, 0.81 mmol) and MeI–$AgBF_4$, in ethanol (10 ml) was added to a solution of sodium ethoxide (52 mg, 0.77 mmol) in ethanol (10 ml) and the mixture was stirred at room temperature for 1 h. The solvent was evaporated off, chloroform (10 ml) was added to the residue, and the precipitated salt was filtered off. The filtrate was dried over $MgSO_4$ and concentrated *in vacuo* to give the crystalline ylide **22** (122 mg, 78% based on **20**). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1510. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.87 (3H, s, COMe), 2.70 (6H, s, SMe_2), 7.0—7.5 (5H, m, aromatic protons). An attempted recrystallization from hexane resulted in partial decomposition of **22**.

2-(Methylthiomethyl)phenylacetone (23), [2-(Methylthiomethoxy)prop-1-enyl]benzene (24), and Ethyl 3-Phenylpropionate (25) DBU (181 mg, 1.2 mmol) was added to a solution of the crude sulfonium salt **21** (224 mg), prepared from **20** (150 mg, 0.83 mmol) and MeI–$AgBF_4$, in dry ethanol (10 ml) and the mixture was heated under reflux for 3 h. After usual work-up, the reaction mixture was chromatographed on silica gel (hexane–ethyl acetate, 60 : 1). The first eluate gave **24** (13 mg, 8% based on **20**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1660. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 2.06 (3H, d, J=1.5 Hz, C=CMe), 2.13 (3H, s, SMe), 5.00 (2H, s, OCH_2S), 5.51 (1H, br s, C=CH), 7.1—7.7 (5H, m, aromatic protons). Exact MS m/z: Calcd for $C_{11}H_{14}OS$: 194.0763. Found: 194.0757.

The second eluate gave **25** (20 mg, 14% based on **20**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.20 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.4—3.2 (4H, m, $ArCH_2CH_2$), 4.10 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.20 (5H, s, aromatic protons). These spectral data were identical with those of an authentic sample purchased from Aldrich Chemical Company, Inc.

The third eluate gave **23** (35 mg, 22% based on **20**) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1720. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.98 (3H, s, SMe), 2.16 (3H, s, COMe), 3.63 (2H, s, $ArCH_2$), 3.85 (2H, s, $ArCH_2$), 7.21 (4H, s, aromatic protons). Exact MS m/z: Calcd for $C_{11}H_{14}OS$: 194.0763. Found: 194.0757.

2-(Methylthiomethyl)phenylacetonitrile (31a) DBU (111 mg, 0.73 mmol) was added to a solution of the sulfonium salt **30a** (120 mg, 0.45 mmol), prepared from **29a**[8] and MeI–$AgBF_4$, in ethanol (5 ml) and the mixture was stirred at room temperature for 3 h. Usual work-up gave **31a** (49 mg, 62%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 2250. ^{1}H-NMR ($CDCl_3$,

60 MHz) δ: 1.95 (3H, s, SMe), 3.65 (2H, s, $ArCH_2$), 3.86 (2H, s, $ArCH_2$), 7.1—7.5 (4H, m, aromatic protons). *Anal.* Calcd for $C_{10}H_{11}NS$: C, 67.76; H, 6.25; N, 7.90. Found: C, 67.70; H, 6.27; H, 7.75.

Diethyl 2-(Methylthiomethyl)phenylmethylphosphonate (31b) DBU (100 mg, 0.66 mmol) was added to a solution of the sulfonium salt **30b** (228 mg, 0.55 mmol), prepared from **29b**[9] and MeI–$AgBF_4$, in ethanol (5 ml) and the mixture was stirred at room temperature for 2 h. Usual work-up gave **31b** (147 mg, 93%) as an oil. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ:1.23 (6H, t, J=7 Hz, $CH_2C\underline{H}_3\times2$), 2.00 (3H, s, SMe), 3.35 (2H, d, J_{PH}=22 Hz, PCH_2), 3.87 (2H, s, SCH_2), 4.00 (4H, dq, J_{PH}=7 Hz, J_{HH}=7 Hz, $C\underline{H}_2CH_3\times2$), 7.18 (4H, s, aromatic protons). *Anal.* Calcd for $C_{13}H_{21}O_3PS$: C, 54.15; H, 7.34. Found: C, 53.83; H, 7.02.

Ethyl 2-Methylphenyl(methylthio)acetate (36a) Using a procedure similar to that described for the preparation of **7a**, the sulfide **33a**[7] (200 mg, 0.95 mmol) was treated with $AgBF_4$ (206 mg, 0.95 mmol) in methyl iodide (5 ml). Excess methyl iodide was removed by decantation, the residue was extracted with acetonitrile (instead of dichloromethane for **7a**), and the solvent was evaporated off to give the sulfonium salt **34a** (285 mg, 97%) as an oil. The salt **34a** thus obtained was dissolved in ethanol (5 ml) and the whole was added to a solution of sodium ethoxide (63 mg, 0.93 mmol) in ethanol (5 ml) at 0 °C. After stirring of the mixture at room temperature for 40 min, the solvent was removed by evaporation. Chloroform was added to the residue and the precipitated salts were filtered off. The filtrate was concentrated *in vacuo* and the residue was chromatographed on silica gel (benzene–ethyl acetate, 30 : 1) to give **36a** (127 mg, 60%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1740. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.25 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.10 (3H, s, SMe), 2.40 (3H, s, ArMe), 4.20 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 4.73 (1H, s, CH), 7.1—7.7 (4H, m, aromatic protons). Exact MS *m/z*: Calcd for $C_{12}H_{16}O_2S$: 224.0869. Found: 224.0842.

Ethyl 2-Methyl-6-(methylthiomethyl)phenylacetate (37a) According to a procedure similar to that described for the preparation of **34a**, the sulfide **36a** (127 mg, 0.57 mmol) was *S*-methylated and the resultant sulfonium salt was heated in boiling ethanol (10 ml) containing DBU (87 mg, 0.57 mmol) for 2 h. Usual work-up gave **37a** (102 mg, 76%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) 1.25 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.00 (3H, s, SMe), 2.33 (3H, s, ArMe), 3.75 (2H, s, $ArCH_2$), 3.85 (2H, s, $ArCH_2$), 4.15 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.07 (3H, s, aromatic protons). Exact MS *m/z*: Calcd for $C_{13}H_{18}O_2S$: 238.1026. Found: 238.1036.

Ethyl 2,6-Dimethylphenylacetate (38a) Using a procedure similar to that described for the preparation of **16**, the sulfide **37a** (101 mg, 0.42 mmol) was treated with Bu_3SnH (0.14 ml, 0.504 mmol) and AIBN (6.6 mg, 0.05 mmol). Usual work-up gave **38a** (49 mg, 60%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) 1.23 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.33 (6H, s, ArMe$\times$2), 3.67 (2H, s, $ArCH_2$), 4.14 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 7.05 (3H, s, aromatic protons). Exact MS *m/z*: Calcd for $C_{12}H_{16}O_2$: 192.1149. Found: 192.1151.

Ethyl (4-Phenoxyphenylmethylthio)acetate (33b) and Ethyl (2-Phenoxyphenylmethylthio)acetate (33c) $TiCl_4$ (0.9 g, 4.74 mmol) was added to a solution of **32**[7] (0.8 g, 4.74 mmol) and diphenyl ether (1.2 g, 7.12 mmol) in dichloromethane (40 ml) at 0 °C and the mixture was stirred at room temperature for 18 h. Work-up as described for the preparation of **6c** gave a *ca.* 4 : 1 mixture of **33b** and **33c** (978 mg, 68%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.28 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$ for **33b,c**), 3.07 (8/5H, s, SCH_2CO for **33b**), 3.16 (2/5H, s, SCH_2CO for **33c**), 3.81 (8/5H, s, $ArCH_2$ for **33b**), 3.88 (2/5H, s, $ArCH_2$ for **33c**), 4.16 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$ for **33b,c**), 6.8—7.5 (9H, m, aromatic protons for **33b,c**). *Anal.* Calcd for $C_{17}H_{18}O_3S$: C, 67.52; H, 6.00. Found: C, 67.96; H, 6.41.

Ethyl 2-Methyl-5-phenoxyphenyl(methylthio)acetate (36b) and Ethyl 2-Methyl-3-phenoxyphenyl(methylthio)acetate (36c) A solution of sodium ethoxide (59 mg, 0.87 mmol) in ethanol (5 ml) was added to a solution of the mixture of sulfonium salts **34b,c** (350 mg, 0.87 mmol), prepared from **33b,c** and MeI–$AgBF_4$, in ethanol (5 ml) at 0 °C and the mixture was stirred at room temperature for 30 min. Usual work-up gave a mixture of **36b,c** (167 mg, 52%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1740. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.20 (12/5H, t, J=7 Hz, $CH_2C\underline{H}_3$ for **36b**), 1.23 (3/5H, t, J=7 Hz, $CH_2C\underline{H}_3$ for **36c**), 2.07 (12/5H, s, SMe for **36b**), 2.12 (3/5H, s, SMe for **36c**), 2.27 (3/5H, s, ArMe for **36c**), 2.37 (12/5H, s, ArMe for **36b**), 4.15 (8/5H, q, J=7 Hz, $C\underline{H}_2CH_3$ for **36b**), 4.18 (2/5H, q, J=7 Hz, $C\underline{H}_2CH_3$ for **36c**), 4.66 (4/5H, s, CH for **36b**), 4.77 (1/5H, s, CH for **36c**), 6.7—7.5 (8H, m, aromatic protons for **36b,c**). Exact MS *m/z*: Calcd for $C_{18}H_{20}O_3S$: 216.1131. Found: 216.1117.

Ethyl 6-Methyl-2-methylthiomethyl-3-phenoxyphenylacetate (37b) and Ethyl 2-Methyl-6-methylthiomethyl-3-phenoxyphenylacetate (37c) $AgBF_4$ (90%) (285 mg, 1.32 mmol) was added to a solution of **36,c** (417 mg, 1.32 mmol) in methyl iodide (15 ml) at 0 °C and the mixture was stirred at room temperature for 3 h, during which time only silver iodide separated out. The precipitates were filtered off and the filtrate was concentrated *in vacuo* to give the corresponding sulfonium salt quantitatively. The salt thus obtained was dissolved in ethanol (30 ml) and the mixture was heated under reflux for 2 h in the presence of DBU (200 mg, 1.32 mmol). Usual work-up gave a mixture of **37b,c** (216 mg, 60% based on **36b**, c) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1730. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.23 (3/5H, t, J=7 Hz, $CH_2C\underline{H}_3$ for **37c**), 1.26 (12/5H, t, J=7 Hz, $CH_2C\underline{H}_3$ for **37b**), 2.03 (3/5H, s, SMe for **37c**), 2.07 (12/5H, s, SMe for **37b**), 2.20 (3/5H, s, ArMe for **37c**), 2.30 (12/5H, s, ArMe for **37b**), 3.73 (2/5H, s, $ArCH_2$ for **37c**), 3.83 (8/5H, s, one of $ArCH_2$ for **37b**), 3.87 (2H, s, $ArCH_2$ for **37b,c**), 4.13 (2/5H, q, J=7 Hz, OCH_2 for **37c**), 4.16 (8/5H, q, J=7 Hz, OCH_2 for **37b**), 6.7—7.6 (7H, m, aromatic protons for **37b,c**). Exact MS *m/z*: Calcd for $C_{19}H_{22}O_3S$: 330.1288. Found: 330.1264.

Ethyl 2,6-Dimethyl-3-phenoxyphenylacetate (38b) Using a procedure similar to that described for the preparation of **16**, the mixtue of **37b,c** (86 mg, 0.26 mmol) was treated with Bu_3SnH–AIBN. Usual work-up gave **38b** (70 mg, 95%) as an oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1735. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 1.24 (3H, t, J=7 Hz, $CH_2C\underline{H}_3$), 2.20 (3H, s, ArMe), 2.33 (3H, s, ArMe), 3.73 (2H, s, $ArCH_2$), 4.16 (2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 6.7—7.5 (7H, m, aromatic protons). Exact MS *m/z*: Calcd for $C_{18}H_{20}O_3$: 284.1411. Found: 284.1422.

2,6-Dimethyl-3-phenoxyphenylacetic Acid (39) A mixture of **38b** (232 mg, 0.82 mmol) and sodium hydroxide (98 mg, 2.45 mmol) in ethanol (1.5 ml) and water (4 ml) was heated under reflux for 1.5 h. After removal of ethanol, the aqueous layer was acidified to pH 1 with concetrated hydrochloric acid and extracted with ethyl ether. The solvent was evaporated off to give **39** (209 mg, 100%), mp 120—121 °C (from hexane–ethyl acetate). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 2300—3400, 1075. ^{1}H-NMR ($CDCl_3$, 60 MHz) δ: 2.20 (3H, s, ArMe), 2.31 (3H, s, ArMe), 3.75 (2H, s, $ArCH_2$), 6.7—7.5 (7H, m, aromatic protons), 9.2—9.8 (1H, br, COOH). *Anal.* Calcd for $C_{16}H_{16}O_3$: C, 74.98; H, 6.29. Found: C, 74.94, H, 6.27.

References and Notes

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Regioselective Monotosylation of Non-protected and Partially Protected Glycosides by the Dibutyltin Oxide Method[1)]

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Tosylation of non-protected glycopyranosides with *p*-toluenesulfonyl chloride in the presence of 4-dimethylaminopyridine, after activiation of the glycosides by dibutyltin oxide, gave mono-*O*-tosylates in good yield. The regioselectivity in this tosylation was different from that in the corresponding benzoylation for some glycosides. The reason for this difference is discussed based on an equilibrium of the tin intermediates and kinetic attack of the tosyl chloride on the intermediates. Thus, by application of this tosylation method to non-protected and partially protected glycosides, various glycoside mono-*O*-tosylates were synthesized regioselectively.

Keywords glycoside; regioselective mono-*O*-tosylation; dibutyltin oxide method; tin intermediate

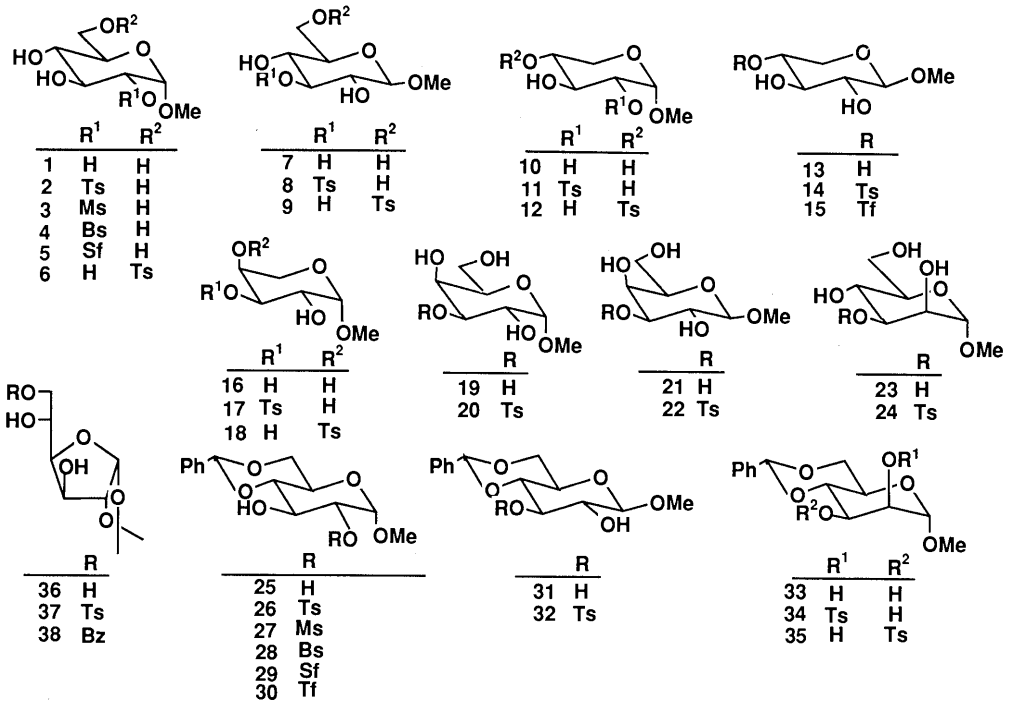
The tosyl group is useful in the field of carbohydrate chemistry as a protecting group,[3)] and for preparation of an epoxide on a glycoside ring, which can be employed as a synthetic intermediate of high reactivity.[4)] However, preparation of a particular mono-*O*-tosylate of glycosides often requires multisteps, *i.e.*, a protection–deprotection procedure, and this fact has limited the utilization of mono-*O*-tosylates in carbohydrate chemistry.

The success of regioselective monoacylation[5)] of non-protected glycosides through activation of a particular hydroxyl group by stannylation with dibutyltin oxide suggests that regioselective mono-*O*-sulfonation could also be achieved by a similar method. However, acylation (benzoylation) and sulfonation (tosylation) of a stannylene intermediate derived from a glycoside sometimes give different results.[6)] This paper described the results of mono-*O*-tosylation of various non-protected and partially protected glycosides, in comparison with previously reported regioselective mono-benzoylations.[5)]

Results and Discussion

The results of tosylation of various glycopyranosides with *p*-toluenesulfonyl chloride (TsCl) after treatment of the glycoside with dibutyltin oxide in boiling methanol are listed in Table I. It should be noted that tosylation is slower than benzoylation (which is usually completed within a few hours in the absence of a basic catalyst),[5)] and usually requires a basic catalyst such as 4-dimethylaminopyridine (DMAP) and overnight reaction to ensure completion. The yield and ratio were determined by chromatographic isolation of each product. Structure determinations are done mainly by carbon-13-nuclear magnetic resonance (^{13}C-NMR) spectral analysis: for example, in the 2-*O*-tosylate **2**, the C-2 signal is shifted downfield by 8.0 ppm and the C-1 and C-3 signals are shifted upfield by 3.2 and 3.7 ppm, respectively, compared to those of the original glycoside **1**.

Non-protected Glycopyranosides Tosylation of Me β-D-Glc[7)] (**7**) and Me β-D-Xyl (**13**) gave the 6-*O*-tosyl and 4-*O*-tosyl derivatives, **9** and **14**, in excellent yields, as in



Ms = methanesulfonyl, Ts = *p*-toluenesulfonyl, Bs = benzenesulfonyl, Sf = *p*-toluenesulfinyl, Tf = trifluoromethanesulfonyl, Bz = benzoyl

Chart 1

TABLE I. Regioselectivity in Monotosylation and Monobenzoylation of Non-protected Glycopyranosides by the Dibutyltin Oxide Method

Substrate	Tosylation					Benzoylation[a]				
	Yield (%)	Composition (%)				Yield (%)	Composition (%)			
	Mono	2-*O*	3-*O*	4-*O*	6-*O*	Mono	2-*O*	3-*O*	4-*O*	6-*O*
Me α-D-Glc (**1**)	50	56	—	—	44	76	100	—	—	—
Me β-D-Glc (**7**)	92	—	—	—	100	86	—	—	—	100
Me α-D-Gal (**19**)	62	—	100	—	—	68	26	51	—	23
Me β-D-Gal (**21**)	78	—	100	—	—	53	—	100	—	—
Me α-D-Man (**23**)	65	—	100	—	—	65	—	100	—	—
Me α-D-Xyl (**10**)	84	38	—	62		82	64	—	36	
Me β-D-Xyl (**13**)	100	—	—	100		78	—	—	100	
Me β-L-Ara (**16**)	94	—	44	56		80	10	61	39	

a) Ref. 5.

TABLE II. ^{13}C-NMR Data for Mono-*O*-sulfonyl Glycopyranosides

Compd.	Hexopyranosides									Pentopyranosides (in Chloroform-*d*)						
	2[a]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**8**[a]	**20**[b]	**22**[b]	**24**[b]	**11**	**12**	**14**	**15**	**17**	**18**	[c]
C-1	98.1	98.7	98.0	99.6	101.3	105.5	102.2	106.3	103.2	97.3	99.0	103.5	103.4	100.1	99.6	98.0
	(−3.2)	(−2.6)	(−3.3)	(−1.7)	(0)	(0)	(+0.5)	(+2.4)	(+1.2)	(−0.1)	(+0.7)	(−0.3)	(−0.4)	(+0.1)	(−0.4)	(−2.0)
C-2	81.7	81.6	81.9	79.8	73.3	72.6	68.0	70.5	71.2	79.5	72.2	72.7	72.8	68.6	69.5	78.6
	(+8.0)	(+7.9)	(+8.2)	(+6.1)	(−0.4)	(−2.4)	(−2.5)	(−0.3)	(−0.5)	(+8.3)	(+1.0)	(0)	(+0.1)	(−0.5)	(+0.4)	(+9.5)
C-3	71.6	71.9	71.6	72.5	75.0	89.6	83.4	85.6	84.7	71.5	71.5	72.7	72.3	81.1	68.5	67.3
	(−3.7)	(−3.4)	(−3.7)	(−2.8)	(−0.3)	(+11)	(+11.8)	(+12.7)	(+13.6)	(−1.7)	(−1.7)	(−3.0)	(−3.4)	(+12)	(−0.6)	(−1.8)
C-4	71.7	71.7	71.7	71.8	71.2	68.6	70.2	69.6	66.3	70.1	78.0	75.7	83.0	66.8	78.9	69.8
	(−0.3)	(−0.3)	(−0.3)	(−0.2)	(−0.8)	(−2.9)	(−0.7)	(+0.8)	(−1.6)	(+0.7)	(+8.6)	(+6.4)	(+14)	(−1.6)	(+10.5)	(+1.4)
C-5	73.9	73.8	73.9	74.0	70.8	77.9	72.6	76.6	75.4	60.8	58.8	62.3	61.9	62.0	60.7	62.0
	(−0.1)	(−0.2)	(−0.1)	(0)	(−3.2)	(+1.3)	(+0.1)	(+1.4)	(+1.7)	(+0.1)	(−1.9)	(−2.4)	(−2.8)	(−0.6)	(−1.9)	(−0.6)
C-6	62.1	62.0	62.1	62.4	71.2	61.8	62.9	62.8	63.3							
	(−0.7)	(−0.8)	(−0.7)	(−0.4)	(+8.4)	(−0.8)	(+0.3)	(+1.7)	(+1.2)							

Parenthetical values indicate shift values from the original glycosides. *a*) In pyridine-d_5. *b*) In methanol-d_4. *c*) Me β-L-Ara 2-*O*-tosylate, prepared by a classical method for comparison.

the case of benzoylation. Trifluoromethanesulfonation (triflation: Tf) of Me β-D-Xyl (**13**) gave the 4-*O*-Tf derivative **15** as well, though in low yield due to the instability of the product.

Me α-D-Gal (**19**), Me β-D-Gal (**21**), and Me α-D-Man (**23**) produced only the 3-*O*-tosyl derivatives, **20**, **22**, and **24**, as the mono-*O*-tosylates,[8] respectively, in accordance with the mechanism proposed for benzoylation: *i.e.*, the formation of a cyclic tin intermediate involving *cis*-vicinal hydroxyls with enhancement of the reactivity of the equatorial hydroxyl group.[5] In these compounds tosylation would have proceeded through the *C1* conformations.

In contrast to the above substrates, Me β-L-Ara (**16**) produced a mixture of the 3-*O* and 4-*O*-tosylates, **17** and **18**, with a slight preference for the latter: the result is different from that of benzoylation and also from benzylation[9] by the dibutyltin oxide method, where the ratio of the 3-*O*-alkyl to 4-*O*-alkyl derivatives is 85 : 15. The present result can be explained by a greater contribution of the *1C* conformer (B) in tosylation. The initially formed *C1* conformer (A) would equilibrate, in a slow tosylation reaction, with the *1C* conformer (B), in which the equatorial 4-*O*-*Sn* bond is the most reactive to a bulky electrophile.

Tosylation of Me α-D-Xyl (**10**) gave a mixture of the 2-*O* and 4-*O*-tosylate, **11** and **12**: the ratio of these compounds, however, is the reverse of that in the case of benzoylation. This result can again be explained by a significant contribution of the *1C* conformer (D), where the 4-*O*–*Sn* bond is the most reactive to a bulky electrophile for steric reasons.

The difference of regioselectivity between benzoylation and tosylation of Me α-D-Glc (**1**) requires a different explanation. The initially formed tin intermediate (E) (benzoylation proceeds through this species)[5] will equilibrate with many species such as E–H in the slow tosylation condition, in which the 6-*O*–*Sn* bond of G and H is the most reactive for steric reasons. Thus, a contribution of G or H, at least partially, would increase the formation of the 6-*O*-tosyl derivative **6**.

From the above results, we conclude that benzoylation (a fast reaction) reflects the relative stability and abundance of the tin intermediates as suggested already,[5] while in tosylation (a slow reaction) the tin intermediate comes into equilibrium with several species, so the contribution of the kinetically most favored species increases the proportion of the product formed through that intermediate, even if it exists in only a small amount.

Partially Protected Glycosides Tosylation of Me 4,6-*O*-benzylidene-α-D-Glc (**25**) by the above method gave the 2-*O*-tosyl derivative **26** in high yield. Methanesulfonation, triflation, benzensulfonation, and *p*-toluenesulfination gave

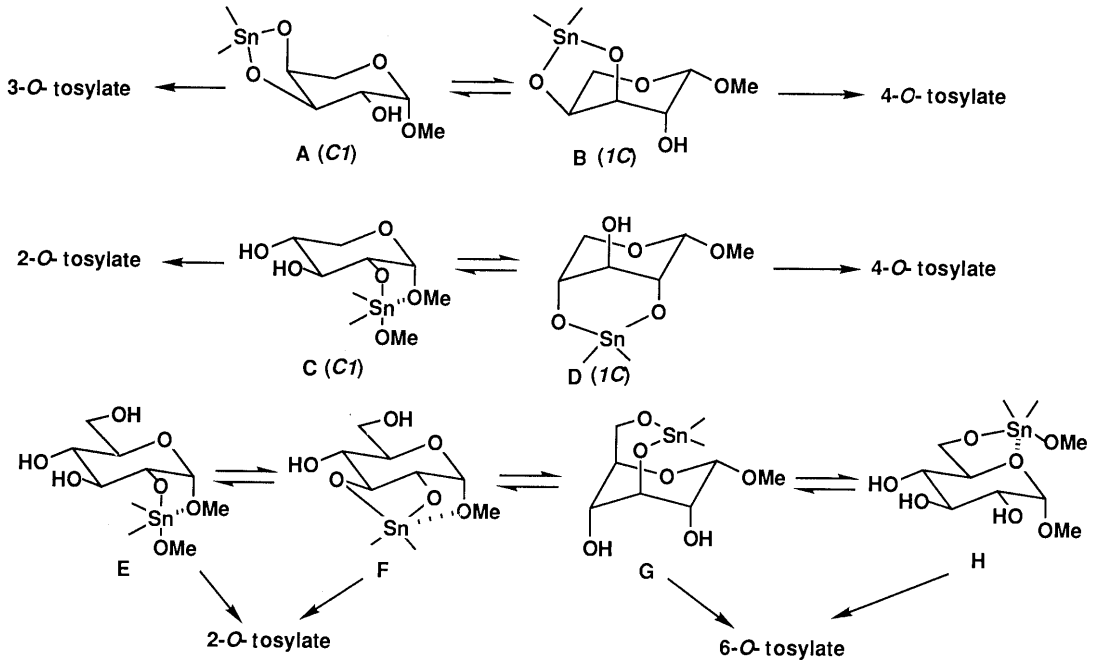


Chart 2

TABLE III. ^{13}C-NMR Data for Partially Protected Glycoside *O*-Sulfonates and *O*-Sulfinate (in Chloroform-*d*)

Compd.	26	27	28	30	29	32	34	35	37
C-1	98.0	98.6	98.1	97.6	99.4	104.3	99.6	101.7	105.9
	(−2.0)	(−1.4)	(−1.9)	(−2.4)	(−0.6)	(+0.5)	(−1.8)	(+0.3)	(0)
C-2	79.4	79.3	79.6	84.1	78.4	73.9	78.6	70.5	86.2
	(+6.6)	(+6.5)	(+6.8)	(+11)	(+5.6)	(−0.9)	(+8.2)	(+0.1)	(−0.2)
C-3	68.6	68.7	68.3	68.1	68.6	82.0	66.8	78.7	74.5
	(−2.7)	(−2.6)	(−3.0)	(−3.2)	(−2.7)	(+9.4)	(−0.8)	(+11.1)	(−0.8)
C-4	80.8	81.4	81.0	81.0	81.0	78.0	78.5	75.5	81.5
	(−0.2)	(+0.4)	(0)	(0)	(0)	(−2.0)	(+0.2)	(−2.8)	(+0.1)
C-5	61.9	61.2	61.9	62.0	62.4	66.1	63.4	63.7	67.0
	(−0.5)	(−1.2)	(−0.5)	(−0.4)	(−0.4)	(+0.4)	(+0.7)	(+1.0)	(−3.6)
C-6	68.1	68.8	69.0	68.6	68.8	68.7	68.6	68.6	74.7
	(−0.8)	(−0.1)	(−0.1)	(−0.3)	(−0.1)	(+0.8)	(+0.6)	(+0.6)	(+11)

Parenthetical value indicate shift values from the original glycosides.

the corresponding sulfonates and sulfinate, **27**—**30**, as well. These products were smoothly hydrolyzed with 50% acetic acid, except for the triflate **30**, to the corresponding Me α-D-Glc 2-*O*-sulfonates or sulfinates **3**—**5** in excellent yields. The *O*-triflate, however, could not be isolated because of its instability.

Me 4,6-*O*-benzylidene-β-D-Glc (**31**) gave, with high regioselectivity, the 3-*O*-tosylate **32**, which was hydrolyzed with 50% acetic acid to Me β-D-Glc 3-*O*-tosylate (**8**) in an excellent yield.

Tosylation of Me 4,6-*O*-benzylidene-α-D-Man (**33**) under the same conditions gave the 3-*O*-tosylate **35** as a single product in high yield. However, when this tosylation was performed without any base catalyst, the product was accompanied with the 2-*O*-tosylate **34**, though the total conversion yield was low. It has been suggested that the dibutylstannylene intermediate derived from **33** is dimeric in non-coordinating solvents such as benzene (in this case, the 2-*O*–*Sn* bond is the most reactive, because it is in an apical orientation), while in coordinating solvents such as dioxane, or in the presence of a base it is possibly monomeric (an equatorial 3-*O*–*Sn* bond is esterified).[10] The present result suggests that, even in coordinating solvent such as dioxane, the cyclic stannylene derivative is not completely monomeric. Complete monomerization of the stannylene intermediate may only be achieved by addition of a powerful coordinating ligand such as DMAP.

Tosylation and benzoylation of 1,2-*O*-isopropylidene-α-D-glucofuranose (**36**) gave the same regioselectivity, where the 6-*O*-tosylate **37** and benzoate **38** were produced in 98% and 75% yields, respectively. Comparisons of the yields with those of direct tosylation and benzoylation (51% and 41%, respectively) clearly indicate that the dibutyltin oxide method is superior to direct tosylation or acylation even for the primary hydroxyl group.

Experimental

Unless otherwise stated, infrared spectra (IR) were taken as KBr disks (data are given in cm^{-1}) and NMR spectra with tetramethylsilane as an internal standard (chemical shifts are given in δ values). Data for the aromatic group and any other protecting group are omitted. For mass spectra (MS) and high-resolution MS (HRMS), major peaks are indicated in *m/z* (%). For other items, see Experimental in ref. 1. Identities were confirmed by mixed melting-point determination (for crystalline compounds), and also by comparisons of thin layer chromatographic (TLC) behavior and ^{1}H-NMR and IR spectra.

Tosylation of Glycosides by the Dibutyltin Oxide Method (General Procedure) A mixture of glycoside (0.3—1 g) and Bu_2SnO (1.0 mol eq) in dry MeOH (10—30 ml) was heated under reflux for 3—10 h. After evaporation of the solvent, the residue was dried, dissolved (or suspended) in dioxane (25—50 ml) and tosylated with TsCl (1.1 mol eq) and DMAP (0.1—0.2 mol eq) for 3—20 h at room temperature with periodic monitoring of the progress of the reaction by TLC. Amounts of materials and reaction times with TsCl are given for each individual experiment. For processing, the mixture was concentrated under reduced pressure below 30 °C and the residue was chromatographed on a silica gel column for separation. Benzene and $CHCl_3$ elution removed tin compounds and the EtOAc eluate gave a mono-*O*-tosylate, which was further purified by medium-pressure liquid chromatography, if necessary. The results for non-protected glycopyranosides are summarized in Table I.

Tosylation of Me α-D-Glc (1) Reaction of **1** (0.3 g) for 11 h gave the 2-*O*-tosylate **2** (142 mg. 28%) and the 6-*O*-tosylate **6**, mp 118—120 °C[11] (116 mg, 22%).

Methyl 2-*O*-Tosyl-α-D-glucopyranoside (**2**): Colorless needles from EtOAc–ether, mp 140—142 °C. IR: 3435, 1366, 1171. ^{1}H-NMR

(pyridine-d_5): 5.11 (1H, d, J=3.8 Hz, H-1), 4.80 (1H, dd, J=3.8, 10 Hz, H-2), 4.6—4.0 (5H, H-3, 4, 5, 6), 3.29 (3H, s, OMe). MS: 317 (M^+ −OMe, 1.6), 161 (100). *Anal.* Calcd for $C_{14}H_{20}O_8S$: C, 48.27; H, 5.79. Found: C, 48.22; H, 5.85.

Tosylation of Me β-D-Glc (7) Reaction of **7** (0.5 g) for 12 h gave the 6-*O*-tosylate **9**, gum,[11] (778 mg, 92%).

Tosylation of Me α-D-Xyl (10) Reaction of **10** (1.0 g) for 16 h gave the 2-*O*-tosylate **11** (625 mg, 32%) and 4-*O*-tosylate **12** (1.01 g, 52%).

Methyl 2-*O*-Tosyl-α-D-xylopyranoside (**11**): Colorless needles from EtOAc–hexane, mp 140—141 °C. IR: 3520, 1355, 1170. ^{1}H-NMR: 4.58 (1H, d, J=3.7 Hz, H-1), 4.28 (1H, dd, J=3.7, 9.8 Hz, H-2), 4.0—3.5 (4H, H-3, 4, 5), 3.27 (3H, s, OMe). MS: 287 (M^+ −OMe, 0.5), 103 (100). *Anal.* Calcd for $C_{13}H_{18}O_7S$: C, 49.05; H, 5.70. Found: C, 49.19; H, 5.77.

Methyl 4-*O*-Tosyl-α-D-xylopyranoside (**12**): Colorless solid. IR ($CHCl_3$): 3560, 1366, 1174. ^{1}H-NMR: 4.67 (1H, d, J=4.5 Hz, H-1), 4.30 (1H, m, H-4), 3.9—3.4 (4H, H-2,3,5), 3.38 (3H, s, OMe). MS: 319 (M^+ −1, 0.8), 287 (M^+ −OMe, 4), 155 (100). HRMS: Calcd for $C_{13}H_{17}O_7S$ (M^+ −1); $C_{12}H_{15}O_6S$ (M^+ −OMe): 319.0849; 287.0588. Found: 319.0839; 287.0605.

Tosylation of Me β-D-Xyl (13) Reaction of **13** (2.0 g) for 2 h gave the 4-*O*-tosylate **14** (3.87 g, 100%).

Methyl 4-*O*-Tosyl-β-D-xylopyranoside (**14**): Colorless needles from EtOAc–hexane, mp 135—136 °C. IR: 3530, 1327, 1170. ^{1}H-NMR: 4.64 (1H, d, J=8.0 Hz, H-1), 4.4—3.8 (5H, H-2,3,4,5), 3.50, (3H, s, OMe). MS: 287 (M^+ −OMe, 1.7), 155 (100). *Anal.* Calcd for $C_{13}H_{18}O_7S$: C, 49.05; H, 5.70. Found: C, 49.15; H, 5.70.

Tosylation of Me β-L-Ara (16) Reaction of **16** (0.5 g) for 16 h gave the 3-*O*-tosylate **17** (396 mg, 41%) and the 4-*O*-tosylate **18** (515 mg, 53%).

Methyl 3-*O*-Tosyl-β-L-arabinopyranoside (**17**): Colorless prisms from EtOAc–hexane, mp 69—72 °C. IR: 3535, 1361, 1175. ^{1}H-NMR: 4.78 (1H, d, J=3.7 Hz, H-1), 4.66 (1H, dd, J=3.4, 9.8 Hz, H-3), 4.16 (1H, m, H-4), 4.00 (1H, dd, J=3.7, 9.8 Hz, H-2), 3.8—3.7 (2H, H-5), 3.41 (3H, s, OMe). MS: 287 (M^+ −OMe, 1.8), 86 (100). *Anal.* Calcd for $C_{13}H_{18}O_7S \cdot H_2O$: C, 46.43; H, 5.99. Found: C, 46.77; H, 6.05.

Methyl 4-*O*-Tosyl-β-L-arabinopyranoside (**18**): Colorless needles from ether–hexane, mp 152—153 °C. IR: 3460, 1368, 1173. ^{1}H-NMR: 4.82 (1H, m, H-4), 4.77 (1H, d, J=3.7 Hz, H-1), 3.88 (1H, dd, J=3.7, 9.8 Hz, H-2), 3.8—3.7 (3H, H-3,5), 3.40 (3H, s, OMe). MS: 287 (M^+ −OMe, 0.4), 155 (100). *Anal.* Calcd for $C_{13}H_{18}O_7S$: C, 49.05; H, 5.70. Found: C, 48.97; H, 5.76.

Sulfonation and Sulfination of Me 4,6-*O*-Benzylidene-α-D-Glc (25) Compound **25** (1 g) was stannylated with Bu_2SnO as described above. The dried tin intermediate was dissolved in dioxane and treated with an appropriate sulfonyl or sulfinyl chloride (1.1 mol eq) and DMAP (0.1—0.2 mol eq) under reflux for 2—4 h, then concentrated to dryness. Chromatography of the residue with benzene and $CHCl_3$ removed tin compounds. Further elution of the column with $CHCl_3$–EtOAc and EtOAc gave the *O*-sulfonate or sulfinate. The following compounds were prepared.

The 2-*O*-Tosylate **26**: Yield 99%. Colorless needles from ether–hexane, mp 158—161 °C (lit. mp 151—153 °C).[12]

The 2-*O*-Methanesulfonate **27**: Yield 93%. Colorless needles from benzene, mp 138—140 °C. IR: 3465, 1329, 1171. ^{1}H-NMR: 4.91 (1H, d, J=3.9 Hz, H-1), 4.47 (1H, dd, J=3.9, 9.2 Hz, H-2), 4.4—3.5 (5H, H-3,4,5,6), 3.44 (3H, s, OMe), 3.11 (3H, s, Ms). MS: 360 (M^+, 20), 107 (100). *Anal.* Calcd for $C_{15}H_{20}O_8S$: C, 50.00; H, 5.60. Found: C, 49.86; H, 5.77.

The 2-*O*-Benzenesulfonate **28**: Yield 100%. Colorless needles from EtOAc–hexane, mp 157—160 °C. IR: 3460, 1361, 1184. ^{1}H-NMR: 4.83 (1H, d, J=3.7 Hz, H-1), 4.40 (1H, dd, J=3.7, 9.3 Hz, H-2), 4.3–3.4 (5H, H-3,4,5,6), 3.33 (3H, s, OMe). MS: 422 (M^+, 0.5), 107 (100). *Anal.* Calcd for $C_{20}H_{22}O_8S$: C, 56.86; H, 5.25. Found: C, 56.50; H, 5.25.

The 2-*O*-Triflate **30**: Yield 90%. Colorless needles from benzene–hexane, mp 110—111 °C. IR: 3485, 1414, 1145. ^{1}H-NMR: 4.95 (1H, d, J=3.9 Hz, H-1), 4.62 (1H, dd, J=3.9, 9.3 Hz, H-2), 4.4—3.6 (5H, H-3,4,5,6), 3.46 (3H, s, OMe). MS: 414 (M^+, 94), 87 (100).

The 2-*O*-*p*-Toluenesulfinate **29**: Yield 100%. Colorless prisms from EtOAc–hexane, mp 164.5—165.5 °C. IR: 3420, 1129. ^{1}H-NMR (400 MHz): 4.95 (1H, d, J=2.8 Hz, H-1), 4.27 (1H, dd, J=4.9, 10.4 Hz, H-6), 4.13—4.11 (2H, H-2,3), 3.85 (1H, dt, J=4.9, 10.4 Hz, H-5), 3.71 (1H, br t, J=10.4 Hz, H-6), 3.48 (1H, m, H-4), 3.45 (3H, s, OMe). MS: 420 (M^+, 0.3), 389 (M^+ −OMe, 0.7), 107 (100). *Anal.* Calcd for $C_{21}H_{24}O_7S \cdot 1/2H_2O$: C, 58.73; H, 5.87. Found: C, 58.49; H, 5.68.

Deprotection of the Benzylidene Derivatives The above benzylidene derivatives were heated in 50% AcOH under reflux for 1 h, then the solvent was evaporated off. Chromatography of the residue gave the deprotected compounds in yields of 85—100%, except for the 2-*O*-triflate.

Methyl 2-*O*-Tosyl-α-D-glucopyranoside (**2**): Yield 100%. Data, see above.

Methyl 2-*O*-Methanesulfonyl-α-D-glucopyranoside (**3**): Yield 100%. Colorless prisms from EtOAc, mp 120—121 °C. IR: 3440, 1364, 1171. ^{1}H-NMR (pyridine-d_5): 5.26 (1H, d, J=4.0 Hz, H-1), 4.86 (1H, dd, J=4.0, 9.0 Hz, H-2), 4.6—4.0 (5H, H-3,4,5,6), 3.40 (3H, s, OMe), 3.37 (3H, s, Ms). MS: 241 (M^+ −OMe, 0.6), 87 (100). *Anal.* Calcd for $C_8H_{16}O_8S$: C, 35.29; H, 5.92. Found: C, 35.19; H, 5.98.

Methyl 2-*O*-Benzenesulfonyl-α-D-glucopyranoside (**4**): Yield 100%. Colorless needles from EtOAc, mp 201—203 °C. IR: 3530, 1356, 1175. ^{1}H-NMR (pyridine-d_5): 5.09 (1H, d, J=3.7 Hz, H-1), 4.80 (1H, dd, J=3.7, 9.4 Hz, H-2), 4.6—4.0 (5H, H-3,4,5,6), 3.36 (3H, s, OMe). MS: 303 (M^+ −OMe, 0.3), 73 (100). *Anal.* Calcd for $C_{13}H_{18}O_8S$: C, 46.70; H, 5.43. Found: C, 46.56; H, 5.48.

Methyl 2-*O*-*p*-Toluenesulfinyl-α-D-glucopyranoside (**5**): Yield 85%. Colorless prisms from acetone–ether, mp 134—137 °C. IR: 3445, 1149. ^{1}H-NMR (400 MHz, pyridine-d_5): 5.43 (1H, br s, H-1), 4.65—4.55 (2H, H-2,3), 4.45 (1H, br d, J=11.9 Hz, H-6), 4.29 (1H, dd, J=4.9, 11.9 Hz, H-6), 4.22—4.13 (2H, H-4,5), 3.46 (3H, s, OMe). MS: 333 (M^+ +1, 0.3), 139 (100). *Anal.* Calcd for $C_{14}H_{20}O_7S \cdot 1/2H_2O$: C, 49.26; H, 6.20. Found: C, 49.49; H, 6.16.

Tosylation of Me 4,6-*O*-Benzylidene-β-D-Glc (31) Compound **31** (800 mg) was tosylated and worked up as described for the sulfonation of **25** to give the 3-*O*-tosylate **32** (949 mg, 77%), as colorless prisms from benzene–hexane, mp 166—167 °C. IR: 3380. 1367, 1173. ^{1}H-NMR: 5.42 (1H, t, J=9.0 Hz, H-3), 4.71 (1H, d, J=7.2 Hz, H-1), 4.4 (1H, m, H-6), 4.1—3.6 (4H, H-2,4,5,6). MS: 436 (M^+, 7), 107 (100). *Anal.* Calcd for $C_{21}H_{24}O_8S$: C, 57.79; H, 5.54. Found: C, 57.85; H, 5.57.

Methyl 3-*O*-Tosyl-β-D-glucopyranoside (8) Compound **32** (200 mg) was deprotected as described above to give **8** (142 mg, 89%), as a gum, from the EtOAc eluate. IR ($CHCl_3$): 3585, 1355, 1171. ^{1}H-NMR (400 MHz, pyridine-d_5): 5.50 (1H, t, J=9.5 Hz, H-3), 4.68 (1H, d, J=7.6 Hz, H-1), 4.44 (1H, dd, J=2.1, 12.2 Hz, H-6), 4.37—4.32 (2H, H-4,6), 3.95 (1H, dd, J=7.6, 9.5 Hz, H-2), 3.85 (1H, ddd, J=2.1, 2.4, 9.4 Hz, H-5), 3.55 (3H, s, OMe). MS: 349 (M^+ +1, 0.4), 317 (M^+ −OMe, 5), 144 (100).

Tosylation of Me 4,6-*O*-Benzylidene-α-D-Man (33) (1) The stannylene derivative of **33** (100 mg), prepared as described above, was tosylated in dioxane (20 ml) with TsCl (1.0 mol eq) and DMAP (0.4 mol eq) to give the 3-*O*-tosylate **35** (150 mg, 97%).

(2) Tosylation of the stannylene derivative of **33** (100 mg) in dioxane with TsCl (1.0 mol eq) (without DMAP) for 12 h gave a mixture of the 2-*O*-tosylate **34** and 3-*O*-tosylate **35** (75 mg, 49%, **34**/**35** ratio was 1:4 on the basis of the NMR spectrum).

The 2-*O*-Tosylate **34**: Colorless prisms from ether, mp 82—84 °C. ^{1}H-NMR: 4.82 (1H, d, J=1.5 Hz, H-1), 4.75 (1H, dd, J=1.5, 3.4 Hz, H-2), 4.3—4.1 (2H), 3.9—3.7 (3H) (H-3,4,5,6), 3.37 (3H, s, OMe). MS: 436 (M^+, 12), 105 (100). *Anal.* Calcd for $C_{21}H_{24}O_8S$: C, 57.79; H, 5.54. Found: C, 57.51; H, 5.66.

The 3-*O*-Tosylate **35**: Colorless needless from CH_2Cl_2–hexane, mp 151—154 °C. IR: 3425, 1353, 1169. ^{1}H-NMR: 4.77 (1H, d, J=1.2 Hz, H-1), 4.77 (1H, dd, J=3.4, 9.5 Hz, H-3), 4.32 (1H, dd, J=1.2, 3.4 Hz, H-2), 4.21 (1H, m, H-5), 4.08 (1H, t, J=9.5 Hz, H-4), 3.8—3.7 (1H, m, H-6), 3.37 (3H, s, OMe). MS: 436 (M^+, 41), 155 (100). *Anal.* Calcd for $C_{21}H_{24}O_8S$: C, 57.79, H, 5.54. Found: C, 57.58; H, 5.58.

Tosylation of 1,2-*O*-Isopropylidene-α-D-glucofuranose (36) (1) The Bu_2SnO Method: Compound **36** (220 mg) was stannylated according to the general procedure and the resulting tin intermediate in dioxane (15 ml) was tosylated with TsCl (229 mg, 1.2 mol eq) (without using DMAP) for 10 min at room temperature. Chromatography of the product gave the 6-*O*-tosylate **37** (366 mg, 98%) as colorless prisms from ether–hexane, mp 103—105 °C. IR: 3530, 1330, 1170. ^{1}H-NMR: 6.14 (1H, d, J=3.5 Hz, H-1), 5.0—4.4 (6H, H-2,3,4,5,6), 2.21 (3H, s, $ArCH_3$), 1.32, 1.52 (each 3H, s, CMe_2). *Anal.* Calcd for $C_{16}H_{22}O_8S$: C, 51.33; H, 5.92. Found: C, 51.41; H, 5.95.

(2) Direct Tosylation: A mixture of **36** (220 mg) and TsCl (229 mg, 1.2 mol eq) in pyridine (2 ml) was stirred for 30 min at room temperature, then weakly acidified with 0.5 N HCl and extracted with EtOAc. Chromatography of the product gave the 6-*O*-tosylate **37** (190 mg, 51%).

Benzoylation of 1,2-*O*-Isopropylidene-α-D-glucofuranose (36) (1) The Bu_2SnO Method: Compound **36** (220 mg) was stannylated and benzoylated with benzoyl chloride (169 mg, 1.2 mol eq) as in the case of tosylation, for 10 min at room temperature. Chromatography of the product gave the 6-*O*-benzoate **38** (244 mg, 75%), as colorless needles from EtOAc, mp 188—191 °C. IR: 3480, 1685. ^{1}H-NMR: 6.30 (1H, d, J=4.0 Hz, H-1), 5.3—4.7 (6H, H-2,3,4,5,6), 1.39, 1.60 (each 3H, s, CMe_2). *Anal.* Calcd for

$C_{16}H_{20}O_7$: C, 59.25; H, 6.22. Found: C, 59.01; H, 6.23.

(2) Direct Benzoylation: Compound **36** (110 mg) in pyridine (2 ml) was benzoylated with benzoyl chloride (1.2 mol eq) for 1.5 h at room temperature to give the 6-*O*-benzoate **38** (80 mg, 49%).

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4) Glycopyranoside mono-*O*-tosylates are also good precursors of branched furanosides [see *a*) ref. 1, and *b*) H. H. Bear, D. J. Astles, H. C. Chin, and L. Siemsen, *Canad. J. Chem.*, **63**, 432 (1985)].
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7) Abbreviations: Me = methyl, Ara = arabinopyranoside, Gal = galactopyranoside, Glc = glucopyranoside, Man = mannopyranoside, Xyl = xylopyranoside.
8) The lower regioselectivity in benzoylation of Me α-D-Gal by the dibutyltin oxide method than in the corresponding tosylation suggests a partial contribution of acyl migration in the former reaction. However, the real reason is still obscure.
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Synthesis and Biological Activities of New 1,4-Benzothiazine Derivatives

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New 2*H*-1,4-benzothiazin-3(4*H*)-one derivatives possessing (4-phenyl-1-piperazinyl)alkyl moieties at the 2-position were synthesized and tested for calcium antagonistic and calmodulin antagonistic activities. Antihypertensive effects in spontaneously hypertensive rats were also evaluated. In general, these compounds were rather weak calcium channel blockers, although, in contrast, many of them had moderate to potent calmodulin antagonistic activity, and 2-[3-(4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzothiazin-3(4*H*)-one derivatives 45, 74 and 75 showed potent antihypertensive effects.

Keywords 1,4-benzothiazine; piperazine; antihypertensive activity; intracellular calcium antagonist; calmodulin antagonist

Calcium ion (Ca^{2+}) is an important intracellular messenger and is involved in the regulation of a variety of cell functions. Calmodulin (CaM) is a ubiquitous intracellular calcium-receptive protein and may play an important role in mechanisms of stimulus-induced cellular responses such as smooth muscle contraction, secretion of thyroid hormone, platelet function, cell proliferation and others.[1] Since the studies on CaM[2,3] much interest has centered around the compounds possessing CaM antagonistic activity and various CaM antagonists have been reported[4] (Fig. 1). Neuroleptic phenothiazine derivatives such as chlorpromazine[5] and trifluoperazine[6] have been reported to be potent CaM antagonists. Recently, CaM antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7)[7] and 3-(2-benzothiazolyl)-4,5-dimethoxy-*N*-[3-(4-phenyl-1-piperazinyl)propyl]benzenesulfonamide (HT-74)[8] were described by Hidaka *et al.* However, these compounds have not been applied clinically as cardiovascular drugs, since phenothiazines such as chlorpromazine and trifluoperazine are very potent central nervous system (CNS) depressants and the W-7 type compounds do not seem to be potent enough as CaM inhibitors to elicit a pharmacological effect *in vivo*.

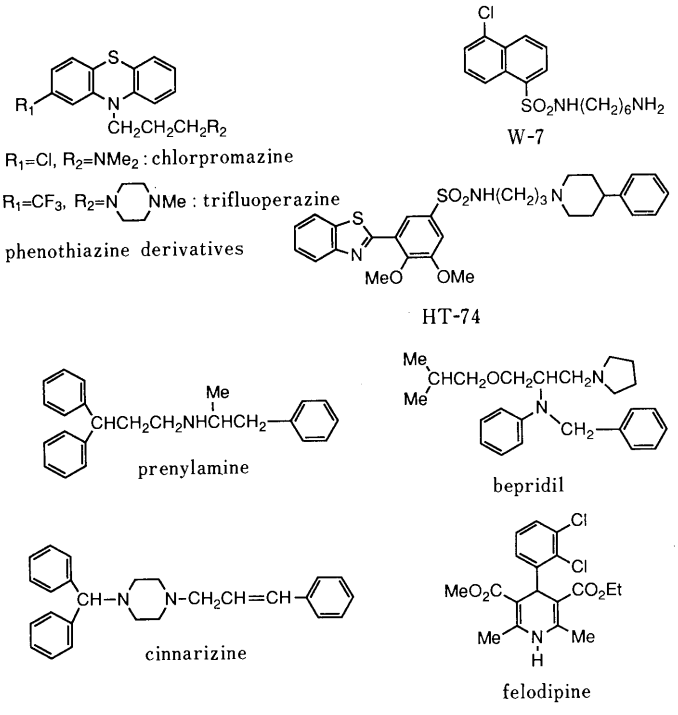


Fig. 1. CaM Antagonists

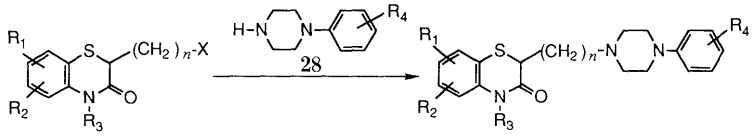
On the other hand, it has been well established that calcium channel blockers are useful therapeutic agents for the treatment of various cardiovascular disorders. Among them, prenylamine,[7] cinnarizine,[9] bepridil[10] and some 1,4-dihydropyridines such as felodipine[11] are also known to possess a CaM antagonistic property. This fact suggests that there is some structural similarity in the drug binding sites of the calcium channel and CaM.

Based on the mentioned observations, we conceived that it would be possible to develop a new type of cardiovascular agent which interacts with CaM and/or calcium channels if the CNS effects of phenothiazines could be removed by an appropriate chemical modification. It seems that the tricyclic ring system in phenothiazine derivatives plays a crucial role in their CNS activity. Therefore, our synthetic effort was first focused on a bicyclic 1,4-benzothiazine skeleton lacking one benzene ring of the phenothiazine structure. Derivatives possessing (4-phenyl-1-piperazinyl)alkyl side chains at the 2-position were of particular interest, since this type of compound has not been explored as far as new drug potential is concerned. This paper describes the synthesis and pharmacological activities of novel 2*H*-1,4-benzothiazin-3(4*H*)-one derivatives.

Chemistry

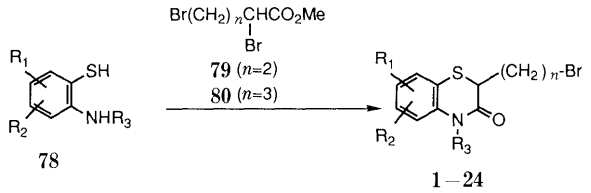
The 2-piperazinylalkyl-2*H*-1,4-benzothiazin-3(4*H*)-one derivatives (**29**—**77**) listed in Table II were prepared by the reaction of 2-haloalkyl-1,4-benzothiazin-3(4*H*)-ones (**1**—**27**) with phenylpiperazines (**28**), and the yields were good (Chart 1).

Compounds **1**—**27** were synthesized *via* the routes shown in Charts 2—4. The reaction of 2-aminothiophenols (**78**) with dibromo esters (**79**, **80**) gave **1**—**24**. Compound **81** obtained from **78** by reaction with diethyl bromomalonate[12] was reduced to **82** which was then chlorinated to give **25** (Chart 2). Methylation of the 2-hydroxyethyl compound **83**[13] followed by chlorination gave **26** (Chart 3). Compound **27** was synthesized starting from the 3-bromopropyl compound (**3**) by introducing one carbon unit as shown in Chart 4.

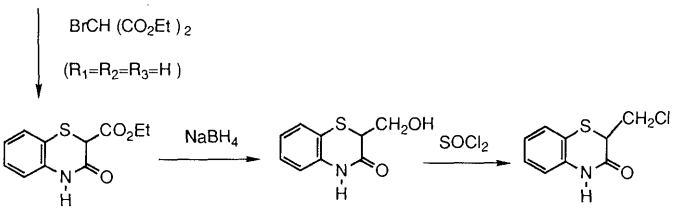


1—27 (X=Br, Cl) 29—77

Chart 1

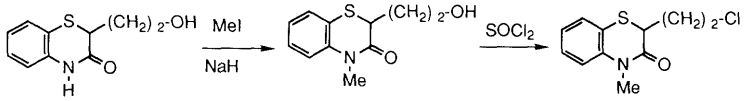


78 1—24



81 82 25

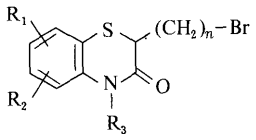
Chart 2



83 84 26

Chart 3

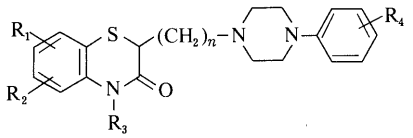
TABLE I. 2-Bromoalkyl-2*H*-1,4-benzothiazin-3(4*H*)-ones (**1**—**24**)[a]



Compd. No.	R_1	R_2	R_3	*n*	mp (°C)	Recrystn. solvent	Yield (%)	Formula[b]
1	H	H	H	2	152—153	MeOH	70.6	$C_{10}H_{10}BrNOS$
2	7-MeO	H	H	2	142—143	MeOH	65.2	$C_{11}H_{12}BrNO_2S$
3	H	H	H	3	103—104	AcOEt	56.9	$C_{11}H_{12}BrNOS$
4	H	H	Me	3	Oil[c]	—	76.2	—
5	5-Me	H	H	3	72—73	AcOEt–hexane	45.6	$C_{12}H_{14}BrNOS$
6	5-Me	6-Me	H	3	95—96	AcOEt–hexane	19.1	$C_{13}H_{16}BrNOS$
7	5-Me	7-Me	H	3	108—109	AcOEt	70.9	$C_{13}H_{16}BrNOS$
8	5-Me	8-Me	H	3	Oil[c]	—	95.5	—
9	5-Et	H	H	3	74—75	MeOH	47.8	$C_{13}H_{16}BrNOS$
10	5-MeO	H	H	3	97—98	MeOH	40.0	$C_{12}H_{14}BrNO_2S$
11	6-Cl	H	H	3	146	MeOH	47.2	$C_{11}H_{11}BrClNOS$
12	6-CF_3	H	H	3	149—150	AcOEt–hexane	17.9	$C_{12}H_{11}BrF_3NOS$
13	6-Me	H	H	3	154—155	CH_2Cl_2–MeOH	37.7	$C_{12}H_{14}BrNOS$
14	6-Me	7-Me	H	3	152—153	MeOH	69.3	$C_{13}H_{16}BrNOS$
15	6-Me	8-Me	H	3	131—132	AcOEt	54.0	$C_{13}H_{16}BrNOS$
16	7-Cl	H	H	3	140—141	AcOEt	42.1	$C_{11}H_{11}BrClNOS$
17	7-Me	H	H	3	126—127	AcOEt–hexane	57.7	$C_{12}H_{14}BrNOS$
18	7-MeO	H	H	3	100—101	AcOEt–hexane	60.3	$C_{12}H_{14}BrNO_2S$
19	5,6-$(CH_2)_4$–		H	3	122—123	MeOH	38.2	$C_{15}H_{18}BrNOS$
20	5,6-(CH=CH)$_2$–		H	3	177—178	CH_2Cl_2–MeOH	50.8	$C_{15}H_{14}BrNOS$
21	6,7-$(CH_2)_3$–		H	3	178—179	MeOH	44.3	$C_{14}H_{16}BrNOS$
22	6,7-$(CH_2)_4$–		H	3	171—172	AcOEt	29.3[d]	$C_{15}H_{18}BrNOS$
23	6,7-OCH_2O–		H	3	168—169	CH_2Cl_2–MeOH	45.0	$C_{12}H_{12}BrNO_3S$
24	7,8-$(CH_2)_4$–		H	3	143—144	AcOEt	52.2	$C_{15}H_{18}BrNOS$

a) Structures of all compounds were confirmed by IR and ^{1}H-NMR spectra. For typical examples, see Experimental. *b*) All compounds were analyzed for C, H and N. Analytical results obtained for these elements were within ±0.4% of the calculated values for the formulae shown. *c*) The oily compound was purified by column chromatography and used for the subsequent reaction. *d*) Overall yield from **110**.

TABLE II. Physical and Biological Properties of 2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzothiazin-3(4*H*)-ones (**29**—**77**)[a]

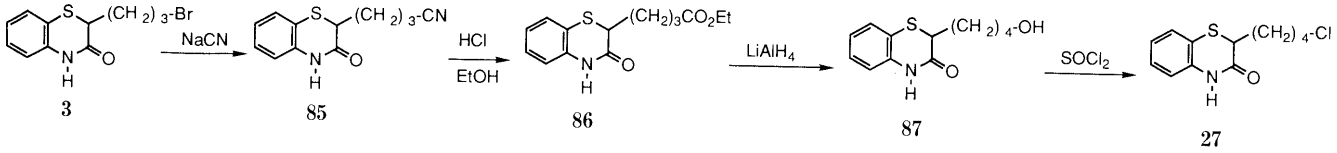


Compd. No.	R_1	R_2	R_3	n	R_4	Yield (%)	mp (°C)	Recrystn. solvent	Formula[b]	Ca^{2+} blocking activity[c] (% inhib. at 10^{-5} M) KCl (60 mM)	CaM antagonistic activity[c] IC_{50}[d] (M)
29	H	H	H	2	H	65.5	145—146	MeOH	$C_{20}H_{23}N_3OS$	20	$>10^{-5}$
30·HCl	H	H	Me	2	H	32.3[e]	208—209	EtOH	$C_{21}H_{25}N_3OS$ ·HCl	40	$>10^{-5}$
31	H	H	H	3	H	59.0	137—138	AcOEt	$C_{21}H_{25}N_3OS$	70	1.1×10^{-6}
32	H	H	H	1	4-F	62.9	192—193	MeOH–CH_2Cl_2	$C_{19}H_{20}FN_3OS$	35	$>10^{-5}$
33	H	H	H	2	4-F	50.5	158—159	MeOH	$C_{20}H_{22}FN_3OS$	60	$>10^{-5}$
34	H	H	H	3	4-F	75.7	120—121	MeOH	$C_{21}H_{24}FN_3OS$	53	$>10^{-5}$
35	H	H	Me	3	4-F	62.5[f]	103—104	AcOEt–hexane	$C_{22}H_{26}FN_3OS$	—	$>10^{-5}$
36	H	H	H	4	4-F	43.2	150—151	MeOH	$C_{22}H_{26}FN_3OS$	30	$>10^{-5}$
37·HCl	H	H	Me	2	3-Cl	41.1[e]	220—222	MeOH–Et_2O	$C_{21}H_{24}ClN_3OS$ ·HCl	17	$>10^{-5}$
38	H	H	H	2	2-MeO	65.2	162—163	MeOH	$C_{21}H_{25}N_3O_2S$	15	6.4×10^{-7}
39·2HCl	H	H	Me	2	2-MeO	50.4[e]	149—151	MeOH–Et_2O	$C_{22}H_{27}N_3O_2S$ ·2HCl·1/2H_2O	0	$>10^{-5}$
40·2HCl	H	H	Et	2	2-MeO	39.0[g]	142—144	MeOH–Et_2O	$C_{23}H_{29}N_3O_2S$ ·2HCl·1/2H_2O	15	8.1×10^{-6}
41·Oxa[h]	H	H	H	3	2-MeO	13.3	202—204	MeOH–CH_2Cl_2	$C_{22}H_{27}N_3O_2S$ ·3/2$C_2H_2O_4$	10	$>10^{-5}$
42·HCl	5-Me	H	H	3	H	56.2	234—236	MeOH	$C_{22}H_{27}N_3OS$ ·HCl	40	$>10^{-5}$
43·2HCl	5-Me	H	H	3	2-F	24.2	121—123	MeOH–iso-Pr_2O	$C_{22}H_{26}FN_3OS$ ·2HCl·1/2H_2O	28	2.5×10^{-6}
44·2HCl	5-Me	H	H	3	3-F	37.2	132—134	EtOH	$C_{22}H_{26}FN_3OS$ ·2HCl·1/2H_2O	48	1.8×10^{-6}
45[i]	5-Me	H	H	3	4-F	88.1	116—117	MeOH	$C_{22}H_{26}FN_3OS$	41	4.1×10^{-7}
46·2HCl	5-Me	H	H	3	3-Cl	53.2	204—206	MeOH–Et_2O	$C_{22}H_{26}ClN_3OS$ ·2HCl·1/2H_2O	50	$>10^{-5}$
47·2HCl	5-Me	H	H	3	4-Cl	55.2	129—130	MeOH	$C_{22}H_{26}ClN_3OS$ ·2HCl	40	$>10^{-5}$
48·2HCl	5-Me	H	H	3	3-CF_3	62.9	132—134	MeOH	$C_{23}H_{26}F_3N_3OS$ ·2HCl	55	$>10^{-5}$
49·HCl	5-Me	H	H	3	2-Me	59.2	240—242	MeOH	$C_{23}H_{29}N_3OS$ ·HCl	11	8.7×10^{-7}
50·2HCl	5-Me	H	H	3	3-Me	65.0	142—144	MeOH	$C_{23}H_{29}N_3OS$ ·2HCl·1/2H_2O	50	1.5×10^{-6}
51	5-Me	H	H	3	4-Me	79.6	149—150	AcOEt	$C_{23}H_{29}N_3OS$	77	8.9×10^{-6}
52·2HCl	5-Me	H	H	3	2-MeO	64.8	168—169	MeOH	$C_{23}H_{29}N_3O_2S$ ·2HCl	33	3.6×10^{-6}
53	5-Me	6-Me	H	3	4-F	82.3	135—136	AcOEt–hexane	$C_{23}H_{28}FN_3OS$	25	$>10^{-5}$
54	5-Me	7-Me	H	3	4-F	66.1	163—164	AcOEt	$C_{23}H_{28}FN_3OS$	70	$>10^{-5}$
55·2HCl	5-Me	7-Me	H	3	2-MeO	58.2	159—161	MeOH–CH_2Cl_2	$C_{24}H_{31}N_3O_2S$ ·2HCl·1/2H_2O	62	$>10^{-5}$
56·2HCl	5-Me	8-Me	H	3	4-F	57.9	131—133	MeOH	$C_{23}H_{28}FN_3OS$ ·2HCl	10	7.0×10^{-6}
57	5-Et	H	H	3	4-F	60.8	117—118	CH_2Cl_2–MeOH	$C_{23}H_{28}FN_3OS$	55	$>10^{-5}$
58·2HCl	5-MeO	H	H	3	H	47.2	209—211	MeOH–Et_2O	$C_{22}H_{27}N_3O_2S$ ·2HCl	20	1.2×10^{-6}
59	5-MeO	H	H	3	2-MeO	72.4	123—124	AcOEt–hexane	$C_{23}H_{29}N_3O_3S$	18	1.2×10^{-6}
60	6-Cl	H	H	3	2-MeO	77.7	191—192	CH_2Cl_2–MeOH	$C_{22}H_{26}ClN_3O_2S$	40	1.2×10^{-8}
61	6-CF_3	H	H	3	4-F	68.4	160—162	MeOH	$C_{22}H_{23}F_4N_3OS$	45	5.0×10^{-6}
62·2HCl	6-Me	H	H	3	4-F	84.2	158—160	MeOH–CH_2Cl_2	$C_{22}H_{26}FN_3OS$ ·2HCl	16	3.9×10^{-6}
63	6-Me	7-Me	H	3	4-F	82.6	161—162	MeOH	$C_{23}H_{28}FN_3OS$	50	$>10^{-5}$
64·2HCl	6-Me	7-Me	H	3	2-MeO	67.0	203—205	MeOH–CH_2Cl_2	$C_{24}H_{31}N_3O_2S$ ·2HCl	40	$>10^{-5}$
65·2HCl	6-Me	8-Me	H	3	4-F	69.4	138—139	MeOH–CH_2Cl_2	$C_{23}H_{28}FN_3OS$ ·2HCl	40 (10^{-6} M)	5.3×10^{-6}
66	7-Cl	H	H	3	2-MeO	59.8	117—118	AcOEt–hexane	$C_{22}H_{26}ClN_3O_2S$	34	4.0×10^{-7}
67·2HCl	7-Me	H	H	3	4-F	65.9	157—159	MeOH	$C_{22}H_{26}FN_3OS$ ·2HCl	20	8.1×10^{-7}
68·2HCl	7-Me	H	H	3	2-MeO	64.4	164—165	MeOH–Et_2O	$C_{23}H_{29}N_3O_2S$ ·2HCl·1/2H_2O	32	6.5×10^{-6}

TABLE II. (continued)

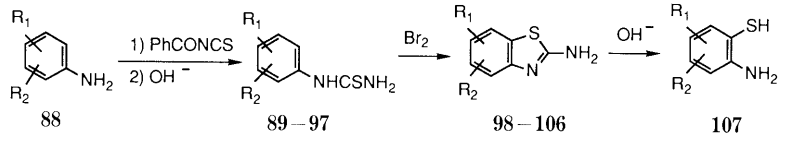
Compd. No.	R_1	R_2	R_3	n	R_4	Yield (%)	mp (°C)	Recrystn. solvent	Formula[b]	Ca^{2+} blocking activity[c] (% inhib. at 10^{-5} M) KCl (60 mM)	CaM antagonistic activity[c] IC_{50}[d] (M)
69	7-MeO	H	H	2	H	72.5	157—158	AcOEt–hexane	$C_{21}H_{25}N_3O_2S$	15	2.4×10^{-6}
70	7-MeO	H	H	3	H	72.7	140—141	AcOEt–hexane	$C_{22}H_{27}N_3O_2S$	10	$>10^{-5}$
71	7-MeO	H	H	3	2-MeO	62.5	123—124	AcOEt–hexane	$C_{23}H_{29}N_3O_3S$	26	1.0×10^{-5}
72·2HCl	5,6-$(CH_2)_4$–		H	3	4-F	60.6	148—149	CH_2Cl_2–MeOH	$C_{25}H_{30}FN_3OS$	35	4.5×10^{-7}
73	5,6-$(CH{=}CH)_2$–		H	3	4-F	71.4	137—138	AcOEt–hexane	$C_{25}H_{26}FN_3OS$	21	3.9×10^{-6}
74[j]	6,7-$(CH_2)_3$–		H	3	4-F	71.9	199—201	$CHCl_3$–AcOEt	$C_{24}H_{28}FN_3OS$	14	5.2×10^{-7}
75	6,7-$(CH_2)_4$–		H	3	4-F	34.8	152—153	$CHCl_3$–AcOEt	$C_{25}H_{30}FN_3OS$	19	5.9×10^{-7}
76·2HCl	6,7-OCH_2O–		H	3	4-F	80.6	175—177	MeOH–CH_2Cl_2	$C_{22}H_{24}FN_3O_3S$ ·2HCl	22	3.5×10^{-6}
77·HCl	7,8-$(CH_2)_4$–		H	3	4-F	83.7	184—186	EtOH–H_2O	$C_{25}H_{30}FN_3OS$ ·HCl·1/2H_2O	—	3.6×10^{-6}
Trifluoperazine											4.1×10^{-6}
W-7											2.2×10^{-4}
HT-74											3.8×10^{-7}

a, b) See footnotes *a*) and *b*), Table I. *c*) For the biological methods, see Experimental. *d*) IC_{50} values were determined by linear regression analysis; the correlation coefficient for each regression line was >0.95. *e*) Prepared from **26**. *f*) Prepared from **4**. *g*) Prepared by ethylation of **38** (see Experimental). *h*) Oxalate. *i*) **45**·2HCl: mp 161—163 °C (MeOH). *j*) **74**·2HCl: mp 152—154 °C (EtOH–H_2O).

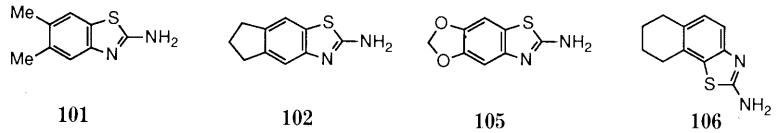


3 85 86 87 27

Chart 4

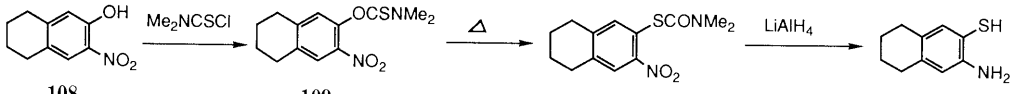


88 89—97 98—106 107



101 102 105 106

Chart 5



108 109 110 111

Chart 6

The starting 2-aminothiophenols (**78**), mostly known compounds, were synthesized according to the procedure in the literature.[14] Some new *N*-unsubstituted 2-aminothiophenols (**107**) were prepared by the route shown in Chart 5. For instance, when the thiourea derivatives (**92**, **93** and **96**, Table V) prepared from 3,4-dimethylaniline, 5-aminoindane and 3,4-methylenedioxyaniline were reacted with bromine, cyclization occurred predominantly in the sterically less hindered course to afford the 2-aminobenzothiazole derivatives **101**, **102** and **105**, respectively, which gave the desired 2-aminothiophenols upon hydrolysis. The structures of **101**, **102** and **105** were supported by proton nuclear magnetic resonance (^{1}H-NMR) spectra in which aromatic protons of these compounds all appeared as two singlets: **101**, δ 7.13 and 7.32; **102**, δ 7.21 and 7.40; **105**, δ 6.92 and 7.13. The cyclization of 5,6,7,8-tetrahydro-2-naphthylthiourea (**97**), on the other hand, took place at the *peri* position to give mainly **106** and a linearly cyclized product relating to **102** could not be isolated. The structure of **106** was determined by ^{1}H-NMR spectrum: the aromatic protons were observed at δ 6.87 and 7.07 as a pair of doublets with coupling constant of 8 Hz. Thus, 3-amino-5,6,7,8-tetrahydro-2-naphthalenethiol (**111**) was prepared from **108** by the alternative route shown in Chart 6. Compound **109** obtained by the reaction of 5,6,7,8-tetrahydro-3-nitronaphthol (**108**) with *N*,*N*-dimethylthiocarbamoyl chloride was converted to compound **111** by the Newman–Kwart rearrangement[15] followed by reduc-

tion with $LiAlH_4$.

These 2-aminothiophenols (**107**, **111**) were generally air-sensitive and easily formed the disulfide compounds. Therefore, crude **107** and **111** were used as starting materials without further purification.

Results and Discussion

The contraction of smooth muscle induced by a high concentration of KCl is known to be due to the influx of Ca^{2+} following the activation of the voltage-dependent calcium channels. Therefore, the compounds (**29**—**77**) prepared in the present study were tested for calcium channel blocking activity by the method described in Experimental, and the results are shown in Table I. Some compounds, especially those bearing an alkyl group at the 5-position (*e.g.*, **48**, **51**, **54**, **55** and **57**) showed moderate calcium blocking effects at 10^{-5} M. However, these effects were rather weak compared to that of the dihydropyridine derivative nicardipine[16] (IC_{50}: 4×10^{-9} M).

The CaM antagonistic activity of **29**—**77** was assayed using a modification of the method of Beavo *et al.*[17] (see Experimental) and is shown in Table II. Although exact relationship between chemical structure and CaM antagonistic activity was not seen, many compounds showed activity comparable to that of trifluoperazine or HT-74. In general, the kind and the position of the substituent R_4 on the phenyl group in the side chain seem to have significant influence on CaM antagonistic activity. In particular, compounds with 2-methoxy or 4-fluoro substituents on the phenyl group tend to show potent CaM antagonistic activity as is seen with **38**, **45**, **60**, **66**, **67**, **72**, **74** and **75**.

Compounds **38**, **45**, **60**, **66**, **72**, **74** and **75** were subsequently evaluated for antihypertensive activity in conscious spontaneously hypertensive rats (SHR). As shown in Table III, these compounds had antihypertensive activity, although the correlation between the CaM antagonistic effects and the antihypertensive effects among the compounds (**38**, **45**, **60**, **66**, **72**, **74** and **75**) were not clear. Furthermore, compounds **45** and **74** inhibited caffeine-induced contractions of rabbit aorta in calcium-free buffer (Table IV), suggesting that these novel compounds may act as intracellular calcium antagonists. Judging from the fact that **45** and **74** have only weak calcium entry blocking activity, intracellular mechanisms are more likely responsible for their pharmacological actions. Although further study is obviously needed to define the precise mechanism of action of these compounds, it is suggested that CaM antagonism contributes to their antihypertensive property in SHR.

TABLE III. Antihypertensive Effects of 2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzothiazin-3(4*H*)-ones in SHR

Compd. No.	Antihypertensive activity[a]	
	Dose (mg/kg, *p.o.*)	Maximum change (mmHg)
38	30	−29
45	10	−45
	3	−25
60	60	−35
66	60	−21
72	30	−35
74	10	−65
	3	−35
75	3	−22

a) For the biological methods, see Experimental. The change in blood pressure is an average of the values from 3 animals.

TABLE IV. Inhibitory Effects (%) of Compounds **45** and **74** on the Caffeine-Induced Contraction of Rabbit Aortic Strips in Ca^{2+}-Free Buffer

Compd. No.	% inhibition		
	Drug concentration (M)		
	10^{-7}	10^{-6}	10^{-5}
45	34±5 (4)	78±5 (3)	84±3 (3)
74	52±5 (3)	78±8 (3)	86±2 (3)

Values represent mean±S.E. The average control contractile response of the rabbit aortic strips was 1.94±0.26 g (mean±S.E. $n=8$). The number of experiments is shown in parentheses.

Experimental

Melting points were determined using a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi IR-260-10 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian EM-390 (90 MHz) spectrometer in the solvent indicated. Chemical shifts are given in ppm relative to Me_4Si as the internal standard. The following abbreviations are used: s=singlet; d=doublet; t=triplet; q=quartet; m=multiplet; br=broad; dd=double doublet. Column chromatography was performed on E. Merck 70—230 mesh silica gel. Evaporation was carried out *in vacuo* on a rotary evaporator.

2-(2-Bromoalkyl)-2*H*-1,4-benzothiazin-3(4*H*)-ones (1—24, Table I) Typical examples are given to illustrate the general procedure.

2-(2-Bromoethyl)-7-methoxy-2*H*-1,4-benzothiazin-3(4*H*)-one (2) Methyl 2,4-dibromobutyrate (**79**, 5.20 g, 20.0 mmol) was added to a solution of 2-amino-5-methoxythiophenol (3.10 g, 20.0 mmol) in dimethylformamide (DMF, 30 ml). The mixture was stirred at room temperature for 2 h and poured into water. The resulting crystals were collected and recrystallized from MeOH to give **2** as colorless prisms (3.94 g, 65.2%), mp 142—143 °C. IR (Nujol): 3190, 1650 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.87—2.63 (2H, m), 3.60 (2H, t, J=6 Hz), 3.68 (1H, dd, J=6, 8 Hz), 3.76 (3H, s), 6.63—6.92 (3H, m), 9.62 (1H, br). *Anal.* Calcd for $C_{11}H_{12}BrNO_2S$: C, 43.72; H, 4.00; N, 4.64. Found: C, 44.09; H, 3.60; N, 4.52.

2-(3-Bromopropyl)-6,7-dimethyl-2*H*-1,4-benzothiazin-3(4*H*)-one (14) Methyl 2,5-dibromovalerate (**80**, 5.48 g, 20.1 mmol) was added to a solution of 2-amino-4,5-dimethylthiophenol (3.06 g, 20.0 mmol) in DMF (50 ml) at room temperature. The mixture was stirred for 14 h, diluted with water and extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was recrystallized from MeOH to give **14** as colorless prisms (4.35 g, 69.3%), mp 152—153 °C. IR (Nujol): 3200, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.37—2.27 (4H, m), 2.42 (6H, s), 3.23—3.40 (1H, m), 3.37 (2H, t, J=6 Hz), 6.64 (1H, s), 7.03 (11H, s), 8.90 (1H, br). *Anal.* Calcd for $C_{13}H_{16}BrNOS$: C, 49.69; H, 5.13; N, 4.46. Found: C, 49.84; H, 4.97; N, 4.75.

2-(3-Bromopropyl)-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]thiazin-3(4*H*)-one (22) (i) A solution of **110** (1.80 g, 6.4 mmol) in anhydrous tetrahydrofuran (THF, 10 ml) was added dropwise to a stirred suspension of $LiAlH_4$ (0.61 g, 16.1 mmol) in anhydrous THF (10 ml) and the mixture was refluxed for 30 min. After cooling, the mixture was quenched with water and acidified with AcOH. The precipitate was removed by filtration and the filtrate was extracted with Et_2O. The extract was washed with water, dried ($MgSO_4$) and concentrated to give 3-amino-5,6,7,8-tetrahydro-2-naphthalenethiol (**111**) as an oil, which was used for the next reaction without further purification.

(ii) A mixture of **111** obtained in (i), **80** (2.30 g, 8.4 mmol) and DMF (20 ml) was stirred at room temperature for 2 h and then at 60 °C for 30 min. The mixture was poured into water and extracted with AcOEt. The extract was washed with saturated NaCl, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (100 g) using hexane–AcOEt (4:1, v/v) as eluant and recrystallized from AcOEt to yield **22** as colorless crystals (0.64 g, 29.3% from **110**), mp 171—172 °C. IR ($CHCl_3$): 3390, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.55—2.25 (8H, m), 2.55—2.83 (4H, m), 3.27—3.50 (3H, m), 6.57 (1H, s), 6.97 (1H, s), 9.00 (1H, br s). *Anal.* Calcd for $C_{15}H_{18}BrNOS$: C, 52.95; H, 5.33; N, 4.12.

Found: C, 52.67; H, 5.25; N, 4.22.

Other compounds listed in Table I were prepared similarly.

2-(Chloromethyl)-2*H*-1,4-benzothiazin-3(4*H*)-one (25) (i) $NaBH_4$ (0.95 g, 25.1 mmol) was added portionwise to a stirred suspension of ethyl 3,4-dihydro-3-oxo-2*H*-1,4-benzothiazine-2-carboxylate[12] (**81**, 3.0 g, 12.6 mmol) in EtOH (50 ml) at room temperature. After stirring for 2 h, the reaction mixture was diluted with water, acidified with dil. AcOH and extracted with Et_2O. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was washed with hexane to give 2-hydroxymethyl-2*H*-1,4-benzothiazin-3(4*H*)-one (**82**) as crystals (1.25 g, 50.8%). Recrystallization from AcOEt gave colorless prisms, mp 156—157 °C. IR (Nujol): 3500—3300, 3200, 1670 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 3.41—3.74 (3H, m), 5.09 (1H, m), 6.83—7.33 (4H, m), 10.83 (1H, br). *Anal.* Calcd for $C_9H_9NO_2S$: C, 55.37; H, 4.65; N, 7.17. Found: C, 55.39; H, 4.68; N, 7.29.

(ii) $SOCl_2$ (0.9 ml, 12.3 mmol) was added to a solution of **82** (1.0 g, 5.1 mmol) in $CHCl_3$ (20 ml), and the mixture was refluxed for 2 h. The mixture was concentrated and extracted with Et_2O. The extract was washed with water, dried ($MgSO_4$) and concentrated. The crude crystals were purified by column chromatography on silica gel (40 g) using hexane–AcOEt (4 : 1, v/v) as eluant to give **25** (0.64 g, 58.7%). Recrystallization from AcOEt–hexane gave colorless needles, mp 152—153 °C. IR (Nujol): 3190, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 3.60—3.93 (3H, m), 6.89—7.37 (4H, m), 9.40 (1H, br). *Anal.* Calcd for C_9H_8ClNOS: C, 50.59; H, 3.77; N, 6.55. Found: C, 50.44; H, 3.53; N, 6.54.

2-(2-Chloroethyl)-4-methyl-2*H*-1,4-benzothiazin-3(4*H*)-one (26) (i) NaH (60% dispersion in oil, 0.80 g, 20.0 mmol) was added to a stirred and ice-cooled solution of 2-hydroxyethyl-2*H*-1,4-benzothiazin-3(4*H*)-one[13] (**83**, 4.18 g, 20.0 mmol) in DMF (50 ml). After stirring for 10 min, methyl iodide (1.24 ml, 20.0 mmol) was added dropwise. The mixture was stirred at room temperature for 1 h, poured into water and extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (100 g) using hexane–AcOEt (2 : 1, v/v) as eluant to give 2-(2-hydroxyethyl)-4-methyl-2*H*-1,4-benzothiazin-3(4*H*)-one (**84**) as an oil (3.90 g, 87.4%). IR (neat): 3600—3250, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.62—2.31 (2H, m), 2.47 (1H, br), 3.43 (3H, s), 3.61 (1H, t, J=7 Hz), 3.77 (2H, m), 6.90—7.41 (4H, m).

(ii) $SOCl_2$ (5 ml, 68.5 mmol) was added to a solution of **84** (4.9 g, 21.9 mmol) in CH_2Cl_2 (20 ml) at room temperature. After stirring for 1 h, the mixture was concentrated and the residue was chromatographed on silica gel (100 g) using hexane–AcOEt (4 : 1, v/v) as eluant to afford **26** as an oil (5.05 g, 95.3%). IR (neat): 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.83—2.55 (2H, m), 3.46 (3H, s), 3.56—3.76 (1H, m), 3.70 (2H, t, J=6 Hz), 6.91—7.43 (4H, m). *Anal.* Calcd for $C_{11}H_{12}ClNOS$: C, 54.64; H, 5.00; N, 5.79. Found: C, 54.54; H, 4.98; N, 5.70.

2-(4-Chlorobutyl)-2*H*-1,4-benzothiazin-3(4*H*)-one (27) (i) NaCN (0.54 g, 11.0 mmol) was added to a stirred solution of **3** (2.86 g, 10.0 mmol) in dimethylsulfoxide (DMSO, 10 ml) at room temperature. After stirring for 3 h, the mixture was diluted with water and the precipitated crystals were collected by filtration. Recrystallization from MeOH gave 2-(3-cyanopropyl)-2*H*-1,4-benzothiazin-3(4*H*)-one (**85**) as prisms (1.55 g, 66.7%), mp 107—108 °C. IR (Nujol): 3190, 2220, 1665 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.62—2.22 (4H, m), 2.23—2.45 (2H, m), 3.35—3.50 (1H, m), 6.85—7.35 (4H, m), 9.17 (1H, br). *Anal.* Calcd for $C_{12}H_{12}N_2OS$: C, 62.05; H, 5.21; N, 12.06. Found: C, 61.78; H, 5.19; N, 11.79.

(ii) A mixture of **85** (1.45 g, 6.2 mmol), 18% ethanolic hydrogen chloride (40 ml) and water (0.3 ml) was refluxed for 24 h. The mixture was diluted with water and extracted with Et_2O. The extract was washed with water, aqueous $NaHCO_3$ and water successively and then dried ($MgSO_4$). Removal of the solvent gave ethyl 3,4-dihydro-3-oxo-2*H*-1,4-benzothiazine-2-butyrate (**86**) as crystals (1.60 g, 91.4%) which were recrystallized from AcOEt to give prisms, mp 78—79 °C. IR (Nujol): 3200, 1730, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, t, J=7 Hz), 1.50—2.20 (4H, m), 2.31—2.47 (2H, m), 3.32—3.51 (1H, m), 3.44 (2H, q, J=7 Hz), 6.72—7.39 (4H, m), 8.20 (1H, br). *Anal.* Calcd for $C_{14}H_{17}NO_3S$: C, 60.19; H, 6.13; N, 5.01. Found: C, 60.33; H, 6.03; N, 5.34.

(iii) A solution of **86** (1.60 g, 5.7 mmol) in Et_2O (30 ml) was added to a stirred and ice-cooled mixture of $LiAlH_4$ (0.46 g, 12.1 mmol) and Et_2O (50 ml). After stirring for 1 h, water was added dropwise to the mixture and the precipitate was filtered through celite. The filtrate was dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (70 g) with hexane–AcOEt (1 : 1, v/v) as eluant to give 2-(4-hydroxybutyl)-2*H*-1,4-benzothiazin-3(4*H*)-one (**87**) as crystals (0.75 g, 55.6%). Recrystallization from AcOEt gave prisms, mp 101—102 °C. IR (Nujol): 3400—3150, 3210, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.44—2.10 (6H, m), 1.80 (1H, br), 3.31—3.71 (3H, m), 6.81—7.33 (4H, m), 9.24 (1H, br). *Anal.* Calcd for $C_{12}H_{15}NO_2S$: C, 60.73; H, 6.37; N, 5.90. Found: C, 60.69; H, 6.35; N, 5.82.

(iv) 2-(4-Chlorobutyl)-2*H*-1,4-benzothiazin-3(4*H*)-one (**27**) was obtained from **87** in 59.0% yield by treatment with $SOCl_2$ in the same manner as that described in the synthesis of **25**. mp 111—112 °C. IR (Nujol): 3190, 1665 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.47—2.06 (6H, m), 3.33—3.57 (3H, m), 6.86—7.34 (4H, m), 9.67 (1H, br). *Anal.* Calcd for $C_{12}H_{14}ClNOS$: C, 56.35; H, 5.52; N, 5.48. Found: C, 56.26; H, 5.41; N, 5.13.

2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzothiazin-3(4*H*)-ones (29—77, Table II) Typical examples are given to illustrate the general procedure.

2-[2-[4-(4-Fluorophenyl)-1-piperazinyl]ethyl]-2*H*-1,4-benzothiazin-3(4*H*)-one (33) A mixture of **1** (1.36 g, 5.0 mmol), 1-(4-fluorophenyl)-piperazine (1.80 g, 10.0 mmol) and AcOEt (20 ml) was heated at 110 °C for 1 h. The mixture was extracted with AcOEt, and the extract was washed with water and dried ($MgSO_4$). After removal of the solvent, the crude crystals were recrystallized from MeOH to give **33** as prisms (0.94 g, 50.5%), mp 158—159 °C. IR (Nujol): 3210, 1665 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.80—2.60 (8H, m), 3.00—3.11 (4H, m), 3.62 (1H, dd, J=7, 8 Hz), 6.70—7.32 (8H, m), 9.46 (1H, br). *Anal.* Calcd for $C_{20}H_{22}FN_3OS$: C, 64.67; H, 5.97; N, 11.31. Found: C, 64.82; H, 6.02; N, 11.25.

4-Ethyl-2-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-2*H*-1,4-benzothiazin-3(4*H*)-one (40) Dihydrochloride NaH (60% dispersion in oil, 0.12 g, 3.0 mmol) was added portionwise to a stirred solution of **38** (1.0 g, 2.6 mmol) in DMF (10 ml). Ethyl iodide (0.48 g, 3.1 mmol) was then added dropwise and the mixture was stirred at room temperature for 2 h. The reaction mixture was poured into ice-water and extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (50 g) using hexane–AcOEt (2 : 1, v/v) as eluant to give **40** as an oil (0.60 g). This oil was treated with 18% methanolic hydrogen chloride to give the dihydrochloride (0.50 g, 39.0%). Recrystallization from MeOH–Et_2O afforded colorless prisms, mp 142—144 °C. IR (Nujol): 2450—2200, 1660 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.13 (3H, t, J=7 Hz), 1.7—2.4 (2H, m), 2.9—3.87 (11H, m), 3.79 (3H, s), 4.02 (2H, q, J=7 Hz), 6.84—7.0 (8H, m). *Anal.* Calcd for $C_{23}H_{29}N_3O_2S \cdot 2HCl \cdot 1/2H_2O$: C, 55.98; H, 6.54; N, 8.54. Found: C, 56.18; H, 6.47; N, 8.62.

2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-5-methyl-2*H*-1,4-benzothiazin-3(4*H*)-one (45) A mixture of **5** (3.0 g, 10.0 mmol), 1-(4-fluorophenyl)piperazine (3.6 g, 20.0 mmol) and AcOEt (30 ml) was heated at 110 °C. The reaction mixture was extracted with AcOEt (100 ml). The extract was washed with water and dried ($MgSO_4$). After removal of the solvent, the residue was purified by column chromatography on silica gel (100 g) using hexane–AcOEt (1 : 1, v/v) as eluant to give **45** as crystals (2.6 g, 88.1%). Recrystallization from MeOH gave colorless prisms, mp 116—117 °C. IR (Nujol): 3220, 1655 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.5—2.1 (4H, m), 2.30 (3H, s), 2.3—2.59 (6H, m), 2.99—3.11 (4H, m), 3.25 (1H, m), 6.7—7.22 (7H, m), 7.98 (1H, br). *Anal.* Calcd for $C_{22}H_{26}FN_3OS$: C, 66.14; H, 6.56; N, 10.52. Found: C, 66.17; H, 6.62; N, 10.49.

Dihydrochloride: **45** was treated with 18% methanolic hydrogen chloride to give the dihydrochloride. Recrystallization from MeOH afforded colorless prisms, mp 161—163 °C. *Anal.* Calcd for $C_{22}H_{26}FN_3OS \cdot 2HCl$: C, 55.93; H, 5.97; N, 8.89. Found: C, 55.56; H, 5.99; N, 8.72.

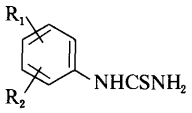
6,7-Cyclopenteno-2-[3-[4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzothiazin-3(4*H*)-one (74) A mixture of **21** (0.98 g, 3.0 mmol), 1-(4-fluorophenyl)piperazine (0.57 g, 3.2 mmol), NEt_3 (0.46 ml, 3.3 mmol) and DMF (10 ml) was stirred at 110 °C for 1 h. The reaction mixture was poured into water and the crystals were collected by filtration, washed well with water and cold MeOH and then dried. Recrystallization from $CHCl_3$–AcOEt gave **74** as colorless prisms (0.95 g, 74.4%), mp 199—201 °C. IR (Nujol): 3190, 1660 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.7 (12H, m), 2.84 (4H, t, J=7 Hz), 3.07 (4H, t, J=7 Hz), 3.3—3.5 (1H, m), 6.75 (1H, s), 6.77—7.07 (4H, m), 7.13 (1H, s), 9.00 (1H, br). *Anal.* Calcd for $C_{24}H_{28}FN_3OS$: C, 67.74; H, 6.63; N, 9.87. Found: C, 67.63; H, 6.55; N, 9.55. **74** was treated with 18% methanolic hydrogen chloride to afford the dihydrochloride. Recrystallization from EtOH–water gave colorless prisms, mp 152—154 °C. *Anal.* Calcd for $C_{24}H_{28}FN_3OS \cdot 2HCl$: C, 57.83; H, 6.07; N, 8.43. Found: C, 57.79; H, 6.07; N, 8.43.

Other compounds listed in Table II were prepared similarly.

***N*-Phenylthioureas (89—97, Table V)** A typical example is given to illustrate the general procedure.

***N*-(5-Indanyl)thiourea (93)** Benzoyl chloride (14.5 ml, 0.12 mol) was

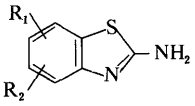
TABLE V. *N*-Phenylthioureas



Compd. No.	R_1	R_2	mp (°C)	Recrystn. solvent	Yield (%)	Formula[a]
89	2-Me	3-Me	230—231	MeOH	89.7	$C_9H_{12}N_2S$
90	2-Me	5-Me	139—140	MeOH–H_2O	57.6	$C_9H_{12}N_2S$
91	2-Et	H	158—159	MeOH–H_2O	53.6	$C_9H_{12}N_2S$
92	3-Me	4-Me	198—199	MeOH–H_2O	60.2	$C_9H_{12}N_2S$
93	3,4-$(CH_2)_3$–		187—188	MeOH	84.6	$C_{10}H_{12}N_2S$
94	2,3-$(CH_2)_4$–		173—174	MeOH–H_2O	72.6	$C_{11}H_{14}N_2S$
95	2,3-$(CH=CH)_2$–		204—205	MeOH	84.2	$C_{11}H_{10}N_2S$
96	3,4-OCH_2O–		207—208	MeOH	79.0	$C_8H_8N_2O_2S$
97	3,4-$(CH_2)_4$–		184—185	MeOH	85.1	$C_{11}H_{14}N_2S$

a) See footnote *b*), Table I.

TABLE VI. 2-Aminobenzothiazoles



Compd. No.	R_1	R_2	mp (°C)	Recrystn. solvent	Yield (%)	Formula[a]
98	4-Me	5-Me	171—172	CH_2Cl_2–MeOH	70.3	$C_9H_{10}N_2S$
99	4-Me	7-Me	153—155	MeOH–H_2O	75.8	$C_9H_{10}N_2S$
100	4-Et	H	118—119	MeOH–H_2O	82.5	$C_9H_{10}N_2S$
101	5-Me	6-Me	179—181	MeOH	58.5	$C_9H_{10}N_2S$
102	5,6-$(CH_2)_3$–		189—191	MeOH	57.9	$C_{10}H_{10}N_2S$
103	4,5-$(CH_2)_4$–		173—174	MeOH–H_2O	95.7	$C_{11}H_{12}N_2S$
104	4,5-$(CH=CH)_2$–		183—186	CH_2Cl_2–MeOH	69.7	$C_{11}H_{10}N_2S$
105	5,6-OCH_2O–		230—232	CH_2Cl_2–MeOH	69.2	$C_8H_6N_2O_2S$
106	6,7-$(CH_2)_4$–		206—208	MeOH	35.8	$C_{11}H_{12}N_2S$

a) See footnote *b*), Table I.

added dropwise to a stirred solution of NH_4SCN (11.4 g, 0.15 mol) in acetone (100 ml) and stirring was continued at 50 °C for 20 min. A solution of 5-aminoindane (16.6 g, 0.13 mol) in acetone (100 ml) was added dropwise and the mixture was stirred at 50 °C for 1 h. The mixture was diluted with water, and the precipitated crystals were collected by filtration and washed with water. These crystals were then added to 2 N NaOH (200 ml), refluxed for 1 h and then poured into water. The crude crystals of **93** were recrystallized from MeOH to give colorless prisms (20.3 g, 84.6%), mp 187—188 °C. IR (Nujol): 3420, 3250, 3175, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.01 (2H, m), 2.68—3.00 (4H, m), 6.93—7.31 (5H, m), 9.48 (1H, br s). *Anal.* Calcd for $C_{10}H_{12}N_2S$: C, 62.47; H, 6.29; N, 14.57. Found: C, 62.40; H, 6.30; N, 14.44.

Other new compounds listed in Table V were prepared similarly.

2-Aminobenzothiazoles (98—106, Table VI) A typical example is given to illustrate the general procedure.

2-Amino-5,6-cyclopentenobenzothiazole (102) Bromine (5.1 ml, 0.10 mol) was added to a suspension of **93** (19.2 g, 0.10 mol) in AcOH (200 ml) at less than 20 °C. After stirring at room temperature for 30 min, Et_2O was added and the precipitate (**102**·HBr) was collected by filtration and washed with Et_2O. These crystals were treated with 25% NH_4OH at room temperature for 1 h to give **102**. Recrystallization from MeOH gave colorless needles (11.0 g, 57.9%), mp 189—191 °C. IR (Nujol): 3440, 3280, 1640 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.00 (2H, m), 2.85 (4H, t, J=8 Hz), 7.18 (2H, br s), 7.21 (1H, s), 7.40 (1H, s). *Anal.* Calcd for $C_{10}H_{10}N_2S$: C, 63.13; H, 5.30; N, 14.72. Found: C, 63.32; H, 5.32; N, 14.62.

Other compounds listed in Table VI were prepared similarly.

2-Aminothiophenols (107) New 2-aminothiophenols (**107**) were synthesized in the manner described in the following example and were used for the next reaction without further purification because of their easy tendency to form the disulfides.

6-Amino-5-indanthiol A mixture of **102** (10.0 g, 52.6 mmol), KOH (60.0 g, 1.07 mol), water (60 ml) and 2-methoxyethanol (60 ml) was refluxed under nitrogen for 2 h. The mixture was poured into ice-water, neutralized with AcOH and extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated to give the crude title compound as a yellow solid (8.2 g, 94.4%), mp 75—77 °C. IR (Nujol): 3430, 3220, 2520, 1610 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.00 (2H, m), 2.77 (4H, t, J=7 Hz), 3.67 (2H, br), 6.58 (1H, s), 7.18 (1H, s).

5,6,7,8-Tetrahydro-2-(*N*,*N*-dimethylthiocarbamoyl)oxy-3-nitronaphthalene (109) A mixture of 5,6,7,8-tetrahydro-3-nitronaphthol (**108**, 0.97 g, 5.0 mmol), *N*,*N*-dimethylthiocarbamoyl chloride (0.62 g, 5.0 mmol), 1,4-diazabicyclo[2.2.2]octane (0.56 g, 5.0 mmol) and DMF (10 ml) was stirred at room temperature for 2 h. The reaction mixture was diluted with water and extracted with Et_2O. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was recrystallized from iso-Pr_2O to give **109** as yellow prisms (0.96 g, 68.6%), mp 123—124 °C. IR ($CHCl_3$): 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.73—1.98 (4H, m), 2.67—2.98 (4H, m), 3.38 (3H, s), 3.44 (3H, s), 6.91 (1H, s), 7.84 (1H, s). *Anal.* Calcd for $C_{13}H_{16}N_2O_3S$: C, 55.70; H, 5.75; N, 9.99. Found: C, 55.76; H, 5.75; N, 9.94.

5,6,7,8-Tetrahydro-2-(*N*,*N*-dimethylcarbamoyl)thio-3-nitronaphthalene (110) **109** (0.60 g, 2.1 mmol) was fused at 190 °C for 40 min. After cooling, the product was purified by column chromatography on silica gel using hexane–AcOEt (1 : 4, v/v) as eluant followed by recrystallization from Et_2O to give **110** as yellow prisms (0.54 g, 90.0%), mp 124 °C. IR ($CHCl_3$): 1660, 1520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.65—1.97 (4H, m), 2.62—2.96 (4H, m), 3.06 (6H, s), 7.37 (1H, s), 7.71 (1H, s). *Anal.* Calcd for $C_{13}H_{16}N_2O_3S$: C, 55.70; H, 5.75; N, 9.99. Found: C, 55.83; H, 5.82; N, 10.02.

Inhibitory Effects on the Contractions Induced by KCl or Caffeine The aorta was excised from New Zealand White rabbits (2—3 kg) and the connective tissue was removed. Then, spirally cut strips of 2 mm × 30 mm were prepared. Each strip was mounted in tissue chambers containing 20 ml of Krebs–Henseleit solution maintained at 37 °C and aerated with 95% O_2–5% CO_2. Tissues were then attached to force displacement transducers and left to equilibrate with a 2 g preload for 2—3 h. The solution in each bath was replaced every 20 min with fresh warmed Krebs–Henseleit solution. In the case of the KCl-induced contraction, 600 μl of 2 M KCl was added (final 60 mM) and the contraction was isometrically recorded on a polygraph (Recti-8s, Nippondenki Sanei). Thirty minutes after washing the tissues, 20 μl of 10^{-2} M drug (final 10^{-5} M, final concentration of DMSO was 0.1%) was added and 30 min later KCl was again added. In the case of the caffeine-induced contraction, following the equilibration period, tissues initially were rinsed with Krebs–Henseleit solution containing 2 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Ca^{2+}-free solution) for 7 min and then treated with 20 mM (final concentration) caffeine, a concentration which gave approximately 75% maximal contractile response. Thereafter, tissues were washed with normal Krebs–Henseleit solution and were allowed to equilibrate for 1 h. Tissues were then rinsed with Ca^{2+}-free solution and pretreated with drugs for 5 min. Two minutes after the addition of drug solution, caffeine was again added.

Percent inhibition was calculated according to the following formula:

$$\%\ \text{inhibition} = [(\text{contraction in the absence of drug}) - (\text{contraction in the presence of drug})] \div (\text{contraction in the absence of drug}) \times 100$$

Assay of CaM Activity CaM activity was assayed using a procedure involving the coupling reaction of phosphodiesterase (PDE) with 5′-nucleotidase and measuring the isotope labeled nucleoside produced from nucleotide-5′-monophosphate. PDE activity was measured by a modification of the method of Beavo *et al.*[17] The standard incubation mixture contained, in a final volume of 0.5 ml, 50 mM Tris–HCl buffer (pH 7.4), 5 mM $MgSO_4$, either 200 μM $CaCl_2$ plus 4 units/ml CaM (Sigma, P-0270) or 3 mM EGTA, cyclic nucleotide PDE (0.02 units/ml) (Sigma, P-0520), 1 μM guanosine 3′,5′-cyclic monophosphate (cGMP) (containing [^{3}H]cGMP as a tracer, purchased from New England Nuclear) and drug solution (dissolved in DMSO; final DMSO concentration was 0.1% or less). After incubation at 37 °C for 15 min, the incubation mixture was boiled for 2 min to stop the reaction of PDE. Then, 5′-nucleotidase from snake venom (Sigma, 50 μg) was added and the mixture was incubated at 37 °C for 10 min. After anion exchange resin (AG® 1-X2, Bio Rad) was added, the radioactivity of the produced [^{3}H]guanosine in the supernatant was counted with a liquid scintillation counter (Aloka LSC-900). The experiment was carried out in duplicate. The inhibitory effect on CaM was expressed in terms of IC_{50} (50% inhibiting concentration). Percent inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100 - [(\text{count in the presence of drug}) - (\text{count in the presence of EGTA})] \div [(\text{count in the absence of drug}) - (\text{count in the presence of EGTA})] \times 100$$

Antihypertensive Activity Male SHR (12 to 13 weeks old, 3 rats per group), whose systolic blood pressure was about 200 mmHg, were used. Blood pressure was measured by the tail cuff method using a plethysmograph. The antihypertensive effect is shown as the reduction in blood pressure (mmHg): the basal value minus the value 1 h after a test compound, prepared as a suspension in gum arabic, was administered orally (2 ml/kg) at various doses (3, 10, 30 or 60 mg/kg).

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Synthesis and Biological Activities of New 2-Substituted 1,4-Benzoxazine Derivatives

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A series of new 1,4-benzoxazine derivatives (XI, XII) possessing (4-phenyl-1-piperazinyl)alkyl moieties at the 2-position and related compounds (XIII) were synthesized and tested for calcium antagonistic, calmodulin antagonistic and antihypertensive activities. Various compounds had *in vitro* calmodulin antagonistic activity superior or comparable to that of trifluoperazine. Among these compounds, tetrahydronaphtho[2,3-*b*][1,4]oxazine derivatives such as 51, 53, 54, 58, 59, 60, 73 and 75 showed potent antihypertensive effects in spontaneously hypertensive rats. Optical isomers of 51 were also synthesized and evaluated biologically. No differences in biological activities were seen between the enantiomers.

Keywords 1,4-benzoxazine; piperazine; calmodulin antagonist; antihypertensive activity; intracellular calcium antagonist; spontaneously hypertensive rat

We reported earlier[1] that 2*H*-1,4-benzothiazin-3(4*H*)-one derivatives possessing a (4-phenyl-1-piperazinyl)propyl moiety at the 2-position had potent antihypertensive activity in spontaneously hypertensive rats (SHR). Although the mode of action of these compounds is not yet fully understood, their intracellular calcium antagonistic property could contribute to their antihypertensive effect.[1] These observations prompted us to synthesize their oxa-analogues, 2*H*-1,4-benzoxazin-3(4*H*)-one derivatives (XI, XII and XIII), in order to explore the potential of intracellular calcium antagonists as a new type of antihypertensive agent.

A number of 2*H*-1,4-benzoxazin-3(4*H*)-one derivatives have been synthesized so far and various pharmacological activities such as central nervous system (CNS) depressant,[2] analgesic,[3] α_2-antagonistic,[4] anthelmintic[5] and aldose reductase inhibitory[6] activity have been reported with this class of molecules. However, those bearing aminoalkyl groups at the 2-position have not yet been investigated as potential cardiovascular agents.[7] This paper describes the synthesis and pharmacological evaluation of 2-(4-phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzoxazin-3(4*H*)-one derivatives.

Chemistry

The 2*H*-1,4-benzoxazin-3(4*H*)-one derivatives (XI, XII and XIII) listed in Tables I—III were prepared according to the procedure shown in Chart 1. The key intermediates, 2-brmoalkyl-1,4-benzoxazin-3(4*H*)-ones (VIII) for the synthesis of XI and XIII were prepared *via* several routes. Reaction of 2-aminophenols (III), obtained from 2-nitrophenols (II) by hydrogenation, with dibromo esters (IV) gave VIII. Acylation of III with acyl chlorides (V) under Schotten–Baumann reaction conditions followed by cyclization in the presence of a base afforded VIII. The reductive cyclization of VI which was prepared by the alkylation of II with IV also yielded VIII. Compounds VIII thus obtained were converted to the desired compounds XI

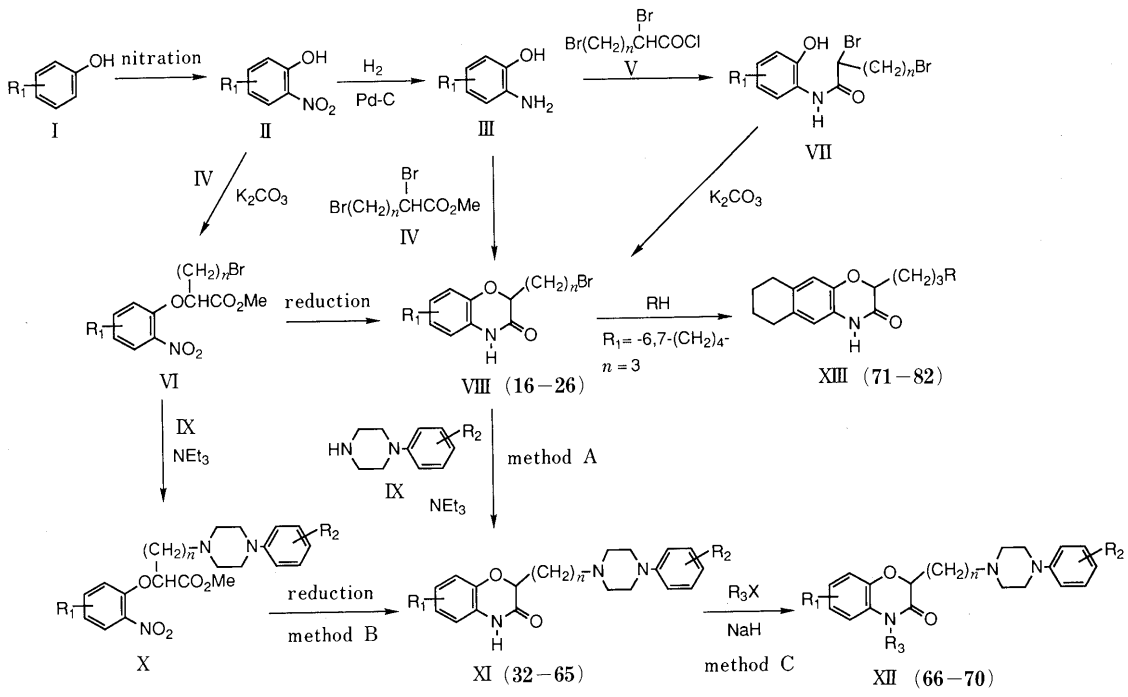


Chart 1

and XIII by reaction with phenylpiperazines (IX) (method A) and appropriate amines (RH), respectively. Compounds XI were alternatively synthesized from VI by first treating with IX to afford X and then reducing X followed by cyclization (method B). The alkylation of XI with alkyl halides (R_3X) in the presence of sodium hydride gave the *N*-alkyl compounds (XII) (method C).

Although the starting 2-nitrophenols (II) and 2-aminophenols (III) are mostly known compounds, commercially unavailable 2-nitrophenols (II) were synthesized by nitration of the phenols (I) using the method reported by Ouertani *et al.*[8] For instance, when 5,6,7,8-tetrahydro-2-naphthol (**1**) was treated with sodium nitrate in the presence of an excess amount of hydrogen chloride and a catalytic amount of $La(NO_3)_3$ in a two phase system of ether/water, the 3-nitrated compound (**2**) and the 1-nitrated compound (**3**) were obtained in a ratio of about 1 to 2 (Chart 2). Compounds **2** and **3** were then converted to the corresponding compounds **51** and **52** (Table I) by method A or B. However, this route was not satisfactory for the synthesis of **51** which is a potent antihypertensive agent (*vide post*), because the desired starting material (**2**) was the minor product and separation of **2** and **3** by column chromatography was rather laborious. Therefore, a more convenient route to **51** was investigated. Bromination of **1** with bromine proceeded selectively at the 1-position to give **4**. Then, **4** was nitrated with nitric acid followed by hydrogenation to give 3-amino-5,6,7,8-tetrahydro-2-naphthol[9] hydrobromide (**6**) which was identical with the one obtained from **2** by hydrogenation in the presence of hydrobromic acid. Compound **6** was converted to **51** following the general route, III→VII→VIII→XI.

Compound **53**, the oxa-analogue of 6,7-cyclopenteno-2-[3-[4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzothiazin-3(4*H*)-one, which was one of the most interesting compounds in the benzothiazine series,[1] was synthesized for comparison.

Nitration of 5-indanol (**7**) following the method of Ouertani *et al.*[8] affroded an inseparable mixture of **8** and **9** in a ratio of approximately 11 to 8 (Chart 3). This mixture was subjected to the reaction sequence, II→VI→VIII→XI or II→VI→X→XI, to yield **53** as crystals. Attempted isolation of **54**, a regioisomer of **53** which was expected to form from **9**, was unsuccesful. Compound **54** was therefore

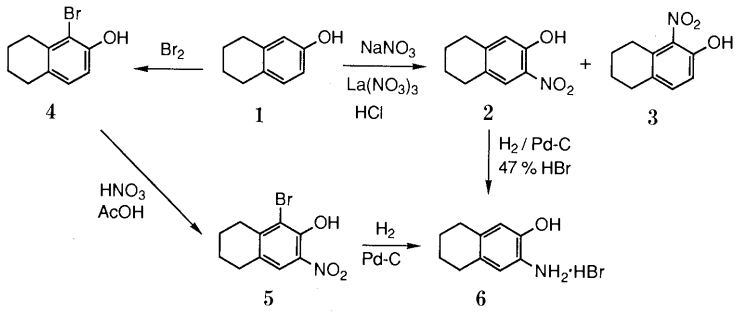


Chart 2

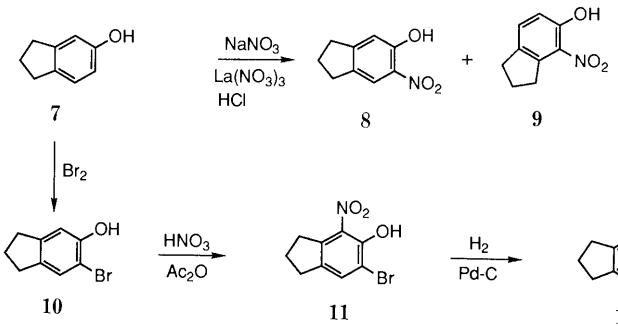
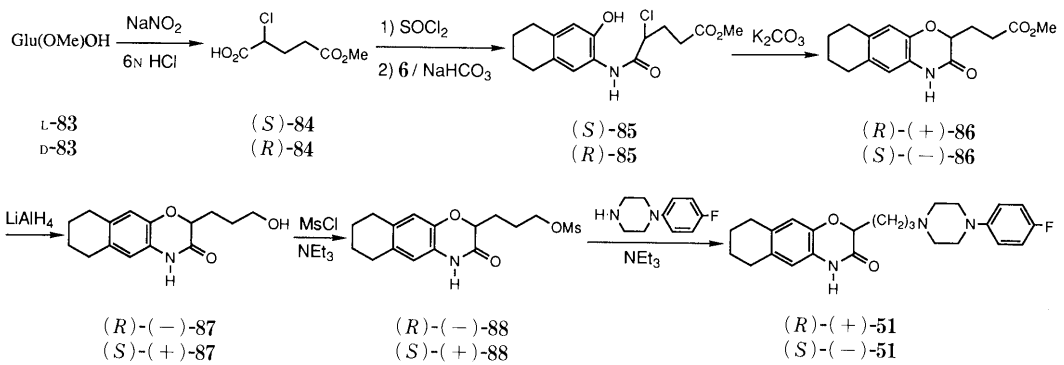


Chart 3



L-83 D-83 (*S*)-84 (*R*)-84 (*S*)-85 (*R*)-85 (*R*)-(+)-86 (*S*)-(−)-86

(*R*)-(−)-87 (*S*)-(+)-87 (*R*)-(−)-88 (*S*)-(+)-88 (*R*)-(+)-51 (*S*)-(−)-51

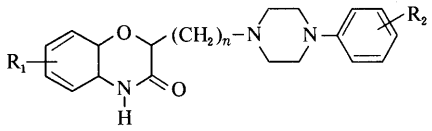
Chart 4

synthesized starting from **12** prepared as shown in Chart 3. Bromination of **7**, in contrast to the case of the tetrahydro-2-naphthol (**1**), gave the 6-bromo compound (**10**) as a single product in a high yield. Nitration of **10** followed by hydrogenation gave 4-amino-5-indanol hydrobromide (**12**).

As these 1,4-benzoxazin-3(4*H*)-one derivatives have an asymmetric carbon at the C-2 position of the oxazine ring, it is of interest to determine if there are differences in the biological activities of the optical isomers. Therefore, the enantiomers of compound **51** were synthesized as the representatives of this series.

Enantiomeric glutamic acid γ-methyl esters (**83**) were chosen as the chiral building blocks to prepare (*R*)- and (*S*)-**51** as shown in Chart 4. Treatment of L-glutamic acid γ-methyl ester (L-**83**) with sodium nitrite in the presence of 6 N HCl afforded the (*S*)-α-chlorocarboxylic acid [(*S*)-**84**]. This type of reaction is known to proceed while retaining

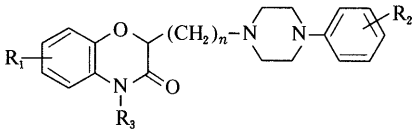
TABLE I. Physical and Biological Properties of 2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzoxazin-3(4*H*)-ones (XI)[a]



Compd. No.	R_1	R_2	n	Method	Yield[b] (%)	mp (°C)	Recrystn. solvent[c]	Formula[d]	Ca^{2+} blocking activity[e] (% inhib. at 10^{-5} M) KCl (60 mM)	CaM antagonistic activity[e] IC_{50}[f] (M)
32	6-NO_2	4-F	3	A	80.6	153—154	EA–E	$C_{21}H_{23}FN_4O_4$	37	1.7×10^{-6}
33	6-Cl	4-F	3	A	72.1	189—190	EA–E	$C_{21}H_{23}ClFN_3O_2$	29	1.7×10^{-6}
34	6-Me	4-F	3	A	94.7	174—175	Et–EA	$C_{22}H_{26}FN_3O_2$	10	5.0×10^{-8}
35	6-Me	3-CF_3	3	A	70.0	144—145	EA–E	$C_{23}H_{26}F_3N_3O_2$	14	$>10^{-5}$
36	6-Me	4-F	2	A	80.4	199—200	EA–E	$C_{21}H_{24}FN_3O_2$	9	1.1×10^{-6}
37	H	4-F	3	A	36.1	142—143	EA–E	$C_{21}H_{24}FN_3O_2$	73	$>10^{-5}$
38	7-CO_2Et	4-F	3	A	34.8	147—148	EA–E	$C_{24}H_{28}FN_3O_4$	33	$>10^{-5}$
39	5-Me-8-iso-Pr	4-F	3	B	19.7	192—193	M–EA	$C_{25}H_{32}FN_3O_2$	−2	1.2×10^{-6}
40	6-iso-Pr-7-Me	4-F	3	B	16.3	174—175	Ch–EA	$C_{25}H_{32}FN_3O_2$	10	1.0×10^{-5}
41	6-CF_3	4-F	3	A	63.5	156—157	Ch–EA	$C_{22}H_{23}F_4N_3O_2$	36	5.6×10^{-6}
42	6-MeO	4-F	3	A	87.2	153—154	M	$C_{22}H_{26}FN_3O_3$	28	3.2×10^{-6}
43	7-MeO	4-F	2	B	17.7	146—147	Et	$C_{21}H_{24}FN_3O_3$	15	1.8×10^{-6}
44	6-iso-Pr-7-Me	4-F	9	B	11.1	108—109	IPE	$C_{31}H_{44}FN_3O_2$	4	$>10^{-5}$
45·2HCl	6-Ph	4-F	3	B	62.7[g,h]	202—204[i]	M–E	$C_{27}H_{28}FN_3O_2$ ·2HCl·1/2H_2O	18	$>10^{-5}$
46·2HCl	6-Cyclohexyl	4-F	3	B	28.7[g]	159—161[j]	M–E	$C_{27}H_{34}FN_3O_2$ ·2HCl	24	$>10^{-5}$
47	8-Cyclohexyl	4-F	3	B	20.2	171—173[k]	EA–E	$C_{27}H_{34}FN_3O_2$	6	$>10^{-5}$
48	6-NH_2	4-F	3	[l]	98.2	158—159	E	$C_{21}H_{25}FN_4O_2$	−2	$>10^{-5}$
49	6-$NHCONMe_2$	4-F	3	[l]	38.8	179—181	MC–EA	$C_{24}H_{30}FN_5O_3$	−1	$>10^{-5}$
50	6-NHCOMe	4-F	3	[l]	71.5	111—112	MC–EA	$C_{23}H_{27}FN_4O_3$ ·1/2H_2O	9	$>10^{-5}$
51	6,7-$(CH_2)_4$–	4-F	3	A B	59.3 43.1[n]	164—165[m]	Ch–EA	$C_{25}H_{30}FN_3O_2$	82	5.2×10^{-7}
52	5,6-$(CH_2)_4$–	4-F	3	B	19.3	176—177	AE	$C_{25}H_{30}FN_3O_2$	3	$>10^{-5}$
53	6,7-$(CH_2)_3$–	4-F	3	A B	90.9 35.4[n]	186—187	MC–EA	$C_{24}H_{28}FN_3O_2$	21	1.9×10^{-6}
54	5,6-$(CH_2)_3$–	4-F	3	A	76.7	171—172	Ch–EA	$C_{24}H_{28}FN_3O_2$	28	2.0×10^{-6}
55·2HCl	6,7-$(CH_2)_4$–	4-F	2	B	22.4[g,n]	232—234	M	$C_{24}H_{28}FN_3O_2$ ·2HCl	−2	$>10^{-5}$
56	6,7-$(CH_2)_4$–	4-F	4	B	31.5	160—161	Ch–EA	$C_{26}H_{32}FN_3O_2$	14	$>10^{-5}$
57	6,7-OCH_2O–	4-F	3	B	77.4[h]	194—195	EA–E	$C_{22}H_{24}FN_3O_4$	24	5.6×10^{-6}
58	6,7-$(CH_2)_4$–	3-F	3	A	54.1	158—159	Ch–EA	$C_{25}H_{30}FN_3O_2$	10	3.2×10^{-7}
59	6,7-$(CH_2)_4$–	H	3	A	53.9	178—179	Ch–EA	$C_{25}H_{31}N_3O_2$	12	1.0×10^{-6}
60·2HCl	6,7-$(CH_2)_4$–	2-MeO	3	A	90.0[g]	185—187[o]	Et	$C_{26}H_{33}N_3O_3$ ·2HCl	8	3.2×10^{-7}
61	6,7-$(CH_2)_4$–	3-CF_3	3	A	71.2	196—197	Ch–EA	$C_{26}H_{30}F_3N_3O_2$	−8	$>10^{-5}$
62	6,7-$(CH_2)_4$–	4-Me	3	A	31.9	180—181	Ch–EA	$C_{26}H_{33}N_3O_2$ ·1/2H_2O	−13	$>10^{-5}$
63	6,7-$(CH_2)_4$–	4-OH	3	A	64.8	217—218	Ch–M	$C_{25}H_{31}N_3O_3$ ·1/2HCl	26	1.3×10^{-6}
64	6,7-$(CH_2)_4$–	3-Cl-4-Me	3	A	45.7	174—175	Ch–EA	$C_{26}H_{32}ClN_3O_2$	10	7.3×10^{-6}
65	6,7-$(CH_2)_4$–	3,4-OCH_2O–	3	A	50.5	152—153	Ch–EA	$C_{26}H_{31}N_3O_4$	32	4.3×10^{-6}
Trifluoperazine										4.1×10^{-6}

a) Structures of all compounds were confirmed by IR and ^{1}H-NMR spectra. For typical examples, see Experimental. *b*) Yield from VIII (method A) and overall yield from II (method B) are shown. *c*) Solvents for recrystallization: A, acetone; Ch, chloroform; E, ethyl ether; EA, ethyl acetate; Et, ethanol; H, hexane; IPE, isopropyl ether; M, methanol; MC, methylene chloride. *d*) All compounds were analyzed for C, H and N and the analytical results were within ±0.4% of the calculated values for the formulae shown. *e*) For the biological methods, see ref. 1. *f*) IC_{50} values were determined by linear regression analysis; the correlation coefficient for each regression line was >0.95. *g*) Yield of free base. *h*) Yield from X. *i*) Free base: mp 173 °C (Ch–EA). *j*) Free base: mp 162—164 °C. *k*) 2HCl salt: mp 232—234 °C (M–E). *l*) For the method, see Experimental. *m*) 2HCl salt: mp 150–151 °C (M). *n*) Yield from VI. *o*) Free base: mp 146—147 °C (Ch–AE).

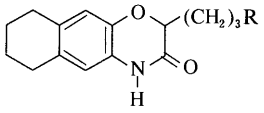
TABLE II. Physical and Biological Properties of 2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzoxazin-3(4*H*)-ones (XII) Synthesized by Method C[a]

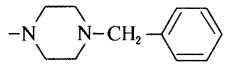
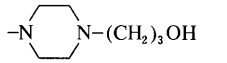
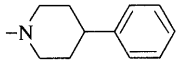
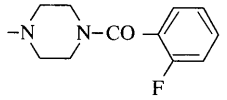
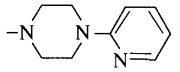
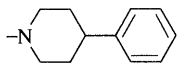
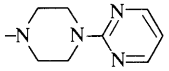
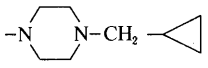
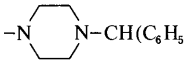
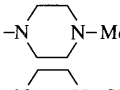
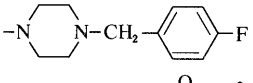
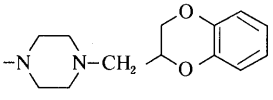


Compd. No.	R_1	R_2	R_3	n	Yield[b] (%)	mp (°C)	Recrystn. solvent[c]	Formula[d]	Ca^{2+} blocking activity[e] (% inhib. at 10^{-5} M) KCl (60 mM)	CaM antagonistic activity[e] IC_{50}[f] (M)
66	6-Me	4-F	CH_2CO_2Et	3	79.7	127	IPE	$C_{26}H_{32}FN_3O_4$	9	$>10^{-5}$
67·3HCl	6-Me	4-F	$CH_2CH_2NMe_2$	3	62.8	166—169	Et	$C_{26}H_{35}FN_4O_2$ ·3HCl·H_2O	13	$>10^{-5}$
68	6,7-$(CH_2)_4$–	4-F	Me	3	63.0	98—99	E	$C_{26}H_{32}FN_3O_2$	20	2.8×10^{-6}
69·2HCl	6,7-$(CH_2)_4$–	4-F	$CH_2CH_2NMe_2$	3	91.7	142—143	M	$C_{29}H_{39}FN_4O_2$ ·2HCl·1/2H_2O	30	$>10^{-5}$
70 Trifluoerazine	6,7-$(CH_2)_4$–	4-F	CH_2CO_2Et	3	82.3	128—129	E	$C_{29}H_{36}FN_3O_4$	12	$>10^{-5}$ 4.1×10^{-6}

a—*f*) See footnotes *a*—*f*) in Table I.

TABLE III. Physical and Biological Properties of Compound XIII Synthesized by Method A[a]



Compd. No.	R	Yield[b] (%)	mp (°C)	Recrystn. solvent[c]	Formula[d]	Ca^{2+} blocking activity[e] (% inhib. at 10^{-5} M) KCl (60 mM)	CaM antagonistic activity[e] IC_{50}[f] (M)
71		63.0	141—142	Ch–EA	$C_{26}H_{33}N_3O_2$	42	5.2×10^{-6}
72·2HCl		78.3	235—239	M	$C_{22}H_{33}N_3O_3\cdot2HCl$	4	$>10^{-5}$
73		56.9	169—170	Ch–EA	$C_{26}H_{30}N_2O_2$	18	6.8×10^{-6}
74		60.4	144—145	Ch–EA	$C_{26}H_{30}FN_3O_3$	16	$>10^{-5}$
75		71.8	150—151	EA	$C_{24}H_{30}N_4O_2$	23	1.5×10^{-6}
76		37.1	154—155	E	$C_{26}H_{32}N_2O_2$	10	$>10^{-5}$
77		74.3	181—182	EA–E	$C_{23}H_{29}N_5O_2$	21	1.0×10^{-5}
78		43.5	107—108	EA–E	$C_{23}H_{33}N_3O_2$	15	$>10^{-5}$
79		76.3	173—174	Ch–EA	$C_{32}H_{37}N_3O_2$	33	$>10^{-5}$
80		46.7	118—119	EA–E	$C_{20}H_{29}N_3O_2$	19	$>10^{-5}$
81·2HCl		87.0	229—230	Et	$C_{26}H_{32}FN_3O_2\cdot2HCl$ ·1/2H_2O	43	$>10^{-5}$
82 Trifluoperazine		44.2	154—157	EA	$C_{28}H_{35}N_3O_4$	22	4.2×10^{-6} 4.1×10^{-6}

a—*f*) See footnotes *a*—*f*) in Table I.

its configuration.[10] Then, (*S*)-**84** was allowed to react with the aminophenol (**6**) *via* the acid chloride and the subsequent cyclization of the resulting acylamide derivative (*S*)-**85** with potassium carbonate gave (*R*)-**86**. This reaction is assumed to proceed *via* an *SN*2 mechanism and thus the configuration is inverted. By the same procedure, (*S*)-**86** was obtained starting from D-glutamic acid γ-methyl ester (D-**83**). The enantiomeric esters, (*R*)-(+)- and (*S*)-(−)-**86**, were reduced with $LiAlH_4$ to the alcohols (*R*)-(−)- and (*S*)-(+)-**87**, which were then mesylated to give (*R*)-(−)- and (*S*)-(+)-**88**, respectively. The reaction of (*R*)-(−)- and (*S*)-(+)-**88** with 1-(4-fluorophenyl)piperazine gave (*R*)-(+)-**51**, mp 155.5—156 °C, $[\alpha]_D^{26}$ +19.9° ($c=0.7$, $CHCl_3$), and (*S*)-(−)-**51**, mp 156—156.5 °C, $[\alpha]_D^{26}$ −19.9° ($c=1.2$, $CHCl_3$), respectively. The optical purities of the enantiomeric **51** were satisfactory as estimated by their nuclear magnetic resonance (^{1}H-NMR, 400 MHz) spectra in the presence of a chiral shift reagent, tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato], europium(III) [Eu(hfc)$_3$] in $CDCl_3$. Each methine proton at C-2 and the NH proton of (±)-**51** was observed as a pair at δ 6.29 and 6.36 and at δ 8.55 and 8.76, respectively. However, these protons of each isomer appeared as single peaks at the expected positions, the higher chemical shifts being attributed to the (*S*)-isomer.

Results and Discussion

The calcium channel blocking and calmodulin antagonistic activity of the new 2*H*-1,4-benzoxazin-3(4*H*)-one derivatives (XI, XII and XIII) are shown in Tables I—III.

As expected from the results[1] obtained with the 1,4-benzothiazin-3(4*H*)-one series, these compounds showed very weak calcium channel blocking effects, while some of them showed potent calmodulin antagonistic activity superior or comparable to that of a phenothiazine derivative, trifluoperazine.[1] Of the compounds with a 3-(4-fluorophenyl-1-piperazinyl)propyl moiety, those bearing an ethoxycarbonyl (**38**), an amino (**48**), or an acylamino (**49**, **50**) group as the R_1 substituent did not show calmodulin antagonistic activity. The compounds having bulky substituents, such as phenyl (**45**) and cyclohexyl (**46**, **47**) as R_1, as well as the unsubstituted compound (**37**) were not calmodulin antagonists. As to the length of the alkylene at the C-2 position, the trimethylene group ($n=3$) seems to be better than others (**34** *vs.* **36**, **40** *vs.* **44**, **51** *vs.* **55** and **56**). In general, compounds with a fused ring at the 5,6- or 6,7-positions of the benzoxazine ring in combination with a 2-methoxyphenyl or 4-fluorophenyl group as an *N*-substituent of the piperazine ring tend to show potent calmodulin antagonistic activity as seen with compounds **51**, **53**, **54** and **60**. Compound **52** with a 5,6-fused six-membered ring was the only exception. In other cases, compounds having a 3-fluoro (**58**), no substituent (**59**), 4-hydroxy (**63**), 3-chloro-4-methyl (**64**) or 3,4-methylenedioxy (**65**) substituent on the benzene ring of the phenylpiperazine moiety also showed potent calmodulin antagonistic activity. Substitution of the nitrogen at the 4-position resulted in a decrease in activity (Table II). Among the other analogues of XI listed in Table III, compound **75** with a 4-(2-pyridyl)-1-piperazinyl group in the side chain retained considerably potent calmodulin antagonistic activity, but introduction of other functional groups generally decreased the activity as compared to that of the corresponding piperazine derivative (**59**).

Several compounds which showed potent calmodulin antagonistic activity were subsequently examined for antihypertensive activity in conscious SHR by the method described in the previous paper,[1] and the results are shown in Table IV. Compounds **51**, **53**, **54**, **58**, **59**, **60**, **73** and **75** having a cycloalkylene ring fused at 5,6- or 6,7-positions of the benzoxazine are particularly interesting, as they showed potent antihypertensive activity in accordance with

TABLE IV. Antihypertensive Effects of 2*H*-1,4-benzoxazin-3(4*H*)-ones in SHR

Compd. No.	Antihypertensive activity[a]	
	Dose (mg/kg *p.o.*)	Maximum change (mmHg)
32	60	43.3
33	30	9.7
34	60	45.0
36	60	39.7
39	60	27.3
41	3	12.0
51	3	30.0
53	3	29.3
54	3	19.7
57	3	26.7
58	3	59.3
59	3	43.3
60	3	49.7
63	3	2.7
65	3	16.3
71	10	11.8
73	3	53.8
75	3	43.0
77	3	19.0

a) See footnote *e*) in Table I. The change in blood pressure is an average of the values from 3 animals.

TABLE V. Antiarrhythmic Activity of **51**

Compd. No.	Dose (mg/kg, *p.o.*)	VT (%)	VF (%)	Mortality (%)
Control	—	100 (16)	100 (16)	38 (16)
51	3	42[a] (12)	25[a] (12)	0[a] (12)

The percentage incidence of reperfusion-induced VT, VF and mortality in control animals and animals pretreated with **51**. The number of animals used in each group is shown in parentheses. *a*) Denotes significant difference ($p<0.05$) from the control group.

TABLE VI. Pharmacological Effects of Compounds (±)-, (*R*)-(+)- and (*S*)-(−)-**51**

Compd. No.	Calmodulin antagonistic activity[a] IC_{50}[b] (M)	Inhibitory effect (%) on caffein-induced contraction[c] (10^{-6} M)	Antihypertensive activity[d] maximum change in systolic blood pressure (3 mg/kg, *p.o.*)
(±)-**51**	5.2×10^{-7}	43± 7 (3)	30
(*R*)-(+)-**51**	5.0×10^{-7}	43±14 (3)	30
(*S*)-(−)-**51**	7.4×10^{-7}	44± 8 (3)	28

a, b) See footnotes *e*) and *f*) in Table I. *c*) For the biological methods, see ref. 1. Values represent mean±S.E. The average control contractile response of the rabbit aortic strips was 1.94±0.26 g (mean±S.E. $n=8$). The number of experiments is shown in parentheses. *d*) See footnote *a*) in Table IV.

their potent calmodulin antagonistic property. Moreover, the antiarrhythmic activity of **51** was evaluated in the anesthetized rat by Winslow's method,[11] and the results are shown in Table V.

Compound **51** antagonized the development of ventricular fibrillation (VF) and ventricular tachycardia (VT), and also reduced the mortality resulting from 5 min of occlusion followed by reperfusion. Compound **51** also inhibited the caffeine induced contraction of rabbit aorta in a calcium-free buffer (Table VI), suggesting that this compound blocks intracellular calcium movement rather than the influx of calcium into the cell. Therefore, intracellular calcium antagonism including calmodulin antagonistic activity may play a major role in the antihypertensive and antiarrhythmic effects[12] of these 1,4-benzoxazines.

The relationship between the absolute configuration of **51** and its biological activities was also examined. However, in contrast to our expectations, there were almost no differences in activity of the racemate and the two enantiomers, as evidenced by the three parameters shown in Table VI: calmodulin antagonism, inhibitory effect on the caffeine induced contraction of rabbit aorta and antihypertensive effect in SHR.

Experimental

Melting points were determined using a Yanagimoto micro melting point apparatus and are uncorrected. Infrared (IR) spectra were taken on a Hitachi IR-260-10 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian EM-390 (90 MHz) spectrometer in the solvent indicated. Chemical shifts are given in ppm relative to Me_4Si as the internal standard. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad. Column chromatography was performed on E. Merck 70—230 mesh silica gel. Evaporation was carried out *in vacuo* on a rotary evaporator.

Synthesis of 2-Nitrophenols (II) Most 2-nitrophenols which are not commercially available were synthesized following the procedure of Ouertani *et al.*[8] A typical example is given to illustrate the general procedure.

5,6,7,8-Tetrahydro-3-nitro-2-naphthol (2)[13] and 5,6,7,8-Tetrahydro-1-nitro-2-naphthol (3) A solution of 5,6,7,8-tetrahydro-2-naphthol (**1**, 29.6 g, 0.20 mol) in Et_2O (600 ml) was added to a stirred mixture of $NaNO_3$ (17.0 g, 0.20 mol), $La(NO_3)_3 \cdot 6H_2O$ (0.87 g, 2.0 mmol) and 6 N HCl (320 ml). The mixture was stirred for 20 h at room temperature (the color of Et_2O layer changed to dark red), diluted with water, and the organic layer was separated. The aqueous layer was extracted with Et_2O, and the combined organics were washed with saturated aqueous NaCl, dried ($MgSO_4$) and concentrated. The oil obtained was purified by column chromatography on silica gel. The first eluate with hexane–AcOEt (9:1, v/v) gave **2** which was triturated with *n*-pentane, filtered and recrystallized from aqueous MeOH to give yellow prisms (9.52 g, 24.6%), mp 89.5—90 °C (lit.[13] mp 89—90 °C). IR (Nujol): 3170, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.64—1.97 (4H, m), 2.59—2.90 (4H, m), 6.80 (1H, s), 7.75 (1H, s), 10.32 (1H, s). The second eluate with hexane–AcOEt (9:1, v/v) gave **3** as a reddish oil (17.72 g, 45.9%). IR (Nujol): 3420, 1605 cm^{-1}. NMR ($CDCl_3$) δ: 1.58—1.92 (4H, m), 2.57—3.08 (4H, m), 6.87 and 7.17 (2H, each d, J=9 Hz).

1-Bromo-5,6,7,8-tetrahydro-2-naphthol (4)[14] A solution of Br_2 (10.8 ml, 0.21 mol) in CCl_4 (100 ml) was added dropwise to a stirred and ice-cooled solution of **1** (29.6 g, 0.20 mol) in CCl_4 (200 ml). After the addition was complete, the ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. The mixture was diluted with water and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water, dried ($MgSO_4$) and concentrated. The residual oil was distilled to yield **4** (43.7 g, 96.2%), bp 115—117 °C (0.15 mmHg), mp 52—53 °C (lit.[14] bp 160 °C (13 mmHg), mp 74 °C). IR (Nujol): 3520 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53—1.99 (4H, m), 2.49—2.90 (4H, m), 5.43 (1H, s). *Anal.* Calcd for $C_{10}H_{11}BrO$: C, 52.89; H, 4.88. Found: C, 52.88; H, 4.85.

1-Bromo-5,6,7,8-tetrahydro-3-nitro-2-naphthol (5)[13] Fumic HNO_3 (d=1.52, 1.87 ml, 45.1 mmol) was added to a stirred solution of **4** (9.77 g, 43.0 mmol) in AcOH (80 ml) at 0 °C. After the addition was complete, the reaction mixture was stirred at room temperature for 1 h, diluted with water and extracted with CH_2Cl_2. The extract was washed with water, dried ($MgSO_4$) and concentrated to give a solid. Recrystallization from AcOEt afforded **5** as yellow prisms (6.52 g, 55.7%), mp 133—133.5 °C (lit.[13] mp 129.5—130 °C). IR (Nujol): 3200 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.62—2.02 (4H, m), 2.48—2.93 (4H, m), 7.77 (1H, s), 10.04 (1H, s). *Anal.* Calcd for $C_{10}H_{10}BrNO_3$: C, 44.14; H, 3.70; N, 5.15. Found: C, 44.11; H, 3.68; N, 5.18.

Nitration of 5-Indanol (7) A solution of **7** (5.40 g, 40.2 mmol) in Et_2O (120 ml) was treated with a mixture of $NaNO_3$ (3.42 g, 40.2 mmol), $La(NO_3)_3 \cdot 6H_2O$ (174 mg, 0.40 mmol) and 6 N HCl (64 ml) in the same manner as described for **1**. Purification by column chromatography on silica gel (200 g) with hexane–AcOEt (9:1, v/v) gave 4.85 g (67.3%) of a solid mixture of 6-nitro- and 4-nitro-5-indanols (**8** and **9**) which could not be separated by column chromatography or thin-layer chromatography (TLC). The approximate ratio (**8**:**9**=11:8) was determined from the ^{1}H-NMR integrals for the OH singlet for each regioisomer. IR (Nujol): 3220, 1640 cm^{-1}. ^{1}H-NMR ($CDCl_3$) data for the aromatic protons and OH group of **8** and **9** are as follows: **8**, δ: 6.91 (1H, s, C_4-H), 7.83 (1H, s, C_7-H), 10.67 (1H, s, OH); **9**, δ: 6.88 (1H, d, J=9 Hz, C_6-H), 7.33 (1H, d, J=9 Hz, C_7-H), 10.58 (1H, s, OH). *Anal.* Calcd for $C_9H_9NO_3$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.24; H, 5.04; N, 7.79.

6-Bromo-5-indanol (10) Compound **10** was prepared in the same manner as described for the synthesis of **4**. Yield: 84.1%, bp 87—88 °C (0.4 mmHg). This oil crystallized on standing, mp 38—39 °C. IR ($CHCl_3$): 3525 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.85—2.27 (2H, m), 2.82 (2H×2, t, J=7.2 Hz), 5.33 (1H, s, OH), 6.88 (1H, s), 7.27 (1H, s). *Anal.* Calcd for C_9H_9BrO: C, 50.73; H, 4.26. Found: C, 50.72; H, 4.07.

6-Bromo-4-nitro-5-indanol (11) Compound **11** was prepared in the same manner as described for the synthesis of **5**. Yield: 52.6%, mp 104 °C (iso-Pr_2O). IR ($CHCl_3$): 3150 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.12 (2H, m), 2.88 (2H, t, J=7.2 Hz), 3.30 (2H, t, J=7.2 Hz), 7.63 (1H, s), 11.13 (1H, s, OH). *Anal.* Calcd for $C_9H_8BrNO_3$: C, 41.89; H, 3.12; N, 5.43. Found: C, 41.94; H, 3.10; N, 5.42.

Synthesis of 2-Aminophenols (III) Typical examples are given to illustrate the general procedure.

3-Amino-5,6,7,8-tetrahydro-2-naphthol[9] Hydrobromide (6) From **2**: A mixture of **2** (8.55 g, 44.3 mmol) and 47% HBr (6.2 ml) in MeOH (300 ml) was hydrogenated in the presence of 10% Pd–C (50% wet, 1.08 g). After the usual workup, the residual oil was crystallized from AcOEt to give **6** (10.5 g, 97.0%), mp 236—238 °C (dec.). IR (Nujol): 3250, 1630 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.50—1.85 (4H, m), 2.39—2.77 (4H, m), 6.72 (1H, s), 6.99 (1H, s), 9.54 (2H, br), 10.15 (1H, br s). *Anal.* Calcd for $C_{10}H_{13}NO \cdot HBr$: C, 49.20; H, 5.78, N, 5.74. Found: C, 48.95; H, 5.75; N, 5.83.

From **5**: A solution of **5** (2.00 g, 7.35 mmol) in MeOH (20 ml)–tetrahydrofuran (THF, 20 ml) was hydrogenated in the presence of 5% Pd–C (50% wet, 0.50 g). After the usual workup, the residual oil was crystallized from Et_2O to give **6** (1.72 g, 95.8%), mp 235—236 °C (dec.). This sample was identical with **6** obtained from **2**.

4-Amino-5-indanol Hydrobromide (12) Hydrogenation of **11** in the same manner as described for the synthesis of **6** gave **12**. Yield 98.7%, mp 262—264 °C (dec.). IR (Nujol): 3240, 1635 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.03 (2H, t, J=7.2 Hz), 2.80 (2H, t, J=7.2 Hz), 2.91 (2H, t, J=7.2 Hz), 6.81 (1H, d, J=8.2 Hz), 7.06 (1H, d, J=8.2 Hz), 9.61 (2H, br), 10.35 (1H, br s). *Anal.* Calcd for $C_9H_{11}NO \cdot HBr$: C, 46.98; H, 5.26; N, 6.09. Found: C, 46.80; H, 5.22; N, 6.13.

Synthesis of VI Typical examples are given to illustrate the general procedure.

Methyl 4-Bromo-2-(5,6,7,8-tetrahydro-3-nitro-2-naphthyloxy)butyrate (13) A mixture of **2** (2.62 g, 13.6 mmol), methyl 2,4-dibromobutylate (3.70 g, 14.2 mmol), K_2CO_3 (2.25 g, 16.3 mmol) and dimethylformamide (DMF, 30 ml) was stirred at room temperature for 5 h. The reaction mixture was worked up to yield the residue which was chromatographed on silica gel with hexane–AcOEt (4:1, v/v) followed by crystallization from Et_2O to give **13** as light yellow crystals (3.34 g, 66.2%), mp 99—100 °C. IR ($CHCl_3$): 1740, 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.63—1.92 (4H, m), 2.35—2.85 (6H, m), 3.57—3.71 (2H, m), 3.77 (3H, s), 4.92 and 4.97 (1H each, d, J=5 Hz), 6.63 (1H, s), 7.59 (1H, s). *Anal.* Calcd for $C_{15}H_{18}BrNO_5$: C, 48.40; H, 4.87; N, 3.76. Found: C, 48.44; H, 4.90; N, 3.77.

Methyl 5-Bromo-2-(5,6,7,8-tetrahydro-3-nitro-2-naphthyloxy)valerate (14) A mixture of **2** (8.0 g, 41.4 mmol), methyl 2,5-dibromovalerate (11.5 g, 42.0 mmol), K_2CO_3 (6.0 g, 43.4 mmol) and DMF (100 ml) was stirred at room temperature for 8 h. The reaction mixture was diluted with

water and extracted with Et_3O. The extract was washed with water, dried ($MgSO_4$) and concentrated. The oil obtained was purified by column chromatography on silica gel with hexane–AcOEt (4 : 1, v/v) to afford **14** as a solid (15.7 g, 98.2%). An analytical sample was obtained by recrystallization from iso-Pr_2O as pale yellow prisms, mp 73—74 °C. IR (Nujol): 1745, 1615 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.69—1.87 (4H, m), 2.08—2.30 (4H, m), 2.60—2.90 (4H, m), 3.40—3.58 (2H, m), 3.76 (3H, s), 4.63—4.82 (1H, m), 6.57 (1H, s), 7.58 (1H, s). *Anal.* Calcd for $C_{16}H_{20}BrNO_5$: C, 49.76; H, 5.22; N, 3.63. Found: C, 49.63; H, 5.26; N, 3.55.

Other compounds (VI) were isolated as oils after column chromatography on silica gel. Therefore, these VI were used for the subsequent reaction without further purification.

Synthesis of 2,5-Dibromo-*N*-(2-hydroxyphenyl)valeramide (VII) A typical example is given to illustrate the general procedure.

2,5-Dibromo-*N*-(2-hydroxy-4,5,6,7-tetrahydronaphthyl)valeramide (15) A solution of 2,5-dibromovaleryl chloride[15] (1.94 g, 7.0 mmol) in AcOEt (6 ml) was added dropwise to a vigorously stirred mixture of **6** (1.70 g, 7.0 mmol), $NaHCO_3$ (1.46 g, 17.4 mmol), AcOEt (15 ml) and water (10 ml) with ice-cooling. After being stirred for 30 min, the reaction mixture was diluted with water and the organic layer was separated. The aqueous layer was extracted with AcOEt. The combined organic layers were washed with water, dried ($MgSO_4$) and concentrated to give **15** as crystals (2.80 g), which was used for the next reaction without further purification. An analytical sample was obtained by recrystallization from AcOEt as colorless crystals, mp 148—149 °C. IR (Nujol): 3350, 3160, 1645 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.58—2.33 (8H, m), 2.43—2.80 (4H, m), 3.51 (2H, t, J=6 Hz), 4.88 (1H, t, J=7 Hz), 6.53 (1H, s), 7.53 (1H, s), 9.28 (1H, s, OH; disappeared on treatment with D_2O), 9.36 (1H, s, NH). *Anal.* Calcd for $C_{15}H_{19}Br_2NO_2$: C, 44.47; H, 4.73; N, 3.46. Found C, 44.34; H, 4.69; N, 3.43.

Other compounds (VII) were prepared similarly and used for the subsequent reaction without further purification.

Synthesis of 2-(2-, 3- or 4-Bromoalkyl)-2*H*-1,4-benzoxazin-3(4*H*)-ones (VIII) Typical examples are given to illustrate the general procedure.

2-(3-Bromopropyl)-6-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (16) From III: A mixture of 2-amino-4-methylphenol (1.23 g, 10 mmol), methyl 2,5-dibromovalerate (2.74 g, 10 mmol), K_2CO_3 (1.38 g, 10 mmol) and acetone (30 ml) was refluxed with stirring for 5 h. The reaction mixture was diluted with water and extracted with AcOEt. The extract was worked up and the residue was chromatographed on silica gel with hexane–AcOEt (4 : 1, v/v). The product was crystallized from iso-Pr_2O to give **16** (0.88 g, 31.0%), mp 157—158 °C. IR (Nujol): 3200, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.87—2.23 (4H, m), 2.28 (3H, s), 3.30—3.54 (2H, m), 4.53 (1H, t, J=6 Hz), 6.59—6.92 (3H, s), 9.14 (1H, br s, NH). *Anal.* Calcd for $C_{12}H_{14}BrNO_2$: C, 50.72; H, 4.97; N, 4.93. Found: C, 50.75; H, 4.88; N, 4.75.

2-(3-Bromopropyl)-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one (17) From VI: A solution **14** (9.50 g, 24.6 mmol) in EtOH (200 ml) was hydrogenated in the presence of 5% Pd–C (50% wet, 1.0 g). The catalyst was filtered off and the filtrate was concentrated. The residual solid was triturated with Et_2O, collected by filtration and recrystallized from AcOEt to give **17** as prisms (6.47 g, 81.1%), mp 139—140 °C. IR ($CHCl_3$): 3390, 1670 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.67—1.89 (4H, m), 2.00—2.29 (4H, m), 2.53—2.80 (4H, m), 3.32—3.56 (2H, m), 4.40—4.59 (1H, m), 6.50 (1H, s), 6.63 (1H, s), 9.23 (1H, s). *Anal.* Calcd for $C_{15}H_{18}BrNO_2$: C, 55.57; H, 5.60; N, 4.32. Found: C, 55.58; H, 5.41; N, 4.29.

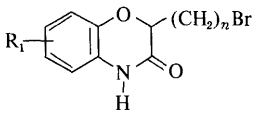
From VII: A mixture of **15** (3.1 g, 12.7 mmol), K_2CO_3 (1.8 g, 13.0 mmol) and DMF (20 ml) was stirred at room temperature for 1.5 h and diluted with water. The precipitate was collected by filtration, washed with water and dried. Recrystallization from AcOEt gave **17** (1.63 g, 72.0%), mp 139—140 °C. This compound was identical with **17** obtained from **14**. *Anal.* Calcd for $C_{15}H_{18}BrNO_2$: C, 55.57; H, 5.60; N, 4.32. Found: C, 55.60; H, 5.76; N, 4.36.

2-(3-Bromopropyl)-6,7-cyclopenteno-2*H*-1,4-benzoxazin-3(4*H*)-one (18) (i) A mixture of 3.85 g (21.5 mmol) of the isomeric mixture of **8** and **9**, methyl 2,5-dibromovalerate (5.90 g, 21.5 mmol), K_2CO_3 (3.00 g, 21.7 mmol) and DMF (30 ml) was stirred at room temperature for 4 h. The mixture was diluted with water and extracted with Et_2O. The extract was washed with saturated NaCl, dried ($MgSO_4$) and concentrated. The residual oil was purified by column chromatography on silica gel (150 g) with hexane–AcOEt (9 : 1, v/v) to give 4.93 g (61.6%) of an oily mixture of methyl 5-bromo-2-(4,5-cyclopenteno-2-nitrophenoxy)valerate (**27**) and methyl 5-bromo-2-(3,4-cyclopenteno-2-nitrophenoxy)valerate (**28**) in a ratio of approximately 1 : 1 (determined from the ^{1}H-NMR integrals of aromatic protons for each regioisomer) which could not be separated by column chromatography or TLC. IR (neat): 1745, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.10—2.30 (6H, m), 2.78—3.14 (4H, m), 3.37—3.58 (2H, m), 3.74 (1.5H × 2, s), 4.61—4.80 (0.5H × 2, m), 6.68 (0.5H, d, J=9 Hz), 6.73 (0.5H, s), 7.22 (0.5H, d, J=9 Hz), 7.66 (0.5H, s).

(ii) The isomeric mixture of **27** and **28** (1.90 g, 5.10 mmol) prepared in (i) in EtOH (30 ml) was hydrogenated in the presence of 10% Pd–C (50% wet, 0.32 g). The catalyst was filtered off and the filtrate was concentrated. The residue was chromatographed on silica gel (100 g) with hexane–AcOEt (2 : 1, v/v) and **18** (0.38 g, 25.3%), mp 154—155 °C, was obtained as colorless plates when crystallized from Et_2O. IR ($CHCl_3$): 3410, 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.85—2.28 (6H, m), 2.68—2.98 (4H, m), 3.35—3.55 (2H, m), 4.50 (1H, t, J=6 Hz), 6.67 (1H, s), 6.78 (1H, s), 9.23 (1H, br s). *Anal.* Calcd for $C_{14}H_{16}BrNO_2$: C, 54.21; H, 5.20; N, 4.52. Found: C, 54.38; H, 5.18; N, 4.66.

2-(3-Bromopropyl)-5,6-cyclopenteno-2*H*-1,4-benzoxazin-3(4*H*)-one (19) (i) 2,5-Dibromovaleryl chloride (1.84 g, 6.6 mmol) in AcOEt (7 ml) was added dropwise to a stirred and ice-cooled mixture of **12** (1.25 g, 5.4 mmol), $NaHCO_3$ (1.06 g, 12.6 mmol), AcOEt (10 ml) and water (7 ml). After being stirred at 0 °C for 30 min, the organic layer was separated and the aqueous layer was extracted with AcOEt. The combined organics were washed with saturated NaCl, dried ($MgSO_4$) and concentrated to yield 2,5-dibromo-1-(2-hydroxy-5,6-cyclopentenophenyl)valeramide as an oil. This was used for the subsequent reaction without further purification.

TABLE VII. 2-Bromoalkyl-2*H*-1,4-benzoxazin-3(4*H*)-ones (VIII)



Compd. No.	R_1	n	Starting material	Yield (%)	mp (°C)	Recrystn. solvent[a]	Formula[b]
16	6-Me	3	III	31.0	157—158	IPE	$C_{12}H_{14}BrNO_2$
17	6,7-$(CH_2)_4$–	3	VI	81.1	139—140	EA	$C_{15}H_{18}BrNO_2$
			VII	72.0			
18	6,7-$(CH_2)_3$–	3	VI	15.6[c]	154—155	E	$C_{14}H_{16}BrNO_2$
19	5,6-$(CH_2)_3$–	3	VII	60.0[d]	155—156	EA	$C_{14}H_{16}BrNO_2$
20	6-NO_2	3	III	27.0	150—151	E	$C_{11}H_{11}BrNO_4$
21	6-Cl	3	III	30.2	157—158	E	$C_{11}H_{11}BrClNO_2$
22	6-Me	2	III	9.6	183—184	IPE	$C_{11}H_{12}BrNO_2$
23	H	3	VI	60.6[e]	90— 92	E	$C_{11}H_{12}BrNO_2$
24	7-CO_2Et	3	VI	78.4[e]	118—119	E	$C_{14}H_{16}BrNO_4$
25	6-CF_3	3	VII	35.6[f]	123—124	IPE	$C_{12}H_{11}BrF_3NO_2$
26	6-MeO	3	VII	62.5[e]	102—103	E	$C_{12}H_{14}BrNO_3$

a, b) See footnotes *c*) and *d*) in Table I. *c*) Overall yield from a mixture of **8** and **9**. *d*) Overall yield from **12**. *e*) Overall yield from II. *f*) Overall yield from III.

(ii) A mixture of the amide obtained in (i), K_2CO_3 (0.91 g, 6.6 mmol) and DMF (15 ml) was stirred at room temperature for 4 h. Water was added and the mixture was extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was recrystallized from AcOEt to give **19** (1.01 g, 60.0%), mp 155—156 °C as colorless crystals. IR ($CHCl_3$): 3400, 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.92—2.35 (6H, m), 2.71—3.00 (4H, m), 3.37—3.59 (2H, m), 4.51 (1H, t, J=6 Hz), 6.74 (1H, d, J=9 Hz), 6.84 (1H, d, J=9 Hz), 8.42 (1H, br). *Anal.* Calcd for $C_{14}H_{16}BrNO_2$: C, 54.21; H, 5.20; N, 4.52. Found: C, 54.05; H, 5.16; N, 4.51.

Other compounds (**20**—**26**) listed in Table VII were prepared similarly from III, VI or VII.

Synthesis of X Typical examples are given to illustrate the general procedure.

Methyl 5-[4-(4-Fluorophenyl)-1-piperazinyl]-2-(2-nitro-4-phenylphenoxy)valerate (29) A mixture of methyl 5-bromo-2-(2-nitro-4-phenylphenoxy)valerate (3.51 g, 8.6 mmol), 1-(4-fluorophenyl)piperazine (1.98 g, 11.0 mmol), NEt_3 (1.40 ml, 10.0 mmol) and DMF (20 ml) was stirred at 80 °C for 2 h. Water was added and the mixture was extracted with AcOEt. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (100 g) with hexane–AcOEt (2 : 3, v/v) as eluant to yield **29**. Recrystallization from Et_2O gave yellow crystals (2.00 g, 35.8%), mp 126—127 °C. IR (Nujol): 1740, 1625 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.60—2.28 (4H, m), 2.63 (2H, t, J=6 Hz), 2.51—2.69 (4H, m), 2.92—3.17 (4H, m), 3.75 (3H, s), 4.89 (1H, t, J=6 Hz), 6.68—7.75 (11H, m), 8.04 (1H, d, J=2.4 Hz). *Anal.* Calcd for $C_{28}H_{30}FN_3O_5$: C, 66.26; H, 5.96; N, 8.28. Found: C, 66.17; H, 5.91; 8.19.

Methyl 5-[4-(4-Fluorophenyl)-1-piperazinyl]-2-(4,5-methylenedioxy-2-nitrophenoxy)valerate (30) A mixture of methyl 5-bromo-2-(4,5-methylenedioxy-2-nitrophenoxy)valerate (0.30 g, 0.8 mmol), 1-(4-fluorophenyl)piperazine (0.23 g, 1.3 mmol), NEt_3 (0.14 ml, 1.3 mmol) and DMF (10 ml) was stirred at 80 °C for 2 h. The mixture was worked up in the same manner as described for **29** to give **30** (0.27 g, 71.2%), mp 98—99 °C (iso-Pr_2O). IR (Nujol): 1735, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.57—2.23 (4H, m), 2.44 (2H, t, J=6 Hz), 2.48—2.67 (4H, m), 2.95—3.17 (4H, m), 3.75 (3H, s), 4.76 (1H, t, J=6 Hz), 6.00 (2H, s), 6.47 (1H, s), 6.70—7.06 (4H, m), 7.40 (1H, s). *Anal.* Calcd for $C_{23}H_{26}FN_3O_7$: C, 58.10; H, 5.51; N, 8.81. Found: C, 58.05; H, 5.41; N, 8.81.

Other compounds (X) prepared similarly were isolated as oils after column chromatography on silica gel. Therefore these X were used for the subsequent reaction without further purification.

Synthesis of 2-(4-Phenyl-1-piperazinyl)alkyl-2*H*-1,4-benzoxazin-3(4*H*)-ones (XI, Table I and Related Compounds XIII, Table III) Typical examples are given to illustrate the general procedure.

2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one (51) Method A: A stirred mixture of **17** (3.1 g, 9.6 mmol), 1-(4-fluorophenyl)piperazine (2.3 g, 13 mmol), NEt_3 (1.5 ml, 11 mmol) and DMF (100 ml) was heated at 80 °C for 1.5 h. The reaction mixture was poured into ice-water and extracted with AcOEt. The organic extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was recrystallized from $CHCl_3$–AcOEt to give **51** as colorless crystals (2.4 g, 59.3%), mp 164—165 °C. IR ($CHCl_3$): 3410, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.55—2.11 (8H, m), 2.43 (2H, t, J=6.0 Hz), 2.48—2.81 (8H, m), 3.02—3.18 (4H, m), 4.53 (1H, t, J=6.0 Hz), 6.47 (1H, s), 6.63 (1H, s), 6.70—7.12 (4H, m), 8.79 (1H, br s). *Anal.* Calcd for $C_{25}H_{30}FN_3O_2$: C, 70.90; H, 7.14; N, 9.92. Found: C, 70.72; H, 7.02; N, 9.88.

Dihydrochloride: A solution of **51** in a small amount of $CHCl_3$–MeOH (1 : 2, v/v) was treated with 20% methanolic hydrogen chloride and concentrated. The crystals obtained were recrystallized from MeOH to give **51**·2HCl as colorless crystals, mp 150—151 °C. IR (Nujol): 3450, 1685 cm^{-1}. NMR (DMSO-d_6) δ: 1.55—2.18 (8H, m), 2.43—2.75 (6H, m), 2.93—3.83 (8H, m), 4.38—4.57 (1H, m), 6.40—7.19 (6H, m), 10.55 (1H, s). *Anal.* Calcd for $C_{25}H_{30}FN_3O_2 \cdot 2HCl$: C, 60.48; H, 6.50; N, 8.46. Found: C, 60.35; H, 6.65; N, 8.42.

Method B: (i) A stirred mixture of **14** (5.00 g, 13.0 mmol), 1-(4-fluorophenyl)piperazine (2.45 g, 13.6 mmol), DMF (40 ml) and NEt_3 (1.99 g, 19.7 mmol) was heated at 80 °C for 2 h. The reaction mixture was diluted with water and extracted with $CHCl_3$. The extract was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel with hexane–AcOEt (1 : 1, v/v) to give methyl 5-[4-(4-fluorophenyl)-1-piperazinyl]-2-(5,6,7,8-tetrahydro-3-nitro-2-naphthyloxy)valerate (**31**) as an oil (4.51 g, 71.8%). IR (neat): 1755, 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.57—2.22 (8H, m), 2.35—2.82 (10H, m), 2.95—3.15 (4H, m), 3.73 (3H, s), 4.80 (1H, t, J=5.7 Hz), 6.55 (1H, s), 6.69—7.05 (4H, m), 7.58 (1H, s).

(ii) Iron powder (1.90 g, 34.0 mmol) was added in a small portion to a stirred solution of **31** (4.10 g, 8.4 mmol) in AcOH (15 ml)–H_2O (2.5 ml). The mixture was stirred at room temperature for 30 min and at 80 °C for an additional 15 min. The insoluble material was filtered off and the filtrate was concentrated, neutralized with aqueous Na_2CO_3 and extracted with $CHCl_3$. The extract was washed with water, dried ($MgSO_4$) and concentrated to afford a solid which was recrystallized from $CHCl_3$–AcOEt to give **51** (1.54 g, 43.1%), mp 164—165 °C. The IR and ^{1}H-NMR spectra of this sample were identical with those of **51** obtained by method A.

6,7-Cyclopenteno-2-[3-[4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (53) Method B: (i) A solution of 2.40 g (6.45 mmol) of the isomeric mixture of **27** and **28** (see preparation of **18**), 1-(4-fluorophenyl)piperazine (1.28 g, 7.10 mmol) and NEt_3 (0.9 ml, 6.5 mmol) in DMF (20 ml) was stirred at 80 °C for 2.5 h. The mixture was diluted with water and extracted with Et_2O. The extract was washed with saturated NaCl, dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography on silica gel (100 g) with Hexane–AcOEt (2 : 3, v/v) to give 2.80 g of an oil.

(ii) A solution of the oil obtained in (i) in AcOH (10 ml)–H_2O (2 ml) was treated with iron powder (1.26 g, 22.6 mmol) and worked up as described for **51**. The residue was chromatographed on silica gel (120 g) using hexane–AcOEt (2 : 3, v/v) as eluant and recrystallized from CH_2Cl_2–AcOEt to give **53** as colorless crystals (0.86 g, 35.4%), mp 186—187 °C. IR ($CHCl_3$): 3400, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.58—2.24 (6H, m), 2.43 (2H, t, J=6.0 Hz), 2.48—2.67 (4H, m), 2.69—2.93 (4H, m), 3.0—3.18 (4H, m), 4.53 (1H, t, J=6.0 Hz), 6.64 (1H, s), 6.72—7.08 (5H, m), 8.84 (1H, br s). *Anal.* Calcd for $C_{24}H_{28}FN_3O_2$: C, 70.39; H, 6.89; N, 10.26. Found: C, 70.37; H, 6.66; N, 10.37.

Other compounds (XI) listed in Table I were prepared similarly. Compounds XIII in Table III were prepared substantially by applying method A.

6-Amino-2-[3-[4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (48) A solution of **32** (2.03 g, 4.90 mmol) in MeOH (80 ml)–THF (20 ml) was hydrogenated in the presence of 10% Pd–C (50% wet, 0.6 g). The catalyst was filtered off and the filtrate was concentrated. The residue was crystallized from Et_2O to give **48** (1.85 g, 98.2%) as a crystalline solid, mp 158—159 °C. IR (Nujol): 3440 and 3350, 3230, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.47—2.09 (4H, m), 2.32—2.70 (6H, m), 2.89—3.21 (4H, m), 3.49 (2H, br s), 4.50 (1H, t, J=6.0 Hz), 6.12—6.38 (2H, m), 6.70—7.07 (5H, m), 8.49 (1H, br s). *Anal.* Calcd for $C_{21}H_{25}FN_4O_2$: C, 65.61; H, 6.55; N, 14.57. Found: C, 65.16; H, 6.46; N, 14.33.

2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-6-(3,3-dimethylureido)-2*H*-1,4-benzoxazin-3(4*H*)-one (49) Dimethylcarbamyl chloride (0.13 ml, 1.41 mmol) was added to a stirred and ice-cooled solution of **48** (0.50 g, 1.30 mmol) in pyridine (10 ml). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for an additional 4 h, diluted with water and extracted with EtOAc. The extract was washed with saturated NaCl, dried ($MgSO_4$) and concentrated to give **49** (0.29 g, 48.9%). Recrystallization from CH_2Cl_2–AcOEt gave pale yellow crystals (0.23 g, 38.8%), mp 179—181 °C. IR ($CHCl_3$): 3475, 3400, 1670. ^{1}H-NMR ($CDCl_3$) δ: 1.56—2.10 (4H, m), 2.33—3.33 (10H, m), 3.00 (6H, s), 4.47 (1H, t, J=6.0 Hz), 6.38 (1H, s), 6.50—7.33 (7H, m), 8.98 (1H, s). *Anal.* Calcd for $C_{23}H_{30}FN_5O_3$: C, 63.28; H, 6.64; N, 15.37. Found: C, 62.92; H, 6.46; N, 15.09.

6-Acetamido-2-[3-[4-(4-fluorophenyl)-1-piperazinyl]propyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (50) Acetic anhydride (0.18 ml) was added to a stirred solution of **48** (0.50 g, 1.30 mmol) in pyridine (5 ml). The reaction mixture was stirred at room temperature for 4 h and poured into ice-water. The resulting crystals were collected by filtration and dried to give **50** (0.46 g, 83.0%). Recrystallization from CH_2Cl_2–AcOEt afforded colorless prisms (0.40 g, 71.5%), mp 111—112 °C. IR (Nujol): 1675 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.49—1.89 (4H, m), 1.98 (3H, s), 2.25—2.63 (6H, m), 2.92—3.13 (4H, m), 4.39—4.58 (1H, m), 6.75—7.40 (8H, m), 9.77 (1H, br s). *Anal.* Calcd for $C_{23}H_{27}FN_4O_3 \cdot 1/2H_2O$: C, 63.43; H, 6.48; N, 12.86. Found: C, 63.57; H, 6.44; N, 12.74.

Synthesis of XII A typical example is given to illustrate the general procedure.

2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-6,7,8,9-tetrahydro-4-methyl-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one (68) Method C: A solution of **51** (0.42 g, 1.0 mmol) in DMF (6 ml) was added dropwise to a stirred and ice-cooled mixture of NaH (60% dispersion in oil, 60 mg, 1.5 mmol) and DMF (4 ml). After stirring for 10 min, methyl iodide (0.10 ml, 1.6 mmol) was added dropwise and the whole mixture was stirred

for an additional 30 min at 0 °C. The reaction mixture was diluted with water and extracted with Et_2O. The organic layer was washed with saturated NaCl, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (70 g) with AcOEt–hexane (3 : 2, v/v) as eluant to give **68** as an oil (0.41 g, 93.6%), which was crystallized from Et_2O to afford colorless crystals (0.28 g, 63.0%), mp 98—99 °C. IR (Nujol): 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.53—2.07 (8H, m), 2.41 (2H, t, J=6.0 Hz), 2.52—2.87 (8H, m), 2.93—3.17 (4H, m), 3.28 (3H, s), 4.50 (1H, t, J=6.0 Hz), 6.60 (1H, s), 6.68 (1H, s), 6.74—7.07 (4H, m). *Anal.* Calcd for $C_{26}H_{32}FN_3O_2$: C, 71.37; H, 7.37; N, 9.60. Found: C, 71.30; H, 7.33; N, 9.65.

Other compounds (XII) listed in Table II were prepared similarly.

(*R*)-(+)-Methyl 3-(3,4,6,7,8,9-Hexahydro-3-oxo-2*H*-naphtho[2,3-*b*]-[1,4]oxazin-2-yl)propionate [(*R*)-(+)-86] (i) A solution of $NaNO_2$ (3.80 g, 55.1 mmol) in water (30 ml) was added dropwise to a stirred and ice-cooled solution of L-glutamic acid γ-methyl ester (L-**83**, 8.06 g, 50.0 mmol) and 6 N HCl (28 ml) at such a rate that the reaction temperature did not exceed 0 °C. The resulting mixture was stirred at 0 °C for 1 h and then extracted with Et_2O. The extract was washed with saturated NaCl, dried ($MgSO_4$) and concentrated to yield the crude (*S*)-2-chloroglutaric acid γ-methyl ester [(*S*)-**84**, 2.69 g, 29.8%] as a colorless oil. This was used for the next reaction without further purification. IR ($CHCl_3$): 1725 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 2.07—2.70 (4H, m), 3.67 (3H, s), 4.46 (1H, dd, J= 5.4, 7.5 Hz), 8.90 (1H, br s).

(ii) A mixture of (*S*)-**84** (0.89 g, 4.9 mmol) and $SOCl_2$ (0.54 ml, 7.4 mmol) was refluxed with stirring for 1 h. The mixture was concentrated to yield the corresponding acid chloride which was used for the next reaction without purification.

(iii) A solution of the acid chloride obtained above in AcOEt (8 ml) was added to a stirred and ice-cooled mixture of **6** (0.80 g, 3.3 mmol), $NaHCO_3$ (0.50 g, 6.0 mmol), AcOEt (8 ml) and water (5 ml). After being stirred at 0 °C for 1 h, the mixture was taken up with AcOEt. The AcOEt layer was washed with saturated NaCl, dried ($MgSO_4$) and concentrated to give (*S*)-methyl 4-chloro-4-(4,5,6,7-tetrahydro-2-hydroxynaphthylcarbamoyl)-butyrate [(*S*)-**85**] as an oil.

(iv) A mixture of (*S*)-**85** obtained in (iii), K_2CO_3 (0.68 g, 4.9 mmol) and DMF (10 ml) was stirred at room temperature for 1 h. Water was added and the resulting precipitate was collected by filtration to give (*R*)-**86**. Recrystallization from CH_2Cl_2–AcOEt gave colorless crystals (0.31 g, 21.7% based on **6**), mp 137—138 °C. $[\alpha]_D^{26}$ +10.3° (c=0.5, $CHCl_3$). IR ($CHCl_3$): 3400, 1720, 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.60—1.93 (4H, m), 2.00—2.87 (8H, m), 3.69 (3H, s), 4.54 (1H, dd, J=5.4, 7.5 Hz), 6.48 (1H, s), 6.63 (1H, s), 8.99 (1H, br). *Anal.* Calcd for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.25; H, 6.67; N, 4.95.

***R*-(−)-6,7,8,9-Tetrahydro-2-(3-hydroxypropyl)-2*H*-naphtho[2,3-*b*]-[1,4]oxazin-3(4*H*)-one [(*R*)-(−)-87]** (*R*)-(+)-**86** (2.16 g, 74.7 mmol) in anhydrous THF (10 ml) was added dropwise to a stirred and ice-cooled suspension of $LiAlH_4$ (0.43 g, 11.3 mmol) in anhydrous THF (20 ml). The mixture was stirred at 0 °C for 1.5 h and neutralized with 6 N HCl. The insoluble material was removed by filtration and washed with AcOEt. The combined filtrate and the washings were partitioned between water and AcOEt. The organic layer was separated, washed with water, dried ($MgSO_4$) and concentrated to yield the crude alcohol (*R*)-**87** (1.84 g, 94.3%). Recrystallization from AcOEt gave colorless crystals, mp 141—141.5 °C $[\alpha]_D^{26}$ −3.1° (c=0.3, $CHCl_3$). IR ($CHCl_3$): 3410, 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.58—2.18 (8H, m), 2.44—2.81 (4H, m), 3.53—3.88 (2H, m), 4.55 (1H, t, J=5.7 Hz), 6.51 (1H, s), 6.67 (1H, s), 8.98 (1H, br s). *Anal.* Calcd for $C_{15}H_{19}NO_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 69.02; H, 7.35; N, 5.55.

***R*-(−)-6,7,8,9-Tetrahydro-2-(3-methanesulfonyloxypropyl)-2*H*-naphtho-[2,3-*b*][1,4]oxazin-3(4*H*)-one [(*R*)-(−)-88]** NEt_3 (0.85 ml, 6.1 mmol) and then methanesulfonyl chloride (0.62 ml, 8.0 mmol) were added dropwise to a solution of (*R*)-(−)-**87** (1.0 g, 3.8 mmol) in CH_2Cl_2 (40 ml) with ice-cooling. The mixture was stirred at 0 °C for 1.5 h and then water was added. The organic layer was washed with water, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (60 g) using hexane–AcOEt (1 : 2, v/v) as eluant and recrystallized from MeOH–AcOEt to yield (*R*)-(−)-**88** as colorless needles (1.01 g, 77.8%), mp 154—155 °C, $[\alpha]_D^{26}$ −9.3° (c=0.3, $CHCl_3$). IR ($CHCl_3$): 3390, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.55—2.25 (8H, m), 2.53—2.82 (4H, m), 2.98 (3H, s), 4.21—4.38 (2H, m), 4.42—4.63 (1H, m), 6.50 (1H, s), 6.64 (1H, s), 8.96 (1H, br s). *Anal.* Calcd for $C_{16}H_{21}NO_5S$: C, 56.62; H, 6.24; N, 4.13. Found: C, 56.70; H, 6.20; N, 4.24.

(*R*)-(+)-2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-6,7,8,9-tetrahydro-2*H*-naphtho[2,3-*b*][1,4]oxazine-3-(4*H*)-one [(*R*)-(+)-51] A mixture of (*R*)-(−)-**88** (0.44 g, 1.3 mmol), 1-(4-fluorophenyl)piperazine (0.35 g, 1.9 mmol), NEt_3 (0.18 ml, 1.3 mmol) and DMF (8 ml) was stirred at 70 °C for 2.5 h. The mixture was concentrated and the residue was subjected to column chromatography on silica gel (60 g). Elution with hexane–AcOEt (1 : 3, v/v) afforded (*R*)-(+)-**51** which was recrystallized twice from MeOH to give colorless needles (0.37 g, 66.5%), mp 153—154 °C, $[\alpha]_D^{26}$ +19.9° (c=0.7, $CHCl_3$). IR ($CHCl_3$): 3415, 1685, 1505 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.59—2.10 (8H, m), 2.32—2.82 (10H, m), 2.98—3.20 (4H, m), 4.54 (1H, t, J=6.0 Hz), 6.47 (1H, s), 6.65 (1H, s), 6.71—7.10 (4H, m), 8.83 (1H, br s). *Anal.* Calcd for $C_{25}H_{30}FN_3O_2$: C, 70.90; H, 7.14; N, 9.92. Found: C, 70.60; H, 7.14; N, 9.89.

The following compounds were prepared starting from D-glutamic acid γ-methyl ester (D-**83**) by the same procedure described for the synthesis of (*R*)-(+)-**51**.

(*S*)-(−)-Methyl 3-(3,4,6,7,8,9-Hexahydro-3-oxo-2*H*-naphtho[2,3-*b*]-[1,4]oxazin-2-yl)propionate [(*S*)-(−)-86] Starting from D-glutamic acid γ-methyl ester (D-**83**), (*S*)-(−)-**86** was obtained *via* (*R*)-**85** in 36.0% yield (based on **6**), mp 138—138.5 °C (AcOEt–Et_2O), $[\alpha]_D^{26}$ −7.8° (c=0.3, $CHCl_3$). IR ($CHCl_3$): 3395, 1720, 1680 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.57—1.93 (4H, m), 2.00—2.82 (8H, m), 3.67 (3H, s), 4.54 (1H, dd, J=5.4, 7.5 Hz), 6.50 (1H, s), 6.63 (1H, s), 9.23 (1H, br s). *Anal.* Calcd for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.01; H, 6.45; N, 4.94.

(*S*)-(+)-6,7,8,9-Tetrahydro-2-(3-hydroxypropyl)-2*H*-naphtho[2,3-*b*]-[1,4]oxazin-3(4*H*)-one [(*S*)-(+)-87] Yield 98.7%, mp 139—140 °C (AcOEt). $[\alpha]_D^{26}$ +1.4° (c=0.5, $CHCl_3$). *Anal.* Calcd for $C_{15}H_{19}NO_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.76; H, 7.37; N, 5.41.

(*S*)-(+)-6,7,8,9-Tetrahydro-2-(3-methanesulfonyloxypropyl)-2*H*-naphtho[2,3-*b*][1,4]oxazin-3(4*H*)-one [(*S*)-(+)-88] Yield 79.7%, mp 153—154 °C (MeOH). $[\alpha]_D^{26}$ +4.8° (c=0.8, $CHCl_3$). *Anal.* Calcd for $C_{16}H_{21}NO_5S$: C, 56.62; H, 6.24; N, 4.13. Found: C, 56.59; H, 6.20; N, 4.14.

(*S*)-(−)-2-[3-[4-(4-Fluorophenyl)-1-piperazinyl]propyl]-6,7,8,9-tetrahydro-2*H*-naphto[2,3-*b*][1,4]oxazin-3(4*H*)-one [(*S*)-(−)-51] Yield 59.6%, mp 156—156.5 °C (MeOH). $[\alpha]_D^{26}$ −19.9° (c=1.2, $CHCl_3$). *Anal.* Calcd for $C_{25}H_{30}FN_3O_2$: C, 70.90; H, 7.14; N, 9.92. Found: C, 70.80; H, 7.10; N, 9.88.

Pharmacological Methods Calcium channel blocking, antihypertensive and calmodulin antagonistic activities and inhibitory effects of caffeine-induced contraction of rabbit aorta were assayed using the methods described earlier.[1]

Antiarrhythmic Activity[11] Male Sprague-Dawley rats (9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and artificially ventilated with room air (stroke volume, 6 ml; 60 strokes/min). The electrocardiogram (Lead II) was recorded from subcutaneous steel needle electrodes. A left thoracotomy was performed, the heart was exteriorized and a 6/0 silk suture placed under the main left coronary artery. The heart was repositioned in the thoracic cavity and the ligature loosely tied around a fine piece of polyethylene tubing. A stabilization period of 15 min was allowed. Drugs or vehicle (distilled water, 10 ml/kg) were orally given 1 h before tightening the ligature. Five minutes later the ligature was released by sliding a scalpel blade over the polyethylene tubing. The incidence of VF, VT and electrical deaths (mortality) were noted.

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Synthesis and Antiulcer Activity of Optical Isomers of 2-(4-Chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic Acid (Rebamipide)[1)]

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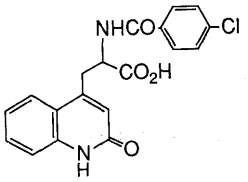
The enantiomers of 2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid [(±)-1, rebamipide, OPC-12759], a new antiulcer agent that enhances mucosal resistance, were synthesized from optically active α-amino acid derivatives of 2(1*H*)-quinolinone. The key intermediates, α-amino acid derivatives, were prepared by asymmetric synthesis and optical resolution. The (+)-1 was about 1.7 times as potent as the (−)-isomer in antiulcer activity against ethanol-induced gastric ulcers.

Keywords rebamipide; OPC-12759; asymmetric synthesis; optical resolution; α-amino acid derivative; 2(1*H*)-quinolinone; enantiomer; antiulcer agent; antiulcer activity

The amino acid derivative 2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid [(±)-**1**, rebamipide, OPC-12759][2)] (Chart 1) is a new antiulcer agent that enhances mucosal resistance. This compound has an asymmetric carbon at the 2-position of the amino acid moiety and therefore has two enantiomers. The pharmacological and pharmacokinetic properties of these isomers pose an interesting problem. We have already reported the synthesis of optically active rebamipide by optical resolution with (−)-brucine.[3)] In order to investigate the efficient synthesis and the pharmacological properties of both enantiomers, optically active rebamipide was synthesized from α-amino acid derivatives of 2(1*H*)-quinolinone obtained by "Shöllkopf's method."[4)] The key intermediates, optically active α-amino acid derivatives also were prepared by optical resolution using D-(−)-mandelic acid. We describe here the synthesis and antiulcer activity of the optical isomers of rebamipide.



1 (OPC-12759)

Chart 1

Synthesis The intermediates, α-amino acid derivatives of 2(1*H*)-quinolinone [(−)-**8** and (+)-**8**], were prepared as shown in Charts 2 and 3. Reaction of 4-bromomethyl-2(1*H*)-quinolinone[2)] with phosphoryl chloride gave 4-bromomethyl- and 4-chloromethyl-2-chloroquinolines (**3** and **4**). 2,5-Dihydro-3,6-dimethoxy-2(*S*)-isopropylpyrazine [(*S*)-**5**][4)] was treated with *n*-butyllithium in tetrahydrofuran (THF) at −78 °C to give a lithio derivative which was alkylated with halides (**3** and **4**) to afford the alkylation product [(−)-**6**] in good yields and acceptable diastereoselectivity. The high diastereoselectivity presumably arose from the preferential addition of an electrophile (**3** and **4**) at the C′-5 trans to the isopropyl group at the residential chiral center C′-2 of (*S*)-**5**. Hydrolysis of compound [(−)-**6**] (with 0.25 N hydrochloric acid) gave the methyl ester [(−)-**7**], which was hydrolyzed with 6 N hydrochloric

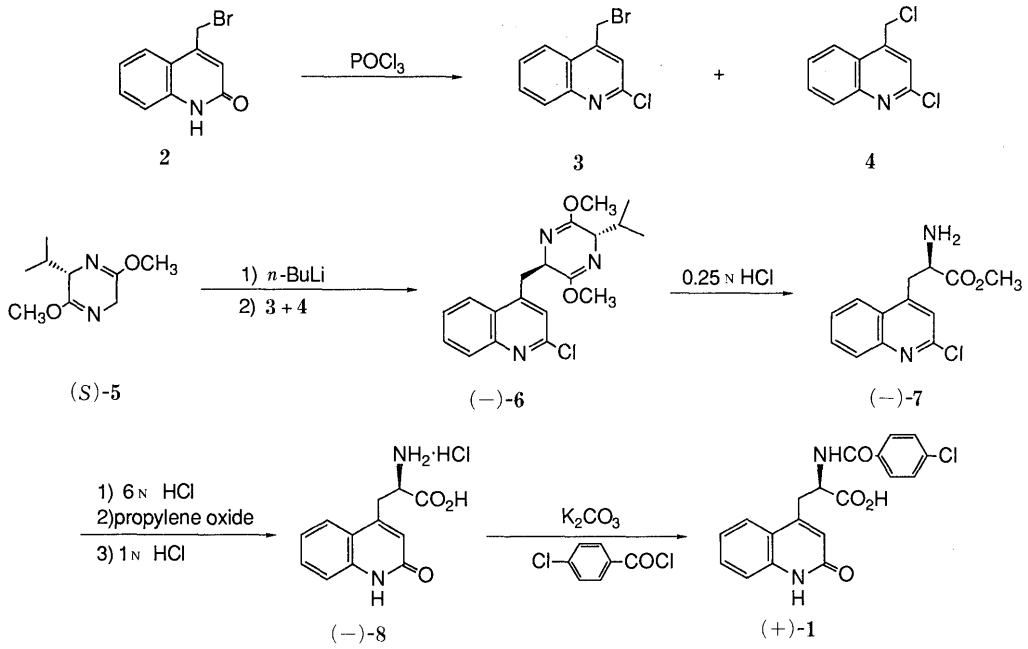


Chart 2

acid to give 2-amino-3-[2(1*H*)-quinolinon-4-yl]propionic acid [(−)-**8**]. The target compound [(+)-**1**] was prepared from (−)-**8** and *p*-chlorobenzoyl chloride using the Shotten–Baumann reaction.

The opposite isomer [(−)-**1**] was obtained similarly from 2,5-dihydro-3,6-dimethoxy-2(*R*)-isopropylpyrazine [(*R*)-**5**], *via* (+)-**6**, which was hydrolyzed with hydrochloric acid. The resulting amino acid [(+)-**8**] was acylated with *p*-chlorobenzoyl chloride to give the desired amide [(−)-**1**]. The optical purities of (+)-**1** and (−)-**1** were determined to be up to 99.5% ee by high-performance liquid chromatography (HPLC) using a chiral stationary phase column.

The key intermediates, (−)-**8** and (+)-**8**, also were synthesized as follows. Esterification of an amino acid derivative (**8**) with methanol–thionyl chloride gave the methyl ester (**9**), which was resoluted with D-(−)-mandelic acid to give **10a** and **10b**. After allowing a mixture of the methyl ester (**9**) and D-(−)-mandelic acid to stir in EtOH, a white crystalline solid was deposited. Recrystallization twice from EtOH gave the salt (**10a**). Another salt (**10b**) was recrystallized from MeOH. Hydrolysis of these isomers with hydrochloric acid gave optically active amino acid derivatives [(−)-**8** and (+)-**8**] (Chart 4). The optical purities of (−)-**8** and (+)-**8** appeared to be 99.5 and 98.4% ee, respectively, as determined by HPLC using a chiral stationary phase column.

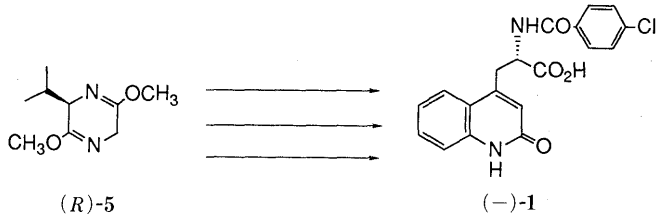
Consequently, we have been able to obtain optically active rebamipide by using three efficient methods. The first method was optical resolution of rebamipide with (−)-brucine. This method required recrystallization many times to obtain a pure enantiomer (98% ee). Next, asymmetric synthesis of the amino acids *via* metalated bis-lactim ethers of 2,5-diketopiperazines provided yields in an essentially optically pure form. However, this method, which uses expensive starting material, is not considered practical for accessing a relatively large amount of product. Finally, optical resolution of the amino acid derivatives was the most convenient method of obtaining the optical isomers of rebamipide.

Antiulcer Activity The antiulcer activities of (+)-, (−)- and (±)-**1** against acetic acid-induced gastric ulcers were reported in the previous paper.[3] Both enantiomers were again evaluated for antiulcer activity using the sensitive method. It was suggested that the mucosal protective effect[5] of rebamipide presumably results from enhancement of the generation of endogenous prostaglandins. Therefore, EtOH-induced gastric ulcers, which have reference to endogenous prostaglandins,[6] were investigated. (+)-, (−)- and (±)-**1** (10—100 mg/kg i.p.) dose-dependently inhibited the formation of gastric lesions induced by absolute EtOH. The ED_{50} values were 17.2, 32.7 and 26.4 mg/kg, respectively (Table I). From these results, it was speculated

Table I. Effect of (−)-, (+)- and (±)-OPC-12759 against Absolute Ethanol-Induced Gastric Necrosis

Drug	Dose mg/kg i.p.	*n*	% inhibition
(−)-OPC-12759	10	10	14.0
	30	10	41.9[a]
	100	10	88.6[a]
(+)-OPC-12759	10	10	19.5
	30	10	80.7[a]
	100	10	100.0[a]
(±)-OPC-12759	10	10	−2.8
	30	10	70.1[a]
	100	10	94.7[a]

a) $p<0.05$ *vs.* control.



(*R*)-5 (−)-1

Chart 3

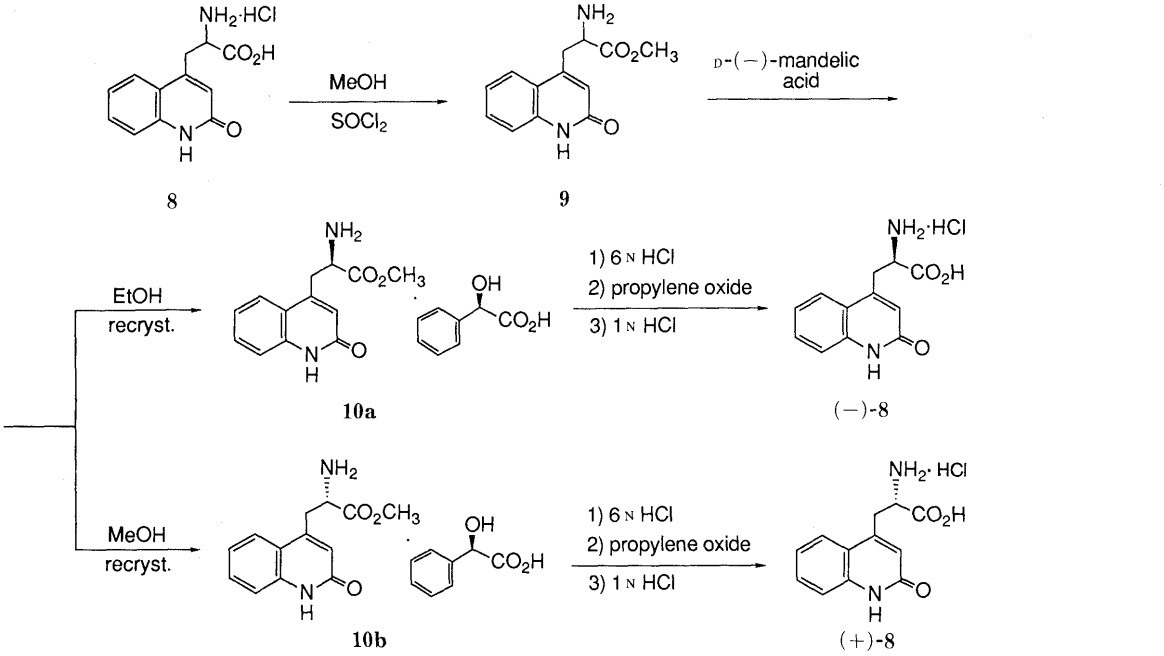


Chart 4

that (+)-**1** was about 1.7-fold were potent than (−)-**1** and about 1.4-fold more potent than (±)-**1**. Both enantiomers showed antiulcer activity against acetic acid-induced gastric ulcers and EtOH-induced gastric ulcers. The pair of enantiomers showed a small difference in activity. Therefore, rebamipide of the racemic mixture was developed.

Experimental

Melting points were determined with a Yamato MP-21 apparatus and are uncorrected. Infrared (IR) spectra were recorded on a JASCO IRA-2 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded in deuteriodimethyl sulfoxide-d_6 (DMSO-d_6) on a Bruker AC-200 spectrometer. Mass spectra (MS) were obtained on a Varian MAT-312 instrument. Optical rotations were measured on a DIP-360 digital polarimeter (Japan Spectroscopic Co., Ltd.).

Reaction of 4-Bromomethyl-2(1*H*)-quinolinone with Phosphoryl Chloride 4-Bromomethyl-2(1*H*)-quinolinone (**2**) (20.0 g, 84 mmol) was added to a stirred and ice-cooled phosphoryl chloride (40 ml). The reaction mixture was heated at 80—90 °C for 40 min with stirring, then allowed to cool. The mixture was poured into ice-H_2O. The precipitates were collected by filtration and dissolved in CH_2Cl_2. The extract was washed with H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was recrystallized from AcOEt–hexane to give 4-bromomethyl- and 4-chloromethyl-2-chloroquinolines [**3** and **4**, **3**:**4** = 1:4 (from NMR), 8.85 g, 48%] as pale yellow needles. mp 83—85 °C. NMR ($CDCl_3$) δ: 4.79 (0.4H, s, bromomethyl), 4.95 (1.6H, s, chloromethyl), 7.46 (1H, d, J=10 Hz), 7.61—7.70 (1H, m), 7.77 (1H, dt, J=1.4, 6.1 Hz), 8.05 (2H, dt, J=1.6, 8.8 Hz). IR (KBr): 3070, 1590, 1510, 1420, 1300, 1150, 1100, 910, 760 cm^{-1}. MS *m/z* (%): 257 (23), 255 [M$^+$ (bromomethyl), 18], 229 (13), 227 (35), 213 (22), 212 (23), 211 [M$^+$ (chloromethyl), 32], 178 (21), 176 (100).

(−)-2-Chloro-4-[2′,5′-dimethoxy-6′-isopropyl-3′,6′-dihydropyrazin-3′-yl)methyl]quinoline [(−)-6] To a stirred solution of 2,5-dihydro-3,6-dimethoxy-2(*S*)-isopropylpyrazine [(*S*)-**5**] (1.3 g, 7.07 mmol) in THF (15 ml) at −78 °C, a 1.6 N solution (4.42 ml, 7.07 mmol) of *n*-butyllithium in hexane was added by syringe and the reaction mixture was stirred for 10 min at the same temperature. Then, a solution of **3** and **4** (1.5 g, 7.07 mmol) in THF (15 ml) was added and the mixture was stirred for 7 h at −78 °C. After removal of the solvent, the residue was poured into H_2O and extracted with Et_2O. The extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluent; CH_2Cl_2:AcOEt = 100:1) to give (−)-**6** (2.06 g, 81%) as colorless oil. $[\alpha]_D^{20} = -31.2°$ (c=0.2, 99.7% MeOH). NMR ($CDCl_3$) δ: 0.62 (3H, d, J=6.8 Hz), 0.97 (3H, d, J=6.8 Hz), 2.09—2.24 (1H, m), 3.21 (1H, dd, J=7.6, 13.4 Hz), 3.53 (3H, s), 3.66—3.78 (1H, m), 3.74 (3H, s), 4.35—4.42 (1H, m), 7.28 (1H, s), 7.50—7.58 (1H, m), 7.65—7.73 (1H, m), 7.99 (1H, d, J=8.5 Hz), 8.15 (1H, d, J=8.5 Hz). IR (neat): 2950, 1700, 1590, 1440, 1300, 1240, 760 cm^{-1}. *Anal.* Calcd for $C_{19}H_{22}ClN_3O_2 \cdot 1/2H_2O$: C, 61.87; H, 6.28; N, 11.39. Found: C, 61.68; H, 6.12; N, 11.16. MS *m/z* (%): 360 (2), 359 (M$^+$, 1), 316 (2), 183 (27), 177 (28), 141 (100).

(+)-2-Chloro-4-[(2′,5′-dimethoxy-6′-isopropyl-3′,6′-dihydropyrazin-3′-yl)methyl]quinoline [(+)-6] Compound [(+)-**6**] (2.17 g, 85%) was prepared by a procedure similar to that used for (−)-**6** with 2,5-dihydro-3,6-dimethoxy-2(*R*)-isopropylpyrazine [(*R*)-**5**] (1.3 g, 7.07 mmol), *n*-butyllithium in hexane (4.42 ml, 7.07 mmol) and **3** and **4** (1.5 g, 7.07 mmol). $[\alpha]_D^{20} = +30.4°$ (c=0.8, 99.7% MeOH). *Anal.* Calcd for $C_{19}H_{22}ClN_3O_2$: C, 63.42; H, 6.16; N, 11.68. Found: C, 63.08; H, 6.11; N, 11.49.

Methyl (−)-2-Amino-3-(2-chloroquinolin-4-yl)propionate [(−)-7] A mixture of (−)-**6** (2.2 g, 6.1 mmol) and 0.25 N HCl (48.4 ml) was stirred at room temperature for 18 h. The reaction mixture was evaporated *in vacuo*. The residue was dissolved in H_2O, adjusted to pH 9 with a 25% ammonia solution and extracted with Et_2O. The extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, eluent; CH_2Cl_2:MeOH = 100:1) and recrystallized from Et_2O to give (−)-**7** (1.3 g, 81%) as pale yellow needles, mp 57—59 °C. $[\alpha]_D^{20} = -31.3°$ (c=0.2, 99.7% MeOH). NMR ($CDCl_3$) δ: 2.11 (2H, br s), 3.20 (1H, dd, J=8.6, 14.1 Hz), 3.61 (1H, dd, J=5.2, 13.8 Hz), 3.70 (3H, s), 3.94 (1H, m), 7.33 (1H, s), 7.56—7.64 (1H, m), 7.70—7.77 (1H, m), 8.05 (2H, d, J=8.0 Hz). IR (KBr): 3360, 2950, 1730, 1590, 1280, 1150, 760 cm^{-1}. *Anal.* Calcd for $C_{13}H_{13}ClN_2O_2$: C, 58.99; H, 4.95; N, 10.58. Found: C, 58.64; H, 4.97; N, 10.49.

Methyl (+)-2-Amino-3-(2-chloroquinolin-4-yl)propionate [(+)-7] Compound [(+)-**7**] (0.2 g, 20%) was prepared by a procedure similar to that used for (−)-**7** with (+)-**6** (1.99 g, 5.5 mmol) and 0.25 N HCl (43.8 ml) as white prisms from Et_2O–hexane), mp 52—54 °C. $[\alpha]_D^{20} = +31.3°$ (c=0.2, 99.7% MeOH). *Anal.* Calcd for $C_{13}H_{13}ClN_2O_2$: C, 58.99; H, 4.95; N, 10.58. Found: C, 58.55; H, 4.89; N, 10.48.

Determination of the Optical Purities of (−)- and (+)-7 Compounds [(−)- and (+)-**7**] were subjected to HPLC (column, Chiralcel OJ, 4.6 mm i.d. × 25 cm; solvent, *n*-hexane : iso-PrOH : diethylamine = 900 : 100 : 1; detection, UV 240 nm). The optical purities were determined to be as follows: (−)-**7**, 75.6% ee; (+)-**7**, 91.6% ee.[7]

(−)-2-Amino-3-[2(1*H*)-quinolinon-4-yl]propionic Acid Hydrochloride [(−)-8] A suspension of (−)-**7** (0.85 g, 3.21 mmol) in 6 N HCl (32 ml) was refluxed for 6 h. After removal of the solvent, the residue was refluxed in propylene oxide (4 ml) and EtOH (10 ml) for 15 min. The precipitated product was isolated by suction and dissolved in 1 N HCl (5 ml). After removal of H_2O, the residue was recrystallized from EtOH–H_2O–Et_2O to give (−)-**8** (0.25 g, 29%) as white granules, mp 242—244 °C. $[\alpha]_D^{20} = -20.2°$ (c=0.1, DMSO). NMR δ: 3.00—3.70 (2H, m), 4.13 (1H, t, J=3.2 Hz), 6.47 (1H, s), 7.22 (1H, t, J=5.0 Hz), 7.34 (1H, d, J=6.4 Hz), 7.52 (1H, t, J=6.0 Hz), 7.76 (1H, d, J=6.0 Hz, 8.00—8.80 (2H, br s), 11.70 (1H, s). IR (KBr): 3450, 1665, 1520, 1410, 1270, 760 cm^{-1}. *Anal.* Calcd for $C_{12}H_{12}N_2O_3 \cdot HCl$: C, 53.64; H, 4.88; N, 10.43. Found: C, 53.48; H, 4.79; N, 10.28.

(+)-2-Amino-3-[2(1*H*)-quinolinon-4-yl]propionic Acid Hydrochloride [(+)-8] Compound [(+)-**8**] (0.17 g, 55%) was prepared by a procedure similar to that used for (−)-**8** with (+)-**7** (0.3 g, 1.1 mmol) and 6 N HCl (12 ml) as white granules, mp 244—246 °C. $[\alpha]_D^{20} = +21.5°$ (c=0.1, DMSO). *Anal.* Calcd for $C_{12}H_{12}N_2O_3 \cdot HCl$: C, 53.64; H, 4.88; N, 10.43. Found: C, 53.53; H, 4.81; N, 10.40.

Determination of the Optical Purities of (−)- and (+)-8 Compounds [(−)- and (+)-**8**] were subjected to HPLC (column, YMC-A3120DS, 4.6 mm i.d. × 25 cm; solvent, 20% MeOH containing [$Cu(CH_3CO_2)_2$: L-phenylalanine = 1 : 2]; detection, UV 295 nm). The optical purities were determined to be as follows: (−)-**8**, 98.2% ee; (+)-**8**, 98.4% ee.

(+)-2-(4-Chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic Acid [(+)-1] A solution of *p*-chlorobenzoyl chloride (82.2 mg, 0.47 mmol) in acetone (25 ml), was added dropwise to a stirred and ice-cooled solution of (−)-**8** (122 mg, 0.45 mmol) and K_2CO_3 (124 mg, 0.9 mmol) in H_2O (2.5 ml) and the reaction mixture was stirred for 2 h. The mixture was acidified with dil. HCl. The resulting precipitates were collected by filtration. Recrystallization from dimethylformamide (DMF)–H_2O gave (+)-**1** (48.1 mg, 29%) as white granules, mp 300—302 °C (dec.). $[\alpha]_D^{20} = +106.2°$ (c=1.0, DMF). NMR δ: 3.15—3.50 (2H, m), 4.60—4.80 (1H, m), 6.43 (1H, s), 7.22 (1H, t, J=7.0 Hz), 7.29 (1H, d, J=3.8 Hz), 7.46—7.55 (1H, m), 7.55 (2H, d, J=8.5 Hz), 7.82 (2H, d, J=8.5 Hz), 8.89 (1H, d, J=8.0 Hz), 11.63 (1H, s), 13.02 (1H, br s). IR (KBr): 3540, 3310, 1670, 1640 cm^{-1}. *Anal.* Calcd for $C_{19}H_{15}ClN_2O_4 \cdot H_2O$: C, 58.69; H, 4.41; N, 7.20. Found: C, 58.58; H, 4.37; N, 7.22.

(−)-2-(4-Chlorobenzoylamino)-[2(1*H*)-quinolinon-4-yl]propionic Acid [(−)-1] Compound [(−)-**1**] (112.5 mg, 55%) was prepared by a procedure similar to that used for (+)-**1** with (+)-**8** (148 mg, 0.55 mmol), *p*-chlorobenzoyl chloride (106 mg, 0.61 mmol) and K_2CO_3 (228 mg, 1.65 mmol) as white granules, mp 301—303 °C. $[\alpha]_D^{20} = -108.0°$ (c=1.0, DMF). *Anal.* Calcd for $C_{19}H_{15}ClN_2O_4 \cdot 1/3H_2O$: C, 60.66; H, 4.18; N, 7.45. Found: C, 60.49; H, 4.39; N, 7.39.

Determination of the Optical Purities (+)- and (−)-1 Compounds (+)- and (−)-**1** were subjected to HPLC [column, Sumipax OA-4000 4.6 mm i.d. × 25 cm (Sumitomo Chemical Co., Ltd.); solvent, acetonitrile : phosphate buffer (pH 5) = 3 : 2; detection, UV 254 nm]. The optical purities were determined to be as follows: (+)-**1**, 99.8% ee; (−)-**1**, 99.6% ee.

Methyl 2-Amino-3-[(1*H*)-quinolinon-4-yl]propionate (9) Thionyl chloride (8.8 g, 74 mmol) was added dropwise to a stirred and ice-cooled suspension of **8** (10 g, 37 mmol)[2] in MeOH (100 ml) and the reaction mixture was refluxed for 3 h. The mixture was evaporated to dryness *in vacuo*. The residue was poured into a $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was recrystallized from MeOH–AcOEt to give **9** (8.5 g, 93%) as white powder, mp 185—186 °C. NMR ($CDCl_3$) δ: 1.59 (2H, br s), 2.97 (1H, dd, J=8.5, 14 Hz), 3.45 (1H, dd, J=5, 14 Hz), 3.89 (1H, dd, J=5, 8.5 Hz), 6.66 (1H, s), 7.20—7.80 (4H, m), 12.75 (1H, br s). IR (KBr): 2950, 2850, 1740, 1670, 1620, 1560, 1440, 1290, 1200, 1180, 760 cm^{-1}. *Anal.* Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.23; H, 5.69; N, 11.36.

(−)-2-Amino-3-[2(1*H*)-quinolinon-4-yl]propionic Acid Hydrochloride [(−)-8] D-(−)-Mandelic acid (3.0 g, 20 mmol) was added to a suspension of **9** (4.9 g, 20 mmol) in EtOH (50 ml) and the reaction mixture was stirred for 1 h at room temperature. The precipitated crystals (7.6 g, 96%) were separated by filtration. Two recrystallizations from EtOH gave the pure salt [**10a**, 1.8 g (23%)], mp 181—181.5 °C. $[\alpha]_D^{20} = -118°$ ($c = 0.2$, DMF). NMR δ: 3.00 (1H, dd, $J = 8.0$, 13.9 Hz), 3.19 (1H, dd, $J = 6.2$, 13.8 Hz), 3.59 (3H, s), 3.79 (1H, dd, $J = 6.2$, 7.9 Hz), 4.90 (1H, s), 5.63 (1H, br s), 6.39 (1H, s), 7.16—7.54 (8H, m), 7.74 (1H, d, $J = 7.2$ Hz), 11.70 (1H, br s). IR (KBr): 3400, 2920, 2880, 1740, 1670, 1640, 1610, 1570, 1510, 1440, 1430, 1270, 1080, 760, 700 cm^{-1}. *Anal.* Calcd for $C_{21}H_{22}N_2O_6$: C, 63.31; H, 5.57; N, 7.03. Found: C, 63.24; H, 5.49; N, 7.03. Then a solution of the salt (1.8 g) in 6 N HCl (20 ml) was refluxed for 5 h. The reaction mixture was concentrated *in vacuo*. The residue was refluxed in propylene oxide (20 ml) and EtOH (50 ml) for 30 min. The precipitated product was isolated by suction and dissolved in 1 N HCl. After removal of H_2O, the residue was recrystallized from H_2O–EtOH–Et_2O to give (−)-**8** (0.6 g, 11%). *Anal.* Calcd for $C_{12}H_{12}N_2O_3 \cdot HCl$: C, 53.64; H, 4.88; N, 10.43. Found: C, 53.37; H, 4.86; N, 10.34. The product was identical with a previous synthetic sample on the basis of NMR, IR, specific rotation and HPLC comparisons.

(+)-2-Amino-3-[2(1*H*)-quinolinon-4-yl]propionic Acid Hydrochloride [(+)-8] D-(−)-Mandelic acid (3.0 g, 20 mmol) was added to a suspension of **9** (4.9 g, 20 mmol) in EtOH (50 ml) and the reaction mixture was stirred for 1 h at room temperature. The precipitated crystals (7.5 g, 95%) were separated by filtration. Two recrystallizations from MeOH gave the pure salt [**10b**, 1.8 g (23%)], mp 158.5—159.5 °C. $[\alpha]_D^{20} = -88.4°$ ($c = 0.2$, DMF). NMR δ: 2.98 (1H, dd, $J = 7.9$, 13.9 Hz), 3.18 (1H, dd, $J = 6.2$, 13.9 Hz), 3.59 (3H, s), 3.76 (1H, dd, $J = 6.2$, 7.9 Hz), 4.92 (1H, s), 4.50—5.70 (1H, br s), 6.39 (1H, s), 7.17—7.54 (8H, m), 7.74 (1H, d, $J = 7.1$ Hz), 11.67 (1H, br s). IR (KBr): 3400, 3150, 3050, 1760, 1670, 1650, 1610, 1550, 1440, 1400, 1280, 1060, 760, 700 cm^{-1}. *Anal.* Calcd for $C_{21}H_{22}N_2O_6$: C, 63.31; H, 5.57; N, 7.03. Found: C, 63.12; H, 5.57; N, 7.02. Then compound [(+)-**8**] (0.8 g, 15%) was prepared by a procedure similar to that used for (−)-**8**. *Anal.* Calcd for $C_{12}H_{12}N_2O_3 \cdot HCl$: C, 53.63; H, 4.88; N, 10.43. Found: C, 53.73; H, 4.86; N, 10.40. The product was identical with a sample prepared by the previous method.

Antiulcer Activity Method: Male Wistar rats weighing between 200 and 250 g were fasted for 24 h, but were allowed water *ad libitum* prior to the study. Test compounds and the vehicle were given intraperitoneally 30 min before the oral administration of 1 ml of absolute EtOH. The animals were killed 1 h after the irritant was given, and the stomachs were removed. After light fixation with formalin, the surface of the gastric mucosa was graded planimetrically. The total surface area damage in each animal was calculated and used as the lesion index. Percentage inhibition was calculated as follows: [(lesion index of control − lesion index test compound)/lesion index of control] × 100. The doses inhibiting absolute EtOH-induced lesions by 50% (ED_{50}) were calculated by linear regression analysis.

References and Notes

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Synthesis, Resolution, and Renal Vasodilation Activity of Novel DA_1 Agonists: 4-(3,4-Dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline Derivatives

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7,8-Dihydroxy-4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline (1) and 4-(3,4-dihydroxyphenyl)-7-hydroxy-8-methyl-1,2,3,4-tetrahydroisoquinoline (2) are potent renal vasodilators which selectively stimulate DA_1 (peripheral dopamine receptor-1) receptors. Especially, (*S*)-(−)-1 is the most potent. Its DA_1 agonist activity is about 10 times stronger than dopamine for increasing renal blood flow in anesthetized dogs. The renal and cardiovascular effects of (*S*)-(−)-1 may be suitable for the treatment of patients with renal insufficiency, heart failure and hypertension.

Keywords DA_1; renal vasodilation; dopamine; optical resolution; 4-phenyltetrahydroisoquinoline

Recently, Massingham *et al.*,[1] through a number of dopamine agonist/antagonist studies, proposed that there exist at least two distinct subtypes of peripheral dopamine receptors. DA_1-dopamine receptors exist postjunctionally in renal and mesenteric arterial beds where their activation leads to direct smooth muscle relaxation. This suggested that this type of activity is useful for renal insufficiency, cardiac failure or hypertension. Thus, we have sought potent and selective DA_1 (peripheral dopamine receptor-1) agonists as therapeutic agents.

Many compounds, *e.g.* benzazepines,[2] octahydrobenzoquinolines,[3] aminotetralines,[4] phenylpiperidines,[5] and tetrahydroisoquinolines,[6] have been synthesized and their DA_1 agonist (or antagonist) activity investigated. On the basis of these studies, we found that (±)-7,8-dihydroxy-4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline (**1**) and (±)-4-(3,4-dihydroxyphenyl)-7-hydroxy-8-methyl-1,2,3,4-tetrahydroisoquinoline (**2**) were potent DA_1 agonists.[7] Therefore we have been interested in the synthesis of the optically active compounds **1** and **2** for evaluating their DA_1 agonist activity.

Compounds **1** and **2** were synthesized by a method shown in Chart 1. Hydroxyamine (**4**), which was derived from veratraldehyde (**3**) *via* cyanohydrin, was reductively condensed with 2,3-dimethoxybenzaldehyde (**5**) to give a secondary amino intermediate (**7**). Compound **7** was cyclized under acid-catalyzed conditions[8] and, following deprotection under the condition of being refluxed in 48% hydrobromic acid or reacted with boron tribromide in dichloromethane at room temperature, gave **1**. Compound **2** was obtained from 3-methoxy-2-methylbenzaldehyde (**6**). The amido intermediate, which was obtained by condensa-

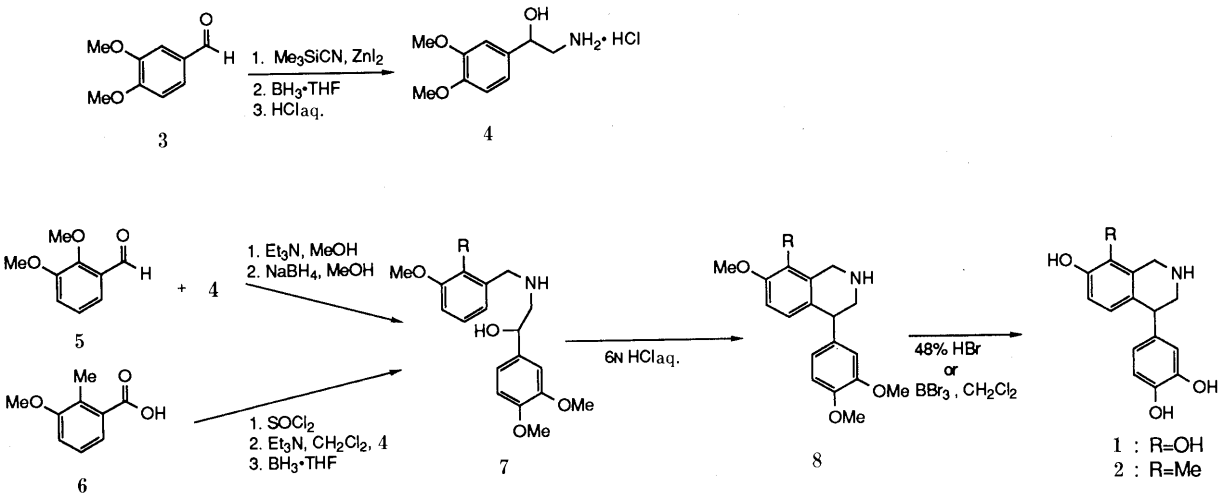


Chart 1

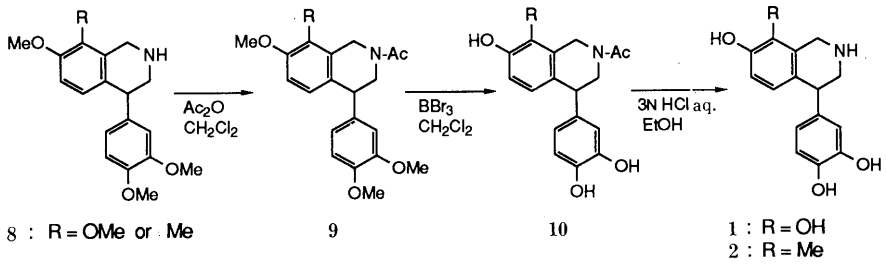
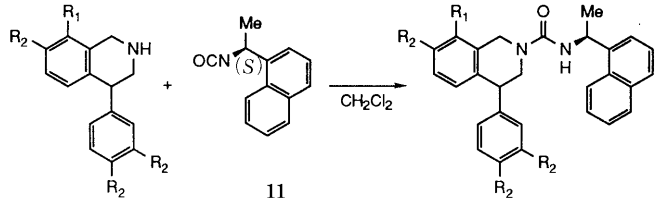


Chart 2



8 : R_1=OMe or Me, R_2=OMe
1 : R_1=R_2=OH
2 : R_1=Me, R_2=OH

Chart 3

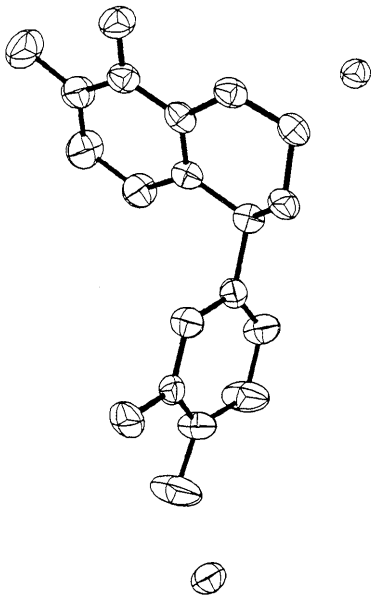


Fig. 1. X-Ray Structure of (−)-**1**·HCl·H_2O

tion of **4** and **6**, was reduced, cyclized, and deprotected to give **2**.

In order to obtain optically active **1** and **2**, a tri- or tetramethoxy precursor (**8**) was resolved, because it was thought that the catecholyl group might be unstable under basic conditions. Compound **8** was recrystallized as diastereomeric hydrogen dibenzoyltartrate,[9] and the salt was treated with aqueous sodium hydroxide to give the free base **8** as crystals. To convert optically active **8** to **1** or **2** with no racemization, and to give **1** or **2** as a hydrochloric acid salt, another route shown in Chart 2 was chosen. After protection of the secondary amine of **8** with an acetyl group, demethylation with boron tribromide and deacetylation with 3 N hydrochloric acid–ethanol produced **1** or **2**. For determination of the optical purities of **1**, **2**, and **8**, each was reacted with (*S*)-1-(1-naphthyl)ethyl isocyanate[10] to form diastereomeric urea derivatives (Chart 3).[11] High performance liquid chromatography (HPLC)[12] determined that **1**, **2**, and **8** were optically pure (at least 99.5% ee, respectively).

The absolute configurations of **1** and **2** were determined by single-crystal X-ray diffraction studies. The structure of (−)-**1** and (−)-**2** are illustrated in Figs. 1 and 2, respectively. As shown in Figs. 1 and 2, the absolute configuration at position 4 of (−)-**1** is *S*, while it is *R* for (−)-**2**. DA_1 agonist activity was evaluated as renal vasodilation which resulted in increased renal blood flow in pentobarbital-anesthetized

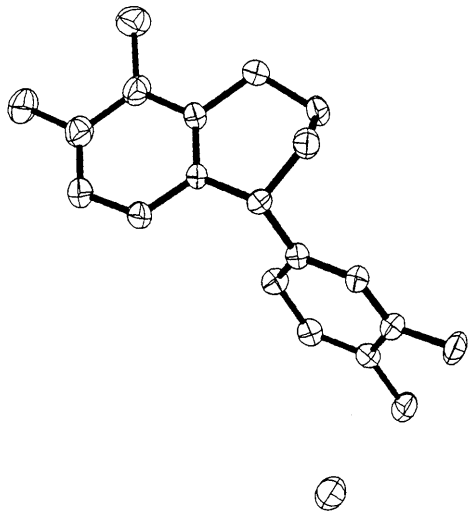


Fig. 2. X-Ray Structure of (−)-**2**·HCl

TABLE I. ED_{20}s of Optically Active 4-(3,4-Dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline Derivatives

	(±)-**1**	(+)-**1**	(−)-**1**	(±)-**2**	(+)-**2**	(−)-**2**	Dopamine
Absolute configuration		*R*	*S*		*S*	*R*	
ED_{20}[a] (μg) i.a.	3.0	—[b]	2.0	5.0	2.2	—[b]	18.9

a) Dose for 20% increase in renal blood flow. *b*) No vasodilation activity.

dogs.[4a] Renal blood flow was measured by an electromagnetic flowmeter. The test compounds were injected into the renal artery. The doses (ED_{20}) of the test compounds that caused a 20% increase in renal blood flow were calculated and compared. Furthermore, it was confirmed that the renal vasodilatory effects of the test compounds were antagonized by a selective DA_1 antagonist, SCH23390.[13]

The biological activities (ED_{20} values) are shown in Table I. Under the same conditions, the ED_{20} value of dopamine is 18.9 μg. Comparing optically active **1** or **2**, it is found that only one enantiomer is active and the other one is inactive. From single-crystal X-ray diffraction studies it has been proved that (+)-**1** and (−)-**2** have *R* absolute configurations at position 4 and (−)-**1** and (+)-**2** have *S*. Therefore, only the *S* enantiomers of both **1** and **2** show DA_1 agonist activity. Carl Kaiser *et al.*[6a] proved that (*S*)-3′,4′-dihydroxynomifensine, which also has a 4-phenyl-1,2,3,4-tetrahydroisoquinoline structure, is nearly 20 times more active than the *R* enantiomer.

Compound (−)-**1**, which has the strongest DA_1 agonist activity in this series, was investigated and the results are as follows[14]: receptor binding assays revealed that (−)-**1** had no affinity for D-2, α_1 and α_2 receptors. In open-chest anesthetized dogs, intravenous infusion of a (−)-**1** (0.1—3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$) dose dependently increased renal blood flow (5—24% from baseline) and cardiac output (0—25%) and decreased mean blood pressure (1—14%), renal (6—30%) and total (1—29%) peripheral resistance with little effect on heart rate or max. *dp/dt*. In anesthetized dogs, intravenous infusion of (−)-**1** (0.1—3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$) increased the glomerular filtration rate (9—63% over baseline), urine flow rate (19—42%) and urinary sodium excretion (56—444%).

Experimental

Melting points were determined with a Yanaco MP-3 apparatus and were not corrected. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL FX90Q or FX100 spectrometer using Me_4Si as an internal standard. The following abbreviations are used; s, singlet, d, doublet, t, triplet, m, multiplet, br, broadened. Mass spectra (MS) were determined with a Hitachi M-80 or JEOL JMS-DX300 spectrometer. Elemental analyses are reported by symbols of the elements and the results were within ±0.3% of the calculated values. Optical rotations were determined with a JASCO DIP-370 polarimeter. HPLC was carried out using a Hitachi L-6000 pump, L-4000 UV detector and D-2500 recorder. Silica gel F_{254} (Merck) thin-layer chromatography (TLC) plates were used. For column chromatography, Kieselgel 60 (Merck) was used. All concentrations by evaporation were carried out *in vacuo*. Single-crystal X-ray analyses for (−)-**1**·HCl·H_2O and (−)-**2**·HCl were carried out on a Rigaku AFC-5R diffractometer.

(±)-[[(2,3-Dimethoxybenzyl)-*N*-amino]methyl]-3,4-dimethoxybenzyl Alcohol: (±)-7 (R=OMe) A mixture of 10.0 g of 1-(3,4-dimethoxyphenyl)-2-aminoethanol hydrochloride (**4**),[15] 7.47 g of 2,3-dimethoxybenzaldehyde (**5**), 6.0 ml of triethylamine and 50 ml of MeOH was stirred at room temperature for 1 h, and 1.62 g of sodium borohydride was added portionwise at room temperature. After the reaction was completed, the mixture was concentrated. The residue was dissolved in toluene and H_2O. The organic layer was washed with H_2O and concentrated. The residual solid was recrystallized from toluene–hexane to give 11.1 g of (±)-**7** (R=OMe). mp 96—97 °C. NMR ($CDCl_3$) δ: 2.54 (3H, m), 3.85 (15H, m), 4.66 (1H, dd), 6.70—7.20 (6H, m). Fast atom bombardment mass spectrum (FAB-MS) *m/z*: 348 (M^++H). *Anal.* Calcd for $C_{19}H_{25}NO_5$: C, 65.69; H, 7.25; N, 4.03. Found: C, 65.71; H, 7.27; N, 3.97.

(±)-7,8-Dimethoxy-4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline: (±)-8 (R=OMe) A mixture of 10.5 g of (±)-**7** (R=OMe) and 105 ml of 6 N HCl was stirred at 60 °C for 2 h. After cooling the mixture, the precipitant was extracted with dichloromethane. The combined extracts were washed with 1 N sodium hydroxide and H_2O and concentrated. The residual solid was recrystallized from ethyl acetate–hexane to give 8.14 g of (±)-**8** (R=OMe). mp 109—110 °C. NMR ($CDCl_3$) δ: 1.72 (1H, s), 2.92—3.41 (2H, m), 3.84 (12H, m), 3.98 (1H, t), 4.14 (2H, s), 6.50—6.84 (5H, m). FAB-MS *m/z*: 330 (M^++H). *Anal.* Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.10; H, 7.08; N, 4.29.

(±)-7,8-Dihydroxy-4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline Hydrobromide: (±)-1·HBr A mixture of 10.3 g of (±)-**8** (R=OMe) and 48.1 ml of 48% hydrobromic acid was heated in a 160 °C bath for 2 h. After cooling to 4 °C, 9.19 g of (±)-**1**·HBr was collected as crystals, mp 230 °C (dec.). NMR (DMSO-d_6) δ: 3.24 (1H, m), 3.52 (1H, m), 6.08 (1H, d), 6.48 (1H, dd), 6.58 (1H, s), 6.68 (1H, d). FAB-MS *m/z*: 274 (M^++H). *Anal.* Calcd for $C_{15}H_{16}BrNO_4$: C, 50.87; H, 4.55; Br, 22.56; N, 3.95. Found: C, 51.02; H, 4.33; Br, 22.82; N, 3.96.

(±)-4-(3,4-Dihydroxyphenyl)-7-hydroxy-8-methyl-1,2,3,4-tetrahydroisoquinoline Hydrobromide: (±)-2·HBr A mixture of 50 g of 3-methoxy-2-methylbenzoic acid (**6**) and 66 ml of thionyl chloride was heated under reflux for 40 min. After cooling, the solution was concentrated and azeotroped with toluene. The residue was dissolved in 253 ml of dichloromethane and added dropwise to a solution of 63.2 g of **4** and 83 ml of triethylamine in 316 ml of dichloromethane under ice-bath cooling. After stirring at 3 °C for 1 h, 1 N HCl was added to the reaction mixture. The organic layer was washed with 1 N sodium hydrogen carbonate and brine, dried ($MgSO_4$) and concentrated. The residual solid was recrystallized from ethyl acetate–hexane to give 76.3 g of crystals, mp 105—109 °C.

To a solution of 74.8 g of the crystals in 763 ml of tetrahydrofuran, 796 mg of 1 M borane in tetrahydrofuran was added dropwise at 10 °C and the solution was heated under reflux for 1.5 h. To the reaction mixture was added 96 ml of MeOH at 4 °C and the mixture was refluxed for 30 min, then 66.3 ml of 12 N HCl was added at 4 °C. The precipitants were collected, 760 ml of 1 N sodium hydrogen carbonate added, and the mixture was extracted with $CHCl_3$. The organic layer was dried ($MgSO_4$) and concentrated. The residual solid was recrystallized from $CHCl_3$–hexane to give 43.0 g of (±)-**7** (R=Me), mp 135—136 °C. FAB-MS *m/z*: 332 (M^++H).

The following experimental procedure was the same as for (±)-**1**·HBr. (±)-**2**·HBr, mp 250 °C (dec.). NMR (DMSO-d_6) δ: 2.04 (3H, s), 3.18 (2H, br), 3.48 (2H, br), 4.12 (1H, t), 4.24 (2H, br), 6.52 (1H, s), 6.60 (4H, dd), 9.30 (3H, br). FAB-MS *m/z*: 272 (M^++H). *Anal.* Calcd for $C_{16}H_{18}BrNO_3$: C, 54.56; H, 5.15; Br, 22.69; N, 3.98. Found: C, 54.46; H, 5.17; Br, 22.65; N, 3.91.

Resolution of (±)-7,8-Dimethoxy-4-(3,4-dimethoxyphenyl)-1,2,3,4-tetrahydroisoquinoline: (+)- and (−)-8 (R=OMe) To a mixture of 100 g of (±)-**8** (R=OMe) and 300 ml of ethanol was added dropwise a solution of 114 g of (−)-dibenzoyl-L-tartaric acid monohydrate in 400 ml of ethanol. After the mixture was stirred at room temperature for 1 h, 139 g of crystals were collected. Recrystallization from ethanol–H_2O (3:1, v/v) gave 79.4 g of **8** (R=OMe) hydrogen dibenzoyl-L-tartrate, mp 181 °C (dec.). $[\alpha]_D^{20}$ −23° (*c*=1, DMF). *Anal.* Calcd for $C_{19}H_{23}NO_4 \cdot C_{18}H_{14}O_8$: C, 64.62; H, 5.42; N, 2.04. Found: C, 64.43; H, 5.48; N, 2.05. 78.9 g of the crystals were added to 390 ml of 1 N sodium hydroxide, and the resulting mixture was extracted with dichloromethane. The extracts were washed with H_2O, dried ($MgSO_4$) and concentrated. The residual solid was recrystallized from ethyl acetate–hexane to give 33.2 g of (+)-**8** (R=OMe), mp 98 °C. $[\alpha]_D^{20}$ +14° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.04; H, 7.06; N, 4.18.

All mother liquors from the previous isolations were combined and concentrated. The residue was added to 640 ml of 1 N sodium hydroxide and the mixture was extracted with dichlormethane. The extracts were washed with H_2O, dried ($MgSO_4$), and concentrated. The residue was treated with an equivalent of (+)- dibenzoyl-D-tartaric acid monohydrate as described for (+)-**8** (R=OMe). After recrystallization, 74.9 g of **8** (R=OMe) hydrogen dibenzoyl-D-tartrate was obtained, mp 181 °C (dec.). $[\alpha]_D^{20}$ +25° (*c*=1, DMF). Then 29.9 g of (−)-**8** (R=OMe) was obtained, mp 98 °C. $[\alpha]_D^{20}$ −14° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{19}H_{23}NO_4$: C, 69.28; H, 7.04; N, 4.25. Found: C, 69.07; H, 7.03; N, 4.12.

(*S*)-(−)-7,8-Dihydroxy-4-(3,4-dihydroxyphenyl)-1,2,3,4-tetrahydroisoquinoline Hydrochloride Hydrate: (*S*)-(−)-1·HCl·H_2O To a solution of 29.7 g of (−)-**8** (R=OMe) in 150 ml of dichloromethane was added dropwise 12.7 ml of acetic anhydride at room temperature. After being stirred at room temperature for 10 min, the solution was concentrated and azeotroped with toluene. The residual solid was added to 300 ml of 1 N sodium hydroxide, and the mixture was extracted with dichloromethane. The extracts were washed with H_2O, dried ($MgSO_4$) and concentrated. The residual solid was recrystallized from ethyl acetate–hexane to give 31.9 g of (+)-**9** (R=OMe), mp 128 °C. $[\alpha]_D^{20}$ +39° (*c*=1, $CHCl_3$). *Anal.* Calcd for $C_{21}H_{25}NO_5$: C, 67.91; H, 6.78; N, 3.77. Found: C, 67.81; H, 6.82; N, 3.72.

To a solution of 31.8 g of (+)-**9** (R=OMe) in 160 ml of dichloromethane was added dropwise 428 ml of 1 M boron tribromide in dichloromethane at −30 °C. After stirring at room temperature for 90 min, the solution was added to 78 ml of MeOH at −30 °C and warmed to room temperature. The solution was concentrated and azeotroped with MeOH. The residue was added to 318 ml of 0.1 N HCl at room temperature. After cooling to 4 °C, 25.2 g of (+)-**10** (R=OH) was collected as crystals, mp 225 °C (dec.). $[\alpha]_D^{20}$ +85° (*c*=1, MeOH). *Anal.* Calcd for: $C_{17}H_{17}NO_5 \cdot 1/4H_2O$: C, 63.84; H, 5.52; N, 4.38. Found: C, 63.87; H, 5.52; N, 4.31.

A mixture of 87.9 g of (+)-**10** (R=OH), 703 ml of 3 N HCl and 703 ml of ethanol was heated under reflux for 43 h. The reaction mixture was concentrated to about 870 ml. After cooling to 4 °C, 75.4 g of (*S*)-(−)-**1**·HCl·H_2O was collected as crystals. $[\alpha]_D^{20}$ −14° (*c*=1, MeOH). *Anal.* Calcd for $C_{15}H_{15}NO_4 \cdot HCl \cdot H_2O$: C, 54.97; H, 5.54; Cl, 10.82; N, 4.27. Found: C, 54.70; H, 5.42; Cl, 10.98; N, 4.34.

(*R*)-(+)-**1**·HCl·H_2O, (*S*)-(+)-**2**·HCl, and (*R*)-(−)-**2**·HCl were obtained as already described.

(*R*)-(+)-**1**·HCl·H_2O; $[\alpha]_D^{20}$ +15° (*c*=1, MeOH). (*S*)-(+)-**2**·HCl: $[\alpha]_D^{25}$ +3.8° (*c*=2, MeOH). (*R*)-(−)-**2**·HCl: $[\alpha]_D^{25}$ −3.7° (*c*=2, MeOH).

Single-Crystal X-Ray Analysis of (−)-1·HCl·H_2O Suitable crystals of (−)-**1**·HCl·H_2O for an X-ray diffraction study were grown from a MeOH–H_2O solution. A crystal of approximate dimensions, 0.4×0.2×0.15 mm, was used for data collection. Diffraction measurements were carried out on a Rigaku AFC-5R diffractometer using graphite-monochromated Cu$K\alpha$ radiation (λ=1.54184 Å). The unit cell dimensions were obtained by least squares of 20 high angle reflections. The crystal data are as follows: $C_{15}H_{18}NO_5Cl$, M_r=327.77, orthorhombic, space group $P2_12_12_1$, a=11.164(2) Å, b=26.054(3) Å, c=5.364(1) Å, Z=4, D_c=1.395 g/cm^3. Intensities were measured in the θ–2θ scan mode with a scanning speed of 8°(2θ)/min. Of 1487 independent reflections with 2θ<125°, 20 weak reflections below the background were considered to be zero reflections. Corrections were made for Lorentz and polarization factors but not for absorption.

The structure was solved by the direct method using the program SHELXS86,[16] and the atomic parameters were refined by the block-diagonal least-squares method. The refinements were performed first isotropically and then anisotropically for non-hydrogen atoms. H atoms attached to O and N atoms were not included in the refinements, but a

TABLE II. Fractional Coordinates and Isotropic Temperature Factors of (−)-**1**·HCl·H_2O

Atom	x	y	z	B(Å^2)
Cl	0.4556 (7)	0.3419 (3)	0.752 (2)	3.5⟨9⟩
N2	0.4702 (6)	0.3995 (3)	0.770 (1)	3.4⟨8⟩
C3	0.3539 (8)	0.4266 (3)	0.797 (2)	3.4⟨8⟩
C4	0.2638 (7)	0.4068 (3)	0.620 (2)	3.0⟨9⟩
C5	0.1590 (8)	0.4358 (3)	0.590 (2)	3.2⟨5⟩
O5	0.1460 (5)	0.4784 (2)	0.736 (1)	4.1⟨11⟩
C6	0.0783 (8)	0.4240 (3)	0.406 (2)	3.8⟨9⟩
O6	−0.0191 (6)	0.4562 (3)	0.383 (2)	5.5⟨24⟩
C7	0.0921 (9)	0.3805 (4)	0.263 (2)	4.7⟨21⟩
C8	0.1927 (8)	0.3498 (3)	0.301 (2)	3.8⟨11⟩
C9	0.2783 (7)	0.3625 (3)	0.477 (2)	2.9⟨4⟩
C10	0.3921 (7)	0.3301 (3)	0.505 (2)	3.0⟨4⟩
C11	0.3672 (7)	0.2722 (3)	0.484 (2)	3.0⟨4⟩
C12	0.2844 (8)	0.2488 (3)	0.645 (2)	3.3⟨6⟩
C13	0.2589 (6)	0.1967 (3)	0.611 (2)	2.9⟨7⟩
O13	0.1786 (6)	0.1734 (2)	0.774 (1)	4.7⟨20⟩
C14	0.3065 (9)	0.1699 (3)	0.428 (2)	3.8⟨13⟩
O14	0.2707 (8)	0.1203 (2)	0.396 (2)	6.5⟨44⟩
C15	0.394 (1)	0.1928 (4)	0.273 (2)	5.7⟨38⟩
C16	0.4206 (9)	0.2448 (3)	0.301 (2)	3.8⟨14⟩
Cl	0.5796 (2)	0.44374 (7)	1.2622 (4)	2.99⟨33⟩
OW	0.3321 (6)	0.0535 (2)	0.018 (1)	4.0⟨9⟩

The B values accompanied with ⟨ ⟩ are the equivalent isotropic temperature factors calculated from anisotropic thermal parameters using the equation $B=8\pi^2(U_1+U_2+U_3)/3$, where U_1, U_2, and U_3 are principal components of the mean square displacement matrix U. Values in ⟨ ⟩ are anisotropicity defined by $(\Sigma(B-8\pi^2U_i)^2/3)^{1/2}$ and those in ( ) are e.s.d.'s; they refer to last decimal places.

TABLE III. Bond Distances (Å) and Angles (°) of (−)-**1**·HCl·H_2O

Bond distance (Å)					
Cl–N2	1.51 (1)	C1–C10	1.53 (1)	N2–C3	1.48 (1)
C3–C4	1.48 (1)	C4–C5	1.40 (1)	C4–C9	1.40 (1)
C5–O5	1.37 (1)	C5–C6	1.37 (1)	C6–O6	1.38 (1)
C6–C7	1.38 (1)	C7–C8	1.39 (1)	C8–C9	1.38 (1)
C9–C10	1.53 (1)	C10–C11	1.54 (1)	C11–C12	1.40 (1)
C11–C16	1.35 (1)	C12–C13	1.40 (1)	C13–O13	1.39 (1)
C13–C14	1.32 (1)	C14–O14	1.36 (1)	C14–C15	1.41 (1)
C15–C16	1.40 (1)				
Bond angle (°)					
N2–C1–C10	107.7 (7)		C1–N2–C3	112.5 (6)	
N2–C3–C4	111.5 (7)		C3–C4–C5	117.1 (7)	
C3–C4–C9	124.3 (7)		C5–C4–C9	118.6 (7)	
C4–C5–O5	117.3 (7)		C4–C5–C6	120.8 (8)	
O5–C5–C6	121.7 (8)		C5–C6–O6	116.4 (8)	
C5–C6–C7	120.7 (9)		O6–C6–C7	122.8 (9)	
C6–C7–C8	118.7 (9)		C7–C8–C9	121.3 (8)	
C4–C9–C8	119.5 (7)		C4–C9–C10	119.8 (7)	
C8–C9–C10	120.6 (7)		C1–C10–C9	111.1 (7)	
C1–C10–C11	110.1 (7)		C9–C10–C11	112.5 (6)	
C10–C11–C12	119.9 (7)		C10–C11–C16	119.4 (7)	
C12–C11–C16	120.5 (8)		C11–C12–C13	118.4 (7)	
C12–C13–O13	118.2 (7)		C12–C13–C14	122.0 (8)	
O13–C13–C14	119.7 (7)		C13–C14–O14	118.6 (8)	
C13–C14–C15	119.4 (9)		O14–C14–C15	121.9 (9)	
C14–C15–C16	120 (1)		C11–C16–C15	119.6 (9)	

rigid model with idealized geometry was employed for H atom refinement. The final R factor was 0.101 for the reflections with $|F_o|>3\sigma|F_o|$. Eight Bijvoet pairs which exhibited the greatest effect of anomalous scattering from the Cl atom were selected. The ratios of $|F_c(hkl)|/|F_c(\bar{h}\bar{k}\bar{l})|$ for the enantiomer shown in Fig. 1 were in agreement with the observed values. Consequently, the absolute configuration of (−)-**1**·HCl·H_2O was determined to be *S*.

The final values of positional parameters, bond distances and bond angles are available as supplementary material (Tables II and III).

Single-Crystal X-Ray Analysis of (−)-2·HCl Suitable crystals of (−)-**2**·HCl for an X-ray diffraction study were grown from a MeOH solution. A crystal of approximate dimensions, 0.3×0.2×0.1 mm, was used for data collection. The crystal data is as follows: $C_{16}H_{18}NO_3Cl$, $M_r=307.78$, monoclinic, space group $P2_1$, $a=8.304(5)$ Å, $b=12.803(3)$ Å, $c=7.468(4)$ Å, $\beta=116.04(5)°$, $Z=2$, $D_c=1.433$ g/cm^3. Of 1117 independent reflections with $2\theta<120°$, 5 weak reflections below the background were considered to be zero reflections.

The structure was solved by the direct method. A rigid model with idealized geometry was employed for H atom refinement. The final R factor was 0.050 for the reflections with $|F_o|>3\sigma|F_o|$. Other details of the experiment and refinement are as for the (−)-**1**·HCl·H_2O.

TABLE IV. Fractional Coordinates and Isotropic Temperature Factors of (−)-**2**·HCl

Atom	x	y	z	B(Å^2)
N1	0.8582 (6)	0.8036 (4)	0.7219 (7)	2.9⟨12⟩
C2	0.7331 (8)	0.8497 (4)	0.8019 (9)	3.0⟨10⟩
C3	0.7332 (7)	0.7817 (5)	0.9685 (8)	2.5⟨7⟩
C4	0.6027 (7)	0.8070 (5)	1.0317 (9)	2.9⟨7⟩
C5	0.5897 (7)	0.7406 (5)	1.1786 (9)	3.1⟨8⟩
C6	0.7039 (7)	0.6574 (5)	1.2578 (8)	3.1⟨9⟩
C7	0.8383 (7)	0.6371 (4)	1.1951 (8)	2.8⟨10⟩
C8	0.8527 (7)	0.7005 (4)	1.0496 (8)	2.3⟨7⟩
C9	1.0036 (7)	0.6779 (4)	0.9889 (9)	2.4⟨5⟩
C10	1.0347 (7)	0.7754 (5)	0.8889 (9)	3.0⟨7⟩
C11	0.4815 (8)	0.9006 (5)	0.954 (1)	3.6⟨12⟩
O12	0.4628 (6)	0.7665 (4)	1.2390 (8)	5.3⟨35⟩
C13	0.9672 (7)	0.5833 (4)	0.8472 (8)	2.3⟨4⟩
C14	1.0926 (7)	0.5608 (5)	0.7766 (8)	2.5⟨7⟩
C15	1.0640 (7)	0.4790 (4)	0.6488 (8)	2.6⟨7⟩
C16	0.9173 (7)	0.4134 (5)	0.5993 (8)	2.7⟨10⟩
C17	0.7921 (7)	0.4356 (4)	0.6654 (8)	2.8⟨7⟩
C18	0.8143 (7)	0.5219 (4)	0.7874 (8)	2.6⟨4⟩
O19	1.1803 (5)	0.4583 (3)	0.5617 (6)	3.2⟨13⟩
O20	0.9033 (5)	0.3276 (3)	0.4802 (6)	3.3⟨15⟩
Cl	0.6280 (2)	0.1541 (2)	0.4612 (2)	3.82⟨87⟩

The B values accompanied with ⟨ ⟩ are the equivalent isotropic temperature factors calculated from anisotropic thermal parameters using the equation $B=8\pi^2(U_1+U_2+U_3)/3$, where U_1, U_2, and U_3 are principal components of the mean square displacement matrix U. Values in ⟨ ⟩ are anisotropicity defined by $(\Sigma(B-8\pi^2U_i)^2/3)^{1/2}$ and those in ( ) are e.s.d.'s; they refer to last decimal places.

TABLE V. Bond Distances (Å) and Angles (°) of (−)-**2**·HCl

Bond distance (Å)					
N1–C2	1.526 (8)	N1–C10	1.493 (8)	C2–C3	1.518 (9)
C3–C4	1.397 (9)	C3–C8	1.381 (8)	C4–C5	1.429 (9)
C4–C11	1.507 (9)	C5–C6	1.375 (9)	C5–O12	1.357 (8)
C6–C7	1.412 (9)	C7–C8	1.402 (8)	C8–C9	1.536 (8)
C9–C10	1.533 (9)	C9–C13	1.549 (8)	C13–C14	1.388 (8)
C13–C18	1.390 (8)	C14–C15	1.366 (8)	C15–C16	1.389 (8)
C15–O19	1.407 (7)	C16–C17	1.363 (8)	C16–O20	1.385 (7)
C17–C18	1.391 (8)				
Bond angle (°)					
C2–N1–C10	110.7 (5)		N1–C2–C3	110.1 (5)	
C2–C3–C4	115.0 (5)		C2–C3–C8	122.2 (5)	
C4–C3–C8	122.8 (6)		C3–C4–C5	116.9 (5)	
C3–C4–C11	122.8 (6)		C5–C4–C11	120.3 (5)	
C4–C5–C6	121.5 (6)		C4–C5–O12	116.1 (5)	
C6–C5–O12	122.4 (6)		C5–C6–C7	119.7 (6)	
C6–C7–C8	120.0 (5)		C3–C8–C7	119.1 (5)	
C3–C8–C9	122.3 (5)		C7–C8–C9	118.6 (5)	
C8–C9–C10	108.9 (5)		C8–C9–C13	114.1 (5)	
C10–C9–C13	109.1 (5)		N1–C10–C9	106.9 (5)	
C9–C13–C14	117.9 (5)		C9–C13–C18	122.6 (5)	
C14–C13–C18	119.5 (5)		C13–C14–C15	119.7 (5)	
C14–C15–C16	120.9 (5)		C14–C15–O19	121.5 (5)	
C16–C15–O19	117.6 (5)		C15–C16–C17	119.8 (6)	
C15–C16–O20	117.8 (5)		C17–C16–O20	122.4 (5)	
C16–C17–C18	120.0 (5)		C13–C18–C17	119.9 (5)	

Thirteen Bijvoet pairs which exhibited the greatest effect of anomalous scattering from the Cl atom were selected. The ratios of $|F_C(hkl)|/|F_C(\bar{h}\bar{k}\bar{l})|$ for the enantiomer shown in Fig. 2 were in agreement with the observed values. The absolute configuration of the inactive enantiomer (−)-**2**·HCl was determined to be *R*.

The final values of positional parameters, bond distances and bond angles are available as supplementary material (Tables IV and V).

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12) HPLC condition: Column, TSK-gel (ODS), 4.6 × 150 mm. Eluent, 30 mM $HClO_4$ aq. sol.–MeOH (for **8**, 7 : 12, v/v; for **1** and **2**, 1 : 1, v/v). Flow rate, 1.0 ml·min^{-1}.
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Agents for the Treatment of Overactive Detrusor. I. Synthesis and Structure–Activity Relationships of 1,1′-Biphenyl Derivatives

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A series of 1,1′-biphenyl-2,6-dicarboxylic acid diesters were synthesized and examined for their inhibitory activity on guinea-pig detrusor muscle contraction at electrical field stimulation *in vitro*. Among them, 6-isopropyl 2-methyl 3-hydroxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate, FR75513 (8a) was one of the potent compounds ($IC_{50} = 3.3 \times 10^{-6}$ g/ml). This compound (8a) exhibited a strong inhibitory activity on detrusor contraction after intravenous administration in anesthetized rats ($ID_{50} = 0.04$ mg/kg).

Keywords overactive detrusor; inhibitory activity; electrical field stimulation; 1,1′-biphenyl-2,6-dicarboxylic acid diester

The overactive detrusor syndrome is a disease which makes the number of micturition extraordinarily large in a single day. Detrusor contraction is mainly mediated by muscarinic receptors in the detrusor and it is well known that anticholinergics alone are unable to completely suppress detrusor contraction (atropine resistance).[1] To overcome this atropine resistance, clinically available agents for the treatment of overactive detrusor have anticholinergic and other pharmacological activities.

For example, terodiline (Mictrol) apparently acts by combined competitive anticholinergic and calcium antagonistic activities.[2] Oxybutynin (Pollakisu), a potent anticholinergic agent, displays potent local anesthetic and spasmolytic activities as well.[3]

On the other hand, nifedipine, a typical calcium antagonist, is more potent in suppressing detrusor contraction at electrical field stimulation *in vitro* than terodiline or oxybutynin, and also suppresses detrusor contraction stimulated by carbachol *in vitro* (Table I). However nifedipine possesses high vascular selectivity.

Recently, attempts to replace the 1,4-dihydropyridine nucleus in nifedipine with other heterocyclic rings such as dihydropyran,[4a] dihydropyridazine,[4b] dihydrothiopyran,[4c] dihydropyrazine,[4d] and dihydropyrimidine[4e] have been carried out, and they also show calcium antagonistic activities. The selectivity of relaxant activity on detrusor muscle to artery muscle of nifedipine might be improved by replacing the 1,4-dihydropyridine nucleus with another skeleton, hence possibly leading to the creation of a new agent for treatment of an overactive detrusor without side effects based on anticholinergic activity.

Our design concepts were as follows; 1) A change in the basic skeleton of nifedipine, 1,4-dihydropyridine, to another skeleton (in this case benzene ring). 2) Introduction of a hydroxyl group to compensate for the decrease of the molecular hydrophilicity due to the replacement described above. 3) Since the unsymmetric esters in 1,4-dihydropyridine tended to show stronger relaxant activity than symmetric esters,[5] ester groups were made unsymmetrical. Using these concepts 6-isopropyl 2-methyl 3-hydroxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate, FR75513 (**8a**) was designed and synthesized (Fig. 1). This compound **8a** possessed weaker inhibitory activity on KCl induced contraction of rat aorta than nifedipine, but had a more potent relaxant activity on detrusor contraction at electrical field stimulation than terodiline or oxybutynin (Table I). In addition to this, the selectivity of the relaxant activity on the detrusor muscle to artery muscle of FR75513 was much improved in comparison to that of nifedipine. The desirable properties of FR75513 as an agent for the treatment of the overactive detrusor made us further investigate this compound. Herein, we report the synthesis of FR75513 and related compounds, pharmacological results *in vitro* and *in vivo* assay, and its structure–activity relationships.

Chemistry Charts 1—3 illustrate the synthetic routes to the present 1,1′-biphenyl related compounds **4**, **6**—**15**.

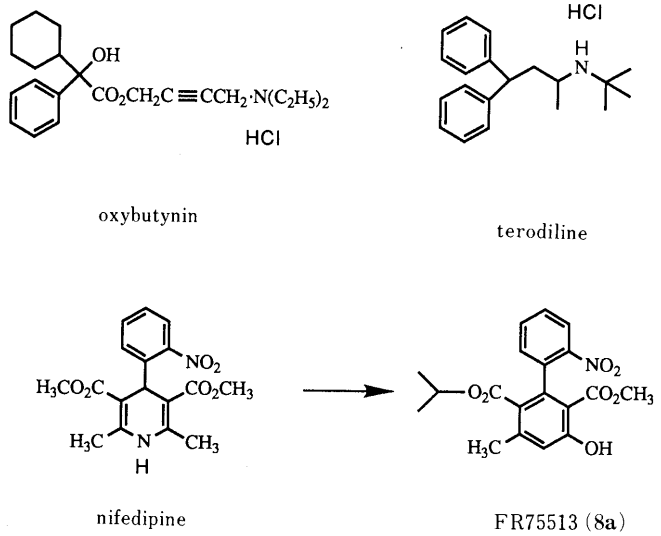


Fig. 1

TABLE I. Effect on Electrically and Carbachol Induced Contractions of G-P Detrusor and KCl Induced Contraction in Rat Aorta[a]

Compd.	G-P detrusor		Rat aorta
	Electrically induced contraction IC_{50} (g/ml)	Carbachol induced contraction IC_{50} (g/ml)	KCl induced contraction IC_{50} (g/ml)
Terodiline	1.4×10^{-5}	9.8×10^{-6}	1.2×10^{-6}
Oxybutynin	2.1×10^{-5}	9.9×10^{-8}	5.4×10^{-7}
Nifedipine	4.7×10^{-7}	1.6×10^{-7}	2.1×10^{-9}
FR75513	3.4×10^{-6}	$> 1.0 \times 10^{-5}$	1.1×10^{-6}

a) The *in vitro* test procedures were carried out as described in the experimental section.

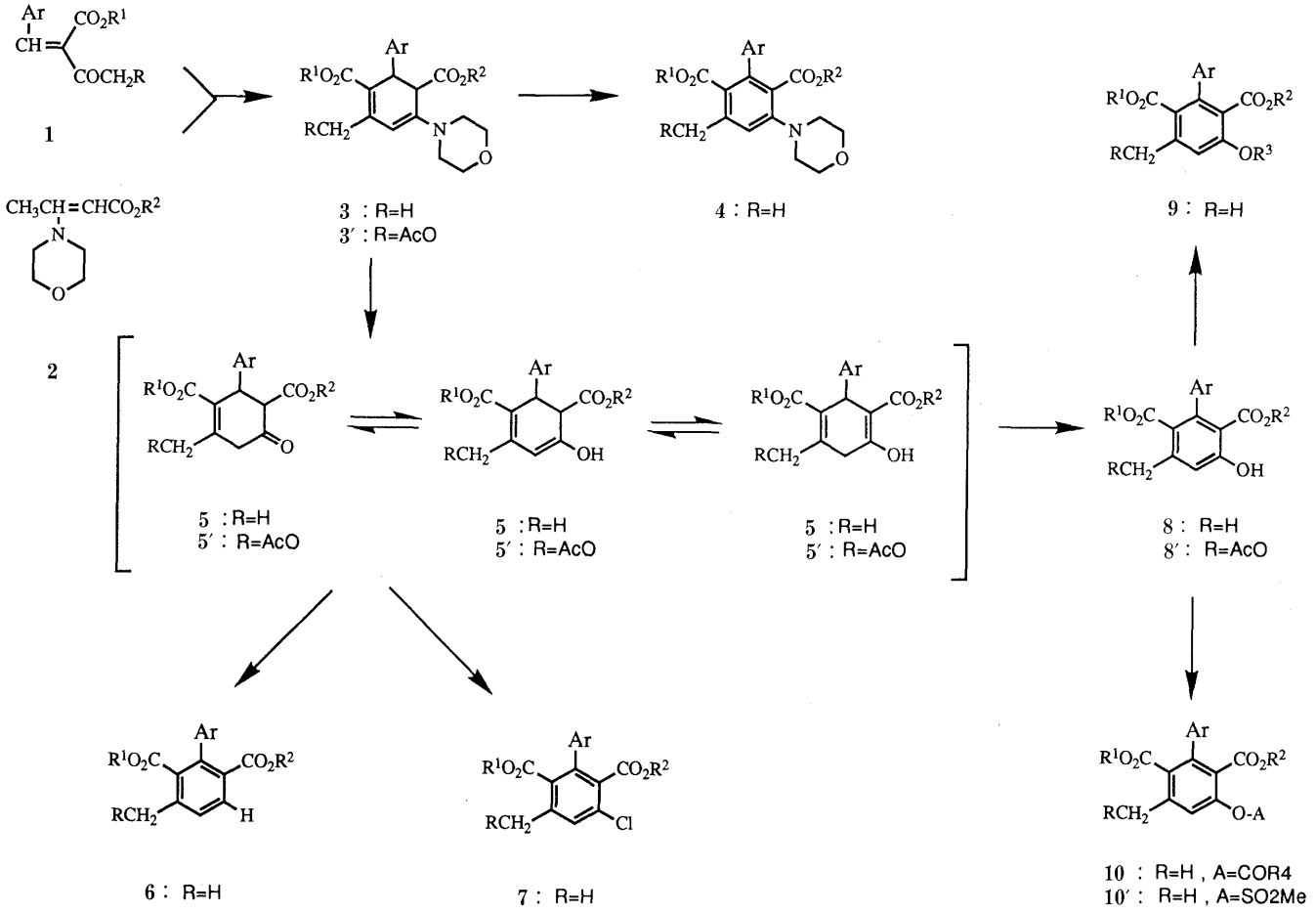


Chart 1

Benzylidene derivative **1**, available by the Knoevenagel condensation of alkyl acetoacetate with the appropriate arylaldehyde, was condensed with morpholinocrotonate **2** to afford the compound **3**, **3′**. Oxidation of **3** afforded morpholino derivative **4**. Hydrolysis of the obtained compound **3**, **3′** afforded a tautomeric mixture of 3-cyclohexenone **5**, **5′**. Oxidation of the tautomeric mixture **5**, **5′** by iodine/sodium acetate or bromine[6] afforded a new phenol derivative **8**, **8′** which has four other substituents in the nucleus. Conversion of the hydroxyl group in compound **8** to a hydrogen atom **6** was achieved by the following procedure. Reduction of **5** with sodium borohydride followed by dehydration of the resulting alcohol with $POCl_3$ and pyridine afforded 2,5-dihydrobenzene-1,3-dicarboxylic acid diester. Oxidation of the dihydrobenzene with iodine/sodium acetate afforded compound **6**. Phosphorous pentachloride treatment of 3-cyclohexenone **5** afforded chloro derivaive **7**. Alkylation of the phenol **8** afforded ether **9** and acylation afforded the corresponding carboxylate and carbonate **10**. Sulfonylation of the phenol **8** afforded sulfonate **10′** (Chart 1).

Deacetylation of the compound **8′** with *p*-toluenesulfonic acid in methanol afforded lactone **11**. Reaction of **8a** with methylamine in ethanol exclusively afforded amide **12**. Removal of the *tert*-butyl group in the ester moiety of **8** with trifluoroacetic acid (TFA) afforded monocarboxylic acid **13**. Dicarboxylic acid **14** was obtained by hydrolysis of diester **8** (Chart 2).

Hydrogenation of the nitro group **9** or hydrogenolysis of the benzyloxy group **8** on the aryl moiety afforded lactam **15** or lactone **15′** which were formed between the amino group or the hydroxy group and the smaller ester group (methyl group). In this case steric effects determined the direction of the cyclization (Chart 3).

Chart 4 illustrates the synthesis of methyl derivative **19**, **21**.

The Sandmeyer reaction of 2-amino-1, 3-dicyano-4, 6-dimethylbenzene (**16**) afforded 2-bromo-1, 3-dicyano-4, 6-dimethylbenzene (**17**). Hydrolysis of the nitrile groups followed by esterification afforded the symmetrical diester **18**, which was coupled with 1-iodo-2-nitrobenzene to afford the 1,1′-biphenyl derivative **19**. In this coupling reaction a dimeric compound was obtained as a minor product.[7] Partial hydrolysis of diester **19** and re-esterification afforded the unsymmetrical diester **21**.

Structure–Activity Relationships (Tables II—V) To study the structure–activity relationships, the parent structure of FR75513 was considered to be comprised of four active regions of importance: the nitrophenyl group (aryl moiety), the ester group, the phenolic hydroxyl group and the methyl group.

Modification of the aryl moiety revealed that substitution at the *ortho* position of the benzene ring in aryl moiety produced higher activity than substitution at the *para* and *meta* positions (**8a,b,c**, Table II). As for the substituents **8a,d—j**, electron withdrawing groups such as nitro and cyano groups **8a,j** had strong activity and electron releasing groups such as methoxy and benzyloxy groups **8f,g**

decreased the activity. In the case where an amino or a hydroxy group is at the ortho position, ring formation between the substituents and the ester group occurs to afford a lactam **15** or lactone **15′**, both of which showed weak activity compared with the parent compound, FR75513 (Table III). Replacement of the benzene ring with heterocycles such as thiophene and pyridine rings **8k—n** also showed a decrease in activity. In the case of pyridine substitution, substitution at the 2- position also showed relatively higher activity than substitution at the 4- and 3-positions.

Concerning modification of the ester groups at the 2- and 6-positions, the unsymmetrical esters **8a,o,q—s,u** exhibited stronger activity than the symmetrical ester **8p** except **8t**. Conversion of the ester function to the carboxylic acid or the amide **12—14** resulted in a nearly complete loss of activity.

Replacement of the phenolic hydroxyl group of the compound **8a** with an alkoxy group **9a—g** or a methanesulfonyloxy group **10′a** decreased the activity. Introduction of an ester **10a,b**, a methyl group **19**, or a chlorine atom **7** instead of the hydroxyl group almost maintained the activity (Table V). In the case of 3,5-dimethyl derivatives **19**, **21**, the symmetric ester **19** was stronger than the unsymmetric ester **21**. Replacement of the hydroxyl group with a hydrogen atom **6** or a carbonate group **10c** slightly decreased the activity.

Replacement of 5-methyl group of the compound **8a** with an acetoxymethyl group **8′a** dramatically decreased the activity. Conversion of **8′a** to the lactone **11** resulted in a complete loss of the activity as seen in 1,4-dihydropyridine lactone derivative[8] (Table IV).

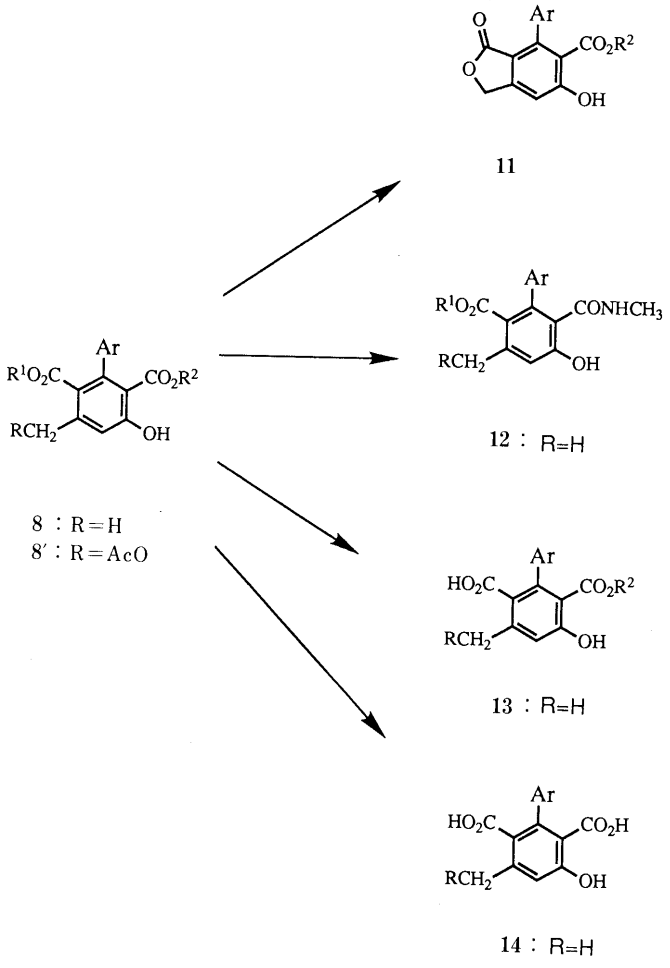


Chart 2

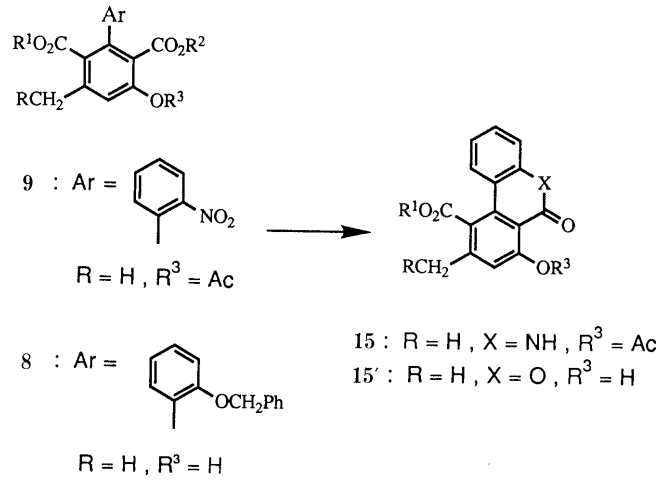


Chart 3

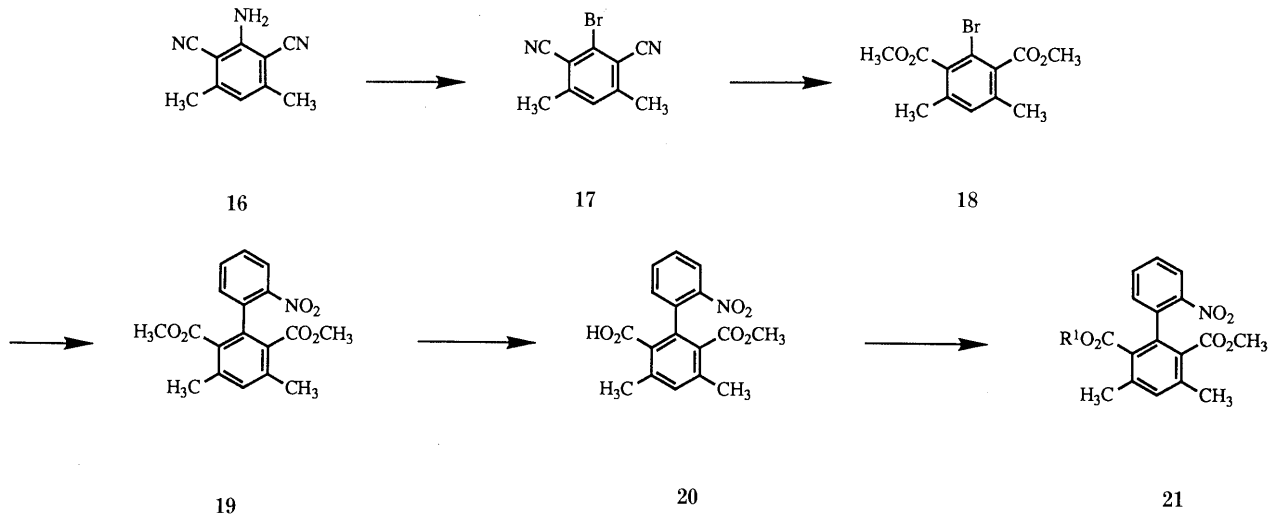
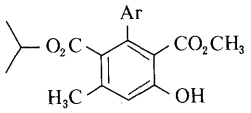


Chart 4

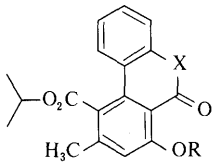
TABLE II. Physical and Pharmacological Properties of 1,1′-Biphenyl-2,6-dicarboxylic Acid Derivatives and Related Compounds



Compd. No.	Ar	mp (°C) Recryst. solv.[a]	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	*In vitro*[b] % inhibition at 10^{-5} g/ml
8a	2-NO_2-Ph	69—71 P.ether	34.4	$C_{19}H_{19}NO_7$	61.12	5.13	3.75	60.76	5.13	4.04	76.2[f]
8b	3-NO_2-Ph	74—76 P.ether	29.4	$C_{19}H_{19}NO_7$	61.12	5.13	3.75	60.71	5.09	3.52	11.8
8c	4-NO_2-Ph	117—119 IPE	18.2	$C_{19}H_{19}NO_7$	61.12	5.13	3.75	60.73	5.19	3.68	−4.3
8d	Ph	71—73[c]	31.3	$C_{19}H_{20}O_5$	69.50	6.14		69.43	6.15		40.0
8e	2-Me-Ph	Oil[d]	46.5	$C_{20}H_{22}O_5$							26.0
8f	2-MeO-Ph	102—104 IPE	5.4	$C_{20}H_{22}O_6$	67.03	6.19		66.61	5.99		10.3
8g	2-$PhCH_2O$-Ph	110.5—104 P.ether	25.1	$C_{26}H_{26}O_6$	71.87	6.03		72.14	6.04		11.6
8h	2-F-Ph	Oil[e]	27.3	$C_{19}H_{19}FO_5$							25.0
8i	2-Cl-Ph	53—55[c]	20.1	$C_{19}H_{19}ClO_5$	62.90	5.28		62.82	5.27		19.4
8j	2-CN-Ph	90—91 IPE	59.8	$C_{20}H_{19}NO_5$	67.98	5.42	3.96	67.87	5.61	4.02	66.1[g]
8k	2-Thienyl	101—102 c-H	17.2	$C_{17}H_{18}O_5S$	61.10	5.43		61.08	5.43		18.1
8l	2-Py·HCl	164 (dec.)[c]	27.5	$C_{18}H_{19}NO_5 \cdot HCl \cdot 0.25H_2O$	58.38	5.58	3.78	58.69	5.32	3.65	38.5
8m	3-Py	106—108 IPE	8.8	$C_{18}H_{19}NO_5 \cdot 0.25H_2O$	64.76	5.89	4.20	64.84	5.68	4.15	7.1
8n	4-Py	175—176 C-IPE	30.8	$C_{18}H_{19}NO_5$	65.64	5.81	4.25	65.39	5.62	4.12	24.0

a) P. ether, petroleum ether; IPE, diisopropyl ether; c-H, cyclohexane; C, chloroform. *b*) The *in vitro* tests (field stimulation) were carried out as described in the experimental section. *c*) Complete evaporation gave analytical sample. *d*) MS *m/z*: 342 (M^+), 310 ($M^+ - OMe$), 283 ($M^+ - O$-iso-Pr). *e*) MS *m/z*: 346 (M^+), 314 ($M^+ - OMe$). *f*) $IC_{50} = 3.3 \times 10^{-6}$ g/ml. *g*) $IC_{50} = 5.3 \times 10^{-6}$ g/ml.

TABLE III. Physical and Pharmacological Properties of 1,1′-Biphenyl-2,6-dicarboxylic Acid Derivatives



Compd. No.	X	R	mp (°C) Recryst.[a] Solv.	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	*In vitro*[b] % inhibition at 10^{-5} g/ml
15	NH	Ac	238—239 IPE–A	52.7	$C_{20}H_{19}NO_5 \cdot 0.25H_2O$	67.12	5.49	3.91	67.21	5.34	3.86	37.5
15′	O	H	142 P.ether	60.0	$C_{18}H_{16}O_5$	69.22	5.16		68.76	4.98		23.8

a) IPE, diisopropyl ether; A, ethyl acetate; P.ether, petroleum ether. *b*) The *in vitro* test were carried out as described in the experimental section.

Pharmacological Activity *in Vivo* and Discussion Among 12 compounds which showed more than 50% inhibition in the *in vitro* test, FR75513 (**8a**) showed the strongest activity and was selected for *in vivo* testing. The results are shown in Table VI.

FR75513 had strong inhibitory activity in i.v. administration ($IC_{50} = 0.04$ mg/kg), but had less activity in i.d. administration. The reason for its poor activity in i.d. administration was considered to be poor i.d. absorption, since this compound administered as polyethyleneglycol suspension exists as a white solid in duodenal lumen 2 h later. The results from FR75513 in that it did not have an anticholinergic property but suppressed the detrusor contraction at electrical field stimulation is different from those of agents on the market for an overactive detrusor. Since it is hoped it will become a new type of agent for the disease, attempts to increase its i.d. absorption were carried out. The results of such experimentation will be published in the following paper.

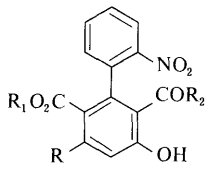
Experimental

All melting points were determined in open glass capillaries on a Thomas-Hoover apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 260-10 infrared spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Hitachi R-90H NMR spectrometer with tetramethylsilane as an internal standard (δ value, ppm). Mass (MS) spectra were recorded on JEOL JMS D-300 mass spectrometer. Elemental analyses were carried out on Perkin-Elmer 2400CHN Elemental Analyzer. Yields are not optimized.

3-(1-Methylethyl) 1-Methyl 4-Methyl-6-morpholino-2-(2-nitrophenyl)-1,2-dihydrobenzene-1,3-dicarboxylate (3a) A typical example is given to illustrate the general procedure.

A mixture of 1-methylethyl 2-acetyl-3-(2-nitrophenyl)-2-propenoate (31.0 g) and methyl 3-morpholino-2-butenoate (20.71 g) in benzene (50 ml) was refluxed for 48 h with continuous azeotropic removal of water using a Dean-Stark apparatus. After cooling, the mixture was evaporated and the residue was triturated with IPE to afford a powder **3a**, (21.70 g). Recrystallization from a mixture of EtOAc and *n*-hexane afforded an analytical sample of the title compound, mp 133—134 °C. *Anal.* Calcd for

TABLE IV. Physical and Pharmacological Properties of 1,1′-Biphenyl-2,6-dicarboxylic Acid Derivatives



Compd. No.	R	R_1	R_2	mp (°C) Recryst. solv.[a]	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	*In vitro*[b] % inhibition at 10^{-5} g/ml
8o	Me	Me	O-iso-Pr	112.5—113.5 IPE	23.4	$C_{19}H_{19}NO_7$	61.12 (61.42)	5.13 5.22	3.75 3.66)	50.0[e]
8p	Me	Me	OMe	125—126 H–IPE	26.5	$C_{17}H_{15}NO_7$	59.13 (59.07)	4.38 4.47	4.06 4.05)	42.1
8q	Me	Cl-$(CH_2)_2$–	OMe	124—125 IPE	43.4	$C_{18}H_{16}ClNO_7 \cdot 0.25H_2O$	54.28 (54.41)	4.18 3.87	3.52 3.68)	69.0[f]
8r	Me	Et	OMe	139—141 H–A	32.1	$C_{18}H_{17}NO_7$	60.17 (60.24)	4.77 4.73	3.90 3.89)	69.4[g]
8s	Me	$PhCH_2$	OMe	123—125 IPE	10.7	$C_{23}H_{19}NO_7$	65.56 (65.84)	4.54 4.49	3.32 3.15)	65.4[h]
8t	Me	*tert*-Bu	OMe	134—136 IPE–P.ether	45.6	$C_{20}H_{21}NO_7$	62.01 (61.65)	5.46 5.34	3.62 3.36)	33.8
8u	Me	Cyclopentyl	OMe	101—102 IPE	23.9	$C_{21}H_{21}NO_7$	63.15 (63.30)	5.30 5.31	3.51 3.30)	56.1[i]
8′a	$AcOCH_2$	iso-Pr	OMe	Oil[c]	34.5	$C_{21}H_{21}NO_9$				6.1
11	–CH_2OCO–		OMe	208—210 MeOH	60.9	$C_{16}H_{11}NO_7 \cdot 0.25H_2O$	57.58 (57.83)	3.47 3.23	4.20 4.06)	−4.9
12	Me	iso-Pr	NHMe	189—190 C–IPE–A	75.5	$C_{19}H_{20}N_2O_6$	61.28 (60.96)	5.41 5.16	7.52 7.75)	12.9
13	Me	H	OMe	179—182[d]	97.0	$C_{16}H_{13}NO_7$	58.01 (57.86)	3.96 3.90	4.23 4.12)	−4.2
14	Me	H	OH	216—219[d]	52.9	$C_{15}H_{11}NO_7 \cdot 0.25H_2O$	55.99 (55.75)	3.60 3.45	4.35 4.15)	13.6

a) IPE, diisopropyl ether; H, *n*-hexane; A, ethyl acetate; P.ether, petroleum ether; C, chloroform. *b*) The *in vitro* tests (field stimulation) were carried out as described in the experimental section. *c*) MS *m/z*: 431 (M^+). *d*) Purification by changing the PH of the alkaline solution to 2 gave analytical sample. *e*) $IC_{50} = 1.0 \times 10^{-5}$ g/ml. *f*) $IC_{50} = 4.0 \times 10^{-6}$ g/ml. *g*) $IC_{50} = 3.6 \times 10^{-6}$ g/ml. *h*) $IC_{50} = 4.0 \times 10^{-6}$ g/ml. *i*) $IC_{50} = 1.9 \times 10^{-6}$ g/ml.

$C_{23}H_{28}N_2O_7$: C, 62.15; H, 6.35; N, 6.30. Found: C, 62.59; H, 6.46; N, 6.27. IR (Nujol) cm^{-1}: 1736, 1690. NMR ($CDCl_3$): 0.80 (3H, d, $J=6$ Hz), 1.12 (3H, d, $J=6$ Hz), 2.39 (3H, s), 2.95—3.30 (4H, m), 3.40—3.90 (5H, m), 3.74 (3H, s), 4.80 (1H, septet, $J=6$ Hz), 4.97 (1H, br s), 5.04 (1H, s) 7.15—8.90 (4H, m).

Other compounds listed in Tables VII and VIII were similarly prepared in accordance with the procedure as described above.

2-(1-Methylethyl) 6-Methyl 3-Methyl-5-morpholino-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (4) To a mixture of **3a** (1.07 g), NaOAc (0.84 g) in MeOH (10 ml) was added I_2 (1.36 g), and refluxed for 30 h. After cooling, the mixture was evaporated. The residue was partitioned between EtOAc and water. The organic layer was separated, washed with aq. $Na_2S_2O_3$, dried over $MgSO_4$, and evaporated. The resulting residue was purified by column chromatography on silica gel with a mixture of $CHCl_3$ and MeOH as eluent to afford **4** (0.79 g), mp 122—124 °C (recrystallized from IPE). IR (Nujol) cm^{-1}: 1715. NMR ($CDCl_3$): 0.78 (3H, d, $J=9$ Hz), 1.00 (3H, d, $J=9$ Hz), 2.44 (3H, s), 2.95—3.25 (4H, m), 3.42 (3H, s), 3.65—3.93 (4H, m), 4.84 (1H, septet, $J=9$ Hz), 6.96 (1H, s), 7.16—7.75 (3H, m), 8.05—8.33 (1H, m).

1-Methyl 3-(1-Methylethyl) 2-(2-Nitrophenyl)-4-methyl-6-oxo-3-cyclohexene-1,3-dicarboxylate (5a) To a solution of **3a** (22.22 g) in tetrahydrofuran (THF) (110 ml) was added 1 N HCl (55 ml), the mixture was stirred for 3 h at room temperature and then saturated with NaCl. The organic layer was separated, washed with brine, dried over $MgSO_4$ and evaporated. The residue was triturated with IPE to afford **5a** (12.08 g). Twice recrystallization from a mixture of IPE and EtOAc gave an analytical sample, mp 128—130 °C. *Anal.* Calcd for $C_{19}H_{21}NO_7$: C, 60.79; H, 5.69; N, 3.73. Found: C, 60.91; H, 5.53; N, 3.73. IR (Nujol) cm^{-1}: 1740, 1710. NMR ($CDCl_3$): 0.82 (3H, d, $J=6$ Hz), 1.12 (3H, d, $J=6$ Hz), 2.25 (3H, s), 3.07 (1H, d, $J=22$ Hz), 3.47 (1H, d, $J=22$ Hz), 3.77 (4H, s), 4.85 (1H, septet, $J=6$ Hz), 5.37 (1H, br s), 6.98—7.98 (4H, m).

Compounds **5a**,**c**,**f**,**j**,**m**,**n**,**p** listed in Table IX were obtained as crystals. Other compounds **5**, **5′** were used in the next reaction without purification.

Method A. 6-(1-Methylethyl) 2-Methyl 3-Hydroxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (8a) A typical example is given to illustrate the general procedure.

To a solution of **5a** (27.6 g) in MeOH (100 ml) were added NaOAc (15.19 g) and I_2 (20.14 g), and stirred for 5 h at room temperature. After removal of MeOH, EtOAc and water were added to the residue. The organic layer was separated, washed with aq. $Na_2S_2O_3$, dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography on silica gel with a mixture of $CHCl_3$ and *n*-hexane as eluent, and crystallization from petroleum ether afforded **8a** (14.65 g), mp 69—71 °C. *Anal.* Calcd for $C_{19}H_{19}NO_7$: C, 61.12; H, 5.13; N, 3.75. Found: C, 60.76; H, 5.13; N, 4.04. IR (Nujol) cm^{-1}: 1720, 1660. NMR ($CDCl_3$): 0.77 (3H, d, $J=6$ Hz), 1.00 (3H, d, $J=6$ Hz), 2.33 (3H, s), 3.40 (3H, s), 4.77 (1H, septet, $J=6$ Hz), 6.93 (1H, s), 7.10—8.30 (4H, m), 11.40 (1H, s).

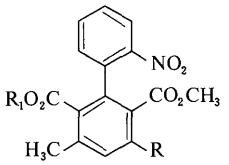
Other compounds listed in Table II and IV (**8a—q**, **i—m**, **o—u**, **8′a**), and compound **4** were simiarly prepared.

Method B. 6-(2-Chloroethyl) 2-Methyl 5-Methyl-3-hydroxy-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (8q) A typical example is given to illustrate the general procedure.

The crude product **5a** (1 g) was dissolved in $CHCl_3$, and a solution of Br_2 (0.13 ml) in $CHCl_3$ (2 ml) was added thereto at 25—27 °C. After stirring for 2 h, ice water was added to the mixture. The organic layer was separated, washed with aq. $Na_2S_2O_3$ and brine, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel with CH_2Cl_2 as eluent to afford **8q** (0.91 g). Crystallization from IPE afforded 0.43 g of **8q**, mp 125—125 °C. *Anal.* Calcd for $C_{18}H_{16}ClNO_7$: C, 54.90; H, 4.10; N, 3.56. Found: C, 54.41; H, 3.87; N, 3.68. IR (Nujol) cm^{-1}: 1710, 1670. NMR ($CDCl_3$): 2.35 (3H, s), 3.30 (2H, t, $J=6$ Hz), 3.40 (3H, s), 3.80—4.30 (2H, m), 6.90 (1H, s), 7.05—7.15 (1H, m), 7.40—7.60 (2H, m), 8.00—8.20 (1H, m), 11.33 (1H, s).

Other compounds listed in Table II (**8h**,**n**) were similarly prepared.

TABLE V. Physical and Pharmacological Properties of 1,1′-Biphenyl-2,6-dicarboxylic Acid Derivatives



Compd. No.	R_1	R	mp (°C) Recryst. solv.[a]	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N	*In vitro*[b] % inhibition at 10^{-5} g/ml
4	iso-Pr		122—124 IPE	74.2	$C_{23}H_{26}N_2O_7 \cdot 0.25H_2O$	61.81 (61.93	5.98 6.13	6.27 6.30)	13.9
6	iso-Pr	H	Oil[c]	18.1	$C_{19}H_{19}NO_6$				54.2[g]
7	Cyclopentyl	Cl	Oil[d]	20.1	$C_{21}H_{20}ClNO_6$				60.6[h]
9a	iso-Pr	OMe	120—122 IPE	67.9	$C_{20}H_{21}NO_7$	62.01 (62.26	5.46 5.73	3.62 3.71)	26.1
9b	iso-Pr	$O-(CH_2)_2-OH$	128—130 IPE	65.9	$C_{21}H_{23}NO_8$	60.43 (60.09	5.55 5.68	3.36 3.00)	25.1
9c	iso-Pr	$O-(CH_2)_2-OEt$	129—133 IPE	71.8	$C_{23}H_{27}NO_8$	62.01 (61.90	6.11 6.25	3.14 2.74)	29.6
9d	iso-Pr	OCH_2CO_2Me	116—118 EtOH	75.1	$C_{22}H_{23}NO_9$	59.32 (68.91	5.20 4.99	3.14 3.40)	7.7
9e	iso-Pr	OCH_2Ph	120—121 A–IPE	55.5	$C_{26}H_{25}NO_7$	67.38 (67.48	5.44 5.29	3.02 3.09)	8.7
9f	iso-Pr	$O-(CH_2)_3-OH$	133—138 IPE	22.6	$C_{22}H_{25}NO_8 \cdot 0.25H_2O$	60.61 (60.45	5.90 5.77	3.21 3.10)	13.8
9g	iso-Pr	$O-(CH_2)_2-NEt_2 \cdot HCl$	146—147 A	38.7	$C_{25}H_{32}N_2O_7 \cdot H_2O$	56.98 (56.90	6.69 6.18	5.32 5.48)	6.1
10a	iso-Pr	OAc	111—112 IPE–Tol	56.2	$C_{21}H_{21}NO_8$	60.72 (60.56	5.10 5.26	3.37 3.29)	70.6[i]
10b	iso-Pr	OCO-*tert*-Bu	Oil[e]	47.4	$C_{25}H_{29}NO_9$				60.0[j]
10c	iso-Pr	OCO_2-iso-Pr	89—90 IPE	49.5	$C_{24}H_{27}NO_9$	60.88 (60.77	5.75 5.61	2.96 2.87)	46.4
10′a	iso-Pr	OMs	Oil[f]	68.2	$C_{20}H_{21}NO_9S$				15.2
19	Me	Me	132—134 H–A	35.0	$C_{18}H_{17}NO_6$	62.97 (62.88	4.99 4.83	4.08 4.11)	70.0[k]
21	Ph-$(CH_2)_2$–	Me	72—75 H–ether	70.2	$C_{25}H_{23}NO_6$	69.27 (69.42	5.35 5.26	3.23 3.23)	44.0

a) A, ethyl acetate; Tol, toluene; H, *n*-hexane. *b*) The *in vitro* tests (field stimulation) were carried out as described in the experimental section. *c*) MS *m/z*: 326, 310. *d*) MS *m/z*: 386, 371. *e*) MS *m/z*: 426, 410. *f*) MS *m/z*: 420, 405. *g*) $IC_{50} = 5.8 \times 10^{-6}$ g/ml. *h*) $IC_{50} = 4.2 \times 10^{-6}$ g/ml. *i*) $IC_{50} = 3.1 \times 10^{-6}$ g/ml. *j*) $IC_{50} = 3.3 \times 10^{-6}$ g/ml. *k*) $IC_{50} = 2.8 \times 10^{-6}$ g/ml.

TABLE VI. Effect of FR75513 on the Urinary Bladder Rhythmic Contractions in Rat Cystometry

Dose (mg/kg)	Inhibition (%)	Duration (min)
0.01 (i.v.)	38	10
0.1 (i.v.)	45	10
1.0 (i.v.)	100	30
100 (i.d.)	14	—

a) The *in vivo* test procedures were carried out as described in the experimental section.

6-(1-Methylethyl) 2-Methyl 5-Methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (6) To a solution of **5a** (3.0 g) in a mixture of THF (15 ml) and MeOH (4 ml) was added a solution of $NaBH_4$ (0.30 g) in water (4 ml) at room temperature. The mixture was stirred for 3 h, and then AcOH (0.6 ml) was added thereto. The resulting mixture was evaporated, and the residue was dissolved in EtOAc. The solution was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel with a mixture of toluene and EtOAc as eluent to afford 3-(1-methylethyl) 1-methyl 2-(2-nitrophenyl)-6-hydroxy-4-methyl-3-cyclohexene-1,3-dicarboxylate (0.60 g) as an oil. IR (neat) cm^{-1}: 3450, 1705. NMR ($CDCl_3$): 0.65 (3H, d, $J=6$ Hz), 1.03 (3H, d, $J=6$ Hz), 2.14 (3H, s), 2.20—2.90 (2H, m), 2.90—3.10 (1H, m), 3.30—3.65 (1H, m), 3.65 (3H, s), 4.10—4.36 (1H, m), 4.73 (1H, septet, $J=6$ Hz), 4.83—5.06 (1H, m), 7.20—7.96 (4H, m).

This product was dissolved in pyridine (2 ml), and then $POCl_3$ (0.12 ml) was added thereto with ice-bath cooling. The mixture was stirred for 1 h at room temperature, and then evaporated. EtOAc and water were added to the residue. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, and evaporated.The residue was purified by column chromatography on silica gel with a mixture of toluene and EtOAc as eluent to afford pale yellow crystals. The crystals were recrystallized from MeOH to afford 3-(1-methylethyl) 1-methyl 4-methyl-2-(2-nitrophenyl)-2,5-dihydrobenzene-1,3-dicarboxylate (0.17 g), mp 103—104 °C. *Anal.* Calcd for $C_{19}H_{21}NO_6$: C, 63.50; H, 5.89; N, 3.90. Found: C, 63.89; H, 5.74; N, 3.92. IR (Nujol) cm^{-1}: 1700. NMR ($CDCl_3$): 1.03 (3H, d, $J=6$ Hz), 1.10 (3H, d, $J=6$ Hz), 2.20 (3H, s), 3.02 (1H, ddd, $J=3.5$, 6, 24 Hz), 3.22 (1H, ddd, $J=3.5$, 6, 24 Hz), 3.57 (3H, s), 4.93 (1H, septet, $J=6$ Hz), 5.66 (1H, br triplet, $J=6$ Hz), 7.06 (1H, t, $J=3.5$ Hz), 7.20—8.00 (4H, m).

A mixture of 3-(1-methylethyl) 1-methyl 4-methyl-2-(2-nitrophenyl)-2,5-dihydrobenzene-1,3-dicarboxylate (0.60 g), NaOAc (0.24 g), and I_2 (0.50 g) in MeOH (3 ml) was stirred for 3 h at room temperature, and then refluxed for 9 h. After cooling, EtOAc and water were added to the mixture. The organic layer was separated, washed with aq. $Na_2S_2O_3$, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel with CH_2Cl_2 as an eluent to afford **6** (0.36 g). IR (neat) cm^{-1}: 1710. NMR ($CDCl_3$): 1.23—1.90 (8H, m), 2.40 (3H, s), 3.63 (3H, s), 4.84—5.10 (1H, m), 7.17—7.37 (2H, m), 7.39—7.57 (2H, m), 8.06 (1H, d, $J=8$ Hz), 8.15—8.34 (1H, m). MS *m/z*: 326 ($M^+ - OMe$), 310 ($M^+ - NO_2$).

6-Cyclopentyl 2-Methyl 3-Chloro-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (7) To a solution of PCl_5 (4.32 g) in $CHCl_3$ (50 ml) was added the crude product [3-cyclopentyl 1-methyl 2-(2-nitrophenyl)-4-methyl-6-

oxo-3-cyclohexene-1,3-dicarboxylate] (3.06 g) with ice-bath cooling. After stirring for 6 d at room temperarure, PCl_5 (1.4 g) was added to the mixture and refluxed for 5 h. After cooling, water and EtOAc were added to the solution. The organic layer was separated, washed with aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography on silica gel with a mixture of *n*-hexane and EtOAc as eluent to afford 3-chloro-6-cyclopentyloxycarbonyl-5-methyl-2′-nitro-(1,1′-biphenyl)-2-carbonyl chloride (1.17 g, higher *Rf*), and 6-cyclopentyl 2-methyl 3-chloro-5-methyl-2′-nitro-(1,1′-biphenyl)-2, 6-dicarboxylate (**7**) (0.64 g, lower *Rf*) as oils.

3-Chloro-6-cyclopentyloxycarbonyl-5-methyl-2′-nitro-(1,1′-biphenyl)-2-carbonyl Chloride: IR (neat) cm^{-1}: 1770, 1710. NMR ($CDCl_3$): 0.93—1.89 (8H, m), 2.42 (3H, s), 4.89—5.14 (1H, m), 7.26—7.51 (1H, m), 7.41 (1H, s), 7.50—7.79 (2H, m), 8.23—8.38 (1H, m). MS *m/z*: 422 (M^+), 386 ($M^+ - Cl$).

7: IR (neat) cm^{-1}: 1710. NMR ($CDCl_3$): 1.25—1.90 (8H, m), 2.43 (3H, s), 3.53 (3H, s), 4.90-5.17 (1H, m), 7.27—7.54 (1H, m), 7.39 (1H, s), 7.54—7.77 (2H, m), 8.16—8.36 (1H, m). MS *m/z*: 386 ($M^+ - OMe$), 3.71 ($M^+ - NO_2$).

6-(1-Methylethyl) 2-Methyl 3-Benzyloxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (9e) A typical example is given to illustrate the general procedure.

A mixture of **8a** (0.45 g), benzylchloride (0.17 g), K_2CO_3 (0.11 g), and KI (0.08 g) in dimethylformamide (DMF) (2.2 ml) was stirred at room temperature overnight. After partition of the reaction mixture between EtOAc and water, the organic layer was separated, washed with water and brine, dried over $MgSO_4$ and evaporated. The crude product was recrystallized from a mixture of EtOAc and IPE to afford pure **9e** (0.31 g), mp 120—121 °C. *Anal.* Calcd for $C_{26}H_{25}NO_7$: C, 67.38; H, 5.44; N, 3.02. Found: C, 67.48; H, 5.29; N, 3.09. IR (Nujol) cm^{-1}: 1720. NMR ($CDCl_3$): 0.77 (3H, d, $J=9$ Hz), 0.98 (3H, d, $J=9$ Hz), 2.93 (3H, s), 3.47 (3H, s), 4.83 (1H, septet, $J=9$ Hz), 5.17 (2H, s), 6.88 (1H, s), 7.10—7.73 (8H, m), 8.00—8.36 (1H, m).

Other compounds **9a—f** listed in Table V were similarly prepared.

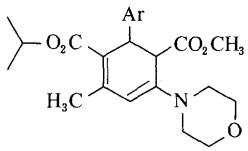
6-(1-Methylethyl) 2-Methyl 5-Methyl-3-(1-methylethoxycarbonyloxy)-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (10c) A typical example is given to illustrate the general procedure.

To a solution of **8a** (0.78 g) and Et_3N (1.5 ml) in $CHCl_3$ (6 ml) was added chloro 1-methylethylcarbonate (0.54 g) in an ice bath, and stirred for 1 h at room temperature. The mixture was washed with aq. $NaHCO_3$ and dil. HCl, dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography on silica gel with a mixture of benzene and EtOAc as eluent and recrystallization from IPE afforded 0.49 g of pure **10c**, mp 89—90 °C. *Anal.* Calcd for $C_{24}H_{27}NO_9$: C, 60.88; H, 5.48; N, 3.05. Found: C, 60.77; H, 5.61; N, 2.87. IR (Nujol) cm^{-1}: 1755, 1715, 1705. NMR ($CDCl_3$): 0.83 (3H, d, $J=6$ Hz), 0.90—1.15 (9H, m), 2.06 (1H, octet, $J=6$ Hz), 2.44 (3H, s), 3.51 (3H, s), 4.06 (2H, d, $J=6$ Hz), 4.85 (1H, septet, $J=6$ Hz), 7.20 (1H, s), 7.16—7.79 (3H, m), 8.10—8.30 (1H, m).

Other compounds **10a, b, 10′a** listed in Table V were similarly prepared.

6-Methyl 5-Hydroxy-7-(2-nitrophenyl)-1(3*H*)-isobenzofuranone-6-carboxylate (11) A mixture of **8′a** (0.63 g) and *p*-TsOH (0.14 g) in MeOH (15 ml) was refluxed for 9 h and cooled. To the solution were added EtOAc and aq. $NaHCO_3$, and the organic layer was separated, and washed with water. The solvent was evaporated, and the residue was washed with toluene. The crude crystals were dissolved in $CHCl_3$–MeOH and the

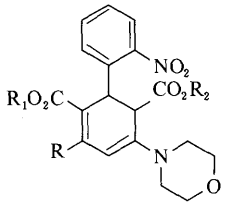
TABLE VII. Physical Properties of 1,2-Dihydrobenzene-1,3-dicarboxylic Acid Derivatives



Compd. No.	Ar	mp (°C) Recryst. solv.[a]	Yield (%)	Formula	Analysis (%) Calcd (Found) C	H	N
3a	2-NO_2-Ph	133—134 IPE–A	43.7	$C_{23}H_{28}N_2O_7$	62.15 (62.59	6.35 6.46	6.30 6.27)
3b	3-NO_2-Ph	148—149 H–A	60.1	$C_{23}H_{28}N_2O_7$	62.15 (62.49	6.35 6.39	6.30 6.27)
3c	4-NO_2-Ph	118—122 IPE	54.2	$C_{23}H_{28}N_2O_7$	62.15 (62.13	6.35 6.41	6.30 5.93)
3d	Ph	125 IPE	29.9	$C_{23}H_{29}NO_5$	69.15 (69.09	7.32 6.85	3.51 3.50)
3e	2-Me-Ph	97—99 H–IPE	21.0	$C_{24}H_{31}NO_5$	69.71 (69.51	7.56 7.26	3.39 3.34)
3f	2-MeO-Ph	122.5—123.5 IPE	14.0	$C_{24}H_{31}NO_6$	67.10 (67.20	7.27 7.20	3.26 2.91)
3g	2-$PhCH_2O$-Ph	108—110 H–A	20.9	$C_{30}H_{35}NO_6$	71.27 (71.35	6.98 6.64	2.77 2.73)
3h	2-F-Ph	Oil[b]	26.3	$C_{23}H_{28}FNO_5$			
3i	2-Cl-Ph	117—118 IPE	38.8	$C_{23}H_{28}ClNO_5$	63.66 (63.95	6.50 6.64	3.23 3.14)
3j	2-CN-Ph	161—164 IPE–A	50.8	$C_{24}H_{28}N_2O_5$	67.91 (67.83	6.65 6.26	6.60 6.44)
3k	2-Thienyl	Oil[c]	30.6	$C_{21}H_{27}NO_5S$			
3l	2-Py	122—124 IPE	41.2	$C_{22}H_{28}N_2O_5$	65.98 (65.81	7.05 6.90	7.00 6.99)
3m	3-Py	126—128 IPE	36.0	$C_{22}H_{28}N_2O_5$	65.98 (65.81	7.05 7.06	7.00 6.98)
3n	4-Py	104—106 IPE	14.3	$C_{22}H_{28}N_2O_5 \cdot 0.25H_2O$	65.25 (65.54	7.09 6.88	6.92 7.00)

a) A, ethyl acetate; H, *n*-hexane. *b*) MS *m/z*: 417 (M^+), 358 ($M^+ - O$-iso-Pr). *c*) MS *m/z*: 405 (M^+), 346 ($M^+ - O$-iso-Pr).

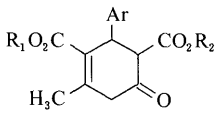
TABLE VIII. Physical Properties of 1,2-Dihydrobenzene-1,3-dicarboxylic Acid Derivatives



Compd. No.	R	R_1	R_2	mp (°C) Recryst. solv.[a]	Yield (%)	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
3o	Me	Me	iso-Pr	Oil[b]	76.2	$C_{23}H_{28}N_2O_7$						
3p	Me	Me	Me	151—152 IPE–A	47.6	$C_{21}H_{24}N_2O_7$	60.57	5.81	6.73	60.50	5.76	6.57
3q	Me	Cl-$(CH_2)_2$–	Me	152.5—154 EtOH	70.2	$C_{22}H_{25}ClN_2O_7$	56.83	5.42	6.03	56.93	5.29	6.03
3r	Me	Et	Me	159—161 H–A	71.2	$C_{22}H_{26}N_2O_7$	61.39	6.09	6.51	61.44	6.01	6.47
3s	Me	$PhCH_2$–	Me	165—167 H–A	53.4	$C_{27}H_{28}N_2O_7$	65.84	5.73	5.69	66.31	5.68	5.66
3t	Me	*tert*-Bu	Me	127—130 H–A	24.2	$C_{24}H_{30}N_2O_7$	62.87	6.59	6.11	62.82	6.79	6.14
3u	Me	Cyclopentyl	Me	168—169 H–A	54.3	$C_{25}H_{30}N_2O_7$	63.82	6.43	5.95	64.12	6.30	5.88
3′a	$AcOCH_2$	iso-Pr	Me	152—156 THF	34.5	$C_{25}H_{30}N_2O_9$	59.75	6.02	5.57	59.91	6.06	5.50

a) A, ethyl acetate; H, *n*-hexane. *b*) MS *m/z*: 444 (M^+), 385 ($M^+ - O$-iso-Pr).

TABLE IX. Physical Properties of 6-Oxo-3-cyclohexene-1,3-dicarboxylic Acid Diesters



Compd. No.	Ar	R_1	R_2	mp (°C) Crystallizing solv.[a]	Yield (%)	Formula	Analysis (%) Calcd C	Calcd H	Calcd N	Found C	Found H	Found N
5a	2-NO_2-Ph	iso-Pr	Me	127—130 A–IPE	72.7	$C_{19}H_{21}NO_7$	60.79	5.64	3.73	60.91	5.53	3.73
5c	4-NO_2-Ph	iso-Pr	Me	157—161 IPE	71.9	$C_{19}H_{21}NO_7$	60.79	5.64	3.73	60.76	5.56	3.73
5f	2-MeO-Ph	iso-Pr	Me	82—84 IPE	54.8	$C_{20}H_{24}O_6$	66.65	6.71		66.33	6.36	
5j	2-CN-Ph	iso-Pr	Me	92—97 IPE	25.4	$C_{20}H_{21}NO_5$	67.59	5.96	3.94	67.72	5.62	4.09
5m	3-Py	iso-Pr	Me	108—111 IPE	46.9	$C_{18}H_{21}NO_5$	65.24	6.39	4.23	64.93	6.36	4.20
5n	4-Py	iso-Pr	Me	122—123 IPE	67.4	$C_{18}H_{21}NO_5$	65.24	6.39	4.23	64.83	6.30	4.21
5p	2-NO_2-Ph	Me	Me	131—134 A–IPE	48.0	$C_{21}H_{25}NO_7$	58.79	4.93	4.03	59.04	4.96	4.06

a) A, ethyl acetate.

insoluble material was removed off by filtration. The filtrate was evaporated and the resulting crystals were washed with EtOAc and MeOH to afford **11** (0.29 g), mp 208—210 °C. *Anal.* Calcd for $C_{16}H_{11}NO_7 \cdot 0.25H_2O$: C, 57.58; H, 3.47; N, 4.20. Found: C, 57.83; H, 3.23; N, 4.06. IR (Nujol) cm^{-1}: 1755, 1660. NMR (DMSO-d_6): 3.40 (3H, s), 5.34 (2H, s), 7.15 (1H, s), 7.20—7.44 (1H, m), 7.63—7.87 (2H, m), 8.10—8.34 (1H, m).

1-Methylethyl 3-Hydroxy-5-methyl-2-methylcarbamoyl-2′-nitro-(1,1′-biphenyl)-6-carboxylate (12) A mixture of **8a** (0.77 g) and $MeNH_2$ (35% in EtOH, 10 ml) was stirred for 10 d at room temperature and diluted with IPE. The resulting precipitates were collected by filtration and recrystallized from $CHCl_3$–EtOAc to afford **12** (0.58 g), mp 189—190 °C. *Anal.* Calcd for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41; N, 7.52. Found: C, 60.96; H, 5.16; N, 7.75. IR (Nujol) cm^{-1}: 1715, 1635, 1625. Conformer A: NMR ($CDCl_3$): 0.94 (3H, d, J=6Hz), 0.98 (3H, d, J=6Hz), 2.30 (3H, s), 2.55 (3H, s), 4.82 (1H, septet, J=6Hz), 6.05 (1H, br s), 6.90 (1H, s), 7.23—7.43 (1H, m), 7.43—7.83 (2H, m), 8.00—8.33 (1H, m), 10.06 (1H, br s). Conformer B: NMR ($CDCl_3$): 0.94 (3H, d, J=6Hz), 0.98 (3H, d, J=6Hz), 2.30 (3H, s), 2.60 (3H, s), 4.82 (1H, septet, J=6Hz), 6.05 (1H, br s), 6.90 (1H, s), 7.23—7.43 (1H, m), 7.43—7.83 (2H, m), 8.00—8.33 (1H, m), 10.06 (1H, br s).

3-Hydroxy-2-methoxycarbonyl-5-methyl-2′-nitro-(1,1′-biphenyl)-6-carboxylic Acid (13) A solution of **8t** (0.21 g) in TFA (0.5 ml) was stirred at room temperature overnight, made alkaline with aq. NaOH and washed with ether. The solution was acidifide with 2 N HCl and stirred for 0.5 h in an ice bath. The resulting precipitates were collected by filtration, washed with cold water and dried to afford **13** (0.14 g) as a powder, mp 170—172 °C. *Anal.* Calcd for $C_{16}H_{13}NO_7$: C, 58.01; H, 3.96; N, 4.23. Found: C, 57.86; H, 3.90; N, 4.12. IR (Nujol) cm^{-1}: 2600—1800, 1720, 1690, 1660. NMR ($CDCl_3$): 2.36 (3H, s), 3.40 (3H, s), 4.50—5.50 (1H, br m), 6.95 (1H, s), 7.10—7.73 (3H, m), 8.05—8.25 (1H, m), 10.3—11.3 (1H, br m).

3-Hydroxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylic Acid (14) To a solution of 2-(1-methylethyl) 6-methyl 3-hydroxy-5-methyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (0.2 g) in acetone (3 ml), 10% NaOH (1 ml) was added and refluxed overnight. After cooling the solution was washed with ether, acidified with 2 N HCl and extracted with $CHCl_3$. The extract was washed with brine, dried over $MgSO_4$, and evaporated. The residue was triturated with a mixture of ether and petroleum ether to afford **14** (0.09 g), mp 216—219 °C. *Anal.* Calcd for $C_{15}H_{11}NO_7 \cdot 0.25H_2O$: C, 55.99: H, 3.60; N, 4.35. Found: C, 55.75; H, 3.45; N, 4.15. IR (Nujol) cm^{-1}: 1650, 1595, 1560, 1520. NMR (DMSO-d_6): 2.33 (3H, s), 3.00—4.00 (2H, br m), 6.90 (1H, s), 7.25 (1H, dd, J=7, 3 Hz), 7.50—7.90 (2H, m), 8.25 (1H, dd, J=7, 3 Hz), 11.0—12.5 (1H, br m).

6-(1-Methylethyl) 8-Hydroxy-6-methyl-9*H*-dibenzo[*b*,*d*]pyran-9-one-6-carboxylate (15′) A solution of **8a** (0.21 g) in MeOH (20 ml) was treated with 10% Pd/C (0.06 g) and stirred under a hydrogen atmosphere for 4 h. The mixture was filtered and the solvent was removed under reduced pressure. The residue was recrystallized from petroleum ether (27 ml) to afford **15′** (0.09 g), mp 142 °C. *Anal.* Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 68.76; H, 4.98. IR (Nujol) cm^{-1}: 1720, 1680. NMR ($CDCl_3$): 1.36 (6H, d, J=6Hz), 2.40 (3H, s), 5.35 (1H, septet, J=6Hz), 6.93 (1H, s), 7.1—7.6 (3H, m), 7.86 (1H, dd, J=1, 7 Hz).

Compound **15** was similarly prepared.

2-Bromo-1,3-dicyano-4,6-dimethylbenzene (17) To a mixture of conc. H_2SO_4 (12 ml) and AcOH (3 ml), $NaNO_2$ (1.04 g) was added portionwise with cooling from an ice bath. The mixture was stirred for 1.5 h at room temperature. Compound **16** (2.57 g) was added portionwise to the reaction mixture, and stirred for 2.5 h with ice bath cooling. The above solution was added to a solution of CuBr (2.2 g) in 48% HBr (15 ml) in an ice bath. After stirring for 0.5 h, ice water was added to the mixture and the resulting precipitates were collected by filtration and recrystallized from a mixture of *n*-hexane and EtOAc to afford 2.32 g of **17**, mp 157—161 °C. *Anal.* Calcd for $C_{10}H_7BrN_2$: C, 51.09; H, 3.00; N, 11.92. Found: C, 51.23; H, 2.85; N, 11.96. IR (Nujol) cm^{-1}: 2210. NMR (DMSO-d_6): 2.56 (6H, s), 7.60 (1H, s).

Dimethyl 2-Bromo-4,6-dimethylbenzene-1,3-dicarboxylate (18) A suspension of **17** (301 mg) in conc. H_2SO_4 (3.2 ml) and water (0.7 ml) was refluxed for 7 h and cooled. To the solution was added a solution of $NaNO_2$ (283 mg) in water (1.5 ml) and heated for 2 h at 80 °C. After cooling, ice water was added to the solution and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was co-evaporated three times with toluene, dissolved in DMF (7 ml), and K_2CO_3 (1.42 g) and MeI (0.8 ml) were added thereto. The mixture was heated at 40 °C for 5 h. After cooling, ice water was added to the mixture, and extracted with CH_2Cl_2. The extract was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel with a mixture of *n*-hexane and CH_2Cl_2 as an eluent to afforded **18** (153 mg), mp 54—56 °C (recrystallized from a mixture of *n*-hexane and EtOAc). *Anal.* Calcd for $C_{12}H_{13}BrO_4$: C, 47.86; H, 4.35. Found: C, 48.13: H, 4.25. IR (Nujol) cm^{-1}: 1720. NMR ($CDCl_3$): 2.30 (6H, s), 3.92 (6H, s), 6.98 (1H, s).

Dimethyl 3,5-Dimethyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (19) To a melt of 1-iodo-2-nitrobenzene (1.90 g) and **18** (6.29 g) was added copper powder (6.42 g). The mixture was heated at 200 °C for 1 h and cooled. To the mixture was added CH_2Cl_2 and the insoluble material was removed by filtration. The filtrate was evaporated and the residue was purified by column chromatography on silica gel with a mixture of *n*-hexane and CH_2Cl_2 as an eluent to afford 0.76 g of **19**, mp 132—134 °C (recrystallized from a mixture of *n*-hexane and EtOAc). *Anal.* Calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.88; H, 4.83; N, 4.11. IR (Nujol) cm^{-1}: 1720. NMR ($CDCl_3$): 2.37 (6H, s), 3.43 (6H, s), 7.03—7.30 (2H, m), 7.32—7.63 (2H, m), 8.03—8.16 (1H, m).

0.30 g of 2,2′,6,6′-tetramethyl 3,3′,5,5′-tetramethyl-(1,1′-biphenyl)-2,2′,6,6′-tetracarboxylate was obtained as a minor product, mp 140—143 °C (recrystallized from a mixture of *n*-hexane and EtOAc). *Anal.* Calcd for $C_{24}H_{26}O_8$: C, 65.15; H, 5.92. Found: C, 65.23; H, 5.79. IR (Nujol) cm^{-1}: 1715. NMR ($CDCl_3$): 2.38 (12H, s), 3.48 (12H, s), 7.01 (2H, s).

3,5-Dimethyl-2-methoxycarbonyl-2′-nitro-(1,1′-biphenyl)-6-carboxylic Acid (20) A mixture of **19** (100 mg) and $Ba(OH)_2 \cdot 8H_2O$ (0.37 g) in dioxane (3.6 ml) and water (3.5 ml) was refluxed at 70 °C for 3 h. After cooling, the solution was neutralized with 5% HCl and extracted with EtOAc. The extract was washed with water and brine, dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography on silica gel with a mixture of CH_2Cl_2 and MeOH as eluent to afford **20** (72 mg), mp 164—168 °C. *Anal.* Calcd for $C_{17}H_{15}NO_6 \cdot 0.5H_2O$: C, 60.35; H, 4.77; N, 4.14. Found: C, 60.53; H, 4.51; N, 4.07. IR (Nujol) cm^{-1}:

2800—2100, 1720, 1685. NMR ($CDCl_3$): 2.35 (3H, s), 2.38 (3H, s), 3.40 (3H, s), 7.03—7.25 (2H, m), 7.30—7.75 (3H, m), 7.95—8.10 (1H, m).

2-Methyl 6-(2-Phenylethyl) 3,5-Dimethyl-2′-nitro-(1,1′-biphenyl)-2,6-dicarboxylate (21) To a suspension of **20** (65 mg) in CH_2Cl_2 (1.5 ml) was added PCl_5 (46 mg) and stirred for 30 min with ice bath cooling. After being stirred for 2.5 h at room temperature, the solution was evaporated and then co-evaporated with benzene. The resulting powder was soon dissolved in CH_2Cl_2 and 2-phenylethylalcohol (0.6 ml) and pyridine (0.5 ml) were added thereto at 0 °C. After stirring at 70 °C for 3 h, the solution was acidified with 5% HCl and extracted with CH_2Cl_2. The extract was washed with water and brine, dried over $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel with a mixture of CH_2Cl_2 and MeOH as eluent to afford **21** (60 mg), mp 72—75 °C (recrystallized from a mixture of ether and *n*-hexane). *Anal.* Calcd for $C_{25}H_{23}NO_6$: C, 69.27; H, 5.35; N, 3.23. Found: C, 69.42; H, 5.26; N, 3.23. IR (Nujol) cm^{-1}: 1720. NMR ($CDCl_3$): 2.30 (3H, s), 2.36 (3H, s), 2.57 (2H, t, $J=7$ Hz), 3.42 (3H, s), 4.02 (1H, t, $J=7$ Hz), 4.06 (1H, t, $J=7$ Hz), 6.93—7.60 (9H, m), 7.93—8.13 (1H, m).

***In Vitro* Studies** Guinea-pigs weighing 320—650 g were killed by blood-letting. The lower abdomen was opened and longitudinally oriented strips of the urinary bladder, 15—20 mm long and 5 mm wide, were excised. The strips were suspended in tissue baths containing 25 ml of Krebs solution. Throughout the experiment, the bathing solution was maintained at 37 °C and continuously aerated with a 95% oxygen and 5% carbon dioxide gas mixture. Bladder strip contactions were recorded isometrically with an electromechanical displacement transducer and a polygraph. All muscle strips were stretched initially to 1 g of tension and allowed to accommodate to this length, and to the bath milieu, for at least 30 min before any durg additions were made. In each instance 15 min intervals were allowed between drug additions. Single strips were exposed only to a single agonist or electrical stimulation and a drug.

Fixed doses of carbacol (10 μM), KCl (30 mM) were used as agonists. To stimulate the bladder strips electrically, two platinum electrodes were placed parallel to each other and 15 mm apart both sides of the tissue preparation. The intensity of square wave stimuli was adjusted to obtain submaximal contractions at a constant frequency of 10 Hz and duration of 1 ms. Usually the electrical intensity was around 10 V, and stimulation was given to the detrusor strips for 5 s every 5 min. The effects of the agonists or the electrical stimuli were examined with variation in concentration of the drugs added to the bath 10 min prior to the administration of the agonists or electrical stimuli.

Male S. D. strain rats, weighing 200—300 g, were killed by bleeding and the thoracic aorta were removed. The helical strips (2.0 × 15 mm) were suspended in an organ bath filled with 25 ml of Tyrode's solution. The strips were connected to a strain gauge and the tension was measured isometrically. The bath solution was bubbled with a mixture of 95% O_2 and 5% CO_2 and was maintained at 37 °C. After the resting tension was adjusted to 0.5 g, the arterial strips were contracted by 30 mM KCl. The test drugs were added in the organ bath cumulatively. At the end of each test, 10^{-4} M of papaverine was added to the organ bath to obtain the maximum relaxation. This effect was taken as a standard for 100% relaxation.

***In Vivo* Studies** Sprague Dawley rats, weighing 220—320 g, were anesthetized with a subcutaneous dose of 1.0 g/kg of urethane and fixed in a supine position. The lower abdomen was opened along the midline to fully expose the urinary bladder. A rubber balloon was inserted into the bladder through a small incision of the wall around the apex, and was connected with a pressure transducer through a polyethylene tube. The bladder was carefully packed with a cotton-wool pad soaked in warm saline and kept warm. The balloon was filled with approximately 1 ml of water, and then pressured. Rhythmic contractions of the urinary bladder became constant at a threshold intravesical pressure between 5 and 15 mmHg, and reached a maximum contraction 50 to 70 mmHg with an amplitude of contraction of 40 to 60 mmHg. After this control period, the drugs were administered intravenously or intraduodenally, and the inhibitory effects were estimated by the reduction in amplitude of the bladder contractions.

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Hydrophobicity Parameters Determined by Reversed-Phase Liquid Chromatography. III.[1] Influence of Stationary and Mobile Phases on the Relationship between the Capacity Factor and the Octanol–Water Partition Coefficient for Pyrazine Derivatives

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Our previous study on the relationship between the logarithm of 1-octanol/water partition coefficient ($\log P$) by the shake-flask method and the logarithm of the capacity factor ($\log k'$) by reversed-phase liquid chromatography for monosubstituted pyrazines (2PR) has been extended to the 2-chloro-6-substituted pyrazine series (6PR) and also to other reversed-phase high-performance liquid chromatographic conditions. Analyses have shown that 6PR behave similarly to 2PR. The influences of stationary and mobile phases were studied using several commercially available reversed-phase columns, and various organic modifiers. The results, from any combination of the stationary phases and the modifiers examined, have presented analogous trends to those previously observed: (1) The $\log P$–$\log k'$ relationship becomes more complicated as the concentration of organic modifier in the mobile phase decreases due to the intervention of the electronic interactions and the retardation effect ascribed to ester and amide substituents. (2) Amphiprotic substituents usually exhibit an acceleration effect and should be treated separately. Practical conditions to be used for predicting the $\log P$ values are proposed.

Keywords reversed-phase HPLC (RPLC); capacity factor; partition coefficient; hydrophobicity; amphiprotic substituent; mobile phase; hydrogen-bond; pyrazine

The hydrophobic property of bioactive compounds, expressed by the logarithm of 1-octanol/water partition coefficient ($\log P$) is one of the most important factors to be considered in quantitative structure–activity relationship (QSAR) studies.[2,3] The logarithm of capacity factor ($\log k'$), measured by reversed-phase high-performance liquid chromatography (RPLC) has increasingly been used for predicting the $\log P$ value as a counterpart of the standard and conventional shake-flask method.[4–8] The RPLC techniques are considered especially useful for estimation of $\log P$ values of highly lipophilic compounds.

Generally, $\log k'$ values for a series of congeners eluted by methanol (MeOH)–water eluents on an alkyl-bonded stationary phase are considered to correlate linearly with $\log P$. However, the linearity is often perturbed by other factors such as hydrogen-bond effects and selective solute–solvent interactions. At the present stage, most successful examples are found in benzene derivatives,[6–9] and little has been studied so far on the relationship between $\log P$ and $\log k'$ for heterocyclic compounds where the important hydrogen-bond effects are expected to be involved. One of the reasons for such paucity is the fact that accumulation of fundamental heterocyclic $\log P$ data has been too meager, except for the pyridines,[10,11] to enable systematic studies in this field. Considering that a number of bioactive compounds have various types of heterocyclic rings as the parent skeleton, we started to measure and evaluate the hydrophobicity parameters in various heteroaromatic systems.

In previous works,[12,13] we measured the $\log k'$ value for a series of monosubstituted pyrazines (2PR), using the Capcell pak C_{18} column and different compositions of MeOH-buffer (pH 9.2) eluents, and analyzed the relationship with our experimentally determined $\log P$ values. It was demonstrated that the $\log k'$ value was formulated by the following general equation in the range from 15 to 70 percent of MeOH (% MeOH).

$$\log k' = a \log P + bHB_{CO} + cHB_{AM} + \rho\sigma_I + \text{const.} \quad (1)$$

where $HB_{CO}=1$ for ester and amide substituents and 0 for the others, $HB_{AM}=1$ for amphiprotic substituents and 0 for the others, and σ_I is Charton's electronic constant.[14] The HB_{CO} and σ_I terms were insignificant or of small significance around 50% MeOH; the plot of $\log k'$ against $\log P$ presented parallel lines belonging to amphiprotics and non-amphiprotics. As the MeOH concentration in the eluents was lowered, the b and ρ values became more positive and more negative, respectively, indicating that the relationship became more complicated. Equation 1 turned out to be insufficient for describing $\log k'$ at 5% MeOH.[1] Analyses with the conventionally used $\log k_W$ parameter (the $\log k'$ value in 100% water) were also attempted. However, neither the experimentally determined nor the extrapolated value mimicked $\log P$.[13,15]

The complicated behavior of the pyrazine derivatives was ascribed to the electron-withdrawing property of the pyrazine ring which affected the over-all hydrogen bonding effect of the molecule.

To establish a RPLC system which could predict reliable $\log P$ values of polar solutes like ours, it would be useful to study the influence of stationary and mobile phases on the relationship between $\log P$ and $\log k'$. In this work we measured the capacity factors for 2PR using alkyl- and phenyl-modified columns as the stationary phase and three different types of solvents as the organic modifier in the mobile phase. The results were analyzed by an analogous treatment. Studies were also extended to the analysis of $\log k'$ values for the 2-chloro-6-substituted pyrazine (6PR) series with the intention of exploring whether Eq. 1 would apply to a disubstituted pyrazines system.

Experimental

Compounds The compounds tested in this work are 2PR and 6PR shown in Table I. The preparation of the compounds has been described elsewhere.[12,16]

Partition Coefficients 1-Octanol/water $\log P$ values, measured by the shake-flask method, were taken from our previous work.[12,16]

RPLC Procedure The apparatus used was the same as previously described.[13] Commercial capcell pak[17] C_{18}, C_8 and phenyl columns

TABLE I. log k' Values for 2PR and 6PR

2PR X	C8[a]			Ph(CP)[b,c]				Ph(CS)[d,e]			C18[b]						
	Mobile phase[f]																
	M15	M30	M50	M15	M30	M50	M70	M30	M50	M70	A5	A15	A30	D5	D10	P5	P15
H	0.007	−0.322	−0.648	−0.033	−0.293	−0.602	−0.791	−0.157	−0.478	−0.759	0.006	−0.466	−0.761	−0.303	−0.517	−0.059	−0.457
F	0.158	−0.119	−0.432	0.074	−0.124	−0.404	−0.670	−0.078	−0.355	−0.703	0.359	0.060	−0.172	0.159	0.033	0.012	−0.311
Cl	0.474	0.158	−0.201	0.368	0.116	−0.222	−0.547	0.164	−0.178	−0.554	0.739	0.368	0.056	0.564	0.402	0.413	0.049
Me	0.342	−0.071	−0.496	0.299	−0.034	−0.431	−0.724	0.046	−0.331	−0.686	0.451	−0.133	−0.520	0.156	−0.093	0.369	−0.130
Et	0.732	0.269	−0.224	0.655	0.275	−0.193	−0.595	0.323	−0.127	−0.553	0.934	0.323	−0.130	0.660	0.402	0.733	0.167
OMe	0.662	0.290	−0.140	0.580	0.291	−0.138	−0.490	0.277	−0.108	−0.506	0.860	0.368	−0.020	0.656	0.453	0.579	0.129
OEt	1.138	0.700	0.181	1.007	0.645	0.127	−0.326	0.596	0.127	−0.350	1.377	0.821	0.340	1.165	0.933	1.035	0.529
OPr	1.641	1.149	0.519	1.478	1.038	0.399	−0.147	0.944	0.374	−0.188	1.948	1.330	0.734	1.727	1.466	1.554	0.992
SMe	0.958	0.538	0.041	0.875	0.524	0.038	−0.396	0.532	0.078	−0.363	1.229	0.699	0.241	1.043	0.816	0.905	0.409
CN	0.023	−0.276	−0.595	0.053	−0.162	−0.466	−0.732	−0.050	−0.349	−0.715	0.378	0.065	−0.169	0.120	−0.021	−0.253	−0.640
Ac	0.350	−0.035	−0.430	0.324	0.030	−0.331	−0.643	0.146	−0.240	−0.613	0.652	0.118	−0.247	0.368	0.143	0.103	−0.392
CO_2Me	0.256	−0.193	−0.634	0.264	−0.078	−0.482	−0.753	0.061	−0.337	−0.703	0.572	−0.094	−0.526	0.216	−0.090	−0.032	−0.603
CO_2Et	0.720	0.210	−0.312	0.652	0.257	−0.223	−0.607	0.358	−0.116	−0.552	1.072	0.344	−0.168	0.713	0.391	0.433	−0.248
$CONH_2$	−0.235	−0.670	−1.126	−0.166	−0.488	−0.880	—	−0.306	−0.707	−1.128	−0.041	−0.725	−1.383	−0.348	−0.649	−0.650	−1.460
$CONMe_2$	—	−0.470	−0.923	—	—	—	—	—	—	—	—	—	—	−0.189	−0.559	—	—
NH_2	−0.110	−0.477	−0.902	−0.085	−0.385	−0.757	−0.988	−0.262	−0.649	−1.068	−0.084	−0.674	−1.201	−0.338	−0.582	—	—
NHMe	0.391	−0.067	−0.551	0.353	−8.029	−0.460	−0.801	0.085	−0.378	−0.806	0.513	−0.143	−0.639	0.264	−0.014	—	—
NMe_2	0.947	0.422	−0.145	0.906	0.456	−0.087	−0.481	0.511	−0.010	−0.467	1.165	0.464	−0.082	0.894	0.584	—	0.280
NHAc	0.121	−0.326	−0.786	0.130	−0.213	−0.635	−0.903	−0.097	−0.516	−0.928	0.348	−0.379	−0.855	0.031	−0.279	−0.167	−0.874

2PR X	C8[d]		Ph(CP)[b]								log P[g]
	Mobile phase[f]										
	TMG5	TMG10	A5	A15	A30	D5	D10	D15	P5	P15	TMG5	TMG10	
H	0.180	0.015	−0.178	−0.509	−0.800	−0.323	−0.484	−0.598	−0.210	−0.499	0.067	−0.040	−0.26
F	0.181	0.070	−0.071	−0.260	−0.437	−0.167	−0.276	−0.358	−0.232	−0.431	0.043	−0.052	0.29
Cl	0.478	0.303	0.233	−0.030	−0.284	0.148	0.011	−0.089	0.103	−0.124	0.324	0.224	0.70
Me	0.500	0.237	0.158	−0.248	−0.611	0.029	−0.165	−0.310	0.131	−0.222	0.427	0.218	0.21
Et	0.762	0.497	0.492	0.046	−0.358	0.389	0.150	−0.010	0.415	0.036	0.691	0.519	0.69
OMe	0.640	0.457	0.374	0.013	−0.329	0.300	0.131	−0.002	0.277	−0.019	0.510	0.401	0.73
OEt	1.010	0.869	0.774	0.339	−0.072	0.703	0.501	0.342	0.655	0.326	0.864	0.740	1.28
OPr	1.434	1.307	1.244	0.718	0.212	1.164	0.910	0.723	1.090	0.728	1.264	1.124	1.84
SMe	0.920	0.778	0.686	0.271	−0.122	0.623	0.425	0.271	0.575	0.239	0.797	0.662	1.17
CN	−0.076	−0.157	−0.028	−0.205	−0.399	−0.152	−0.256	−0.339	−0.326	−0.571	−0.054	−0.159	−0.01
Ac	0.112	0.013	0.188	−0.169	−0.483	0.063	−0.118	−0.248	−0.052	−0.399	0.160	0.026	0.20
CO_2Me	0.001	−0.140	0.150	−0.262	−0.653	−0.006	−0.222	−0.384	−0.095	−0.534	0.147	−0.007	−0.23
CO_2Et	0.378	0.222	0.514	0.042	−0.389	0.359	0.115	−0.064	0.241	−0.216	0.467	0.294	0.28
$CONH_2$	−0.457	−0.572	−0.273	−0.709	−1.057	−0.387	−0.587	−0.753	−0.501	−0.973	−0.225	−0.384	−0.50
$CONMe_2$	−0.355	−0.500	—	—	—	—	—	—	—	—	—	—	−0.80
NH_2	−0.054	−0.216	−0.191	−0.567	−0.949	−0.313	−0.483	−0.620	−0.235	−0.547	−0.041	−0.175	−0.05
NHMe	0.505	0.301	0.241	−0.199	−0.636	0.156	−0.067	−0.238	0.218	−0.165	0.441	0.265	0.56
NMe_2	0.968	0.675	0.730	0.205	−0.272	0.630	0.375	0.174	0.660	0.190	0.909	0.684	0.93
NHAc	−0.054	−0.298	0.019	−0.434	−0.881	−0.120	−0.340	−0.508	−0.176	−0.618	0.048	−0.117	−0.03

6PR X	C18[d]				Ph(CS)[d]			log P[h]	log k_w[i]	
	Mobile phase[f]									
	M15	M30	M50	M70	M30	M50	M70			
H	0.603	0.311	−0.106	−0.477	0.164	−0.178	−0.554	0.70	0.87	(−0.999)
F	0.924	0.636	0.265	−0.182	0.335	0.001	−0.415	1.16	1.24	(−0.999)
Cl	1.236	0.927	0.495	0.012	0.533	0.153	−0.293	1.53	1.58	(−0.999)
Me	0.978	0.579	0.120	−0.302	0.377	−0.036	−0.443	1.04	1.30	(−0.999)
OMe	1.479	1.100	0.582	0.049	0.729	0.259	−0.229	1.65	1.88	(−1.000)
OEt	1.974	1.535	0.919	0.270	1.052	0.487	−0.089	2.22	2.45	(−1.000)
OPr	—	2.045	1.278	0.515	1.421	0.730	0.062	2.71	3.19[j]	(−1.000)
CN	0.770	0.448	0.025	−0.455	0.343	−0.025	−0.448	0.79	1.11	(−1.000)
CO_2Me	0.859	0.383	−0.093	−0.525	0.391	−0.039	−0.453	0.47	1.16[k]	(−0.994)
$CONH_2$	0.478	0.052	−0.414	−0.885	0.089	−0.308	−0.715	0.28	0.82	(−0.998)
NH_2	0.648	0.264	−0.224	−0.710	0.159	−0.268	−0.729	0.95	1.01	(−1.000)
NMe_2	1.857	1.290	0.626	0.005	0.975	0.381	−0.174	1.95	2.33	(−0.999)
NHAc	0.933	0.484	−0.029	−0.520	0.385	−0.086	−0.534	1.10	1.30	(−0.999)

a) Aqueous phase: buffer (pH 9.2). *b*) Aqueous phase: buffer (pH 7.4). *c*) Capcell Pak Phenyl. *d*) Aqueous phase: water. *e*) Cosmosil Phenyl. *f*) Organic modifier, M: methanol, A: acetonitrile D: dioxane, P: 1-propanol, TMG: trimethylene glycol, the figure represents the volume % of organic modifier. *g*) Taken from ref. 12. *h*) Taken from ref. 16. *i*) Derived from the linear extrapolation using the data on C18 column for M15—M70. Figures in parentheses are correlation coefficients. *j*) Derived from the linear extrapolation using the data for M30—M70. *k*) Not exactly linear in the range of M15 to M70. The log k_w value derived from the linear extrapolation using the data for M30—M70 is 1.01 (−1.000).

(4.6 mm × 15 cm, Shiseido) [C18, C8 and Ph(CP), respectively] and a Cosmosil 5Ph packed column (4.6 mm × 15 cm, Nakalai tesque) [Ph(CS)] were used without further treatment. Commercial high performance liquid chromatographic (HPLC) grade MeOH, acetonitrile, dioxane and 1-propanol were used without further purification. As an aqueous buffer, 0.01 M sodium borate (pH 9.2) or phosphate buffer (pH 7.4) was used. Eluents were prepared by volume. Appropriate quantities of samples were dissolved in MeOH and 1—2 μl was injected at 25 °C. The flow-rate was 0.2—1.5 ml/min. The capacity factor ($\log k'$) was obtained as previously.[13]

In the beginning of this series of studies, the buffer solutions of pH 9.2 were used because of the necessity of measuring stronger bases (pyridines) simultaneously.[13] Since preliminary examination showed that the eluents at pH 7.4 and 9.2 gave practically equivalent retention data for the pyrazine series, measurements were done afterward at pH 7.4. Measurements for 6PR were done using a MeOH–water mobile phase system because it was shown that the use of buffered solutions was not needed for the compounds used.

Results and Discussion

To examine the effects of stationary phases on the retention data, the series of 2PR was chromatographed on three different stationary phases, C8, Ph(CP) and Ph(CS), with eluents containing 15 to 70% MeOH. The $\log k'$ values obtained are listed in Table I with all the other $\log k'$ values obtained in this study. As demonstrated in our previous work,[13] the possibilities of partial ionization of the solutes and the silanol–solute interactions could be excluded under the conditions we used.

Preliminary analyses of the retention data using Eq. 1 had suggested that the H-acceptors other than ester and amide groups also exerted small but significant hydrogen-bond effects in phenyl-columns. Such an effect usually became involved at very high water contents even on the C18 column. Therefore, the general formula to be investigated was as follows.

$$\log k' = a \log P + bHB_{CO} + cHB_{AM} + \rho\sigma_I + dHB_A + \text{const.} \quad (2)$$

where the parameters except for HB_A are the same as in Eq. 1, therefore,

$HB_{CO}=1$: CO_2R, $CONH_2$, $CONMe_2$, NHAc; 0 for the others

$HB_{AM}=1$: NH_2, NHMe, NHAc, $CONH_2$; 0 for the others

$HB_A=1$: OR, SMe, CN, Ac, NMe_2, NH_2, NHMe; 0 for the others

The significance of each term was tested statistically. Among the correlations obtained for 2PR with different columns, those considered to be most reasonable are summarized in Table II along with our previous results with the C18 column.

We tried to extend our procedure to the 6PR series. The retention data was taken with C18 and Ph(CS) columns (Table I), and was analyzed similarly. Although the number of samples was not enough, good correlations that were similar to those for 2PR were yielded. The results are given in Table III. Since the corresponding coefficients of each term in the correlations for 2PR and 6PR coincided within the 95% interval for 30—70% MeOH, the analyses were done with the combined data set to give very good fits as shown in the lower part in Table III. Again an almost linear relationship was observed at 50% MeOH, so far as amphiprotics were treated separately (Fig. 1).

The $\log k_W$ values for 6PR, derived from the extrapolation using the data on the C18 column (Table I), are included in Table I. Most of them were higher than the $\log P$ values, as was the case with 2PR.[13]

Next, we investigated the influence of mobile phases. The $\log k'$ values for 2PR were measured on C18 and Ph(CP) columns, using acetonitrile, dioxane and 1-propanol as the organic modifiers. Again, Eq. 2 worked well, though the data was limited to narrow ranges of the mobile phase compositions because the retention times at higher organic modifier concentrations were too short to measure. The results are presented in Table IV.

The fact that Eq. 2 applied to $\log k'$ values for various combinations of stationary- and mobile-phases indicates that our treatment is reasonable in spite of using the discrete-type parameters as a first approximation. Examples where the hydrogen-bond effects are successfully described by indicator variables, have often been found not only in the $\log P$–$\log k'$ relationships[4,18] but also in structure–activity relationships.[19]

Inspection of the obtained correlations presents the

TABLE II. Correlations for 2PR on Various Columns by Eq. 2

Column	Mobile[a] phase	a	b	c	ρ	d	Const	n	r	s	F
C18[b]	M15	0.824	0.414	−0.319	−0.371		0.292	19	0.991	0.078	186.7
	M30	0.744	0.237	−0.305			−0.147	19	0.996	0.050	565.7
		0.747	0.248	−0.315	−0.137		−0.113	19	0.997	0.045	513.6
	M50	0.582		−0.349			−0.511	19	0.992	0.060	503.2
		0.620	0.100	−0.361			−0.549	19	0.995	0.047	543.2
	M70	0.495		−0.348			−0.887	17	0.991	0.051	376.7
C8	M15	0.794	0.340	−0.248	−0.404		0.242	18	0.993	0.066	218.1
	M30	0.697	0.228	−0.286	−0.203		−0.165	19	0.995	0.054	317.1
	M50	0.564	0.088	−0.305			−0.577	19	0.995	0.045	485.5
Ph(CP)[c]	M15	0.669	0.438	−0.287	−0.506	0.169	0.155	18	0.996	0.047	292.8
	M30	0.587	0.307	−0.278	−0.256	0.123	−0.153	18	0.998	0.031	550.0
	M50	0.471	0.120	−0.245			−0.496	18	0.995	0.035	468.5
	M70	0.309	0.064	−0.222			−0.746	17	0.991	0.032	239.7
Ph(CS)[d]	M30	0.479	0.322	−0.271	−0.294	0.137	−0.039	18	0.995	0.040	226.7
	M50	0.402	0.140	−0.240			−0.391	18	0.991	0.040	268.9
	M70	0.293	0.082	−0.296			−0.726	18	0.991	0.036	253.4

a) The figure represents the % MeOH. *b*) The data taken from ref. 13. *c*) Capcell pak phenyl. *d*) Cosmosil phenyl.

TABLE III. Correlations for 6PR by Eq. 2

System	Column	Mobile[a] phase	*a*	*b*	*c*	*ρ*	*d*	Const	*n*	*r*	*s*	*F*
6PR	C18	M15	0.780	0.446	−0.349	−0.212	0.177	0.124	12	0.994	0.072	97.1
		M30	0.811	0.241	−0.268			−0.256	13	0.994	0.076	230.1
		M50	0.607		−0.243			−0.451	13	0.990	0.075	249.8
			0.638	0.136	−0.301			−0.507	13	0.994	0.060	265.9
		M70	0.460		−0.300			−0.762	13	0.989	0.066	220.4
	Ph(CS)[b]	M30	0.517	0.372	−0.271		0.163	−0.222	13	0.993	0.059	135.6
		M50	0.382	0.238	−0.266		0.091	−0.440	13	0.996	0.032	272.3
		M70	0.286	0.154	−0.260			−0.724	13	0.995	0.027	297.8
2PR+6PR[c]	C18	M30	0.754	0.229	−0.302			−0.156	31	0.996	0.061	1020.4
		M50	0.632		−0.283			−0.515	31	0.990	0.082	712.8
			0.664	0.144	−0.324			−0.566	31	0.995	0.058	701.0
		M70	0.518		−0.294			−0.879	29	0.987	0.074	495.4
	Ph(CS)[b]	M30	0.473	0.334	−0.288	−0.260	0.157	−0.060	30	0.994	0.047	416.5
		M50	0.383	0.200	−0.262		0.074	−0.426	30	0.997	0.029	931.3
		M70	0.294	0.126	−0.265			−0.732	29	0.992	0.035	487.5

a) The figure represents the % MeOH. *b*) Cosmosil Phenyl. *c*) Combination of mono(2PR)- and di(6PR)-substituted pyrazines.

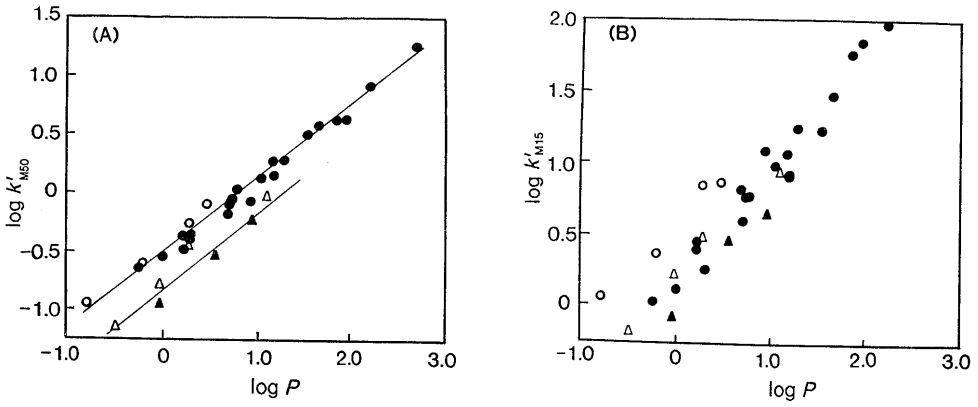

Fig. 1. Relationship between log *P* and log *k'* with C18 Column for 2PR and 6PR

(A): Relationship between log *P* and log k'_{M50}. (B): Relationship between log *P* and log k'_{M15}. Non-hydrogen bonders and H-acceptors are represented by the circles (●, $HB_{CO}=0$; ○, $HB_{CO}=1$) and amphiprotics by the triangles (▲, $HB_{CO}=0$; △, $HB_{CO}=1$).

TABLE IV. Correlations for 2PR from Various Mobile Phase Systems by Eq. 2

Column	Mobile[a] phase	*a*	*b*	*c*	*ρ*	*d*	Const	*n*	*r*	*s*	*F*
C18	A5	0.813	0.583	−0.418	−0.222	0.172	0.277	18	0.993	0.077	162.8
	A15	0.770	0.240	−0.416	0.345		−0.255	18	0.993	0.069	243.0
	A30	0.610		−0.536	0.614		−0.612	18	0.989	0.088	203.2
	D5	0.855	0.484	−0.373		0.138	−0.032	19	0.996	0.057	423.1
	D10	0.837	0.359	−0.386		0.115	−0.227	19	0.996	0.058	420.0
	P5	0.850	0.238	−0.383	−0.692		0.156	15	0.999	0.030	1244.8
	P15	0.768		−0.517	−0.453		−0.320	16	0.995	0.064	433.0
Ph(CP)[b]	A5	0.612	0.422	−0.260	−0.447	0.163	0.016	18	0.992	0.062	140.4
	A15	0.545	0.171	−0.228			−0.332	18	0.987	0.062	178.6
	A30	0.408		−0.274	0.333		−0.677	18	0.992	0.045	292.3
	D5	0.649	0.392	−0.234	−0.404	0.156	−0.116	18	0.995	0.052	214.8
	D10	0.601	0.299	−0.229	−0.222	0.132	−0.305	18	0.997	0.039	340.7
	D15	0.572	0.210	−0.219		0.093	−0.459	18	0.996	0.037	437.3
	P5	0.642	0.274	−0.185	−0.718	0.115	−0.026	18	0.995	0.051	221.4
	P15	0.622		−0.143	−0.397		−0.344	18	0.995	0.048	416.9

a) Organic modifier A: acetonitrile, D: dioxane, P: 1-propanol; the figure represents the volume % of the organic modifiers. *b*) Capcell Pak Phenyl.

following trends.

(a) The sign of the coefficient of HB_{AM} was always negative in all the cases. This feature is shown in Fig. 1 through the downward deviation of the points for amphiprotic substituents. This can be explained, as was discussed in our previous paper.[13] The electron-

withdrawing property of the pyrazine ring enhances the H-donating ability of amphiprotic groups like NHR, $CONH_2$ and NHAc so that such H-donors would behave in a similar manner as phenols which are well known to present an acceleration effect relative to neutral benzenoid compounds[6,20]; the acidic hydrogen in the amphiprotic substituents would undergo hydrogen-bonding more effectively with octanol than any stationary phase examined because of the higher basicity of octanol. Hence, the amphiprotic compounds appear to be more hydrophobic in the octanol/water system than in the HPLC system.

(b) The compounds in the 2PR and 6PR series were well incorporated in the same correlations in the range of 30 to 70% MeOH as shown in Table III, which means that no significant modification of hydrogen-bond effects arises from the introduction of the non-hydrogen bonding second substituent (Cl) into the 2PR. This conforms to our previous finding[16] that the electronic interactions between two substituents in 6PR were small enough to enable the prediction of their log P values by the summation of the π values of the corresponding 2PR as far as the substituents examined were concerned. The situation would, however, not be the same in such a case where one or both of the substituents are amphiprotic(s) of pronounced hydrogen-bond ability.

(c) High linearities between the log k' values for C8 and C18 stationary phases were observed to hold, indicating that both the stationary phases behave similarly. Likewise, the two kinds of Ph-columns from different suppliers also showed a resemblance in retention behavior, except that Ph(CP) is somewhat more dependent on log P than Ph(CS) because, for a given mobile phase, the slope of the log P term for Ph(CP) is higher than that for Ph(CS).

(d) The contributions of electronic effects, represented by ρ values, became more negative as the concentration of organic modifier in the mobile phases was lowered. As mentioned above, the HB_A term also became significant in highly water-rich mobile phases. These results suggest that a RPLC system with an eluent of low organic modifier concentrations discriminates subtle changes in the hydrogen bonding properties of solutes more effectively than eluents with intermediate modifier concentrations. The reason why attempts to incorporate the 2PR and 6PR series into a common correlation failed at 15% MeOH would be understood on this basis. The difference in polarity between the mobile and stationary phases becomes more pronounced as the water content in the mobile phase increases. It should also be mentioned that the change in the stationary phase structure tends to occur as the composition of eluents approaches the pure water.[5] Such concomitant effects would make the retention mechanism more complicated in highly water-rich eluents.

It is of interest to observe positive ρ values for acetonitrile (A15 and A30 on C18, and A30 on Ph(CP)). This would mean that the ρ value, being negative at 100% water, increases continuously as the organic modifier-content increases. Since the ρ value obtained with the C18-phase varied with % CH_3CN in a similar manner to that with Ph(CP), it seems that the $\rho\sigma_I$ term is mostly ascribed to the solute–solvent interactions. McCormick and Karger[21] reported that acetonitrile was extracted into the stationary phase (octyl bonded Hypersil) to a much larger extent than MeOH. It might then be possible that the solute–solvent interaction in the stationary phases also plays a role in retention behavior when the modifier is acetonitrile. More comprehensive study would be required to elucidate the physical meaning of the coefficients.

(e) A retardation effect ascribed to ester and amide groups (ester effect) was commonly observed with water-rich mobile phases. In the previous paper,[13,22] we speculated that this ester effect was rather due to the interaction with the mobile phase but not with the stationary phase including silanol effects. The present finding that the reproducible ester effect exists regardless of the stationary phases, seems to supply additional support to our view. Although the origin of this effect remains to be clarified, one possibility is that the ester and amide groups are more sensitive to the change in the solvent system than other ordinary H-acceptors in the sense that ester type functional groups have two H-accepting sites. It would be interesting to extend the measurements to higher contents of organic modifiers to see whether a negative coefficient of the HB_{CO} term could be obtained using an appropriate model system which gives retention times long enough to allow the measurements. To know whether this effect is specific for α-substituted azines or not would also useful. Studies to elucidate this problem are under way.

(f) The HB_A term was significant, in some cases, only with Ph-columns but not with alkyl-columns, suggesting that the Ph-phases are more discriminating between non-hydrogen-bonders and H-acceptors than the alkyl-phases relative to the octanol/water partitioning system. Therefore, the Ph-columns are supposed to be inferior in simulating log P for our solutes. The situation is shown in Fig. 2, where the relationship between log P and log k' obtained using the Ph(CS) clumn and 50% aqueous methanol as the eluent is illustrated. Comparison between Figs. 1A and 2 reveals that the C18 stationary phase gives a higher linearity than the Ph(CS) one. Thus and coworkers have reported that the retention for various benzenoid compounds on a phenyl-bonded stationary phase (Phenyl-Bondapack) with aqueous MeOH as the eluent correlates better with the log P values than that on a C18-modified phase (Hypersil ODS).[8] However, the preliminary experiments on monosubstituted benzenes under our RPLC conditions again showed that the Ph-columns were more discriminating between H-acceptors and non-hydrogen bonders than the C18 column (the data

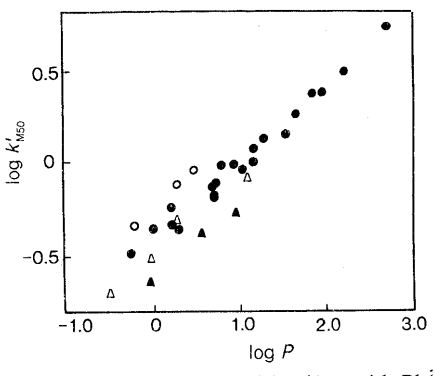

Fig. 2. Relationship between log P and log k'_{M50} with Ph(CS) Column for 2PR and 6PR

For Symbols see Fig. 1.

not shown). Especially, with MeOH as the modifier, the relative contribution of the $\log P$ term to $\log k'$ was usually higher in C18 than phenyl-ones (Tables II and III). All of these support the preference of C18 over Ph-phases.

This work has revealed that $\log k'$ values for 2PR and 6PR obtained from various combinations of stationary- and mobile-phase systems correlate very well with $\log P$ by the general Eq. 2. In all cases, the retention mechanism was shown to vary systematically: it became more complicated with the decrease in organic modifier content. The electronic interactions between the ring-N atoms and the substituents are thought to play an important role in governing the retention mechanism. The solutes having amphiprotic substituents usually exhibited the acceleration effect compared to non-amphiprotic compounds with the same $\log P$. The results led us to conclude that care should be taken to treat the RPLC capacity factor in connection with the $\log P$ value in heterocyclic systems. The fact that the $\log P$–$\log k'$ linearity is usually broken by other disturbing factors in highly water rich eluents strongly demonstrates that the conventional $\log k_W$ approach is not effective in predicting the $\log P$ values in the present cases. It is rather more practical to use the RPLC system consisting of the C18 column and the mobile phase containing about 50% MeOH, where the disturbing terms (σ_I, HB_{CO} and HB_A terms) are negligible or of small significance and hence $\log k'$ depends almost linearly on $\log P$, as far as amphiprotic substituents are treated separately, as shown in Fig. 1A.

To elucidate the effect of amphiprotics as well as ester groups on the retention behavior, analyses with a number of additional data would be required to parametrize the hydrogen-bonding effects in heteroaromatic systems. Our future work is being directed towards this problem.

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Development of Selective Inhibitors against Plasma Kallikrein[1)]

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Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Nishi-ku, Kobe 651–21, Japan, Kobe Research Projects on Thrombosis and Haemostasis, Saiseikai Hyogo Hospital,[b] Chuo-ku, Kobe 651, Japan, School of Allied Medical Sciences, Kobe University,[c] Suma-ku, Kobe 654–01, Japan, and Life Science Research Lab., Showa Denko Co., Ltd.,[d] Ohta-ku, Tokyo 146, Japan. *Received May 17, 1991*

Specific plasma kallikrein inhibitors were designed and synthesized and their structure–activity relationship was studied. *trans*-4-Aminomethylcyclohexanecarbonyl(Tra)-lysyl-4-ethoxycarbonylanilide inhibited plasma kallikrein and plasmin with IC_{50} values of 23 and 210 μM, respectively, indicating that this compound is fairly specific to plasma kallikrein. Tra-arginyl-4-ethoxycarbonylanilide inhibited plasma kallikrein and plasmin with IC_{50} values of 16 and 480 μM, respectively. Tra-homoarginyl-4-carboxyanilide inhibited plasma kallikrein and plasmin with IC_{50} values of 14 μM and 1 mM, respectively. Finally, Tra–Arg(Mts)-4-acetylanilide (ACA) exhibited potent and selective inhibitory activity against plasma kallikrein (IC_{50} value for plasma kallikrein: 2 μM and for plasmin: 42 μM).

Keywords plasma kallikrein; competitive inhibitor; specific inhibitor; design; synthesis; structure–activity relationship

It is well known that the action of plasma kallikrein (PK) liberates bradykinin (BK) from high molecular weight kininogen.[2)] It was also reported that PK can activate factor XII,[3)] prourokinase[4)] and plasminogen[5)] and may enhance blood polymorphonuclear leukocyte chemotaxis.[6)] These results suggest that PK has a broad spectrum of activities, but as yet, detailed studies of the role of PK remain to be performed.

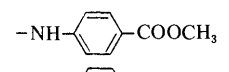
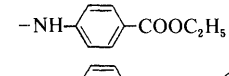
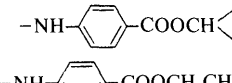
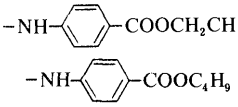
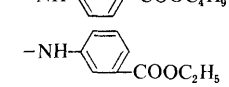
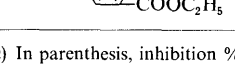
Aprotinin (Trazylol) is an inhibitor of both kallikrein and plasmin and it is widely used as a drug in the treatment of various diseases and as a tool for studying proteases.[7,8)] However, it should be noted that, though aprotinin is able to inhibit grandular kallikrein (GK), it is neither potent nor selective enough to inhibit PK.[8)] Soybean trypsin inhibitor (SBTI), a potent inhibitor of PK, can not inhibit GK. However, SBTI inhibits plasmin as well as PK, meaning that SBTI is not a selective inhibitor of PK.

Under these circumstances, our studies were directed to the synthesis of a specific plasma kallikrein inhibitor with the objective of obtaining a valuable and powerful tool in the study of the role of plasma kallikrein and of development of new types of clinical therapy. This paper deals with the synthesis of Lys, Arg and Hag derivatives, examination of their inhibitory activity against plasma kallikrein, plasmin, urokinase and thrombin and studies on structure–inhibitory activity relationship.

In our previous report,[9)] it was reported that *trans*-4-aminomethylcyclohexanecarbonyl(Tra)–Lys-4-methoxycarbonylanilide (**1**) inhibited plasma kallikrein with an IC_{50} value of 50 μM and plasmin with an IC_{50} value of >500 μM, indicating that this compound is ten fold more selective against plasma kallikrein. Thus, we synthesized Tra–Lys-4-methoxycarbonylanilide derivatives substituted methyl ester group by other alkyl ester groups and their inhibitory activities were examined as summarized in Table I.

As can be seen in Table I, 3-ethoxycarbonylanilide derivative (**6**) as well as 4-ethoxycarbonylanilide derivative (**2**) exhibited inhibitory activity against plasma kallikrein rather than plasmin. The bulkiness of the alkyl ester group did not affect the inhibitory activity against plasma kallikrein and plasmin. All the above derivatives exhibited more potent inhibitory activity against plasma kallikrein than plasmin, indicating that an alkoxycarbonyl group on

TABLE I. IC_{50} Values of NH_2CH_2–  –CO–Lys–R for Various Enzymes

	R	P. Kall S-2302	Pl S-2251	UK S-2444	TH S-2238
1		50	>500 (17%)[a)]	310	>500 (0%)
2		23	210	110	ND
3		40	>200 (16%)	100	>400 (0%)
4		27	>500 (39%)	59	>1000 (0%)
5		50	>300 (11%)	110	>400 (0%)
6		60	>500 (31%)	150	>500 (0%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

TABLE II. IC_{50} Values of NH_2CH_2––CO–Arg–R for Various Enzymes

	R	P. Kall S-2302	Pl S-2251	UK S-2444	TH S-2238
7	$-NHCH_3$	>1000 (11%)[a)]	>1000 (12%)	ND	ND
8		36	420	110	>1000 (20%)
9		27	>1000 (38%)	340	>1000 (0%)
10		16	480	90	>1000 (20%)
11		15	370	50	>1000 (17%)
12		>1000 (32%)	>1000 (40%)	>1000 (28%)	>1000 (12%)
13		>1000 (42%)	>1000 (43%)	>1000 (34%)	>1000 (0%)
14		200	350	>500 (17%)	>1000 (27%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

the phenyl ring of the C-terminal anilide moiety is suitable for the interaction between inhibitor and plasma kallikrein.

It is well known that plasmin preferentially hydrolyzes the lysylpeptide bonds in physiological substrates, while thrombin, plasma kallikrein, urokinase and factor Xa preferentially hydrolyze the arginylpeptide bonds.[10] In fact, D-Ile–Phe–Lys–*p*NA is a good substrate for plasmin and D-Ile–Phe–Arg–*p*NA is for plasma kallikrein. D-Ile–Phe–Lys–CH_2Cl inhibited plasmin preferentially, while D-Ile–Phe–Arg–CH_2Cl inhibited plasma kallikrein preferentially.[11] So, Arg derivatives instead of Lys were synthesized in order to obtain plasma kallikrein inhibitors and their inhibitory activities are summarized in Table II.

As expected, Tra–Arg-4-ethoxycarbonylanilide (**10**) and Tra–Arg-4-isobutyloxycarbonylanilide (**11**) inhibited plasma kallikrein more strongly than plasmin.

As illustrated in Fig. 1, these compounds inhibited plasma kallikrein competitively. Tra–Arg-4-carboxyanilide (**9**) inhibited plasma kallikrein with an IC_{50} value similar to those of the corresponding ester derivatives, while its inhibitory activity against plasmin decreased compared with the corresponding alkyl ester compound, resulting in an increase of discrepancy between the inhibitory activity against plasma kallikrein and plasmin. The inhibitory activity of Tra–Arg–ACA (**8**) against plasma kallikrein and plasmin exhibited a similar tendency to that of ethoxycarbonylanilide derivatives (**10**). In Tra–Arg-piperidine amide derivatives, only Tra–Arg–BPP (**14**) slightly inhibited plasmin but did not inhibit thrombin, although N^{α}-protected-Arg-piperidine amide derivatives inhibited thrombin very strongly and selectively.[12] These results indicate that the combination of Tra and Arg is not suitable for thrombin inhibitors.

Next, homoarginine (Hag) derivatives were synthesized and their inhibitory activity against plasmin, plasma kallikrein, urokinase and thrombin were examined and the results are summarized in Table III. Tra–Hag-4-methoxycarbonylanilide (**18**) and Tra–Hag-4-carboxyanilide (**17**) inhibited plasma kallikrein with similar IC_{50} values (18 and 14 μM, respectively) and the latter inhibitor exhibited weaker inhibitory activity against plasmin than the former one (IC_{50} values: > 1 mM and 610 μM, respectively). These phenomena are similar to those of Arg derivatives described above. Tra–Hag-piperidine amide derivatives (**19**—**21**) also did not inhibit thrombin. The combination of Tra and Hag is not suitable for thrombin inhibitors.

As stated above, in our inhibitor series, substitution of Lys by Arg [Tra–Lys-4-ethoxycarbonylanilide (**2**) and Tra–Arg-4-ethoxycarbonylanilide (**10**)] did not dramatically increase inhibitory activity against plasma kallikrein,

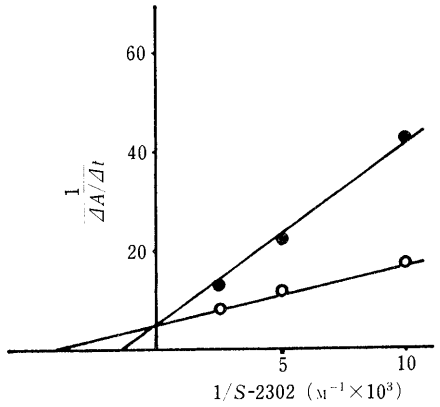


Fig. 1. Mode of Inhibition of Tra–Arg–NH–⟨⟩–$COOC_2H_5$ (**10**) against Plasma Kallikrein

●: with inhibitor (20 μM); ○: without inhibitor.

TABLE III. IC_{50} Values of NH_2CH_2–⟨H⟩–CO–Hag–R for Various Enzymes

	R	P. Kall S-2302	PI S-2251	UK S-2444	TH S-2238
15	$-NHCH_3$	>1000 (24%)[a]	>1000 (13%)	580	>1000 (0%)
16	–NH–⟨⟩–$COCH_3$	270	>1000 (8%)	>500 (15%)	>1000 (0%)
17	–NH–⟨⟩–COOH	14	>1000 (48%)	95	>1000 (0%)
18	–NH–⟨⟩–$COOCH_3$	18	610	85	>1000 (0%)
19	–N⟨⟩–CH_3	>1000 (39%)	>1000 (27%)	880	>1000 (0%)
20	–N⟨⟩–$COOC_2H_5$	>1000 (47%)	>1000 (35%)	>1000 (48%)	>1000 (0%)
21	–N⟨⟩–CH_2–⟨⟩	>500 (36%)	650	>250 (30%)	>500 (28%)

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

TABLE IV. IC_{50} Values of Arg Derivatives for Various Enzymes

	Compound	P. Kall S-2302	PI S-2251	UK S-2444	TH S-2238
22	NH_2CH_2–⟨H⟩–CO–Arg(Mts)–CO_2–CH_2CCl_3	38	4.7	600	230
23	NH_2CH_2–⟨H⟩–CO–Arg(Mts)–NH–⟨⟩–$COCH_3$	2.0	42	370	>400 (21%)[a]

a) In parenthesis, inhibition % at the concentration described (μM) is indicated.

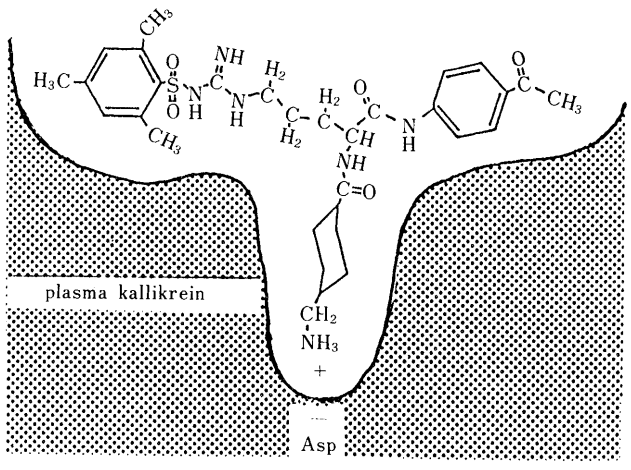


Fig. 2. Schematic Representation of Interaction of Tra–Arg(Mts)–ACA (**23**) with Enzyme

although in the case of chloromethylketone derivatives, Arg derivatives inhibited plasma kallikrein much more strongly than Lys derivatives.[11] Arg and Hag piperidine amide derivatives did not inhibit thrombin, indicating that these derivatives could manifest inhibitory activity against plasma kallikrein by quite a different mechanism from that of Arg derivatives reported previously.[12] In order to gain further insight into the inhibitory mechanism, Tra–Arg(Mts)–ACA (**23**) and Tra–Arg(Mts)–NHNH–Troc (**22**), in which basisity of Arg is decreased by protection of the guanidino group by Mts (mesitylenesulfonyl), were synthesized and their inhibitory effect on plasma kallikrein, plasmin, urokinase and thrombin was examined. As summarized in Table IV, Tra–Arg(Mts)–ACA (**23**) inhibited plasma kallikrein with an IC_{50} value of 2.0×10^{-6} M, while it inhibited plasmin with an IC_{50} value of 4.2×10^{-5} M. Tra–Arg(Mts)–NHNH–Troc (**22**) inhibited plasma kallikrein and plasmin with IC_{50} values of 3.8×10^{-5} and 4.8×10^{-6} M, respectively. From these results, it is deduced that the positively charged amino group of Tra moiety might interact with the negatively charged group of the active center of enzymes as illustrated in Fig. 2. Previously, Muramatsu and Fujii prepared various esters of ε-aminocaproic acid and *trans*-4-aminomethylcyclohexanecarboxylic acid, which exhibited inhibitory effects on trypsin, plasmin, plasma kallikrein and thrombin.[13] In these cases, an amino group of ε-aminocaproic acid or Tra also interacted with a negatively charged group of enzymes. In our previous report[9] about Lys derivatives, both Lys derivatives in which an ε-amino group of Lys or an amino group of Tra was protected, could inhibit plasmin. In the above Arg and Hag derivatives, it is deduced that either the guanidino group of Arg and Hag or the amino group of Tra can interact with the negatively charged group of the active center of enzymes and the amino group of Tra is more suitable for the interaction than the guanidino group of Arg and Hag.

These results provided us with a rational method for designing potent and specific inhibitor against not only plasma kallikrein but also plasmin and thrombin.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DPI-360 (Japan Spectroscopic Co., Ltd.). On thin-layer chromatography (TLC) (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5, Rf^6, Rf^7 and Rf^8 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (89 : 10 : 1), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase), *n*-BuOH, AcOH, pyridine and H_2O (4 : 1 : 1 : 2), *n*-BuOH, AcOH, pyridine and H_2O (1 : 1 : 1 : 1), $CHCl_3$ and ether (4 : 1) and *n*-PrOH, H_2O, AcOEt and conc. NH_4OH (5 : 1 : 2 : 1), respectively.

General Procedure for Preparation of Boc–Lys(Z)–R (R: See Table V) A mixed anhydride [prepared from 3.0 g of Boc–Lys(Z)–OH (3.0 g, 7.9 mmol) and ethyl chloroformate (0.76 ml, 7.9 mmol)] in THF (80 ml) was added to an ice-cold solution of 4-alkoxycarbonylaniline [prepared from 4-alkoxycarbonylaniline hydrochloride (7.9 mmol) and Et_3N (1.1 ml, 7.9 mmol)] in *N,N*-dimethylformamide (DMF) (30 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration and reprecipitated from AcOEt and petroleum ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table V.

General Procedure for Preparation of Z–Tra–Lys(Z)–R (R: See Table VI) An acid chloride [prepared from Z–Tra–OH (0.77 g, 3.5 mmol) and $SOCl_2$ (0.80 ml, 6.7 mmol)] in $CHCl_3$ (10 ml) was added to an ice-cold solution of H–Lys(Z)-4-alkoxycarbonylanilide·HCl [prepared from Boc–Lys(Z)-4-alkoxycarbonylanilide (0.4 mmol) and 5.6 N HCl/dioxane (0.70 ml)] in DMF (8 ml) containing Et_3N (0.11 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VI.

General Procedure for Preparation of H–Tra–Lys–R (R: See Table VII) Z–Tra–Lys(Z)-4-alkoxycarbonylanilide (0.03 mmol) in MeOH (4 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to afford crystals. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VII.

General Procedure for Preparation of Z–Arg(NO_2)–R (R: See Table VIII) PCl_3 (0.12 ml, 1.35 mmol) was added to a solution of the corresponding amine component [prepared from corresponding hydrochloride (2.7 mmol) and Et_3N (0.38 ml, 2.7 mmol)] in dry pyridine (25 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and then at room temperature for 30 min. Z–Arg(NO_2)–OH (0.95 g, 2.7 mmol) was added to the above solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration and reprecipitated from AcOEt and ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table VIII.

General Procedure for Preparation of Z–Tra–Arg(NO_2)–R (R: See Table IX) An acid chloride [prepared from Z–Tra–OH (0.72 g, 3.3 mmol) and $SOCl_2$ (1.6 ml, 13.3 mmol)] in $CHCl_3$ (20 ml) was added to an ice-cold solution of H–Arg(NO_2)–R·HBr [prepared from the corresponding N^{α}–Z-protected compound (0.83 mmol) and 25% HBr–AcOH (8 ml, 2.5 mmol)] in DMF (15 ml) containing Et_3N (0.24 ml, 1.7 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and

TABLE V. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Boc–Lys(Z)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
–NH–⟨⟩–$COOC_2H_5$	12.6	95—99	−1.8° (DMF)	$C_{28}H_{37}N_3O_7$	63.8 (63.9	7.02 7.12	7.97 8.23)	0.50	0.95
–NH–⟨⟩–$COOCH(CH_3)_2$	73.9	Amorphous	−15.8° ($CHCl_3$)	$C_{29}H_{39}N_3O_7$	64.4 (64.6	7.21 7.33	7.76 7.68)	0.68	0.76
–NH–⟨⟩–$COOCH_2CH(CH_3)_2$	51.4	Amorphous	−15.7° ($CHCl_3$)	$C_{30}H_{41}N_3O_7$	64.9 (65.1	7.58 7.62	7.56 7.35)	0.60	
–NH–⟨⟩–$COOC_4H_9$	44.7	Amorphous	−25.9° ($CHCl_3$)	$C_{30}H_{41}N_3O_7$	64.9 (64.9	7.58 7.40	7.56 7.37)	0.61	
–NH–⟨⟩–$COOC_2H_5$	21.6	Amorphous	−11.3° (MeOH)	$C_{28}H_{37}N_3O_7 \cdot 0.5H_2O$	62.7 (62.9	7.09 6.98	7.83 7.62)	0.60	0.67

TABLE VI. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Tra–Lys(Z)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
–NH–C$_6$H$_4$–COOC$_2$H$_5$	16.8	169—173	−1.4° (MeOH)	$C_{39}H_{48}N_4O_8 \cdot H_2O$	65.2 (65.5	6.96 7.11	7.80 8.13)	0.70	
–NH–C$_6$H$_4$–COOCH(CH$_3$)$_2$	31.0	163—171	−23.5° ($CHCl_3$)	$C_{40}H_{50}N_4O_8$	67.3 (67.0	7.00 7.13	7.84 7.89)	0.69	0.56
–NH–C$_6$H$_4$–COOCH$_2$CH(CH$_3$)$_2$	30.7	136—141	−17.3° ($CHCl_3$)	$C_{41}H_{52}N_4O_8 \cdot 3H_2O$	62.9 (62.9	7.41 7.40	7.16 7.29)	0.57	
–NH–C$_6$H$_4$–COOC$_4$H$_9$	55.4	145—149	−24.5° ($CHCl_3$)	$C_{41}H_{52}N_4O_8 \cdot H_2O$	66.0 (65.7	7.24 7.43	7.50 7.71)	0.56	0.82
–NH–C$_6$H$_4$–COOC$_2$H$_5$	84.0	148—152	−0.7° (DMF)	$C_{39}H_{48}N_4O_8$	66.9 (66.8	6.85 7.03	8.00 8.04)	0.83	0.86

TABLE VII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of NH_2CH_2–C$_6$H$_4$–CO–Lys–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^5	Rf^6
2 –NH–C$_6$H$_4$–COOC$_2$H$_5$	55.5	46—50	−7.3° (MeOH)	$C_{23}H_{36}N_4O_4 \cdot 1.5H_2O$	60.2 (59.9	8.49 8.23	12.2 12.5)	0.54	
3 –NH–C$_6$H$_4$–COOCH(CH$_3$)$_2$	60.7	52—54	−16.5° (MeOH)	$C_{24}H_{38}N_4O_4 \cdot H_2O$	62.1 (61.7	8.62 8.33	12.1 12.1)	0.49	0.78
4 –NH–C$_6$H$_4$–COOCH$_2$CH(CH$_3$)$_2$	83.4	128—135	−12.7° (MeOH)	$C_{25}H_{40}N_4O_4 \cdot 5H_2O$	54.6 (54.6	9.38 9.29	9.80 10.1)	0.51	0.78
5 –NH–C$_6$H$_4$–COOC$_4$H$_9$	68.7	Amorphous	−18.6° (MeOH)	$C_{25}H_{40}N_4O_4$	65.2 (65.5	8.75 8.76	12.2 12.1)	0.59	0.87
6 –NH–C$_6$H$_4$–COOC$_2$H$_5$	66.3	Amorphous	−8.3° (MeOH)	$C_{23}H_{36}N_4O_4 \cdot 2H_2O$	59.0 (59.1	8.54 8.33	12.0 12.1)	0.54	

TABLE VIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Arg(NO_2)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
–NHCH$_3$	10.7	Oil	−12.4° ($CHCl_3$)	$C_{15}H_{22}N_6O_5$	49.2 (49.0	6.07 6.21	22.9 22.6)	0.52	0.55
–NH–C$_6$H$_4$–COCH$_3$	65.2	150—151	+15.0° (DMF)	$C_{22}H_{26}N_6O_6$	56.2 (56.2	5.53 5.38	17.9 17.7)	0.60	0.62
–NH–C$_6$H$_4$–COOC$_2$H$_5$	74.0	79—85	+12.4° (DMF)	$C_{23}H_{28}N_6O_7$	55.2 (55.5	5.60 5.60	16.8 16.5)		0.37
–NH–C$_6$H$_4$–COOCH$_2$CH(CH$_3$)$_2$	63.5	99—104	+11.2° (DMF)	$C_{25}H_{32}N_6O_7$	56.9 (56.9	6.06 6.03	15.9 15.7)		0.47
–N(C$_4$H$_3$N$_2$)–CH$_3$	34.6	Oil	−36.9° ($CHCl_3$)	$C_{20}H_{30}N_6O_5$	55.3 (55.7	6.91 7.12	19.4 19.1)	0.51	0.58
–N(C$_4$H$_3$N$_2$)–CO$_2$C$_2$H$_5$	63.7	Oil	−27.0° ($CHCl_3$)	$C_{22}H_{32}N_6O_7$	53.7 (53.5	6.50 6.40	17.1 16.8)	0.50	0.51
–N(C$_4$H$_3$N$_2$)–CH$_2$–C$_6$H$_5$	47.6	Oil	−30.8° ($CHCl_3$)	$C_{26}H_{34}N_6O_5$	61.2 (61.2	6.66 7.00	16.5 16.5)	0.46	0.48

concentrated to a small volume. Ether was added to the residue to give crystals, which were collected by filtration and reprecipitated from AcOEt and ether. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table IX.

General Procedure for Preparation of H–Tra–Arg–R (R: See Table X) Z–Tra–Arg(NO_2)–R (0.2 mmol) in AcOH–H_2O (5 ml–5 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, water was added to the residue and the solution was lyophilized to give a hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table X.

Z–Arg(Mts)-4-Methoxycarbonylanilide PCl_3 (0.36 g, 4 mml) was added to a solution of 4-methoxycarbonylaniline (1.2 g, 8 mmol) in dry pyridine (40 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and at room temperature for 30 min. Z–Arg(Mts)–OH [prepared from Z–Arg(Mts)-OH·CHA (4.8 g, 8 mmol) and 1 N HCl (8 ml) as usual] was added to the above solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate,

TABLE IX. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z–Tra–Arg(NO_2)–R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^1	Rf^2
$-NHCH_3$	44.3	115—119	−3.9° (DMF)	$C_{23}H_{33}N_7O_6$	54.9 (54.7	6.62 6.48	19.5 19.6)	0.60	0.62
$-NH-C_6H_4-COCH_3$	78.3	172—176	+2.7° (DMF)	$C_{30}H_{39}N_7O_7$	59.1 (58.9	6.40 6.48	16.1 15.9)	0.52	0.68
$-NH-C_6H_4-COOC_2H_5$	50.9	95—100	−78.8° ($CHCl_3$)	$C_{31}H_{41}N_7O_8 \cdot 0.5H_2O$	57.4 (57.6	6.48 6.43	15.2 15.1)		0.52
$-NH-C_6H_4-COOCH_2CH(CH_3)_2$	50.2	86—92	−20.5° ($CHCl_3$)	$C_{33}H_{45}N_7O_8$	59.4 (59.5	6.74 7.02	14.7 14.6)		0.46
$-N(C_5H_4)-CH_3$	78.5	Oil	−40.9° ($CHCl_3$)	$C_{28}H_{43}N_7O_6$	58.7 (58.4	7.58 7.71	17.1 17.2)	0.53	0.62
$-N(C_5H_4)-CO_2C_2H_5$	51.5	Oil	−30.8° ($CHCl_3$)	$C_{38}H_{45}N_7O_8$	57.1 (57.4	7.13 7.42	15.1 14.9)	0.58	0.55
$-N(C_5H_4)-CH_2-C_6H_5$	66.2	Oil	−35.4° ($CHCl_3$)	$C_{34}H_{47}N_7O_6 \cdot 3.5H_2O$	57.3 (57.2	7.63 7.38	13.8 14.0)	0.54	0.58

TABLE X. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of $NH_2CH_2-C_6H_{10}-CO-Arg-R$

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC Rf^5	Rf^8
7 $-NHCH_3$	69.0	Amorphous	−7.0° (MeOH)	$C_{15}H_{30}N_6O_2 \cdot 2AcOH \cdot H_2O$	49.1 (49.3	8.67 8.81	18.1 17.8)	0.10	0.38
8 $-NH-C_6H_4-COCH_3$	59.1	Amorphous	−17.3° (MeOH)	$C_{22}H_{34}N_6O_3 \cdot 2AcOH \cdot H_2O$	54.9 (54.6	7.80 7.94	14.8 14.8)	0.10	0.51
9 $-NH-C_6H_4-CO_2H$	82.7	Amorphous	−11.4° (MeOH)	$C_{21}H_{32}N_6O_4 \cdot 2AcOH \cdot H_2O$	52.7 (52.7	7.44 7.28	14.7 14.6)	0.10	0.52
10 $-NH-C_6H_4-COOC_2H_5$	50.9	Amorphous	−12.8° (MeOH)	$C_{23}H_{36}N_6O_4 \cdot 2AcOH \cdot 1.5H_2O$	53.4 (53.4	7.25 7.49	13.8 13.6)	0.45	
11 $-NH-C_6H_4-COOCH_2CH(CH_3)_2$	89.4	Amorphous	−8.0° (MeOH)	$C_{25}H_{40}N_6O_4 \cdot 2AcOH \cdot 2H_2O$	54.1 (54.3	8.07 8.02	13.0 13.2)	0.52	
12 $-N(C_5H_4)-CH_3$	75.6	Amorphous	−19.1° (MeOH)	$C_{20}H_{38}N_6O_2 \cdot 2AcOH \cdot H_2O$	54.1 (53.8	9.08 9.24	15.8 16.0)	0.10	0.36
13 $-N(C_5H_4)-CO_2C_2H_5$	68.4	Amorphous	−23.2° (MeOH)	$C_{22}H_{40}N_6O_4 \cdot 2AcOH \cdot H_2O$	52.7 (52.5	8.84 8.82	14.2 14.2)	0.10	0.42
14 $-N(C_5H_4)-CH_2-C_6H_5$	78.2	Amorphous	−13.5° (MeOH)	$C_{26}H_{42}N_6O_2 \cdot 2AcOH \cdot 1.5H_2O$	62.9 (62.7	7.31 7.11	15.1 15.3)	0.10	0.38

which was collected by filtration and reprecipitated from AcOEt and petroleum ether, yield 4.4 g (88.2%), mp 87—89°C, $[\alpha]_D^{26}$ −14.5° (c=1.0, MeOH), Rf^1 0.54, Rf^2 0.45. *Anal.* Calcd for $C_{31}H_{37}N_5O_7S$: C, 59.7; H, 6.00; N, 11.2. Found: C, 59.6; H, 5.91; N, 11.5.

Z–Tra–Arg(Mts)-4-Methoxycarbonylanilide The title compound was prepared by the same way as described in the synthesis of Z–Tra–Arg(NO_2)–R from an acid chloride [prepared from Z–Tra–OH (1.1 g, 5.2 mmol) and $SOCl_2$ (2.4 ml, 5.2 mmol)] and H–Arg(Mts)-4-methoxycarbonylanilide·HCl [prepared from the corresponding N^{α}–Z-derivative (1.2 g, 2 mmol) in MeOH (100 ml) by catalytic hydrogenation in the presence of 1 N HCl (2 ml)]. The crude product in $CHCl_3$ (3 ml) was applied to a silica gel column (2.2×17 cm), equilibrated and eluted with $CHCl_3$ (350 ml), followed by 1% MeOH in $CHCl_3$. Individual fractions (50 ml each) were collected. The solvent of the effluent (tube Nos. 13—29) was removed by evaporation. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.8 g (60%), mp 114—116 °C, $[\alpha]_D^{26}$ −5.6° (c=1.1, MeOH), Rf^1 0.64, Rf^2 0.56. *Anal.* Calcd for $C_{39}H_{50}N_6O_8S \cdot 0.5H_2O$: C, 60.7; H, 6.61; N, 10.9. Found: C, 60.9; H, 6.53; N, 11.0.

Z–Tra–Arg(Mts)-4-Carboxyanilide A solution of Z–Tra–Arg(Mts)-4-methoxycarbonylanilide (0.38 g, 0.5 mmol) in MeOH (40 ml) containing 1 N NaOH (1.5 ml) was stirred at room temperature for 30 min and at room temperature for 2 h. The pH of the reaction mixture was adjusted to 7 with AcOH. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration, yield 0.33 g (88.2%), mp 134—135 °C, $[\alpha]_D^{26}$ −5.0° (c=1.0, MeOH), Rf^1 0.44, Rf^2 0.33. *Anal.* Calcd for $C_{38}H_{48}N_6O_8S \cdot 0.75H_2O$: C, 59.9; H, 6.49; N, 11.0. Found: C, 60.0; H, 6.49; N, 11.0.

H–Tra–Arg-4-Carboxyanilide (9) Z–Tra–Arg(Mts)-4-carboxyanilide (0.26 g, 0.35 mmol) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of *m*-cresol (0.38 ml, 10 eq) in the ice-bath for 90 min and ether was added to the solution to give a precipitate, which was collected by centrifugation and dried over KOH pellets *in vacuo*. The resulting powder in H_2O (3 ml) was treated with Amberlite IRA 45 (acetate form). The solution was applied to a column of Sephadex G-25 (1.4×140 cm), equilibrated and eluted with 3% AcOH. Individual fractions (5 g each) were collected and the solvent of the effluent (tube Nos. 50—55) was removed by lyophilization to afford hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table X.

General Procedure for Preparation of Z–Hag(NO_2)–R (R: See Table XI) PCl_3 (0.23 ml, 2.5 mmol) was added to a solution of the corresponding amine component (5 mmol) in dry pyridine (20 ml) cooled with ice-salt. The reaction mixture was stirred at the same temperature for 15 min and at room temperature for 30 min. Z–Hag(NO_2)–OH (1.8 g, 5 mmol) was added to the above solution and the reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was

TABLE XI. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z- or Boc-Hag(NO_2)-R

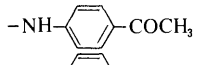
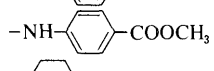
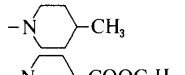
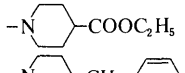
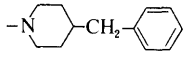
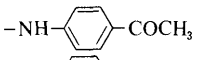
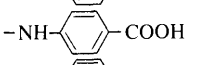
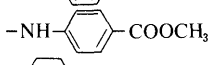
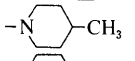
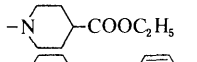
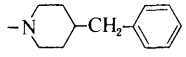
Compound		Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
N^{α}	R					C	H	N	Rf^1	Rf^2
Z	$-NHCH_3$	34.5	Amorphous	$-10.8°$ ($CHCl_3$)	$C_{16}H_{24}N_6O_5 \cdot 2H_2O$	46.1 (46.3	6.79 6.58	20.2 20.1)	0.33	
Z		24.2	Amorphous	$-11.6°$ ($CHCl_3$)	$C_{23}H_{28}N_6O_6 \cdot 2H_2O$	53.1 (53.0	6.21 6.19	16.1 16.3)	0.25	
Z		20.5	Amorphous	$-20.6°$ ($CHCl_3$)	$C_{23}H_{28}N_6O_7 \cdot H_2O$	53.3 (53.5	5.84 5.85	16.2 16.0)	0.26	
Boc		20.1	Amorphous	$-8.6°$ ($CHCl_3$)	$C_{18}H_{34}N_6O_5$	52.2 (52.1	8.29 8.11	20.3 20.5)	0.32	0.52
Boc		15.5	Amorphous	$-10.2°$ ($CHCl_3$)	$C_{20}H_{36}N_6O_7$	50.9 (50.7	7.70 7.56	17.8 17.9)	0.40	0.58
Z		20.5	Amorphous	$-21.7°$ ($CHCl_3$)	$C_{27}H_{36}N_6O_5 \cdot 1.5H_2O$	58.8 (58.6	7.14 7.15	15.2 15.3)	0.38	

TABLE XII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of Z-Tra-Hag(NO_2)-R

Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^1	Rf^2
$-NHCH_3$	32.6	Amorphous	$-15.2°$ ($CHCl_3$)	$C_{24}H_{37}N_7O_6$	55.5 (55.2	7.19 7.08	18.9 18.8)	0.52	
	75.2	76—81	$-26.2°$ ($CHCl_3$)	$C_{31}H_{41}N_7O_7$	59.7 (59.7	6.64 6.65	15.7 15.9)	0.28	
	36.0	195—198	$-31.8°$ (MeOH)	$C_{30}H_{39}N_7O_8$	57.6 (57.6	6.30 6.18	15.7 15.8)	0.29	0.10
	46.9	142—145	$-12.5°$ (MeOH)	$C_{31}H_{41}N_7O_8$	58.2 (58.0	6.47 6.62	15.3 15.5)	0.56	0.41
	92.1	86—94	$-5.2°$ ($CHCl_3$)	$C_{29}H_{45}N_7O_6$	59.3 (59.5	7.73 7.75	16.7 16.8)	0.70	0.63
	47.7	Amorphous	$-8.5°$ ($CHCl_3$)	$C_{31}H_{47}N_7O_8$	57.7 (57.9	7.35 7.38	15.2 15.6)	0.41	
	56.6	Amorphous	$-26.5°$ ($CHCl_3$)	$C_{35}H_{49}N_7O_6$	63.3 (63.6	7.46 7.45	14.8 14.7)	0.25	

extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. The crude product in $CHCl_3$ (5 ml) was applied to a silica gel column (3.6 × 20 cm), equilibrated and eluted with $CHCl_3$ (200 ml), followed by 1% MeOH in $CHCl_3$ (200 ml), 1.5% MeOH in $CHCl_3$ (200 ml) and 2% MeOH in $CHCl_3$ (500 ml). The solvent of the 2% MeOH in $CHCl_3$ effluent was removed by evaporation. Petroleum ether was added to the residue to give an amorphous powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XI.

General Procedure for Preparation of Boc-Hag(NO_2)-R (R: See Table XI) A mixed anhydride [prepared from Boc-Hag(NO_2)-OH (1.0 g, 3 mmol) and ethyl chloroformate (0.3 ml, 3 mmol) as usual] in THF (20 ml) was added to an ice-cold solution of the corresponding amine component (3 mmol) in THF (20 ml). After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford an amorphous powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XI.

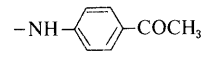
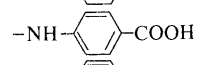
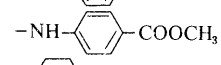
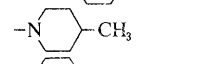
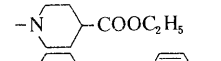
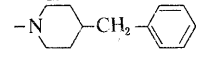
General Procedure for Preparation of Z-Tra-Hag(NO_2)-R (R: See Table XII) An acid chloride [prepared from Z-Tra-OH (1.8 g, 6 mmol) and $SOCl_2$ (2.8 ml, 6 mmol)] in $CHCl_3$ (5 ml) was added to an ice-cold solution of H-Hag(NO_2)-R·HBr or ·HCl [prepared from the corresponding N^{α}-Z- or Boc-derivative (3 mmol) by 25% HBr-AcOH or 5.6 N HCl-dioxane as usual] in DMF (20 ml) containing Et_3N (0.84 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to yield a precipitate, which was collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XII.

Z-Tra-Hag(NO_2)-4-Carboxyanilide Z-Hag(NO_2)-4-methoxycarbonylanilide (0.51 g, 0.8 mmol) was dissolved in MeOH (50 ml) containing 1 N NaOH (8 ml, 8 mmol) under cooling with ice. The reaction mixture was stirred at the same temperature for 30 min and at room temperature for 3 h. The pH of the reaction mixture was adjusted to 7 with AcOH. After removal of the solvent, the residue was extraced with AcOEt. The extract was washed with 3% AcOH and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give a precipitate, which was collected by filtration. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* value was summarized in Table XII.

General Procedure for Preparation of H-Tra-Hag-R (R: See Table XIII) The corresponding protected compound (0.16 mmol) in AcOH-H_2O (8 ml—8 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, the residue was dissolved in water and lyophilized to give a hygroscopic powder. Yield, mp, $[\alpha]_D$ value, elemental analysis and *Rf* values are summarized in Table XIII.

Boc-Tra-Arg(Mts)-NHNH-Troc A mixed anhydride [prepared from Boc-Tra-OH (0.39 g, 1.5 mmol) and ethyl chloroformate (0.14 ml, 1.5 mmol) as usual] in THF (20 ml) was added to an ice-cold solution of H-Arg(Mts)-NHNH-Troc·HCl [prepared from Z-Arg(Mts)-NHNH-Troc[14] (0.51 g, 0.75 mmol) by catalytic hydrogenation] in DMF (20 ml) containing Et_3N (0.11 ml, 0.75 mmol). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration. The crude product in $CHCl_3$ (2 ml) was applied to a column of silica gel (1.6 × 10 cm), equilibrated and eluted with $CHCl_3$ (300 ml), followed by 1% MeOH in $CHCl_3$ (250 ml) and 3% MeOH in $CHCl_3$ (350 ml). The

TABLE XIII. Yield, Melting Point, Optical Rotation, *Rf* Values and Analytical Data of NH_2CH_2-⟨H⟩-CO-Hag-R

	Compound R	Yield (%)	mp (°C)	$[\alpha]_D$ (Solvent)	Formula	Elemental analysis Calcd (Found) C	H	N	TLC *Rf*[8]
15	$-NHCH_3$	91.7	Amorphous	−19.6° (MeOH)	$C_{16}H_{32}N_6O_2 \cdot 2CH_3COOH$	52.2 (52.1)	8.75 (8.68)	18.2 (18.0)	0.37
16		39.2	Amorphous	−10.5° (MeOH)	$C_{23}H_{36}N_6O_3 \cdot 2CH_3COOH$	57.4 (57.1)	7.85 (7.91)	14.9 (14.7)	0.47
17		80.3	Amorphous	−11.8° (MeOH)	$C_{22}H_{34}N_6O_4 \cdot 2CH_3COOH$	55.2 (55.2)	7.49 (7.18)	14.8 (14.8)	0.47
18		55.2	Amorphous	−21.5° (MeOH)	$C_{23}H_{36}N_6O_4 \cdot 2CH_3COOH \cdot 1.5H_2O$	53.4 (53.5)	7.82 (7.74)	13.8 (13.9)	0.45
19		50.8	Amorphous	−12.8° (MeOH)	$C_{21}H_{40}N_6O_2 \cdot 2CH_3COOH$	56.8 (56.5)	9.15 (9.25)	15.9 (15.7)	0.42
20		73.3	Amorphous	−15.7° (MeOH)	$C_{23}H_{42}N_6O_4 \cdot 2CH_3COOH$	55.3 (55.1)	8.59 (8.68)	14.3 (14.1)	0.42
21		67.6	Amorphous	−15.3° (MeOH)	$C_{27}H_{44}N_6O_2 \cdot 2CH_3COOH$	61.6 (61.5)	8.67 (8.81)	13.9 (13.6)	0.47

solvent of the 3% MeOH in $CHCl_3$ effluent was removed by evaporation. Ether was added to the residue to afford a precipitate, which was collected by filtration, yield 0.2 g (34.0%), mp, 71—74 °C, $[\alpha]_D^{26}$ −18.0° (c=1.0, $CHCl_3$), Rf^1 0.22, Rf^3 0.78. *Anal.* Calcd for $C_{31}H_{48}Cl_3N_7O_8S \cdot H_2O$: C, 46.4; H, 6.23; N, 12.2. Found: C, 46.5; H, 6.06; N, 11.9.

H-Tra-Arg(Mts)-NHNH-Troc (22) Boc-Tra-Arg(Mts)-NHNH-Troc (0.14 g, 0.18 mmol) was dissolved in 7.3 N HCl–dioxane (0.25 ml, 1.8 mmol). After 5 min, dioxane (0.25 ml) was added to the solution. The reaction mixture was stirred at room temperature for 60 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 0.11 g (82.4%), amorphous powder, $[\alpha]_D^{26}$ −13.0° (c=1.3, MeOH), Rf^4 0.46, Rf^5 0.85. *Anal.* Calcd for $C_{26}H_{40}Cl_3N_7O_6S \cdot HCl \cdot H_2O$: C, 42.3; H, 5.82; N, 13.3. Found: C, 42.3; H, 5.71; N, 13.5.

Z-Tra-Arg(Mts)-ACA Z-Arg(Mts)-ACA was first prepared by the same way as described in the synthesis of Z-Arg(Mts)-4-methoxycarbonylanilide from PCl_3 (0.02 g, 0.27 mmol), 4-acetylaniline (0.07 g, 0.54 mmol) and Z-Arg(Mts)-OH [prepared from Z-Arg(Mts)-OH·CHA (0.32 g, 0.54 mmol) and 1 N HCl (0.54 ml, 0.54 mmol)], yield 0.17 g (52.0%), mp 162—164 °C. Reaction of an acid chloride [prepared from Z-Tra-OH (0.6 g, 2.8 mmol) and $SOCl_2$ (1.3 g, 11.2 mmol)] and H-Arg(Mts)-ACA·HBr [prepared from the corresponding N^{α}-Z-derivative (0.43 g, 0.7 mmol) obtained above and 25% HBr-AcOH (0.7 ml, 2.1 mmol)] gave the title compound, yield 0.19 g (37.8%), mp 136—138 °C, $[\alpha]_D^{26}$ +8.0° (c=1.0, DMF), Rf^1 0.49, Rf^2 0.25. *Anal.* Calcd for $C_{39}H_{50}N_6O_7S$: C, 62.0; H, 6.75; N, 11.1. Found: C, 61.9; H, 6.54; N, 10.8.

H-Tra-Arg(Mts)-ACA (23) Z-Tra-(Mts)-ACA (0.08 g, 0.11 mmol) was dissolved in 25% HBr-AcOH (0.11 ml, 0.33 mmol). After 5 min, AcOH (0.11 ml) was added to the solution. The reaction mixture was stirred at room temperature for 40 min. Ether was added to the solution to yield a white precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 0.068 g (90.1%), amorphous, $[\alpha]_D^{26}$ −3.7° (c=1.3, MeOH), Rf^4 0.18, Rf^5 0.54. *Anal.* Calcd for $C_{44}H_{44}N_6O_5S \cdot HBr \cdot 2.5H_2O$: 50.4; H, 6.84; N, 11.4. Found: C, 50.2; H, 6.59; N, 11.5.

Assay Procedure Amidolytic activity of enzymes was measured according to the procedure described previously.[12] The IC_{50} value was taken as the concentration of the inhibitor which decreased the absorbance at 405 nm by 50% compared with the absorbance without an inhibitor. (P. Kall, plasma kallikrein; Pl, plasmin; UK, urokinase; TH, thrombin).

References and Notes

1) The customary L-configuration for amino acid residues is omitted; only D-isomers are indicated. Standard abbreviations for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Mts, mesitylenesulfonyl; CHA, cyclohexylamine; ACA, 4-acetylanilide; BPP, 4-benzylpiperidine; Hag, homoarginine, TFMSA, trifluoromethanesulphonic acid; AcOH, acetic acid; *n*-BuOH, *n*-butanol; AcOEt, ethyl acetate; Troc, 2,2,2-trichloroethyloxycarbonyl.
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Studies on Antiulcer Drugs. I. Synthesis and Antiulcer Activities of Imidazo[1,2-*a*]pyridinyl-2-oxobenzoxazolidines-3-oxo-2*H*-1,4-benzoxazines and Related Compounds

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A series of imidazo[1,2-*a*]pyridinyl-2-oxobenzoxazolidines (4a—i), -3-oxo-2*H*-1,4-benzoxazines (5a—q), their thio-analogues (4j—p and 5r—t) and 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridinyl derivatives (8 and 9) were synthesized and tested for anti-stress ulcer activity in rats. Several compounds were found to be more active than the reference compounds, zolimidine, cimetidine and sucralfate. Among them, compound 4e, 5i and 5l also exhibited potent protective activity against ethanol-induced gastric lesion. The synthesis and structure–activity relationships of these compounds are discussed.

Keywords anti-stress ulcer activity; ethanol-induced gastic lesion; cytoprotective activity; imidazo[1,2-*a*]pyridinyl-2-oxobenzoxazolidine; imidazo[1,2-*a*]pyridinyl-3-oxo-2*H*-1,4-benzoxazine; zolimidine; cimetidine; sucralfate; structure–activity relationship

It is well known that peptic ulcers are the outcome of an imbalance between the defensive factors (forces of mucosal resistance) and the aggressive factors (gastric acid and pepsin secretion).[1] In the last two decades, research of many antisecretory agents has been carried out in an effort to inhibit the latter factors, and as a result, a number of anti-cholinergic drugs, histamine H_2-receptor antagonists and recently proton potassium adenosine triphosphatase (ATPase) inhibitor (omeprazole)[2] have been marketed. These drugs are very useful in the treatment of gastric and duodenal ulcers, but it has been pointed out that the relapse of these ulcers occurs in a high ratio following the cessation of treatment with such antiacids. Although no clear cause for this has yet been proven, it has been empirically considered that the protection of the stomach against various injuries is advantageous to maintenance therapy for peptic ulcers. Therefore, numerous efforts have been continued to find a novel class of antiulcer agent which strengthens defensive mechanisms.

Some derivatives incorporating an imidazo[1,2-*a*]pyridine ring have been described as exhibiting cytoprotective activity.[3-5]

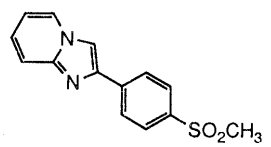
As a start for our studies on antiulcer durgs, we focused our attention on the activity of zolimidine (**1**), which was reported to protect against experimentally-induced lesions of the gastric mucosa in various animal species[3a] and to indicate its therapeutic benefit in peptic ulcer patients without marked side effects.[3b-d]

Oxobenz(o)-azolidine and -azine groups have been extensively introduced into various drugs, for example, anti-inflammatory,[6] antihypertensive,[7] cardiotonic,[8] and central nervous system affecting agents.[9] Thus, we wanted to combine this versatile pharmacophore with the imidazo-[1,2-*a*]pyridine nuclei.

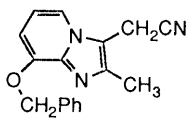
To our knowledge, no compound consisting of a two bicyclic hetero-ring system has been reported in the field of antiulcer drug treatment. In this paper, we describe the synthesis and pharmacological activities of a series of imidazo[1,2-*a*]pyridinyl-2-oxobenzoxazolidines, imidazo-[1,2-*a*]pyridinyl-3-oxo-2*H*-1,4-benzoxazines and related compounds.

Chemistry The desired compounds listed in Tables I and II were synthesized by the routes as shown in Charts 2—5.

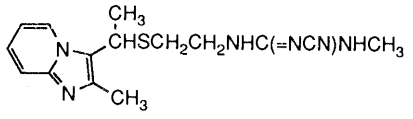
The 2-oxobenzoxazolidine and thiazolidine derivatives (**4**) were prepared according to methods similar to those described in the literature. Friedel–Crafts acylation[7-10] of 2-oxobenz(o)-azolidines (**6**) with α-bromoacylbromide in the presence of aluminum chloride gave the corresponding α-bromoketones (**7**). Condensation[11] of these haloketones (**7**) with the appropriate substituted 2-aminopyridines afforded the desired imidazo[1,2-*a*]pyridine derivatives (**4**) (Chart 2). The *N*-alkylated derivatives on the benzoxazolidine and thiazolidine group (**4g** and **4n**) were prepared by alkylation of **4d** and **4l** with the corresponding alkyl halides in the presence of sodium hydride in *N*,*N*-di-



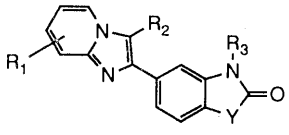
zolimidine (1)



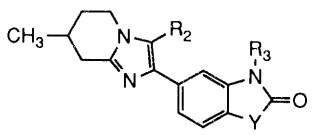
SCH-28080 (2)



3

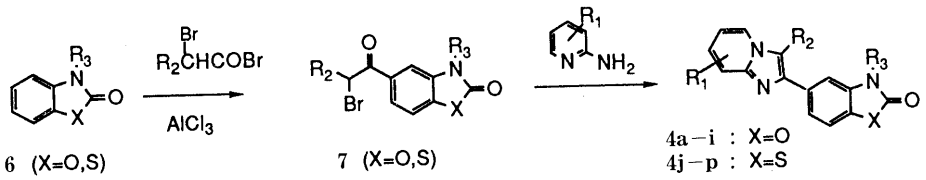


4 : Y = O, S
5 : Y = -O-CH-, -S-CH-
R4 R4



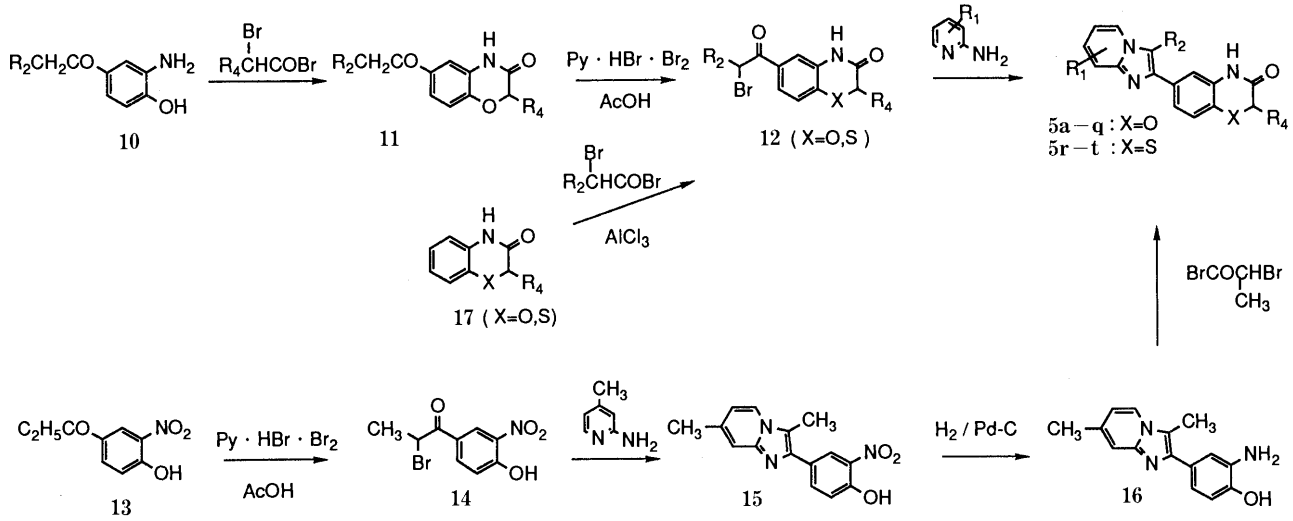
8 : Y = O
9 : Y = -O-CH-
CH3

Chart 1



6 (X=O,S) 7 (X=O,S)

Chart 2



10 11 12 (X=O,S) 17 (X=O,S) 13 14 15 16

Chart 3

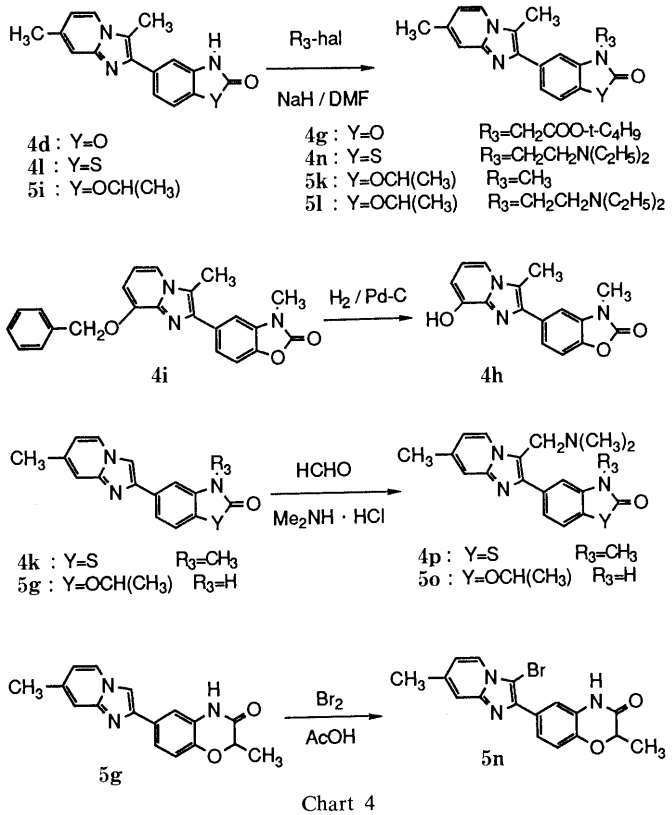


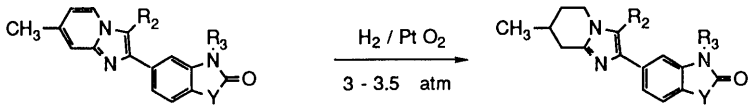
Chart 4

methylformamide (DMF). The 8-hydroxy derivative (**4h**) was obtained by debenzylation of **4i** using 10% palladium on carbon under hydrogen (1 atm) at room temperature. The Mannich base **4p** was prepared by the treatment of **4k** with formaline and dimethylamine hydrochloride in acetic acid[11a,b] (Chart 4). Hydrogenation of **4e** and **4f** by using platinum dioxide as a catalyst at 3 to 3.5 atm at room temperature afforded 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridine derivatives (**8a** and **8b**)[11d] (Chart 5).

The 3-oxo-2*H*-1,4-benzoxazine derivatives were obtained by three different pathways as outlined in Chart 3. 4-Acyl-2-aminophenol (**10**) was treated with α-bromoacyl bromide to afford 6-acyl-3-oxo-2*H*-1,4-benzoxazine (**11**), which was brominated with pyridinium bromide perbromide in the presence of hydrogen bromide in acetic acid to yield the corresponding α-bromoketones (**12**). In the second method, starting from 4′-hydroxy-3′-nitropropiophenone (**13**), bromination of **13** with pyridinium bromide perbromide, followed by treatment with 4-methyl-2-aminopyridine, gave 4-imidazo[1,2-*a*]pyridinyl-2-nitrophenol (**15**). Catalytic reduction of the nitro group over 10% palladium on carbon at room temperature gave *o*-aminophenol (**16**), followed by cyclization with α-bromopropionyl bromide afforded the desired product (**5i**). The third method was performed according to a synthetic route similar to that of azolidines (**4**). The bromo derivative at the 3-position on the imidazo[1,2-*a*]pyridine nuclei (**5n**) was obtained by the bromination of **5g** with bromine.[11a,c] The preparation of *N*-alkylated derivatives (**5k** and **5l**), Mannich base (**5o**) and 5,6,7,8-tetrahydro-derivatives (**9a—c**) were carried out in a manner similar to that of **4g**, **4p** and **8**. The 3-oxo-2*H*-1,4-benzothiazines (**5r—t**) were also obtained in a manner similar to that of benzothiazolidines (**4j—n**) from substituted benzothiazines (**17**) as the starting materials.

Pharmacological Results and Discussion

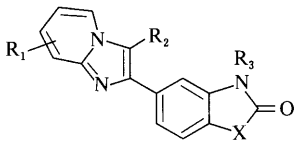
The imidazo[1,2-*a*]pyridine derivatives (**4** and **5**) and their 5,6,7,8-tetrahydro-analogues (**8** and **9**) obtained in this study were evaluated for antiulcer activity at a dose of 32 mg/kg



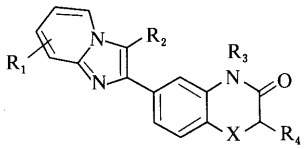
4e	Y=O	R_2=CH_3	R_3=CH_3	8a	Y=O	R_2=CH_3	R_3=CH_3
4f	Y=O	R_2=CH_3	R_3=iso-C_3H_7	8b	Y=O	R_2=CH_3	R_3=iso-C_3H_7
5g	Y=OCH(CH_3)	R_2=H	R_3=H	9a	Y=OCH(CH_3)	R_2=H	R_3=H
5i	Y=OCH(CH_3)	R_2=CH_3	R_3=H	9b	Y=OCH(CH_3)	R_2=CH_3	R_3=H
5k	Y=OCH(CH_3)	R_2=CH_3	R_3=CH_3	9c	Y=OCH(CH_3)	R_2=CH_3	R_3=CH_3

Chart 5

TABLE I. Physical Data of Imidazo[1,2-*a*]pyridines (**4** and **5**)



4



5

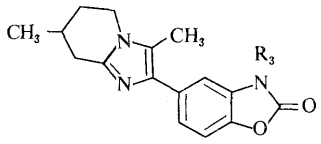
Compd. No.	R_1	R_2	R_3	R_4	X	Yield (%)	mp (°C) (Recryst. solvent)[a]	Formula	Analysis (%) Calcd (Found) C	H	N
4a	H	CH_3	CH_3		O	69.2	231—232 (D–W)	$C_{16}H_{13}N_3O_2$	68.81 (68.89)	4.69 4.61	15.04 15.01)
4b	H	CH_3	iso-C_3H_7		O	61.9	195—197 (A–T)	$C_{18}H_{17}N_3O_2$	70.34 (70.49)	5.57 5.57	13.67 13.63)
4c	6-CH_3	CH_3	CH_3		O	77.7	247—249 (A–T)	$C_{17}H_{15}N_3O_2$	69.61 (69.71)	5.15 5.10	14.33 14.43)
4d	7-CH_3	CH_3	H		O	25.1	276—278 (T–W)	$C_{16}H_{13}N_3O_2$ ·H_2O	64.63 (64.72)	5.41 5.03	14.13 14.14)
4e	7-CH_3	CH_3	CH_3		O	58.6	240 (EA–T)	$C_{17}H_{15}N_3O_2$	69.61 (69.55)	5.15 5.23	14.33 14.21)
4f	7-CH_3	CH_3	iso-C_3H_7		O	65.6	179—180 (EA–I)	$C_{19}H_{19}N_3O_2$	71.01 (70.68)	5.96 5.91	13.07 13.37)
4g	7-CH_3	CH_3	CH_2COO-*tert*-C_4H_9		O	71.1	198—200 (EA)	$C_{22}H_{23}N_3O_4$	67.16 (67.22)	5.89 5.86	10.68 10.59)
4h	8-OH	CH_3	CH_3		O	63.4	>280 (A–T)	$C_{16}H_{13}N_3O_3$	65.08 (65.07)	4.44 4.32	14.23 14.26)
4i	8-OCH_2Ph	CH_3	CH_3		O	54.5	210 (AN)	$C_{23}H_{19}N_3O_3$	71.68 (71.45)	4.97 4.93	10.90 11.06)
4j	H	CH_3	CH_3		S	50.8	206—208 (EA)	$C_{16}H_{13}N_3OS$	65.10 (65.14)	4.44 4.38	14.23 14.08)
4k	7-CH_3	H	CH_3		S	72.1	264—265 (EA)	$C_{16}H_{13}N_3OS$	65.10 (65.28)	4.44 4.41	14.23 14.15)
4l	7-CH_3	CH_3	H		S	42.3	252—254 (A–EA)	$C_{16}H_{13}N_3OS$	65.10 (64.82)	4.44 4.55	14.23 14.08)
4m	7-CH_3	CH_3	CH_3		S	63.4	204—206 (EA–T)	$C_{17}H_{15}N_3OS$	66.00 (65.73)	4.89 4.80	13.58 13.42)
4n	7-CH_3	CH_3	$CH_2CH_2N(C_2H_5)_2$		S	26.4	272 (I–M)	$C_{22}H_{26}N_4OS$ ·2HCl·$3H_2O$	50.67 (50.26)	6.57 6.61	10.74 10.64)
4o	7-CH_3	C_2H_5	CH_3		S	49.5	276—281 (A)	$C_{18}H_{17}N_3OS$ ·HCl·$3/2H_2O$	55.88 (55.96)	5.47 5.42	10.88 10.63)
4p	7-CH_3	$CH_2N(CH_3)_2$	CH_3		S	69.0	167—168 (EA)	$C_{19}H_{20}N_4OS$	64.75 (64.73)	5.72 5.71	15.90 15.70)
5a	H	H	H	CH_3	O	50.2	241—243 (C)	$C_{16}H_{13}N_3O_2$	68.81 (68.53)	4.69 4.55	15.04 14.98)
5b	H	CH_3	H	H	O	38.1	280—282 (A–EA)	$C_{16}H_{13}N_3O_2$	68.81 (68.65)	4.69 4.58	15.04 14.83)
5c	H	CH_3	H	CH_3	O	48.6	216—217 (A–E)	$C_{17}H_{15}N_3O_2$ ·H_2O	65.58 (65.96)	5.50 5.22	13.50 13.36)
5d	H	CH_3	H	C_2H_5	O	49.3	197—198 (C–E)	$C_{18}H_{17}N_3O_2$ ·H_2O	66.45 (66.09)	5.89 5.87	12.91 13.03)
5e	H	C_2H_5	H	CH_3	O	55.4	237—239 (A)	$C_{18}H_{17}N_3O_2$	70.34 (69.96)	5.57 5.56	13.67 13.50)
5f	6-CH_3	CH_3	H	CH_3	O	72.9	232 (D–W)	$C_{18}H_{17}N_3O_2$ ·$7/5H_2O$	65.36 (65.15)	5.97 5.95	12.70 12.43)
5g	7-CH_3	H	H	CH_3	O	79.5	255—256 (C)	$C_{17}H_{15}N_3O_2$ ·$2/3H_2O$	66.87 (67.00)	5.17 5.26	13.76 13.80)

TABLE I. (continued)

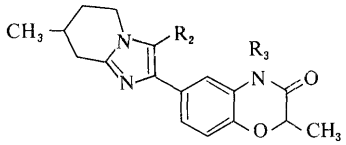
Compd. No.	R_1	R_2	R_3	R_4	X	Yield (%)	mp (°C) (Recryst. solvent)[a]	Formula	Analysis (%) Calcd (Found) C	H	N
5h	7-CH_3	CH_3	H	H	O	48.5	239—241 (C–E)	$C_{17}H_{15}N_3O_2 \cdot H_2O$	65.58 (65.21	5.50 5.35	13.50 13.33)
5i	7-CH_3	CH_3	H	CH_3	O	32.5	254—256 (EA)	$C_{18}H_{17}N_3O_2$	70.34 (70.38	5.57 5.59	13.67 13.67)
5j	7-CH_3	CH_3	H	C_2H_5	O	25.5	194—195 (EA)	$C_{19}H_{19}N_3O_2 \cdot H_2O$	67.24 (67.20	6.24 6.18	12.38 12.20)
5k	7-CH_3	CH_3	CH_3	CH_3	O	43.0	180—182 (EA)	$C_{19}H_{19}N_3O_2$	71.01 (70.79	5.96 6.01	13.07 12.98)
5l	7-CH_3	CH_3	$CH_2CH_2N(C_2H_5)_2$	CH_3	O	45.8	135—136 (EA–I)	$C_{24}H_{30}N_4O_2$	70.91 (70.74	7.44 7.43	13.78 14.03)
5m	7-CH_3	C_2H_5	H	CH_3	O	53.9	223—225 (C–E)	$C_{19}H_{19}N_3O_2$	71.01 (70.82	5.96 5.94	13.07 13.00)
5n	7-CH_3	Br	H	CH_3	O	72.0	229—230 (T)	$C_{17}H_{14}BrN_3O_2$	54.86 (55.17	3.79 3.76	11.29 10.94)
5o	7-CH_3	$CH_2N(CH_3)_2$	H	CH_3	O	66.5	183—184 (A–H)	$C_{20}H_{22}N_4O_2 \cdot H_2O$	65.20 (65.59	6.57 6.62	15.21 14.86)
5p	8-CH_3	CH_3	H	CH_3	O	62.8	249—250 (D–W)	$C_{18}H_{17}N_3O_2 \cdot 1/5H_2O$	69.53 (69.55	5.64 5.59	13.51 13.43)
5q	8-C_2H_5O	CH_3	H	CH_3	O	46.0	180—181 (D–W)	$C_{19}H_{19}N_3O_3 \cdot H_2O$	64.21 (64.33	5.96 6.03	11.82 11.73)
5r	H	CH_3	H	CH_3	S	45.3	229—231 (C–E)	$C_{17}H_{15}N_3OS \cdot 1/5H_2O$	65.24 (65.21	4.95 4.83	13.43 13.34)
5s	H	CH_3	CH_3	H	S	68.0	191 (E–EA)	$C_{17}H_{15}N_3OS$	66.00 (66.01	4.89 4.82	13.58 13.93)
5t	7-CH_3	CH_3	H	CH_3	S	68.1	259—261 (D–W)	$C_{18}H_{17}N_3OS$	66.85 (67.10	5.30 5.27	12.99 12.61)

a) A, EtOH; AN, MeCN; C, CH_2Cl_2; D, DMF; E, Et_2O; EA, EtOAc; H, hexane; I, diisopropyl ether; M, MeOH; T, THF; W, H_2O.

TABLE II. Physical Data of 5,6,7,8-Tetrahydroimidazo[1,2-*a*]pyridines (**8** and **9**)



8



9

Compd. No.	R_2	R_3	Yield (%)	mp (°C) (Recryst. solvent)[a]	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
8a		CH_3	49.0	213—215 (I–M)	$C_{17}H_{19}N_3O_2$	68.67	6.44	14.13	68.71	6.42	14.29
8b		iso-C_3H_7	52.7	164—166 (A–I)	$C_{19}H_{23}N_3O_2$	70.13	7.12	12.91	69.80	7.05	13.06
9a	H	H	82.9	215—218 (A–I)	$C_{17}H_{19}N_3O_2 \cdot 1/2H_2O$	66.65	6.58	13.72	66.52	6.61	13.55
9b	CH_3	H	74.7	221—222 (E–EA)	$C_{18}H_{21}N_3O_2 \cdot 6/5H_2O$	64.92	7.08	12.62	64.87	6.94	12.60
9c	CH_3	CH_3	66.5	112—115 (EA–I)	$C_{19}H_{23}N_3O_2 \cdot H_2O$	66.45	7.34	12.24	66.21	7.24	12.33

a) A, EtOH; E, Et_2O; EA, EtOAc; I, diisopropyl ether; M, MeOH.

p.o. in restraint and water-immersion stressed rats (stress ulcer). The results were compared with those of reference compounds, zolimidine (**1**), cimetidine and sucralfate, and are summarized in Table III.

In the series of 2-oxobenzoxazolidines, **4a** and **4e** exhibited excellent anti-stress ulcer activity. 6- and 8-substitution on the imidazo[1,2-*a*]pyridine ring (**4c** and **4h**) resulted in diminished activity. Introduction of a bulky substituent at the *N*-position of the benzoxazolidine ring (**4b**, **4f** and **4g**) tended to decrease activity. The activity of **4d**, the *N*-demethylated derivative of **4e**, was also relatively low. Among the 2-oxobenzothiazolidines, **4m**, which is the thio-isostere of **4e**, had high activity. Replacement of the 3-methyl group on the imidazo[1,2-*a*]pyridine ring of **4m** with a *N*,*N*-dimethylaminomethyl group (**4p**) showed lower activity, and the derivative with an ethyl group (**4o**) showed total disappearence of the activity. Compound **4j** without substitution on the pyridine component was less active than **4m**, but the degree of activity was comparable to that of cimetidine.

Next, the activities of 3-oxo-2*H*-1,4-benzoxazines and 3-oxo-2*H*-1,4-benzothiazines were examined. In this series, the most active compound was **5i**, which revealed complete inhibition of stress ulcers at the test dose. 6-Methyl (**5f**) and

TABLE III. Anti-ulcer Activity of Imidazo[1,2-*a*]pyridine Derivatives against Restraint and Water-Immersion Stressed Rats ($n=5$)

Compd. No.	Anti-stress ulcer activity (% inhibition) 32 mg/kg *p.o.*	Compd. No.	Anti-stress ulcer activity (% inhibition) 32 mg/kg *p.o.*
4a	88.1[a]	**5i**	98.8[a]
4b	45.0	**5j**	24.4
4c	44.9	**5k**	61.5
4d	52.2	**5l**	90.7[a]
4e	92.5[a]	**5m**	74.7
4f	60.5	**5n**	30.1
4g	−162.5	**5o**	19.1
4h	12.0	**5p**	59.7
4j	69.2	**5q**	−183.1
4m	80.5[a]	**5r**	54.5
4n	51.8	**5s**	31.0
4o	5.1	**5t**	66.7
4p	56.4	**8a**	74.7[a]
5a	2.2	**8b**	76.8[a]
5b	20.7	**9a**	62.2
5c	23.7	**9b**	74.2
5d	80.8[a]	**9c**	63.8
5e	48.9	Zolimidine	48.2
5f	57.5	Cimetidine	69.4[a]
5g	32.2	Sucralfate	45.6[b]
5h	58.3		

a) $p<0.01$. *b*) 100 mg/kg *p.o.*

TABLE IV. Gastric Cytoprotective Activity of **4e**, **5i** and **5l** in Rats ($n=5$)

Compd. No.	Dose (mg/kg *p.o.*)	Ethanol-induced gastric lesion (% inhibition)
4e	32	80.7[b]
	10	69.6[b]
	3.2	57.5[c]
5i	32	86.0[b]
	10	52.3[c]
	3.2	−20.6
5l	32	95.0[a]
	10	75.2[c]
	3.2	19.8
Zolimidine	32	64.9[c]
Cimetidine	100	40.3
Sucralfate	100	50.3

a) $p<0.001$. *b*) $p<0.01$. *c*) $p<0.05$.

8-methyl (**5p**) derivatives, positional isomers of the methyl group at the pyridine ring of **5i**, showed relatively low activities. The 8-ethoxy derivative (**5q**) exhibited an adverse effect. The activity of the 3-ethyl-7-methyl derivative (**5m**) was more potent than that of reference compounds. However, replacement of the substituent at the 3-position with a bromo (**5n**) or an *N*,*N*-dimethylaminomethyl group (**5o**) reduced the activities markedly. Introduction of an *N*,*N*-diethylaminoethyl group onto the *N*-position of the benzoxazine ring (**5l**) maintained high activity, but the activity of the *N*-methylated derivative (**5k**) was moderate. Conversion of the methyl group at the 2′-position on the benzoxazine ring (R_4) of **5i** into a hydrogen (**5h**) or an ethyl group (**5j**) tended to decrease activity. The activities of unsubstituted derivatives on the pyridine ring (**5a**—**c**, **5e**, **5r** and **5s**) were markedly reduced in comparison to that of the 7-methyl derivatives (**5i** and **5t**) except for the case of **5d** *versus* **5j**.

With regard to structure–activity relationships, these results indicate that the 3,7-dimethyl group is more favorable as a substituent on the imidazo[1,2-*a*]pyridine nuclei. On the other hand, the introduction of a bulky group at the *N*-position of the benz(o)-azolidine ring tends to cause the decrease of activity. As further study on the substitution at the *N*-position on the benz(o)-azine moiety is not yet complete, the influence in the case is still unclear.

Next, 5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridinyl derivatives (**8** and **9**) were evaluated. These compounds revealed good activities which were equipotent with that of cimetidine. No clear structure–activity relationship was apparent between these compounds and the parent compounds (**4e**, **4f**, **5g**, **5i** and **5k**).

Finally, the cytoprotective activities of three compounds (**4e**, **5i** and **5l**) were examined on the ethanol-induced gastric lesion (ethanol ulcer) in rats. The tested compounds were administered orally at a dose range from 3.2 to 32 mg/kg. The results were compared with those of reference compounds, zolimidine (32 mg/kg), cimetidine (100 mg/kg) and sucralfate (100 mg/kg), and are listed in Table IV. These evaluated compounds showed good activities, and the potencies at 32 mg/kg were superior to that of reference compounds.

In conclusion, we obtained several imidazo[1,2-*a*]pyridine derivatives having potent anti-stress ulcer activities (**4a**, **4e**, **4m**, **5d**, **5i** and **5l**). As an additional pharmacological profile, **4e**, **5i** and **5l** revealed cytoprotective activity against ethanol ulcer. The potencies of antiulcer activities of these compounds were superior to that of the prototype compound zolimidine and the therapeutic agents cimetidine and sucralfate.

Experimental

The melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The infrared (IR) spectra were taken with Hitachi 260-10 spectrometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with JNM-PMX 60 spectrometer using tetramethylsilane as an internal standard.

5-(3,7-Dimethylimidazo[1,2-*a*]pyridin-2-yl)-2-oxobenzoxazolidine (4d) A solution of 5-(2-bromopropionyl)-2-oxobenzoxazolidine (**7b**) (5.4 g, 20 mmol) and 2-amino-4-methylpyridine (6.5 g, 60 mmol) in MeCN (100 ml) was refluxed with stirring for 1 h. After the solvent was evaporated *in vacuo*, the residue was mixed with H_2O and AcOEt. The mixture was adjusted to pH 0.8 with 10% HCl and the resulting precipitate was collected by filtration. The obtained solid was suspended in a mixture of H_2O and AcOEt, and brought to pH 8.0 with 20% aqueous K_2CO_3. The crystals which separated were recrystallized from aqueous tetrahydrofuran (THF) to afford **4d** (1.4 g, 25.1%). IR (Nujol): 3500, 1760 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.37 (3H, s), 2.61 (3H, s), 6.79 (1H, dd, $J=2$, 7 Hz), 7.17 (1H, d, $J=8$ Hz), 7.33 (1H, d, $J=2$ Hz), 7.59 (1H, dd, $J=2$,8 Hz), 7.64 (1H, d, $J=2$ Hz), 8.21 (1H, d, $J=7$ Hz).

5-(8-Hydroxy-3-methylimidazo[1,2-*a*]pyridin-2-yl)-3-methyl-2-oxobenzoxazolidine (4h) A solution of **4i** (1.6 g, 4.2 mmol) in a mixture of EtOH (200 ml) and THF (100 ml) was treated with 10% Pd–C (50% wet, 0.5 g) under atmospheric pressure of H_2 at room temperature. After the catalyst was filtered off, the solvent was evaporated *in vacuo*. The residue was recrystallized from a mixture of EtOH and THF to afford **4h** (0.78 g, 63.4%). IR (Nujol): 1770, 1615, 1550 cm^{-1}. ^{1}H-NMR (CF_3COOH) δ: 2.76 (3H, s), 3.63 (3H, s), 7.33—7.90 (5H, m), 8.10 (1H, t, $J=7$ Hz).

5-(3-*N*,*N*-Dimethylaminomethyl-7-methylimidazo[1,2-*a*]pyridin-2-yl)-3-methyl-2-oxobenzothiazolidine (4p) A mixture of **4k** (1.9 g, 6.4 mmol), 36% aqueous HCHO (1.07 g, 12.8 mmol), 50% aqueous Me_2NH (1.16 g 12.8 mmol) and AcOH (770 mg, 12.8 mmol) in MeOH (10 ml) was stirred at 55—60 °C for 3 h. After the solvent was evaporated *in vacuo*, the residue was dissolved with 1 N HCl (10 ml), H_2O (10 ml) and AcOEt (30 ml). The aqueous layer was separated, adjusted to pH 7.0 with 20% aqueous K_2CO_3 and extracted with AcOEt. The extract was dried over $MgSO_4$ and

evaporated *in vacuo*. The residue was recrystallized from AcOEt to afford **4p** (1.56 g, 69.0%). IR (Nujol): 1670, 1570 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.16 (6H, s), 2.38 (3H, s), 3.45 (3H, s), 3.88 (2H, s), 6.78 (1H, dd, J=2,7 Hz), 7.35 (1H, d, J=8 Hz), 7.40 (1H, d, J=2 Hz), 7.89 (1H, dd, J=2, 8 Hz), 8.12 (1H, d, J=2 Hz), 8.40 (1H, d, J=7 Hz).

2-Bromo-4′-hydroxy-3′-nitropropiophenone (14) Pyridinium bromide perbromide (19.2 g, 60 mmol) was added in portions to a solution of 4′-hydroxy-3′-nitropropiophenone (**13**) (10.8 g, 55 mmol) and 30% HBr–AcOH (10 ml) in AcOH (50 ml) at room temperature with stirring. After being stirred for 1 h, the mixture was poured into H_2O. The resulting precipitate was collected by filtration, washed with H_2O and recrystallized from a mixture of AcOEt and ether to afford **14** (14.3 g, 94.1%), mp 62—64 °C. IR (Nujol): 3150, 1690, 1620, 1535 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.80 (3H, d, J=7 Hz), 5.78 (1H, q, J=7 Hz), 7.30 (1H, d, J=9 Hz), 8.20 (1H, dd, J=2, 9 Hz), 8.55 (1H, d, J=2 Hz).

3,7-Dimethyl-2-(4-hydroxy-3-nitrophenyl)imidazo[1,2-*a*]pyridine (15) This compound was prepared from **14** in a manner similar to that described for **5i**. Yield 35.9%, mp 176—179 °C (from AcOEt–THF). IR (Nujol): 1630, 1530, 1350 cm^{-1}. ^{1}H-NMR (CF_3COOH) δ: 2.69 (3H, s), 2.74 (3H, s), 7.45 (1H, d, J=7 Hz), 7.46 (1H, d, J=9 Hz), 7.70 (1H, s), 7.92 (1H, dd, J=2, 9 Hz), 8.30 (1H, d, J=7 Hz), 8.46 (1H, d, J=2 Hz).

2-(3-Amino-4-hydroxyphenyl)-3,7-dimethylimidazo[1,2-*a*]pyridine (16) A solution of **15** (2.6 g, 9.2 mmol) in a mixture of EtOH (200 ml) and THF (100 ml) was hydrogenated under atmospheric pressure of H_2 over 10% Pd–C (50% wet, 2.0 g) at room temperature. After the catalyst was removed by filtration, the filtrate was evaporated *in vacuo*. The residue was recrystallized from a mixture of AcOEt and THF to afford **16** (1.25 g, 53.9%), mp 251—253 °C. IR (Nujol): 3370, 1620 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.35 (3H, s), 2.55 (3H, s), 6.72 (1H, dd, J=2, 8 Hz), 6.75 (1H, d, J=8 Hz), 6.88 (1H, d, J=7 Hz), 7.13 (1H, d, J=2 Hz), 7.25 (1H, s), 8.17 (1H, d, J=7 Hz).

3,4-Dihydro-6-(3,7-dimethylimidazo[1,2-*a*]pyridin-2-yl)-2-methyl-3-oxo-2*H*-1,4-benzoxazine (5i) 2-Bromopropionyl bromide (1.30 g, 6.0 mmol) was added dropwise to a solution of **16** (1.27 g, 5.0 mmol) and Et_3N (1.0 g, 10.0 mmol) in THF (100 ml) at room temperature and the mixture was stirred for 2 h. After being stirred at 60—70 °C for an additional 2 h, the solvent was evaporated *in vacuo* and the residue was mixed with AcOEt and H_2O. The organic layer was separated, washed with brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on silica gel by eluting with a mixture of $CHCl_3$ and MeOH (9 : 1) to give **5i** (0.5 g, 32.5%) after recrystallization from AcOEt. IR (Nujol): 1690, 1605 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.45 (3H, d, J=7 Hz), 2.37 (3H, s), 2.60 (3H, s), 4.71 (1H, q, J=7 Hz), 6.79 (1H, dd, J=2,7 Hz), 7.03 (1H, d, J=8 Hz), 7.31 (1H, d, J=2 Hz), 7.33 (1H, dd, J=2,8 Hz), 7.44 (1H, d, J=2 Hz), 8.20 (1H, d, J=7 Hz), 10.70 (1H, s).

3,4-Dihydro-2,4-dimethyl-6-(3,7-dimethylimidazo[1,2-*a*]pyridin-2-yl)-3-oxo-2*H*-1,4-benzoxazine (5k) NaH (50% dispersion in mineral oil, 0.6 g, 12.5 mmol) was added in portions to a solution of **5i** (2.5 g, 8.1 mmol) in DMF (25 ml) at room temperature and the mixture was stirred for 2 h. MeI (1.7 g, 12.0 mmol) was added and the resulting mixture was further stirred for 3 h. The solvent was evaporated *in vacuo*. The residue was suspended in H_2O and acidified with 10% aqueous HCl. The aqueous solution was washed with AcOEt, and then adjusted to pH 7.5 with aqueous K_2CO_3. The resulting precipitate was extracted with $CHCl_3$. The extract was washed with brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was recrystallized from AcOEt to afford **5k** (1.1 g, 43.0%). IR (Nujol): 1680, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.47 (3H, d, J=7 Hz), 2.37 (3H, s), 2.60 (3H, s), 3.37 (3H, s), 4.75 (1H, q, J=7 Hz), 6.78 (1H, dd, J=2,7 Hz), 7.03 (1H, d, J=8 Hz), 7.32 (1H, d, J=2 Hz), 7.43 (1H, dd, J=2, 8 Hz), 7.52 (1H, d, J=2 Hz), 8.20 (1H, d, J=7 Hz).

6-(3-Bromo-7-methylimidazo[1,2-*a*]pyridin-2-yl)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazine (5n) Pyridinium bromide perbromide (1.44 g, 4.5 mmol) was added in portions to a solution of **5g** (1.20 g, 4.1 mmol) in a mixture of AcOH (9 ml) and THF (7 ml) at room temperature with stirring. After being stirred for 2 h, the reaction mixture was poured into H_2O and neutralized to pH 7.0 with 20% aqueous K_2CO_3. The resulting precipitate was extracted with AcOEt and the extract was dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the residue was recrystallized from THF to afford **5n** (1.10 g, 72.0%). IR (Nujol): 1685, 1610 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.46 (3H, d, J=7 Hz), 2.40 (3H, s), 4.74 (1H, q, J=7 Hz), 6.94 (1H, dd, J=2, 7 Hz), 7.08 (1H, d, J=8 Hz), 7.43 (1H, d, J=2 Hz), 7.68 (1H, dd, J=2,8 Hz), 7.70 (1H, d, J=2 Hz), 8.26 (1H, d, J=7 Hz), 10.81 (1H, s).

3,4-Dihydro-6-(3,7-dimethyl-5,6,7,8-tetrahydroimidazo[1,2-*a*]pyridin-2-yl)-2-methyl-3-oxo-2*H*-1,4-benzoxazine (9b) A solution of **5i** (2.46 g, 8.0 mmol) in MeOH (200 ml) was hydrogenated over PtO_2 (0.35 g) at 3 to 3.5 atm pressure of H_2 for 6 h at room temperature. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo* to give a residue. An aqueous solution of the residue was adjusted to pH 8.0 with 20% aqueous K_2CO_3 and extracted with AcOH. The extract was washed with brine, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from a mixture of AcOEt and ether to afford **9b** (1.86 g, 74.7%). IR (KBr): 3400, 1680, 1605 cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.06 (3H, d, J=6 Hz), 1.42 (3H, d, J=7 Hz), 1.52—1.64 (1H, m), 1.94—2.01 (2H, m), 2.18—2.36 (1H, m), 2.27 (3H, s), 2.83 (1H, dd, J=4, 16 Hz), 3.68 (1H, dt, J=5, 12 Hz), 3.88—3.95 (1H, m), 4.64 (1H, q, J=7 Hz), 6.78 (1H, d, J=8 Hz), 7.11 (1H, dd, J=2, 8 Hz), 7.23 (1H, d, J=2 Hz), 10.63 (1H, br s).

Restraint and Water-Immersed Stress Ulcer The stress ulcer was produced according to the method described by Takagi and Okabe.[12] 7 week old male SD rats, weighing 200 to 260 g, were fasted for 24 h before the experiment. A suspension of the test drug in 0.1% methylcellulose (MC) was administered orally to the group of five rats 30 min before stress initiation. The rats were immobilized in a stress cage and immersed vertically in a water bath at 22 °C to the height of the xiphoid process of the rats. 7 h later, the animals were sacrificed. The isolated stomachs were cut open along the greater curvature and examined for ulcers generated in the glandular portion. The ulcer index was calculated as the sum of the area (mm^2) of each ulcer in the stomach. The inhibitory ratio (%) was obtained by comparing the ulcer index with that of the control group.

Ethanol-Induced Gastric Lesion The cytoprotective effect against absolute ethanol was examined by the method of Robert *et al.*[13] 6 week old male SD rats, weighing 150 to 210 g, were fasted for 24 h before the experiment. A suspension of the test drug in 0.1% MC was administered orally to a group of five rats. After 30 min, absolute ethanol (1 ml) was given orally, and the animals were sacrificed 1 h later. The stomachs were isolated and cut open along the greater curvature. The ulcer index was calculated as the sum of the length (mm) of each lesion in the glandular portion. The inhibitory ratio (%) was obtained by comparing the ulcer index with that of the control group.

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Acylated Iridoid Glycosides from *Buddleja japonica* HEMSL.

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Sixteen new acylated iridoid glycosides, called buddlejosides A_1—A_{16}, were isolated from *Buddleja japonica* HEMSL., together with four known iridoid glycosides; 6-vanilloyl-ajugol, 6-feruloyl-ajugol, verbascoside A, and 6-*O*-[α-L-(4-*O*-feruloyl)-rhamnopyranosyl]-catalpol. Structures of the new compounds were determined on the basis of chemical and spectroscopic evidence. Buddlejosides A_1 was an ajugol derivative acylated with monoterpenic acid, A_2 was an sinuatol and A_3—A_{16} were 6-rhamnopyranosyl-catalpol derivatives acylated with cinnamic acid derivative and acetic acid.

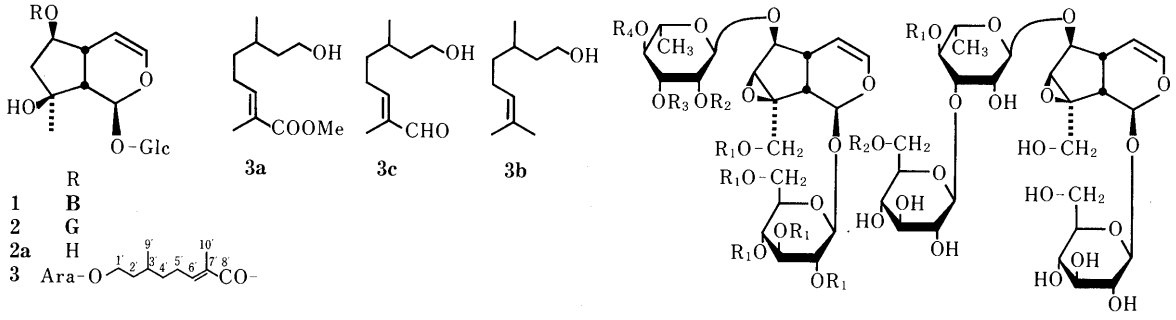
Keywords *Buddleja japonica*; Buddlejaceae; iridoid glycoside; *trans*-cinnamic acid derivative; *cis*-cinnamic acid derivative

Some *Buddleja* spp. are known to be toxic to fish and to contain caryophyllane-type sesquiterpenoids called buddledins,[1] phenylethanoid glycosides[2-4] or iridoid glycosides.[3,4] Houghton and Hikino reported the isolation of acteoside, echinacoside and linarin as the anti-hepatotoxic active compounds of *B. officinalis*, *B. globosa* and *B. americana*.[3]

We investigated the polar constituents of *B. japonica* HEMSL. and isolated twenty acylated iridoid glycosides.

The suspension of the methanolic extract of the air dried whole plants in water was extracted with ether and the water layer was passed through a Diaion HP-20 column. The adsorbed material was successively eluted with 50% methanol and 100% methanol. The 100% methanolic eluate afforded twenty iridoid glycosides after repeated chromatography. Four known compounds (**1**, **2**, **5**, **8**) were identified by comparison with reported data.[5-7] New iridoid glycosides, buddlejosides A_3—A_{16}, were 6-rhamnopyranosyl-catalpol derivatives.

Buddlejoside A_1 (**3**), $C_{30}H_{48}O_{15}\cdot H_2O$, was obtained as an amorphous powder. Its proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra suggested that **3** was also an acylated ajugol derivative like **1** and **2**, showing a singlet methyl signal at δ 1.37 and two olefinic proton signals at δ 4.95 (1H, dd, J=6, 2 Hz) and 6.21 (1H, dd, J=6, 2 Hz). Alkaline hydrolysis afforded ajugol (**2a**) and acid hydrolysis afforded arabinose and glucose as the sugar moiety. In the ^{13}C-NMR spectrum, ten carbon signals were observed other than arabinose and ajugol moiety and three downfield shifted carbon signals (δ 128.7; 144.3; 169.5) suggested the presence of α,β-unsaturated ester group. A secondary methyl [δ 0.94 (3H, d, J=6.5 Hz)], a vinyl methyl [δ 1.83 (3H, d, J=1 Hz)], a downfield shifted olefinic proton [δ 6.80 (1H, qt, J=1, 7.5 Hz)] and two proton signals attached to an oxygenated carbon [δ 3.57 (1H, m); 3.85 (1H, m)] were observed in the ^{1}H-NMR spectrum of **3**. At the last two proton signals, the nuclear Overhauser effects (NOEs) were observed by irradiation of the doublet methyl signal at δ 0.94 and of the anomeric



	R
1	B
2	G
2a	H
3	Ara-O

	R_1	R_2	R_3
4	H	A	D
4a	A	A	A

	R_1	R_2	R_3	R_4
5	H	H	H	D
5a	H	H	H	H
5b	A	A	A	D
6	H	A	H	D
7	H	H	A	D
8	H	H	D	H
8a	A	A	D	A
9	H	A	D	H
10	H	A	H	E
10a	A	A	A	E
11	H	H	A	E
12	H	H	H	H
12a	A	A	A	H
13	H	A	H	H
14	H	H	A	H
15	H	H	A	I
16	H	H	A	G
16a	A	A	A	G

	R_1	R_2
17	D	F
17a	H	H
18	D(G)	G(D)
19	C(G)	G(C)
20	E	F

A : acetyl
B : vanilloyl
C : *trans*-*p*-coumaroyl
D : *trans*-*p*-methoxycinnamoyl
E : *cis*-*p*-methoxycinnamoyl
F : *trans*-caffeoyl
G : *trans*-feruloyl
H : *trans*-3, 4-dimethoxycinnamoyl
I : *cis*-3, 4-dimethoxycinnamoyl

Chart 1

TABLE I. ^{1}H-NMR Chemical Shifts and Coupling Constants in Methanol-d_4

	4	5	5a	6	7
Aglycone moiety					
1	4.94 (1H, d, J=7 Hz)	5.09 (1H, d, J=9.5 Hz)	5.08 (1H, d, J=9.5 Hz)	5.08 (1H, d, J=10 Hz)	5.11 (1H, d, J=9 Hz)
3	6.34 (1H, dd, J=6, 2 Hz)	6.38 (1H, dd, J=6, 1.5 Hz)	6.36 (1H, dd, J=6, 1.5 Hz)	6.39 (1H, dd, J=6, 2 Hz)	6.40 (1H, dd, J=6, 1.5 Hz)
4	5.11 (1H, dd, J=6, 4 Hz)	5.06 (1H, dd, J=6, 4 Hz)	5.06 (1H, dd, J=6, 4.5 Hz)	5.06 (1H, dd, J=6, 5 Hz)	
5	2.84 (1H, m)	2.42 (1H, m)	2.39 (1H, m)	2.44 (1H, m)	2.48 (1H, m)
6	4.48 (1H, m)	4.03 (1H, d, J=8 Hz)	4.00 (1H, br d, J=8 Hz)	4.03 (1H, dd, J=8, 1 Hz)	4.06 (1H, d, J=8 Hz)
7	5.88 (1H, br s)	3.65 (1H, br s)	3.62 (1H, br s)	3.64 (1H, br s)	3.69 (1H, br s)
9	2.92 (1H, br t, J=7 Hz)	2.57 (1H, dd, J=9.5, 8 Hz)	2.54 (1H, dd, J=9.5, 8 Hz)	2.57 (1H, dd, J=10, 8 Hz)	2.60 (1H, dd, J=9, 8 Hz)
10	4.20 (1H, br d, J=15 Hz)	3.81 (1H, d, J=13 Hz)	3.80 (1H, d, J=13 Hz)	3.81 (1H, d, J=13 Hz)	3.84 (1H, d, J=13 Hz)
	4.38 (1H, br d, J=15 Hz)	4.15 (1H, d, J=13 Hz)	4.14 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.17 (1H, d, J=13 Hz)
Sugar moiety (Glucose)					
1	4.68 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.80 (1H, d, J=8 Hz)
2	3.22 (1H, t, J=8 Hz)	3.26 [a]	3.25 (1H, t, J=8 Hz)		3.3 [a]
3	3.27 [a]	3.40 (1H, t, J=9 Hz)	3.39 (1H, t, J=8.5 Hz)		3.43 (1H, t, J=9 Hz)
4	3.27 [a]	3.25 [a]	3.24 (1H, t, J=8.5 Hz)		3.3 [a]
5	3.3 [a]	3.3 [a]			3.3 [a]
6	3.64 (1H, dd, J=12, 5 Hz)	3.63 (1H, dd, J=12, 6 Hz)	3.62 (1H, dd, J=12, 6 Hz)	3.62 (1H, dd, J=12, 6 Hz)	3.66 (1H, dd, J=12, 6 Hz)
	3.86 (1H, dd, J=12, 2 Hz)		3.91 (1H, dd, J=12, 2 Hz)	3.92 (1H, dd, J=12, 2 Hz)	3.94 (1H, br d, J=12 Hz)
(Rhamnose)					
1	4.90 (1H, d, J=1.5 Hz)	5.00 (1H, br s)	4.93 (1H, d, J=1.5 Hz)	5.02 (1H, d, J=1.5 Hz)	5.04 (1H, br s)
2	5.03 (1H, dd, J=3.5, 1.5 Hz)	3.93 (1H, dd, J=3, 1.5 Hz)	3.85 (1H, dd, J=3.5, 1.5 Hz)	5.12 (1H, dd, J=3.5, 1.5 Hz)	4.11 (1H, dd, J=3, 1.5 Hz)
3	4.07 (1H, dd, J=9.5, 3.5 Hz)	3.88 [a]	3.67 (1H, dd, J=9.5, 3.5 Hz)	4.13 (1H, dd, J=9.5, 3.5 Hz)	5.25 (1H, dd, J=9.5, 3 Hz)
4	5.03 (1H, t, J=9.5 Hz)	5.08 (1H, t, J=9.5 Hz)	3.39 (1H, t, J=9.5 Hz)	5.04 (1H, t, J=9.5 Hz)	5.29 (1H, t, J=9.5 Hz)
5	3.95 (1H, dd, J=10, 6 Hz)				
6	1.19 (3H, d, J=6 Hz)	1.17 (3H, d, J=6 Hz)	1.25 (3H, d, J=6 Hz)	1.18 (3H, d, J=6 Hz)	1.22 (3H, d, J=6 Hz)
(Glucose)					
1					
2					
3					
4					
5					
6					
Ester moiety (Acetyl)					
2	2.15 (3H, s)			2.16 (3H, s)	2.02 (3H, s)
(Cinnamoyl at rhamnose)					
β	6.42 (1H, d, J=16 Hz)	6.43 (1H, d, J=16 Hz)		6.43 (1H, d, J=16 Hz)	6.37 (1H, d, J=16 Hz)
γ	7.69 (1H, d, J=16 Hz)	7.68 (1H, d, J=16 Hz)		7.70 (1H, d, J=16 Hz)	7.67 (1H, d, J=16 Hz)
2	7.57 (1H, d, J=9 Hz)	7.56 (1H, d, J=8.5 Hz)		7.57 (1H, d, J=9 Hz)	7.56 (1H, d, J=8.5 Hz)
3	6.95 (1H, d, J=9 Hz)	6.95 (1H, d, J=8.5 Hz)		6.96 (1H, d, J=9 Hz)	6.96 (1H, d, J=8.5 Hz)
5	6.95 (1H, d, J=9 Hz)	6.95 (1H, d, J=8.5 Hz)		6.96 (1H, d, J=9 Hz)	6.96 (1H, d, J=8.5 Hz)
6	7.57 (1H, d, J=9 Hz)	7.56 (1H, d, J=8.5 Hz)		7.57 (1H, d, J=9 Hz)	7.56 (1H, d, J=8.5 Hz)
OMe	3.83 (3H, s)	3.83 (3H, s)		3.83 (3H, s)	3.83 (3H, s)
(Cinnamoyl at glucose)					
β					
γ					
2					
3					
5					
6					
OMe					

a) Overlapped with other signals.

proton signal of arabinose at δ 4.18. **3** was treated with acetyl chloride–methanol (1:20) to give **3a**, which was decided on as to be *R* configuration by comparison with a chiral synthetic sample in high-performance liquid chromatography (HPLC) using a chiral column. From these data, buddlejoside A_1 was decided on as to be **3**. An absolute configuration of arabinose was not clear because of too small a yield.

Buddlejoside A_2 (**4**), $C_{33}H_{42}O_{16}\cdot 3/2H_2O$ was obtained as an amorphous powder. Its ultraviolet (UV) spectrum showed the absorption maxima at 227 (4.15), 299 (sh 4.30) and 312 (4.35) nm ($\log\varepsilon$) indicating the presence of cinnamoyl residue in the molecule. Alkaline hydrolysis gave *p*-methoxycinnamic acid and sinuatol acetate (**4a**) followed by acetylation.[8] Acid hydrolysis gave rhamnose and glucose as the sugar moiety. The ^{1}H- and ^{13}C-NMR spectra suggested that an acetyl and a *p*-methoxycinnamoyl group were located at C-2 and C-4 of the rhamnosyl residue. A carbonyl carbon signal (δ 170.7) of the acetyl group was long-range coupled with H-2 signal (δ 5.74) of rhamnose and a carbonyl carbon signal (δ 167.1) of the *p*-methoxycinnamoyl group was long-range coupled with H-4 signal (δ 5.84) of rhamnose in a pyridine-d_5 solution. These data led us to conclude the structure of buddlejoside A_2 was to be **4**.

Buddlejosides A_3 (**6**), $C_{33}H_{42}O_{17}\cdot 1/2H_2O$ and A_4 (**7**),

TABLE I. (continued)

	8	9	10	11	12
Aglycone moiety					
1	5.10 (1H, d, J=9.5 Hz)	5.09 (1H, d, J=10 Hz)	5.07 (1H, d, J=10 Hz)	5.10 (1H, d, J=9 Hz)	5.09 (1H, d, J=9.5 Hz)
3	6.38 (1H, dd, J=6, 2 Hz)	6.38 (1H, dd, J=6, 2 Hz)	6.37 (1H, dd, J=6, 1.5 Hz)	6.39 (1H, dd, J=6, 1.5 Hz)	6.38 (1H, dd, J=6, 2 Hz)
4	5.11 (1H, dd, J=6, 4 Hz)	5.10 (1H, dd, J=6, 5 Hz)	4.99 [a]	5.02 (1H, dd, J=6, 1 Hz)	5.01 (1H, dd, J=6, 2 Hz)
5	2.46 (1H, m)	2.48 (1H, m)	2.41 (1H, m)	2.45 (1H, m)	2.42 (1H, m)
6	4.05 (1H, dd, J=8, 1 Hz)	4.05 (1H, dd, J=8, 1 Hz)	4.00 (1H, d, J=8 Hz)	4.03 (1H, d, J=8 Hz)	4.03 (1H, br d, J=8 Hz)
7	3.66 (1H, br s)	3.65 (1H, br s)	3.62 (1H, br s)	3.67 (1H, br s)	3.65 (1H, br s)
9	2.57 (1H, dd, J=9.5, 8 Hz)	2.59 (1H, dd, J=10, 8 Hz)	2.56 (1H, dd, J=9, 8 Hz)	2.58 (1H, dd, J=9, 8 Hz)	2.57 (1H, dd, J=9.5, 8 Hz)
10	3.82 (1H, d, J=13 Hz)	3.81 (1H, d, J=13 Hz)		3.83 (1H, d, J=13 Hz)	3.82 (1H, d, J=13 Hz)
	4.15 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.14 (1H, d, J=13 Hz)	4.16 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)
Sugar moiety (Glucose)					
1	4.78 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.76 (1H, d, J=8 Hz)	4.79 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)
2			3.25 [a]	3.27 [a]	3.25 [a]
3	3.40 (1H, t, J=9 Hz)		3.39 (1H, t, J=9 Hz)	3.41 (1H, t, J=9 Hz)	3.40 (1H, t, J=8.5 Hz)
4			3.25 [a]	3.27 [a]	3.25 [a]
5			3.3 [a]	3.3 [a]	
6	3.63 (1H, dd, J=12, 6 Hz)	3.61 (1H, dd, J=12, 5 Hz)	3.62 (1H, dd, J=12, 6 Hz)	3.64 (1H, dd, J=12, 6 Hz)	3.63 (1H, dd, J=12, 6 Hz)
	3.92 (1H, dd, J=12, 2 Hz)	3.92 (1H, dd, J=12, 2 Hz)	3.91 (1H, dd, J=12, 2 Hz)	3.93 (1H, dd, J=12, 2 Hz)	3.91 (1H, dd, J=12, 2 Hz)
(Rhamnose)					
1	4.97 (1H, d, J=1.5 Hz)	5.01 (1H, d, J=1.5 Hz)	5.00 (1H, br s)	5.01 (1H, br s)	5.00 (1H, br s)
2	4.09 (1H, dd, J=3.5, 1.5 Hz)	5.31 (1H, dd, J=3.5, 1.5 Hz)	5.10 (1H, dd, J=3, 1.5 Hz)	4.08 (1H, dd, J=3, 1.5 Hz)	3.92 (1H, dd, J=3.5, 1.5 Hz)
3	5.11 (1H, dd, J=9.5, 3.5 Hz)	5.21 (1H, dd, J=9.5, 3.5 Hz)	4.05 (1H, dd, J=9.5, 3 Hz)	5.13 (1H, dd, J=9.5 Hz)	
4	3.68 (1H, t, J=9.5 Hz)	3.62 (1H, t, J=9.5 Hz)	4.98 (1H, t, J=9.5 Hz)	5.23 (1H, t, J=9.5 Hz)	5.08 (1H, t, J=9.5 Hz)
5					
6	1.31 (3H, d, J=6 Hz)	1.32 (3H, d, J=6 Hz)	1.15 (3H, d, J=6 Hz)	1.18 (3H, d, J=6 Hz)	1.18 (3H, d, J=6 Hz)
(Glucose)					
1					
2					
3					
4					
5					
6					
Ester moiety (Acetyl)					
2		2.11 (3H, s)	2.15 (3H, s)	2.02 (3H, s)	
(Cinnamoyl at rhamnose)					
β	6.47 (1H, d, J=16 Hz)	6.36 (1H, d, J=16 Hz)	5.87 (1H, d, J=13 Hz)	5.81 (1H, d, J=13 Hz)	6.46 (1H, d, J=16 Hz)
γ	7.73 (1H, d, J=16 Hz)	7.65 (1H, d, J=16 Hz)	6.97 (1H, d, J=13 Hz)	6.98 (1H, d, J=13 Hz)	7.67 (1H, d, J=16 Hz)
2	7.56 (1H, d, J=9 Hz)	7.56 (1H, d, J=9 Hz)	7.72 (1H, d, J=9 Hz)	7.70 (1H, d, J=8.5 Hz)	7.23 (1H, d, J=2 Hz)
3	6.96 (1H, d, J=9 Hz)	6.95 (1H, d, J=9 Hz)	6.89 (1H, d, J=9 Hz)	6.91 (1H, d, J=8.5 Hz)	
5	6.96 (1H, d, J=9 Hz)	6.95 (1H, d, J=9 Hz)	6.89 (1H, d, J=9 Hz)	6.91 (1H, d, J=8.5 Hz)	6.97 (1H, d, J=8 Hz)
6	7.56 (1H, d, J=9 Hz)	7.56 (1H, d, J=9 Hz)	7.72 (1H, d, J=9 Hz)	7.70 (1H, d, J=8.5 Hz)	7.19 (1H, dd, J=8, 2 Hz)
OMe	3.83 (3H, s)	3.83 (3H, s)	3.81 (3H, s)	3.83 (3H, s)	3.86 (6H, s)
(Cinnamoyl at glucose)					
β					
γ					
2					
3					
5					
6					
OMe					

$C_{33}H_{42}O_{17}\cdot H_2O$ showed similar ^{1}H- and ^{13}C-NMR spectra to those of **5** except for the presence of an acetyl group [**6**: δ 2.16 (3H, s); 20.9, 172.2. **7**: δ 2.02 (3H, s); 20.9, 172.0]. Acetylation, alkaline and acid hydrolysis of these two compounds afforded the same reaction products **5b**, **5a**[9] + *p*-methoxycinnamic acid and rhamnose + glucose, respectively. The ^{1}H- and ^{13}C-NMR spectra showed the acylated position to be C-2 and C-4 of rhamnose in **6** and to be C-3 and C-4 of rhamnose in **7** by downfield shifts and acylation shifts. These data gave the structure **6** and **7** for buddlejosides A_3 and A_4, respectively.

Buddlejoside A_5 (**9**), $C_{33}H_{42}O_{17}\cdot H_2O$ gave peracetate **8a** and the ^{1}H- and ^{13}C-NMR spectra of **9** indicated the presence of an acetyl group [δ 2.11 (3H, s); 20.7, 171.7] and showed that **9** was acylated at C-2 and C-3 of rhamnose. So, the structure of buddlejoside A_5 was decided on as to be **9**.

Buddlejosides A_6 (**10**), $C_{33}H_{42}O_{17}\cdot 1/2H_2O$ and A_7 (**11**), $C_{33}H_{42}O_{17}\cdot H_2O$ consisted of a *cis-p*-methoxycinnamic acid [δ 5.87; 6.97 (each 1H, d, J=13 Hz)] and gave the same acetate **10a**. In the ^{13}C-NMR spectrum of **10**, the long-range couplings were observed between H-2 signal of rhamnose and C-1 signal of acetyl group and between H-4 signal of rhamnose and carbonyl carbon signal of *cis-p*-methoxycinnamoyl group. So, acetyl group was attached to C-2 and C-3 of rhamnose in buddlejosides A_6 and A_7, respectively.

Buddlejosides A_8 (**12**), $C_{32}H_{42}O_{17}\cdot 3/2H_2O$, A_9 (**13**),

TABLE I. (continued)

	13	14	15	16	17
Aglycone moiety					
1	5.08 (1H, d, J=10 Hz)	5.10 (1H, d, J=9 Hz)	5.09 (1H, d, J=9.5 Hz)	5.10 (1H, d, J=9.5 Hz)	4.95 (1H, d, J=9.5 Hz)
3	6.38 (1H, dd, J=6, 1.5 Hz)	6.39 (1H, dd, J=6, 1.5 Hz)	6.38 (1H, dd, J=6, 2 Hz)	6.39 (1H, dd, J=6, 2 Hz)	6.29 (1H, dd, J=6, 2 Hz)
4	5.04 [a]	5.08 (1H, dd, J=6, 5 Hz)	5.03 (1H, dd, J=6, 4.5 Hz)	5.09 (1H, dd, J=6, 4 Hz)	5.02 (1H, dd, J=6, 5 Hz)
5	2.43 (1H, m)	2.46 (1H, m)	2.44 (1H, m)	2.46 (1H, m)	2.27 (1H, m)
6	4.03 (1H, br d, J=8 Hz)	4.05 (1H, br d, J=8 Hz)	4.02 (1H, br d, J=8 Hz)	4.05 (1H, br d, J=8 Hz)	3.65 (1H, d, J=8 Hz)
7	3.64 (1H, br s)	3.67 (1H, br s)	3.65 (1H, br s)	3.67 (1H, br s)	3.37 (1H, br s)
9	2.57 (1H, dd, J=9, 7 Hz)	2.58 (1H, dd, J=9, 8 Hz)	2.57 (1H, dd, J=9.5, 8 Hz)	2.58 (1H, dd, J=9.5, 7 Hz)	2.41 (1H, dd, J=9.5, 8 Hz)
10	3.81 (1H, d, J=13 Hz)	3.82 (1H, d, J=13 Hz)	3.81 (1H, d, J=13 Hz)	3.82 (1H, d, J=13 Hz)	3.73 (1H, d, J=13 Hz)
	4.15 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.15 (1H, d, J=13 Hz)	4.11 (1H, d, J=13 Hz)
Sugar moiety (Glucose)					
1	4.77 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)
2	3.25 [a]	3.25 [a]	3.25 [a]	3.25 [a]	3.27 [a]
3	3.40 (1H, t, J=8.5 Hz)	3.40 (1H, t, J=9 Hz)	3.40 (1H, t, J=9 Hz)	3.40 (1H, t, J=8.5 Hz)	3.41 (1H, t, J=8 Hz)
4	3.25 [a]	3.25 [a]	3.25 [a]	3.25 [a]	3.3 [a]
5	3.3 [a]	3.3 [a]	3.3 [a]		3.35 (1H, m)
6	3.63 (1H, dd, J=12, 6 Hz)	3.63 (1H, dd, J=12, 6 Hz)	3.62 (1H, dd, J=12, 6 Hz)	3.63 (1H, dd, J=12, 6 Hz)	3.67 (1H, dd, J=12, 5.5 Hz)
	3.91 (1H, dd, J=12, 2 Hz)	3.92 (1H, dd, J=12, 2 Hz)	3.91 (1H, dd, J=12, 2 Hz)	3.92 (1H, dd, J=12, 2 Hz)	3.95 (1H, dd, J=12, 2 Hz)
(Rhamnose)					
1	5.02 (1H, d, J=1.5 Hz)	5.02 (1H, d, J=1.5 Hz)	4.99 (1H, d, J=1.5 Hz)	5.02 (1H, d, J=1.5 Hz)	4.86 (1H, br s)
2	5.12 (1H, dd, J=3, 1.5 Hz)	4.08 (1H, dd, J=3, 1.5 Hz)	4.07 (1H, dd, J=3, 1.5 Hz)	4.08 (1H, dd, J=3, 1.5 Hz)	4.09 (1H, dd, J=3.5, 1.5 Hz)
3		5.22 (1H, dd, J=9.5, 3 Hz)	5.13 (1H, dd, J=9.5, 3 Hz)	5.22 (1H, dd, J=9.5, 3 Hz)	4.05 (1H, dd, J=9.5, 3.5 Hz)
4	5.04 (1H, t, J=9.5 Hz)	5.28 (1H, t, J=9.5 Hz)	5.23 (1H, t, J=9.5 Hz)	5.28 (1H, t, J=9 Hz)	5.19 (1H, t, J=9.5 Hz)
5		4.00 (1H, m)			3.91 (1H, dd, J=10, 6 Hz)
6	1.19 (3H, d, J=6 Hz)	1.20 (3H, d, J=6 Hz)	1.18 (3H, d, J=6 Hz)	1.20 (3H, d, J=6 Hz)	1.14 (3H, d, J=6.5 Hz)
(Glucose)					
1					4.44 (1H, d, J=8.5 Hz)
2					3.22 (1H, t, J=8.5 Hz)
3					
4					
5					3.57 (1H, m)
6					4.39 (1H, dd, J=12, 7 Hz)
					4.51 (1H, dd, J=12, 2 Hz)
Ester moiety (Acetyl)					
2	2.16 (3H, s)	2.01 (3H, s)	1.99 (3H, s)	2.01 (3H, s)	
(Cinnamoyl at rhamnose)					
β	6.47 (1H, d, J=16 Hz)	6.40 (1H, d, J=16 Hz)	5.80 (1H, d, J=13 Hz)	6.35 (1H, d, J=16 Hz)	6.40 (1H, d, J=16 Hz)
γ	7.69 (1H, d, J=16 Hz)	7.64 (1H, d, J=16 Hz)	6.95 (1H, d, J=13 Hz)	7.63 (1H, d, J=16 Hz)	7.66 (1H, d, J=16 Hz)
2	7.23 (1H, d, J=2 Hz)	7.22 (1H, d, J=2 Hz)	7.75 (1H, d, J=2 Hz)	7.20 (1H, d, J=2 Hz)	7.56 (1H, d, J=9 Hz)
3					6.94 (1H, d, J=9 Hz)
5	6.97 (1H, d, J=8 Hz)	6.97 (1H, d, J=8.5 Hz)	6.92 (1H, d, J=8 Hz)	6.81 (1H, d, J=8 Hz)	6.94 (1H, d, J=9 Hz)
6	7.19 (1H, dd, J=8, 2 Hz)	7.17 (1H, dd, J=8.5, 2 Hz)	7.22 (1H, dd, J=8, 2 Hz)	7.09 (1H, dd, J=8, 2 Hz)	7.56 (1H, d, J=9 Hz)
OMe	3.86 (6H, s)	3.86 (6H, s)	3.84 (3H, s); 3.85 (3H, s)	3.89 (3H, s)	3.82 (3H, s)
(Cinnamoyl at glucose)					
β					6.32 (1H, d, J=16 Hz)
γ					7.60 (1H, d, J=16 Hz)
2					7.07 (1H, d, J=2 Hz)
3					
5					6.80 (1H, d, J=8 Hz)
6					6.97 (1H, dd, J=8, 2 Hz)
OMe					

$C_{34}H_{44}O_{18}\cdot 2H_2O$ and A_{10} (**14**), $C_{34}H_{44}O_{18}\cdot 1/2H_2O$ consisted of a *trans*-3,4-dimethoxycinnamic acid. From the ^{1}H-NMR spectrum of **12**, *trans*-3,4-dimethoxycinnamic acid was attached to C-4 of rhamnose. The ^{1}H-NMR spectra of the last two compounds indicated the presence of an acetyl group [**13**: δ 2.16 (3H, s), **14**: δ 2.01 (3H, s)] and showed the acylated position to be C-2 and C-4 of rhamnose in **13** and to be C-3 and C-4 of rhamnose in **14**. These three compounds gave the same acetate **12a**. So, the acetyl group was attached to C-2 of rhamnose in **13** and to C-3 of rhamnose in **14**.

Buddlejosides A_{11} (**15**), $C_{34}H_{44}O_{18}\cdot 3/2H_2O$ and A_{12} (**16**), $C_{33}H_{42}O_{18}\cdot 2H_2O$ were both di-esters of 6-rhamnopyranosyl catalpol at C-3 and C-4 of rhamnose. The ^{1}H-NMR spectrum of **15** showed the presence of an acetyl [δ 1.99 (3H, s)] and a *cis*-3,4-dimetoxycinnamoyl group [δ 5.80 and 6.95 (each 1H, d, J=13 Hz); 6.92 (1H, d, J=8 Hz); 7.22 (1H, dd, J=8, 2 Hz); 7.75 (1H, d, J=2 Hz)]. The presence of the C–H long-range couplings determined the position of two acyl groups as shown in structure **15**. Alkaline hydrolysis and the NMR data of **16** indicated the presence of an acetyl and a feruloyl group. The peracetate **16a** was identical to the peracetate of 6-*O*-[α-L-(4-*O*-feruloyl)-rhamnopyranosyl]-catalpol.[7] So, an acetyl and a feruloyl group was respectively attached to C-3 and C-4 of rhamnose.

Buddlejosides A_{13} (**17**), $C_{46}H_{56}O_{24}\cdot 2H_2O$, A_{14} (**18**), $C_{47}H_{58}O_{24}\cdot 2H_2O$, A_{15} (**19**), $C_{46}H_{56}O_{24}\cdot 5/2H_2O$ and A_{16}

TABLE I. (continued)

	17a	18	19	20
Aglycone moiety				
1	5.08 (1H, d, J=9.5 Hz)	4.93 (1H, d, J=9.5 Hz)	4.93 (1H, d, J=9.5 Hz)	4.94 (1H, d, J=9.5 Hz)
3	6.37 (1H, dd, J=6, 1.5 Hz)	6.29 (1H, dd, J=6, 2 Hz)	6.29 (1H, dd, J=6, 2 Hz)	6.29 (1H, dd, J=6, 2 Hz)
4	5.06 (1H, dd, J=6, 5 Hz)	5.00 (1H, dd, J=6, 4 Hz)	5.00 (1H, dd, J=6, 5 Hz)	4.96 (1H, dd, J=6, 5 Hz)
5	2.40 (1H, m)	2.26 (1H, m)	2.25 (1H, m)	2.26 (1H, m)
6	4.01 (1H, br d, J=8 Hz)	3.66 (1H, dd, J=8, 1 Hz)	3.66 (1H, d, J=8 Hz)	3.65 (1H, d, J=8 Hz)
7	3.64 (1H, br s)	3.38 (1H, br s)	3.38 (1H, br s)	3.37 (1H, br s)
9	2.55 (1H, dd, J=9.5, 8 Hz)	2.30 (1H, dd, J=9.5, 8 Hz)	2.29 (1H, dd, J=9.5, 8 Hz)	2.38 (1H, dd, J=9.5, 8 Hz)
10	3.80 (1H, d, J=13 Hz)	3.72 (1H, d, J=13 Hz)	3.71 (1H, d, J=13 Hz)	3.72 (1H, d, J=13 Hz)
	4.14 (1H, d, J=13 Hz)	4.06 (1H, d, J=13 Hz)	4.06 (1H, d, J=13 Hz)	4.10 (1H, d, J=13 Hz)
Sugar moiety (Glucose)				
1	4.77 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.77 (1H, d, J=8 Hz)	4.78 (1H, d, J=8 Hz)
2				3.27 [a]
3		3.40 (1H, t, J=9 Hz)	3.40 (1H, t, J=9 Hz)	3.41 (1H, t, J=9 Hz)
4				3.3 [a]
5				3.35 [a]
6	3.62 (1H, dd, J=12, 6.5 Hz)	3.66 (1H, dd, J=12, 6 Hz)	3.65 (1H, dd, J=12.5, 5.5 Hz)	3.67 (1H, dd, J=12, 6 Hz)
	3.91 (1H, dd, J=12, 2 Hz)	3.94 (1H, dd, J=12, 2 Hz)	3.94 (1H, dd, J=12.5, 2 Hz)	3.95 [a]
(Rhamnose)				
1	4.95 (1H, d, J=1.5 Hz)		4.88 (1H, br s)	4.93 (1H, d, J=1.5 Hz)
2	4.14 (1H, dd, J=3.5, 1.5 Hz)	4.08 (1H, dd, J=3.5, 1.5 Hz)	4.08 (1H, dd, J=3.5, 1.5 Hz)	4.04 (1H, dd, J=3.5, 1.5 Hz)
3	3.77 (1H, dd, J=9.5, 3.5 Hz)		4.05 (1H, dd, J=9.5, 3.5 Hz)	3.95 [a]
4		5.20 (1H, t, J=9.5 Hz)	5.19 (1H, t, J=9.5 Hz)	5.12 (1H, t, J=9.5 Hz)
5				3.88 (1H, m)
6	1.27 (3H, d, J=6.5 Hz)	1.14 (3H, d, J=6 Hz)	1.13 (3H, d, J=6.5 Hz)	1.12 (3H, d, J=6.5 Hz)
(Glucose)				
1	4.54 (1H, d, J=8 Hz)	4.45 (1H, d, J=8 Hz)	4.44 (1H, d, J=8 Hz)	4.35 (1H, d, J=8 Hz)
2			3.21 (1H, t, J=8.5 Hz)	3.22 (1H, t, J=8 Hz)
3				3.3 [a]
4				3.3 [a]
5		3.57 (1H, m)	3.57 (1H, m)	3.53 (1H, m)
6	3.72 (1H, dd, J=12, 4 Hz)	4.41 (1H, dd, J=12, 7 Hz)	4.41 (1H, dd, J=12, 7 Hz)	4.38 (1H, dd, J=12, 7 Hz)
	3.83 (1H, dd, J=12, 2.5 Hz)	4.50 (1H, dd, J=12, 2 Hz)	4.50 (1H, dd, J=12, 2 Hz)	4.49 (1H, dd, J=12, 2 Hz)
Ester moiety (Acetyl)				
2				
(Cinnamoyl at rhamnose)		(*p*-Methoxycinnamoyl)	(*p*-Coumaroyl)	
β		6.40 (1H, d, J=16 Hz)	6.40 (1H, d, J=16 Hz)	5.87 (1H, d, J=13 Hz)
γ		7.66 (1H, d, J=16 Hz)	7.67 (1H, d, J=16 Hz)	6.95 (1H, d, J=13 Hz)
2		7.56 (1H, d, J=9 Hz)	7.47 (1H, d, J=9 Hz)	7.68 (1H, d, J=9 Hz)
3		6.95 (1H, d, J=9 Hz)	6.80 (1H, d, J=9 Hz)	6.89 (1H, d, J=9 Hz)
5		6.95 (1H, d, J=9 Hz)	6.80 (1H, d, J=9 Hz)	6.89 (1H, d, J=9 Hz)
6		7.56 (1H, d, J=9 Hz)	7.47 (1H, d, J=9 Hz)	7.68 (1H, d, J=9 Hz)
OMe		3.82 (3H, s)		3.80 (3H, s)
(Cinnamoyl at glucose)		(Feruloyl)	(Feruloyl)	
β		6.41 (1H, d, J=16 Hz)	6.36 (1H, d, J=16 Hz)	6.31 (1H, d, J=16 Hz)
γ		7.67 (1H, d, J=16 Hz)	7.64 (1H, d, J=16 Hz)	7.60 (1H, d, J=16 Hz)
2		7.18 (1H, d, J=2 Hz)	7.18 (1H, d, J=2 Hz)	7.07 (1H, d, J=2 Hz)
3				
5		6.84 (1H, d, J=8 Hz)	6.84 (1H, d, J=8 Hz)	6.80 (1H, d, J=8 Hz)
6		7.12 (1H, dd, J=8, 2 Hz)	7.12 (1H, dd, J=8, 2 Hz)	6.98 (1H, dd, J=8, 2 Hz)
OMe		3.90 (3H, s)	3.89 (3H, s)	

(**20**), $C_{46}H_{56}O_{24}\cdot 7/2H_2O$ were tri-glycosides having two acyl groups. One acyl group was located at C-4 of rhamnose and the other at C-6 of glucose judging from the ^{1}H-NMR spectra. Sugar analysis and the ^{1}H-NMR data suggested the presence of two glucose and one rhamnose, while alkaline hydrolysis gave tri-glycoside **17a** and two cinnamic acid derivatives (**17**: *trans-p*-methoxycinnamic acid + *trans*-caffeic acid, **18**: *trans-p*-methoxycinnamic acid + *trans*-ferulic acid, **19**: *trans-p*-coumaric acid + *trans*-ferulic acid, **20**: *cis-p*-methoxycinnamic acid + *trans*-caffeic acid). In the ^{1}H-NMR spectrum of **17**, NOEs were observed at H-3 signal of rhamnose [δ 4.05 (1H, dd, J=9.5, 3.5 Hz)] and at H-5 signal of glucose [δ 3.57 (1H, m)] on irradiation at the glucosyl anomeric proton signal [δ 4.44 (1H, d, J=8.5 Hz)], and the H-5 signal of glucose was correlated with the signals at δ 4.39 (1H, dd, J=12, 7 Hz) and 4.51 (1H, dd, J=12, 2 Hz) which were assigned to acylated methylene protons of glucose. From the observation of H–H and C–H long-range couplings, *trans-p*-methoxycinnamic acid and *trans*-caffeic acid were attached to C-4 of rhamnose and C-6 of terminal glucose, respectively. By the same method, *cis-p*-methoxycinnamic acid and *trans*-caffeic acid were attached to C-4 of rhamnose and C-6 of terminal glucose in **20**. The position of two acyl groups was not decided in **18** and **19** because of too small a yield.

TABLE II. ^{13}C-NMR Chemical Shifts in Methanol-d_4

	4	5	5a	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aglycone moiety																		
1	98.3	95.1	95.2	95.1	95.1	95.2	95.1	95.1	95.1	95.2	95.1	95.1	95.1	95.1	95.2	95.2	95.1	95.1
3	142.1	142.3	142.2	142.4	142.3	142.2	142.3	142.4	142.3	142.3	142.4	142.4	142.4	142.4	142.1	142.2	142.1	142.1
4	105.3	103.4	103.7	103.2	103.0	103.6	103.4	103.2	103.3	103.4	103.2	103.3	103.3	103.4	103.4	103.4	103.4	103.3
5	44.2	37.3	37.4	37.2	37.2	37.3	37.2	37.2	37.2	37.3	37.2	37.2	37.2	37.2	37.2	37.2	37.1	37.1
6	89.7	84.1	83.6	84.8	84.3	83.8	84.3	84.6	84.1	84.1	84.8	84.3	84.2	84.3	85.0	84.7	84.6	84.7
7	126.9	59.4	59.4	59.5	59.4	59.3	59.3	59.4	59.3	59.4	59.5	59.4	59.3	59.4	59.9	59.6	59.6	59.7
8	150.0	66.5	66.6	66.5	66.5	66.6	66.5	66.5	66.5	66.6	66.5	66.5	66.5	66.5	66.2	66.3	66.3	66.2
9	48.2	43.3	43.3	43.3	43.2	43.3	43.2	43.2	43.2	43.3	43.3	43.2	43.2	43.3	43.3	43.3	43.2	43.2
10	61.4	61.5	61.5	61.4	61.4	61.5	61.4	61.4	61.4	61.5	61.4	61.4	61.4	61.4	61.5	61.5	61.4	61.5
Sugar moiety (Glucose)																		
1	99.9	99.7	99.8	99.7	99.6	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.8	99.8	99.7
2	74.3	74.8	74.9	74.8[c]	74.7	74.8[d]	74.8	74.8	74.8	74.8	74.8	74.8	74.8	74.8	74.7[l]	74.8[n]	74.8[q]	74.7[u]
3	78.3	78.6	78.7	78.6	78.5	78.6	78.6	78.6	78.6	78.6	78.6	78.6	78.6	78.6	78.5	78.6	78.6	78.5
4	71.5	71.7	71.8	71.7	71.7	71.8	71.8	71.8	71.7	71.8	71.7	71.7	71.8	71.8	72.0[m]	72.0[o]	72.0[r]	72.0[v]
5	77.9	77.5	77.7	77.6	77.6	77.7	77.6	77.7	77.6	77.7	77.6	77.6	77.7	77.7	77.6	77.7	77.6	77.6
6	62.6	62.9	63.0	62.9	62.9	62.9	62.9	62.9	62.9	62.9	62.9	62.9	62.9	62.9	62.9	63.0	62.9	62.9
(Rhamnose)																		
1	97.9	100.4	100.4	97.7	100.2	100.2	97.6	97.6	100.1	100.4	97.7	100.2	100.2	100.2	100.4	100.3	100.2	100.2
2	74.3	72.4	72.3[b]	74.1[c]	70.0	70.3[e]	71.6[f]	74.8[g]	69.9	72.4	74.1	70.0	69.9	70.1	71.7[m]	71.8[o]	71.7[r]	71.7[v]
3	68.4[a]	70.3	72.4[b]	68.3	73.0	75.4[d]	71.5f)	68.1[h]	73.2	70.3	68.3	73.1[i]	73.2[j]	73.1[k]	79.5	79.3	79.3	79.1
4	75.3	75.3	73.9	75.1[c]	72.2	71.3	72.9	74.1[g]	72.0	75.3	75.1	72.3[i]	72.1[j]	72.2[k]	73.7	73.8	73.7	73.5
5	68.3[a]	68.3	70.2	68.3	68.3	70.2[e]	70.2	68.3[h]	68.1	68.3	68.3	68.3	68.2	68.3	68.2	68.3	68.3	68.0
6	18.0	17.9	18.0	17.9	17.8	18.0	18.0	17.9	17.8	17.9	17.9	17.8	17.8	17.8	17.8	17.9	17.8	17.8
(Glucose, terminal)																		
1															105.4	105.4	105.4	105.4
2															74.8[l]	74.9[n]	74.8[q]	74.8[u]
3															77.6	77.6	77.6	77.6
4															72.0[m]	72.1[o]	72.0[r]	72.0[v]
5															75.5	75.6	75.6	75.5
6															64.7	64.8	64.7	64.7
Ester moiety (Acetyl)																		
1	172.2			172.2	172.0		171.7	172.2	172.0		172.2	172.0	172.0	172.1				
2	20.9			20.9	20.9		20.7	20.9	20.9		20.9	20.9	20.9	20.9				
(Cinnamoyl at rhamnose)																		
α	168.4	168.7		168.4	168.0	168.6	168.2	167.3	168.0	168.7	168.4	168.0	167.8	168.2	168.7	168.7[p]	168.8[s]	167.3
β	115.9	116.1		115.8	115.3	116.3	115.8	117.3	116.9	116.4	116.2	115.6	115.0	114.7	116.1	116.2	115.1[t]	117.9
γ	146.7	146.5		146.7	147.0	146.3	146.6	145.5	146.0	146.8	147.0	147.3	146.4	147.8	146.6	146.6	147.0	145.5
1	128.3	128.3		128.3	128.0	128.4	128.3	128.7	128.6	128.8	128.7	128.5	129.0	127.5	128.4	128.5	127.2	128.8
2	131.1	131.0		131.1	131.1	131.0	131.0	133.4	133.4	111.6	111.6	111.5	111.9	111.8	131.1	131.1	131.1	133.4
3	115.4	115.4		115.4	115.4	115.4	115.4	114.3	114.4	150.8	150.7	150.7	149.7	150.8	115.4	115.4	116.8	114.3
4	163.2	163.2		163.2	163.2	163.2	163.2	162.1	162.2	152.9	152.9	153.0	151.9	149.4	163.1	163.2	161.3	162.0
5	115.4	115.4		115.4	115.4	115.4	115.4	114.3	114.4	112.6	112.6	112.5	116.8	116.5	115.4	115.4	116.8	114.3
6	131.1	131.0		131.1	131.1	131.1	131.0	133.4	133.4	124.1	124.1	124.3	126.5	124.4	131.1	131.1	131.3	133.4
OMe	55.9	55.9		55.9	55.9	55.9	55.9	55.8	55.8	56.5×2	56.9×2	56.4, 56.5	56.4, 56.5	56.5	55.9	55.9		55.8
(Cinnamoyl at glucose)																		
α															169.0	169.0[p]	169.0[s]	169.0
β															115.0	115.6	115.5[t]	115.5
γ															147.1	147.1	147.0	147.1
1															127.7	127.8	127.7	127.7
2															115.1	112.2	112.2	115.1
3															149.6	150.7	150.6	149.6
4															146.9	149.5	149.4	146.8
5															116.7	116.8	116.8	116.7
6															123.0	124.0	123.9	123.0
OMe																56.6	56.5	

a—*v*) Assignments may be interchanged within each column.

Experimental

General Procedure Optical rotations were measured with a Jasco DIP-360 digital polarimeter. UV spectra were taken on a Hitachi U-3410 spectrophotometer. Fast atom bombardment mass spectra (FAB-MS) were measured on a Jeol JMS-SX102 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol GSX-500 and GSX-270. Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Gas chromatography (GC) was done on a Hitachi G-3000 gas chromatograph. HPLC was done on a Jasco model 800 instrument.

Isolation Air dried whole plants of *B. japonica* HEMSL. (1.2 kg) collected in October 1990 in Shizuoka, Japan, were extracted twice with MeOH under reflux. The extract was concentrated under reduced pressure and the residue was suspended in H_2O. This suspension was extracted with ether to give a green gum (20 g). The H_2O layer was passed through a Diaion HP-20 column and the adsorbed material was eluted with 50% MeOH and 100% MeOH successively to give a brown gum (50% MeOH eluate 25 g, 100% MeOH eluate 11 g). The 100% MeOH eluate was chromatographed on a silica gel column using chloroform–MeOH (9 : 1—8 : 2) as eluent to give fraction 1—15. From fraction 4—11, iridoid glycosides were isolated by HPLC [YMC D-ODS-7, 20 mm × 25 cm, CH_3CN–H_2O (2 : 8—3 : 7)]: **1** (35 mg), **2** (67 mg), **3** (20 mg), **4** (17 mg), **5** (17 mg), **6** (84 mg), **7** (125 mg), **8** (8 mg), **9** (29 mg), **10** (50 mg), **11** (23 mg), **12** (15 mg), **13** (21 mg), **14** (92 mg), **15** (24 mg), **16** (16 mg), **17** (23 mg), **18** (8 mg), **19** (9 mg), **20** (23 mg).

Buddlejoside A$_1$ (3) Amorphous powder, $[\alpha]_D^{20}$ −103.5° (c = 1.01, MeOH). UV λ_{max}^{MeOH} nm (log ε): 218 (3.95). *Anal.* Calcd for $C_{30}H_{48}O_{15}\cdot H_2O$: C, 54.04; H, 7.56. Found: C, 54.15; H, 7.55. FAB-MS *m/z*: 671 $[M+Na]^+$, 649 $[M+H]^+$. ^{1}H-NMR (CD_3OD): δ 0.94 (3H, d, J = 6.5 Hz, H_3-9′), 1.30 (1H, m, H-4′), 1.37 (3H, s, H_3-10), 1.43 (1H, m, H-2′), 1.48 (1H, m, H-4′), 1.67 (2H, m, H-2′, H-3′), 1.83 (3H, d, J = 1 Hz, H_3-10′), 1.97 (1H, dd, J = 14.5, 4 Hz, H-7β), 2.21 (1H, dd, J = 14.5, 6.5 Hz, H-7α), 2.23 (2H, m, H_2-5′), 2.56 (1H, dd, J = 9, 2 Hz, H-9), 2.88 (1H, br dd, J = 9, 2 Hz, H-5), 3.20 (1H, t, J = 8 Hz, H-2 of glc), 3.38 (1H,

t, $J=8$ Hz, H-3 of glc), 3.57 (overlapped, H-1'), 3.67 (1H, dd, $J=12$, 5.5 Hz, H-6 of glc), 3.85 (overlapped, H-1'), 4.18 (1H, d, $J=7$ Hz, H-1 of ara), 4.66 (1H, d, $J=8$ Hz, H-1 of glc), 4.95 (1H, dd, $J=6$, 2 Hz, H-4), 5.50 (1H, d, $J=2$ Hz, H-1), 6.21 (1H, dd, $J=6$, 2 Hz, H-3), 6.80 (1H, qt, $J=1$, 7.5 Hz, H-6'). ^{13}C-NMR (CD_3OD): δ (aglycone, 1—10) 93.4, 141.0, 104.7 (or 104.6), 39.2, 80.4, 47.8, 79.0, 51.5, 26.1. (ester, 1'—10') 68.5, 37.5, 30.5, 36.8, 27.1, 144.3, 128.7, 169.5, 19.7, 12.4. (glucose, 1—6) 99.3, 74.7, 78.2, 71.6, 78.0, 62.8. (arabinose, 1—5) 104.6 (or 104.7), 72.4, 74.3, 69.6, 66.8.

Buddlejoside A_2 (4) Amorphous powder, $[\alpha]_D^{20}$ −136.0° ($c=0.25$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 227 (4.15), 299 (sh 4.30), 312 (4.35). *Anal.* Calcd for $C_{33}H_{42}O_{16}\cdot 3/2H_2O$: C, 54.92; H, 6.28. Found: C, 54.62; H, 6.12. FAB-MS *m/z*: 717 $[M+Na]^+$, 695 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II. ^{1}H-NMR (C_5D_5N): δ 5.74 (1H, dd, $J=3.5$, 1.5 Hz, H-2 of rham), 5.84 (1H, t, $J=9.5$ Hz, H-4 of rham). ^{13}C-NMR (C_5D_5N): δ 167.1 (C-α of *p*-methoxycinnamate), 170.7 (C-1 of acetate).

Buddlejoside A_3 (6) Amorphous powder, $[\alpha]_D^{20}$ −136.0° ($c=0.82$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 226 (4.14), 293 (sh 4.34), 311 (4.46). *Anal.* Calcd for $C_{33}H_{42}O_{17}\cdot 1/2H_2O$: C, 55.07; H, 6.02. Found: C, 54.93; H, 6.07. FAB-MS *m/z*: 733 $[M+Na]^+$, 711 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_4 (7) Amorphous powder, $[\alpha]_D^{20}$ −218.0° ($c=0.32$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 226 (4.15), 299 (sh 4.34), 311 (4.39). *Anal.* Calcd for $C_{33}H_{42}O_{17}\cdot H_2O$: C, 54.39; H, 6.09. Found: C, 54.56; H, 5.94. FAB-MS *m/z*: 733 $[M+Na]^+$, 711 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_5 (9) Amorphous powder, $[\alpha]_D^{20}$ −109.4° ($c=0.80$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 226 (4.11), 298 (sh 4.33), 311 (4.38). *Anal.* Calcd for $C_{33}H_{42}O_{17}\cdot H_2O$: C, 54.39; H, 6.09. Found: C, 54.39; H, 6.37. FAB-MS *m/z*: 733 $[M+Na]^+$, 711 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_6 (10) Amorphous powder, $[\alpha]_D^{20}$ −127.2° ($c=1.84$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 225 (sh 4.12), 297 (sh 4.15), 308 (4.19). *Anal.* Calcd for $C_{33}H_{42}O_{17}\cdot 1/2H_2O$: C, 55.07; H, 6.02. Found: C, 54.89; H, 6.07. FAB-MS *m/z*: 733 $[M+Na]^+$, 711 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_7 (11) Amorphous powder, $[\alpha]_D^{20}$ −167.8° ($c=0.45$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 225 (sh 4.10), 299 (sh 4.17), 310 (4.21). *Anal.* Calcd for $C_{33}H_{42}O_{17}\cdot H_2O$: C, 54.39; H, 6.09. Found: C, 54.29; H, 6.11. FAB-MS *m/z*: 733 $[M+Na]^+$, 711 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_8 (12) Amorphous powder, $[\alpha]_D^{20}$ −139.4° ($c=0.33$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 215 (sh 4.27), 233 (4.17), 242 (sh 4.11), 297 (4.18), 323 (4.28). *Anal.* Calcd for $C_{32}H_{42}O_{17}\cdot 3/2H_2O$: C, 52.96; H, 6.25. Found: C, 52.89; H, 6.35. FAB-MS *m/z*: 721 $[M+Na]^+$, 699 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_9 (13) Amorphous powder, $[\alpha]_D^{20}$ −142.7° ($c=0.41$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 216 (4.31), 235 (4.21), 295 (4.22), 324 (4.34). *Anal.* Calcd for $C_{34}H_{44}O_{18}\cdot 2H_2O$: C, 52.57; H, 6.23. Found: C, 52.38; H, 6.09. FAB-MS *m/z*: 763 $[M+Na]^+$, 741 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{10} (14) Amorphous powder, $[\alpha]_D^{20}$ −140.4° ($c=1.04$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 216 (sh 4.19), 237 (4.11), 325 (4.30). *Anal.* Calcd for $C_{34}H_{44}O_{18}\cdot 1/2H_2O$: C, 54.47; H, 6.06. Found: C, 54.30; H, 6.06. FAB-MS *m/z*: 763 $[M+Na]^+$, 741 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{11} (15) Amorphous powder, $[\alpha]_D^{20}$ −157.9° ($c=0.19$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 231 (sh 4.10), 297 (4.03), 324 (4.12). *Anal.* Calcd for $C_{34}H_{44}O_{18}\cdot 3/2H_2O$: C, 53.19; H, 6.17. Found: C, 53.08; H, 5.94. FAB-MS *m/z*: 763 $[M+Na]^+$, 741 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{12} (16) Amorphous powder, $[\alpha]_D^{20}$ −169.4° ($c=0.31$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 217 (sh 4.19), 236 (4.09), 244 (sh 4.05), 296 (sh 4.14), 327 (4.31). *Anal.* Calcd for $C_{33}H_{42}O_{18}\cdot 2H_2O$: C, 51.97; H, 6.08. Found: C, 52.15; H, 5.92. FAB-MS *m/z*: 749 $[M+Na]^+$, 727 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{13} (17) Amorphous powder, $[\alpha]_D^{20}$ −183.1° ($c=0.62$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 221 (4.43), 232 (sh 4.33), 248 (sh 4.09), 299 (sh 4.54), 314 (4.58). *Anal.* Calcd for $C_{46}H_{56}O_{24}\cdot 2H_2O$: C, 53.69; H, 5.88. Found: C, 53.64; H, 5.90. FAB-MS *m/z*: 1015 $[M+Na]^+$, 993 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{14} (18) Amorphous powder, $[\alpha]_D^{20}$ −158.2° ($c=0.49$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 219 (sh 4.44), 230 (sh 4.39), 299 (sh 4.51), 315 (4.56). *Anal.* Calcd for $C_{47}H_{58}O_{24}\cdot 2H_2O$: C, 54.12; H, 5.99. Found: C, 54.27; H, 5.96. FAB-MS *m/z*: 1029 $[M+Na]^+$, 1007 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{15} (19) Amorphous powder, $[\alpha]_D^{20}$ −118.9° ($c=0.66$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 219 (sh 4.46), 230 (sh 4.38), 299 (sh 4.36), 318 (4.43). *Anal.* Calcd for $C_{46}H_{56}O_{24}\cdot 5/2H_2O$: C, 53.23; H, 5.92. Found: C, 53.20; H, 6.06. FAB-MS *m/z*: 1015 $[M+Na]^+$, 993 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Buddlejoside A_{16} (20) Amorphous powder, $[\alpha]_D^{20}$ −156.3° ($c=0.08$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 216 (sh 4.44), 230 (sh 4.29), 247 (sh 4.09), 300 (sh 4.36), 313 (4.37). *Anal.* Calcd for $C_{46}H_{56}O_{24}\cdot 7/2H_2O$: C, 52.32; H, 6.01. Found: C, 52.38; H, 5.79. FAB-MS *m/z*: 1015 $[M+Na]^+$, 993 $[M+H]^+$. ^{1}H- and ^{13}C-NMR: Tables I and II.

Acid Hydrolysis of 3, 4, 6, 7, 9—20 A solution of a glycoside (*ca.* 0.1 mg) in 5% H_2SO_4 (1 drop) was heated in a boiling water bath for 30 min. The solution was passed through an Amberlite IRA-45 column and concentrated to give a residue, which was reduced with $NaBH_4$ (*ca.* 1 mg) for 1 h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and the residue was concentrated to dryness. Boric acid was removed by co-distillation with MeOH and the residue was acetylated with acetic anhydride and pyridine (1 drop each) overnight at room temperature. The reagents were evaporated off *in vacuo*. From **3** arabinitol acetate and glucitol acetate were detected and from **4**, **6**, **7**, **9—20** rhamnitol acetate and glucitol acetate were detected by GC. Conditions: column, Supelco capillary column SP-2380 (0.25 mm × 30 m); column temperature, 250 °C; carrier gas, N_2; t_R, rhamnitol acetate, 5.0 min; arabinitol acetate, 6.3 min; glucitol acetate, 11.2 min.

Alkaline Hydrolysis of 3—20 **5** (8 mg) was treated with 0.1% NaOH aq. (1 ml) for 6 h at room temperature in the atmosphere of N_2. The reaction mixture was passed through an Amberlite IR-120 column and eluted with MeOH. The eluate was purified by HPLC [YMC D-ODS-7, 20 mm × 25 cm, 30% CH_3CN] to give **5a** (3 mg) as amorphous powder. ^{1}H-NMR: Table I. **17** (2 mg) was treated with 1% NaOH (3 drops) for 40 min at room temperature in N_2 atmosphere and the reaction mixture was passed through an Amberlyst 15 column. The methanolic eluate was concentrated and then partitioned between ethyl acetate and H_2O. From the H_2O layer, **17a** (1 mg) was obtained as an amorphous powder. ^{1}H-NMR: Table I. FAB-MS *m/z*: 693 $[M+Na]^+$, 672 $[M+H]^+$. **4** (2 mg) was treated in the same way as **17** and the hydrolysate was acetylated in the usual manner to give **4a** (1 mg). **4a**: ^{1}H-NMR ($CDCl_3$): δ 1.24 (3H, d, $J=6.5$ Hz, H_3-6 of rham), 1.98, 2.01, 2.028, 2.033, 2.06, 2.10, 2.12, 2.15 (each 3H, s, OAc), 2.85 (1H, m, H-5), 3.09 (1H, br t, $J=6$ Hz, H-9), 3.71 (1H, ddd, $J=10$, 4, 2 Hz, H-5 of glc), 3.94 (1H, m, H-5 of rham), 4.18 (1H, dd, $J=12.5$, 2 Hz, H-6 of glc), 4.25 (1H, dd, $J=12.5$, 4 Hz, H-6 of glc), 4.36 (1H, m, H-6), 4.73 (2H, br s, H_2-10), 4.82 (1H, d, $J=1.5$ Hz, H-1 of rham), 4.86 (1H, d, $J=8$ Hz, H-1 of glc), 4.93 (1H, dd, $J=6$, 3 Hz, H-4), 4.98 (1H, d, $J=5$ Hz, H-1), 5.03 (1H, dd, $J=9.5$, 8 Hz, H-2 of glc), 5.07 (1H, t, $J=9.5$ Hz, H-4 of rham), 5.11 (1H, t, $J=9.5$ Hz, H-4 of glc), 5.19 (1H, dd, $J=3.5$, 1.5 Hz, H-2 of rham), 5.23 (1H, t, $J=9.5$ Hz, H-3 of glc), 5.26 (1H, dd, $J=9.5$, 3.5 Hz, H-3 of rham), 5.87 (1H, br t, $J=2$ Hz, H-7), 6.19 (1H, dd, $J=6$, 2 Hz, H-3). **3**, **6—20** (each *ca.* 0.05 mg) was treated with 1% NaOH aq. (2 drops) for 30 min at room temperature in N_2 atmosphere and the reaction mixture was passed through an Amberlyst 15 column. **5a** (t_R 8.0 min) was detected by HPLC (YMC R-ODS-7, 4.6 mm × 25 cm, 3% CH_3CN, 1.3 ml/min) from **6—16**, **17a** (t_R 11.3 min) from **17—20** and **2a** (t_R 25.2 min) from **3**. Cinnamic acid derivatives were detected by HPLC (330 nm, 1.3 ml/min): *trans-p*-methoxycinnamic acid (YMC R-ODS-7, 4.6 mm × 25 cm, 30% CH_3CN + 0.05% trifluoroacetic acid (TFA), t_R 14.5 min) from **4—9**, **17**, **18**; *cis-p*-methoxycinnamic acid (YMC R-ODS-7, 4.6 mm × 25 cm, 30% CH_3CN + 0.05% TFA, t_R 13.2 min) from **10**, **11**, **20**; *trans-p*-coumaric acid (YMC R-ODS-7, 4.6 mm × 25 cm, 22.5% CH_3CN + 0.05% TFA, t_R 8.3 min) from **19**; *trans*-caffeic acid (YMC R-ODS-7, 4.6 mm × 25 cm, 22.5% CH_3CN + 0.05% TFA, t_R 4.8 min) from **17**, **20**; *trans*-ferulic acid (YMC R-ODS-7, 4.6 mm × 25 cm, 22.5% CH_3CN + 0.05% TFA, t_R 8.3 min) from **16**, **18**, **19**; *trans*-3,4-dimethoxycinnamic acid (Zorbax Pro-10 C-8, 4.6 mm × 25 cm, 17.5% CH_3CN + 0.05% TFA, t_R 17.7 min) from **12—14**; *cis*-3,4-dimethoxycinnamic acid (Zorbax Pro-10 C-8, 4.6 mm × 25 cm, 17.5% CH_3CN + 0.05% TFA, t_R 16.1 min) from **15**.

Methanolysis of 3 **3** (*ca.* 0.1 mg) was refluxed with acetyl chloride–MeOH (1 : 20) (0.5 ml) for 30 min. The reagents were evaporated off and (*R*)-**3a** was detected by HPLC (Sumipax OA-2000, 4.6 mm × 25 cm, hexane, t_R 35.3 min).

Preparation of (*R*)-3a and (*S*)-3a A solution of (*R*)-citronellol (100 mg) and selenium dioxide (100 mg) in ethanol (2 ml) was refluxed for 1 h. After evaporation of the solvent, the reaction product was purified by thin layer chromatography (TLC) [Silica gel PF_{254}, benzene–acetone (8 : 2)]

to give **3c** (15 mg) as a colorless oil. **3c**: ^{1}H-NMR ($CDCl_3$): δ 0.96 (3H, d, J=7 Hz, H_3-9), 1.76 (3H, br s, H_3-10), 3.72 (2H, m, H_2-1), 6.49 (1H, qt, J=1, 8 Hz, H-6), 9.40 (1H, s, H-8). ^{13}C-NMR ($CDCl_3$): δ (1—10) 60.8, 39.6, 29.3, 35.6, 26.5, 154.8, 139.3, 195.3, 19.3, 9.2. A solution of silver nitrate (20 mg) in H_2O (0.5 ml) was added to the reaction mixture of **3c** (10 mg) in 10% NaOH aq. (1 ml) and stirred overnight at room temperature. The reaction product was extracted with ethyl acetate after acidification with dil. HCl and purified by TLC [Silica gel PF_{254}, benzene–acetone–AcOH (80 : 19 : 1)]. The methyl ester (*R*)-**3a** (3 mg) (t_R 35.3 min) was obtained as colorless oil after treatment with CH_2N_2. (*S*)-**3a** (t_R 34.7 min) was obtained from (*S*)-citronellol in the same way. **3a**: ^{1}H-NMR (CD_3OD): δ 0.93 (3H, d, J=6.5 Hz, H_3-9), 1.32 (2H, m, H-2, H-4), 1.47 (1H, m, H-4), 1.50 (2H, m, H-2, H-3), 1.83 (3H, d, J=1 Hz, H_3-10), 2.22 (2H, m, H_2-5), 3.59 (2H, m, H_2-1), 3.71 (3H, s, COOMe), 4.85 (1H, br s, OH), 6.76 (1H, qt, J=1, 8 Hz, H-6). ^{13}C-NMR (CD_3OD): δ (1—10, OMe) 60.9, 40.5, 30.4, 36.9, 27.1, 144.0, 128.4, 170.0, 19.3, 12.5, 52.2.

Acetylation of 5—14 and 16 Each glycoside (1 mg) was treated with acetic anhydride (1 drop) and pyridine (1 drop) overnight at room temperature and the reagents were evaporated off to give an acetate. From **5**—**7**, **5b** was obtained as an amorphous powder. **5b**: ^{1}H-NMR ($CDCl_3$): δ 1.25 (3H, d, J=6 Hz, H_3-6 of rham), 1.96, 2.02, 2.03, 2.05, 2.11, 2.13, 2.18 (each 3H, s, OAc), 2.52 (1H, m, H-5), 2.63 (1H, dd, J=9.5, 8 Hz, H-9), 3.58 (1H, br s, H-7), 3.69 (1H, m, H-5 of glc), 3.85 (3H, s, OMe), 3.94 (1H, dd, J=8, 1 Hz, H-6), 3.99 (1H, d, J=13 Hz, H-10), 4.0 (overlapped, H-5 of rham), 4.16 (1H, dd, J=12, 4 Hz, H-6 of glc), 4.33 (1H, dd, J=12, 2 Hz, H-6 of glc), 4.77 (1H, d, J=9.5 Hz, H-1), 4.82 (1H, d, J=13 Hz, H-10), 4.96 (overlapped, H-1 and H-2 of glc), 4.97 (overlapped, H-1 of rham), 5.09 (1H, dd, J=6, 4.5 Hz, H-4), 5.15 (1H, t, J=9 Hz, H-4 of glc), 5.23 (1H, t, J=8.5 Hz, H-3 of glc), 5.23 (1H, t, J=9.5 Hz, H-4 of rham), 5.32 (1H, dd, J=3.5, 1.5 Hz, H-2 of rham), 5.43 (1H, dd, J=9.5, 3.5 Hz, H-3 of rham), 6.26 (1H, d, J=16 Hz, H-β), 6.33 (1H, dd, J=6, 2 Hz, H-3), 6.92 (2H, d, J=9 Hz, H-3′, H-5′), 7.49 (2H, d, J=9 Hz, H-2′, H-6′), 7.65 (1H, d, J=16 Hz, H-γ). From **8** and **9**, **8a** was obtained as an amorphous powder. **8a**: ^{1}H-NMR ($CDCl_3$): δ 1.24 (3H, d, J=6 Hz, H_3-6 of rham), 2.02, 2.03, 2.04, 2.05, 2.11, 2.13, 2.17 (each 3H, s, OAc), 2.52 (1H, m, H-5), 2.63 (1H, dd, J=9.5, 8 Hz, H-9), 3.58 (1H, br s, H-7), 3.69 (1H, m, H-5 of glc), 3.84 (3H, s, OMe), 3.93 (1H, dd, J=8, 1 Hz, H-6), 3.98 (1H, d, J=13 Hz, H-10), 3.99 (overlapped, H-5 of rham), 4.17 (1H, dd, J=12, 4 Hz, H-6 of glc), 4.33 (1H, dd, J=12, 2 Hz, H-6 of glc), 4.77 (1H, d, J=9.5 Hz, H-1), 4.82 (1H, d, J=13 Hz, H-10), 4.97 (overlapped, H-1 and H-2 of glc), 4.98 (1H, br s, H-1 of rham), 5.09 (1H, dd, J=6, 5 Hz, H-4), 5.15 (1H, t, J=9 Hz, H-4 of glc), 5.18 (1H, t, J=9.5 Hz, H-4 of rham), 5.23 (1H, t, J=9 Hz, H-3 of glc), 5.35 (1H, dd, J=3.5, 1.5 Hz, H-2 of rham), 5.45 (1H, dd, J=9.5, 3.5 Hz, H-3 of rham), 6.22 (1H, d, J=16 Hz, H-β), 6.32 (1H, dd, J=6, 2 Hz, H-3), 6.90 (2H, d, J=9 Hz, H-3′, H-5′), 7.47 (2H, d, J=9 Hz, H-2′, H-6′), 7.60 (1H, d, J=16 Hz, H-γ). From **10** and **11**, **10a** was obtained as an amorphous powder. **10a**: ^{1}H-NMR ($CDCl_3$): δ 1.23 (3H, d, J=6.5 Hz, H_3-6 of rham), 1.95, 2.02, 2.03, 2.05, 2.10, 2.13, 2.16 (each 3H, s, OAc), 2.50 (1H, m, H-5), 2.62 (1H, dd, J=9.5, 8 Hz, H-9), 3.56 (1H, br s, H-7), 3.69 (1H, dt, J=10, 3 Hz, H-5 of glc), 3.84 (3H, s, OMe), 3.91 (1H, br d, J=8 Hz, H-6), 3.94 (1H, m, H-5 of rham), 3.98 (1H, d, J=13 Hz, H-10), 4.16 (1H, dd, J=12.5, 3.5 Hz, H-6 of glc), 4.33 (1H, dd, J=12.5, 3 Hz, H-6 of glc), 4.76 (1H, d, J=9.5 Hz, H-1), 4.81 (1H, d, J=13 Hz, H-10), 4.95 (overlapped, H-1 and H-2 of glc, H-1 of rham), 5.05 (1H, dd, J=6, 5 Hz, H-4), 5.15 (1H, t, J=9.5 Hz, H-4 of glc), 5.17 (1H, t, J=9.5 Hz, H-3 of glc), 5.22 (1H, t, J=10 Hz, H-4 of rham), 5.31 (1H, dd, J=3.5, 1.5 Hz, H-2 of rham), 5.35 (1H, dd, J=10, 3.5 Hz, H-3 of rham), 5.77 (1H, d, J=13 Hz, H-β), 6.32 (1H, dd, J=6, 2 Hz, H-3), 6.89 (2H, d, J=9 Hz, H-3′, H-5′), 6.91 (1H, d, J=13 Hz, H-γ), 7.73 (2H, d, J=9 Hz, H-2′, H-6′). From **12**—**14**, **12a** was obtained as an amorphous powder. **12a**: ^{1}H-NMR ($CDCl_3$): δ 1.26 (3H, d, J=6 Hz, H_3-6 of rham), 1.97, 2.02, 2.04, 2.05, 2.11, 2.13, 2.18 (each 3H, s, OAc), 2.52 (1H, m, H-5), 2.63 (1H, dd, J=9.5, 8 Hz, H-9), 3.58 (1H, br s, H-7), 3.69 (1H, dt, J=10, 3 Hz, H-5 of glc), 3.93 (6H, s, OMe × 2), 3.93 (1H, br d, J=8 Hz, H-6), 3.99 (1H, d, J=13 Hz, H-10), 4.01 (1H, m, H-5 of rham), 4.16 (1H, dd, J=12.5, 3.5 Hz, H-6 of glc), 4.33 (1H, dd, J=12.5, 3 Hz, H-6 of glc), 4.77 (1H, d, J=9.5 Hz, H-1), 4.82 (1H, d, J=13 Hz, H-10), 4.97 (overlapped, H-1 and H-2 of glc, H-1 of rham), 5.09 (1H, dd, J=6, 5 Hz, H-4), 5.15 (1H, t, J=9.5 Hz, H-4 of glc), 5.23 (overlapped, H-3 of glc, H-4 of rham), 5.32 (1H, dd, J=3.5, 1.5 Hz, H-2 of rham), 5.44 (1H, dd, J=10, 3.5 Hz, H-3 of rham), 6.27 (1H, d, J=16 Hz, H-β), 6.32 (1H, dd, J=6, 2 Hz, H-3), 6.88 (1H, d, J=8 Hz, H-5′), 7.06 (1H, d, J=2 Hz, H-2′), 7.12 (1H, dd, J=8, 2 Hz, H-6′), 7.64 (1H, d, J=16 Hz, H-γ). From **16**, **16a** was obtained as an amorphous powder and identical to the acetate of 6-*O*-[α-L-(4-*O*-feruloyl)-rhamnopyranosyl]-catalpol. **16a**: ^{1}H-NMR ($CDCl_3$): δ 1.26 (3H, d, J=6.5 Hz, H_3-6 of rham), 1.97, 2.02, 2.04, 2.05, 2.11, 2.13, 2.18, 2.33 (each 3H, s, OAc), 2.51 (1H, m, H-5), 2.63 (1H, dd, J=9.5, 8 Hz, H-9), 3.58 (1H, br s, H-7), 3.69 (1H, dt, J=10, 3 Hz, H-5 of glc), 3.88 (3H, s, OMe), 3.94 (1H, br d, J=8 Hz, H-6), 3.99 (1H, d, J=13 Hz, H-10), 4.02 (1H, m, H-5 of rham), 4.16 (1H, dd, J=12.5, 3.5 Hz, H-6 of glc), 4.33 (1H, dd, J=12.5, 3 Hz, H-6 of glc), 4.77 (1H, d, J=9.5 Hz, H-1), 4.82 (1H, d, J=13 Hz, H-10), 4.97 (overlapped, H-1 and H-2 of glc, H-1 of rham), 5.09 (1H, dd, J=6, 5 Hz, H-4), 5.15 (1H, t, J=9.5 Hz, H-4 of glc), 5.23 (1H, t, J=9.5 Hz, H-3 of glc), 5.23 (overlapped, H-4 of rham), 5.32 (1H, dd, J=3.5, 1.5 Hz, H-2 of rham), 5.44 (1H, dd, J=10, 3.5 Hz, H-3 of rham), 6.32 (1H, dd, J=6, 2 Hz, H-3), 6.34 (1H, d, J=16 Hz, H-β), 7.07 (1H, d, J=8 Hz, H-5′), 7.11 (1H, d, J=2 Hz, H-2′), 7.13 (1H, dd, J=8, 2 Hz, H-6′), 7.65 (1H, d, J=16 Hz, H-γ).

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Lignoids from Albizziae Cortex. II.[1] A Biodegradation Pathway of Syringaresinol

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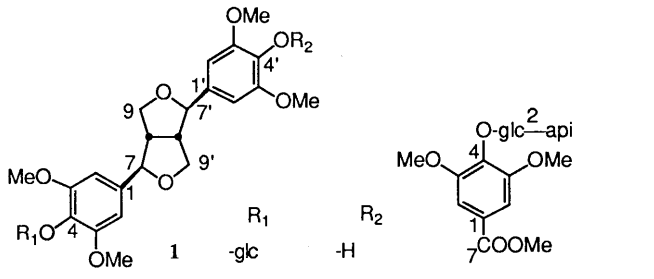
Five new lignoid glycosides including a novel lignan were isolated from Albizziae Cortex, the dried stem bark of *Albizzia julibrissin* DURAZZ. Three of them appear to be key metabolites of a biodegradation pathway for syringaresinol.

Keywords *Albizzia julibrissin*; Leguminosae; lignan; lignoid glycoside; syringaresinol; 5,5′-dimethoxy-7-oxolariciresinol; glaberide I; syringic acid; biodegradation; metabolite

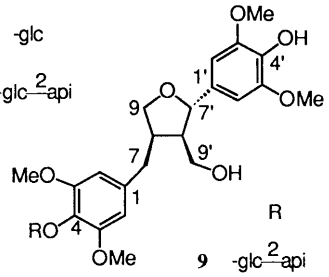
The dried stem bark of *Albizzia julibrissin* DURAZZ (Albizziae Cortex) is used as a tonic in China and Japan. In the course of our studies on the constituents of leguminous plants,[1] we attempted to clarify the ingredients of this crude drug, and reported the structure of three new syringaresinol glycosides (**3**—**5**) which include triglycoside (**4**) and tetraglycoside (**5**).[2] Syringaresinol diglucoside (**2**) which is responsible for the pharmacological effect as a tonic was also obtained.

In connection with this study, we have now obtained five new glycosides (**6**—**10**) which contain key intermediates in a biodegradation pathway of syringaresinol. This paper deals with the structural elucidation of these compounds and a consideration of the syringaresinol metabolism in the plant.

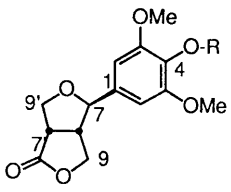


	R_1	R_2
1	-glc	-H
2	-glc	-glc
3	-glc-2-api	-H
4	-glc-2-api	-glc
5	-glc-2-api	-glc-2-api

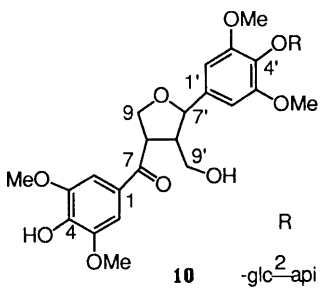
6



	R
9	-glc-2-api
9a	H



	R
7	-glc
7a	H
8	-glc-2-api
8a	H



	R
10	-glc-2-api
10a	H

Compound **6** was obtained as a white amorphous powder, $[\alpha]_D$ −57.9° (MeOH). In the negative fast atom bombardment-mass spectrum (FAB-MS), **6** showed $[M-H]^-$ ion at *m/z* 505. In the infrared (IR) spectrum of **6**, absorptions of an ester carbonyl group (1720 cm^{-1}) and hydroxy group were observed. Upon acidic hydrolysis of **6**, apiose and glucose were identified by thin layer chromatography (TLC). Although signals due to the glycosidic moiety were in good agreement with those of **3**, signals due to C-1 and -2 of the aglycone moiety were shifted in the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum (Table I) of **6**. Furthermore, a methoxycarbonyl group was observed. In the ^1H-nuclear magnetic resonance (^1H-NMR) spectrum of

TABLE I. ^{13}C-NMR Spectral Data for Compounds **6**—**10** (δ: ppm, in DMSO-d_6)

	6	**7**	**8**	**9**	**10**
C-1	124.9	136.0	136.0	139.7	126.5
C-2	107.0	104.2	104.3	105.9	106.3
C-3	152.3	153.0	152.8	152.7	147.5
C-4	138.3	133.8	134.0	133.2	141.5
C-5	152.3	153.0	152.8	152.7	147.5
C-6	107.0	104.2	104.3	105.9	106.3
C-7	165.7	85.5	85.5	32.7	197.5
C-8		45.9	46.0	42.0	48.6
C-9		70.5	70.5	72.0	69.9
C-1′				130.9	137.6
C-2′				103.7	104.3
C-3′				147.9	152.6
C-4′				134.6	133.4
C-5′				147.9	152.6
C-6′				103.7	104.3
C-7′		178.8	178.8	81.8	82.7
C-8′		47.7	47.7	52.4	53.2
C-9′		69.8	69.8	58.7	60.0
OMe	56.3 (×2)	56.5 (×2)	56.5 (×2)	56.3 (×2)	56.2 (×2)
				56.0 (×2)	56.0 (×2)
COOMe	52.1				
Glc-1	100.1	102.7	100.7	100.6	100.5
Glc-2	77.0	74.2	77.1	77.1	77.0
Glc-3	76.0	76.6	76.3	76.2	76.1
Glc-4	69.9	70.0	70.1	70.1	69.9
Glc-5	76.9	77.3	76.9	76.8	76.7
Glc-6	60.7	61.0	61.1	61.0	60.8
Api-1	108.4		108.6	108.5	108.4
Api-2	76.7		76.9	76.8	76.7
Api-3	79.2		79.4	79.5	79.2
Api-4	73.9		74.1	74.0	73.9
Api-5	64.4		64.7	64.6	64.5

6, a singlet signal ascribable to C-2 and -6 appeared in the downfield (δ 7.23), suggesting that the methoxycarbonyl group was located at C-1. Since the signals of C-3 and -4 were basically consistent with those of **3** in the ^{13}C-NMR spectrum, the aglycone was deduced to be syringic acid methyl ester. Consequently, the structure of **6** was determined to be syringic acid methyl ester 4-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside.

Compounds **7** and **8** showed the $[M-H]^-$ peak at *m/z* 441 and 573, respectively in the negative FAB-MS. Both compounds had the absorption of γ-lactone in the IR spectra. Both were hydrolyzed enzymatically to yield an aglycone (**7a**), $[\alpha]_D$ 0° (MeOH), which was identified as glaberide I[3] based on electron impact mass spectrum (EI-MS) (*m/z* 280) and ^{1}H-NMR spectra. Glaberide I was first isolated from *Ilex pubescens* and its structure elucidated by X-ray analysis, but it showed no optical activity. It is a member of a very rare class of lignoid, the only one known from plants. In the ^{13}C-NMR spectra, the sugar moieties of **7** and **8** were identical with those of **2** and **3**, respectively. Therefore, the structures of **7** and **8** were established as glaberide I 4-*O*-β-D-glucopyranoside and glaberide I 4-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside, respectively. These are the first glycosides of 6-oxo-2-phenyl-3,7-dioxabicyclo[3.3.0]octane derivative.

Compound **9** showed $[M-H]^-$ peak at *m/z* 713 which was increased by 2 mass units more than that of **3** in the negative FAB-MS. Upon enzymatic hydrolysis of **9**, the aglycone was identified as (+)-5,5′-dimethoxylariciresinol[4] based on various data. In the ^{13}C-NMR spectrum of **9**, signals due to the sugar units were consistent with those of **8**, whereas signals due to the upper unit (C-1′—6′) were in agreement with those of **1**. Consequently, the structure of **9** was concluded to be (+)-5,5′-dimethoxylariciresinol 4-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside.

Compound **10** gave $[M-H]^-$ ion peak at *m/z* 727 which was increased by 14 mass units more than that of **9** in the negative FAB-MS. In the ^{1}H-NMR spectrum of **10**, the singlet signal ascribable to C-2 and -6 was observed downfield (δ 7.32), suggesting that a carbonyl group is located at C-7. In addition, the aglycone (**10a**) derived through an enzymatic hydrolysis also showed a similar signal at δ 7.31 in the ^{1}H-NMR and gave a molecular ion peak at *m/z* 434 which was increased by 14 mass units more than that of **9a** in the EI-MS. Since signals due to the upper unit (C-1′—6′, glc-1′—6′ and api-1′—5′) were in accordance with those of **5** in the ^{13}C-NMR spectrum of **10**, the structure of **10** was elucidated to be 5,5′-dimethoxy-7-oxolariciresinol 4′-*O*-β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside. This alycone (**10a**) was recently reported to be a metabolite of syringaresinol by wood-rot fungi (*Corilolus versicolor*)[5]; however, it is the first example of isolation from a plant and **10** is a novel glycoside.

Meanwhile, Kamaya and Higuchi[6] reported the experiments of a metabolism of syringaresinol by white-rot fungi (*Phanerochaete* (*P.*) *chrysosporium*). It seemed that these reactions are resposible for lignin biodegradation and metabolism of lignin structure by wood destroying fungi. In that paper, they indicated that the non-phenolic model (syringaresinol dimethyl ether) was little metabolized, but

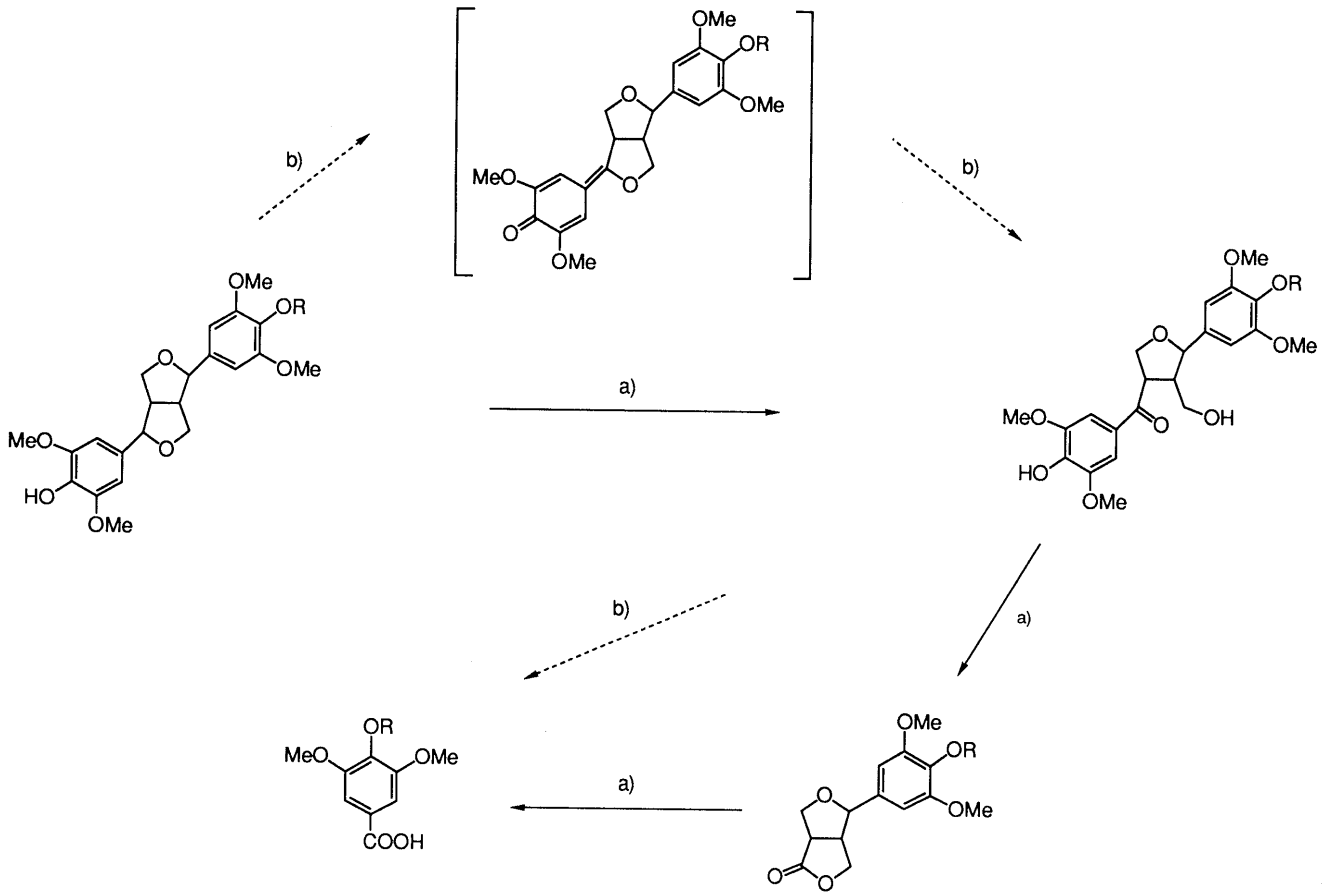


Chart 1. Proposed Degradation Pathway for Syringaresinol by Fungi, a) *Phanerochaete chrysosporium* [R=Me] or b) *Coriolus versicolor* [R=H]

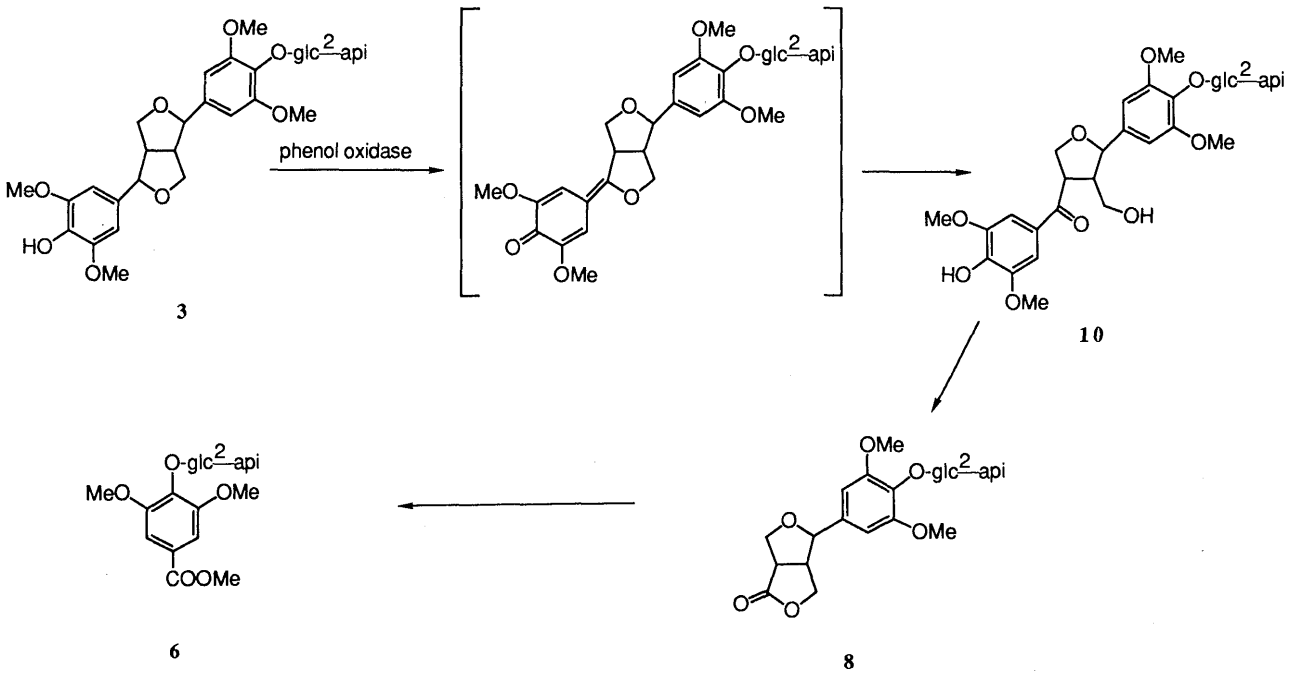


Chart 2. Proposed Biodegradation Pathway of Syringaresinol Apioglucoside in *Albizzia julibrissin*

phenolic models (syringaresinol and its monomethyl ether) were degraded mainly *via* alkyl–aryl cleavage, by the fungal phenol-oxidizing enzymes. They also presented a proposed degradation pathway for syringaresinal monomethyl ether by *P. chrysosporium* (Chart 1).

Katayama and Fukuzumi[5] presented a similar degradation pathway of syringaresinol by another fungi, *Coriolus versicolor* (Chart 1). They clarified that syringaresinol was oxidized by the phenol oxidase to initially have a quinone structure.

It can be pointed that our compounds (**3**, **10**, **8** and **6**) have an important significance. Therefore, we propose an alternative biodegradation pathway of syringaresinol (Chart 2).

In Kamaya's experiment with used syringaresinol 4′-*O*-monomethyl ether, syringic acid monomethyl ether was finally obtained. This meant that the unprotected lower unit was broken by phenol oxidase in a microorganism. In our experiment, compound **3**[7] which was protected at C-4′ as glycoside was also broken from the unprotected lower unit, then converted to compound **6** *via* **10** and **8** by phenol oxidase in the plant itself. It is noteworthy that our compounds have a natural protecting group, apiosyl(1→2)glucoside at C-4 (or C-4′). Since syringaresinol and syringic acid are popular in nature, it is reasonable that the metabolic (biodegradation) pathway as shown in Fig. 2 in the presence of phenol oxidase might be common in plants.

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. The optical rotations were measured with a JASCO DIP-360 automatic digital polarimeter. The IR spectra were recorded with a Hitachi IR spectrometer, model 270-30. The ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX 400 NMR spectrometer and chemical shifts are given on a δ(ppm) scale with tetramethylsilane as an internal standard. The EI- and FAB-MS were measured with a JEOL DX-300 spectrometer. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck). Column chromatography was carried out on Kieselgel 60 (70—230 and 230—400 mesh, Merck), Sephadex LH-20 (Pharmacia) and Bondapak C_{18} (Waters) MCI gel CHP 20P (Mitsubishi Chemical Ind.). Mixed glycosidases (from *Turbo cornutus*) were purchased from Seikagaku Kogyo and hesperidinase was from Sigma Co., Ltd.

Extraction and Isolation Albizziae Cortex was purchased from Uchida Wakanyaku Co., Ltd., Tokyo and 2.0 kg was extracted with MeOH twice under reflux. The combined extract (126 g) was concentrated and partitioned with 1-BuOH and H_2O. The aqueous extract (51 g) was subjected to Bondapak C_{18} column chromatography using 0%→100% MeOH to give fractions 1 to 4. Fractions 1—3 were separated by MCI gel CHP 20P (0%→100% MeOH), Sephadex LH-20 (0%→100% MeOH) and silica gel ($CHCl_3$–MeOH–H_2O=8:2:0.2→7:3:0.5) to provide compounds **1** (0.005%), **2** (0.004%), **3** (0.014%), **4** (0.011%), **5** (0.004%), **6** (0.004%), **7** (0.002%), **8** (0.006%), **9** (0.002%) and **10** (0.005%).

Compound **6**: White amorphous powder, $[\alpha]_D^{25}$ −57.9° (c=0.14, MeOH). IR (KBr): 1720 ($\nu_{C=O}$)cm^{-1}. UV λ_{max} (MeOH)nm (log ε): 264 (4.06). Negative FAB-MS m/z: 505 $[M-H]^-$. ^{1}H-NMR (in DMSO-d_6) δ: 7.23 (2H, s, H-2, 6), 5.33 (1H, s, api H-1), 5.18 (1H, d, J=7 Hz, glc H-1), 3.81 (6H, s, 3, 5-OMe), 3.84 (3H, s, 1-COOMe). ^{13}C-NMR: Table I.

Solvolysis of 6 A solution of **6** in 1 N HCl–MeOH was heated under reflux for 30 min and the reaction mixture was neutralized by 3% KOH–MeOH. After filtration and evaporation *in vacuo*, the residue taken in a small amount of $CHCl_3$–MeOH was analyzed by TLC ($CHCl_3$–MeOH–H_2O=8:2:0.2). Methylapioside (Rf 0.37); methylglucoside (Rf 0.14).

Compound **7**: White amorphous powder, $[\alpha]_D^{25}$ −9.1° (c=0.19, MeOH), IR (KBr): 1790 ($\nu_{C=O}$)cm^{-1}. UV λ_{max}^{MeOH} nm (log ε): 271 (2.73). Negative FAB-MS m/z: 441 $[M-H]^-$. ^{1}H-NMR (in DMSO-d_6) δ: 6.69 (2H, s, H-2, 6), 4.89 (1H, d, J=7 Hz, glc H-1), 4.70 (1H, d, J=6 Hz, H-7), 4.50 (1H, dd, J=7, 10 Hz, H-9$_{eq}$), 4.22 (1H, t, J=9 Hz, H-9′$_{eq}$), 4.41 (1H, dd, J=2, 10 Hz, H-9$_{ax}$), 3.98 (1H, dd, J=3, 9 Hz, H-9′$_{ax}$), 3.58 (1H, m, H-8′), 3.19 (1H, m, H-8). ^{13}C-NMR: Table I.

Enzymatic Hydrolysis of 7 A solution of **7** (7 mg) and hesperidinase in citrate–phosphate buffer (pH 5.0) was incubated at 37 °C for 5 d and after filtration mixture was subjected to MCI gel CHP 20P. The MeOH eluate was separated by silica gel to give **7a** (2 mg), white amorphous powder, $[\alpha]_D^{25}$ 0° (c=0.12, MeOH). IR (KBr): 1775 ($\nu_{C=O}$)cm^{-1}. EI-MS m/z: 280 $[M]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 6.57 (2H, s, H-2,6), 5.55 (1H, br s, 4-OH), 4.59 (1H, d, J=7 Hz, H-7), 4.51 (1H, dd, J=7, 10 Hz, H-9$_{eq}$), 4.39 (1H, t, J=9 Hz, H-9′$_{eq}$), 4.34 (1H, dd, J=2, 10 Hz, H-7$_{ax}$), 4.20 (1H, dd, J=4, 9 Hz, H-9′$_{ax}$), 3.91 (6H, s, 3, 5-OMe), 3.40—3.54 (1H, m, H-8′), 3.05—3.18 (1H, m, H-8).

Compound **8**: White amorphous powder, $[\alpha]_D^{25}$ −40.4° (c=0.20, MeOH). IR (KBr): 1770 ($\nu_{C=O}$)cm^{-1}. UV λ_{max} (MeOH)nm (log ε): 271

(2.53). Negative FAB-MS *m/z*: 573 $[M-H]^-$. ^{1}H-NMR (in DMSO-d_6) δ: 6.67 (2H, s, H-2, 6), 5.32 (1H, s, api H-1), 4.95 (1H, d, $J=7$ Hz, glc H-1), 4.70 (1H, d, $J=6$ Hz, H-7), 4.59 (1H, dd, $J=6$, 10 Hz, H-9$_{eq}$), 4.23 (1H, t, $J=9$ Hz, H-9′$_{eq}$), 4.40 (1H, dd, $J=2$, 10 Hz, H-9$_{ax}$), 3.98 (1H, dd, $J=4$, 9 Hz, H-9′$_{ax}$), 3.58 (1H, m, H-8′), 3.18 (1H, m, H-8). ^{13}C-NMR: Table I.

Enzymatic Hydrolysis of 8 A solution of **8** (10 mg) and hesperidinase in citrate–phosphate buffer (pH 5.0) was incubated at 37 °C for 10 d and after filtration the reaction mixture was separated as above to give **8a** (3 mg), white amorphous powder, $[\alpha]_D^{25}$ 0° ($c=0.20$, MeOH). IR (KBr): 1775 ($\nu_{C=O}$) cm^{-1}. EI-MS *m/z*: 280 $[M]^+$, ^{1}H-NMR (in $CDCl_3$) δ: 6.57 (2H, s, H-2, 6), 5.52 (1H, br s, 4-OH), 4.59 (1H, d, $J=7$ Hz, H-7), 4.51 (1H, dd, $J=7$, 10 Hz, H-9$_{eq}$), 4.39 (1H, t, $J=9$ Hz, H-9′$_{eq}$), 4.34 (1H, dd, $J=2$, 10 Hz, H-9$_{ax}$), 4.20 (1H, dd, $J=4$, 9 Hz, H-9′$_{ax}$), 3.91 (6H, s, 3, 5-OMe), 3.43—3.49 (1H, m, H-8′), 3.08—3.14 (1H, m, H-8).

Compound **9**: White amorphous powder, $[\alpha]_D^{25}$ −2.7° ($c=0.40$, MeOH). UV λ_{max} (MeOH) nm (log ε): 277 (3.65). Negative FAB-MS *m/z*: 713 $[M-H]^-$. ^{1}H-NMR (in DMSO-d_6) δ: 8.07 (1H, s, 4′-OH), 6.58 (2H, s, H-2′, 6′), 6.44 (2H, s, H-2, 6), 5.33 (1H, s, api H-1), 4.93 (1H, d, $J=7$ Hz, gle H-1), 3.73 (12H, s, 3, 3′, 5, 5′-OMe). ^{13}C-NMR: Table I.

Enzymatic Hydrolysis of 9 A solution of **9** (10 mg) and glycosidases from *Turbo cornutus* in citrate–phosphate buffer (pH 5.0) was incubated at 37 °C for 5 d and after filtration the reaction mixture was subjected to MCI gel CHP 20P. The MeOH eluate was separated by silica gel to give **9a** (2 mg), white amorphous powder, $[\alpha]_D^{20}$ +14.4° ($c=0.09$, MeOH). EI-MS *m/z*: 420 $[M]^+$. ^{1}H-NMR (in $CDCl_3$) δ: 6.57 (2H, s, H-2′,6′), 6.42 (2H, s, H-2, 6) 4.79 (1H, d, $J=7$ Hz, H-7′), 4.06 (2H, dd, $J=7$, 8 Hz, H-9$_{eq}$), 2.93 (1H, dd, $J=5$, 14 Hz, H-7b), 2.54 (1H, dd, $J=11$, 14 Hz, H-7a), 3.88, 3.89 (each 6H, s, 3, 3′, 5, 5′-OMe), 2.73 (1H, m, H-8), 2.43 (1H, quintet, $J=7$ Hz, H-8′).

Compound **10**: White amorphous powder, $[\alpha]_D^{20}$ −46.4° ($c=0.58$, MeOH). UV λ_{max} (MeOH) nm (log ε): 287 (4.73), 301 (3.88). Negative FAB-MS *m/z*: 727 $[M-H]^-$. ^{1}H-NMR (in DMSO-d_6) δ: 7.32 (2H, s, H-2, 6), 6.67 (2H, s, H-2′, 6′), 4.59 (2H, d, $J=7.3$ Hz, H-9), 5.33 (1H, s, api H-1), 4.95 (1H, d, $J=7.3$ Hz, glc H-1), 3.84 (6H, s, 3, 5-OMe), 3.75 (6H, s, 3′, 5′-OMe). ^{13}C-NMR: Table I.

Enzymatic Hydrolysis of 10 A solution of **10** (8 mg) and glycosidases from *Turbo cornutus* in citrate–phosphate buffer (pH 5.0) was incubated at 37 °C for 3 d and after filtration the reaction mixture was separated as above to give **10a** (4 mg), white amorphous powder, $[\alpha]_D^{20}$ +1.1° ($c=0.09$, MeOH). EI-MS *m/z*: 434 $[M]^+$. ^{1}H-NMR (in $CDCl_3$) δ: 7.31 (2H, s, H-2, 6), 6.68 (2H, s, H-2′, 6′), 4.68 (1H, d, $J=9$ Hz, H-7′), 4.31 (1H, t-like, $J=8$ Hz, H-9$_{eq}$), 4.22 (2H, m, H-9$_{ax}$ and H-8), 3.81 (1H, dd, $J=11$, 4 Hz, H-9′a), 3.71 (1H, dd, $J=11$, 6 Hz, H-9′b), 3.98, 3.92 (each 6H, s, 3, 3′, 5, 5′-OMe), 2.89 (1H, m, H-8′).

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References and Notes

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Biologically Active Constituents of *Arnebia euchroma*: Structure of Arnebinol, an Ansa-Type Monoterpenylbenzenoid with Inhibitory Activity on Prostaglandin Biosynthesis

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Three phenolic compounds were isolated from the roots of *Arnebia euchroma* as inhibitors of *in vitro* prostaglandin biosynthesis. Two known compounds were identified as shikonofurans and des-*O*-methyllasiodiplodin. The other new compound was named arnebinol and its structure was elucidated as a novel ansa-type monoterpenylbenzenoid derivative.

Keywords *Arnebia euchroma*; Boraginaceae; arnebinol; shikonofuran; des-*O*-methyllasiodiplodin; inhibitor; prostaglandin

In the course of studies to clarify bioactive principles of medicinal plants used in traditional medicines,[1] we have identified a number of natural products possessing inhibitory effect on an *in vitro* prostaglandin (PG) biosynthesizing enzyme system (PG synthetase).[1c-e] Gingerols and diarylheptanoids have been isolated as the inhibitors of PG synthetase, respectively from the rhizomes of *Zingiber officinale* and *Alpinia officinarum* which have been used as medicinal drugs in the traditional Japanese Kampo medicine.[1c,d] The efficacy of such an inhibitor of PG biosynthesis was proved in a pre-clinical test by Hirai and Tamura on paeony root bark.[2] Administration of an aqueous extract of its root bark to humans resulted in a lowering of platelet aggregation in parallel with a decrease of thromboxane A_2 (TXA_2) synthesis in platelets. The inhibitory effect of the extract on arachidonate cyclooxygenase was seen in a decrease of the level of HHT (12-hydroxy-5,8,10-heptadecatrienoic acid), while the formation of 15-HETE (15-hydroxyeicosatetraenoic acid), a product of lipoxygenase, was not affected. Paeonol, the main constituent of paeony root bark inhibited *in vitro* PG biosynthesis at a level comparable to aspirin.[3,4] The results indicate that paeonol inhibited platelet cyclooxygenase to decrease platelet aggregation induced by arachidonic acid.[2] In our subsequent studies on the inhibitors of PG biosynthesis, cinnamoylphenols, isoflavonoids and a benzoic acid derivative were isolated from *Dalbergia odorifera*,[3] cinnamoyl-β-phenethylamines from *Mucuna birdiwoodiana*,[5] and a dihydrostylbene from *Allium chinensis*.[6] Some of them also inhibited *in vitro* platelet aggregation induced by arachidonic acid.[3,5,6]

The roots of *Arnebia euchroma* (ROYLE) JOHNST (Japanese name nanshikon) have been used as a medicinal drug for antipyretic and antibacterial purposes in traditional oriental medicines.[7] In previous communications we briefly reported on the identification and structural elucidation of several constituents possessing inhibitory activity to PG synthetase from this material.[8] This paper describes in detail the isolation, identification and structural elucidation of three phenolic compounds, shikonofuran B and C (**1**), arnebinol (**2**) and des-*O*-methyllasiodiplodin (**4**).

A hot aqueous extract of *A. euchroma* roots inhibited PG synthetase reaction by 35% at a concentration of 750 μg/ml. A preliminary fractionation of the aqueous extract with XAD-2 followed by inhibition testing of the fractions revealed that bioactive compounds contained in the extract were not naphthoquinone pigments such as alkannin (**5**), the main constituents of this medicinal plant, since the most active fraction eluted from the XAD-2 column with methanol contained no naphthoquinones (Chart 1). In spite

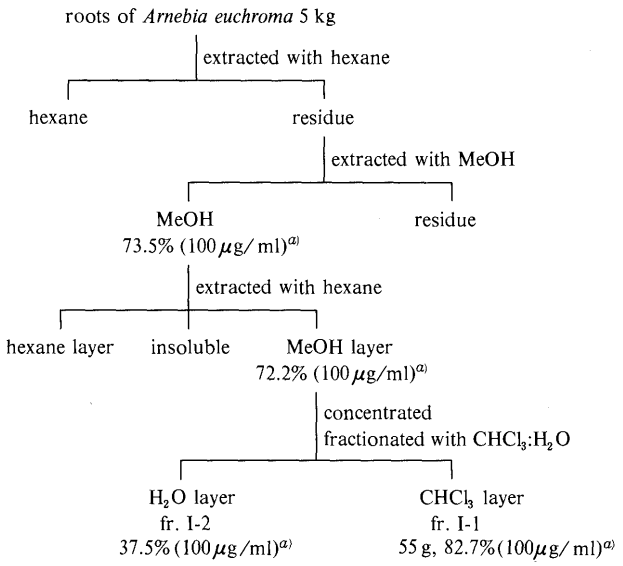


Chart 1. Extraction of *A. euchroma* Roots and Fractionation of Extract

a) Inhibition % for *in vitro* PG biosynthesis.

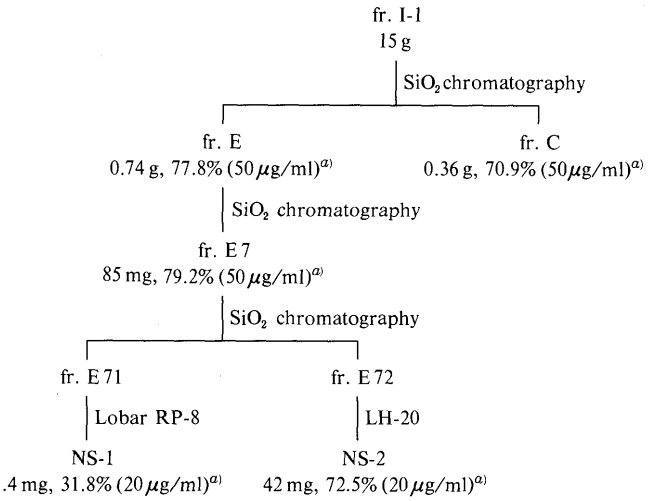


Chart 2. Chromatographic Separation of Active Fractions

a) Inhibition % for *in vitro* PG biosynthesis.

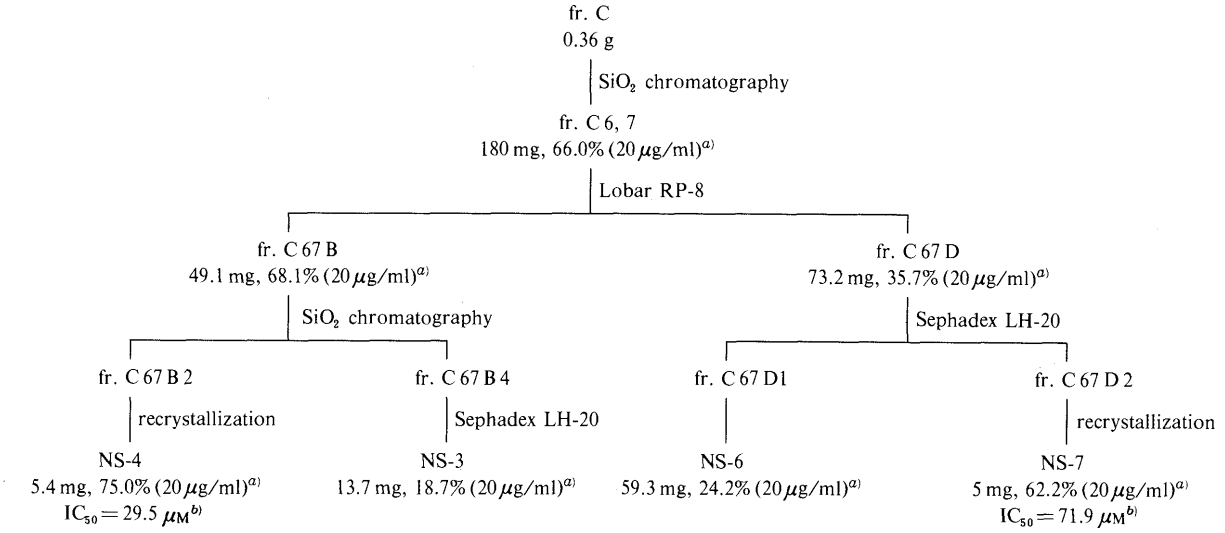
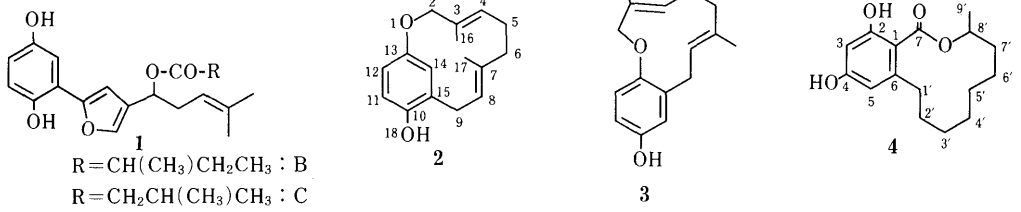


Chart 3. Chromatographic Separation of Active Fractions

a) Inhibition % for *in vitro* PG biosynthesis. *b*) IC_{50} values for *in vitro* PG biosynthesis.



of extensive fractionation and separation on a hot aqueous extract from 5 kg of roots of *A. euchroma*, no fruitful results have so far been obtained in isolating active compounds. A small scale extraction with organic solvents followed by inhibition testing revealed that the active compounds were effectively extracted by organic solvents and that chloroform extract exhibited the strongest inhibition to PG biosynthesis.

Based on these observations 5 kg of *A. euchroma* roots was extracted with hexane to remove naphthoquinones and then extracted with methanol. The methanol extracts were fractionated with hexane and methanol to remove heavily coloured pigments. The chloroform fraction (fr. I-1) that was obtained upon further fractionation of the methanol layer with chloroform and water was highly active as shown in Chart 1. In the following separation and fractionation process each fraction was monitored by testing its inhibitory activity against PG synthetase. For example, silica-gel column chromatography gave two fractions (frs. C and E) of relatively high activity (Chart 2). The extensive separation of the fr. C by repeated column chromatographies with silica-gel, Lobar RP-8 and Sephadex LH-20 finally afforded four compounds, tentatively designated as NS-3, NS-4, NS-6 and NS-7 (Chart 3). The fr. E also afforded two compounds, NS-1 and NS-2, of which NS-1 could not be characterized due to its small yield (Chart 2). It is clear from that the isolated compounds cannot represent all the activity present in the fr. I-1. Many other compounds contained in the chloroform soluble fraction (fr. I-1) must contribute to the overall activity.

By chemical and spectral investigations NS-2 was identified as a mixture of shikonofuran B and C (**1**), which had been isolated from the roots of *Lithospermum erythrorhizon*.[9] Gas chromatography-mass spctra (GC-MS) analysis of the methyl esters of fatty acids obtained by methanolysis of shikonofurans (**1**) clarified that the ester group consists of α-methylbutyrate and isovalerate in a ratio of 1.4 : 1. Thus, NS-2 is a mixture of shikonofurans B and C (**1**) in a ratio of 1.4 : 1.

A colourless compound NS-4 which was named arnebinol (**2**) was obtained in 0.0004% yield and its IC_{50} value for PG synthetase was 29.5 μM. High resolution mass spectra (HR MS) gave an element composition of $C_{16}H_{20}O_2$. Its spectral data indicated NS-4 to be a new compound. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum that was measured at room temperature gave signals corresponding to the protons of 1,2,4-substituted benzene, two methyls on olefinic carbons, two olefinic methines and four aliphatic methylenes. Of the four aliphatic methylenes only one gave a broad but clear triplet (δ 2.34, J = 7 Hz), while the others gave two pairs of extremely broad singlets (δ 2.14, 2.49 and δ 3.07, 3.30) and a broad singlet (δ 4.52) (Fig. 1A and Table I). The broad signals of the methylenes indicate that a steric hindrance preventing the free rotation of the three methylenes causes broadening of the signals by an equilibrium between different conformations. In the NMR spectrum taken at a low temperature (239 K), the three methylenes appear as sharp signals, which prove the conformational equilibrium of arnebinol (**2**) (Fig. 1A). Proton homonuclear decoupling and proton correlation spectroscopy (^{1}H-COSY) data clarified two C_5 partial structures of isopentenyl skeleton, which were combined into a C_{10} partial structure with geranyl skeleton according to biogenetical considerations. Each olefinic methine proton couples with adjacent methylene

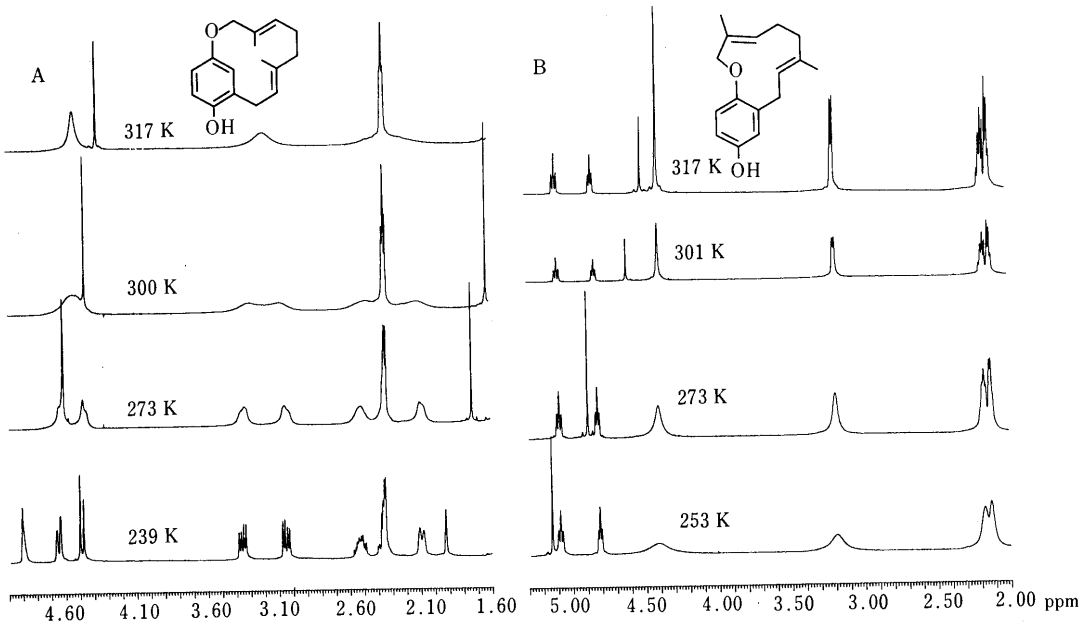


Fig. 1. ^{1}H-NMR Spectra of Arnebinol (A, **2**) and Isoarnebinol (B, **3**) at Varied Temperatures

TABLE I. NMR Assignment of Arnebinol (**2**)

Atom No.	^{1}H-NMR (δ)	^{13}C-NMR (δ)[a]
2	4.52 (2H, br s)	—[b]
3	—	140.2
4	5.51 (1H, br t, J=7 Hz)	127.0
5	2.14, 2.49 (1H×2, br s×2)	25.3[c]
6	2.34 (2H, br t, J=7 Hz)	25.8[c]
7	—	133.6
8	5.67 (1H, br t, J=7 Hz)	124.2
9	3.07, 3.30 (1H×2, br s×2)	39.3
10	—	146.8
11	6.59 (1H, d, J=8.5 Hz)	118.2
12	6.55 (1H, dd, J=3, 8.5 Hz)	115.1
13	—	151.8
14	7.44 (1H, d, J=3 Hz)	115.2
15	—	132.6
16	1.50 (3H, s)	14.8
17	1.24 (3H, s)	12.6

a) ^{13}C-NMR signals were assigned based on CH-COSY. *b*) Overlapped with solvent signal. *c*) Signals had no correlation in CH-COSY and assignments may be interchangable.

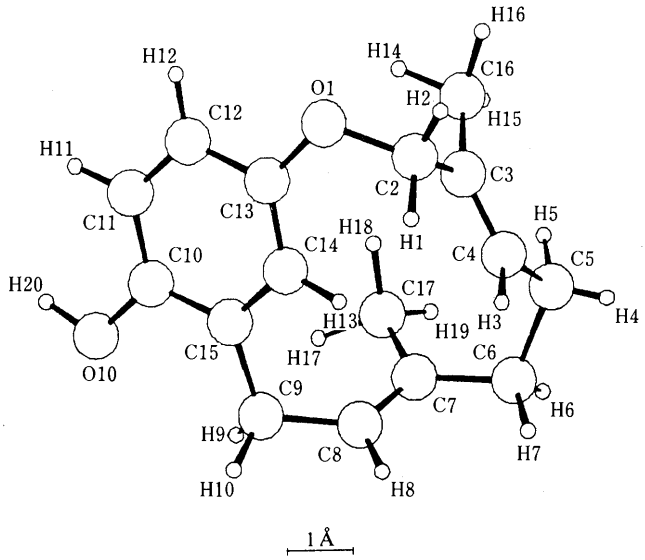


Fig. 2. Drawing of Arnebinol (**2**) with Coordinates of X-Ray Analysis

protons as well as with allylic methyl protons with significant coupling constants. The methylene protons at δ 2.34 (C-6) coupled with the allylic methine proton at δ 5.67 (C-8) with a very small coupling constant causing broadening in the signals. This allows complete assignment of ^{1}H-NMR signals of the monoterpene moiety. The NMR data and biogenetical considerations indicated that the configuration of the two double bonds should be *E*.

Geranylhydroquinone (**6**) and alliodorol (**7**), monoterpenylhydroquinnones, have been isolated from Boraginaceae plants and are postulated to be the precursors of other phenolic and quinonic compounds occurring in these plants.[10] This suggested that arnebinol (**2**) is a dehydroderivative of alliodorol (**7**) which forms an ether linkage with one of the oxygen atoms of the hydroquinone. There were two possible structures (**2**,**3**) for arnebinol. However, the presence of steric hindrance that prevented free rotation of the methylenes indicated that the ansa-type structure (**2**) with a 12-membered ether ring was the more probable. The structure of arnebinol (**2**) was unambiguously established by X-ray analysis. Arnebinol (**2**) gave a crystal suitable for X-ray analysis from benzene with a space group of $P2_1$. The structure was solved by the direct method and refined by least-squares. The final R value was 0.052 with anisotropic temperature factors for carbon and oxygen atoms, and isotropic temperature factors for hydrogen atoms. A drawing of the structure from X-ray analysis is shown in Fig. 2.

The biogenesis of shikonofuran (**1**) and arnebinol (**2**) is a very interesting subject. Shikonin, alkannin (**5**) and shikonofuran (**1**) have been suggested to be derived from geranylhydroquinone (**6**) *via* a hydroxylated geranyl-hydroquinone (**8**), though further modification is required to form a furan and naphthoquinone group.[11] The

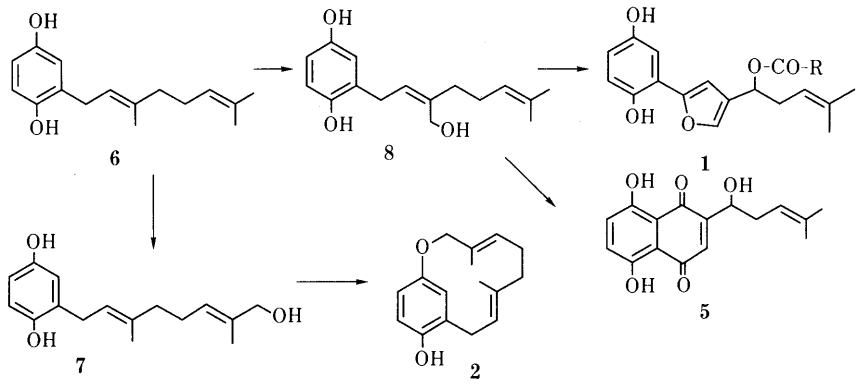


Chart 4. Biogenesis of Shikonofurane (**1**), Arnebinol (**2**) and Alkannin (**5**)

biogenesis of arnebinol (**2**) is readily accounted for by the dehydration reaction of alliodorol (**7**) which has a hydroxy group at pro-*E* methyl of geranylhydroquinone. Mori *et al.* attempted to synthesize arnebinol (**2**) according to the biogenetic scheme (Chart 4) and obtained arnebinol (**2**) and unnatural isoarnebinol (**3**) by the cyclization of a derivative of alliodorol.[12] The dynamic NMR spectra of arnebinol (**2**) and isoarnebinol (**3**) that were measured at different temperatures clearly demonstrate arnebinol (**2**) to be the more hindered compound. The methylene groups of isoarnebinol (**3**) rotate freely above 28 °C (301 K), while the rotation of the methylene groups of arnebinol (**2**) is hindered up to 44 °C (317 K) (Fig. 1). At a low temperature (239 K) arnebinol (**2**) gave a spectrum of one conformation in a solution.

NS-7 was obtained as colourless crystals in yield of 0.0004% and its IC_{50} value for the inhibition of *in vitro* PG biosynthesis was 71.5 μM. Molecular formula was given by HR MS as $C_{16}H_{22}O_4$. The ^{13}C-NMR spectrum gave signals arising from seven methylene groups, indicating NS-7 is not a compound deriving from geranylhydroquinone (**6**) as are shikonofuran (**1**) and arnebinol (**2**). The ^{1}H-NMR spectrum gave signals assignable to two aromatic protons at *meta* position in a benzene ring, a secondary methyl appearing as a doublet and a methine showing ddq coupling pattern, suggesting NS-7 is a polyketide macrolide. It was finally identified by direct comparison of samples as des-*O*-methyllasiodiplodin (**4**) which had been isolated from the fungus *Lasiodiplodia theobromae*.[13] Lasiodiplodin, a methyl ether of des-*O*-methyllasiodiplodin, was isolated from the plant, *Euphorbia apendens* (Euphorbiaceae), as a potent antileukemic substance.[14] However, the polyketides of macrolide structure are typical of fungal metabolites and not often isolated from plants. Des-*O*-methyllasiodiplodin (**4**) may derive from a fungus grown on the plant by symbiosis or just contamination.

The inhibitory activities of shikonofurans (**1**), arnebinol (**2**) and des-*O*-methyllasiodiplodin (**4**) support our view on the inhibitory activity of phenolic compounds to *in vitro* PG biosynthesis. They belong to the rather more potent phenolic inhibitors so far observed in our studies.[1c-e,3-5] The presence of free phenolic and lipophilic groups is their common structural feature as in gingerols, diarylheptanoids, cinnamoyl-phenols and isoflavonoids. As far as our studies on the inhibitors of PG biosynthesis from plant origin are concerned, α-cyperone, a sesquiterpene, obtained from *Cyperus rhotondus* is the sole exception.[1e]

Experimental

NMR spectra were measured on a JEOL FX-100 or FX-400 spectrometer, MS on a JEOL JMS-DX300, infrared (IR) on a JASCO model 701 G and ultraviolet (UV) on a Hitachi spectrometer model 100-60. GC-MS was performed on a Shimadzu LKB GC-MS 9000 combined with a GC-MS PAC 300 computer system and Chromatopac 4B Integrator. Melting points were determined on a Yanagimoto micro melting point apparatus and are not corrected.

Assay of PG Biosynthesis Bioassay of PG biosynthesis was carried out with radioactive arachidonic acid and a microsomal enzyme preparation from rabbit kidney medulla as described in a previous paper.[1e]

Preliminary Fractionation of a Hot Aqueous Extract The roots of *Arnebia euchroma* (nanshikon) purchased from Uchida Wakanyaku Co. (Tokyo) were extracted with 15 ml water per g of material at 90 °C for 6 h. A solution of the extract obtained upon filtration while still hot was concentrated *in vacuo*, frozen and dried to give the hot aqueous extract, which showed 35% inhibition against the PG biosynthesizing enzyme system at a concentration of 750 μg/ml. The extract (4 g) was dissolved in water (40 ml), applied on a XAD-2 column (110 g: 26 mm × 350 mm) and eluted with water at a flow rate of 2 ml/min. The column was successively eluted with 50% MeOH (1350 ml), MeOH (850 ml) and acetone (1050 ml), H_2O and 50% MeOH eluents showed no significant activities, while the MeOH and acetone fractions inhibited PG biosynthesis by 97 and 75.6%, respectively, at a concentration of 750 μg/ml.

Preliminary Extraction Experiment with Organic Solvents The roots of *A. euchroma* (50 g) were successively extracted twice with 500 ml each of hexane, $CHCl_3$, MeOH and water. Extracts were evaporated to dryness *in vacuo* and their inhibitory activities tested. The dried $CHCl_3$ extract was obtained in 0.7% yield and inhibited PG synthetase by 73.5% at a concentration of 200 μg/ml.

Extraction and Fractionation The roots of *A. euchroma* (5 kg) were extracted with boiling hexane (30 l × 6 times) to remove naphthoquinone pigments and then extracted with MeOH (30 l × 4 times) at 60 °C. A small portion of the MeOH extract was evaporated to dryness and tested for inhibition of PG biosynthesis. The dried extract inhibited PG biosynthesis 73.4% at a concentration of 100 μg/ml. The MeOH solution was concentrated to 4 l *in vacuo* and fractionated with hexane (6 l) after adding a small volume of water. This process was repeated 6 times to give an upper hexane layer, a middle layer containing insoluble material and a lower MeOH layer, The MeOH layer was concentrated to 1 l *in vacuo* and fractionated with $CHCl_3$ and water (2 : 1, 3 l) three times. The $CHCl_3$ layer was evaporated to dryness to give an extract (55 g), which showed 82.7% inhibition (100 μg/ml) against the PG biosynthesizing enzyme system. The dried $CHCl_3$ extract (15 g) was subjected to chromatography over a silica-gel (500 g) and successively eluted with benzene, benzene–acetone (49 : 1, 19 : 1, 1 : 1, v/v) and MeOH. The two fractions eluted with benzene–acetone (49 : 1) exhibited the strongest inhibitory effect, 77.8% and 70.9% inhibition at 50 μg/mg, respectively, and were designated as fr. C (360 mg) and fr. E (740 mg).

Isolation of Shikonofuran (NS-2 : 1) The fr. E (240 mg) was further fractionated with a silica gel column (24 g) using $CHCl_3$, $CHCl_3$–MeOH and MeOH as eluents. The most active fr. E7 (85 mg) was obtained by $CHCl_3$ elution and inhibited PG biosynthesis by 79.2% at a 50 μg/ml concentration. The fr. E7 was further separated with a silica-gel column

TABLE II. Fractional Coordinates, B_{eq}(Å^2), U_{eq}(Å^2) and $U(ij)$(Å^2) for Non Hydrogen Atoms with Estimated Standard Deviations in Parentheses ($\times 10^4$)

Atom	x	y	z	B_{eq}	U_{eq}	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
O(1)	−1094 (4)	−134 (0)	2009 (5)	48400 (400)	613 (5)	812 (16)	279 (12)	797 (19)	89 (12)	517 (15)	9 (13)
C(2)	−2230 (5)	−517 (3)	3592 (7)	42900 (600)	543 (7)	696 (24)	277 (16)	689 (25)	108 (16)	359 (21)	99 (17)
C(3)	−4065 (5)	−126 (3)	3394 (6)	35900 (500)	454 (6)	624 (19)	259 (15)	499 (18)	2 (15)	213 (15)	6 (14)
C(4)	−4782 (5)	234 (3)	5109 (7)	35700 (400)	453 (6)	571 (18)	287 (16)	521 (17)	4 (13)	234 (14)	19 (14)
C(5)	−6552 (5)	700 (3)	5240 (8)	46300 (600)	587 (8)	524 (18)	370 (18)	894 (30)	−53 (16)	310 (19)	−96 (20)
C(6)	−6387 (5)	1715 (3)	6220 (7)	41600 (600)	527 (7)	525 (20)	380 (20)	707 (25)	−7 (15)	342 (18)	−68 (18)
C(7)	−5103 (5)	2335 (3)	5100 (6)	33100 (400)	420 (5)	480 (14)	305 (16)	493 (17)	45 (12)	201 (13)	−29 (13)
C(8)	−3585 (4)	2607 (3)	6048 (5)	33500 (400)	425 (5)	542 (17)	314 (15)	436 (15)	12 (14)	196 (13)	−64 (15)
C(9)	−2143 (5)	3115 (3)	4954 (7)	39400 (500)	498 (6)	566 (19)	309 (15)	645 (21)	−77 (16)	272 (17)	−84 (15)
C(10)	−506 (4)	2851 (3)	1575 (5)	34000 (400)	431 (6)	404 (18)	370 (16)	531 (19)	−12 (13)	153 (14)	52 (15)
C(11)	90 (6)	2235 (3)	29 (7)	39600 (500)	502 (6)	519 (17)	448 (19)	564 (19)	18 (16)	260 (15)	70 (16)
C(12)	−109 (5)	1240 (3)	183 (6)	39000 (500)	494 (6)	533 (18)	408 (19)	566 (20)	72 (16)	270 (17)	1 (16)
C(13)	−865 (5)	857 (3)	1967 (6)	36200 (500)	458 (6)	503 (17)	287 (16)	608 (20)	73 (14)	246 (15)	17 (14)
C(14)	−1367 (4)	1470 (3)	3556 (6)	34100 (400)	432 (6)	451 (15)	337 (16)	526 (19)	43 (13)	213 (14)	29 (14)
C(15)	−1283 (4)	2469 (3)	3357 (5)	31400 (400)	398 (5)	393 (13)	338 (16)	475 (16)	−27 (12)	136 (12)	−9 (13)
C(16)	−4953 (8)	−200 (4)	1238 (7)	58100 (800)	736 (0)	985 (32)	698 (32)	527 (22)	−51 (30)	58 (22)	−179 (24)
C(17)	−5601 (6)	2588 (4)	2819 (7)	49700 (600)	629 (8)	676 (24)	613 (26)	598 (21)	23 (24)	30 (19)	78 (23)
O(10)	−385 (4)	3834 (2)	1440 (5)	46800 (400)	592 (5)	791 (18)	336 (13)	680 (17)	−65 (14)	323 (14)	44 (13)

$T=\exp\{-2\pi^2(a^{*2}U_{11}h^2+b^{*2}U_{22}k^2+c^{*2}U_{33}l^2+2a^*b^*U_{12}hk+2a^*c^*U_{13}hl+2b^*c^*U_{23}kl)\}$.

$U_{eq}=\frac{1}{3}\sum_i\sum_j U_{ij}a_i^*a_i^*\boldsymbol{a}_i\boldsymbol{a}_j$.

(20 g) and two active fractions (frs. E71 and E72) were eluted with benzene. Fraction, E71 was finally separated with a Lobar (RP-8) column by eluting with MeOH–H_2O (8.5 : 1.5) to give NS-1 (1.4 mg), which inhibited PG biosynthesis 31.8% (20 μg/ml). Fraction E72 was further chromatographed on LH-20 to give an active compound designated as NS-2 (**1**: 42 mg). The final yield of NS-2 was 0.003% and its inhibition to PG biosynthesis at a concentration of 20 μg/ml was 72.5%.

Isolation of NS-3 (Arnebifuranone)[8c] and NS-4 (Arnebinol: 2) The fr. C (360 mg) obtained in the first silica-gel column chromatography was further fractionated on a silica-gel (36 g) column. Fractions C6 and C7 which were eluted with hexane–benzene (1 : 2) and benzene gave a mixture of several of several compounds (180 mg) of relatively high activity (66% inhibition at a concentration of 20 μg/ml). The mixture (180 mg) was further separated by a Lobar RP-8 column with 85% MeOH to give fr. C67B (49 mg) and fr. C67D (73 mg), which inhibited PG biosynthesis 68.1% and 35.7% (20 μg/ml), respectively. Fraction C67B was separated into two active fractions with a silica-gel (3 g) column using benzene as a solvent. One of the two fractions; fr. C67B2, was first washed with hexane and recrystallized from 20% MeOH to give colourless crystals of NS-4 (5.4 mg), which inhibited PG biosynthesis 75.0% at a concentration of 20 μg/ml. The other fraction, fr. C67B4, was chromatographed on a Sephadex LH-20 (15 × 1.7 cm) and NS-3 (14 mg) was obtained as an orange oil by collecting a yellow band eluted with MeOH; it inhibited PG biosynthesis 18.7% at a concentration of 20 μg/ml.

Isolation of NS-6 (Arnebinone)[8b] and NS-7 (Des-*O*-methyllasiodiplodin: 4) The fr. C67D (73 mg) described above was further separated with a Sephadex LH-20 column (7 × 1.6 cm) to give two active fractions, fr. C67D1 and fr. C67D2. NS-6 (60 mg) was obtained as an orange oil from the former fraction and the latter gave NS-7 (5 mg) as colourless crystals from MeOH. It inhibited PG biosynthesis 62.2% at a concentration of 20 μg/ml.

Shikonofuran B and C (NS-2 : 1) A slightly yellow oil. HR MS: Calcd for $C_{21}H_{26}O_5$ 358.1779, Found 358.1760. MS *m/z* (rel. int.): 358 (M^+, 2.4), 256 (37.4), 85 (63.7), 57 (100). ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 0.88 (3H, t, J=7 Hz), 1.16 (3H, d, J=7 Hz), 1.63 (3H, s), 1.70 (3H, s), 2.44 (1H, m), 2.57 (2H, t, J=7 Hz), 5.08 (1H, br t, J=7 Hz), 5.78 (1H, t, J=7 Hz), 6.43 (1H, br s), 6.64 (1H, dd, J=2.5, 9 Hz), 6.70 (1H, br s), 6.83 (1H, d, J=9 Hz), 7.00 (1H, d, J=2.5 Hz), 7.43 (1H, br s). Inhibition of PG biosynthesis: 72.5% (20 μg/ml).

Identification of Fatty Acids of the Ester Group in Shikonofurans (1) A sample of shikonofuran (1 mg) in 3% HCl/MeOH was sealed in a glass tube and heated at 100 °C for 3 h. Water (100—200 μl) and hexane (300 μl) were added to the cooled reaction mixture. After vortexing the hexane layer was subjected to GC-MS analysis. Column: DEGS (GL Science) 0.28 mm × 1.5 m, carrier gas: N2 (fllumn rate 20 ml/min), column temp.: 50 °C, inj. temp: 120 °C, retention times: methyl α-methylbutyrate 5.36 min; methyl isovalerate 5.59 min. Peak ratio of methyl α-methylbutyrate and methyl isovalerate was 1.4 : 1.

TABLE III. Fractional Coordinates ($\times 10^3$), B_{eq} (Å^2) and U_{eq} (Å$^2 \times 10^2$) for Hydrogen Atoms with Estimated Standard Deviations in Parentheses

Atom	x	y	z	B_{eq}	U_{eq}
H(C2)	−159 (4)	−36 (4)	520 (6)	4.0 (1)	5.3 (1.1)
H′(C2)	−219 (5)	−116 (4)	337 (7)	5.0 (1)	5.9 (1.3)
H(C4)	−401 (6)	19 (4)	661 (7)	4.0 (1)	5.4 (1.1)
H(C5)	−729 (8)	41 (5)	606 (11)	8.0 (0)	9.9 (0.0)
H′(C5)	−716 (5)	75 (4)	359 (7)	5.0 (1)	6.7 (1.4)
H(C6)	−774 (7)	205 (5)	595 (10)	7.0 (0)	9.5 (0.0)
H′(C6)	−581 (6)	158 (4)	792 (8)	5.0 (1)	6.4 (1.3)
H(C8)	−331 (6)	274 (5)	765 (7)	5.0 (1)	6.7 (1.3)
H(C9)	−245 (5)	370 (4)	448 (7)	4.0 (1)	5.5 (1.3)
H′(C9)	−123 (5)	338 (4)	611 (7)	5.0 (1)	6.3 (1.3)
H(C11)	55 (6)	252 (5)	−127 (8)	5.0 (0)	8.9 (0.0)
H(C12)	20 (7)	83 (5)	−104 (9)	6.0 (1)	8.2 (1.7)
H(C14)	−173 (4)	117 (3)	498 (5)	3.0 (1)	4.0 (0.9)
H(C16)	−415 (8)	7 (5)	4 (11)	9.0 (0)	11.1 (0.0)
H′(C16)	−593 (6)	4 (5)	92 (8)	7.0 (1)	8.8 (1.7)
H″(C16)	−492 (12)	−89 (7)	56 (13)	12.0 (0)	14.6 (0.0)
H(C17)	−504 (5)	326 (4)	237 (6)	5.0 (1)	6.5 (1.3)
H′(C17)	−521 (5)	202 (4)	176 (7)	6.0 (1)	7.0 (1.4)
H″(C17)	−703 (5)	266 (4)	255 (6)	5.0 (1)	6.4 (1.2)
H(O10)	−7 (7)	407 (4)	7 (8)	5.0 (1)	6.2 (1.3)

Arnebinol (NS-4 : 2) Colourless needls, mp 163—164 °C (MeOH–water). HR MS: Calcd for $C_{16}H_{20}O_2$ 244.1464, Found 244.1428. UV λ_{max}^{EtOH} nm (log ε): 213 (4.42), 294.2 (3.83). ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data are in Table I. MS *m/z* (rel. int.): 244 (M^+, 65.3), 229 (14.4), 161 (84.6), 121 (100), 55 (51.2). Inhibition of PG biosynthesis: 75.0% (20 μg/ml); IC_{50} = 29.5 μM.

Crystal Data of Arnebinol (2) Space group: $P2_1$. a=7.633 (4), b= (13.868 (7), c=6.300 (3) Å, β=92.98 (5), Z=2, D_X=1.11 g cm^{-3}, V= 666.0 Å^3.

X-Ray Analysis of Arnebinol (2) All reflection data was recorded on a Philips PW 1100 four circle diffractometer with graphite monochromated Cu$K\alpha$ radiation. A total of 1315 reflection was recorded within a range of 3—78°. The structure was solved by the direct method (MULTAN) and refined by block-diagonal least-squares (BLS). A final R value was 0.052 with anisotropic temperature factors for 16 carbon and 2 oxygen atoms, and isotropic temperarure factors for 20 hydrogen atoms.

Des-*O*-methyllasiodiplodin (NS-7 : 4) Colourless needles of mp 128—129 °C (MeOH–water). HR MS: Calcd for $C_{16}H_{22}O_4$ 278.1519, Found 278.1507. UV λ_{max}^{EtOH} nm: 224, 303. IR ν_{max}^{KBr} cm^{-1}: 3340, 1617, 1580, 1460, 1380, 1260, 1140, 1102, 1087, 1013, 920, 837, 713. ^{1}H-NMR ($CDCl_3$,

100 MHz) δ: 1.31 (3H, d, J=6 Hz, C-9′), 1.34—1.67 (10H, m, C-2′-C6′), 1.71—1.86 (2H, m, C-7′), 2.43, 3.21 (1H×2, m×2, C-1′), 5.11 (1H, ddq, J=8, 6, 1.5 Hz, C-8′), 5.34 (1H, br s, OH), 6.16 (1H, d, J=2.5 Hz, C-3), 6.21 (1H, d, J=2.5 Hz, C-5), 12.0 (1H, br s, OH). ^{13}C-NMR ($CDCl_3$, 25 MHz) δ: 20.0 (C-9′), 21.1, 24.0, 24.5, 27.1, 30.7 (C-2′-C-6′), 31.0 (C-7′), 33.4 (C-1′), 75.0 (C-8′), 101.3 (C-3), 105.5 (C-1), 110.6 (C-5), 149.3 (C-6), 159.9 (C-2), 165.3 (C-4), 171.7 (C-7).

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Biologically Active Constituents of *Arnebia euchroma*: Structures of New Monoterpenylbenzoquinones: Arnebinone and Arnebifuranone

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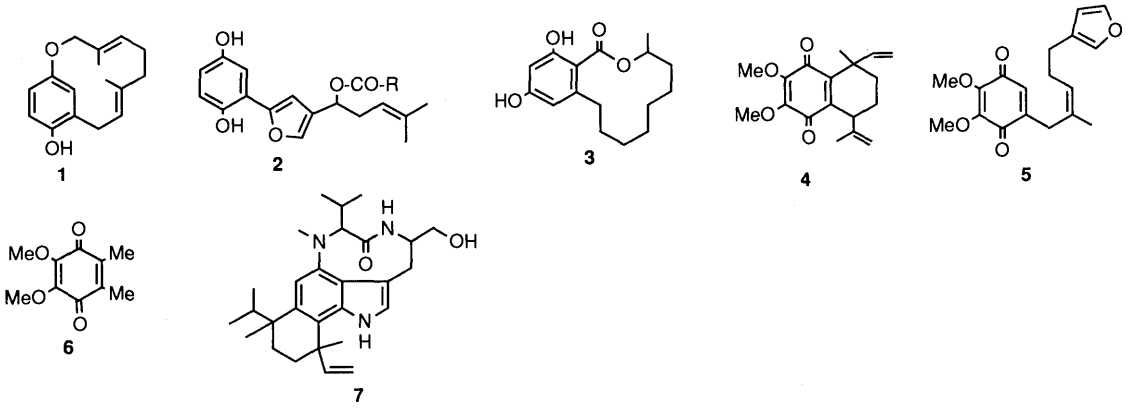
Two quinonic compounds, arnebinone and arnebifuranone, were isolated from the roots of *Arnebia euchroma* and their structures were elucidated on the basis of spectral evidence. Arnebinone is a monoterpenyl-benzoquinoe in which the monoterpene moiety forms a fused ring to the benzoquinone. Arnebifuranone is another monoterpenylbenzoquinone with a furan ring containing side chain which is bonded to the benzoquinone at the head carbon of C_{10} moiety originating from the geranyl moiety of geranylhydroquinone.

Keywords *Arnebia euchroma*; arnebinone; arnebifuranone; prostaglandin; inhibitor

During our studies on the constituents of medicinal plants which act as modulators of arachidonate cascade enzymes,[1] we have isolated three phenolic and two quinonic compounds possessing inhibitory activity against prostaglandin (PG) biosynthesis from the roots of *Arnebia euchroma* (Japanese name, nanshikon),[2] which has been used in traditional Kampo medicine for antipyretic and antibacterial purpose.[3] A phenolic compound, arnebinol (**1**) is a monoterpenylbenzenoid of a unique ansa-type structure.[2a,d] Shikonofuran (**2**)[2b,d] is another phenolic monoterpenylbenzenoid isolated from the roots of *Lithospermum erythrorhizone*, Japanese name, koshikon.[4] The third active phenolic compound is a polyketide macrolide, des-*O*-methyllasiodiplodin (**3**),[2a,d] which is the known metabolite of a fungus, *Lasiodiplodia theobroma*,[5] and its methyl ether, lasiodiplodin, has been isolated from an Euphorbiaceae plant as a compound having anti-leukemia activity.[6] The three potent phenolic inhibitors so far isolated from *A. euchroma* roots have a common structural feature required for the inhibitory activity to PG biosynthesis.[6] The presence of at least one phenol group and a lipophilic moiety is essential for the phenolic inhibitors of PG biosynthesis, as it is seen in the structures of gingerols and diarylheptanoids isolated as potent inhibitors of PG biosynthesis from Zingiberaceae plants.[1a,b] This paper describes details of the structural elucidation of the two quinonic compounds, arnebinone (NS-6: **4**) and arnebifuranone (NS-3: **5**), which were isolated along with the phenolic inhibitors (**1**—**3**) from *A. euchroma* roots.[2b,c]

Arnebinone (**4**) (NS-6) was obtained as an orange oil, which solidified to give yellowish orange crystals when kept at a low temperature. Many attempts to obtain crystalline arnebinone (**4**) were unsuccessful. It inhibited an *in vitro* PG biosynthesis by 24.5% at a concentration of 20 μg/ml and belongs to a rather weak inhibitor of PG biosynthesis probably due to the lack of phenolic group. Arnebinone (**4**) is optically active and its high resolution mass spectra (HR MS) gave an element composition of $C_{18}H_{22}O_4$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of arnebinone (**4**) gave signals assignable to olefinic ABX type signals at δ 4.96 (1H, d, J=17 Hz), 4.98 (1H, d, J=11 Hz) and 5.79 (1H, dd, J=11, 17 Hz), indicating the presence of a vinyl group. The signals at δ 4.72 (1H, s), 4.90 (1H, s) and 1.76 (3H, s) were assignable to an isopropyridene group. The ^{1}H-NMR spectrum showed additional signals assignable to a methyl group at δ 1.00 (3H, s), a methine group at δ 2.23 (1H, dd, J=6, 9 Hz) and two methylene groups at δ 2.32—2.64 (4H, m). No aromatic protons were observed, but a signal corresponding to two methoxy groups appeared at δ 4.00 (6H, s). Ultraviolet (UV) absorption maxima at 277 and 410 nm suggested its quinonic nature, since UV absorption maxima of aurantiogliocladin (**6**), 275 and 407 nm,[7] are very similar to those of arnebinone (**4**). This indicates the presence of 2,3-dimethoxy-5,6-disubstituted benzoquinone chromophore. Thus the basic carbon skeleton of arnebinone (**4**) should consist of C_{16} which corresponds to a monoterpenylbenzenoid with a condensed monoterpene moiety to the benzoquinone ring. Teleosidin (**7**) congeners have a condensed monoterpene ring with a vinyl and



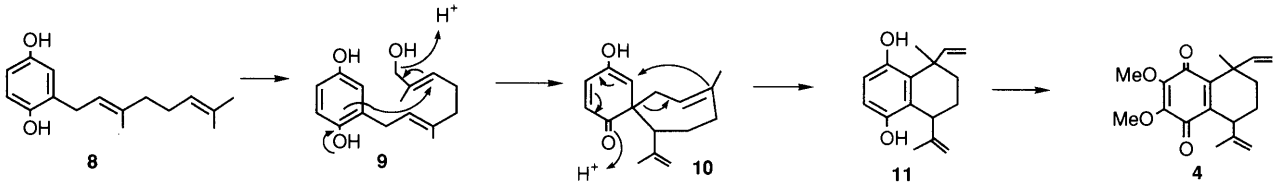


Chart 1. Biogenetic Scheme of Arnebinone (**4**)

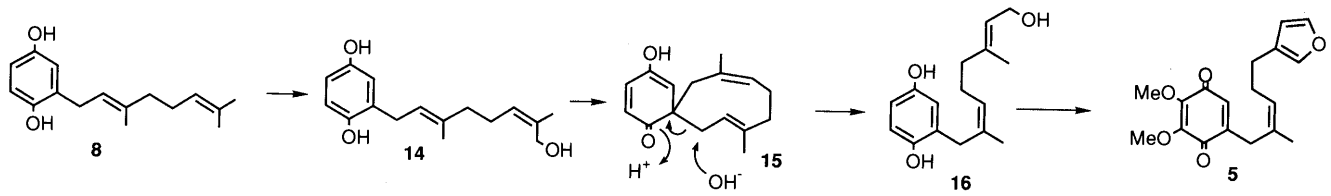
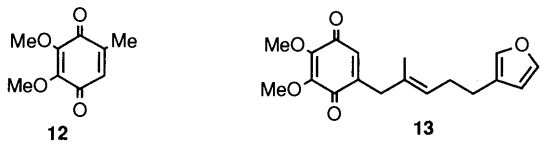


Chart 2. Biogenetic Scheme of Arnebifuranone (**5**)

an isopropyl group, and gave a precedence of condensed monoterpenoid structure similar to arnebinone (**4**), except for an extra-methyl probably derived from a C_1 unit.[8] Biogenesis of arnebinol (**1**) was readily explained by the cyclization of alliodorol (**9**), which derived from geranylhydroquinone (**8**).[9] A biogenetic scheme starting from geranylhydroquinone (**8**) *via* alliodorol (**9**) reasonably accounts for formation of the arnebinone structure (**4**). If we assume a highly strained spyrane intermediate (**10**) which is formed by cyclization of alliodorol (**9**), the spyrane ring undergoes rearrangement by a driving forth of ring strain to give a hydroquinone having a condensed monoterpene ring (**11**) (Chart 1). Functional groups required for the structure of arnebinone (**4**) can be reasonably accommodated.

The other quinonic compound NS-3 was obtained as an orange oil and called arnebifuranone (**5**). HR MS gave an element composition of $C_{18}H_{20}O_5$. It is an unstable compound. When a purified sample was kept at room temperature, small additional signals were observed in the ^{1}H-NMR spectrum. The signals were attributable to an other compound which was presumably formed by the isomerization of arnebifuranone (**5**). Sharp singlet signals at δ4.00 (3H, s) and δ4.02 (3H, s) in the ^{1}H-NMR spectrum indicated the presence of two methoxy groups and its UV absorption maxima, 266 and 410 nm, were very similar to those of 2-methyl-5,6-dimethoxybenzoquinone (**12**),[7] indicating that arnebifuranone (**5**) belonged to 2,3-dimethoxybenzoquinone as arnebinone (**4**). Since the basic carbon skeleton of arnebifuranone (**5**) is C_{16}, it was suggested that it belonged to a monoterpenylbenzenoid as the other phenolic and quinonic compounds isolated from the *A. euchroma* roots. The presence of a mono-substituted furan ring, a methyl on an olefinic carbon, an olefinic methine and three methylenes was indicated by ^{1}H- and ^{13}C-NMR spectra and their connectivity was established by decoupling data and biogenetic consideration (Chart 2). MS gave fragments supporting this structure (**5**). In a previous communication we proposed the structure of arnebifuranone (**5**) with *E* double bond configuration (**13**), without detailed investigation of this double bond configuration.[2c] However, the configuration of arnebifuranone (**5**) has been amended to *Z* by the unambiguous synthesis of *E* isomer, isoarnebifuranone (**13**).[10] The



chemical shift values of the methine and methyl protons are significantly different between the two isomers as discussed by Foland *et al.*[10] Arnebifuranone (**5**) is an unstable compound, as mentioned above, and gradually isomerized to give another compound showing small additional signals in ^{1}H- and ^{13}C-NMR spectra. It became quite clear by the synthesis of isoarnebifuranone (**13**) that additional small signals in ^{1}H-NMR spectrum of arnebifuranone (**5**) correspond exactly to those of unnatural isoarnebifuranone (**13**). It is therefore evident that the unstable double bond of *Z* configuration in arnebifuranone (**5**) gradually isomerized to more stable *E* configuration to give isoarnebifuranone (**13**).

The structure of arnebifuranone (**5**) is quite unique since the monoterpene moiety is linked to the benzoquinone ring by the head methyl but not by the tail methylene as in other monoterpenylbenzenoids of Boraginaceae origin. This is accounted for by the biogenetic scheme shown in Chart 2 where a flip of monoterpene bonding occurs by the cleavage of a C–C bond in a hypothetical sprane intermediate (**15**) formed by the cyclization of isoalliodorol (**14**). It is quite clear that the constituents of *A. euchroma* roots so far isolated in this study seem to originate from geranylhydroquinone (**8**) except for des-*O*-methyllasiodiplodin (**3**). Geranylhydroquinone (**8**) seems to be an important intermediate in the biosynthesis of various secondary metabolites in Boraginaceae plants.[9]

Experimental

NMR spectra were measured on a JEOL FX-400 spectrometer, MS on a JEOL JMS-DX 300, IR on a JASCO model 701G and UV on a Hitachi spectrometer model 100-60. Optical rotation was measured on a JASCO DIP-140 digital polarimeter.

Assay of PG Biosynthesis Bioassay of PG biosynthesis was carried out with radioactive arachidonic acid and a microsomal enzyme preparation from rabbit kidney medulla as described previously.[1e]

Separation and Isolation of Arnebinone (4) and Arnebifuranone (5) Separation and isolation of arnebinone and arnebifuranone was described in a previous paper.[2d]

Arnebinone (**4**): $[\alpha]_D^{20}$ $-4°$ (c=0.01, dioxane). HR MS: Calcd for

$C_{18}H_{22}O_4$ (M^+) 302.1518, Found 302.1518. UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 277 (4.11), 410 (2.76). IR: 3080, 1730, 1656, 1647, 1610, 995, 910. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.00 (3H, s), 1.76 (3H, s), 2.23 (1H, dd, $J=6$, 9 Hz), 2.32—2.64 (4H, m), 4.00 (6H, s), 4.72 (1H, br s), 4.90 (1H, br s), 4.96 (1H, d, $J=17$ Hz), 4.98 (1H, d, $J=11$ Hz), 5.79 (1H, dd, $J=11$, 17 Hz). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 20.1 (CH_3), 24.6 (CH_3), 26.3 (CH_2), 34.5 (CH_2), 38.1 (C), 47.5 (CH), 61.1 ($CH_3\times2$), 111.5 (CH_2), 113.2 (CH_2), 138.5 (C), 139.3 (C), 144.3 (C), 144.4 (C), 145.5 (C), 146.1 (CH), 183.5 (C), 183.6 (C). MS *m/z* (rel. int.): 302 (M^+, 33.9), 287 (100), 255 (21.7), 247 (26.0), 219 (32.1), 93 (41.1), 91 (35.5), 77 (29.4). Inhibition of *in vitro* PG biosynthesis: 24.2% (20 μg/ml).

Arnebifuranone (**5**): HR MS: Calcd for $C_{18}H_{20}O_5$ (M^+) 316.1312, Found 316.1312; Calcd for $C_{13}H_{15}O_4$ 235.0971, Found 235.0951; Calcd for $C_9H_9O_4+H$ 182.0580, Found: 182.0486; Calcd for $C_9H_{11}O$ 135.0811, Found: 135.0791; Calcd for C_5H_5O 81.0341, Found 81.0338. UV λ_{max}^{EtOH}: 266, 404. IR ν_{max}^{Nujol} cm^{-1}: 1655, 1600, 1500, 1450, 1380, 1025, 870. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.65 (3H, d, $J=1.2$ Hz), 2.21 (2H, td, $J=7.4$, 7.4 Hz), 2.46 (2H, br t, $J=7.4$ Hz), 3.10 (2H, d, $J=2.0$ Hz), 4.00 (3H, s), 4.02 (3H, s), 5.46 (1H, br t, $J=7.4$ Hz), 6.24 (1H, t, $J=2$ Hz), 6.25 (1H, br s), 7.20 (1H, br s), 7.33 (1H, dd, $J=1.5$, 1.5 Hz). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 23.4 (CH_3), 24.7 (CH_2), 28.6 (CH_2), 30.7 (CH_2), 61.0 (CH_3), 61.1 (CH_3), 110.8 (CH), 124.3 (C), 128.8 (CH), 130.0 (C), 130.3 (CH), 130.3 (C), 130.8 (C), 138.8 (CH), 142.6 (CH), 145.0 (C), 184.0 (C), 184.2 (C).

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Deoxyribonucleic Acid Strand Break Induced by the Hydroxy Radical-Generating Cyclic Peroxide, 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol

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Deoxyribonucleic acid (DNA) strand break caused by a synthetic cyclic peroxide, 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) was studied by both ethidium bromide fluorescence quenching and agarose gel electrophoresis. The Bd-mediated DNA strand break occurred dependently on temperature and also on Bd concentration. The reaction proceeded at a temperature higher than 30 °C (decomposition temperature of Bd), indicating that the reactive species generated by Bd-decomposition are responsible for the reaction. The reaction was protected by 1,4-diazabicyclo[2.2.2]octane and NaN_3, and also moderately by OH radical scavengers such as Na-benzoate and NaBr. Possible involvement of active oxygen radicals including OH radical in the DNA strand break is discussed in relation to the mechanism of Bd decomposition.

Keywords synthetic peroxide; hydroxy radical; DNA strand break; peroxide decomposition; radical generation

Introduction

Cell damaging effects of oxygen radicals are well recognized in many biological systems.[1] The deoxyribonucleic acid (DNA) strand break is one of the critical effects of the oxygen radicals in the cell killing process.[2–4] The active oxygen radicals, on the other hand, play a role in tumor cell necrosis by anticancer drugs.[5,6] Thus, if one can control the generation of active oxygen radicals at the localized site such as in tumor, new chemotherapeutic methodology could be developed for hypoxic tumor cell necrosis. In this sense, we focused our attention on organic peroxides as the hydroxy radical source and studied the degradation of synthetic cyclic peroxides, 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ols.[7–10] We found that the cyclic peroxides oxidatively degraded cytochrome c *via* radical mediated reaction. Further, a water mediated decomposition of the peroxides accompanied OH radical generation which was determined by electron spin resonance using dimethylpyrroline N-oxide as a spin trapping reagent.

Since the OH radicals play a central role in DNA strand break,[2–4,11,12] it is of interest to know if DNA break is caused by the synthetic cyclic peroxides. We studied here the DNA breaking reaction caused by 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) *in vitro*.

Materials and Methods

Bd was synthesized by the ozonolysis of naphthalene in ethanol according to the method of Bailey *et al.*[13] and purified by repeating recrystalization in cold diethylether at 40 °C as described previously.[8,9] The purity of Bd was more than 90% when it was determined by nuclear magnetic resonance (NMR) spectroscopy.

Calf thymus DNA and λ phage DNA (*Escherichia coli* GM 119) were purchased from Sigma Co., Ltd. Restriction enzyme, *Hind*III from Toyobo Co., Ltd., ethidium bromide (ETB) from Wako Chemicals Co., Ltd. and agarose (type S) for electrophoresis from Nippon Gene Co., Ltd., respectively. Other reagents were all purchased from Wako Chemicals Co., Ltd., and were all reagent grade. A gas chromatography (GC) contents analysis kit was purchased from Yamasa Sho-yu Co., Ltd.

DNA strand break was determined either by the ETB fluorescence quenching method[14] or agarose gel electrophoresis.[15]

For the DNA–ETB intercalation method, an aliquot of Bd solution (10 mM in acetonitrile) was added to 1.0 ml of aqueous solution containing 20 μg of DNA. For the control, the same volume of acetonitrile was substituted for Bd solution. After 40 min of reaction at 40 °C, the reaction mixture was diluted with 2.0 ml of 1 mM ETB aqueous solution containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and shaken to mix, then the fluorescence from the DNA-ETB complex was determined at 590 nm by excitation at 510 nm using Shimadzu Fluorescence spectrophotometer, model RF-502.

For the analysis of DNA strand break by agarose gel electrophoresis, a λ phage DNA was dissolved in a metal free distilled water to make 1 mg/ml solution. To the solution containing 10 μg of DNA, an aliquot of Bd solution (100 mM methanol) was added and the mixture was incubated in a water bath at 40 °C for a specified time period with or without scavengers (total volume of the reaction mixture was 50 μl). Aliquots of the reaction mixture were applied on a 0.5% agarose gel and electrophoresis was run under the electrical field of 20 V. Buffers used for gel preparation and running consist of 89 mM Tris/2 mM EDTA/89 mM borate (pH 8.0). DNA and the fragments on the gel were visualized with ETB fluorescence on a transilluminator at 302 nm.

Results

Impairment of DNA is proved by the DNA–ETB intercalation method,[14] which takes advantage of the characteristic fluorescence of the DNA–ETB complex and its inhibition by Bd-mediated alterations of the DNA base pair region. ETB fluorescence excited at 510 nm was markedly enhanced when mixed with DNA. The fluorescence intensity (Em_{max} at 590 nm) was saturated at approximately 20 μg DNA/ml when 0.3 mM ETB was present. Hence, 20 μg DNA/ml was used for the following studies. The fluorescence was, however, inhibited when the DNA was pretreated with Bd without changing the spectral shape (not shown), indicating that DNA base-pair region is altered.[14] Thus, the fluorescence change at 590 nm was used as a monitor wavelength for DNA strand break.

Since Bd decomposition is temperature dependent,[7–10] DNA was treated with Bd at various temperatures for 40 min and then the DNA–ETB fluorescence intensity was determined (Fig. 1). The fluorescence intensity gradually decreased with increased temperature and became saturated at approximately 30 °C. This temperature range is well consistent with the Bd decomposition temperature determined previously by ESR[7,8] and high performance liquid chromatography (HPLC).[9] It was also shown that the time course of Bd-induced fluorescence inhibition examined at 40 °C was well consistent with that of Bd decomposition in an aqueous medium at the same temperature, that completed by 20 min (not shown). Therefore, it is indicated that the DNA damage is strongly dependent on the Bd decomposition.

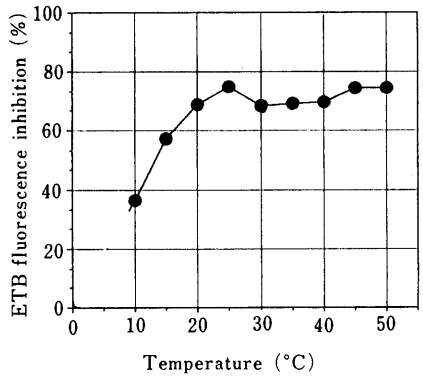

Fig. 1. Temperature Dependence of Bd-Induced DNA Strand Break

Calf thymus DNA (20 μg) was treated with Bd at the final concentration of 0.25 mM for 40 min at the temperatures indicated. The ETB–DNA fluorescence was determined at 590 nm (excitation at 510 nm) after the reaction mixture was diluted with ETB aqueous solution. Relative inhibition % was obtained to the control sample omitting only Bd. Each data points are the average of 2—3 determinations.

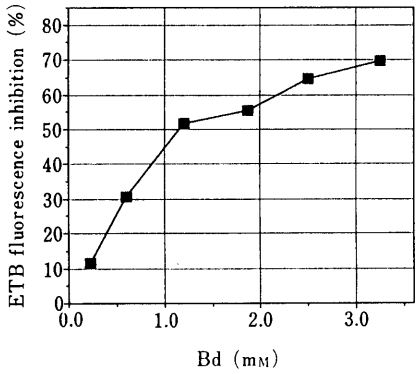

Fig. 2. Bd Concentration Dependence of DNA Strand Break

Calf thymus DNA was treated with BD at the concentrations indicated at 40 °C for 40 min, then the ETB–DNA fluorescence intensity was determined as described in Fig. 1.

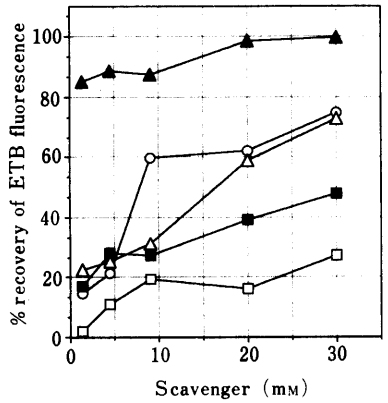

Fig. 3. Protective Effects of Reactive Oxygen Radical Scavengers on Bd-Induced DNA Strand Break

Calf thymus DNA was incubated with 2.5 mM Bd at 40 °C for 40 min in the presence and absence of increased concentrations of radical scavengers. All the radical scavengers were added as an aqueous solution. ETB–DNA fluorescence was determined as described in Fig. 1.

Bd dependence of the DNA–ETB fluorescence inhibition was examined with the DNA pretreated with increased concentrations of Bd at 40 °C for 40 min. As Fig. 2 clearly shows, the fluorescence was inhibited dependently on Bd concentration. Since Bd is known to mediate cytochrome c degradation *via* the oxygen radicals generated by its

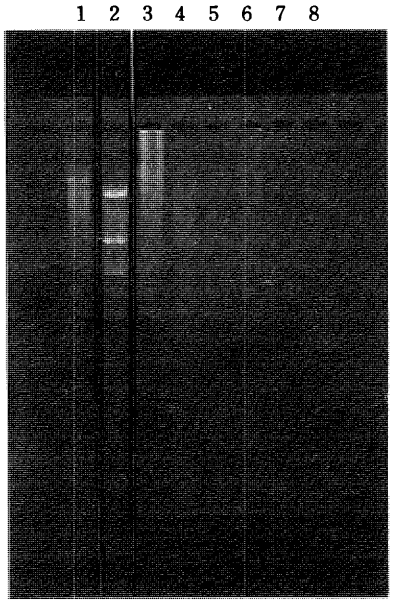

Fig. 4. Time Course of λ Phage DNA Break in the Presence of Bd

DNA cleavage was determined on agarose gel electrophoresis. Cleavage pattern was determined at 302 nm after ETB staining. Lanes: (1) methanol control without Bd (incubation for 180 min), (2) *Hind*III digestion, and λ phage DNA treated with 10 mM Bd at 40 °C for (3) 0, (4) 20, (5) 40, (6) 60, (7) 120, (8) 180 min, respectively.

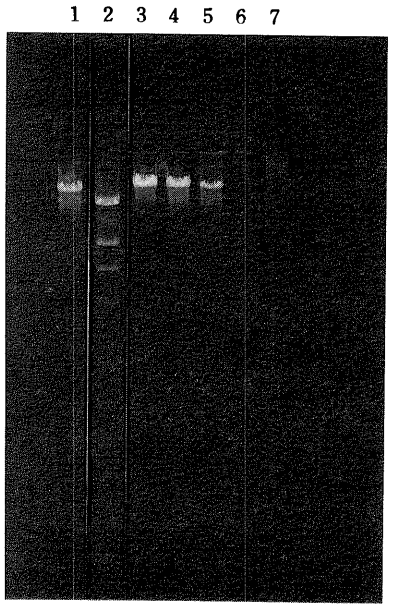

Fig. 5. Effect of Bd Concentration on λ Phage DNA Break

Lanes: (1) control, (2) *Hind*III digestion, and λ phage DNA treated with (3), 0.5, (4) 1.0, (5) 2.0, (6) 5.0 and (7) 10 mM Bd at 40 °C for 1 h.

decomposition,[10] it is also suggested that the inhibition of DNA–ETB fluorescence is due to the DNA alteration caused by the reactive oxygen radicals generated on Bd decomposition.

Protective effects of reactive oxygen radical scavengers were studied for the Bd-induced quenching of the DNA–ETB fluorescence. All the hydroxy radical scavengers examined protected the Bd-induced fluorescence quenching in their concentration dependent manner (Fig. 3). Na-benzoate and NaBr were active and inhibited the reaction for approximately 70% at 30 mM although mannitol was rather ineffective. On the other hand, 1,4-

diazabicyclo[2.2.2]octane (DABCO) inhibited the reaction almost completely.

Fluorescence quenching is affected not only by DNA strand break or other structural alteration of DNA but also artificial quenching. In order to confirm that Bd actually causes DNA strand break, agarose gel electrophoresis was carried out on Bd-treated λ phage DNA. As is shown in Fig. 4, DNA break occurred after Bd treatment. The ETB fluorescence completely disappeared after 20 to 40 min of reaction, that is, the time range of Bd decomposition.[8,9]

The electrophoretic study also showed that the DNA strand break occurs dependently on Bd concentration (Fig. 5). ETB fluorescence at the original DNA mass gradually decreased dependently on Bd concentration and almost completely disappeared at a concentration higher than 5 mM. It is noted that the reaction products which could not penetrate the gel matrix appeared at the initial stage of Bd treatment or with low Bd concentration. It is possible that crosslinking of DNA occurs. The DNA is torn into pieces afterward with a higher Bd concentration or after a long reaction period. Since any characteristic fluorescent fragment with specific molecular weight appeared on the gel, it is expected that the breaks which occurred in the DNA strand are non-specific. Non-specificity of the Bd-mediated DNA break is also suggested by the GC contents change in the Bd treated λ phage DNA determined by HPLC. The GC contents were not significantly changed after Bd treatment (Table I). However, it is notable that both the relative amounts of d-CMP and d-AMP significantly decreased after the reaction. These results indicate that the major DNA damage occurs in the deoxyribose region rather than in the base region.

Protective effects of radical scavengers on the reaction revealed that DABCO and NaN_3 prevented the DNA scission (Fig. 6). The effects of OH radical scavengers are again variable as in the case of Bd-induced ETB fluorescence protection in Fig. 3. Na-benzoate inhibited the Bd-mediated DNA break but mannitol and dimethyl sulfoxide (DMSO) were ineffective.

TABLE I. GC Contents Change after Bd Treatment

	Relative molar ratio (%)				GC contents (%)
	d-GMP	d-CMP	d-AMP	d-TMP	
Untreated	21.85±0.41	35.36±0.16	21.98±0.16	20.81±0.28	57.79±0.74
+10 mM Bd	29.16±0.40	30.07±0.33	18.84±0.06	21.93±0.14	59.23±0.06

DNA samples were incubated with nuclease P1 (0.1 mg/ml in 1/35 M CH_3COONa/0.2 mM $ZnCl_2$, pH 5.3) at 50 °C for 1 h. The liberated mononucleotides were analyzed by HPLC (Nippon Bunko model Twinkle) equipped with reverse phase column (Hitachi #3056) using 5 mM K_2HPO_4/5 mM KH_2PO_4 as an elution solvent.

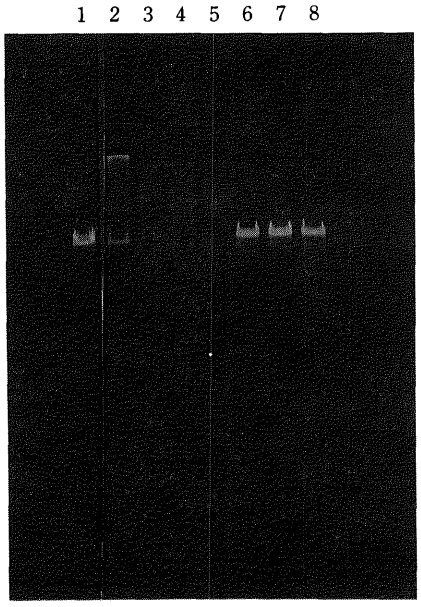

Fig. 6. Inhibition of Bd-Induced DNA Break by Reactive Oxygen Radical Scavengers

λ phage DNA was treated with 10 mM Bd at 40 °C in the presence or absence of radical scavengers for 1 h. Lanes: (1) control, (2) with Bd at 4 °C, (3) with Bd at 40 °C, (4) +mannitol (30 mM), (5) +DMSO (30 mM), (6) +Na-benzoate (30 mM), (7) +DABCO (30 mM) and (8) +NaN_3 (30 mM).

Discussion

Present studies revealed that Bd, a synthetic cyclic peroxide, causes DNA strand break. The reactive oxygen species responsible for the DNA break are considered to be the radicals generated on Bd decomposition because the reactions were totally dependent on Bd decomposition conditions previously reported[7-9] and were protected by the reactive oxygen radical scavengers, as was consistent with our previous papers dealing with the Bd reactions on cytochrome c.[10]

Scavenger studies revealed that singlet oxygen scavengers, DABCO and NaN_3, and also OH radical scavengers, Na-benzoate and NaBr, significantly prevented the Bd-mediated DNA breaks determined by the EBT intercalation and agarose gel electrophoresis. However, it is unlikely that singlet oxygen is the major species responsible for the DNA strand break because the previous study on the mechanism of Bd decomposition supports direct generation of OH-radical but not singlet oxygen in the Bd aqueous medium.[9,16] In addition, we observed that DABCO causes a rapid decrease of Bd absorption at 250 nm, that is, Bd decomposition (unpublished observation), thus the inhibitory action of DABCO may not be due to its singlet oxygen scavenging activity. Further, NaN_3 is reactive not only to singlet oxygen but also to the OH radical.[14] Hence, it is likely that the OH radical is most plausible species involved in the DNA break. Although the effect of OH radical scavengers are divergent such that mannitol and DMSO were ineffective, it is well known that the inhibitory action of the OH scavenger is variable depending on the characteristics of the OH radical generating system.[17]

Since the OH radical is extraordinarily reactive, it does not survive more than a few collisions following its formation.[18] Therefore, the site specific DNA strand break by the OH radical occurs when the OH radical generating compounds or systems interact specifically with DNA in such a case as the ternary metal complexes of Cu-phenanthroline-reducing agent–O_2[19] and else.[20] In the present study, though the DNA strand break occurred as well as in other OH generating systems, Bd did not give rise to any specific fragmentation pattern of DNA on the agarose gel. Moreover, it required rather high concentrations (>5 mM) for the significant breaking of DNA. These discrepancies could be mainly attributed to the difference in the DNA-chemicals interaction.

Although the involvement of radical species other than the OH radical can not be ruled out, the present studies suggest that further modification of the cyclic peroxide to improve specific interaction with DNA could provide an effective DNA strand breaking chemical system.

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An Acidic Polysaccharide Having Activity on the Reticuloendothelial System from the Root of *Astragalus mongholicus*

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An acidic polysaccharide, designated as AMon-S, was isolated from the roots of *Astragalus mongholicus* BUNGE. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 7.6×10^4. It showed significant reticuloendothelial system-potentiating activity in a carbon clearance test. It is composed of L-arabinose: D-galactose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 18 : 18 : 1 : 1, in addition to small amounts of *O*-acetyl groups and peptide moiety. A part of the hexuronic acid residues exist as the methyl esters. Methylation analysis, carbon-13 nuclear magnetic resonance and periodate oxidation studies enabled elucidation of its structural features and revealed mainly α-arabino-β-3,6-galactan type structural units.

Keywords *Astragalus mongholicus*; root; AMon-S; polysaccharide structure; acidic arabinogalactan; immunological activity; reticuloendothelial system

The root of *Astragalus mongholicus* BUNGE (Leguminosae) is a representative material of a traditional Chinese crude drug used as a tonic, a diuretic and an anti-perspirant under the name of Huang-gi in China (Japanese name, Ougi). Although many triterpenoid oligoglycosides have been isolated from the roots of *Astragalus membranaceus*,[1] *A. sieversianus*[2] and *A. ernestii*,[3] no published report has referred to saponins as constituents of *A. mongholicus* root. On the other hand, several polysaccharides have been obtained from the root of this plant[4-8] in addition to β-sitosterol, linoleic and linolenic acids.[9] Some of these polysaccharides have been shown to stimulate the phagocytic activity by i.p. injection.[5,7] However, their structural features have not yet been elucidated so far. The present paper describes the isolation, structural analysis and immunological activity on the reticuloendothelial system (RES) of a novel acidic polysaccharide from the water extract of the root of *A. mongholicus*.

Materials and Methods

Isolation of Polysaccharide The material was imported from China. The sliced roots (1143 g) were extracted with hot water (11.4 l) under stirring for 30 min in a boiling water bath. After suction filtration, the filtrate (7.74 l) was added to 1% sodium sulfate (78 ml); 5% cetyltrimethylammonium bromide (240 ml) was then added to the solution. After centrifugation, the supernatant obtained was poured into two volumes of ethanol. The precipitate was dissolved in water, then dialyzed, concentrated and lyophilized. Yield, 11.58 g. Half of this fraction was dissolved in water and applied to a column (4.5 × 45 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 which had been pretreated as described in a previous report.[10] After elution with water (840 ml), the column was eluted with 0.2 M ammonium acetate. Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[11] The eluates obtained from tubes 16 to 43 were combined, dialyzed, concentrated and lyophilized. Yield, 202 mg. This fraction (fr. A, 135 mg) was dissolved in 1/15 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and applied to a column (1.5 × 39 cm) of Con A-Sepharose (Pharmacia Co.). The column was equilibrated and eluted with the same buffer at 4 °C, and fractions of 10 ml were collected. The eluates obtained from tubes 4 to 11 were combined, dialyzed and then concentrated. After two repetitions of this affinity chromatography, the solution corresponding to 404 mg of fr. A was applied to a column (5 × 77 cm) of Toyopearl HW60F. The column was equilibrated and eluted with 0.1 M Tris–HCl buffer (pH 7.0), and fractions of 20 ml were collected. The eluates obtained from tubes 39 to 43 were combined, dialyzed, concentrated and rechromatographed using a column (2.6 × 97 cm) of Toyopearl HW60F. The column was equilibrated and eluted with the same Tris–HCl buffer, and fractions of 10 ml were collected. The eluates obtained from tubes 30 to 35 were combined, dialyzed, concentrated and applied to a column (2.6 × 94 cm) of Sephadex G-25. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 22 to 24 were combined, concentrated and lyophilized. AMon-S (43 mg) was obtained as a white powder.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel tubes (4 × 142 mm each) and 5 mM Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate-Schiff (PAS) procedure and with Coomassie blue reagent. AMon-S gave a clear band at a distance 63 mm from the origin.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 97 cm) of Toyopearl HW60F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Phagocytic Activity This was measured as described in a previous report.[12] The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 and 40 mg/kg body weight) once a day. The 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. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF). The comparison of results was performed by means of Student's t-test.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[13] The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylaminoalditol derivatives.[14] GC was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of Components Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described previously.[12] Hexuronic acid was estimated by the *m*-hydroxybiphenyl method.[15] The ratio of galacturonic acid and glucuronic acid was determined by GC of the alditol acetates derived from hexuronic acids by chromatographic separation of the hydrolyzate followed by successive reduction and acetylation in the manner described in a previous report.[16]

Determination of *O*-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described previously.[17]

Determination of *O*-Methyl Groups in Methyl Esters This was performed by GC after saponification using ethanol as an internal standard as described previously.[18]

Nuclear Magnetic Resonance (NMR) NMR spectrum was recorded on a JEOL JNM-GX270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30 °C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.[19] The reduction was

TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a)]	Main fragments (*m/z*)
1,4-Ac_2-2,3,5-Me_3-L-arabinitol	0.68	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3-Me_2-L-arabinitol	1.14	43, 87, 101, 117, 129, 189
1,5-Ac_2-2,3,4,6-Me_4-D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,5-Ac_2-2,3,4,6-Me_4-D-galactitol	1.10	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac_3-2,4,6-Me_3-D-galactitol	1.38	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3,6-Me_3-D-galactitol	1.46	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac_3-2,3,4-Me_3-D-galactitol	1.60	43, 87, 99, 101, 117, 129, 161, 189
1,3,5,6-Ac_4-2,4-Me_2-D-galactitol	2.01	43, 87, 117, 129, 189

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,4-Ac_2-2,3,5-Me_3- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

repeated three times under the same conditions. Yield was 22 mg from 30 mg of AMon-S.

Methylation The sample (8 mg) was dissolved in dimethyl sulfoxide (0.8 ml) under heating, then finely powdered sodium hydroxide (40 mg) and methyl iodide (0.2 ml) were added to the solution. The whole was stirred at room temperature for 1 h. All procedures were carried out under nitrogen. Water and chloroform (3 ml each) were then added to the reaction mixture, and the whole was extracted three times with chloroform (3 ml each). The combined extract was washed three times with water (12 ml each), then dried over sodium sulfate. The filtrate was concentrated to dryness, and the residue was dissolved in chloroform–methanol mixture (2 : 1), then applied to a column (1 × 20 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 4 to 6 were combined and concentrated. The infrared (IR) spectra of final residues showed no hydroxyl group absorption. Yield, 6.0 mg from the origin and 9.3 mg from the carboxyl-reduced product.

Analysis of Methylated Products The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[20] The partially methylated alditol acetates obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) using a fused capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) and with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX303 mass spectrometer. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GC and the main fragments in MS are listed in Table I.

Periodate Oxidation Periodate oxidation followed by reduction with sodium borohydride was performed as described previously.[21] Oxidation was completed after 84 h. Yield of the product was 9 mg from 9.5 mg of AMon-S. Determination of the surviving component sugars was carried out as described above.

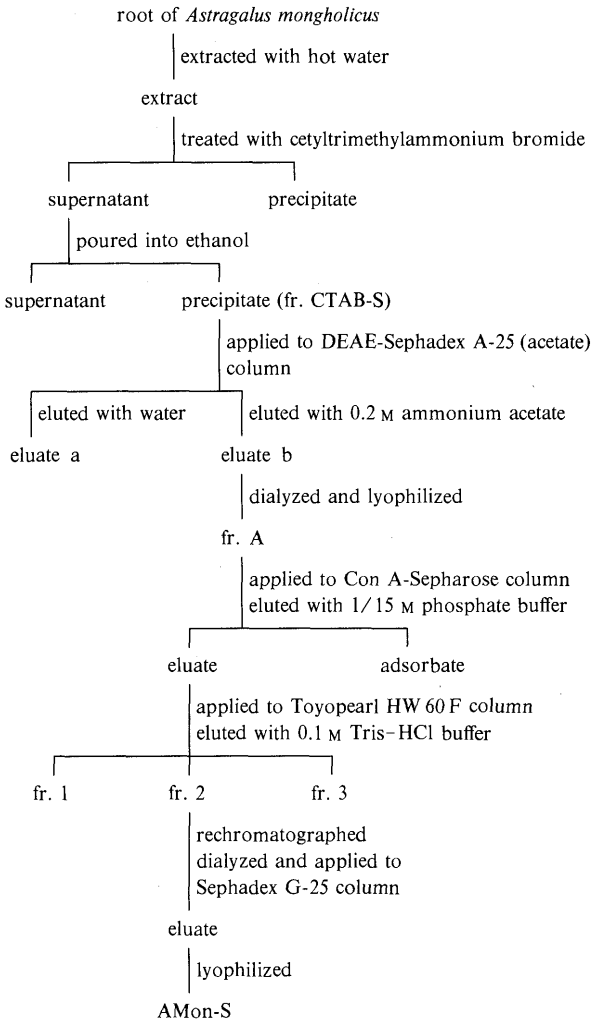

Fig. 1. Isolation of AMon-S

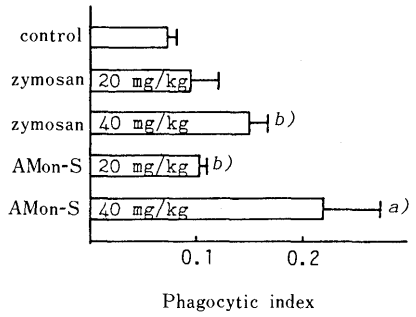

Fig. 2. Effect of AMon-S on Carbon Clearance Index in ICR Mice
Significantly different from the control, *a*) $p<0.005$, *b*) $p<0.001$.

Results

The hot water extract obtained from the root of *Astragalus mongholicus* was treated with cetyltrimethylammonium bromide in the presence of small amounts of sodium sulfate. The supernatant obtained was poured into ethanol, then the precipitate was applied to a column chromatography of DEAE-Sephadex A-25. The eluate with 0.2 M ammonium acetate was subjected to affinity chromatography on Con A-Sepharose. The passed-through fraction was applied to gel chromatography on a Toyopearl HW60F column. AMon-S was obtained after rechromatography, followed by dialysis and gel chromatography with Sephadex G-25. The isolation method of the polysaccharide is summarized in Fig. 1.

The polysaccharide gave a single band on PAGE, and gave a single peak on gel chromatography. It had $[\alpha]_D^{23}$ $-44.6°$ (H_2O, $c=0.1$). Gel chromatography gave a value of 7.6×10^4 for the molecular mass.

The effect of AMon-S on the RES was demonstrated by a modification[12] of the *in vivo* carbon clearance test[22] using zymosan as a positive control. As shown in Fig. 2, the phagocytic index was significantly increased, suggesting activation of the RES by i.p. injection of AMon-S.

AMon-S is composed L-arabinose, D-galactose, D-galacturonic acid and D-glucuronic acid and a peptide moiety. Quantitative analyses showed that it contained 40.6% arabinose, 48.8% galactose, 2.9% galacturonic acid, 3.0% glucuronic acid and 4.7% peptide moiety. The molar ratio of these component sugars was 18 : 18 : 1 : 1.

The carbon-13 NMR (^{13}C-NMR) spectrum of AMon-S showed signals at δ 21.77 and 178.27 ppm, suggesting the presence of *O*-acetyl group. In addition, the ^{13}C-NMR spectrum showed a signal at δ 57.04 ppm, suggesting the presence of *O*-methyl groups as carboxylic acid methyl esters. The presence of these groups was confirmed by GC of the hydrolyzate, and the contents of acetyl and methoxyl groups were 2.6% and 0.41%, respectively. Thus existence of about 40% of the hexuronic acid residues in the polysaccharide as methyl esters is evident.

Further, the ^{13}C-NMR spectrum showed five anomeric carbon signals at δ 100.64, 105.33, 105.90, 110.14 and 111.94 ppm. The first and the second were assigned to the anomeric carbons of α-D-galactopyranosyluronic acid and β-D-glucopyranosyluronic acid, respectively.[16,23] The signal at δ 105.90 ppm was assigned to the anomeric carbon of β-D-galactopyranose, and the signals at δ 110.14 and 111.94 ppm were assigned to the anomeric carbons of α-L-arabinofuranose.[16,24]

The carboxyl groups of hexuronic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[25] Both the original polysaccharide and the carboxyl-reduced derivatives were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[26] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. The hexuronic acid methyl esters were removed from the hydrolysis products of the methylated native sample by treatment with anion-exchange resin. Analysis by GC-MS[27] gave the results shown in Table II. The results indicated that D-galacturonic acid and D-glucuronic acid residues in the native polysaccharide produced 2,3,6-tri-*O*-methyl D-galactose and 2,3,4,6-tetra-*O*-methyl D-glucose in the methylation products obtained from the carboxyl-reduced derivative, respectively.

AMon-S was deacetylated,[21] and both the original polysaccharide and the *O*-deacetylated product were separately subjected to periodate oxidation followed by reduction with sodium borohydride. The periodate oxidation-reduction product from the original sample contained 7.9% arabinose and 39.0% galactose. All hexuronic acid residues were completely destroyed by this treatment. The deacetylated product gave no arabinose and a similar amount of galactose to that of the original sample after periodate oxidation. These results suggested that the *O*-acetyl groups must be located at some arabinose residues.

Based on the accumulated evidence described above, it can be concluded that AMon-S has the structural features shown in Chart 1.

TABLE II. Methylation Analysis of AMon-S and Its Carboxyl-Reduced Derivative

Methylated sugars (as alditol acetates)	Molar ratio	
	Original	Carboxyl-reduced
2,3,5-Me_3-L-arabinose	9	9
2,3-Me_2-L-arabinose	9	9
2,3,4,6-Me_4-D-glucose	—	1
2,3,4,6-Me_4-D-galactose	1	1
2,4,6-Me_3-D-galactose	5	5
2,3,6-Me_3-D-galactose	1	2
2,3,4-Me_3-D-galactose	1	1
2,4-Me_2-D-galactose	10	10

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of AMon-S

(nine)[a] α-L-Araf 1→
(nine)[a] →5 α-L-Araf 1→
(one)[a] β-D-Galp 1→
(five)[a] →3 β-D-Galp 1→
(one)[a] →4 β-D-Galp 1→
(one)[a] →6 β-D-Galp 1→

(ten)[a] →3 β-D-Galp 1→
↓
6
(one)[a] →4 α-D-GalpA 1→
(one)[a] β-D-GlcpA 1→

a) Number of residues. Araf, arabinofuranose; Galp, galactopyranose; GalpA, galactopyranosyluronic acid; GlcpA, glucopyranosyluronic acid.

Discussion

The polysaccharide fraction having a phagocytosis-enhancing effect has been isolated from the roots of *Astragalus mongholicus* by water extraction.[5] This fraction was mainly composed of two polysaccharides, called astragalans I and II, and it has been reported that the former possessed immunological effects on mice.[7] Astragalan I was composed of arabinose, galactose and glucose. In addition, two polysaccharides, designated AG-1 and AH-1, were reported as immunologically active polysaccharides obtained from the water extract of this crude drug.[8] AG-1 was a glucan having α-1,4- and α-1,6-linkages, and AH-1 was composed of hexuronic acid, glucose, rhamnose and arabinose. However, both the details of activity of these two polysaccharides and the structural features of astragalan I and AH-1 are yet unknown so far.

The novel acidic polysaccharide, AMon-S, obtained from this crude drug evidently has α-1,5-linked L-arabino-β-3,6-branched D-galactan type structural units as its major part. As other examples of the RES-activating polysaccharides from Oriental crude drugs, we have already reported saposhnikovans A and C from the root and rhizome of *Saposhnikovia divaricata*,[12,21] MVS-IIIA, -IVA and -VI from the seed of *Malva verticillata*,[28-30] cinnaman AX from the bark of *Cinnamomum cassia*,[31] ukonans A, B and C from the rhizome of *Curcuma longa*,[32-34] glycyrrhizans UA, UB, UC and GA from the root of *Glycyrrhiza uralensis*[35,36] and the stolon of *G. glabra* var. *glandulifera*,[16] and eucommans A and B from the bark of *Eucommia ulmoides*.[37,38] Among these RES-activating polysaccharides we obtained, the majority possess mainly α-L-arabino-β-3,6-D-galactan type structural features. Saposhnikovan A, MVS-IIIA, -IVA and -VI, ukonans A and B, glycyrrhizans UA and GA belong to this group. In addition, both ukonan C and glycyrrhizan UC are essentially arabino-3,6-galacto-glucan.

Other RES-activating polysaccharides reported to belong to the arabino-3,6-galactan group are sanchinan-A from the root of Panax *notoginseng*,[39] polysaccharide F from *Echinacea purpurea* cell culture,[40] and PS-I, -II and -III from the flower of *Calendula officinalis*.[41]

The component galactose and hexuronic acid residues in the structure of AMon-S are closely similar to those in glycyrrhizan GA.[16] However, AMon-S possesses only two kinds of arabinose units and no rhamnose, so its structural features are simpler than glycyrrhizan GA. The presence of terminal β-D-glucuronic acid residues in these two polysaccharides is characteristic, and AMon-S is the second example having terminal glucuronic acid units among the known RES-activating acidic polysaccharides.

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Microbiologically Modified Chiral Synthon. III. 4,9-Dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin for Formal Total Syntheses of Certain Sesquiterpenoids[1)]

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Microbiological enantioselective transformation of 4,9-dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin, (±)-1 was accomplished with various yeasts, *e.g. Rhodotorula rubra*. With properly selected microorganisms, (+)-4,9*S*-dimethyl-5*S*-hydroxy- (2a) and (−)-4,9*R*-dimethyl-5*S*-hydroxy-3-oxo-$\Delta^{4(10)}$-octalin (3b), (−)-4,9*R*-dimethyl-3*S*-hydroxy- (6b) and (+)-4,9*S*-dimethyl-3*S*-hydroxy-5-oxo-$\Delta^{4(10)}$-octalin (7a) were obtained with high optical purity.

These compounds have now become available for the total syntheses of sesquiterpenoids such as tuberiferine and temisin.

Keywords 3,5-dioxooctalin; 5*S*-hydroxy-3-oxooctalin; 3*S*-hydroxy-5-oxooctalin; microbiological transformation; enantioselective reduction; yeast; *Kloeckera saturnus*; lipase; *Rhodotorula rubra*

In recent years, the most significant development in the field of synthetic organic chemistry of natural products has been made in stereospecific, stereoselective and chemospecific functional group transformations. Most natural products with many chiral centers in their molecules are known to be optically active. Generally, these biological and pharmacological activities are indeed quite different from those of enantiomers. We require optically active products rather than racemates, in the case of total syntheses of natural products and medical supplies with many chiral carbones.

Two spproaches including chemical and biological methods are commonly used in enantioselective syntheses. Biological syntheses using microorganisms and enzymes are useful from the viewpoints of ease and efficiency of reaction and their self-multiplying ability. The most striking differences between enzymes and chemical catalysts lie in their substrate specificities. They catalyze specific reactions for one or just a few structurally related compounds, and distinguish almost absolutely some specific structural feature among various stereoisomers or regioisomers. However, it is possible to screen for various synthetic non-natural products and substrates by the use of microorganisms and/or enzymes. The results and scope of this biological strategy for organic syntheses have been extensively evaluated. The substrate has been used in a higher concentration, from below one percent to several percent. Therefore, the biological reactions performed by microorganisms or catalyzed by enzymes have been regarded as essentially equivalent to those carried out in conventional organic chemistry. The high optical purity must be made possible by microorganisms and enzymes with a high stereoselective ability and chemospecific reactivities, which cannot be obtained by using conventional chemical reagents. The kinetic resolution by biological reactions produced the optically active products as well as the recovered optically active substrate, which can also be useful. Numerous reviews[3)] are available for the hydrolysis using lipases and the reduction or oxidation by microorganisms.

We now intend to study the above chiral syntheses of biologically and pharmacologically active natural and non-natural products. These optically active natural products such as eudesmanolide-type sesquiterpenolids bearing α-methylene-γ-lactones[4-11)] as well as non-natural related compounds may be expected to exhibit antitumor activities.[12)] However, in order to investigate the biological activities of these compounds, it appears important that fairly large samples should be obtained by use of the biological approaches. Instead of many conventional racemic sesquiterpenoids syntheses, we examined the synthesis of the optically active synthons by biological enantioselective reactions such as reduction with microorganisms and hydrolysis with enzymes. The use of these biological methods to prepare chiral alcohols has been widespread and very efficient. There are numerous examples[13)] of biological reduction of acyclic ketones, but only a few reports[14)] of bicyclic ketone reduction.

We communicated earlier the reduction of bicyclic ketones by microorganisms (yeasts).[15,16)] These optically active bicyclic compounds were useful for the syntheses of the optically active natural products.[15b,16b)] The reduction of 4-methoxycarbonyl-9-methyl-3,8-dioxo-$\Delta^{4(10)}$-octalin afforded the optically pure (+)-8*S*-hydroxy-4-methoxycarbonyl-9-methyl-3-oxo-$\Delta^{4(10)}$-octalin by the specialized yeasts.[15)] Both the normal-type ketol and the ent-type diketone were key intermediates for the syntheses of sesquiterpenoids and diterpenoids. The saturated ketone of 4,9-dimethyl-3,7-dioxo-$\Delta^{4(10)}$-octalin was reduced to afford the normal-type and ent-type synthons using selected yeasts.[16)] On the other hand, the use of a specified lipase for enantioselective hydrolysis is an appealingly a convenient method, since the reaction is quick and yields a relatively large amount of product with high optical purity. Then through a single enantioselective hydrolysis, *trans*-7-acetoxy-4,9-dimethyl-3-oxo-$\Delta^{4(10)}$-octalin afforded the corresponding optically pure ketol.[17)] In the hydrolysis of a simpler monocyclic acetate, only one yielded the pure optical product.[17)] In general, it was considered that biological reduction of α,β-unsaturated ketones was difficult because the resonance stability occurs in its carbon–oxygen double bond. However, the α,β-unsaturated ketone of the C(15)-position and the ω-chain of the building block of prostaglandin (PG)[18)] and 4-methoxycarbonyl-3,8-dioxo-$\Delta^{4(10)}$-octalin[15)] were biologically reduced to afford the corresponding allyl alcohol.

We examined the enantioselective hydrolysis of the *trans*-(**4**)[19)] and *cis*-5-acetoxy-4,9-dimethyl-3-oxo-$\Delta^{4(10)}$-

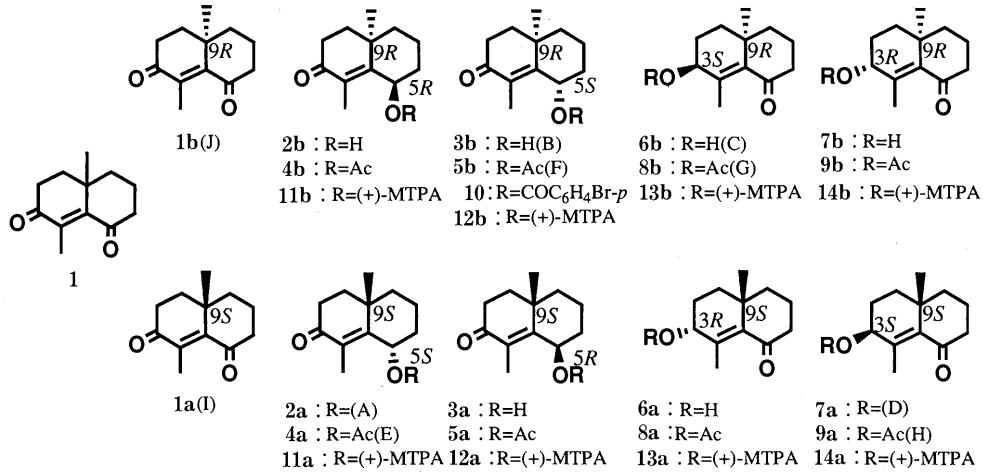

Chart 1

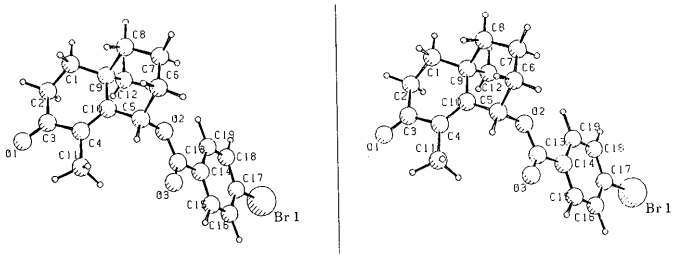

Fig. 1. Stereoscopic Drawing of *p*-Bromobenzoate (**10**) of (−)-4,9*R*-Dimethyl-5*S*-hydroxy-3-oxo-$\Delta^{4(10)}$-octalin (**3b**)

octalin (**5**)[19] with seventeen kinds of commercially available lipases, but recovered solely the starting material. Steric interference occurs between the C(4)-methyl and the C(5)-acetoxyl in (±)-**4**, and a 1,3-diaxial interaction also occurs between the angular methyl and the C(5)-acetoxyl in (±)-**5**. After the screening of various microoganisms, the reduction of (±)-diketone (**1**)[19] with *Rhodotorula rubra* produced the four possible ketols (A+B+C+D), which were sensitive to air, to afford the diketones. The corresponding four ketol acetates (E+F+G+H) were subjected to a silica gel chromatography; they were separated into the less polar fraction yielding E, the more polar fraction containing (F+G+H) and the (+)-diketone (27% yield), $[\alpha]_D$ +84.0°. The acetate (E) and the acetate mixture (F+G+H) were each treated with potassium carbonate to afford the optically active ketol (A) (7.5% yield), $[\alpha]_D$ +126.7°, and a mixture of the ketols (B+C+D), respectively. The ketols were successively separated by repeated chromatography into the ketol (B), $[\alpha]_D$ −43.5°, the ketol (C), $[\alpha]_D$ −156.4° and the ketol (D), $[\alpha]_D$ +88.1° in 24%, 5.4% and 9.4% yield, respectively. The absolute configuration of the main product (B) was determined by X-ray analysis of its *p*-bromobenzoate (**10**) to be 5*S*,9*R* (hence B=**3b**) (Fig. 1). The final atomic coordinates and equivalent isotropic temperature factors are given in Table IV. The structure of the molecule is illustrated in Fig. 1 by the stereoscopic drawing plotted by the PLUTO program.[20] Figure 2 is another view of the molecule showing the planarity of the C(2), C(3), O(1), C(4), C(11), C(10), C(5) and C(9) atoms and the orientation of the C(9)–C(12) bond and the *p*-bromobenzoate group with respect to the molecular plane. The above mentioned

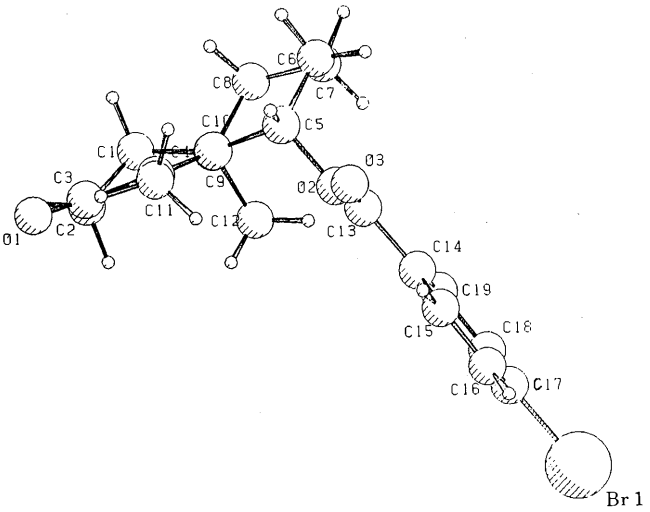

Fig. 2. Another View of the Molecule (**10**)

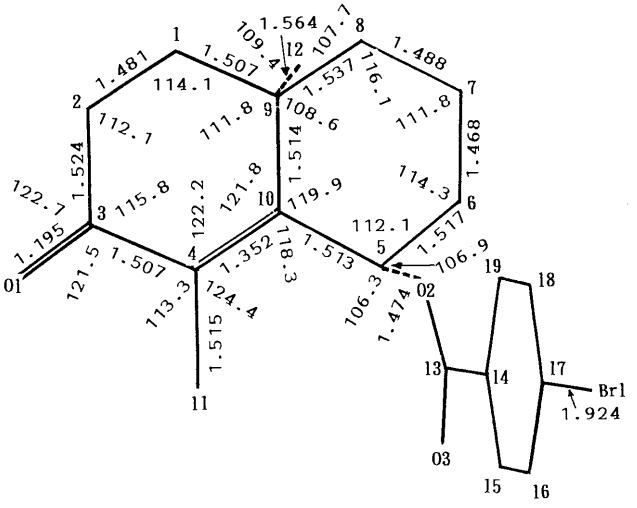

Fig. 3. Bond Lengths (Å) and Valency Angles (°)

molecular plane is planar within the root mean square displacement of the atoms from the least-squares plane of 0.059 Å. As is clear from Fig. 2, C(1), C(5), and C(8) are puckered from the plane so that rings A and B adopt an

envelope and a chair form, respectively. In Tables VI and VII, the bond lengths and valency angles are listed, and Fig. 3 illustrates some important values found the skeleton of the molecule. The values show general agreement with the chemical structure. The ketol (A) was oxidized with Jones reagent to provide the diketone (I), $[\alpha]_D$ +166.8°, which was identical except for the sign of the rotation with 9*R*-diketone (**1b**) (J), $[\alpha]_D$ −168.4° obtained by Jones oxidation of the aforegoing ketol (B). Since the sign of the optical rotation in I was opposite to that in **1b**, the absolute configuration of I and the above recovered diketone was found to be 9*S* (hence I=**1a**), respectively. The stereochemistry of the C(5)-hydroxy group in A was found to be equatorial because the C(5)-hydroxy group of **3b** was just as axial as in **10**. Therefore, the absolute configuration of A was determined to be 5*S*,9*S* (hence A=**2a**). Ketols (C) and (D) were oxidized to provide the corresponding diketones (J), $[\alpha]_D$ −169.0° and (I), $[\alpha]_D$ +153.6°, respectively. The absolute configuration of their angular methyl groups were determined in the same way as for the ketol (A). The stereochemistry of the C(3)-hydrogen atom in C was found to be equatorial; the signal of the proton nuclear magnetic resonance spectrum (^{1}H-NMR) appeared at δ 4.00 as dd with coupling constants of 2.6 and 2.9 Hz. Thus the absolute configuration of C was determined to be 3*S*,9*R* (hence C=**6b**). The stereochemistry of the C(3)-hydrogen atom in D was found to be axial; the ^{1}H-NMR signal due to the C(3)-hydrogen atom appeared at δ 4.07 as dd with *J* values of 5.5 and 8.4 Hz. Therefore, the absolute configuration of D was determined to be 3*S*,9*S* (hence D=**7a**).

In order to determine the opticalpurity of the reduction products, the racemic *trans*-5-hydroxy-3-ketone (**2**)[21] and *cis*-5-hydroxy-3-ketone (**3**)[21] were treated with (+)-α-methoxy-α-trifluoromethylphenylacetic acid chloride [(+)-MTPACl][22] to give the corresponding (+)-MTPA esters (**11**) and (**12**). The two NMR signals due to the C(4)-methyl group at δ 1.75 and δ 1.65 for **11** and those due to each angular methyl group at δ 0.96 and δ 1.10 for **12** appeared in each different field. The racemic *cis*-3,9-hydroxy-5-ketone (**7**)[23] gave the (+)-MTPA ester **14** as usual. The NMR signal due to the angular methyl appeared in distinctly different fields at δ 0.99 and δ 1.03 for **14**. The first ketol 5*S*,9*S*-(**2a**) (A) was converted to the corresponding (+)-MTPA ester (**11**) (δ 1.65), which was found to be 97% ee by taking account of the small signal (δ 1.75) due to its enantiomer (**11b**). The second ketol 5*S*,9*R*-(**3b**) (B) was converted to the corresponding (+)-MTPA ester (**12b**) (δ 0.96), whose optical purity was found to be more than 99% ee. Although the racemic sample of the third ketol (±)-**6** was not obtained, the optical purity of the (+)-MTPA ester of the microbial reduction product 3*S*,9*R*- (**6b**) (C) (δ 0.97 for C(9)-methyl) was found to be 91% ee by taking account of the small signal for the C(9)-methyl due to its enantiomer 3*R*,9*S*-(**13a**). The optical purity of the fourth ketol 3*S*,9*S*-(**7a**) (D) was found to be 96% ee [(+)-MTPA ester (**14b**) (δ 1.03) and its enantiomer (**14a**) (δ 0.99)], as usual. Thus the relation ship between the absolute configuration and the chemical shift was established. The result of the enantioselective reduction of (±)-diketone (**1**), using the specified yeast, is shown in Table I.

In conclusion, with the properly selected microorganism, the enantioselective reduction of 4,9-dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin (±)-(**1**) afforded the four optically pure ketols in both cases. The ent-type, 9*R*-ketol (**3b**) was obtained as the main product in reasonable yield by reduction of *Rhodotorula rubra*, and another normal-type, 9*S*-ketol (**7a**) was acquired in a good yield by reduction of *Kloeckera saturnus*. Since the two racemic sesquiterpenoids, tuberiferine[24] and temsin,[25] have been synthesized starting from (±)-(**1**), the synthesis of the optically active compound, *i.e.* (+)-3*S*-hydroxy-4,9*S*-dimethyl-5-oxo-$\Delta^{4(10)}$-octalin (**7a**), may be regarded as implicating the formal total syntheses of the two optically active sesquiterpenoids starting from the normal-type chiral synthon. The ent-type chiral key intermediate (**3b**) will be available for the syntheses of various optically active natural products, such as (−)-frullanolide[5] and (+)-*cis*-β-cyclocostunolide.[5]

TABLE I. Microbiological Reduction of 4,9-Dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin (**1**)

Yeasts	Products		Recovery
	5-OH	3-OH	
Rhodotorula rubra	**2a** (7.5%, 97% ee)	**6b** (5.4%, 91% ee)	**1a**
	3b (24%, >99% ee)	**7a** (9.4%, 96% ee)	(27%, 50% ee)
Kloekera saturnus	**2a** (1.6%, 94% ee)	**6b** (8.5%, 88% ee)	**1b**
	3b (15%, 98% ee)	**7a** (16.3%, 95% ee)	(34.4%, 8% ee)

Experimental

Melting points were measured with a Kofler micro-melting point apparatus and are uncorrected. Infrared (IR) spectra were measured in a chloroform ($CHCl_3$) solution on a JASCO A-3 spectrophotometer. All the 400 MHz ^{1}H-NMR spectra were determined on a JEOL FX 400 instrument in 5—10% (w/v) solutions of deuterochloroform ($CDCl_3$) with tetramethylsilane (Me_4Si) as an internal reference. Both gas chromatography-mass (GC-MS) and high resolution mass (MS) spectrometries were carried out on a JEOL JMS-D-300 (JMA-200 data analysis system) mass spectrometer. Optical rotations were measured on a JASCO polarimeter in the $CHCl_3$ solution otherwise stated.

Enantioselective Reduction of 4,9-Dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin (1) with *Rhotorula rubra* i) A test tube (200 × 25 i.d. mm) contained 10 ml of a culture medium comprising 5% glucose, 0.1% potassium dihydrogen phosphate (KH_2PO_4), 0.1% ammonium sulfate ($(NH_4)_2SO_4$), 0.05% urea, 0.05% magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.05% calcium chloride ($CaCl_2 \cdot 2H_2O$), 0.1% manganese chloride ($MnCl_2 \cdot 4H_2O$), and 0.1% zinc sulfate ($ZnSO_4 \cdot 7H_2O$), and tap water (pH 6.5). It was incubated with *Rhodotorula rubra*. The yeast was cultured at 30 °C for 3 d with continuous shaking. Then 1 ml of the seed culture was transferred to 1 l each of the same medium. After cultivation for 3 d, two 325 mg of the substrate (**1**) were added and cultivation was continued for an additional 3 d under the same conditions. ii) These reaction mixtures were filtered with the aid of celite, and the filtrate was extracted with ethyl acetate (EtOAc). The EtOAc extract was dried over anhydrous $MgSO_4$. Removal of the solvent gave an oily product (711.7 mg). Pyridine (10 ml) was added to a mixture of the reaction product, acetic anhydride (Ac_2O) (1.83 g) and dimethylaminopyridine (DMAP) (10 mg). Then the reaction mixture was stirred for 17.5 h at room temperature. After the addition of water (H_2O), the reaction mixture was extracted with ether. The ether extract was washed with saturated aqueous sodium chloride (NaCl), dried over anhydrous $MgSO_4$ and concentrated to give an oily product (E+F+G+H), which was chromatographed on silica gel (70 g) to give the less polar fraction (E, 61 mg, 7.5%), the more polar fraction (F+G+H, 320 mg) and diketone (176 mg, 27%) from hexane–EtOAc (1 : 1) elute. Diketone (**1a**): mp 35—36 °C. MS Calcd for $C_{12}H_{16}O_2$ (M^+, *m/z*): 192.115, Found: 192.114. $[\alpha]_D^{27}$ +84° (*c*=2.5, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1694, 1685. ^{1}H-NMR ($CDCl_3$) δ: 1.148 (3H, s, 9-CH_3), 1.741 (3H, s, 4-CH_3), iii) Metanol (MeOH) (0.3 ml) was added to a mixture of acetate (E) (40 mg) and potassium carbonate (K_2CO_3) (5 mg). Then the reaction mixture was stirred for 5.5 h at room temperature. After the addition of H_2O, the

reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give compound (A), (30 mg, 92% yield). A: MS Calcd for $C_{12}H_{18}O_2$ (M^+, m/z): 194.130, Found: 194.130. $[\alpha]_D^{27}$ +126.67° (c=3.0, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3400, 3330, 1660, 1646, 1616, 1593. ^{1}H-NMR ($CDCl_3$) δ: 1.217 (3H, s, 9-CH_3), 1.933 (3H, s, 4-CH_3), 4.729 (1H, dd, J=4 Hz, 5-H). iv) MeOH (0.5 ml) was added to the acetate mixture (F+G+H) (320 mg) and K_2CO_3 (10 mg). The reaction mixture was stirred for 6 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product, which was subjected to preparative thin layer chromatography (TLC) (silica gel, 20×20 cm; solvent, hexane–EtOAc (1:1)] and was chromatographed on silica gel (hexane–EtOAc (4:1)] to provide the ketol (B) (157 mg, 24.2% yield), (C) (36 mg, 5.4% yield) and (D) (62 mg, 9.4% yield). B: mp 67—70 °C. MS Calcd for $C_{12}H_{18}O_2$ (M^+, m/z): 194.131, Found: 194.131. $[\alpha]_D^{27}$ −43.5° (c=1.7, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3425, 1673, 1661, 1608. ^{1}H-NMR ($CDCl_3$) δ: 1.406 (3H, s, 9-CH_3), 1.850 (3H, s, 4-CH_3), 4.946 (1H, t, J=3 Hz, 5-H). C: mp 92—99 °C. MS Calcd for $C_{12}H_{18}O_2$ (M^+, m/z): 194.131, Found: 194.126. $[\alpha]_D^{27}$ −156.4° (c=2.2, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3420, 1678, 1632. ^{1}H-NMR ($CDCl_3$) δ: 0.973 (3H, s, 9-CH_3), 1.862 (3H, s, 4-CH_3), 3.989 (1H, br s, 3-H). D: mp 100—102 °C. MS Calcd for $C_{12}H_{18}O_2$ (M^+, m/z): 194.131, Found: 194.127. $[\alpha]_D^{27}$ +88.1° (c=2.6, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3425, 1673, 1618. ^{1}H-NMR ($CDCl_3$) δ: 1.039 (3H, s, 9-CH_3), 1.814 (3H, d, J=1 Hz, 4-CH_3), 4.063 (1H, dd, J=6, 14 Hz, 3-H).

Preparation of 5*S*,9*S*-Benzoate (10) from 5*S*-Hydroxy-4,9*S*-dimethyl-3-oxo-$\Delta^{4(10)}$-octalin (2) Pyridine (0.5 ml) was added to a mixture of the ketol (B) (22 mg), *p*-bromobenzoyl chloride (50 mg) and DAMP (10 mg); then the reaction mixture was stirred for 2 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with ether. The ether extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give the oily product, which was subjected to preparative TLC (silica gel, 20×20 cm; solvent, hexane–EtOAc (1:1)) to provide *p*-bromobenzoate (**10**) (26 mg, 61% yield) which was crystallized from hexane, $[\alpha]_D^{27}$ +77.7° (c=1.09, $CHCl_3$). MS Calcd for $C_{19}H_{21}O_3Br$, (M^+, m/z): 378.063, Found: 378.063. IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1716, 1678, 1616, 1594.

Collection of the Crystal Data and Intensity Data The crystals of *p*-bromobenzoate (**10**) were grown in a hexane solution as colorless plates. An X-ray specimen of the approximate dimensions 0.15×0.30×0.04 mm was chosen and set on a Philips PW1100 diffractometer. The lattice constants and intensity data were obtained by measurement using graphite monochromated CuK_α radiation. The crystal data is summarized in Table II. Intensities were measured using a $\theta-2\theta$ scan method with the scan speed θ=4°/min. Scans were repeated twice if the total counts in the first scan were less than 3000. The background was measured at each end of half the scan time. A total of 2239 reflections were measured in the 2θ range of 6° through 120° of which 180 were symmetry eqivalent reflections and 788 were Friedel reflections. The R_F[26] values were 0.047 for the former and 0.077 for the latter kind of reflections.

The number of independent observed structure factors was 1267 taking the averages of equivalent reflections, which corresponds to about 80% of the number theoretically possible.

Crystal Structure Analysis The crystal structure was solved by the heavy

Table II. Crystal Data of the Molecule of **10**

(−)-5-*p*-Bromebenzoyl-4,9*R*-dimethyl-5*S*-hydroxy-3-oxo-$\Delta^{4(10)}$-octalin: $C_{19}H_{21}O_3Br$, M.W. 377.3
Crystal system : orthorhombic
Space group : $P2_12_12_1$, Z=4
Lattice constants: a=9.953 (5), b=20.408 (11), c=8.979 (5) Å, U=1824 Å^3, D_x=1.374 g cm^{-3}, μ for CuK_α=31.7 cm^{-1}

Table III. Determination of the Absolute Configuration by Comparison of the Calculated and Observed Values of $\{F(hkl)/F(\bar{h}\bar{k}\bar{l})\}^2$

h	k	l	$\{F(hkl)/F(\bar{h}\bar{k}\bar{l})\}^2$ Cal.	Obs.	(S.T.D.)	h	k	l	$\{F(hkl)/F(\bar{h}\bar{k}\bar{l})\}^2$ Cal.	Obs.	(S.T.D.)
1	1	1	0.85	0.77	(0.04)	2	8	3	0.82	0.53	(0.28)
4	10	1	0.84	0.65	(0.30)	7	9	3	1.23	1.29	(0.10)
1	2	2	0.84	0.71	(0.12)	1	15	3	0.85	0.73	(0.07)
8	2	2	0.85	0.82	(0.08)	5	15	3	0.82	0.77	(0.13)
3	6	2	1.35	1.46	(0.19)	2	1	4	1.23	1.24	(0.09)
1	8	2	1.30	1.76	(0.22)	8	1	4	0.84	0.66	(0.17)
4	9	2	1.19	1.61	(0.17)	1	6	4	1.55	1.54	(0.19)
7	12	2	1.15	2.49	(0.17)	4	3	5	0.85	0.56	(0.12)
6	14	2	1.19	3.69	(0.32)	7	4	5	1.35	1.79	(0.14)
3	17	2	1.22	1.78	(0.23)	1	6	6	1.17	1.85	(0.12)
4	5	3	0.81	0.69	(0.11)	4	8	8	0.85	0.46	(0.42)
1	8	3	1.22	1.79	(0.16)						

Table IV. Fractional Atomic Coordinates (x, y and z) and Equivalent Isotropic Temperature Factors (B_{eq} in Å)

No.	Atom	x 10^5	y 10^5	z 10^5	B_{eq} Å^2
1	Br1	76529 (14)	−24574 (9)	−26070 (21)	6.65 (0.03)
No.	**Atom**	**x 10^4**	**y 10^4**	**z 10^4**	**B_{eq} Å^2**
2	C1	−103 (18)	759 (8)	−88 (23)	7.4 (0.3)
3	C2	58 (19)	1044 (8)	−1594 (26)	8.8 (0.4)
4	C3	−525 (17)	603 (8)	−2801 (22)	7.6 (0.3)
5	C4	−434 (12)	−123 (7)	−2511 (20)	5.3 (0.2)
6	C5	2 (14)	−1104 (6)	−990 (16)	4.5 (0.2)
7	C6	−456 (18)	−1309 (8)	551 (19)	6.4 (0.3)
8	C7	137 (20)	−926 (8)	1769 (18)	7.3 (0.3)
9	C8	−60 (18)	−209 (7)	1549 (18)	6.5 (0.3)
10	C9	446 (15)	73 (7)	64 (18)	5.4 (0.3)
11	C10	−25 (14)	−368 (7)	−1187 (16)	4.7 (0.2)
12	C11	−840 (21)	−537 (11)	−3837 (21)	9.1 (0.4)
13	C12	2015 (15)	101 (9)	138 (22)	7.1 (0.3)
14	C13	1646 (14)	−1774 (7)	−2108 (17)	5.2 (0.2)
15	C14	3138 (12)	−1949 (6)	−2187 (16)	4.3 (0.2)
16	C15	3571 (14)	−2333 (7)	−3317 (16)	5.2 (0.2)
17	C16	4901 (13)	−2482 (7)	−3472 (14)	4.9 (0.2)
18	C17	5779 (12)	−2234 (6)	−2462 (18)	4.8 (0.2)
19	C18	5414 (14)	−1834 (6)	−1301 (16)	4.6 (0.2)
20	C19	4054 (14)	−1674 (7)	−1138 (17)	4.9 (0.2)
21	O1	−978 (17)	813 (7)	−3938 (18)	12.1 (0.4)
22	O2	1420 (9)	−1303 (5)	−1133 (11)	5.2 (0.2)
23	O3	830 (10)	−2047 (6)	−2868 (15)	9.1 (0.3)
No.	**Atom**	**x 10^3**	**y 10^3**	**z 10^3**	**B_{eq} Å^2**
24	HC1	−116 (15)	74 (7)	14 (17)	8.0 (4.0)
25	H′C1	33 (18)	105 (8)	78 (20)	12.0 (6.0)
26	HC2	115 (15)	110 (7)	−182 (16)	9.0 (4.0)
27	H′C2	−37 (12)	152 (5)	−166 (13)	5.0 (3.0)
28	HC5	−63 (10)	−135 (5)	−178 (11)	4.0 (3.0)
29	HC6	−24 (11)	−180 (5)	61 (13)	5.0 (3.0)
30	H′C6	−151 (14)	−126 (7)	51 (16)	8.0 (4.0)
31	HC7	115 (12)	−106 (5)	178 (13)	5.0 (3.0)
32	H′C7	−30 (13)	−109 (6)	280 (17)	8.0 (4.0)
33	HC8	−109 (16)	−13 (8)	126 (19)	11.0 (5.0)
34	H′C8	9 (16)	4 (7)	255 (23)	9.0 (4.0)
35	HC11	−2 (16)	−84 (7)	−425 (18)	11.0 (5.0)
36	H′C11	−160 (15)	−87 (7)	−357 (17)	9.0 (4.0)
37	H″C11	−121 (13)	−22 (6)	−473 (14)	6.0 (3.0)
38	HC12	242 (16)	−35 (6)	28 (16)	9.0 (4.0)
39	H′C12	237 (14)	43 (6)	113 (14)	7.0 (3.0)
40	H″C12	233 (18)	29 (7)	−75 (18)	11.0 (5.0)
41	HC15	286 (10)	−252 (6)	−405 (11)	5.0 (3.0)
42	HC16	523 (11)	−283 (5)	−436 (12)	4.0 (3.0)
43	HC18	615 (11)	−161 (5)	−56 (13)	4.0 (3.0)
44	HC19	372 (12)	−133 (5)	−19 (14)	6.0 (3.0)

Equivalent positions

x	y	z
1/2−x	−y	1/2+z
1/2+x	1/2−y	−z
−x	1/2+y	1/2−z

TABLE V. Anisotropic Thermal Parameter for Each Atom

No.	Atom	U11	U22	U33	U12	U13	U23
		U(ij)'s are multiplied by 10^4					
1	Br1	595 (8)	981 (11)	951 (11)	97 (10)	96 (11)	−175 (13)
		U(ij)'s are multiplied by 10^3					
2	C1	92 (13)	66 (10)	124 (16)	7 (10)	−6 (13)	−10 (12)
3	C2	95 (13)	58 (10)	182 (21)	5 (10)	17 (16)	30 (13)
4	C3	83 (11)	98 (12)	106 (15)	4 (10)	18 (13)	46 (12)
5	C4	52 (8)	73 (9)	77 (10)	−3 (7)	0 (13)	14 (10)
6	C5	62 (9)	48 (8)	59 (9)	10 (7)	−3 (8)	−1 (7)
7	C6	95 (12)	70 (10)	77 (11)	−2 (10)	7 (11)	10 (10)
8	C7	127 (15)	83 (11)	70 (11)	18 (12)	3 (12)	3 (10)
9	C8	108 (14)	60 (9)	82 (11)	16 (10)	−5 (12)	−16 (9)
10	C9	57 (9)	59 (9)	89 (12)	5 (8)	2 (9)	3 (9)
11	C10	50 (8)	65 (9)	65 (9)	0 (7)	−7 (8)	3 (8)
12	C11	119 (17)	148 (18)	79 (13)	11 (16)	−1 (14)	41 (14)
13	C12	55 (10)	95 (12)	118 (15)	−1 (10)	−15 (11)	−23 (12)
14	C13	69 (9)	61 (8)	66 (10)	5 (8)	−2 (9)	−1 (8)
15	C14	55 (7)	50 (7)	58 (9)	13 (6)	−4 (8)	−12 (7)
16	C15	64 (9)	66 (10)	66 (9)	10 (8)	−2 (8)	−3 (8)
17	C16	70 (8)	65 (9)	50 (7)	28 (9)	8 (7)	−7 (8)
18	C17	48 (7)	63 (8)	71 (9)	9 (6)	8 (9)	37 (9)
19	C18	68 (9)	47 (8)	59 (9)	3 (7)	−2 (8)	−24 (7)
20	C19	53 (8)	64 (9)	68 (10)	7 (7)	−6 (8)	−17 (8)
21	O1	173 (15)	141 (12)	148 (13)	−1 (12)	−30 (13)	79 (11)
22	O2	53 (6)	73 (6)	73 (7)	14 (5)	0 (6)	−17 (6)
23	O3	62 (6)	143 (10)	142 (12)	8 (7)	−16 (8)	−93 (10)
		U(ij)'s are multiplied by 10^2					
24	HC1	11 (6)					
25	H'C1	15 (7)					
26	HC2	12 (5)					
27	H'C2	6 (4)					
28	HC5	5 (3)					
29	HC6	6 (4)					
30	H'C6	10 (5)					
31	HC7	7 (4)					
32	H'C7	10 (5)					
33	HC8	14 (7)					
34	H'C8	12 (5)					
35	HC11	14 (6)					
36	H'C11	12 (6)					
37	H''C11	8 (4)					
38	HC12	12 (6)					
39	H'C12	8 (4)					
40	H''C12	14 (7)					
41	HC15	6 (3)					
42	HC16	5 (3)					
43	HC18	6 (4)					
44	HC19	7 (4)					

Temperature factor T is the form of

$$T=\exp\{-2\pi^2(U11h^2a^{*2}+U22k^2b^{*2}+U33l^2c^{*2}+2U12hka^*b^*+2U13hla^*c^*+2U23klb^*c^*)\}.$$

atom method and refined by the method of least-squares with block diagonal matrix approximations. The final R value was 0.075 including 21 hydrogen atoms, found on the difference electron-density map, which were assumed to have isotropic temperature factors. The absolute configuration was determined by taking into account the anomalous dispersion effects of bromine, oxygen and carbon atoms for CuK_α radiation. Pairs of Friedel reflections were selected for cases, where the calculated and observed values of $|F(hkl)|/|F(\bar{h}\bar{k}\bar{l})|$ differed more than 5% from unity and where the difference between the observed $|F(hkl)|$ and $|F(\bar{h}\bar{k}\bar{l})|$ is greater than 2σ of $F(hkl)$. Comparison of the calculated and observed values of $\{|F(hkl)|/|F(\bar{h}\bar{k}\bar{l})|\}^2$ for these pairs listed in Table III indicated

TABLE VI. Bond Lengths (Å) (a) and Bond Lengths Including Hydrogen Atoms (Å) (b)

(a) Atom 1 Atom 2	Length (S.T.D.)	(b) Atom 1 Atom 2	Length (S.T.D.)
Br1–C17	1.924 (12)	C1 –HC1	1.07 (15)
C1 –C2	1.481 (30)	C1 –H'C1	1.07 (17)
C1 –C9	1.507 (21)	C2 –HC2	1.11 (15)
C2 –C3	1.524 (27)	C2 –H'C2	1.06 (11)
C3 –C4	1.507 (22)	C5 –HC5	1.07 (10)
C3 –O1	1.195 (25)	C6 –HC6	1.03 (11)
C4 –C10	1.352 (22)	C6 –H'C6	1.05 (15)
C4 –C11	1.515 (26)	C7 –HC7	1.05 (12)
C5 –C6	1.517 (22)	C7 –H'C7	1.08 (14)
C5 –C10	1.513 (19)	C8 –HC8	1.07 (16)
C5 –O2	1.474 (17)	C8 –H'C8	1.04 (19)
C6 –C7	1.468 (24)	C11–HC11	1.09 (16)
C7 –C8	1.488 (22)	C11–H'C11	1.05 (15)
C8 –C9	1.537 (23)	C11–H''C11	1.09 (13)
C9 –C10	1.514 (21)	C12–HC12	1.02 (14)
C9 –C12	1.564 (21)	C12–H'C12	1.17 (12)
C13–C14	1.529 (19)	C12–H''C12	0.93 (16)
C13–O2	1.319 (17)	C15–HC15	1.04 (10)
C13–O3	1.198 (19)	C16–HC16	1.11 (10)
C14–C15	1.354 (19)	C18–HC18	1.09 (11)
C14–C19	1.426 (20)	C19–HC19	1.15 (12)
C15–C16	1.365 (19)		
C16–C17	1.357 (19)		
C17–C18	1.373 (20)		
C18–C19	1.400 (20)		

TABLE VII. Valency Angles in Degrees (Not Including Hydrogen Atoms)

Atom 1 Atom 2 Atom 3	Angle (S.T.D.)	Atom 1 Atom 2 Atom 3	Angle (S.T.D.)
C2 –C1 –C9	114.1 (15)	C8 –C9 –C12	107.7 (13)
C3 –C2 –C1	112.1 (16)	C14–C13–O2	111.5 (11)
C4 –C3 –C2	115.8 (15)	C14–C13–O3	121.6 (13)
C4 –C3 –O1	121.5 (16)	O2 –C13–O3	126.9 (14)
C2 –C3 –O1	122.7 (17)	C15–C14–C13	118.7 (12)
C10–C4 –C3	122.2 (14)	C15–C14–C19	121.3 (12)
C10–C4 –C11	124.4 (14)	C13–C14–C19	119.9 (12)
C3 –C4 –C11	113.3 (14)	C16–C15–C14	120.9 (13)
C6 –C5 –C10	112.1 (12)	C17–C16–C15	118.3 (13)
C6 –C5 –O2	106.9 (11)	C18–C17–Br1	116.7 (10)
C10–C5 –O2	106.3 (11)	C18–C17–C16	123.9 (13)
C7 –C6 –C5	114.3 (14)	Br1 –C17–C16	119.4 (10)
C8 –C7 –C6	111.8 (15)	C19–C18–C17	118.3 (13)
C9 –C8 –C7	116.1 (14)	C4 –C10–C5	118.3 (13)
C10–C9 –C1	111.8 (13)	C4 –C10–C9	121.8 (13)
C10–C9 –C8	108.6 (12)	C5 –C10–C9	119.9 (12)
C10–C9 –C12	111.2 (12)	C5 –O2 –C13	115.0 (10)
C1 –C9 –C8	107.9 (13)	C14–C19–C18	117.2 (13)
C1 –C9 –C12	109.4 (13)		

the absolute configuration as shown in Fig. 1.

Conversion of Ketols (A—D) into 4,9*S* or 4,9*R*-Dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin (I or J) (General Procedure) Jones reagent (2—5 drops) was added to a stirred solution of A—D in acetone (1—2.5 ml); this mixture was cooled in an ice-salt bath for 30 min. After the addition of isopropyl alcohol and sodium hydrogen carbonate ($NaHCO_3$), the reaction mixture was filtered and concentrated to an oil; this was subjected to TLC (silica gel, 20 × 20 cm; solvent, hexane–EtOAc (1 : 1)) to provide the diketone.

The diketone (I) (25 mg, 89% yield from A (28 mg)). I: $[\alpha]_D^{25}$ +166.8° (c=3.5, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1694, 1685, 1610.

The diketone (J) [(−)-**1b**] [25 mg, 70% yield from B (35 mg)] **1b**: $[\alpha]_D^{26}$ −168.4° (c=2.5, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1694, 1685, 1610.

The diketone (J) (11 mg, 85% yield from C (13 mg)]. J: $[\alpha]_D^{32}$ −169° (c=1.1, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1694, 1685, 1610.

The diketone (I) (14 mg, 78% yield from D (18 mg)]. I: $[\alpha]_D^{32}$ +154°

($c=1.4$, $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ (cm^{-1}): 1694, 1685, 1610.

Preparation of (+)-MTPA Ester (11 and 12) from Racemic 5,9-*trans*- and *cis*-Ketol (2 and 3) (General Procedure) Pyridine (0.3 ml) was added to a mixture of 5-hydroxy (**2** and **3**), (+)-MTPACl (20 mg) and DMAP (10 mg). Then the reaction mixture was stirred for 16 h at room temperature. After the addition of H_2O, the reaction mixture was extracted withs ether. The ether extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give (+)-MTPA ester which was subjected to preparative TLC (silica gel, 20 × 20 cm; solvent, hexane–EtOAc (2 : 1)) to provide the MTPA ester. The MTPA ester (**11**) (22 mg, 95% yield from **2** (11 mg)]. **11**: ^{1}H-NMR ($CDCl_3$) δ: 1.225 (3H, s, 9-CH_3), 1.649, 1.746 (each 3H, s, 4-CH_3), 3.509 (1H, d, $J=1.2$ Hz, –OCH_3), 3.533 (3H, d, $J=1.2$ Hz, –OCH_3), 6.108 (1H, m, 5-H). The MTPA ester (**12**) (14 mg, 48% yield from **3** (14 mg)). **12**: ^{1}H-NMR ($CDCl_3$) δ: 0.956, 1.105 (each 3H, s, 9-CH_3), 1.866, 1.879 (each 3H, s, 4-CH_3), 3.489 (1H, d, $J=1.2$ Hz, –OCH_3), 3.556 (3H, d, $J=0.98$ Hz, –OCH_3), 6.294 (each 1H, br s, 5-H).

Preparation of (+)-MTPA Ester (14) from Racemic 3,9-*cis*-Ketol (7) i) Pyridine (2 ml) was added to mixture of racemic ketols (**2**) and (**3**) (1.077 g), Ac_2O (1.698 g) and DMAP (10 mg). Then the reaction mixture was stirred for 6.5 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give crude acetate (1.195 g, 92% yield). ii) Abs. MeOH (10 ml) was added to a mixture of the above acetate (793 mg), sodium borohydride ($NaBH_4$) (63.5 mg) and cerium chloride ($CeCl_3 \cdot 7H_2O$) (1.252 g). Then the reaction mixture was stirred for 6.5 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with ether. The ether extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product which was subjected to chromatography (silica gel (20 g), solvent, hexane–EtOAc (19 : 1)) to provide a mixture of 3,9-ketol (593 mg, 74% yield). 3,9-Ketol: ^{1}H-NMR ($CDCl_3$) δ: 1.166, 1.219 (each 3H, s, 9-CH_3), 1.778 (3H, s, 4-CH_3), 1.847 (3H, d, $J=0.97$ Hz, 4-CH_3), 2.021, 2.059 (each 3H, s, –OCH_3), 5.139, 5.605, 5.881, 6.014 (each 1H, m, 5-H). iii) *N*,*N*-dimethylformamide (DMF) (3 ml) was added to a mixture of 3,9-ketol (533 mg) and imidazol (374 mg). To the mixture was added *tert*-butyldimethylsilyl chloride (TBDMSCl) (374 mg) in an ice bath, then the reaction mixture was stirred at room temperature for 1 h and 40 min. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product which was subjected to chromatography [silica gel (20 g), solvent, and hexane–EtOAc (99 : 1)] to provide the coresponding sillilated ketone (574 mg, 72% yield). iv) MeOH (1 ml) was added to a mixture of the sillilated ketone (472 mg) and K_2CO_3 (10 mg). Then the reaction mixture was stirred for 4.5 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product, which was subjected to chromatography [silica gel (80 g), solvent, hexane–EtOAc (19 : 1)] to provide the sillilated ketol (220 mg, 53% yield) and the starting material (212 mg, 45% yield). v) MeOH (0.5 ml) was added to a mixture of the sillilated ketol (50 mg) and *p*-toluenesulfonic acid (*p*-TsOH) (10 mg). Then the reaction mixture was stirred for 30 min at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product which was subjected to preparative TLC [solvent, hexane–EtOAc (2 : 1)] to provide 3,9-*cis*-ketol (**6**) (14 mg, 45% yield). vi) The MTPA ester (**13**) was obtained in the same manner as described for the MTPA esters (**11** or **12**). The MTPA ester (**13**) [3 mg, 48% yield from 3,9-*cis*-ketol (**6**) (3 mg)]. **13**: ^{1}H-NMR ($CDCl_3$) δ: 0.990, 1.035 (each 3H, s, 9-CH_3), 1.477 (each 3H, d, $J=0.92$ Hz, 4-CH_3), 1.669 (3H, s, 4-CH_3), 3.548, 3.565 (each 1H, d, $J=1.2$ Hz, –OCH_3), 5.501 (each 1H, m, 3H).

Preparation of (+)-MTPA Esters (11a, 12b, 13b or 14a) from Ketols (A—D) (General Procedure) The MTPA esters (**11a** or **12b** or **13b** or **14a**) were obtained in the same manner as described for the MTPA esters (**11** or **12**). The MTPA ester (**11a**) [16 mg, 95% yield from ketol (A) (6 mg)]. **11a**: ^{1}H-NMR ($CDCl_3$) δ: 1.225 (3H, s, 9-CH_3), 1.649, 1.745 (each 3H, s, 4-CH_3), 3.509, 3.533 (each 3H, d, $J=1.2$ Hz, –OCH_3), 6.094 (1H, m, 5-H). The optical purity of **11a** (hence A) was found to be 97% ee. The MTPA ester (**12b**) [16 mg, 72% yield from ketol (B) (10 mg)]. **12b**: ^{1}H-NMR ($CDCl_3$) δ: 0.956 (3H, s, 9-CH_3), 1.866 (3H, s, 4-CH_3), 3.556 (3H, d, $J=0.98$ Hz, –OCH_3), 6.294 (1H, br s, 5-H). The optical purity of **12b** (hence B) was found to be more than 99% ee. The MTPA ester (**13b**) [4 mg, 95% yield from ketol (C) (3 mg)]. **13b**: ^{1}H-NMR ($CDCl_3$) δ: 0.990, 0.969 (each 3H, s, 9-CH_3), 1.649, 1.745 (each 3H, s, 4-CH_3), 3.509, 3.533 (each 3H, d, $J=1.2$ Hz, –OCH_3), 6.094 (1H, m, 3-H). The optical purity of **13b** (hence C) was found to be 91% ee. The MTPA ester (**14a**) (16 mg, 85% yield from ketol (D) (9 mg)]. **14a**: ^{1}H-NMR ($CDCl_3$) δ: 0.990, 1.035 (each 3H, s, 9-CH_3), 1.476, 1.669 (each 3H, s, 4-CH_3), 3.548, 3.565 (each 3H, d, $J=1.2$ Hz, –OCH_3), 5.517 (1H, m, 3-H). The optical purity of **14a** (hence D) was found to be 97% ee.

Enantioselective Reduction of 4,9-Dimethyl-3,5-dioxo-$\Delta^{4(10)}$-octalin (1) with *Kloekera saturnus* i) After cultivation, the reaction mixture was worked up in the same way as in the case of *Rhodotorula rubra* to afford the oily product (617.5 mg). After the acetylation, an oily product (**4a**+**5b**+**8b**+**9a**) was obtained. The mixture product was chromatographed on silica gel (75 g) to give the less polar fraction **4a** (13 mg, 1.6%), the more polar fraction (**5b**+**8b**+**9a**, 320 mg) and (–)-diketone (**1**) (224 mg, 34.4%) from hexane–EtOAc (1 : 1) elute. (–)-Diketone (**1**): mp 58—59 °C. $[\alpha]_D^{25}$ –13.6° ($c=1.7$, $CHCl_3$). ii) MeOH (0.5 ml) was added to a mixture of acetate **4a** (13 mg) and K_2CO_3 (5 mg). The reaction mixture was stirred for 24.5 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give crude **2a** (7 mg, 65% yield). **2a**: $[\alpha]_D^{32}$ +75.0° ($c=1.2$, $CHCl_3$). iii) MeOH (0.5 ml) was added to the acetates (**5b**+**8b**+**9a**) (320 mg) and K_2CO_3 (10 mg). Then the reaction mixture was stirred for 16 h at room temperature. After the addition of H_2O, the reaction mixture was extracted with EtOAc. The EtOAc extract was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$ and concentrated to give an oily product, which was subjected to preparative TLC [silicagel, 20 × 20 cm; solvent, hexane–EtOAc (1 : 1)] and was chromatographed on silica gel [hexane–EtOAc (4 : 1)] to provide the ketol **3b** (97 mg, 15% yield), **6b** (55.4 mg, 8.5% yield) and **7a** (106.1 mg, 16.3% yield). **3b**: mp 73—76 °C. $[\alpha]_D^{32}$ –41.1° ($c=1.8$, $CHCl_3$). **6b**: mp 85—88 °C. $[\alpha]_D^{32}$ –156.7° ($c=1.3$, $CHCl_3$). **7a**: mp 94—97 °C. $[\alpha]_D^{32}$ +88.1° ($c=1.2$, $CHCl_3$).

Preparation of (+)-MTPA Esters (11a, 12b, 13b or 14a) from Ketols (2a, 3b, 6b, or 7a) The MTPA esters (**11a** or **12b** or **13b** or **14a**) were obtained in the same manner as described for the MTPA esters (**11** or **12**). The MTPA ester **11a** (7 mg, 48% yield from ketol **2a** (7 mg)]. **11a**: ^{1}H-NMR ($CDCl_3$) δ: 1.225 (3H, s, 9-CH_3), 1.649, 1.745 (each 3H, s, 4-CH_3), 3.509, 3.533 (each 3H, d, $J=1.2$ Hz, –OCH_3), 6.094 (1H, m, 5-H). The optical purity of **11a** (hence **2a**) was found to be 94% ee. The MTPA ester **12b** [12 mg, 57% yield from ketol **3b** (10 mg)]. **12b**: ^{1}H-NMR ($CDCl_3$) δ: 0.956 (3H, s, 9-CH_3), 1.866 (3H, s, 4-CH_3), 3.556 (3H, d, $J=0.98$ Hz, –OCH_3), 6.294 (1H, br s, 5-H). The optical purity of **12b** (hence **3b**) was found to be 98% ee. The MTPA ester **13b** (16 mg, 76% from yield ketol **6b** (10 mg)). **13b**: ^{1}H-NMR ($CDCl_3$) δ: 0.990. 0.969 (each 3H, s, 9-CH_3), 1.649, 1.745 (each 3H, s, 4-CH_3), 3.509, 3.533 (each 3H, d, $J=1.2$ Hz, –OCH_3), 6.094 (1H, m, 3-H). The optical purity of **13b** (hence **6b**) was found to be 91% ee. The MTPA ester **14a** [14 mg, 67% yield from ketol **7a** (10 mg)]. **14a**: ^{1}H-NMR ($CDCl_3$) δ: 0.990, 1.035 (each 3H, s, 9-CH_3), 1.476, 1.669 (each 3H, s, 4-CH_3), 3.548, 3.565 (each 3H, d, $J=1.2$ Hz, –OCH_3), 5.517 (1H, m, 3-H). The optical purity of **14a** (hence **7a**) was found to be 95% ee.

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The Effects of Coexisting Lipids on the Action of *Bacillus thuringiensis* Phosphatidylinositol-Specific Phospholipase C toward Liposomal Substrate

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The hydrolytic activity of phosphatidylinositol (PI)-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was studied in detail toward mixed liposomes consisting of PI and one of other phospholipids and cholesterol. Among PI-liposomes, small unilamellar vesicles (SUV) were the most sensitive to PI-PLC; the enzymatic hydrolysis of PI in SUV was not less than 10-fold that in large unilamellar vesicles (LUV) or in multilamellar vesicles (MLV). Thus, in a survey of the effects of coexisting lipids on PI-PLC activity, PI-SUV was used. Phosphatidylcholine (PC) was stimulative for the enzyme activity toward PI-SUV at any molar ratio of PC to PI. Also, the effects of the addition of sphingomyelin (SM), phosphatidylethanolamine (PE) and cholesterol on the enzymatic hydrolysis of PI were studied in detail on the basis of concentration of total lipids or PI.

Keywords phosphatidylinositol (PI); liposomal PI; PI-specific phospholipase C (PI-PLC); *Bacillus thuringiensis*

Bacterial phosphatidylinositol (PI)-specific phospholipases C (EC 3.1.4.10, PI-PLCs) which catalyze the hydrolysis of PI to yield diacylglycerol and *myo*-inositol 1,2-cyclic phosphate are now being used in order to investigate glycosylphosphatidylinositol (GPI)-anchored proteins, since most of these membrane proteins bearing several enzymatic and antigenic activities have been shown to be solubilized by the action of these PI-PLCs.[2–4] Especially PI-PLC from *Bacillus thuringiensis* has become widely used in the study on GPI-anchored proteins. Although recent studies on complementary deoxyribonucleic acid (cDNA) cloning have disclosed an amino acid sequence of this enzyme,[5,6] the mechanism of action of this enzyme toward membranous PI was not sufficiently clarified. In spite of biological significance in the pathway of signal transduction, PI is contained as one of the minor components in the biomembranes, corresponding to only 5—10% of the total phospholipids. Therefore, the action of PI-PLC on membranous PI must be significantly influenced by the interaction of PI with other lipid constituents in these membranes. Recently, we have found that the action of PI-PLC toward the PI-detergent mixed micelles is significantly influenced by coexisting lipids.[7] We further investigated the effects of coexisting membranous lipids on the action of *B. thuringiensis* PI-PLC in the liposome level, since phospholipid liposomes are usually regarded as the models proximate to biological membranes.

Materials and Methods

Materials PI was purified from an autolysate of baker's yeast, by batch operation with DEAE-cellulose or silicic acid column chromatography.[8,9] Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from egg yolk, and sphingomyelin (SM), from bovine brain by silicic acid column chromatography.[9] According to the method described in the previous report,[10] PI-PLC was purified from the culture broth of *B. thuringiensis* IAM 12077 in a homogenous state, as indicated by polyacrylamide gel electrophoresis. All other chemicals used were of analytical reagent grade, unless otherwise stated.

Enzyme Assay The activity of PI-PLC was determined by the method reported previously.[11]

Preparation of Liposomes Large unilamellar vesicles (LUV) containing PI were prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos.[12] According to the method of Bangham *et al.*,[13] using a voltex mixer, multilamellar vesicles (MLV) were prepared by mixing well the organic solvent-free PI or the mixture of PI/one of other lipids (PC, PE, SM and cholesterol), with an appropriate amount of the borate buffer prepared by a 10-fold dilution of borate-buffered saline (pH 8.0) which was made isotonic by the addition of 0.75% NaCl to 0.05 M borate. According to the method of Huang[14] and Kinoshita *et al.*,[15] small unilamellar vesicles (SUV) were prepared by sonicating the multilamellar vesicles for 30 min with Tomy UR-200 Ultrasonifier at 0—5 °C and then by centrifuging at 25000 × *g* for 30 min. The resulting supernatants were used as small unilamellar vesicles.

The Action of PI-PLC on Liposomes The reaction mixtures containing 200 munits or 100 munits of PI-PLC, 0.02% bovine serum albumin and PI-LUV, PI-MLV, PI-SUV or PI/one of other lipids containing SUV in 40 mM borate buffer, pH 7.5, were incubated at 37 °C for 15 min. The reaction was terminated by the addition of $CHCl_3$–CH_3OH–HCl (66 : 33 : 1) and the mixtures were centrifuged at 3000 × *g* for 5 min. Then, aliquots of the upper layer were withdrawn from the mixtures and subjected to phosphate analysis according to the method of Eibl and Lands,[16] after oxidation of organic phosphate by the method of Fiske and Subbarow.[17]

Results

The Enzymatic Activity of Hydrolysis of PI Vesicles by *B. thuringiensis* PI-PLC Prior to examination of the effects of other lipids on PI-PLC activity toward liposomal PI, we prepared three types of liposomes; MLV, LUV and SUV, which were different from one another in their size and physical state. Table I shows the hydrolytic activity of PI-PLC toward these liposomes at 2 mM PI in the absence of other lipids. Among these liposomes, SUV was the most sensitive to enzymatic hydrolysis by PI-PLC; the extent of PI hydrolysis in SUV was not less than 10-fold that in LUV or MLV. Thus, we used PI-SUV as the substrate vesicles in all the following experiments, although the activity of PI-PLC toward PI-SUV was about one-eighth that toward PI/sodium deoxycholate (SDC) mixed micelle in the routine system of enzyme assay. In the following experimental runs, the molar concentration of PI was not always kept constant. Instead, the molar ratios of other lipids *versus* PI in SUV vesicles were varied from 1/3 to 3.

The Effects of Choline-Containing Phospholipids, PC and SM, on PI-PLC Activity toward PI-SUV Figure 1 shows

TABLE I. The Enzymatic Hydrolysis of Phosphatidylinositol Vesicles by PI-PLC

Liposomes	Activity (nmol/min)
Multilamellar vesicles (MLV)	0.20
Large unilamellar vesicles (LUV)	0.14
Small unilamellar vesicles (SUV)	2.5

PI-PLC (200 munits) was added to the assay mixture.

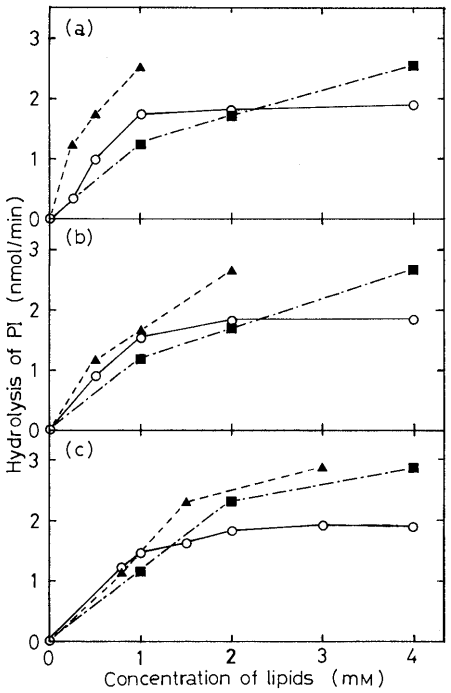

Fig. 1. Effect of PC Addition on PI-PLC Activity toward PI-Liposomes

The molar ratios of PC to PI were 3, 1 and 1/3 in (a), (b) and (c), respectively. (○) PI-liposome without other lipids; (■, ▲) PC/PI mixed liposomes. PI-PLC activity was plotted against total lipid concentration (■) or against PI concentration (▲) in PC/PI mixed liposomes.

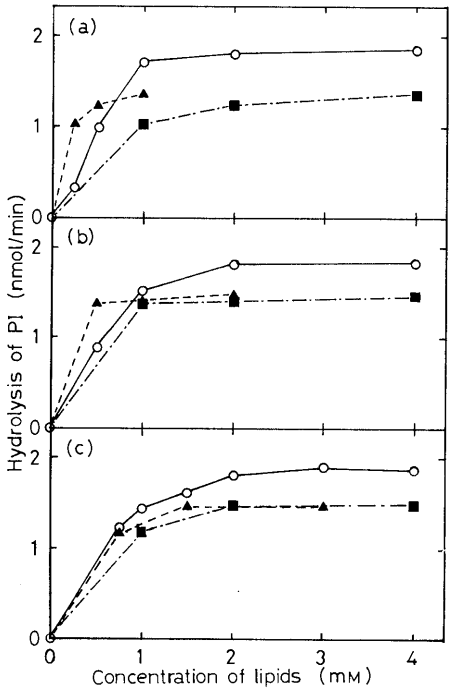

Fig. 2. Effect of SM Addition on PI-PLC Activity toward PI-Liposomes

The molar ratios of SM to PI were 3, 1 and 1/3 in (a), (b) and (c), respectively. (○) PI-liposome without other lipids; (■, ▲) SM/PI mixed liposomes. PI-PLC activity was plotted against total lipid concentration (■) or against PI concentration (▲) in SM/PI mixed liposomes.

the effects of coexisting PC on PI-PLC activity toward PI-SUV. The effects of PC were analyzed on the basis of concentration of PI alone or total lipids (PC+PI). Throughout Figs. 1a, 1b and 1c, the added PC stimulated the enzyme activity toward liposomal PI, on the basis of

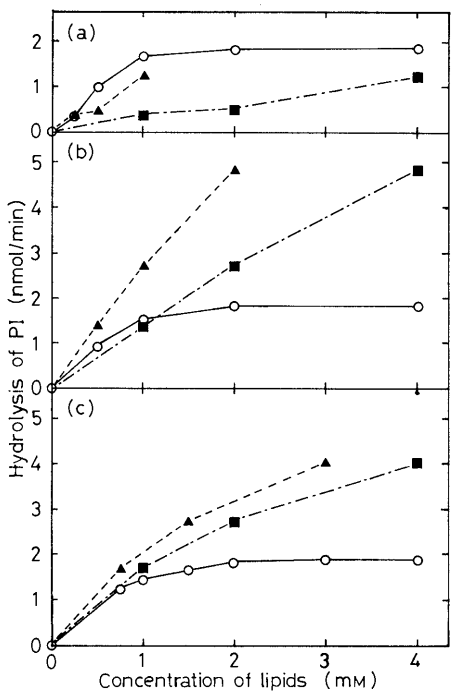

Fig. 3. Effect of PE Addition on PI-PLC Activity toward PI-liposomes

The molar ratios of PE to PI were 3, 1 and 1/3 in (a), (b) and (c), respectively. (○) Pi-liposome without other lipids; (■, ▲) PE/PI mixed liposomes. PI-PLC activity was plotted against total lipid concentration (■) or against PI concentration (▲) in PE/PI mixed liposomes.

PI concentration in the reaction mixture. On the basis of total lipid concentration (PC+PI), however, the enzyme activity toward 1 mM mixed liposome was lower than that toward 1 mM pure PI liposome. At the total lipid concentration of 2 mM, the enzyme activity toward mixed liposomes in which the molar ratios of PC/PI were 3 and 1, was comparable to that toward PI-liposome (Figs. 1a and 1b), whereas the activity toward mixed liposome at the molar ratio of 1/3 was higher than that toward PI-liposome (Fig. 1c).

A ceramide phospholipid, SM, has a phosphorylcholine residue as the polar head common to PC. At a PI concentration lower than 1 mM, the enzyme activity toward liposomal PI was stimulated in the presence of SM, on the basis of the PI concentration (Figs. 2a and 2b). On the basis of total lipid concentration (SM+PI), however, the enzyme activity toward mixed SM/PI liposome became apparently lower than that toward pure PI-liposome (Figs. 2a—c), in contrast with the results obtained by the addition of PC to PI-liposome. Especially at the molar ratio of SM/PI=3, the extent of PI hydrolysis corresponded to approx. 2/3 that in PI-liposome (Fig. 2a).

The Effect of PE on PI-PLC Activity toward PI-SUV

The addition of PE to the substrate liposomes caused a fairly unique effect on PI-PLC activity. At the molar ratio of PE/PI=3, the hydrolytic activity of PI-PLC toward liposomal PI was significantly inhibited in the presence of PE at any concentration, on the basis of both total lipid (PE+PI) and PI concentrations (Fig. 3a). On the other hand, the hydrolysis of liposomal PI was enhanced in the presence of equimolar PE on the basis of PI concentration and of total lipid concentration more than 1 mM (Fig. 3b). Especially, a 2.5-fold stimulation of the enzyme activity was observed on the addition of 2 mM PE to 2 mM PI. This

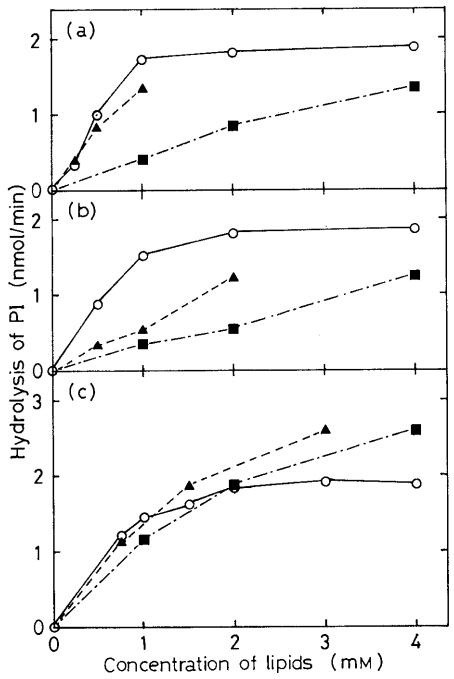

Fig. 4. Effect of Cholesterol Addition on PI-PLC Activity toward PI-Liposomes

The molar ratios of cholesterol to PI were 3, 1 and 1/3 in (a), (b) and (c), respectively. (○) PI-liposome without other lipids; (■, ▲) cholesterol/PI mixed liposomes. PI-PLC activity was plotted against total lipid concentration (■) or against PI concentration (▲) in cholesterol/PI mixed liposomes.

stimulative effect of PE exceeded that of PC or SM. Also, at the molar ratio of 1/3, the enzymatic hydrolysis of liposomal PI was stimulated by coexisting PE on the basis of both total lipid and PI concentrations (Fig. 3c).

The Effect of Cholesterol on PI-PLC Activity toward PI-SUV Among the lipids examined in this study, cholesterol is the sole lipid which has no phosphorous in its molecule. Thus, the effect of the addition of cholesterol on PI-PLC activity was slightly complicated as compared with that of the phospholipid addition. At the molar ratio of cholesterol/PI = 3, the hydrolytic activity of PI-PLC toward liposomal PI seemed to be inhibited by coexisting cholesterol on the basis of total lipid concentration (cholesterol + PI), but the activity in the presence of cholesterol was actually comparable to that in the absence of cholesterol on the basis of the PI concentration (Fig. 4a). In contrast, the hydrolytic activity of PI-PLC was significantly inhibited by the addition of equimolar cholesterol to PI-liposomes, on the basis of both total lipid and PI concentrations (Fig. 4b). At the molar of ratio of 1/3, however, the enzymatic hydrolysis of liposomal PI was slightly stimulated at more than 2 mM on the basis of total lipid concentration and at more than 1 mM on the basis of PI concentration (Fig. 4c).

Discussion

From the present study, the PI-PLC activity toward liposomal PI was proved to be significantly influenced by the size of the substrate vesicles. As expected from the study of detergent-dispersed PI-micelles,[11] PI in SUV was much more sensitive to the hydrolysis by PI-PLC than that in larger vesicles such as MLV and LUV. Because of the large numbers and surface area, finely dispersed small vesicles such as SUV must be more accessible to PI-PLC than the other two vesicles.

The enhancement of PI-hydrolysis by the addition of PE at the molar ratio of PE/PI $\leqq$ 1 might also be explained by the size of the substrate vesicles. The molecular shape of PE is generally accepted to be cone-shaped[18] whereas PE forms a micelle having a hexagonal (H_{II}) structure. Thus, coexisting PE might act on the PI-liposomes so as to produce a number of small vesicles, promoting the hydrolysis of PI. On the other hand, when excess PE was added to the PI-liposome (PE/PI = 3), the outer surface of liposome was significantly perturbed by the cone-shaped PE molecule. Probably, this disorder must exert an inhibitory effect on PI-PLC activity toward liposomal PI.

The enhancement of PI-PLC activity by added PC might be caused by the dilution of the negative surface charge of PI-liposomes. The molecular form of PC has a cylindrical shape[18] which might not interfere with the physical state of liposomal PI except for the dilution of the negative surface charge. Since PI is an anionic phospholipid, the surface of PI-liposome is negatively charged in the reaction medium at pH 7.5. In addition, PI-PLC of *B. thuringiensis* has an acidic pI, 4.9.[10] Therefore, the molecule of PI-PLC is also negatively charged at pH 7.5. Probably, the repulsive force between negatively charged PI-liposome and PI-PLC must be weakened in the presence of PC, one of the neutral phospholipids. As a result of the dilution of the negative charge, the enzymatic hydrolysis of liposomal PI must have been significantly stimulated by coexisting PC. In another study on the activity of PI-PLC toward micellar PI,[7] we examined the effects of the same four lipids (PC, SM, PE and cholesterol) on PI-PLC activity toward detergent (SDC or Triton X-100)/PI mixed micelles and got similar results. PC added to these mixed micelles also enhanced the enzymatic hydrolysis of PI owing to the promotion of PI dispersion.

The influence of the addition of PC on PI-PLC activity at the molar ratio PC/PI = 3, was essentially similar to that at the molar ratio = 1 (Figs. 1a and 1b). Thus the physical state of liposomal PI in the presence of excess PC was not so far from that in the presence of equimolar PC, as regards the accessibility to PI-PLC. Two models for the enzyme mechanism of PI-PLC might be considered: In the first model, the enzyme attached to the substrate moves on the surface of the PI-liposome, as shown in the scooting model for the mechanism of phospholipase A_2.[19] In another model, the enzyme mainly moves from one vesicle to another, being not firmly attached to the liposomal surface. Since the stimulation of enzyme activity toward PI-liposome was not significantly altered in the presence of excess PC as compared with that in the presence of equimolar PC, the possibility for the first model must be excluded. Also, the fact that PI-PLC activity toward smaller vesicles such as PI-SUV was much higher than the activity toward PI-MLV or PI-LUV supports the second model.

SM is a choline-containing, neutral phospholipid, having a cylindrically shaped molecule similar to PC.[18] However, the effect of SM addition on PI-PLC activity toward liposomal PI was different from that of PC addition. On the basis of total lipid concentration, the enzyme activity toward PI-liposome was significantly inhibited by coexisting SM. Probably, a sphingosine base existing in the hydro-

phobic moiety of SM is more or less responsible for the inhibition, since PC, another choline-containing phospholipid, enhanced the enzymatic hydrolysis of PI. Also, an amide bond and a hydroxyl group existing in the neighboring carbon atoms in the sphingosine moiety of SM might have some contribution to this inhibition.

Since cholesterol has a molecular shape similar to PE, the addition of cholesterol might cause significant perturbation of the surface of PI-liposome. Also, cholesterol has been shown to reduce fluidity of the phospholipid bilayer containing unsaturated fatty acids.[20] These two characteristics inevitably reflect the complicated effect of the addition of cholesterol on PI-PLC activity toward PI-liposomes. Thus, at a higher molar ratio of cholesterol this lipid exhibited an inhibitory effect on PI-PLC activity on the basis of total lipid concentration because of the reduction of membrane fluidity in PI-liposomes. At a lower molar ratio, cholesterol seems to probably exert some membrane-perturbing effect favorable for PI-PLC action toward PI-liposomes.

Biomembranes have been shown to be negatively charged in general, and PI is one of the minor, anionic components in biomembranes. Thus, in the present study, the mixed liposomes having the molar ratios of other lipids/PI = 3 are the nearest models for the biomembranes. Therefore, the data obtained by the addition of other lipids in such an excess will contribute to studies on the action of PI-PLC toward biomembranes, especially in studies on GPI anchors. On the other hand, the date obtained by the addition of a smaller amount of these lipids will serve as a reference for the enzyme assay using commercial PI preparations which contain a significant amount of contaminating lipids examined for their effect in the present study.

In the eucaryotic cells, PI plays a crucial role in the anchoring of membrane proteins as well as in signal transduction, although its contents in the membranes are generally low. In studies on the functions of PI, bacterial PI-PLCs have been used as valuable tools. Thus, the sensitivity of GPI anchors to bacterial PI-PLCs is critical in studies on these protein anchors, since liberation by PI-PLCs is one of the potent hallmarks for the presence of GPI anchors in membrane proteins. In the outer surface of eucaryotic cells, the protein-linked, glycosylated PIs are surrounded by a vast amount of PC which has been known to be localized mainly in the outer leaflet of the membrane-lipid bilayer.[21] In the present study, we showed that the presence of PC always favors PI-PLC-catalyzed hydrolysis of PI on the surface of liposomes which are generally used as membrane models. Therefore, this is probably the case with GPI anchors surrounded by PC and other phospholipids in the plasma membrane. Also, the presence of some phospholipids such as PE in a moderate quantity may serve to segregate protein-linked PIs into small areas or vesicles, allowing them to be more accessible to PI-PLC. Thus, the results in the present study will provide a fundamental model for the survey on the mode of action of PI-PLC toward GPI anchors of the membrane proteins.

The physical forms and the characteristics of PI-containing liposomes must also be significantly influenced by fatty acid composition. The effect of the molecular species of PI on PI-PLC activity is now being investigated for PIs which contain different fatty acids in their diacylglycerol moieties (to be reported elsewhere).

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Steroidogenic Activity of Synthetic Hybrid Molecules Composed of Human Chorionic Gonadotropin and Either the A or B Chain of Lectin Ricin or Horseradish Peroxidase

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Human chorionic gonadotropin (hCG) is a glycoprotein consisting of noncovalently bound α- and β-subunits which shows hormonal activity (stimulatory effect on testosterone production) toward rat Leydig cells. To modify the hormonal activity of hCG, hybrid molecules composed of hCG and other glycoproteins, either the A or B chain of lectin ricin (hCG-A and hCG-B) through disulfide bridges and horseradish peroxidase (hCG-HRP) through Schiff's base, were synthesized. Hormonal activity and the effect of these hybrids on [^{125}I]hCG binding to rat Leydig cells were compared to those of native hCG. Modification of hCG resulted in a significant decrease in hormonal activity for hCG-HRP but not for hCG-A to approximately 1/100 and 1/10 that of native hCG, respectively. On the other hand, hCG-B unexpectedly showed hormonal activity similar to that of native hCG. These hybrids inhibited the binding of ^{125}I-labeled hCG to rat Leydig cells with potencies of 1/10, 1/100 and 1/500 that of hCG for hCG-B, hCG-A and hCG-HRP, respectively. These results indicate that the B chain of ricin, the active component hCG-B, participated in stimulating testosterone production according to its own nature. Data which indicate that hybrids consisting of hCG subunits and the B chain of ricin (α-B and β-B) stimulated testosterone significantly more than hybrids consisting of hCG subunits and A chain (α-A and β-A) support the above finding. When asialofetuin was added to the assay system as an inhibitor of binding of the B chain to galactose residues attached to the cell surface membrane, the inhibition of hCG-B with the binding of [^{125}I]hCG to rat Leydig cells was depressed and testosterone production provoked by hCG-B was repressed to the level induced by hCG-A. Furthermore, a hybrid consisting of hCG and enzymatically deglycosylated B chain stimulated testosterone production, but its activity was significantly weaker than that of hCG-B with the level approaching that of hCG-HRP. These results suggested that three kinds of binding of hCG-B to rat Leydig cells participate in expression of hormonal activity of hCG-B as follows: (1) binding of the hCG moiety to its receptor, (2) binding of the B chain moiety, as lectin, to terminal galactose residues attached to cell-surface membrane, and (3) binding of a lectin-like protein(s) in the cell-surface membrane to oligosaccharides of the B chain moiety.

Keywords human chorionic gonadotropin; hCG; hybrid protein; hCG-ricin subunit hybrid; lectin; ricin; steroidogenic activity

Human chorionic gonadotropin (hCG) is a member of the glycoprotein hormone family which includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). These four hormones are composed of a common α-subunit combined noncovalently with a hormone-specific β-subunit that confers receptor binding specificity.[1] Both the α- and β-subunits of hCG contain two Asn-linked complex type oligosaccharide chains with microheterogeneity,[2,3] while the β-subunit contains four additional Ser-linked oligosaccharides.[4] It is accepted that the Asn-linked oligosaccharide moieties of glycoprotein hormones play an important role in signal transduction.[5–13] Thus, enzymatically or chemically deglycosylated hCG (dghCG) as well as other glycoprotein hormones become drastically decreased in their post-receptor biological activity, *i.e.*, the stimulating effects of adenosine 3′,5′-cyclic monophosphate (cAMP) and testosterone production by testicular Leydig cells. The β-subunits of hCG and LH are closely related in the amino acid sequence, except that the β-subunit of LH is deficient in the carboxyl terminal peptide present on the β-subunit of hCG in which four Ser-linked oligosaccharide chains are attached, and it is accepted that these two hormones bind to the same receptor (LH/CG receptor) and elicit identical biological responses.[1] The LH/CG receptor is present on testicular Leydig cells and various ovarian cells, and recently the structures of LH/CG receptors from rat luteal cells[14] and porcine testicular cells[15] were deduced from their complementary deoxyribonucleic acid (DNA). It was revealed that seven transmembrane domains exist in the receptor which show homology with the corresponding regions of other G protein-coupled receptors. However, the exact mechanism of how hCG interacts with cell membranes containing LH/CG receptor resulting in the activation of hormone-bound receptor still remains to be clarified.

In our previous study, we reported on a synthetic hybrid (hCG-A) prepared with hCG and the A chain of ricin.[16] Ricin is a toxic glycoprotein from castor beans consisting of subunits linked by the single disulfide bridge, A and B chains. Predominantly, one xylomannose type and two oligomannose type Asn-linked oligosaccharide chains are connected to the A and B chains, respectively.[17] The B chain has galactose recognition sites by which ricin binds to cell-surface receptors, glycoproteins and/or glycolipids containing terminal galactose residues. The receptor bound ricin is internalized and then the free A chain, which is released through cleavage of the intersubunit disulfide bridge, inactivates the 28S ribosomal ribonucleic acid (rRNA) of eukaryotic ribosomes enzymatically.[18] Consequently, cellular protein synthesis is inhibited and cell death occurs. In the beginning of the earlier study, we prepared hCG-A as a hormonotoxin, and it was revealed that hCG-A acts not only as the hormonotoxin but also as a hormone to rat Leydig cells. It was supposed that hCG sufficiently maintained rather than lost its hormonal activity after modification with the A chain of ricin. The participation of lectin-like sites on the cell-surface in the binding of the hormone was previously assumed and a soybean lectin-like region is found in the sequence of

cloned hCG receptor.[14] Thus, the hypothesis which arose postulated that the hormonal activity of hCG-A was not lost completely in spite of modification, possibly because of participation of the oligosaccharide chain of A chain introduced to hCG. This hypothesis led us to compare the hormonal activity of hCG-A with hCG-B, since the B chain has two Asn-linked oligosaccharide chains in addition to galactose recognition sites resembling lectin activity.

In this study, we have synthesized hybrids composed of hCG disulfide-bridged to other glycoproteins, either the A or B chain of ricin (hCG-A and hCG-B). hCG cross-linked with horseradish peroxidase (hCG-HRP) was also prepared as a control hybrid through Shiff's base, since oligosaccharide moieties of HRP were broken by periodate oxidation, thus hCG-HRP resembles hCG modified with a simple protein. Furthermore, hCG subunit hybrids connected with ricin subunits (α-A, β-A, α-B and β-B) were also prepared. Data obtained in this study showed that the B chain moiety of hCG-B participates uniquely in the hormonal activity.

Experimental

Materials Wistar rats (250—300 g) were obtained from Ohtsubo Animal Co. (Nagasaki). Reagents were obtained from the following sources: *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) from Pharmacia; HRP and collagenase (230 U/mg) from Wako Chemical Co.; 3-isobutyl-1-methyl-xanthine, bovine serum albumin, α-mannosidase (α-mannoside mannohydrolase from jack beans, EC 3.2.1.24) and asialofetuin from Sigma; endoglycosidase H (*endo-β-N*-acetyl-glucosaminidase H from *Streptomyces griceus*, EC 3.2.1.96) from Seikagaku Kogyo Co.; and Na[^{125}I] (3700 MBq/ml) and Testosterone RIA kit from Amersham; and cAMP RIA kit from Yamasa Co. All other chemicals were of reagent grade and obtained from commercial sources.

Proteins hCG and its subunits were purified from crude hCG (3300 IU/mg; Organon Inc.) by the published methods.[19,20] dghCG was prepared by incubation with trifluoromethane sulfonic acid for 2 h at 0 °C according to the method of Sojar and Bahl.[21]

Ricin was purified from Chinese castor beans according to the method of Hara *et al.*[22] Ricin A and B chains were prepared as described by Fulton *et al.*[23] with some modifications. The extinction coefficients ($E^{1\%}_{280\,nm}$) for ricin, ricin A chain and B chain were found to be 14.4, 7.0 and 14.9, respectively.[22,23] Deglycosylated B chain (dgB) was prepared according to the method of Foxwell *et al.*[24] as follows. Ricin B chain at 2 mg/ml in 0.1 M sodium citrate (pH 5.5) containing 0.2 M galactose, 0.25% 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride was incubated with 10 mU/ml endoglycosidase H for 24 h, after which 0.5 U/ml of α-mannosidase (pretreated with 1 mM phenylmethylsulfonyl-fluoride) was added and the incubation was continued for a further 16 h at 30 °C. After treatment, the mixture was filtered at a flow rate of 0.5 ml/min through a column (1.5 ml) of coenzyme A-Sepharose equilibrated with 10 mM phosphate-buffered saline (PBS) (pH 7.0), containing 0.1 M galactose. Eluted fractions were collected and dialyzed against 10 mM PBS, then filtered through a 1.5 ml Sepharose 4B column equilibrated with 10 mM PBS. Bound material was eluted from the column with PBS containing 0.2 M galactose. This preparation was used as dgB.

Preparation of Hybrids Hybrids of hCG or hCG subunits cross-linked to ricin subunits by disulfide bridges were synthesized using SPDP according to the published method described for synthesis of hCG-A.[16] Typically, purified hCG or hCG subunits dissolved in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl were incubated to derivatize with a threefold molar equivalent of SPDP for 30 min at room temperature. Under this condition, the average number of 3-(2-pyridyldithio)propionyl groups introduced into hCG and its subunits per mole was determined to be 2 by measurement of the absorbance at 343 nm due to free pyridine-2-thione generated on reduction of the product with dithiothreitol.[25] After excess reagents were removed by gel-filtration on Sephadex G-25 equilibrated with 0.05 M sodium phosphate buffer (pH 7.6) containing 0.15 M NaCl, the derivatized hCG or hCG subunits were incubated with ricin A chain, B chain or dgB, which were completely reduced, at a molar ratio of 1 : 1.5. After incubation for 16 h at 25 °C, mixtures containing the resultant hybrids were concentrated with Centricon 10 (Amicon Co.).

A hybrid of hCG-HRP was prepared by the periodate oxidation method[26] as follows. HRP (1 mg/0.5 ml) was incubated with 0.1 ml of 0.1 M $NaIO_4$ for 20 min at room temperature. The HRP–aldehyde solution was then dialyzed against 1 mM acetate buffer (pH 4.5) overnight. hCG (1 mg) was added to the HRP–aldehyde solution, the pH of which was adjusted to 9.5 by addition of 20 μl of 0.2 M sodium carbonate buffer, and incubated for 2 h at room temperature. To stabilize the cross-link of hCG-HRP, reduction with sodium borohydride was performed for 2 h at 4 °C. hCG-HRP solution was dialyzed against PBS, then concentrated with Centricon 10.

Each hybrid was purified with a gel permeation chromatography column of YMC-pack 200 Diol (8 × 500 mm, Yamamura Chem. Co.) equilibrated with PBS at a flow rate of 0.5 ml/min. Molar ratios of hybrid components in each fraction were analyzed by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method described previously.[16] SDS-PAGE was performed according to the method of Laemmli[27] under non-reducing (8% gel) and reducing (13% gel) conditions for detection of cross-linked hybrids and their components, respectively. The proteins separated in the gel were stained using a silver staining kit (Wako Chem.) or immunostained by subunit specific antibodies and goat anti-rabbit immunoglobulin G (IgG) ^{125}I-labeled whole antibody (330 kBq/μg, NEN) after blotting the proteins[28] in the gel onto a Durapore (Millipore) filter. Finally, hybrid fractions with molar ratios of hCG or its subunits and other proteins from 1 : 1 to 1 : 2 were pooled, concentrated and stored at −80 °C until use.

Assaying of Testosterone and cAMP Leydig cells from rat testes were suspended in medium 199 containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) and 0.1% bovine serum albumin (pH 7.3). After preincubation at 37 °C for 60 min in a 5% CO_2 incubator, the Leydig cells (8 × 10^5/0.2 ml) were incubated with either the hCG, hCG hybrids or subunit hybrids in the presence of 0.125 mM 3-isobutyl-1-methyl-xanthine at 34 °C for 2 h under 5% CO_2–95% O_2 with shaking at 100 cycles/min. For assaying testosterone, an aliquot of the incubation mixture was transferred onto ice and then centrifuged at 3000 rpm for 5 min. The supernatant was saved for radioimmunoassay of testosterone. To determine the amount of cAMP produced, an aliquot of the incubation mixture was mixed with 1/10 volume of 1 N HCl, followed by boiling for 3 min. After centrifugation, the supernatant was stored at −80 °C until use for radioimmunoassay of cAMP.

Binding Experiments hCG was iodinated by the chloramine T method of Greenwood *et al.*[29] using 18.5 MBq Na[^{125}I] and 40 μg of hCG in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl. Preparation with specific radioactivity of 1.1 MBq/μg was obtained. In the competitive assay, Leydig cells (1.6 × 10^6 cells/0.1 ml) were incubated with hybrids or hCG in the presence of [^{125}I]hCG (4 × 10^4 cpm) at 37 °C for 2 h. The incubation mixture was transferred onto ice and cells were collected onto a GF/C filter (Whatman) presoaked with 4% bovine serum albumin, washed with PBS containing 1% bovine serum albumin (3 ml × 4) and ethanol (3 ml × 2), after which the radioactivity bound to the cells was measured. The mean radioactivity bound to the cells in the absence of inhibitors was 8000 cpm, which was approximately 30% of maximal binding.

Protein Assay Protein quantities were determined by the method of Bradford[30] with bovine thyroglobulin as the standard.

Results

Hormonal Activity of hCG-Hybrids We have synthesized three hCG-hybrids composed of hCG cross-linked with glycoproteins, either the A or B chain of ricin through disulfide bridges (hCG-A and hCG-B) and with HRP through Schiff's base (hCG-HRP). In addition, dghCG was prepared. Hybrids were purified with gel permeation chromatography and their composition was analyzed by SDS-PAGE followed by both protein staining and immunostaining. Each hybrid having a hCG/glycoprotein ratio of 1 : 1 to 2 was purified. Analysis by SDS-PAGE also showed that purified hCG-hybrids contained no unconjugated free hCG (data not shown). To determine the hormonal activity of purified hCG-hybrids, stimulation of

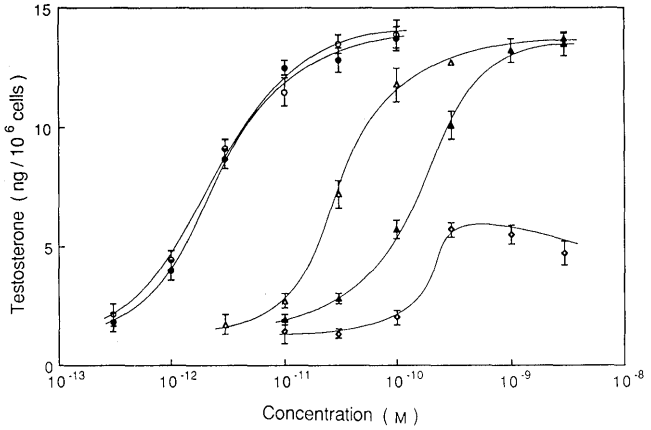

Fig. 1. Stimulation of Testosterone Production in Rat Leydig Cells by hCG, dghCG and hCG-Hybrids

Leydig cells were incubated with the indicated doses of hCG (○), dghCG (◇), hCG-A (△), hCG-B (●), and hCG-HRP (▲) for 2 h and then testosterone production was determined as described under Experimental. Each point is the mean ± S.E. of four separate experiments.

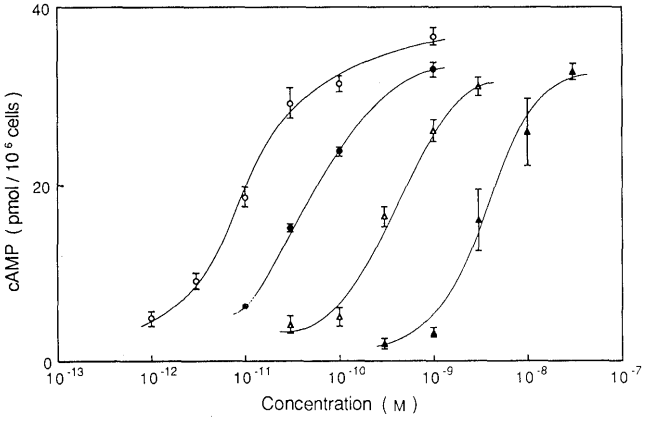

Fig. 2. Stimulation of cAMP Accumulation in Rat Leydig Cells by hCG and hCG-Hybrids

Leydig cells were incubated with the indicated doses of hCG (○), hCG-A (△), hCG-B (●), and hCG-HRP (▲) for 2 h and then cAMP was determined as described under Experimental. Each point is the mean ± S.E. of four separate experiments.

TABLE I. Hormonal Activity of hCG and hCG-Hybrids

	ED_{50} (M)	
	Testosterone	cAMP
hCG	$2.3 \pm 0.3 \times 10^{-12}$	$9.6 \pm 0.4 \times 10^{-12}$
hCG-A	$2.8 \pm 0.5 \times 10^{-11}$	$3.9 \pm 0.4 \times 10^{-10}$
hCG-B	$2.3 \pm 0.3 \times 10^{-12}$	$4.8 \pm 0.2 \times 10^{-11}$
hCG-HRP	$1.7 \pm 0.4 \times 10^{-10}$	$3.8 \pm 1.1 \times 10^{-9}$

The concentrations required for 50% stimulation of testosterone and cAMP production for hCG and hCG-hybrids are calculated from the data of Figs. 1 and 2.

testosterone and cAMP production by rat Leydig cells was examined. As shown in Figs. 1 and 2, hCG as well as each hybrid stimulated testosterone and cAMP production dose-dependently with maximal response, but, consistent with reports of several investigators,[6-9] dghCG did not. The concentrations required for 50% stimulation (ED_{50}) of testosterone and cAMP production are summarized in Table I. It was shown that, due to the modification of hCG, the relative potencies of testosterone production

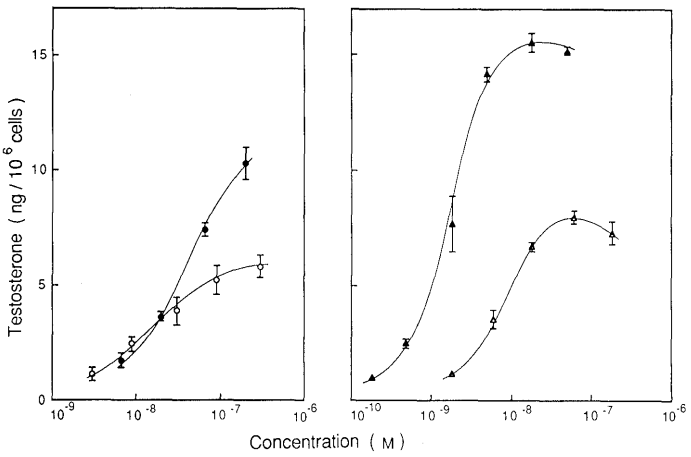

Fig. 3. Effect of Subunit-Hybrids on Testosterone Production in Rat Leydig Cells

Leydig cells were incubated with the indicated doses of subunit-hybrids composed of either α-subunit (left panel) or β-subunit (right panel) with either A (open symbols) or B chain (closed symbols) for 2 h and then testosterone production was determined as described under Experimental. Each point is the mean ± S.E. of four separate experiments.

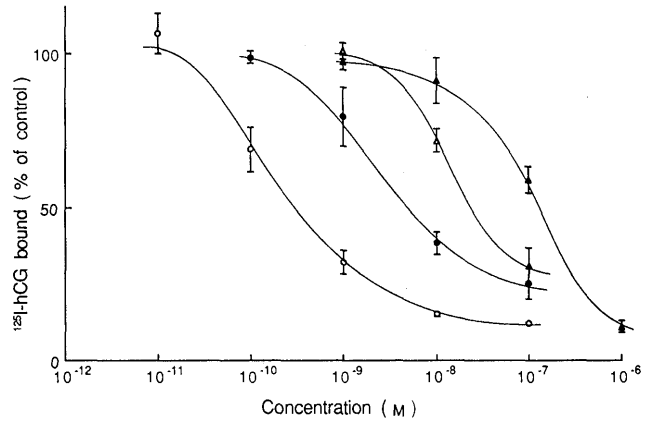

Fig. 4. Receptor Binding Activity of hCG and hCG-Hybrids

Rat Leydig cells were incubated with a fixed amount of [^{125}I]hCG (4×10^5 cpm) in the presence of the indicated doses of unlabeled hCG (○), hCG-A (△), hCG-B (●), and hCG-HRP (▲) for 2 h at 37 °C. The radioactivity bound to the cells was determined as described under Experimental. The data are calculated as percentages of [^{125}I]hCG bound maximally. Each point is the mean ± S.E. of five separate experiments.

were decreased for hCG-HRP and hCG-A, to 1/100 and 1/10 that of native hCG, respectively. Similarly, stimulation of cAMP production by these hybrids was decreased. On the other hand, the ED_{50} of hCG-B for testosterone production was nearly the same as that of hCG. The dose–response curve of testosterone production by hCG was not changed by the addition of free B chain in the range of concentration of 10^{-10}—10^{-8} M. These results indicated that, compared to the HRP moiety, the A chain and the B chain moieties of hybrids, especially B chain, may participate in stimulating the testosterone production.

Stimulation of Testosterone Production by Subunit-Hybrids To determine more exactly the effect of B chain on testosterone production, the subunit-hybrids consisting of α- or β-subunit of hCG and ricin A or B chain were prepared, and their steroidogenic activities were compared (Fig. 3). The stimulating activities of free α- and β-subunits of hCG and free A and B chains were not detected in the

range of concentration indicated in the figure (data not shown). Although the stimulating activities of all subunit-hybrids were very low compared to those of hCG and hCG-B, the hybrids in which either the α- or β-subunit of hCG cross-linked to the B chain (α-B and β-B hybrids) stimulated testosterone production more effectively than hybrids cross-linked to the A chain.

Receptor Binding Properties of the Hybrids Inhibition by unlabelled hCG and various hybrids of the binding of a fixed amount of [^{125}I]hCG to rat Leydig cells is shown in Fig. 4. hCG as well as hCG-hybrids inhibited the binding of [^{125}I]hCG to rat Leydig cells competitively, although the concentrations required for 50% inhibition were about 10-, 100- and 500-fold higher than that of native hCG for hCG-B, hCG-A and hCG-HRP, respectively. Thus, hCG-B was the most potent inhibitor of [^{125}I]hCG binding among the various hybrids. The [^{125}I]hCG binding was not affected in the presence of free A and B chains or HRP in the concentration range of 10^{-10}—10^{-8} M.

Effect of B Chain Moiety on the Binding of hCG-B Ricin B chain differs from the A chain and HRP in that it has galactose recognition sites by which ricin binds to cell-surface receptors, glycoproteins and/or glycolipids containing terminal galactose residues. Further, two high-mannose type oligosaccharide chains are bound to the ricin B chain, which are recognizable by cell-surface lectin. Actually, a lectin-like structure has been presumed to be present in the LH/CG receptor.[14] Thus, to determine the effect of the B chain moiety on the binding of hCG-B, two experiments were performed (Fig. 5). First, the effect of asialofetuin, which is a galactose-terminating glycoprotein, on the inhibition by hCG-B of the binding of [^{125}I]hCG for rat Leydig cells was examined. In the presence of asialofetuin, the inhibition curve of [^{125}I]hCG binding by the hCG-B hybrid shifted to the right, reaching a level similar to that of the hCG-A hybrid. The effect of asialofetuin was examined in the range of concentration of 0.5 to 3.0 mg/ml and the maximal effect was observed beyond the concentration of 1.0 mg/ml. The inhibition by hCG, hCG-A and hCG-HRP of the binding of [^{125}I]hCG to rat Leydig cells was not affected by asialofetuin (data not shown).

Second, to exclude the effects of oligosaccharide chains of the B chain moiety, hCG-hybrid constituted with hCG and dgB (hCG–dgB) was prepared. As described under Experimental, dgB used for the synthesis of hCG–dgB was Sepharose 4B-bound dgB, indicating that the galactose-binding activity of dgB was similar to that of native B chain. As shown in Fig. 5, the inhibition curve of hCG–dgB with the binding of the [^{125}I]hCG to rat Leydig cells shifted significantly to the right from that of hCG-B. In the presence of asialofetuin, the inhibition curve by hCG–dgB shifted much more to the right, reaching a level similar to that of hCG-HRP.

Effect of the B Chain Moiety on the Stimulation of Testosterone Production by hCG-B The effect of asialofetuin on the stimulation of testosterone production by hCG-B was examined. As shown in Fig. 6, testosterone production provoked by hCG-B was inhibited in the presence of asialofetuin. Figure 6 also shows that, although the hCG–dgB hybrid stimulated testosterone dose-dependently, the steroidogenic activity of the hCG–dgB hybrid was less than that of hCG-B both in terms of the relative and the maximal testosterone producing potencies. Furthermore, in the presence of asialofetuin, the steroidogenic activity of hCG–dgB was further decreased, and the level of its activity did not differ from that of hCG-HRP.

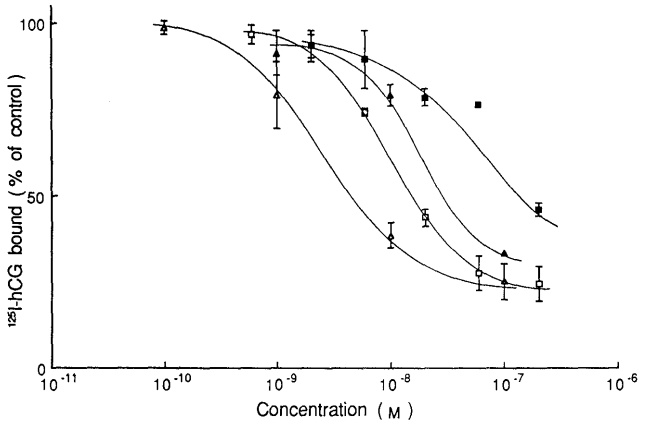

Fig. 5. Effect of B Chain Moiety on the Inhibition by hCG-B of the Binding of [^{125}I]hCG to Rat Leydig Cells

Inhibition of either hCG-B (△, ▲) or hCG–dgB (□, ■) with the binding of [^{125}I]hCG to rat Leydig cells in the presence (closed symbols) or absence (open symbols) of asialofetuin (1 mg/ml) was determined by the method described in the legend of Fig. 4. Each point is the mean ± S.E. of five separate experiments.

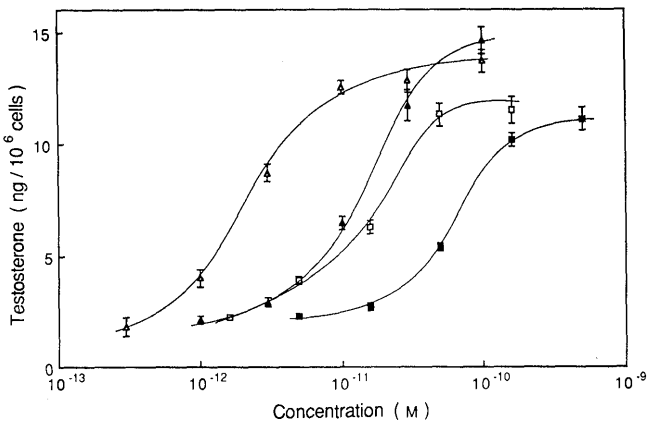

Fig. 6. Effect of B Chain Moiety on the Stimulation of Testosterone Production by hCG-B

Stimulation of testosterone production of either hCG-B (△, ▲) or hCG–dgB (□, ■) in the presence (closed symbols) or absence (open symbols) of asialofetuin (1 mg/ml) was determined by the method described in the legend to Fig. 1. Each point is the mean ± S.E. of four separate experiments.

Discussion

To modify the hormonal activity of the glycoprotein hormone hCG, we synthesized hCG hybrid molecules composed of hCG and other glycoproteins. The hCG-A and hCG-B hybrids were synthesized by cross-linking hCG with either the A or B chain of lectin ricin through disulfide bridges. The oligosaccharide moieties of the A and B chains in these hybrids were not chemically modified. On the other hand, the hCG-HRP hybrid was synthesized by cross-linking hCG with HRP through Shiff's base using a periodate oxidation and reduction method. Thus, the oligosaccharide moieties of HRP were chemically broken. Hormonal activities, the potencies stimulating both testosterone and cAMP productions in rat Leydig cells, of

these hCG hybrids were compared to those of native hCG. hCG-HRP stimulated both testosterone and cAMP production with maximal responses, but their ED_{50} increased significantly (100- and 400-fold, respectively) compared to those of native hCG. This may not be caused by reduction treatment of hCG with sodium borohydride, since borohydride treated-hCG stimulated testosterone production in a similar manner as native hCG. On the other hand, hCG-A stimulated testosterone and cAMP production with increased ED_{50}, but not as significantly as hCG-HRP. hCG-B clearly showed a nearly identical ED_{50} for testosterone production to that of native hCG, although the ED_{50} for cAMP was slightly higher. Thus, it has been demonstrated that the potentials for cAMP and testosterone production by hCG were reduced by modification with other proteins, and only hCG modified with B chain (hCG-B) revealed nearly the same potency as native hCG. Every hybrid inhibited the binding of [^{125}I]hCG to rat Leydig cells in parallel with the potencies of their steroidogenic activity, suggesting that all hybrids bind to Leydig cells predominantly through the hCG receptor. Furthermore, the hybrids composed of either the α- or β-subunits of hCG and the B chain of ricin (α-B and β-B hybrids) stimulate testosterone production more efficiently than hybrids composed of A chain. These results prompted us to investigate the role of the B chain moiety in hCG-B with respect to the steroidogenic activity and binding of hCG-B to rat Leydig cells.

The B chain of ricin has galactose recognition sites (lectine activity) by which ricin binds to cell-surface receptors, glycoprotein and/or glycolipids containing terminal galactose residues. To study the participation of the B chain moiety on the steroidogenic activity of hCG-B, the effects of asialofetuin, which is a galactose-terminating glycoprotein and binds to the B chain, on stimulation of testosterone production and inhibition of [^{125}I]hCG binding to rat Leydig cells by hCG-B were determined. Asialofetuin inhibited both activities induced by hCG-B but not for hCG itself. These results suggested that the B chain of ricin consisting of the hCG-B hybrid binds to Leydig cells *via* galactose-residues of membranes, and that additional steroidogenic activity of the hCG-B hybrid may be mediated through recognition of galactose-residues, if present, in the hCG receptor or on membrane glycoprotein and/or glycolipid in the vicinity of the hCG receptor. Furthermore, in the presence of asialofetuin, the dose–response curve of testosterone production by hCG-B and the inhibition curve of hCG-B with binding of [^{125}I]hCG approached those of hCG-A, indicating that the higher steroidogenic activity of hCG-B compared to hCG-A may be due to the lectin activity of the B chain.

Two high-mannose type oligosaccharide chains ($Man_{6-7}GlcNAc_2$ linked to Asn-95 and $Man_{5-7}GlcNAc_2$ to Asn-135) are attached to the ricin B chain.[17] To determine the effect of sugar chains attached to the B chain on the binding of hCG-B to the cells, a hybrid composed of hCG–dgB was prepared. Steroidogenic activity and inhibitory activity with [^{125}I]hCG binding of hCG–dgB decreased significantly compared to hCG-B. These activities of hCG–dgB were further decreased in the presence of asialofetuin, and their levels approached those of hCG-HRP. It was assumed that the activity of hCG–dgB in the presence of asialofetuin became equivalent to that of hCG-HRP, since the sugar chains of HRP in hCG-HRP are broken by periodate oxidation and HRP itself has no lectin-like activity. The A chain of ricin contains the single xylomannose type oligosaccharide chain at Asn-10,[17] according to the above assumption, and this oligosaccharide chain may interact with the Leydig cell membrane. This might explain why the effect of hCG-A was moderate between hCG-B and hCG-HRP. It has been well established that the oligosaccharide moieties of hCG are essential for the full expression of its biological activities. Recently, Matzuk *et al.* indicated that the oligosaccharide chain at Asn-52 of the α-subunit plays an important role in signal transduction using the technique of site-directed mutagenesis.[12] The present results may indicate that, by a modification of hCG with other proteins, the oligosaccharide moieties of hCG may be masked and their interaction with receptors may be blocked, resulting in a loss of biological activity. However, when hCG is modified with proteins containing intact oligosaccharide chain(s), *i.e.*, either the B or A chain of ricin, the introduced oligosaccharide moieties take on the role of the hCG moieties. Further, it is suggested that the structures of the introduced oligosaccharide moieties are not necessarily the same as those of hCG.

Wang *et al.*[31,32] purified placental hCG (SP-hCG), which contains incompletely formed Asn-linked oligosaccharide chains, oligomannose type, for each subunit and lacks O-linked oligosaccharide chains for the β-subunit. They indicated that the SP-hCG increased cAMP accumulation and testosterone production by rat Leydig cells to the same levels as that induced by hCG. We confirmed their results using hCG reconstituted with purified placental subunits.[33] They also indicated that the mannose moieties are essential structural components of the hormone in terms of its biological activity, since the biological activity of SP-hCG was markedly reduced following treatment with endoglycosidase H or α-mannosidase.[32] Recently, Thotakura *et al.* showed that Asn-linked oligosaccharide chains from various glycoproteins, *i.e.*, oligosaccharides prepared from hCG, transferrin, fetuin, α_1-acid glycoprotein and ovalbumin, inhibit the binding of hCG to its receptor.[13] The data obtained here may be consistent with their suggestion that Asn-linked oligosaccharide chains from various glycoproteins (not only those for hCG) perturb hCG-receptor interactions through a putative carbohydrate binding site on the receptor.[13,32] Thus, a lectin-like site must be present on the receptor itself or on another membrane protein as previously reported by Thotakura *et al.*[13] or Calvo and Ryan,[34] respectively. Actually, a soybean lectin-like structure is found in the sequence of cloned hCG receptor.[14]

Dufau *et al.* reported that aggregation or dimerization of the LH/CG receptor could promote clustering and/or cross-linking of receptors in the membrane favoring the initial transduction steps in the action of these hormones.[35] Podesta *et al.* demonstrated the receptor capping which may be associated with receptor-mediated testosterone production with an immunofluorescence technique using monoclonal antibodies against the lutropin receptor.[36] Luborsky *et al.* showed that LH/CG receptor microaggregation is directly related to receptor occupancy and

may be dependent on cAMP levels, since prostaglandin F2α reduces receptor microaggregation, while addition of dibutyryl cAMP increases it.[37] In agreement with the examples cited above, the present study might suggest that local aggregation induced by binding of the B chain moiety in hCG-B hybrid, through its lectin activity, may contribute to stimulating testosterone production.

In conclusion, the hybrid consisting of hCG and the B chain of ricin stimulates testosterone and cAMP production by rat Leydig cells. The hCG-B binds to the LH/CG receptor on Leydig cell membrane probably through the hCG moiety. In addition, the B chain moiety binds to galactose-residues of the hCG receptor or a near neighbor of the receptor. Further, we suggest that the oligosaccharides of the B chain moiety, Asn-linked oligomannose type, differing from the complex type oligosaccharide chains attached to native hCG, bind to a membrane lectin and may play an important role in signal transduction of hCG.

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Removal of Hydrogen Sulfide by *Hyphomicrobium neptunium* ATCC 15444

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Hydrogen sulfide (H_2S) was efficiently removed from air by *Hyphomicrobium neptunium* ATCC 15444. When the loading of H_2S was less than 68.3 mmol of H_2S per liter-culture per day, the H_2S was completely removed. At this point, the inlet maximum concentration of H_2S was 850 ppm. This H_2S removal effect was strongly dependent on the pH of the medium and the existence of Mg^{2+}, Ca^{2+} in the medium which were growth factors of *H. neptunium* ATCC 15444.

Keywords *Hyphomicrobium neptunium*; hydrogen sulfide; deodorization; sulfur compound; malodorous component

Introduction

Sulfur-containing volatile compounds such as hydrogen sulfide, methyl mercaptan, dimethyl sulfide and dimethyl disulfide are major malodorous components which are produced by waste treatment facilities and factories. These malodorous components exceed the odor threshold at low concentrations. Especially, since hydrogen sulfide (H_2S) has a strong toxicity toward the human body, the removal of this gas is very important for public health. Various methods have been used for the removal of H_2S. The biological deodorizing method is now attracting attention because of its low operating cost and high ability as compared with physical or chemical processes such as washing with chemicals, absorption with activated carbon, and incineration.[1-5] However, there is little knowledge on the identifiable bacteria which cause the degradation of H_2S. Furusawa *et al.*[6] have demonstrated that fibrous peat is an effective deodorizing agent of H_2S. Furthermore, Wada *et al.*[7] have indicated that the deodorizing ability of the peat is dependent on the growth of *Thiobacillus intermedius*. On the other hand, Kanagawa and Mikami,[8] and Tanji *et al.*[9] have shown that *Thiobacillus thioparus* TK-m more effectively degrades H_2S. But, there are no reports on other identifiable species of bacteria which can remove H_2S. Recently, we found *Hyphomicrobium neptunium* ATCC 15444 could remove higher concentrations of H_2S as compared with the above bacteria. In this paper, therefore, we described that *H. neptunium* ATCC 15444 efficiently removed H_2S from air.

Materials and Methods

Bacterial Strain and Growth Condition The bacterial strain used throughout this study was *H. neptunium* ATCC 15444. This strain was pre-cultured at 30 °C in a liquid medium (pH 8.0) containing 0.2% of polypeptone (Nihon Seiyaku), 0.1% of yeast extract (Difco), 0.1% of $CaCl_2 \cdot 2H_2O$ and 0.3% of $MgCl_2 \cdot 6H_2O$. In the early stationary phase of growth, a 2 ml-portion of the culture broth was added to 250 ml of the same medium in 500 ml sakaguchi flasks. The flasks were shaken at 30 °C for 18 h, and the bacterial culture, which was in the early stationary phase of growth, was used for a deodorizing test.

Deodorizing Test A schematic diagram of the deodorizing apparatus used in this study is shown in Fig. 1. The culture bottle (70 mm × 200 mm) was filled with 400 ml of the culture of *H. neptunium* ATCC 15444. Hydrogen sulfide gas was generated by feeding solutions of Na_2S (1—3%) and HCl (0.12—0.36 N) by peristaltic pumps to the top of a H_2S generator. The gas was diluted with air and supplied to the culture bottle. All experiments were carried out at room temperature.

Gas Analysis Inlet and outlet concentrations of H_2S in air were measured by gas detector tubes (Gastec). The relative error of the tube to the standard methylene blue method is nominally ±10%. The lowest detection limit of the tube was 0.2 ppm.

Results

Pattern of Removal of H_2S The characteristic pattern of H_2S removal by *H. neptunium* ATCC 15444 is shown in Fig. 2. The inlet H_2S concentration was fixed around 300 ppm and air was allowed to pass through the culture of bacteria in the bottle at a flow rate of about 0.5 l/min/400 ml. The outlet concentration of H_2S gradually decreased from the time zero and eventually became less than the detection limit.

H_2S Removal by Killed Bacteria To prove that the removal of H_2S is dependent on live cells of *H. neptunium* ATCC 15444, an experiment was carried out using killed bacteria. As shown in Fig. 3, *H. neptunium* ATCC 15444, which was killed by heating at 121 °C for 15 min, could not efficiently remove H_2S as compared to the live bacteria. Furthermore, the other killed bacteria, which were killed by ultraviolet (UV) irradiation (254 nm) for 30 min or by

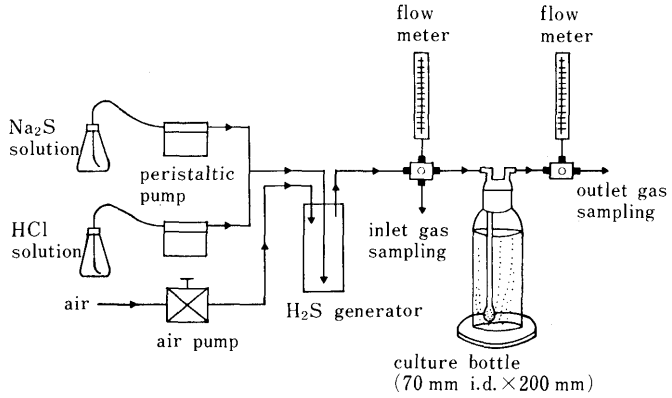

Fig. 1. Schematic Diagram of Laboratory-Scale Deodorizing System

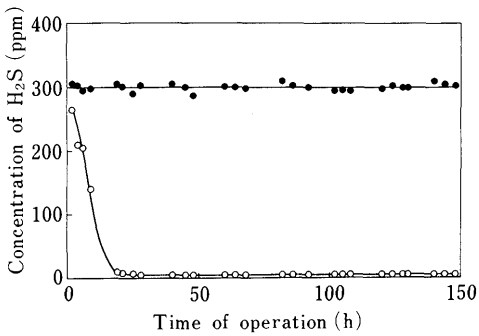

Fig. 2. Time Course of H_2S Removal by *H. neptunium* ATCC 15444

The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. Symbols: ●, inlet H_2S concentration; ○, outlet H_2S concentration.

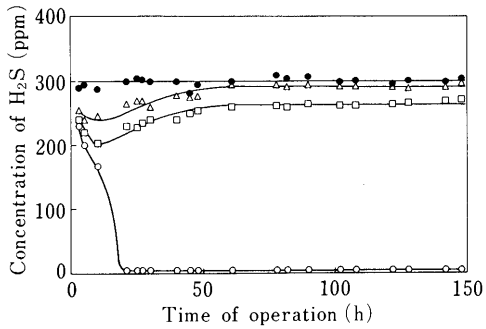

Fig. 3. Time Course of H_2S Removal by Live or Killed Cells of *H. neptunium* ATCC 15444

The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. Symbols: ●, inlet H_2S concentration; ○, H_2S removal by live cells of *H. neptunium* ATCC 15444; □, H_2S removal by killed cells of *H. neptunium* ATCC 15444; △, H_2S removal by only medium.

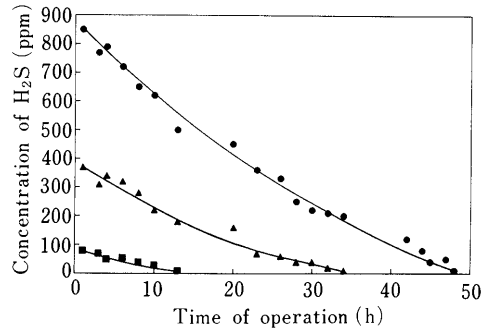

Fig. 4. Relationship between Concentration of H_2S and Acclimation Period

The inlet H_2S concentration was fixed around 850, 370 or 80 ppm, respectively. Symbols indicated the outlet concentration of H_2S under inlet concentrations of ●, 850 ppm; ▲, 370 ppm; ■, 80 ppm.

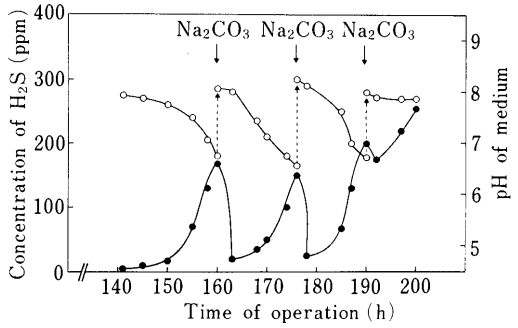

Fig. 5. Effect of pH on H_2S Removal by *H. neptunium* ATCC 15444

The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. The adjustment of pH was carried out by the addition of 10% Na_2CO_3 solution. Symbols: ●, outlet H_2S concentration; ○, pH of culture medium.

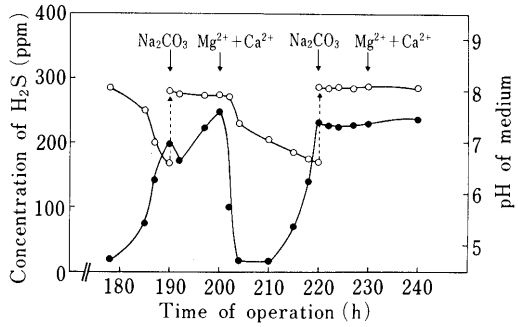

Fig. 6. Effect of Mg^{2+} and Ca^{2+} on H_2S Removal by *H. neptunium* ATCC 15444

The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. The addition of Mg^{2+} and Ca^{2+} was carried out adding 10% $CaCl_2 \cdot 2H_2O$ solution and 30% $MgCl_2 \cdot 6H_2O$ solution. The adjustment of pH was carried out by the addition of 10% Na_2CO_3 solution. Symbols: ●, outlet H_2S concentration; ○, pH of culture medium.

antibiotic treatment (streptomycin; 100 μg/ml) for 24 h, also had the same inefficiency. The medium itself also did not have the ability to remove H_2S. These results indicated that the H_2S removal was caused by live cells of *H. neptunium* ATCC 15444.

Acclimation Period It is important to know the acclimation period required for the stable removal of H_2S under various loading concentrations. Figure 4 shows the relationship between concentration of H_2S and the acclimation period. The time required for the acclimation was increased according to the inlet concentration of H_2S. After only 48 h of acclimation, as much as 850 ppm of H_2S was completely removed by *H. neptunium* ATCC 15444. This result indicated that *H. neptunium* ATCC 15444 could completely remove H_2S very rapidly at relatively short periods of operation.

Effects of pH, Mg^{2+} and Ca^{2+} of Medium on H_2S Removal by *H. neptunium* ATCC 15444 The breakdown was observed about 145 h after H_2S loading (Fig. 5). To clarify the effect of pH on H_2S removal, the pH value of bacterial culture medium was also calibrated at the same time. The H_2S removal rate at 160 h was about 44%, and the pH of the medium also decreased to 6.7 with a breakdown of deodorization. When the pH of medium was re-adjusted to about 8.0 by the addition of 10% Na_2CO_3 solution, the H_2S removal rate suddenly recovered until about 93%. However, this effect could continue for only a few hours, and the breakdown was observed again with the decrease of pH. When a third adjustment of pH was performed, the H_2S removal was scarcely recovered although the pH value was around 8.0, and the H_2S removal rate at 200 h decreased to 15%. The bacterial growth at this point was in the early phase of decline.

Therefore, following pH adjustment, the effect of Mg^{2+} and Ca^{2+} on H_2S removal was studied. When 4 ml of 10% $CaCl_2 \cdot 2H_2O$ solution and 4 ml of 30% $MgCl_2 \cdot 6H_2O$ solution were added into the culture bottle at 200 h, the H_2S removal rate was recovered again until about 95% a few hours later (Fig. 6). This effect could continue for about 7 h, and the breakdown was observed again with the decrease of the pH value of the medium. The H_2S removal rate at 220 h decreased to about 22%, and the pH also decreased to 6.6. Furthermore, although the pH adjustment and the addition of Mg^{2+}, Ca^{2+} were performed, the ability to remove H_2S from air was not recovered again. Although the bacterial growth was recovered by the addition of Mg^{2+} and Ca^{2+} at 200 h, the phase of decline was observed again after 210 h. However, the adjustment of pH and the addition of Mg^{2+}, Ca^{2+} at 220 and 230 h did not recover the bacterial growth.

Relationship among pH, Mg^{2+}, Ca^{2+} of Medium and Breakdown of H_2S Removal The breakdown was recovered by an adjustment of pH or an addition of Mg^{2+}, Ca^{2+}. To clarify the relationship between pH, Mg^{2+}, Ca^{2+} of medium and H_2S removal by *H. neptunium* ATCC 15444, the deodorizing tests were performed under various culture

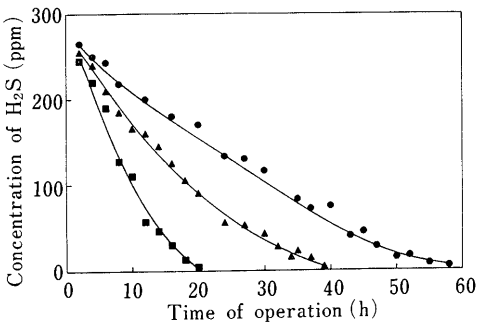

Fig. 7. Relationship between pH of Culture Medium and H_2S Removal by *H. neptunium* ATCC 15444

The pH of culture medium was adjusted around 8.0, 7.0 or 6.0, respectively, by the addition of 10% Na_2CO_3 or 1 N HCl solution before the deodorizing tests. The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. Symbols indicated the pH of culture medium: ■, pH 8.0; ▲, pH 7.0; ●, pH 6.0.

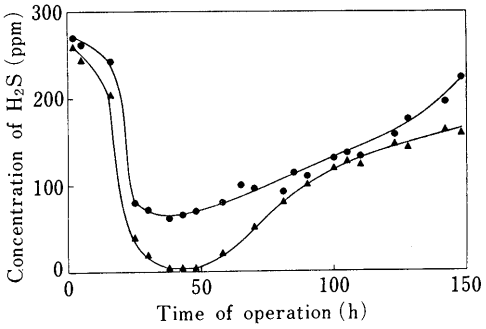

Fig. 8. Relationship between Mg^{2+}, Ca^{2+} in Culture Medium and H_2S Removal by *H. neptunium* ATCC 15444

H. neptunium ATCC 15444 was grown in a medium containing either Mg^{2+} or Ca^{2+}, and the bacterial culture was used for the deodorizing test. The inlet H_2S concentration was fixed around 300 ppm at a flow rate of 0.5 l/min. Symbols: ▲, H_2S removal by *H. neptunium* ATCC 15444 cultured in the Mg^{2+}-free medium; ●, H_2S removal by *H. neptunium* ATCC 15444 cultured in the Ca^{2+}-free medium.

medium conditions. Figure 7 shows the relationship between starting pH of culture medium and H_2S removal by *H. neptunium* ATCC 15444. Culture mediums were adjusted to pH 8.0, 7.0 and 6.0, respectively, before the deodorizing tests, and then the H_2S removal by each culture medium was performed. When the culture medium of pH 8.0 was used, the complete H_2S removal was observed about 20 h later. On the other hand, the culture mediums of pH 7.0 and 6.0 required about 39 and 57 h, respectively, for the complete H_2S removal. After the complete H_2S removal, the pH value of each culture medium became around 7.6—8.0 with bacterial growth, and these H_2S removal effects could continue for at least 100 h (data not shown).

The relationship between Mg^{2+}, Ca^{2+} in culture medium and H_2S removal by *H. neptunium* ATCC 15444 was also studied. *H. neptunium* ATCC 15444 could grow by the addition of either Mg^{2+} or Ca^{2+}. The level of growth was similar to that in the complete medium including both Mg^{2+} and Ca^{2+}. When the Mg^{2+}-free midium (pH 8.0) was used for culture of *H. neptunium* ATCC 15444, the complete H_2S removal was observed 38 h later (Fig. 8). However, the effect could continue for only 12 h, and then the breakdown started. When the Ca^{2+}-free medium (pH 8.0) was used for culture of *H. neptunium* ATCC 15444, the complete H_2S removal was not observed. The maximum H_2S removal rate (80.0%) was observed 38 h later, and then the breakdown was gradually observed. On the other hand, the medium itself including either Mg^{2+} or Ca^{2+} did not have the ability to remove H_2S.

Discussion

When the air containing H_2S was supplied to a culture of *H. neptunium* ATCC 15444, a significant deodorizing effect was observed. However, this deodorizing effect was not observed when killed-bacteria or only medium was used. This result indicated that the H_2S removal was dependent on metabolism including an enzyme system such as sulfide oxidase of *H. neptunium* ATCC 15444.

Kanagawa and Mikami[8] have reported that a culture of *T. thioparus* TK-m can degrade H_2S at the removal rate of 1.76 mmol/l/d (at 0 °C, 1 atm). Furthermore, Tanji *et al.*[9] have reported a more effective H_2S removal (17.4 mmol/l/d) using *T. thioparus* TK-m immobilized on polypropylene pellets. On the other hand, our experiment using a culture of *H. neptunium* ATCC 15444 showed almost complete H_2S removal at the loading of 68.3 mmol/l/d, indicating that the decomposition capability was about 39-fold higher than that of *T. thioparus* TK-m. Therefore, the immobilization of *H. neptunium* ATCC 15444 would be expected to get a more efficient deodorizing effect.

This deodorizing effect was strongly dependent on conditions of a culture medium. That is, the pH value of the medium and the existence of Mg^{2+}, Ca^{2+} in the medium were important for the continuous and significant deodorizing effect. *H. neptunium* ATCC 15444 can grow in the pH range of 6.0—9.5, requires Mg^{2+} or Ca^{2+} as a growth factor, and shows the best growth in the medium of pH 8.0 including both Mg^{2+} and Ca^{2+}.[10] Although the H_2S removal rate was not recovered after a third adjustment of pH, this would be dependent on the survival rate of bacteria, actually the bacterial growth at this point was in the early phase of decline. The addition of Mg^{2+} and Ca^{2+} as growth factors could recover the H_2S removal rate with the bacterial growth, however, the second addition of Mg^{2+} and Ca^{2+} did not recover the H_2S removal. This result would mean that the bacterial growth was already in the late phase of decline. The H_2S removal rate would not be recovered unless the culture medium was replaced by fresh medium. Therefore, it would seem that the level of deodorizing effect was directly dependent on that of survival of *H. neptunium* ATCC 15444, indicating that a more continuous deodorizing effect would be gotten by continuing to control the medium condition.

Our observations demonstrated that *H. neptunium* ATCC 15444 could effectively remove H_2S from air. A study is in progress to analyze sulfur compounds produced with H_2S removal.

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Effect of Enzymatic Sulfation on Biochemical and Pharmacological Properties of Catecholamines and Tyrosine-Containing Peptides

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Substrate specificity of a novel sulfotransferase produced by *Eubacterium* A-44 isolated from human feces has been studied. Phenolic drugs, catecholamines, were good acceptors of this bacterial enzyme. With regard to dopamine, sulfation mostly occurred at the 4-aromatic hydroxy group.

We also investigated the effects of enzymatic sulfation on pharmacologically active phenolic compounds. Sulfation of phenolic compounds generally led to inactivation (*e.g.* tyramine and Leu-enkephalin), with the exception of cholecystokinin (CCK) and some gastrointestinal peptides. Proteolytic hydrolysis *in vitro* did not occur at the C-terminal of the sulfated tyrosine residues of peptides such as Leu-enkephalin and kyotorphin. These results suggest that the sulfation by bacterial enzyme plays an important role in detoxification, activation and stability of phenolic compounds in the human body.

Keywords sulfotransferase; *Eubacterium*; sulfation; dopamine; Leu-enkephalin; protease

Introduction

Sulfate conjugation catalyzed by sulfotransferase (E.C. 2.8.2.1) is an important metabolic pathway for catecholamine and many phenolic drugs. The enzyme is found in various mammalian organs[1-6] and the sulfate donor substrate is known to be 3′-phosphoadenosine-5′-phosphosulfate (PAPS).

In humans, catecholamine neurotransmitters, such as dopamine and norepinephrine, were considered to be inactivated by monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT).[7] However, it has been clarified that sulfotransferase has a relatively high affinity for dopamine and norepinephrine[8] in the human brain and that sulfate conjugation of catecholamine is quite important in human central nervous system (CNS).[9-11] On the other hand, sulfate conjugation is generally considered to be a metabolic pathway for inactivation or detoxification of phenolic compounds. But, cholecystokinin (CCK), which was discovered as a gastrointestinal peptide hormone, is sulfated at its tyrosine residue. The activity is 600-fold higher than that of non-sulfated CCK[12] and sulfation is absolutely necessary for the pharmacological activity of CCK. Therefore, sulfotransferase is considered to take another role in the activation of phenolic compounds and tyrosine-containing peptides. In addition, Huttner reported that tyrosine sulfation might be an important process for the secretion of peptides and proteins from cells.[13] However, the biological role of sulfation has been established in only a few cases.

We have previously reported that *Eubacterium* A-44, a predominant bacteria in the human intestine, produces a novel type of sulfotransferase.[14-19] This enzyme catalyzed stoicheometric transfer of a sulfate group from phenolic sulfate esters, but not from PAPS, to phenolic compounds. In this paper, we describe further characterization of the enzyme using various phenolic compounds as sulfate acceptors. Furthermore, in order to investigate the functional aspects of sulfation of tyrosine residues, we examined the resistance of several sulfated peptides to various proteases.

Materials and Methods

Chemicals *p*-Nitrophenol (PNP) and dopamine-HCl were purchased from Wako Pure Chemical Industry (Japan). *p*-Nitrophenyl sulfate (PNS), 4-methylumbelliferyl sulfate (MUS), chymotrypsin, aminopeptidase, carboxypeptidase and pronase were from Sigma Chemical (U.S.A.). Tyramine was from Nacalai Tesque, Inc. (Japan). Kyotorphin was from Funakoshi Pharmaceuticals (Japan). Leu-enkephalin was kindly donated by Dr. T. Morikawa, Fuji Pharmaceutical Ind. (Japan). Leu-enkephalin-*O*-sulfate and kyotorphin-*O*-sulfate were prepared by enzymatic sulfation according to previous reports.[17,18] All other chemicals were of analytical reagent grade.

Enzyme Preparation Sulfotransferase obtained from *Eubacterium* A-44 was partially purified according to the same procedures as previously described.[15] A harvested bacterial suspension was disrupted by a sonicator (Wakenyaku Co., Ltd., Japan), to obtain a crude extract, which was fractionated with 40—60% saturation of ammonium sulfate, dialyzed and purified by diethylaminoethyl (DEAE)-cellulose column chromatography.

Enzyme Activity Assay The assay mixture contained 30 μl of 50 mM PNS, 0.29 ml of 20 mM phenolic acceptor (tyramine was used as a control), 0.21 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 0.1 ml of enzyme solution in a final volume of 0.63 ml. The mixture was incubated for 15 min at 37 °C and the reaction was terminated by adding 0.40 ml of 1 N NaOH, and the absorbance at 405 nm was measured by a spectrophotometer (Shimadzu, UV-240, Japan). One unit of enzyme activity was defined as the amount required to catalyze the formation of 1.0 μmol of *p*-nitrophenol per minute. Specific activity was defined in terms of units per mg protein. The activity assay for some yellowish colored acceptors was performed by using MUS as a donor and the reaction product, 4-methylumbelliferone was measured fluorometrically at ex. 330 nm and em. 450 nm (Shimadzu, RF-500, Japan).

High Performance Liquid Chromatography (HPLC) Sulfated products of sulfotransferase reaction and amino acid fragments of protease digestions were identified by HPLC equipped with a reverse phase C 18 column (Microsorb (RAININ, U.S.A.), Develosil (Nomura, Japan)) and a HPLC pump system (Gilson, U.S.A.). The solvent system used was indicated in each legend to figures.

Protein Determination Protein concentrations were estimated by the method of Lowry *et al.*[20] using bovine serum albumin as a standard.

Synthesis of Dopamine-*O*-sulfate Dopamine-4- and -3-sulfate esters were prepared according to the method of Harbeson *et al.*[21] The former was synthesized as 1/20 scale and the yield was 14.1% (5.21 mg). Dopamine-3-sulfate was synthesized by a modified procedure. Dopamine-HCl, 900 mg (10.5 mmol), was added to a solution of triethylamine, 0.657 ml (10.5 mmol), in 4.5 ml dimethylformamide (DMF). Then benzylchloroformate, 3.33 ml (0.66 ml × 5), was added to the mixture in an ice bath. The mixture was allowed to stand for 4 h with stirring. The reaction mixture was filtered and ethylacetate was added and the resulting mixture was washed three times with cold 1 N HCl. The organic layer was concentrated to give a colorless oil. This oil was crystallized from EtOAc–hexane. A white solid *N*-carbobenzyloxy dopamine 300 mg (1.05 mmol) was suspended in 3 ml DMF with *tert*-BuOK 200 mg (40 mg × 5) and benzyl bromide 0.17 ml (0.57 ml × 3, 1.43 mmol) was

added dropwise; and the mixture was stirred for 6 h. After the mixture was washed at least three times with ether–1 N HCl, the organic layer was concentrated and purified with a silica column (silicic acid 1.5 g + Merck saulen 1.5 g, 16 i.d. × 33 mm) and thin layer chromatography (TLC) (silica gel plate, Merck 60F254, 0.5 cm, 20 × 20 cm) using a solvent $CHCl_3$. *N*-Carbobenzyloxy-4-benzyl dopamine was then recrystallized from CH_2Cl_2–hexane. 91.6 mg (0.24 mmol) of the solid in 15.5 ml tetrahydrofuran (THF) was added to pyridine–sulfur trioxides complex 386.4 mg (Aldrich, 243 mmol) and stirred for 14 h. The solvent was removed under vacuum and purified with TLC using the solvent 20% MeOH–$CHCl_3$. Deprotection was accomplished by dissolving the solid 85.6 mg (0.187 mmol) in 2.8 ml MeOH and stirring with a mixture of ammonium formate 250 mg (3.97 mmol), 10% palladium carbon 140 mg and 40 μl acetic acid. After stirring for 1 h, the mixture was filtered with suction to remove solids, and concentrated and crystallized from H_2O–EtOH. Dopamine-3-sulfate 1.5 mg was recrystallized from the same solvent. The synthesized dopamine-4-sulfate and dopamine-3-sulfate were identified by nuclear magnetic resonance (NMR), infrared (IR) and mass (MS) spectrometry.

Pharmacological Assays Effect of tyramine and tyramine-*O*-sulfate on norepinephrine-induced chronotropic and inotropic responses of isolated rat atria have been studied according to the method of Johansson.[22] On the other hand, the effect of sulfation of Leu-enkephalin on the guinea-pig longitudinal-myenteric plexus preparation was carried out as described by Kosterlitz *et al.*[23] The inotropic effects were driven at 0.1 Hz by electrical field stimulation. Square wave pulses of 1 V and 1 ms were delivered to platinum electrodes on a muscle holder.

Results

Substrate Specificity Using PNS or MUS as a donor substrate, acceptor specificities of some phenolic drugs and hormones were determined as shown in Table I. Phenol was a good acceptor and was sulfated as well as tyramine. *p*-Acetamidophenol was rapidly sulfated, but *o*- and *m*-acetamidophenols were hardly sulfated. Salicylic acid was not sulfated but salicylamide was, which suggests that formation of a hydrogen bond between free carboxylic acid and the *o*-hydroxyl group hinder the sulfation of the phenol. Naloxone, 5-hydroxyindole-3-acetic acid and folic acid were weak substrates, and pyridoxine, tetracycline and minocycline were ineffective as acceptor substrates. Among catecholamines, norepinephrine, epinephrine, dopamine were good acceptors but dopa and octopamine were weaker acceptors. 3,3′,5-Triiodothyronine (T_3) was more sulfated than thyroxine (T_4), probably the steric hindrance of iodine at 5′ position. Serotonin was sufficiently sulfated (we corrected the previous results[18] that serotonin was not sulfated).

Furthermore, the reaction products in the system of dopamine as an acceptor and PNS as a donor were identified by HPLC, as shown in Fig. 1. After 2 h incubation, one major and one minor peak of dopamine-*O*-sulfate were detected. The amount ratios of these peaks were 0.85 and 0.15, respectively. In order to identify which peaks are dopamine-3-sulfate or -4-sulfate, authentic compounds were synthesized. As a result of HPLC analysis, a major peak was identified to be dopamine-4-sulfate and the other minor one was dopamine-3-sulfate, which shows that the sulfation by this enzyme occurred predominantly at the *p*-hydroxyl group of dopamine. Dopamine-3-sulfate incubated with the enzyme without any addition of acceptors resulted in the formation of dopamine-4-sulfate (data not shown). These finding suggested that the sulfate group at *m*-position is intramolecularly transferred to *p*-position of dopamine by the enzyme.

Pharmacological Activities Tyramine, which was a good acceptor of sulfotransferase, has been known to enhance norepinephrine-induced chronotropic and inotropic responses of isolated rat atria. Tyramine-*O*-sulfate was inactive in both of them (data not shown). Similarly, the effect of sulfation of Leu-enkephalin on guinea-pig longitudinal-myenteric plexus preparation was examined. The inotropic response was suppressed about 46% by 3×10^{-7} M enkephalin, but it was conversely increased about

TABLE I. Substrate Specificity

Acceptors	Activity (%)
Phenol	101
Acetamidophen	
o-Acetamidophenol	3
m-Acetamidophenol	1
p-Acetamidophenol	129
Salicylic acid	0
Salicylamide	14
Naloxone	5
5-Hydroxyindole-3-acetic acid	6
Pyridoxine (VB_6)	0
Folic acid (5 mM)	2[a]
Tetracycline	0[a]
Minocycline	0[a]
Tyramine	100
Norepinephrine	106
Epinephrine	91
Dopamine	170
Dopa	27
Octopamine	17
Phenylephrine	1
Thyroxine (T_4) (0.16 mM)	2
3,3′,5-Triiodothyronine (T_3) (0.16 mM)	36
Serotonin	100

Specific activity of the enzyme was 5.21 U/mg protein in a PNS–tyramine system, which was taken as 100%. Final acceptor concentrations were 9.2 mM except folic acid, T_4 and T_3 whose final concentrations were indicated in parentheses. *a*) The activities were measured fluorometrically using MUS as a donor.

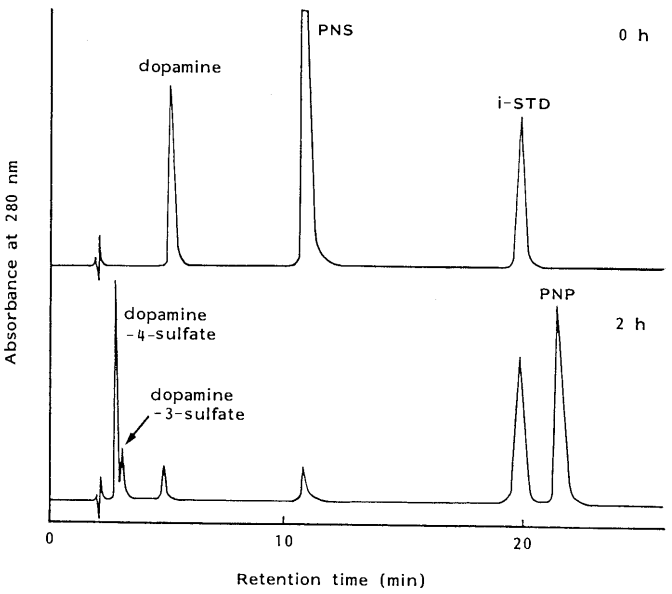

Fig. 1. HPLC Chromatograms of Sulfated Dopamine

HPLC conditions: column, Microsorb C-18 5 μm (4.5 × 250 mm); solvent, 10% MeOH/0.1% TFA; flow rate, 1.0 ml/min; wavelength, 280 nm. A reaction mixture contained 0.1 ml of 5 mM dopamine, 0.1 ml of 5 mM PNS, 0.2 ml of 0.1 M Tris–HCl buffer (pH 8.0) and 0.2 ml of enzyme (5.21 U/mg protein, 50 μg). After incubation of the reaction mixture at 37 °C for 2 h, 90 μl of a reaction mixture and 10 μl of 5 mM tryptophan (i-STD) were injected into HPLC.

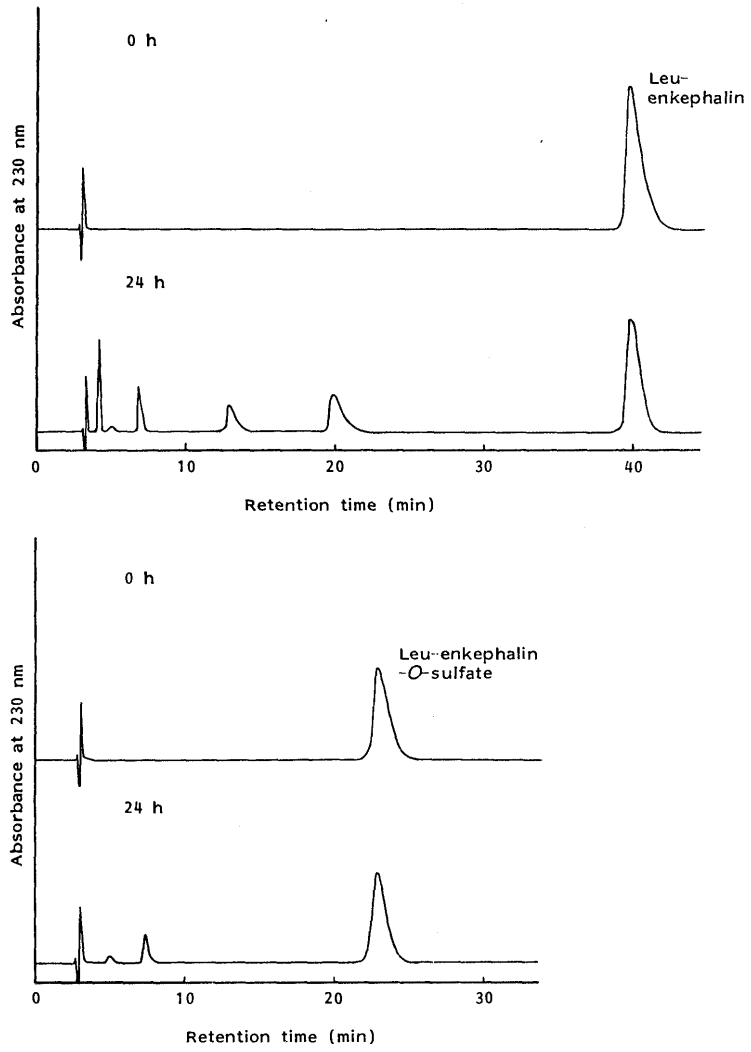

Fig. 2. HPLC Chromatograms of Chymotryptic Digests of Leu-Enkephalin and Leu-Enkephalin-*O*-sulfate

HPLC conditions were the same as Fig. 1, with the exception of the solvent; 20% MeOH/0.1% TFA and wavelength 230 nm. Chymotrypsin (α-chymotrypsin, from bovine pancreas Type I-S) 2 μg was used for each experiment. Final concentrations of Leu-enkephalin and Leu-enkephalin-*O*-sulfate were 1.67 mM.

12% by the following addition of 10^{-5} M enkephalin-*O*-sulfate. Furthermore, the response which was once increased by 10^{-5} M enkephalin-*O*-sulfate, was suppressed by the addition of 3×10^{-7} M enkephalin.

Sensitivity of the Proteases We examined the effects of chymotrypsin, aminopeptidase, carboxypeptidase and pronase on the digestion of several peptides which were sulfated by this bacterial sulfotransferase. In the preliminary experiments, Ac–Tyr–NH_2 and Bz–Tyr–NH_2 were sulfated by the enzyme and then incubated with chymotrypsin for 24 h. Chymotryptic hydrolysis of both sulfated tyrosine amides decreased half and one-tenth, respectively, of that of both original tyrosine amides. Leu-enkephalin and Leu-enkephalin-*O*-sulfate were hydrolyzed by chymotrypsin (Fig. 2). The former was hydrolyzed to amino acid fragments as is expected from the specificity of chymotrypsin. Tyrosine and leucine were detected at 4 and 5 min, respectively, on HPLC analysis. Three other peptides were detected at 7, 13 and 20 min which may correspond to Gly–Gly–Phe, Gly–Gly–Phe–Leu and Tyr–Gly–Gly–Phe, respectively (Fig. 2 upper). However, Leu-enkephalin-*O*-sulfate was hydrolyzed to leucine and Tyr(OSO_3H)–Gly–Gly–Phe which were detected at the retention time of 5 and 8 min (Fig. 2 lower). These results show that the Tyr(OSO_3H)–Gly bond was not hydrolyzed by chymotrypsin. Furthermore, the effects of aminopeptidase, which is known as enkephalin-degradating peptidase, were analyzed. Leu-enkephalin was rapidly hydrolyzed to tyrosine and Gly–Gly–Phe–Leu which was detected at 21 min in HPLC (Fig. 3 left) and confirmed with DABITC's (4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyanate) method for N-terminal amino acid analysis. On the other hand, Leu-enkephalin-*O*-sulfate was completely stable against aminopeptidase digestion under the same condition. When a 10-fold higher amount of aminopeptidase was added to the sulfated peptide solution and incubated longer, a small amount of digested peptide, Gly–Gly–Phe–Leu, was detected (Fig. 3 right). Digestibility of Tyr(OSO_3H)–Gly-bond by aminopeptidase was calculated to be only 2.5 percent of that of Tyr–Gly-residue. Kyotorphin and its sulfate ester were treated with carboxypeptidase B (Fig. 4). Kyotorphin was hydrolyzed about 40% to tyrosine and arginine after 24 h of incubation. But the sulfate ester was completely resistant to the protease. Also, pronase (10 tyrosine unit for 5×10^{-5} M substrate) was used for hydrolysis of kyotorphin and its

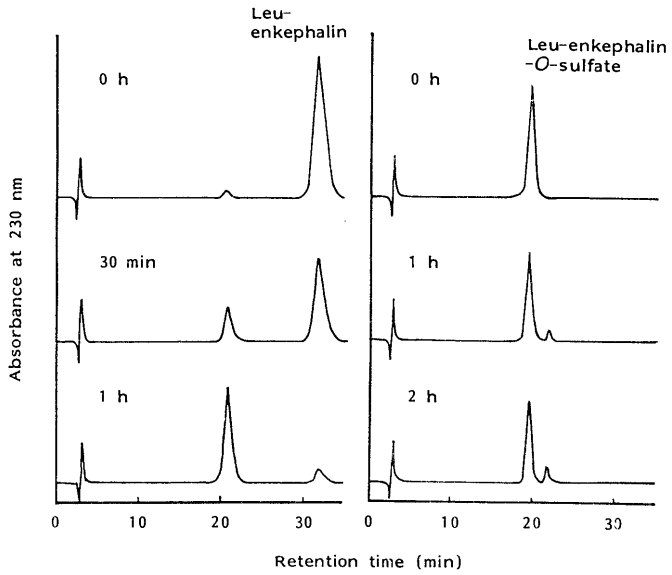

Fig. 3. HPLC Chromatograms of Aminopeptidase Digests of Leu-Enkephalin and Leu-Enkephalin-*O*-sulfate

HPLC conditions: column, Develosil ODS 7 μm (4.6 × 250 mm); solvent, 40% MeOH/0.1% TFA; flow rate, 1.0 ml/min; wavelength, 230 nm. Aminopeptidase (leucine aminopeptidase, cytosol, from porcine kidney Type III-CP) 4 μg (200 U/mg protein) was used for Leu-enkephalin degradation and 40 μg was used for Leu-enkephalin-*O*-sulfate. Final concentrations of Leu-enkephalin and Leu-enkephalin-*O*-sulfate were 3.3×10^{-4} M.

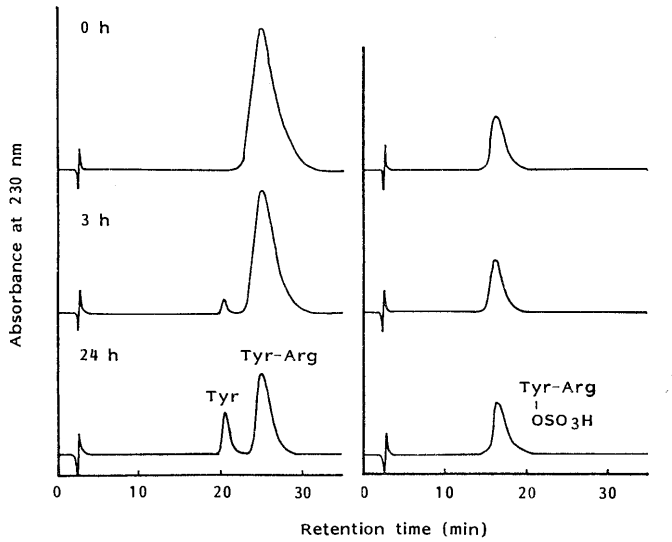

Fig. 4. HPLC Chromatograms of Carboxypeptidase B Digests of Kyotorphin and Kyotorphin-*O*-sulfate

HPLC conditions were the same as Fig. 3, with the exception of the solvent; 5% MeOH/0.1% TFA. Carboxypeptidase B (from porcine pancreas) 1.1 μg (130 U/mg protein) was used for each measurement. Final concentrations of kyotorphin and kyotorphin-*O*-sulfate were 5×10^{-5} M.

sulfate ester and the results were the same as the effect of carboxypeptidase B (data not shown).

Discussion

Several investigators have studied the elucidation of the functions of O-sulfation of phenolic compounds by phenol sulfotransferase which is distributed in various animal organs.[24,25] The enzyme requires only PAPS as a sulfate donor. Recently, we isolated a novel type of sulfotransferase obtained from an anaerobic bacterium of human intestine because the donor of this bacterial enzyme is not PAPS, but phenol sulfate esters.[14] The present results show that some drugs and catecholamines are good acceptors of this enzyme (Table I). By human brain phenolsulfotransferase, dopamine was mainly sulfated at the 3-position of hydroxy residues.[26] However, our results of dopamine and *o*, *m*, *p*-acetamidophenol showed that this bacterial enzyme catalyzes sulfation at the 4-position of aromatic hydroxy groups. This finding also supports that this enzyme is a novel type of sulfotransferase.

Our observation indicated that pharmacological activity of tyramine was inactivated by enzymatic sulfation. Tyramine is the metabolite of tyrosine which is formed by decarboxylase in human intestinal bacteria. Since tyramine is known to be a false neurotransmitter of dopaminergic neuron,[27] sulfation by bacterial sulfotransferase might be a detoxification pathway of tyramine in the human intestine. Furthermore, our finding showed that enkephalin-*O*-sulfate was not the opiate agonist. This result is the same as the biological activity of Leu-enkephalin inhibited by tyrosine sulfation in the brain.[28] Similarly, angiotensin-II was sulfated at its tyrosine residue by this bacterial sulfotransferase, and the sulfated angiotensin-II was less potent than angiotensin-II for contraction of isolated smooth muscles.[29] These findings suggest that sulfation of phenolic compounds, or some tyrosine-containing peptides, by sulfotransferase represented the detoxification reaction in human intestine. On the other hand, some peptide hormones (CCK, caerulein, phyllocaerulein) show characteristic biological activities only in sulfated forms.[30,31] CCK-8 (nonsulfate) was a good acceptor of this enzyme.[16] Therefore, this bacterial sulfotransferase is considered to take part not only in the detoxification, but also in the activation, of endogenous and exogenous phenolic functional compounds.

In addition, biological functions of peptides were inactivated by enzymatic degradation in the body.[32] It is well known that the physiological activity of enkephalin is transient because of its rapid degradation by blood and brain proteases.[33] It has been observed that chymotryptic cleavage *in vitro* does not occur at sulfated tyrosine residues in caerulein and in yolk protein 2.[34] We examined the sensitivity of *O*-sulfated peptides to proteolytic cleavage, and found that the C-terminal side of sulfated tyrosine of test peptides was resistant to proteolytic digestion. Therefore, it is expected that when the tyrosine residues of hormonal peptides were sulfated by sulfotransferase, these peptides would not be degradated immediately in the body, suggesting that tyrosine-sulfation plays an important role in the stability of peptides in the body.

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Pharmacokinetics of Caffeine and Its Metabolites in Horses after Intravenous, Intramuscular or Oral Administration

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The pharmacokinetics of caffeine (CAF) and its metabolites, dimethylxanthines, were examined in horses administered 2.5 mg/kg of CAF intravenously (i.v.), intramusculary (i.m.), or orally (*p.o.*). The plasma samples were extracted by Extrelut® and the concentrations of CAF and metabolites were determined by high performance liquid chromatography (HPLC) with a short column. The pharmacokinetics of CAF after bolus i.v. injection were described by the assumption of a two-compartment model, and those of CAF after i.m. or *p.o.* administration were done by the assumption of a one-compartment model. The biologic half lives of CAF were 15.5, 18.6, and 16.4 h after administering i.v., i.m. and *p.o.*, respectively. The extent of the bioavailability of the *p.o.* administration was determined as 1.04 times the dose. The differences in pharmacokinetic parameters were not statistically significant among administration routes. A straight correlation existed between the logarithms of body weights of different species of animals and those of their biologic half lives of CAF. Therefore, the biologic half life of CAF in an animal might be predictable as a function of its body weight.

Keywords caffeine; pharmacokinetics; dimethylxanthine; horse; metabolite

Introduction

Caffeine (CAF), which is not only consumed as a drug but also contained in many beverages, has pharmacological effects as a central nervous stimulant, cardiotonic and diuretic. CAF is utilized in horses in the clinical treatment of colic, heat stroke, disturbances of circulation and fatigue. In addition, CAF has been used as a doping drug in races. Although the use of this drug in horse races is prohibited by law, due to its widespread usage, it has been detected in doping tests at our laboratory.

The pharmacokinetics of CAF were reported in men,[1] monkeys,[2] rabbits,[3] rats,[1a,4] mice,[5] dogs,[6] and Chinese hamsters,[7] and are known to be variable in these species. Greene *et al.* reported the pharmacokinetics of CAF in horses,[8] but not those of its metabolites. CAF and its metabolites have been determined in biological fluid.[9] CAF has eighteen metabolites,[10] in which we extracted CAF and theophylline (TPL), theobromine (TBM) and paraxanthine (PXT) of dimethylxanthins in horse plasma, using Extrelut®. CAF and dimethylxanthines extracted were separated in a short time by high performance liquid chromatography (HPLC) with a short column.

In this paper the pharmacokinetics of CAF and dimethylxanthines in horses after intravenous (i.v.), intramuscular (i.m.) or oral (*p.o.*) administration were investigated.

Experimental

Materials Twenty percentage CAF-sodium benzoate for injection, CAF, TPL and TBM were obtained from Fuso Yakuhin Kogyo Inc., Sanko Seiyaku Kogyo Inc., Wako Pure Chemical Industries Ltd. and Tokyo Kasei Kogyo Co., Ltd., respectively. PXT and β-hydroxyethyltheophylline as internal standard (I.S.) were from Sigma Chemical Co. The reagents obtained from Nacalai Tesque Inc. were of HPLC grade and special grade. Extrelut® was from Merck.

The mixture solutions of CAF, TPL, TBM and PXT (0.2—200 μg/ml in methanol) and the I.S. solution (100 μg/ml in methanol) were stored at −20 °C.

Methods of Experiments with Animals Healthy riding horses which had free access to water and were fed three times a day were used and animal parameters in the present study are shown in Table I. CAF at a dose of 2.5 mg/kg was administered i.v., i.m. and *p.o.* Each group consisted of 4 or 5 horses. The 20% CAF-sodium benzoate was injected *via* the jugular vein or the jugular muscle at a dose of 2.5 mg/kg of CAF when converted to CAF. In the case of *p.o.* administration, 2.5 mg/kg of CAF was challenged with 2 l of water by a catheter. All administrations were performed 3 h after the morning feeding. The horses were kept in a stall for one day after the treatments and were used for riding purposes the day after. Each administration was carried out at an interval longer than four weeks. Blood samples were collected into heparinized tubes from the jugular vein and the plasma separated immediately. The plasma samples were stored at −20 °C until assay.

Methods of Assay Two ml of plasma and 4 ml of 0.4 M phosphate buffer (pH 5.3) were added to the test tubes containing 2 μg of I.S. Three ml of the mixture was put onto an Extrelut column which was a glass column (12 mm i.d.) packed with one quarter of a package of 20 ml Extrelut. After 10 min, the column was eluted with 25 ml of chloroform, and then the elute was evaporated at 40 °C. The residue was dissolved in 200 μl of a mobile phase of HPLC and a 20 μl of aliquot was injected into HPLC.

The Hitachi 655 HPLC system with a pre-column filter of 2 μm mesh and Cosmosil $3C_{18}$ packed column (50 × 4.6 mm i.d.; Nacalai Tesque Co., Ltd.) was used. The mobile phase was 1% triethylamine solution (adjusted

TABLE I. Animal Parameters

Horse No.	Strain	Sex	Age (year)	Body weight (kg)
1	HB	F	7	501
2	HB	F	6	554
3	AA	G	8	530
4	TB	F	4	450
5	TB	G	9	550
6	AA	F	4	488
7	AA	F	4	448
8	TB	F	4	460

HB, half-bred; AA, anglo-arab; TB, thoroughbred; F, female; G, gelding.

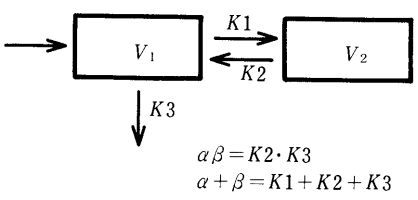

Fig. 1. Two Compartment Open Model

$K1$, $K2$, $K3$: Rate constant of disposition. V_1, V_2: Volume of each compartment.

to pH 5.3 with phosphoric acid) and tetrahydrofuran (98.75 : 1.25 v/v). The flow rate of the mobile phase was 1.0 ml/min at 40 °C. The ultraviolet (UV) absorbance (Hitachi 638 UV detector) was monitored at 274 nm.

Pharmacokinetic Analysis of Data The pharmacokinetic parameters are as follows. CL_{tot}: Total body clearance. D: Dose of CAF. F: Extent of bioavailability of CAF. K_a: Absorption rate constant of CAF. K_e: Elimination rate constant of CAF. V_d: Distribution volume of CAF. Cl_m: Metabolic clearance of CAF. K_{em}: Elimination rate constant of metabolite. V_m: Distribution volume of metabolite.

A schematic representation of the two compartment open model is shown in Fig. 1. After bolus i.v. injection, the plasma concentration of CAF in systemic circulation is described by Eq. 1 based on the assumption of a two-compartment model.

$$B(t)=\{D/V_1(\alpha-\beta)\}\{(\alpha-K2)\cdot\exp(-\alpha t)+(K2-\beta)\cdot\exp(-\beta t)\} \quad (1)$$

After i.m. or *p.o.* administration, the plasma concentration of CAF is described by Eq. 2 based on the assumption of the one compartment model.

$$B(t)=\{K_aDF/V_d(K_a-K_e)\}\{\exp(-K_et)-\exp(-K_at)\} \quad (2)$$

The plasma concentrations of metabolites are described by Eq. 3.

$$B_m(t)=\int_0^t B(t-\theta)(Cl_m/V_m)\cdot\exp(-K_{em}\theta)d\theta \quad (3)$$

In this equation, Cl_m and V_m can't be calculated separately. Cl_m/V_m is replaced with K, and then Eq. 3 can be Eq. 4.

$$B_m(t)=\int_0^t B(t-\theta)K\cdot\exp(-K_{em}\theta)d\theta \quad (4)$$

The experimental values measured in this study were put into Eqs. 1 and 2 and the parameters, *i.e.*, V_1, α, β, $K2$, V_d, K_a, K_e, for the phamacokinetics of CAF were computed from the equations using the no-linear least square method. K and K_{em} for CAF metabolites were calculated by the same method as the pharmacokinetic analysis of CAF, applying the parameters for $B(t)$ which were obtained from Eqs. 1 and 2 to Eq. 4.

Results and Discussion

Determination of CAF and its Metabolites The recoveries of TPL and PXT which were extracted from Extrelut decreased at pH 8 or above, whereas those of CAF, TBM, and I. S. were independent of the pH at the range of pH 4.3—9.0. Therefore, the plasma samples were extracted at pH 5.3. The recoveries of CAF, dimethylxanthines and I.S. (0.1 and 1.0 μg/ml plasma) were 90.3—99.7%, and the coefficients of variations in one day were 0.5—3.9%. Other metabolites such as monomethylxanthines and uric acids were not extracted by the present method. The minimal detectable amount of CAF and its metabolites was 0.01 μg/ml. The calibration curves of CAF and its metabolites were linear at the range of 0.01—5.0 μg/ml.

CAF and dimethylxanthines were separated in 8 min by

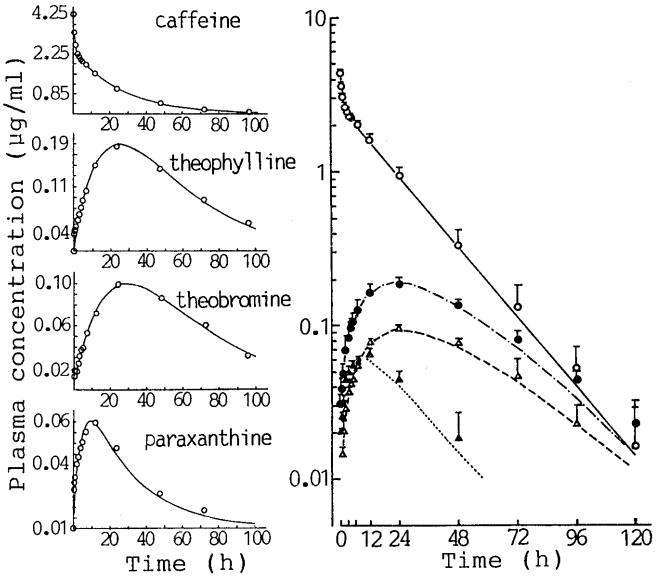

Fig. 3. The Time Courses of Plasma Concentration of CAF and Its Metabolites after Bolus i.v. Administration of CAF

CAF-sodium benzoate was administered at the dose of 2.5 mg/kg of CAF when converted to CAF. The left figures show the results of horse No. 4 and the right figure represents the means ± S.D. of four horses (Nos. 1, 2, 3 and 4). The lines were produced by Eqs. 1 and 4, in the text. CAF (—○—), TPL (—●—), TBM (---△---) and PXT (---▲---).

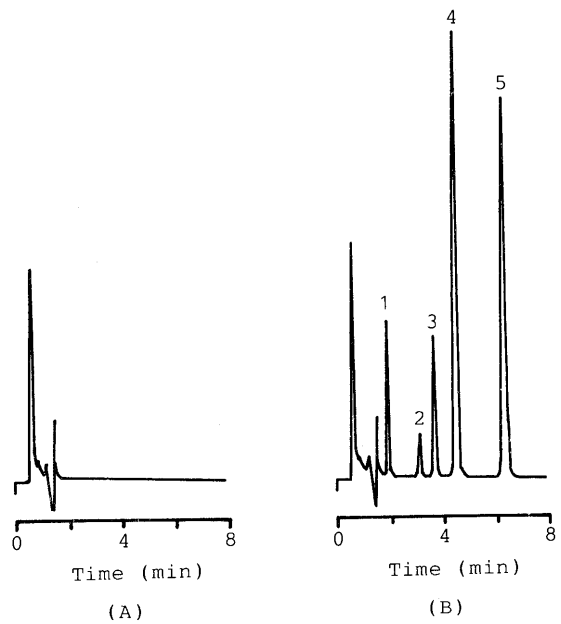

Fig. 2. HPLC Chromatograms of Blank Plasma (A) and CAF-Administered Plasma (B)

TBM (1), PXT (2), TPL (3), I.S. (4) and CAF (5).

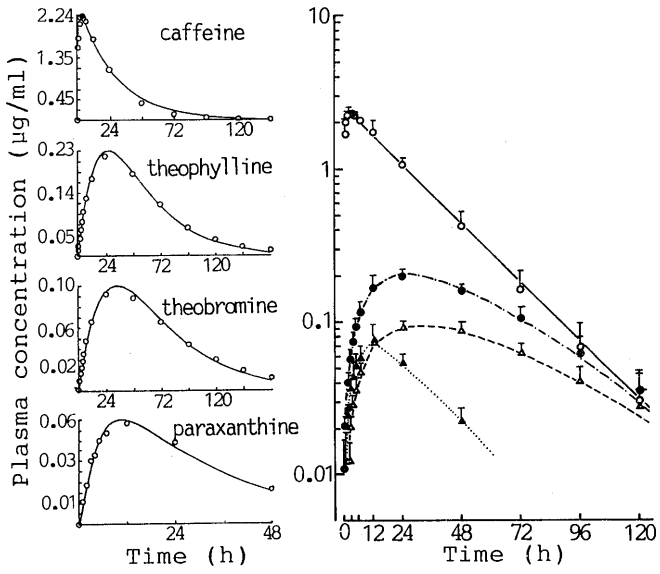

Fig. 4. The Time Courses of Plasma Concentration of CAF and Its Metabolites after i.m. Administration of CAF

CAF-sodium benzoate was administered at the dose of 2.5 mg/kg of CAF when converted to CAF. The left figures show the results of horse No. 4 and the right figure represents the means ± S.D. of five horses (Nos. 3, 4, 5, 6 and 7). See also legend to Fig. 3.

a short column as shown in Fig. 2.

Pharmacokinetics of CAF in Horses Figures 3, 4 and 5 show the time courses of the plasma concentrations of CAF and its metabolites after i.v., i.m. or *p.o.* administration of 2.5 mg/kg of CAF. Symbol marks in the figures represent experimental values, and lines are produced by Eqs. 1, 2 and 4. The simulated curves fitted to the experimentally observed values. These results demonstrate that the pharmacokinetics of CAF and its metabolites can be expressed by these equations, and that no rate limited procedure was found in the absorption of CAF, the production of metabolites or the elimination of CAF and metabolites at this dose.

The pharmacokinetic parameters of CAF and its metabolites in horses, which were calculated from the data shown in Figs. 3, 4 and 5 and the equations, are shown in Tables II, III and IV. The differences in the parameters were not statistically significant except V_d between i.m. and *p.o.* administrations. It is likely that the difference in V_d was due to apparent values brought by the calculation since the Cl_{tot} of the i.m. administration was not different from that of the *p.o.* administration, although V_d equals Cl_{tot}/K_e. The present study was not carried out at a cross-over and V_d was affected by small variations of the measured values. The difference of V_d may result from the variations of V_d in different horses.

The ratio of average area under the blood concentration curve (*AUC*) of CAF after *p.o.* to that after i.v. was 1.04, hence the extent of bioavailability was estimated to be approximately 1. Consequently, CAF was considered to be almost completely absorbed and transported into systemic circulation. This hypothesis was confirmed by the following results. The differences in K and the ratio of (*AUC* of metabolite)/(*AUC* of CAF) in all of the three determined metabolites were not statistically significant between i.v. and *p.o.* administrations. Even after *p.o.* administration, most of the metabolites of CAF detected in the plasma were considered to be from CAF in systemic circulation and the first pass effect may be negligible.

The results presented in this study reveal that the pharmacokinetic behavior of CAF in the horse is only slightly influenced by the route of administration.

Figure 6 shows a straight correlation between the logarithms of the body weights and of the biologic half lives of CAF in mammalian species. Inspite of the different species, as shown in this figure, biologic half life $T_{1/2}$ might

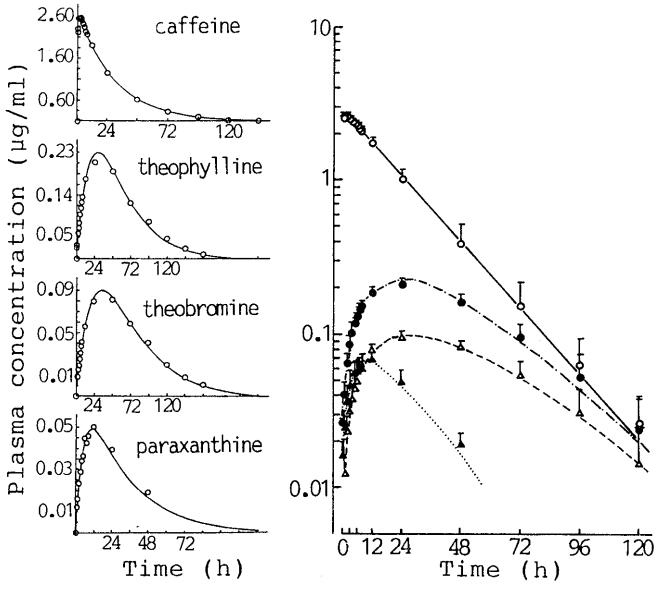

Fig. 5. The Time Courses of Plasma Concentration of CAF and Its Metabolites after *p.o.* Administration of CAF

CAF at the dose of 2.5 mg/kg was administered. The left figures show the results of horse No. 6 and the right figure represents the means ± S.D. of four horses (Nos. 2, 5, 6, and 8). See also legend to Fig. 3.

TABLE II. Pharmacokinetic Parameters of CAF and Its Metabolites after i.v. Administration of CAF

Compd.	Parameter	Horse No. 1	2	3	4	Mean ± S.D.
CAF	α (h^{-1})	2.50	2.13	1.68	2.26	2.14 ± 0.30
	$T_{1/2\alpha}$ (h)	0.28	0.33	0.42	0.31	0.34 ± 0.05
	β (h^{-1})	0.0549	0.0497	0.0398	0.0382	0.0457 ± 0.0069
	$T_{1/2\beta}$ (h)	12.6	13.9	17.4	18.1	15.5 ± 2.3
	$K2$ (h^{-1})	1.202	0.984	1.005	1.149	1.085 ± 0.093
	V_1 (l/kg)	0.413	0.415	0.526	0.460	0.454 ± 0.046
	V_d (l/kg)	0.862	0.898	0.881	0.905	0.887 ± 0.017
	AUC (μg·h/ml)	52.8	56.0	71.3	72.3	63.1 ± 8.8
	Cl_{tot} (ml/h·kg)	47.3	44.6	35.1	34.6	40.4 ± 5.6
TPL	K (h^{-1})	0.0104	0.0095	0.0068	0.0076	0.0086 ± 0.0014
	K_{em} (h^{-1})	0.0481	0.0414	0.0425	0.0398	0.0430 ± 0.0031
TBM	K (h^{-1})	0.0043	0.0036	0.0034	0.0038	0.0038 ± 0.0004
	K_{em} (h^{-1})	0.0410	0.0332	0.0314	0.0297	0.0338 ± 0.0043
PXT	K (h^{-1})	0.0089	0.0077	0.0083	0.0065	0.0079 ± 0.0009
	K_{em} (h^{-1})	0.278	0.222	0.219	0.213	0.233 ± 0.026

TABLE III. Pharmacokinetic Parameters of CAF and Its Metabolites after i.m. Administration of CAF

Compound	Parameter	Horse No. 3	4	5	6	7	Mean ± S.D.
CAF	K_a (h^{-1})	2.06	1.56	3.26	2.60	1.04	2.10 ± 0.78
	K_e (h^{-1})	0.0344	0.0355	0.0419	0.0322	0.0467	0.0381 ± 0.0054
	$T_{1/2}$ (h)	20.2	19.6	16.5	21.6	14.9	18.6 ± 2.5
	V_d (l/kg)	1.003	1.015	0.936	0.955	0.915	0.965 ± 0.038
	AUC (μg·h/ml)	72.6	69.5	63.8	81.4	58.6	69.2 ± 7.8
	Cl_{tot} (ml/h·kg)	34.4	36.0	39.2	30.7	42.7	36.6 ± 4.1
TPL	K (h^{-1})	0.0076	0.0097	0.0096	0.0064	0.0114	0.0090 ± 0.0017
	K_{em} (h^{-1})	0.0381	0.0419	0.0472	0.0294	0.0480	0.0409 ± 0.0068
TBM	K (h^{-1})	0.0034	0.0033	0.0038	0.0021	0.0046	0.0034 ± 0.0008
	K_{em} (h^{-1})	0.0268	0.0255	0.0291	0.0186	0.0404	0.0281 ± 0.0071
PXT	K (h^{-1})	0.0088	0.0071	0.0086	0.0057	0.0073	0.0075 ± 0.0011
	K_{em} (h^{-1})	0.190	0.218	0.183	0.354	0.142	0.217 ± 0.073

TABLE IV. Pharmacokinetic Parameters of CAF and Its Metabolites after *p.o.* Administration of CAF

Compd.	Parameter	Horse No. 3	5	6	8	Mean±S.D.
CAF	K_a (h^{-1})	5<	4.51	2.86	4.38	3.92±0.75[a]
	K_e (h^{-1})	0.0457	0.0468	0.0322	0.0500	0.0437±0.0068
	$T_{1/2}$ (h)	15.2	14.8	21.5	13.9	16.4±3.0
	V_d (l/kg)	0.861	0.845	0.883	0.933	0.881±0.033
	AUC (μg·h/ml)	63.5	63.2	82.8	53.6	65.8±10.6
	Cl_{tot} (ml/h·kg)	39.4	39.6	30.3	46.6	39.0±6.7
TPL	K (h^{-1})	0.0085	0.0103	0.0082	0.0125	0.0099±0.0017
	K_{em} (h^{-1})	0.0422	0.0478	0.0384	0.0527	0.0453±0.0054
TBM	K (h^{-1})	0.0037	0.0040	0.0025	0.0043	0.0036±0.0007
	K_{em} (h^{-1})	0.0287	0.0323	0.0253	0.0402	0.0316±0.0055
PXT	K (h^{-1})	0.0079	0.0086	0.0046	0.0082	0.0073±0.0016
	K_{em} (h^{-1})	0.182	0.233	0.209	0.233	0.212±0.019

a) Mean±S.D. of three horses (Nos. 5, 6, and 8).

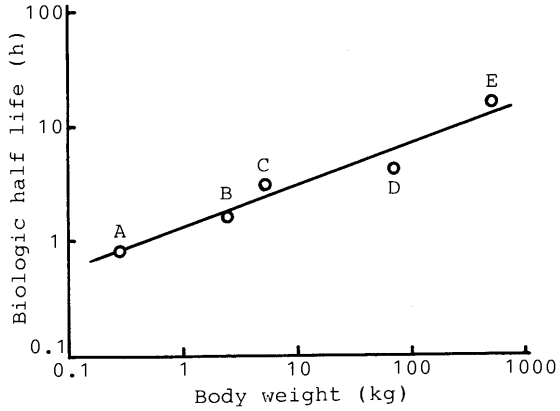

Fig. 6. Biologic Half Life of CAF in Mammalian Species as a Function of Body Weight

Rat (A), rabbit (B), monkey (C), human (D) and horse (E). A, B, C and D are referred to Bonati, *et al.*[10]

be describable with Eq. 5 as a function of body weight W.

$$T_{1/2}=AW^B \quad (5)$$

In this equation, A and B are constants. When the unit of $T_{1/2}$ is h and that of W is kg, the values of A and B calculated by the least square method were 1.315 and 0.369, respectively and the correlation coefficient was 0.969. It is suggested that the biologic half life of CAF can be calculated by the body weight of an animal.

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Multiple and Irreversible Binding of *cis*-Diamminedichloroplatinum(II) to Human Serum Albumin and Its Effect on Warfarin Binding

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Irreversible bindings of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) to human serum albumin (HSA) were investigated in a pH 7.4 buffer containing 0.1 M NaCl at various molar ratios (*cis*-DDP/HSA) up to 60 over a 14 d period (37 °C). The metal binding seemed to reach a plateau when incubated at less than 10 times excess of *cis*-DDP. As the molar ratio increased, the reaction rate was relatively fast within the first day, followed by a moderate increase in the metal binding. When incubated at 60 times excess of *cis*-DDP, the metal bound as much as 20 mol per mol of HSA in 14 d. Fluorescence quenching of the metal-bound protein suggested that the tryptophan residue was gradually exposed to a hydrophilic environment as the metal binding increased. Furthermore, *cis*-DDP cleaved disulfide bonds at the ratio of 1 mol of disulfide bond per 5.3 mol of the metal binding. It was therefore suggested that the metal binding also occurred at several sites other than the disulfide bond. Warfarin binding to the metal-bound protein, examined by fluorescence changes, also decreased with increasing metal binding or cleavage of the disulfide bonds. Thus, *cis*-DDP bound to multiple sites in addition to the lone sulfhydryl group (Cys-34), suggesting that massive conformational changes of the protein took place.

Keywords *cis*-diamminedichloroplatinum(II); human serum albumin; protein binding; irreversible drug binding; disulfide bond; conformational change; warfarin

Introduction

cis-Diamminedichloroplatinum(II) (*cis*-DDP) has been widely used as a potent antitumor drug.[1] The drug is a unique metal complex with a square planar structure, and may be regarded as a bifunctional alkylating agent and an intercalater for deoxyribonucleic acid (DNA). *cis*-DDP reacts with naturally occurring small molecules, cysteine and methionine, as well as biomacromolecules such as proteins, enzymes and DNA.[2-5] The mechanism of the reaction with these substances is attributed to a strong nucleophilic displacement reaction for one or both of the coordinated chlorides *via* the aquated forms.[6-8]

The reaction of *cis*-DDP with human serum albumin (HSA) attracts attention since the drug is usually administered intravenously and the binding may decrease the effective concentration of the free drug which is responsible for antitumor activity.[9,10] Also, *cis*-DDP may cause intrinsic conformational changes of proteins and modification in association with certain drugs because of its irreversible binding which should be distinguished from ordinary reversible weak protein bindings.[3]

Gonias and Pizzo reported that bovine serum albumin bound 1 mol of platinum per mol of the protein, and that significantly increased binding was not found with higher concentrations of *cis*-DDP or longer incubation periods.[9] Momburg *et al.* reported that when *cis*-DDP was incubated with HSA at an equal molar ratio in a phosphate buffer solution (pH 7.4, 0.1 M NaCl), the lone sulfhydryl group of the protein was involved in the drug binding and another site which was not identified was possible.[10] Furthermore, from fluorescence spectral measurements of *cis*-DDP–HSA and HSA alone, no significant modification of the protein conformation was concluded. Also, no affinity change was found in warfarin binding to *cis*-DDP–HSA.

However, Lempers *et al.* reported that a monofunctional platinum compound [PtCl(dien)]Cl reacts not only with the glutathione sulfhydryl group but also with oxidized glutathione and causes cleavage of the disulfide bond.[11] Since disulfide bonds play a very important role in maintaining the unique structures of proteins and enzymes and, therefore, their biological activities, it is important to clarify whether *cis*-DDP also breaks disulfide bonds in biomacromolecules.

The present study is concerned with whether *cis*-DDP reacts with HSA at more than one or two binding sites in relation to the cleaving activity of the drug to the disulfide bond of the protein. Furthermore, warfarin binding was examined as a measure of the modification of HSA molecules caused by *cis*-DDP binding.

Experimental

Materials *cis*-DDP was purchased from Aldrich Chemical Co., Milwaukee and used as received. HSA (fraction V, lot no. 76F-9353) was used after being purified by the method of Chen.[12] Disodium 2-nitro-5-thiosulfobenzoate (NTSB) was synthesized according to the method of Thannhauser *et al.*[13] Potassium warfarin (racemate) was a gift from Eisai Co., Tokyo and used as received. All other chemicals were of reagent grade.

Binding of *cis*-DDP to HSA *cis*-DDP was dissolved in a phosphate buffer solution (0.05 M, pH 7.4) with NaCl (0.1 M). HSA was also dissolved in the same medium. Both solutions were mixed at various molar ratios of *cis*-DDP to HSA up to 60 where the final protein concentration was always adjusted to become 1×10^{-5} M. Each mixed solution was filtered through a 0.2 μm sterilized filter (Toyo Roshi, Tokyo) and placed in sterilized test tubes with a screw cap. This operation was always conducted in a glove box to avoid contamination. The mixed solutions were incubated at 37 °C over a 14 d period, being protected from light, during which one of the tubes was separately taken with time for various analyses.

Separation of Unbound *cis*-DDP and HSA At an appropriate time interval, 0.5 ml of the incubation mixture was removed and the HSA and unbound *cis*-DDP fractions were separated by gel filtration. The HSA fraction was obtained by passing it through a column (1.0 cm i.d. × 18 cm) of Sephadex G-25 (Pharmacia, Uppsala) equilibrated with a pH 7.4 phosphate buffer containing 0.1 M NaCl. The fraction of *cis*-DDP was assayed in each 2.0 ml fraction and the sum of the amount in each fraction was assumed to be the total amount of unbound *cis*-DDP in the mixture.

Dialysis of *cis*-DDP and HSA Reaction Mixture The reaction mixtures incubated at various molar ratios (up to 60) for 14 d were processed with Sephadex G-25. The HSA fraction (2 ml) was dialyzed against an external pH 7.4 buffer (2 ml) at 25 °C for 24 h using seamless cellulose tubings (30/32, Union Carbide, New York), and the amount of *cis*-DDP in the dialysate was assayed. No apparatus binding of *cis*-DDP was detected in the range of the concentration examined.

Fluorescent Spectroscopic Measurements The fluorescence spectra of the tryptophan in HSA were recorded on a spectrofluorophotometer (RF-520, Shimadzu, Kyoto) at an excitation wavelength of 300 nm. Relative fluorescence intensity at the emission maximum wavelength of 350 nm was followed with an incubation time at various molar ratios of the components (HSA, 1×10^{-5} M; *cis*-DDP, 1×10^{-5}—4×10^{-4} M). The medium is the same as that used in the binding study.

Determination of Cleaved Disulfide Bond The number of cleaved disulfide bonds of HSA was estimated using the NTSB method.[13] HSA solution (0.5 ml) was mixed with an NTSB assay solution (3 ml) with a freshly-prepared Na_2SO_3 solution (0.4 M). After 10 min incubation, the absorbance at 412 nm was monitored against an appropriate blank. The concentration of the cleaved disulfide bond was calculated by the use of the extinction coefficient of 2-nitro-5-thiobenzoic acid (1.36×10^4 $M^{-1} \cdot cm^{-1}$).

Analyses of *cis*-DDP and HSA *cis*-DDP was analyzed by the method of Ayres and Meyer with some modification.[14] In brief, 0.3 ml of HCl (11.3 N) was added to each fractionated *cis*-DDP solution (2.0 ml). And then 0.6 ml of a 1.0 M $SnCl_2$ solution in 3.4 N HCl was added. The mixed solution was incubated at room temperature for 15 min and the absorbance at 403 nm was recorded on a spectrophotometer. Native HSA concentration was determined using an extinction coefficient $E^{0.1\%}_{1\,cm}$ of 0.531 at 278 nm.[15]

Warfarin Binding to HSA Fluorometric titration was carried out as follows: 3 ml of HSA solution (2×10^{-6} M) incubated with *cis*-DDP at various molar ratios (0—40) for 14 d was placed in a 1 cm fluorescence quartz cell (25 °C), followed by the addition of warfarin solution giving a final concentration ranging from 0.5 to 15×10^{-6} M using a microsyringe. The mixture was excited at 335 nm and the emission was measured at 378 nm using a spectrofluorophotometer (RF-520, Shimadzu, Kyoto).

Results and Discussion

***cis*-DDP Binding to HSA** Figure 1 shows the binding of *cis*-DDP to HSA as a function of time incubation at various molar ratios of *cis*-DDP to HSA over a 14 d period. These results indicate that the reaction between *cis*-DDP and HSA is relatively slow and proceeds further beyond the first covalent binding to the lone sulfhydryl group (Cys-34) of the HSA and a second unknown binding.[10] The characteristics of the time course were: (1) the binding reaction seemed to consist of two stages, the initial faster binding and the subsequent rather moderate increase of the binding, (2) this tendency was little noticeable as the molar ratio decreased, and (3) the binding reactions appeared not to proceed to a common equilibrium, at least over an incubation period of 14 d.

However, such extra bindings of *cis*-DDP may not always be due to covalent binding to the protein. In order to clarify whether the backward reaction of bound *cis*-DDP occurs, HSA fractions which were separated from the mixture and incubated at various molar ratios for 14 d, were dialyzed. As shown in Table I, little metal was detected in the acceptor compartment even after 48 h dialysis, indicating that either the backward reaction is extremely slow or the binding results in the formation of very stable covalent bond.

The results suggest that the binding reaction appears to proceed to a first transient stage of binding, and subsequently, more binding sites available for reaction are exposed to *cis*-DDP, possibly due to conformational changes of the protein.

Fluorescence Study Tryptophan fluorescence is most frequently examined among the three intrinsic aromatic fluors in HSA molecules to obtain information about conformational changes. When HSA is excited at 300 nm, the resulting fluorescence efficiency reflects changes of the microenvironment of tryptophan residue.[16] Figure 2 shows typical changes of fluorescence intensity of the reaction mixture in which *cis*-DDP and HSA were incubated at various molar ratios for 14 d. The fluorescence intensity decreased and the spectrum slightly shifted to red with an increasing molar ratio of the components. The larger the molar ratio, the more the quenching of tryptophan fluo-

TABLE I. Dialysis for HSA Incubated with *cis*-DDP

cis-DDP/HSA	Number of bound platinum (mol/mol of HSA)	Platinum concentration $\times 10^5$ (M)	
		Donor chamber	Acceptor chamber
5	2.59	1.15	0.03
10	4.40	2.63	0.00
20	8.84	5.21	0.05
40	15.1	12.4	0.00
60	19.9	20.4	0.06

HSA (1.0×10^{-5} M) was incubated with *cis*-DDP (5.0×10^{-5}—6.0×10^{-4} M) at pH 7.4, 37 °C for 14 d. HSA fractions were dialyzed immediately after separated by Sephadex G-25.

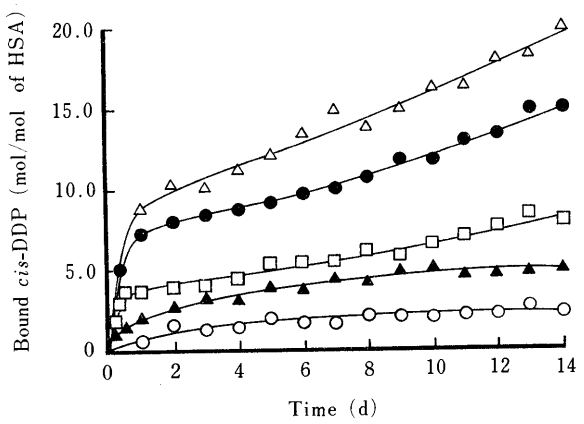

Fig. 1. Kinetics of *cis*-DDP Binding to HSA Incubated at Various Molar Ratios at 37 °C

HSA, 1.0×10^{-5} M. *cis*-DDP ($\times 10^5$ M): ○, 5; ▲, 10; □, 20; ●, 40; △, 60.

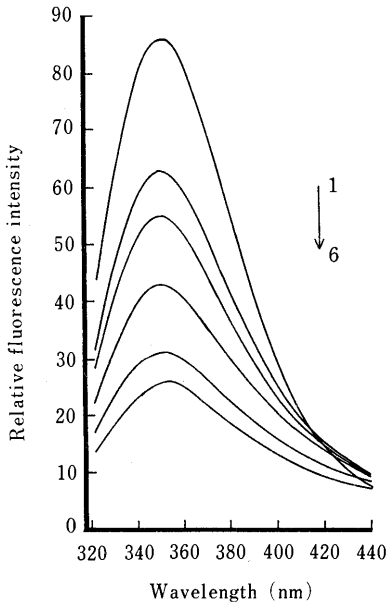

Fig. 2. Fluorescence Spectral Changes of HSA Incubated with *cis*-DDP at Various Molar Ratio for 14 d

HSA, 1.0×10^{-5} M. *cis*-DDP ($\times 10^5$ M): 1, 0; 2, 5; 3, 10; 4, 20; 5, 40; 6, 60. Excitation at 300 nm.

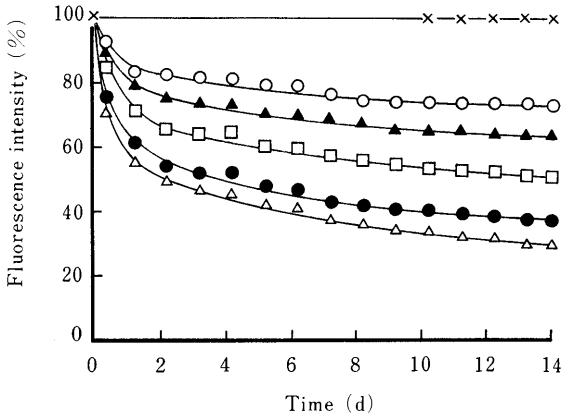

Fig. 3. Time Courses of Relative Fluorescence Changes of HSA Incubated with *cis*-DDP at Various Molar Ratios

HSA, 1.0×10^{-5} M. *cis*-DDP ($\times 10^{5}$ M): ×, 0; ○, 5; ▲, 10; □, 20; ●, 40; △, 60. Excitation at 300 nm and emission at 350 nm.

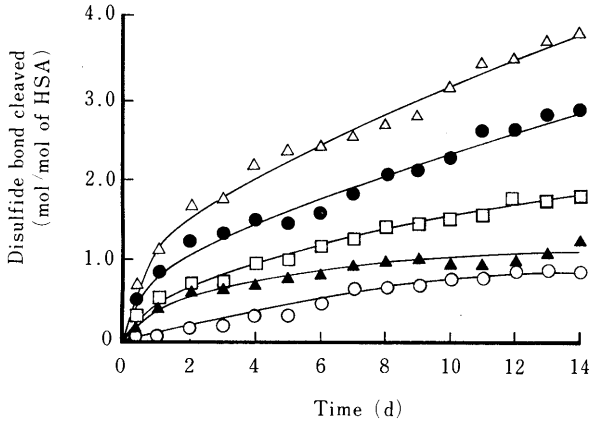

Fig. 4. The Number of the Cleaved Disulfide Bond of HSA with Time

HSA, 1.0×10^{-5} M. *cis*-DDP ($\times 10^{5}$ M): ○, 5; ▲, 10; □, 20; ●, 40; △, 60.

rescence.

According to empirical rules for fluorescent spectra of proteins,[16] the tryptophan (Trp-214) residue of HSA is considered to be brought to a more hydrophilic environment as a result of *cis*-DDP bindings. It may not always be ruled out that the binding occurs at the tryptophan residue or its proximity. Instead, it is very probable that the multiple bindings of *cis*-DDP result in tryptophan exposure to a polar environment, if the bindings are responsible for massive unfolding of the protein.

To interrelate the fluorescence changes to the binding data, the time course of fluorescence quenching was similarly followed under the same conditions. Figure 3 shows the relative fluoresence changes with incubation time at various molar ratios. No change of the fluorescence intensity was observed for the control HSA solution with very good clarity over an incubation period of 14 d. The decreasing time courses of the intensity appears to match well the time courses of *cis*-DDP binding to HSA, indicating that the unfolding of the tryptophan proximity of the protein is closely related to the *cis*-DDP binding. The results also raise a question as to whether the protein undergoes intrinsic conformational changes with *cis*-DDP binding. Especially, if disulfide bond cleavage is involved, remarkable conformational changes of the protein may be quite likely.

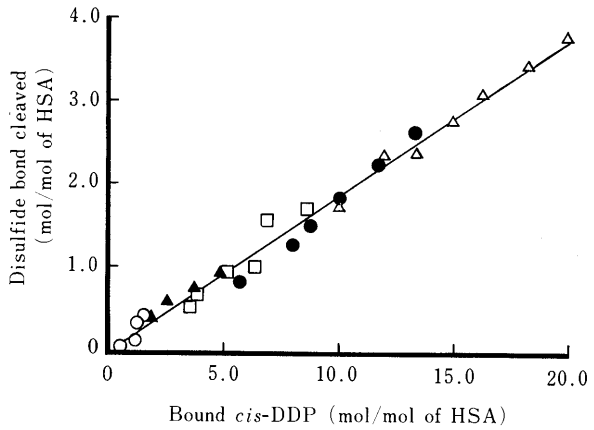

Fig. 5. Relationship between Bound *cis*-DDP and Cleaved Disulfide Bond

Data was taken from Figs. 1 and 4. *cis*-DDP/HSA: ○, 5; ▲, 10; □, 20; ●, 40; △, 60. A linear regression analysis gave $Y = 0.188X - 0.001$ ($r = 0.991$).

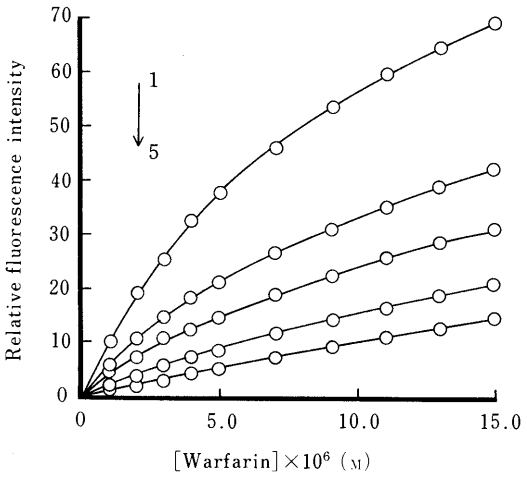

Fig. 6. Relative Fluorescence Changes of Warfarin with *cis*-DDP Bound HSA

Metal-bound HSA (2.0×10^{-6} M throughout) was used after the protein was incubated with *cis*-DDP at different molar ratios for 14 d. *cis*-DDP/HSA: 1, 0; 2, 10; 3, 20; 4, 40; 5, 60. Excitation at 335 nm and emission at 378 nm.

Disulfide Bond Cleavage Figure 4 shows changes in the number of the cleaved disulfide bond of HSA incubated with *cis*-DDP at various molar ratios. HSA has one sulfhydryl group (Cys-34) and 17 disulfide bridges.[17] However, the number of the disulfide bond of native HSA experimentally determined was 14.7 even when the protein was maximally cleaved by sodium sulfite (with 4.3 M guanidine thiocyanate).[18] Because NTSB inherently reacts with a sulfhydryl group, the contribution of the intact sulfhydryl group (Cys-34) was subtracted from the number of cleaved disulfide bond experimentally determined. The time course should be reviewed in connection with the data shown in Figs. 1 and 3. The characteristics of these three different results were very similar to each other, although the fluorescence quenching and the number of cleaved disulfide bond appeared to reach respective plateaus with time while the binding number of *cis*-DDP incubated at the higher molar ratios (20—60) seemed to have an upward tendency.

The relationship between the bound *cis*-DDP and the cleaved disulfide bond of the HSA was plotted, as shown

in Fig. 5. A linear relationship having a slope of about 0.188 ($r=0.991$) was obtained regardless of the initial drug to protein molar ratios. This indicates that as the result of *cis*-DDP bindings, the disulfide bond was cleaved at the ratio of one disulfide bond per 5.3 mol of *cis*-DDP binding in the range of the total bond cleavage up to about 4. However, both numbers may not always match each other in the relationship between the metal binding and the cleaved disulfide bond, if *cis*-DDP binds only to cleaved disulfide bonds in addition to Cys-34. Amino acids which are coordinated with platinum(II) complexes include methionine and histidine other than cysteine.[19] It is therefore very probable that these amino acid residues are also responsible for the multiple bindings of *cis*-DDP.

Binding of Warfarin to *cis*-DDP Bound HSA Warfarin has been known to bind to a site near Trp-214.[20] The ability of *cis*-DDP bound HSA to bind warfarin was examined by fluorescence changes to provide further information around the warfarin binding site. Warfarin has a weak fluorescence at 378 nm when excited at 335 nm, and the addition of HSA induced an increase in fluorescence intensity when warfarin binds to a single site on the protein.[21] Figure 6 shows the relative fluorescence changes of warfarin with different *cis*-DDP bound HSAs as a function of the warfarin concentration. The incubation mixtures were used for this fluorescence measurement without removing unbound *cis*-DDP because preliminary tests confirmed that warfarin fluorecence enhanced by HSA was not interfered with by unbound *cis*-DDP. The intensity changes were therefore considered to be solely due to the magnitude of warfarin binding, which was greatly decreased with increasing metal bindings are increasing cleaved disulfide bonds. While the warfarin binding to HSA is the result of hydrogen bonding and hydrophobic interaction at a site near Trp-214,[22] the warfarin binding site was likely to be considerably perturbed as the disulfide bonds were cleaved, being exposed to polar environment. These results are consistent with the fluorescence quenching of the tryptophan residue of HSA (Fig. 3).

It was reported that when *cis*-DDP was incubated with HSA[10] or bovine serum albumin[9] at an equimolar ratio, the molar binding ratio was 1 : 1. Our results demonstrated that further metal bindings occurred, depending on the drug concentration and time of incubation. It should be especially noted that the multiple metal bindings resulted in the cleavage of disulfide bonds, since the bonds can be considered to be one of the most important linkages for maintaining the integrity and biological function of many proteins. These exhaustive bindings of *cis*-DDP to HSA and the resulting conformational changes suggest that similar effects may produce serious impairment of the biological activity of other essential proteins and enzymes, especially if they occur in a very small amount in the body. The binding mode of *cis*-DDP to the disulfide bond of HSA and the location and order of the disulfide bonds which are attacked by *cis*-DDP remains to be proven.

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Enhanced Rectal Absorption of Insulin in Rabbits from Hollow-Type Suppositories Containing Insulin and Glyceryl-1-monooctanoate[1)]

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The absorption of two kinds of insulin (from porcine or bovine pancreas) from the rectum of rabbits after the administration of hollow-type suppositories containing insulin and glyceryl-1-monooctanoate (GMO) as an absorption-enhancing agent was investigated. Two types of suppositories were employed: type I containing insulin in an aqueous solution (approx. 25 IU/mg/100 μl citric buffer solution at pH 3.0) in the cavity of the suppository and GMO mixed with a base material (Witepsol H-15), and type II containing insulin in a crystalline form in the same amount as in type I. Without GMO, the insulin and glucose levels in plasma were unchanged, whereas a marked increase in the plasma levels of insulin and a decrease of glucose concentrations were found following coadministration of insulin and GMO by the type I suppository. Similar enhancement of rectal absorption of insulin was obtained from porcine and bovine sources. In the case of the crystalline insulin, despite the use of the same amount of GMO, porcine insulin was more efficiently absorbed than bovine insulin by the type II suppository. GMO enhances the absorption of insulin in an aqueous solution or a crystalline form, and the dissolution rate of insulin may be an important factor in the rectal absorption of insulin.

Keywords rectal insulin absorption; absorption-enhancing agent; glyceryl-1-monooctanoate; hollow-type suppository; crystalline insulin; insulin solution; insulin dissolution rate

Introduction

A number of problems, including the inconvenience of injection dosage forms for long-term administration, are associated with the use of insulin, an efficient hypoglycemic peptide drug, used for the treatment of diabetes. Therefore, the development of acceptable alternative methods of insulin administration has been the aim of many investigative efforts. Attempts to achieve rectal absorption of insulin promoted by absorption-enhancing agents (absorption enhancers) such as surfactants,[2,3)] enamines,[4)] bile salts,[5)] sodium salicylate[6)] and sodium tauro-24,25-dihydrofusidate[7)] in animals and humans suggest that the rectal administration of insulin offers promising insulin therapy.

With respect to the rectal dosage form of insulin, a lower bioavailability of insulin was observed when it was dispersed in a glyceride suppository base compared to a crystalline suspension in a gelatin microenema.[8)] Moreover, the difference in drug release between insulin and the absorption enhancer from a conventional suppository has been reported.[9)]

In the present investigation, a new insulin formulation comprising a hollow-type suppository[10)] containing insulin in its cavity and an absorption enhancer in the body (oleaginous base) of the suppository was prepared. The advantages of the use of this suppository are that it can contain either powdered or solution forms of peptide drugs, and it can eliminate the effect of the heating process on the nature of peptides during the preparation of the suppository. Furthermore, earlier studies in our laboratory have indicated that drugs are released more rapidly from a hollow-type suppository than from a conventional suppository.[11,12)] Consequently, drugs, for instance indomethacin[11)] and propranolol,[12)] are absorbed more efficiently from a hollow-type suppository than from a conventional type. These results suggest that the hollow-type suppository is a better possible dosage vehicle than a conventional suppository for the rectal absorption of insulin.

We chose glyceryl-1-monooctanoate (GMO)[13)] as a convenient absorption enhancer to prepare the hollow-type suppository since it mixes well with some of the commercially available oleaginous suppository bases. Two types of hollow suppositories, containing insulin in either aqueous solution or crystalline form, were administered rectally, and the rectal absorption of insulin in rabbits from these suppositories was evaluated.

Materials and Methods

Materials Porcine insulin (26.1 IU/mg containing approx. 0.5% zinc) and bovine insulin (24.5 IU/mg containing approx. 0.5% zinc) were obtained from Sigma, St. Louis, MO, U.S.A. GMO (Poemu M-100®) and a suppository base, Witepsol H-15 (H-15), were kindly supplied by Riken Vitamin, Tokyo, Japan and Hüls Troisdorf, Troisdorf, Germany, respectively. All other reagents used were of analytical grade.

Preparation of Suppositories The two types of hollow suppositories (approximately 2 g) shown in Fig. 1 were prepared using H-15 and GMO by the fusion-process method. Suppository I (type I) containing insulin in a solution form and suppository II (type II) containing insulin in a crystalline form in their cavities were prepared using H-15 mixed with GMO in the body of the suppository. For the control experiments, suppositories containing insulin of the same forms as in types I and II were prepared using H-15 without GMO. The preparation method reported by Watanabe *et al.*[12)] was employed as follows. A sample of H-15 with

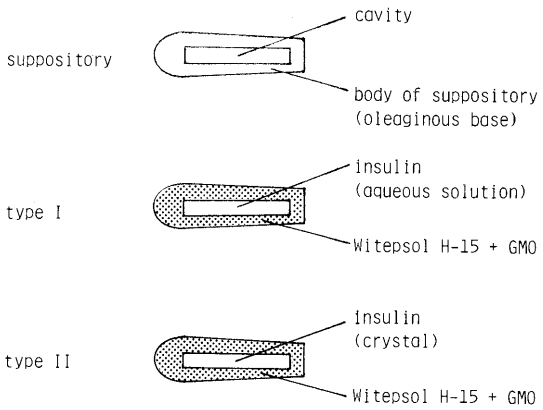

Fig. 1. Schematic Illustration of Hollow-Type Suppositories Containing Insulin with or without GMO

The weight of the suppository is approximately 2 g. For contents of insulin and GMO, see Table I.

various amounts of GMO was melted at approximately 45 °C, and the mixture was poured into a mold equipped with an adapter[12,14] for the preparation of hollow-type suppositories at approximately 30 °C. It was allowed to stand for 2 h at room temperature to solidify. After construction of a hollow cavity of the solidified H-15 and GMO, insulin in a crystalline form or an aqueous solution form was added. The insulin and GMO contents are listed in Table I. When insulin was used in a crystalline form, the dose of insulin was accurately weighed and added to each cavity. For the insulin in an aqueous solution (freshly prepared), crystalline insulin was dissolved at the appropriate concentration in a pH 3.0 citrate buffer solution[15] and 100 μl of insulin solution were added to each cavity. The opening at the hind part of the suppository was sealed with the base material, melted. All suppositories which had been stored in a refrigerator overnight after preparation were examined.

Animal Experiments Male albino rabbits weighing 2.8—3.3 kg with free access to water were fasted for one night prior to each experiment. Each suppository was administered into the rectum according to the method described in our previous reports.[12,16] After rectal administration of the suppository, 2 ml blood samples were taken from the auricular vein by a syringe containing ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) at predetermined time intervals. These samples were centrifuged at 3000 rpm for 15 min to separate plasma. Each plasma sample was stored at −30 °C until assays were performed for insulin and glucose.

Determination of Insulin and Glucose in Plasma The plasma insulin level was determined by the enzyme immunoassay (EIA) method employing an EIA Insulin Test-S kit (Medical & Biological Laboratories, Nagoya, Japan) based on the two-site binding immunoassay. This kit is calibrated for measurement of insulin concentration in the range 3 to 200 μIU/ml. When insulin concentration exceeded the range of the calibration curve, plasma samples were diluted with the buffer solution (a component part of this kit) and insulin was measured in the diluted samples. Multiplying the determined concentration by the dilution multiple, we obtained the actual insulin level in plasma. The assay of glucose in plasma was performed using a glucose-test kit (Wako Pure Chemicals, Tokyo, Japan) based on the *o*-toluidine–boric acid method.

Pharmacokinetic Analysis The peak plasma insulin level (C_{max}) and the peak concentration time (t_{max}) were obtained from individual plasma insulin concentration–time curves. The area under the individual plasma insulin concentration–time curves from 0 to 6 h after rectal administration (AUC_{0-6}) was calculated using the trapezoidal rule. Plasma glucose concentrations after insulin administration were expressed as a mean value of observed concentration.

Statistical analysis of the results was made by the one-way analysis of variance and Dunnett's tests. A significant difference was estimated using $p=0.05$ as the minimal level of significance.

Release Measurement *in Vitro* Insulin release from hollow-type suppositories *in vitro* was determined according to the method described in our previous report[12] using an instrument (model TMS-103, Toyama Sangyo, Osaka, Japan) for measuring drug release from a suppository. The dissolution medium was 250 ml of an isotonic phosphate buffer solution (PBS, pH 7.4) at a constant temperature (37 °C). The medium was stirred at 100 rpm with a polytef-coated magnetic stirring bar placed at the bottom of the sink. A sample of 2 ml of the dissolution medium was taken, and the medium was replenished with the same volume of PBS. Insulin in the medium was assayed by means of an EIA kit following dilution with PBS.

TABLE I. Contents of Insulin and GMO Added in Hollow-Type Suppositories

Source	Insulin Form	Insulin Dose (IU)	Insulin Amount (mg)	GMO (mg)	Volume (μl)
Porcine	Aqueous solution[a]	26.1	1	0	100
		26.1	1	30	100
		26.1	1	100	100
		26.1	1	300	100
		8.2	0.3	100	100
		2.6	0.1	100	100
	Crystal	26.1	1	0	—
		26.1	1	100	—
Bovine	Aqueous solution[a]	24.5	1	0	100
		24.5	1	30	100
		24.5	1	100	100
		24.5	1	300	100
		7.7	0.3	100	100
		2.5	0.1	100	100
	Crystal	24.5	1	0	—
		24.5	1	100	—

a) Insulin was dissolved in a citrate buffer solution (pH 3.0).

Results and Discussion

Plasma Insulin and Glucose Levels after Rectal Administration of Hollow-Type Suppositories Containing Insulin in Aqueous Solution To evaluate the enhancing effect of GMO on the rectal absorption of insulin after the coadministration of insulin and GMO, 1 mg of porcine insulin (26.1 IU) and bovine insulin (24.5 IU) in each suppository was used. To determine the dose, 26.1 or 24.5 IU (approx. 8 IU/kg) of insulin in the suppository, we referred to the reports of Liversidge *et al.*[17] and Aungst *et al.*[18]

Figure 2 illustrates the mean semilog plasma insulin concentration–time curves after rectal administration of type I suppositories containing insulin in an aqueous solution (porcine, 26.1 IU/100 μl or bovine, 24.5 IU/100 μl) in the cavity and various amounts of GMO in the suppository base. The plasma glucose levels observed after administration of type I are shown in Fig. 3. Plasma insulin and glucose concentrations did not change from the physiological levels (endogenous levels represented by the straight line with signs (×) in Figs. 2A and 3A, respectively) observed when the blank suppository containing a pH 3.0 citrate buffer solution without insulin was administered. Plasma insulin and glucose levels (unfilled circles in Figs. 2 and 3) after the administration of suppositories containing insulin without GMO were no different from those obtained by blank suppositories in the control experiments. No significant difference in the AUC_{0-6} values of insulin between the blank suppository and the suppository containing insulin without GMO was found (Fig. 4).

On the other hand, the extent of insulin absorption was indeed significantly enhanced by GMO. Very high plasma levels (C_{max}: 1198 ± 221 μIU/ml (porcine), 1132 ± 226 μIU/ml (bovine)) of insulin and a maximum decrease in plasma glucose concentrations (from 149 to 55 mg/dl (porcine), from 135 to 43 mg/dl (bovine)) were obtained after the coadministration of insulin and 300 mg of GMO (represented by the dotted line with filled circles). When the coadministered amount of GMO was decreased to 30 mg (one-tenth), the insulin concentrations decreased but still were determinable (C_{max}: 105 ± 73 μIU/ml (porcine), 96 ± 24 μIU/ml (bovine)). A decrease in glucose levels (116 ± 25 mg/dl (porcine) and 80 ± 16 mg/dl (bovine) at the minimum values) was also observed.

The mean values of AUC_{0-6} of the two insulins are shown as plain (porcine) and filled (bovine) columns in Fig. 4. In comparing porcine insulin with bovine insulin, there was no statistically significant difference between the mean of AUC_{0-6} observed after the coadministration of insulin and GMO. The absorption-enhancing effect of GMO should have increased by increasing the amount in the suppository. However, no significant difference in AUC_{0-6} was obtained using GMO at amounts between 100 and 300 mg. GMO of 100 mg (a sufficient amount to enhance rectal insulin

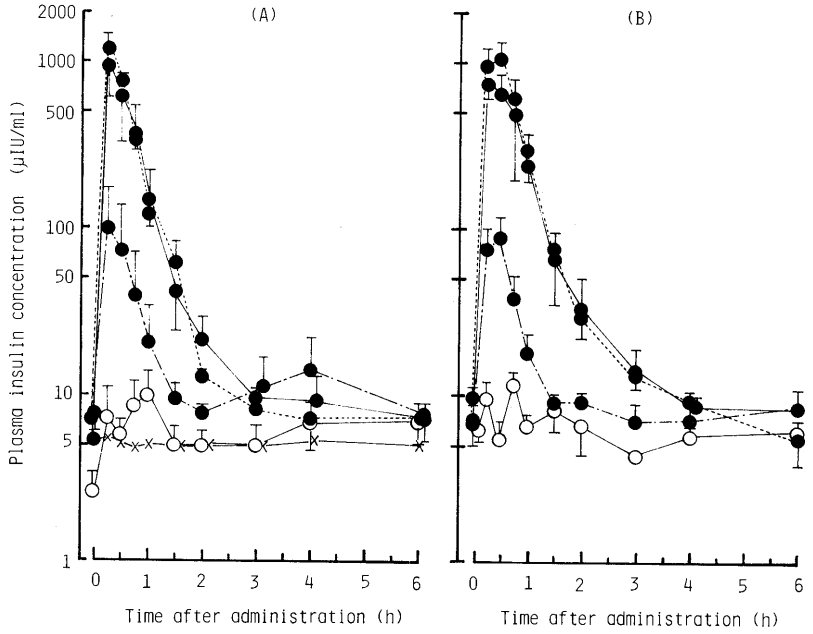

Fig. 2. Mean Plasma Concentration of Insulin Following the Rectal Administration of Hollow-Type Suppositories Containing Porcine (A) or Bovine (B) Insulin in Citrate Buffer Solution (pH 3.0) and Various Amounts of GMO in Rabbits

Each point represents the mean ± S.E. (vertical bar) of 3—5 rabbits. Contents of porcine and bovine insulin in suppositories are 26.1 and 24.5 IU, respectively. ●---●, insulin with 300 mg GMO; ●—●, insulin with 100 mg GMO; ●-·-·-●, insulin with 30 mg GMO; ○—○, insulin without GMO; ×—×, without insulin or GMO (blank suppository).

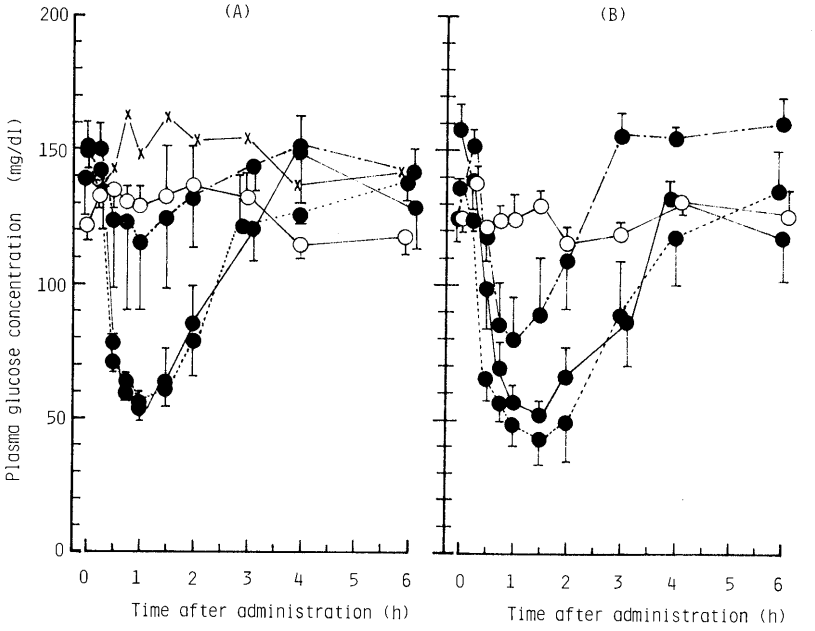

Fig. 3. Mean Plasma Glucose Levels Following Rectal Administration of Hollow-Type Suppositories Containing Porcine (A) or Bovine (B) Insulin in Citrate Buffer Solution (pH 3.0) and Various Amounts of GMO in Rabbits

Each point represents the mean ± S.E. (vertical bar) of 3—5 rabbits. Contents of porcine and bovine insulin in suppositories are 26.1 and 24.5 IU, respectively. ●---●, insulin with 300 mg GMO; ●—●, insulin with 100 mg GMO; ●-·-·-●, insulin with 30 mg GMO; ○—○, insulin without GMO; ×—×, without insulin or GMO (blank suppository).

absorption) was therefore used in hollow-type suppositories in the subsequent experiments.

Plasma Insulin Concentrations after Administration of Suppositories Containing Various Amounts of Insulin in Aqueous Solution High levels of insulin in plasma and a marked decrease in plasma glucose concentration were obtained after the coadministration of insulin (approx. 25 IU) and GMO. To evaluate the plasma insulin concentrations after the administration of insulin in lesser amounts and GMO (100 mg), type I suppositories containing either a porcine insulin solution (8.2 and 2.6 IU/100 μl) or bovine insulin solution (7.7 and 2.5 IU/100 μl) were administered into the rectum. As shown in Fig. 5A, even though the amount of porcine insulin was decreased to 2.6 IU (one-tenth), insulin levels were still obtained (C_{max}: 60 ± 29 μIU/ml). In this case, the minimum glucose level in plasma was 88 ± 26 mg/dl for porcine insulin (Fig. 5B). Concerning a comparison of the C_{max} and AUC_{0-6} values

between porcine and bovine insulin, no significant difference was recognized when insulin in an aqueous solution was administered (Table II).

For rectal insulin delivery using a suppository, various doses of insulin, for instance 2 or 5 IU/kg in rabbits[19] and 1 or 2 IU/kg in humans,[6] were administered with absorption enhancers. With respect to the insulin concentration in plasma or serum after rectal administration, various levels have been reported in human subjects[6] and experimental animals; for example rats,[7,8] rabbits,[19-21] normal[9,21,22] and depancreatized[8,23] dogs. However it is difficult to define the optimal insulin concentration in blood from these reports, since various doses of insulin with absorption enhancers were rectally administered. In our observation in rabbits, high levels of insulin in plasma following the rectal administration of the hollow-type suppository containing insulin solution and GMO were obtained. It is possible to regulate plasma insulin concentrations using the hollow-type suppositories containing insulin at various doses (approx. 2.5—8 IU (0.8—2.7 IU/kg)) combined with at least 100 mg of GMO. Even low contents of insulin in these suppositories, for instance 0.1 mg (approx. 2.5 IU), were effective.

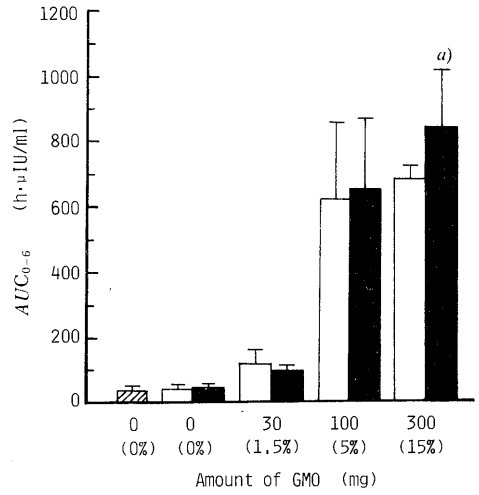

Fig. 4. The *AUC* of Insulin Following Rectal Administration of Hollow-Type Suppositories Containing Porcine or Bovine Insulin in Citrate Buffer Solution (pH 3.0) and Various Amounts of GMO

Column: ▨, without insulin (blank suppository); □, porcine insulin (26.1 IU/100 μl); ■, bovine insulin (24.5 IU/100 μl). The percentages shown in parentheses represent the concentration of GMO in H-15 used to prepare type I suppositories. Each value represents the mean ± S.E. of 3—5 experiments. Statistically significant difference: *a*) $p<0.05$ in 300 mg GMO *vs.* 0 and 30 mg GMO.

Plasma Insulin Concentrations from Hollow-Type Suppositories Containing Crystalline Insulin The hollow-type suppository can contain insulin in a crystalline form instead of aqueous solution. To better understand the effect of the added form of insulin in a suppository on the absorption of insulin, rectal insulin absorption using the crystalline form was evaluated. The plasma insulin concentrations after administration of type II suppositories containing crystal-

Table II. Pharmacokinetic Parameters of Insulin in Rabbits Following Rectal Administration of Hollow-Type Suppositories Containing Various Amounts of Insulin

Insulin			AUC_{0-6}	C_{max}	t_{max}
Source	Form	Dose (IU)	(h·μIU/ml)	(μIU/ml)	(min)
Porcine	Aq. solution	2.6	61 ± 14	60 ± 29	15 ± 0
		8.2	140 ± 24	174 ± 17	20 ± 5
		26.1	610 ± 254	952 ± 344	15 ± 0
	Crystal	26.1	348 ± 148	458 ± 199	27 ± 7
Bovine	Aq. solution	2.5	71 ± 11	63 ± 24	15 ± 0
		7.7	108 ± 11	155 ± 17	20 ± 5
		24.5	652 ± 232	970 ± 265	21 ± 6
	Crystal	24.5	96 ± 20[a)]	60 ± 23[a,b)]	57 ± 17[a,b)]

GMO: 100 mg. Each value represents the mean ± S.E. of 3—5 rabbits. Statistically significant difference: *a*) $p<0.05$ in bovine insulin crystal (24.5 IU) *vs.* bovine insulin solution (24.5 IU), *b*) $p<0.05$ in bovine insulin crystal (24.5 IU) *vs.* porcine insulin solution (26.1 IU).

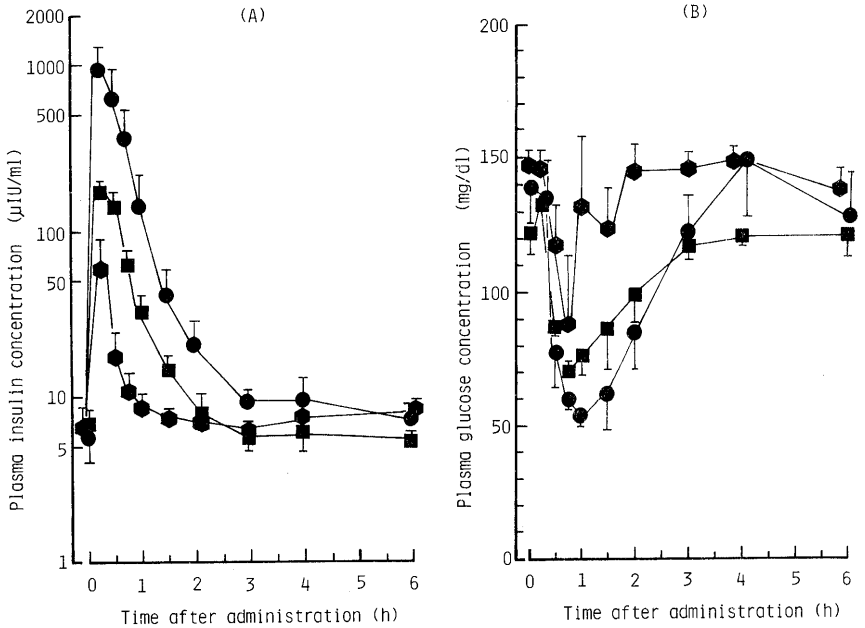

Fig. 5. Mean Plasma Concentrations of Insulin (A) and Glucose (B) Following Rectal Administration of Hollow-Type Suppositories Containing Various Amounts of Porcine Insulin in Citrate Buffer Solution and GMO (100 mg) in Rabbits

Insulin amount: ●—●, 26.1 IU (1.0 mg); ■—■, 8.2 IU (0.3 mg); ●—●, 2.6 IU (0.1 mg). Each point represents the mean ± S.E. (vertical bar) of 3—5 rabbits.

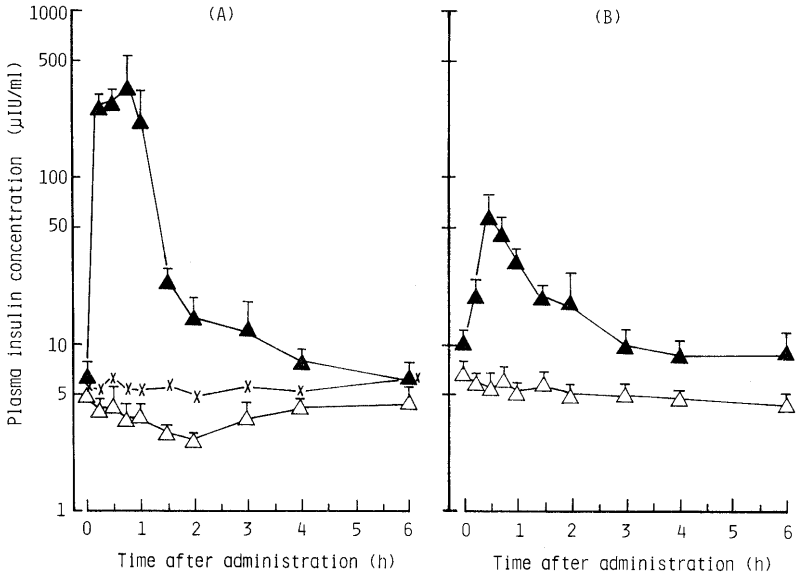

Fig. 6. Mean Plasma Concentrations of Insulin Following Rectal Administration of Hollow-Type Suppositories Containing Porcine (A) and Bovine (B) Insulin in Crystalline Form and GMO in Rabbits

Contents of porcine and bovine insulin in suppositories are 26.1 and 24.5 IU, respectively. ▲—▲, insulin crystal with 100 mg GMO; △—△, insulin crystal without GMO; ×—×, without insulin or GMO. Each point represents the mean ± S.E. (vertical bar) of 3—5 rabbits.

line insulin in their cavities and GMO (100 mg) in the suppository base were determined (Fig. 6). With regard to the dose of insulin, 1 mg of insulin (porcine, 26.1 IU: bovine, 24.5 IU) in suppositories was employed. Suppositories containing less than 1 mg of insulin in crystalline form were not examined bacause of the difficulty of weighing out the insulin accurately into each cavity. Without GMO, no increase in plasma insulin concentration occurred from the suppository containing crystalline insulin applied in the same amount as in the type II suppository (represented by the straight line with unfilled triangles in Fig. 6).

In the case of porcine insulin (Fig. 6A), insulin was efficiently absorbed with GMO (100 mg). Insulin concentrations following the rectal coadministration of insulin in the crystalline form and GMO were similar to the case of the insulin solution (shown by the straight line with filled circles in Fig. 2A). As shown in Table II, the mean values of AUC_{0-6} (348 ± 148 h·μIU/ml) and t_{max} (27 ± 7 min) obtained for the crystalline form were not significant compared to the values (610 ± 254 h·μIU/ml and 15 ± 0 min) obtained using the insulin (26.1 IU) solution.

Interestingly, the results using bovine insulin differed from those employing porcine insulin. Plasma insulin levels observed after the administration of the type II suppository containing bovine insulin in a crystalline form were significantly lower (Fig. 6B) than those obtained using the solution containing the same dose (24.5 IU) of insulin solution for type I (represented by the straight line with filled circles in Fig. 2B). A significant decrease (approximately one-seventh, $p < 0.05$) in AUC_{0-6} was proven and t_{max} was markedly delayed (from 21 ± 6 to 57 ± 17 min) following the use of crystalline bovine insulin (Table II).

Similar values of AUC_{0-6}, C_{max} and t_{max} between porcine and bovine insulin were obtained using the solution form, while a significant difference in those pharmacokinetic parameters between the two insulins was observed when the crystalline form was used. With respect to insulin release from the suppositories *in vitro*, insulin was rapidly released

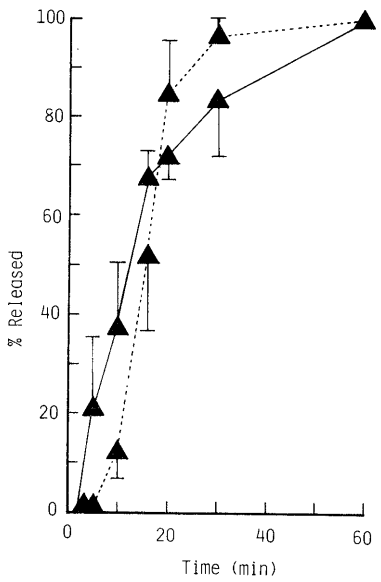

Fig. 7. Release Profiles of Insulin from Hollow-Type Suppositories Containing Insulin in Crystalline Form and GMO *in Vitro* at 37 °C

Insulin: ▲—▲, porcine; ▲---▲, bovine. Dissolution medium was a phosphate buffer solution. Each point represents the mean ± S.E. of three experiments.

from both types I and II suppositories after melting of the body (oleaginous base) of the suppository. However, a difference in the percentage of insulin released (% released) between porcine and bovine insulin crystals from suppositories was observed (Fig. 7). Porcine insulin was released more rapidly than bovine insulin. Kasama *et al.*[24] suggested that the difference in dissolution between porcine and bovine insulin crystals is probably related to the configurational differences between the two insulins arising from the differences in amino acid residues at positions 8 and 10 of the A-chain[24] and physical characteristics, such as amorphousness and crystal size.[25] The difference in pharmacokinetic parameters obtained between porcine and bovine insulin in crystalline form may be caused by the

difference in the dissolution rate of insulin in rectal fluid in the neutral pH region. It is probable that insulin absorption varies after rectal administration of the crystalline form.

Concerning the mechanism of the absorption-enhancing action of GMO, an effect on the transcellular absorption pathway may be involved,[26] as has been described in the case of glycerylmonooleate, which was shown to destabilize the membrane structure.[27]

Conclusions

We have found that when GMO was coadministered, plasma insulin concentrations significantly increased following the use of hollow-type suppositories containing porcine or bovine insulin. These results demonstrate that the two insulins are efficiently absorbed through the rectum of rabbits when either the aqueous solution or the crystalline form of insulin is used. However, the dissolution rate of insulin may be an important factor in the rectal absorption of insulin coadministered with GMO. To render easy delivery of peptide drugs and absorption enhancers to the rectum, the hollow-type suppository containing peptide drug and GMO is useful as a practical rectal dosage form.

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The Solid Dispersion of Benzodiazepins with Phosphatidylcholine.[1] The Effect of Substituents of Benzodiazepins on the Formation of Solid Dispersions

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Solid dispersions of four benzodiazepins, nitrazepam (NZP), nimetazepam (NMP), diazepam (DZP) and medazepam (MZP), with phosphatidylcholine (PC) were prepared to investigate the effect of functional groups of benzodiazepins. These benzodiazepins were present in an amorphous state immediately after preparation of the solid dispersion. The limit mole fractions for the amorphous state were 0.50 (NZP), 0.25 (NMP), 0.25—0.40 (DZP) and 0.25 (MZP). Infrared spectra and thermal analysis suggested an interaction, probably a hydrogen bond, between PC and NZP. In contrast, no interaction was suggested between PC and NMP, DZP or MZP, because they have no functional donor groups for the hydrogen bond. NZP solid dispersion showed no change after 1 year, but NMP, DZP and MZP solid dispersion showed recrystallization of drugs after 1 year. Thus, it was considered that some interaction between PC and the drug was needed to prevent the recrystallization. Dissolution of NZP in pH 7.0 phosphate buffer solution was much faster from NZP solid dispersion than from NZP crystals, and there was no aging effect of the solid dispersion. Dissolution of DZP also improved with the formation of a solid dispersion, but the dissolution rate became slower with time.

Keywords solid dispersion; phosphatidylcholine; benzodiazepin; aging; thermal analysis; X-ray diffraction pattern; dissolution

Solid dispersion is a method for improving the dissolution rate of poorly water soluble drugs by dispersing the drug in a carrier as an ultramicrocrystal or amorphously.[2] The bioavailability of poorly water soluble drugs, which limited absorption from the gastro-intestine track by dissolution rate, was improved,[3] and the dose could be reduced.[4] Some cases showed no aging effect,[3a] though some cases showed a drug condition change, agglomeration or recrystallization, with time and improvement of the dissolution rate decreased.[5] The reason for the different aging effect must be clarified. Thakker *et al.*[6] reported that nabilone and polyvinylpyrrolidone (PVP) interacted with a hydrogen bond in solid dispersion, and polymorphism was prevented for more than 2 years. Dohrty and York[7] defined a furosemid–PVP interaction in solid dispersion, and a hydrogen bond may account for the formation and stability of the amorphous form in solid dispersion.

We have been studying the solid dispersion, the carrier of which is phosphatidylcholine (PC), and reported indomethacin (IM), ketoprofen (KP), flurubiprofen (FP),[8] phenytoin (PHT),[9] and phenobarbital (PB)[10] solid dispersions. These drugs exist in an amorphous state in solid dispersion and show no recrystallization with time. Infrared (IR) spectra and differential scanning calorimetry (DSC) curves suggested a weak interaction, probably a hydrogen bond, between the drug and PC. However, the interactions between PC and IM, KP or FP with a carbonyl group were different from those between PC and PHT or PB with an oxopyrimidin ring. On the other hand, the limit mole fractions of PHT and PB for the amorphous state were quite different, 0.33 and 0.75, respectively, in spite of similar chemical structures. So the steric structure also affects the formation of a solid dispersion.

In this report, four benzodiazepins which have the same skeleton but different substituents were used to investigate the effect of functional groups on the formation of solid dispersions without taking into account steric factors.

Experimental

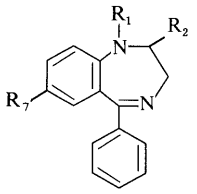
Materials PC was purified by the method described in an earlier report.[11] Its purity was above 95% and the fatty acid composition was palmitic acid : stearic acid = 15 : 85. The structure and abbreviations of benzodiazepins are listed in Table I. Nitrazepam (NZP) and nimetazepam (NMP) were the kind gift of Toyo Jozo Co., Ltd. and Sumitomo Pharmaceutical Ltd., respectively. Diazepam (DZP) and medazepam (MZP) were provided by Nippon Bulk Yakuhin Co., Ltd. Other chemicals were of a reagent grade.

Preparation of Solid Dispersion The required amounts of drug and PC were weighed out and dissolved in xylene. The xylene was then evaporated off *in vacuo*, sometimes warming to 80 °C. The glassy film thus obtained was crushed and sieved with an 80-mesh screen. This powder was placed *in vacuo* again to remove residue xylene. The solid dispersion thus obtained was described as drug–PC (mole fraction of drug). Drugs were obtained from xylene and sieved with an 80-mesh screen. The physical mixture (P-mix) was prepared by mixing drug and PC with a spatula. It was stored in a desiccator over silica gel at 25±2 °C.

Physicochemical Properties of Solid Dispersion IR spectra were obtained on an IR spectrophotometer (Japan Spectroscopic, Ltd., FT/IR-8000) by the diffuse reflectance method with no dilution. The base lines were corrected. Also, the IR spectra of the $CHCl_3$ solution (5×10^{-2} M) were measured. DSC curves and X-ray diffraction patterns were measured by the same condition as previously reported.[10] Measurements were performed within 1 week after preparation. To investigate the aging effect, the solid dispersions packed in sample tubes were stored in the dark in a desiccator over silica gel at 25±2 °C. After 1, 3, 6 and 12 months, the X-ray diffraction patterns were measured.

Dissolution Studies The dissolution patterns of NZP and DZP were tested in a JP XI dissolution test apparatus. As test solution, 500 ml of pH 7.0 phosphate buffer solution (PBS) was kept at 37±0.1 °C with stirring at 100 rpm by a paddle. A sample equivalent to 10 mg of the

TABLE I. Benzodiazepins Used in This Study



Drug	Abbreviation	R_1	R_2	R_7
Nitrazepam	NZP	H	=O	NO_2
Nimetazepam	NMP	CH_3	=O	NO_2
Diazepam	DZP	CH_3	=O	Cl
Medazepam	MZP	CH_3	H, H	Cl

drug was dispersed in a test solution. An aliquot of the test solution was withdrawn periodically and immediately filtered through a 0.20 μm membrane filter (Dismic-25, Advantec Toyo). The same volume of PBS was added to the test solution. The NZP concentration was determined by the absorbance at 258 nm after dilution with pH 7.0 PBS. The DZP concentration was determined by the absorbance at 241 nm after dilution with 0.1 N HCl solution. The solubilities were determined by dispersing the equivalent to 25 mg of the drug in 50 ml of pH 7.0 PBS at 37±0.1 °C. The test was performed within 1 week after preparation. All studies were done in triplicate.

Results and Discussion

IR Spectra NZP–PC and MZP–PC were a slightly yellow powder, and NMP–PC and DZP–PC were white powder.

The IR spectra of solid dispersions and P-mix were compared. Each P-mix showed the sum of the drug and PC spectra. NZP–PC showed some difference from the P-mix (Fig. 1). PC the phosphate band at 1170 cm^{-1} showed some change. The NZP amide NH bands at 3350 and 3225 cm^{-1}, and NZP absorption at 1917 and 1025 cm^{-1} disappeared. NZP absorption at 1620 and 1342 cm^{-1} showed some change. These changes were also observed in $CHCl_3$ solution. The NZP amide C=O band at 1712 and 1685 cm^{-1} was observed only at 1685 cm^{-1} in P-mix. On the other hand, this band was observed only at 1715 cm^{-1} in NZP–PC. This band in $CHCl_3$ solution is at 1699 cm^{-1}. The above changes indicated not only an amorphous state of NZP but also some interaction between NZP and PC.

NMP and DZP also have an amide, but NMP–PC showed no evident difference from the P-mix, and DZP–PC sometimes showed some difference at 1700 cm^{-1} only immediately after preparation. MZP–PC showed the same spectra with P-mix.

DSC Curves Figure 2 shows the DSC curves of solid dispersions and related materials. Heating was carried out under 200 °C because PC decomposes above 200 °C even if its atmosphere is N_2. The melting point of NZP is above 200 °C (225 °C), so its DSC curve is not shown. The P-mix of NZP and PC showed an endothermic peak at 85 °C, which was considered the phase transition of PC (anhydride–monohydrate).[12] The base line drifted at above 150 °C, which suggested that some change occurred. The appearance was observed with a micro melting point apparatus, and melting occurred gradually from 150 °C. NZP–PC (0.25) showed endothermic peaks at 63 and

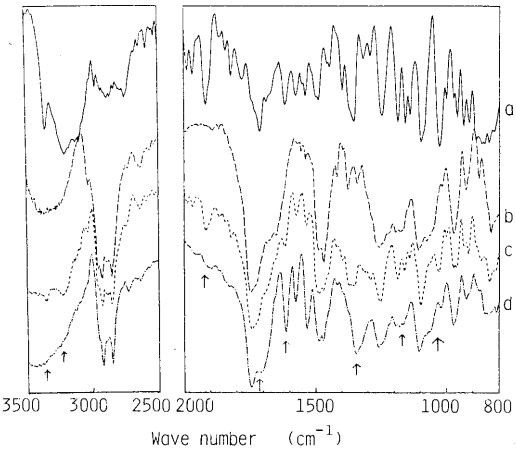


Fig. 1. IR Spectra of NZP–PC and Related Materials
a, NZP; b, PC; c, P-mix (0.50); d, NZP–PC (0.50).

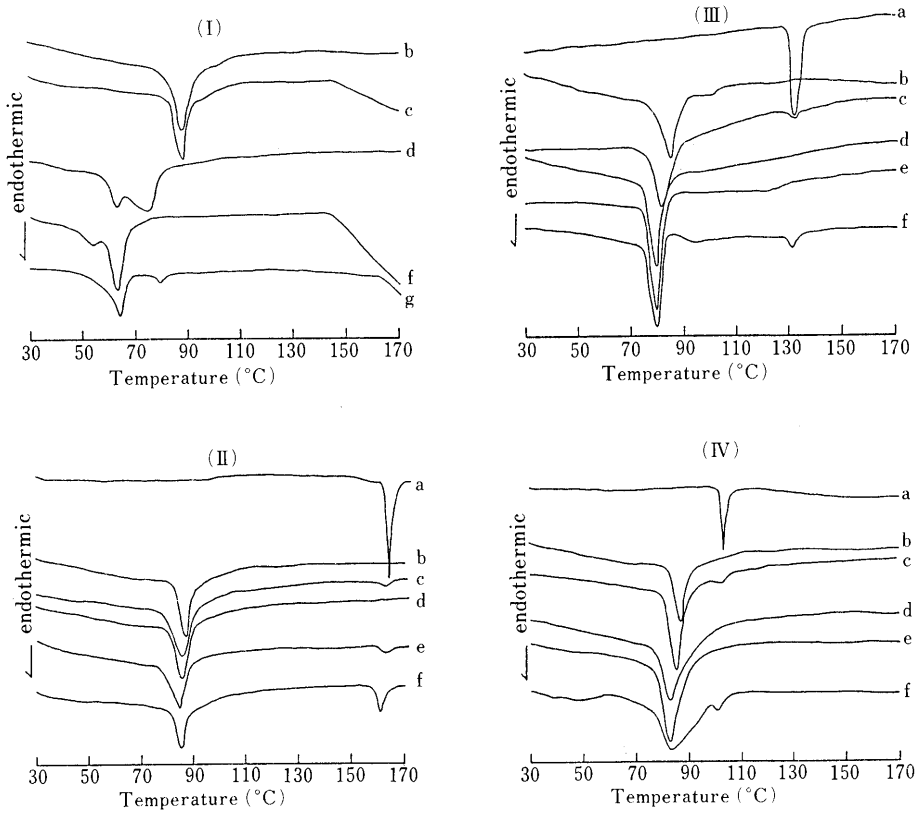


Fig. 2. DSC Curves of Solid Dispersions and Related Materials
(I) NZP; (II) NMP; (III) DZP; (IV) MZP. a, drug; b, PC; c, P-mix (0.25); d, solid dispersion (0.25); e, 0.33; f, 0.50; g, 0.67.

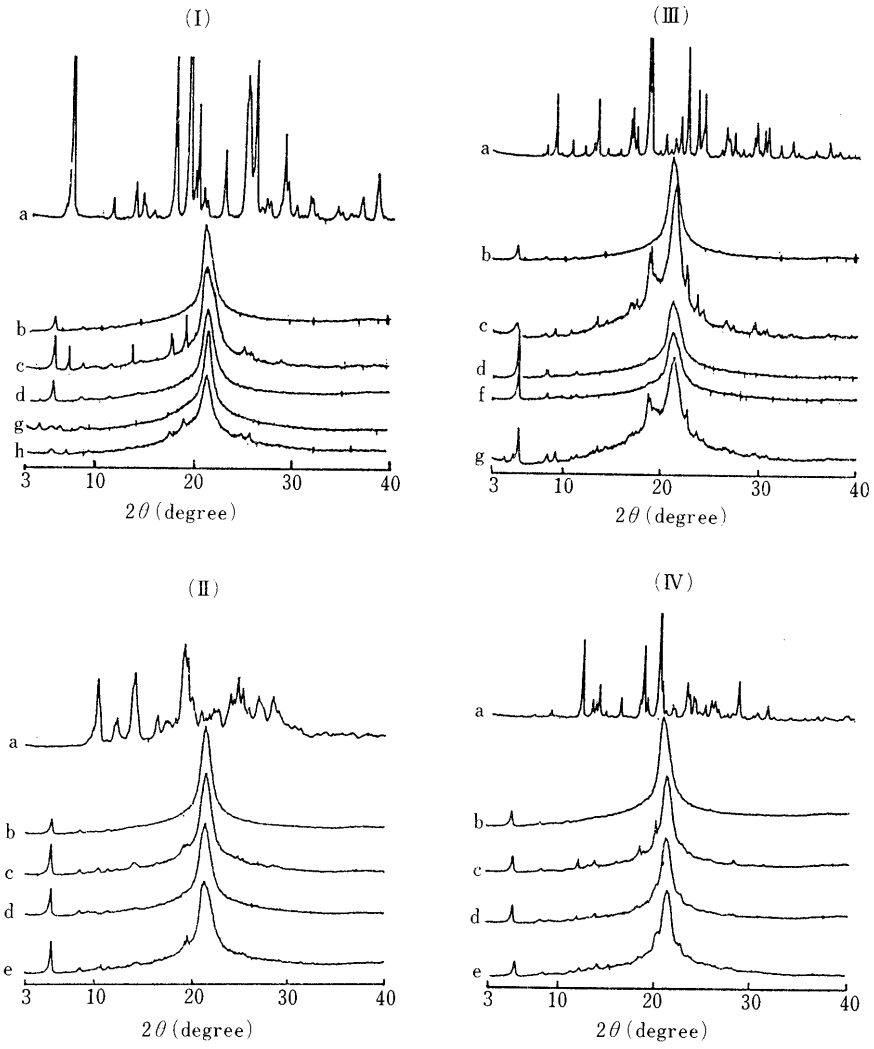


Fig. 3. X-Ray Diffraction Patterns of Solid Dispersions and Related Materials

(I) NZP; (II) NMP; (III) DZP; (IV) MZP. a, drug; b, PC; c, P-mix (0.25); d, solid dispersion (0.25); e, 0.33; f, 0.40; g, 0.50; h, 0.67.

75 °C, and NZP–PC (0.50) at 54 and 63 °C. By increasing the mole fraction of drug, a lowering of the peak temperature was observed. This phenomenon was also observed in DSC curves of IM–PC, KP–PC and FP–PC, and the degree of temperature depression was almost the same if the mole fraction of the drug was the same.[8] Therefore, it was suggested that NZP and PC had some interaction so that the PC phase transition temperature was lowered. NZP–PC (0.67) showed endothermic peaks at 63 and 78 °C, and the degree of depression of the phase transition temperature was not significant as with NZP–PC (0.50).

In contrast, NMP–PC, DZP–PC and MZP–PC showed an endothermic peak at 85 °C, the same as PC only, and they showed no base line drift at high temperature. It was suggested that NMP, DZP and MZP have no interaction with PC which affects the PC phase transition, as does NZP. An NMP melting peak was observed if the mole fraction of NMP was above 0.33, and DZP and MZP melting peaks were observed for mole fractions above 0.50. The drug crystals remained in solid dispersion in these cases.

X-Ray Diffraction Patterns Figure 3 shows the X-ray diffraction patterns of solid dispersions and related materials. NZP–PC showed only PC signals, and the amorphous state of NZP in solid dispersion was suggested. NZP–PC (0.50) also showed no NZP signals, suggesting the

TABLE II. The Change in the X-Ray Diffraction Patterns of Solid Dispersions

	Time after preparation (months)				
	0	1	3	6	12
NZP–PC (0.25)	−	−	−	−	−
(0.50)	−	−	−	−	−
(0.67)	+	+	+	+	+
NMP–PC (0.10)	−	−	−	−	−
(0.25)	−	±	±	±	±
(0.33)	+	+	+	+	+
DZP–PC (0.10)	−	−	±	±	+
(0.25)	−	−	+	+	+
(0.33)	±	+	+	+	+
(0.40)	±	+	+	+	+
(0.50)	+	+	+	+	+
MZP–PC (0.10)	−	+	+	+	+
(0.25)	±	+	+	+	+
(0.33)	+	+	+	+	+

−, no drug signals; ±, drug signals were sometimes observed; +, drug signals were always observed.

amorphous state of NZP. However some signals which did not fit with either PC or NZP were observed at $2\theta=4.4°$ ($d=20.1$ Å), 6.6° (13.4 Å) and 8.8° (10.0 Å). These signals were also observed in the X-ray diffraction patterns of PB–PC (0.50). The long space of the lamellar phase of PC, in which the acyl chains are C_{18}, was reported 60—63 Å (anhydride–monohydrate).[13] 62 Å/n ($n=4$, 6, 8) signals were observed in our case, and these signals changed into 80 Å/n ($n=4$, 6, 8), probably due to arranging NZP into a PC lattice. NZP–PC (0.67) showed both NZP and PC signals. If the mole fraction of NZP was under 0.50, NZP was considered to be present in an amorphous state.

In the case of NMP, NMP–PC (0.33) showed NMP signals clearly. DZP–PC (0.25) showed only PC signals. DZP–PC (0.33) and (0.40) showed only PC signals in some cases, but both PC and DZP signals in other cases, and the reproducibility of the preparation was poor. DZP–PC (0.50) consistently showed both PC and DZP signals. The patterns of MZP–PC (0.25) were observed in two cases, for PC signals only and for both PC and MZP signals. MZP–PC (0.33) exhibited both PC and MZP signals.

As mentioned above, the limit mole fractions of the drugs present in the amorphous state were about 0.50 (NZP), 0.25 (NMP), 0.25—0.40 (DZP) and 0.25 (MZP). However, reproducibility was poor in the cases of DZP and MZP, unlike that of other drugs which had been investigated. Next, the changes of X-ray diffraction patterns with time were studied, and the results are shown in Table II. NZP–PC showed no change after 1 year (Fig. 4I). However, in the cases of NMP–PC, DZP–PC and MZP–PC, the solid dispersion which had shown no drug signals immediately after preparation, showed drug signals after some months. The change of DZP–PC (0.25) is shown in Fig. 4II as a typical example.

To sum up their physicochemical properties, the solid dispersions were divided into two groups; one was NZP and the other was NMP, DZP and MZP. The amorphous state of NZP is stable with time, but those of NMP, DZP and MZP are unstable and recrystallization occurs.

NZP has H as R_1 and =O as R_2, a lactum ring. This structure contributes to the hydrogen bond. Actually, the IR spectra show some change in amide, and the PC phase transition temperature was lowered. This indicates an interaction between NZP and PC. In the case of NMP, in which R_1 substituent is replaced with CH_3, its tertiary amide group, C=O, does not contribute to the hydrogen bond. The nitro group at the 7-position contributes little to the hydrogen bond. The IR spectra show no change in either the amide band (1680 cm^{-1}) or the nitro band (1520, 1340 cm^{-1}), and DSC curves show the PC phase transition peak at the same temperature as PC alone. Thus, it was considered that there was no interaction between PC and NMP. DZP and MZP were also considered to have no interaction with PC for the same reason.

For the above reason, NZP is present in an amorphous state in NZP–PC like IM, PHT or PB; however, NMP, DZP and MZP are temporarily present in an amorphous state by coprecipitation with PC, but there is no interaction for stabilizing the amorphous state, so those drugs change into a crystal form, which is a more stable energy state.[2]

Dissolution Studies It became apparent that NZP was present in an amorphous state in NZP–PC but NMP, DZP and MZP were recrystallized over time even if they were present in an amorphous state immediately after preparation. Although it is not necessary for drugs to exist in an amorphous state, they will be undesirable for use if their dissolution patterns result in recrystallization. Therefore, the dissolution pattern was tested for NZP–PC and DZP–PC as typical examples of recrystallizing drugs.

The solubility of NZP was 35 μg/ml. It raised above

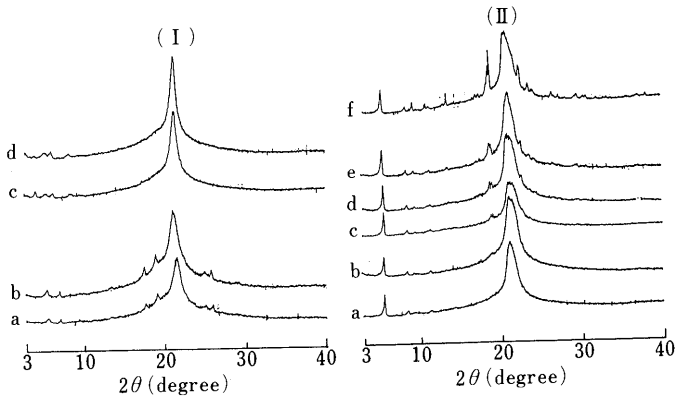


Fig. 4. Change of X-Ray Diffraction Patterns of NZP–PC and DZP–PC as a Function of Time

(I) NZP–PC: a, 0.67; b, 0.67 after 12 months; c, 0.50; d, 0.50 after 12 months. (II) a, DZP–PC (0.25); b, after 1 month; c, after 3 months; d, after 6 months; e, after 12 months; f, P-mix (0.25).

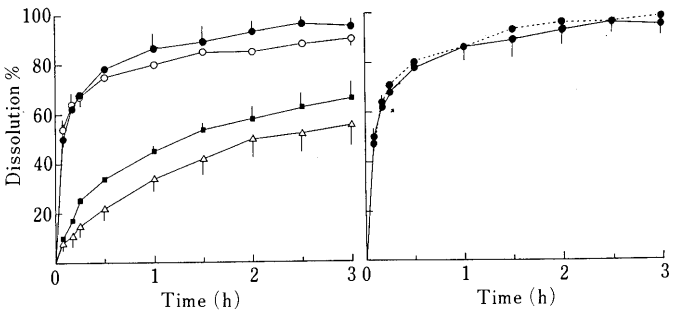


Fig. 5. Dissolution Patterns of NZP in a pH 7.0 Phosphate Buffer Solution

△, NZP crystals; ■, P-mix (0.50); ○, NZP–PC (0.25); ●, 0.50. —, immediately after preparation; ---, after 12 months. Each point represents the mean±S.D. of three experiments.

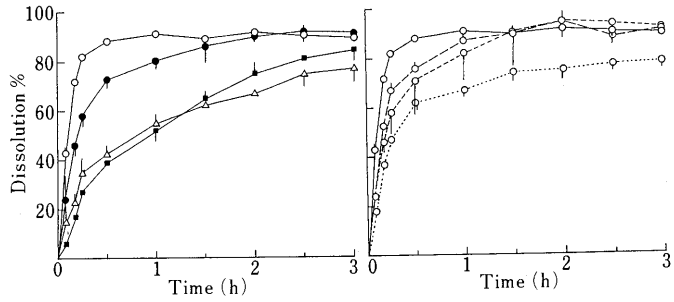


Fig. 6. Dissolution Patterns of DZP in pH 7.0 Phosphate Buffer Solution

△, DZP crystals; ■, P-mix (0.40); ○, DZP–PC (0.25); ●, 0.40. ——, immediately after preparation; – – –, after 1 months; ------, after 3 months; ·····, after 12 months. Each point represents the mean±S.D. of three experiments.

100 μg/ml with NZP–PC, though recrystallization of NZP in solution was very fast. DZP–PC also temporarily raised the solubility of DZP from 50 to 73 μg/ml.

Figure 5 shows the dissolution patterns of NZP. NZP–PC (0.25) and (0.50) showed similar dissolution patterns; 50% of NZP dissolved within 5 min, 80% within 30 min. The dissolution rate was much higher than that from NZP crystals. These improved dissolution patterns showed no change after 1 year. It was suggested that NZP–PC may be used to improve the NZP dissolution rate.

Figure 6 shows the dissolution patterns of DZP. DZP dissolved 40% within 5 min and 80% within 30 min from DZP–PC (0.25). From DZP crystals, only 14% of the DZP dissolved within 5 min and 40% within 30 min. Thus, the dissolution rate of DZP was improved by the formation a solid dispersion, as well as NZP–PC. DZP–PC (0.40) also improved the dissolution rate of DZP, but not as much as DZP–PC (0.25).

The change of dissolution pattern with time was investigated with DZP–PC (0.25). After 1 month, while DZP signals were not observed in X-ray diffraction patterns, the dissolution slowed compared with the rate immediately after preparation. The dissolution became slower and slower with time, and after 1 year, DZP dissolved only 19% within 5 min, which was half the rate immediately after preparation. Thus, it was considered that the improvement of the dissolution rate by forming a solid dispersion was not maintained for a long time in the case of DZP, which recrystallized with time.

In conclusion, some interaction between PC and a drug is required to keep the drug in an amorphous state. And some functional group is needed. If the amorphous state of the drug is stable, the improvement of the dissolution rate shows no change with time. In this case, solid dispersion is efficiently improves the dissolution of a drug which does not dissolve well in water.

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The Nature of Hydrolysis of Novel Methacryloyl Polymeric Prodrugs Prepared by Mechanochemical Solid State Polymerization

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The alkaline hydrolysis of several powdered methacryloyl polymeric prodrugs (1P—3P) prepared by mechanochemical polymerization, each of which contains acetaminophen (1), 7-theophyllineacetic acid (2) and 5-fluorouracil (3) as a pendant drug group has been examined in a heterogeneous system on its comparison with that of the corresponding polymeric prodrugs prepared by conventional radical-initiated polymerizations. The rate of hydrolysis depended largely on the structural features of the polymer hydrolyzates; carboxyl groups are formed with the progress of hydrolysis of 1P and 3P so that the rate of hydrolysis is markedly lowered due to suppression of a subsequent nucleophilic attack of hydroxyl anions, and the reaction ceased before its completion. In contrast, the hydrolysis of 2P was completed within several hours and finally the suspended powders had been completely solubilized, since non-acidic hydroxylethyl groups are formed as a polymer hydrolyzate. Thus, the kinetics for hydrolysis of 2P was shown to be well correlated with the powder dissolution rate, which is known as the Hixson–Crowell cube root law. It has also been observed that the rate of hydrolysis of 1Pm was higher than that of 1Pr. This fact has been ascribed to the difference in the stereochemical configuration (tacticity) of the polymer main chain between 1Pm and 1Pr, where m and r denote mechanochemical and radical-initiated, respectively.

Keywords polymeric prodrug; drug release; mechanochemical polymerization; hydrolysis; acetaminophen; 5-fluorouracil; 7-theophyllineacetic acid

The chemotherapeutic utility of polymeric drugs has recently been the focus of intense research, since polymeric drugs possess unique properties distinct from those of lower-molecular-weight drug substances.[1–4] The design of polymeric drugs can be devided into two types: polymers acting themselves as genuine drugs due entirely to their polymer properties, and a second class of polymeric drugs consisting of drugs with a polymeric carrier and biologically active groups linked to the main polymer chain as a pendant group which could either be linked directly or with a spacer group of some desired chain length, but can be released from the polymeric carrier by hydrolytic, enzymatic or oxidative cleavages. Thus, this type of polymeric drug can be regarded as polymeric prodrugs. The majority of polymeric drugs thus far investigated are polymeric prodrugs, and the synthetic polymers are widely used as drug carriers because the properties of these molecules can be readily modified by varying their structures.[3]

In principle, the polymeric prodrugs can be prepared in two different ways.[1–3] One approach is substitution of reactive groups of the polymers with functional groups of biologically active compounds. It is often observed, however, that some undesired side reactions also occur in such reactions, and the substitution reactions of this type sometimes do not proceed to completion due to severe steric hindrance by neighboring side-chain groups, although such problems can be removed by spacing the reactive group of several carbon atoms from the polymer main chain. The other approach is the polymerization of drug molecules with polymerizable functional groups such as vinyl groups. This approach has the advantage that the monomer can be highly purified and then be polymerized or copolymerized with any number of desirable comonomers. All these reactions, however, are usually carried out in the liquid state, thus require the need for work-up of the reaction mixture such as product separation and purification.

On the other hand, we have recently reported the detailed electron spin resonance (ESR) study on mechanochemical solid state polymerization of acrylamide[5] and the nature of mechanoradical formation of several conventional polymers.[6] Part of the motivation for these investigations originated from the need for the mechanoradical information related to our ESR studies of plasma-induced solid state radicals[7] and their pharmaceutical applications.[8] Subsequently, we have presented the first example of polymeric prodrugs prepared by mechanochemical solid state polymerizations of several selected methacryloyl vinyl monomers which were specially synthesized from bioactive compounds such as acetaminophen, (**1**), (analgesic and antipyretic agent), 7-theophyllineacetic acid, (**2**) (smooth muscle relaxant) and 5-fluorouracil (5-FU), (**3**) (antineoplastic agent), as well as the scope and limitations of mechanochemical polymerizations of vinyl monomers based on quantum chemical consideration.[9]

Several important conclusions have been reached from a series of such studies. It was shown that there was a monomer selectivity for efficiency of the mechanochemical solid state polymerizations, although all the monomers examined underwent conventional radical-initiated solution polymerizations. However, the appropriately designed methacryloyl derivatives of the drugs underwent facile mechanochemical solid state polymerizations to give the corresponding polymeric prodrugs essentially quantitatively. Thus, this method eliminates the need for any work-up of the reaction mixture. One of the most striking properties observed in such polymers is that the resulting polymeric prodrugs are of very low heterogeneity (narrow molecular weight distribution)[9] represented by $\bar{M}_w/\bar{M}_n$, which is of great value in pharmaceuticals for highly functionalized polymeric prodrugs. Thus, if one designs the mechanochemically polymerizable vinyl derivative of drugs along the line of the structural criteria derived from the quantum chemical consideration,[9] the present reactions seem applicable to a wide variety of vinyl monomers of an important class of bioactive compounds with different physicochemical properties, and provides a novel and simple

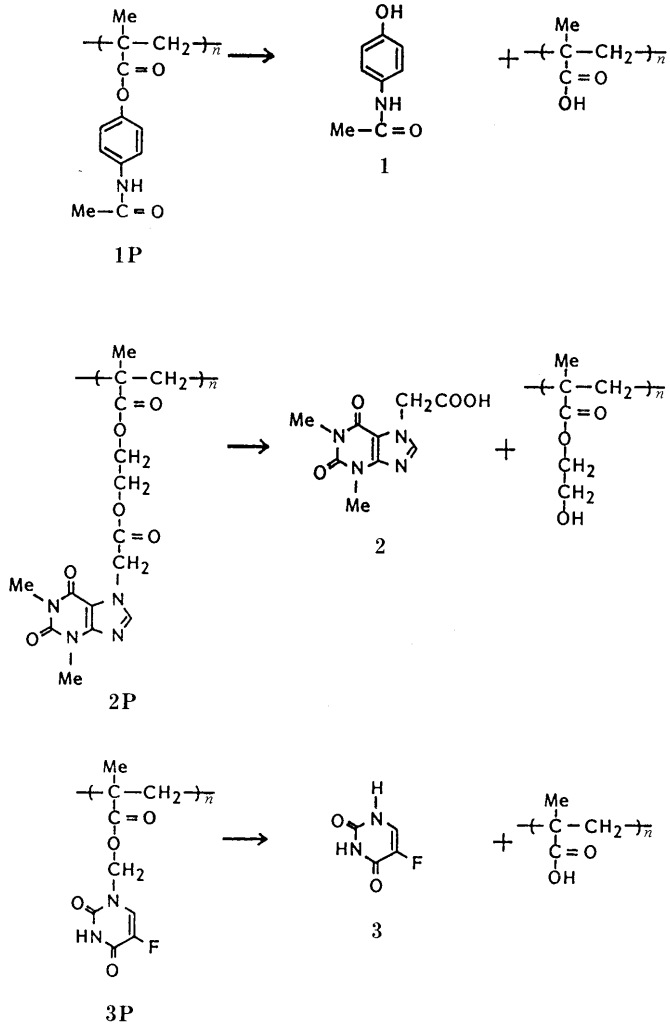

Chart 1

methodology for syntheses of polymeric prodrugs through a totally dry process.

In this connection, we have studied for the first time the hydrolysis of the above-mentioned mechanochemically polymerized prodrugs for the drug release as shown in Chart 1, and report the detailed nature of such reactions on its comparison with those prepared by conventional radical-initiated solution polymerizations.

Experimental

Materials Methacryloyl derivatives of bioactive compounds, *i.e.* *p*-methacryloyloxy acetanilide (**1M**), methacryloyloxyethyl-7-theophylline-acetate (**2M**), and 1-methacryloyloxymethyl-5-fluorouracil (**3M**), and the corresponding mechanochemically polymerized prodrugs (**1Pm**, **2Pm**, and **3Pm**) were prepared according to the method previously reported.[9] The powdered samples for the hydrolysis test were obtained as follows: After drying *in vacuo* for 5 h at room temperature, the polymeric prodrugs were pulverized for a prescribed period of time and screened with a 200 mesh sieve. $\bar{M}_n = ca.$ 30000, $\bar{M}_w/\bar{M}_n = 1.08$ for **1Pm** (30000), $\bar{M}_n = ca.$ 27000, $\bar{M}_w/\bar{M}_n = 1.07$ for **2Pm** (27000) and $\bar{M}_n = ca.$ 29000, $\bar{M}_w/\bar{M}_n = 1.10$ for **3Pm** (29000). The corresponding polymeric prodrugs (**1Pr**, **2Pr** and **3Pr**) were also prepared by conventional radical-initiated solution polymerization in a similar manner previously reported,[9] and pulverized for a prescribed period of time, and screened with a 200 mesh sieve; **1Pr** (82000); $\bar{M}_n = ca.$ 82000, $\bar{M}_w/\bar{M}_n = 1.67$, **1Pr** (30000); $\bar{M}_n = ca.$ 30000, $\bar{M}_w/\bar{M}_n = 1.10$, **2Pr** (50000); $\bar{M}_n = ca.$ 50000, $\bar{M}_w/\bar{M}_n = 1.67$, **2Pr** (34000); $\bar{M}_n = 34000$, $\bar{M}_w/\bar{M}_n = 1.28$, **2Pr** (27000); $\bar{M}_n = 27000$, $\bar{M}_w/\bar{M}_n = 1.11$, **3Pr** (83000); $\bar{M}_n = 83000$, $\bar{M}_w/\bar{M}_n = 1.57$, **3Pr** (29000); $\bar{M}_n = 29000$, $\bar{M}_w/\bar{M}_n = 1.08$. The mean particle size of powdered samples of **2Pr** (50000), **2Pr** (34000), **2Pr** (27000) and **2Pm** (27000) was measured in ethylacetate by centrifugal automatic particle analyzer (Horiba, CAPA-500 Japan): **2Pr** (50000); 2.52 μm, **2Pr** (34000); 2.53 μm, **2Pr** (27000); 2.56 μm, **2Pm** (27000); 2.33 μm.

Molecular Weight Measurement Molecular weight of polymeric prodrugs was measured by gel permeation chromatograph (GPC, Shimadzu LC-6A), equipped with a refractive index detector (Shimadzu, RID-6A), gel column (Shodex, KD-800M and KD-80M) and a data analyzer (Shimadzu, Chromatopac C-R4A) under the following conditions; elution solvent, dimethylformamide (DMF) containing 0.01 M LiBr; flow rate, 0.7 ml/min; column temperature, 40 °C. The calibration for the molecular weight determination was made by a standard specimen of polyethylene oxide.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectral Measurement ^{1}H-NMR spectra were recorded on a JEOL JNM-GX270 FT-NMR spectrometer in either dimethyl sulfoxide-d_6 (DMSO-d_6) or $CDCl_3$. Tetramethylsilane (TMS) was used as an internal standard. The relative areas of the α-methyl proton resonance peaks represent the stereoregular triad probabilites.[10] The observed peak areas, however, are partially superposed to each other so that the relative peak intensities were determined by the deconvolution of the observed peaks using Lorentzian functions.

Method of Hydrolysis The hydrolysis of powdered polymeric prodrugs (5.0—6.0 mg) was conducted in 10 ml of aqueous buffer–organic solvent mixture (1 : 1, v/v) in a heterogeneous system; A phosphate buffer–acetonitrile solution (pH 12) at 60 °C for **1P**, a carbonate buffer–acetonitrile solution (pH 10) at 37 °C for **2P** and a phosphate buffer–dioxane mixture (pH 12) at 60 °C for **3P**. The reaction progress was monitored by high performance liquid chromatograph (HPLC, Shimadzu LC-6A) under the following conditions: for **1** and **2**, elution solvent, H_2O–acetonitrile (1 : 1, v/v); flow rate, 0.4 ml/min; column, Asahi-pak ODP-50 and ODP-50G; column temperature, 37 °C; detector, ultraviolet (UV) 245 nm for (1) and 273 nm for **2**. For **3**; elution solvent, H_2O–acetonitrile (1 : 1, v/v); flow rate, 1.2 ml/min; column, Shim-pack Diol-150; column temperature, 37 °C; detector, UV 266 nm. Quantities of the hydrolyzate in solution were determined from the comparison of the HPLC peak intensity with those of the calibration lines obtained previously. Each of the drugs, **1**—**3**, was separately confirmed to be stable under the prescribed conditions of each hydrolysis. Structural identification of drugs, **1**—**3**, as the product of hydrolyzates were made by infrared (IR), UV and ^{1}H-NMR spectral measurements of the isolated product after the work-up. For **3P**, the hydrolysis takes place so as to produce 1-hydroxymethyl-5-fluorouracil, which is known to convert rapidly to 5-fluorouracil, **3**.[11]

Results and Discussion

Hydrolysis of 1Pm—3Pm on Its Comparison with Those of 1Pr—3Pr Figure 1 shows the progressive changes of hydrolysis of **1Pm—3Pm** as a function of reaction time, together with those of **1Pr—3Pr** under each prescribed condition as shown in the Experimental section.

It is seen from Fig. 1a that although the rates of hydrolyses are very low in both **1Pm** and **1Pr**, the rate of **1Pm** is apparently higher than that of **1Pr**, but tends to gradually level off to *ca.* 80% completion in both cases. Since the molecular weight, the heterogeneity and mean particle size of **1Pm** and **1Pr** are nearly identical with each other, the difference in the rate of hydrolysis must be caused by the difference in the method of polymerization.

On the other hand, it is seen from Fig. 1b that the gross features of the hydrolysis profiles of **2Pm** (27000) and **2Pr** (27000) are more or less the same. However, the rate of hydrolyses of **2P** are much higher than those of **1P**, and the hydrolyses have proceeded to 100% completion. Furthermore, it can also be seen that the rate of hydrolyses depends on the polymer molecular weight (*vide infra*). Thus, comparison of the nature of hydrolyses between **1P** and **2P** demonstrates an interesting contrast.

First, the powders of **2P** in the suspended solution disappeared when the reaction was completed. The dif-

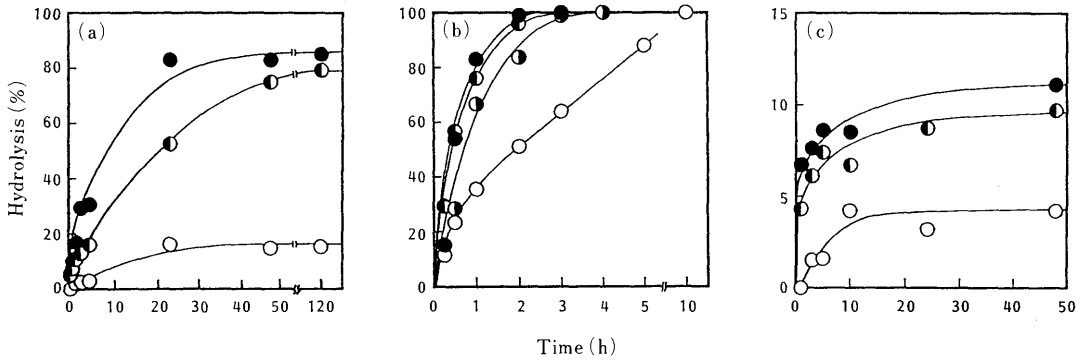

Fig. 1. Hydrolysis Profile of Polymeric Prodrugs, **1P**—**3P**, as a Function of Reaction Time

(a) Polymeric prodrugs containing acetaminophen, **1P**. ●, **1Pm** (30000); ◐, **1Pr** (30000); ○, **1Pr** (82000). (b) Polymeric prodrugs containing 7-theophyllineacetic acid, **2P**. ●, **2Pm** (27000); ◐, **2Pr** (27000); ◑, **2Pr** (34000); ○, **2Pr** (50000). (c) Polymeric prodrugs containing 5-fluorouracil, **3P**. ●, **3Pm** (29000); ◐, **3Pr** (29000); ○, **3Pr** (83000).

ference in the rate of hydrolysis between **1P** and **2P** can be rationalized in terms of such that the carboxyl groups are produced with progress of hydrolyses of **1P**, which should be completely dissociated under the present basic media causing the retardation of subsequent nucleophilic reactions, whereas in the case of **2P** such carboxyl groups are not produced during the course of reactions, since the hydrolysis occurs exclusively so as to produce hydroxylethyl groups as a side chain of the polymer hydrolyzate. We believe this is the essential reason for the observed difference in the susceptibility of hydrolysis between **1P** and **2P**. Such a pronounced effect on retardation of the hydrolysis has further emerged in the case of **3P** as can be seen in Fig. 1c. The rates of hydrolysis are highly suppressed in both cases of **3Pm** and **3Pr**, and the hydrolysis has leveled off to only *ca.* 10% progress even at an elevated temperature (60 °C). The result can be interpreted by the combined factors such that the polymeric prodrug, **3P**, not only produces a carboxyl group as a result of hydrolysis in the polymer hydrolyzate, but also has a dissociable hydrogen (pK_a = 7.3) at 3-position of the uracil ring, both hydrogens being completely dissociated under the present basic media, resulting in intense retardation of the hydrolysis as observed.

Stereochemical Configuration of Mechanochemical Polymerization It is a well-known fact that the proportion of the stereochemical configuration (tacticity) of polymethacrylic esters varies with the method of polymerization such as anion polymerization, radical polymerization.[10,12] In γ-ray induced solid state polymerization, the ratio of syndiotactic configuration in the polymethacrylic acid formed increases as the polymerization proceeds, and the polymer is mainly of syndiotactic configuration, although it depends on the temperature, the reaction time and the molecular weight.[13-15]

It has also been reported that the rate of hydrolysis of polymethylmethacrylate (PMMA) and polyisopropylacrylate (PiPrA) varies with the difference in the stereochemical configuration and that the hydrolyses of isotactic and heterotactic configurations are faster than the syndiotactic configuration in such polymers (*ca.* 2—5 times).[16,17]

Thus, we have compared the tacticity of the present polymeric prodrugs, **1Pm** and **2Pm**, prepared by mechanochemical polymerization with those by radical polymerization, **1Pr** and **2Pr**. In fact, the tacticity ratios of **1Pm** and **2Pm** are apparently different from **1Pr** and **2Pr**, as evidenced by NMR spectral measurement as shown in Fig. 2.

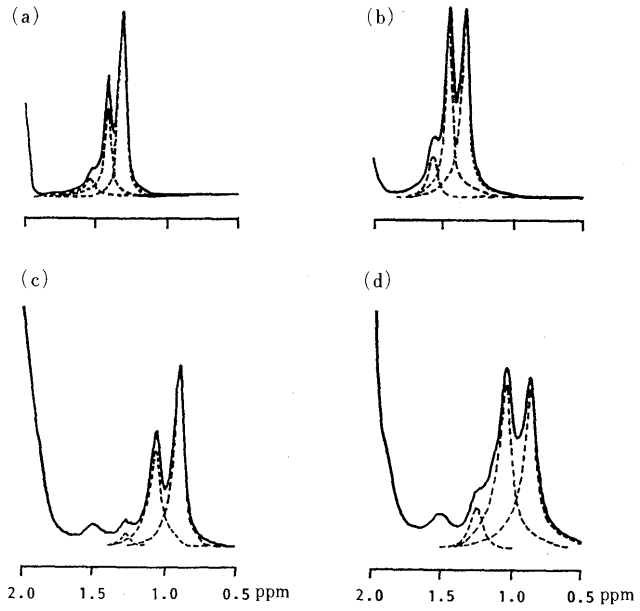

Fig. 2. Deconvolution of ^{1}H-NMR Spectra of α-Methyl Peaks in Polymeric Prodrugs, **1P** (a, b) and **2P** (c, d)

(a) **1Pr** (30000), (b) **1Pm** (30000), (c) **2Pr** (27000), (d) **2Pm** (27000).

TABLE I. Stereochemical Configuration of Polymers by Mechanochemical and Radical Polymerization

Compound	Method of polymerization	Tacticity (%)		
		Iso-	Hetero-	Syndio-
1Pm (30000)	Mechanochemical	19	41	41
1Pr (30000)	Radical-initiated	9	38	53
2Pm (27000)	Mechanochemical	11	50	39
2Pr (27000)	Radical-initiated	6	35	59

2.

Three kinds of α-methyl peaks of methacrylic polymers in NMR spectra are known to be assignable to iso-(i), hetero-(h), and syndiotactic (s) triads in order of increasing magnetic field. The proportions of tacticity deduced by the deconvolution of the partially superposed α-methyl peaks of the NMR spectra are listed in Table I.

It is clear that the ratios of i-, h- and s-triads of **1Pr** and **2Pr** are similar to each other, and s-triads is a predominant configuration, in accord with the result of PMMA reported

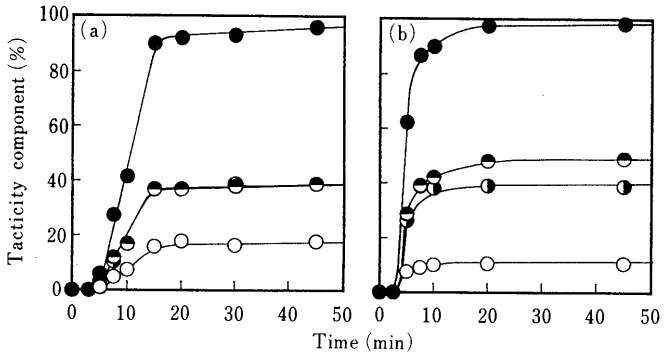

Fig. 3. Progressive Changes of Polymer Conversion with Each Tacticity in the Course of Mechanochemical Polymerization of **1M** (a) and **2M** (b)

(a) Mechanochemical polymerization of **1M**. ●, polymer conversion; ○, isotactic; ◓, heterotactic; ◑, syndiotactic. (b) Mechanochemical polymerization of **2M**. ●, polymer conversion; ○, isotactic; ◓, heterotactic; ◑, syndiotactic.

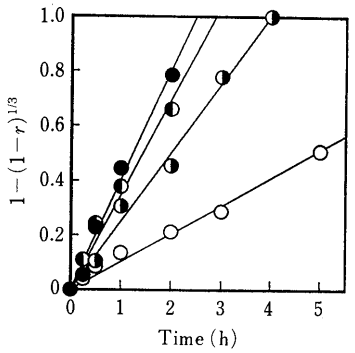

Fig. 4. Plots of $1-(1-r)^{1/3}$ as a Function of Reaction Time for **2P**

●, **2Pm** (27000); ◐, **2Pr** (27000); ◑, **2Pr** (34000); ○, **2Pr** (50000).

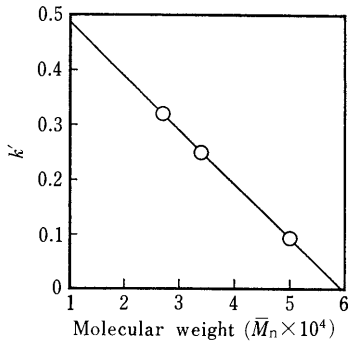

Fig. 5. Plots of Proportionality Constants (k') of **2Pr** in Fig. 4 as a Function of Molecular Weight

by F. A. Bovey.[10] On the other hand, it can be seen that **1Pm** and **2Pm** are of more atactic configuration in nature due to the increase in h-triads. Based on these results, the fact that **1Pm** was hydrolyzed more easily than **1Pr** as shown in Fig. 1 can be rationalized in terms of the predominant formation of atactic configuration in **1Pm**. We have further examined the progressive changes of polymer conversion with tacticity in the course of mechanochemical polymerization of **1M** and **2M**, and the results are shown in Fig. 3.

It is seen that each line feature is similar in nature to that of the total polymer conversion in both cases, indicating that the tacticity proportion remained unchanged with the progress of polymerization. Separate experiments have also shown that the progressive changes in tacticity of **1Pr** and **2Pr** remained essentially unchanged in the course of polymer degradation. Thus, the result is consistent with the fact that the greater part of mechanochemical polymerization proceeds by mechanoradical-initiated polymerization.[9]

Nature of Hydrolysis The rate of hydrolysis of polymers in the heterogeneous system must be discussed on the basis of many factors, and is not amenable to simple rationalization, since the factors (structure of hydrolyzate, particle size, powder morphology, molecular weight and reaction conditions) at play may act in opposing ways.

As described in a previous section, although the hydrolyses of **1P** and **3P** have ceased before its completion, **2P** did undergo the hydrolysis to completion and the powder size gradually decreased with the progress of the reaction and finally disappeared completely. Thus, the detailed nature of hydrolysis of **2P** was further explored, as a representative example.

The rate of powder dissolution itself does not represent the rate of polymer hydrolysis, since partially hydrolysized polymers would become a soluble polymer resulting in the decrease in the particle size. However, the above-mentioned fact clearly indicates that the nature of hydrolysis of **2P** is closely associated with the solubility of the polymer hydrolyzates.

It is well known that the rate of powder dissolution, assuming that a powder consists of monodispersed spherical particles, can be effectively described based on the cube root of the weight of the particles known as the Hixson–Crowell cube root law and expressed by Eq. 1.[18]

$$W_0^{1/3} - W_t^{1/3} = kt \quad (1)$$

in which W_0 is the original mass of the particles, W_t is the mass of particles at given time of t, k is the cube root powder dissolution rate constant, and t is the reaction time for dissolution.[18]

In fact, plots of $1-(1-r)^{1/3}$ as a function of the reaction time for all the polymers of **2P** exhibited a straight line as shown in Fig. 4, where r is the conversion ratio of the hydrolysis based on the quantities of drug hydrolyzates, (**2**). This demonstrates that the rate of hydrolysis is well correlated with the decrease in the particle size, and the slope in Fig. 4 should be the proportionality constant (k') of dissolution rate constant (k).

Furthermore, it can be reasonably assumed that the rate of polymeric powder dissolution should be associated with the molecular weight of the polymers, *i.e.* the length of polymer main chain, in the powders of the same particle size, since soluble polymer of higher-molecular-weight would be removed more slowly from the bulk powder than that of lower-molecular-weight. This view accounts for the result that the rate of hydrolysis has been lowered as the molecular weight increases as shown in Fig. 1. Thus, plots of each of the proportionality constants (k') of **2Pr** in Fig. 4 as a function of the molecular weight also give a straight line, as shown in Fig. 5, reinforcing the above-mentioned view for the effect of the molecular weight.

Thus, all these results clearly demonstrated that the rate of hydrolysis of polymer prodrugs in the heterogeneous system can be controlled by not only the structural feature of the polymer hydrolyzate but also the molecular weight of the polymeric prodrugs, and provides a basis for future synthetic design of polymeric prodrugs with a desired rate of the drug release.

Conclusion

The conclusion drawn from the present study for the nature of hydrolysis of powdered methacryloyl type of polymeric prodrugs in a heterogeneous system can be summarized as follows: The gross rate of alkaline hydrolysis depended largely on the structural features of the polymers after the hydrolysis; when carboxyl groups remain in the polymer hydrolyzates as in the case of **1P** and **3P**, the rate of hydrolysis is markedly lowered due to the retardation of a subsequent nucleophilic attack of hydroxyl anions. Such polymeric prodrugs with higher-molecular-weight also cause the lowering of dissolution of the polymer hydrolyzates so that the reaction has ceased before its completion. Unlike the case of **1P** and **3P**, the rate of hydrolysis of **2P** is markedly high and the reaction is completed, since non-acidic hydroxylethyl groups are formed in the resulting polymer hydrolyzates and the powders of polymeric prodrugs are completely solubilized when the reaction is completed. Thus, the rate of hydrolysis has been well correlated with the rate of powder dissolution, evidenced by the examination of the Hixson–Crowell cube root law.

It has also been observed in **1P** that the rate of hydrolysis of **1Pm** was higher than that of **1Pr**. The result has been interpreted by the difference in the stereochemical configuration (tacticity) between **1Pm** and **1Pr**, the former being of atactic configuration to a greater extent.

We are now actively elaborating these initial studies and the nature of hydrolysis of the hydrophilic polymeric prodrugs will be the subject of a forthcoming paper.

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Synthesis and Crystal Structure of Platinum(II) Dichlorodimethylsulfoxide Complex of Tenonitrozole, a 2-Amino-5-nitrothiazole Derivative, an Antimycotic and Trichomonacid Agent

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The crystal structure of *trans*-dichlorodimethylsulfoxide–Tenonitrozole (synthesized from *cis*-$PtCl_2(DMSO)_2$ and tenonitrozole in methanol) was determinated by X-ray analysis.

Keywords X-ray analysis; crystal structure determination; platinum(II) complex; (2′-thenoyl)-2-amino-5-nitrothiazole.

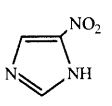
Farrell *et al.* have described the synthesis and properties of some nitroimidazole complexes of platinum(II) and palladium(II): *cis*-(MCl_2L_2) complexes were obtained from L = 5-nitroimidazole ligands whereas *trans*-(MCl_2L_2) were synthesized for 2-nitroimidazole and misonidazole ligands.[1]

On the other hand, the 2-amino-5-nitrothiazole is characterizd by several possible centres of coordination, the nitrogen ring, the nitro group, the sulphur ring, π electrons of the ring and the exocyclic nitrogen. Therefore, metal complexes of this ligand with Co(II), Ni(II), Cu(II), Cd(II), Cu(I), Ag(I)[2] or with palladium salts[3] and rhodium(II) carboxylates[4] were synthesized. The crystal structure of the later complex was confirmed by three dimensional X-ray structure analysis.

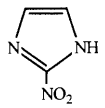
To our knowledge, the complexation by metal ions of tenonitrozole (which includes an aminonitrothiazole moiety) has not been described. We report here the synthesis and crystal structure of dichlorodimethylsulfoxide platinum(II) with tenonitrozole, the chemical formula of which is (2′-thenoyl)-2-amino-5-nitrothiazole.

Tenonitrozole is an antimycotic and trichomonacid drug. It may be solvated (or not) by dimethylformamide, the crystal structures of which were solved by our group.[5,6] We thought it would be of interest to extend our research on Pt(II) complexes.

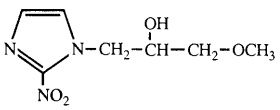
Equimolar amounts of each reactant (Tenonitrozole and *cis*-$PtCl_2(DMSO)_2$) were used and dissolved in methanol at 60 °C. The mixture was mechanically stirred for 5 h at the same temperature. The precipitate was filtered, washed with a methanol solution and then dried with diethyloxide and air. The neutral adduct was recrystallized in dichloromethane.



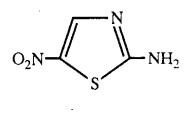
5-nitroimidazole



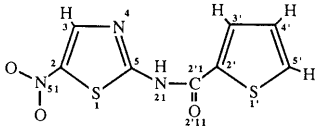
2-nitroimidazole



misonidazole



2-amino-5-nitrothiazole



tenonitrozole

Anal. Calcd for $C_{10}H_{11}Cl_{12}N_3O_4PtS_3$ (yellow parallelepipeds): C, 20.04; H, 1.85; N, 7.01. Found: C, 19.92; H, 1.83; N, 6.95. *cis*-$PtCl_2(DMSO)_2$ was synthesized by a method similar to that reported by Kukushkin *et al.*[7]

The crystallographic data was as follows: space group $Pna2_1$; $Z=4$; cell constants: $a=10.120(1)$, $b=22.660(1)$, $c=7.6312(3)$ Å; density, $D_x=2.27$, $D_m=2.23(2)$ g·cm^{-3}. All data were collected on an Enraf Nonius CAD4 diffractometer using graphite-monochromatized Mo$K\alpha$ radiation.

The structure was solved by using the standard Patterson techniques to find the Pt atom and the normal sequence of least-squares and difference Fourier cycles to locate all other atoms with Crystals.[8] Atomic scattering factors were taken from "International Tables for X-Ray crystallography" and anomalous corrections were applied to the curves for platinum and chloride. 1600 reflections satisfying the criterion $F>3\sigma(F)$ were retained; a final refinement led to $R=0.034$ and $R_w=0.037$. The computations were carried out on a VAX 11/725 computer. The final atomic coordinates are listed in Table II. Bond lengths and

TABLE I

Atom	*x*	*y*	*z*	*U*(eqv)
Pt	0.1679 (5)	0.24217 (2)	−0.0002 (5)	0.0327
Cl(1)	0.0328 (6)	0.2673 (2)	0.2495 (9)	0.0591
Cl(2)	0.2788 (5)	0.2206 (2)	−0.2366 (8)	0.0526
S(1)	0.1718 (5)	0.4409 (2)	−0.1031 (8)	0.0405
S(1′)	0.4498 (5)	0.4616 (2)	0.522 (1)	0.0524
S(11)	0.1723 (5)	0.1508 (2)	0.1151 (8)	0.0393
O(2′11)	0.296 (1)	0.4674 (5)	0.186 (2)	0.0519
O(12)	0.055 (1)	0.1161 (6)	0.090 (2)	0.0566
O(511)	−0.002 (2)	0.4002 (7)	−0.533 (3)	0.0815
O(512)	0.072 (2)	0.4823 (6)	−0.432 (2)	0.0712
N(3)	0.145 (1)	0.3272 (5)	−0.080 (2)	0.0332
N(21)	0.255 (1)	0.3692 (6)	0.164 (2)	0.0417
N(51)	0.050 (2)	0.4303 (8)	−0.420 (2)	0.0503
C(2)	0.195 (1)	0.3750 (6)	0.002 (4)	0.0283
C(4)	0.091 (2)	0.3422 (7)	−0.239 (2)	0.0369
C(5)	0.098 (2)	0.3990 (8)	−0.267 (2)	0.0418
C(2′)	0.364 (2)	0.4064 (7)	0.418 (2)	0.0423
C(3′)	0.371 (1)	0.3530 (6)	0.509 (4)	0.0395
C(4′)	0.443 (2)	0.3599 (8)	0.674 (3)	0.0463
C(5′)	0.492 (2)	0.4162 (8)	0.691 (2)	0.0466
C(2′1)	0.304 (2)	0.4178 (8)	0.247 (2)	0.0422
C(23)	0.310 (2)	0.1113 (7)	0.035 (3)	0.0518
C(24)	0.215 (2)	0.1509 (8)	0.344 (2)	0.0417

U(eqv) = 1/3 trace (*U*).

Table II. Bond Lengths (Å) and Angles (°) with Their e.s.d.'s in Parentheses

Distance (Å)		Angles (°)	
Pt–Cl(1):	2.3761 (7)	Cl(2)–Pt–Cl(1):	178.6 (3)
Pt–Cl(2):	2.216 (7)	S(11)–Pt–Cl(1):	86.2 (2)
Pt–S(11):	2.253 (5)	S(11)–Pt–Cl(2):	95.0 (2)
Pt–N(3):	2.03 (1)	N(3)–Pt–Cl(1):	88.2 (4)
S(1)–C(2):	1.71 (2)	N(3)–Pt–Cl(2):	90.5 (4)
S(1)–C(5):	1.74 (2)	N(3)–Pt–S(11):	174.2 (5)
C(2)–N(3):	1.35 (2)	C(5)–S(1)–C(2):	85.5 (11)
N(3)–C(4):	1.38 (2)	S(1)–C(2)–N(3):	115.4 (19)
C(4)–C(5):	1.31 (2)	C(2)–N(3)–C(4):	111.3 (15)
C(5)–N(51):	1.45 (2)	N(3)–C(4)–C(5):	111.0 (16)
S(11)–O(12):	1.44 (1)	C(4)–C(5)–S(1):	116.7 (15)
S(11)–C(13):	1.76 (2)	N(51)–C(5)–C(4):	126.1 (19)
S(11)–C(14):	1.80 (2)	N(51)–C(5)–S(1):	117.2 (14)
N(51)–O(511):	1.22 (2)	C(5)–N(51)–O(511):	116.3 (16)
N(51)–O(512):	1.20 (2)	O(511)–N(51)–O(512):	118.3 (18)
C(2)–N(21):	1.38 (3)	O(512)–N(51)–C(5):	125.2 (18)
N(21)–C(2′1):	1.36 (2)	S(1)–C(2)–N(21):	124.4 (16)
C(2′1)–O(2′11):	1.22 (2)	N(3)–C(2)–N(21):	120.1 (14)
C(2′1)–C(2′):	1.46 (3)	C(2)–N(21)–C(2′1):	119.8 (15)
C(2′)–C(3′):	1.40 (3)	N(21)–C(24)–C(2′):	115.1 (16)
C(3′)–C(4′):	1.47 (3)	N(21)–C(2′1)–O(2′11):	122.7 (17)
C(4′)–C(5′):	1.38 (2)	O(2′11)–C(2′1)–C(2′):	122.2 (17)
C(5′)–S(1′):	1.71 (2)	C(2′1)–C(2′)–S(1′):	119.6 (14)
S(1′)–C(2′):	1.72 (2)	C(2′1)–C(2′)–C(3′):	128.1 (17)
		S(1′)–C(2′)–C(3′):	112.0 (15)
		C(2′)–C(3′)–C(4′):	111.1 (14)
		C(3′)–C(4′)–C(5′):	111.2 (16)
		C(4′)–C(5′)–S(1′):	113.4 (15)
		C(5′)–S(1′)–C(2′):	92.2 (9)
		Pt–S(11)–O(12):	114.2 (6)
		Pt–S(11)–C(13):	111.8 (7)
		Pt–S(11)–C(14):	113.0 (7)
		O(12)–S(11)–C(13):	109.2 (9)
		O(12)–S(11)–C(14):	109.1 (9)
		C(13)–S(11)–C(14):	98.5 (11)

Table III. Torsion Angles (°)

	In the title complex	In the free ligand
N(3)–C(2)–N(21)–C(2′1):	−179 (2)	179.0 (3)
S(1)–C(2)–N(21)–C(2′1):	−2 (2)	−0.9 (4)
C(2)–N(21)–C(2′1)–C(2′):	180 (2)	179.6 (6)
C(2)–N(21)–C(2′1)–O(2′1):	1 (3)	−0.3 (7)
N(21)–C(2′1)–C(2′)–C(3′):	−4 (3)	15.1 (5)
N(21)–C(2′1)–C(2′)–S(1′):	171 (1)	167.5 (2)

distances are given in Table III.[9] The figure was drawn by using the program ORTEP.[10]

The platinum atom is in nearly-square planar coordination and is bonded to tenonitrozole through the N(3) position of the thiazole ring, to sulfur of the dimethylsulfoxide (DMSO) molecule, and to two chlorine atoms in a *trans*-square configuration (Figure). The Pt–Cl(2) bond is shorter that of the Pt–Cl(1) and falls below the range of the reported values for the Pt(II)–Cl bond in the related compounds (2.30—2.42 Å).[11] The other distances, Pt–N and Pt–S, are as expected from previous X-ray studies. The

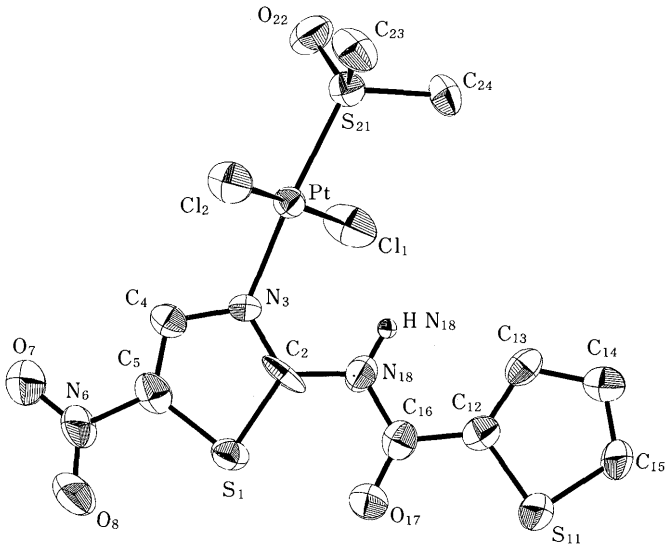


Fig. 1

"mean plane" of the heterocyclic thiazole ring is twisted 119.4° with respect to the $PtCl_2NS$ "mean plane": this value is in accordance with that found by Farrell *et al.*[1] in *trans*-dichloro bis(misonidazole) platinum(II) (dihedral angle: 124°). In the title compound, the NO_2 group lies almost in the "mean plane" of the thiazole ring with a dihedral angle of 5.4°, while in the complex,[1] the same NO_2 group lies out of the "mean plane" of the imidazole ring with a loss of coplanarity.

As shown by the torsion angles (Table III), the ligand in the title complex exhibits the same conformation as in the free ligand.

The closest non-bonded contact between non-hydrogen atoms in the unit-cell is: 2.91(2) Å.

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Solvent Effect in the Oxygenation of *cis*-Stilbene Catalyzed by Non-porphyrin and Porphyrin Iron Complexes

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A solvent effect in the oxygenation reactions of *cis*-stilbene in the mixed solvents, MeCN–C_6H_6, MeCN–CCl_4, and MeCN–CH_2Cl_2, catalyzed by a non-porphyrin iron(II) complex $Fe(MeCN)_6\cdot(ClO_4)_2$ and tetraphenylporphinato iron(III) chloride, resulted in significant changes of the product ratio.

Keywords oxygenation; *cis*-stilbene; solvent effect; mixed solvent; MeCN–C_6H_6; MeCN–CCl_4; MeCN–CH_2Cl_2; $Fe(MeCN)_6\cdot(ClO_4)_2$; tetraphenylporphinato iron(III) chloride

Although a large number of studies on the oxygenation reactions of alkenes catalyzed by enzymes and enzyme modelds for mono-oxygenase have been reported,[1] little attention has yet been paid to solvent effects. Several solvents have been used, namely, methylene chloride, methanol, benzene, chlorobenzene, acetonitrile, water, and their mixtures, for studies on the oxygenation reactions of stilbenes, which have been used as a mechanistic probe for investigations of the catalytic mechanism in mono-oxygenase models.[2,3] We report herein the presence of a solvent effect in the oxygenation reactions of *cis*-stilbene catalyzed by non-porphyrin and porphyrin iron complexes, resulting in significant change of the product ratio.

Experiments on the oxygenation reaction of *cis*-stilbene by a simple model reagent system for mono-oxygenase, $Fe(MeCN)_6^{2+}$–H_2O_2–Ac_2O in MeCN,[4] were carried out under various conditions as described in Table I. The oxygenation reaction of *cis*-stilbene afforded several products, namely, *erythro*- and *threo*-1,2-diphenyl-1,2-di-acetoxyethane (*erythro*-DA and *threo*-DA; **7** and **8**), de-oxybenzoin (DEB; **9**), diphenyl-acetaldehyde (DPA; **10**), diphenylcarbinol acetate (DCA; **12**), benzophenone (BP; **13**), and benzaldehyde (BA; **14**), which are similar to those obtained from *cis*-stilbene with perfluoroiodosylbenzene (C_6F_5IO) catalyzed by the electron-deficient tetraphen-ylporphinato metallo(III) complexes in CH_2Cl_2[2i] except for formation of *cis*- and *trans*-stilbene oxides, because the present reagent system is strongly acidic and contains acetic anhydride and acetic acid, so that the epoxides, if formed, would be transformed to **7** and **8**.

It has been proposed that the active species of the reagent system, $Fe(MeCN)_6^{2+}$–H_2O_2–Ac_2O, may be shown as $Fe^{IV}=O\leftrightarrow Fe^{III}\text{-}O\cdot$ or $Fe^{V}=O\leftrightarrow Fe^{IV}\text{-}O\cdot$.[4] Thus, the for-mation mechanism of several products in this oxygenation reaction of *cis*-stilbene may be postulated to be as shown in Chart 1, according to the previous proposal in the case of the oxygenation reaction catalyzed by porphyrin iron(III) complexes.[2i] That is, *erythro*- and *threo*-DA (**7** and **8**) are formed from the acyclic carbonium ion **3**, which is produced through the pathway (a) or (b) or (c), or from the epoxides **4** by the reaction with acetic anhydride or acetic acid in the presence of the Lewis acid [$Fe(MeCN)_6(ClO_4)_2$]. From **3**, hydride migration yields DEB **9** and phenyl migration provides DPA **10** through the carbonium ions **5** and **6**.

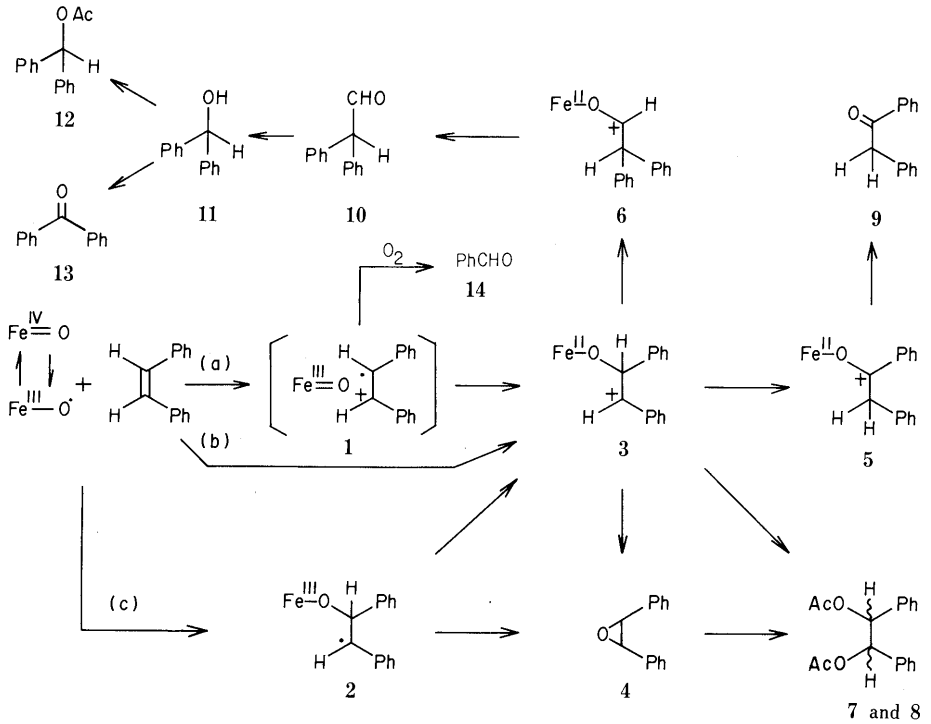

Chart 1

TABLE I. Oxygenation of *cis*-Stilbene Catalyzed by Iron(II) Acetonitrile Solvate in Acetonitrile

Run	Molar ratio[a]			Product distribution (%)[b]							Product yield (mol%)[c]
	$Fe(MeCN)_6^{2+}$	H_2O_2	*cis*-Stilbene	*erythro*-DA **7**	*threo*-DA **8**	DEB **9**	DPA **10**	DCA **12**	BP **13**	BA **14**	
1	0.1	1	1	6.9	3.5	8.6	38.8	3.8	8.1	0.2	36.4
2	0.5	1	1	8.4	10.4	6.8	21.3	8.8	10.4	0.3	32.2
3	1	1	1	7.1	12.3	5.2	19.4	12.0	11.6	0.1	39.1
4	1	2	1	11.1	11.9	5.1	0.0	16.1	26.7	0.4	55.0
5	1	3	1	12.9	7.4	4.6	0.0	8.9	37.0	0.8	83.5
6	0.1	2	1	9.2	7.3	7.9	31.5	5.3	10.2	0.1	47.6
7	0.2	2	1	12.1	8.2	4.4	0.0	16.6	24.7	0.3	40.7

a) Reactions employed 2 mmol of *cis*-stilbene under various conditions in acetonitrile. *b*) Determined by gas-liquid chromatography (GLC) analysis on a 1 m, 10% Chromosorb W, SE-30 column (80—100 mesh) at 120—240 °C (2 °C/min). *c*) Product yields were based on *cis*-stilbene used.

TABLE II. Product Distribution in the Oxygenation of *cis*-Stilbene Catalyzed by Non-porphyrin and Porphyrin Iron Complexes[a]

Run	Solvent (v/v %)	Product distribution (%)[b]							Product yield (mol%)[c]
		erythro-DA **7**	*threo*-DA **8**	DEB **9**	DPA **10**	DCA **12**	BP **13**	BA **14**	
1	MeCN	9.2	7.3	7.9	31.5	5.3	10.2	0.1	47.6
2	MeCN–CCl_4 (1 : 2)	14.3	20.3	13.0	20.7	5.2	13.7	0.2	26.1
3	MeCN–CH_2Cl_2 (1 : 2)	19.3	26.6	—	8.7	18.9[d]	21.3	0.1	14.7
4	MeCN–C_6H_6 (1 : 2)	7.2	18.6	9.5	18.0	7.2	10.3	1.7	21.4[e]

a) Reactions were carried out under the following conditions; $Fe(MeCN)_6^{2+}$: H_2O_2 : *cis*-stilbene = 0.1 : 2 : 1 (molar ratio) in the solvent (30 ml). *b*) See Table I. *c*) Based on starting material. *d*) In the present case, DCA and DEB could not be distinguished. *e*) Benzene consumed 29.8% of H_2O_2 in the oxygenation reaction.

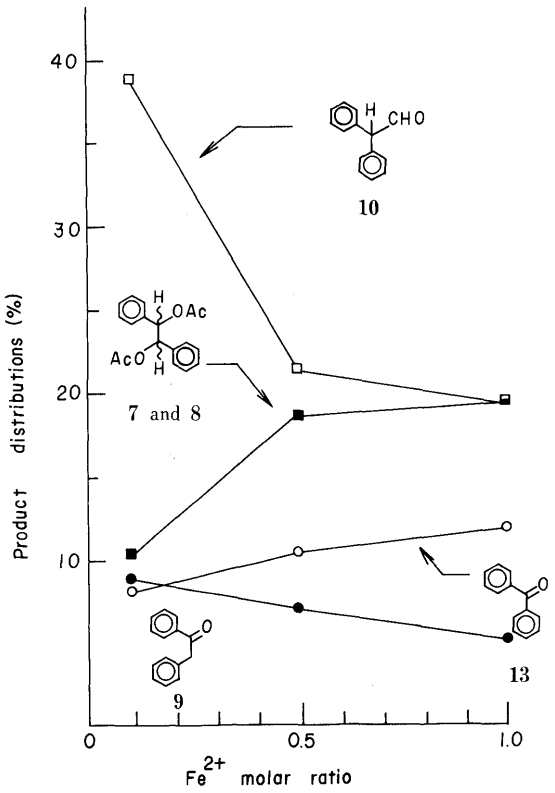

Fig. 1. Plot of Product Distribution (Runs 1, 2, and 3 in Table I) against Iron(II) Molar Ratio

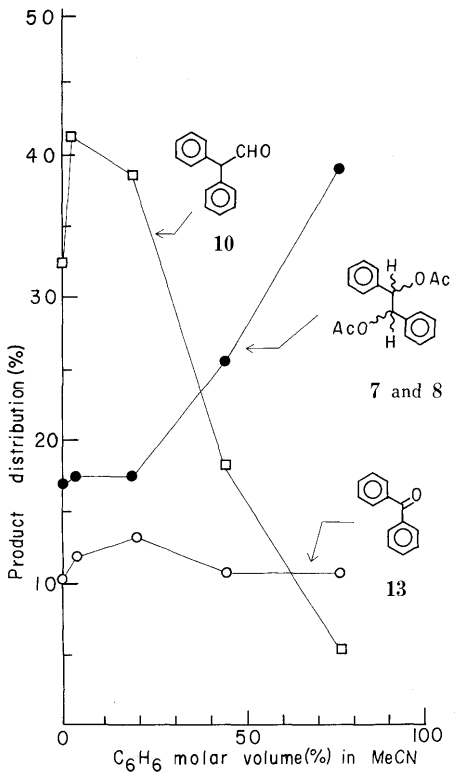

Fig. 2. Plot of Product Distribution against Benzene Molar Volume in the Solvent in the Oxygenation of *cis*-Stilbene by $Fe(MeCN)_6^{2+}$–Ac_2O–H_2O_2

Further oxidation of **10** gave diphenylcarbinol, which transforms to DCA **12** and BP **13**. The reaction of **1** with O_2 results in the formation of benzaldehyde.

Table I shows the results of oxygenation reactions of *cis*-stilbene catalyzed by the iron(II) acetonitrile solvate employing various molar ratios of $Fe(MeCN)_6^{2+}$, H_2O_2, and *cis*-stilbene; the product ratios and yields varied significantly (Fig. 1). These results show that (a) the yield

TABLE III. Oxygenations of *cis*-Stilbene Catalyzed by Iron(II) Acetonitrile Solvate in Acetonitrile–Benzene

Run	Molar ratio[a]			Solvent (v/v %)	Product distribution (%)[b]						Product yield (mol%)[c]
	$Fe(MeCN)_6^{2+}$	H_2O_2	*cis*-Stilbene		DA (*erythro*- + *threo*-) **7**, **8**	DEB **9**	DPA **10**	DCA **12**	BP **13**	BA **14**	
1	0.1	2	1	MeCN–C_6H_6 (30: 0)	16.5	7.9	31.5	5.3	10.2	0.1	47.6
2	0.1	2	1	MeCN–C_6H_6 (28: 2)	17.6	8.9	42.4	8.7	11.6	1.0	45.5[d]
3	0.1	2	1	MeCN–C_6H_6 (20:10)	17.4	6.9	38.6	6.3	13.2	2.0	42.8[d]
4	0.1	2	1	MeCN–C_6H_6 (10:20)	25.8	9.3	18.0	7.2	10.3	1.7	21.4[d]
5	0.1	2	1	MeCN–C_6H_6 (2:28)	39.2	14.3	6.2	7.2	10.5	0.5	32.1[d]

a) Reactions employed 1 mmol of *cis*-stilbene under aerobic conditions. *b*) Product distributions were determined by GLC analysis on a 1 m, 10% Chromosorb W, SE-30 column (80—100 mesh) at 120—240 °C (2 °C/min). *c*) Product yields were based on *cis*-stilbene. *d*) Phenol acetate was formed in yields of 0.03 mmol (4.1 mg, run 2), 0.18 mmol (24.5 mg, run 3), 0.4 mmol (55 mg, run 4), and 0.9 mmol (120 mg, run 5).

TABLE IV. Oxygenations of *cis*-Stilbene Catalyzed by Iron(II) Acetonitrile Solvate in Acetonitrile–Tetrachloromethane

Run	Molar ratio[a]			Solvent (v/v %)	Product distribution (%)[b]						Product yield (mol%)[c]
	$Fe(MeCN)_6^{2+}$	H_2O_2	*cis*-Stilbene		DA (*erythro*- + *threo*-) **7**, **8**	DEB **9**	DPA **10**	DCA **12**	BP **13**	BA **14**	
1	1	3	1	MeCN–CCl_4 (30: 0)	20.3	4.6	0	8.9	37.0	0.8	83.5
2	1	3	1	MeCN–CCl_4 (31: 2)	24.1	4.1	0	7.1	32.6	1.7	49.8
3	1	3	1	MeCN–CCl_4 (23:10)	31.3	4.4	0	7.1	29.6	1.4	44.9
4	1	3	1	MeCN–CCl_4 (13:20)	51.9	2.6	0	5.0	5.4	1.0	30.1
5	1	3	1	MeCN–CCl_4 (5:28)	51.0	3.6	0	7.2	5.8	0.8	42.6

a) Reactions employed 1 mmol of *cis*-stilbene under aerobic conditions. *b*) See Table III. *c*) Product yields were based on *cis*-stilbene.

TABLE V. Product Distribution in the Oxygenation of *cis*-Stilbene Catalyzed by Porphyrin Iron(III) Complexes

Run	Solvent (v/v %)	Product distribution (%)[a]					Product yield[b] (mol%)
		cis-Oxide **4**	DEB **9**	DPA **10**	BP **13**	BA **14**	
1	MeCN	44.4	39.2	5.5	3.2	1.9	33.9
2	CCl_4	70.4	18.1	9.3	—	0.2	35.3
3	CH_2Cl_2	33.6	47.7	10.4	—	0.2	5.4
4	C_6H_6	57.9	32.6	6.9	—	1.0	50.9
5	MeCN–CCl_4 (3:8)	73.7	13.6	9.1	—	0.3	22.4
6	MeCN–CH_2Cl_2 (3:8)	39.8	42.7	10.5	—	0.2	17.5
7	MeCN–C_6H_6 (3:8)	33.8	52.6	7.7	—	0.5	15.9

a) Determined by GLC analysis on a 1 m, 10% Chromosorb W, SE-30 column (80—100 mesh) at 120—240 °C (2 °C/min). *b*) Based on starting material. Reagent; TPPFe(III)Cl : PhIO : *cis*-stilbene = 0.05 : 1 : 1 (molar ratio) in the solvent (11 ml). All reactions were carried out under aerobic conditions.

of the phenyl migration product DPA **10** was increased when lower ratios of the iron(II) solvate relative to the substrate were used (runs 1, 2, and 6), (b) the yields of *erythro*- and *threo*-DA **7** and **8** were increased by the use of larger amounts of H_2O_2 in the presence of an equimolar ratio of the iron(II) solvate and the substrate (runs 4 and 5). Such observations suggest that the selectivity to form the reaction products depends on the conditions used, namely, (a) conditions favoring the migration reactions give DEB **9** and DPA **10** and (b) other conditions give the non-migration product **7** or **8**.

During our investigations, we found that the reaction products were changed significantly by the replacement of the solvent MeCN with mixed solvents such as MeCN–CCl_4,

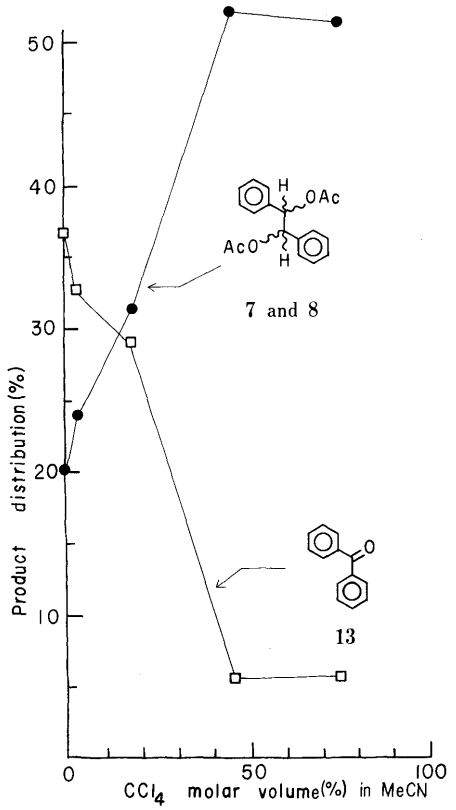

Fig. 3. Plot of Product Distribution against Carbon Tetrachloride Molar Volume in the Solvent in the Oxygenation of *cis*-Stilbene by $^1Fe(MeCN)_6^{2+}$–Ac_2O–H_2O_2

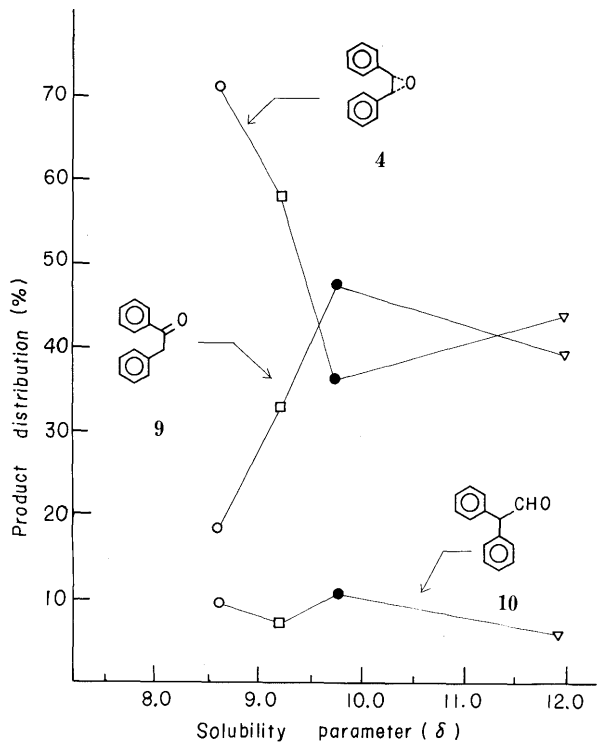

Fig. 4. Plot of Product Distribution in the Oxygenation Reaction of *cis*-Stilbene Catalyzed by TPPFe(III)Cl against Solubility Parameter in Various Solvents

—○—, CCl_4=δ 8.6; —□—, C_6H_6=δ 9.2; —●—, CH_2Cl_2=δ 9.7; —▽—, CH_3CN=δ 11.9.

MeCN–CH_2Cl_2, and MeCN–C_6H_6, as shown in Table II. Detailed experiments on the oxygenation reactions in the mixed solvents MeCN–C_6H_6 and MeCN–CCl_4 were carried out, and the results are shown in Tables III and IV and Figs. 2 and 3. These results clearly show that an increment of the non-polar solvents C_6H_6 (molar volume 89.30 ml/mol) and CCl_4 (molar volume 97.10 ml/mol) relative to MeCN (molar volume 52.78 ml/mol) causes a decrement of formation of the migration products, such as DPA **10** or BP **13**, compared with the non-migration products *erythro*- and *threo*-DA **7** and **8**.

Further, we investigated oxygenation reactions of *cis*-stilbene with iodosylbenzene (PhIO) catalyzed by tetraphenylporphinato iron(III) chloride [TPPFe(III)Cl] in several solvents in connection with the above oxygenation reactions catalyzed by the non-phorphyrin iron complex. In these reactions, we also found that the reaction products changed significantly depending on the solvents used (Table V). The relationship between product distributions and solubility parameters[5] of the solvents is shown in Fig. 3, from which it is clear that solvents having a higher solubility parameter (δ) cause an increment of formation of the hydride-rearranged product DEB **9** (Fig. 3).

These observations of a solvent effect in the oxygenation reactions of *cis*-stilbene catalyzed by non-porphyrin and porphyrin iron complexes, causing significant changes of product ratio, may support the proposed reaction mechanism and suggest that solvents having higher solubility parameters stabilize the carbonium ion intermediates such as **3**, **5**, and **6** by solvation and enhance migration reactions to give the rearrangement products **9**, **10**, **12**, and **13**.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were recorded with a JASCO A-100 grating spectrometer, proton nuclear magnetic resonance (^{1}H-NMR) spectra with a JEOL JNM-FX 100 spectrometer with tetramethylsilane as an internal standard ($CDCl_3$ solution), and mass spectra (MS) with a JEOL LMS-D 300 spectrometer. Gas chromatographic (GC) analyses were performed on a Hitachi 163 analyzer using a flame ionization detector, and quantification was accomplished by comparison of peak areas with those of authentic and synthetic samples. Thin-layer and column chromatographies were performed on Merck Kieselgel 60F-254 and Wakogel C-200 silica gel (100 mesh), respectively. $Fe(ClO_4)_2\cdot 6H_2O$ and *cis*-stilbene were commercial products. *cis*-Stilbene oxide was obtained by the reaction of *cis*-stilbene (0.36 g, 2 mmol), *m*-chloroperbenzoic acid (0.39 g, 2.2 mmol), and K_2HPO_4 (0.05 g) in CH_2Cl_2 (30 ml) with stirring overnight at room temperature; the reaction mixture was worked up in a usual manner, and the product was purified by silica gel column chromatography.

Oxygenation Reaction of *cis*-Stilbene by the Reagent System Fe(MeCN)$_6$(ClO$_4$)$_2$–Ac$_2$O–H$_2$O$_2$ General Procedure: A solution of 30% H_2O_2 (2.4 ml, 22.2 mmol) in MeCN (5 ml) was added over 3 min to a solution of $Fe(ClO_4)_2\cdot 6H_2O$ (1.34 g, 3.7 mmol), Ac_2O (18 ml), and *cis*-stilbene (1.34 g, 7.4 mmol) in MeCN (85 ml) with stirring, keeping the solution temperature at *ca.* 30 °C. The reaction mixture was poured into ice-water, and the organic layer was washed with 5% aqueous $NaHCO_3$ and water and then dried and concentrated. The residue was subjected to silica gel column chromatography with CH_2Cl_2–hexane (1 : 2 v/v%) [eluate A: 0.70 g, Rf=0.5] and CH_2Cl_2–hexane (1 : 1 v/v%) [eluate B: 0.41 g, Rf=0.41]. Eluate A was further subjected to silica gel column chromatography with hexane and CH_2Cl_2–hexane (1 : 10 v/v%) as the eluents to give benzophenone (BP, **13**), diphenyl carbinol acetate (DCA, **12**), and deoxybenzoin (DEB, **9**). Further chromatographic purification of eluate B on silica gel using CH_2Cl_2–hexane (10 : 1 v/v%) as an eluent afforded *erythro*-1,2-diacetoxy-1,2-diphenylethane (DA, **7**) and *threo*-1,2-diacetoxy-1,2-diphenylethane (DA, **8**). These products were identified by comparison of their physical data and retention times in gas chromatography (GC) analysis with those of an authentic samples. 1,2-Diphenyl acetaldehyde (DPA, **10**) obtained under certain reaction condi-

tions (Table I) was also identified by comparison of its physical data and retention time on GC analysis with those of an authentic sample, and by comparison of the 2,4-dinitrophenylhydrazones, mp 146—147 °C.

Oxygenation Reactions of *cis*-Stilbene by the Reagent System Fe(MeCN)$_6$(ClO$_4$)$_2$–Ac$_2$O–H$_2$O$_2$ in the Mixed Solvents The oxygenation reactions in the mixed solvents, MeCN–CCl_4, MeCN–CH_2Cl_2, and MeCN–C_6H_6 at various ratios as described in Tables II, III, and IV, were carried out by the general procedure as described above except for the solvent, MeCN. The products were identified and quantified by GC analysis on a 1 m, SE-30 column (80—100 mesh) of 10% Chromosorb W at 120—240 °C (2 °C/min). Under these conditions, the peaks of benzophenone (BP, **13**), diphenyl acetaldehyde (DDA, **10**), diphenylcarbinol acetate (DCA, **12**), deoxybenzoin (DEB, **9**), *erythro*-1,2-diphenyl-1,2-diacetoxyethane (*erythro*-DA, **7**), and *threo*-1,2-diphenyl-1,2-diacetoxyethane (*threo*-DA, **8**) were observed at the retention times of 28.6, 30.9, 33.2, 34.2, 45.5 and 46.5 min, respectively. Benzaldehyde (BA, **14**) was identified and quantified by GC analysis on a 3 m, DEGS column (80—100 mesh) of 10% Chromosorb W at 150 °C.

Oxidation of Diphenyl Acetaldehyde (10) by Fe(MeCN)$_6$(ClO$_4$)$_3$ Diphenyl acetaldehyde (392 mg, 2 mmol) was added to a solution of Fe(MeCN)$_6$(ClO$_4$)$_3$ prepared from Fe(ClO$_4$)$_3$·H$_2$O (1,032 mg, 2 mmol) and Ac_2O (3 ml) in MeCN (17 ml). After 20 min, the solution was poured into ice water and extracted with CH_2Cl_2. The organic layer was washed with diluted $NaHCO_3$, brine, and water and then dried and concentrated to give **13**.

Oxygenation of *cis*-Stilbene with Iodosylbenzene Catalyzed by Tetraphenylporphinato Iron(III) Chloride General Procedure: Iodosylbenzene (PhIO, 110 mg, 0.5 mmol) was added with stirring to a solution of tetraphenylporphinato iron(III) chloride (16 mg, 0.025 mmol) and *cis*-stilbene (90 mg, 0.5 mmol) in a solvent (MeCN or CCl_4 or CH_2Cl_2, 11 ml) at room temperature under aerobic conditions. After 2 h, the solution was subjected directly to GC analysis on a 1 m, SE-30 column (80—100 mesh) of 10% Chromosorb W at 120—240 °C (2 °C/min) for identification and quantification of the products.

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Degradation of 3-Aryl-2-hydroxyiminopropionic Acids into Arylacetonitriles Using 1,1′-Carbonyldiimidazole or 2,2′-Oxalyldi(*o*-sulfobenzimide)

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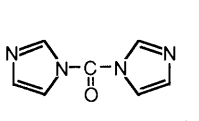
1,1′-Carbonyldiimidazole (1) is a useful reagent for the preparation of arylacetonitriles (9) from 3-aryl-2-hydroxyiminopropionic acids (8), and 2,2′-oxalyldi (*o*-sulfobenzimide) (2) can also be used for this purpose under essentially neutral conditions.

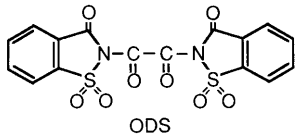
Keywords 1,1′-carbonyldiimidazole; 2,2′-oxalyldi (*o*-sulfobenzimide); arylacetonitrile; 3-aryl-2-hydroxyiminopropionic acid; dehydration; decarboxylation; degradation

1,1′-Carbonyldiimidazole (CDI: **1**)[1] is sometimes utilized for activation of the carbonyl group of carboxylic acids (**3**) in the synthesis of amides (**5**). In addition it can also be applied to the dehydration of aldoximes (**6**) to form nitriles (**7**).[2] In an earlier publication,[3] we showed that 2,2′-oxalyldi (*o*-sulfobenzimide) (ODS: **2**) is an efficient and reliable condensing reagent for the preparation of amides (**5**) under essentially neutral conditions.

The role of arylacetonitriles (**9**) as valuable intermediates for the preparation of 2-arylethylamines[4] and arylacetic acids[5] is well recognized. A well established method for the preparation of **9** is the decarboxylation and dehydration of 3-aryl-2-hydroxyiminopropionic acids (**8**) using acetic anhydride.[5a] However, a disadvantage of this method is the difficulty in separating the desired arylacetonitriles (**9**), which resemble acetic anhydride or aqueous acetic acid very closely in boiling point. To overcome these difficulties in the synthesis of arylacetonitriles (**9**), we examined the ability of CDI (**1**) or ODS (**2**) to degrade 3-aryl-2-hydroxyiminopropionic acids (**8**) into the corresponding arylacetonitriles (**9**).



CDI
1



ODS
2

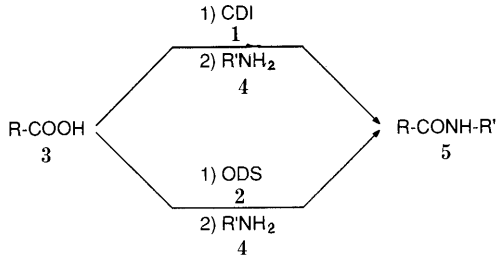


Chart 1

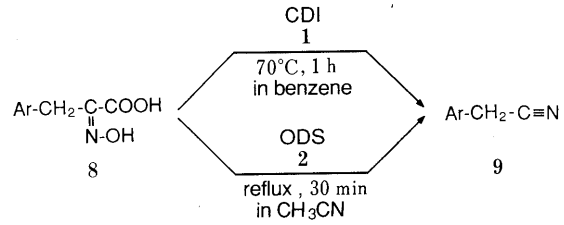


Chart 2

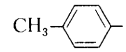
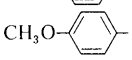
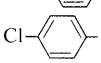
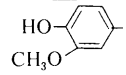
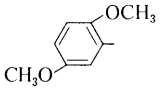
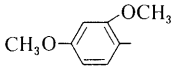
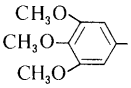
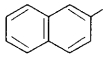
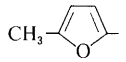
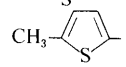
We found that 3-phenyl-2-hydroxyiminopropionic acid (**8a**) reacts readily with CDI (**1**) at 70 °C for 1 h in benzene to afford phenylacetonitrile (**9a**) with accompanying effervescence in 91% yield. This led us to examine the possibility that CDI (**1**) or ODS (**2**) may be generally effective as a reagent for the conversion of **8** into **9** by dehydration and decarboxylation. Incidentally, the critical reagent CDI (**1**) is prepared from the reaction of imidazole with phosgene in tetrahydrofuran.[1b] Unfortunately, the toxicity of phosgene means that the preparation of CDI (**1**) in the average laboratory is quite troublesome. On the other hand, ODS (**2**) is easily prepared from the reaction of *o*-sulfobenzimide (saccharin) in benzene with oxalyl chloride in the presence of triethylamine at room temperature.

The purpose of this paper is to describe a simple method for the preparation of arylacetonitriles (**9**) using CDI (**1**) or ODS (**2**). Continuing from the degradation of 3-phenyl-2-hydroxyiminopropionic acid (**8a**) into phenylacetonitrile (**9a**) using CDI (**1**) mentioned above, we prepared **9a** directly from the reaction of **8a** with ODS (**2**). This degradation was performed within 30 min in refluxing acetonitrile and the product (**9a**) was obtained in 77% yield, whereas the reaction giving a 70% yield of **9a** required 2 h at 70 °C in benzene because ODS (**2**) is not sufficiently soluble in benzene.

This degradation method using CDI (**1**) or ODS (**2**) was found to be satisfactory for several structurally different 3-aryl-2-hydroxyiminopropionic acids (**8b—o**). The transformation of 3-(4-substituted phenyl)-, 3-(3,4-, 2,4- or 2,5-disubstituted phenyl)-, 3-(3,4,5-trisubstituted phenyl)-, and 3-(1- or 2-naphthyl)-2-oximinopropionic acids (**8b—k**) into the corresponding acetonitriles (**9a—k**) was also accomplished in acceptable yields as shown in Table I. Moreover, it was ascertained that the heteroaromatic compound 3-(2-furyl)-2-hydroxyiminopropionic acid (**8l**) readily reacted with CDI (**1**) or ODS (**2**) to give 2-furylacetonitrile (**9l**) in 75% or 70% yield, respectively. A similar result was observed with 3-(5-methyl-2-furyl)-, 3-(2-thenyl)-, and 3-(5-methyl-2-thenyl)-2-hydroxyiminopropionic acids (**8m—o**), which were converted into the corresponding arylacetonitriles (**9m—o**) in 71—86% yields.

All of the arylacetonitriles (**9a—o**) prepared in our experiments are known compounds. The structures of **9a—o** were established mainly by infrared (IR) spectroscopic

TABLE I. Substituted Acetonitriles (**9**) Prepared

Product	Ar	Yield (%)[a]		bp (°C)/Torr or [mp (°C)]		IR (cm^{-1})
		CDI (**1**)	ODS (**2**)	Found	Reported	ν-CN (phase)
9a		91	77	80—81/3	98—100/9[4d]	2254 (neat)
9b		95	84	84—87/3	107—109/9[4d]	2254 (neat)
9c		93	86	105—108/3	74—76/0.2[4d]	2252 (neat)
9d		94	90	117—120/3	140/13[6]	2252 (neat)
9e		90	85	113—116/16	133—143/24[7]	2252 (neat)
9f		83	80	140—143/0.2	135—140/0.05[4c]	2252 (neat)
9g		88	87	[53—54][b]	[56—57][8]	2254 (KBr)
9h		94	89	[74—75][b]	[76][9]	2248 (KBr)
9i		85	83	[75—77][c]	[77][10]	2244 (KBr)
9j		97	87	106—109/0.3	183—187/15[11]	2252 (neat)
9k		97	83	[79—82][d]	[77][11]	2254 (KBr)
9l		75	75	88—90/27	64—65/9[12]	2254 (neat)
9m		86	71	91—93/15	82—85/12[13]	2256 (neat)
9n		88	81	109—111/15	89—100/9[4d]	2254 (neat)
9o		94	86	107—110/17	110—112/20[14]	2252 (neat)

a) Yield of isolated product **9** based on **8**. *b*) Recrystallized from benzene–pet. ether. *c*) Recrystallized from EtOH–water. *d*) Recrystallized from benzene.

studies. The IR spectra of **9a—o** show characteristic absorption bands for the cyano group at near 2250 cm^{-1}. These results, which demonstrate the effectiveness of this method, are summarized in Table I.

Regarding the arylacetonitrile (**9a—o**) yields from our procedure, the data of Table I show that not only CDI (**1**), but also ODS (**2**) can form **9a—o** through degradation of the 3-aryl- or 3-heteroaryl-2-hydroxyiminopropionic acids (**8a—o**). In general, the procedure which utilized CDI (**1**) gave each of the products (**9a—o**) in a slightly higher yield than that which used ODS (**2**). However, CDI (**1**), through commercially available, is too expensive for utilization on a large scale, whereas ODS (**2**) is readily preparable at low cost was nonhygroscopic crystals.[3] With regard to cost and performance relationships, we suggest that ODS (**2**) is more convenient to handle on a laboratory scale than CDI (**1**). In addition, the application of CDI (**1**) to the degradation produces basic imidazole as a by-product, which might cause serious difficulties in the synthesis of base-sensitive arylacetonitriles. In such a case, simple replacement of CDI (**1**) by ODS (**2**) is useful because saccharin formed as a by-product is essentially neutral.

As shown in Chart 3, the mechanism of this degradation most probably involves nucleophilic attack of the hydroxy group of **8** on the carbonyl carbon of CDI (**1**) to give an intermediate (**11**), which undergoes internal rearrangement (path A). In a like manner, the reaction of **8** with ODS (**2**) proceeds *via* the formation of an intermediate (**13**) and the subsequent concerted elimination of CO_2, CO, and saccharin takes place under heating to afford the corresponding arylacetonitrile (**9**) as a final product (path B).

In conclusion, the degradation reaction described herein has the following advantages over the previously reported preparation methods for arylacetonitriles (**9**) using acetic anhydride as the dehydrating reagent: (i), the reagent CDI (**1**) or ODS (**2**) can be employed for the degradation of 3-aryl-2-hydroxyiminopropionic acids (**8a—o**) into **9a—o** with no additive by means of a one-pot procedure, which is simple; (ii), reaction condition are mild with short reaction times, and (iii), using simple chromatographic techniques, the prepared arylacetonitriles (**9**) can usually be isolated in good yields.

Experimental

Melting points were measured on a Yanagimoto melting point apparatus. Boiling points refer to the oven temperature of a Kugelrohr apparatus.

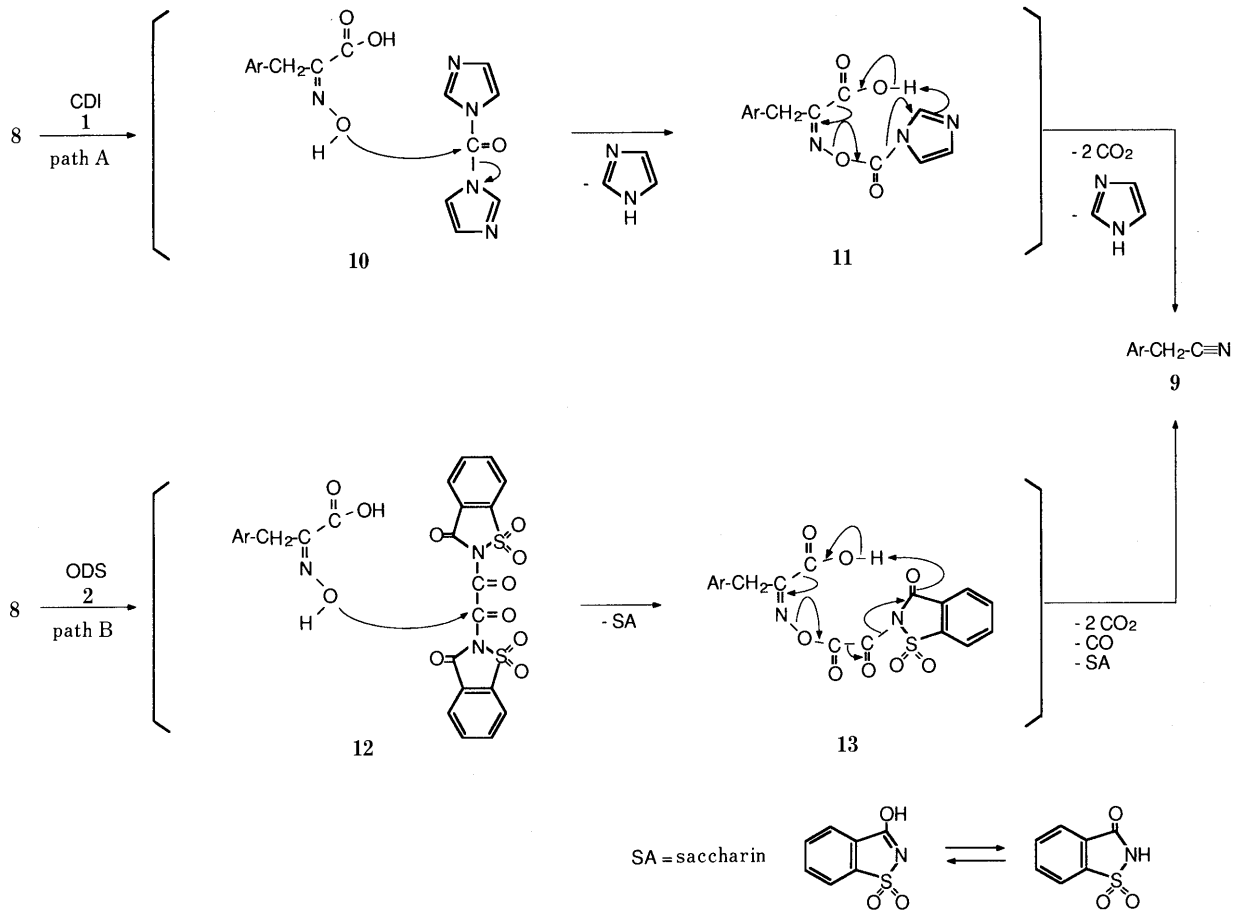


Chart 3

All melting and boiling points are uncorrected. IR spectra were recorded on a Hitachi 270-30 IR spectrophotometer.

Materials CDI (**1**) from Aldrich Chemical Company, Inc. was used without further purification. ODS (**2**) was prepared from the reaction of saccharin with oxalyl chloride in the presence of triethylamine according to a previous report.[3] Distilled benzene and acetonitrile were subjected to successive drying over 4 Å molecular sieves. 3-Aryl-2-hydroxyiminopropionic acids (**8a**—**o**) except **8g** and **8k** were prepared as previously described; **8a**,[15] **8b**,[14] **8c**,[16] **8d**,[15] **8e**,[17] **8f**,[5a] **8h**,[18] **8i**,[10] **8j**,[14] **8l**,[12] **8m**,[5b] **8n**,[5b] and **8o**.[14] 3-Aryl-2-hydroxyiminopropionic acids (**8a**—**o**) used in our experiments are mixtures of (*E*)- and (*Z*)-isomers.

3-(2,5-Dimethoxyphenyl)-2-hydroxyiminopropionic Acid (8g) This compound (**8g**) was prepared according to a published procedure.[5a] Hydroxylamine hydrochloride (4.3 g, 62 mmol) in water (5 ml) was allowed to react with sodium ethoxide [prepared from sodium (1.6 g, 69 mg atom) and anhydrous EtOH (40 ml)]. After removal of inorganic material by filtration, the filtrate containing free hydroxylamine was added to 2,5-dimethoxyphenylthiopyruvic acid (4.8 g, 20 mmol) and the mixture was refluxed for 1 h. The solvent was removed under reduced pressure and the residue was dissolved in 5% NaOH (100 ml). The aqueous phase was filtered through a sintered glass filter and the filtrate was cooled to −15 °C, then acidified with 10% HCl (90 ml). The precipitate was washed with water and dried *in vacuo*; the yield was 4.6 g (96%); crystals (benzene–AcOEt), mp 162—163 °C. *Anal.* Calcd for $C_{11}H_{13}NO_5$: C, 55.23; H, 5.48; N, 5.86. Found: C, 55.05; H, 5.46; N, 5.96. IR ν_{max}^{KBr} cm^{-1}: 1701 (CO).

3-(2-Naphtholyl)-2-hydroxyiminopropionic Acid (8k) Treatment of 2-naphtholylthiopyruvic acid[19] (4.6%, 20 mmol) with hydroxylamine [prepared from hydroxylamine hydrochloride (4.3 g, 62 mmol) and Na metal (1.6 g, 69 mg atom)] as described for the preparation of **8g** gave **8k**; the yield was 3.2 g (69%); crystals (benzene–AcOEt), mp 170—175 °C. *Anal.* Calcd for $C_{13}H_{11}NO_3$: C, 68.11; H, 4.84; N, 6.11. Found: C, 68.05; H, 4.96; N, 6.04. IR ν_{max}^{KBr} cm^{-1}: 1701 (CO).

Preparation of Arylacetonitriles (9) Using CDI (1) General Procedure CDI (**1**) (3.3 g, 20 mmol) was added to a solution of the appropriate 3-substituted 2-hydroxyiminopropionic acid (**8**) (20 mmol) in benzene (50 ml). The mixture was stirred at room temperature for 15 min, then heated at 68—70 °C for 1 h. After cooling to room temperature, the resultant mixture was poured into ice water (30 ml) and the organic layer was extracted with 50 ml of benzene in several portions. The combined extracts were washed with 1% $NaHCO_3$ (15 ml), 1% HCl (15 ml), and water (15 ml), then dried over anhydrous sodium sulfate. The organic layer was evaporated under reduced pressure to give the crude acetonitrile (**9**), which was developed on a silica gel column with benzene. Concentration of the first eluate (100 ml) gave **9**, which was further purified by distillation or recrystallization.

Preparation of Arylacetonitriles (9) Using ODS (2) General Procedure ODS (**2**) (8.4 g, 20 mmol) was added to a solution of the appropriate 3-substituted 2-hydroxyiminopropionic acid (**8**) (20 mmol) in acetonitrile (70 ml). The mixture was stirred at room temperature for 15 min, then refluxed for 30 min. After cooling to room temperature, the solvent was removed under reduced pressure to give a residue, which was treated with benzene, with stirring. Precipitated saccharin was removed by filtration. Evaporation of the benzene afforded crude **9**, which was purified by column chromatography on silica gel as described for the preparation of **9** using CDI (**1**). The yield, melting point or boiling point, and spectroscopic data for the substituted acetonitriles (**9a**—**o**) prepared are given in Table I.

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Cynanformosides A and B, Two New Pregnane Glycosides, from the Aerial Part of *Cynanchum formosanum*

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Two new pregnane glycosides, cynanformosides A and B, together with α-amyrin acetate, taraxerol, chrysoeriol, and isorhamnetin were isolated from the aerial part of *Cynanchum formosanum*. The structures of the new pregnane glycosides have been elucidated by spectroscopic and chemical methods.

Keywords *Cynanchum formosanum*; Asclepiadaceae; steroid; cynanformoside A; cynanformoside B; pregnane glycoside

Extensive studies on the constituents[1-6] of some species of *Cynanchum* (*C.*), as well as the immunopotentiating activities[6b,7] of C/D-*cis*-polyoxypregnane glycosides, have been reported. In connection with our interest in pregnane-type steroids, chemical studies on the constituents of *C. formosanum* (MAXIM.) HEMSL. ex FORBES *et* HEMSL., a folk medicine used as an expectorant,[8] were undertaken in our laboratory.

The methanol extract of the aerial part of *C. formosanum* was partitioned between petroleum ether and 80% aqueous methanol. The aqueous methanol layer was evaporated under reduced pressure to afford an aqueous solution which was extracted successively with chloroform and butanol. After repeated purification on silica gel and Sephadex LH-20 columns, triterpenoids(α-amyrin acetate[9] and taraxerol[10]), pregnane glycosides [cynanformosides A (**1a**) and **B** (**1b**)], and flavonoids (chrysoeriol[11] and isorhamnetin[12])were isolated from the petroleum ether extract, chloroform extract, and butanol extract, respectively. This paper deals with the structural elucidation of cynanformosides A (**1a**) and B (**1b**).

Cynanformoside A (**1a**), mp 183 °C (dec.), $[\alpha]_D^{24}$ −15.5° (c=1.00 in $CHCl_3$), has the molecular formula $C_{28}H_{46}O_8$ on the basis of elemental analysis and its infrared (IR) spectrum shows hydroxyl group (3407 cm^{-1}) and olefinic group (3053 and 1636 cm^{-1}) absorptions. The presence of a steroidal glycoside with a 2-deoxysugar was indicated by the positive Liebermann–Burchard and Keller–Kiliani reactions.[13] The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1a** (Table I) showed seven methine proton signals at δ 3.10—3.62 (5H, m, 3-, 12-, 3′-, 4′-, and 5′-H), 4.04 (1H, q, J=6.4 Hz, 20-H), and 4.57 (1H, dd, J=9.1, 1.3 Hz, 1′-H), four methyl signals at δ 0.98 and 1.13 (each 3H, s, 18- and 19-H), 1.17 (3H, d, J=6.4 Hz, 21-H), and 1.30 (3H, d, J=6.0 Hz, 6′-H), one methoxyl signal at 3.36 (3H, s), and one olefinic proton signal at δ 5.38 (1H, br s, 6-H). The coupling constant of the anomeric proton (1′-H) revealed that the sugar is β-linkage. The mass spectrum (MS) of **1a** exhibited the (M^+ − sugar) peak at m/z (%) 348 (20) and other fragment peaks at 330 (M^+ − sugar − H_2O, 13), 312 (M^+ − sugar − $2H_2O$, 29), 303 (M^+ − sugar − CH_3CHOH, 46), 145 [(sugar − OH)$^+$, 100], 113 [(sugar − OH–CH_3OH)$^+$, 47]. The peaks at 303, 145, and 113 suggested the presence of a 1-hydroxyethyl group at C-17[14] and oleandrose[15] (or cymarose). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopic data (Table II) of **1a** were also consistent with the proposed structure. The acetylation of **1a** with Ac_2O and pyridine at room temperature for 1 d afforded a triacetate (**1c**) [mp 211—214 °C; IR ν_{max}^{KBr}: 3441 and 1731 cm^{-1}; ^{1}H-NMR δ: 1.95, 2.05, 2.06 (each 3H, s)]. The result shows that the structure of **1a** contains three secondary hydroxyl groups. A comparison of the physical data of **1a** and utendin-3-*O*-β-D-cymaropyranoside (**1d**) isolated from *Marsdenia formosana*[16] showed that they are different compounds with similar structures except for oleandrosyl instead of cymarosyl at 3-C. The existence of the oleandrosyl moiety in **1a** was confirmed by comparison of the chemical shifts of 1′-H (δ 4.57) and CH_3O- (δ 56.5) with those (δ 4.80, 59.0)[3a,6b,17] in cymarosyl. In addition, the signal of 4′-H

TABLE I. ^{1}H-NMR Data (δ-Value) for **1a**, **1b** and **1d** ($CDCl_3$)

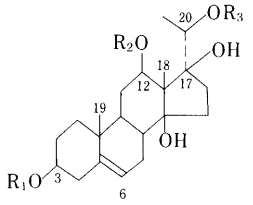
H	1a[a]	1b[a]	1d[b]
3	3.46—3.60 m	3.54 m	
6	5.38 br s	5.38 br s	5.40 br s
12	3.46—3.60 m	3.41 dd (9.9, 5.7)	
18	0.98 s	0.96 s	1.00 s
19	1.13 s	1.16 s	1.14 s
20	4.04 q (6.4)[c]	5.15 q (6.5)	
21	1.16 d (6.0)	1.24 d (7.5)	1.20 d (6.0)
1′	4.57 dd (9.1, 1.3)	4.57 dd (8.4, 1.9)	4.80 dd (9.0, 3.0)
6′	1.30 d (6.4)	1.29 d (6.0)	1.28 d (6.0)
CH_3O–	3.36 s	3.38 s	3.42 s
CH_3CO–		2.02 s	

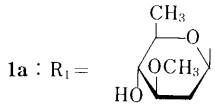
a) 300 MHz. *b*) 60 MHz. *c*) Figures in parentheses are coupling constants in Hz.

TABLE II. ^{13}C-NMR Data (δ-Value) for **1a** and **1b** (75 MHz, $CDCl_3$)

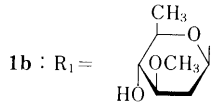
C	1a	1b	C	1a	1b
1	37.2 t	38.6 t	16	29.4 t	29.5 t
2	31.0 t	31.1 t	17	87.7 s	87.5 s
3	75.5 d	75.6 d	18	8.1 q	7.0 q
4	38.5 t	38.6 t	19	19.4 q	19.3 q
5	139.0 s	139.2 s	20	72.4 d	75.2 d
6	122.0 d	122.0 d	21	16.7 q	14.8 q
7	31.4 t	31.7 t	CH_3CO–		21,3 q
8	35.6 d	36.1 d	CH_3CO–		169.8 s
9	42.6 d	42.8 d	1′	97.6 d	97.7 d
10	36.8 s	36.7 s	2′	35.6 t	35.7 t
11	26.1 t	26.1 t	3′	80.8 d	80.8 d
12	71.6 d	71.6 d	4′	77.4 d	77.6 d
13	56.2 s	56.8 s	5′	70.4 d	69.8 d
14	88.3 s	88.0 s	6′	17.9 q	17.8 q
15	32.3 t	32.1 t	CH_3O–	56.5 q	56.1 q

Assignments established by DEPT and off-resonance methods. s, singlet; d, doublet; t, triplet; q, quartet.

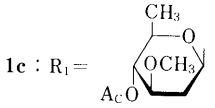




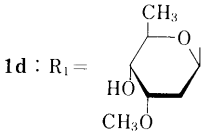
1a : R_1 = $R_2=R_3=H$



1b : R_1 = $R_2=H$, $R_3=Ac$



1c : R_1 = $R_2=R_3=Ac$



1d : R_1 = $R_2=R_3=H$

1e : $R_1=R_2=R_3=H$

in **1c** appears at δ 4.63 with a large coupling constant (t, $J=9.3$ Hz). On acid hydrolysis with aqueous 0.025 M H_2SO_4–1,4-dioxane, **1a** gave an aglycone and a sugar. The resulting aglycone (mp 248—250 °C) was identical co-thin layer chromatography ((co-TLC) and mixed melting point) with an authentic sample of natural utendin (**1e**)[18] which was obtained by the acidic hydrolysis of **1d**.[16] The sugar fraction was also identified as oleandrose by comparison of the thin-layer chromatographic (TLC) behavior with that of an authentic sample. As a result of the above experiments, cynanformoside A is proved to be utendin-3-*O*-β-oleandropyranoside.

Cynanformoside B (**1b**), mp 212—214 °C, $[\alpha]_D^{20}$ −17.5° ($c=1.00$ in $CHCl_3$), has the molecular formula $C_{30}H_{48}O_9$ on the basis of elemental analysis its IR spectrum, which showed the presence of hydroxyl group (3400 cm^{-1}), olefinic group (3051 and 1637 cm^{-1}), and acetoxyl group (1720 cm^{-1}) absorptions. It also exhibited positive Liebermann–Burchard and Keller–Kiliani reactions. Prominent MS peaks indicative of a 1-acetoxyethyl group were observed at *m/z* 43 (acetyl cation) and 87 (acetoxyethyl cation). Further substantiation was obtained from the MS peaks of this compound at 390 (M^+ − oleandrose), 372 (M^+ − oleandrose − H_2O), 354 (M^+ − oleandrose − $2H_2O$), 330 (M^+ − oleandrose − AcOH), 303 (M^+ − oleandrose − CH_3CHOAc), 145 [(oleandrose − OH)$^+$], and 113 [oleandrose − OH–CH_3OH)$^+$]. The peaks at *m/z* 303, 145, and 113 suggested the presence of acetoxyl at C-20[14] and oleandrosyl.[15] The ^{1}H-NMR spectrum of **1b** (Table I) also showed seven methine signals, four methyl signals, one acetoxyl signal, one methoxyl signal, and one olefinic signal. Compound **1b** showed similar ^{1}H-NMR signals to **1a** except for the low-field shift of the quartet signal at δ 5.15 (20-H) and an additional acetyl group. This fact suggests that **1b** is a monoacetate derivative of **1a**, and the acetoxyl group of **1b** must be located at C-20. Hydrolysis of **1b** with 5% methanolic sodium hydroxide afforded **1a** as a neutral product. The ^{13}C-NMR data (Table II) of **1b** were also consistent with the structure of **1b**. On the basis of the above evidence, the structure of cynanformoside B can be assigned as 20-*O*-acetylutendin-3-*O*-β-oleandropyranoside (**1b**).

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter at room temperature. IR spectra were recorded on a JASCO A-102 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were run on a Bruker AM 300 in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given in δ values and coupling constants (J) are given in hertz (Hz). Electron impact mass spectrum (EI-MS) was taken on a JEOL JMS-100 spectrometer.

Extraction and Isolation The air dried aerial parts of *C. formosanum* (2.3 kg) were extracted with methanol (5 l) five times (6 h for every time) under reflux. The combinded extracts were evaporated under reduced pressure to give a residue, which was partitioned between petroleum ether (500 ml) and 80% aqueous methanol (500 ml). The latter layer was evaporated to about 100 ml of aqueous solution, then 200 ml of water was added. The aqueous solution was partitioned with choloroform (300 ml) and butanol (300 ml), successively. Every fraction was evaporated to give a residue, and the weights of the petroleum ether fraction, chloroform fraction, butanol fraction, and the last aqueous fraction were 36.1 g, 14.6 g, 43.7 g, and 59.3 g, respectively. After repeated purification on silica gel and Sephadex LH-20 columns, α-amyrin acetate (202 mg) and taraxerol (13 mg) from the petroleum ether fraction, cynanformoside B (**1b**, 11 mg) and cynanformoside A (**1a**, 15 mg) from the chloroform fraction, and chrysoerial (18 mg) and isorhamnetin (8 mg) from the butanol fraction were isolated.

Cynanformoside A (1a) mp 183 °C (dec.) (from chloroform–hexane), $[\alpha]_D^{24}$ −15.5° ($c=1.00$ in $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3407, 3053, 1636, 1407, 1365, 1272, 1069, 988, 962, 806, 737. MS *m/z* (%): 348 (M^+ − oleandrose, 20), 330 (13), 312 (29), 303 (46), 285 (24), 268 (19), 145 (100), 113 (47). *Anal.* Calcd for $C_{28}H_{46}O_8$: C, 65.85; H, 9.08. Found C, 65.75; H, 9.05.

Cynanformoside B (1b) mp 212—214 °C (from chloroform–hexane), $[\alpha]_D^{20}$ −17.5° ($c=1.00$ in $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3415, 3051, 1720, 1637, 1260, 1160, 1100, 1065, 1030, 980, 963, 845. MS *m/z* (%): 390 (M^+ − oleandrose, 5), 372 (2), 354 (12), 330 (11), 303 (11), 297 (14), 294 (13), 276 (16), 251 (13), 224 (19), 145 (100), 113 (65), 95 (6), 87 (11), 43 (8). *Anal.* Calcd for $C_{30}H_{48}O_9$: C, 65.19; H, 8.75. Found C, 65.24; H, 8.69.

α-Amyrin Acetate[9] mp 228 °C (from chloroform–methanol). IR ν_{max}^{KBr} cm^{-1}: 1730, 1450, 1370, 1250. MS *m/z* (%): 468 (9.5, M^+), 408 (3), 218 (100), 203 (36), 189 (31.5), 175 (10.5), 161 (21), 147 (28.4), 133 (35.7), 122 (26.3), 107 (23.1). ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, s), 0.84 (3H, s), 0.89 (9H, s), 0.95 (3H, s), 0.98 (3H, s), 1.04 (3H, s), 2.02 (s, OAc), 4.47 (dd, $J=7.4$, 9.1 Hz, 3α-H), 5.11 (t, $J=3.0$ Hz, 12-H). ^{13}C-NMR ($CDCl_3$) δ: 38.5 (C-1), 23.6 (C-2), 80.9 (C-3), 37.7 (C-4), 55.3 (C-5), 18.3 (C-6), 32.9 (C-7), 40.0 (C-8), 47.6 (C-9), 36.8 (C-10), 17.5 (C-11), 124.3 (C-12), 139.6 (C-13), 42.1 (C-14), 26.6 (C-15), 26.6 (C-16), 33.7 (C-17), 59.1 (C-18), 39.6 (C-19), 39.6 (C-20), 31.2 (C-21), 41.5 (C-22), 28.9 (C-23), 16.7 (C-24), 15.7 (C-25), 16.9 (C-26), 23.3 (C-27), 28.1 (C-28), 23.4 (C-29), 21.4 (C-30), 170.9 (MeC=O), 21.3 (MeC=O).

Taraxerol[10] mp 280—282 °C (from chloroform–methanol). IR ν_{max}^{KBr} cm^{-1}: 3500, 1480, 1390, 1380, 1040. MS *m/z* (%): 426 (9.1, M^+), 411 (12.7), 393 (4.6), 302 (34.5), 287 (54.5), 269 (34), 257 (19.1), 245 (15.5), 231 (13.6), 218 (21.8), 204 (100), 189 (50), 175 (25.5), 161 (23.6), 147 (36.4), 135 (80), 121 (56.4), 107 (59.1). ^{1}H-NMR ($CDCl_3$) δ: 0.79 (3H, s), 0.89 (3H, s), 0.91 (6H, s), 0.93 (3H, s), 0.95 (3H, s), 1.01 (3H, s), 1.25 (3H, s), 3.18 (m, 3-H), 5.51 (dd, $J=3.2$, 8.1 Hz, 15-H).

Chrysoeriol[11] mp 280—282 °C (from chloroform–methanol). IR ν_{max}^{KBr} cm^{-1}: 3600—3349, 1644, 1617, 1593, 1559, 1501, 1429. UV λ_{max}^{MeOH} nm (log ε): 241 sh (3.89), 249 (3.9), 268 (3.9), 345 (4.0); +NaOMe: 265, 405; +$AlCl_3$: 276, 358, 386; +$AlCl_3$/HCl: 276, 355, 383, +NaOAc: 274, 320, 362; +NaOAc/H_3BO_3: 252, 269, 347. MS *m/z* (%): 300 (100), 285 (0.3), 272 (8.2), 257 (19.5), 229 (11.7), 153 (6.5), 148 (3.4), 124 (2.1), 105 (4.3). ^{1}H-NMR (DMSO-d_6) δ: 12.4 (5-OH), 8.1 (OH), 7.49 (2H, d, $J=8.6$ Hz),

6.92 (1H, d, J=8.6 Hz), 6.72 (1H, s), 6.43 (1H, d, J=2.0 Hz), 6.16 (1H, d, J=2.0 Hz), 3.89 (3H, s).

Isorhamnetin[12] mp 308—310 °C (from chloroform–methanol). IR ν_{max}^{KBr} cm^{-1}: 3229, 1650, 1609, 1552, 1501. UV λ_{max}^{MeOH} nm (log ε): 254 (3.2), 262 sh (3.1), 294 (4.0), 340 sh (3.2), 367 (3.6); +NaOMe: 275, 323, 413; +$AlCl_3$: 266, 304, 358, 428; +$AlCl_3$/HCl: 266, 302, 357, 426; +NaOAc: 255, 274, 320, 379; +NaOAc/H_3BO_3: 254, 294, 369. MS m/z (%): 316 (100), 301 (10.5), 287 (10), 273 (8), 257 (4), 245 (11), 217 (4.5), 153 (2.5), 123 (4.5), 108 (4). ^{1}H-NMR (DMSO-d_6) δ: 12.43 (5-OH), 10.7 (OH), 9.69 (OH), 9.37 (OH), 7.73 (d, J=2.0 Hz, 2′-H), 7.68 (dd, J=8.4, 2.0 Hz, 6′-H), 6.92 (d, J=8.4 Hz, 5′-H), 6.45 (d, J=2.0 Hz, 8-H), 6.17 (d, J=2.0 Hz, 6-H), 3.84 (3H, s, Me).

Acetylation of Cynanformoside A (1a) A solution of cynanformoside A (**1a**, 5 mg) in pyridine (0.2 ml) and acetic anhydride (0.2 ml) was left for 1d at room temperature. The reaction mixture was treated by the usual method and purified by silica gel column chromatography (10% EtOAc in hexane as the eluent) to give a triacetate (**1c**) (5 mg) [mp 211—214 °C (from MeOH). IR ν_{max}^{KBr} cm^{-1}: 3441, 1731, 1259, 1238, 1105, 1060, 1035, 1018, 973, 802. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, s, 18-H), 1.16 (3H, d, J=6.2 Hz, 6′-H), 1.20 (3H, s 19-H), 1.21 (3H, d, J=6.0 Hz, 21-H), 1.95, 2.05, 2.06 (each 3H, s, -OCOMe), 3.29 (3H, s, -OMe), 4.49—4.61 (3H, m, 1′-, 12-, 20-H), 4.63 (1H, t, J=9.3 Hz, 4′-H), 5.37 (1H, br s, 6-H)].

Mild Hydrolysis of Cynanformoside A (1a) with Acid Cynanformoside A (**1a**) (10 mg) in 80% aqueous 1,4-dioxane (1.2 ml) was mixed with 0.05 M H_2SO_4 (1.2 ml) and warmed for 30 min at 50 °C.[18] Dioxane was then removed under reduced pressure. The aqueous concentrate was repeatedly extracted with chloroform and the organic layer washed in turn with H_2O, 3% Na_2CO_3, and H_2O, dried over Na_2SO_4, and evaporated to afford utendin (**1e**) (colorless needles from $CHCl_3$–MeOH, mp 248—250 °C)[19] (5 mg). The aqueous layer of the hydrolysate was neutralized with Amberlite IR-45 and concentrated to dryness under reduced pressure. The residue was identified as oleandrose by TLC comparison ($CHCl_3$: MeOH = 9 : 1), with an authentic sample.

Alkaline Hydrolysis of Cynanformoside B (1b) Cynanformoside B (**1b**) (10 mg) was dissolved in 5% methanolic NaOH (1 ml) at room temperature overnight. After addition of H_2O (1 ml), MeOH was removed under reduced pressure. The aqueous concentrate was extracted with $CHCl_3$, dried over Na_2SO_4, filtered and evaporated to dryness, yielding a product (7 mg) which was identical with cynanformoside A (**1a**).

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Triazolo[4,5-*d*]pyrimidines. XI.[1] Halogen–Metal Exchange Reaction of 5-Halo-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidines with Butyllithium

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The amino group at the 5-position on the 3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (triazolopyrimidine) ring was converted into a halogen atom by treatment with isopentyl nitrite in halomethanes in satisfactory yields.

The 5-halotriazolopyrimidines not having a substituent at the 7-position reacted with butyllithium to give 7-butyl-6,7-dihydro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidines by addition of butyllithium across the C^7,N^6-double bond. In the case of the 7-substituted 5-halotriazolopyrimidines, the halogen–metal exchange reaction proceeded and the resultant 5-lithio compound reacted with electrophiles to give the 5-substituted triazolopyrimidines.

Keywords 5-halo-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine; butyllithium; halogen–metal exchange reaction; addition; electrophile; 5-substituted 3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine

It is well known[2] that lithio compounds of heteroarenes are useful intermediates to introduce carbon chains into heteroarenes. In the preceding paper, we reported[1] that the halogen–metal exchange reaction between the iodine atom at the 7-position on the 3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (triazolopyrimidine) ring and butyllithium (BuLi) proceeded and the resultant 7-lithio compound smoothly reacted with electrophiles such as aldehydes and ketones to give the 7-substituted triazolopyrimidines.

As a continuation of that work, we have investigated the lithiation of 5-halotriazolopyrimidines to introduce electrophiles into the 5-position on the triazolopyrimidine ring. In the present paper, we describe the preparation of 5-halotriazolopyrimidines and their reaction with BuLi.

It was reported[3] that an amino group on heteroarenes is converted easily into a halogen atom by treatment with pentyl nitrite in halomethanes, such as tribromomethane and diiodomethane. We therefore examined the preparation of 5-bromo- (**4a**, **b**) and 5-iodo-3-phenyl-3*H*-1,2,3-triazolo-[4,5-*d*]pyrimidines (**3a**, **b**) from the 5-amino derivatives (**2a**, **b**).

When a solution of 5-amino-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**2a**) and isopentyl nitrite in diiodomethane was heated at 85 °C for 1 h, 5-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**3a**) was obtained in 65% yield. Similarly, 5-iodo-7-(*N*-methylanilino)- (**3b**), 5-bromo- (**4a**) and 5-bromo-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidines (**4b**) were prepared from the 5-amino derivatives (**2a** and **2b**) under the same conditions in satisfactory yields, as shown in Table I.

Next, in order to synthesize the 5-lithio compound, the 5-halotriazolopyrimidines (**1a**, **3a** and **4a**) not having a substituent at the 7-position were subjected to the reaction with BuLi. We have shown[1] that halogen–metal exchange reaction of 7-iodo- and 7-bromo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine with BuLi in the presence of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) proceeds to give the 7-lithio compound, and reaction of 7-chloro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine with BuLi gives

TABLE I. Yields, Melting Points, and Elemental Analysis for **3a**, **b** and **4a**, **b**

Compd.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
3a	65	144—145	$C_{10}H_6IN_5$	37.17 (37.46)	1.87 1.83	21.68 21.86)
3b	32	187—190	$C_{17}H_{13}IN_6$	47.68 (48.05)	3.06 3.09	19.62 19.54)
4a	51	143—145	$C_{10}H_6BrN_5$	43.50 (43.67)	2.19 2.18	25.37 25.50)
4b	37	171—174	$C_{17}H_{13}BrN_6$	53.56 (54.01)	3.44 3.34	22.04 22.26)

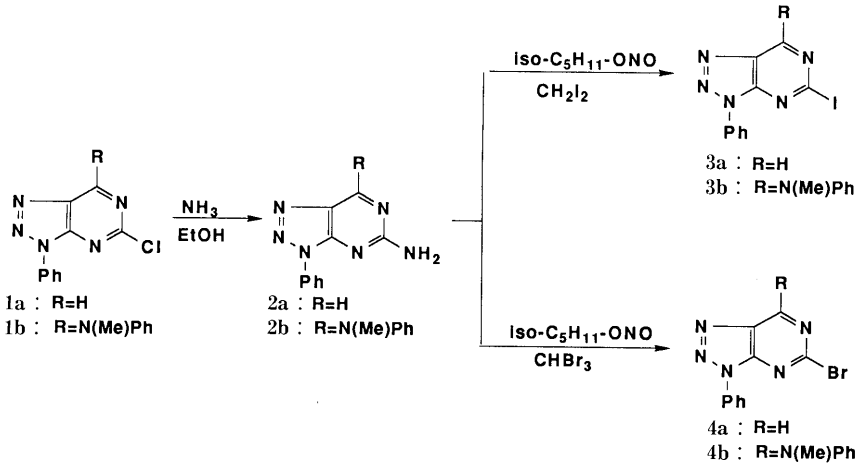

Chart 1

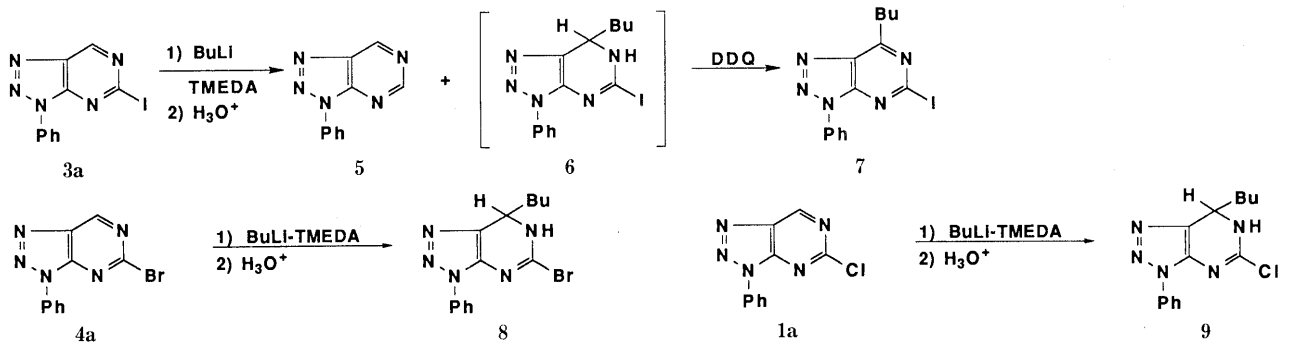

Chart 2

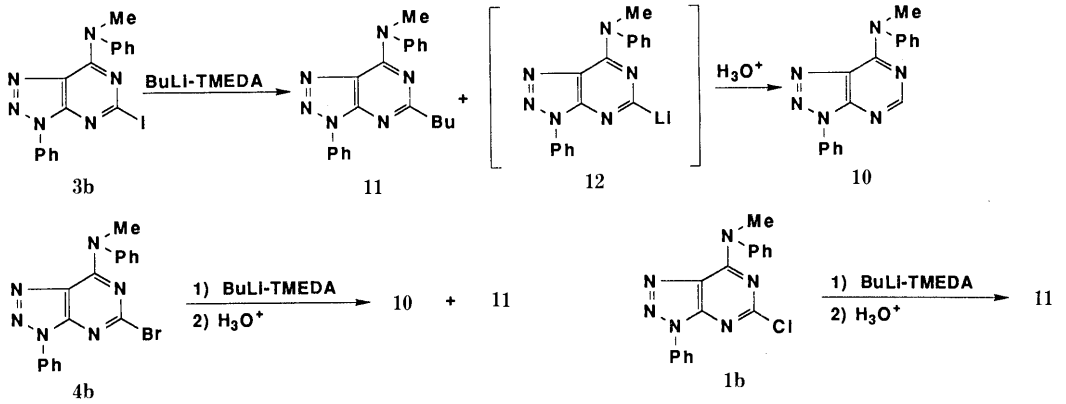

Chart 3

the ring fission product, 5-amino-1-phenyl-1*H*-triazole-4-carbonitrile. When a solution of **3a** with BuLi in the presence of TMEDA was stirred at −100 °C, followed by quenching with aqueous ammonium chloride, halogen–metal exchange reaction took place in the same way as observed for 7-iodotriazolopyrimidine, giving 3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**5**), though in low yield, together with 7-butyl-5-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**7**) which was formed from the crude addition product (**6**) by oxidation with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ). On the other hand, **1a** and **4a** underwent only the addition reaction of BuLi across the C^7,N^6-double bond, differently from 7-chloro- and 7-bromotriazolopyrimidines,[1] and the addition products (**9** and **8**) were obtained.

Then, the reactions of the 5-halotriazolopyrimidines having a substituent at the 7-position with BuLi were investigated. In the treatment of 5-iodo-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**3b**) with BuLi under the same conditions, 7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**10**), formed through the halogen–metal exchange reaction, was obtained in 73% yield, together with the substituted product, 5-butyl-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**11**). This result shows that the lithio compound (**12**) is undoubtedly formed in the initial step of the reaction. Similarly, the 5-bromo derivative (**4b**) reacted with BuLi to give **10** in 73% yield, together with **11**. However, in the case of **1b** with BuLi, only the substitution occurred to give compound **11** in 57% yield.

The application of this lithiation to introduce electrophiles into the 5-position of the triazolopyrimidine ring

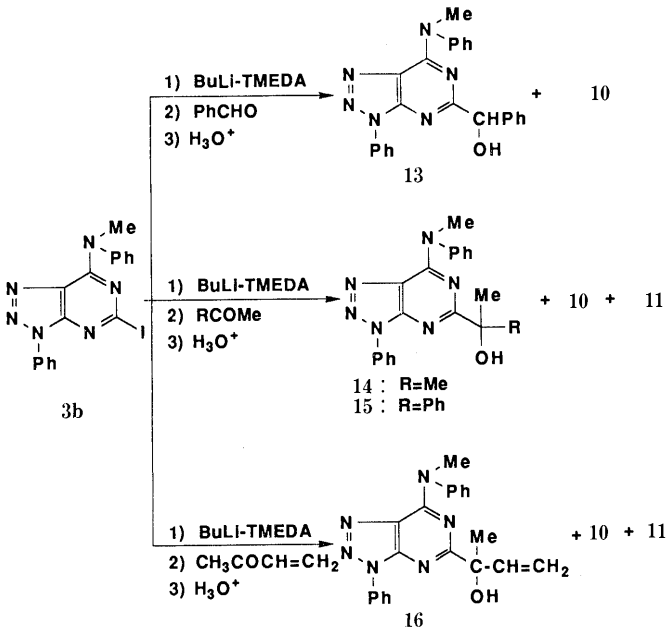

Chart 4

was investigated.

The 5-lithio compound (**12**), prepared from **3b** with BuLi in the presence of TMEDA, reacted smoothly with benzaldehyde to give 5-(α-hydroxybenzyl)-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimine (**13**) in 54% yield, together with **10**. Similarly, reactions of **12** with ketones, such as acetone, acetophenone and methyl vinyl ketone, under the same conditions gave the corresponding 5-substituted compounds (**14**—**16**) in 35—54% yields.

TABLE II. Yields, Melting Points, and Elemental Analysis for **12**—**16**

Compd.	Yield (%)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
13	54	150—152	$C_{24}H_{20}N_6O$	70.57	4.94	20.58
				(70.62	4.99	20.61)
14	41	155—157	$C_{20}H_{20}N_6O$	66.65	5.59	23.32
				(66.73	5.62	23.24)
15	35	119—122	$C_{25}H_{22}N_6O$	71.07	5.25	19.89
				(70.90	5.23	19.90)
16	40	115—117	$C_{21}H_{20}N_6O$	67.72	5.41	22.57
				(67.89	5.51	22.73)

The 5-lithio compound, prepared easily from the 7-substituted 5-iodotriazolopyrimidine with BuLi, reacted smoothly with aldehydes and ketones. This reaction provides a convenient method for introduction of electrophiles into the 5-position on the triazolopyrimidine ring.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were measured with a JASCO IR-700 diffraction grating IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz and 23 °C with a Hitachi R-24B high resolution ^{1}H-NMR spectrometer. Chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, dd = double doublet, t = triplet, br s = broad singlet, m = multiplet.

5-Amino-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (2a) A solution of **1a**[4] (5 g, 22 mmol) and NH_3 gas (2 g) in EtOH (100 ml) was heated at 135 °C in a sealed tube for 1.5 h. The solvent was removed under reduced pressure. The residue was diluted with H_2O and extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography with $CHCl_3$ and recrystallized from MeOH to give colorless needles (**2a**), mp 174—176 °C. Yield 3.4 g (75%). *Anal.* Calcd for $C_{10}H_8N_6$: C, 56.59; H, 3.80; N, 39.61. Found: C, 56.57; H, 3.69; N, 39.37. IR ν_{max}^{KBr} cm^{-1}: 3414, 3310 (NH_2). ^{1}H-NMR ($CDCl_3$): 5.04—5.74 (2H, br s, NH_2), 7.19—7.65 (3H, m, N^3–Ph), 7.88—8.22 (2H, m, N^3–Ph), 8.98 (1H, s, C^7-H).

5-Amino-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]-pyrimidine (2b) A solution of **1b**[4] (3 g, 8.9 mmol) and NH_3 gas (12 g) in EtOH (150 ml) was heated at 140 °C in a sealed tube for 4 h. The solvent was removed under reduced pressure. The residue was diluted with H_2O and extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography with $CHCl_3$ and recrystallized from MeOH to give colorless prisms (**2b**) mp 189—192 °C. Yield 2.5 g (88%). *Anal.* Calcd for $C_{17}H_{15}N_7$: C, 64.34; H, 4.76; N, 30.90. Found: C, 64.26; H, 4.86; N, 30.75. IR ν_{max}^{KBr} cm^{-1}: 3484, 3296 (NH_2). ^{1}H-NMR ($CDCl_3$): 3.82 (3H, s, N(CH_3) Ph), 4.79—5.12 (2H, br s, NH_2), 7.12—7.64 (8H, m, N^3–Ph and N(CH$_3$)Ph), 7.82—8.18 (2H, m, N^3–Ph).

5-Iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (3a) Isopentyl nitrite (15 ml) was added to a suspension of **2a** (1.5 g, 7.1 mmol) in CH_2I_2 (45 ml) under a nitrogen atmosphere. The mixture was stirred at 85 °C for 1 h. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless needles (**3a**), mp 144—145 °C. Yield 1.5 g (65%).

5-Iodo-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (3b) Isopentyl nitrite (30 ml) was added to a suspension of **2b** (3 g, 9.5 mmol) in CH_2I_2 (90 ml) under a nitrogen atmosphere. The mixture was stirred at 85 °C for 15 min. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless prisms (**3b**), mp 187—190 °C. Yield 1.3 g (32%).

5-Bromo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (4a) Isopentyl nitrite (15 ml) was added to a susupension of **2a** (1.5 g, 7.1 mmol) in $CHBr_3$ (45 ml) under a nitrogen atmosphere. The mixture was stirred at 85 °C for 30 min. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless prisms (**4a**), mp 143—145 °C. Yield 1 g (51%).

5-Bromo-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]-pyrimidine (4b) Isopentyl nitrite (20 ml) was added to a suspension of **2b** (2 g, 6.3 mmol) in $CHBr_3$ (60 ml) under a nitrogen atmosphere. The mixture was stirred at 80 °C for 5 min. The solvent was removed under reduced pressure. The crude product was purified by SiO_2 column chromatography with petroleum benzin–benzene (1 : 1) and recrystallized from petroleum benzin–benzene to give colorless prisms (**4b**), mp 171—174 °C. Yield 0.9 g (37%).

TABLE III. ^{1}H-NMR Spectral Data for **3a**, **b** and **4a**, **b**

Compd.	^{1}H-NMR ($CDCl_3$) δ
3a	7.32—7.86 (3H, m, N^3-Ph), 7.96—8.39 (2H, m, N^3-Ph), 9.27 (1H, s, C^7-H)
3b	3.70 (3H, s, N(CH_3)Ph), 6.85—7.69 (8H, m, N^3-Ph, N(CH_3)Ph), 7.75—8.19 (2H, m, N^3-Ph)
4a	7.24—7.70 (3H, m, N^3-Ph), 7.89—8.19 (2H, m, N^3-Ph), 9.24 (1H, s, C^7-H)
4b	3.76 (3H, s, N(CH_3)Ph), 7.03—7.58 (8H, m, N^3-Ph, N(CH_3)Ph), 7.72—8.09 (2H, m, N^3-Ph)

Reaction of 3a with BuLi A solution of TMEDA (235 mg, 2 mmol) in tetrahydrofuran (THF) (15 ml) was cooled to −100 °C under a nitrogen atmosphere and treated with BuLi (1.62 M in hexane) (1.4 ml, 2.2 mmol). After 5 min, a solution of **3a** (323 mg, 1 mmol) in THF (10 ml) was added dropwise to the solution over 10 min at −100 °C. After 30 s, aqueous NH_4Cl was added, and the mixture was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography. The first fraction eluted with benzene gave **3a**. Yield 39 mg (12%). The second fraction eluted with benzene gave 3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**5**) as pale yellow needles, mp 112—114 °C (lit.[5] mp 114—115 °C). Yield 11 mg (6%). ^{1}H-NMR ($CDCl_3$): 7.39—7.82 (3H, m, N^3–Ph), 8.00—8.39 (2H, m, N^3–PH), 9.20 (1H, s, C^5-H), 9.55 (1H, s, C^7-H). The fraction eluted with $CHCl_3$ was dissolved in THF (5 ml). DDQ (100 mg, 0.44 mmol) was added to the solution and the solution was stirred at room temperature for 2 h. The solvent was removed under reduced pressure. The residue was diluted with H_2O and extracted with benzene. The crude product was purified by SiO_2 column chromatography with benzene to give 7-butyl-5-iodo-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**7**) as colorless needles from MeOH, mp 102—104 °C. Yield 15 mg (4%). *Anal.* Calcd for $C_{14}H_{14}IN_5$: C, 44.34; H, 3.72; N, 18.47. Found: C, 44.40; H, 3.44; N, 18.51. ^{1}H-NMR ($CDCl_3$): 0.76—2.21 (7H, m, C^7-$CH_2C_3H_7$), 3.33 (2H, t, J = 7.0 Hz, C^7–$CH_2C_3H_7$), 7.25—7.74 (3H, m, N^3–Ph), 7.85—8.23 (2H, m, N^3–Ph).

Reaction of 4a with BuLi A solution of **4a** (300 mg, 1.1 mmol) in THF (10 ml) was treated with BuLi and TMEDA as described for the reaction of **3a** with BuLi. A similar work-up gave 7-butyl-5-bromo-6,7-dihydro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**8**) as colorless prisms from petroleum benzin–benzene, mp 109—112 °C. Yield 250 mg (69%). *Anal.* Calcd for $C_{14}H_{16}BrN_5$: C, 50.32; H, 4.83; N, 20.95. Found: C, 50.53; H, 4.92; N, 21.12. IR ν_{max}^{KBr} cm^{-1}: 3152 (NH). ^{1}H-NMR ($CDCl_3$): 0.41—2.28 (9H, m, C_4H_9), 4.85—5.34 (1H, m, C^7-H), 6.99 (1H, br s, NH), 7.09—7.50 (3H, m, N^3–Ph), 7.60—8.12 (2H, m, N^3–Ph).

Reaction of 1a with BuLi A solution of **1a** (232 mg, 1 mmol) in THF (10 ml) was treated with BuLi and TMEDA as described for the reaction of **3a** with BuLi. A similar work-up gave 7-butyl-5-chloro-6,7-dihydro-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**9**) as colorless needles from petroleum benzin–benzene, mp 104—106 °C. Yield 285 mg (98%). *Anal.* Calcd for $C_{14}H_{16}ClN_5$: C, 58.03; H, 5.57; N, 24.17. Found: C, 57.82; H, 5.51; N, 24.22. IR ν_{max}^{KBr} cm^{-1}: 3160 (NH). ^{1}H-NMR ($CDCl_3$): 0.60—2.21 (9H, m, C_4H_9), 4.99—5.32 (1H, m, C^7-H), 6.16—6.59 (1H, br s, NH), 7.21—7.64 (3H, m, N^3–Ph), 7.72—8.07 (2H, m, N^3–Ph).

Reaction of 3b with BuLi A solution of TMEDA (120 mg, 1 mmol) in THF (7 ml) was cooled to −78 °C under nitrogen atmosphere and treated with BuLi (1.62 M in hexane) (0.6 ml, 1 mmol). After 5 min, a solution of **3b** (200 mg, 0.47 mmol) in THF (5 ml) was added dropwise to the solution over 10 min at −78 °C. After 3 min, aqueous NH_4Cl was added, and the mixture was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography. The fraction eluted with benzene gave 5-butyl-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**11**) as colorless needles from petroleum benzin, mp 89—91 °C. Yield 17 mg (10%). *Anal.* Calcd for $C_{21}H_{22}N_6$: C, 70.37; H, 6.19; N, 23.45. Found: C, 69.94; H, 6.07; N, 23.38. ^{1}H-NMR ($CDCl_3$): 0.68—2.12 (7H, m,

TABLE IV. IR and ^{1}H-NMR Spectral Data for **13**—**16**

Compd.	IR ν_{max}^{KBr} cm^{-1} (OH)	^{1}H-NMR ($CDCl_3$) δ
13	3452	3.85 (3H, s, N(CH_3)Ph), 4.66 (1H, d, J=6.0 Hz, CH(O$\underline{H}$)Ph), 5.60 (1H, d, J=6.0 Hz, C$\underline{H}$(OH)-Ph), 6.96—7.75 (13H, m, N^3-Ph, N(CH_3)$\underline{Ph}$, CH(OH)$\underline{Ph}$), 7.86—8.23 (2H, m, N^3-Ph)
14	3436	1.57 (6H, s, C(CH_3)$_2$OH), 3.94 (3H, s, N(CH_3)Ph), 4.60 (1H, br s, CH(CH_3)$_2$O$\underline{H}$), 7.20—7.70 (8H, m, N^3-Ph, N(CH_3)$\underline{Ph}$), 8.09—8.36 (2H, m, N^3-Ph)
15	3430	1.93 (3H, s, C(CH_3)Ph), 3.94 (3H, s, N(CH_3)Ph), 5.30 (1H, s, C(O$\underline{H}$)CH_3), 6.82—7.81 (13H, m, N^3-Ph, N(CH_3)$\underline{Ph}$, C(CH_3)$\underline{Ph}$), 7.95—8.26 (2H, m, N^3-Ph)
16	3424	1.65 (3H, s, C(CH_3)OH), 3.93 (3H, s, N(CH_3)Ph), 4.77 (1H, s, C(CH_3)O$\underline{H}$), 5.01 (1H, dd, J_{AB}=2.0 Hz, J_{AX}=10.0 Hz, CH_X=C$\underline{H_A}$-(H_B)), 5.38 (1H, dd, J_{AB}=2.0 Hz, J_{BX}=17.0 Hz, CH_X=CH_A($\underline{H_B}$)), 6.24 (1H, dd, J_{AX}=10.0 Hz, J_{BX}=17.0 Hz, C$\underline{H_X}$=CH_A(H_B)), 7.05—7.62 (8H, m, N^3-Ph, N(CH_3)$\underline{Ph}$), 7.89—8.24 (2H, m, N^3-Ph)

C^5-$CH_2C_3H_7$), 2.83 (2H, t, J=7.0 Hz, C^5-$\underline{CH_2}C_3H_7$), 3.82 (3H, s, N(CH_3)Ph), 6.97—7.60 (8H, m, N^3-Ph and N(CH_3)$\underline{Ph}$), 7.92—8.26 (2H, m, N^3-Ph). The fraction eluted with benzene–$CHCl_3$ gave 7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (**10**) as colorless prisms, mp 108—110 °C. Yield 103 mg (73%). *Anal.* Calcd for $C_{17}H_{14}N_6$: C, 67.53; H, 4.67; N, 27.80. Found: C, 67.52; H, 4.69; N, 27.52. ^{1}H-NMR ($CDCl_3$): 3.96 (3H, s, N(CH_3)Ph), 7.09—7.80 (8H, m, N^3–Ph and N(CH_3)$\underline{Ph}$), 7.94—8.28 (2H, m, N^3–Ph), 8.39 (1H, s, C^5-H).

Reaction of 4b with BuLi A solution of **4b** (202 mg, 0.52 mmol) in THF (5 ml) was treated with BuLi and TMEDA as described for the reaction of **3b** with BuLi. A similar work-up gave **11** in 12% (22 mg) yield and **10** in 73% (114 mg) yield.

Reaction of 1b with BuLi A solution of **1b** (336 mg, 1 mmol) in THF (5 ml) was treated with BuLi and TMEDA as described for the reaction of **3b** with BuLi. A similar work-up gave **11**. Yield 205 mg (57%).

5-(α-Hydroxybenzyl)-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (13) A solution of TMEDA (120 mg, 1 mmol) in THF (7 ml) was cooled to −78 °C under a nitrogen atmosphere and treated with BuLi (1.62 M in hexane) (0.6 ml, 1 mmol). After 5 min, a solution of **3b** (200 mg, 0.47 mmol) in THF (5 ml) was added dropwise to the solution over 10 min at −78 °C. After 3 min, a solution of benzaldehyde (500 mg, 4.7 mmol) in THF (5 ml) was dropped into the mixture at −78 °C and then the whole was stirred at −78—−10 °C for 1.5 h. Aqueous NH_4Cl was added, and the reaction mixture was extracted with $CHCl_3$. The crude product was purified by SiO_2 column chromatography. The fraction eluted with benzene gave **13** as colorless prisms, mp 150—152 °C. Yield 103 mg (54%). The fraction eluted with benzene–$CHCl_3$ gave **10**. Yield 16 mg (11%).

5-(2-Hydroxy-2-propyl)-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (14) A solution of **3b** (200 mg, 0.47 mmol) in THF (5 ml) and acetone (273 mg, 4.7 mmol) was treated with BuLi and TMEDA as described for **13**. A similar work-up gave **14** as colorless needles from petroleum benzin–benzene, mp 155—157 °C, in 41% (70 mg) yield, together with **10** in 25% (36 mg) yield and **11** in 11% (19 mg) yield.

5-(1-Hydroxy-1-phenylethyl)-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (15) A solution of **3b** (200 mg, 0.47 mmol) in THF (5 ml) and acetophenone (564 mg, 4.7 mmol) was treated with BuLi and TMEDA as described for **13**. A similar work-up gave **15** as colorless needles from petroleum benzin–benzene, mp 119—122 °C, in 35% (70 mg) yield, together with **11** in 38% (54 mg) yield.

5-(2-Hydroxy-3-buten-2-yl)-7-(*N*-methylanilino)-3-phenyl-3*H*-1,2,3-triazolo[4,5-*d*]pyrimidine (16) A solution of **3b** (428 mg, 1 mmol) in THF (10 ml) and methyl vinyl ketone (700 mg, 10 mmol) was treated with BuLi and TMEDA as described for **13**. A similar work-up gave **16** as colorless needles from petroleum benzin, mp 115—117 °C, in 40% (70 mg) yield, together with **10** in 15% (48 mg) yield and **11** in 9% (32 mg) yield.

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Chemical Transformation of (+)-Dehydroabietic Acid Leading to a Formal Synthesis of (+)-Coleon A

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(*R*)-6-Hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (3), prepared from (+)-dehydroabietic acid (2), was converted into (*R*)-9-acetoxy-6-benzoyloxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5,8(3*H*)-trione (10) and its 9-acetyl compound (11) by a series of reactions: sodium borohydride reduction, acetylation, alkaline hydrolysis, benzoyl peroxide oxidation, Jones oxidation, and *m*-chloroperbenzoic acid oxidation. The trione 10 was further transformed into (*R*)-5,6,8,9-tetraacetoxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (15) by alkaline hydrolysis and reductive acetylation. Since the conversion of 15 into coleon A (1) has already been reported, the present work can be regarded as a new formal synthesis of coleon A.

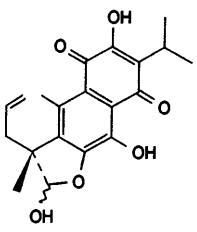
Keywords coleon A; synthesis; highly oxygenated 1,10-secoabietane; *Coleus igniarius* (Labiatae); diterpene; benzoyl peroxide oxidation; naphthol derivative

Coleon A (**1**), a rare highly oxygenated 1,10-secoabietane derivative, has been isolated from the leaves of *Coleus igniarius* SCHWEINF (Labiatae) by Eugster *et al.*[1-3] In previous papers,[4,5] we have reported the novel conversion of (+)-dehydroabietic acid (**2**) into coleon A (**1**) *via* the lactone intermediate, (*R*)-6-hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (**3**). In order to obtain further information on the introduction of oxygen functions into the naphthol skeleton, we have recently investigated the oxidation of some simple naphthol compounds with benzoyl peroxide, and these results were also reported in the preceding paper.[6] As an extension of the previous work, we now describe a new synthetic route to coleon A which involves the oxidation of highly substituted naphthol compounds with benzoyl peroxide.

The lactone **3** prepared from **2** was reduced with sodium borohydride in ethanol and the resulting mixture of C-2 epimeric alcohols (**4**) was immediately treated with acetic anhydride in the presence of boron trifluoride etherate to give the corresponding 2,6-diacetoxy-9-acetyl derivative (**5**). The infrared (IR) spectrum of **5** indicated the presence of acetoxyl (1748 cm^{-1}) and acetyl (1672 cm^{-1}) groups, and its proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed singlet signals at δ 2.68 (3H) due to an acetyl group and at δ 7.58 (1H) and 8.36 (1H) due to the C-5 and C-8 aromatic protons. Hydrolysis of the acetoxyl groups in **5** with sodium hydrogencarbonate in refluxing aqueous methanol afforded the hemiacetal derivative (**6**). This was oxidized with benzoyl peroxide in chloroform at room temperature and then with Jones reagent to give two bis(benzoyloxy) keto lactones (**7** and **8**) in 30% and 8% yields from **3**. The ^{1}H-NMR spectrum of **7** showed a singlet signal due to a C-8 olefinic proton at δ 7.15, while that of **8** showed the corresponding signal at a lower filed (δ 7.54). These spectral data suggested that the C-7 and C-8 double bond in **8** is conjugated with a carbonyl group. Thus, the structures of **7** and **8** were assigned to be (*R*)-9-acetyl-6,6-bis(benzoyloxy)-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5(3*H*,6*H*)-dione and (*R*)-9-acetyl-5,5-bis(benzoyloxy)-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,6(3*H*,5*H*)-dione, respectively.

Similarly, (*R*)-9-acetyl-6-hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (**9**),[5] prepared from **3**, was also oxidized with benzoyl peroxide in dichloromethane at room temperature to give **7** and **8** in 75% and 9% yields, respectively. In order to introduce oxygen functions at the C-8 and C-9 positions, a mixture of the acetyl compounds (**7** : **8** = *ca.* 8/1) was refluxed with *m*-chloroperbenzoic acid and *p*-toluenesulfonic acid monohydrate in dichloromethane. Purification of the crude product gave two *p*-quinones, **10** and **11**, in 18% and 13% yields, respectively. Each of **7** and **8** was also oxidized with *m*-chloroperbenzoic acid to give a similar result. The IR spectrum of **10** showed absorption bands at 1813 (γ-lactone), 1770 (acetoxyl), 1739 (benzoyloxyl), and 1660 cm^{-1} (*p*-quinone). The spectrum of **11** showed absorption bands at 1812 (γ-lactone), 1738 (benzoyloxyl), 1702 (acetyl), and 1662 cm^{-1} (*p*-quinone). Hydrolysis of the acetyl *p*-quinone (**11**) with sodium hydrogencarbonate in refluxing aqueous methanol produced the corresponding hydroxy *p*-quinone (**12**),[5] which was then submitted to reductive acetylation with zinc powder and acetic anhydride in pyridine to give the corresponding triacetate (**13**). Transformation of **13** into the desired tetraacetoxy lactone (**15**) by Baeyer–Villiger oxidation using *m*-chloroperbenzoic acid was attempted, but only the starting **13** was recovered. Subsequently, the acetoxy *p*-quinone (**10**) was also hydrolyzed with sodium hydrogencarbonate in refluxing aqueous methanol and the resulting dihydroxy *p*-quinone (**14**) was then treated with zinc powder and acetic anhydride in pyridine to give the tetraacetoxy lactone (**15**) in 79% yield from **10**.

Since the conversion of **15** into coleon A (**1**) has already



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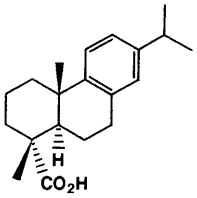

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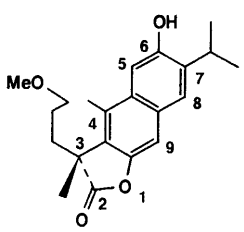

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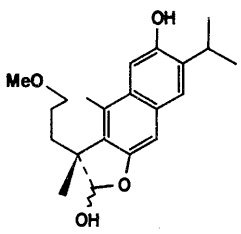

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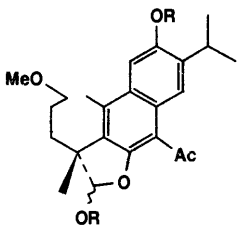

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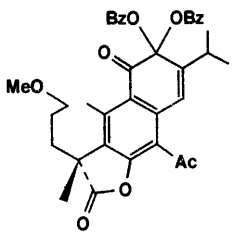
6 : R=Ac



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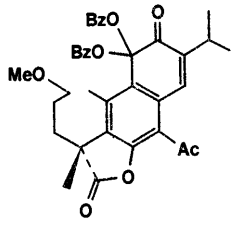

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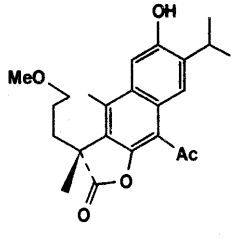

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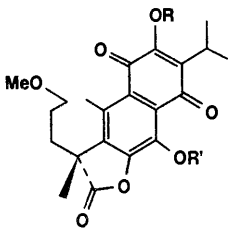
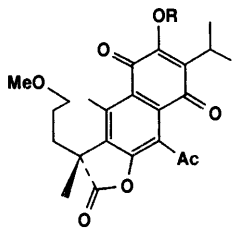
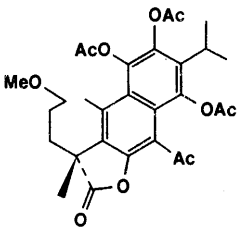


10 : R=Bz, R'=Ac
14 : R=R'=H



11 : R=Bz
12 : R=H



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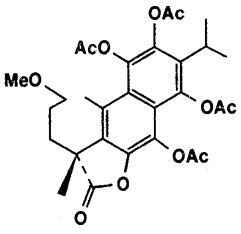

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been reported,[5] the present work can be regarded as a new formal synthesis of (+)-coleon A.

Experimental

The IR spectra and optical rotations were measured in chloroform, and the ^{1}H-NMR spectra in deuteriochloroform at 90 MHz with tetramethylsilane as an internal standard, unless otherwise stated. The column chromatography was performed using Merck silica gel (0.063 mm).

The C-2 Epimeric Mixture of 9-Acetyl-2,3-dihydro-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,6-diol (6) Sodium borohydride (0.83 g) was added portionwise to a stirred solution of (*R*)-6-hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]-furan-2(3*H*)-one (**3**)[5] (3.41 g) in ethanol (140 ml) with cooling in an ice-water bath. The mixture was stirred at this temperature for 1 h and then at room temperature for 15 h. After the excess hydride had been decomposed with acetone, the mixture was concentrated *in vacuo*, poured into dilute hydrochloric acid, and extracted with chloroform. The chloroform extract was washed with brine, dried over sodium sulfate, and evaporated *in vacuo* to give a mixture of the crude C-2 epimeric alcohols (**4**) (3.40 g). IR (Nujol): 3350 (br) cm^{-1}.

Boron trifluoride etherate (3.40 ml) was added to a stirred solution of the crude **4** (3.40 g) in acetic anhydride (68 ml) at −5 °C over a period of 5 min. The mixture was stirred at room temperature for 5 min, poured into a mixture of ice and aqueous sodium hydrogencarbonate, and extracted with chloroform. The chloroform extract was washed with brine, dried, and evaporated *in vacuo* to give a crude epimeric mixture (*ca.* 3 : 1) at C-2 of the 2,6-diacetoxy-9-acetyl derivative (**5**) (3.92 g). IR: 1748, 1672 cm^{-1}. ^{1}H-NMR δ: 1.27 (6H, d, J=7 Hz, $-CH(C\underline{H}_3)_2$), 1.46 (minor) and 1.55 (major) (3H, each s, C_3-CH_3), 2.07 (major) and 2.10 (minor) (3H, each s, C_2-$OCOCH_3$), 2.37 (3H, s, C_6-$OCOCH_3$), 2.62 (3H, s, C_4-CH_3), 2.68 (3H, s, C_9-$COCH_3$), 3.09 (1H, m, $-C\underline{H}(CH_3)_2$), 3.23 (major) and 3.28 (minor) (3H, each s, $-OCH_3$), 3.27 (major) and 3.33 (minor) (2H, each t, J=6 Hz, $-C\underline{H}_2OCH_3$), 6.47 (minor) and 6.57 (major) (1H, each, s, C_2-H), 7.58 (1H, s, C_5-H), 8.36 (1H, s, C_8-H).

A mixture of the crude **5** (3.92 g) and sodium hydrogencarbonate (4.20 g) in methanol (390 ml) and water (84 ml) was refluxed for 2 h. The mixture was concentrated *in vacuo* to *ca.* 100 ml, diluted with brine, and extracted with ether. The ether extract was washed with brine, dried, and evaporated *in vacuo* to give a crude epimeric mixture (**6**) (3.40 g), which was used without purification in the next reaction. IR: 3600, 3320 (br), 1658 cm^{-1}.

(*R*)-9-Acetyl-6,6-bis(benzoyloxy)-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5(3*H*,6*H*)-dione (7) and (*R*)-9-Acetyl-5,5-bis(benzoyloxy)-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,6(3*H*,5*H*)-dione (8) a) A solution of the above crude **6** (3.40 g) and benzoyl peroxide (4.67 g) in chloroform (46 ml) was stirred at room temperature for 26 h. After the addition of ether (200 ml), acetic acid (2.0 ml), and aqueous potassium iodide (20%, 50 ml), the mixture was further stirred at room temperature for 3 h and then washed successively with water, aqueous sodium thiosulfate, aqueous sodium hydrogencarbonate, and brine. The dried solution was evaporated *in vacuo*. The residue was dissolved in acetone (150 ml) and then oxidized with Jones reagent (2.5 mol dm^{-3}, 16 ml) at 0—5 °C for 15 min. After the usual work-up, the crude product was chromatographed on silica gel (500 g), using ether–benzene (3 : 97) as an eluent, to give oily **7** (1.87 g, 30.1% yield from **3**), $[\alpha]_D$ +39° (c=2.44). IR: 1809, 1725, 1690 cm^{-1}. ^{1}H-NMR δ: 1.12 (6H, d, J=7 Hz, $-CH(C\underline{H}_3)_2$, 1.66 (3H, s, C_3-CH_3), 2.69 (6H, s, C_4-CH_3 and C_9-$COCH_3$), 2.88 (1H, m, $-C\underline{H}(CH_3)_2$), 3.22 (3H, s, $-OCH_3$), 3.26 (2H, t, J=6 Hz, $-C\underline{H}_2OCH_3$), 7.15 (1H, br s, C_8-H), *ca.* 7.3—7.7 (6H, m) and 8.07 (4H, dd, J=2, 8 Hz) (2-C_6H_5). MS *m/z*: 624 (M^+). *Anal.* Calcd for $C_{37}H_{36}O_9$: C, 71.14; H, 5.81. Found: C, 70.97; H, 5.88.

Further elution gave oily **8** (0.52 g, 8.4% yield from **3**), $[\alpha]_D$ +54° (c=1.85). IR: 1810, 1732, 1688 cm^{-1}. ^{1}H-NMR δ: 1.24 (6H, d, J=7 Hz, $-CH(C\underline{H}_3)_2$), 1.51 (3H, s, C_3-CH_3), 2.69 (6H, s, C_4-CH_3 and C_9-$COCH_3$), 2.80 (3H, s, $-OCH_3$), 3.00 (2H, m, $-C\underline{H}_2OCH_3$), 3.12 (1H, m, $-C\underline{H}(CH_3)_2$), 7.35—7.75 (6H, m), 8.04 and 8.06 (4H, each dd, J=2, 8 Hz) (2-C_6H_5), 7.54 (1H, d, J=1.5 Hz, C_8-H). MS *m/z*: 624 (M^+). *Anal.* Calcd for $C_{37}H_{36}O_9$: C, 71.14; H, 5.81. Found: C, 71.41; H, 5.96.

b) A solution of (*R*)-9-acetyl-6-hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (**9**)[5] (3.76 g) and benzoyl peroxide (5.33 g) in dichloromethane (110 ml) was stirred at room temperature for 54 h. After work-up as described in a), the crude product was chromatographed on silica gel (600 g), using ether–benzene (2 : 98) as an eluent, to give oily **7** (4.55 g, 74.5%) and **8** (0.56 g, 9.2%). The IR and ^{1}H-NMR spectra of **7** and **8** were identical with those of authentic samples.

(*R*)-9-Acetoxy-6-benzoyloxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5,8(3*H*)-trione (10) and (*R*)-9-Acetyl-6-benzoyloxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]-furan-2,5,8-(3*H*)-trione (11) a) A solution of a mixture of **7** and **8** (*ca.* 8 : 1 ratio, 1.220 g), *m*-chloroperbenzoic acid (90%, 1.23 g), and *p*-toluenesulfonic acid monohydrate (244 mg) in dichloromethane (60 ml) was refluxed for 8 h. The solution was cooled, diluted with ether, and then washed successively with aqueous potassium iodide, aqueous sodium thiosulfate, aqueous sodium hydrogencarbonate, and brine. The dried solution was evaporated *in vacuo*. The residue was chromatographed on silica gel (100 g), using ether–benzene (2 : 98) as an eluent, to give oily **10** (192 mg, 18.4%), $[\alpha]_D$ +36° (c=1.95). IR: 1813, 1770, 1739, 1660 cm^{-1}. ^{1}H-NMR δ: 1.28 (6H, d, J=7 Hz, $-CH(C\underline{H}_3)_2$), 1.63 (3H, s, C_3-CH_3), 2.46 (3H, s, C_9-$OCOCH_3$), 2.67 (3H, s, C_4-CH_3), 3.19 (3H, s, $-OCH_3$),

3.22 (2H, t, $J=6$ Hz, $-C\underline{H}_2OCH_3$), 3.30 (1H, m, $-C\underline{H}(CH_3)_2$), *ca.* 7.4—7.8 (3H, m) and 8.18 (2H, dd, $J=2$, 8 Hz) ($-C_6H_5$). *Anal.* Calcd for $C_{30}H_{30}O_9$: C, 67.40; H, 5.66. Found: C, 67.11; H, 5.93.

Further elution gave oily **11** (133 mg: 13.1%), $[\alpha]_D$ +21° ($c=1.72$). IR: 1812, 1738, 1702, 1662 cm^{-1}. ^{1}H-NMR δ: 1.28 (6H, d, $J=7$ Hz, $-CH(C\underline{H}_3)_2$), 1.61 (3H, s, C_3-CH_3), 2.60 (3H, s, C_9-$COCH_3$), 2.70 (3H, s, C_4-CH_3), 3.18 (3H, s, $-OCH_3$), 3.22 (2H, t, $J=6$ Hz, $-C\underline{H}_2OCH_3$), 3.29 (1H, m, $-C\underline{H}(CH_3)_2$), *ca.* 7.4—7.8 (3H, m) and 8.17 (2H, dd, $J=2$, 8 Hz) ($-C_6H_5$). *Anal.* Calcd for $C_{30}H_{30}O_8$: C, 69.48; H, 5.83. Found: C, 69.52; H, 6.01.

b) A solution of **7** (1.690 g), *m*-chloroperbenzoic acid (90%, 1.86 g), and *p*-toluenesulfonic acid monohydrate (169 mg) in chloroform (85 ml) was refluxed for 17 h to give **10** (212 mg, 14.7%) and **11** (167 mg, 11.9%).

c) A solution of **8** (192.7 mg), *m*-chloroperbenzoic acid (90%, 212.6 mg), and *p*-toluenesulfonic acid monohydrate (193 mg) in chloroform (9.6 ml) was refluxed for 17 h to give **10** (24.6 mg, 14.9%) and **11** (6.5 mg, 4.1%).

(*R*)-9-Acetyl-6-hydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5,8-(3*H*)-trione (12) A mixture of **11** (253.4 mg) and sodium hydrogencarbonate (845 mg) in methanol (50 ml) and water (10 ml) was refluxed for 15 min. After removal of the methanol *in vacuo*, the residue was diluted with brine and extracted with ether. The ether extract was washed with brine and evaporated *in vacuo*. The residue was dissolved in methanol (18 ml) and dilute hydrochloric acid (6 mol dm^{-3}, 3.6 ml) was then added. The mixture was refluxed for 15 min, concentrated *in vacuo*, and extracted with ether. The ether extract was washed with brine, dried, and evaporated *in vacuo*. The crude product was chromatographed on silica gel (20 g), using ether–benzene (6.94) as an eluent, to give oily **12**[5] (61.5 mg, 30.4%), $[\alpha]_D$ +26° ($c=0.35$). IR: 3360, 1820, 1712, 1652 cm^{-1}. *Anal.* Calcd for $C_{23}H_{26}O_7$: C, 66.65; H, 6.32. Found: C, 66.40; H, 6.30.

(*R*)-5,6,8-Triacetoxy-9-acetyl-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (13) A mixture of **12** (220.1 mg), acetic anhydride (0.6 ml), and zinc powder (100 mg) in pyridine (1.0 ml) was stirred at 0—5 °C for 1 h. The mixture was diluted with ether and washed successively with aqueous sodium hydrogencarbonate, dilute hydrochloric acid, and brine. The dried solution was evaporated *in vacuo*. The residue was chromatographed on silica gel (25 g), using ether–benzene (2 : 8) as an eluent, to give oily **13** (56.3 mg, 19.5%), $[\alpha]_D$ +16° ($c=1.11$). IR: 1815, 1784, 1712 cm^{-1}. ^{1}H-NMR δ: 1.26 (6H, d, $J=7$ Hz, $-CH(C\underline{H}_3)_2$), 1.60 (3H, s, C_3-CH_3), 2.28 (3H, s) and 2.35 (6H, s) (3-$OCOCH_3$), 2.59 (3H, s, C_9-$COCH_3$), 2.68 (3H, s, C_4-CH_3), 3.05 (1H, m, $-C\underline{H}(CH_3)_2$), 3.18 (3H, s, $-OCH_3$), 3.22 (2H, t, $J=6$ Hz, $-C\underline{H}_2OCH_3$). *Anal.* Calcd for $C_{29}H_{34}O_{10}$: C, 64.19; H, 6.32. Found: C, 64.07; H, 6.37.

(*R*)-5,6,8,9-Tetraacetoxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2(3*H*)-one (15) A mixture of **10** (192.1 mg) and sodium hydrogencarbonate (302 mg) in methanol (38 ml) and water (7.5 ml) was refluxed for 15 min. The mixture was cooled, acidified with dilute hydrochloric acid, and then refluxed for 15 min. After removal of the methanol *in vacuo*, the residue was extracted with ether. The ether extract was washed successively with aqueous sodium hydrogencarbonate and brine, dried, and evaporated *in vacuo* to give crude (*R*)-6,9-dihydroxy-7-isopropyl-3-(3-methoxypropyl)-3,4-dimethylnaphtho[2,3-*b*]furan-2,5,8-(3*H*)-trione (**14**) (142.7 mg). IR: 3340, 1812, 1650, 1622 cm^{-1}.

A solution of the crude **14** (142.7 mg) and acetic anhydride (2.5 ml) in pyridine (2.5 ml) was stirred at room temperature for 30 min and then cooled in an ice-water bath. After the addition of zinc powder (42 mg), the mixture was further stirred at this temperature for 5 min and at room temperature for 1 h, diluted with ether, and then filtered. The filtrate was washed successively with aqueous sodium hydrogencarbonate, dilute hydrochloric acid, and brine. The dried solution was evaporated *in vacuo*. The residue was chromatographed on silica gel (20 g), using ether–benzene (15 : 85) as an eluent, to give **15** (158.0 mg, 78.7% yield from **10**), mp 243 °C (from methanol), $[\alpha]_D$ +12° ($c=0.65$). IR: 1809, 1775, 1648 cm^{-1}. ^{1}H-NMR δ: 1.27 (6H, br d, $J=7$ Hz, $-CH(C\underline{H}_3)_2$), 1.63 (3H, s, C_3-CH_3), 2.35 and 2.40 (each 6H and s, 4-$OCOCH_3$), 2.66 (3H, s, C_4-CH_3), 3.17 (3H, s, $-OCH_3$), 3.21 (2H, t, $J=6$ Hz, $-C\underline{H}_2OCH_3$), 3.21 (1H, m, $-C\underline{H}(CH_3)_2$). The IR and ^{1}H-NMR spectra of **15** were identical with those of an authentic sample.[5] *Anal.* Calcd for $C_{29}H_{34}O_{11}$: C, 62.35; H, 6.14. Found: C, 62.56; H, 6.17.

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Structure Elucidation of Glycosidic Antibiotics, Glykenins, from *Basidiomycetes* sp. IV. Structure of Glykenin III

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Three new glycosidic antibiotics, glykenin (GK)-III A, B, and C (2a—c), were isolated as a mixture from a strain of *Basidiomycetes* sp. and identified as glycosides of C_{26}-fatty acids and a diacetylated trisaccharide composed of glucose and two xyloses. The locations of the two acetyl groups were elucidated by secondary ion mass spectrometry (SIMS), double quantum filter (DQF), and relayed chemical shift correlation spectroscopy (COSY) spectral analysis.

Keywords *Basidiomycetes* sp.; glykenin III; glycosidic antibiotic; SIMS; double quantum filter ^{1}H COSY; relayed COSY

Glycosidic antibiotics known as glykenins (GK), which are produced by a *Basidiomycetes* sp., exhibit inhibitory activity against gram-positive bacteria. GK is composed of two major components (GK-III and GK-IV) and five minor components (GK-I, GK-II, and GK-V—VII), as determined by silica gel thin layer chromatography (TLC). In the previous papers,[1,2] we reported the structures of deacetyl GK (DG-A—C, **1a—c**), and the basic structures of GK, which are composed of unusual tetrahydroxylated long chain (C_{26}) fatty acids as aglycones, and trisaccharides. We describe here the precise structure elucidation of the major component GK-III (**2a—c**) by the use of two-dimensional nuclear magnetic resonance (NMR) methods, and the structural relationship of **1a—c** and **2a—c** (Chart 1).

The major component GK-III was isolated from the ethyl acetate extract of the culture broth of a strain of *Basidiomycetes* sp. in glucose–glutamine medium by Sephadex LH-20 chromatography and repeated chromatography on silica gel ($CHCl_3$: MeOH : 50% AcOH = 65 : 15 : 5). Although TLC, mass spectrometry and ^{1}H-NMR examinations suggested that GK-III was pure, analysis by high-performance liquid chromatography (HPLC) of the peracetyl phenacyl ester derivative (**3a—c**) of GK-III using CH_3CN : MeOH : H_2O = 9.0 : 0.5 : 0.5 as the mobile phase indicated the presence of three peaks, which were identical with those obtained from the peracetyl phenacyl esters of **1a—c** as shown in Fig. 1. The retention times of **3a—c** were 11.1, 11.5, and 10.0 min, respectively. These results revealed that GK-III is a mixture of three components derived from DG-A—C, which represent the basic structures of GK. The three components of GK-III differ in the aglycone parts, and they were proved to be regio- and stereoisomers of hydroxy groups by chemical transformation.[1,2]

GK-III is an amorphous powder and showed molecular ions at *m/z* 969 $(M-H)^-$ and *m/z* 993 $(M+Na)^+$ in the negative and positive secondary ion mass spectrometry (SIMS) using glycerol as a liquid matrix, respectively. GK-III showed an absorption at 1730 cm^{-1} in the infrared (IR) spectrum, and showed ^{1}H-NMR signals at 2.07 and 2.08 ppm, indicating that it possesses two acetyl groups. Separation of the three components by normal and reversed-phase chromatographies was not successful due

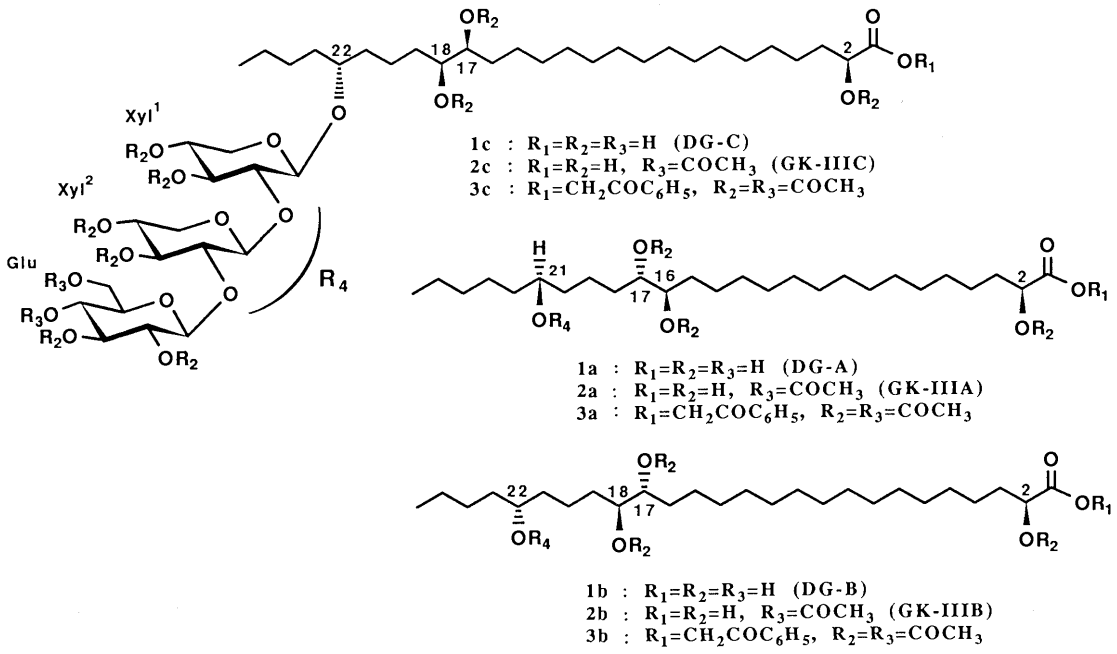

Chart 1. Structures of DG, GK-III and Peracetyl Phenacyl Esters

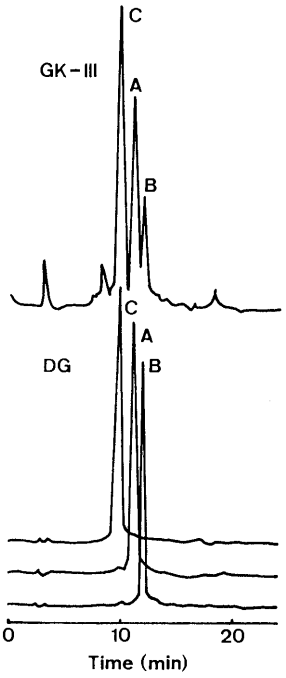

Fig. 1. HPLC Analyses of the Peracetyl Phenacyl Esters of GK-III (**2**) and DG-A—C (**1a**, **1b**, and **1c**)

to the structural similarity of the aglycone parts. The NMR spectrum of GK-III, however, appeared to be that of a single compound and therefore we decided to analyze GK-III as it was. We employed the double quantum filter (DQF) and relayed proton chemical shift correlation spectroscopy (^{1}H COSY) techniques[3,4] to determine the positions of the two acetyl groups. Using these techniques, we could elucidate the connectivities of many overlapped methine proton signals of the sugar moiety as follows.

In the DQF ^{1}H COSY spectrum, the anomeric proton (H-1) of glucose (Glc) was connected with Glc-H-2 by cross peaks, and Glc-H-2 was correlated with Glc-H-3. Glc-H-4, the lower-field-shifted signal (4.85 ppm), was easily assigned from Glc-H-3 based on the cross peaks and was correlated with Glc-H-5. The chemical shifts of Glc-H-6 and Glc-H-6′ assigned from Glc-H-5 were 4.14 and 4.22 ppm, which were lower-field signals. In the relayed COSY, Glc-H-1 was connected with Glc-H-2 and Glc-H-3 and in the 2-relayed COSY, with Glc-H-4, as shown in Fig. 2. In addition, starting from Glc-H-4, connections were observed between Glc-H-4 and Glc-H-1, H-2, H-3, H-5, H-6, and H-6′ in 2-relayed COSY. As mentioned above, the lower-field-shifted signals connected with acetyl groups, 4.85, 4.14 and 4.22 ppm, were assigned to Glc-H-4, Glc-H-6 and Glc-H-6′, respectively. The proton signals of xylose-1 (Xyl^1) were upfield signals, and the xylose-2 (Xyl^2) moiety showed similar signals to Xyl^1. It was confirmed by DQF, relayed and 2-relayed COSY spectra that no acetyl group was present on the two xylose moieties.

The fragmentations in negative SIMS also suggested the positions of the two acetyl groups. The fragment ions of GK-III include *m/z* 927, 723, 591 and 459 (aglycone-H)$^-$, indicating the sequential elimination of the three sugar moieties. The mass differences between of the fragment ions, corresponded to the loss of ketene (Δ 42), glucose and keten (Δ 42+162), xylose-2 (Δ 132), and xylose-1 (Δ 132), respectively. The fragment ions of DG-C (**1c**) at *m/z* 723, 591 and 459 indicated only the sequential elimination of the three sugars. These data support the conclusion that the two acetyl groups are present on the glucose moiety in the structures of GK-III (Fig. 3).

Based on the analyses described above, the structures of GK-III A—C were determined to be as shown in Chart 1.

Experimental

IR spectra were recorded on a Hitachi 215 spectrometer, and ultraviolet (UV) spectra were measured on a Hitachi 200-10 spectrophotometer. NMR spectra were recorded on JEOL JNM-GX270 (270 MHz for ^{1}H-NMR), and JNM-GX400 (400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR) spectrometers, and chemical shifts are given on the ppm (δ) scale with tetramethylsilane as an internal standard. Mass spectra (MS) were taken on a Hitachi M-80 spectrometer and a JEOL HX-110. Column chromatographic separations were carried out using Sephadex LH-20 (Pharmacia) or Silica gel 60 (Nacalai). Analytical TLC was performed on precoated silica gel 60 plates (Merck, Art. 5715) and RP-18F_{254S} (Merck, Art. 15685). HPLC was performed on a JASCO Trirotar V using a Develosil ODS column.

Isolation and Purification The fermentation broth of GK was extracted 3 or 4 times with AcOEt. The organic layer was concentrated and the residue was chromatographed on the Sephadex LH-20 column with MeOH to give GK complex (9.88 g). GK complex (9.88 g) was chromatographed on a silica gel column using $CHCl_3$: MeOH : 50% AcOH (65 : 15 : 5), and repeated chromatographies using $CHCl_3$: MeOH : 50% AcOH (65 : 10 : 5) gave GK-III (12.5 mg), GK-IV (51.6 mg), and a mixture of minor components (90.8 mg).

GK-III: Amorphous powder. UV λ_{max}^{MeOH} nm: end absorption. IR ν_{max}^{KBr} cm^{-1}: 3600—3200, 1730. SIMS (positive) *m/z*: 993 $(M+Na)^+$. SIMS (negative) *m/z*: 969 $(M-H)^-$. Positive high-resolution fast atom bombardment mass spectrum (FAB-MS): Found 1015.5050. Calcd for $C_{46}H_{81}O_{21}Na_2$ $(M+2Na-H)^+$ 1015.5065. ^{1}H-NMR (400 MHz, CD_3OD) δ: 4.85 (1H, t, J=9.8 Hz, G-3), 4.72 (1H, d, J=8.1 Hz, G-1), 4.61 (1H, d, J=7.1 Hz, X^2-1), 4.41 (1H, d, J=7.1 Hz, X^1-1), 4.22 (1H, dd, J=4.9, 11.7 Hz, G-6′), 4.19 (1H, m, aglycone-2), 4.14 (1H, dd, J=1.9, 11.7 Hz, G-6), 3.86 (2H, dd, J=4.9, 10.7 Hz, X^2-5′, X^1-5′), 3.78 (1H, m, aglycone-22), 3.72 (1H, m, G-5), 3.58 (1H, t, J=9.8 Hz, G-3), 3.56 (1H, t, J=9.5 Hz, X^2-3), 3.51 (2H, t, J=9.8 Hz, X^1-3 and m, X^2-4), 3.50 (1H, m, X^1-5), 3.43 (1H, t, J=9.5 Hz, X^2-2), 3.35 (1H, t, J=9.8 Hz, G-2), 3.27 (1H, t, J=9.8 Hz, X^1-2), 3.26 (2H, m, aglycone-17, 18), 3.18 (2H, dd, J=3.9, 9.8 Hz, X^1-4, X^2-5). 2.08 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 1.53, 1.29 (CH_2), 0.92 (3H, t, J=6.5 Hz, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 172.8, 171.9, 105.5, 104.2, 102.5, 84.2, 83.6, 80.2, 77.3, 77.1, 76.0, 75.9, 75.7, 75.4, 75.3, 75.2, 73.6, 71.9, 70.9, 70.7, 66.8, 66.4, 63.5, 37.4, 35.9, 35.5, 34.6, 34.0, 33.5, 30.9, 30.8, 30.7, 30.5, 28.4, 27.1, 26.2, 24.2, 23.8, 22.9, 22.6, 20.9, 14.6.

The Peracetyl Phenacyl Esterifications A methanolic solution (0.5 ml) of the mixture of GK-III A—C (2.8 mg) was treated with 1% NaOH in MeOH (23 μl) and stirred for 1 h at room temperature. The residue obtained by evaporation *in vacuo* was dissolved in *N,N*-dimethylformamide. Dicyclohexyl 18-crown-6 (2.2 mg) and phenacyl bromide (1.7 mg) were added to the solution, and the whole was stirred for 4 h. The solvent was removed *in vacuo* and the residue was chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (65 : 15 : 5) to give the phenacyl esters (2.2 mg). The phenacyl esters (2.2 mg) were acetylated with acetic anhydride (0.1 ml) and pyridine (0.1 ml) and the products obtained was chromatographed on Sephadex LH-20 (MeOH) to give a mixture of peracetyl phenacyl esters (3.1 mg).

Peracetyl Phenacyl Ester of GK-III: Oil. UV λ_{max}^{MeOH} nm: 242. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1365, 1240. SIMS (positive) *m/z*: 1489 $(M+Na)^+$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.90 (2H, d, J=2.4 Hz, aromatic proton), 7.62 (1H, t, J=7.5 Hz, aromatic proton), 7.49 (2H, t, J=7.4 Hz, aromatic proton), 5.50 (1H, d, J=16.2 Hz, CH_2), 5.28 (1H, d, J=16.2 Hz, CH_2), 5.18 (1H, t, J=9.5 Hz, G-4), 5.16 (1H, t, J=9.0 Hz, X^1-3), 5.11 (1H, t, J=9.5 Hz, G-3), 5.09 (1H, m, aglycone-2), 5.07 (1H, t, J=8.8 Hz, X^2-3), 5.01 (1H, m, aglycone-18), 4.98 (1H, m, aglycone-17), 4.95 (1H, dd, J=9.5, 8.1 Hz, G-2), 4.87 (1H, m, X^1-4), 4.84 (1H, m, X^2-4), 4.64 (1H, d, J=6.4 Hz, X^2-1), 4.54 (1H, d, J=8.1 Hz, G-1), 4.43 (1H, d, J=7.0 Hz, X^1-1), 4.35 (1H, dd, J=12.2, 2.2 Hz, G-6′), 4.23 (1H, dd, J=12.2, 3.9 Hz, G-6), 4.05 (1H, dd, J=12.5, 5.1 Hz, X^2-5′), 3.96 (1H, dd,

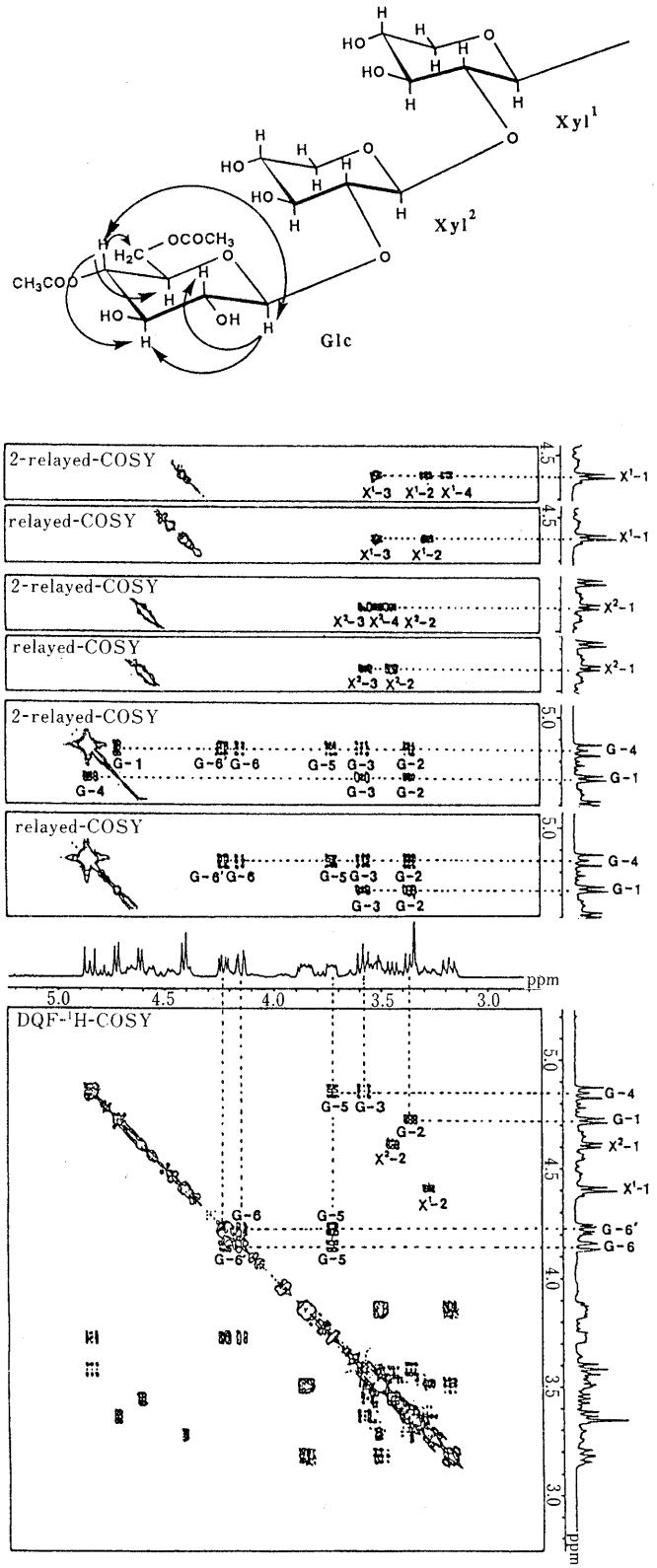

Fig. 2. Multiple-Relayed COSY Spectra of GK-III

The arrows in the structure show the correlated protons from H-1 and H-4 in glucose.

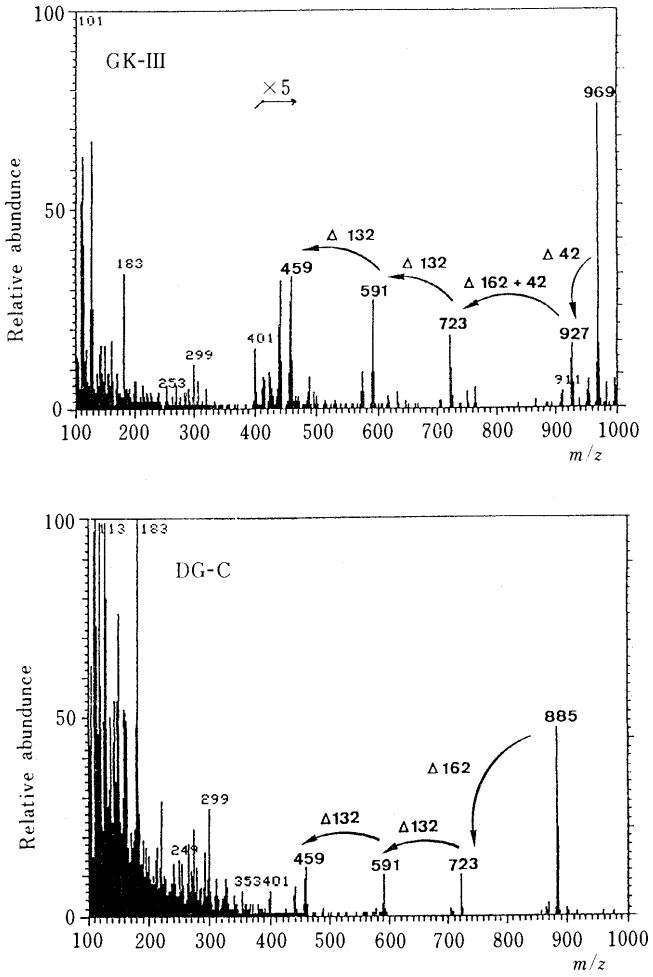

Fig. 3. Negative Ion Liquid SI Mass Spectra (Glycerol) of GK-III (**2**) and DG-C (**1c**)

J=11.7, 5.4 Hz, X^1-5'), 3.64 (1H, m, G-5), 3.59 (1H, dd, J=9.0, 6.8 Hz, X^1-2), 3.55 (1H, m, aglycone-22), 3.52 (1H, dd, J=8.8, 6.4 Hz, X^2-2), 3.39 (1H, dd, J=11.7, 9.0 Hz, X^1-5), 3.33 (1H, dd, J=12.5, 7.6 Hz, X^1-5), 2.17, 2.15, 2.10, 2.08, 2.07, 2.06, 2.03, 2.02, 2.01, 2.00, 1.99 (3H×11, s, $COCH_3$), 0.91 (3H, t, J=7.5 Hz, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 193.6, 172.6, 172.3, 172.2, 171.8, 171.6, 171.5, 171.1, 170.0, 135.3, 135.1, 130.1, 128.9, 126.1, 101.2, 101.1, 100.8, 79.3, 77.9, 76.9, 74.2, 74.1, 73.6, 73.4, 73.2, 72.4, 71.9, 70.9, 70.1, 69.8, 68.3, 67.5, 62.3, 61.9, 61.4, 35.0, 33.2, 32.0, 30.9, 30.7, 30.2, 29.1, 26.3, 25.6, 24.0, 21.5, 21.2, 21.1, 21.0, 20.7, 14.7.

In the same manner, DG-A (1.5 mg), DG-B (1.2 mg), and DG-C (3.2 mg) were derivatized to give the peracetyl phenacyl esters A (1.8 mg), B (1.1 mg), and C (3.8 mg).

Peracetyl Phenacyl Ester of **1a**: Oil. $[\alpha]_D^{20}$ +0.37° (c=0.7, MeOH), UV λ_{max}^{MeOH} nm: 242. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1365, 1240. SIMS (positive) m/z: 1489 $(M+Na)^+$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.90 (2H, d, J=7.1 Hz, aromatic proton), 7.62 (1H, t, J=7.4 Hz, aromatic proton), 7.49 (2H, t, J=7.1 Hz, aromatic proton), 5.50 (1H, d, J=16.4 Hz, CH_2), 5.28 (1H, d, J=16.4 Hz, CH_2), 5.18 (1H, t, J=9.3 Hz, G-4), 5.15 (1H, t, J=9.0 Hz, X^1-3), 5.12 (1H, m, aglycone-2), 5.11 (1H, t, J=9.3 Hz, G-3), 5.07 (1H, t, J=8.8 Hz, X^2-3), 5.01 (1H, m, aglycone-17), 4.99 (1H, m, aglycone-16), 4.95 (1H, dd, J=9.3, 8.1 Hz, G-2), 4.86 (1H, m, X^1-4), 4.83 (1H, m, X^2-4), 4.63 (1H, d, J=6.4 Hz, X^2-1), 4.54 (1H, d, J=8.1 Hz, G-1), 4.43 (1H, d, J=7.1 Hz, X^1-1), 4.36 (1H, dd, J=12.2, 1.9 Hz, G-6'), 4.22 (1H, dd, J=12.2, 3.7 Hz, G-6), 4.05 (1H, dd, J=12.7, 5.1 Hz, X^2-5'), 3.95 (1H, dd, J=11.7, 5.6 Hz, X^1-5'), 3.62 (1H, m, G-5), 3.58 (1H, dd, J=9.0, 7.1 Hz, X^1-2), 3.55 (1H, m, aglycone-21), 3.51 (1H, dd, J=8.8, 6.4 Hz, X^2-2), 3.38 (1H, dd, J=11.7, 9.3 Hz, X^1-5), 3.32 (1H, dd, J=12.7, 7.8 Hz, X^2-5), 2.17, 2.15, 2.10, 2.07, 2.06, 2.04, 2.04, 2.03, 2.01, 2.00, 1.99 (3H×11, s, $COCH_3$), 0.92 (3H, t, J=7.0 Hz, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 193.6, 172.6, 172.3, 172.2, 171.8, 171.6, 171.5, 171.1, 170.0, 135.5, 135.1, 130.1, 128.9, 126.1, 101.2, 101.1, 100.9, 79.6, 77.9, 76.9, 74.3, 74.2, 74.2, 73.5, 73.2, 72.3, 71.9, 70.9, 70.0, 69.8, 68.2, 67.9, 62.3, 61.9, 61.4, 35.2, 33.4, 32.3, 30.9, 30.7, 30.0, 26.6, 26.0, 25.6, 23.9, 22.3, 21.5, 21.2, 21.1, 20.7, 14.7.

Peracetyl Phenacyl Ester of **1b**: Oil. $[\alpha]_D^{20}$ −0.08° (c=0.8, MeOH). UV λ_{max}^{MeOH} nm: 242. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1365, 1240. SIMS (positive) m/z 1489 $(M+Na)^+$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.90 (2H, d, J=7.3 Hz, aromatic proton), 7.62 (1H, t, J=7.5 Hz, aromatic proton),

7.49 (2H, t, $J=7.3$ Hz, aromatic proton), 5.50 (1H, d, $J=16.2$ Hz, CH_2), 5.28 (1H, d, $J=16.2$ Hz, CH_2), 5.19 (1H, t, $J=9.5$ Hz, G-4), 5.16 (1H, t, $J=9.2$ Hz, X^1-3), 5.11 (1H, t, $J=9.5$ Hz, G-3), 5.10 (1H, dd, $J=8.1$, 4.6 Hz, aglycone-2), 5.07 (1H, t, $J=8.9$ Hz, X^2-3), 4.99 (1H, m, aglycone-18), 4.96 (1H, m, aglycone-17), 4.95 (1H, dd, $J=9.5$, 8.1 Hz, G-2), 4.87 (1H, m, X^1-4), 4.83 (1H, m, X^2-4), 4.63 (1H, d, $J=6.4$ Hz, X^2-1), 4.55 (1H, d, $J=8.1$ Hz, G-1), 4.44 (1H, d, $J=6.8$ Hz, X^1-1), 4.37 (1H, dd, $J=12.2$, 1.9 Hz, G-6′), 4.22 (1H, dd, $J=12.2$, 3.7 Hz, G-6), 4.05 (1H, dd, $J=12.8$, 5.0 Hz, X^2-5′), 3.95 (1H, dd, $J=11.6$, 5.3 Hz, X^1-5′), 3.62 (1H, ddd, $J=9.8$, 3.8, 2.3 Hz, G-5), 3.59 (1H, dd, $J=9.2$, 6.8 Hz, X^1-2), 3.55 (1H, m, aglycone-22), 3.51 (1H, dd, $J=8.9$, 6.4 Hz, X^2-2), 3.38 (1H, dd, $J=11.6$, 9.0 Hz, X^1-5), 3.33 (1H, dd, $J=12.8$, 7.6 Hz, X^2-5), 2.17, 2.15, 2.10, 2.06, 2.05, 2.04, 2.04, 2.02, 2.01, 2.00, 1.99 ($3H\times11$, s, $COC\underline{H}_3$), 0.92 (3H, t, $J=7.1$ Hz, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 193.4, 172.3, 172.2, 172.0, 171.6, 171.3, 170.9, 170.0, 135.4, 135.0, 129.9, 128.9, 126.1, 101.2, 101.1, 100.9, 79.6, 77.9, 76.9, 74.3, 74.2, 74.2, 73.4, 72.4, 72.2, 71.9, 70.9, 70.0, 69.8, 68.2, 67.7, 62.3, 61.9, 35.2, 34.9, 32.2, 30.9, 30.7, 30.4, 30.2, 30.0, 28.0, 26.5, 25.9, 24.0, 22.2, 21.4, 21.2, 21.0, 20.5, 14.7.

Peracetyl Phenacyl Ester of **1c**: Oil. $[\alpha]_D^{20}$ $-1.49°$ ($c=0.4$, MeOH). UV λ_{max}^{MeOH} nm: 242. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1365, 1240. SIMS (positive) m/z: 1489 $(M+Na)^+$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.90 (2H, d, $J=7.4$ Hz, aromatic proton), 7.62 (1H, t, $J=7.5$ Hz, aromatic proton), 7.49 (2H, t, $J=7.4$ Hz, aromatic proton), 5.50 (1H, d, $J=16.2$ Hz, CH_2), 5.28 (1H, d, $J=16.2$ Hz, CH_2), 5.18 (1H, t, $J=9.5$ Hz, G-4), 5.16 (1H, t, $J=9.0$ Hz, X^1-3), 5.11 (1H, t, $J=9.5$ Hz, G-3), 5.09 (1H, m, aglycone-2), 5.07 (1H, t, $J=8.8$ Hz, X^2-3), 5.01 (1H, m, aglycone-18), 4.98 (1H, m, aglycone-17), 4.95 (1H, dd, $J=9.5$, 8.1 Hz, G-2), 4.88 (1H, m, X^1-4), 4.84 (1H, m, X^2-4), 4.64 (1H, d, $J=6.4$ Hz, X^2-1), 4.55 (1H, d, $J=8.1$ Hz, G-1), 4.44 (1H, d, $J=6.8$ Hz, X^1-1), 4.35 (1H, dd, $J=12.2$, 2.2 Hz, G-6′), 4.23 (1H, dd, $J=12.2$, 3.9 Hz, G-6), 4.05 (1H, dd, $J=12.5$, 5.1 Hz, X^2-5′), 3.97 (1H, dd, $J=11.7$, 5.4 Hz, X^1-5′), 3.64 (1H, ddd, $J=9.5$, 3.9, 2.2 Hz, G-5), 3.59 (1H, dd, $J=9.0$, 6.8 Hz, X^1-2), 3.55 (1H, m, aglycone-22), 3.52 (1H, dd, $J=8.8$, 6.4 Hz, X^2-2), 3.39 (1H, dd, $J=11.7$, 9.0 Hz, X^1-5), 3.33 (1H, dd, $J=12.5$, 7.6 Hz, X^2-5), 2.17, 2.15, 2.10, 2.08, 2.07, 2.06, 2.03, 2.02, 2.01, 2.00, 1.99 ($3H\times11$, s, $COC\underline{H}_3$), 0.91 (3H, t, $J=7.9$ Hz, CH_3). ^{13}C-NMR (100 MHz, CD_3OD) δ: 193.6, 172.3, 171.8, 171.6, 171.5, 171.1, 170.0, 135.5, 135.1, 130.1, 128.9, 126.1, 101.2, 101.1, 100.8, 79.3, 77.9, 76.9, 74.2, 74.0, 73.7, 73.4, 73.2, 72.4, 71.9, 70.9, 70.1, 69.9, 68.3, 67.6, 62.3, 61.9, 61.4, 34.9, 32.3, 31.7, 30.9, 30.7, 30.2, 28.2, 26.3, 25.9, 24.0, 21.5, 21.2, 21.1, 21.0, 20.7, 14.7.

The HPLC Analyses The HPLC analyses were performed using a column of Develosil ODS-5 (260×4.6 mm, Nomura Kagaku). The solvent system was CH_3CN–MeOH–H_2O (9.0 : 0.5 : 0.5) and the flow late was 1 ml/min. The HPLC analysis of the peracetyl phenacyl ester of GK-III showed three peaks at $t_R=11.1$, 11.5, and 10.0 min, which coincided with the respective peaks of **1a**—**c**.

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Intramolecular Michael Reaction in Diastereoselective Synthesis of *dl*-Griseofulvin

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The diastereoselective cyclization of 2a to 5a, which was employed in our novel synthesis of *dl*-griseofulvin (7a), was studied in detail. It was found that the use of the metallic bases resulted in excellent diastereoselectively, whereas organic bases are ineffective for diastereoselective cyclization. Consequently, diastereoselective cyclization of 2 to 5 was proposed to proceed through the intramolecular Michael reaction under chelation control.

Keywords reaction mechanism; *dl*-griseofulvin; diastereoselective Michael reaction; chelation control

We have previously reported[1a,b] a novel synthesis of *dl*-griseofulvin (**7a**) which involves diastereoselective intramolecular cyclization of **2a** to **5a** as a key reaction (Chart 1). This method has significant advantages over other approaches in terms of suitability for large-scale production, simplicity, and safety, and should also be applicable to the synthesis of congeners of **7a**. However, the reaction mechanism for the diastereoselective cyclization of **2a** to **5a** by treatment with activated alumina remains unknown. This paper describes a detailed investigation of the diasteroselective cyclization.

We have assumed that the cyclization of **2a** to **5a** by treatment with activated alumina[2] might proceed as shown in Chart. 1. The elimination of methanol (MeOH) from **2a** affords **3a**, which is subsequently converted into the enolate **4a**. The enolate **4a** undergoes stereoselective Michael reaction under chelation control to generate the cyclized product **5a** with high diastereoselectivity.

Stork and Tomasz[3a,b] diastereospecifically synthesized *dl*-griseofulvin by the reaction of **1a** with 1-methoxy-4-hexen-1-yn-3-one (**8**) in the presence of potassium *tert*-butoxide in 5% yield (Chart 2). They proposed that this reaction proceeds through the double Michael reaction and that in the latter Michael reaction, the enolate **9**, which is similar to the intermediate **4a**, is diastereospecifically formed under kinetic control and converted to *dl*-griseofulvin. However, a detailed examination was not made.

We first attempted to isolate a possible intermediate **3a**, but without success. However, the corresponding intermediate **3b** could be isolated in the case of an analogous compound without a substituent on the benzene ring (Chart 3). Treatment of **2b**, prepared by the reaction of 3(2*H*)-benzofuranone (**1b**)[4] with 5-methoxy-1-methylsulfinyl-1-methylthio-1-hexen-3-one (**10**) with activated alumina in refluxing ether afforded a 9:1 mixture of **5b** and **6b**. On the other hand, treatment of **2b** with sodium hydride (NaH) afforded pure **3b** in 34% yield. The structure **3b** was confirmed by the proton nuclear magnetic resonance (^{1}H-

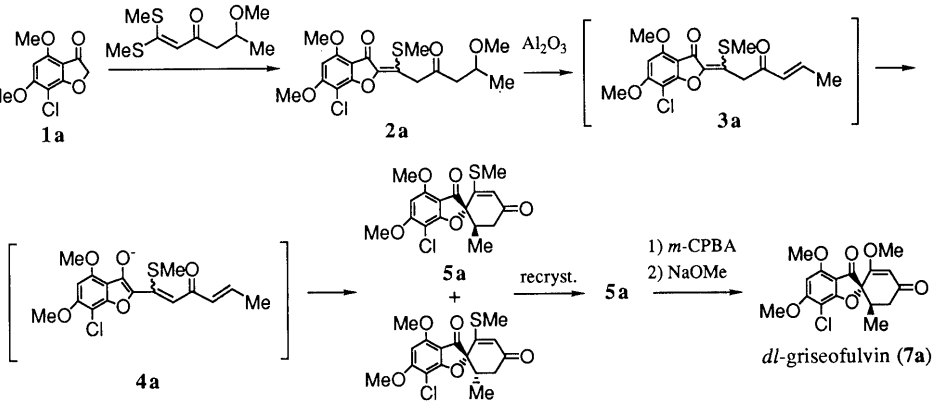


Chart 1

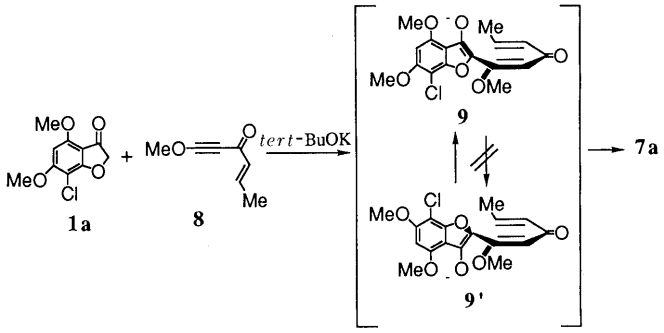


Chart 2

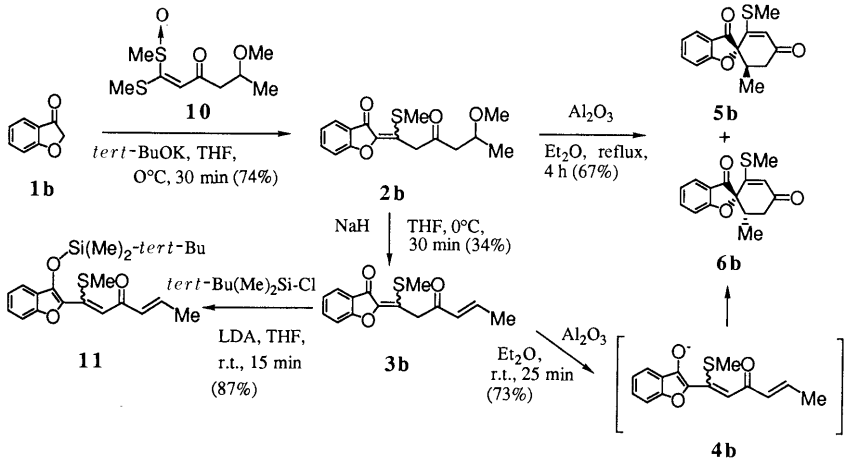
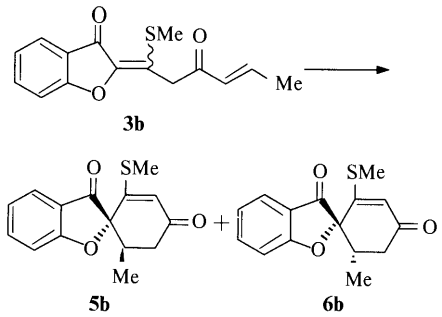


Chart 3

TABLE I. The Reaction of **3b** with Various Bases



Run	Base	Reaction condition			Product (**5b**+**6b**)	
		Solvent	Temp. (°C)	Time (h)	Yield (%)	de (%)[a]
1	LiH	THF	r.t.	10	55	93
2	LDA[b]	THF	r.t.	11	70	91
3	NaH	THF	r.t.	18	63	93
4	Al_2O_3	Et_2O	r.t.	0.5	73	93
5	Al(O-iso-Pr)$_3$	CH_2Cl_2	r.t.	18	70	93
6	KH	THF	0	4	0	—
7	Ti(O-iso-Pr)$_4$	CH_2Cl_2	r.t.	24	0	—
8	DMAP[c]	CH_2Cl_2	r.t.	78	13	29
9	Triton B[d]	Pyridine	r.t.	18	8	23
10	DBU[e]	THF	r.t.	12	50	51

a) Diastereomeric excess of **5b** was determined by HPLC analysis. *b*) Lithium diisopropylamide. *c*) 4-Dimethylaminopyridine. *d*) Trimethylbenzylammonium hydroxide. *e*) 1,8-Diazabicyclo[5.4.0]undec-7-ene.

NMR, 500 MHz) spectrum of **3b**. The methylene group and vinyl group signals appeared at δ 4.49 ppm as a singlet and at δ 6.30 and 7.05 ppm, each as a quartet of doublets, respectively.

Using **3b** in place of **3a**, we investigated the mechanism of diastereoselective cyclization of **3** to **5**. When **3b** was treated with alumina or NaH, the cyclization occurred even at room temperature to give *dl*-**5b** with 93% de, as expected (Chart 3). This result suggested that **3b** is easily transformed to **4b**, which subsequently undergoes diastereoselective cyclization to **5b**. The formation of **4a** from **3a** (Chart 1) was confirmed by the following indirect means. Treatment of **3b** with lithium diisopropylamide (LDA) followed by reaction with *tert*-butyltrimethylsilyl chloride (TBDMS-Cl) gave **11** in 87% yield (Chart 3). The structure of **11** was

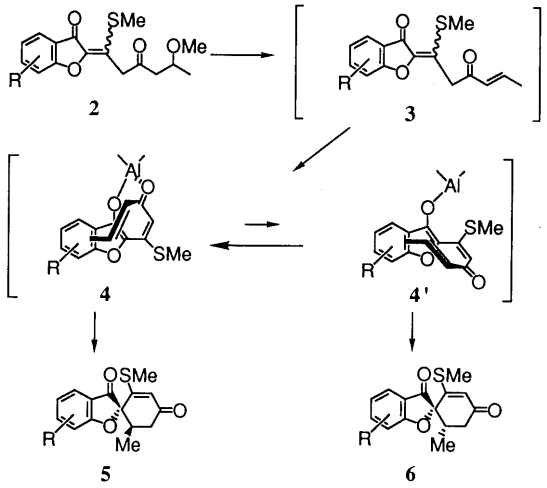


Chart 4

confirmed by the ^{1}H-NMR spectrum: signals due to vinyl protons appear at δ 6.85 ppm as a singlet and at δ 6.27 and 6.92 ppm each as a quartet of doublets. This result implied that the treatment of the intermediate **3b** with base affords **4b**, in which an enolate ion exists on the oxygen atom at the 3-position of benzofuran, and that compounds **4b** cyclized to **5b** through the intramolecular Michael reaction.

Using **3b** and various base catalysts, we next examined whether the diastereoselective cyclization of **3b** to **5b** proceeded under kinetic control or chelation control (Table I). When lithium or sodium base was used, **5b** was generated with high chemical yield and excellent diastereomeric excess (runs 1—5). However, treatment of **3b** with potassium or titanium base gave not the cyclized product **5b** but many decomposed products (runs 6 and 7). When a nonmetallic organic base, such as 4-dimethylaminopyridine (DMAP), benzyltrimethylammonium hydroxide (Triton B), or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), was used, **5b** was formed in poor chemical yield and low diastereoselectivity (runs 8—10). In those reactions, the diastereomeric excess was determined by high performance liquid chromatogaphy (HPLC) analysis. The structure of the major product **5b** was identified based on the similarities of the NMR spectral patterns to those of **5a**, which was converted to *dl*-griseofulvin in our previous work.[1b]

It was found that metallic bases are effective for diastereoselective cyclization, whereas nonmetallic bases give **5b** in poor chemical yield and low diastereoselectivity. These findings suggested that the conversion of **2** to **5** proceeds *via* diastereoselective cyclization of **4** under chelation control, as shown in Chart 4. When **2** was treated with a metallic base, elimination of MeOH occurred to give **3**. Two types of conformers **4** and **4'** can be formed from **3** in the presence of a base. In the case of using a metallic base, intermediate **4** might be preferentially formed under chelation control and converted to **5**.

Consequently, it was concluded that the diastereoselective cyclization of **2a** to **5a** proceeds through the Michael reaction of **4a** not under kinetic control but under chelation control.

Experimental

Melting points are uncorrected. Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer. Fast atom bombardment mass spectra (FAB-MS) were recorded on a VG-70SE spectrometer. ^{1}H-NMR spectra were run on a Hitachi R-24 spectrometer (60 MHz) or on a Varian VXR-500 (500 MHz) instrument. Analytic HPLC was performed with a Shimadzu SPD-6A instrument on a Chemcosorb 5Si-U (Chemco) column fitted with an ultraviolet (254 nm) detector. Merck Silica gel 60 (230—400 mesh) was employed for column chromatography. Extracts were dried over anhydrous $MgSO_4$.

2-[5-Methoxy-1-(methylthio)-3-oxohexylidene]-3(2*H*)-benzofuranone (2b) *m*-Chloroperbenzoic acid (5.8 g, 33.6 mmol) was added to a mixture of 1,1-bis(methylthio)-5-methoxy-1-hexen-3-one (6.3 g, 28.6 mmol)[1b] and CH_2Cl_2 (600 ml). The mixture was stirred at 0 °C for 30 min, then made basic with saturated $KHCO_3$ solution and the organic layer was separated. The organic layer was washed with 10% Na_2SO_3 solution and water, and dried. Removal of the solvent gave 5-methoxy-1-(methylsulfinyl)-1-(methylthio)-1-hexen-3-one (**10**) as a crude product. A mixture of **10** and dry tetrahydrofuran (THF) (10 ml) was added dropwise to a mixture of 3(2*H*)-benzofuranone (**1b**; 2.9 g, 21.6 mmol),[4] *tert*-BuOK (3.2 g, 28.5 mmol), and dry THF (108 ml). After being stirred at 0 °C for 15 min, the reaction mixture was made acidic with 10% HCl solution and extracted with AcOEt. The organic layer was washed with water and dried. The solvent was evaporated off and the residue was chromatographed on an SiO_2 column with a mixture of AcOEt and hexane (1 : 5) to give 4.9 g (74%) of **2b** as an oily product. *Anal.* Calcd for $C_{16}H_{18}O_4S$: C, 62.73; H, 5.92. Found: C, 62.49; H, 6.02. IR ν (neat): 1720, 1680 cm^{-1}. ^{1}H-NMR (60 MHz, CCl_4) δ: 1.16 (3H, d, J=6 Hz), 2.42 (3H, s), 2.51—2.78 (2H, m), 3.25 (3H, s), 3.52—3.95 (1H, m), 4.03, 4.87 (each 1H, each d, J=17 Hz), 6.90—7.28 (2H, m), 7.35—7.78 (2H, m). FAB-MS (positive ion mode) m/z: 307 [(M+1)$^+$].

Reaction of 2b with Al_2O_3 A suspension of **2b** (300 mg, 0.98 mmol), activated Al_2O_3 (3g),[2] and Et_2O (24 ml) was heated at reflux for 4 h. The solvent was removed and the residue was chromatographed on an SiO_2 column with a mixture of AcOEt and hexane (1 : 4). The first eluate gave 160 mg (60%) of *erythro*-6'-methyl-2'-(methylthio)spiro[benzofuro-2(3*H*), 1'-[2]-cyclohexene]-3,4'-dione (**5b**), mp 159—161 °C (CH_2Cl_2 and hexane). *Anal.* Calcd for $C_{15}H_{14}O_3S$: C, 65.70; H, 5.14. Found: C, 65.43; H, 5.02. IR ν (Nujol): 1715, 1655 cm^{-1}. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 0.89 (3H, d, J=6 Hz), 2.22 (3H, s), 2.45—3.19 (3H, m), 5.94 (1H, s), 7.02—7.40 (2H, m), 7.59—7.90 (2H, m). FAB-MS (positive ion mode) m/z: 275 [(M+1)$^+$]. The second eluate gave 20 mg (7%) of *threo*-6'-methyl-2'-(methylthio)spiro[benzofuro-2(3*H*),1'-[2]-cyclohexene]-3,4'-dione (**6b**). *Anal.* Calcd for $C_{15}H_{14}O_3S$: C, 65.70; H, 5.14. Found: C, 65.52; H, 4.98. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.13 (3H, d, J=6 Hz), 2.26 (3H, s), 2.42—2.97 (3H, m), 6.02 (1H, s), 7.08—7.54 (2H, m), 7.59—7.94 (2H, m). FAB-MS (positive ion mode) m/z: 275 [(M+1)$^+$].

2-[1-(Methylthio)-3-oxo-4-hexenylidene]-3(2*H*)-benzofuranone (3b) NaH (64% dispersion in material oil, 360 mg, 9.6 mmol) was added portionwise to a mixture of **2b** (950 mg, 3.1 mmol) and dry THF (32 ml) at 0 °C for 30 min. The mixture was poured into ice water and then made acidic with 10% HCl solution and extracted with AcOEt. The organic layer was washed with water and dried. The solvent was evaporated off. Recrystallization of the residue from AcOEt gave 290 mg (34 %) of **3b**, mp 160—162 °C. *Anal.* Calcd for $C_{15}H_{14}O_3S$: C, 65.70; H, 5.14. Found: C, 65.42; H, 5.09. IR ν (Nujol): 1680, 1630 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.93 (3H, dd, J=1.52, 6.84 Hz), 2.47 (3H, s), 4.49 (2H, s), 6.30 (1H, qd, J=1.52, 15.63 Hz), 7.05 (1H, qd, J=6.84, 15.63 Hz), 7.13—7.25 (2H, m), 7.57—7.72 (2H, m). FAB-MS (positive ion mode) m/z: 275 [(M+1)$^+$].

2-[1-(Methylthio)-3-oxo-4-hexenylidene]-3-*tert*-butyldimethylsiloxybenzofuran (11) Butyllithium (1.37 M in hexane, 0.77 ml, 1.05 mmol) was added dropwise to a mixture of diisopropylamine (0.17 ml, 1.2 mmol) and dry THF (2.4 ml) at −78 °C and the whole was stirred at 0 °C for 30 min, then added dropwise to a mixture of **3b** (260 mg, 0.95 mmol) and dry THF (8 ml) at −78 °C. The reaction mixture was stirred at room temperature for 1 h. *tert*-Butyldimethylsilyl chloride (262 mg, 1.7 mmol) was added, the whole was stirred at room temperature for 15 min, then poured into ice water and extracted with AcOEt. The organic layer was washed with water and dried. The solvent was evaporated off. The residue was chromatographed on an SiO_2 column with Et_2O and petroleum ether (1 : 1) to give 320 mg (87%) of **11**. IR ν (Nujol): 1660, 1617 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.15 (6H, s), 1.04 (9H, s), 1.91 (3H, dd, J=1.68, 6.89 Hz), 2.28 (3H, s), 6.27 (1H, qd, J=1.68, 15.57 Hz), 6.85 (1H, s), 6.92 (1H, qd, J=6.89, 15.57 Hz), 7.24—7.36 (2H, m), 7.42—7.55 (2H, m).

Cyclization of 3b with Various Bases: General Procedure A suspension of **3b** (200 mg, 0.68 mmol), activated Al_2O_3 (2 g), and Et_2O (17 ml) was stirred at room temperature for 25 min, then chromatographed on an SiO_2 column with a mixture of AcOEt and hexane (1 : 4) to give 145 mg (73%) of a mixture of **5b** and **6b**. Diastereo excess of **5b** was determined by HPLC to be 93% de.

Acknowledgment We are grateful to the SC-NMR Laboratory of Okayama University for 500 MHz proton-NMR experiments.

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Further Studies on the Structure of Retigeranic Acid

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Retigeranic acid, $C_{25}H_{38}O_2$, a sesterterpene isolated from the lichen, *Lobaria retigera* group, *L. isidiosa* VAIN. var. *subisidiosa* ASAH., was separated by high performance liquid chromatography into two components, retigeranic acid A (minor) (1) and B (major) (2). Crystals of both components were subjected to X-ray crystallographic analysis. The crystal parameters are monoclinic space group $P2_1$, $Z=4$ with $a=24.10(3)$, $b=12.86(1)$, $c=7.412(9)$ Å, $\beta=96.5(1)°$ for 1 and $a=24.067(17)$, $b=12.614(9)$, $c=7.457(5)$ Å, $\beta=96.30(7)°$ for 2.

Retigeranic acids A and B were proved to be *S* and *R* stereoisomers at C-18, respectively. The absolute configurations of other parts of the structure of retigeranic acids are assumed to be identical, as they have previously been determined by the X-ray anomalous dispersion method on the *p*-bromoanilide derived from native retigeranic acid.

Keywords retigeranic acid A; retigeranic acid B; *Lobaria isidiosa* var. *subisidiosa*; X-ray cyrstallography

Retigeranic acid, $C_{25}H_{38}O_2$, isolated first from the lichens of *Lobaria retigera* group by Seshadri *et al.*[2)] and then from *L. isidiosa* VAIN. var. *subisidiosa* ASAH. by the present authors (S.S. and Y.I.), was formulated (**1**) as a novel sesterterpene on the basis of chemical and X-ray crystallographic studies.[3,4)]

Recently, Corey *et al.*[5)] attempted to synthesize (±)-retigeranic acid as a novel model sesterterpene. The methyl ester of the sample of retigeranic acid supplied by one of us (S.S.) to Prof. Corey was found by him to be a mixture of major and minor components on high performance liquid chromatography (HPLC). It has become clear that retigeranic acid itself is a mixture of stereochemical isomers, which have now been designated retigeranic acids A (minor) and B (major), and can be separated by HPLC on an octadecyl silica(ODS) column using a gradient solvent system (3% AcOH–CH_3CN) (Fig. 1). Shortly after the publication of Corey's work, Paquette and his associates[6)] reported a total synthesis of (−)-retigeranic acid A and its 4β-H epimeric isomer. The latter was not identical with (−)-retigeranic acid B. Corey's synthesis[5)] proved the structure of the minor component (*A*) to be as formulated previously for retigeranic acid.[3,4)]

Retigeranic acid A, $C_{25}H_{38}O_2$, mp 220—222 °C, $[\alpha]_D^{23}$ −86.5° (EtOH), M^+ 370, and retigeranic acid B, $C_{25}H_{38}O_2$, mp 188—190 °C, $[\alpha]_D^{23}$ −30.4° (EtOH), M^+ 370, gave superimposable ultraviolet (UV) absorptions (max 250 nm) suggesting stereochemically isomeric structures. The stereochemical structures of retigeranic acids A and B have been investigated by means of X-ray crystallography, though the stereochemistry of native retigeranic acid had previously been evaluated using a single crystal of its *p*-bromoanilide.[4)]

X-Ray Crystallography Crystals of *A* and *B* were grown in the *A* and *B* fractions from HPLC (Fig. 1), respectively. Crystals of *AB* were similarly grown from an intermediate fraction between the *A* and *B* peaks. For comparison, the crystal structure of the *p*-bromoanilide was refined using new data collected in the same way as for the *A*, *B* and *AB* crystals. *p*-Bromoanilide crystals were obtained from the samples prepared in 1972.[3)]

The X-ray diffraction intensities and crystal data were obtained on a Philips PW1100 diffractometer using Cu $K\alpha$ radiation monochromated by a graphite plate. Integrated intensities were measured by the θ–2θ scan method with the scan rate of $4°\theta\,min^{-1}$. Background was measured at both ends of the scan for half the scan time.

Due to the small size of the X-ray specimens, especially of the crystals of *A* and *AB*, the maximum 2θ angles were limited to below 100°. The crystal data and a summary of the structure determination are given in Table I.

The structure of the *B* crystal was solved by the direct method based on MULTAN[7)] and refined by the block-diagonal-matrix least-squares method. All hydrogen atoms except carboxyl hydrogen were found on the difference electron-density map and located at the calculated positions. They were included in the refinement assuming isotropic thermal vibrations.[8)]

The unit cell of the *B* crystal contains two kinds of crystallographically independent molecules which occupy sites 1 and 2, respectively. In Fig. 2, the atoms of the molecule at site 1 are denoted by means of the letter A, such as C1A and C2A, and those at site 2 are indicated as C1B and C2B. If the absolute configurations at C2, C3, C6, C7, C10, C14 and C15 are assumed to be identical with the corresponding configurations in the *p*-bromoanilide, for which the absolute configurations were determined by the X-ray anomalous dispersion method,[3,4)] the configurations at C18A and C18B are reversed as compared with C18 of the *p*-bromoanilide.

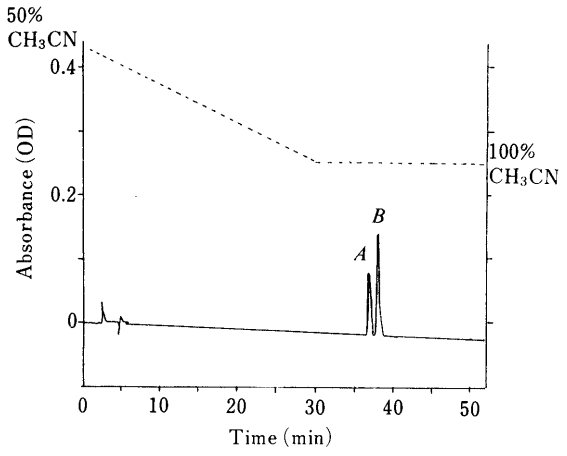


Fig. 1. HPLC of Retigeranic Acid

Column: ORD 4.6 mm × 250 mm. Mobile phase: 3% AcOH–CH_3CN. Flow rate: 1 ml/min. UV detection: 253 nm. *A*: retigeranic acid A, *B*: retigeranic acid B.

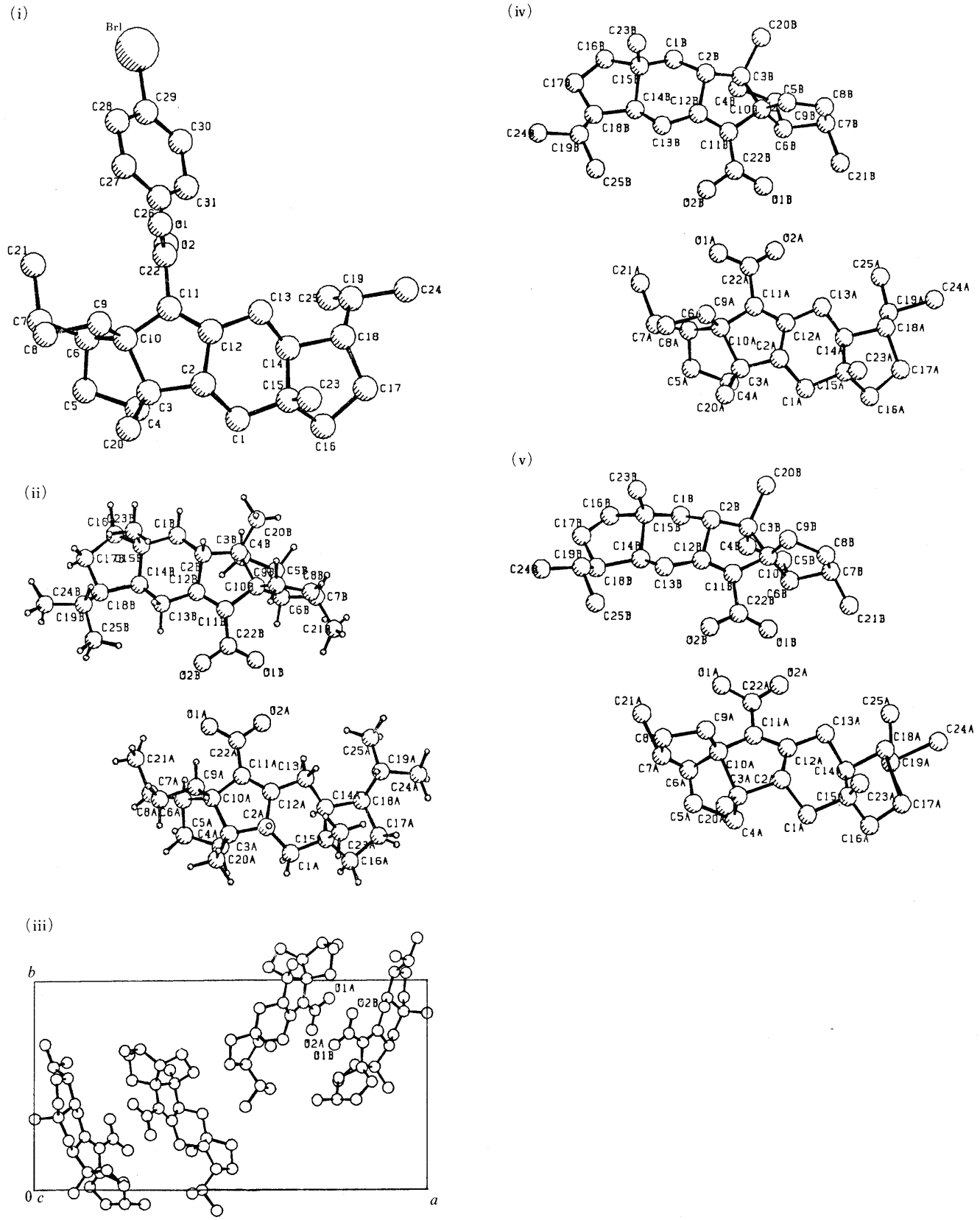


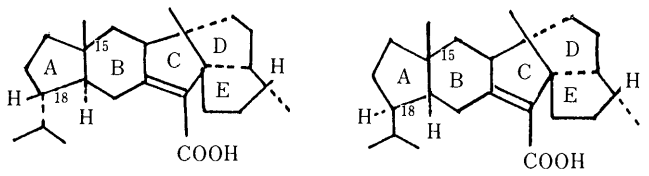
Fig. 2. Molecular Structure Found in the Crystals of the i) *p*-Bromoanilide, ii) *B* Crystal, iii) *c*-Axis Projection of the Unit Cell of the *B* Crystal, iv) *A* Crystal, and v) *AB* Crystal.

i) Molecules take *S* configuration at C18. ii) Molecules at site 1 are *R*, and those at site 2 are *R*. iii) O1A and O2A are the carboxyl oxygen atoms of the molecule at site 1. O1B and O2B are those of the molecule at site 2. These two molecules form an H-bonded dimer. iv) Site 1, *S*; site 2, *R*. v) Site 1, mixture of *S* and *R* (random arrangement of two kinds of cells, one contains *S* and the other *R* molecule, both at site 1; site 2, *R*. The figures were drawn by the PLUTO program.[9]

TABLE I. Crystal Data and Summary of Structure Determination

Crystal	*A*	*B*	*AB*	*p*-Bromoanilide
Crystallized from fraction	*A* (Shown in Fig. 1)	*B*	Intermediate of *A* and *B*	
Chemical formula		$C_{25}H_{38}O_2$		$C_{31}H_{42}NOBr$
Molecular weight		370.6		524.6
Crystal system		Monoclinic		Monoclinic
Space group		$P2_1$		$P2_1$
Cell dimensions				
a (Å)	24.10 (3)	24.067 (17)	24.10 (4)	13.148 (4)
b (Å)	12.86 (1)	12.614 (9)	12.69 (2)	9.410 (3)
c (Å)	7.412 (9)	7.457 (5)	7.441 (12)	11.429 (4)
β (°)	96.5 (1)	96.30 (7)	96.3 (2)	101.82 (3)
U (Å^3)	2282	2250	2262	1384
Z	4	4	4	2
D_x (g cm^{-3})	1.08	1.094	1.09	1.259
μ for Cu $K\alpha$ (cm^{-1})	4.8	4.8	4.8	21.8
No of reflections used for refinement	1133	2581	1244	2050
$2\theta_{max}$ (°) in the direction of				
a^*	75	125	60	120
b^*	75	100	90	110
c^*	75	90	75	120
Final R value	0.150	0.062	0.220	0.086
No of atoms refined C, N and				
O (anisotropic)	54[a]	54	54[a]	34 (incl. Br)
H (isotropic)	0	74	0	0
Configuration at C18 (isopropyl bearing C, Fig. 2)				
Site 1	*S*	*R*	Mixture of *S* and *R*	*S*[b]
Site 2	*R*	*R*	*R*	

a) Isotropic temperature factor for individual atom was assigned. *b*) The unit cell contains only one site. The molecule with *S* configuration corresponds to retigeranic acid A, and the *R* molecule to retigeranic acid B.



retigeranic acid A (**1**) retigeranic acid B (**2**)

S at C18 *R* at C18

Chart 1

Thus in both molecules of the *B* crystal, the isopropyl group is β (*R* configuration at C18), while it is α (*S* configuration) in the *p*-bromoanilide crystal (see Table I and Fig. 2).

The structures of the *A* and *AB* crystals were refined in the same way as *B* assuming the same crystal structure as *B* but neglecting all hydrogen atoms. In the course of the refinement, some atoms, especially those of C18 and the isopropyl group were moved as much as 1.66 Å (C18A of *A* crystal) and 1.41 Å (C18A of *AB* crystal). The r.m.s. shifts of C18 and the isopropyl atoms in the *A* crystal were 0.94 Å for site 1 and 0.45 Å for site 2 molecules, and those in the *AB* crystal were 0.90 Å and 0.15 Å, respectively.

As a result of the refinement, the molecule at site 1 of the *A* crystal turned out to have *S* configuration, while that of the *AB* crystal was intermediate between *R* and *S*. Possibly a fraction of *R* molecule remains at site 1 in the *AB* crystal. However, a definitive conclusion is difficult because of the limited data obtained for the *A* and *AB* crystals. It is rather surprising that the molecules at site 2 always held *R* configuration throughout the *A*, *B* and *AB* crystals.

Discussion

The X-ray crystallographic analysis showed that retigeranic acids A and B are epimers at the carbon bearing the isopropyl group, as formulated by **1** and **2**, respectively.

The stereochemistry of retigeranic acid B (major) was established as *R* at C18, and that of retigeranic acid A (minor) as *S* at C18, which was mainly deduced from the data obtained from the *p*-bromoanilide crystals. The present X-ray crystallographical data obtained from the HPLC fraction *A* (minor) may suggest the occurrence of a molecular compound of retigeranic acid, *S* and *R* (1 : 1) at C18, though conclusive evidence has not yet been obtained due to the shortage of material.

According to Corey's synthetic scheme for retigeranic acid, it is quite clear that he synthesized the racemic methyl ester of retigeranic acid A (**1**). He assumed that retigeranic acid B possibly differs at the methyl-bearing carbon of ring E. Paquette and his associates also synthesized (−)-retigeranic acid A (**1**) and an epimer having β-H at the ring A/B juncture.

In our previous X-ray crystallographic study[3,4] on native retigeranic acid, the structure (**1**) was deduced from the experimental data on a single crystal of the *p*-bromoanilide, $C_{31}H_{42}NOBr$, mp 276—278 °C, $[\alpha]_D^{23}$ −56.2°. It would be reasonable to assume that the *p*-bromoanilide of the minor partner (**1**) of native retigeranic acid crystallized out more readily from the acetonic solution under the conditions of the previous experiment.

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Marine Terpenes and Terpenoids. XIII.[1] Isolation of a New Dihydrofuranocembranoid, Sarcophytonin E, from the *Sarcophyton* sp. Soft Coral of Okinawa

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A new dihydrofuranocembranoid, sarcophytonin E (1a), was isolated from the *Sarcophyton* sp. soft coral of Chatan, Okinawa. The structure of 1a was derived from the spectroscopic data, and was confirmed by correlation with the known compound 16-deoxosarcophine (2a). Compound 1a was found to be converted, on storage, to the butenolide 3a. The proton nuclear magnetic resonance spectra of the 2-methoxy-2-trifluoromethylphenylacetic acid esters of 1a and 3a indicated that they are enantiomerically pure, and showed our previous assumption, that the cembranoids isolated from this soft coral are enantiomeric mixtures, to be incorrect.

Keywords soft coral; *Sarcophyton* sp.; cembranoid; dihydrofuranocembranoid; sarcophytonin E

Previously we isolated three new cembranoids, sarcophytonins B, C and D, together with the known compounds cembrene C, sarcophine (**2b**), 16-deoxosarcophine (**2a**) and sarcophytonin A (**4**), from the *Sarcophyton* sp. soft coral, collected off the coast of Chatan, Okinawa.[1,2] Subsequent study of the minor components of this soft coral resulted in the isolation of a new compound designated sarcophytonin E (**1a**). The proton and the carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of **1a** showed signals due to the dihydrofuran ring (^{13}C-NMR, δ 78.3 (t), 127.8, 133.5 (each s), 83.7 (d); ^{1}H-NMR, δ 4.51, 2H, m, 5.58, 1H, m), in close analogy with those of sarcophytonin A (**4**, ^{13}C-NMR, δ 78.4, 127.2, 134.0, 84.1; ^{1}H-NMR, δ 4.48, 5.52).[3] Other signals due to the cembrane ring were also common between these two compounds except that one of the trisubstituted double bonds in **4** was converted to a terminal methylene and a secondary alcohol (^{13}C-NMR, δ 70.3 (d), 109.5 (t), 155.0 (s)). Sarcophytonin E monoacetate (**1b**) was found to be identical with the allylic alcohol monoacetate which was derived previously from 16-deoxosarcophine (**2a**) by hydrolysis, acetylation and dehydration.[2]

Dihydrofurano-type cembranoids are quite susceptible to autoxidation and afford degradation products, even during storage at -30 °C in the dark. In the preceding paper, we described in detail this autoxidation process, of which the conversion to the butenolide derivatives was the major pathway.[1] Probably, traces of peroxidic radical initiator impurities caused the formation of C-16 hydroperoxide, which, on dehydration, led to the butenolides. Purified sarcophytonin E was found, similarly, to give several oxidation products, of which the butenolide **3a** was the major product (*ca.* 40%, see Experimental).

The specific rotation ($+120°$) of the major component, sarcophytonin A (**4**), reported in the previous paper,[2] was different from that ($-92°$) of **4** which we first obtained from *S. glaucum* of Ishigaki Island, Okinawa.[3] This compound was subsequently reported as a deoxygenation product of several sarcophytonin A epoxides,[4,5] but the reported specific rotations varied considerably in both sign and magnitude ($+239°$ to $-210°$). Similarly, the specific rotation of 16-deoxosarcophine (**2a**), isolated from our Chatan material, was $+129°$ while the recorded values were diverse ($+157°$ to $-191°$).[4] These facts indicated that dihydrofuranocembranoid derivatives of this type exist in both (2*R*)- and (2*S*)-enantiomeric form and led us to conclude that our cembranoids, isolated from the Chatan material, and showing smaller magnitude of rotation, are composed of enantiomeric mixtures.[2] This time we prepared 2-methoxy-2-trifluoromethylphenylacetic acid (MTPA) esters (**1c**, **3c** and **3d**), in an attempt to determine the enantiomeric ratio of **1a** and **2a**. The (*S*)-MTPA ester **1c** was prepared by conversion of **2a** isolated from the soft coral used in this study. The (*S*)- and (*R*)-MTPA esters **3c** and **3d** were prepared from **3a** which was obtained by autoxidation of natural **1a**. However, examination of the ^{1}H-NMR spectra of these MTPA esters indicated that they consisted of single sets of signals corresponding to enantiomerically pure compounds, thus indicating that our previous assumption was incorrect. The large range of reported magnitudes of the specific rotations, at least for 16-deoxosarcophine (**2a**) and probably for sarcophytonin A (**4**), appears to be simply a result of inaccurate measurement.

The MTPA esters **3c** and **3d** were expected to reveal the absolute configuration of the (+)- and (−)-16-deoxosarcophine, which were assigned originally from the interpretation of the circular dichroism (CD) data of the butenolide derivative (+)-sarcophine.[6,7] In principle, the plane which involves the C-7 carbinyl methine and the ester carbonyl group is supposed to bisect the average plane of the cembrane ring, and affords $\Delta\delta$'s having opposite sign for the chemical shifts of the protons in the

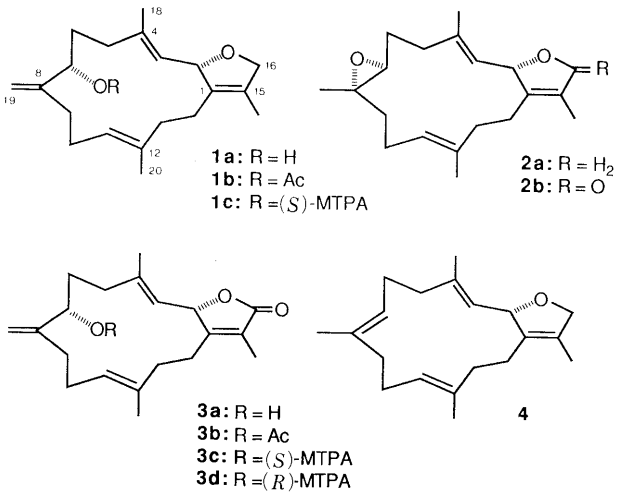

Chart 1

two segments.[8] In fact, even **3c** and **3d** exhibited small but distinctly different patterns of ^{1}H-NMR spectra, the $\Delta\delta$'s were observed for protons at C-2, C-3, and C-11 all having positive sign (+0.011, +0.091, and +0.021 ppm, respectively), so the attempted confirmation of the absolute configuration was unsuccessful. Apparently, the characteristic conformational movement of the cembrane ring makes the straightforward application of the MTPA method inappropriate for these compounds.

Experimental

Melting points were determined on Kofler hot stage and are uncorrected. Optical rotations were determined in $CHCl_3$ on a JASCO DIP-370 digital polarimeter. NMR spectra were determined in $CDCl_3$ solution on a JEOL JMN GX-400 spectrometer at 400 MHz (^{1}H) and on a JEOL JMN FX-90Q spectrometer at 22.5 MHz (^{13}C) with tetramethylsilane as an internal standard. Mass spectra (MS) were determined on a JEOL JMS D300 mass spectrometer. Chromatography was done by flash column chromatography[9] using silica gel (Wako gel C-300, 200—300 mesh, Wako Pure Chemical Industries).

Isolation of Sarcophytonin E (1a) The separation and purification of major known cembranoid derivatives from 20.9 g of the crude extract of the *Sarcophyton* sp. collected in October 1986 in Chatan, Okinawa, were described in the previous paper.[2] The cembranoid mixture obtained from the extract by chromatography with 15% ethyl acetate–hexane (250 mg) was used. It was separated into subfraction 1 (*ca.* 100 mg), which was composed of **2b**, and subfraction 2 (*ca.* 100 mg) with 2.5% Et_2O in $CHCl_3$. Subfraction 2 was purified with 2.5% Et_2O in $CHCl_3$ giving 84.2 mg of **1a**.

Sarcophytonin E (1a) Oil, $[\alpha]_D^{25}$ +120° (c=2.06). ^{1}H-NMR δ: 1.59, 1.65 (each 3H, br s, 17, 20-H_3), 1.82 (3H, br s, 18-H_3), 4.16 (1H, br d, J=10.5 Hz, 7-H), 4.51 (2H, m, 16-H_2), 4.90 (1H, br s, 19-H_2), 5.05 (1H, br t, J=7.0 Hz, 11-H), 5.07 (1H, br t, J=1.5 Hz, 19-H), 5.19 (1H, br d, J=10.5 Hz, 3-H), 5.58 (1H, m, 2-H). ^{13}C-NMR δ: C-1 (133.5), C-2 (83.7), C-3, 11 (125.0, 127.3), C-4 (138.9), C-5, 9, 13 (34.0, 36.3, 36.9), C-6, 10 (30.1, 32.1), C-7 (70.3), C-8 (155.0), C-12 (136.0), C-14 (24.7), C-15 (127.8), C-16 (78.3), C-17 (10.1), C-18, 20 (15.3, 15.5), C-19 (109.5). MS m/z: 302 (M^+), 287, 269, 243. High-resolution MS [Found (Calcd)] m/z: $C_{20}H_{30}O_2$ (M^+), 302.2273 (302.2245).

Sarcophytonin E Acetate (1b) Acetylation of **1a** by a usual method (Ac_2O–pyridine, room temperature, overnight) gave **1b** ($[\alpha]_D^{20}$ +90° (c=0.28)), which was identical with that ($[\alpha]_D$ +86°) prepared from 16-deoxosarcophine **2a**,[2] by direct comparison of their ^{1}H-NMR spectra and thin-layer chromatographic (TLC) behavior.

16-Oxosarcophytonin E (3a) A five-month-old sample of **1a**, stored in the dark at −30 °C, was found to have decomposed. The product was subjected to silica gel column chromatography with ethyl acetate–hexane (2:8), giving 14.6 mg of unchanged **1a** and 31 mg of **3a**. Oil, $[\alpha]_D^{20}$ +120° (c=0.60). ^{1}H-NMR δ: 1.62 (3H, s, 20-H_3), 1.84 (3H, t, J=1.5 Hz), 1.89 (3H, s), 4.09 (1H, br d, J=11.0 Hz, 7-H), 4.93, 5.07 (each 1H, s, 19-H_2), 5.00 (1H, d, J=10.0 Hz, 3-H), 5.12 (1H, br t, J=7.0 Hz, 11-H), 5.61 (1H, dq, J=10.0, 1.5 Hz, 2-H). ^{13}C-NMR δ: C-1 (162.9), C-2 (78.9), C-3 (121.5), C-4 (143.7), C-5, 9, 13 (33.9, 36.0, 36.8), C-6, 9 (30.0, 32.2), C-7 (70.5), C-8 (154.5), C-11 (125.9), C-14 (26.3), C-15 (122.9), C-16 (174.8), C-17 (9.0), C-18, 20 (15.5, 16.0), C-19 (110.0). MS m/z: 316 (M^+), 298, 283, 270, 255. High-resolution MS [Found (Calcd)] m/z: $C_{20}H_{28}O_3$ (M^+), 316.2028 (316.2039).

16-Oxosarcophytonin E Acetate (3b) Oil, $[\alpha]_D^{20}$ +83° (c=0.84). ^{1}H-NMR δ: 1.66 (3H, s, 20-H_3), 1.83 (3H, t, J=1.5 Hz), 1.91 (3H, s), 2.07 (3H, s, OAc), 4.85 (1H, d, J=10.0 Hz, 3-H), 4.89, 4.92 (each 1H, br s, 19-H_2), 5.16 (1H, br t, J=6.5 Hz, 11-H), 5.26 (1H, br d, J=10.5 Hz, 7-H), 5.59 (1H, dq, J=10.0, 1.0 Hz, 2-H). MS m/z: 358 (M^+), 316, 298, 283. High-resolution MS [Found (Calcd)] m/z: $C_{22}H_{30}O_4$ (M^+), 358.2163 (358.2144).

***(S)*- and *(R)*-MTPA Esters of 16-Oxosarcophytonin (3c and 3d) and *(S)*-MTPA Ester of Sarcophytonin E (1c)** (a) A solution of **3a** (7 mg) in CH_2Cl_2 (0.5 ml) was treated at room temperature with (*S*)-MTPA (30 mg), dicyclohexylcarbodiimide (DCC, 30 mg) and dimethylaminopyridine (10 mg) for 2 h. The mixture was charged on a column of silica gel. Elution with ethyl acetate–hexane (2:8) gave the (*S*)-MTPA ester (**3c**, 7.4 mg) as an oil, $[\alpha]_D^{20}$ −8° (c=1.47). ^{1}H-NMR δ: 1.675 (3H, s, 20-H_3), 1.870 (6H, s, 17, 18-H_3), 2.77 (1H, ddd, J=14.5, 11.5, 7.5 Hz), 3.504 (3H, d, J=1.5 Hz), 4.712 (1H, d, J=10.0 Hz, 3-H), 4.892, 4.933 (each 3H, s, 19-H_2), 5.204 (1H, br dd, J=7.5, 6.5 Hz, 11-H), 5.382 (1H, br d, J=10.5 Hz, 7-H), 5.578 (1H, dq, J=10.0, 1.5 Hz, 2-H).

(b) Treatment of 6 mg of **3a** with (*R*)-MTPA according to the same procedure as in (a) gave the (*R*)-MTPA ester (**3d**, 6 mg). Oil, $[\alpha]_D^{20}$ +30° (c=1.25). ^{1}H-NMR δ: 1.678 (3H, s, 20-H_3), 1.846, 1.880 (each 3H, s, 17, 18-H_3), 2.730 (1H, m), 3.495 (1H, d, J=1.0 Hz), 4.621 (1H, d, J=10.0 Hz, 3-H), 4.830, 4.870 (each 1H, br s, 19-H_2), 5.183 (1H, br dd, J=7.0, 6.5 Hz, 11-H), 5.344 (1H, br d, J=10.5 Hz, 7-H), 5.567 (1H, dq, J=10.0, 1.5 Hz, 2-H).

(c) Compound **1a** (0.2 mg) prepared from **2a** as described in a previous report,[2] was treated with (*S*)-MTPA according to the same procedure as in (a) to afford 0.2 mg of the (*S*)-MTPA ester (**1c**) as an oil, $[\alpha]_D^{21}$ −10° (c=0.040; due to the low concentration of the sample, this specific rotation is not reliable). ^{1}H-NMR δ: 1.66, 1.67 (each 3H, br s, 17, 20-H_3), 1.80 (3H, br s, 18-H_3), 2.55 (1H, m), 3.53 (3H, d, J=1.0 Hz), 4.51 (2H, br s, 16-H_2), 4.89, 4.91 (each 1H, br s, 19-H_2), 4.94 (1H, d, J=10.0 Hz, 3-H), 5.14 (1H, m, 11-H), 5.40 (1H, br d, J=10.5 Hz, 7-H), 5.54 (1H, m, 2-H).

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Synthesis of Benzofuro[3,2-*f*]phthalazine Derivatives

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The reaction of hydrazine with dibenzofuran-1,2-dicarboxylic anhydrides followed by treatment with acetic acid resulted in the formation of 1,4-dioxo-1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazines. Dibenzofuran-1,2-dicarboxylic anhydrides were prepared by the Diels–Alder reaction of 2-alkenyl benzofurans and maleic anhydride followed by oxidative aromatization. Chlorination of 1,4-dioxo-1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazines and subsequent nucleophilic substitution afforded 1,4-dialkoxybenzofuro[3,2-*f*]phthalazines. These compounds were subjected to cytotoxicity assay against L1210 mice leukemia as analogues of methoxyellipticines.

Keywords dibenzofuran-1,2-dicarboxylic anhydride; cyclization; chlorination; nucleophilic substitution; 1,4-dialkoxybenzofuro[3,2-*f*]phthalazine; cytotoxicity

There has been great interest in the development of heterocyclic structures like pyridocarbazoles which possess marked antitumor activity. Thus, ellipticine and derivatives[1-8] exert their antitumor effects at a molecular level through the intercalation of deoxyribonucleic acid (DNA), as has been demonstrated in several studies,[9-16] and 9-hydroxy-2,5,11-trimethyl-6*H*-pyrido[4,3-*b*]carbazolium acetate (ellipticinium acetate) was used in the treatment of breast cancers.[17,18]

Therefore we have been most interested in developing new tetracyclic heterocycles, benzofuro[3,2-*f*]phthalazines, which consist of a dibenzofuran ring fused to a pyridazine ring, and we have studied their cytotoxic activity against L1210 leukemia in mice.

The key step in the benzofuro[3,2-*f*]phtalazine synthesis is the Diels–Alder addition of maleic anhydride to 2-alkenylbenzofurans. The 1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarboxylic anhydrides were obtained following the method described by Davidson and Elix.[19]

5-Methoxysalicylic aldehyde was converted by condensation with chloroacetone to 2-acetyl-5-methoxybenzofuran **1c** following the procedure of Stoermer used for the compound **1b**.[20] Compound **1a** was obtained by the reaction of dimethylformamide on 2-lithiobenzofuran.[21]

The carbonyl compounds **1** underwent the Wittig reaction with methyltriphenylphosphonium bromide to 2-alkenylbenzofurans **2**, *i.e.* 2-vinylbenzofuran **2a** and 2-isopropenylbenzofurans **2b**[22] and **2c**.

Since 2-acetyl-5-methoxybenzofuran **1c** is less reactive toward the Wittig reagent, the synthesis of 5-methoxy-2-isopropenylbenzofuran **2c** has proved difficult. We found, however, that the reaction was achieved after refluxing for 3 h and standing at room temperature for 16 h. If triphenylphosphinoxide was removed by distillation *in vacuo*, the final product **2c** was broken down, and it was necessary to subject the crude reaction product to chromatography on silica gel. Elution with dichloromethane afforded **2c** in good yield (84%).

The Diels–Alder reaction is applicable to the dienes like 2-alkenylbenzofurans **2** containing intra- and exocyclic double bonds, and the reaction with maleic anhydride gave 1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarboxylic anhydrides **3a**, **3b**[23] and **3c** in good yields.

Several attempts were made in order to obtain dioxo-octahydrobenzofurao[3,2-*f*]phthalazines by bimolecular cyclization between hydrazine hydrate and anhydrides **3**, but the reaction failed. The reaction products are 1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarbohydrazides like **5′**, as are suggested by their infrared (IR) spectra which show absorption bands between 3220 and 3320 cm^{-1} belonging to NH streching vibrations of hydrazide groups. The attempts to cyclize hydrazides **5′** either by refluxing in acetic acid or by heating failed too.

The aromatization reaction of tetrahydrodibenzofuran

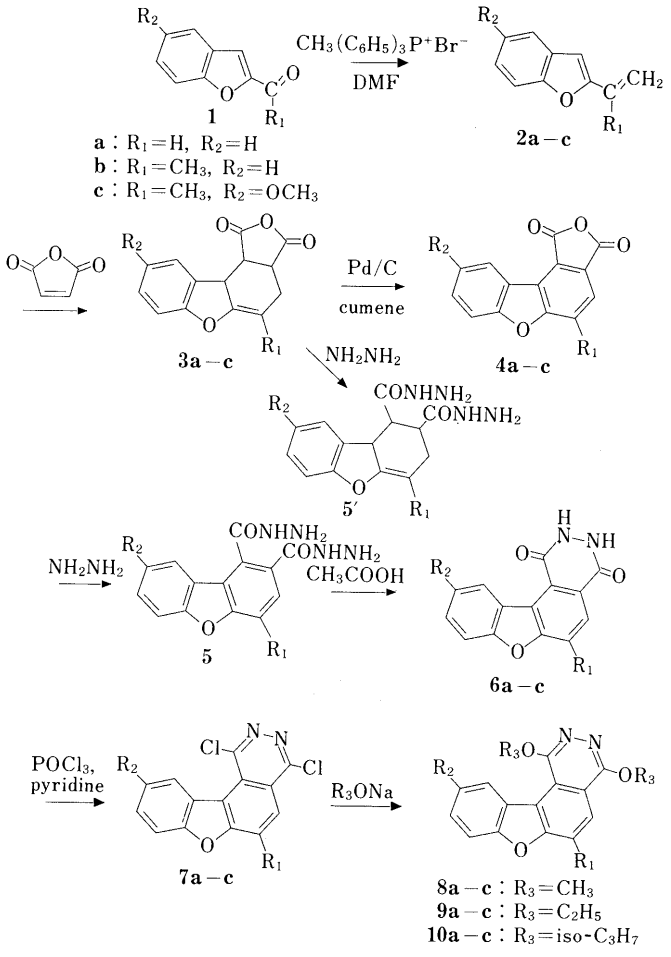

Chart 1

anhydrides **3** proceeded successfully after oxidization by refluxing for 10 h in cumene in the presence of 10% palladium on carbon. The bimolecular cyclization of dibenzofuran anhydrides **4** was realized in two steps. After refluxing in hydrazine hydrate, anhydrides **4** were converted to the respective dicarbohydrazide intermediates **5** which can be isolated by removing the reagent *in vacuo* and identified by IR spectra analysis. The IR spectra show absorptions between 3160 and 3350 cm^{-1} (NH bonds) and at 1650 cm^{-1} (CO bonds). The proton nuclear magnetic resonance (^{1}H-NMR) cannot be realized because of a lack of solubility. Intramolecular cyclization of the crude dicarbohydrazide intermediates was realized on refluxing in acetic acid to afford 1,4-dioxo-1,2,3,4-tetrahydrobenzofuro-[3,2-*f*]phthalazines **6**.

The dioxo derivatives **6** may be single tautomers, dilactim or dilactam structures, or equilibrium mixtures of lactam–lactim structures. The IR and ^{1}H-NMR spectra confirmed the dilactam tautomers. The IR spectra were recorded on a solid state (KBr disks) and revealed a very strong CO absorption band between 1650 and 1660 cm^{-1}, excluding the dilactim tautomer. The ^{1}H-NMR spectra showed a significant deshielded effect on the H11 protons relative to analogous H9 protons of the aromatic anhydrides **4** ($\Delta\delta = 0.7$ to 1.4 ppm). These results are consistent with the anisotropic influence of the carbonyl group C1, which induced the deshielding of H11 they are also consistent with the more spatial vicinity of the lactam CO and the H11 protons in diones **6** relative to the CO group and H9 protons in anhydrides **4**.

1,4-Dichlorobenzofurophthalazines **7** could be obtained after chlorination reactions of 1,4-dioxo,1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazines **6** with phosphoryl chloride in the presence of a catalytic amount of pyridine. The tautomeric equilibria of dioxotetrahydrobenzofurophthalazines **6** were displaced to the bis-lactim forms to realize dichlorination reactions after refluxing for at least 3 to 5 h. Since 1,4-dichlorobenzofurophthalazines **7** show very poor solubilities in usual solvents, we have synthesized 1,4-dialkoxybenzofurophthalazines with the intention of facilitating the biological tests on L1210 leukemia. Usually, the nucleophilic substitution reactions of chloropyridazines in which halogens are located on the α carbons near nitrogen atoms proceed readily. Surprisingly, the reactions of dichlorobenzofurophthalazines **7** with sodium alcoholates required considerably drastic conditions, namely, heating for 6 h at 150°C under pressure. Under these conditions, the reactions were carried to completion to produce in moderate yields 1,4-dimethoxybenzofurophthalazines **8**, 1,4-diethoxybenzofurophthalazines **9**, and 1,4-diisopropoxybenzofurophthalazines **10**.

The studies of the antitumor activity of the dioxo, dichloro and dialkoxy derivatives were done in the Institute of Cancerology and Immuno Genetic (Villejuif France) on L1210 mice leukemia. The tested derivatives did not show any activity, probably because of their low solubility.

Experimental

Melting points were determined on a Kofler apparatus and are uncorrected. IR spectra were recorded in KBr disks with a Perkin Elmer 257 spectrometer. NMR spectra were obtained with a Varian EM (90 MHz) using dimethyl sulfoxide-d_6 (DMSO-d_6). Chemical shifts and coupling constants (J) were measured in ppm (δ) and hertz (Hz) with respect to tetramethylsilane (TMS). The electron impact mass spectra (MS) were recorded on a JEOL D300 instrument.

2-Vinyl Benzofuran (2a) A mixture of 2-formyl benzofuran (**1a**) (10 g, 68 mmol) and methyltriphenylphosphonium bromide (32.8 g) in anhydrous ether (300 ml) and dimethylformamide (150 ml) was stirred for 4 h at room temperature under a nitrogen atmosphere after the addition of sodium ethoxide (12.6 g). The mixture was diluted with water (250 ml) and extracted with ether. The organic layer was washed with water, dried and concentrated *in vacuo*. The oil was distilled under reduced pressure (5 mmHg) to give the title compound (**2a**). (7 g, 70.6%). *Anal.* Calcd for $C_{10}H_8O$: C, 83.31; H, 5.59. Found: C, 83.07; H, 5.78. IR ν_{max}^{KBr} cm^{-1}: 1610, 1550 (C=C), 1450, 1285, 1255, 1200, 1025, 950, 815, 755.

2-Isopropenyl-5-methoxybenzofuran (2c) The mixture of 2-acetyl-6-methoxybenzofuran (**1c**) (19 g, 0.1 mol) and methyltriphenyl phosphonium bromide (53.6 g) in anhydrous ether (500 ml) and dimethylformamide (250 ml) was stirred under nitrogen. After the addition of sodium ethoxide (20.4 g), the solution was heated for 3 h at 65 °C, then stirred at room temperature for 20 h. The mixture was diluted with water (500 ml) and extracted with ether. The combined extracts were washed with water, dried and concentrated *in vacuo*. The residue was subjected to chromatography on silica gel using dichloromethane for elution to afford the title compound (**2c**) (15.5 g, 84%), mp 45—46 °C. *Anal.* Calcd for $C_{12}H_{12}O_2$: C, 76.57; H, 6.43; O, 17.00. Found: C, 76.27; H, 6.29; O, 16.68. IR ν_{max}^{KBr} cm^{-1}: 1610, 1595 (C=C), 1475, 1220, 1150, 1035, 910, 855, 815. ^{1}H-NMR δ: 2.05 (3H, CH_3), 3.78 (3H, OCH_3), 5.10—5.68 (2H, CH_2), 6.50 (2H, H6, H7), 6.60 (1H, H3), 7.27 (1H, H4).

8-Methoxy-4-methyl-1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarboxylic Anhydride (3c) A solution of **2c** (11 g, 58.8 mmol) and maleic anhydride (6 g, 61 mmol) in benzene (200 ml) was heated under reflux for 72 h. The solvent was evaporated under reduced pressure to give the title compound (**3c**) (9.1 g, 54%), mp 190—191 °C (ether). *Anal.* Calcd for $C_{16}H_{14}O_5$: C, 67.12; H, 4.93. Found: C, 66.88; H, 4.76. IR ν_{max}^{KBr} cm^{-1}: 1765, 1715 (C=O), 1485, 1205, 1155, 1120, 940, 820, 735. ^{1}H-NMR δ: 1.72 (3H, s, CH_3), 2.42 (2H, m, H3a, 3b), 3.63 (4H, m, OCH_3, H9b), 4.1 (2H, m, H1, 2), 6.78—6.90 (2H, d, J=8 Hz, H6,7), 7.10 (1H, d, J=2.5 Hz, H9).

Dibenzofuran-1,2-dicarboxylic Anhydride (4a) A mixture of 1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarboxylic anhydride (**3a**) (2.2 g, 9 mmol) in cumene was oxidized over 10% palladium on carbon (1.5 g) after refluxing for 10 h. The catalyst was removed by filtration and washed with boiling cumene. The filtrate was evaporated *in vacuo* and the precipitate was washed with ether to give the title compound (**4a**) (1 g, 46%), mp 225—226 °C (benzene). *Anal.* Calcd for $C_{14}H_6O_4$: C, 70.59; H, 2.54. Found: C, 70.46; H, 2.65. IR ν_{max}^{KBr} cm^{-1}: 1830, 1760 (C=O), 1580, 1340, 1300, 1220, 1170, 1140, 900, 785. ^{1}H-NMR δ: 7.80 (4H, m, H3, 4, 7,8), 8.18 (1H, dd, J=8, 2.5 Hz, H6), 8.60 (1H, dd, J=8, 2.5 Hz, H9).

4-Methyldibenzofuran-1,2-dicarboxylic Anhydride (4b) By use of the method described for the preparation of **4a**, a mixture of 4-methyl-1,2,3,9b-tetrahydrodibenzofuran-1,2-dicarboxylic anhydride (**3b**) (3 g, 11.7 mmol) with 10% palladium on carbon (2 g) in cumene was refluxed for 20 h to afford the title compound (**4b**) (1.6 g, 54%), mp >260 °C (benzene). *Anal.* Calcd for $C_{15}H_8O_4$: C, 71.43; H, 3.20. Found: C, 71.08; H, 3.21. IR ν_{max}^{KBr} cm^{-1}: 1830, 1770 (C=O), 1445, 1265, 1205, 1130, 1100, 880, 770, 755, 730.

8-Methoxy-4-methyldibenzofuran-1,2-dicarboxylic Anhydride (4c) By use of the method described for the preparation of **4a** a mixture of **3c** (3 g, 10.5 mmol) was refluxed in cumene with 10% palladium on carbon (2 g), for 10 h to give the compound (**4c**) (1.5 g, 50.7%), mp 225—226 °C (benzene). *Anal.* Calcd for $C_{16}H_{10}O_5$: C, 68.08; H, 3.57. Found: C, 68.17; H, 3.50. IR ν_{max}^{KBr} cm^{-1}: 1835, 1760 (C=O), 1600, 1485, 1280, 1200, 905, 750. ^{1}H-NMR δ: 2.57 (3H, s, CH_3), 3.76 (3H, s, OCH_3), 7.13 (1H, dd, J=8, 2.5 Hz, H7), 7.58 (1H, d, J=8 Hz, H6), 7.62 (1H, d, J=2.5 Hz, H9), 7.80 (1H, s, H3).

4-Methyl-1,2,3-9b-tetrahydrodibenzofuran-1,2-dicarbohydrazide (5'a) A solution of **3a** (1 g, 4 mmol) in hydrazine hydrate (100 ml) was refluxed for 2 h. The solvent was removed *in vacuo* and the residue was washed with water to afford the title compound (**5'a**) (0.9 g, 75%), mp >260 °C. *Anal.* Calcd for $C_{15}H_{20}N_4O_3$: C, 59.20; H, 6.62; N, 18.41. Found: C, 59.39; H, 6.55; N, 18.66. IR ν_{max}^{KBr} cm^{-1}: 3320, 3160 (NH), 1650 (C=O), 1280, 1260, 1180, 1000, 750.

1,4-Dioxo-1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazine (6a) A solution of **4a** (1.7 g, 7 mmol) in hydrazine hydrate (150 ml) was refluxed for 3 h. The solvent was removed *in vacuo* and the residue washed with ethanol. The precipitate was refluxed for 30 min in acetic acid (100 ml), then after cooling, filtered and washed with water to afford the title compound (**6a**) (1.1 g, 61%), mp 244—245 °C. *Anal.* Calcd for $C_{14}H_8N_2O_3$: C, 66.66; H, 3.20. Found: C, 66.36; H, 2.91. IR ν_{max}^{KBr} cm^{-1}: 3170 (NH), 1660 (C=O),

1500, 1455, 1330, 980, 750. ^{1}H-NMR δ: 7.25—7.75 (3H, m, H8, 9, 10), 8.13 (2H, s, H5, 6), 9.26 (1H, dd, J=8, 2.5 Hz, H11).

1,4-Dioxo-6-methyl-1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazine (6b) A solution of **4b** (5 g, 20 mmol) was refluxed for 1.5 h in hydrazine hydrate (250 ml), then the precipitate was refluxed for 1 h in acetic acid as described previously to give the title compound (**6b**) (4.7 g, 89%), mp >260 °C. *Anal.* Calcd for $C_{15}H_{10}N_2O_3$: C, 67.66; H, 3.79; N, 10.52. Found: C, 67.55; H, 3.77; N, 10.45. IR ν_{max}^{KBr} cm^{-1}: 3150 (NH), 1650 (C=O), 1500, 1330, 1220, 1100, 875, 810, 750. ^{1}H-NMR δ: 2.66 (3H, s, CH_3), 6.97—7.90 (4H, m, H5, 8, 9, 10), 9.26 (1H, d, J=8 Hz, H11).

10-Methoxy-6-methyl-1,4-dioxo-1,2,3,4-tetrahydrobenzofuro[3,2-*f*]phthalazine (6c) By use of the method described for the preparation of **6a**, **4c** (3 g, 10 mmol) was refluxed for 4 h in hydrazine hydrate (200 ml), then the residue was refluxed for 4 h in acetic acid to afford the title compound (**6c**) (2 g, 63.5%), mp >260 °C. *Anal.* Calcd for $C_{16}H_{12}N_2O_4$: C, 64.86; H, 4.08; N, 9.46. Found: C, 64.90; H, 4.22; N, 9.21. IR ν_{max}^{KBr} cm^{-1}: 3240, 3130 (NH), 1650 (C=O), 1450, 1330, 1200, 1050, 890, 820. ^{1}H-NMR δ: 2.77 (3H, s, CH_3), 3.93 (3H, s, OCH_3), 7.18 (1H, dd, J=8, 2.5 Hz, H9), 7.68 (1H, d, J=8 Hz, H8), 8.00 (1H, s, H5), 8.98 (1H, d, J=2.5 Hz, H11).

1,4-Dichlorobenzofuro[3,2-*f*]phthalazine (7a) In a solution of phosphoryl chloride (120 ml) and pyridine (1 ml), **6a** (1 g, 3.9 mmol) was added, then the mixture was refluxed for 3 h. After concentration under reduced pressure, the residue was diluted with water and made alkaline with an ammonium hydroxide solution, then filtered and washed with water to afford the title compound (**7a**) (0.5 g, 44%), mp 208—209 °C (acetone). *Anal.* Calcd for $C_{14}H_6Cl_2N_2O$: C, 58.15; H, 2.09; Cl, 24.25. Found: C, 58.06; H, 2.22; Cl, 24.36. IR ν_{max}^{KBr} cm^{-1}: 1620, 1575 (C=C, C=N), 1460, 1440, 1370, 1335, 1260, 1230, 850. MS *m/z*: 292 (11), 290 (67), 288 (M^+, 100%), 262 (7), 260 (11), 218 (39), 197 (10).

1,4-Dichloro-6-methylbenzofuro[3,2-*f*]phthalazine (7b) By the use of the method described for the preparation of **7a**, a solution of phosphoryl chloride (120 ml) and pyridine (10 ml) was refluxed for 3 h with **6b** (1 g, 3.7 mmol) to give the title compound (**7b**) (0.8 g, 57%), mp 242—243 °C (acetone). *Anal.* Calcd for $C_{15}H_8Cl_2N_2O$: C, 59.43; H, 2.60; N, 9.24; Cl, 23.39. Found: C, 59.22; H, 2.61; N, 9.42; Cl, 23.32. IR ν_{max}^{KBr} cm^{-1}: 1610, 1595 (C=C, C=N), 1360, 1330, 1180, 1160, 760. MS *m/z*: 302 (M^+, 100%), 273 (5), 239 (16), 232 (33), 231 (19), 206 (7), 203 (8), 176 (13).

1,4-Dichloro-10-methoxy-6-methylbenzofuro[3,2-*f*]phthalazine (7c) Compound **6c** (1 g, 3.4 mmol) was refluxed for 5 h in a mixture of phosphoryl chloride (100 ml) and pyridine (1 ml), as already described for the compound **7a**, to give the title compound (**7c**) (0.65 g, 58%), mp 222—223 °C (acetone). *Anal.* Calcd for $C_{16}H_{10}Cl_2N_2O_2$: C, 57.66; H, 3.02; N, 8.44; Cl, 21.27. Found: C, 57.80; H, 3.09; N, 8.38; Cl, 21.22. IR ν_{max}^{KBr} cm^{-1}: 1615, 1595, 1560 (C=C, C=N), 1480, 1435, 1295, 1210, 1165, 850, 825, 775. MS *m/z*: 332 (M^+, 100%), 317 (19), 289 (11), 262 (19), 254 (19), 247 (20), 219 (24).

General Procedure for the Synthesis of 1,4-Dialkoxybenzofurophthalazines In a sealed tube, the 1,4-dichlorobenzofurophthalazine was heated with alcohol and sodium alkoxide at 160 °C for 6 h. After cooling, the solvent was removed *in vacuo* and the residue was diluted with water and washed to give the 1,4-dialkoxy derivative.

1,4-Dimethoxybenzofuro[3,2-*f*]phthalazine (8a) Compound **7a** (1 g, 3.3 mmol), MeOH (300 ml) and sodium methoxide (1.4 g, 26 mmol) gave the title compound (**8a**) (0.5 g, 52%), mp 188—189 °C (MeOH). *Anal.* Calcd for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 10.00. Found: C, 68.45; H, 4.36; N, 10.04. IR ν_{max}^{KBr} cm^{-1}: 2980, 2950 (CH_3), 1590, 1545 (C=C, C=N), 1460, 1365, 1235, 1070, 770, 760. ^{1}H-NMR δ: 4.10 (3H, s, OCH_3), 4.23 (3H, s, OCH_3), 7.33—7.80 (3H, m, H8—10), 8.15 (2H, s, H5, 6), 8.62 (1H, dd, J=8, 2.5 Hz, H11).

1,4-Dimethoxy-6-methylbenzofuro[3,2-*f*]phthalazine (8b) Compound **7b** (1 g, 3.3 mmol), MeOH (300 ml) and sodium methoxide (1.4 g, 26 mmol) gave the title compound (**8b**) (0.55 g, 57%), mp 228—229 °C (MeOH). *Anal.* Calcd for $C_{17}H_{14}N_2O_3$: C, 69.37; H, 4.80; N, 9.52. Found: C, 69.17. H, 4.91; N, 9.54. IR ν_{max}^{KBr} cm^{-1}: 2950 (CH_3), 1650, 1590, 1550 (C=C, C=N), 1460, 1380, 1240, 1000, 755. MS *m/z*: 294 (M^+, 100%), 293 (72), 265 (42), 234 (16), 233 (21), 208 (13), 180 (25). ^{1}H-NMR δ: 2.60 (3H, s, CH_3), 4.03 (3H, s, OCH_3), 4.15 (3H, s, OCH_3), 7.3—7.83 (4H, m, H5, 8, 9, 10), 8.45 (1H, dd, J=8, 2.5 Hz, H11).

1,4,10-Trimethoxy-6-methylbenzofuro[3,2-*f*]phthalazine (8c) Compound **7c** (1 g, 3 mmol), MeOH (300 ml) and sodium methoxide (1.4 g, 26 mmol) afforded the title compound (**8c**) (0.5 g, 51%), mp 195—196 °C (MeOH). *Anal.* Calcd for $C_{18}H_{16}N_2O_4$: C, 66.64; H, 4.97; N, 8.64. Found: C, 66.52; H, 5.04; N, 8.78. IR ν_{max}^{KBr} cm^{-1}: 2950 (CH_3), 1590, 1550 (C=C, C=N), 1400, 1250, 1230, 1205, 1020, 800, 700. MS *m/z*: 324 (M^+, 100%), 309 (62), 295 (31), 293 (23), 281 (14), 263 (11), 210 (18), 195 (14). ^{1}H-NMR δ: 2.33 (3H, s, CH_3), 3.68 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 6.87 (1H, dd, J=8, 2.5 Hz, H9), 7.32 (1H, d, J=8 Hz, H8), 7.37 (1H, s, H5), 7.43 (1H, d, J=2.5 Hz, H11).

1,4-Diethoxybenzofuro[3,2-*f*]phthalazine (9a) From **7a** (1 g, 3.3 mmol), EtOH (300 ml) and sodium ethoxide (1.77 g, 26 mmol) we obtained the title compound (**9a**) (0.5 g, 46.7%), mp 158—159 °C (EtOH). *Anal.* Calcd for $C_{18}H_{16}N_2O_3$: C, 70.11; H, 5.23; N, 9.08. Found: C, 70.00; H, 5.19; N, 9.04. IR ν_{max}^{KBr} cm^{-1}: 2980, 2930 (CH_3), 1585, 1545 (C=C, C=N), 1420, 1370, 1340, 1235, 1150, 1070, 920, 810, 690. ^{1}H-NMR δ: 1.42 (3H, t, J=6 Hz, CH_3), 1.58 (3H, t, J=6 Hz, CH_3), 4.47 (2H, q, J=6 Hz, OCH_2), 4.63 (2H, q, J=6 Hz, OCH_2), 7.28—7.77 (3H, m, H8, 9, 10), 8.11 (2H, s, H5, 6), 8.72 (1H, dd, J=8, 2.5 Hz, H11).

1,4-Diethoxy-6-methylbenzofuro[3,2-*f*]phthalazine (9b) Compound **7b** (1 g, 3.3 mmol), EtOH (300 ml) and sodium ethoxide (1.77 g, 26 mmol) gave the title compound (**9b**) (0.55 g, 52%), mp 220—221 °C (EtOH). *Anal.* Calcd for $C_{19}H_{18}N_2O_3$: C, 70.79; H, 5.63; N, 8.69. Found: C, 71.04; H, 5.86; N, 8.40. IR ν_{max}^{KBr} cm^{-1}: 2950 (CH_3), 1610, 1550 (C=C, C=N), 1415, 1380, 1340, 1325, 1235, 1020, 920, 775. MS *m/z*: 350 (M^+, 17), 335 (3), 308 (17), 293 (9), 266 (100), 251 (8), 250 (30), 234 (22), 233 (13), 208 (40), 180 (30).

1,4-Diethoxy-10-methoxy-6-methylbenzofuro[3,2-*f*]phthalazine (9c) Compound **7c** (1 g, 3 mmol), EtOH (300 ml) and sodium ethoxide (1.77 g, 26 mmol) afforded the title compound (**9c**) (0.6 g, 56%), mp 190—191 °C (EtOH). *Anal.* Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.02; H, 5.62; N, 7.92. IR ν_{max}^{KBr} cm^{-1}: 2990, 2960 (CH_3), 1615, 1590, 1555 (C=C, C=N), 1470, 1435, 1380, 1340, 1230, 1205, 1040, 925, 790. MS *m/z*: 352 (M^+, 36%), 337 (93), 324 (44), 309 (31), 296 (30), 281 (12), 210 (17). ^{1}H-NMR δ: 1.36 (6H, t, J=6 Hz, CH_3), 2.48 (3H, s, CH_3), 3.68 (3H, s, OCH_3), 4.33 (4H, q, J=6 Hz, OCH_2), 6.90 (1H, dd, J=8, 2.5 Hz, H9), 7.40 (1H, d, J=8 Hz, H8), 7.60 (1H, s, H5), 7.83 (1H, d, J=2.5 Hz, H11).

1,4-Diisopropoxybenzofuro[3,2-*f*]phthalazine (10a) Compound **7a** (1 g, 3.3 mmol), propan-2-ol (300 ml) and sodium isopropoxide (2.14 g, 26 mmol) gave the title compound (**10a**) (0.5 g, 42.8%), mp 88—89 °C (iso-PrOH). *Anal.* Calcd for $C_{20}H_{20}N_2O_3$: C, 71.41; H, 5.99; N, 14.27. Found: C, 71.60; H, 6.15; N, 14.37. IR ν_{max}^{KBr} cm^{-1}: 2980, 2940 (CH_3), 1590, 1545 (C=C, C=N), 1450, 1320, 1235, 1115, 830, 750. ^{1}H-NMR δ: 1.48 (6H, d, J=6 Hz, CH_3), 1.61 (6H, d, J=6 Hz, CH_3), 5.47 (1H, m, J=6 Hz, OCH), 5.69 (1H, m, J=6 Hz, OCH), 7.33—7.78 (3H, m, H8, 9, 10), 8.15 (2H, s, H5, 6), 8.85 (1H, dd, J=8, 2.5 Hz, H11).

1,4-Diisopropoxy-6-methylbenzofuro[3,2-*f*]phthalazine (10b) Compound **7b** (1 g, 3.3 mmol), propan-2-ol (300 ml) and sodium isopropoxide (2.14 g, 26 mmol) gave the title compound (**10b**) (0.65 g, 56%), mp 150—151 °C (iso-PrOH). *Anal.* Calcd for $C_{21}H_{22}N_2O_3$: C, 71.98; H, 6.33; N, 8.00. Found: C, 71.68; H, 6.17; N, 7.94. IR ν_{max}^{KBr} cm^{-1}: 2980, 2930 (CH_3), 1615, 1585, 1555 (C=C, C=N), 1410, 1315, 1110, 945, 740. MS *m/z*: 350 (M^+, 18), 335 (29), 308 (18), 293 (9), 267 (20), 266 (100), 251 (8), 250 (30), 208 (36), 180 (27). ^{1}H-NMR δ: 1.42 (6H, d, J=6 Hz, CH_3), 1.53 (6H, d, J=6 Hz, CH_3), 2.60 (3H, s, CH_3), 5.42 (1H, m, J=6 Hz, OCH), 5.62 (1H, m, J=6 Hz, OCH), 7.25—7.68 (3H, m, H8, 9, 10), 7.83 (1H, s, H5).

1,4-Diisopropoxy-10-methoxy-6-methylbenzofuro[3,2-*f*]phthalazine (10c) Compound **7c** (1 g, 3 mmol), propan-2-ol (300 ml) and sodium isopropoxide (2.14 g, 26 mmol) afforded the title compound (**10c**) (0.55 g, 48%), mp 128—129 °C (iso-PrOH). *Anal.* Calcd for $C_{22}H_{24}N_2O_4$: C, 69,45; H, 6.36; N, 7.36. Found: C, 69.40; H, 6.39; N, 7.30. IR ν_{max}^{KBr} cm^{-1}: 2975, 2935 (CH_3), 1590, 1550, (C=C, C=N), 1470, 1410, 1320, 1230, 1185, 1100, 1035, 935, 795. MS *m/z*: 380 (M^+, 25), 365 (9), 338 (20), 323 (7), 297 (20), 296 (96), 281 (15), 253 (6), 238 (7), 210 (14). ^{1}H-NMR δ: 1.43 (6H, d, J=6 Hz, CH_3), 1.56 (6H, d, J=6 Hz, CH_3), 2.65 (3H, s, CH_3), 3.87 (3H, s, OCH_3), 5.48 (1H, m, J=6 Hz, OCH), 5.75 (1H, m, J=6 Hz, OCH), 7.14 (1H, dd, J=8, 2.5 Hz, H9), 7.65 (1H, d, J=8 Hz, H8), 7.92 (1H, s, H5), 8.25 (1H, d, J=2.5 Hz, H11).

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Syntheses and Biological Activities of Endothelin-1 Analogs

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Endothelin-1 analogs replaced by various amino acids at position 21, namely [X^{21}]-ET-1, were synthesized, and their agonistic vasoconstrictor activity on rat thoracic aortic strips and receptor binding activity on rat brain membrane fraction were examined to elucidate their structure-activity relationship. The vasoconstrictor activities of [Tyr^{21}]- and [Phe^{21}]-ET-1 were one order of magnitude smaller than that of ET-1, and those of [His^{21}]-, [Gly^{21}]-, [Ser^{21}]-, [Ala^{21}]- and [Lys^{21}]-ET-1 were more than two orders of magnitude smaller than that of ET-1. On the other hand, the replacements by Ile, Glu, Gln and Pro resulted in distinguished losses of the vasoconstrictor activities. In addition, preincubation with these analogs did not blunt ET-1-induced vasoconstriction and showed no antagonistic activity. The binding inhibitory activities of these analogs against ^{125}I-ET-1 were approximately conformable to the vasoconstrictor activities with only a slight exception. These findings demonstrate that the phenyl group at position 21 is important for both the vasoconstrictor activity and the receptor binding activity.

Keywords endothelin-1 analog; solid phase peptide synthesis; biological activity; vasoconstriction; receptor binding

Introduction

Endothelin (ET)-1, an endothelium-derived vasoconstrictor peptide,[1] is composed of 21 amino acid residues with two intramolecular disulfide linkages, and provokes a potent and sustaining constriction of various smooth muscles, not only in blood vessels but in other organs, from a variety of species.[2] The magnitudes of ET-1 induced constriction dependent on extra- and intracellular Ca^{2+}. ET-1 receptors were found in vascular smooth muscle cell and membrane fragments of other tissues of various species.[3] To date three ETs, ET-1, -2 and -3, have been reported[4] and the classification of the receptors into subclasses by specificities against ET-1, ET-2 and ET-3 were reported.[5]

Synthesis and disulfide structure determination of ET-1 were reported by Kumagaye *et al.*[6] Moreover, sarafotoxin S-6b,[7] and VIC[8] were shown to have similar vasoconstrictor activity with a high degree of homology to the ET family. Recently, relationships of structure–vasoconstrictor activity and structure–receptor binding activity of ET-related peptides were reported by several groups[9-12] and it has been inferred that C-terminal Trp as well as the loop structure linked by the disulfide bonds of ET-1 molecule is essential for these activities.

We synthesized ET-1 analogs, namely [X^{21}]-ET-1, and examined their biological activities using rat aortic strip and rat brain membrane fraction.

Materials and Methods

All chemicals were purchased from Applied Biosystems Inc.

Carboxypeptidase Y was purchased from Boehringer Mannheim GmbH, Mannheim. Solid phase peptide synthesis was carried out on Applied Biosystems Model 430 A peptide synthesizer (software version 1.40 for the symmetric anhydride procedure) with 500 μmol of Boc-X-phenylacetamidomethyl (PAM)-resin as the solid support. Reverse phase high performance liquid chromatography (RP-HPLC) was performed on Shimazu liquid chromatograph Model LC-9A for an analitical scale and LC-8A for a preparative scale. Amino acid analysis was carried out on a Beckman amino acid analyzer Model 6300E. The constriction was measured isometrically with a force displacement transducer (Model T-30-240, Orientec) coupled to a polygraph (Model Rectigraph-8K, NEC San-ei) through an amplifier (Model RPM-6004, Nihonkohden). Radioactivities were counted by Aloka autowell gamma system ARC-251.

Solid phase peptide synthesis was carried out with 2 mmol of Boc-L-amino acids and side-chain protecting groups used were as follows; (Cys(MBzl), Ser(Bzl), Asp(OBzl), Lys(ClZ), Glu(OBzl), Tyr(Brz), His(DNP) and Trp(For)).

The final protected peptide-resin *ca.* 600 mg was treated with 1 ml of thiophenol and 14 ml of *N,N*-dimethylformamide (DMF) for 3 h at room temperature (RT). The resultant peptide-resin was filtered and washed with DMF (5 ml × 3) and CH_2Cl_2 (5 ml × 3). It was treated with 0.12 ml of ethanedithiol, 0.48 ml of *m*-cresol, 1.8 ml of dimethylsulfide, 3 ml of trifluoroacetic acid (TFA), and 0.6 ml of trifluoromethansulfonic acid (TFMSA) for 3 h at −2 °C and collected by filtration. After washing with DMF (5 ml × 3) and CH_2Cl_2 (5 ml × 5), the peptide was cleaved from the resin with 0.36 ml of thioanisol, 0.18 ml of ethanedithiol, 0.18 ml of dimethyl sulfide, 0.72 ml of *m*-cresol, 3.42 ml of TFA, and 1.16 ml of trimethylsylyl trifluoromethanesulfonate (TMSOTf) for 2 h at 0 °C.

Cold ether was added to precipitate the peptide, and collected by filtration. After washing with ether, the solid was dissolved in 15 ml of TFA, and filtered into the cold ether for the second precipitation. The precipitate was collected by filtration and washed with ether. The solid was subjected to air oxidation in 800 ml of 0.2 M ammonium trifluoroacetate solution, pH 7.50 containing 5 M guanidine hydrochloride at RT for 2 d.

The solution was adsorbed to a column of 10 g of YMC-ODS, washed with 0.1% aq. TFA, eluted with 80% aq. CH_3CN containing 0.1% TFA, and then purified repeatedly ten times by preparative RP-HPLC on a Waters μBondasphere column (19 × 150 mm) with a linear gradient of aq. CH_3CN (27—34%) containing 0.1% TFA for 60 min at a flow rate of 10 ml/min. The fractions containing desired peptides were collected, evaporated, and lyophylized to give a colorless powder.

Homogeneity of the final product was confirmed by RP-HPLC on a μBondasphere column (3.9 × 150 mm) with the same linear gradient at a flow rate of 0.6 ml/min for 40 min and by amino acid analysis after hydrolysis of the peptide with 6 N HCl in an evacuated and sealed tube at 110 °C for 24 h.

The type of S–S linkage (Cys^1–Cys^{15}, Cys^3–Cys^{11}) was determined by comparing the elution HPLC profile of carboxypeptidase-digeste with that of the standard ET-1, (ET(1-16)).

Bioassay of Agonistic Vasoconstrictor Activities Thoracic aortae were isolated from Wistar male rats (300 to 350 g) killed by decapitation. Adherent fat and connective tissues were removed from the vessels, which were cut into 3 mm ring segments. The ring preparations were mounted between stainless hooks in a 3 ml organ bath filled with Klebs–Henseleit solution (113 mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.5 mM glucose) maintained at 37 °C and aerated with 95% O_2–5% CO_2, and then equilibrated at 2 g of passive tension for one hour. The constriction was measured isometrically with a force displacement transducer coupled to a polygraph through an amplifier. Constrictions in 80 mM KCl were measured as an increase in isometric tension at 30 min intervals until a steady contractile response was obtained. After washing, the rings were exposed to various concentrations (4×10^{-5} to 4×10^{-9} M) of the

synthetic ET-1 analogs, which were added to the bath cumulatively at 10 to 20 min intervals after a contractile plateau was observed at each dose. The resultant constriction responses were expressed as a percentage of the response to 80 mM KCl. The analogs were dissolved in phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T) at various concentrations. Aliquots of those 1 mg/ml solution were stored at −20 °C in Nunc tubes and used as needed without re-freezing ($n=3$—5).

Bioassay of Antagonistic Activities against ET-1-Induced Vasoconstriction Approximate maximum concentrations of each synthetic analog, which showed no significant effects, were determined. Namely, those of three analogs containing 21-aromatic amino acid residue, [Tyr^{21}]-, [Phe^{21}]- and [His^{21}]-ET-1, were 10, 10 and 100 ng/ml, respectively. On the contrary, those of the following eight analogs were 1 μg/ml (in case of [Gly^{21}]-, [Ser^{21}]-, [Ala^{21}]- and [Lys^{21}]-ET-1) and 10 μg/ml (in case of [Pro^{21}]-, [Ile^{21}]-, [Gln^{21}]- and [Glu^{21}]-ET-1). The bioassay was performed similarly by using isolated rat aortic rings. After preincubation with the preceding cocentrations of those analogs for 10 min, 10 ng/ml of ET-1 was added. The resultant constrictions were compared statistically with ET-1-induced (10 ng/ml) one ($n=3$—5).

ET-1 Receptor Binding Assays Brains were obtained from Wistar male rats killed by decapitation. Tissues were homogenized in a 10-fold volume of 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose, 3 mM ethylenediamine tetraacetic acid (EDTA), 5 U/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF). After centrifugation at 1000 × *g* for 20 min, the resultant supernatant was further centrifuged at 25000 × *g* for 40 min. The pellet was resuspended in 50 mM Tris–HCl, pH 7.4 containing 5 U/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 0.1 mM *p*-APMSF at a final concentration of 5 mg/ml protein and stored as aliquots at −40 °C. Aliquots (7.5 μl) of brain membrane homogenates were incubated at 25 °C for 90 min with 5.5×10^{-11} M ^{125}I-ET-1 and various concentrations of [X^{21}]-ET in 50 mM Tris–HCl, pH 7.4 containing 0.5% bovine serum albumin (BSA) [buffer B] (total volume 200 μl). Reactions were terminated by the addition of 2 ml of ice cold buffer B and filtrated *in vacuo* through HA filters (pore size 0.45 μm: Millipore). Non-specific binding was determined in the presence of 4×10^{-7} M non-radioactive ET-1. The filters were washed twice with 5 ml of buffer B and their radioactivities were counted by a gamma counter.

Results and Discussion

In light of some recent studies of the structure–vasoconstrictor activity relationship of ET-1 related peptides, it can be inferred that C-terminal Trp^{21} is essential for them to exert the activity. However, as to position 21 of the ET-1 analogs, the vasoconstrictor activities linked with the receptor binding activities of the ET-1 analogs systematically replaced by various amino acids have not been reported.

[X^{21}]-ET-1 was synthesized by the Boc-based solid phase method to examine its biological activities. After treatment with thiophenol, TFMSA,[13] TMSOTf,[14] the fully deprotected tetrahydro-[X^{21}]-ET-1 was subjected to air oxidation. Elution profiles of the products by RP-HPLC showed two major peaks, linked by different disulfide linkages, which were separated with a linear gradient of 27—34% aq. CH_3CN containing 0.1% TFA.

[Cys^{1-11}, Cys^{3-15}]-peptides (type-B) were eluted earlier than [Cys^{1-15}, Cys^{3-11}]-peptides (type-A) in all of these peptides, in a ratio of approximately 1 : 3 (type B/type A), and the formation of [Cys^{1-3}, Cys^{11-15}]-peptides were almost negligible. The types of intrachain S–S linkages were determined by comparing the RP-HPLC profile of carboxypeptidase Y digest of these peptides with that of the standard ET-1, namely ET-1(1—16) linked at positions 1—15 and 3—11 by the method of Kimura.[8] Amino acid ratios in acid hydrolysates of these peptides are shown in Table I.

The dose–response relationship of the vasoconstrictor effects were shown in Fig. 1. In many cases, these analogs-induced maximum tensions were comparable to that of ET-1. An exception was the [Tyr^{21}]-ET-1 induced one which was approximately 40% of the native ET-1 induced one.

The estimated 50% effective concentrations (EC_{50}) of these analogs are shown in Table II. Compared with the vasoconstrictor activity of ET-1, those of [Tyr^{21}]- and [Phe^{21}]-ET-1 were one order of magnitude smaller, and these findings corresponded to the result by Nakajima *et al.*[11] That of [His^{21}]-ET-1 was two orders of magnitude smaller. On the contrary, those of [Gly^{21}]-, [Ser^{21}]-, [Ala^{21}]-, [Lys^{21}]-ET-1, and [Cys^{1}–Cys^{11}, Cys^{3}–Cys^{15}]-ET-1 (type-B) were three orders of magnitude smaller, and those of [Pro^{21}]-, [Ile^{21}]-, [Glu^{21}]- and [Gln^{21}]-ET-1 were more than three orders of magnitude smaller.

In addition, preincubation with the following concentrations of these synthetic ET-1 analogs did not blunt the ET-1 (10 ng/ml)-induced vasoconstriction significantly in the Student's *t*-test. ($p<0.05$, $n=3$ to 5) (data are not shown); [Tyr^{21}]- and [Phe^{21}]- (each 10 ng/ml); [His^{21}]- (100 ng/ml); [Gly^{21}]-, [Ser^{21}]-, [Ala^{21}]- and [Lys^{21}]- (each 1 μg/ml); and [Pro^{21}]-, [Ile^{21}]-, [Glu^{21}]- and [Gln^{21}]-ET-1 (each 10 μg/ml).

TABLE I. Amino Acid Ratios in Acid Hydrolysates of ET-1 Analogs ([X^{21}]-ET-1)

X	Trp	Ile	Phe	Lys	Tyr	Pro	Glu	Ser	Gly	Gln	His	Ala
Asp	2.03 (2)	1.95 (2)	2.20 (2)	2.00 (1)	2.03 (2)	2.07 (2)	2.00 (2)	2.00 (2)	2.03 (2)	2.04 (1)	2.05 (2)	2.00 (2)
Ser	2.83 (3)	2.75 (3)	3.10 (3)	2.71 (3)	2.75 (3)	2.78 (3)	2.83 (3)	3.20 (3)	2.71 (3)	2.79 (3)	2.72 (3)	2.71 (3)
Glu	1.13 (1)	1.04 (1)	1.11 (1)	1.08 (1)	1.03 (1)	1.10 (1)	2.14 (1)	1.08 (1)	1.07 (1)	2.07 (1)	1.12 (1)	1.13 (1)
Gly									1.07 (1)			1.08 (1)
Ala												3.46 (4)
Cys	2.72 (4)	3.14 (4)	2.92 (4)	3.41 (4)	2.87 (4)	3.40 (4)	3.10 (4)	3.44 (4)	3.30 (4)	2.77 (4)	3.35 (4)	0.96 (1)
Val	1.03 (1)	0.90 (1)	0.91 (1)	1.00 (1)	0.94 (1)	0.99 (1)	0.92 (1)	0.98 (1)	0.98 (1)	0.88 (1)	0.97 (1)	1.01 (1)
Met	1.00 (1)	0.97 (1)	1.01 (1)	1.03 (1)	0.89 (1)	1.02 (1)	0.89 (1)	1.03 (1)	1.02 (1)	0.91 (1)	1.01 (1)	1.90 (2)[a]
Ile	1.96 (2)[a]	2.97 (3)[a]	2.03 (2)[a]	1.89 (2)[a]	1.92 (2)[a]	1.99 (2)[a]	1.92 (2)[a]	1.93 (2)[a]	1.93 (2)[a]	1.95 (2)[a]	1.92 (2)[a]	2.18 (2)
Leu	1.94 (2)	2.04 (2)	2.16 (2)	2.16 (2)	2.04 (2)	2.08 (2)	2.13 (2)	2.10 (2)	2.09 (2)	2.07 (2)	2.09 (2)	0.85 (1)
Tyr	1.01 (1)	0.84 (1)	0.78 (1)	0.95 (1)	1.77 (2)	0.94 (1)	0.88 (1)	1.76 (2)	0.87 (1)	0.78 (1)	0.88 (1)	1.06 (1)
Phe	1.05 (1)	0.99 (1)	2.06 (2)	1.06 (1)	1.00 (1)	1.06 (1)	1.08 (1)	1.00 (1)	1.07 (1)	1.10 (1)	1.08 (1)	1.16 (1)
His	1.00 (1)	0.79 (1)	0.88 (1)	1.03 (1)	1.06 (1)	1.91 (1)	0.88 (1)	1.06 (1)	1.01 (1)	1.00 (1)	1.94 (2)	0.87 (1)
Lys	1.00 (1)	1.00 (1)	1.00 (1)	1.89 (2)	1.00 (1)	1.00 (1)	0.94 (1)	1.00 (1)	1.00 (1)	0.95 (1)	1.00 (1)	
Trp	0.99 (1)[b]											

Residues per molecule from the sequence in parentheses are shown. Acid hydrolysis was performed with 5.7 M HCl at 110 °C for 24 h in the presence of 0.2% phenol. *a*) Obtained from 72 h hydrolysis. *b*) Obtained from 24 h hydrolysis with 4 M methanesulfonic acid.

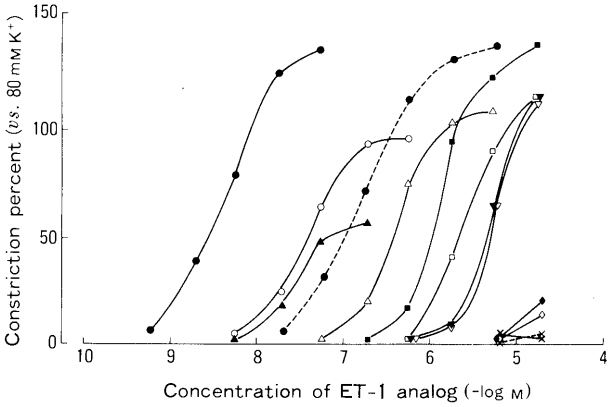

Fig. 1. The Dose–Response Relationship of ET-1 Analogs on Rat Thoracic Aorta

The tension developed by ET-1 analogs was expressed as a percent of the maximum response to 80 mM K^+. Data show the dose-response curve for ET-1 (—●—), ET-1 type B (---●---), Phe^{21}-ET-1 (—○—), Tyr^{21}-ET-1 (—▲—), His^{21}-ET-1 (—△—), Gly^{21}-ET-1 (—■—), Ser^{21}-ET-1 (—□—), Lys^{21}-ET-1 (—▼—), Ala^{21}-ET-1 (—▽—), Ile^{21}-ET-1 (—◆—), Glu^{21}-ET-1 (—◇—), Pro^{21}-ET-1 (—×—) and Gln^{21}-ET-1 (---×---).

TABLE II. Vasoconstrictor Activities and Receptor Binding Activities of ET-1 Analogs ([X^{21}]-ET-1)

X	Constriction EC_{50} (M)	Binding IC_{50} (M)
Trp	4.9×10^{-9}	1.8×10^{-10}
Tyr	2.8×10^{-8}	5.6×10^{-10}
Phe	4.1×10^{-8}	3.5×10^{-9}
His	4.0×10^{-7}	2.8×10^{-9}
Type B	1.9×10^{-7}	3.0×10^{-9}
Gly	1.4×10^{-6}	5.0×10^{-9}
Ser	3.2×10^{-6}	2.0×10^{-9}
Ala	4.9×10^{-6}	8.9×10^{-8}
Lys	5.2×10^{-6}	2.8×10^{-7}
Ile	$>2.0\times10^{-5}$	3.5×10^{-9}
Pro	$>2.0\times10^{-5}$	1.1×10^{-6}
Glu	$>2.0\times10^{-5}$	5.6×10^{-8}
Gln	$>2.0\times10^{-5}$	$>1.0\times10^{-5}$

Radioligand binding studies of ET-1 and [X^{21}]-ET-1 analogs were by the method of Ambar *et al.*,[15] and were shown in Fig. 2. The estimated concentration of half maximal inhibition (IC_{50}) of ET-1 and these analogs are shown in Table II. [Tyr^{21}]-ET-1 was less potent and [Ser^{21}]-, [His^{21}]-, [Ile^{21}]-, [Phe^{21}]- and [Gly^{21}]-ET-1 were far less potent. Other analogs, [Gly^{21}]-, [Ala^{21}]-, [Lys^{21}]- and [Pro^{21}]-ET-1, were two to three orders of magnitude smaller than ET-1, while [Gln^{21}]-ET-1 was ineffective in inhibiting the binding of ^{125}I-ET-1 at concentrations up to 10^{-6} M.

As to the magnitude of the substituent effect of the analogs, the binding inhibitory activities of these analogs against ^{125}I-ET-1 were approximately comformable to the vasoconstrictor activities with only a slight exception. These coordinations could be implicated to the lack of antagonistic activity of the analogs. These findings lead us to the assumption that C-terminal amino acid residue

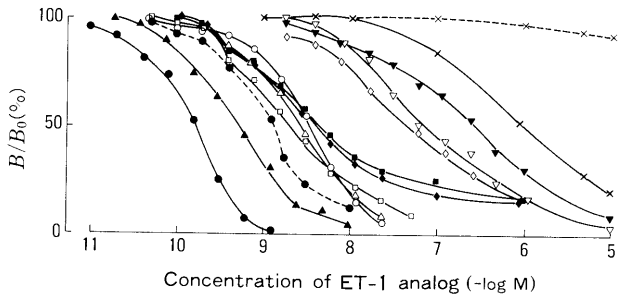

Fig. 2. The Dose–Response Relationship of ET-1 Analogs of Binding Assay

Rat brain membrane was incubated with ^{125}I-ET-1 in the presence of unlabeled ET-1 (—●—), ET-1 type B (---●---), Phe^{21}-ET-1 (—○—), Tyr^{21}-ET-1 (—▲—), His^{21}-ET-1 (—△—), Gly^{21}-ET-1 (—■—), Ser^{21}-ET-1 (—□—), Lys^{21}-ET-1 (—▼—), Ala^{21}-ET-1 (—▽—), Ile^{21}-ET-1 (—◆—), Glu^{21}-ET-1 (—◇—), Pro^{21}-ET-1 (—×—) and Gln^{21}-ET-1 (---×---).

could be a crucial part of the affect of the receptor-binding affinity through the influence of steric and electric effects and conclusively to determine the bioability.

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Pharmacological Properties of Galenical Preparation. XV.[1] Pharmacokinetics Study of Evocarpine and Its Metabolite in Rats

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It is known that when methanol extract of Evodia fruit is orally administered, 5-(1,4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl) pentanoic acid (EVCA) is excreated as a matabolite in rat urine. In this study, we separated Evodia fruit extract into major alkaloids administered each alkaloid individally to male Wistar rats. Consequently, it was demonstrated that the original substance of the metabolite are evocarpine and its analogues, dihydroevocarpine and 1-methyl-2-undecenyl-4(1*H*)-quinolone. Investigation of a blood sample after oral administration of evocarpine by high performance liquid chromatography confirmed that the substance was absorbed without alteration.

Pharmacokinetics of evocarpine after intravenous injection was expressed in a one-compartment model, showing a linear elimination of plasma evocarpine up to a dosage of 75 mg/kg. Total plasma clearance (*CL*), volume of distribution (*Vd*), and half-life ($T_{1/2}$) of evocarpine were 60 ml/min·kg, 3.2 l/kg and 0.6 h^{-1}, respectively. Metabolic ratio of evocarpine into EVCA after intravenous injection was 15.4%, and absorption ratio of the unaltered compound calculated from the levels of *AUC* after oral administration and intravenous injection was 4.7%. In this paper, it is shown that evocarpine is absorbed amount 100% when it is administered orally.

Keywords Evodia fruit; *Evodia officinalis;* Rutaceae; evocarpine; 5-hydroxy-1,4-dihydro-2-quinolin-2-yl pentanoic acid; metabolite; pharmacokinetic parameter

Evodia fruit is made by drying unripe fruit of *Evodia officinalis* DODE (Rutaceae), and is combined with Go-shuyu-to which is administered to counteract cold of the extremities, the alimentary system, and the hemicrania complicated by a reduction of metabolism.[2] The active components of Evodia fruit in Chinese medicine have not yet been identified, and we thus focused on the fact that certain of its components must be absorbed into the body in order to show physiological activity such as improvement of reduced metabolism. We earlier showed,[2] we showed that 5-(1,4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl) pentanoic acid (EVCA) is excreted specifically into urine after oral administration of methanol (MeOH) extract of Evodia fruit. One feature of EVCA with quinolone structure is similar to that of evocarpine, a main alkaloid in the fruit.

This time, we separated an extract of Evodia fruit into several fractions and administered each fraction to male Wistar rats. Six alkaloid components were individually administered. Consequently, it was clarified that the original substance of the metabolite are evocarpine and its analogues, dihydroevocarpine[3] and 1-methyl-2-undecenyl-4(1*H*)-quinolone.[3]

It was also learned that evocarpine exists in blood in an unaltered form after oral administration. We also studied on the pharmacokinetics of evocarpine and its metabolite EVCA in rats.

Experimental

Crude Drug Evodia fruit which conforms to Japan Pharmacopeia was obtained from a market in Osaka in 1987. The original material was identified as unripe fruit of *Evodia officinalis* DODE, judging from its external form.[4]

Standard Sample Hydroxyevodiamine,[5] evodiamine,[6] rutaecarpine,[6] evocarpine,[3] dihydroevocarpine[3] and 1-methyl-2-undecynyl-4(1*H*)-quinolone[3] which were separately isolated and purified from the Evodia fruit.[7] EVCA, which was isolated and purified from rat urine following administration of an extract of Evodia fruit, was also used.[2]

Experimental Animals Male Wistar rats 5 weeks old (Shizuoka Laboratory Animal Center) were housed under ordinary conditions for one week acclimation. Then, those with body weight of 130 to 150 g were used for the experiment.

Confirmation of EVCA in Urine 1) Preparation of Reagent Solution: Evodia fruit powder (100 g) was extracted twice with 500 ml boiled MeOH. To the total extracted fluid, after vacuum concentration, was added 1% NH_4OH and AcOEt, and the solution was then partitioned. After further extraction with 1% AcOH from the AcOEt layer, the organic solvent was exsiccated. To the exsiccant was added MeOH and hexane and the solution was partitioned (Chart 1). Each fraction obtained thus was vacuum exsiccated, and the extract converted into the weight of crude drugs was suspended at a concentration of 1.5 g/ml with 2% Tween 80. Hydroxyevodiamine, evodiamine and rutaecarpine were also suspended at a concentration of 30 mg/ml with 2% Tween 80, whereas evocarpine, dihydroevocarpine and 1-methyl-2-undecenyl-4(1*H*)-quinolone were made into an emulsion with 2% Tween 80.

2) Administration of the Drugs and Collection of Urine Samples: Each rat was kept in a separate metabolic cage under conditions of room temperature of 24±1 °C and 12 h cycle of light and dark for 1d. Subsequently, 5 ml/kg of each fraction and alkaloid solution was orally administered. Thereafter, the animals were allowed free access to food and water, and urine samples were collected for up to 12 h after the administration.

3) Preparation of Urine Sample: To the total urine sample obtained (about 5 ml) was added MeOH to make 50 ml. The mixture was centrifuged at 3000 rpm for 10 min and the supernatant obtained was then filtered through a 0.45 μm filter to prepare the sample for high performance liguid chromatography (HPLC).

4) Condition of HPLC Analysis of Urine Sample: Device for sending fluid: Shimadzu LC-6A system. Detector: Shimadzu RF-535. Data processor: Shimadzu C-R4A. Column: Inertsil ODS-2 (Gasukuro Kogyo, 5 μm, 4.6×250 mm). Mobile phase: MeOH/water=30/70—100/0, linear gradient, 35 min. Flow rate: 1.0 ml/min. Temperature of the column: 40 °C. Detection: Ex=300 nm, Em=700 nm. Injection volume: 10 μl.

Determination of Evocarpine in Blood 1) Administration of the Drugs and Collection of Blood Sample: All the administered solutions were combined in one emulsion with 2% Tween 80. Each group of 10 rats was given intravenously 25 mg/kg (25 mg/ml), 50 mg/kg (50 mg/ml) or 75 mg/kg (75 mg/ml) evocarpine; or orally 75 mg/kg (75 mg/ml), 225 mg/kg (225 mg/ml) or 370 mg/kg (370 mg/ml). After a designated time period, the cervical artery was cut under ether anesthesia, and a blood sample was collected.

2) Preparation of Blood Sample: The 5 ml blood sample with heparin was centrifuged at 3000 rpm for 10 min. Plasma (2 ml) accurately taken was diluted with water (5 ml), charged in Sep-Pak, washed with 5 ml of water, and eluted with MeOH to make exactly 5 ml. The eluate was filtered through a 0.45 μm filter for HPLC.

3) Condition of HPLC Analysis of Blood Sample: Device for sending fluid: Shimadzu LC-6A system. Detector: Shimadzu SPD-6A. Data processer: Shimadzu C-R4A. Column: Inertsil ODS-2 (Gasukuro Kogyo,

5 μm, 4.6×250 mm). Mobile phase: MeOH/water=82/18. Flow rate: 1.0 ml/min. Temperature of the column: 40 °C. Detection: 320 nm. Injection volumn: 50 μl.

4) Calibration Curve: Standard solutions for the calibration curve were made by the addition of 5 different concentrations of original solution to normal rat plasma (2 ml) to become 0.02—20.00 μg/ml. The solution treatment was similar to that for the blood sample. The peak area was obtained from chromatography, and an equation for the calibration curve was obtained by the least squares method. A favorable linear relation with 0.999 of correlation coefficient was obtained in a range of 0.02—20.00 μg/ml, and the regression equation was $y=310.3x+152.1$ (y: peak area of evocarpine, x: concentration of evocarpine in blood). Accuracy of the quantitative analysis was 94—103%.

Determination of EVCA in Urine 1) Administration and Collection of Urine: One group of 6 rats was placed in a metabolic cage and housed for 1 d. Evocarpine was administered intravenously with 75 mg/kg or orally with 225 mg/kg. After the administration, food and water were taken freely, and urine samples were collected at the following intervals: 0—3, 3—6, 6—9, 9—12, 12—15 and 15—18 h.

2) Determination of EVCA in Urine: MeOH (10 ml) was added into the total urine obtained (about 1 ml), and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter for HPLC.

3) Condition of HPLC Analysis of Urine Sample: Same as confirmation of EVCA in urine.

4) Calibration Curve: Five kinds of different concentrations of original solution within 0.01—2.00 mg/ml were made by control urine, and was used as sample fluid for calibration curve. The solution was treated similarly to that for the urine sample. The peak area was obtained from chromatography, and an equation for the calibration curve was obtained by the least squares method. A favorable linear relation with 0.999 of correlation coefficient was obtained in a range of 0.02—2.00 mg, and the regression equation was $y=142.1x\times 59.9$ (y; peak area of EVCA, x; EVCA amount in urine). Accuracy of the quantitative analysis was 97—102%.

Results

Evodia Fruit Alkaloid Metabolite in Urine MeOH extract (33.9 g) obtained from 100 g Evodia fruit was separated into the 7 fractions of A to G shown in Chart 1. Excretion of EVCA into urine was recognized when fraction, A, C, E or F was administered. Analysis of F fraction was under the same HPLC condition as that in the previous report[7] revealed that the main components included evodiamine and rutaecarpine which are alkaloids of the indolquinazolin type, and evocarpine, dihydroevocarpine and 1-methyl-2-undecenyl-4(1*H*)-quinolone which are alkaloids of the quinolone type.

Thus, these alkaloids were orally administered separately.

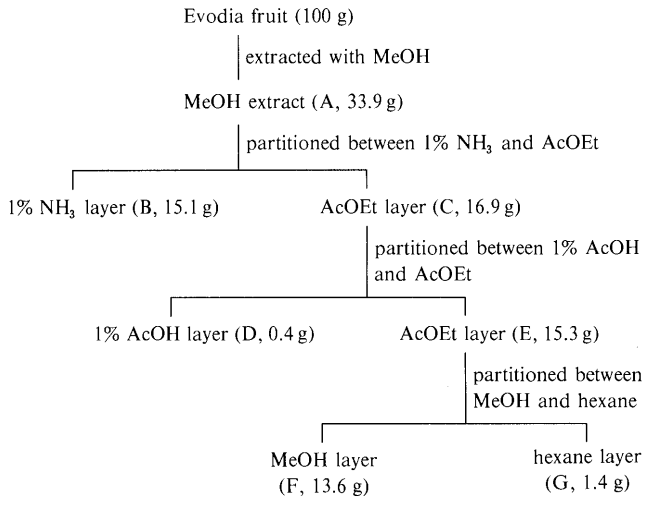

Chart 1

The metabolite was detected in the urine only when the evocarpine or the analogues were administered orally (Fig. 1).

Pharmacokinetics of Evocarpine In analysis of rat blood by HPLC after oral administration of evocarpine, one component which was not found prior to the administration was confirmed. This component was eluted at t_R similar to that with evocarpine. The ultraviolet (UV) spectrum also completely corresponded when it was measured using a three dimensional detector. Moreover, the part corresponding to this component which was collected by HPLC completely coincided in mass spectrum (MS) measurement.

These findings, confirmed that evocarpine exists in an unaltered form in rat blood after its oral administration.

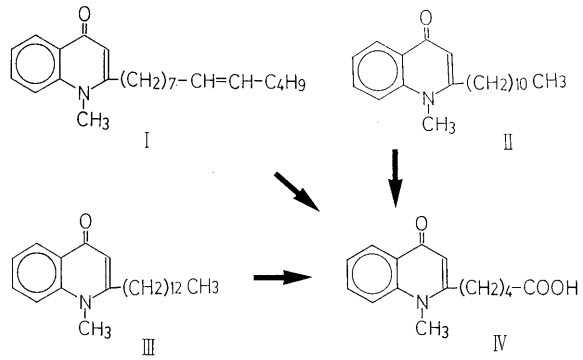

Fig. 1. Goshuyu Alkaloids and Their Metabolite in Urine

I: evocarpine, II: 1-methyl-2-undecenyl-4(1*H*)-quinolone, III: dihydroevocarpine, IV: 5-(1,4-dihydro-1-methyl-4-oxo-2-quinolin-2-yl) pentanoic acid.

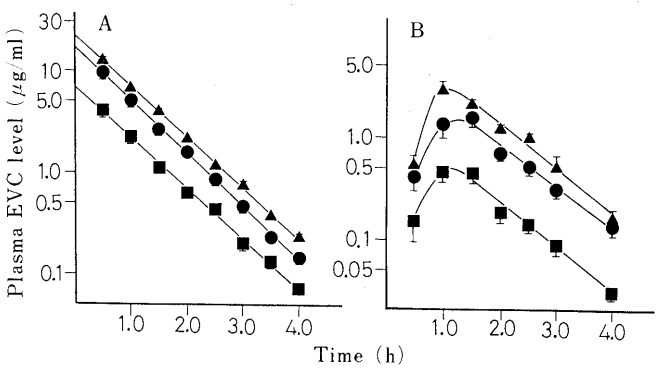

Fig. 2. Time Courses of Evocarpine (EVC) Concentration in Plasma of Rats

A, i.v.; ▲, 75; ●, 50; ■, 25 mg/kg ($n=5$). B, *p.o.*; ▲, 370; ●, 225; ■, 75 mg/kg ($n=10$).

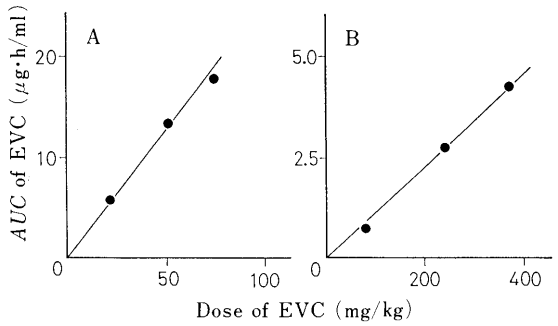

Fig. 3. Relationship between *AUC* of Evocarpine (EVC) and Dose of Evocarpine

A, i.v.; B, *p.o.*

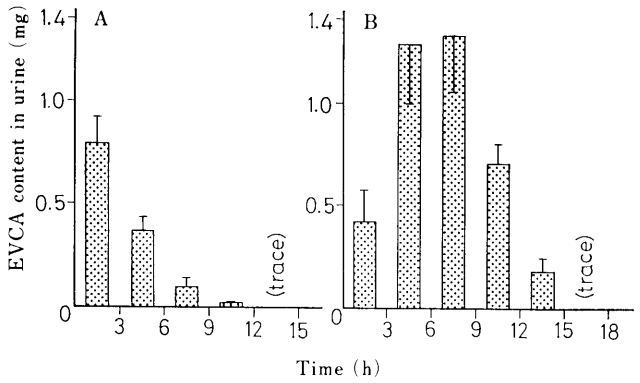

Fig. 4. Time Courses of EVCA Content in Urine of Rats ($n=6$)
A, i.v.; B, *p.o.*

Figure 2 shows change of plasma concentration of evocarpine with intravenous injection in contrast with oral administration. As seen, the change was a monophasic reduction. Thus, pharmacokinetics of evocarpine can be analyzed using a one-compatment model in rats.

The amount of evocarpine administered and the area under the plasma concentration–time curve (*AUC*) were calculated by trapezoid method from a graph up to 4.0 h; thereafter it was calculated by integral calculation and summed. Figure 3 shows the relationship between the administered amount of evocarpine and *AUC*, in which a linear relationship passing through the origin was recognized. This result indicated that it would be reasonable to assume that the process of disappearance of evocarpine followed first-order kinetics with an intravenous injection of less than 75 mg/kg or an oral administration of less than 370 mg/kg.

Figure 4 shows changes in the amount of EVCA was excreted in urine following intravenous injection of 75 mg/kg of evocarpine or oral administration of 225 mg/kg. Excretion lasted 12 h (twenty times as long as half-life), after which little evocarpine was believed to exist in the blood kinetically.

Discussion

We previously reported that EVCA was excreted in the urine when MeOH extract of Evodia fruit was administered to rat.[2] This time, it was clarified that the metabolite originated from evocarpine, a major alkaloid, and the analogues, dihydroevocarpine and 1-methyl-2-undecenyl-4(1*H*)-quinolone. It was also found that regardless of the presence or absence of double bonds in side chain, these compounds are metabolized to the compound having 5 carbon side chain with the terminal carboxyl group.

This experiment proved that pharmacokinetics of evocarpine in rat can be approximated by a one-compartment model. Thus, the kinetic parameters of evocarpine are shown in Table I. Distribution volume was very large, approximately 3.2 l/kg, indicating that a large quantity transferred to the tissue. The half-life is less than 1 h, suggesting that the metabolism occurs very easily. However, excretion of

TABLE I. Pharmacokinetic Parameters of Evocarpine in Rat

Dose (mg/kg)	V_d (l/kg)	$T_{1/2}$ (h)	*CL* (ml/min kg)	*AUC* (μg·h/ml)	EVCA amount (mg/kg)
i.v.					
25	3.22	0.60	62.1	6.0	—
50	3.12	0.58	62.4	13.5	8.7
75	3.33	0.56	69.3	17.8	—
p.o.					
75	—	0.68	54.6	0.83	—
225	—	0.73	51.3	2.63	29.1
370	—	0.68	54.5	4.39	—

EVCA into urine continues for 12 h (twenty times as long as half-life) at which time little evocarpine is believed to exist kinetically in blood. This time difference indicates that evocarpine may not be metabolized into EVCA in a single process, but by several processes.

The metabolic ratio into EVCA with intravenous injection of evocarpine was 15.4%; the number of molecules of EVCA divided by evocarpine. *AUC* of evocarpine was 0.25 μg·h/ml per 1 mg/kg administered by intravenous injection, and 0.012 μg·h/ml by oral administration, from which bioavailability of evocarpine was calculated to be 4.7%. Total plasma clearance was that *AUC* divided by the dose.

Applying this to evocarpine (225 mg/kg), 10.5 mg is transferred into blood in an unaltered form in oral administration of 225 mg/kg, and about 1.2 mg (15.4%) of the total amount is assumed to be excreted into urine. The actually excreted amount of EVCA into urine, however, is approximately 29.1 mg. The difierence of 27.9 mg/kg is assumed to have originated from an intermediate metabolite transferred into blood after first being metabolized in the intestine or liver, and 27.9 mg of EVCA corresponds to about 240 mg of unaltered form. Therefore, the absorption of evocarpine from the intestinal tract is assumed to be almost 100%. We are now investigating the pharmacological activity of evocarpine and the metabolite, and absorption and metabolism of other alkaloids.

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Purification and Some Properties of Inducible Lysine Decarboxylase from *Vibrio parahaemolyticus*

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Inducible lysine decarboxylase from *Vibrio parahaemolyticus* AQ 3627 was purified to apparent homogeneity and characterized. The enzyme displayed a molecular weight of 531000 by gel filtration and 79000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme required pyridoxal phosphate as a cofactor, and the pH optimum was 5.5. The K_m value for L-lysine was 3.2 mM, and the enzyme was inhibited by 6-aminocaproic acid and α-fluoromethyl analogs of cadaverine. δ-Hydroxylysine and *S*-aminoethyl-L-cysteine was active as substrates to a lesser extent than L-lysine. The amino-terminal amino acid sequence was determined to be Met–Asn–Ile–Phe–Ala–Ile–Leu. These properties were compared with those of other bacterial lysine decarboxylases.

Keywords lysine decarboxylase; *Vibrio parahaemolyticus*; purification; property; amino-terminal sequence; enzyme induction

Lysine decarboxylase (LDC), which catalyzes the formation of cadaverine and CO_2 from L-lysine, is produced by certain bacteria in response to growth in an acid environment,[1] and it has been suggested that bacteria use this reaction either to increase intracellular pH[2] or to control the intracellular concentration of CO_2, which would otherwise be exceedingly low under acidic conditions.[3] In addition to the acidic conditions, the presence of its substrate is known to favor LDC induction.[2] As a consequence of these observations, Moeller[4] and others[5] have developed qualitative, colorimetric tests for the presence of inducible LDC, which are being used routinely for the differentiation of genera and species within the bacterial families. Despite the widespread utilization of the inducible LDC in *Vibrio* species for this purpose,[6] it has not been enzymologically well-characterized.

In this paper, we describe the purification and characterization of the inducible LDC from *V. parahaemolyticus*, and compare some properties of this enzyme with those of the corresponding enzymes from *Escherichia coli* and *Hafnia alvei* (formerly designated *Bacterium cadaveris*).

Materials and Methods

Chemicals Cadaverine analogs as inhibitors of LDC[7] were kindly supplied by Merrell Dow Institute (Strasboug Cedex, France); standard proteins for sodium dodecylsufate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration and pyridoxal phosphate (PLP) were from Sigma; hydroxyapatite and lysine analogs were from Wako; L-[U-^{14}C]lysine (6.36 GBq/mmol) was from ICN. All other chemicals were of analytical or reagent grade.

Bacterial Strain and Growth Conditions The strain used was *V. parahaemolyticus* AQ3627, which was supplied by the Research Institute for Microbial Disease, Osaka University (Osaka). It was precultured at 37 °C for 12 h in medium A [0.45% yeast extract (Difco); 0.1% Bactocasitone (Difco); basal inorganic salts[8]; 2% NaCl; final pH 7.5]. Several 2 l Erlenmeyer flasks, each containing 1 l of medium A supplemented with L-lysine and glucose at final concentration of 50 mM and 0.5%, respectively, were inoculated with 20 ml of the preculture and then incubated at 37 °C with a rotary shaker. The concentrations of L-lysine and glucose were as recommended by Moeller.[4] After 6 h growth, the cells were harvested by centrifugation for 10 min at 5300 × *g* at 4 °C and washed once with ice-cold 2% NaCl containing 10 mM $MgCl_2$. Cell pellets were stored at −70 °C before enzyme purification. No change in enzyme activity was noted for at least one month.

Enzyme Assay The standard assay mixture (total volume 1 ml) contained 100 mM sodium acetate buffer (pH 5.5), 10 mM L-lysine, 0.1 mM PLP and enzyme protein (0.1—100 μg). After incubation at 37 °C for 10 or 20 min, the reaction was stopped by the addition of 0.4 ml of 20% $HClO_4$. Cadaverine formed was determined by a gas liquid chromatography (GLC) method[9] using 1,7-diaminoheptane as an internal standard. Under the conditions used, the reaction was proportional to the amount of enzyme added. The decarboxylation of δ-hydroxylysine (DL and DL allo), *S*-aminoethyl-L-cysteine, N^{α}- and N^{ε}-acetyl-L-lysine was followed manometrically by measuring the CO_2 evolution (Warburg apparatus). Enzyme assays involving α-fluoromethylcadaverines were carried out by a modification of the method of Boeker and Fischer[10] in which radioactive CO_2 released from L-[^{14}C]lysine (10 mM, *ca.* 1.1×10^4 dpm/μmol) was measured, since these compounds interfered with the GLC analysis of cadaverine. One unit of activity was defined as the amounts of enzyme that catalyzed the formation of 1 mmol cadaverine/h under standard conditions. Protein was determined by the method of Lowry with bovine serum albumin as the standard.

Purification of LDC All operations were done at 4 °C or in an ice bath, and all dialyses were done against 100 volumes of the specified buffers with two changes for 12 h. The final procedures adopted for the purification of LDC are shown in Table II. The frozen cells (43 g) were thawed and suspended in 200 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 0.04 mM PLP, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 0.1% Tween 80 (buffer A), and sonicated with an ultrasonic homogenizer. Cell debris was removed by centrifugation at 40000 × *g* for 30 min. A small portion of the supernatant was dialyzed against buffer A to assay the activity (crude extract). The supernatant was fractionated with solid ammonium sulfate, and the protein precipitated between 50—60% saturation was collected by centrifugation, dissolved in buffer A and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sepharose CL-6B column (2.6 × 20 cm) equilibrated with 10 mM Tris–HCl (pH 7.5) containing 1 mM DTT (buffer B). After washing with 200 ml of the same buffer, the column was eluted with a linear gradient of NaCl, 0—1 M in 500 ml of the same buffer. Fractions (8 ml) were collected in tubes containing 80 μl of 4 mM PLP. The active fractions were pooled and dialyzed against buffer B containing 0.04 mM PLP. This preparation was rechromatographed in the same way except that a linear gradient of NaCl, 0—0.75 M, was applied. The pooled active fraction was dialyzed against 20 mM potassium phosphate buffer (pH 7.5) containing 0.04 mM PLP and 1 mM DTT and then applied to a hydroxyapatite column (2.1 × 10 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.5). After washing the column with 3 column volumes of the same buffer, the enzyme was eluted with a 200 ml gradient of 20—200 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. Fractions (5 ml) were collected in tubes containing 50 μl of 4 mM PLP. The pooled active fraction, which had been concentrated to 3 ml using a Centricon 30 (Amicon), was applied to a Sephacryl S-300 column (2.1 × 85 cm) equilibrated with buffer B containing 0.04 mM PLP, 0.4 M NaCl and 0.02% sodium azide. The peak fraction (2 ml) was dialyzed for 4 h against buffer B containing 0.04 mM PLP and 0.02% sodium azide and then stored at 4 °C.

Electrophoretic Analysis and Gel Filtration SDS-PAGE was done by an 8.5% polyacrylamide slab gel according to the method of Laemmli.[11] Before loading, the enzyme sample was heated for 3 min with 0.1% SDS and 10% glycerol in a boiling-water bath. The gel was stained for protein with Coomassie brilliant blue R-250. The relative mobility was determined for the molecular weight markers as well as for the enzyme. Analytical gel filtration for the molecular weight determination of the native enzyme was done using a Sephacryl S-300 (2.1 × 90 cm) calibrated with the standard proteins, as described by Andrews.[12] The column was equilibrated and eluted with buffer B containing 0.04 mM PLP, 0.4 M NaCl and 0.02%

sodium azide.

Amino-Terminal Amino Acid Sequencing This was determined with an Applied Biosystems model 477A protein sequencer.

Results and Discussion

In order to define a good enzyme source for purification, LDC activity was determined in cells grown under various medium conditions (Table I). At an initial pH of 7.5, glucose relatively strongly induced the enzyme with a concomitant drop of the medium pH, whereas L-lysine did not induce it at all. However, at an initial pH of 6.0, L-lysine alone produced and appreciable induction, but to a lesser extent than glucose. Simultaneous addition of L-lysine and glucose resulted in a maximal induction with a similar specific activity, regardless of the initial pH of the medium. These results suggest that the low pH of the medium, which would perturb intracellular pH homeostasis, or lowering the intracellular and extracellular pH by the acid products of glucose fermentation may contribute to LDC induction. When the bacterium was grown for 6 h at an initial pH of 7.5, the total (intra- and extracellular) cadaverine content increased from 0.47 in the absence of L-lysine and glucose to 88.1 μmol/mg cell protein in the presence of both. In the latter case, more than 95% of cadaverine produced was detected in the medium. Recently, Ruzgiene *et al.*[13] reported the factors affecting LDC induction in *Vibrio* species.

The purification of LDC from *V. parahaemlyticus* AQ 3627 is summarized in Table II. The purified enzyme had a specific activity of 19.5 units/mg of protein, and showed 279-fold purification over the crude extract, with about 30% recovery. The purified enzyme gave a single protein band on SDS-PAGE (Fig. 1).

Stability of the enzyme was enhanced by the presence of PLP in the solution. Freezing and thawing almost completely inactivated the enzyme. The purified enzyme could be stored at 1 °C in the presence of 1 mM DTT, 40 μmol PLP and 0.02% sodium azide for at least 2 weeks without any appreciable loss of activity.

The molecular weight of the native enzyme was determined to be 531000 by gel filtration (Fig. 2), and the band on SDS-PAGE was estimated to have a molecular

TABLE I. Effects of Glucose, L-Lysine and pH on LDC Induction in *V. parahaemolyticus*

Addition to the medium	LDC activity (μmol/mg protein/h)[a]	
	Initial pH of the medium	
	7.5	6.0
None	0.49 (7.43)	2.32 (6.35)
L-Lysine	0.52 (7.41)	15.0 (7.05)
Glucose	13.8 (6.63)	31.2 (5.27)
L-Lysine + glucose	71.4 (7.18)	70.1 (6.91)

a) LDC activity was determined with cells grown for 6 h in the absence or presence of L-lysine (50 mM) and/or glucose (0.5%). Values shown in parentheses represent the pHs of the media after 6 h growth.

TABLE II. Purification of Inducible LDC from *V. parahaemolyticus*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	2087	146	0.070	100	1
Ammonium sulfate (50—60%) precipitation	834	171	0.205	117	2.9
1st DEAE-Sepharose CL-6B	334	137	0.410	94	5.9
2nd DEAE-Sepharose CL-6B	16.0	119	7.44	82	106
Hydroxyapatite	5.5	88	16.0	60	229
Sephacryl S-300	2.3	45	19.5	31	279

A typical purification from 43 g of wet weight cells is shown.

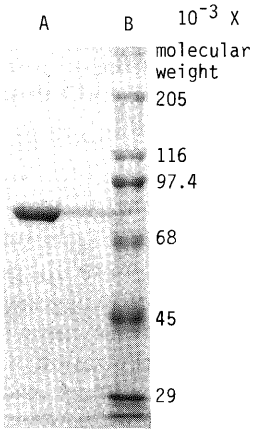

Fig. 1. SDS-PAGE of the Purified LDC

A, purified enzyme (6 μg); B, standard proteins (from the top: myosin; β-galactosidase, phosphorylase b; bovine serum albumin; egg albumin; carbonic anhydrase).

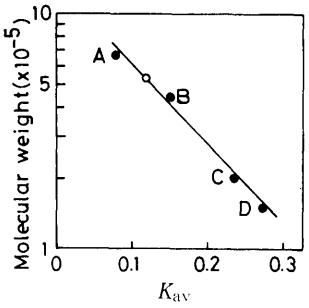

Fig. 2. Estimation of the Molecular Weight of the Purified Enzyme by Gel Filtration

The standard proteins and their molecular weights were: A, thyroglobulin (669000); B, apoferritin (443000); C, β-amylase (200000); D, alcohol dehydrogenase (150000). ○: the purified enzyme.

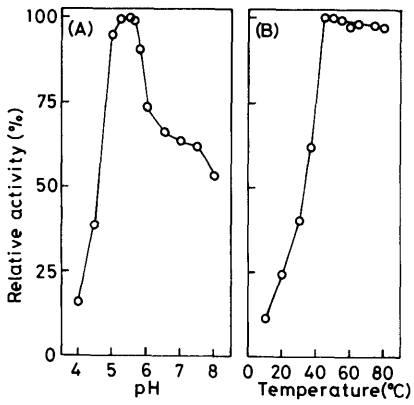

Fig. 3. Effects of pH (A) and Temperature (B) on the Purified LDC Activity

The standard assay mixture containing 0.3 μg of the purified enzyme was incubated at various pHs and temperatures as indicated. The buffers used were: 100 mM sodium acetate buffer (pH 4.0—5.6); 100 mM sodium phosphate buffer (pH 5.8—8.0).

weight of 79000. The molecular weight of this band was unaltered when the protein was treated with 2-mercaptoethanol before electrophoresis. Thus, the active form may be composed of seven subunits of identical size which are not linked by disulphide bonds. Similarly, a heptameric structure has been suggested for the *H. alvei* LDC when calibrated by gel filtration.[15a]

The effects of pH and temperature on enzyme activity were investigated. The enzyme was most active at pH 5.5, as shown in Fig. 3A, but, unlike that from *E. coli*,[2b] showed no bell-shaped curve. The maximum activity, which was about 1.6-fold higher than that observed at 37 °C, was seen at 45—50 °C (Fig. 3B). Furthermore, when the enzyme was incubated at various temperatures for 10 min at pH 5.5, and then the remaining activity was measured under the standard assay conditions, 65% of its initial activity was retained after heating even at 80 °C. A similar heat stability was reported for the *E. coli* LDC.[2b]

D-Lysine, L-ornithine and lysine analogs at 10 mM were tested as substrates under the standard assay conditions. *S*-Aminoethyl-L-cysteine and δ-hydroxylysine were decarboxylated at rates of 23% and 17% that observed for L-lysine, whereas D-lysine, L-ornithine, N^{α}- and N^{ε}- acetyl-L-lysine were not. Lineweaver-Burk plots gave a K_m of 3.2 mM for L-lysine and a V_{max} of 20.5 mmol cadaverine/mg protein/h.

Overnight dialysis of the purified enzyme against buffer B (devoid of PLP) resulted in about 60% irreversible loss of activity. When PLP was omitted from the assay mixture, the activity of such a dialyzed preparation decreased to 30% of the control activity in the presence of 0.1 mM PLP. These results indicate that PLP is indispensable for both stability and full activity of the enzyme.

The effect of various inhibitors on enzyme activity was examined by adding them to the incubation mixture (Table III). At a final concentration of 5 mM, 6-aminocaproic acid and carboxymethoxylamine, a PLP-dependent enzyme inhibitor, produced a more than 80% inhibition of the *V. parahaemolyticus* LDC. It is of interest that the *V. parahaemolyticus* LDC, as well as the *E. coli* LDC,[2b] is inhibited by 6-aminocaproic acid which lacks an α-amino group expected to be important in the interaction with the enzyme. On the other hand, at a final concentration of 1 mM, α-monofluoromethylcadaverine (MFMC) inhibited the enzyme to a much lesser extent than the other α-fluoromethylcadaverines. However, when preincubated with MFMC at the same concentration in the absence of the substrate, the enzyme was inhibited by about 80%. The same treatment of the enzyme with α-difluoromethylcadaverine (DFMC) or α-trifluoromethylcadaverine (TFMC) brought about no appreciable inhibition. Moreover, TFMC appeared to be more potent that DFMC in inhibiting the enzyme. These results are in agreement with the finding[7] that MFMC acts on the *E. coli* LDC as an enzyme-activated irreversible inhibitor and two other α-fluoromethylcadaverines as competitive inhibitors.

Addition of EDTA, DTT, Mg^{2+} or Ca^{2+} (all 1 mM) to the assay mixture did not affect enzyme activity. DTT was required for stabilizing the enzyme during purification and storage.

The amino-terminal sequence of the enzyme was determined to be Met–Asn–Ile–Phe–Ala–Ile–Leu.

TABLE III. Effect of Inhibitors on Inducible LDC from *V. parahaemolyticus*

Inhibitor	Concentration (mM)	Relative activity (%)[a]
None (Control)	—	100
6-Aminocaproic acid	1	42
	5	23
Carboxymethoxylamine	1	37
	5	9
α-Monofluoromethylcadaverine	1	92
	1	16[b]
α-Difluoromethylcadaverine	1	77
	1	77[b]
	2	59
α-Trifluoromethylcadaverine	1	53
	1	47[b]
	2	12

Each assay was carried out with 0.3 μg of the purified enzyme. *a*) The values are means of triplicate measurements, which are expressed as percentages of the control activity. *b*) The standard assay mixture without L-lysine as the substrate was preincubated with individual inhibitors for 20 min at 37 °C, and then the activity was assayed after addition of the substrate to the reaction mixture.

TABLE IV. Comparison of Some Properites and Amino-Terminal Sequences of LDCs from Different Bacteria

LDC source	K_m (mM)	pH optimum	Mol.wt. (native) ($\times 10^{-3}$)	Subunit mol.wt. ($\times 10^{-3}$)	Amino-terminal sequence
V. parahaemolyticus	3.2	5.5	531	79	Met–Asn–Ile–Phe–Ala–Ile–Leu–
E. coli	1.5	5.7	780	80	Met–Asn–Val–Ile–Ala–Ile–Leu–
H. alvei	1.7	5.8	542	77	Met–Asn–Ile–Ile–Ala–Ile–Leu–

Table IV shows some properties and the amino-terminal sequences of the inducible LDCs from *E. coli*[2b,14] and *H. alvei*[15] in Enterobacteriaceae, together with those of the *V. parahemolyticus* LDC. There are several similarities among the enzymes from these bacteria, including cofactor requirement, optical pH, subunit molecular weight and amino-terminal sequence. However, the molecular structure of the enzyme in *V. parahaemolyticus* is somewhat different from that in *E. coli*, but rather similar to that in *H. alvei*. The molecular weights of the native enzymes of *V. parahaemolyticus* and *H. alvei*, as judged by gel filtration, are 531000 and 545000, respectively, and hence they may consist of seven identical subunits, whereas that of *E. coli* is characterized by a decameric structure with a molecular weight of 780000, consisting of ten identical subunits with a molecular weight similar to *V. parahaemolyticus*. The K_m value of the *V. parahaemolyticus* LDC was higher than those of the *E. coli* and *H. alvei* LDCs. Moreover, the amino-terminal sequence of the *V. parahaemolyticus* LDC differs from that of *H. alvei* LDC only in the fourth position, but differs from that of *E. coli* in the third and fourth positions. These substitutions in amino acids, however, may be caused by only one base pair exchange, and the amino-terminal regions appear to be well conseverd among these species to remain rich in hydrophobic amino acids.

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Electron Spin Resonance Studies of Phosphatidylcholine Interacted with Cholesterol and with a Hopanoid in Liposomal Membrane

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The effects of bacteriohopane-32-ol (Monol) on liposomal membrane composed of dipalmitoylphosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (egg PC) were compared with those of cholesterol (Chol) in the change of fluidity using a spin label. The fluidity change close to the polar head groups caused by temperature increase in the DPPC membrane containing Monol was different from that of Chol. Chol had a condensing effect on DPPC membrane, whereas Monol had only a slight effect except when used at 20 mol%. Near the hydrophobic end, Chol incorporation into DPPC led to fluidization below transition temperature (*Tm*) and condensation above *Tm*. Monol incorporation into DPPC had only a fluidizing effect below *Tm*. On the other hand, in egg PC membrane Chol had the condensing effect at any temperature, whereas Monol had only slight effect. These results suggest that Monol may have a role in supporting constant membrane fluidity under drastic conditions.

Keywords hopanoid; cholesterol; liposome; ESR spectrum; spin label; membrane fluidity

Sterols are indispensable components of the membranes in eukaryotic cells and play functional important roles in the membranes.[1] Lacking the sterols normally in prokaryotes, the existence of other molecules functional equivalent to sterols has been assumed. Hopanoids, which belong to the triterpene family, are found in strains widely scattered through numerous taxonomic groups such as cyanobacteria, methylotrophs, purple non-sulphur bacteria and gram-negative chemoheterotrophs.[2] According to their structural features and the characteristics of their biosynthetic pathway, hopanoids have been considered to be phylogenetic precursors of sterols and to act as membrane reinforcers on prokaryotic systems as sterols do in the membranes of eukaryotes.[3] Bacteriohopane-32-ol (Monol) used in this study is one of the hopanoids derived from many hopanepolyols[4] and has structural similarities to cholesterol (Chol) (Fig. 1). We recently observed that Monol can be incorporated into the lipid phase of dipalmitoylphosphatidylcholine (DPPC) liposomes and that it stabilizes the liposomal membranes.[5]

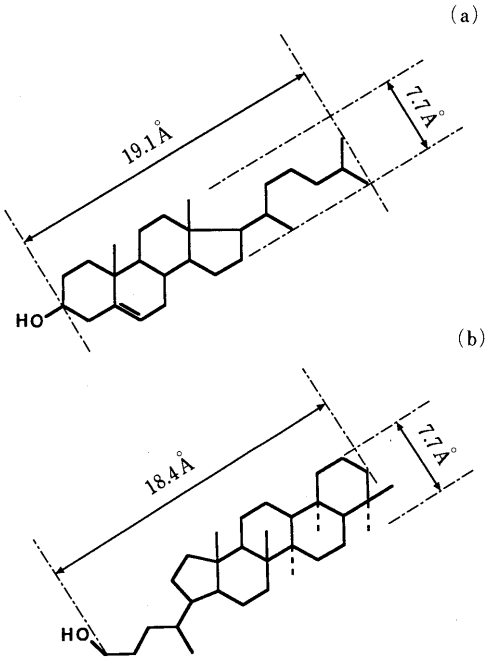

Fig. 1. Comparison of the Dimensions of Chol (a) and Monol (b) and Structural Equivalents

In this study, we compared the reinforcing effect of Monol on the liposomal membranes composed of DPPC or egg yolk phosphatidylcholine (egg PC) using the electron spin resonance (ESR) method.

Materials and Methods

Lipids DPPC and egg PC were purchased from Nippon Oil & Fats Co., Ltd. 5-Doxyl stearic acid (5-SASL) and 16-doxyl stearic acid (16-SASL) were obtained from Aldrich Chemical Co. Chol from Wako Pure Chemical Industries, Ltd. was recrystallized from C_2H_5OH–H_2O (2 : 1, v/v). Monol was prepared as described previously.[5]

Preparation of Liposomes PC (10 μmol), various amounts of Chol or Monol (2.5, 5, 7.5 μmol) and spin probes (PC : probe = 150 : 1, mol/mol) were dissolved in $CHCl_3$ and the solvent was evaporated *in vacuo* to form a thin homogeneous film in a tube. Phosphate buffered saline (pH 7, 0.5 ml) was added and the mixture was vigorously vortexed for 1 min and then sonicated for 10 min at 50 °C.

ESR Measurement ESR spectra were recorded with a JEOL JES-FE spectrometer (X-band, 100 kHz field modulation, 0.63 *mT* modulation width) equipped with a temperature controller.

Results and Discussion

Effect of Monol on DPPC Membranes Figure 2 shows typical spectra of 5-SASL (a) and 16-SASL (b) at various temperatures in liposomes composed of DPPC. A maximum splitting value, which is directly related to the viscosity of

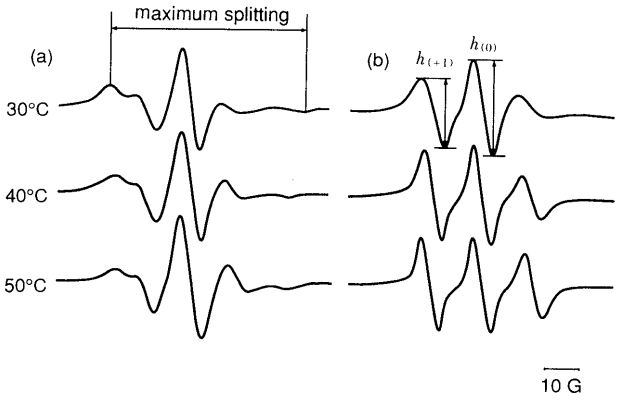

Fig. 2. ESR Spectra of 5-SASL (a) and 16-SASL (b) Embedded in the Liposomes Composed of DPPC

The temperatures of ESR measurements are indicated to the left of the spectra.

the environment, has been used as a convenient parameter to monitor the rotational motional freedom of the nitroxide radical group of 5-SASL. The nitroxide radical of 5-SASL is located close to the polar groups of phospholipids in the liposomal membranes. As can be seen from Fig. 2a, the splitting value decreased with increasing temperature, indicating a decrease of alkyl chain order near the phospholipid head groups. The fluidity of bilayer near the hydrophobic end can be examined using a spin probe of 16-SASL. The ESR spectra of 16-SASL in the DPPC liposomes were characterized by three peaks as shown in Fig. 2b. In these spectra, the ratio of the low-field peak height to the central one ($h_{(+1)}/h_{(0)}$) can be used as an empirical parameter for the membrane fluidity.[6-8] An increase of the values of this parameter reflects an increase in the mobility of the nitroxide radical near the hydrophobic end of the acyl chains.

ESR spectra of 5-SASL and 16-SASL in liposomes containing Monol of 20 mol% to DPPC concentration are presented at various temperatures in Fig. 3. On the basis of these spectra, the effects of Monol on DPPC membranes can be estimated. Figure 4 shows the effects of various amounts of Chol or Monol on the splitting value as a function of temperature. The incorporation of Chol into the DPPC liposomes decreased the membrane fluidity and affected the phase transition observed at about 40 °C in the liposomes composed of DPPC alone (control) (Fig. 4a). When Monol was incorporated instead of Chol, the change of the membrane fluidity was similar to that of the control except for the presence of 20 mol% Monol (Fig. 4b). In the liposomes containing 20 mol% Monol, the fluidity was higher than that of the control below the transition temperature (*Tm*) of DPPC and the fluidity change was slight even at higher temperatures. This Monol content is close to the content of hopanoids detected in *Bacillus acidocaldarius* under cultivation at high temperatures.[9] *B. acidocaldarius* is a unique bacterium which grows in the pH range from 2 to 6 at temperatures from 45 to 70 °C.[10] Therefore, its content of about 20 mol% may have an important role in maintaining bacterial membranes that are subjected to lysis-inducing stress.

Figure 5 shows the change of the $h_{(+1)}/h_{(0)}$ value as a function of temperature in the presence of various amounts of Chol or Monol. The phase transition could be observed at about 40 °C in the control. However, the change of the membrane fluidity near the hydrophobic end of the acyl chains caused by Chol was different from that observed near the polar groups of the phospholipids (Fig. 4). At temperatures below *Tm*, the fluidity of the membrane was increased by the addition of Chol (fluidizing effect). On the other hand, at temperatures above *Tm*, the fluidity was lower than that of control (condensing effect) (Fig. 5a). Such a contradictory effect of Chol has been reported by many researchers[11] and seems to be due to the formation of intermediate gel state resulting from a hydrophobic interaction of Chol with the fatty acyl chains of the saturated PC.[12] When Monol was incorporated, the fluidizing effect was observed below *Tm*, but no condensing effect above *Tm* was produced (Fig. 5b). These results indicate that Monol has no effect on the membrane fluidity at liquid crystalline state but that it affects the molecular order of the liposome membranes composed of saturated PC in its own specific manner. Both Chol and Monol molecules possess a quasi-planar, rigid and amphiphilic structure (Fig. 1).[3] Monol seems to be oriented parallel to the fatty acyl chains of the phospholipids in an inverted orientation compared to Chol, because its hydroxy group is located in the side chain. It can be considered that there are hydrogen bonds between the hydroxyl groups at the side chain of Monol and the ester carbonyl groups of the saturated PC. Furthermore, Monol has methyl groups on both sides of the plane of the skeleton, whereas methyl groups of sterol are directed only on one side of its ring system.[13] The hydrogen bonds and these methyl groups may produce a specific effect on the membrane composed of saturated PC. These considerations support that the membrane fluidity

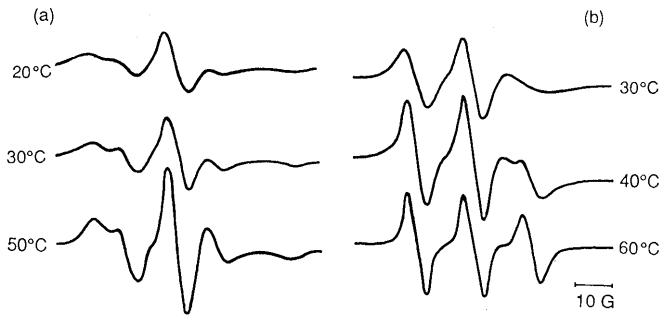

Fig. 3. Temperature Dependence of ESR Spectra of 5-SASL (a) and 16-SASL (b) Embedded in the DPPC Liposomes Containing 20 mol% of Monol

All are plotted at the same instrument sensitivity so that their spectra intensities can be directly compared. (The spectrum at 60 °C in (b) was drawn on a scale of half intensity.)

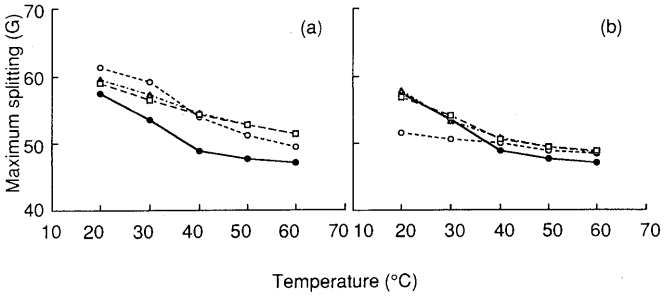

Fig. 4. Change of the Maximum Splitting of 5-SASL in the DPPC Liposomes Containing Chol (a) or Monol (b) as a Function of Temperature

Content of Monol: —●—, 0 mol%; ---○---, 20 mol%; -·-△-·-, 33.3 mol%; --□--, 42.9 mol%.

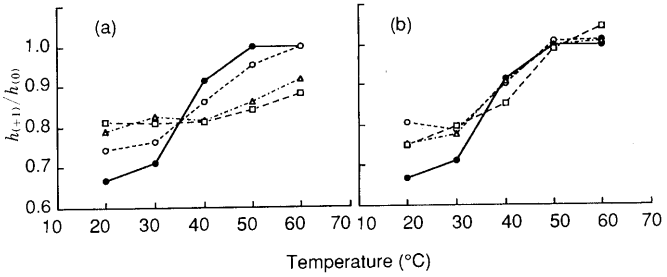

Fig. 5. Change of the Ratio of Peak Height of 16-SASL in the DPPC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

near the polar groups at the lower temperatures showed little change by Monol addition (Fig. 4b). As the ring system is somewhat shifted towards the ends of the acyl chains of DPPC, the lower condensation effect of Monol at the higher temperatures would be due to a perturbation of the interactions of the ring system of Monol with the acyl chains.[13]

Stabilization of DPPC Membranes by Monol We recently reported that a release of entrapped 6-carboxyfluorescein (6-CF) from DPPC-DCP (dicethylphosphate) liposomes at 37 °C was increased by the addition of Monol at 20 mol%.[5] In the present study, the fluidity of DPPC liposome membranes containing 20 mol% Monol was higher than that of the control below *Tm*. The destabilization of the DPPC-DCP liposomes by Monol correlates well with this fluidizing effect of Monol on the DPPC bilayer.

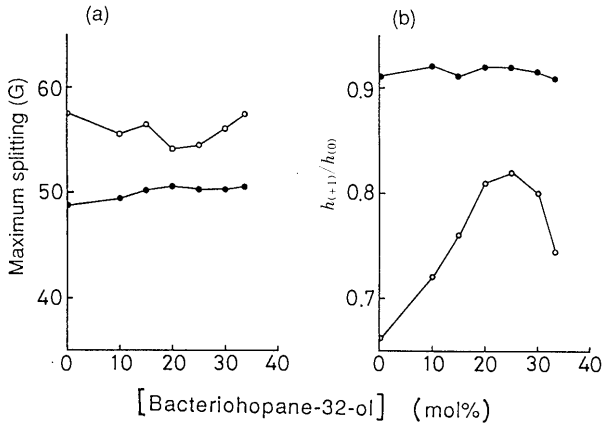

Fig. 6. Change of the Order Parameters in 5-SASL (a) and 16-SASL (b) Embedded in the DPPC Liposomes Containing Various Amounts of Monol

—○—, at 20 °C; —●—, at 40 °C.

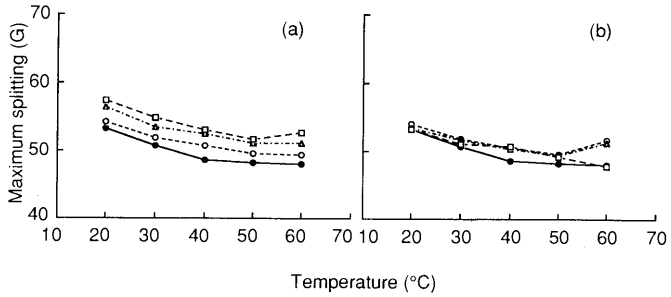

Fig. 7. Change of the Maximum Splitting of 5-SASL in the Egg PC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

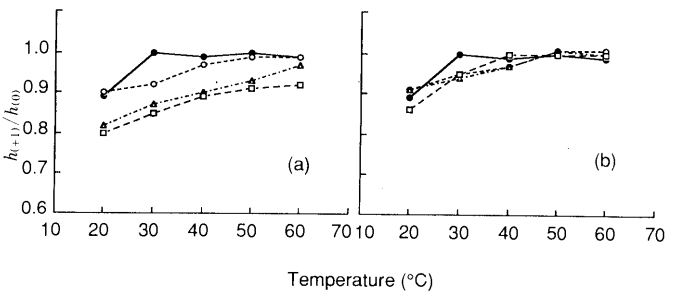

Fig. 8. Change of the Ratio of Peak Height of 16-SASL in the Egg PC Liposomes Containing Chol (a) and Monol (b) as a Function of Temperature

Symbols are the same as in Fig. 4.

To clarify the peculiar effect of 20 mol% content of Monol on the DPPC membrane, ESR spectra of 5-SASL and 16-SASL were measured under various conditions. The order parameters, the maximum splitting and $h_{(+1)}/h_{(0)}$ values were plotted against Monol concentration at two temperatures (Fig. 6). As shown in Fig. 6a, a trough could be observed around 20 mol% Monol at 20 °C. At this ratio, Monol should hold most loose packing near the phospholipid head groups interacting with its side chain. At 40 °C, however, the maximum splitting values were increased by addition of Monol, suggesting the condensing effect of this material above *Tm*.

Changes of the $h_{(+1)}/h_{(0)}$ value in the 16-SASL spectra as a function of Monol concentration are shown in Fig. 6b. A crest can be observed around 25 mol% Monol at 20 °C. The bulky ring system extending to the acyl chain ends of DPPC presumably decreases the acyl chain order around this mole ratio. At 40 °C, Monol did not affect the $h_{(+1)}/h_{(0)}$ value at any concentrations.

Effect of Monol on Egg PC Membranes Next, in order to elucidate the function of Monol, the effects of Monol on the liposome membranes composed of egg PC were compared with those of Chol. Figure 7 shows the change of the maximum splitting value of 5-SASL in egg PC liposomes as a function of temperature in the presence of various amounts of Chol or Monol. The increase of Chol content lowered the fluidity independent of temperature (Fig. 7a). The influence of Monol on the membrane fluidity is less than that of Chol (Fig. 7b). The peculiar effect of Monol observed in the DPPC membranes at the concentration of 20 mol% was not obvious in the egg PC membranes. When 16-SASL was used as a spin probe, comparative results with those shown in Fig. 7 were obtained (Fig. 8). In the egg PC liposomes, the contradictory effect of Chol shown in Fig. 5a was not observed. Monol seems to have a comparatively little influence on the fluidity of the membranes composed of unsaturated PC. The difference of the fluidity between the DPPC and egg PC membranes has resulted from the difference in their molecular packings with Chol or Monol in the hydrocarbon region of the bilayer.[11] This may be due to the fit between the ring systems of Monol and the kinks of unsaturated acyl chains in egg PC molecules. From these results, it can be said that Monol has some role in the homeostatic regulation of the membrane fluidity. This fluidity is considered to be a crucial factor in regulating certain cellular processes such as permeability of small molecules,[13] proliferation,[14,15] fusion[16] and endocytosis.[17] A decrease[18,19] or increase[20] of the membrane fluidity or activation resulted in strong inhibition of the activities of membrane bound enzymes. The incorporation of Monol into the membrane allows the fluidity to remain constant and support its normal functions under drastic conditions such as at high temperatures.

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Release of Nicotinamide from Fatty Acid–Nicotinamide Equimolar Complexes[1)]

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The release behavior of nicotinamide (NAA) from fatty acid (FA)–NAA equimolar complexes was examined in a JP XI dissolution test apparatus in JP XI disintegration test medium No. 1 (pH 1.2) at 37 °C where the carbon number of FA is 14—18. The time required for 50% or 80% of NAA to release (T_{50} or T_{80}) was measured, and the effect of the constituent FA on T_{50} or T_{80} was investigated. The values of T_{50} or T_{80} for FA–NAA formed with odd-numberd FA were larger than those for FA–NAA formed with even-numbered FA whose alkyl chain length is one more carbon number longer, though the values of T_{50} of T_{80} increased rather regularly with an increase of the alkyl chain length for only even-numbered or odd-numbered FA. The values of T_{50} and T_{80} for FA–NAA formed with heptadecanoic acid (C17–NAA) were about 36 and 102 min, respectively, suggesting that C17–NAA may be applicable to the preparation of a sustained-release drug formulation.

Keywords nicotinamide; fatty acid; complex; equimolar complex; release; sustained-release

Nicotinamide (NAA) is well-known as being biosynthesized to nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate in organisms. In addition, NAA is known as an antipellagra substance. 25—200 mg/d of NAA is applied to clinical trials for pellagra, 0.5—1 g/d of NAA is applied in serious cases.

The side effects of NAA are weaker than that of nicotinic acid which have a similar therapeutic efficacy. However, side effects such as a disturbance of the stomach and intestine and a decline of hepatic function is generated when large doses of NAA are administered. Furthermore, *in vivo* utilization of glucose is disturbed by the administration of large doses of NAA. These side effects are caused by a sudden increase of the serum concentration of nicotinic acid or NAA. For the purpose of suppression of the side effects, a sustained-release dosage of nicotinic acid derivatives is now in the process of development: for example, a patent with a slow-release drug product containing a nicotinic acid derivative as the major component and a water soluble polymer as the carrier was reported.[2)]

On the other hand, it has been reported[3)] that NAA forms a crystalline complex with a fatty acid (FA) whose molar ratio of FA to NAA is 1 : 1, FA–NAA. Such a complex formed with FA and a water soluble drug has been found for thiamine disulfide (TDS), and the release behavior of TDS from the complex has also been examined.[4)] In the studies, it is suggested that the complexes formed with FA may be applicable to the preparation of a sustained-release drug formulation. FA–NAA is, therefore, expected as a sustained-release drug. It is required to know the release behavior of NAA from FA–NAA for pharmaceutical applications. From these points of view, the release rates of NAA from FA–NAA were determined. The effect of particle size on the release rate has been investigated,[4a)] and the particle size of 48—60 mesh is suggested to be suitable at least for a subsequent study. The particle size of FA–NAA in this study was, therefore, set at a limit of 48—60 mesh.

Experimental

Materials NAA, tetradecanoic acid (C14), pentadecanoic acid (C15), hexadecanoic acid (C16), heptadecanoic acid (C17) and octadecanoic acid (C18) were the same as those used previously.[3)] The melting points of NAA, C14, C15, C16, C17 and C18 are 128—129, 53—55, 52—54, 64—65, 60—62 and 69—71 °C, respectively. FA–NAA were prepared as previously described.[3)] Purities of FA–NAA were examined with a melting point-measuring apparatus equipped with a microscope (×100), and it was confirmed that no extra free FA and/or NAA was present. The melting points of C14–NAA, C15–NAA, C16–NAA, C17–NAA and C18–NAA were 73—75, 77—79, 79—80, 80—82 and 83—85 °C, respectively. Crystals of FA–NAA were passed through 48 and 60 mesh sieves, and the particle of 48—60 mesh was collected.

Measurement of Release Rate The release of NAA from FA–NAA was tested as previously described[4)] in a JP XI dissolution test apparatus (paddle method) in 500 ml of JP XI disintegration test medium No. 1 (pH 1.2) at an agitation speed of 200 rpm at 37 °C. About 29—33 mg of each FA–NAA (this corresponds to about 10 mg of NAA) was used in the test. Aliquots of 5 ml of sample solution were withdrawn at appropriate time intervals, and the volume was kept constant by adding the same volume of fresh medium at the same temperature. The sample solution was filtered immediately through a glass filter, and the absorbance was determined. FA is insoluble in aqueous acidic solvent, and a solid residue remains after NAA is released. All release experiments were carried out at least in triplicate and the results were highly reproducible. The release rates are shown as the times required for 50% or 80% of NAA to release (T_{50} or T_{80}).

Quantitative Analysis of NAA The concentration of NAA was determined spectrophotometrically at a wavelength of 261 nm. The relationship between concentration and absorbance was found to obey Beer's law, and the molar absorptivity (ε_{261}) was obtained as $5.80 \times 10^3\ mol^{-1}\ dm^3\ cm^{-1}$.

Solubility of NAA NAA dissolved immediately in the acidic test medium. It is considered that the dissolution rate of NAA is negligible to determine the release rate of NAA from FA–NAA.

Results and Discussion

Effect of FA on the Release Rate of NAA The effect of the constituent FA on the release behavior of NAA from FA–NAA was examined, and the results are shown in Fig. 1. The percentages of released NAA were calculated with respect to the total concentration of NAA which is theoretically contained in the 1 : 1 complex, FA–NAA. As can be seen in Fig. 1, NAA was released to about 95% from C14–NAA and about 88% from C15–NAA—C18–NAA, while TDS was released[4)] to the extent of about 100% from all $(FA)_6$ (TDS).

Recently, we have confirmed that the equimolar complex FA–NAA consists of six molecules of FA and six molecules of NAA. The results were reported promptly.[5)] Furthermore, it was suggested[6)] that six molecules of NAA are included in the $(FA)_6$ host structure which consists of six molecules of FA. On the other hand, one molecule of TDS is included in $(FA)_6$.[4b)] One reason for the difference in the released percentage of drug between FA–NAA and

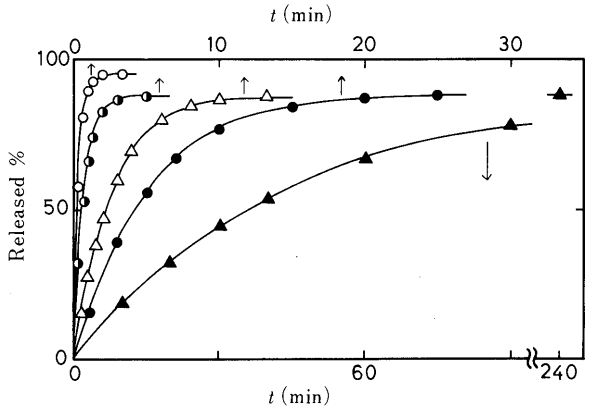

Fig. 1. Release Behavior of NAA from FA–NAA with Varying Alkyl Chain Lengths

Carbon numbers in FA: ○, 14; ◑, 16; ●, 18; △, 15; ▲, 17. Particle size: 48—60 mesh. Temperature: 37 °C.

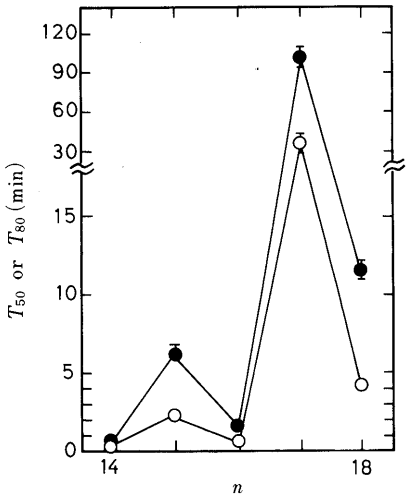

Fig. 2. Effect of FA on the Release Rate (T_{50} or T_{80}) of NAA from FA–NAA

Time required for 50% or 80% of NAA to release; ○, T_{50}; ●, T_{80}. Particle size: 48—60 mesh. Temperature: 37 °C.

$(FA)_6$(TDS) may be related to the compositions of FA–NAA and $(FA)_6$(TDS).

The values of T_{50} and T_{80} are plotted against the carbon numbers of FA, n, in Fig. 2. To indicate the variation in measured values, the difference between minimum and maximum values is shown by a bar in Fig. 2. Where no bar is shown, it lies within the symbol. The relationship between release time (T_{50} or T_{80}) and n was a zig-zag one, though the values of T_{50} or T_{80} increased rather regularly with an increase of n for only even-numbered or odd-numbered FA. This is a similar tendency to that reported for the release of TDS from $(FA)_6$ (TDS).[4] The delayed release rate for FA–NAA composed of odd-numbered FA may be considered to be due to that the interaction between odd-numbered FA and NAA is stronger than that between even-numbered FA and NAA. This is also reflected in the melting points of FA–NAA: the melting points of FA–NAA formed from even-numbered FA are about 15—20 °C higher than those of the original even-numbered FA, while the melting points of FA–NAA formed from odd-numbered FA are about 20—25 °C higher than those of the original odd-numbered FA. Furthermore, this is related to that the crystal structure of FA–NAA formed with odd-numbered FA is a little different from that formed with even-numbered FA.[3,6] In addition, the phenomenon that the release rate decreases as the carbon number of either even-numbered or odd-numbered FA increases is considered to be related to the hydrophobic character of FA. Namely, the hydrophobicity of FA becomes stronger as the carbon number of FA increases, leading to poor wettability by the test medium and consequent delayed release.

It is suggested from the *in vitro* release behaviors shown in Figs. 1 and 2 that FA–NAA formed with FA which have longer alkyl chains may be applicable to the preparation of sustained-release drug products. Regarding the clinical applications of FA–NAA, it is suggested that a sudden increase of the serum concentration of NAA, which brings about the side effects, can be suppressed by the administration of FA–NAA instead of NAA. FA–NAA is expected as a new drug product under the present situation that a sustained-release dosage of nicotinic acid derivatives is in the process of development.

Conclusion

The rate of release of NAA from FA–NAA decreased regularly with increasing n in only even-numbered or odd-numbered FA. The values of T_{50} or T_{80} for FA–NAA formed with odd-numbered FA was larger than that for FA–NAA formed with even-numbered FA whose alkyl chain length is one more carbon number longer.

It is concluded that FA–NAA as a sustained-release drug product could be clinically useful, through the investigation of the optimum dosage form of FA–NAA and extensive preliminary examinations in experimental animals and eventually human volunteers will be required.

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Antifungal Activity of Fumaric Acid in Mice Infected with *Candida albicans*

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An examination was made on the effect of fumaric acid on an experimental systemic candidiasis. Male ICR mice were innoculated into the tail veins with 10^6 yeast cells of *Candida albicans* and treated with daily intraperitoneal injections of fumaric acid at the dose of 40 mg/kg/d. The results indicated that the administration of fumaric acid was effective in prolonging the survival of animals after the fungal challenge and prevented one-fifth of the treated animals from dying of candidiasis.

Keywords fumaric acid; *Candida albicans*; systemic mycosis; antifungal activity

Introduction

In our pharmacological studies on the extract of *Capsella burse-pastoris* (Cruciferae), fumaric acid (FA) was isolated and identified as the component of the herb responsible for inhibiting the growth of subcutaneously transplanted Ehrlich tumors in mice and gastric ulceration in rats.[1,2] Thereafter, we found that FA reduced the toxic symptoms in mice and rats injected with mitomycin C or aflatoxin B_1[3–5] and that FA suppressed hepatocarcinogenesis in rats fed 3′-methyl-4-(dimethylamino)azobenzene or thioacetamide[6,7] or hepatocarcinogenesis in mice fed thioacetamide.[8] The present study indicates that FA has a different chemotherapeutic activity, *i.e.*, an antifungal activity in a model system of candidiasis, one of the most frequent human mycoses.

Materials and Methods

Animals used were male ICR mice weighing approximately 20 g. They were supplied from Clea Japan Inc., Tokyo, housed 5 to each cage, and maintained on a basal diet CE-2 (Clea Japan Inc.) and water *ad libitum*. The strain of *Candida albicans* used was ATCC 48130. Suspensions of living yeasts were prepared from fresh cultures grown 2 d on Sabrouraud dextrose agar slants at 37 °C. Yeast cells were harvested from the slants with a platinum loop, washed with saline (0.9% NaCl solution), and resuspended in saline. Then, 5×10^6 per ml cell suspension was prepared by using a Thoma's hemocytometer. Each mouse was innoculated into the tail vein with 0.2 ml of cell suspension. On the following day, mice were divided into two groups. One group received daily intraperitoneal injections of 40 mg/kg of FA (Nakarai Chemicals Ltd., Kyoto) dissolved in saline (5 mg/ml) and the other (control) group received those of saline (8 ml/kg). The observation of the death of the animals was made for 44 d. In a separate experiment, 2 animals were randomly taken from each group at 16 d after challenge, and lung, liver, spleen and kidneys were examined histologically. Tissue samples for microscopic examination were fixed in 10% formaline, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

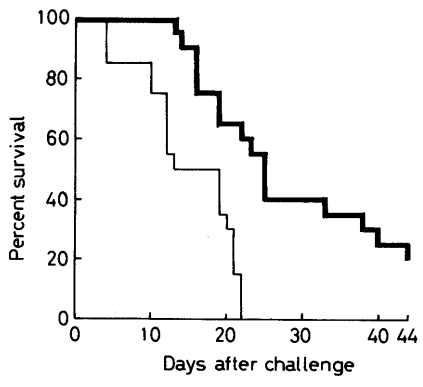

Fig. 1. Effect of FA on the Survival Time of Mice with Candidiasis

Groups of 20 mice were challenged with *Candida albicans*. They were treated 1 d after challenge with daily intraperitoneal injections of saline (8 ml/kg) or FA (40 mg/kg). The survival rate (percent survival) is indicated by a solid line for the saline-injected control group and by a bald line for the FA-injected group.

Results and Discussion

Figure 1 shows the effect of FA on the survival of mice after being challenged with *Candida albicans*. A total of 40 animals, 20 animals per group, were used for the experiment. All animals of the control group injected with saline were dead within 22 d after challenge. The injections of FA delayed the death of animals and prevented 4 animals (20% of a total of 20 animals) from dying of fungal infection after 44 d.

Histological examination was made on the animals killed at 16 d after challenge. Responses of the tissues to the fungal infection were noted in the animals of the control group; presence of secretions and focal bleeding in the bronchi of the lung (Fig. 2a), infiltration of neutrophils in the liver and kidneys (Figs. 2b and c), and a marked development of immature lymphoblasts in the white pulp of the spleen (Fig. 2d). In contrast, no such pathological responses were noted in the tissues of the animals of the FA-treated group except a slight development of immature lymphoblasts in the white pulp of the spleen (Figs. 2e—h).

The presnet findings indicate that FA effectively retarded the development of systemic candidiasis. There are a few clinically useful drugs for systemic mycoses. The classical and representative examples are amphotericin B, 5-fluorocytocin and ketokonazol.[9] However, each one has its limitation, *e.g.*, side effects, risk of resistance, or poor penetration into certain components of the body.[9] FA is a simple dicarboxylic acid and is expected to have a low toxicity in chemotherapeutic use. FA has the ability to reduce toxic symptoms in mice and rats injected with mitomycin C and aflatoxin B_1,[3–5] and is expected to be useful for the combination therapy.

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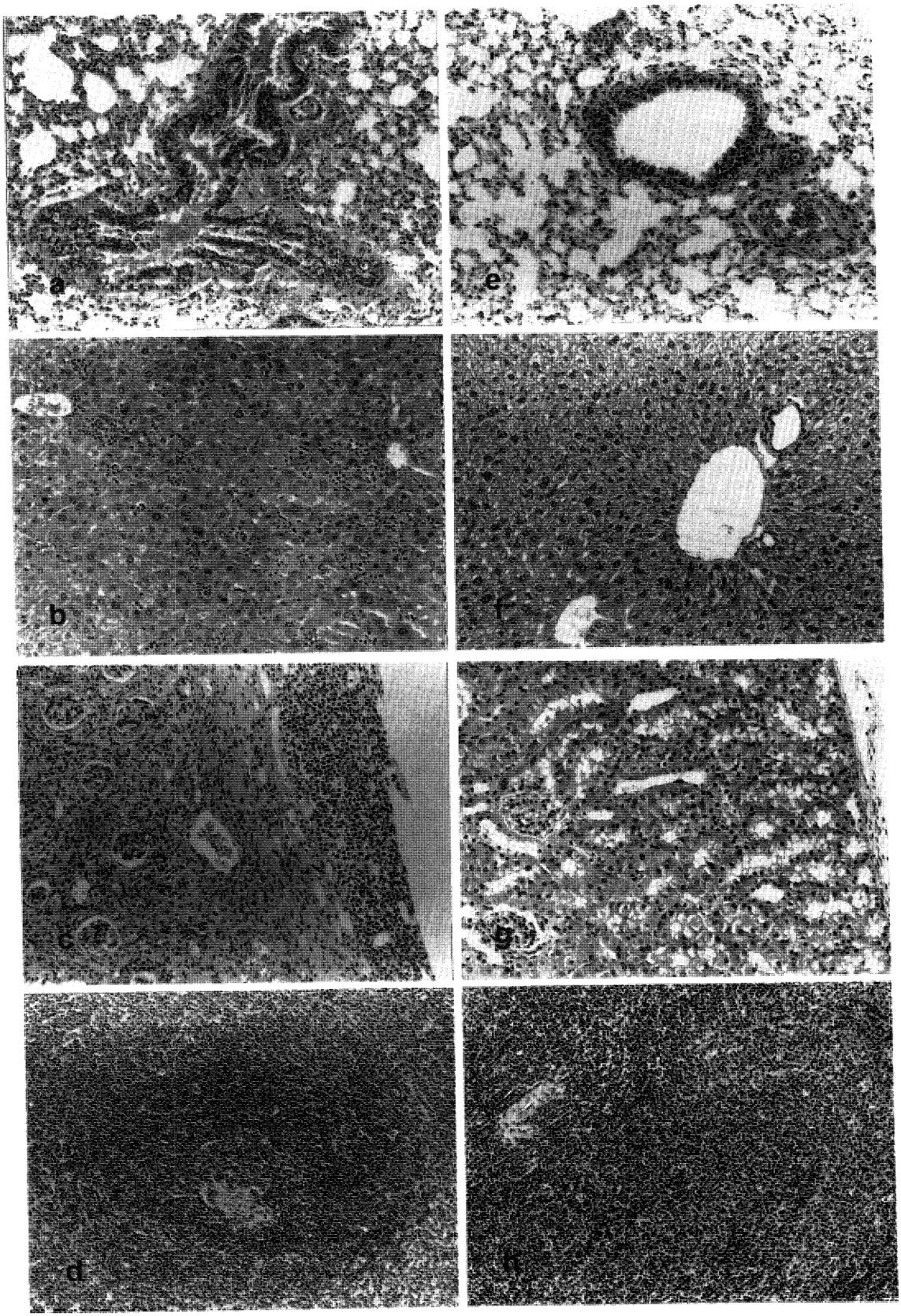

Fig. 2. Micrographs of the Tissues of Mouse Treated with Daily Injections of Saline or FA for 16 d after Challenge with *Candida albicans*

Figures 2a—d are micrographs of the tissues for the saline-injected control mouse. × 100. a) Lung; secretions and focal bleeding are seen in the bronchus. b) Liver; infiltration of neutrophils into the sinusoids. c) Kidney; infiltration of neutrophils into the fibrous membrane of the capsule. d) Spleen; a marked development of immature lymphoblasts in the white pulp. Figures 2 e—h are micrographs of the tissues for the FA-injected mouse. × 100. e) Lung, f) liver, and g) kidney; no significant pathological findings. h) Spleen; a slight development of lymphoblasts in the white pulp.

A SPIDER TOXIN BINDING PROTEIN FROM BOVINE BRAIN: ITS PURIFICATION AND N-TERMINAL AMINO ACID SEQUENCE DETERMINATION

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A 60kDa spider toxin binding protein from bovine brain was solubilized with digitonin and purified up to 5800-folds over starting crude homogenate. The purification procedure entailed DEAE-cellulose, concanavalin-A affinity, 1-naphthylacetyl spermine affinity and high performance liquid chromatography. The purified protein owned a very high affinity for ligand ^{125}I-JSTX-3 binding Kd 15.6nM and Bmax 6.5nM. The amino acid composition of the protein was determined. The N-terminal amino acid sequence analysis yielded a unique sequence:NH_2-X-Pro-X-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-X-

KEYWORDS spider toxin binding protein; glutamate receptor; 1-naphthylacetyl spermine affinity chromatography; N-terminal amino acid analysis; JSTX-3; excitatory postsynaptic potential

The development of polyamine containing toxin has been reported from spiders and wasps which exhibit pharmacological glutamate receptor(GluR) antagonism properties at synapses where quisqualate type receptors may be predominant .[1-5] But recently the action of these polyamine toxin on N-methyl-D-aspartate(NMDA)-type receptor has also been reported .[6,7] A number of synthetic analogs of spider toxin have been synthesized by Asami *et al.*[8]; among the synthetic analogs, 1-naphthylacetyl spermine (1-NA-Spm) retained the most potent reversible blocking action on GluR. This is the simplest type of GluR antagonist currently available and seems promising for its reversible properties to use as molecular probes for affinity chromatography ligand for these classes of GluR. We developed a polyclonal antibody (Ab-30) in guinea pigs against 1-NA-Spm binding protein in polyacrylamide gel bands. This Ab-30 blocked excitatory postsynaptic potentials in a similar way to that of polyamine toxin and immuno-reacted with 60kDa protein in both vertebrates and invertebrates.[9] This communication reports the purification of this 60kDa protein from bovine brain and its amino acid composition and N-terminal sequence.

Taking into account the immunoreactivity of solubilized protein, a procedure to purify immunoreactive protein has been developed from bovine brain. It should be noted that the activity of a specific GluR blocker polyamine containing Joro spider toxin (JSTX-3) in binding the fractions obtained during purification steps was also done simultaneously as a double check to purify the toxin binding protein. 980mg of Solubilized protein (solubilization buffer 50mM Tris-HCl, pH 7.4 + 2% digitonin + PIC[2mM EDTA + 2mM benzamidine-HCl + 1μM pepstatin + 10μg/ml bacitracin + 20μg/ml soyaben trypsin inhibitor + 10μg/ml antipin + 10μg/ml Chymostatin]) from bovine brain P-2 fraction[10] was loaded onto a (1.5x20cm) pre-equilibrated (20mM Tris-HCl buffer + PIC + 0.01% lubrol) DEAE-cellulose column. Following extensive washing of the column, the bound

material was eluted with a stepwise elution by 0.2M NaCl and 0.8M NaCl in equilibration buffer. The 0.8M NaCl fraction retained Ab-30 immunoreactivity as well as 99% of JSTX-3 binding activity. This active fraction was dialyzed, concentrated with centricon-30 (1mg/ml) and further purified with concanavalin-A-sepharose affinity chromatography. The bound glycomacromolecules were eluted from the column with 0.1M acetate buffer, pH 5.0, containing 2.5%(w/v) D-mannose and 2.5% (w/v) methyl-D-mannopyranoside. The eluent immediately dialyzed against equilibration buffer, concentrated as before and containing 94% of the total binding activity. This fraction was incubated with 1-NA-Spm-formyl-cellulofine affinity resin overnight with gentle stirring. After extensive washing with equilibration buffer and 0.2M NaCl, the bound protein was then eluted with 1M NaCl in equilibration buffer. The 1M NaCl fraction was dialyzed, concentrated as before. This fraction contained only immunoreactive protein and represented 14% total JSTX-3 binding activity of the initial fraction. The low yield of JSTX-3 binding activity at this step may be due to the discarding of non-specific binding protein mostly in this step. Probably this non-specific high ionic interaction of binding properties cannot be discarded in the previous two steps. But poor elution from the column or non-specific absorption or denaturation of the protein cannot be ruled out. This 1M NaCl eluted fraction was further purified to homogeneity(Fig. 1A) by high performance liquid chromatography (HPLC) using isocratic elution with buffer 25mM phosphate buffer containing 0.01% lubrol and 150mM NaCl (column TSK gel G3000 PW [7.5x600mm] at room temperature, flow rate 0.7ml per ml). The high performance liquid chromatography fraction contained 12% of the total binding activity. This fraction also retained immunoreactivity with Ab-30 (Fig. 1B). Through the four - step purification , the specific binding (JSTX-3 bound per mg of protein) increased from 17pmol to 99×10^3 pmol/mg, and the fold of purification attended about 5800-fold. If JSTX-3 binding protein is a polypeptide having molecular weight 60kDa by SDS-PAGE, this protein had a stoichiometry of JSTX-3 binding that was equivalent to 7mol of JSTX-3 per mol of 60kDa protein. E. Michaelis and his group cloned a putative hetero-oligomeric NMDA receptor complex; among the peptides, 60kDa protein contained the glutamate-binding site.[11] Identical molecular weight NMDA ligand binding protein has been reported elsewhere.[12,13]

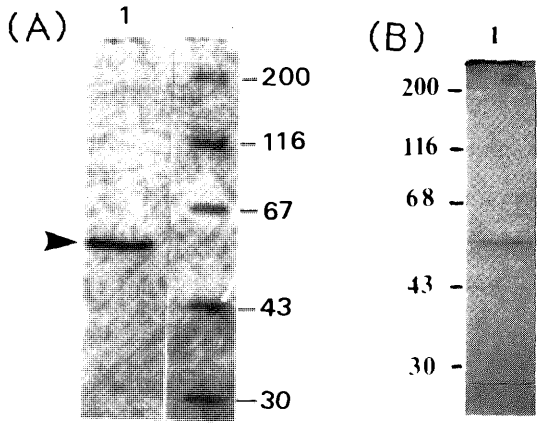

Fig. 1. A- SDS-Polyacrylamide Gel Electrophoresis of HPLC Purified Spider Toxin Binding Protein; 1: 5μg of Purified Protein(First Developed with Coomassie Blue and then with Silver Staining). B- Immunostaining with Antibody Ab-30.

The Bmax and Kd value determination by Scatchard plot revealed that 60kDa protein have very high affinity for JSTX-3 binding. Kd and Bmax values were found to be 15.6nM and 6.5nM respectively.

The amino acid composition of the protein was determined by post-column o-phthalaldehyde[14] derivation method. The results are shown in Table I and reveal that the protein contained relatively large amounts of Gly and Asp. This is an acidic protein having pI value 4.55. This protein contains the smallest amounts of Met and Cys. The average hydrophobicity of the protein was calculated and found to be 990 cal/mol .[15] The

contributions of Trp and sugar chain residues to the hydrophobicity were excluded. This result indicated the relative hydrophobic nature of the protein.

The N-terminal amino acid sequence of the protein was determined up to 15 residues, as presented here: X-Pro-X-Val-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-X-. The peptide sequence was analyzed with software developed by the Genetics computer group NBRF and SWISS-PROT, and no identity to other entries in the database was found. Furthermore, the determined N-terminal amino acid sequence of the toxin binding protein had no precedent among kainate receptor(s) reported elsewhere.[16 - 19] Therefore, we concluded this is the first N-terminal sequence information for a polyamine spider toxin binding protein or receptor from any source. Molecular biology techniques can now be employed for determination of the complete amino acid sequence, thus allowing further study on the structure/function of the polyamine toxin binding protein in glutamate-receptor-ion channel complex.

Table I. Amino Acid Composition Analysis of 60kDa Polyamine Toxin Binding Protein

Amino acid	% mol present	Amino acid	% mol present
Cys	1.6	Asp	13.0
Thr	5.5	Ser	6.8
Glu	4.2	Pro	6.6
Gly	13.3	Ala	6.4
Val	7.1	Met	1.7
Ile	5.2	Leu	6.4
Tyr	3.7	Phe	4.7
His	2.4	Lys	9.1
Arg	2.8	Trp	ND

Cys determined as cysteinic acid; ND-Not determined.

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TENUIFOLIOSES A-F, OLIGOSACCHARIDE MULTI-ESTERS FROM THE ROOTS OF *POLYGALA TENUIFOLIA* WILLD.

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From the roots of *Polygala tenuifolia* WILLD. six new oligosaccharides, named tenuifolioses A-F, were isolated and their structures were elucidated by spectroscopic data and chemical evidence. These oligosaccharides were esterified with acetic, benzoic, *p*-coumaric and ferulic acid.

KEYWORDS *Polygala tenuifolia*; Polygalaceae; tenuifoliose; oligosaccharide; acetate; benzoate; *p*-coumarate; ferulate

The root of *Polygala tenuifolia* WILLD. is a famous crude drug used as an expectorant and a tonic in China and Japan and is studied on saponins[1] and xanthones.[2] But there are few studies on the other type constituents of this crude drug, so we investigated with a view to isolating the biologically active substances other than saponin and xanthone. Now we wish to report the isolation and the structural elucidation of six new multi-acylated oligosaccharides, named tenuifolioses A-F (**1-6**).

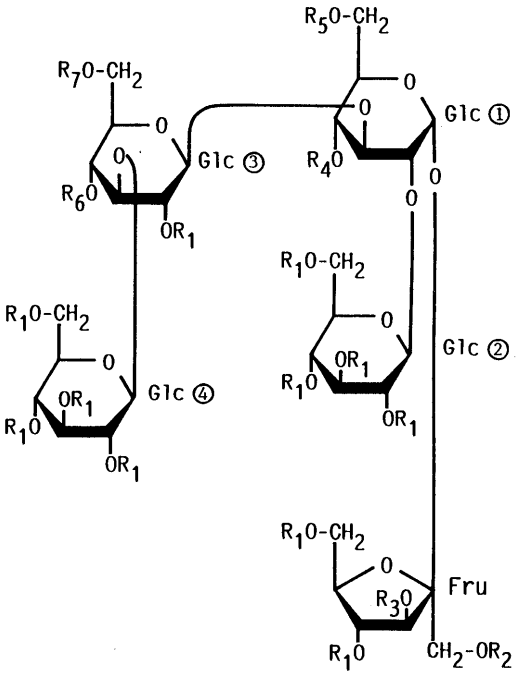
The water extract of the roots of *P. tenuifolia* WILLD. was chromatographed on MITSUBISHI Diaion HP-20 and the adsorbed material was eluted with 50% MeOH aq., 70% MeOH aq. and 100% MeOH, successively. The 70% MeOH aq. eluate was rechromatographed on silica gel using $CHCl_3$-MeOH system to give four fractions (Fr. 1-4). Six oligosaccharides were isolated by HPLC using an ODS column from Fr. 2 and 3.

Tenuifoliose A (**1**), $[\alpha]_D^{25}$ -32.8°, $C_{62}H_{76}O_{35}$,[3] was obtained as an amorphous powder, and it showed $[M + Na]^+$ and $[M + H]^+$ ions at *m/z* 1403 and 1381, respectively, in the FAB-MS. **1** gave glucose and fructose in the ratio 4:1 on acid hydrolysis , while it gave pentasaccharide **1a**[4] and an acid mixture composed of benzoic, *p*-coumaric and ferulic acid on alkaline hydrolysis.[5] The ^{1}H-NMR spectrum showed three acetyl methyl signals (δ 1.53, 1.99, 2.02), one α-linked glucosyl anomeric proton signal [δ 5.90 (1H, d, *J*=3.5 Hz)], three β-linked ones [δ 4.46 (1H, d, *J*=8 Hz), 4.53 (1H, d, *J*=8 Hz), 4.59 (1H, d, *J*=8 Hz)] and the signals due to a benzoyl, a *p*-coumaroyl and a feruloyl residue.[6] So, **1** was assumed to be a hexa-acylated pentasaccharide. Detailed proton spin decoupling experiments, difference NOE experiments on irradiation at each anomeric proton signal and ^{1}H-^{13}C COSY spectrum led us to presume the sugar linkages and the acylated sites of tenuifoliose A (**1**) as shown. The position of each acyl residue was decided by observation of $^3J_{(COCH)}$ using a long-range selective proton decoupling method. On acetylation, **1** gave peracetate **1b**[7], which had two aromatic and fourteen aliphatic acetoxyl signals in the ^{1}H-NMR spectrum. The glycosylation and acylation shifts in the ^{13}C-NMR spectrum of tenuifoliose A supported structure **1**.

The ^{1}H-NMR spectra of tenuifolioses B (**2**), C (**3**), D (**4**) and E (**5**)[3,8] showed that these four compounds were composed of a pentasaccharide **1a**, a benzoic acid, a *p*-coumaric acid, a ferulic acid and one or two acetic acids. On acetylation **2-5** gave the same peracetate **1b** and pentasaccharide **1a** on alkaline hydrolysis as in the case of **1**. Therefore the positions of benzoyl, *p*-coumaroyl, feruloyl and one or two acetyl residues were the same as **1**.

In the ^{1}H-NMR spectrum of **2**, two acetyl methyl signals were observed at δ 1.54 and 1.99, while H_2-6 of Glc ① shifted up-field at δ 3.58 and 3.67 compared with those of **1**. Therefore, two acetyl groups were located at C-4 and C-6 of Glc ③.

The ^{1}H-NMR spectrum of **3** showed one acetyl methyl signal at δ 1.59 and up-field shifted carbinyl at δ 3.32 and hydroxymethyl proton signals at δ 3.57, 3.68 which were assigned to H-4 of Glc ③ and H_2-6 of Glc ①, respectively. So, one acetyl group located at C-6 of Glc ③.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1 :	H	C	B	D	A	A	A	H	H
1a:	H	H	H	H	H	H	H	H	H
1b:	A	C	B	D	A	A	A	A	A
2 :	H	C	B	D	H	A	A	H	H
3 :	H	C	B	D	H	H	A	H	H
4 :	H	C	B	D	A	H	A	H	H
5 :	H	C	B	D	A	H	H	H	H
6 :	H	C	B	D	A	A	A	H	Rham
6b:	A	C	B	D	A	A	A	A	$Rham(OAc)_3$

A : $-CO-CH_3$

B : $-CO-C_6H_5$

C : $-CO-CH=CH-C_6H_4-OR_8$

D : $-CO-CH=CH-C_6H_3(OMe)-OR_9$

Table II. ^{13}C-NMR Data of 1 - 6 in CD_3OD

	1	2	3	4	5	6
Glc ①						
1	92.7	92.9	93.2	92.8	92.3	92.7
2	81.3	81.4	81.5	81.3	80.9	81.3
3	79.3	79.5	79.3	79.0	79.7	79.2
4	70.3	70.2	70.3	70.3	71.0	70.4
5	69.6	72.2	72.3	69.6	69.7	69.6
6	64.4	62.1	62.1	64.3	64.4	64.3
Glc ②						
1	105.4	105.3	105.4	105.3	105.4	105.3
2	75.2	75.3	75.4	75.2	75.3	75.3
3	78.5	78.4	78.5	78.4	78.5	78.4
4	71.5	71.5	71.6	71.5	71.1	71.5
5	77.7	77.7	77.7	77.6	77.7	77.7
6	63.0	63.0	63.1	63.0	63.0	63.0
Glc ③						
1	104.1	104.1	104.2	104.1	104.5	104.1
2	75.4	75.3	75.4	75.2	74.9	75.2
3	83.4	83.4	87.2	87.2	87.1	83.4
4	69.6	69.6	69.7	69.5	70.0	69.6
5	72.3	72.3	74.4	74.3	77.1	72.4
6	63.0	63.0	64.3	64.1	62.7	63.0
Glc ④						
1	105.4	105.3	105.1	105.0	105.0	105.3
2	75.7	75.7	74.8	74.6	75.3	75.7
3	78.4	78.4	78.5	78.3	78.3	78.4
4	71.5	71.5	71.6	71.5	71.5	71.5
5	77.9	77.8	78.0	77.9	78.0	77.8
6	62.8	62.8	62.6	62.6	62.6	62.8
Fru						
1	66.3	66.3	66.0	65.9	65.8	66.3
2	103.7	103.6	103.8	103.8	103.9	103.7
3	80.4	80.4	80.2	80.1	79.9	80.4
4	74.0	73.6	73.6	73.9	73.9	74.0
5	84.7	84.5	84.5	84.6	84.6	84.6
6	63.9	63.2	63.2	63.8	63.8	63.8
Rham						
1						100.7
2						71.9
3						72.1
4						73.7
5						71.0
6						18.0
Ac (R_5)						
1	172.5			172.5	172.5	172.5
2	20.8			20.8	20.8	20.8
Ac (R_6)						
1	171.9	171.9				171.9
2	21.1	21.1				21.1
Ac (R_7)						
1	172.3	172.3	172.5	172.4		172.2
2	20.3	20.3	20.5	20.4		20.3

Table I. ^1H-NMR Data of 1 - 6 in CD_3OD and Yields

	1	2	3	4	5	6
Yield	0.14 %	0.062 %	0.020 %	0.072 %	0.011 %	0.018 %
Glc ①						
1	5.90 (d,3.5)	5.88 (d,3.5)	5.85 (d,3.5)	5.89 (d,3.5)	5.87 (d,3.5)	5.90 (d,3.5)
2	3.83 (dd,9,3.5)	3.79 (dd,9.5,3.5)	3.79 (dd,9.5,3.5)	3.85 *a)*	3.83 (dd,9,3.5)	3.85 *a)*
3	3.98 (t,9)	3.98 (t,9.5)	3.98 (t,9.5)	3.98 (t,9.5)	4.04 (t,9.5)	3.98 (t,9)
4	5.03 (t,9.5)	5.03 (t,9.5)	5.02 (t,9.5)	5.04 (t,9.5)	5.02 (t,9.5)	5.03 (t,9)
5	4.43 (m)	4.25 (ddd,9.5,4,1.5)	4.22 (m)	4.41 (m)	4.39 (ddd,9.5,5,3)	4.43 (m)
6	4.15 (dd,12.5,6)	3.58 (dd,12,5)	3.57 (dd,12.5,5.5)	4.15 (dd,12,5.5)	4.15 (dd,12,5.5)	4.16 (dd,12.5,5.5)
	4.20 (dd,12.5,2)	3.67 (dd,12,2)	3.68 (dd,12.5,1.5)	4.19 (dd,12,3)	4.19 (dd,12,2)	4.21 (dd,12.5,2)
Glc ②						
1	4.59 (d,8)	4.59 (d,8)	4.58 (d,7.5)	4.60 (d,8)	4.58 (d,8)	4.60 (d,8)
Glc ③						
1	4.53 (d,8)	4.50 (d,8)	4.49 (d,8)	4.54 (d,8)	4.48 (d,8)	4.53 (d,8)
2	3.30 (dd,9,8)	3.30 (dd,9,8)	3.23 (t,9)	3.25 *a)*	3.23 *a)*	3.31 *a)*
3	3.61 (t,9)	3.60 (t,9.5)	3.37 (t,9)	3.37 (t,9)	3.38 (t,9)	3.61 (t,9)
4	4.73 (t,9.5)	4.74 (t,9.5)	3.32 *a)*	3.35 *a)*	3.28 *a)*	4.73 (t,9)
5	3.20 (td,3,9.5)	3.18 (td,3,10)	3.10 (ddd,9,3,1.5)	3.13 (td,3,9.5)	3.05 (ddd,9.5,4.5,2)	3.20 (td,2,10)
6	3.68 (dd,12,2)	3.68 (dd,12,2)	4.01 (dd,12,2)	4.01 (dd,12,2)	3.45 (dd,12,5)	3.66 (dd,12,2)
	3.98 (dd,12,3.5)	3.98 (dd,12,3.5)	4.06 (dd,12,3.5)	4.07 (dd,12,4)	3.62 (dd,12,2)	3.98 (dd,12,3.5)
Glc ④						
1	4.46 (d,8)	4.46 (d,8)	4.47 (d,8)	4.48 (d,8)	4.47 (d,8)	4.47 (d,8)
Fru						
1	4.29 (d,12)	4.30 (d,12)	4.23 (d,12)	4.23 (d,12)	4.19 (d,12)	4.30 (d,12)
	4.60 (d,12)	4.63 (d,12)	4.70 (d,12)	4.69 (d,12)	4.70 (d,12)	4.61 (d,12)
3	5.75 (d,8)	5.73 (d,8)	5.72 (d,8)	5.75 (d,8)	5.75 (d,8)	5.75 (d,8)
4	4.43 (t,9)	4.51 (t,8)	4.50 (t,8)	4.44 (t,8)	4.43 (t,8)	4.43 (t,8)
5	4.09 (ddd,8,7,3)	4.06 (ddd,8,6,3)	4.03 (m)	4.09 (m)	4.07 (ddd,8,7,3)	4.09 (m)
6	3.84 (dd,12,3)	3.86 (dd,12.5,3)	3.84 (dd,12.5,3)	3.86 *a)*	3.84 (dd,12.5,3)	3.83 *a)*
	3.89 (dd,12,6)	3.90 (dd,12.5,6)	3.88 (dd,12.5,6)	3.88 (dd,12.5,6)	3.89 (dd,12.5,6)	3.87 (dd,12.5,6)
Rham						
1						5.49 (d,1.5)
2						4.11 (dd,3.5,1.5)
3						3.93 *a)*
4						3.49 (t,9.5)
5						3.75 (m)
6						1.24 (d,6.5)
Ac (R_5)	2.02 (s)			2.07 (s)	2.07 (s)	2.08 (s)
Ac (R_6)	1.99 (s)	1.99 (s)				1.99 (s)
Ac (R_7)	1.53 (s)	1.54 (s)	1.59 (s)	1.59 (s)		1.50 (s)

a) Overlapped with other signals.

The ^{1}H-NMR spectrum of **4** showed the presence of two acetyl groups at C-6 of Glc ① and C-6 of Glc ③, and that of **5** showed only one acetyl group at C-6 of Glc ① by down-field shifts.

Tenuifoliose F (**6**), $[\alpha]_D^{25}$ -52.3°, $C_{68}H_{86}O_{39}$,[3] was obtained as an amorphous powder, and it showed an $[M + Na]^+$ ion at *m/z* 1549 in the FAB-MS. **6** gave glucose, fructose and rhamnose in the ratio 4:1:1 on acid hydrolysis, while **6** gave pentasaccharide **1a** and the same acid mixture as in the case of **1** on alkaline hydrolysis. The ^{13}C-NMR spectrum revealed the presence of rhamnose other than **1**. On acetylation **6** gave peracetate **6b**[9], and its ^{1}H-NMR spectrum showed the presence of one aromatic and seventeen aliphatic acetoxyl signals. An NOE was observed at H-5 of feruloyl residue by irradiation at the anomeric proton signal of rhamnose, suggesting **6** to be a phenolic rhamnoside. The rhamnosyl anomeric configuration was α from the $J_{C1\text{-}H1}$ (171.3 Hz). These data led us to conclude the structure of **6**.

From *P. amarella* , a tetrasaccharide hexa-ester amarelloside,[10] and from *P. chamaebuxus*, three sucrose di-esters[11] were isolated, but tenuifolioses A-F (**1-6**) were the first examples of pentasaccharide multi-esters from *Polygala* species.

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3) The molecular formula was determined by elemental analysis.

4) Amorphous powder, $[\alpha]_D^{27}$ +4.8° (H_2O), FAB-MS *m/z*: 851 $[M + Na]^+$. ^{1}H-NMR (D_2O): δ 4.01 (1H, t, *J*=8.5 Hz, H-3 of Glc ①), 4.13 (1H, t, *J*=9.5 Hz, H-3 of Glc ③), 4.20 (1H, d, *J*=9 Hz, H-1 of Glc ④), 4.69 (1H, d, *J*=8 Hz, H-1 of Glc ③), 4.82 (1H, d, *J*=8 Hz, H-1 of Glc ②), 5.52 (1H, d, *J*=3 Hz, H-1 Glc ①). ^{13}C-NMR (D_2O): δ 60.8, 61.4 (2C), 61.6, 61.7, 63.1, 68.5, 69.0, 70.3, 70.5, 72.8, 74.0, 74.2 (2C), 74.7, 76.3 (2C), 76.5, 76.7 (3C), 79.5, 80.2, 82.3, 85.4, 92.7, 102.6, 103.6, 103.9, 104.7. Dioxane (δ 3.73, 67.3) was used as an internal standard.

5) These acids were identified by HPLC. Conditions: Column, YMC R-ODS-7 4.6 x 250 mm; Solvent, CH_3CN-H_2O (22.5 : 77.5) + 0.05% TFA; Flow rate, 1.0 ml/min; Detector, UV 270 nm; t_R, 8.4 min (*p*-coumaric acid), 10.0 min (ferulic acid), 17.2 min (benzoic acid).

6) NMR data of ester moieties other than acetates. ^{1}H-NMR (CD_3OD): δ (benzoate) 7.61 (2H, t, *J*=8 Hz, H-3, H-5), 7.69 (1H, br t, *J*=8 Hz, H-4), 8.21 (2H, br d, *J*=8 Hz, H-2, H-6), (*p*-coumarate) 6.31 (1H, d, *J*=16 Hz, H-β), 6.80 (2H, d, *J*=8.5 Hz, H-3, H-5), 7.35 (2H, d, *J*=8.5 Hz, H-2, H-6), 7.64 (1H, d, *J*=16 Hz, H-γ), (ferulate) 3.93 (3H, s, OMe), 6.26 (1H, d, *J*=16 Hz, H-β), 6.85 (1H, s, *J*=8Hz, H-5), 7.05 (1H, dd, *J*=8, 2 Hz, H-6), 7.17 (1H, d, *J*=2 Hz, H-2), 7.61 (1H, d, *J*=16 Hz, H-γ). ^{13}C-NMR (CD_3OD): δ (benzoate) 130.0 (C-3, C-5), 131.1 (C-1, C-2, C-6), 134.8 (C-4), 167.1 (C-α), (*p*-coumarate) 114.9 (C-β), 116.9 (C-3, C-5), 127.0 (C-1), 131.2 (C-2, C-6), 146.8 (C-γ), 161.3 (C-4), 168.3 (C-α), (ferulate) 56.4 (OMe), 111.5 (C-2), 115.1 (C-β), 116.6 (C-5), 124.4 (C-6), 127.5 (C-1), 147.1 (C-γ), 149.4 (C-3), 150.9 (C-4), 167.8 (C-α). These carbon signals were assigned by a ^{1}H-^{13}C COSY and a long-range ^{1}H-^{13}C COSY spectra.

7) Amorphous powder, FAB-MS *m/z*: 1950 $[M + Na]^+$, 1928 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$): δ 1.75, 1.96, 1.97, 2.08, 2.10, 2.15 (each 3H, s, aliphatic OAc), 2.32, 2.33 (each 3H, s, aromatic OAc), 2.00, 2.01, 2.05, 2.08 (each 6H, s, aliphatic OAc).

8) **2**: Amorphous powder, $[\alpha]_D^{25}$ -35.9°, $C_{60}H_{74}O_{34}$, FAB-MS *m/z*: 1361 $[M + Na]^+$, 1339 $[M + H]^+$. **3**: Amorphous powder, $[\alpha]_D^{25}$ -8.3°, $C_{58}H_{72}O_{33}$, FAB-MS *m/z*: 1319 $[M + Na]^+$, 1297 $[M + H]^+$. **4**: Amorphous powder, $[\alpha]_D^{25}$ -10.5°, $C_{60}H_{74}O_{34}$, FAB-MS *m/z*: 1361 $[M + Na]^+$, 1339 $[M + H]^+$. **5**: Amorphous powder, $[\alpha]_D^{25}$ -2.2°, $C_{58}H_{72}O_{33}$, FAB-MS *m/z*: 1319 $[M + Na]^+$, 1297 $[M + H]^+$.

9) Amorphous powder, FAB-MS *m/z*: 2180 $[M + Na]^+$, 2158 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$): δ 1.75, 1.96, 1.97, 2.03, 2.08, 2.10, 2.20 (each 3H, s, aliphatic OAc), 2.00, 2.01, 2.06 (each 6H, s, aliphatic OAc), 2.08 (12H, s, aliphatic OAc), 2.32 (3H, s, aromatic OAc).

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IMMOBILIZATION OF YEAST AND THE FUNCTION OF IMMOBILIZED YEAST

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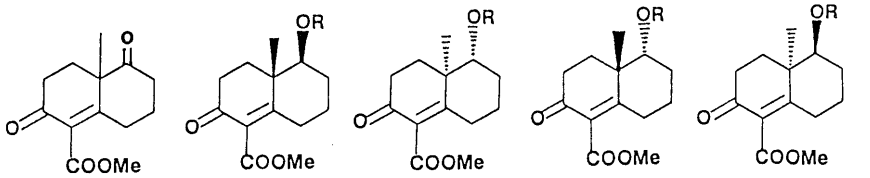
Hansenula anomala, which catalyzes an asymmetric reduction, was immobilized in bulk or spherical crosslinked polymers. The catalytic activity of the yeast for the enantioselective reduction of 3,8-dioxo-4-methoxycarbonyl-9-methyl-Δ4(10)-octalin (1ab) was severely affected by the immobilization conditions, such as the crosslink density, and by hydrophobicity of polymers and other components used for immobilization, oxygen concentration in the medium, etc. In some immobilized systems, the ratio of resulting enantiomers was inverted from that in a free yeast system.

KEYWORDS yeast; immobilization; enantioselective reduction; hydrogel; suspension polymerization

Reduction of 3,8-dioxo-4-methoxycarbonyl-9-methyl-$\Delta^{4(10)}$-octalin (1ab) catalyzed by free Hansenula anomala results in the formation of only the enantiomer (+)-8S-hydroxy-4-methoxycarbonyl-9S-methyl-3-oxo-$\Delta^{4(10)}$-octalin (2a) (Chart 1).[3)]



1ab 2a:R=H 3a:R=(+)MTPA 2b:R=H 3b:(+)MTPA 2c:R=H 3c:R=(+)MTPA 2d:R=H 3d:(+)MTPA

Chart 1

There have been many studies on the immobilization of cells in polymers to prepare bioreactors,[4~6)] but few papers have dealt with the immobilization of yeasts for enantioselective reduction. In this study, Hansenula anomala was immobilized in polymer mass or particles and the effect of immobilization on the enantioselective reduction was investigated.

The immobilization in polymer mass was carried out by bulk copolymerizations in the presence of Hansenula anomala. The standard procedure [7)] is: Preparation of dispersion (A) of 10 g of Hansenula anomala in 20 ml water, 30 ml aqueous solution (B) of acrylamide (AAm, 7.5 g) and methylenebisacrylamide (MBAAm, 0.4 g) and 2 ml solution (C) of 0.255 g 3-dimethylaminopropionitrile and 0.05 g potassium persulfate, followed by bulk polymerization in which A, B and C were mixed and kept at 25°C for 24 h.

The resulting gel was cut into 5 × 5 × 5 mm cubes. Reduction of lab was carried out as described in a previous paper[3] although immobilized Hansenula anomala was employed in the present system instead of free Hansenula anomala. Polymer cubes containing 10 mg of Hansenula anomala were added to each reaction system. After a 3-day reaction, the determination and identification of the reaction products were done by NMR of (+)-α-methoxy-α-(trifluoromethyl)phenyl acetates of products.

First, Hansenula anomala was immobilized in poly(AAm-co-MBAAm) bulk gels having different crosslink densities. These samples were used for the catalytic reduction. The results are shown in Fig. 1. It was found that appropriate crosslink density or appropriate MBAAm/AAm ratio is necessary to keep the yield and selectivity high. But even in the best case (MBAAm/AAm = 0.9/7.5), immobilized Hansenula anomala no longer exhibited its perfect selectivity in enantioselective reduction.

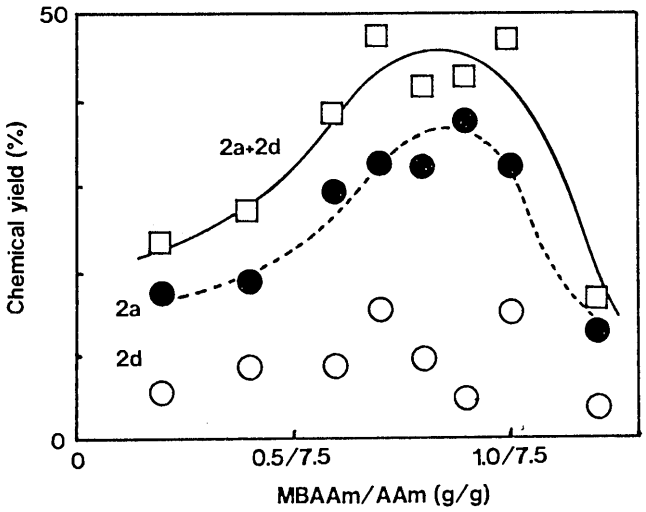


Fig. 1. Dependence of Microbiological Reduction on Crosslink Density in Carrier for Hansenula anomala

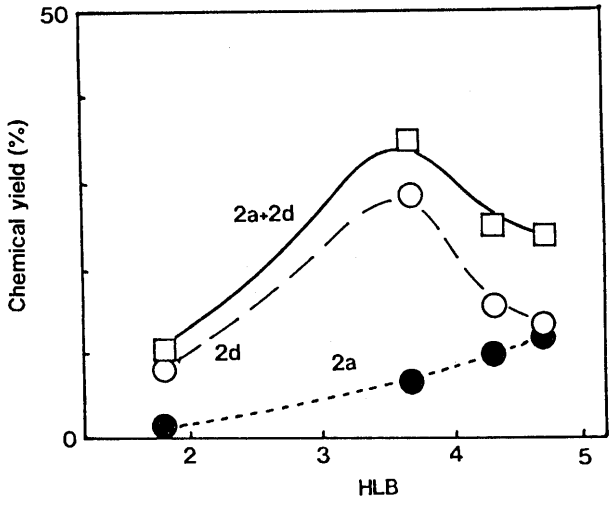


Fig. 2. Dependence of Microbiological Reduction on HLB of Surfactant Used for Inverse Suspension Polymerization

Some polymers other than crosslinked poly-AAm were used as carriers for immobilization of Hansenula anomala. They were crosslinked polyacryloylpyrrolidine (APr), poly-N-methylacrylamide (NMAAm), their copolymers and others. The chemical yield and 2a/2d for these carrier systems are summarized in Table 1. Among the polymers employed, the copolymer of 1:1 acrylamide and N-methylacrylamide gave the best result (yield 52%, 2a/2d 92/8), perhaps due to its suitable hydrophilicity.

Table 1. Dependence of Microbiological Reduction on the Carriers for Hansenula anomala

Polymer	Chemical yield (%)	Diketone recovery (%)	2a/2d	Optical purity (% e.e.)
APr	23	73	50/50	2a > 99, 2b > 99
NMAAm	50	52	62/38	2a > 99, 2b > 99
NMAAm/AAm (1:1)	52	41	92/08	2a > 99, 2b > 99
NMAAm/AAm (1:2)	51	47	71/29	2a > 99, 2b > 99
AAm	41	40	78/22	2a > 99, 2b > 99
(Free)	36	54	100/00	2a > 99

e.e.; enantiomeric excess.

Hansenula anomala-immobilized gel spheres were prepared by inverse suspension polymerization in water-in-oil emulsion.[8,9] The water phase contained Hansenula anomala, monomers and initiator. The oil phase was composed of hexane/chloroform solution with emulsifier. The polymerization resulted in the formation of gel spheres having 100 - 700 μm diameters. The inverse suspension polymerization damaged the viability or the ability of the yeast to reduce the substrate asymmetrically. The damage was severe, especially when Hansenula anomala came in contact, in the course of immobilization, with hydrophobic compounds such as hexane and emulsifiers having low Hydrophilic Lipophilic Balance (HLB).

Figure 2 shows the dependence of enantioselective reactivity of immobilized Hansenula anomala on the HLB of solbitan-type emulsifier used for the suspension polymerization. The 2a/2d increased with increasing HLB of emulsifier. Use of solbitan monoolate (HLB 1.8) resulted in large particles. The large size, and consequent small surface/volume ratio, would be responsible for low reaction yields.

Some of the immobilized Hansenula anomala gave 2a/2d < 1. If there is an immobilized system giving 2a << 2d, it will be useful for the preparation of unusual enantiomers. Among the systems employed, the systems of poly-hydroxymethyl acrylamide bulk gel and spherical poly-AAm gel prepared using sorbitan monoolate gave the smallest 2a/2d (= 13/87). As a reference, Hansenula anomala was immobilized in calcium arginate spheres by the usual procedure.[10] This system gave 2a/2d = 94/6.

The effects of components used for immobilization of Hansenula anomala on enantioselective reactivity of free Hansenula anomala were studied. The reactivity was measured in aqueous dispersion of Hansenula anomala in the presence of each component. In aqueous systems of AAm monomer and four emulsifiers, 2a/2d was 100/0. But the yield of products depended on the HLB of emulsifiers. Low HLB emulsifiers and hexane caused low yield of 2a. Highly hydrophobic compounds seem to suppress the smooth transfer of reactant and product through the polymer phase. Coexistence of polyacrylamide particles in the reaction system caused a decrease in 2a/2d even if Hansenula anomala was not immobilized intentionally. This might be attributed to attachment of a small fraction of cells on the polymer or some toxicity of the polymer.[11]

In order to complete suspension polymerization, oxygen should be excluded. But it was found that a certain amount of oxygen must remain during the course of polymerization to keep the enantioselective reactivity of immobilized Hansenula anomala.

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KNI-102, A NOVEL TRIPEPTIDE HIV PROTEASE INHIBITOR CONTAINING ALLOPHENYLNORSTATINE AS A TRANSITION-STATE MIMIC[1)]

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HIV-1 protease inhibitors containing allophenylnorstatine[Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]-Pro (*syn* diastereomer) as a transition-state mimic were established to be potent and highly selective. Z-Asn-Apns-Pro-NHBu^t (KNI-102) is the only tripeptide exhibiting substantial anti-HIV activity and may be of minimum size for potent, selective inhibition of HIV protease. Ready availability due to its simple chemical structure and stability should make it valuable for studies of the development of metabolically stable anti-AIDS drugs.

KEYWORDS HIV protease; HIV protease inhibitor; HIV; peptide synthesis; hydroxymethylcarbonyl isostere; transition-state mimic; phenylnorstatine; allophenylnorstatine; AIDS

The human immunodeficiency virus type-1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), codes for a virus-specific aspartic protease responsible for processing the *gag* and *gag-pol* polyproteins and for the proliferation of the retrovirus. The HIV-1 protease functions as a homodimer and can recognize Phe-Pro and Tyr-Pro sequences as the cleavage site, but mammalian aspartic proteases do not have such specificity. These features provided a basis for the rational design of selective HIV protease-targeted drugs for the treatment of AIDS and related complex.[2)]

Several HIV-1 protease inhibitors[3)] have been discovered based on the transition-state analogue concept which was known to be effective in studies of inhibitors of aspartic proteases such as renin and pepsin. These inhibitors contain a critical hydroxyl group as a transition-state mimic which interacts with the catalytically active aspartic acid carboxyl groups of protease, and the stereochemistry of the hydroxyl group[4)] is important for the inhibition.

In the previous paper,[5)] we have described potent and selective HIV-1 protease inhibitors containing a hydroxymethylcarbonyl (HMC) isostere as a transition-state mimic (Fig.1). Unexpectedly, the (2*S*)-hydroxymethylcarbonyl (HMC) inhibitor (KNI-93; **1S**: *syn*[4)] diastereomer) containing allophenylnorstatine (Apns) was more active against HIV-1 protease than the *anti* [4)] diastereomer (KNI-122, **1A**) containing phenylnorstatine (Pns), in contrast to the case of renin inhibitors[6)] which show a preference for the *anti* diastereomer [*e.g.*, cyclohexylnorstatine (Chns)-containing inhibitors; Fig.2].

However, a preferred configuration of the hydroxyl group in a series of HMC inhibitors of HIV protease remains to be established. Also, long-chain peptides are unsuitable for a metabolically stable anti-HIV drug, and it is necessary to minimize the number of natural peptide bonds and reduce the molecular weight. We therefore sought the minimum size required for potent inhibition and the preferred hydroxyl group configuration in HMC-inhibitors, and can now report a novel tripeptide, Z-Asn-Apns-Pro-NHBu^t (KNI-102; **6S**: *syn* diastereomer) which exhibits a potent, selective inhibitory activity against HIV-1 protease (Fig.3, Table 1). In contrast, its *anti* diastereomer, Z-Asn-Pns-Pro-NHBu^t (**6A**) exhibits little inhibitory activity,

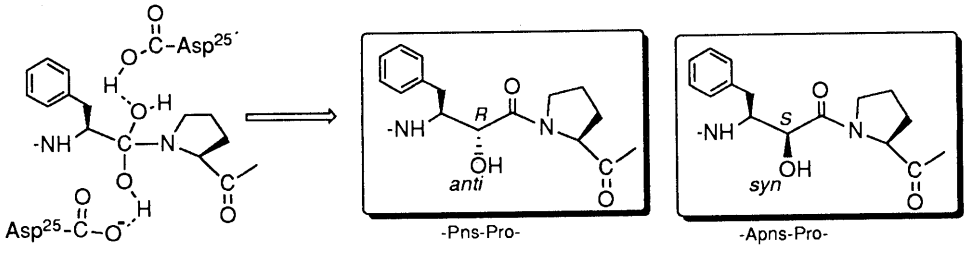

Fig.1. The Phe-Pro Transition State in HIV-1 Protease and P_1-P_1' Pns-Pro and Apns-Pro with the Hydroxymethylcarbonyl (HMC) Isostere Mimicking the Transition State
Pns = phenylnorstatine = (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; Apns = allophenylnorstatine = (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid.

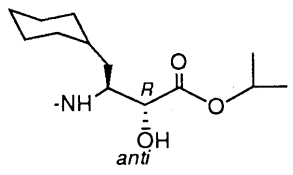

Fig.2. P_1-P_1' Chns Isopropyl Ester in Renin Inhibitors
Cyclohexylnorstatine (Chns)= (2*R*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid.

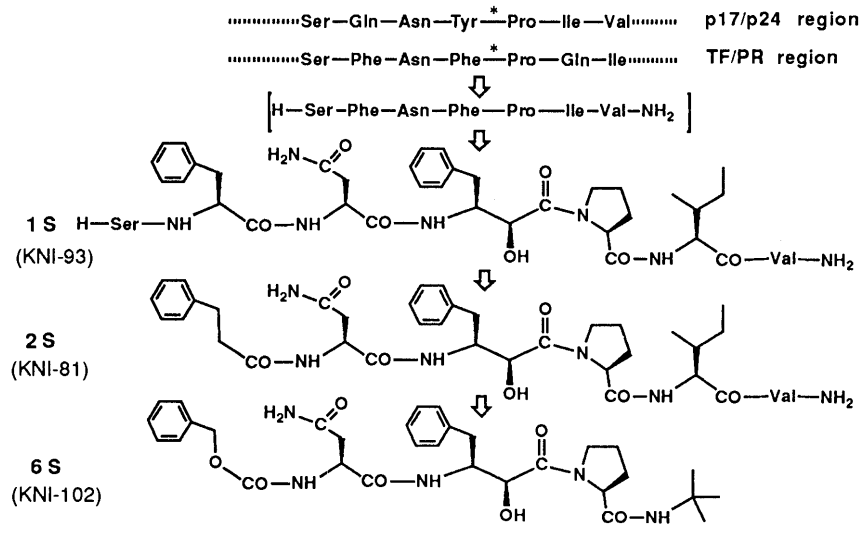

Fig.3. Design of Substrate-Based Inhibitors of HIV Protease

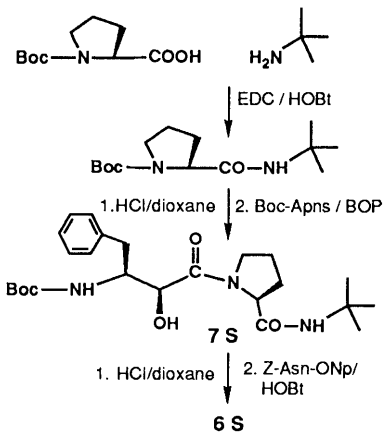

Chart 1. Synthesis of KNI-102 (**6S**)
Boc = t-butoxycarbonyl; BOP = benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; Z = benzyloxycarbonyl; ONp = *p*-nitrophenyl ester; EDC= 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt=N-hydroxybenzotriazole.

that is, HMC-Pro inhibitors show a preference for the *syn* diastereomer exceptionally.

As previously described,[5] we focused on Phe-Pro scissile site which was a unique structure for HIV-1 protease in the design of substrate-based HIV protease inhibitors (Fig.3), and incorporated the HMC isostere as a transition-state mimic at P_1 site in a heptapeptide amide, Ser-Phe-Asn-Phe-Pro-Ile-Val-NH_2, similar to the TF/PR and p17/p24 sequences. In order to obtain smaller inhibitors, we deleted P_4 Ser and replaced P_3 Phe with the isosteric 3-phenylpropionic acid. Moreover, we replaced P_3 Phe with the isosteric benzyloxycarbonyl group, and deleted P_3' Val and replaced P_2' Ile with the isosteric t-butyl amine. We also designed symmetric-type inhibitors containing HMC structure at the symmetric axis based on the dimeric character of HIV protease.

These peptides except for **6S** and **6A** were synthesized by the efficient solid-phase method.[5,7] Compound **6S** was conveniently synthesized by the solution method in a stepwise manner (Chart 1). After the acidolytic removal of the Boc group, Boc-Apns was condensed by the means of BOP reagent without the protection of the hydroxy group to give **7S** in almost quantitative yield. After the coupling reaction of Z-Asn-ONp at the last stage, pure **6S** was obtained readily. Compound **6A** was prepared by the same procedure. The protease inhibitory activities of HMC isostere-containing peptides (Table I) were examined by using the chemically synthesized [$Ala^{67,95}$]-HIV-1 protease[5] and the synthetic substrate, Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2.[8]

Compound **2S** (KNI-81) containing Apns, in which P_4 Ser was deleted and P_3 Phe was replaced by the isosteric 3-phenylpropionic acid, exhibited substantial inhibitory activity, and was more active than the *anti* diastereomer (**2A**). Replacement of P_2 Asn with Ser[9] (compound **3S**) decreased the inhibitory activity, but compound **3S** was also more active than the *anti* diastereomer (**3A**). Furthermore, incorporation of phenylacetyl group at P_3 site (compounds **4A** and **4S**)

Table I. Protease Inhibitory Activities of HMC Compounds (IC_{50}, nM)

No	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	HIV Protease	Pepsin
1A (KNI-122)	Ser-	Phe-	Asn-	Pns-	Pro-	Ile-	Val-NH_2[5]	100	>10,000
1S (KNI-93)	Ser-	Phe-	Asn-	Apns-	Pro-	Ile-	Val-NH_2[5]	5	>10,000
2A		Pp-	Asn-	Pns-	Pro-	Ile-	Val-NH_2[5]	3,000	>10,000
2S (KNI-81)		Pp-	Asn-	Apns-	Pro-	Ile-	Val-NH_2	468	>10,000
3A		Pp-	Ser-	Pns-	Pro-	Ile-	Val-NH_2[5]	>10,000	N.D.
3S		Pp-	Ser-	Apns-	Pro-	Ile-	Val-NH_2	1,594	N.D.
4A		Pa-	Ser-	Pns-	Pro-	Ile-	Val-NH_2	>10,000	N.D.
4S		Pa-	Ser-	Apns-	Pro-	Ile-	Val-NH_2	5,041	N.D.
5A		Pp-	Asn-	Chns-	Pro-	Ile-	Val-NH_2[5]	>10,000	N.D.
5S		Pp-	Asn-	Achns-	Pro-	Ile-	Val-NH_2	1,999	N.D.
6A		Z-	Asn-	Pns-	Pro-$NHBu^t$			>10,000	N.D.
6S (KNI-102)		Z-	Asn-	Apns-	Pro-$NHBu^t$			89	>100,000
8A		Val-	Val-	Pns-	Phe-	Val-	Val-NH_2[5]	350	4,000
8S		Val-	Val-	Apns-	Phe-	Val-	Val-NH_2	2,600	>10,000

Pp = 3-phenylpropionyl; Pa = phenylacetyl; Achns = allocyclohexylnorstatine = (2*S*,3*S*)-3-amino-4-cyclohexyl-2-hydroxybutyric acid; Bu^t=t-butyl; N.D. = not determined.

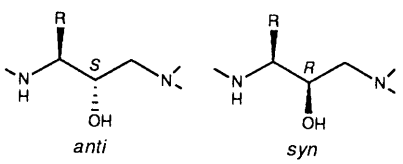

Fig. 4. Hydroxyethylamine Isostere

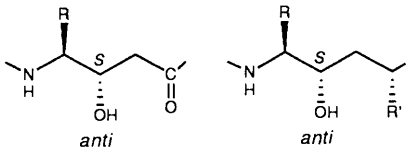

Fig.5. Statine-Type Isostere **Fig.6.** Hydroxyethylene Isostere

decreased the potency, and also in this case, the *syn* configuration of the hydroxyl group was preferred. Replacement of P_1 Apns with allocyclohexylnorstatine (Achns)[10] (compound **5S**) reduced the inhibitory potency, and also in this case, the Achns-containing inhibitor was more active than the Chns-containing inhibitor (**5A**), in contrast to the case of renin inhibitors.[6] The Apns-containing tripeptide **6S** (KNI-102), in which P_3 Phe was replaced by the isosteric benzyloxycarbonyl group, P_3' Val deleted and P_2' Ile replaced by the isosteric t-butylamine, exhibited a higher activity compared to the pentapeptide **2S**. It was surprising that such a small compound as **6S** was more potent than a longer compound **2S**. On the other hand, the Pns-containing tripeptide **6A** exhibited little activity even at a concentration of 5μM.

Thus it became clear that the *syn* configuration of the hydroxyl group was preferred in both cases of low molecular-weight and long-chain HMC-Pro inhibitors of HIV protease. In contrast, symmetric-type inhibitors containing P_1' Phe showed a preference for the *anti* diastereomer (**8A**), in agreement with the case of renin inhibitors.[6] This difference of HMC-Pro inhibitors from symmetric-type inhibitors may be based on the unique conformation of the proline residue at P_1' site.

In the case of hydroxyethyl-Pro-type inhibitors of HIV protease (Fig.4),[11,12] the preference shifted from the *syn* diastereomer in short-chain inhibitors to the *anti* diastereomer in long inhibitors, in contrast to the series of HMC-Pro inhibitors. The discrepancy between the HMC-Pro inhibitors and the hydroxyethyl-Pro inhibitors seems to be due to the conformational difference between the constrained peptide bond and the relatively flexible methylene-amine bond. A preference for the *syn* hydroxyl group shown in this series of HMC-Pro inhibitors is exceptional among various inhibitors of aspartic proteases such as HIV protease, renin and pepsin, which implies the uniqueness of the HMC-Pro structure.

As shown in Table I, HMC-Pro inhibitors of HIV protease did not practically inhibit pepsin but the symmetric-type compound containing P_1' Phe (**8A**) did inhibit pepsin,[5] which showed that Apns-Pro inhibitors of HIV protease were selective. Especially, the tripeptide containing Apns-Pro (**6S**; KNI-102) was a potent and highly selective HIV protease inhibitor, causing no inhibition of pepsin even at a concentration of 80μM. Since dipeptides Boc-Pns-Pro-$NHBu^t$ and Boc-Apns-Pro-$NHBu^t$ (**7S**) exhibited little inhibitory activity (IC_{50} > 10,000 nM; not shown in Table I), the tripeptide, KNI-102 (**6S**) was identified to be of minimum size required for potent inhibition. None of four peptide bonds in KNI-102 are natural type, and KNI-102 exhibits substantial anti-HIV activity whereas long-chain peptides have little antiviral activity,[13,14] which implies that the tripeptide is relatively stable in the cell.

In conclusion, Apns-Pro inhibitors (*syn* diastereomer) of HIV-1 protease were established to be potent and highly selective. KNI-102 is the only tripeptide exhibiting substantial anti-HIV activity and may be of minimum size for potent, selective inhibition of HIV protease. Ready availability due to its simple chemical structure and stability should make it valuable for studies of the development of metabolically stable anti-AIDS drugs.

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SYNTHESIS OF THE 2',3'-DIDEOXYNUCLEOSIDE DERIVATIVES OF THE SPECIFIC BINDING PEPTIDE PART OF CD4

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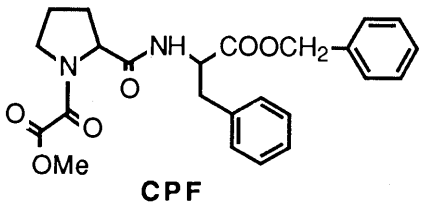
The 2',3'-dideoxynucleoside derivatives of the specific binding peptide part of CD4 to HIV envelope protein gp120 were synthesized.

KEYWORDS 2',3'-dideoxynucleoside derivative; glycoprotein CD4; envelope protein gp120; anti-HIV activity

Infection by human immunodeficiency virus type-1 (HIV-1) was found to be initiated when its envelope protein gp120 binds to the T-cell surface glycoprotein CD4 as its receptor.[1] The amino acid Ser^{42}-Ser^{49} region of the CD4 V1 domain was reported to be potent to bind to gp120.[2] Recently, Robert W. Finberg *et al.* have reported that *N*-carbomethoxycarbonyl-prolyl-phenylalanyl benzyl esters (CPFs) as the modified derivatives of the amino acid Ser^{42}-Phe^{43} region of the CD4 V1 domain blocked the binding of CD4 to gp120.[3]

-Ser^{42}-Phe-Leu-Thr-Lys-Gly-Pro-Ser^{49}-

Ser^{42}-Ser^{49} of CD4 V1 domain



CPF

The reverse transcriptase inhibitors such as the triphosphates of 3'-azido-3'-deoxythymidine (AZT) and the other 2',3'-dideoxynucleosides are currently the most effective chemotherapeutic agents for treatment of HIV infection.

Although AZT is at present the only drug used clinically for treatment of acquired immunodeficiency syndrome (AIDS), it displays severe bone-marrow toxicity with only short-term benefit[4], and resistant virus strains[5] are now emerging. From these facts we describe the synthesis of the AZT and 2',3'-dideoxynucleoside (ddN; *e.g.*, dideoxyuridine: ddU, dideoxycytidine: ddC, dideoxyinosine: ddI and dideoxyadenosine: ddA) (Fig. 1) derivatives having the peptide moiety to both bind specifically to the gp120 and inhibit the reverse transcriptase.

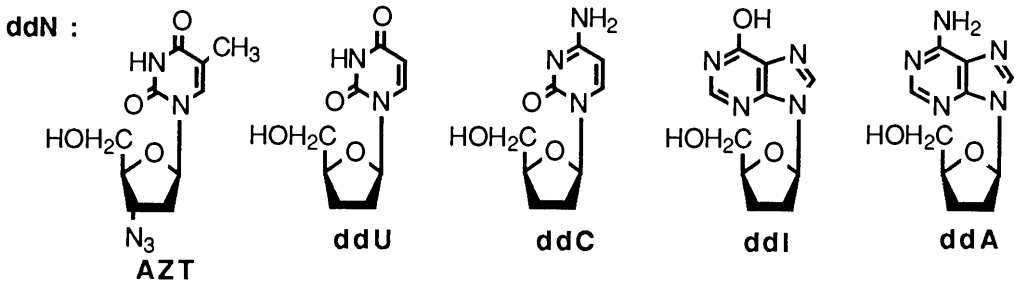


Fig. 1

We designed a structure constituting a link of oxalyl between ddNs and the specific binding peptide part of CD4.

The peptide part was synthesized from Boc-Pro-OH and H-D-Phe-OBzl•Tos-OH by diethyl phosphorocyanidate (DEPC)-triethylamine (TEA) coupling method.[6] The protected peptide Boc-Pro-D-Phe-OBzl was treated with HCl/AcOEt to give H-Pro-D-Phe-OBzl•HCl. Similarly, the octapeptide Ser^{42}-Ser^{49} of CD4 V1 domain was synthesized as shown in Chart 1.

Synthesis of the octapeptide Ser^{42}-Ser^{49}

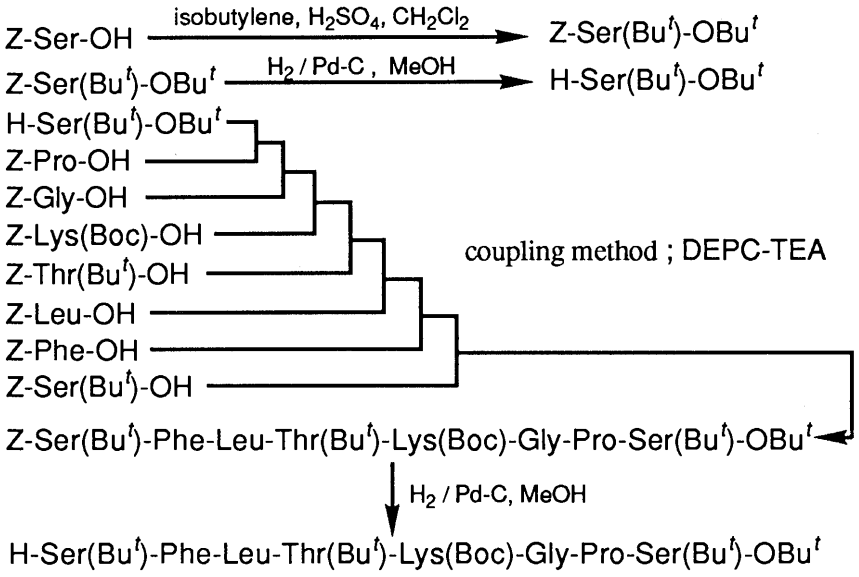


Chart 1

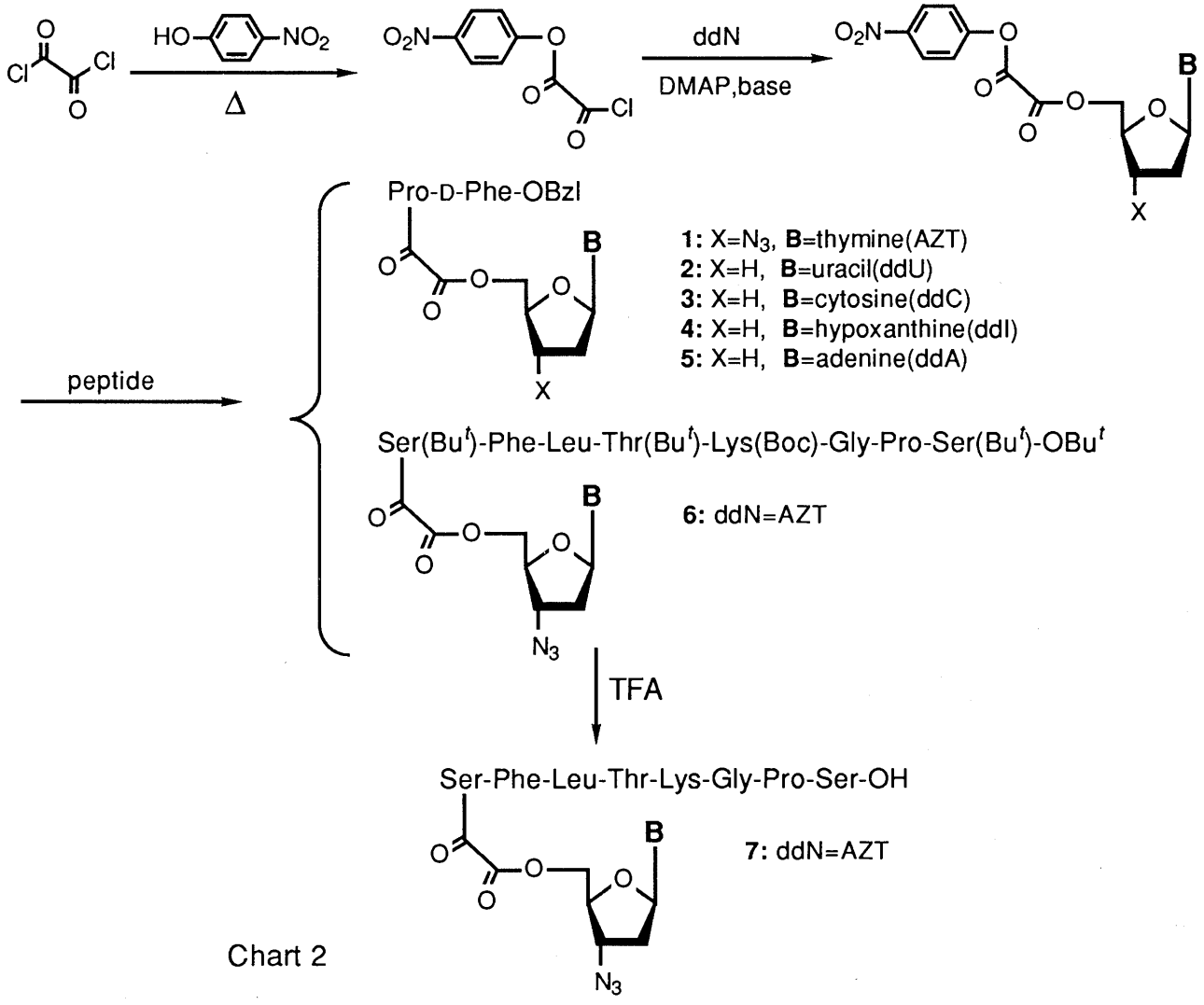


Chart 2

To obtain *p*-nitrophenyl chloroglyoxylate, *p*-nitrophenol was added to excess oxalyl chloride and the reaction mixture was refluxed for 16 h.[7] The purified chloroglyoxylate ester was treated with a variety of ddN in dry pyridine in the presence of 4-dimethylaminopyridine for 24-36 h at room temperature, followed by amidation with the corresponding peptide derivatives. Column chromatographic purification(SiO_2;$CHCl_3$:MeOH=10:1-6:1) of the reaction mixture afforded compounds(**1**-**6**) and succesive treatment of **6** with trifluoroacetic acid (TFA) gave the deprotected compound **7** (Chart 2).[8]

Preliminary examination of the biological activity revealed that compounds **1** and **3** show a significant inhibition of HIV infection.[9]

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8)**1**.(yield 32%) mp 75-78°C, $[\alpha]_D^{22}$ -38.2°(*c*=0.99;$CHCl_3$), FABMS(*m/z*) 674$(M+H)^+$, **2**.(yield 25%) mp 71-74°C, $[\alpha]_D^{22}$ -42.8°(*c*=0.31; $CHCl_3$), FABMS(*m/z*) 619$(M+H)^+$, **3**.(yield 12%) mp 72-76°C, $[\alpha]_D^{22}$ -9.3°(*c*=0.20;$CHCl_3$), FABMS(*m/z*) 618$(M+H)^+$, **4**.(yield 21%) mp 75-77°C, $[\alpha]_D^{22}$ -35.2° (*c*=0.20;$CHCl_3$), FABMS(*m/z*) 643$(M+H)^+$, **5**.(yield 24%) mp 72-75°C, $[\alpha]_D^{22}$ -8.5°(*c*=0.20;$CHCl_3$), FABMS (*m/z*) 642$(M+H)^+$, **6**.(yield 14%) mp 169-174°C, $[\alpha]_D^{22}$ -28.3°(*c*=0.28;$CHCl_3$), FABMS(*m/z*) 1482$(M+H)^+$, **7**.(yield 96%) mp 127-132°C, $[\alpha]_D^{22}$ -38.5°(*c*=0.28;$CHCl_3$:MeOH=1:1), FABMS(*m/z*) 1158$(M+H)^+$.

9)The detailed results of these biological activities will be published separately by Prof. Hiroo Hoshino *et.al.* (Gunma University).

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A FACILE CHEMOENZYMATIC ROUTE TO ENANTIOMERICALLY PURE 4,5-DISUBSTITUTED-2-HEXENOATE DERIVATIVES

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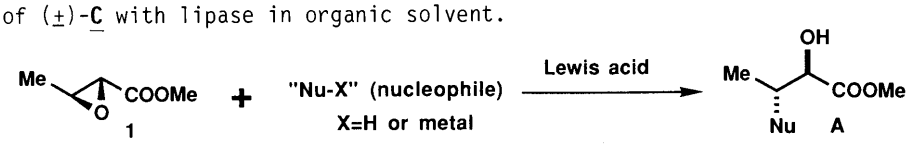
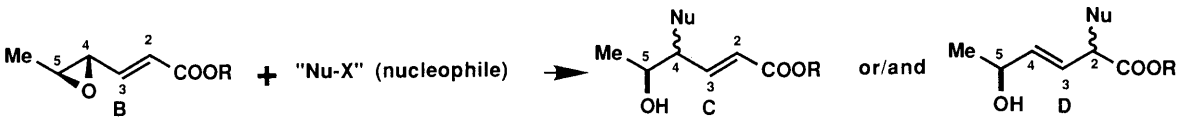
The reaction of 4,5-epoxy-2-hexenoate **2** and various nucleophiles in the presence of $BF_3.Et_2O$ predominantly gave the (4,5)-5-hydroxy-4-substituted compounds. Among them, (±)-(4,5)-anti-5-hydroxy-4-thiophenoxy ester **17** was enantioselectively esterified with acylating reagent in the presence of lipase "PL 266" from Alcaligenes sp. to provide the (4S,5R)-5-acetoxy ester **20** and the (4R,5S)-**17** quantitatively.

KEYWORDS 4,5-epoxy-2-hexenoate; epoxy ring opening; boron trifluoride etherate; lipase; enantioselective esterification

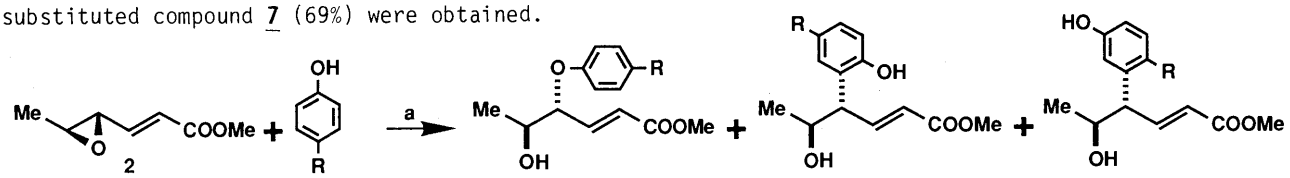
In connection with our work directed towards the synthesis of optically active 2,3-disubstituted butyrate derivatives **A** which were obtained by the reaction of an optically active 2,3-epoxy butyrate **1** and nucleophilic reagents in the presence of Lewis acid,[1] the reaction of 4,5-epoxy-2-hexenoate **B** having a vinylogous structure of **1** and various nucleophiles has aroused our interest. The reactions of **B** and alcohols[2] or acetoacetate[3] or methylcopper reagent,[4] producing the 4,5- or/and 2,5-disubstituted compounds (**C** or **D**), have been reported, but regio- and stereo-selectivities have not been thoroughly studied yet. Optically active 4,5-anti-disubstituted-2-hexenoate derivatives **C** are expected to be a chiral intermediate for the synthesis of biologically active compounds such as amino sugars or their related compounds.[2] We now report the regioselective synthesis of (±)-(4,5)-anti **C** and enantioselective esterification of (±)-**C** with lipase in organic solvent.



"Nu-X" ; O-nucleophile (alcohol, phenol), C-nucleophile (indol, $Et_2AlC{\equiv}CSiMe_3$)



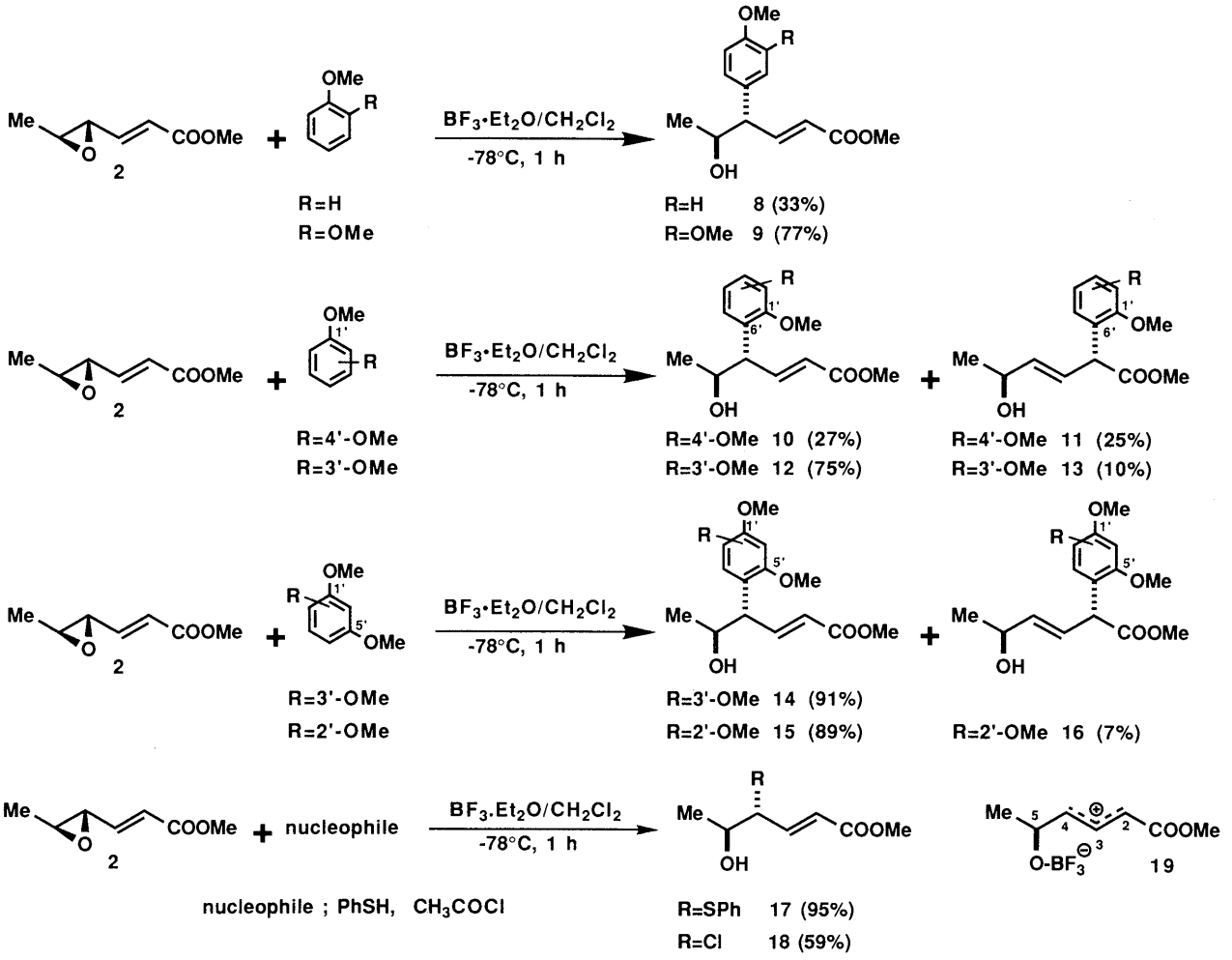
The Reaction of Methyl 4,5-Epoxy-2-hexenoate 2 with Various Nucleophiles The reaction of **2** (10 m mol) with p-methoxyphenol (5 m mol) in the presence of $BF_3.Et_2O$ (10 m mol) gave the 4,5-anti-4-aryl ether (O-substituted compound) **3** (25%)[5] and the 4,5-anti-4-aryl substitution products (C-substituted compounds **4** (48%)[6], **5** (6%)[6]. When p-cresol was used, the O-substituted compound **6** (17%) and the C-substituted compound **7** (69%) were obtained.



R=OMe R=OMe 3 (25%) R=OMe 4 (48%) R=OMe 5 (6%)
R=Me R=Me 6 (17%) R=Me 7 (69%)

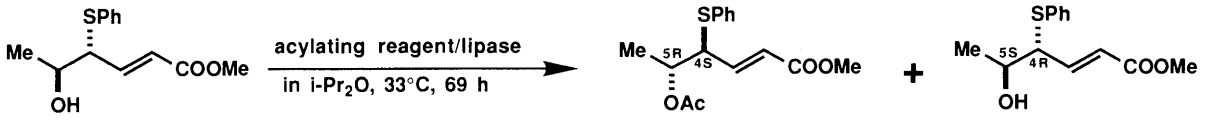
a; $BF_3.Et_2O/CH_2Cl_2$, -78°C, 1 h

For the purpose of obtaining selectively the C-substituted compounds, the reaction of 2 with various carbon nucleophiles was examined. When anisole, 1,2-dimethoxybenzene and 1,3,5-trimethoxybenzene were employed, the 4,5-anti-4-aryl substitution products (8 (33%), 9 (77%) and 14 (91%)) were predominantly obtained. The yield was found to rise as the nucleophilicity of aromatic ring increased. On the other hand, the reaction of 2 with other polymethoxybenzenes yielded the 2,5-anti-2-carbon substituted products[7] as well as the 4,5-anti-4-carbon substituted compounds. The reaction of 2 with thiophenol or with acetyl chloride afforded regioselectively the 4-substituted compounds (17 (95%) or 18 (59%)), respectively.



In the reaction of 2 with nucleophiles in the presence of $BF_3.Et_2O$, the possibility of forming oxo-π-allyl complex 19 from 2 are considered. If the complex formation takes place, then the nucleophilic attack from the less hindered side (anti-direction) should give the 4,5-anti-4-substituted compounds or the 2,5-anti-2-substitution products. In the reaction of 2 and the methylcopper reagents, it was reported that the harder nucleophiles attack predominantly the C_4-position while the softer reagents react predominantly at the C_2-position.[4] In the present case, it is difficult to explain the regioselectivity quantitatively on the basis of a delicate difference in softness among the used nucleophiles.

Enantioselective Esterification of (±)-4-Thiophenoxy Ester 17 with Lipase The treatment of (±)-17 and acylating reagents (isopropenyl acetate or phenylthioacetate) with lipase "PL 266" from Alcaligenes sp. in isopropyl ether provided quantitatively the (4S,5R)-5-acetoxy ester 20 (98-99% ee) and the (4R,5S)-5-hydroxy ester 17 (97-99% ee). The stereochemistry of (4R,5S)-17 was determined by comparison with authentic sample (4S,5R)-17, prepared by the reaction of the known (4R,5R)-2[8] and thiophenol in the presence of $BF_3.Et_2O$.

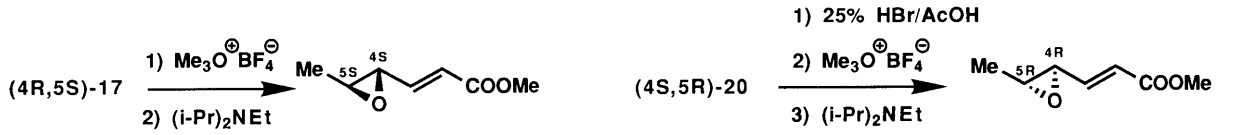


(±)-17 (4S,5R)-20 (4R,5S)-17

Entry	substrate (mg)	Lipase	Acylating reagent	Product (yield, %; optical purity, % ee)	
1	(103)	PL 266 (*Alcaligenes* sp.)	A	(4S,5R)-20 (50.2%, 98% ee)	(4R,5S)-17 (49.7%, >99% ee)
2	(97)	PL 679 (*Alcaligenes* sp.)	A	(4S,5R)-20 (61.1%, 58% ee)	(4R,5S)-17 (34.6%, 90% ee)
3	(107)	PL 266 (*Alcaligenes* sp.)	B	(4S,5R)-20 (49.5%, 99% ee)	(4R,5S)-17 (49.8%, 97% ee)
4	(109)	PL 679 (*Alcaligenes* sp.)	B	(4S,5R)-20 (41.5%, 95% ee)	(4R,5S)-17 (57.8%, 67% ee)

A ; isopropenyl acetate B ; PhSAc

Treatment of (4R,5S)-17 with $Me_3O^+BF_4^-$ was followed by epoxy formation with $(i\text{-}Pr)_2NEt$ to afford the (4S,5S)-2 in 58% overall yield. In this process, the isomerization to the undesired cis epoxy ester was partly observed, but no loss of optical purity was detected. The (4S,5R)-5-acetoxy ester 20 was also converted into the (4R,5R)-2. These conversions mean that chiral synthesis of the 4,5-disubstituted-2-hexenoate derivatives is achieved by applying the reaction of (±)-2 and various nucleophiles to the reaction using the chiral 4,5-epoxy-2-hexenoate 2.



ACKNOWLEDGEMENT This work was supported by the Grant for "Biodesign Research Program" from RIKEN to H. A.

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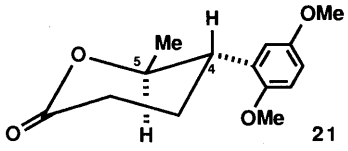
5) Satisfactory analytical data were obtained for all new compounds.
6) The structure of 4 and 5 were determined by NMR analysis (NOE experiment) and chemical correlation. Methylation of 4 and 5 provided the same dimethoxy compound 10 which was converted in the δ-lactone 21.
The coupling constant of C_4-axial proton and C_5-axial proton of 21 was 10 Hz, clearly indicating that the starting 4 or 5 possessed anti-configuration.



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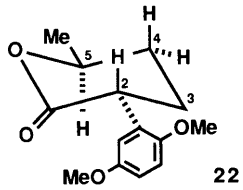

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7) In order to determine the structure of this type compound, for example, compound 11 was converted into the δ-lactone 22. The coupling constants due to the C_2-axial proton and C_5-axial proton were $J_{2a,3a}$= 11 Hz , $J_{2a,3e}$=7 Hz and $J_{5a,4a}$=11 Hz, $J_{5a,4e}$=2 Hz, respectively.
These data clearly indicate that the starting 11 possessed 2,5-anti-configuration.
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NEW HYDROXYL PROTECTING GROUPS OF A SAFETY-CATCH TYPE REMOVABLE BY REDUCTIVE ACIDOLYSIS[1)]

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New hydroxyl protecting groups of a safety-catch type, *i.e.*, 4-methylsulfinylbenzyloxycarbonyl (Msz) group for Tyr and 4-methylsulfinylbenzyl (Msob) ether for Thr, have been developed. O-Msz and O-Msob groups are stable under both acidic and basic conditions and can be removed by a one-pot reaction involving reductive acidolysis using tetrachlorosilane-trifluoroacetic acid (TFA)-scavengers. Using these new protecting groups, a 17 residue-peptide, γ-endorphin, was successfully synthesized by the efficient solid phase method.

KEYWORDS solid phase peptide synthesis; 4-methylsulfinylbenzyloxycarbonyl (Msz); 4-methylsulfinylbenzyl (Msob); safety-catch type protecting group; reductive acidolysis; fluoride ion deprotection

Recently, we have developed a safety-catch type of protecting group: 4-methylsulfinylbenzyl (Msob) ester[2-4)] for carboxyl and 4-methylsulfinylbenzyloxycarbonyl (Msz)[3)] for amino groups. The safety-catch type of protecting group can be smoothly removed by a one-pot reaction involving reductive acidolysis using tetrachlorosilane-trifluoroacetic acid (TFA)-scavengers,[3,4)] which is a mild acidolysis system having strong reductivity. In this paper, we report new hydroxyl protecting groups of the safety-catch type removable by reductive acidolysis, *i.e.*, Tyr(Msz) and Thr(Msob).

The preparation of Boc-Tyr(Msz)-OH (Boc=*t*-butoxycarbonyl) is illustrated in Fig. 1. 4-Methylthiobenzyl 4'-nitrophenyl carbonate (Mtz-ONp, Mtz=4-methylthiobenzyloxycarbonyl) (**2**) (mp 78-79°C, *Anal.* Calcd for $C_{15}H_{13}NO_5S$: C, 56.42; H, 4.10; N, 4.39. Found; C, 56.38; H, 4.18; N, 4.29.) was

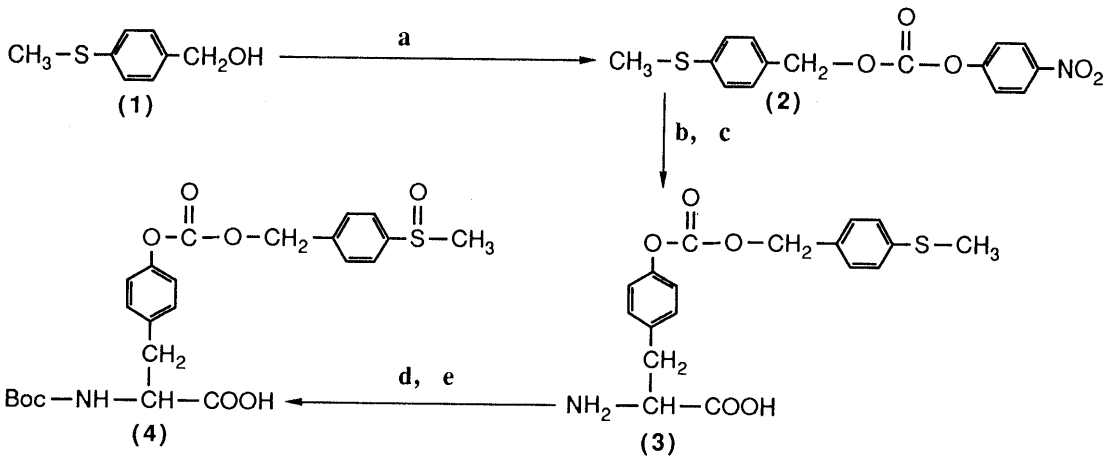

Fig. 1. Preparation of Boc-Tyr(Msz)-OH
Reagents: **a**, 4-nitrophenyl chloroformate / pyridine; **b**, Tyr•1/2Cu, $NaHCO_3$ / DMF-H_2O; **c**, EDTA•2Na / H_2O; **d**, Boc_2O, Et_3N / tetrahydrofurane-H_2O; **e**, $NaBrO_2$•$3H_2O$ / AcOEt-H_2O.

prepared in 70% yield by the reaction of 4-methylthiobenzyl alcohol (**1**) and 4-nitrophenyl chloroformate in pyridine at 25°C for 4h. Mtz-group was introduced to phenolic moiety of tyrosine by the reaction of copper complex of tyrosine with Mtz-ONp (**2**) in 70% aqueous DMF (25°C, 24h) followed by treatment with aqueous ethylenediaminetetraacetic acid (EDTA) disodium salt to give H-Tyr(Mtz)-OH (**3**) [mp 213-216°C, $[\alpha]_D^{26}$ -16.07° (c=0.56, 50% AcOH)] in 68% yield. Boc-Tyr(Msz)-OH (**4**) [mp 61-64°C, $[\alpha]_D^{26}$ -167.82 (c=0.44, MeOH), *Anal.* Calcd for $C_{23}H_{27}NO_8S \cdot 3/4H_2O$: C, 56.26; H, 5.54; N, 2.85. Found: C, 56.45; H, 5.79; N, 2.88.] was obtained in 50% yield from H-Tyr(Mtz)-OH (**3**) by the conventional *t*-butoxycarbonylation with $(Boc)_2O$ and following oxidation using sodium bromite trihydrate[5] in AcOEt-H_2O at 25°C for 30min. Boc-Thr(Msob)-OH [mp 114-116°C, $[\alpha]_D^{26}$ -176.47° (c=0.60, MeOH), *Anal.* Calcd for $C_{17}H_{25}NO_6S \cdot H_2O$: C, 52.42; H, 6.47; N, 3.60. Found: C, 52.48; H, 6.78; N, 3.50.] and Boc-Ser(Msob)-OH [mp 69-70°C, $[\alpha]_D^{26}$+34.15° (c=0.41, MeOH) *Anal.* Calcd for $C_{16}H_{23}NO_6S \cdot 1/2H_2O$: C, 52.44; H, 6.33; N, 3.82. Found: C, 52.44; H, 6.50; N, 3.82.] were prepared using Msob-Br and NaI in essentially the same manner as described by Futaki et al.[6]

The stabilities of Tyr(Msz), Thr(Msob) and Ser(Msob) under various conditions were examined by TLC. These protecting groups were sufficiently stable under the acidic condition [TFA-anisole (25°C, 24h), or 0.5M methanesulfonic acid / dichloromethane : dioxane (9:1) containing 2% anisole (25°C, 24h)][7] and the basic condition [5% Et_3N / dimethylformamide (DMF) (25°C, 24h), or 5% diisopropylethylamine / DCM (25°C, 24h)]. O-Msz or O-Msob group was rapidly cleaved with reductive acidolysis system [1M $SiCl_4$ (10eq) / TFA-anisole (10eq), or 1M $SiCl_4$ (10eq) / TFA-anisole, thioanisole, ethanedithiol (EDT) (10eq each) (25°C, 10min)] to give a sole product on TLC. The O-Msz group of Tyr was also cleaved by saponification [1N NaOH / MeOH (25°C, 24h)], while the Msob ether of Thr or Ser was kept completely intact under this condition.

In Merrifield type solid phase peptide synthesis, it is inevitable that slight cleavage of semi-permanent protecting groups occurs during repetitive TFA treatment. Thus, we have compared the stabilities of Msz and Msob groups to TFA with those of conventional benzyl alcohol type protecting groups in detail using an amino acid analyzer. As shown in Fig. 2, Msz group of Tyr was most stable among the phenolic protecting groups tested, *i.e.*, benzyl (Bzl), *o*-bromobenzyloxycarbonyl (Br-Z), 2,6-dichlorobenzyl (Cl_2Bzl) and Msob[8] groups. The Msob ethers of Ser and Thr were about 10 times more stable than the corresponding Bzl ethers. The high stability of Msz and Msob groups seemed to be advantageous, especially for the solid phase synthesis of long chain peptides.

In order to demonstrate the usefulness of the new hydroxyl protecting groups of the safety-catch type, we synthesized γ-endorphin consisting of 17 amino acid residues (Fig. 3). The peptide backbone was constructed on the phenacyl (Pac) resin[9] by the efficient method[10] of solid phase peptide synthesis, which consists of selective N^{α}-deprotection by dilute methanesulfonic acid, *in situ* neutrali-

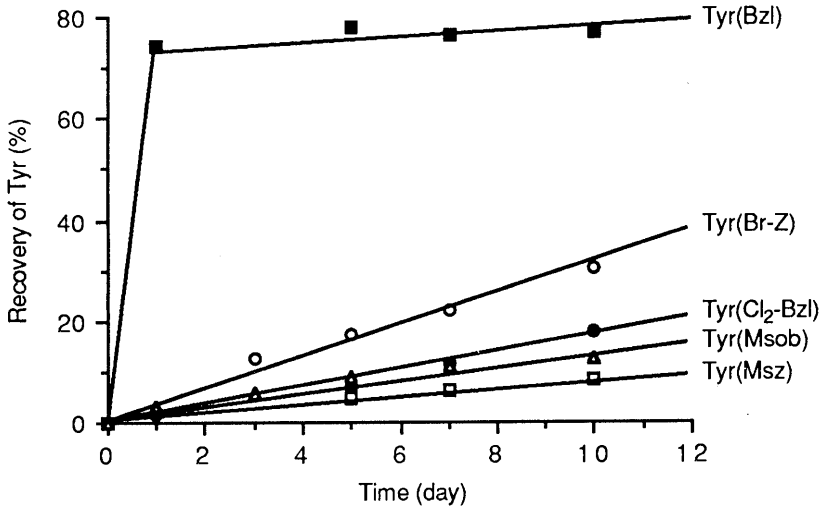

Fig. 2. Stability of Msz and Msob Group in TFA-Anisole (25°C)

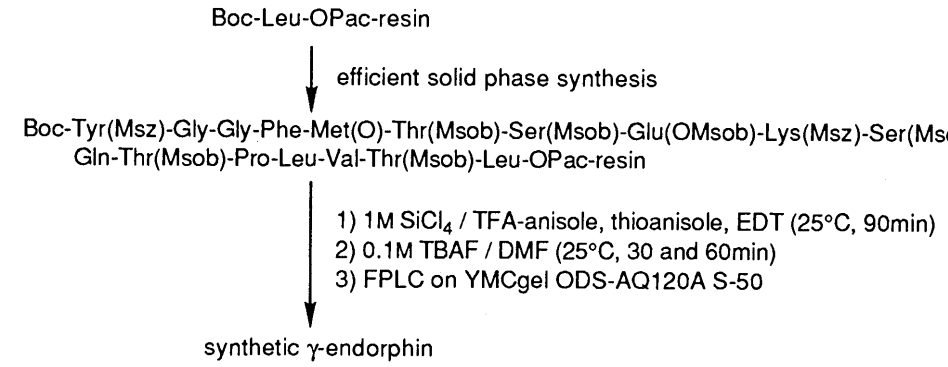

Fig. 3. Synthesis of γ-Endorphin

zation, and rapid coupling reaction using benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate. Together with Boc-Tyr(Msz), Boc-Thr(Msob) and Boc-Ser(Msob), Boc-amino acid derivatives bearing safety-catch type protecting groups were employed, *i.e.*, Boc-Glu(OMsob)[11] and Boc-Lys(Msz).[3] After the completion of the peptide chain elongation, the protected peptide-resin was treated with 1M $SiCl_4$ / TFA-anisole, thioanisole, EDT at 25°C for 90min to remove all of the side chain protecting groups. After washing the resin, the deprotected peptide was cleaved from the Pac resin by treating twice (30min and the 60min) with 0.1M tetra-*n*-butylammonium fluoride trihydrate (TBAF) / DMF at 25°C.[7,10,11] The product was purified by fast protein liquid chromatography (FPLC, Pharmacia) on a column packed with YMC-gel ODS-AQ 300A (S-50) (20x500mm). The homogeneous peptide was obtained in 49% yield from the starting resin. The purity of synthetic γ-endorphin was confirmed by analytical HPLC on YMC AM302 column (4.6x150mm) [retention time; 19.53min, on a gradient elution with MeCN (10-60% in 30min) in 0.1% aqueous TFA, 0.7ml/min] and by amino acid analysis after acid hydrolysis with 6N HCl-phenol [amino acid ratios (theoretical numbers in parentheses): Leu 2.00 (2), Thr 2.86 (3), Ser 1.87 (2), Glu 2.04 (2), Gly 2.02 (2), Val 0.99 (1), Met 0.94 (1), Tyr 0.97 (1), Phe 0.98 (1), Lys 0.99 (1), Pro 0.98 (1), Rec. of Leu 61%].

These excellent results demonstrate the usefulness of the new hydroxy protecting groups of the safety-catch type removable by reductive acidolysis in practical solid phase peptide synthesis.

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A CONVENIENT SYNTHESIS OF A SIMPLE COUMARIN

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Reaction of salicylaldehydes (**1**) with carbethoxymethylenephosphorane in diethylaniline under reflux gave coumarins (**3**) in excellent yields. Methoxy substituent at C_4 on salicylaldehyde (**1**) facilitated the formation of coumarin from *trans*-cinnamate (2), which is first prepared by reaction of **1** with Wittig reagent.

KEYWORDS coumarin synthesis; Wittig reaction; salicylaldehyde; methoxy group effect; reaction temperature; mechanism

Coumarins are widely distributed in nature and have been reported to exhibit various biological and/or pharmaceutical activities.[1] Although various synthetic routes to coumarins, especially to simple coumarins (**3**), are known,[2] much effort is still being devoted to exploring new synthetic methods[3] because of their lack of generality and/or efficiency.

Recently we developed a new synthesis method of salicylaldehydes (**1**) *via* formation of benzofuran by CsF-mediated Claisen rearrangement of aryl propargyl ether followed by oxidative cleavage of furan ring[4] (see Chart 1). Mali *et al.* reported a new coumarin synthesis by Wittig reaction of **1** with carbethoxymethylenephosphorane without solvent at 210-215°C.[5] We planned to re-examine the generality and efficiency of this method by Mali *et al.* in connection with synthetic studies on Reisch's coumarin.[6] Herein we present an improved and practical synthetic method of synthesizing simple coumarins (**3**).

Since coumarins contained in *Rutaceae* plants[7] usually have a methoxy group at C_7 ring, reaction of 4-methoxysalicylaldehyde (**1a**) with carbethoxymethylenephosphorane at various temperatures was first examined. The results are summarized in Table I, showing that yield of coumarin (**3a**) rises as reaction temperature becomes high. Thus, reaction of **1a** with phosphorane in diethylaniline under reflux, for only 15 min, gave **3a** in 95.2% yield, whereas reaction of **1a** in pyridine under reflux, even for 4h, gave **3a** in 9.0% yield (see runs 5 and 3 in Table I). Next, in order to compare our method (in diethylaniline at 215°C) with Mali's method (neat at 215°C), reaction of **1b** and **1f** with phosphorane was carried out. The results (see runs 3, 4, 8, and 9 in Table II, and also runs 1 and 2 in Table II) show that reaction by our method occurs readily to give a somewhat higher yield, and isolation of products was much easier. The additional results (see runs 5, 6, and 7 in Table II) indicate that our method is practically useful for the synthesis of simple coumarins (**3**). Furthermore, in connection with synthetic studies on Reisch's coumarin,[6] reaction of 4-formyl-5-hydroxy-7-methoxy-2-methylbenzo[b]furan (**4**) under our reaction conditions for 30 min was carried out to give the corresponding coumarin (**5**) in 80.4% yield.[8] The methoxy substituent at C_4 on salicylaldehyde (**1**), *i.e.* on cinnamate (2) which is first prepared by the Wittig reaction of **1** with phosphorane, facilitated the formation of coumarin ring in comparison with the methoxy group at other positions.

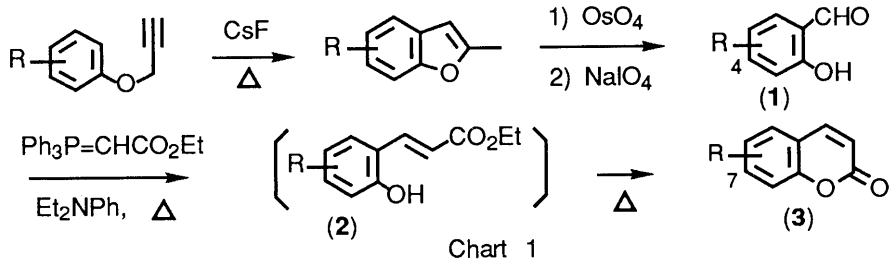


Chart 1

Table I. The Results of Reaction of 4-Methoxysalicylaldehyde (**1a**) with Carbethoxymethylenephosphorane at Various Temperatures

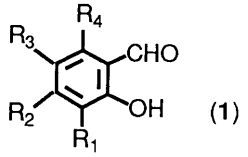
Run	Solvent	Temperature	Time	Product(%)[a] **2a / 3a**
1	Benzene	r.t.	4h	87.9 / 4.6
2	Benzene	Reflux(80°C)	4h	90.7 / 7.4
3	Byridine	Reflux(115°C)	4h	88.9 / 9.0
4	Xylene	Reflux(140°C)[b]	4h	43.2 / 41.8
5	Diethylaniline	Reflux(215°C)	15min	0 / 95.2

a) Isolated yield.
b) Heating of **2a** in diethylaniline at 140°C for 4h gave **3a** in 41.5% yield with the recovery of **2a** in 36.2% yield.

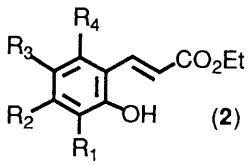
Table II. The Results of Reaction of Salicylaldehydes (**1**) with Carbethoxymethylenephosphorane at 210-215°C

Run	Starting material	Reaction conditions Solvent	Time	Product (%)[a] **2 / 3**
1	**1a**	Diethylaniline	15min	0 / 95.2
2	**1a**	- - -	5h	0 / 71[b]
3	**1b**	Diethylaniline	30min	0 / 90
4	**1b**	- - -	10min	0 / 74.8
5	**1c**	Diethylaniline	40min	0 / 95.2
6	**1d**	Diethylaniline	2.5h	0 / 93.4
7	**1e**[c]	Diethylaniline	4h	0 / 89.2
8	**1f**	Diethylaniline	6h	10.5 / 80.8
9	**1f**	- - -	6h	4.5 / 70.1

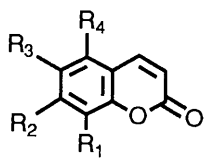
a) Isolated yield.
b) See ref. 5c).
c) Perkin reaction of **1c** with Ac_2O-AcONa gave **3c** only in 43.3% yield.



(1)



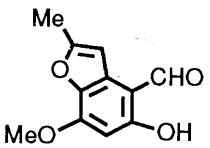
(2)



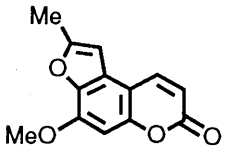
(3)

a: $R_1=R_3=R_4=H$, $R_2=OMe$
b: $R_1=R_2=OMe$, $R_3=H$, $R_4=Me$
c: $R_1=R_4=H$, $R_2=OMe$, $R_3=OPr^i$
d: $R_1=R_2=R_4=H$, $R_3=OMe$
e: $R_1=R_2=R_3=R_4=H$
f: $R_1=OMe$, $R_2=R_3=R_4=H$

Chart 2



(4)



(5)

This could be explained by supposing that C_4-methoxy group favours the isomerization of *trans*-cinnamate (*trans*-**2a**) to *cis*-cinnamate (*cis*-**2a**), which cyclized irreversibly to coumarin (**3**) as shown in Chart 3.[9] Synthetic studies on natural coumarins using our method are now in progress.

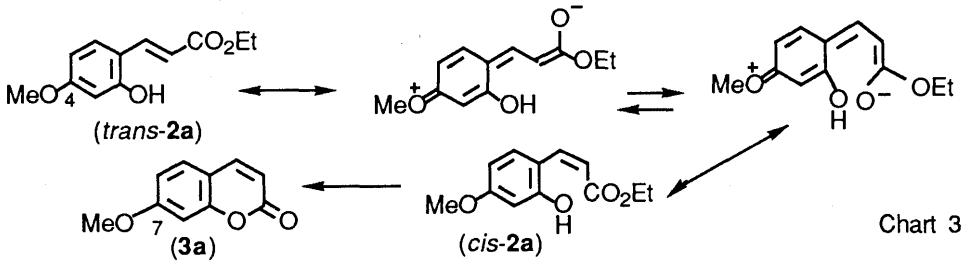


Chart 3

A representative experimental procedure for preparing coumarin is as follows.

Reaction of **1a** with Phosphorane A solution of salicylaldehyde (**1a**) (1 mmol) and carbethoxymethylenephosphorane (1.2 mmol) in diethylaniline (10 ml) was heated at 210-215°C for 15 min under an argon atmosphere. Reaction mixture was diluted with water and extracted with ether. The ether extract was thoroughly washed with 5% HCl and brine, dried over $MgSO_4$ and concentrated *in vacuo*. Chromatography on SiO_2 gave coumarin (**3a**) in 95.2% yield.

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8) Compound (**4**) was synthesized from 5-formyl-7-methoxy-2-methylbenzo[b]furan[4] by a following sequence of reactions: a) 30% H_2O_2-HCO_2H, b) 5% NaOH, c) benzyl chloride-K_2CO_3 in dimethylformamide (DMF), d) DMF-$POCl_3$, e) H_2-5% Pd/C in AcOEt. Details will be reported elsewhere.

9) Reaction of **1e** having no methoxy group at C_4, with phosphorane in xylene under reflux for 4h gave cinnamate (**2e**) and coumarin (**3e**) in 82.8% and 9.6% yields, respectively, whereas reaction of **1a** having methoxy group at C_4 under the same conditions gave **2a** and **3a** in 43.2% and 41.8% yields, respectively. Interestingly, reaction of **1c** having C_4-OMe group, with phosphorane in xylene, provided only coumarin (**3c**) in 86.7% yield on refluxing for 11h.

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