

Novel Cycloartane Saponins from *Corchorus depressus* L.

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Two novel bidesmosidic cycloartane-type glycosides, depressosides C and D were isolated from *Corchorus depressus* L. Their structures were elucidated as (22*R*)-16 β ,22-epoxy-3 β ,26-dihydroxy-9,19-cyclolanost-24*E*-ene 3, 26-di-*O*- β -D-glucopyranoside and (22*R*,24*S*)-22,25-epoxy-3 β ,16 β ,24-trihydroxy-9,19-cyclolanostane 3, 24-di-*O*- β -D-glucopyranoside, respectively on the basis of chemical evidence and detailed spectroscopic studies.

Key words *Corchorus depressus*; Tiliaceae; cyclolanostane; glycoside; depressoside

Corchorus depressus L. (Tiliaceae) is a medicinal plant growing wild in sandy, clayey and saline areas of Pakistan.¹⁾ It is commonly known as “Bhaupali” and has been used in folk medicine in the Subcontinent. The herbalist doctors sell it under the name “Munderi.” Its leaves are used as an emollient and cooling agent. The mucilage is used for the treatment of gonorrhea and applied as a poultice for healing wounds.²⁾ The decoction of seeds and leaves is used as a tonic and the infusion is used as a fever drink.³⁾ Chemical analysis of this species has led to the isolation of flavonoids,⁴⁾ and α -amyirin derivatives.⁵⁾ Recently we have reported the isolation and structure elucidation of two new cycloartane-type glycosides, depressoside A and B.⁶⁾ The present paper describes the isolation and structure elucidation of two novel bidesmosidic cycloartane-type triterpene saponins named as depressosides C and D (**1**–**2**).

Results and Discussion

The whole plants of *Corchorus depressus* were extracted with EtOH. The EtOH extract was partitioned among hexane, CHCl₃, EtOAc, *n*-BuOH and H₂O. The *n*-BuOH soluble portion was subjected to silica gel column chromatography. The polar fractions obtained were further chromatographed on Sephadex LH-20 to afford a saponin mixture. Repeated column chromatography of this saponin mixture followed by flash chromatography on RP-18 silica yielded two pure saponins, depressosides C and D (**1**, **2**). Compound **1** was isolated as an amorphous powder. The positive ion FAB mass spectrum of **1** exhibited a quasi-molecular ion peak at m/z 803; calculated for C₄₂H₆₈O₁₃Na [M+Na]⁺. The fragment ions at m/z 641 [M+Na–162]⁺, 623 [M+Na–162–H₂O]⁺ and m/z 461 [M+Na–2×162–H₂O]⁺, indicated the successive loss of two hexose units from the molecule. The negative ion FAB-MS showed the pseudomolecular ion peak at m/z 779 [M–H][–], and the fragment ions at m/z 599 [M–H–162–H₂O][–] and m/z 437 [M+H–2×162–H₂O][–], confirming the presence of two hexoses in the molecule. The fragment ion at m/z 456 (calculated for C₃₀H₄₈O₃) in the EI-MS of the intact saponin was attributed to the aglycone **1a**.

The ¹H-NMR spectrum of **1** (CD₃OD, 500 MHz) contained signals characteristic of a cyclopropyl methylene group as a pair of doublet at δ 0.31 and δ 0.64 ($J=4.0$ Hz, H₂-19), five tertiary methyl groups at δ 0.88 (3H, s, H₃-29), 0.94 (3H, s, H₃-30), 1.08 (3H, s, H₃-28), 1.15 (3H, s, H₃-18), and at δ 1.70 (3H, s, H₃-27), and a secondary methyl group at δ 1.0 (3H, d, $J=6.6$ Hz, H₃-21). Furthermore the ¹H-NMR spec-

trum of **1** clearly showed two anomeric proton doublets at δ 4.23 (1H, d, $J=7.7$ Hz) and δ 4.48 (1H, d, $J=7.6$ Hz), indicative of two β linked sugars and a broad triplet at δ 5.56 (1H, t, $J=7.2$ Hz, H-24), ascribable to an olefinic proton.

The ¹³C-NMR spectrum of **1** (CD₃OD, 125 MHz), displayed a total of 42 carbon signals, of which 30 were attributed to the triterpenoid moiety and 12 to the saccharide portion. The ¹³C-NMR, ¹³C-DEPT and heteronuclear multiple quantum coherence (HMQC) spectra revealed that the 30 signals of the aglycone consisted of six methyls, ten methylenes, eight methines and six quaternary carbons, indicating a genin different from the previously reported depressogenin.⁶⁾ Acid hydrolysis of **1** failed to yield the genuine sapogenol **1a** (see experimental section). The extensive use of different 1D and 2D-NMR techniques and MS studies led to the determination of structure of the saponin in general and the aglycone part in particular. The assignments of all the ¹H- and ¹³C-signals in **1** were successfully carried out using ¹H–¹H shift correlation spectroscopy (COSY), HMQC, nuclear Overhauser enhancement spectroscopy (NOESY) and heteronuclear multiple bond correlation (HMBC) experiments (Table 1).

Comparison of the ¹³C-NMR spectrum of **1** with those of depressoside A and depressogenin showed good general agreement except for the signals pertaining to the ring D and the side chain (C-20–C-27). The chemical shift value at δ 90.89 (CH) was attributed to the C-3 to which one of the sugar residues was connected. The existence of unsaturation was revealed by the presence of easily recognizable signals at δ 124.97 (CH) and δ 134.64 (C). The remaining three downfield signals at δ 90.80 (CH), 85.04 (CH) and δ 75.83 (CH₂) were assigned to two oxymethine and an oxymethylene carbons, respectively. The olefinic carbon at δ 124.97 (C-24) was correlated to the proton at δ 5.56 (H-24). The vinyl proton (H-24) showed long range correlations with a vinyl methyl carbon at δ 14.31 (C-27) and anoxymethylene carbon at δ 75.83 (C-26) in the HMBC spectrum (Fig. 1). The vinyl methyl protons at δ 1.70 (H₃-27) in turn showed long range correlations with olefinic carbons at δ 134.64 (C-25) and δ 124.97 (C-24) and to an oxymethylene carbon at δ 75.83 (C-26) in the same spectrum. This showed that both methyl and oxymethylene groups are attached to the olefinic carbon and are located at the terminal of the side chain. The pronounced downfield shift observed for C-26 (δ 75.83) relative to the corresponding signal at δ 61.1, reported for thalictogenin A (3 β ,22*S*,26-trihydroxycycloart-24-ene),⁷⁾ indicated that the second sugar moiety was attached at C-26. This was further

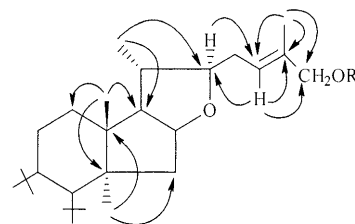
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Table 1. ^{13}C (125 MHz) and ^1H -NMR (500 MHz) Spectral Data of **1** from 1D and 2D-NMR Experiments in CD_3OD

Carbon	DEPT	δ_{C}	δ_{H} (J, Hz)
1	CH_2	33.02	1.25, 1.52
2	CH_2	31.8	1.70, 2.05
3	CH	90.89	3.21 dd (4.4, 11.5)
4	C	42.00	—
5	CH	48.88	1.33 dd (3.5, 12.5)
6	CH_2	21.70	0.81, 1.60
7	CH_2	27.46	1.05, 1.32
8	CH	49.10	1.69 dd (4.4, 12.6)
9	C	20.83	—
10	C	27.10	—
11	CH_2	27.92	1.14, 2.05
12	CH_2	33.23	1.45, 1.67
13	C	44.98	—
14	C	52.64	—
15	CH_2	43.52	1.45, 1.80
16	CH	85.04	4.42 ddd (6.1, 7.6, 7.6)
17	CH	63.51	2.14 dd (5.6, 8.2)
18	CH_3	22.59	1.15 s
19	CH_2	31.32	0.31 d (4.0) 0.64 d (4.0)
20	CH	39.51	1.95 m
21	CH_3	18.88	1.00 d (6.6)
22	CH	90.80	3.43 ddd (4.5, 7.0, 8.2)
23	CH_2	32.39	2.32 ddd (4.5, 6.0, 12.5) 2.40 ddd (6.0, 7.0, 12.5)
24	CH	124.97	5.56 br t (7.0)
25	C	134.64	—
26	CH_2	75.83	4.05 d (11.6) 4.21 d (11.6)
27	CH_3	14.31	1.70 s
28	CH_3	26.04	1.08 s
29	CH_3	15.43	0.88 s
30	CH_3	20.17	0.94 s
Glc I			
1'	CH	104.16	4.48 d (7.6)
2'	CH	81.87	4.05 dd (7.6, 9.1)
3'	CH	77.84	3.65 t (9.1)
4'	CH	71.81	3.28 t (9.1)
5'	CH	77.28	3.25 ddd (2.5, 5.5, 9.1)
6'	CH_2	62.85	3.68 dd (5.2, 12.0) 3.82 d (2.5, 12.0)
Glc II			
1''	CH	102.80	4.23 d (7.7)
2''	CH	75.13	3.20 dd (7.7, 9.2)
3''	CH	78.21	3.65 t (9.2)
4''	CH	71.30	3.41 t (9.2)
5''	CH	77.82	3.32 ddd (2.5, 5.4, 9.2)
6''	CH_2	62.80	3.64 dd (5.4, 11.8) 3.85 dd (2.5, 11.8)

supported from the subsequent upfield shift for C-25 and C-27 carbon signals resonating at δ 134.64 and δ 14.31 compared to the corresponding signals in thalictogenin A, which were observed at δ 137.7 and δ 22.2, respectively.

In the ^1H - ^1H COSY spectrum the vinyl proton (H-24) showed cross peaks with two geminal methylene protons at δ 2.32 (H-23 α) and 2.40 (H-23 β) which were connected to a downfield methine proton at δ 3.43 (ddd, $J=4.5, 7.0, 8.2$, H-22). This methine proton (H-22) had a correlation to the carbon signal at δ 90.80 in the HMQC spectrum, indicating the presence of an oxygen atom at C-22. The H-22 (δ 3.43) methine proton also showed connectivity with H-20 (δ 1.95). The latter in turn showed couplings with the H_3 -21 protons at δ 1.00 and H-17 at δ 2.14. The downfield signal observed at

Fig. 1. Most Significant HMBC Correlations Observed in the Aglycone Part of **1**

δ 4.42 (ddd, $J=6.1, 7.6, 7.6$ Hz) was assigned to H-16 α on the basis of its COSY interactions with H-17 (δ 2.14) as well as with H-15 α (δ 1.45) and H-15 β (δ 1.80). The H-16 (δ 4.42) proton signal showed connectivity to the carbon resonance at δ 85.04 in the HMQC spectrum. A comparative study of the ^{13}C -NMR spectrum of **1** with that of $3\beta,16\beta,22$ -trihydroxycycloart-24-ene,^{8,9} showed a significant lowfield shift for the C-16 and C-22 signals resonating at δ 85.04 and δ 90.80 compared to δ 71.78 and δ 74.40, respectively. A downfield shift was also observed for C-17, C-20 and C-21 carbon signals which appeared at δ 63.51, δ 39.51 and δ 18.88 compared to δ 53.06, δ 36.07 and δ 14.65, respectively. These results were consistent with a five membered ring including C-16, C-17, C-20, C-22 and an oxygen atom between C-16 and C-22 as reported for some dammarane-type glycosides *e.g.* lotosides,¹⁰ mabiosides¹¹ and jujubasaponins.¹² The upfield shift for C-15 (δ 43.52) was in good agreement with that reported for actein (16,23-epoxy cycloartane).¹³

Assignments of the ^1H - and ^{13}C -NMR signals in rings D, E and the side chain based on one bond ^1H - ^1H COSY and ^1H - ^{13}C COSY correlations were further confirmed through two and three bonds ^1H - ^{13}C couplings observed in the HMBC spectrum (Fig. 1). The H_3 -21 methyl protons at δ 1.0 showed long range correlations with the carbons at δ 39.5 (C-20), δ 63.51 (C-17) and at δ 90.80 (C-22), indicating that C-17, C-21, and C-22 were connected with C-20. The methyl signal at δ 1.15 (H_3 -18) exhibited long range correlations with the carbons at δ 33.23 (C-12), δ 44.98 (C-13), δ 52.64 (C-14) and δ 63.51 (C-17), and the methyl signal at δ 0.94 (H_3 -30) showed long range correlations with the carbons at δ 49.10 (C-8), δ 44.98 (C-13), δ 52.64 (C-14), and at δ 43.52 (C-15), indicating that C-12, C-14, C-17, and C-18 were connected with C-13 and C-8, C-13, C-15, and C-30 were connected with C-14.

The presence of two sugar residues was established by the observation of anomeric carbons at δ 102.80 and δ 104.16. A combination of ^1H - ^1H COSY, HMQC, HMBC and NOESY experiments allowed unambiguous assignments of all ^1H and ^{13}C signals in the sugar moieties, identified as two β -D-glucopyranoses. Starting from the anomeric protons of each sugar unit, all the hydrogens within each spin system were assigned by COSY spectrum. On the basis of assigned protons, the ^{13}C resonances of each sugar moiety were identified by HMQC and further confirmed by the HMBC. The absence of any glycosylation shift for the ^{13}C -NMR resonances of both the glucopyranosyl units, suggested that **1** was a bidesmosidic saponin. Finally the sites of attachment of the glucose moieties on the aglycone of **1** were established by the HMBC spectrum. The long range correlations observed

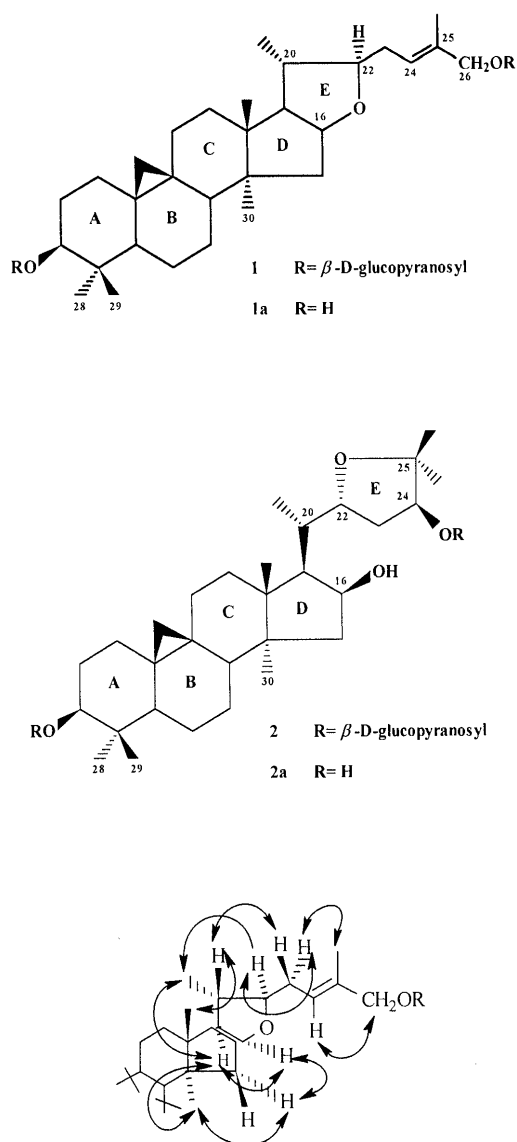


Fig. 2. Cross Peaks from the NOESY Experiment for Compound 1

between H-1' (δ 4.48) of the glucose and C-3 (δ 90.89) of the aglycone and between H-1'' of the second glucose unit at δ 4.23 and C-26 (δ 75.83), confirmed that the glucose moieties were linked to the C-3 and C-26 positions of the aglycone.

The stereochemistry of **1** was established from the NOESY and NOE difference experiments (Fig. 2). The correlations observed between H₃-21/H-17, H₃-30/H-17 and between H₃-18/H-20, established the usual H-17 α and H-20 β configuration of the cycloartane derivatives. The cross peaks observed between H₃-21/H-16, H-22, H-23 α , between H₃-30/H-16, H-15 α , and between H-17/H-16, indicated the configurations at C-16, and C-22 to be 16 β 22 R , respectively. In addition, the NOEs observed between H-16/H-17, H15 α and between H-22/H-23 α , H₃-21 further supported the 16 β , 22 R configurations at C-16 and C-22, respectively. Comparison of the ¹³C-NMR data of depressoside C with known compounds having a similar 16,22-epoxy side chain^{10–12} also confirmed the 22 R configuration in **1**. The NOE correlations observed between the olefinic protons (H-24) and the oxymethylene protons (H₂-26) suggested the *E*-geometry of the double bond. On the basis of the above evidence the structure of **1**

was established as (22 R)-16 β ,22-epoxy-3 β ,26-dihydroxy-9,19-cyclolanost-24 E -ene 3, 26-di-*O*- β -D-glucopyranoside. To the best of our knowledge, this study is the first instance of the isolation of a cycloartane-type saponin from natural products possessing a 16,22-epoxy ring in the side chain.

Compound **2** was isolated as an amorphous powder. The positive ion FAB-MS of **2** exhibited a quasi-molecular ion peak at m/z 821 [M+Na]⁺, and a molecular ion peak at m/z 799 [M+H]⁺ (Calcd for C₄₂H₇₁O₁₄). The fragment ions at m/z 659 [M+Na-162]⁺ and at m/z 497 [M+Na-2 $\times$ 162]⁺, indicated the presence of two hexose units in the molecule. The negative ion FAB-MS showed the pseudomolecular ion peak at m/z 797 [M-H]⁻ and the fragment ions at m/z 635 [M-H-162]⁻, 599 [M-H-162-2H₂O]⁻, 455 [M-H-2 $\times$ 162-H₂O]⁻, also indicating the presence of two hexoses in the molecule.

The ¹H-NMR (CD₃OD, 400 MHz) spectrum of **2** showed signals for six tertiary methyl groups at δ 0.89 (3H, s, H₃-29), 0.95 (3H, s, H₃-30), 1.07 (3H, s, H₃-28), 1.17 (3H, s, H₃-18), 1.22 (3H, s, H₃-27), 1.30 (3H, s, H₃-26), a secondary methyl group at δ 0.92 (3H, d, J =7.0 Hz, H₃-21), and a cyclopropyl methylene group (ABq at δ 0.35 and 0.59, J =4.0 Hz, H₂-19), indicating a cycloartane skeleton as the aglycone. The ¹H-NMR spectrum of **2** also contained two anomeric proton signals at δ 4.32 (1H, d, J =7.7 Hz) and δ 4.48 (1H, d, J =7.6 Hz), suggesting the presence of two β -linked sugars in the molecule. The ¹³C-NMR (CD₃OD, 100 MHz) spectrum of **2** exhibited 42 carbon resonances, of which 30 were attributed to the aglycone. The remaining 12 signals were in good accordance for the presence of two terminal β -D-glucopyranoses, anomeric carbons were observed at δ 104.17 and δ 105.45. The ¹³C-NMR resonances arising from the sapogenol moiety were very similar to those already reported for depressogenin⁶) except for the signals assigned to C-3 (δ 91.01) and C-24 (δ 86.85), exhibiting significant glycosylation shifts. Full assignments of the ¹H and ¹³C signals of **2** were secured by ¹H-¹H COSY, HMQC and HMBC spectra (Table 2). Acid hydrolysis of **2** yielded D-glucose as the only sugar residue and the aglycone **2a** which was identical to the already reported aglycone depressogenin, confirmed by direct comparison of TLC, EI-MS and ¹H-NMR data. Recently two novel cycloartane derivatives, cyclokiridol and isocyclokiridol, possessing the same 22,25-epoxy side chain have been reported.¹⁴) Comparison of the ¹³C-NMR data of depressogenin with cyclokiridol (22 S) and isocyclokiridol (22 R) supported the previously assigned 22 R configuration at C-22 chiral center.

The position of the sugar residues was unambiguously defined by ¹³C-NMR compared with that of the aglycone, depressogenin. The signals of the oxygenated carbons C-3 and C-24 appeared at lower field than the corresponding signals in depressogenin, while the oxymethine carbons C-16 (δ 73.05) and C-22 (δ 81.67) appeared at almost the same chemical shifts. Therefore the sugar moieties must be located at the C-3 and C-24 positions. This was further confirmed from HMBC experiment. A cross peak due to long range correlations between C-3 (δ 91.01) of the aglycone and H-1' of Glc I (δ 4.48) confirmed that the glucose residue was linked to C-3 of the aglycone. The cross peak between C-24 (δ 86.85) and H-1'' of Glc II (δ 4.32) confirmed the location of the second glucose unit at C-24. Thus, **2** was identified as

Table 2. ^{13}C (100 MHz) and ^1H -NMR (400 MHz) Spectral Data of **2** from 1D and 2D-NMR Experiments in CD_3OD

Carbon	DEPT	δ_{C}	δ_{H} (J, Hz)
1	CH_2	33.10	1.15, 1.53
2	CH_2	30.25	1.75, 2.05
3	CH	91.01	3.23 dd (4.4, 11.3)
4	C	42.04	—
5	CH	48.88	1.31 dd (3.4, 12.2)
6	CH_2	22.06	0.82, 1.62
7	CH_2	27.26	1.02, 1.32
8	CH	49.46	1.60 dd (4.4, 12.0)
9	C	21.06	—
10	C	27.42	—
11	CH_2	27.35	1.13, 2.05
12	CH_2	34.35	1.38, 1.65
13	C	47.89	—
14	C	47.02	—
15	CH_2	47.40	1.36, 1.95
16	CH	73.05	4.53 ddd (5.6, 8.0, 8.1)
17	CH	52.47	1.97 dd (7.0, 12.0)
18	CH_3	19.41	1.17 s
19	CH_2	30.91	0.35 d (4.0) 0.59 d (4.0)
20	CH	33.51	2.30 m
21	CH_3	16.19	0.92 d (7.0)
22	CH	81.67	4.05 ddd (2.5, 7.5, 7.5)
23	CH_2	35.31	2.20, 2.30
24	CH	86.85	4.02 dd (3.5, 7.0)
25	C	84.14	—
26	CH_3	26.03	1.30 s
27	CH_3	23.37	1.22 s
28	CH_3	26.35	1.07 s
29	CH_3	15.48	0.89 s
30	CH_3	20.63	0.95 s
Glc I			
1'	CH	104.17	4.48 d (7.6)
2'	CH	81.84	4.08 dd (7.6, 9.0)
3'	CH	78.30	3.65 t (9.0)
4'	CH	71.66	3.25 t (9.0)
5'	CH	77.25	3.30 ddd (2.5, 5.5, 9.0)
6'	CH_2	63.15	3.68 dd (5.5, 11.6) 3.85 dd (2.5, 11.6)
Glc II			
1''	CH	105.45	4.32 d (7.7)
2''	CH	75.50	3.20 dd (7.7, 9.2)
3''	CH	77.87	3.68 t (9.2)
4''	CH	71.99	3.40 t (9.2)
5''	CH	77.76	3.35 ddd (2.5, 5.3, 9.2)
6''	CH_2	63.71	3.71 dd (5.3, 12.0) 3.89 dd (2.5, 12.0)

(22*R*,24*S*)-22,25-epoxy-3 β ,16 β ,24-trihydroxy-9,19-cyclolanostane 3, 24-di-*O*- β -D-glucopyranoside.

Experimental Section

General Experimental Procedures Melting points were determined on a Buchi-535 melting point apparatus and are uncorrected. Optical rotations were taken with a JASCO DIP-360 automatic digital polarimeter. The IR spectra were recorded on a JASCO IRA-I spectrophotometer. The ^1H - and ^{13}C -NMR spectra were measured with Bruker AM-400/500 spectrometers in CD_3OD using tetramethylsilane (TMS) as an internal standard. 2D-NMR experiments included COSY-45°, HMQC, NOESY, 2D-J-RESOLVED, DEPT and HMBC. Coupling constants (*J*-Values) are given in Hertz (Hz). The FAB-MS were recorded in glycerol matrix on a Jeol JMS HX-110 mass spectrometer. EI-MS was recorded with a Finnigan MAT-312 double focusing mass spectrometer. Kieselgel 60 (35–70 mesh, Merck) and Sephadex LH-20 were used for column chromatography, silica gel 60 F_{254} (Merck) was used for TLC. Flash chromatography was carried out with a Eyla Flash Chromatography EF-10 instrument. The flash column was packed with RP-18 silica (Merck).

Plant Material The plant material was collected from Karachi in June, 1995. A voucher specimen of the plant is deposited at the herbarium of Department of Botany, University of Karachi.

Extraction and Isolation Air dried plants of *Corchorus depressus* L. were cut into small pieces and were extracted with 95% EtOH. The solvent was removed by rotary evaporation and the dark brown extract obtained was dissolved in H_2O and extracted with hexane, CHCl_3 , EtOAc and finally with *n*-BuOH. The *n*-BuOH extract was concentrated *in vacuo* to give 182 g of a solid residue. This solid residue was then subjected to silica gel (1.6 kg) column chromatography, elution was carried out with a gradient of CHCl_3 in MeOH. The fractions eluted with CHCl_3 -MeOH (4:1) gave a mixture of crude saponins. This saponin mixture was further subjected to Sephadex LH-20 column chromatography using a water-methanol (1:1) system as eluent. The fractions obtained were again subjected to repeated silica gel column chromatography, eluting with a CHCl_3 -MeOH- H_2O (79.5:20:0.5) system to give a mixture of two saponins. Reversed phase flash chromatography using Lichrospher RP-18 silica (20 g, cat. No. 16105, EM Science) and the elution with MeOH- H_2O (1:1), afforded **1** (25 mg) and **2** (50 mg). The purity of the compounds was checked on HPTLC plates using an *n*-BuOH-AcOH- H_2O (12:3:5) system. The spots were visualized by spraying with ceric ammonium sulfate reagent followed by heating.

Depressoside C (**1**): Amorphous solid, mp 173–175°C (decomp.), $[\alpha]_{\text{D}}^{28} -85.97^\circ$ ($c=0.164$, MeOH). IR (KBr) cm^{-1} : 3350–3450 (OH), 2160 (C=C). FAB-MS (Positive mode) m/z : 803 $[\text{M}+\text{Na}]^+$, 641 $[\text{M}+\text{Na}-162]^+$, 623 $[\text{M}+\text{Na}-162-\text{H}_2\text{O}]^+$, 461 $[\text{M}+\text{Na}-2\times 162-\text{H}_2\text{O}]^+$. FAB-MS (Negative mode) m/z : 779 $[\text{M}-\text{H}]^-$, 617 $[\text{M}-\text{H}-162]^-$, 599 $[\text{M}-\text{H}-162-\text{H}_2\text{O}]^-$, 455 $[\text{M}-\text{H}-2\times 162]^-$, 437 $[\text{M}-\text{H}-2\times 162-\text{H}_2\text{O}]^-$. EI-MS m/z : 600 $[\text{M}-162-\text{H}_2\text{O}]^+$ (2.8), 456 $[\text{aglycone}]^+$ (5.2), 438 $[\text{aglycone}-\text{H}_2\text{O}]^+$ (16.1), 370 (92.8), 312 (72.9), 294 (36.9), 230 (73.3), 120 (80.0), 106.0 (100). ^1H - and ^{13}C -NMR (CD_3OD): Table 1.

Depressoside D (**2**): Amorphous solid, mp 213–215°C, $[\alpha]_{\text{D}}^{25} -24.96^\circ$ ($c=0.45$, MeOH). IR (KBr) cm^{-1} : 3350–3450 (OH). FAB-MS (Positive mode) m/z : 821 $[\text{M}+\text{Na}]^+$, 799 $[\text{M}+\text{H}]^+$, 659 $[\text{M}+\text{Na}-162]^+$, 641 $[\text{M}+\text{Na}-162-\text{H}_2\text{O}]^+$, 497 $[\text{M}+\text{Na}-2\times 162]^+$, 479 $[\text{M}+\text{Na}-2\times 162-\text{H}_2\text{O}]^+$. FAB-MS (Negative mode) m/z : 797 $[\text{M}-\text{H}]^-$, 635 $[\text{M}-\text{H}-162]^-$, 599 $[\text{M}-\text{H}-162-2\text{H}_2\text{O}]^-$, 455 $[\text{M}-\text{H}-2\times 162-\text{H}_2\text{O}]^-$. ^1H - and ^{13}C -NMR (CD_3OD): Table 2.

Acid Hydrolysis of **1 and **2**** A sample of **1** (15 mg) was dissolved in MeOH and was refluxed for 4 h with 1 M HCl. After cooling the reaction mixture, MeOH was evaporated *in vacuo*. Distilled water (3 ml) was added and the solution was extracted with EtOAc. The aqueous layer was neutralized with Ag_2CO_3 , filtered and evaporated *in vacuo*. The residue obtained was compared with authentic sugar samples on silica gel cards using the solvent system EtOAc-MeOH-HOAc- H_2O (11:2:2:2). The spots were visualized with aniline phthalate reagent which indicated that the only sugar unit present was D-glucose. The EtOAc layer was concentrated *in vacuo*. The residue on TLC, indicated the presence of several artifact sapogenols, which could not be isolated or identified. D-Glucose and depressogenin **2a** were obtained when **2** was treated in the same way.

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References and Notes

- Ali S. I., Nasir E., "Flora of Pakistan," No. 75, Ferozsons, Karachi, Pakistan, 1974, p. 20.
- Perry L., Metzger J., "Medicinal Plants of East and Southeast Asia," MIT Press, Cambridge, England, 1980, p. 407.
- Wahid A., Siddiqui H. H., "A Survey of Drugs," 2nd Ed., IHMMR, Delhi, India, 1961.
- Harsh M. L., Nag T. N., *Geobios*, **15**, 32 (1988).
- Khan M. S. Y., Javed K., Khan M. H., Shamsi M. A., Siddiqui A. A., *Phytochemistry*, **30**, 1989–1992 (1991).
- Ahmad V. U., Ali A., Ali Z., Baqai F. T., Zafar F. N., *Phytochemistry*, **49**, 829–834 (1998).
- Yoshimitsu H., Hayashi K., Shinju K., Kinjo J., Yahara S., Nakano K., Murakami K., Tomimatsu T., Nohara T., *Chem. Pharm. Bull.*, **40**, 2465–2468 (1992).
- Gromova A. S., Lutsikii V. I., Zinchenko S. V., Trofimova N. N., Semenov A. A., Nakhova N. A., *Khim. Priir. Soedin.*, **1993**, 103–109; *Chemistry of Natural Compounds (Engl. Transl.)*, **29**, 87–91 (1993).
- Gromova A. S., Lutsikii V. I., Semenov A. A., Deniskeno V. A., Isakov V. V., *Khim. Priir. Soedin.*, **1984**, 213–219; *Chemistry of Natural Compounds (Engl. Transl.)*, **20**, 197–202 (1984).

- 10) Renault J.-H., Ghedira K., Thepenier P., Lavaud C., Zeches-Hanrot M., Men-Olivier L. L., *Phytochemistry*, **44**, 1321—1327 (1997).
- 11) Oulad-Ali A., Guillaume D., Weniger B., Jiang Y., Anton R., *Phytochemistry*, **36**, 445—448 (1994).
- 12) Yoshikawa K., Shimono N., Arihara S., *Chem. Pharm. Bull.*, **40**, 2275—2278 (1992).
- 13) Kusano A., Takahira M., Shibano M., In Y., Ishida T., Miyase T., Kusano G., *Chem. Pharm. Bull.*, **46**, 467—472 (1998).
- 14) Kimura Y., Akihisa T., Yasukawa K., Takase S., Tamura T., Ida Y., *Chem. Pharm. Bull.*, **45**, 415—417 (1997).

Chemical States and Lattice Dynamics of α -Diimine Fe^{2+} Complexes Intercalated into γ -Zirconium Phosphate

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The α -diimine Fe^{2+} complexes, $[\text{Fe}(\text{phen})_3]^{2+}$, $[\text{Fe}(\text{bpy})_3]^{2+}$, and $[\text{Fe}(\text{terpy})_2]^{2+}$, (phen: 1,10-phenanthroline, bpy: 2,2'-bipyridyl, terpy: $\alpha, \alpha', \alpha''$ -tripyridine) were intercalated into zirconium dihydrogenphosphate phosphate dihydrate (γ -zirconium phosphate, $\gamma\text{-ZrP}$), $\text{Zr}(\text{PO}_4)(\text{H}_2\text{PO}_4) \cdot 2\text{H}_2\text{O}$. The rate of the intercalation, the molar ratio of Fe to Zr, was found to be 3.82–7.76%. Mössbauer spectra indicated that one part of $[\text{Fe}(\text{phen})_3]^{2+}$ and $[\text{Fe}(\text{bpy})_3]^{2+}$ changed from a low-spin Fe^{2+} to high-spin Fe^{2+} state on intercalation, but $[\text{Fe}(\text{terpy})_2]^{2+}$ did not change in chemical state. The lattice dynamics of the complexes and the intercalation compounds were investigated in terms of the temperature dependence of the area intensity on the Mössbauer spectra. A linear relationship was established for all the complex salts and the intercalation compounds investigated between the $\ln[A(T)/A(82)]$ and absolute temperature, T , where $A(T)$ and $A(82)$ show the intensities of a doublet at T and 82 K of the Mössbauer spectra, respectively. From the slope of the linear relation, the Θ^2M values, which were derived based on the Debye approximation of lattice vibration, were evaluated for the complex salts and the intercalation compounds. The Fe^{2+} complexes showed Θ^2M values of 1.27 to 2.32×10^6 , whereas the intercalation compounds showed very similar values to each other, ranging from 2.19 to 2.39×10^6 , irrespective of different α -diimine ligands. The results were explained in terms of the characteristic layered structure of zirconium phosphate, and by the tight bond between the α -diimine Fe^{2+} complexes and the host $\gamma\text{-ZrP}$.

Key words intercalation; Mössbauer Study; α -diimine Fe^{2+} -complex; lattice dynamics; γ -zirconium phosphate

Recent studies on the intercalation of ions or small molecules into various layered compounds have shown new chemical properties on ion exchange, redox reactions, and a novel drug carrier system.^{1–4)} Because of its excellent stability toward temperature, oxidizing agents and radiation, investigation of the intercalation of crystalline zirconium phosphate (ZrP) has been attractive. The intercalation of metal ions into layered structure of a crystalline ZrP has been studied as well as the intercalation of polar organic molecules such as alcohols, glycols, and amine.^{5–7)} Among these studies, the ion-exchange properties of zirconium phosphate towards divalent transition metal ions have been investigated extensively by Allulli *et al.*⁵⁾ in relation to the chemical composition and structure.

Studies of the crystal structures of ZrP have revealed that three crystalline phases are present depending on the preparation, *i.e.*, α -ZrP is known as mono hydrate, $\text{Zr}(\text{HPO}_4)_2 \cdot \text{H}_2\text{O}$, β -ZrP as anhydrous $\text{Zr}(\text{HPO}_4)_2$, and γ -ZrP as $\text{Zr}(\text{PO}_4)(\text{H}_2\text{PO}_4) \cdot 2\text{H}_2\text{O}$, respectively.^{8,9)} The ion-exchange and uptake behaviors of alkaline metal ions have been investigated using $\gamma\text{-ZrNH}_4\text{H}(\text{PO}_4)_2$.^{10,11)} Allulli *et al.* studied the ion-exchange mechanism of divalent transition metal ions on one half converted zirconium phosphate, $\text{ZrMH}(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$ ($M = \text{Na}$ and Li), and found that the ion exchange occurs in two steps, from 0 to 80% and from 80 to 100% conversion of the exchange.¹²⁾ Ferragina *et al.* studied in detail the intercalation of 2,2'-bipyridyl into α -ZrP as well as the subsequent uptake of 3d transition metal ions such as Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , and Zn^{2+} into the intercalate.^{13–14)}

Alonzo *et al.* studied the uptake of iron in $\alpha\text{-Zr}(\text{HPO}_4)_2(\text{bpy})_{0.25} \cdot 1.5\text{H}_2\text{O}$, $\alpha\text{-Zr}(\text{HPO}_4)_2(\text{phen})_{0.5} \cdot 2\text{H}_2\text{O}$ and $\alpha\text{-Zr}(\text{HPO}_4)_2(\text{bmp})_{0.5} \cdot 2.5\text{H}_2\text{O}$, and found by Mössbauer spectroscopy that low-spin tris-chelate, $[\text{Fe}(\text{phen})_3]^{2+}$, is

formed partly in the case of the uptake of iron by phen intercalate.¹⁵⁾ Tomita *et al.* examined the intercalation condition of phen and its derivatives into $\gamma\text{-ZrP}$, as well as the uptake of Fe^{2+} ions into the intercalates. They compared the chemical states of ^{57}Fe ions in the intercalates with those in the direct intercalation of trisphenanthroline Fe^{2+} chelates into $\gamma\text{-ZrP}$ by means of Mössbauer spectroscopy.¹⁶⁾

Alonzo *et al.* found that *in situ* iron-amine coordination occurs in the case of iron-bipyridyl- and iron-phenanthroline- γ -zirconium phosphate by means of Mössbauer spectroscopy, and found that low spin tris-chelates $[\text{Fe}(\text{bipy})_3]^{2+}$ and $[\text{Fe}(\text{phen})_3]^{2+}$ are formed between the layers of the host.¹⁷⁾ They also extended their work to examine the uptake rates of diimines (2,2'-bipyridyl (2,2'-bipy), 2,4'-bipyridyl (2,4'-bipy), and 4,4'-bipyridyl (4,4'-bipy) into $\gamma\text{-ZrP}$ under various conditions such as temperature, ligands, and solvents.¹⁸⁾

In the present study, α -diimine Fe^{2+} complexes such as $[\text{Fe}(\text{phen})_3]^{2+}$, $[\text{Fe}(\text{bpy})_3]^{2+}$, and $[\text{Fe}(\text{terpy})_2]^{2+}$ were intercalated into $\gamma\text{-ZrP}$. It has been shown that the interlayer separation is 7.6 Å for the α -form, 9.28 Å for β -, and 12.2 Å for the γ -form.⁸⁾ In the present work, the γ -form with the largest interlayer separation was used. Although temperature dependence of resonance intensity for ionically bonded compounds and molecular compounds has been investigated, little is known regarding intercalation compounds.^{19–22)} The temperature dependence of the Mössbauer intensity of α -diimine Fe complex intercalated into ZrP was studied in order to clarify the chemical bond between the guest and host, and also the effects of the ligand mass.

Experimental

Chemicals 1,10-Phenanthroline, 2,2'-bipyridyl and $\alpha, \alpha', \alpha''$ -tripyridine were obtained from Kanto Chemical Co., Inc., $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$, NaH_2PO_4 .

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2H₂O and (NH₄)₂Fe(SO₄)₂·6H₂O from Wako Pure Chemicals, and other reagents used were in extra pure grade.

Preparation of the Host Material γ -ZrP (γ -Zr(PO₄)(H₂PO₄)·2H₂O) was prepared by following the method described in the literature.^{23,24} Reagent grade ZrOCl₂·8H₂O (8 g) was dissolved in 25 ml of water, and the solution was added gently to 50 ml of 3 N HCl solution containing NaH₂PO₄·2H₂O of 62.5 g under constant stirring and refluxing until the solution was changed to slurry. The gelatinous reaction mixture was transferred into a Pyrex glass tube and subjected to hydrothermal treatment in an autoclave at 185 °C for one week. After filtration, the crystalline γ -ZrP was washed with 1 N HCl on filter paper. It was washed with water fully to remove Cl⁻ ions and then dried in a silica gel desiccator. The prepared γ -ZrP was identified by X-ray diffractometry.^{24,25} Further characterization of γ -ZrP was performed by thermogravimetric analysis (TG) of Seiko Denshi Co., Ltd. The programmed rate of heating was 1 °C/min.

Preparation of Fe²⁺ Complexes [Fe(phen)₃]X₂ (X = Cl⁻ and ClO₄⁻) and [Fe(bpy)₃]Cl₂ were prepared by the procedure reported in the literature.^{26–28} [Fe(terpy)₂]Br₂·4H₂O was prepared by a method analogous to that for [Fe(phen)₃]Cl₂·H₂O.

Intercalation of Fe²⁺ Complexes into γ -ZrP Intercalation of Fe²⁺ complexes into γ -ZrP was performed in a 100 ml Pyrex glass vial at 50 °C for 48 h based on a suspension method under constant stirring. The molar ratio of the Fe²⁺ complex to γ -ZrP was 1 : 2 for all the complexes investigated. A saturated water solution of Fe²⁺ complex was used for intercalation. After intercalation, the intercalation compound was separated by filtration, then washed with water. The prepared intercalation compounds were stored in silica gel desiccator until measurement.

Measurement X-Ray diffraction patterns of the intercalation compounds were measured with Ni-filtered CuK_α radiation (40 kV, 20 mA) using a Geigerflex RAD-C diffractometer of Rigaku-Denki Co., Ltd.

⁵⁷Fe Mössbauer spectra were measured using a Wissel Mössbauer spectrometer in a constant acceleration mode against a moving 370 MBq ⁵⁷Co (Rh) source. The velocity scale was calibrated using ⁵⁷Fe-enriched iron foil at room temperature. A Heli-Tran LT-3-110 Cryochip from Air Product Co., Ltd. was used to measure Mössbauer spectra at various temperatures ranging from 315 to 82 K. Mössbauer spectra were fitted with a least square fitting program of Lorentzian functions.

Neutron Activation Analysis Neutron activation analysis was applied in order to determine the atomic ratio of Fe to Zr in the intercalation compounds. Accurately weighted intercalation compounds (*ca.* 100 mg) were sealed in a polyethylene sheet and irradiated for 24 h with a thermal neutron flux of 10¹¹ n·cm⁻²·s⁻² under a 100 kW operation at F-24 of the TRIGA II Nuclear Reactor at the Atomic Energy Institute of Rikkyo University. Reference materials used were 100 mg of prepared γ -ZrP and 15 mg of (NH₄)₂Fe(SO₄)₂·6H₂O, which were irradiated simultaneously with the intercalation compounds. The γ -rays of ⁵⁹Fe and ⁹⁵Zr of the intercalation compounds and the reference materials were measured with a high-purity Ge detector after cooling radioactivities for a week, and the atomic ratios of Fe to Zr in the prepared intercalation compounds were evaluated.

Results and Discussion

X-Ray Diffraction Patterns The X-ray diffraction patterns (XRD) of the intercalation compounds are shown in Fig. 1. The interlayer distance along the c-axis of γ -ZrP is 12.19 Å, whereas those intercalated with [Fe(phen)₃]²⁺, [Fe(bpy)₃]²⁺, and [Fe(terpy)₂]²⁺ were enlarged up to 19.66, 18.86, and 18.70 Å, respectively. The distance observed on [Fe(phen)₃]²⁺ intercalation is consistent with 19.9 Å, as reported by Tomita *et al.*¹⁶ The diffraction peak of 12.19 Å was completely disappeared in all the intercalation compounds. The increment of interlayer distance may be related to the packing mode of guest molecules. In the present case, the interlayer distances observed for three intercalated compounds are obviously greater than 15.2 Å reported for the bis-type γ -ZrP/[Cu(bpy)₂]²⁺.²⁹ Regarding the intercalation of Cu²⁺ ions, Tomita *et al.* concluded from the electronic spectra that Cu²⁺ ions incorporated into γ -ZrP/bpy host were exchanged with residual protons in P-OH groups.²⁹ In fact, it was observed in the present experiments that the pH values of the

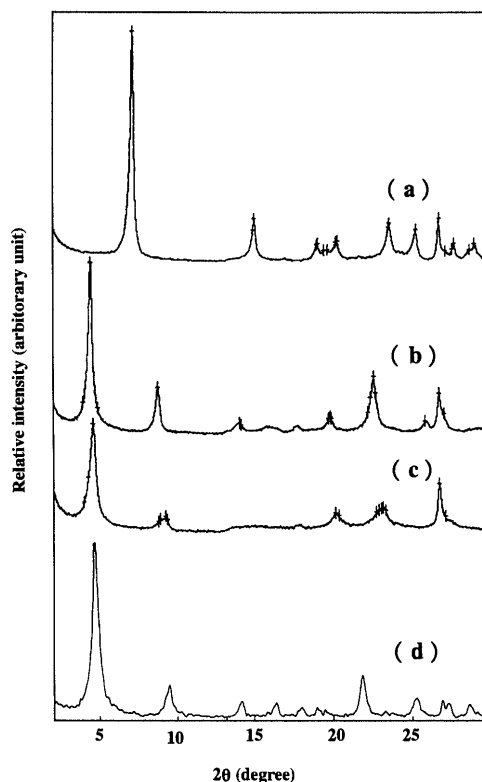


Fig. 1. X-ray Diffraction Patterns of (a) γ -ZrP, (b) γ -ZrP/[Fe(phen)₃]²⁺, (c) γ -ZrP/[Fe(bpy)₃]²⁺, and (d) γ -ZrP/[Fe(terpy)₂]²⁺

Table 1. Molar Ratio of Iron to Zirconium in Intercalation Compounds and the Lattice Parameter, Θ^2M , Evaluated from the Debye Model for α -Diimine Fe²⁺ Complexes and Those Intercalated into γ -Zirconium Phosphate

	(Fe/Zr)×10 ⁻²	Θ^2M ×10 ⁻⁶
[Fe(phen) ₃](ClO ₄) ₂ ·H ₂ O		1.27
[Fe(bpy) ₃]Cl ₂ ·7H ₂ O		1.36
[Fe(terpy) ₂]Br ₂ ·4H ₂ O		2.32
γ -ZrP/[Fe(phen) ₃] ²⁺	7.76	2.31(4.56) ^a
γ -ZrP/[Fe(bpy) ₃] ²⁺	3.82	2.19(4.23) ^a
γ -ZrP/[Fe(terpy) ₂] ²⁺	6.62	2.39

a) Values in parenthesis are in high-spin Fe²⁺ which formed on intercalation.

saturated solution of [Fe(phen)₃]²⁺ containing γ -ZrP changed from 7.0 to 2.0 when the Fe complex ions were intercalated into the host. This indicates that the intercalation of tris-type Fe chelates proceeds *via* ion exchange.

Ratio of the Host to Guest in Intercalation Compounds

The molar ratio of Fe to Zr in the intercalation compounds was determined by neutron activation analysis, and the results are listed in the second column of Table 1. The molar ratio of Fe to Zr was found to be 3.82 to 7.76% when the α -diimine complexes, [Fe(phen)₃]²⁺, [Fe(bpy)₃]²⁺ and [Fe(terpy)₂]²⁺ were intercalated. The effects of the anions on the uptake of [Fe(phen)₃]X₂ (X = Cl, Br and ClO₄) into γ -ZrP were examined in water at 50 °C for 48 h, since the solubility is largely different among the perchlorate, chloride and bromide. In the intercalation experiments, the saturated solution of each Fe²⁺ complex salt was used at 50 °C. The results, however, showed that the intercalation (uptake) did not depend on the counter anions of the complex. These results are similar to those reported so far for the bis-type copper complex²⁹ and other divalent transition elements,¹³ although the

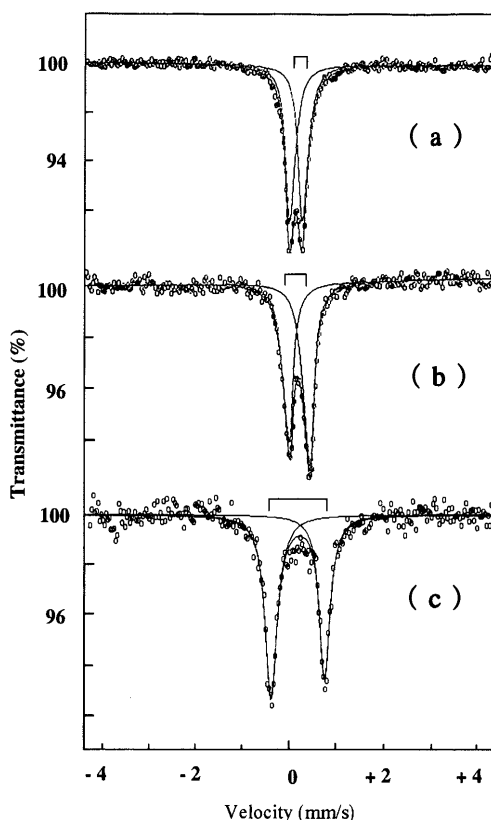


Fig. 2. Mössbauer Spectra at Room Temperature of (a) $[\text{Fe}(\text{phen})_3](\text{ClO}_4)_2 \cdot \text{H}_2\text{O}$, (b) $[\text{Fe}(\text{bpy})_3]\text{Cl}_2 \cdot 7\text{H}_2\text{O}$, and (c) $[\text{Fe}(\text{terpy})_2]\text{Br}_2 \cdot 4\text{H}_2\text{O}$

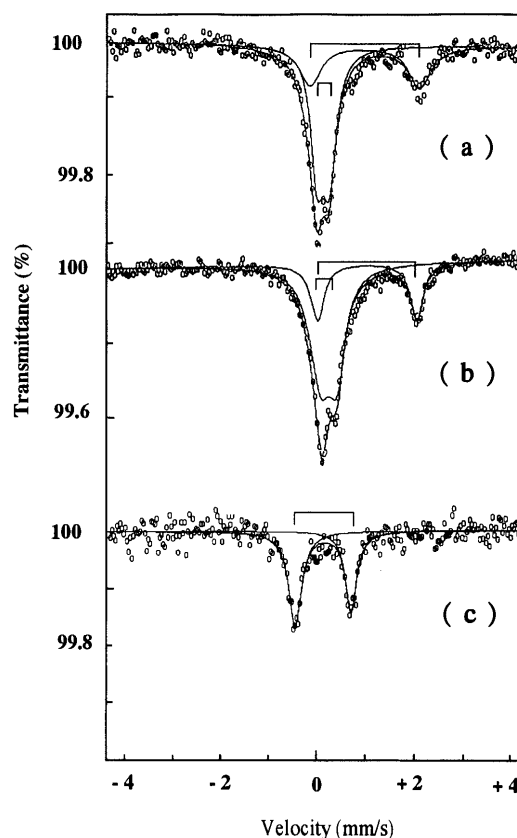


Fig. 3. Mössbauer Spectra at Room Temperature of (a) $\gamma\text{-ZrP}/[\text{Fe}(\text{phen})_3]^{2+}$, (b) $\gamma\text{-ZrP}/[\text{Fe}(\text{bpy})_3]^{2+}$, and (c) $\gamma\text{-ZrP}/[\text{Fe}(\text{terpy})_2]^{2+}$

geometrical sizes of the cations are different.

Chemical States of Iron in Intercalation Compounds

Mössbauer spectra at room temperature of $[\text{Fe}(\text{phen})_3](\text{ClO}_4)_2 \cdot \text{H}_2\text{O}$, $[\text{Fe}(\text{bpy})_3]\text{Cl}_2 \cdot 7\text{H}_2\text{O}$ and $[\text{Fe}(\text{terpy})_2]\text{Br}_2 \cdot 4\text{H}_2\text{O}$ are shown in Fig. 2, and those of $[\text{Fe}(\text{phen})_3]^{2+}$, $[\text{Fe}(\text{bpy})_3]^{2+}$ and $[\text{Fe}(\text{terpy})_2]^{2+}$ intercalated into $\gamma\text{-ZrP}$ are in Fig. 3. Based on the Mössbauer spectra, one part of $[\text{Fe}(\text{phen})_3]^{2+}$ and $[\text{Fe}(\text{bpy})_3]^{2+}$ changed to high-spin Fe^{2+} on intercalation, while $[\text{Fe}(\text{terpy})_2]^{2+}$ did not change. The spectral areas of 62.9% for $[\text{Fe}(\text{phen})_3]^{2+}$ and 71.7% for $[\text{Fe}(\text{bpy})_3]^{2+}$ remained as original low-spin Fe^{2+} under the present intercalation conditions. Mössbauer parameters of the intercalation compounds showed that the low-spin Fe^{2+} states had no remarkable influence on intercalation. In the case of terpy complex, the $\alpha, \alpha', \alpha''$ -tripyridine acts as a tridentate ligand, and the bond between the Fe-ion and the ligand is more stable because of the chelate effects. It is, therefore, concluded that the terpy complex was more stable than other phen and bpy complexes on intercalation under the present condition.

Temperature Dependence of Mössbauer Spectra for α -Diimine Fe^{2+} Complex Salts and Intercalation Compounds

The logarithmic resonance area ratio, $\ln[A(T)/A(82)]$, of the spectra for $[\text{Fe}(\text{phen})_3](\text{ClO}_4)_2 \cdot \text{H}_2\text{O}$, $[\text{Fe}(\text{bpy})_3]\text{Cl}_2 \cdot 7\text{H}_2\text{O}$, $[\text{Fe}(\text{terpy})_2]\text{Br}_2 \cdot 4\text{H}_2\text{O}$ are plotted against absolute temperature in Fig. 4, where $A(82)$ is the resonance area of the quadrupole doublet of the low spin Fe^{2+} at 82 K, and $A(T)$ the resonance area at T K. For each Fe^{2+} complex salt and intercalation compound, a good linear relationship was established for the investigated temperature range from 82 to 314 K. $[\text{Fe}(\text{terpy})_2]\text{Br}_2 \cdot 4\text{H}_2\text{O}$ showed the most mild slope in

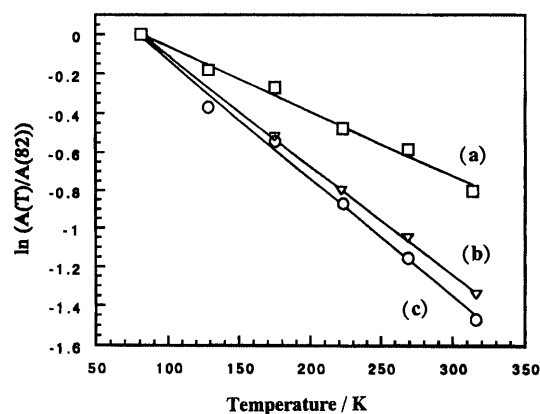


Fig. 4. Logarithmic Resonance Area Ratio, $\ln[A(T)/A(82)]$, of (a) $[\text{Fe}(\text{terpy})_2]\text{Br}_2 \cdot 4\text{H}_2\text{O}$, (b) $[\text{Fe}(\text{bpy})_3]\text{Cl}_2 \cdot 7\text{H}_2\text{O}$, and (c) $[\text{Fe}(\text{phen})_3](\text{ClO}_4)_2 \cdot \text{H}_2\text{O}$ Plotted against Absolute Temperature

these complex salts investigated, whereas $[\text{Fe}(\text{phen})_3](\text{ClO}_4)_2 \cdot \text{H}_2\text{O}$ the steepest. The intensity of a Mössbauer spectrum depends on the intermolecular bond strength such as van der Waals interaction, hydrogen bond, or ionic bond. Therefore, it also depends on the anions of the complex. For the effective thickness of the absorber to be thin enough, the relative value of the recoil-free fraction, f_a , is given by an expression as

$$f_a = \exp(-3E_\gamma^2 T / Mc^2 k \Theta^2) \quad (1)$$

where E_γ is the Mössbauer transition energy, M the effective mass of the resonance absorber, k the Boltzmann constant, c the velocity of light, Θ the characteristic Debye tempera-

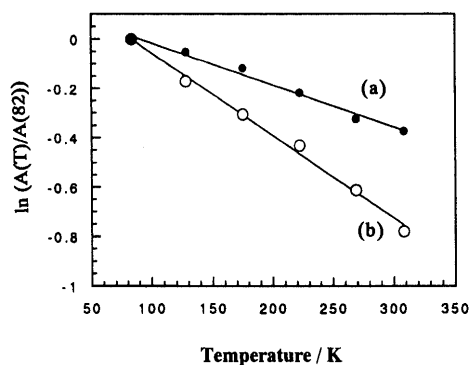


Fig. 5. Logarithmic Resonance Area Ratio, $\ln[A(T)/A(82)]$, of (a) Low-Spin Fe^{2+} and (b) the High-Spin Fe^{2+} Doublets Observed in $\gamma\text{-ZrP}/[\text{Fe}(\text{phen})_3]^{2+}$ Plotted against Absolute Temperature

$A(82)$ and $A(T)$ indicate the resonance areas of the quadruple doublet of Fe^{2+} at 82 and T K, respectively.

ture.^{19–22)}

Since the relative value of f_a can be replaced by a given resonance area, A , of a spectrum, Eq. (1) can be written as

$$d \ln A / dT = -3E_{\gamma}^2 / Mc^2 k \Theta^2 \quad (2)$$

In the Debye model for lattice vibration, the characteristic Debye temperature, Θ , is related to $h\nu_D/k$, where ν_D is the characteristic frequency of lattice. If the intermolecular force constant is denoted as α , then ν_D is proportional to $(\alpha/M)^{1/2}$. From the slope of the lines in Fig. 4, the $\Theta^2 M$ values for the complex salts were evaluated and are listed in Table 1, together with those for the intercalation compounds. The values for the complex salts show characteristic intermolecular interactions, resulting in different intermolecular bond strength.

The $\Theta^2 M$ values for the low-spin Fe^{2+} of the intercalation compounds showed larger values than those for the neat complex salts, and lie in a closer range than those for the neat complex salts. By the analogy for organotin compounds,^{21,22)} the $\Theta^2 M$ values in the present case will show the bond strength between the intercalated tris-type Fe^{2+} complex and $\gamma\text{-ZrP}$. The chemical bond is, of course, essentially ionic in character; i.e., in $\text{Zr}(\text{PO}_4)(\text{H}_2\text{PO}_4) \cdot 2\text{H}_2\text{O}(\gamma\text{-ZrP})$, H^+ ions are replaced by $[\text{Fe}(\text{phen})_3]^{2+}$. Similar $\Theta^2 M$ values were obtained among three intercalation compounds. This indicates that the ionic character of the bond between the guest Fe^{2+} α -diimine complex and the host $\gamma\text{-ZrP}$ is nearly the same, irrespective of different ligands. Therefore, the recoilless fraction which affects the intensity of the spectrum may be governed mainly by the two-dimensional strong chemical bond between $\gamma\text{-ZrP}$ and Fe^{2+} α -diimine complexes. The complexes act as pillars between the $\gamma\text{-ZrP}$ layers.

The logarithmic area ratio, $\ln[A(T)/A(82)]$, of high-spin and low-spin Fe^{2+} ions on the Mössbauer spectra of $\gamma\text{-ZrP}/[\text{Fe}(\text{phen})_3]^{2+}$ is plotted against absolute temperature in Fig. 5. It is shown that the slope of the high-spin Fe^{2+} is

more mild than that of the low-spin Fe^{2+} . The $\Theta^2 M$ values for the high-spin Fe^{2+} of $\gamma\text{-ZrP}/[\text{Fe}(\text{phen})_3]^{2+}$ and $\gamma\text{-ZrP}/[\text{Fe}(\text{bpy})_3]^{2+}$ were found to be 4.56 and 4.23×10^{-6} , respectively. This fact can be attributed to the greater ionic radius of the high-spin Fe^{2+} . The Mössbauer parameters of high-spin Fe^{2+} species observed in the intercalation compounds suggest a bis-type α -diimine complex formed during intercalation, although the species is difficult to identify chemically at present.

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References

- 1) Uematsu T., Saitoh T., Shimazu S., Hashimoto H., Hinode H., Taniguchi M., *J. Mater. Science Lett.*, **7**, 47–50 (1988).
- 2) Kanzaki Y., Abe M., *Bull. Chem. Soc. Jpn.*, **64**, 1846–1853 (1991).
- 3) Menendez A., Barcena M., Jaimerez E., Garcia J. R., Rodriguez J., *Chem. Mater.*, **5**, 1078–1084 (1993).
- 4) Watanabe Y., Kanzaki Y., Fujii M., Matsumoto Y., Shiozaki I., Tanaka T., Matsumoto M., *Chem. Pharm. Bull.*, **42**, 163–166 (1994).
- 5) Allulli S., Ginestra A. L., Massucci M. A., Pelliccioni M., Tomassini N., *Inorg. Nucl. Chem. Lett.*, **10**, 337–341 (1974).
- 6) Alberti G., Bertrani R., Casciola M., Costantino U., Gupta J. P., *J. Inorg. Nucl. Chem.*, **38**, 843–848 (1976).
- 7) Costantino U., *J. Chem. Soc. Dalton*, **1979**, 402–405.
- 8) Clearfield A., Blessing R. H., Stynes J. A., *J. Inorg. Nucl. Chem.*, **30**, 2249–2258 (1968).
- 9) Poojary D. M., Shpeizer B., Clearfield A., *J. Chem. Soc. Dalton Trans.*, **1995**, 111–113.
- 10) Kobayashi E., Yamazaki S., *Bull. Chem. Soc. Jpn.*, **56**, 1632–1636 (1983).
- 11) Kobayashi E., *Bull. Chem. Soc. Jpn.*, **56**, 3756–3760 (1983).
- 12) Alluli S., Ferragina C., Ginestra A. L., Massucci M. A., Tomassini N. T., *J. Chem. Soc. Dalton Trans.*, **1977**, 1879–1884.
- 13) Ferragina C., Ginestra A. L., Massucci M. A., Patrono P., Tomlinson A. A. G., *J. Phys. Chem.*, **89**, 4762–4769 (1985).
- 14) Ferragina C., Massucci M. A., Patrono P., Ginestra A. L., Tomlinson A. A. G., *J. Chem. Soc. Dalton Trans.*, **1986**, 265–271.
- 15) Alonzo G., Bertazzi N., Cafarelli P., Ferragina C., Ginestra A. L., Massucci M. A., Patrono P., *Annal. di Chimica*, **81**, 655–671 (1991).
- 16) Tomita I., Sasaki K., Hasegawa Y., Takeda M., Takahashi M., *J. Incl. Phenom. Mol. Recognit. Chem.*, **14**, 317–326 (1992).
- 17) Alonzo G., Bertazzi N., Ferragina C., Ginestra A. L., Massucci M. A., Patrono P., *J. Mater. Chem.*, **7**, 135–142 (1997).
- 18) Ferragina C., Cafarelli P., Stefans A. De, Mattei G., *Mater. Res. Bull.*, **34**, 1039–1053 (1999).
- 19) Matsubara S., Katada M., Sato K., Motoyama I., Sano H., *J. Phys.*, **C2**, 363–366 (1979).
- 20) Katada M., Harber R. H., *J. Phys.*, **C2**, 663–665 (1979).
- 21) Katada M., Uchida Y., Iwai K., Sano H., Sakai H., Maeda M., *Bull. Chem. Soc. Jpn.*, **60**, 911–917 (1987).
- 22) Katada M., Fujita M., Yamada H., Kawata S., Sano H., *Nucl. Instr. and Method Phys. Res., Sect. B* **B76**, 310–312 (1993).
- 23) Costantino U., *J. Inorg. Nucl. Chem.*, **43**, 1895–1902 (1981).
- 24) Clearfield A., Grace J. M., *J. Inorg. Nucl. Chem.*, **41**, 879–884 (1979).
- 25) Yamanaka S., Tanaka M., *J. Inorg. Nucl. Chem.*, **41**, 45–48 (1979).
- 26) Wicholas M., Drago R. S., *J. Am. Chem. Soc.*, **90**, 6946–6950 (1968).
- 27) Madeja K., König E., *J. Inorg. Nucl. Chem.*, **25**, 377–384 (1963).
- 28) Miyoshi K., Taura T., Shimada C., Yoneda H., *Bull. Chem. Soc. Jpn.*, **48**, 1783–1785 (1975).
- 29) Tomita I., Takeo C., Hasegawa Y., *J. Incl. Phenom. Mol. Recognit. Chem.*, **9**, 315–325 (1990).

Human Intestinal Bacteria Capable of Transforming Secoisolariciresinol Diglucoside to Mammalian Lignans, Enterodiols and Enterolactone

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Seven metabolites were isolated after anaerobic incubation of secoisolariciresinol diglucoside (**1**) with a human fecal suspension. They were identified as (–)-secoisolariciresinol (**2**), 3-demethyl-(–)-secoisolariciresinol (**3**), 2-(3-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butane-1,4-diol (**4**), didemethylsecoisolariciresinol (**5**), 2-(3-hydroxybenzyl)-3-(3,4-dihydroxybenzyl)butane-1,4-diol (**6**), enterodiols (**7**) and enterolactone (**8**).

Furthermore, two bacterial strains, *Peptostreptococcus* sp. SDG-1 and *Eubacterium* sp. SDG-2, responsible for the transformation of **1** to a mammalian lignan **7**, were isolated from a human fecal suspension. The former transformed **2** to **3** and **5**, as well as **4** to **6**, and the latter transformed **5** to **6** and **7**.

Key words biotransformation; enterodiols; enterolactone; *Eubacterium* sp.; *Peptostreptococcus* sp.; secoisolariciresinol diglucoside

Fiber-rich foods, such as flaxseed, are consumed in varying quantities in many parts of the world. Such widely used materials are of great concern with regard to their potential biological benefits. Flaxseed is the most significant dietary source of the plant lignan secoisolariciresinol diglucoside (SDG, **1**).¹⁾ Compound **1** was found to be a precursor of phytoestrogenic lignans, enterodiols (**7**) and enterolactone (**8**), which have been detected in the serum, urine and bile of humans and several animals ingesting flaxseed.²⁾ Adlercreutz *et al.* reported that vegetarians excreted large amounts of **7** and **8**, and noted a significant correlation between the excretion of these compounds and the consumption of fiber-rich foods such as flaxseed, grains and legumes.^{3,4)} Compounds **7** and **8** were found to have estrogenic activity and to play an important role in the prevention of breast cancer incidence in vegetarians.^{5–8)} Jacobs *et al.* reported that hepatic microsomes from rats and humans were able to oxidize **7** and **8**,⁹⁾ and that similar derivatives were excreted in the urine of humans ingesting flaxseed.¹⁰⁾ However, these derivatives accounted for less than 5% of the total urinary metabolites. Generally, before being absorbed to the body fluids, orally ingested dietary lignans are expected to undergo metabolism by intestinal bacteria. Borriello *et al.*¹¹⁾ reported that human intestinal bacteria were able to transform **1** to **7** and **8** *in vitro*. However, it should be pointed out that the pathway to the formation of these metabolites was not clearly explained, and the intestinal bacteria capable of transforming **1** to **7** and **8** have not been isolated from human feces so far. In order to clarify the transformation of **1** by intestinal bacteria, the present study was designed to a) isolate intestinal bacterial metabolites of **1** after anaerobic incubation with a human fecal suspension, and b) to isolate bacterial strains capable of transforming **1** to **7** and **8** from human feces.

Results

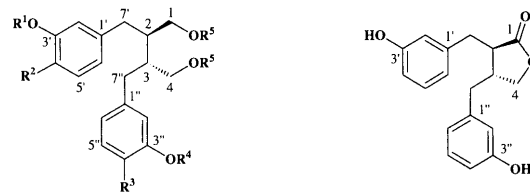
Transformation of Secoisolariciresinol Diglucoside (SDG, **1) by a Human Fecal Suspension** After anaerobic incubation of SDG (**1**) with a human fecal suspension, the culture was extracted with BuOH and then purified by silica gel, Sephadex LH-20 and RP-18 column chromatography to afford seven metabolites (**2–8**) (Chart 1). The structures of

these metabolites were determined by NMR and MS spectroscopy.

Compound **2** (EI-MS m/z 362 $[M]^+$) was obtained as one of the major metabolites after incubation for 12 h. The ¹H- (see Experimental) and ¹³C-NMR (Table 1) spectral data of **2** were in good agreement with those reported for secoisolariciresinol, an aglycone of **1**.¹²⁾

Compound **3** showed a molecular ion peak at m/z 348 $[M]^+$ in its EI-MS spectrum, 14 mass units less than that of **2**, suggesting that **3** was a demethylation product of **2**. This was confirmed by the presence of a singlet signal for *O*-methyl protons at δ 3.76 and signals for 6 non-equivalent aromatic protons at δ 6.43, 6.56, 6.57, 6.63, 6.63 and 6.66 in the ¹H-NMR spectrum. Therefore, the structure of **3** was identified as 3-demethyl-(–)-secoisolariciresinol previously isolated from the needles of the Himalayan yew, *Taxus baccata*.¹³⁾ Compound **3** has been previously reported as a possible precursor of **7**.¹⁴⁾

Most of the signals in the ¹H- and ¹³C-NMR spectra of **4** were similar to those of **3** (see Experimental and Table 1) except for additional signals for an aromatic methine at δ 6.60 (δ_C 113.7). The spin-coupling patterns in the aromatic region of the ¹H-NMR spectrum showed the presence of 1,3,4-trisubstituted and 1,3-disubstituted phenyl groups. Furthermore, the EI-MS spectrum of **4** showed a molecular ion peak at m/z 332 $[M]^+$, 16 mass units less than that of **3**, suggesting



- 1 $R^1=R^4=Me$, $R^2=R^3=OH$, $R^5=Glc$
- 2 $R^1=R^4=Me$, $R^2=R^3=OH$, $R^5=H$
- 3 $R^1=R^5=H$, $R^2=R^3=OH$, $R^4=Me$
- 4 $R^1=R^2=R^5=H$, $R^3=OH$, $R^4=Me$
- 5 $R^1=R^4=R^5=H$, $R^2=R^3=OH$
- 6 $R^1=R^2=R^4=R^5=H$, $R^3=OH$
- 7 $R^1=R^2=R^3=R^4=R^5=H$

8

Chart 1. Structures of **1** and Its Metabolites (**2–8**)

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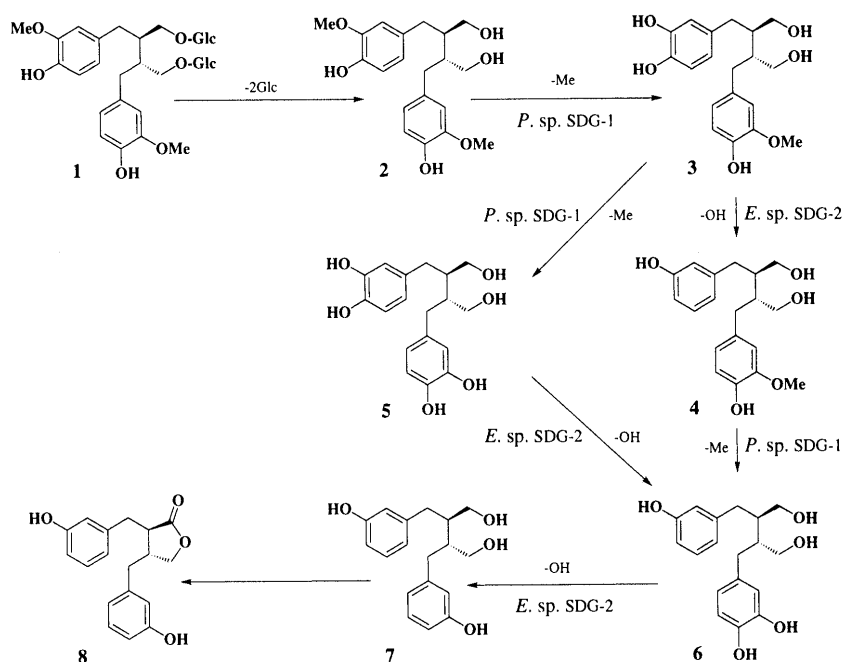


Chart 2. Possible Pathway for the Transformation of **1** by *P. sp.* Strain SDG-1 and *E. sp.* Strain SDG-2, Human Intestinal Bacteria

that **4** was produced by *p*-dehydroxylation of **3**. The structure of **4** was determined to be 2-(3-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butane-1,4-diol.

The molecular ion peak of **5** (m/z 334 $[M]^+$) in its EI-MS spectrum showed 28 mass units less than that of **2**. The ^1H -NMR spectrum of **5** showed close resemblance to that of **2**, but signals for two methoxy groups in **2** were not seen in the spectrum of **5**. These findings indicated that **5** was formed by complete demethylation of two methoxy groups in **2**. The structure of **5** was consequently identified as didemethylsecosolaricresinol, previously reported as a precursor of **8**.¹⁴⁾

Compound **6** was a metabolite considered to be formed by *p*-dehydroxylation of **5**, as confirmed by EI-MS (m/z 318 $[M]^+$, 16 mass unit less than that of **5**) and NMR, and its structure was established as 2-(3-hydroxybenzyl)-3-(3,4-dihydroxybenzyl)butane-1,4-diol, previously identified by GC/MS as a metabolite of **7** in the urine of humans ingesting flaxseed.¹⁰⁾ Furthermore, this compound was claimed as a metabolic intermediate in the bacterial transformation of **1** to **7**.¹⁰⁾

Compounds **7** and **8** were identified as enterodiol and enterolactone, respectively, by direct comparison of their spectral data with those of authentic samples.^{15,16)} After prolonged incubation of **1** for 3 d with a human fecal suspension, **8** was obtained in a yield of 90%, as determined by HPLC.

Isolation of Bacterial Strains Capable of Transforming **2 and **5** from a Human Fecal Suspension** In order to clarify the transformation of **1** by human intestinal bacteria, trials were made to isolate bacterial strains capable of transforming **1** from a human fecal suspension. After repeated culture of the human fecal suspension in GAM broth containing **2** or **5** (1 mM) under anaerobic conditions, two colonies (strains SDG-1 and SDG-2) responsible for demethylation and dehydroxylation, respectively, were isolated. The biochemical characteristics of strains SDG-1 and SDG-2 are shown in Table 2. Strain SDG-1 was strictly anaerobic, Gram-positive,

Table 1. ^{13}C -NMR (100 MHz) Spectral Data of Compounds **2**–**6**

C	2 ^{a)}	3 ^{b)}	4 ^{b)}	5 ^{b)}	6 ^{b)}
1	60.3	61.9 ^{c)}	61.7 ^{c)}	61.8	61.8 ^{c)}
2	43.7	44.2 ^{d)}	44.2	44.6	44.4 ^{d)}
3	43.7	44.5 ^{d)}	44.2	44.6	44.7 ^{d)}
4	60.3	62.0 ^{c)}	61.8 ^{c)}	61.8	61.9 ^{c)}
1'	132.4	133.9 ^{e)}	143.8	133.9	144.0
2'	111.8	117.2	116.9	117.2	117.0
3'	146.7	146.0	158.1	146.0	158.2
4'	143.7	144.2	113.7 ^{d)}	144.2	113.8
5'	114.3	116.1	130.1	116.1	130.2
6'	121.5	121.4	121.4	121.3	121.4
7'	35.8	35.8 ^{f)}	36.3	35.7	36.3
1''	132.4	133.8 ^{e)}	133.7	133.9	133.9
2''	111.8	113.4	113.4 ^{d)}	117.2	117.2
3''	146.7	148.8	148.7	146.0	146.1
4''	143.7	145.4	145.4	144.2	144.3
5''	114.3	115.8	115.8	116.1	116.1
6''	121.5	122.6	122.5	121.3	121.4
7''	35.8	36.0 ^{f)}	35.9	35.7	35.7
-OMe	55.8	56.3	56.3		

a, b) Chemical shifts in δ ppm, measured in: a) CDCl_3 , b) $\text{MeOH}-d_4$. c–f) Assignments with the same superscript may be interchangeable within the same column.

and elliptical cocci. Strain SDG-1 hydrolyzed esculin and starch, but was not able to produce indole, H_2S or nitrite. The strain produced acetic acid and lactic acid from D-glucose. Strain SDG-1 grew well in GAM broth (A_{max} value of 0.43 at 540 nm). The cells cultured on an EG agar plate were mainly in chains, while those on a BL agar plate were singly or in pairs. Accordingly, strain SDG-1 was considered to belong to the genus *Peptostreptococcus*. Strain SDG-1 fermented amygdalin, salicin, esculin, and most carbohydrates tested, but did not ferment inulin or inositol. Therefore, strain SDG-1 was concluded to be a *Peptostreptococcus* sp. with characteristics similar to those reported for *P. productus* isolated from human fecal samples (Table 3),¹⁷⁾ and was tentatively named *P. sp.* strain SDG-1. However, *P. sp.* strain SDG-1 did

Table 2. Biochemical Characteristics of Strains SDG-1 and SDG-2

Characteristic	Strain SDG-1	Strain SDG-2
Aerobic growth	—	—
Gram stain	+	+
Major product from PYFG	Acetic acid, lactic acid	ND
Growth in 20% bile	—	—
Esculin hydrolysis	+	—
Starch hydrolysis	+	+
Indole production	—	—
H ₂ S production	—	—
Nitrate reduction	—	+
Genus	<i>Peptostreptococcus</i>	<i>Eubacterium</i>

Symbols: +: positive; -: negative; ND, not detected.

Table 3. Comparative Fermentation Reactions of Strains SDG-1 and SDG-2

Compound	Strain SDG-1	<i>Peptostreptococcus productus</i> ¹⁷⁾	Strain SDG-2	<i>Eubacterium lentum</i> ¹⁸⁾
Amygdalin	+	+	—	—
Arabinose	+	+	—	—
Cellobiose	+	+	—	—
Dextrin	—	+	—	—
Esculin	+	±	—	—
Fructose	+	+	—	—
Galactose	+	+	—	—
Glucose	+	+	—	—
Glycerol	—	—	—	—
Glycogen	—	—	—	—
Inositol	—	—	—	—
Inulin	—	—	—	—
Lactose	+	+	—	—
Maltose	+	+	—	—
Mannitol	—	+	—	—
Mannose	—	+	—	—
Melibiose	+	+	—	—
Melezitose	+	+	—	—
Rhamnose	—	—	—	—
Ribose	—	v	—	—
Raffinose	+	+	—	—
Starch	—	v	—	—
Salicin	+	+	—	—
Sorbitol	—	+	—	—
Sucrose	+	+	—	—
Trehalose	+	+	—	—
Xylose	+	+	—	—

Symbols: +, positive reaction (below pH 5.5); —, negative reaction (pH 6.0 or above); v, sometimes positive, sometimes negative; ±, almost positive (negative) but may exhibit a weak reaction.

not utilize dextrin, mannitol, mannose or sorbitol, in contrast with *P. productus*.

In a similar procedure, strain SDG-2 capable of transforming **5** to **6** and **7** was also isolated from the human fecal suspension. This strain was strictly anaerobic, Gram-positive, and non-sporeforming rods (Table 2). The strain reduced nitrate and hydrolyzed starch, but did not ferment carbohydrates, nor produce acids from media containing D-glucose. Indole and H₂S were not produced by this strain. Similar characteristics were reported for *Eubacterium lentum*.¹⁸⁾ The growth of strain SDG-2 in PYFG and GAM broth was very poor (A_{540} : 0.04 at 540 nm), but the growth was significantly enhanced by the addition of arginine to GAM broth (A_{540} : 0.11 at 540 nm in the presence of 1% arginine), similar to the

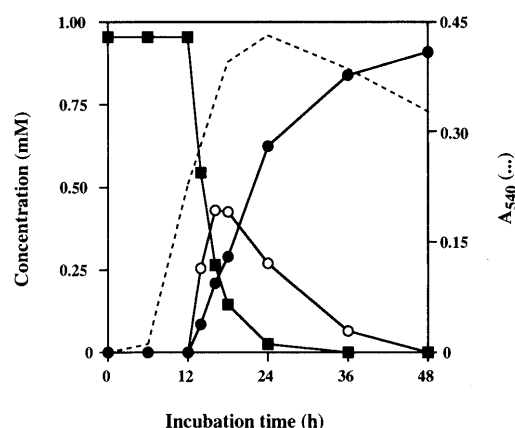


Fig. 1. Time Course of Transformation of **2** (■) to **3** (○) and **5** (●) by *P. sp.* Strain SDG-1

Bacterial growth (---) was monitored by measuring turbidity at 540 nm.

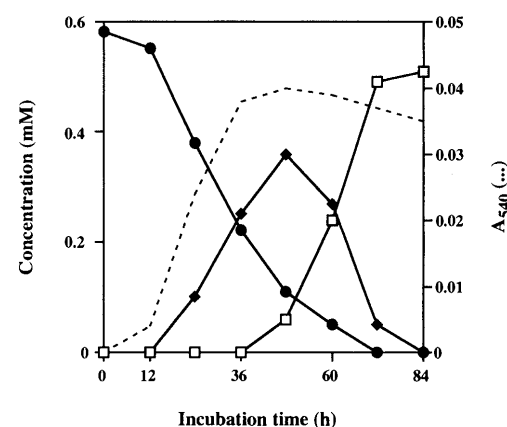


Fig. 2. Time Course of Transformation of **5** (●) to **6** (◆) and **7** (□) by *E. sp.* Strain SDG-2

Bacterial growth (---) was monitored by measuring turbidity at 540 nm.

case of *E. lentum*.¹⁹⁾ Consequently, the isolated strain SDG-2 was concluded to be a *Eubacterium. sp.* and was named *E. sp.* strain SDG-2.

Transformation of (–)-Secoisolariciresinol (2**) by *P. sp.* Strain SDG-1** This strain showed high ability to transform **2** to **3** and **5** after incubation for 16 h (Fig. 1). The maximal production of **3** was observed 16 h after the start of incubation, then the amount was gradually decreased. This was accompanied by a gradual increase in the amount of **5** after 12 h. Further transformation of **5** to another metabolite was not demonstrated by this strain. This observation suggested that **5** was produced in a manner of sequential demethylation via **3**.

Similarly, *P. sp.* strain SDG-1 was capable of transforming **4** to **6** (data not shown). However, tetra-*O*-methylsecoisolariciresinol was not converted, even after a long period of incubation (data not shown), suggesting that the presence of a free hydroxy group vicinal to the methoxy group (as in **2**–**4**) is necessary for demethylation of these compounds.

Transformation of Didemethylsecoisolariciresinol (5**) by *E. sp.* Strain SDG-2** Compound **5** was slowly transformed to **6** and **7** by anaerobic incubation with *E. sp.* strain SDG-2 in GAM broth (Fig. 2). Transformation of **5** to **6** was started after 12 h of incubation, and the amount was slowly increased to reach a maximum after 48 h, then gradually de-

creased. A further metabolite (**7**) was detected more than 60 h after incubation.

In addition, the transformation of **3** to **4** was also demonstrated by this strain (data not shown).

Discussion

The present study provided evidence that human intestinal bacteria is capable of producing phytoestrogenic lignans, **7** and **8**, and other metabolic intermediates from one of the major dietary lignans, **1** after anaerobic incubation *in vitro*. The diphenolic structures of **7** and **8** share similarities with several estrogen-like substances, such as dienoestrol, diethylstilboestrol and its dihydroderivative hexoestrol, which accounts for the hormone-like effects of these compounds.²⁰ Epidemiological studies showed the low urinary excretion of **7** and **8** in patients with breast cancer, suggesting that the consumption of some lignans may be related with the incidence of such cancer.^{3,4} Furthermore, the United States National Cancer Institute considers these compounds to be potential anti-cancer agents.³

Compounds **7** and **8** were excreted in the urine of normal rats, but not in the urine of germ-free rats or humans who were administered antibiotics.² However, in our *in vitro* experiments, the transformation of **7** to **8** was not observed by incubation with a rat fecal suspension, even after prolonged incubation for one week. In contrast, **8** was the end product of **1** and **7** by incubation with a human fecal suspension. This may be due to the difference in bacterial flora between both fecal suspensions used.

Transformation of **1**, **2** or **7** was effectively achieved by the human fecal suspension under anaerobic conditions, while similar results were not obtained under aerobic conditions (compound **2** was a sole metabolic product from **1**) or when a sterilized fecal suspension was used. These findings suggest that the conversion of **1** to **7** and **8** is not the result of spontaneous chemical reactions, but due to the metabolic reaction of viable intestinal bacteria under anaerobic conditions.

Compound **8** was also reported to be detected after incubation of a plant lignan, matairesinol, with a human fecal suspension.

To date, the metabolic pathway from **1** to **7** and **8** by intestinal bacteria had not been determined, and intestinal bacteria responsible for this transformation had not yet been isolated from human feces. However, Setchell and co-workers proposed a possible metabolic pathway for the formation of **7** and **8** from **1** by incubation with a human faecal flora, and claimed that dehydroxylation was the first step, followed by demethylation.¹¹ From the present experiment, it seems reasonable that demethylation occurs prior to dehydroxylation since demethylated metabolites **2**, **3** and **5** were formed from **1** after incubation for a short period of time (20–30 h), whereas dehydroxylated ones **4**, **6**–**8** were obtained later than 48 h. In addition, no transformation of **2** or **4** was observed by a newly isolated strain of *E. sp.* SDG-2 having dehydroxylation ability.

Several of these metabolites, such as **6**–**8**, may find their way to the general circulation of humans who ingest flaxseed or dietary lignans, as recently reported by Jacobs *et al.*¹⁰ All the possible precursors in the transformation from **1** to **7** (**3** and **5**) suggested by Kurzer *et al.*¹⁴ were isolated as intermediates in this experiments, and these may be further metabo-

lized by tissue enzymes to be excreted into the urine.

In conclusion, several intestinal bacterial metabolites that can be considered intermediates in the pathway from **1** to **7** and **8** were isolated, in which demethylation was suggested to occur prior to dehydroxylation. Two bacterial strains, *P. sp.* SDG-1 and *E. sp.* SDG-2, responsible for the demethylation and dehydroxylation of the metabolic intermediates were isolated from human feces.

The present study emphasizes the role of human intestinal bacteria in the activation of dietary lignans, and suggests that, following the oral consumption of fiber-rich foods, lignan glycosides may undergo metabolism by intestinal bacteria to give mammalian lignans. Some of the metabolic intermediates may be excreted into the bile as conjugates which will eventually be exposed to intestinal bacteria again in the large intestine, which harbours numerous bacteria capable of metabolizing these compounds to mammalian lignans.

Trials to isolate bacterial strains from human feces capable of transforming **7** to **8** are currently underway in our laboratories.

Experimental

General Optical rotations were measured in MeOH solutions using a JASCO DIP-360 automatic polarimeter at 25 °C. UV spectra were taken on a Shimadzu UV-2200 UV-VIS spectrophotometer (Kyoto, Japan) in MeOH solution. ¹H- and ¹³C-NMR spectra were taken on a JOEL JNM GX-400 with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were measured with a JEOL JMS DX-300 mass spectrometer at an ionization voltage of 70 eV. For anaerobic cultivation, an anaerobic incubator EAN-140 (Tabai Co., Osaka, Japan) was used. A gas chromatograph (Shimadzu Co., Kyoto, Japan) fitted with a DB-1 column [0.25 (i.d.) × 30 m] (J & W Scientific, U.S.A.) (column temperature: 140 °C, carrier gas: He) coupled to an Automass System II Mass spectrometer (JEOL, Japan) was used for determination of fatty acids. HPLC was performed on a CCPM-II (Tosoh, Tokyo, Japan) equipped with a Tosoh UV-8020 spectrometer and a Shimadzu C-R 6A chromatopac (Shimadzu, Kyoto, Japan). Column, TSKgel ODS-80T_M (Tosoh Co., Tokyo, Japan, 250 mm × 4.6 mm i.d.); mobile phase, CH₃OH (solvent system A) and H₂O containing 0.1% TFA (solvent system B) in a gradient mode (A from 33 to 50% in 60 min, and then isocratic for 10 min); flow rate, 1.0 ml/min; detection, UV 280 nm. Kieselgel 400 (Wako Pure Chemical Co., Osaka, Japan) and Sephadex LH-20 (Pharmacia, Sweden) were used for column chromatography. Merck Kieselgel 60 F₂₅₄ (layer thickness 0.25 mm) were used for TLC, with solvent system A, CHCl₃–MeOH (10:1). Spots were detected under a UV lamp and after spraying with anisaldehyde/H₂SO₄ reagent, followed by heating.

Chemicals and Media Secoisolariciresinol diglucoside (**1**) was provided by Suntory Co. and purified in our laboratory. Methylation of **2** with Me₂SO₄ was carried out by the usual method.²¹ General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan). Peptone yeast extract flides (PYF) broth was prepared according to the procedure of Mit-suoka.²²

Preparation of a Human Fecal Suspension A fresh fecal sample (5 g), obtained from a healthy subject, was homogenized with K-phosphate buffer (50 ml, pH 7.3), and the sediments were removed by filtration through gauze. The suspension was made up to 100 ml with the same buffer and was used as a human fecal suspension in this experiment.

Preparation of a Rat Fecal Suspension A fresh fecal sample (5 g), obtained from male Wistar strain rats (250 g; Sankyo Lab. Service, Tokyo, Japan), was homogenized in K-phosphate buffer (50 ml, pH 7.3). After filtration through gauze, the suspension was made up to 100 ml with the same buffer and used in this experiment.

Incubation of Secoisolariciresinol Diglucoside (1**) with a Human Fecal Suspension** Secoisolariciresinol diglucoside (**1**, 300 mg) dissolved in PYF broth (250 ml) was incubated with a human fecal suspension (50 ml) at 37 °C in an anaerobic incubator for 15 h. The mixture was then extracted with BuOH, saturated with H₂O (300 ml × 3), and the BuOH layer was evaporated *in vacuo* to give a residue (950 mg). The residue was applied to a column of silica gel, and elution was started with CHCl₃, then with CHCl₃ with increasing percentages of methanol. Similar fractions were pooled to give fr. A and B. Purification of fr. A and B through Sephadex LH-20 (eluting with

95% aq. MeOH) and then RP-18 (eluting with 50% aq. MeOH) gave **2** (68 mg) and **4** (10 mg), respectively.

Compound **1** (500 mg) in GAM broth (500 ml) was anaerobically incubated with a human fecal suspension (50 ml) under anaerobic conditions for 30 h. The mixture was then extracted and purified as mentioned above to afford **3** (6 mg), **5** (9 mg), **6** (4 mg) and **7** (70 mg). On the other hand, anaerobic incubation of **1** (50 mg) with a human fecal suspension in GAM broth (50 ml) for 3 d yielded **8** (7 mg) after repeated column chromatography.

Incubation of 2–7 with a Human Fecal Suspension A human fecal suspension (2 ml) containing a test compound (0.8 mM) was anaerobically incubated at 37°C for 48 h. The mixture (200 μ l) was then extracted with BuOH saturated with H₂O (200 μ l). Ten μ l of the BuOH layer was applied on TLC, which was then developed with solvent system A. Identification was made by direct comparison of *R_f* values with those of authentic samples.

Compound 2 Amorphous powder. EI-MS *m/z*: 362 [M]⁺. ¹H-NMR (400 MHz, CDCl₃) δ : 1.84 (2H, m, H-2, 3), 2.66 (H, m, H-7', 7''), 3.46 (4H, m, H-1, 4), 3.80 (6H, s, OCH₃), 6.60 (4H, overlapped, H-2', 2'', 5', 5''), 6.75 (2H, dd, *J*=8.4, 3.0 Hz, H-6', 6'').

Compound 3 Amorphous powder. EI-MS *m/z*: 348 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 1.91 (2H, m, H-2, 3), 2.60 (4H, m, H-7', 7''), 3.53 (2H, dd, *J*=11.1, 5.1 Hz, H_a-1, H_a-4), 3.59 (2H, dd, *J*=11.1, 4.8 Hz, H_b-1, H_b-4), 3.76 (3H, s, -OCH₃), 6.43 (1H, dd, *J*=8.0, 1.9 Hz, H-6'), 6.56 (1H, dd, *J*=8.0, 1.9 Hz, H-6''), 6.57 (1H, d, *J*=1.9 Hz, H-2'), 6.63 (1H, d, *J*=1.9 Hz, H-2''), 6.63 (1H, d, *J*=8.0 Hz, H-5''), 6.66 (1H, d, *J*=8.0 Hz, H-5').

Compound 4 Amorphous powder. [α]_D 24.4° (*c*=0.16, MeOH). UV (MeOH) nm: 227, 280. EI-MS *m/z*: 332 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 1.95 (2H, m, H-2, 3), 2.63 (4H, m, H-7', 7''), 3.55 (2H, ddd, *J*=11.1, 5.7, 2.7 Hz, H_a-1, H_a-4), 3.62 (2H, ddd, *J*=11.1, 6.8, 2.4 Hz, H_b-1, H_b-4), 3.78 (3H, s, -OCH₃), 6.57 (1H, dd, *J*=8.0, 1.9 Hz, H-6'), 6.60 (3H, overlapped, H-2', 4', 6'), 6.65 (1H, d, *J*=1.9 Hz, H-2''), 6.68 (1H, d, *J*=8.0 Hz, H-5''), 7.04 (1H, t, *J*=8.0 Hz, H-5').

Compound 5 Amorphous powder. EI-MS *m/z*: 334 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 1.86 (2H, m, H-2, 3), 2.51 (4H, m, H-7', 7''), 3.45 (2H, dd, *J*=11.1, 5.3 Hz, H_a-1, H_a-4), 3.56 (2H, dd, *J*=11.1, 3.8 Hz, H_b-1, H_b-4), 6.41 (2H, dd, *J*=8.0, 2.2 Hz, H-6', 6''), 6.55 (2H, dd, *J*=2.2 Hz, H-2', 2''), 6.60 (2H, d, *J*=8.0 Hz, H-5', 5'').

Compound 6 Amorphous powder. EI-MS *m/z*: 318 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 1.94 (2H, m, H-2, 3), 2.56 (2H, m, H-7' or H-7''), 2.62 (2H, m, H-7'' or H-7'), 3.50 (2H, ddd, *J*=11.1, 6.0, 3.6 Hz, H_a-1, H_a-4), 3.61 (2H, ddd, *J*=11.1, 7.0, 4.1 Hz, H_b-1, H_b-4), 6.45 (1H, dd, *J*=8.0, 2.1 Hz, H-4'), 6.58 (1H, ddd, *J*=8.0, 2.1, 2.1 Hz, H-6'), 6.59 (1H, s, H-2''), 6.59 (1H, t, *J*=2.1 Hz, H-2'), 6.61 (1H, d, *J*=8.0 Hz, H-6''), 6.64 (1H, d, *J*=8.0 Hz, H-5''), 7.03 (1H, t, *J*=8.0 Hz, H-5').

Compound 7 Amorphous powder. EI-MS *m/z*: 302 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 1.98 (2H, m, H-2, 3), 2.64 (4H, m, H-7', 7''), 3.53 (2H, dd, *J*=11.1, 5.3 Hz, H_a-1, H_a-4), 3.63 (2H, dd, *J*=11.1, 3.8 Hz, H_b-1, H_b-4), 6.60 (2H, ddd, *J*=7.8, 2.2, 1.2 Hz, H-4', 4''), 6.62 (4H, overlapped, H-2', 2'', 6', 6''), 7.05 (2H, t, *J*=7.8 Hz, H-5', 5'').

Compound 8 Amorphous powder. [α]_D 34.8° (*c*=0.12, MeOH). EI-MS *m/z*: 298 [M]⁺. ¹H-NMR (400 MHz, MeOH-*d*₄) δ : 2.50 (2H, m, H_a-7'', 3), 2.59 (1H, m, H-2), 2.61 (1H, m, H_b-7''), 2.91 (1H, m, H_a-7'), 3.00 (1H, m, H_b-7'), 3.87 (1H, dd, *J*=9.1, 7.6 Hz, H_a-4), 4.14 (1H, dd, *J*=9.1, 7.0 Hz, H_b-4), 6.47 (1H, t, *J*=1.6 Hz, H-2''), 6.59 (1H, t, *J*=1.6 Hz, H-2'), 6.60 (1H, ddd, *J*=7.9, 1.6, 1.6 Hz, H-6''), 6.70 (1H, m, H-4'), 6.73 (1H, dt, *J*=7.9, 1.6, 1.6 Hz, H-6'), 6.74 (1H, m, H-4''), 7.15 (1H, t, *J*=7.9 Hz, H-5''), 7.18 (1H, t, *J*=7.9 Hz, H-5').

Isolation of Bacterial strains Capable of Transforming 2 and 5 from Human Fecal Suspension A bacterial suspension from fresh feces of a healthy man was repeatedly cultured in GAM broth containing **2** or **5** (1 mM) and incubated at 37°C in an anaerobic incubator. Five μ l of the culture was seeded on BL agar plates. Colonies were picked up and screened for the ability to transform **2** and **5**, respectively.

Time Course of Transformation of 2 by P. sp. SDG-1 GAM broth (4 ml) containing **2** (a final concentration of 1 mM) was incubated at 37°C with a bacterial suspension of *P. sp.* SDG-1 (100 μ l) in an anaerobic incubator.

A 150 μ l portion was taken out at intervals and extracted with BuOH saturated with H₂O (150 μ l). The BuOH extract was evaporated *in vacuo* to give a residue. The residue was dissolved in MeOH (150 μ l) and the MeOH extract was analyzed by HPLC. Bacterial growth in GAM broth was monitored by measuring turbidity at 540 nm.

Time Course of Transformation of 5 by E. sp. SDG-2 GAM broth (6 ml) containing **5** (a final concentration of 0.8 mM) was incubated at 37°C with a bacterial suspension of *E. sp.* SDG-2 (100 μ l) under anaerobic conditions. Samples were taken at intervals and analyzed by HPLC, as mentioned above.

Fermentation Test Carbohydrate fermentation tests were carried out according to the method of Sneath *et al.*²³⁾ Fatty acids obtained in PYF broth containing D-glucose were determined using gas chromatography.

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References

- 1) Axelson M., Sjövall J., Gustafsson B. E., Setchell K. D. R., *Nature* (London), **298**, 659–660 (1982).
- 2) Axelson M., Setchell K. D. R., *FEBS Letters*, **123**, 337–342 (1981).
- 3) Adlercreutz H., Fotsis T., Heikkinen R., Dwyer J. T., Woods M., Goldin B. R., Gorbach S. L., *Lancet* ii, **1982**, 1295–1299.
- 4) Adlercreutz H., Höckerstedt K., Bannwart C., Hämäläinen E., Fotsis T., Bloigu S., "Progress in Cancer Research and Therapy, Vol. 35: Hormones and Cancer 3," ed. by Bresciani F., King R. J. B., Lippman M. E., Raynaud J. P., Raven Press, Ltd., NY, 1988, pp. 409–412.
- 5) Armstrong B., Doll R., *Int. J. Cancer*, **15**, 617–631 (1975).
- 6) Miller A. B., *Cancer*, **39**, 2704–2708 (1977).
- 7) Wynder E. L., *Cancer*, **46**, 899–904 (1980).
- 8) Reddy B. S., Cohen L. A., McCoy G. D., Hill P., Weisburger J. H., Wynder E. L., *Adv. Cancer Res.*, **32**, 237–345 (1980).
- 9) Jacobs E., Metzler M., *J. Agric. Food Chem.*, **47**, 1071–1077 (1999).
- 10) Jacobs E., Kulling S. E., Metzler M., *J. Steroid Biochem. Molec. Bio.*, **68**, 211–218 (1999).
- 11) Borriello S. P., Setchell K. D. R., Axelson M., Lawson A. M., *J. Appl. Bacteriol.*, **58**, 37–43 (1985).
- 12) Agrawal P. K., Rastogi R. P., *Phytochemistry*, **21**, 1459–1461 (1982).
- 13) Das B., Takhi M., Srinivas K. V. N. S., Yadav J. S., *Phytochemistry*, **33**, 1489–1491 (1993).
- 14) Wang C. F., Makela T., Hase T., Adlercreutz H., Kurzer S. K., *J. Steroid Biochem. Molec. Biol.*, **50**, 205–212 (1994).
- 15) Setchell K. D. R., Lawson A. M., Conway E., Taylor N. F., Kirk D. N., Cooley G., Farrant R. D., Wynn S., Axelson M., *Biochem. J.*, **197**, 447 (1980).
- 16) Cooley G., Farrant R. D., Kirk D. N., Patel S., Wynn S., Buckingham M. J., Hawkes G. E., Hursthouse M. B., Galas A. M. R., Lawson A. M., Setchell K. D. R., *J. Chem. Soc. Perkin Trans. II*, **1984**, 489.
- 17) Sneath P. H., Mair N. S., Sharpe M. S., Holt J. G., "Bergey's Manual of Systematic Bacteriology," Williams and Wilkins, Baltimore, Vol. 2, pp. 1091–1092 (1984).
- 18) Moore W. E. C., Cato E. P., Holdeman L. V., *Int. J. Syst. Bacteriol.*, **21**, 299–303 (1971).
- 19) Sperry J. F., Wilkings T. D., *J. Bacteriol.*, **127**, 780–784 (1976).
- 20) Setchell K. D. R., Lawson A. M., Metcalf F. L., Adlercreutz H., Kirk D. N., Axelson M., *Nature* (London), **287**, 740–742 (1980).
- 21) Nonaka G. I., Ezaki E., Hayashi K., Nishioka I., *Phytochemistry*, **22**, 1659–1661 (1983).
- 22) Mitsuoka T., "A Color Atlas of Anaerobic Bacteria," Shobunsha Co., Tokyo, 1980, p. 325.
- 23) Sneath P. H., Mair N. S., Sharpe M. S., Holt J. G., "Bergey's Manual of Systematic Bacteriology," Williams and Wilkins, Baltimore, pp. 1418–1434 (1986).

Synthesis of New Cardiosensitive M₂ Muscarinic Receptor Antagonists

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A series of 5*H*-dibenz[*b,f*]azepine derivatives was prepared and evaluated for binding affinities to muscarinic receptors *in vitro*. Among them, compound 8 showed a high affinity for human recombinant M₂ receptors ($K_i=2.6$ nM), a low affinity for M₄ receptors (39-fold less than for M₂ receptors) and a very low affinity for M₁ and M₃ receptors (119- and 112-fold less than for M₂ receptors, respectively). The high M₂ selectivity of 8 may be attributed to the olefinic bond of the azepine ring. Functional experiments showed 8 to be a competitive antagonist with high affinity to the cardiac ($pA_2=7.1$) and low affinity to the intestinal muscarinic receptors ($IC_{50}=0.54$ μ M). *In vivo* experiments confirmed the *in vitro* M₂ selectivity of 8. Acetylcholine-induced bradycardia was dose-dependently antagonized in rats after both intravenous and intraduodenal administration of 8. In rats, cholinergic functions mediated by M₁ or M₃ receptors (salivary secretion, pupil diameter, gastric emptying, intestinal transit time) were not affected by the oral administration of 8 even at doses as high as 30 times the antibradycardic effective dose. Furthermore, 8 had no analgesic activity in mice, indicating poor central nervous system penetration. In dogs, nocturnal bradycardia was dose-dependently inhibited by the oral route with a duration of action of about 24 h. Compound 8 appears to be a promising cardiosensitive antimuscarinic agent for the treatment of dysfunctions of the cardiac conduction system such as sinus or nodal bradycardia ("sick-sinus syndrome") and atrioventricular block.

Key words 5*H*-dibenz[*b,f*]azepine derivative; M₂ muscarinic receptor; muscarinic antagonist; M₂ selectivity; bradycardia

Acetylcholine acts through receptors located on the cell membrane surface. These receptors are classified as nicotinic or muscarinic receptors on the basis of their response to alkaloid nicotine and muscarine. Muscarinic receptors modulate a number of important functions including nerve-to-nerve transmission, smooth-muscle contraction, and exocrine and endocrine secretion. They play a major role in many pathologic conditions.¹⁾

Four muscarinic receptor subtypes have been characterized pharmacologically (M₁, M₂, M₃ and M₄), while five molecular forms have been identified and cloned (*m*₁, *m*₂, *m*₃, *m*₄ and *m*₅).²⁾ Muscarinic receptors are coupled to G-proteins, which in turn modulate the activity of a different second messenger (phospholipase C and adenylyl cyclase).³⁾ The use of muscarinic agonists or antagonists has been limited by their low specificity. Due to the fact that muscarinic receptors are found in many tissues, a therapeutic effect in one tissue is often accompanied by unwanted effects in another.⁴⁾

The cardiac conduction system and cardiac muscle have postsynaptic M₂ receptors that mediate bradycardia and decrease cardiac contractility.⁵⁾ Cortex and hippocampus have presynaptic M₂ receptors (autoreceptors) which, when stimulated, inhibit the release of acetylcholine.⁶⁾ The loss of presynaptic muscarinic receptors has been implicated in the etiology of Alzheimer's disease.⁷⁾ In the heart, the overactivation of M₂ muscarinic receptors *via* the vagus nerve is believed to play a major role in the etiology of the autonomic (neuromediated) sick sinus syndrome.^{8,9)} Thus, M₂ muscarinic receptor antagonists may be useful in the treatment of sinus node dysfunction caused by vagus hypertone, and may postpone or replace permanent endocardial pacing for patients with contraindications.¹⁰⁾ Atropine, a non-selective muscarinic receptor antagonist, was shown to be effective in increasing the heart rate of patients with sick sinus syndrome.¹¹⁾ However,

its use is limited by the short duration of action and the occurrence of unwanted side effects such as dry mouth, mydriasis, constipation and urinary retention caused by the antagonism of other subtypes.

Different groups have reported the synthesis of selective M₂ antagonists: Boehringer Ingelheim with AF-DX 116 (1) and AQ-RA 741 (2) derivatives (Fig. 1),^{12,13)} Yamanouchi with YM-47244 and YM-55758 derivatives,^{14–17)} George Washington University with DIBA (3) and DIBD derivatives,^{18,19)} University of Bologna with methoctramine and tripitramine derivatives,^{20–23)} University of Bonn with W84 and WDUO derivatives²⁴⁾ and Schering-Plough with SCH 57790 derivatives.²⁵⁾ However, most of the reports do not provide information on the selectivity of M₂ antagonists *versus* M₄ receptors which has recently been recognized to play an important role in respiratory physiology.²⁶⁾ In addition, many reports used putative receptor subtypes from tissue or cell preparations that do not express a single receptor subtype. We have synthesized a new group of selective M₂ muscarinic receptor antagonists (4) by modification of DIBA (3), as depicted in Fig. 2. We replaced the amide bond of the tricyclic ring system of DIBA with the bioisoster olefinic bond^{27–29)} differently substituted. In addition, we have explored the effect of increased flexibility of the side chain of DIBA by cleavage of the piperidine ring.¹⁴⁾ We have also determined the optimal distances between the carbonyl carbon and the two amine groups (Fig. 2).

Receptor binding affinities of the compounds were evaluated using human recombinant muscarinic receptors subtypes M₁, M₂, M₃ and M₄.

Chemistry

5*H*-Dibenz[*b,f*]azepine derivatives 8–17 and 19–24 (Tables 1 and 2) were prepared according to Chart 1. The key

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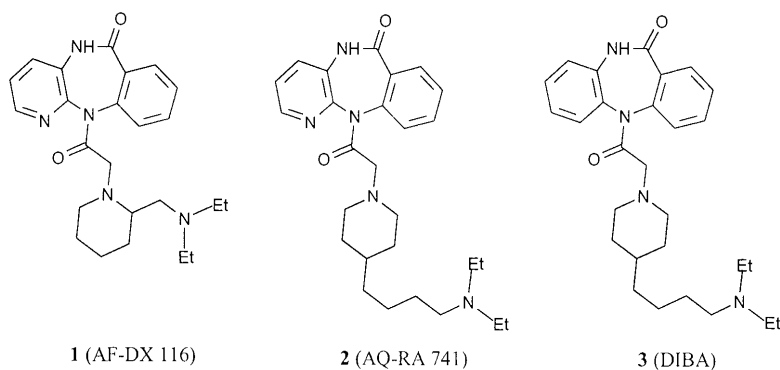


Fig. 1. Chemical Structure of AF-DX 116, AQ-RA 741 and DIBA

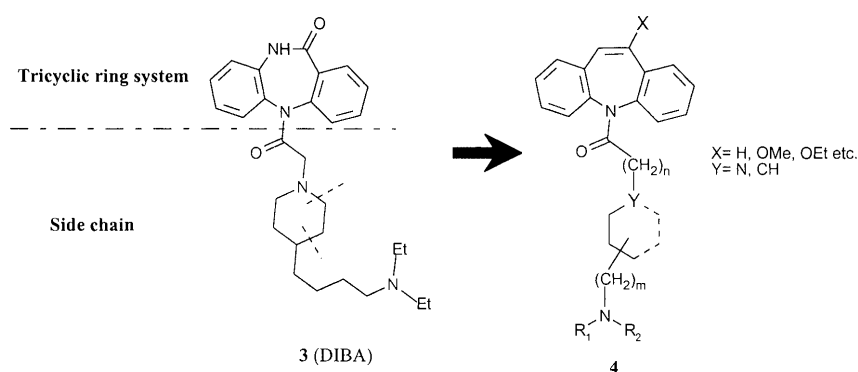
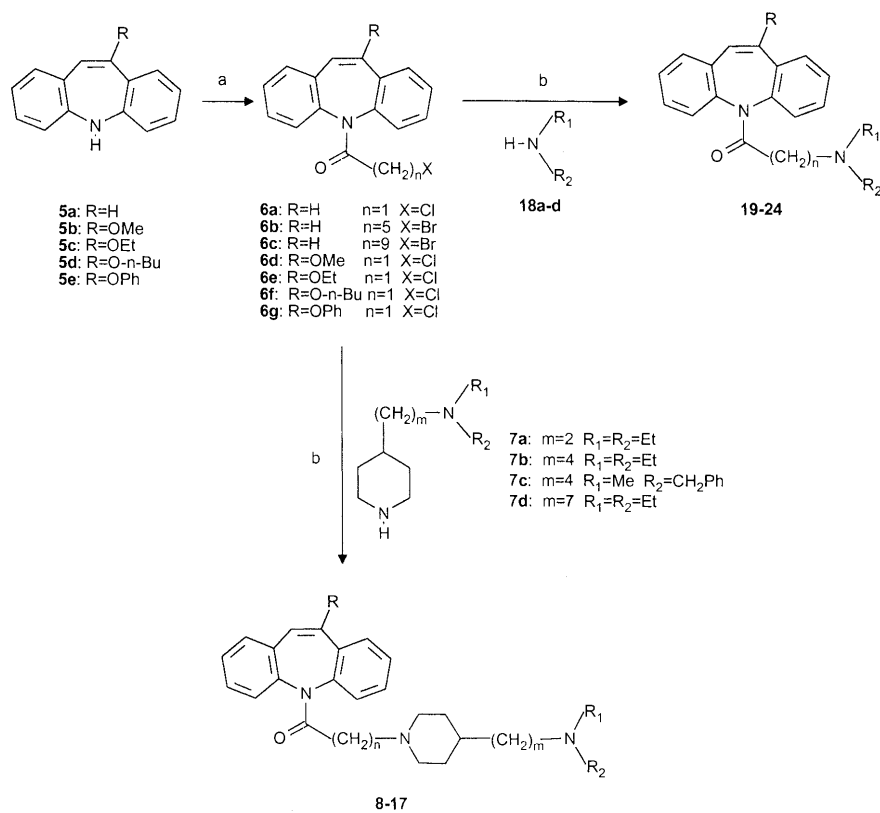


Fig. 2. Structural Variations within the Tricyclic Ring and the Side Chain of DIBA

reagents: a) ClCO(CH₂)_nX, *N,N*-dimethylaniline, THF, Δ; b) Na₂CO₃, NaI, CH₃CN, ΔChart 1. Preparation of the 5*H*-Dibenz[*b,f*]azepine Derivatives 8—17 and 19—24

intermediates **6a—g** were prepared from the corresponding *o*-haloacyl chlorides and the appropriate dibenz[*b,f*]azepines **5**. Reaction of **6a—g** with different amine com-

pounds, **7a—d** and **18a—d**, in acetonitrile in the presence of sodium carbonate afforded target compounds. Alkoxy-dibenz[*b,f*]azepine **5b—d** were prepared according to the

method reported by Haász,³⁰⁾ while the 5-phenoxy-dibenz[*b,f*]azepine **5e** was synthesized starting from 5-acetyl-10-bromo-5*H*-dibenz[*b,f*]azepine **25**,³¹⁾ as shown in Chart 2. Treatment of compound **25** with *t*-BuOK in the presence of phenol yields **5e**, probably *via* a hetaryne intermediate.³²⁾

Compounds **8**—**17** were obtained by the corresponding **6a**—**g** and 4-(dialkylaminoalkyl)piperidines **7a**—**d**.³³⁾

Chart 3 shows the preparation of **7b,c** starting from *N*-benzyl-piperidone **26**. **26** was treated with triethyl phosphonocrotonate (Horner–Emmons reaction) to afford **27**, which was easily hydrogenated and benzylated to give **28b**. Conversion of ethylester **28b** to alcohol **29a**, followed by reaction with methanesulfonyl chloride, gave **29b**. Treatment of compound **29b** with diethylamine, followed by debenzylation, gave piperidine derivative **7b**. Compound **29b** was also used to prepare compound **7c**. **29b** was treated with methylamine

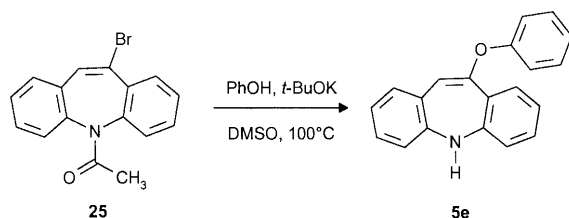


Chart 2. Preparation of 10-Phenoxy-5*H*-dibenz[*b,f*]azepine **5e**

and acylated with benzoyl chloride to afford **32a**, which was easily reduced and debenzylated to give piperidine derivative **7c**.

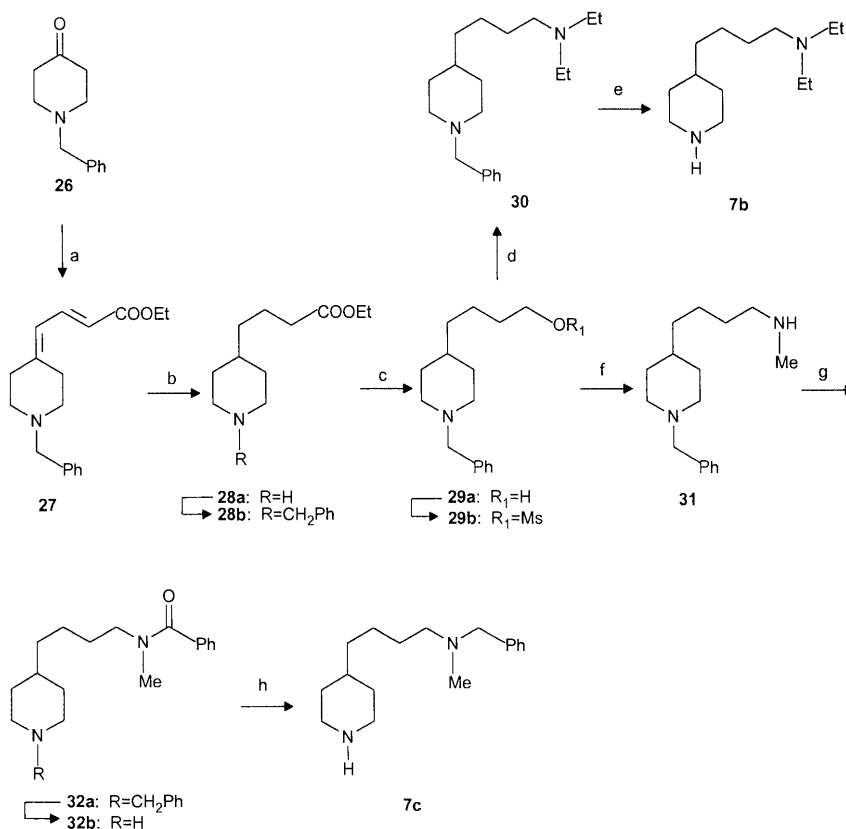
The synthetic route for preparation of the piperidine derivative **7d** is illustrated in Chart 4. Pyridine derivative **35** was obtained by the alkylation of 4-picolylnsodium with bromoderivative **34**. Catalytic reduction of compound **35** over platinum oxide, followed by reduction with lithium aluminium hydride, gave **7d**.

Chart 5 reports the synthesis of a corresponding hydrogenated analog of compound **8**, starting from iminodibenzyl derivative **37** and amine **7b**.

Pharmacological Results and Discussion

Receptor Binding The muscarinic receptor binding affinities of test compounds were assessed using human recombinant M_1 , M_2 , M_3 and M_4 receptors. The results, expressed as pK_i values and selectivity ratios for M_2 muscarinic receptors over M_1 , M_3 and M_4 muscarinic receptors (M_1/M_2 , M_3/M_2 and M_4/M_2 , respectively), are presented in Tables 1 and 2. Compound **1** was used as a reference compound.

Initially, the effects of the replacement of the amide bond of the 5*H*-dibenzo[*b,e*][1,4]diazepin-11-one ring of **3** with the olefinic bond were investigated (Table 1). Compound **8** displayed a strong affinity for M_2 receptors ($K_i = 2.6 \pm 1.1$ nM) with an extreme selectivity *versus* the other receptors subtypes (119-, 112- and 39-fold for M_1 , M_3 and M_4 receptors,



reagents: a) $(EtO)_2P(O)CH_2CH=CHCOOEt$, EtOH, EtONa, r.t. b) i) H_2 , Pd/C 10%, EtOH, r.t. ii) $PhCH_2Cl$, Na_2CO_3 , DMF, r.t. c) i) $LiAlH_4$, THF, N_2 , $0^\circ C$ ii) $MsCl$, Et_3N , THF, $0^\circ C$ d) Et_2NH , CH_3CN , Δ e) $HCOONH_4$, Pd/C 10%, MeOH, Δ , N_2 f) $MeNH_2$ 40%, CH_3CN , r.t. g) i) $PhCOCl$, NaOH 1N, CH_3COCH_3 , r.t. ii) $HCOONH_4$, Pd/C 10%, MeOH, Δ , N_2 h) $LiAlH_4$, THF, N_2 , Δ .

Chart 3. Preparation of Piperidine Derivatives **7b**—**c**

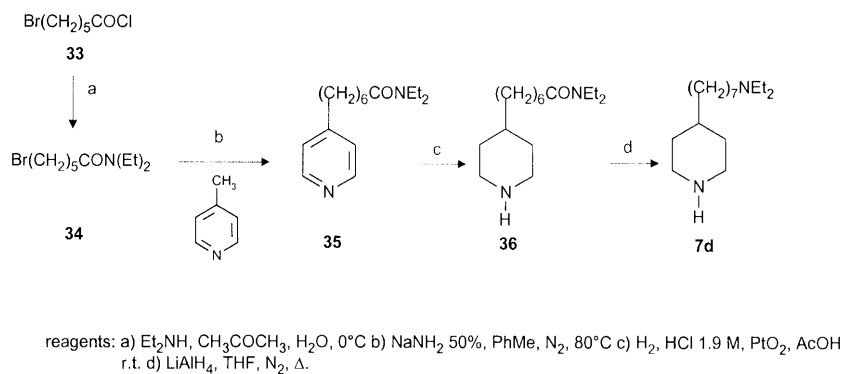


Chart 4. Preparation of Piperidine Derivative 7d

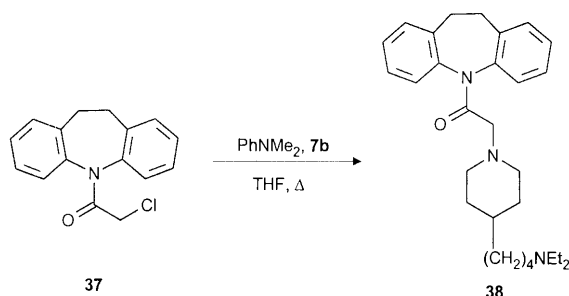


Chart 5. Preparation of Iminodibenzyl Derivative 38

respectively). For reference, pK_i of compound **3** are 8.40, 9.52, 7.96 and 8.70 for M₁, M₂, M₃ and M₄ receptors, respectively, the selectivity ratios for M₁/M₂, M₃/M₂ and M₄/M₂ being 13, 37 and 7, respectively.³⁴⁾

A comparison of **9**–**11** demonstrated that alkoxy substitution at position 11 of the dibenz[*b,f*]azepine skeleton decreases the affinity for M₂ receptors proportionally to the steric hindrance of the chain (BuO **11** < EtO **10** < MeO **9**). The introduction in this position of a phenoxy moiety (**12**) further decreases the affinity for the M₂ receptors. Saturation of the olefinic bond (**38**) reduces the activity on M₂ receptors, although selectivity for the other receptor subtypes remains high.

We next studied the influence of the side chain on the dibenz[*b,f*]azepine skeleton (Table 2). A comparison of compounds **8**, **13** and **14** indicates that the highest M₂ affinity and selectivity is obtained with 4 carbon atoms in the (diethylamino)alkyl chain at position 4 of the piperidine ring. Replacement of the terminal diethylamino moiety with a *N*-benzyl-*N*-methyl group resulted in a reduction of the activity for both the piperidine (**15**) and linear alkyl derivatives (**22**). An attempt to distance the piperidine ring from dibenz[*b,f*]azepine did not increase the affinity for the M₂ receptors, and strongly decreased the selectivity (**16**–**17**). Opening the piperidine ring and eliminating the nitrogen atom decreased the activity on M₂ receptors (**19**). Shortening the resulting aliphatic chain did not improve the activity (**20** and **21**). Cyclization of the diethylamino moiety of **21** in a piperidine (**23**) or *N*-methylpiperazine (**24**) ring didn't significantly increase the activity.

In Vitro Functional Activity Functional activities of compounds **8**, **9** and **38** on muscarinic receptors were further evaluated in *in vitro* organ preparations. In field stimulated guinea pig left atria, all the three compounds antagonized the methacholine-induced reduction of contractile response, a

muscarinic M₂ receptor mediated agonistic effect. Increasing concentrations of the compounds produced a right shift of the concentration–response curves of the relaxing effects produced by methacholine. The corresponding pA_2 values for **8**, **9** and **38** were 7.08, 6.20 and 5.61, respectively. The functional activity of **8**, **9** and **38** on M₃ receptors was evaluated in guinea-pig ileum preparations. All three compounds antagonized in a concentration-dependent fashion the acetylcholine-induced contractile response of the guinea-pig ileum preparation. Compounds showed a low potency, with an estimated IC₅₀ in the μ M range of 0.54, 6.79 and 1.19 μ M for **8**, **9** and **38**, respectively. These functional *in vitro* studies confirm that **8**, **9** and **38** have high affinity for M₂ muscarinic receptors and low affinity for M₃ muscarinic receptors.

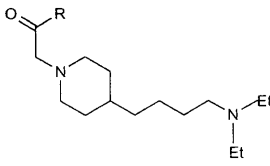
In Vivo Antibradycardic Activity Antibradycardic activity of **8** was evaluated *in vivo* in comparison to AF-DX 116. The effects of **8** on acetylcholine-induced bradycardia in rats were evaluated after intravenous and intraduodenal administration. Acetylcholine administration induced a decrease in heart rate and blood pressure. Both **8** and AF-DX 116 dose-dependently counteracted the acetylcholine-induced bradycardia and hypotension. The calculated ED₅₀ for bradycardia of **8** and AF-DX 116 were 55 ± 4 and 13 ± 2 μ g/kg, respectively. The corresponding values for hypotension were 80 ± 1 and 56 ± 1 μ g/kg, respectively. In the intraduodenal studies, acetylcholine administration induced a dose-dependent reduction of both heart rate (−129 ± 12, −224 ± 12 and −284 ± 10 beats/min after 10, 30 and 100 μ g/kg, respectively) and blood pressure (−45 ± 2, −50 ± 2, and −71 ± 4 mmHg, respectively). Compound **8** dose-dependently counteracted both the bradycardia (Fig. 3) and the hypotension induced by acetylcholine. The effects of AF-DX 116 were not significantly different from those of **8**.

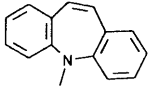
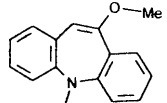
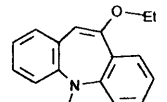
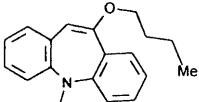
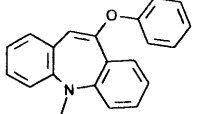
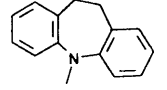
The hemodynamic effects of **8** in dogs were evaluated after oral administration. Figure 4 shows the time courses of mean heart rate for the 24-h period after placebo and two doses of **8**. Compared to the placebo, compound **8** at the dose of 10 mg/kg produced a significant increase in heart rate for the entire observation period.

These *in vivo* studies demonstrated that compound **8** is able to dose-dependently reverse both pharmacologically-induced and physiological bradycardia in different animal models.

In Vivo Functional Selectivity Compound **8** was investigated in rats for potential effects caused by the antagonism of other muscarinic receptor subtypes (mainly M₃). Multiples of the antibradycardic dose were used (15, 50 and 150 mg/

Table 1. Binding Affinities of a Number of (4-Diethylamino)butylpiperidine Derivatives for M₁, M₂, M₃, and M₄ Muscarinic Receptors and Corresponding Selectivity Ratios *versus* M₂ Receptors



Compd. No.	R	Binding affinity, pK _i				Selectivity ratio		
		M ₁	M ₂	M ₃	M ₄	M ₁ /M ₂	M ₃ /M ₂	M ₄ /M ₂
8		6.51	8.59	6.54	7.00	119.2	111.5	38.5
9		6.44	7.70	6.38	7.00	18.0	21.0	5.0
10		5.80	7.47	6.25	6.40	47.1	16.5	11.8
11		<6.00	7.23	<6.00	<6.00	>16.9	>16.9	>16.9
12		<6.00	6.92	<6.00	<6.00	>8.3	>8.3	>8.3
38		6.09	7.92	5.96	6.43	67.5	91.7	30.8
1	AF-DX 116	5.54	7.15	5.11	5.92	40.8	108.5	16.9

kg). Studies were carried out in comparison with atropine and AF-DX 116. Mean percent changes, compared to the controls, of the rate of gastric emptying, intestinal transit time, salivary secretion and pupil diameter after the different treatments are listed in Table 3. As expected, atropine significantly delayed the rate of gastric emptying (−84%) and intestinal transit time (−40%), inhibited oxotremorine-stimulated salivary secretion (−54%), and increase pupil diameter (+855%). At the lower dose of 15 mg/kg, compound **8** and AF-DX 116 had no effects of all these functions. At the medium dose (50 mg/kg) AF-DX 116 significantly affected gastric emptying (−74%), intestinal transit (−25%) and pupil diameter (+176%), while **8** only influenced the rate of gastric emptying (−48%). At the highest doses of 150 mg/kg, both compounds delayed gastric emptying and intestinal transit time. At this dose, AF-DX 116 also influenced pupil diameter (+631%), while compound **8** had no effects.

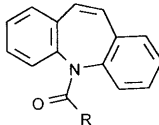
These studies show that in rats the antibradycardic effective doses of **8** have virtual no effects on physiological functions mediated mainly by M₃ and M₁ cholinergic receptors. The compound appears to be more selective than AF-DX 116, especially as far as the pupil diameter is concerned, where a favorable M₁/M₂ selectivity may play an important role.³⁵⁾

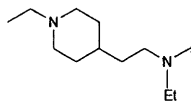
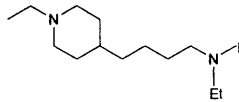
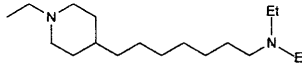
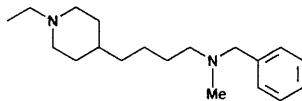
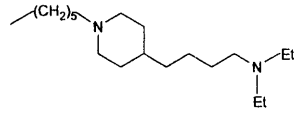
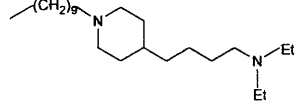
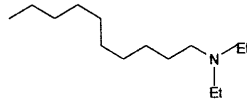
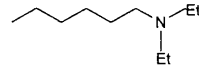
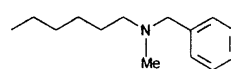
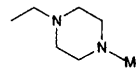
CNS Penetration It is well known that centrally-acting cholinergic agents increase the pain threshold.³⁶⁾ There is evidence that muscarinic receptors of the M₂ subtype are presynaptic autoreceptors that modify the release of acetylcholine through a negative feedback mechanism. Blocking these receptors by selective antagonists may therefore lead to increased acetylcholine release. Thus, a centrally-acting M₂ antagonist should display significant antinociceptive activity. The central analgesic activity of compounds **8**, **9** and **38** (5 mg/kg s.c.) were evaluated in mice. The study was carried out in comparison with oxotremorine (0.2 mg/kg s.c.) and AF-DX 116 (5 mg/kg s.c.). Mean licking latency before treatments was around 25 s. Oxotremorine produced a significant prolongation of licking latency (70 s), with maximum effect 30–45 min after administration. Both AF-DX 116 and compounds **8**, **9** and **38** produced weak and transient effects (Fig. 5), suggesting poor penetration of the blood brain barrier.

Conclusions

A series of 5*H*-dibenz[*b,f*]azepine derivatives having a lateral amino group was synthesized on the basis of bioisosterism of the double bond and amide bond. Among them, **8** (MF 10058) showed a high affinity to the human recombi-

Table 2. Binding Affinities of a Number of 5*H*-Dibenz[*b,f*]azepine Derivatives with Different Amine Chains for M₁, M₂, M₃, and M₄ Muscarinic Receptors and Corresponding Selectivity Ratios *versus* M₂ Receptors



Compd. No.	R	Binding affinity, p <i>K</i> _i				Selectivity ratio		
		M ₁	M ₂	M ₃	M ₄	M ₁ /M ₂	M ₃ /M ₂	M ₄ /M ₂
13		<6.00	6.44	<6.00	<6.00	>2.8	>2.8	>2.8
8		6.51	8.59	6.54	7.00	119.2	111.5	38.5
14		6.74	7.05	6.34	7.07	2.0	5.1	0.9
15		<6.00	7.26	<6.00	6.60	>18.2	>18.2	4.5
16		7.89	8.05	7.28	8.06	1.4	5.8	1.0
17		7.74	7.85	6.55	7.31	1.1	20.0	3.5
19		6.96	7.42	6.62	6.85	2.9	6.3	3.7
20		<6.00	6.92	<6.00	<6.00	>8.3	>8.3	>8.3
21		<6.00	<6.00	<6.00	<6.00	nt	nt	nt
22		<6.00	7.21	6.32	6.40	16.1	7.7	6.3
23		<6.00	6.92	6.88	7.04	>8.3	1.1	0.8
24		6.62	<6.00	6.64	6.82	<0.2	<0.2	<0.1

nant M₂ receptors ($K_i=2.6$ nmol/l), a low affinity for the M₄ receptors (39-fold less than for M₂ receptors) and a very low affinity for M₁ and M₃ receptors (119- and 112-fold less than for M₂ receptors). Functional experiments confirmed **8** to be a competitive antagonist with high affinity to the cardiac ($pA_2=7.1$) and low affinity to the intestinal muscarinic receptors ($ED_{50}=0.54$ μ M). *In vivo* experiments confirmed the *in vitro* M₂ selectivity. Acetylcholine-induced bradycardia was dose-dependently antagonized in rats after both intravenous

and intraduodenal administration. In rats, cholinergic functions mediated by M₁ or M₃ receptors (salivary secretion, pupil diameter, gastric emptying, intestinal transit time) were not affected by the oral administration of **8**, even at doses as high as 30 times the antibradycardic effective dose. Nocturnal bradycardia was dose-dependently inhibited in dogs by the oral route, with a duration of action of about 24 h. Compound **8** appears to be a promising cardioselective antimuscarinic agent for the treatment of dysfunctions of the cardiac

conduction system such as sinus or nodal bradycardia ("sick-sinus syndrome") and atrioventricular block.

Experimental

All reagents and solvents were from commercial suppliers and used with no further purification. Proton magnetic resonance spectra ($^1\text{H-NMR}$) were recorder on a Bruker AC 200 (4.7 T) instrument. Chemical shifts (δ) are reported in parts per million relative to the internal standard deuterated chloroform ($\delta=7.26$ ppm). The following abbreviations are used: s=singlet, d=doublet, dd=double doublet, td=triple doublet, q=quartet, m=multiplet, br=broad. IR spectra were recorder on a Perkin Elmer Spectrum RX FT-IR System spectrometer in the range $4000\text{--}600\text{ cm}^{-1}$. Reaction products were purified, when necessary, by flash column chromatography on silica gel (J. T.

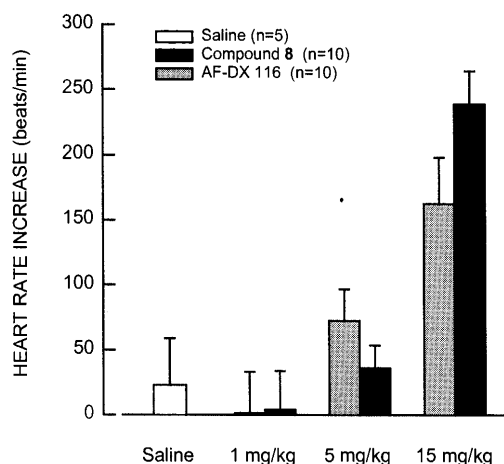


Fig. 3. Dose-Response Effects (Mean $\pm$ S.E.M.) of Compound 8 and AF-DX 116, Administered by Intraduodenal Route, on the Acetylcholine-Induced Bradycardia in Rat

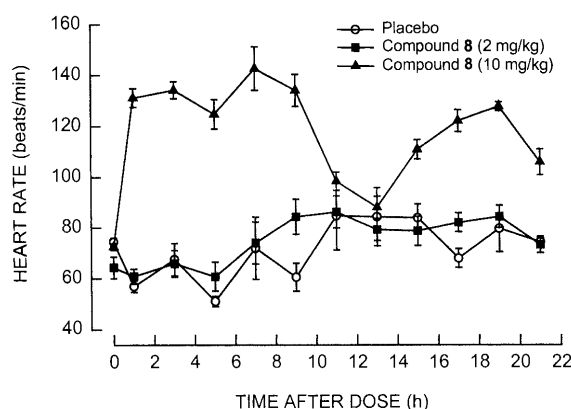


Fig. 4. Time Course of Heart Rate (Mean $\pm$ S.E.M.) in Two Dogs Receiving Placebo and Different Oral Doses of Compound 8

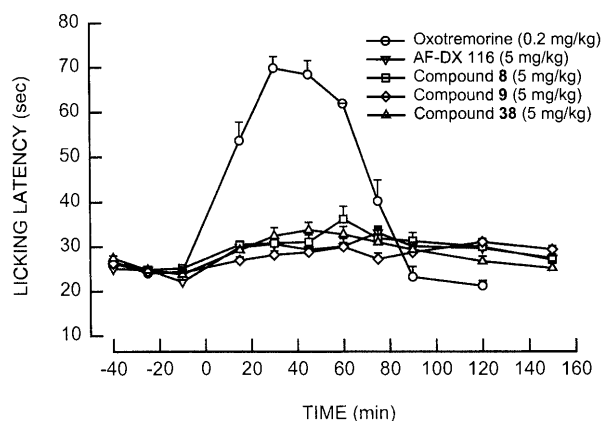


Fig. 5. Licking Latencies (Mean $\pm$ S.E.M.) after Subcutaneous Administration of Oxotremorine (0.2 mg/kg), AF-DX 116 (5 mg/kg) and Compounds 8, 9, and 38 (5 mg/kg) to Groups of 12–17 Mice

Table 3. Percent Changes (Mean $\pm$ S.E.M.) Compared to Controls of Rate of Gastric Emptying, Intestinal Transit Time, Salivary Secretion and Pupil Diameter after Atropine and Different Doses of AF-DX 116 and Compound 8

	Gastric emptying	Intestinal transit	Salivary secretion	Pupil diameter
Atropine	$-84\pm10^{**}$	$-40\pm6^{**}$	$-54\pm7^{**}$	$855\pm17^{**}$
AF-DX 116				
15 mg/kg	0 ± 8	6 ± 5	-22 ± 8	12 ± 5
50 mg/kg	$-74\pm11^{**}$	$-25\pm8^*$	-18 ± 9	$176\pm67^*$
150 mg/kg	$-87\pm13^{**}$	$-36\pm5^{**}$	-12 ± 9	$631\pm90^{**}$
Compound 8				
15 mg/kg	7 ± 9	-2 ± 5	2 ± 10	49 ± 33
50 mg/kg	$-48\pm19^{**}$	-19 ± 8	-13 ± 13	4 ± 2
150 mg/kg	$-107\pm14^{**}$	$-31\pm9^{**}$	-5 ± 12	7 ± 8

* $p<0.05$ vs. controls; ** $p<0.01$ vs. controls.

Baker 230–400 mesh). Analytical thin-layer chromatography (TLC) was carried out on precoated glass plates (Macherey-Nagel Durasil-25 UV₂₅₄), and visualized with UV light at 254 nm and/or I_2 vapor.

10-Phenoxy-5H-dibenz[b,f]azepine (5e) To a solution of phenol (0.43 g, 4.5 mmol) in 8 ml of anhydrous dimethyl sulfoxide was added potassium *t*-butoxide (1.02 g, 4.5 mmol). The solution was stirred at room temperature for 30 min, then 5-acetyl-10-bromo-5H-dibenz[b,f]azepine **25** (0.95 g, 3.0 mmol) was added and the mixture was stirred at 100°C for 20 h. After cooling to room temperature, the reaction mixture was poured into water (100 ml) and extracted with diethyl ether (4×25 ml). The combined organic phases were washed with 2 N NaOH, dried over Na_2SO_4 and concentrated to give 0.17 g of **5e** as a yellow oil in 20% yield. $^1\text{H-NMR}$ (CDCl_3) δ : 5.17 (1H, s br, NH), 6.23 (1H, s br, $\text{CH}=\text{O-Ph}$), 6.55–7.42 (13H, m).

Ethyl trans-4-(N-Benzyl-4'-piperidinyliden)-2-butenate (27) 1-Benzyl-4-piperidone (17.1 ml, 92.2 mmol) and triethyl 4-phosphonocrotonate (25.63 g, 92.2 mmol) were dissolved in 60 ml of absolute ethanol and cooled to $0\text{--}5^\circ\text{C}$, under a nitrogen atmosphere. Metallic sodium (2.76 g, 119.9 mmol) dissolved in 150 ml of absolute ethanol was added dropwise to the cooled solution at below 5°C for 40 min. The solution was stirred at 5°C for 45 min, then kept for 1 h at room temperature. The reaction mixture was diluted with 600 ml of brine and extracted with diethyl ether (5×100 ml). The combined extracts were washed with water, dried over Na_2SO_4 , the solvent was evaporated *in vacuo*, and the residue purified by flash column chromatography eluting with petroleum ether– Et_2O (1:1, v/v) to afford 16.6 g of **27** as a yellow oil in 63% yield. IR (neat) cm^{-1} : 2920, 2800, 1710 (C=O), 1635, 1610. $^1\text{H-NMR}$ (CDCl_3) δ : 1.29 (3H, t, $J=7.0$ Hz), 2.25–2.60 (8H, m), 3.51 (2H, s), 4.19 (2H, q, $J=7.0$ Hz), 5.80 (1H, d, $J=15.2$ Hz, $\text{CH}=\text{COOEt}$), 5.96 (1H, d, $J=10.9$ Hz, $\text{CH}=\text{CH}=\text{CH}=\text{COOEt}$), 7.20–7.35 (5H, m), 7.56 (1H, dd, $J=15.2, 10.9$ Hz, $\text{CH}=\text{CH}=\text{CH}=\text{COOEt}$).

Ethyl 4-(4'-Piperidinyl)butanoate (28a) A suspension of **27** (16.48 g, 57.7 mmol) and 1.6 g of 10% palladium on activated carbon in 150 ml of ethanol was hydrogenated at atmospheric pressure and room temperature for 8 h. The reaction mixture was filtered through Celite[®], and the solvent was evaporated off under reduced pressure. The residue was dissolved in 100 ml of diethyl ether and the solvent was washed with brine, dried over Na_2SO_4 ,

Table 4. Physical Data for α -Haloacyldibenzazepine Derivatives **6a**–**g** and **37**

Compd. (No.)	R	n	X	Yield (%)	$^1\text{H-NMR } \delta$ (in CDCl_3 , J in Hz)	Ref.
6a	H	1	Cl	>98	3.72 (1H, d, $J=12.3$ Hz, $\text{COCHH}'\text{Cl}$), 3.96 (1H, d, $J=12.3$ Hz, $\text{COCHCHH}'\text{Cl}$), 6.95 (2H, dd, $J=15.3, 11.5$ Hz, CH=CH), 7.31–7.50 (8H, m)	40)
6b	H	5	Br	96	1.20–1.81 (6H, m, $-(\text{CH}_2)_5-$), 1.92 (1H, m, COCHH'), 2.23 (1H, m, COCHH'), 3.32 (2H, t, $J=6.9, -\text{CH}_2-\text{Br}$), 6.95 (2H, dd, $J=15.2, 12.2$ Hz, CH=CH), 7.25–7.50 (8H, m)	—
6c	H	9	Br	94	1.07–2.03 (15H, m, $-(\text{CH}_2)_7-$ and COCHH'), 2.20 (1H, m, COCHH'), 3.38 (2H, t, $J=6.8$ Hz, $-\text{CH}_2-\text{Br}$), 6.95 (2H, dd, $J=16.3, 13.0$ Hz), 7.30–7.55 (m, 8H)	—
6d	OMe	1	Cl	>98	3.73 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.80 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.88 (3H, d, $J=4.5$ Hz, OCH_3), 3.97 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 4.03 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 6.15 (1H, d, $J=13.8$ Hz, CH=C-O-Me), 7.26–7.55 (7H, m), 7.77 (1H, t br, $J=7.7$ Hz)	—
6e	OEt	1	Cl	>98	1.48 (3H, m, CH_2CH_3), 3.73 (0.5H, d, $J=6.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.80 (0.5H, d, $J=6.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.94–4.17 (3H, m, CH_2CH_3 and $\text{COCHH}'\text{Cl}$), 6.14 (1H, d, $J=11.5$ Hz, CH=C-OEt), 7.26–7.55 (7H, m), 7.79 (1H, t br, $J=7.3$ Hz)	—
6f	OBu	1	Cl	94	1.02 (3H, t, $J=6.8$ Hz, $\text{O}(\text{CH}_2)_3\text{CH}_3$), 1.48–1.67 (2H, m, $\text{O}(\text{CH}_2)_2\text{CH}_2\text{CH}_3$), 1.74–1.95 (2H, m, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 3.73 (0.5H, d br, $J=6.11$ Hz, $\text{COCHH}'\text{Cl}$), 3.89 (0.5H, d br, $J=6.11$ Hz, $\text{COCHH}'\text{Cl}$), 3.90–4.09 (3H, m, $\text{CH=C-OCH}_2(\text{CH}_2)_2\text{CH}_3$ and $\text{COCHH}'\text{Cl}$), 7.26–7.55 (7H, m), 7.77 (1H, m)	—
6g	OPh	1	Cl	65	3.82 (1H, d, $J=13.3$ Hz, $\text{COCHH}'\text{Cl}$), 4.02 (1H, d, $J=13.3$ Hz, $\text{COCHH}'\text{Cl}$), 6.58 (1H, s, CH=C-OPh), 6.95–7.55 (12H, m), 7.77 (1H, dd, $J=22.2, 7.5$ Hz)	—
37	—	—	—	>98	2.85 (2H, m, $\text{CHH}'-\text{CHH}'$), ca. 3.4 (2H, m br, $\text{CHH}'-\text{CHH}'$), 4.02 (2H, dd, $J=18.7, 11.5$ Hz, $\text{COCHH}'\text{Cl}$), 7.11–7.40 (8H, m)	41)

filtered, and evaporated *in vacuo* to give 11.2 g of **28a** as a yellow oil in 97% yield. IR (neat) cm^{-1} : 3280 (NH), 2910, 2840, 1725 (C=O). $^1\text{H-NMR}$ (CDCl_3) δ : 1.22 (3H, t, $J=7.0$ Hz), 1.55–1.78 (9H, m), 2.25 (2H, t, $J=6.8$ Hz), 2.53 (2H, td, $J=11.6, 2.6$ Hz, NCH_{ax}), 3.01 (2H, d, $J=11.6$ Hz, NCH_{eq}), 4.09 (2H, q, $J=6.8$ Hz).

Ethyl 4-(*N*-Benzyl-4'-piperidinyl)butanoate (28b) Sodium carbonate (5.9 g, 55.4 mmol), benzylchloride (6.4 ml, 55.4 mmol) and a catalytic amount of sodium iodide were added to **28a** (11.04 g, 55.4 mmol) dissolved in 60 ml of dimethylformamide. The reaction mixture was stirred at room temperature for 6 h, diluted in 400 ml of 0.1 N HCl and extracted with diethyl ether (3×80 ml). The aqueous layer was basified with 40% aqueous NaOH and extracted with diethyl ether (4×100 ml). The combined organic phases were washed with water, dried over Na_2SO_4 , filtered and evaporated to give 13.85 g of **28b** as a yellow oil in 86% yield. IR (neat) cm^{-1} : 2927, 2840, 2800, 1736 (C=O), 1454. $^1\text{H-NMR}$ (CDCl_3) δ : 1.15–1.35 (8H, m), 1.50–1.70 (4H, m), 1.91 (2H, t br $J=10.4$ Hz, NCH_{ax}), 2.27 (2H, t, $J=7.1$ Hz), 2.86 (2H, d, $J=11.2$ Hz, NCH_{eq}), 3.46 (2H, s, CH_2Ph), 4.11 (2H, q, $J=6.8$ Hz), 7.20–7.35 (5H, m).

4-(*N*-benzyl-4'-piperidinyl)-1-butanol (29a) A solution of **28b** (13.8 g, 47.8 mmol) in 60 ml of anhydrous tetrahydrofuran was added dropwise, under a nitrogen atmosphere, to a suspension of lithium aluminium hydride (2.7 g, 71.7 mmol) in 40 ml of anhydrous tetrahydrofuran while maintaining the reaction temperature below 10 °C. The mixture was stirred for 1 h at room temperature, cooled to 0 °C and then hydrolyzed by the addition of 1 N NaOH. The resulting suspension was filtered and the filtrate evaporated. The residue dissolved in 100 ml of diethyl ether was washed with water and the organic layer was dried over Na_2SO_4 , filtered and evaporated *in vacuo* to give 11.3 g of **29a** as yellow oil in 96% yield. IR (neat) cm^{-1} : 3339 (O–H), 2928, 1454. $^1\text{H-NMR}$ (CDCl_3) δ : 1.10–1.70 (11H, m), 1.91 (2H, t br $J=11.1$ Hz, NCH_{ax}), 2.86 (2H, d, $J=10.9$ Hz, NCH_{eq}), 3.47 (2H, s, CH_2Ph), 3.61 (2H, t, $J=5.7$ Hz), 7.20–7.35 (5H, m).

4-[4-(Diethylamino)butyl]-*N*-benzylpiperidine (30) Triethylamine (6.4 ml, 45.8 mmol) and **29a** (11.23 g, 45.4 mmol) were dissolved in 60 ml of tetrahydrofuran and cooled to 0 °C. Methanesulfonyl chloride (3.6 ml, 45.8 mmol) dissolved in 20 ml of tetrahydrofuran was added dropwise while the reaction temperature was maintained below 5 °C. The mixture was stirred for 45 min at this temperature and then warmed to room temperature. The resulting suspension was diluted with 60 ml of diethyl ether and the insoluble precipitate was filtered. The solvent was evaporated *in vacuo* to give 15.20 g of **29b** as a yellow oil. The compound was characterized only by IR and reacted immediately. IR (neat) cm^{-1} : 2928, 1454, 1355 ($\nu_{\text{as}} \text{SO}_2$), 1176 ($\nu_{\text{s}} \text{SO}_2$). Methanesulfonate derivative **29b** (15.03 g, 46.2 mmol) and diethylamine (19.1 ml, 184.8 mmol) dissolved in 130 ml of acetonitrile were refluxed for 6 h. After cooling to room temperature, the mixture was diluted with 250 ml of brine and extracted with diethyl ether (4×80 ml). The combined organic phases were washed with water, dried over Na_2SO_4 , filtered and evaporated *in vacuo* to give 12.94 g of **30** as a brownish oil in 94%

yield. IR (neat) cm^{-1} : 2928, 2840, 2790, 1454. $^1\text{H-NMR}$ (CDCl_3) δ : 0.99 (6H, t, $J=7.0$ Hz), 1.10–1.70 (11H, m), 1.91 (2H, m, NCH_{ax}), 2.38 (2H, t, $J=8.4$ Hz), 2.50 (4H, q, $J=7.0$ Hz), 2.85 (2H, d br, $J=10.0$ Hz, NCH_{eq}), 3.46 (2H, s, CH_2Ph), 7.20–7.35 (5H, m).

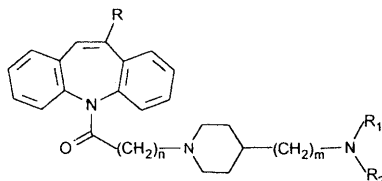
4-[4-(Diethylamino)butyl]piperidine (7b) A mixture of **30** (12.9 g, 42.7 mmol), ammonium formate (13.5 g, 213.5 mmol) and 10% palladium on activate carbon (13 g) in 160 ml of anhydrous methanol was refluxed for 30 min under nitrogen atmosphere. After cooling to room temperature, the reaction mixture was filtered through Celite[®] and the solvent evaporated off to afford 8.13 g of **7b** as a yellow oil in 90% yield. IR (neat) cm^{-1} : 3278 (N–H), 2929, 1466, 1446. $^1\text{H-NMR}$ (CDCl_3) δ : 0.99 (6H, t, $J=7.1$ Hz), 1.05–1.75 (12H, m), 2.38 (2H, t, $J=8.1$ Hz), 2.50 (4H, q, $J=7.1$ Hz), 2.55 (2H, m, NCH_{ax}), 3.02 (2H, dt, $J=12.2, 2.8$ Hz, CH_{eq}).

5-(Chloroacetyl)-10-methoxy-5*H*-dibenz[*b,f*]azepine (6d) To a solution of 10-methoxy-5*H*-dibenz[*b,f*]azepine **5b** (4 g, 20.7 mmol) in tetrahydrofuran (40 ml) were added *N,N*-dimethylaniline (2.75 ml, 21.7 mmol) and chloroacetylchloride (1.73 ml, 21.7 mmol), and the solution was refluxed for 1 h. The reaction was quenched with 200 ml of brine and extracted with diethyl ether (3×80 ml). The combined organic phases were washed with 0.1 N HCl (2×100 ml) in order to eliminate *N,N*-dimethylaniline, with 0.1 N NaOH (2×100 ml) to eliminate chloroacetylacetic acid, then with water. The organic layer was dried over Na_2SO_4 , filtered and evaporated *in vacuo* to give 5.67 g of **6d** as a yellowish powder in quantitative yield. IR (nujol) cm^{-1} : 3040, 1670 (C=O). $^1\text{H-NMR}$ (CDCl_3) δ : 3.73 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.80 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 3.88 (3H, d, $J=4.5$ Hz, OCH_3), 3.97 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 4.03 (0.5H, d, $J=5.1$ Hz, $\text{COCHH}'\text{Cl}$), 6.15 (1H, d, $J=13.8$ Hz, CH=C-O-Me), 7.26–7.55 (7H, m), 7.77 (1H, t br, $J=7.7$ Hz).

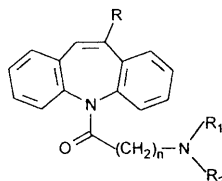
Compounds **6a**–**c** and **6e**–**g** were prepared in the same fashion as described for **6d**. Physical data for these compounds are listed in Table 4.

5-{4-[4-(Diethylamino)butyl]-1-piperidinyl}acetyl-5*H*-dibenz[*b,f*]azepine (8) Sodium carbonate (1.36 g, 12.8 mmol), **6a** (3.46 g, 12.8 mmol) and a catalytic amount of sodium iodide were added to 4-[4-(diethylamino)-butyl] piperidine **7b** (3 g, 14.1 mmol) in 70 ml of acetonitrile. The mixture was refluxed for 1.5 h, diluted in 150 ml of 2 N HCl, and extracted with diethyl ether (2×80 ml). The aqueous layer was basified with 40% aqueous NaOH and extracted with diethyl ether (3×80 ml). The combined organic phases were washed with water, dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography elution with petroleum ether–acetone–triethyl amine (16:4:0.5, v/v/v). After solvent evaporation, the residue was dissolved in diethyl ether and washed with water (3×100 ml). The organic layer was dried over Na_2SO_4 , filtered and evaporated *in vacuo* to give 5.04 g of **8** as a light yellow oil in 88% yield. IR (neat) cm^{-1} : 3024, 2929, 1676 (C=O), 1490, 1462, 1442. $^1\text{H-NMR}$ (CDCl_3) δ : 0.99 (6H, t, $J=6.9$ Hz, NCH_2CH_3), 1.05–2.00 (13H, m, CH_2 , CH and NCH_{ax}), 2.37 (2H, m, CH_2NEt_2), 2.50 (4H, q, $J=6.5$ Hz, NCH_2CH_3), 2.68 (2H, d, $J=9.8$ Hz, NCH_{eq}), 2.76 (1H, d, $J=14.3$ Hz, COCHH'), 3.07 (1H, d,

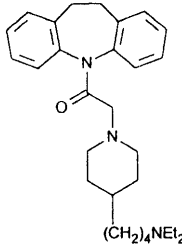
Table 5. Analytical and Spectral Data for Compounds **8–17**, **19–24** and **38**



8-17



19-24



38

Compd. (No.)	R	R ₁	R ₂	n	m	Formula	Analysis (%)			Yield (%)	¹ H-NMR δ (in CDCl ₃)
							Calcd	Found			
							C	H	N		
8	H	Et	Et	1	4	C ₂₉ H ₃₉ N ₃ O	78.16 (78.06)	8.82 8.90	9.43 9.37)	88	0.99 (6H, t, <i>J</i> =6.9 Hz, NCH ₂ CH ₃), 1.05—2.00 (13H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.37 (2H, m, CH ₂ NEt ₂), 2.50 (4H, q, <i>J</i> =6.5 Hz, NCH ₂ CH ₃), 2.68 (2H, d, <i>J</i> =9.8 Hz, NCH ₂ CH ₃), 2.76 (1H, d, <i>J</i> =14.3 Hz, COCHH'), 3.07 (1H, d, <i>J</i> =14.3 Hz, COCHH'), 6.91 (2H, dd, <i>J</i> =13.7, 11.7 Hz, CH=CH), 7.30—7.50 (8H, m)
9	OMe	Et	Et	1	4	C ₃₀ H ₄₁ N ₃ O ₂	75.75 (75.61)	8.69 8.70	8.83 8.78)	92	1.00 (6H, t, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 1.08—2.03 (13H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.39 (2H, t, <i>J</i> =7.1 Hz, CH ₂ NEt ₂), 2.52 (4H, q, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 2.60—3.15 (4H, m, COCH ₂ N and NCH ₂ CH ₃), 3.86 (3H, d, <i>J</i> =3.8 Hz, OCH ₃), 6.11 (1H, d, <i>J</i> =7.3 Hz, CH=COCH ₃), 7.20—7.48 (7H, m), 7.72 (1H, m)
10	OEt	Et	Et	1	4	C ₃₁ H ₄₃ N ₃ O ₂	76.03 (75.88)	8.85 8.92	8.58 8.60)	67	1.00 (6H, t, <i>J</i> =6.9 Hz, NCH ₂ CH ₃), 1.08—2.05 (16H, m, CH ₂ , CH, OCH ₂ CH ₃ and NCH ₂ CH ₃), 2.37 (2H, t, <i>J</i> =7.1 Hz, CH ₂ NEt ₂), 2.49 (4H, q, <i>J</i> =6.9 Hz, NCH ₂ CH ₃), 2.60—3.15 (4H, m, COCH ₂ N and NCH ₂ CH ₃), 4.05 (2H, q br, OCH ₂ CH ₃), 6.11 (1H, d, <i>J</i> =5.1 Hz, CH=C—OEt), 7.20—7.46 (7H, m), 7.75 (1H, m)
11	OBu	Et	Et	1	4	C ₃₃ H ₄₇ N ₃ O ₂	76.55 (76.53)	9.15 9.20	8.12 8.10)	71	1.00 (9H, m, NCH ₂ CH ₃ and O(CH ₂) ₃ CH ₃), 1.05—2.05 (17H, m, CH ₂ , CH, OCH ₂ (CH ₂) ₂ CH ₃ and NCH ₂ CH ₃), 2.37 (2H, t, <i>J</i> =6.9 Hz, CH ₂ NEt ₂), 2.49 (4H, q, <i>J</i> =6.6 Hz, NCH ₂ CH ₃), 2.60—3.15 (4H, m, COCH ₂ N and NCH ₂ CH ₃), 4.00 (2H, s br, OCH ₂ (CH ₂) ₂ CH ₃), 6.11 (1H, m, CH=COBu), 7.15—7.48 (7H, m), 7.72 (1H, m)
12	OPh	Et	Et	1	4	C ₃₅ H ₄₃ N ₃ O ₂	78.12 (77.99)	8.06 8.10	7.81 7.75)	67	1.00 (6H, t, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 1.05—2.10 (13H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.38 (2H, t, <i>J</i> =6.9 Hz, CH ₂ NEt ₂), 2.51 (4H, q, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 2.60—2.96 (3H, m, COCHH' and NCH ₂ CH ₃), 3.17 (1H, m, COCHH'), 6.54 (1H, d, <i>J</i> =11.6 Hz, CH=C—OPh), 6.95—7.49 (12H, m), 7.72 (1H, dd, <i>J</i> =23.1, 7.4 Hz)
13	H	Et	Et	1	2	C ₂₇ H ₃₅ N ₃ O	77.66 (77.50)	8.45 8.50	10.06 10.00)	77	1.00 (6H, t, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 1.05—2.05 (9H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.39 (2H, m, CH ₂ NEt ₂), 2.49 (4H, q, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 2.65 (2H, d, <i>J</i> =9.0 Hz, NCH ₂ CH ₃), 2.78 (1H, d, <i>J</i> =14.7 Hz, COCHH'), 3.07 (1H, d, <i>J</i> =14.7 Hz, COCHH'), 6.92 (2H, dd, <i>J</i> =13.5, 11.1 Hz, CH=CH), 7.30—7.45 (8H, m)
14	H	Et	Et	1	7	C ₃₂ H ₄₅ N ₃ O	78.80 (78.75)	9.30 9.31	8.62 8.59)	61	1.00 (6H, t, <i>J</i> =7.1 Hz, NCH ₂ CH ₃), 1.05—2.05 (19H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.37 (2H, m, CH ₂ NEt ₂), 2.49 (4H, q, <i>J</i> =7.1 Hz, NCH ₂ CH ₃), 2.65 (2H, d, <i>J</i> =9.7 Hz, NCH ₂ CH ₃), 2.78 (1H, d, <i>J</i> =15.1 Hz, COCHH'), 3.07 (1H, d, <i>J</i> =15.1 Hz, COCHH'), 6.92 (2H, dd, <i>J</i> =12.5, 11.0 Hz, CH=CH), 7.30—7.45 (8H, m)
15	H	Bn	Me	1	4	C ₃₃ H ₃₉ N ₃ O	80.28 (80.07)	7.96 8.00	8.51 8.49)	95	1.00—2.00 (13H, m, CH ₂ , CH and NCH ₂ CH ₃), 2.15 (3H, s, NCH ₃), 2.32 (2H, t, <i>J</i> =6.8 Hz, CH ₂ NMeBn), 2.66 (2H, d, <i>J</i> =9.1 Hz, NCH ₂ CH ₃), 2.76 (1H, d, <i>J</i> =14.3 Hz, COCHH'), 3.07 (1H, d, <i>J</i> =14.3 Hz, COCHH'), 3.44 (2H, s, NCH ₂ Ph), 6.92 (2H, dd, <i>J</i> =15.7, 11.8 Hz, CH=CH), 7.20—7.45 (8H, m)
16	H	Et	Et	5	4	C ₃₃ H ₄₇ N ₃ O	78.99 (78.80)	9.44 9.55	8.37 8.25)	71	1.00 (6H, t, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 1.08—1.98 (20H, m, CH ₂ , CH, COCHH' and NCH ₂ CH ₃), 2.20 (3H, m, COCHH' and CH ₂ N(CH ₂ CH ₂) ₂ CH), 2.38 (2H, t, <i>J</i> =6.9 Hz, CH ₂ NEt ₂), 2.50 (4H, q, <i>J</i> =6.8 Hz, NCH ₂ CH ₃), 2.82 (2H, d br, <i>J</i> =10.2 Hz, NCH ₂ CH ₃), 6.92 (2H, dd, <i>J</i> =15.8, 11.3 Hz, CH=CH), 7.27—7.45 (8H, m)
17	H	Et	Et	9	4	C ₃₇ H ₅₅ N ₃ O	79.66 (79.45)	9.94 9.99	7.53 7.50)	34	1.00 (6H, t, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 1.07—2.00 (28H, m, CH ₂ , CH, COCHH' and NCH ₂ CH ₃), 2.20 (3H, m, COCHH' and CH ₂ N(CH ₂ CH ₂) ₂ CH), 2.40 (2H, t, <i>J</i> =7.0 Hz, CH ₂ NEt ₂), 2.51 (4H, q, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 2.88 (2H, d br, <i>J</i> =10.6 Hz, NCH ₂ CH ₃), 6.92 (2H, dd, <i>J</i> =16.5, 11.3 Hz, CH=CH), 7.27—7.45 (8H, m)

Table 5. Continued

Compd. (No.)	R	R ₁	R ₂	n	m	Formula	Analysis (%)			Yield (%)	¹ H-NMR δ (in CDCl ₃)
							Calcd	Found			
							C	H	N		
19	H	Et	Et	9	—	C ₂₈ H ₃₈ N ₂ O	80.34 (80.02)	9.15 9.19	6.69 6.58)	38	1.00 (6H, t, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 1.07—1.60 (14H, m, COCH ₂ (CH ₂) ₇), 1.90 (1H, m, COCHH'), 2.19 (1H, m, COCHH'), 2.37 (3H, t, <i>J</i> =7.3 Hz, CH ₂ NEt ₂), 2.50 (4H, q, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 6.92 (2H, dd, <i>J</i> =16.6 Hz, 11.5, CH=CH), 7.27—7.50 (8H, m)
20	H	Et	Et	5	—	C ₂₄ H ₃₀ N ₂ O	79.52 (79.11)	8.34 8.31	7.73 7.80)	45	0.97 (6H, t, <i>J</i> =7.0 Hz, NCH ₂ CH ₃), 1.15—1.60 (6H, m, COCH ₂ (CH ₂) ₃), 1.90 (1H, m, COCHH'), 2.16 (1H, m, COCHH'), 2.31 (3H, t, <i>J</i> =7.0 Hz, CH ₂ NEt ₂), 2.45 (4H, q, <i>J</i> =7.0 Hz, N(CH ₂ CH ₃) ₂), 6.95 (2H, dd, <i>J</i> =16.1, 11.6 Hz, CH=CH), 7.30—7.45 (8H, m)
21	H	Et	Et	1	—	C ₂₀ H ₂₂ N ₂ O	78.40 (78.21)	7.24 7.19	9.14 9.09)	91	0.87 (6H, t, <i>J</i> =7.2 Hz, NCH ₂ CH ₃), 2.53 (4H, qd, <i>J</i> =7.3, 2.5 Hz, NCH ₂ CH ₃), 2.94 (1H, d, <i>J</i> =15.6 Hz, COCHH'), 3.23 (1H, d, <i>J</i> =15.6 Hz, COCHH'), 6.95 (2H, dd, <i>J</i> =15.8, 11.2 Hz, CH=CH), 7.30—7.45 (8H, m)
22	H	Bn	Me	5	—	C ₂₈ H ₃₀ N ₂ O	81.91 (81.94)	7.37 7.45	6.82 6.74)	73	1.12—1.58 (6H, m, COCH ₂ (CH ₂) ₃), 1.92 (1H, m, COCHH'), 2.13 (3H, s, NCH ₃), ca. 2.2 (1H, m, COCHH'), 2.27 (2H, m, CH ₂ NCH ₂ Bn), 3.43 (2H, s, NCH ₂ Ph), 6.91 (2H, dd, <i>J</i> =16.8, 11.0 Hz, CH=CH), 7.23—7.45 (13H, m)
23	H	—(CH ₂) ₅ —	—	1	—	C ₂₁ H ₂₂ N ₂ O	79.21 (78.92)	6.96 7.12	8.80 8.74)	84	1.23—1.54 (6H, m, —(CH ₂) ₃ —), 2.28 (4H, t, <i>J</i> =5.4 Hz, CH ₂ —N—CH ₂), 2.78 (1H, d, <i>J</i> =15.3 Hz, COCHH'), 3.05 (1H, d, <i>J</i> =15.3 Hz, COCHH'), 6.93 (2H, d, <i>J</i> =1.7 Hz, CH=CH), 7.27—7.45 (8H, m)
24	H	N-methyl Piperazine	—	1	—	C ₂₁ H ₂₃ N ₃ O	75.65 (75.01)	6.95 7.02	12.60 12.51)	87	2.23 (3H, s, NCH ₃), 2.38 (8H, s br, N[CH ₂ CH ₂] ₂ N), 2.76 (1H, d, <i>J</i> =14.1 Hz, COCHH'), 3.08 (1H, <i>J</i> =14.1 Hz, COCHH'), 6.95 (2H, dd, <i>J</i> =12.3, 11.1 Hz, CH=CH), 7.30—7.45 (8H, m)
38	—	—	—	—	—	C ₂₉ H ₄₁ N ₃ O	77.81 (77.58)	9.23 9.39	9.17 9.42)	84	1.00 (6H, m, NCH ₂ CH ₃), 1.05—2.15 (13H, m, CH ₂ , CH and NCH ₂), 2.37 (2H, m, CH ₂ NEt ₂), 2.50 (4H, q, <i>J</i> =7.1 Hz, NCH ₂ CH ₃), 2.60—3.55 (8H, m, COCH ₂ N, CH ₂ —CH ₂ and NCH ₂), 7.10—7.45 (8H, m)

J=14.3 Hz, COCHH'), 6.91 (2H, dd, *J*=13.7, 11.7 Hz, CH=CH), 7.30—7.50 (8H, m).

Compounds **9**—**17**, **19**—**24** and **38** were prepared in the same fashion as described for **8**. Physical data for these compounds are listed in Table 5.

4-[4-(Methylamino)butyl]-*N*-benzylpiperidine (31) To a stirred solution of methanesulfonate derivative **29b** (1.65 g, 5.1 mmol) in acetonitrile (7 ml) was added methylamine 40% in water (102 mmol), and the mixture was stirred for 15 h at room temperature. The reaction was poured in brine (80 ml), extracted with diethyl ether (3×50 ml), dried over Na₂SO₄, and the solvent was removed *in vacuo* to afford 1.35 g of **31** as a yellow oil in 98% yield. IR (neat) cm⁻¹: 3250 (NH), 2900, 2830, 2780, 1440. ¹H-NMR (CDCl₃) δ : 1.14—1.70 (11H, m), 1.90 (2H, t br, *J*=11.5 Hz, NCH₂), 2.41 (3H, s), 2.54 (2H, *J*=7.1 Hz, t), 2.85 (2H, d br, *J*=11.4 Hz, NCH₂), 3.46 (2H, s, CH₂Ph), 7.20—7.35 (5H, m).

4-[4-(*N*-Benzoyl-*N*-methylamino)butyl]-1-benzylpiperidine (32a) To a stirred solution of **31** (1.30 g, 5.0 mmol) in acetone (7 ml) was added 1 N NaOH (5.5 mmol), then a solution of benzoyl chloride (0.61 ml, 5.25 mmol) in acetone (3 ml) was added dropwise. The reaction mixture was stirred at room temperature for 2 h and then poured in brine (20 ml), made alkaline with 1 N NaOH and extracted with diethyl ether (3×20 ml). The combined organic phases were dried over Na₂SO₄ and concentrated *in vacuo*. The residue was purified by flash column chromatography elution with CHCl₃—MeOH (98:2, v/v) to afford 1.50 g of **32a** as a light yellow oil in 82% yield. IR (neat) cm⁻¹: 2900, 2840, 1620 (C=O), ¹H-NMR (CDCl₃) δ : 1.10—2.00 (13H, m), 2.85—3.10 (5H, m), 3.20 (1H, t br, *J*=7.0 Hz), 3.48 (2H, s, CH₂Ph), 3.51 (1H, m), 7.20—7.35 (5H, m), 7.37 (5H, s).

4-[4-(*N*-Benzoyl-*N*-methylamino)butyl]piperidine (32b) The title compound was prepared as described for **7b**, and was produced in 91% yield. IR (neat) cm⁻¹: 3260 (NH), 2890, 2830, 1615 (C=O). ¹H-NMR (CDCl₃) δ : 0.90—1.75 (11H, m), 2.07 (1H, s br, NH), 2.56 (2H, t br, *J*=10.0 Hz, NCH₂), 2.86—3.10 (5H, m, NCH₂ e NCH₃), 3.20 (1H, t br, *J*=5.7 Hz), 3.50 (1H, t br, *J*=5.7 Hz), 7.35 (5H, s).

4-[4-(*N*-Benzyl-*N*-methylamino)butyl]piperidine (7c) The title compound was prepared as described for **29a**, heating at reflux for 1 h, in 92% yield. IR (neat) cm⁻¹: 2910, 2840, 2780, 1450. ¹H-NMR (CDCl₃) δ : 0.90—

1.80 (12H, m), 2.17 (3H, s), 2.34 (2H, t, *J*=6.4 Hz), 2.56 (2H, td, *J*=11.1, 2.8 Hz, NCH₂), 3.03 (2H, dt, *J*=11.1, 2.8 Hz, NCH₂), 3.45 (2H, s, CH₂Ph), 7.20—7.35 (5H, m).

***N,N*-Diethyl-6-bromohexanamide (34)** A solution of 6-bromohexanoyl chloride (2.0 g, 9.37 mmol) in acetone (4 ml) was added dropwise to a solution of diethylamine (4.9 ml, 46.85 mmol) in water (8 ml) at 0 °C. The mixture was stirred for 2 h at 0 °C, then poured in brine (40 ml) and extracted with diethyl ether (3×30 ml). The combined extract was washed with water, dried over Na₂SO₄ and concentrated *in vacuo* to give 2.2 g of **34** as a yellow oil in 94% yield. IR (neat) cm⁻¹: 2920, 1610 (C=O), 1420. ¹H-NMR (CDCl₃) δ : 1.09 (3H, t, *J*=7.0 Hz, CONCH₂CH₃), 1.14 (3H, t, *J*=7.0 Hz, CONCH₂CH₃), 1.35—1.75 (4H, m), 1.87 (2H, m), 2.29 (2H, t, *J*=7.8 Hz), 3.20—3.46 (6H, m).

***N,N*-Diethyl-7-(4-pyridyl)heptanamide (35)** A suspension of 50% sodium amide in toluene (0.75 g, 9.60 mmol) was added to a solution of 4-picoline (0.93 ml, 9.60 mmol) in anhydrous toluene (10 ml) in a nitrogen atmosphere. The mixture was stirred for 30 min at 80 °C, cooled to room temperature, then **34** (1.6 g, 6.40 mmol) in toluene (6 ml) was added. The resulting solution was stirred at 80 °C for 6 h, then poured in brine (30 ml) and the organic layer was separated. The water layer was extracted with diethyl ether (3×20 ml). The combined extracts were washed with water, dried over Na₂SO₄ and concentrated. The residue was purified by a flash chromatography column with petroleum ether—acetone (65:35, v/v) to afford 0.51 g of **35** as a yellow oil in 30% yield. ¹H-NMR (CDCl₃) δ : 1.10 (3H, t, *J*=7.0 Hz), 1.15 (3H, t, *J*=7.0 Hz), 1.27—1.80 (8H, m), 2.26 (2H, t, *J*=7.0 Hz, CH₂CONEt₂), 2.58 (2H, t, *J*=7.0 Hz, CH₂C₃H₄N), 3.32 (4H, m), 7.08 (2H, dd, *J*=4.1, 1.3 Hz), 8.45 (2H, dd, *J*=4.1, 1.6 Hz).

***N,N*-Diethyl-7-(4-piperidyl)heptanamide (36)** Pyridyl derivative **35** (0.47 g) was dissolved in 1.9 M in methanol HCl (20 ml) and stirred at room temperature for 40 min. The solvent was evaporated, the residue was dissolved in acetic acid, and PtO₂ (25 mg) was added. The mixture was maintained under a hydrogen atmosphere at room temperature and atmospheric pressure for 24 h. After the catalyst was filtered off and the filtrate was basified with 32% NaOH, it was then extracted with diethyl ether. The combined extracts were washed with water, dried over Na₂SO₄ and concentrated to af-

ford 0.41 g of **36** in 85% yield. $^1\text{H-NMR}$ (CDCl_3) δ : 1.09 (3H, t, $J=7.0$ Hz), 1.15 (3H, t, $J=7.0$ Hz), 1.20–1.80 (16H, m), 2.27 (2H, t, $J=7.8$ Hz, $\text{CH}_2\text{CONEt}_2$), 2.55 (2H, td, $J=11.4$, 2.2 Hz, NCH_2ax), 3.02 (2H, dt, $J=11.4$, 2.1 Hz, NCH_2eq), 3.32 (4H, m, NCH_2CH_3).

4-[4-(Diethylamino)heptyl]piperidine (7d) The title compound was prepared as described for **29a**, heating at reflux for 2 h, and was produced in 96% yield. $^1\text{H-NMR}$ (CDCl_3) δ : 1.01 (6H, t, $J=6.9$ Hz), 1.10–1.80 (18H, m), 2.39 (2H, t, $J=7.7$ Hz, CH_2NEt_2), 2.51 (4H, q, $J=6.9$ Hz, NCH_2CH_3), 2.55 (2H, m, NCH_2ax), 3.03 (2H, dt, $J=11.8$, 2.8 Hz, NCH_2eq).

Receptor Binding Assay Membranes of Hamster ovarian cells expressing human M_1 , M_2 , M_3 or M_4 receptors were suspended in a 10 mM Tris–HCl solution at pH 7.2 containing 2 mM EDTA (plus 10% sucrose in the case of M_1 , M_3 and M_4 receptors). Aliquots of the membrane fractions (0.02 ml) were incubated in duplicate, with 10 increasing concentrations (from 10^{-10} to 10^{-5} M) of the reference (4-DAMP) or test compounds. The radioligand [^3H]N-methylscopolamine ([^3H]NMS) was added to the membrane preparations, which were then incubated for 60 min at 25 °C or 27 °C. Apparent equilibrium dissociation constants (K_d) for [^3H]NMS binding were 0.28, 0.29, 0.19, 0.13 nM for M_1 , M_2 , M_3 , M_4 receptors, respectively. Maximum receptor density (B_{max}) was 64, 65, 4400, 1200 fmol/mg protein for M_1 , M_2 , M_3 , M_4 receptors, respectively.

The assay was terminated by rapid filtration under suction using a 96-well harvester. After 4–5 washes with ice-cold buffer, the filters were put in plastic bags with 25 ml scintillant cocktail, and the bags were sealed. The radioactivity was counted using a liquid scintillation counter. Affinity constants (K_i) of the test compounds for muscarinic receptor subtypes were estimated with non-linear regression.

Guinea-Pig Atrium Isolated left atria were prepared from male or female Duncan Hartley derived guinea pigs weighing 325 ± 25 g. Animals were sacrificed by CO_2 overexposure. Each atria was placed under 1 g tension in a 10 ml bath containing McEwen's solution at pH 7.4, bubbled with 95% O_2 /5% CO_2 at 32 °C, and subjected to field stimulation by 70% maximum voltage, 2.5 Hz at 0.5 ms pulse width. The left atria was connected to an isometric transducer and two pen recorders and allowed to equilibrate for 60 min before the field stimulation was initiated. Each tissue was accepted for experimental use only if 1 g or more of tension was obtained. A cumulative relaxation–response curve to methacholine was then generated with consecutive applications of 9 concentrations in 3-fold increments ranging from 1 nM to 10 μM at 2-min intervals for a total of 18 min to establish the maximal response. In 4 separate tissues, similar methacholine concentration–response experiments were carried out in the presence of test compound concentration (low, middle and high), following a 15-min incubation period. pA_2 values for the test compounds were calculated by linear regression of the corresponding Schild plots.

Guinea-Pig Ileum Segments (2–3 cm) of proximal ileum were suspended in 30 ml Tyrode solution at 37 ± 1 °C. The preparation was connected to an isometric strain gauge and was kept at a resting tension of 1 g. The changes in tension were registered through a polygraphic recorder. The ileum was stimulated with a sub-maximal concentration of acetylcholine (0.03 μM) every 4–5 min. The drugs were prepared in physiological saline solution. The activity of test compounds under investigation was expressed as the percent inhibition of the contraction induced by acetylcholine. The IC_{50} was calculated by non-linear regression.

Antibradycardic Activity in Rats Male Sprague–Dawley rats weighing 270 $\pm$ 5 g were used in the study. Animals (two per cage) were housed for a minimum of 5 d before the experiments under a 12-h light-dark cycle at room temperature (20 ± 2 °C) and 55% minimum humidity. Rats had free access to commercial chow and tap water. Animals were anesthetized with urethane (1.5 g/kg, i.p.) and their body temperature was maintained at 37 °C with a heating pad. A 2 cm incision was made in the middle of the neck and a tracheotomy tube was inserted. The carotid aorta was isolated and connected with a catheter to a pressure transducer. Heart rate and blood pressure were recorded and analyzed with the software HEM (Notocord, Croissy sur Seine, France). Another catheter was introduced in the left jugular vein for acetylcholine perfusion. A third catheter was introduced in the right jugular vein for vehicle, test or reference compound administration. After a 10 min stabilization period, acetylcholine (50 $\mu\text{g}/\text{min}/\text{kg}$) was continuously infused intravenously until stabilization of the blood pressure. The vehicle (sterile saline, 1 ml/kg) was administered as an intravenous bolus. Test compounds were then injected as boluses at increasing concentrations (10, 50, 250 $\mu\text{g}/\text{kg}$). Finally, atropine (10 $\mu\text{g}/\text{kg}$) was injected intravenously. At the end of the experiments, rats were killed by urethane overdose. Four animals were used for each dose of the test compounds. ED_{50} values were estimated by non-linear regression.

In the intraduodenal studies, male Sprague–Dawley rats weighing 315 ± 3 g were housed two per cage under a 12-h light-dark cycle at room temperature (20 ± 2 °C) and 55% minimum humidity for a minimum of 5 d before the experiments. Animals had free access to commercial rat chow and tap water. Rats were anesthetized with urethane (1.5 g/kg, i.p.) and body temperature was maintained at 37 °C with a heating pad. A 2 cm incision was made at the middle of the neck and a tracheotomy tube was inserted. A catheter, filled with sterile heparin solution (5 U/ml), was inserted in the carotid aorta and connected to a pressure transducer. Heart rate and blood pressure were recorded and analyzed with the software HEM (Notocord, Croissy sur Seine, France). After a 10 min stabilization period, three boluses of acetylcholine (10, 30 and 100 $\mu\text{g}/\text{kg}$) were administered intravenously at 5 min intervals. Then, vehicle (sterile saline, 1 ml/kg) or test compounds (1, 5 or 15 mg/kg) were administered through a catheter inserted in the duodenum. Forty-five minutes later, a second series of boluses of acetylcholine (10, 30 and 100 $\mu\text{g}/\text{kg}$) were administered intravenously, again at 5 min intervals. At the end of experiments, rats were killed by urethane overdose. Ten animals were used for each dose of test compounds. Five rats were employed for the vehicle administration. Statistical comparisons were carried out using one-way analysis of variance for completed randomized block.

Hemodynamic Effects in Dogs Two beagle dogs (11.5 and 12.4 kg) participated in the study. Animals were housed in individual stainless steel boxes of standard dimensions (1.2 m²). These animals were placed in an air-conditioned (17–21 °C) animal house kept at relative humidity between 45% and 65% with non-recycled filtered air changed approximately 10 times per hour. The artificial day/night cycle involved 12 h light and 12 h darkness with light on at 7:30 a.m. A telemetric transmitter was implanted under general anesthesia and aseptic conditions for measurement of blood pressure and heart rate. Implantation was performed under anesthesia induced by thiopental (20 mg/kg i.v.) and then halothane (1–1.5%, i.v.). The telemetric transmitter was implanted in the left flank. The electrodes of the transmitters were placed in lead 2. The sensor catheter of transmitters was introduced into the femoral artery. Telemetric transmitters were implanted at least 10 d before use. The measurements were done in the animal room by means of RLA2000 biotelemetry receivers. Using telemetry, the animals remained free to move about during the entire period of measurement. Systolic and diastolic blood pressure and heart rate were measured during 15-s periods at regular intervals of 5 min. Hemodynamic variables were recorded for 24 h after dosing. Any gross behavioral or autonomic changes observed during the experiment were recorded. Placebo and compound **8** (2 and 10 mg/kg) were administered according to a randomized four-way cross-over design with a washout period of at least one week. Treatments were administered orally in gelatin capsules at approximately 10:30 p.m.. Statistical comparisons were carried out using repeated analysis of variance measurements.

Gastric Emptying in Rats Gastric emptying was evaluated by measuring the rate of disappearance on an aqueous suspension of phenol red from the stomach.³⁷⁾ Compound **8** and AF-DX 116 were examined at doses of 15, 50 and 150 mg/kg by the oral route and administered to groups of 8 animals. Atropine (20 mg/kg) was used as positive control drug. Wistar rats (200–300 g) were used in this study. Animals were housed in cages of standard dimensions with sawdust. The animal house was kept at a temperature of 19–23 °C and a relative humidity of 45–65% with non-recycled filtered air changed approximately 10 times per hour. A 12-h day–night cycle was adopted with light on at 7:30 a.m. Rats received commercial food. Tap water was available *ad libitum* in polycarbonate feeder bottles with a stainless steel nipple. The day prior to the study, animals were kept on a water only fast in cages with grid floors in order to minimize coprophagia. On the study day, animals were dosed as defined by the randomization plan. Sixty minutes after test compound administration, animals were orally given a 1.5 ml suspension of 0.05% phenol red in 2.5% carboxymethylcellulose (kept at 37 ± 0.5 °C). Ten minutes after the administration of phenol red, animals were sacrificed by pentobarbital overdosing (i.p.). Following midline laparotomy and ligation of the pylorus, the stomach was cut longitudinally and placed in 30 ml of a 0.9% NaCl solution. Twenty-four hours afterwards, the stomach was removed and the volume adjusted to 36 ml with 0.9% NaCl, and 4 ml of 1 M NaOH was added. After homogenization, the mixture was centrifuged at 3000 rpm for 10 min. Phenol red concentrations were measured by spectrophotometry at 550 nm.

Intestinal Transit Time in Rats Intestinal transit time was evaluated by measuring the distance covered in the intestines by a suspension of orally administered vegetal charcoal.³⁸⁾ Compound **8** and AF-DX 116 were examined at doses of 15, 50 and 150 mg/kg administered by the oral route to groups of 8 animals. Atropine (20 mg/kg *p.o.*) was used as a positive control drug. Wistar rats (200–300 g) were used in this study. Animals were housed

in cages of standard dimensions with sawdust. The animal house was kept at a temperature of 19–23 °C and a relative humidity of 45–65% with non-recycled filtered air changed approximately 10 times per hour. A 12-h day-night cycle was adopted (7:30 a.m.–7:30 p.m.). Rats received commercial food. Tap water was available *ad libitum* in polycarbonate feeder bottles with a stainless steel nipple. The day prior to the study, animals were kept on a water only fast in cages with grid floors in order to minimize coprophagia. On the study day, animals were dosed as defined by the randomization plan. Sixty minutes after test compound administration, animals were orally given a 2 ml suspension of 10% charcoal in 2.5% carboxymethylcellulose (kept at 37 °C ± 0.5). Fifteen minutes after the administration of charcoal, animals were sacrificed by cervical dislocation. Following midline laparotomy, the intestines were rapidly removed from the pylorus to the extremity of the cecum and spread out on a glass plate. The total length of the intestine and the distance covered by the charcoal were then measured immediately afterwards.

Salivary Secretion in Rats Salivary secretion was evaluated by measuring the weight gained by sublingual foam cubes after the subcutaneous administration of oxotremorine.³⁷⁾ Compound **8** and AF-DX 116 were examined at doses of 15, 50, and 150 mg/kg by the oral route and administered to groups of 8 animals. Atropine (10 mg/kg *p.o.*) was used as a positive control drug. Male Sprague-Dawley rats (200–300 g) were randomly allocated to test treatments. Fifty minutes after test compound or vehicle administration, rats were injected subcutaneously with oxotremorine (0.5 mg/kg). Ten minutes after oxotremorine administration, the mouth of the animals was opened with gentle pressure on a snare, allowing placement in the oral cavity of a pre-weighed, absorbent foam cube. Foam cubes were held for 10 s and immediately after re-weighed. The difference between the first and second weight represents the saliva secreted.

Pupil Diameter in Rats Pupil diameter was evaluated under constant bright light with a dissecting microscope.³⁹⁾ Compound **8** and AF-DX 116 were examined at doses of 15, 50 and 150 mg/kg by the oral route and administered to groups of 8 animals. Atropine (0.3 mg/kg *p.o.*) was used as a positive control drug. Sprague-Dawley rats (200–250 g) were randomly allocated to test treatments. Pupil diameter was measured just before and 60 min after test compound or vehicle administration.

In all *in vivo* functional selectivity studies, statistical comparisons were carried out using one-way analysis of variance for a completed randomized block.

Analgesic Activity in Mice The modified hot-plate procedure described by O'Callaghan and Holtzman was adopted.^{41–43)} Male Swiss albino mice (20–30 g) were used. Animals were kept at 22 ± 1 °C with a 12-h light-dark cycle, and allowed access to food and water *ad libitum*. Mice were placed on a stainless steel thermostat plate set at 50 ± 0.1 °C. A plastic cylinder was used to confine the mice to the heated surface of the hot-plate. The licking latency was defined as the time elapsing from thermal exposure to the licking of the fore or hind paws. A cut-off time of 75 s was adopted for licking latency in order to avoid unethical suffering and injury of the animals. Mean baseline licking latency was determined with three measurements before test compound administration. Test compounds were administered by a subcutaneous route to groups of 12–17 animals. Compounds **8**, **9**, **38** and AF-DX 116 were evaluated at doses of 5 mg/kg. Oxotremorine (0.2 mg/kg *s.c.*) was used as a positive control drug. After test compound administration, licking latencies were measured at 15 min intervals for 150 min. Statistical comparisons were carried out using repeated analysis of variance measurements.

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References

- Goyal R. K., *N. Engl. J. Med.*, **321**, 1022–1029 (1989).
- Wess J., *Crit. Rev. Neurobiol.*, **10**, 69–99 (1996).
- Caulfield M. P., *Pharmacol. Ther.*, **58**, 319–379 (1993).
- Eglen R. M., Watson N., *Pharmacol. Toxicol.*, **78**, 59–68 (1996).
- van Zwieten P. A., Doods H. N., *Cardiovasc. Drugs Ther.*, **9**, 159–167 (1995).
- Rouse S. T., Thomas T. M., Levey A. I., *Life Sci.*, **60**, 1031–1038 (1997).
- Stillman M. J., Shukitt-Hale B., Galli R. L., Levy A., Lieberman H. R., *Brain Res. Bull.*, **41**, 221–226 (1996).
- Piwowska W., Mroczek-Czernecka D., Baciorek B., *Jpn. Heart J.*, **29**, 639–648 (1988).
- Sethi K. K., Balachandrar J., Jaishankar S., Gupta M. P., *Int. J. Cardiol.*, **12**, 233–242 (1986).
- van Zwieten P. A., Doods H. N., *Cardiovasc. Drugs Ther.*, **9**, 159–167 (1995).
- Hluchy J., Milovsky V., Pavlovic M., Uhliarikova H., Makovini M., *Int. J. Cardiol.*, **33**, 357–364 (1991).
- Engel W. W., Eberlein W. G., Mihm G., Hammer R., Trummelitz G., *J. Med. Chem.*, **32**, 1718–1724 (1989).
- Eberlein W. G., Engel W., Mihm G., Rudolf K., Wetzel B., Entzeroth M., Mayer N., Doods H. N., *Trends Pharmacol. Sci.*, **1989**, Suppl., 50–54.
- Watanabe T., Kinoyama I., Kakefuda A., Okazaki T., Takizawa K., Hirano S., Shibata H., Yanagisawa I., *Chem. Pharm. Bull.*, **45**, 996–1007 (1997).
- Watanabe T., Kakefuda A., Kinoyama I., Takizawa K., Hirano S., Shibata H., Yanagisawa I., *Chem. Pharm. Bull.*, **45**, 1458–1469 (1997).
- Watanabe T., Kakefuda A., Tanaka A., Takizawa K., Hirano S., Shibata H., Yamagiwa Y., Yanagisawa I., *Chem. Pharm. Bull.*, **46**, 53–68 (1998).
- Watanabe T., Kinoyama I., Takizawa K., Hirano S., Shibamura T., *Chem. Pharm. Bull.*, **47**, 672–677 (1999).
- Cohen V. I., Baumgold J., Jin B., De La Cruz R., Rzeszutarski W. J., Reba R. C., *J. Med. Chem.*, **36**, 162–165 (1993).
- Cohen V. I., Jin B., Gitler M. S., De La Cruz R., Boulay S. F., Sood V. K., Zeeberg B. R., Reba R. C., *Eur. J. Med. Chem.*, **30**, 61–69 (1995).
- Bolognesi M. L., Minarini A., Budriesi R., Cacciaguerra S., Chiarini A., Spampinato S., Tumiatti V., Melchiorre C., *J. Med. Chem.*, **41**, 4150–4160 (1998).
- Minarini A., Bolognesi M. L., Budriesi R., Canossa M., Chiarini A., Spampinato S., Melchiorre C., *J. Med. Chem.*, **37**, 3363–3372 (1994).
- Melchiorre C., Bolognesi M. L., Chiarini A., Minarini A., Spampinato S., *J. Med. Chem.*, **36**, 3734–3737 (1993).
- Melchiorre C., Quaglia W., Picchio M. T., Giardina D., Brasili L., Angeli P., *J. Med. Chem.*, **32**, 79–84 (1989).
- Nassif-Makki T., Trankle C., Zlotos D., Bejeuhr G., Cambareri A., Pfletschinger C., Kostenis E., Mohr K., Holzgrabe U., *J. Med. Chem.*, **42**, 849–858 (1999).
- Lachowicz J. E., Lowe D., Duffy R. A., Ruperto V., Taylor L. A., Guzik H., Brown J., Berger J. G., Tice M., McQuade R., Kozlowski J., Clader J., Strader C. D., Murgolo N., *Life Sci.*, **64**, 535–539 (1999).
- Chelala J. L., Kilani A., Miller M. J., Martin R. J., Ernsterberger P., *Pharmacol. Toxicol.*, **83**, 200–207 (1998).
- Fincham C. I., Higginbottom M., Hill D. R., Horwell D. C., O'Toole J. C., Ratcliffe G. S., Rees D. C., Roberts E., *J. Med. Chem.*, **35**, 1472–1484 (1992).
- Chandrakumar N. S., Yonan P. K., Stapelfeld A., Savage M., Rorbacher E., Contreras P. C., Hammond D., *J. Med. Chem.*, **35**, 223–233 (1992).
- Anwer M. K., Sherman D. B., Spatola A. F., *Int. J. Pept. Protein Res.*, **36**, 392–399 (1990).
- Haasz F., Galamb V., *Syn. Commun.*, **24**, 683–687 (1994).
- Geigy J. R., Brit. Patent 943277 (1963) [*Chem. Abstr.*, **61**, P1815e (1964)].
- Das B. P., Boykin D. W., *J. Med. Chem.*, **14**, 56–58 (1971).
- Phillips A. P., *J. Am. Chem. Soc.*, **79**, 2836–2838 (1957).
- Gitler M. S., Reba R. C., Cohen V. I., Rzeszutarski W. J., Baumgold J., *Brain Res.*, **582**, 253–260 (1992).
- Noronha-Blob L., Kachur J. F., *J. Pharmacol. Exp. Ther.*, **256**, 562–567 (1991).
- Hartvig P., Gillberg P. G., Gordh T., Jr., Post C., *Trends Pharmacol. Sci.*, **1989**, Suppl. 75–79.
- Erni W., Ritschel W. A., *Arzneimittelforschung*, **27**, 1043–1045 (1977).
- Fichelle J., *J. Pharmacol.*, **2**, 85–86 (1971).
- Richter W., *Acta Pharmacol. Toxicol.*, **24**, 243–254 (1966).
- Zhuravin I. A., Bures J., *Exp. Brain Res.*, **83**, 687–690 (1991).
- O'Callaghan J. P., Holtzman S. G., *J. Pharmacol. Exper. Ther.*, **192**, 497–505 (1975).
- Geigy J. R., Brit. Patent 848032 (1960) [*Chem. Abstr.*, **55**, P8436g (1961)].
- Eberlein W. G., Trummelitz G., Engel W. W., Schmidt G., Pelzer H., Mayer N., *J. Med. Chem.*, **30**, 1378–1382 (1987).

Release of Nortriptyline Hydrochloride from Oil–Water Microemulsions

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The release of nortriptyline hydrochloride from oil-in-water (o/w) microemulsions (isopropyl myristate as oil, propylene glycol as cosurfactant, polysorbate 80 as surfactant and phosphate buffer, pH 7.4, as the continuous phase) containing increasing concentrations of polyethylene glycol 400, used to facilitate the diffusion of a drug from the inner oily phase of the microemulsion to the outer aqueous phase of such a dispersion system, was studied by determining the permeability constants of the drug through hydrophilic and lipophilic membranes separating the o/w microemulsions from the receiving aqueous phase (phosphate buffer pH 7.4). The permeability of nortriptyline hydrochloride from microemulsions through the lipophilic membrane increased as the concentration of polyethylene glycol 400 in the disperse system increased. The apparent permeability constant for nortriptyline hydrochloride, from the microemulsion without polyethylene glycol, was $1.36 \times 10^{-3} \text{ cm} \cdot \text{h}^{-1}$, it increased up to $7.80 \times 10^{-3} \text{ cm} \cdot \text{h}^{-1}$ in the presence of polyethylene glycol at a concentration of 50% (v/v) of the initial volume of the aqueous phase.

Key words oil–water microemulsion; nortriptyline hydrochloride; partition coefficient; lipophilic/hydrophilic membrane; permeability constant

The design and development of new drug delivery systems with a view to enhancing the efficacy of existing drugs is an ongoing process in pharmaceutical research. Recently, a great deal of dosage form development activity has focused on the employment of microemulsions, clear isotropic systems of infinite stability¹⁾ as transdermal delivery systems.^{2–4)}

The present work relates to oil–water (o/w) microemulsions containing nortriptyline hydrochloride. It is well known that when nortriptyline hydrochloride, a tricyclic antidepressant, is administered orally, its bioavailability is very low^{5,6)} and also varies with individuals⁷⁾ due to extensive first-pass metabolism in the liver. For this reason, a novel controlled release dosage form of nortriptyline hydrochloride, intended for transdermal administration, has been developed in order to diminish the daily dose taken by the patients and to facilitate dosage compliance by the patient.

The aim of this study was to develop nortriptyline hydrochloride microemulsions that might be suitable for the transdermal administration of this drug. The drug lipophilicity allowed its formulation into the dispersed phase of the microemulsions, which could act as a long-acting reservoir of the drug. The ability of polyethylene glycol 400 to act as a solubility modifier of nortriptyline hydrochloride, and hence as a diffusion modifier of such drug, was also evaluated by determining the *in vitro* permeability constants of the drug from such disperse systems, through two different membranes, hydrophilic and lipophilic, in order to find any differences that might occur on the release profiles of the drug related to the membrane nature. Such permeability rates were compared with those obtained in the permeability studies carried out through the same types of membranes, using an aqueous phase as a vehicle for the drug, as described by many other authors.^{8,9)}

Materials and Methods

Nortriptyline hydrochloride (HCl) was provided by Synthelabo S.A. (Madrid, Spain). Isopropyl myristate (IPM) was obtained from Merck (Darmstadt, Germany). Polysorbate 80 was supplied by Sigma Chemicals (Madrid, Spain). Propylene glycol (PG) and polyethylene glycol 400 (PEG

400) were used, as obtained from Panreac (Madrid, Spain). A cellulosic Membrane MF VCWP 14250 with a pore size of $0.1 \mu\text{m}$ and a thickness of 0.1 mm was from Millipore (Madrid, Spain). Hydrophilic Membrane D-9652 (cellulose) was from Sigma (Madrid, Spain).

Instruments: UV-Visible spectrophotometer Beckman DU®-7 (Fullerton, CA, U.S.A.). Photon correlation spectrophotometer Malvern S Zetamaster.

Microemulsion Preparation a) In the Presence of Nortriptyline HCl: An oil–water microemulsion was prepared using polysorbate 80 as a surfactant, IPM as oil and propylene glycol as a cosurfactant. Phosphate buffer (11.7 ml, 0.2 M, pH 7.4) was added to a 50 ml test tube containing 2.2 ml of IPM, 2.7 ml of propylene glycol and a weighed amount of polysorbate 80 (8.6 g). Nortriptyline HCl was added to IPM, yielding a final concentration of 7 mg per ml of microemulsion.

b) In the Presence of Increasing Amounts of PEG 400: The dispersed system had the same composition as reported above. Different percentages of PEG 400 spanning 10 to 50% (v/v) of the initial volume of the aqueous phase (11.7 ml) were added to the microemulsion. The volume of the aqueous phase was maintained by substituting phosphate buffer with PEG 400.

The dispersed systems obtained were analyzed through cross polaroids and studied by photon correlation spectroscopy (PCS), after their dilution (1:2, v/v) with the external phase, in order to determine whether they were microemulsion-type systems. Statistical analysis of the data obtained from the PCS studies was performed by the CONTIN program. The mean droplet size measured for all the disperse systems studied was of approximately $112 \pm 52 \text{ nm}$, lower than most of the values reported by Constantinides¹⁰⁾ for microemulsion droplet sizes, and, since they were homogeneous, isotropic as well as transparent, they were deemed to be true microemulsions. The addition of increasing amounts of PEG 400 to the microemulsion not only maintained its inner structure, but also led to reduction of the droplet size of the disperse systems, probably due to the co-surfactant role that such molecules may play.¹⁾

Qualitative tests, including dye solubility tests and dilution tests, were used to determine the type of microemulsion. The first method involved the addition of a water- (methyl orange) or oil- (Sudan III) soluble dye to the microemulsion. Thus, intense staining of the external phase after the addition of water-soluble dye indicated an o–w microemulsion. The addition of oil-soluble dye to the same microemulsion resulted in staining of the droplets of the internal phase.

The dilution tests involved observation of the microemulsion following its dilution with IPM or aqueous phase to see whether separation occurred. Since water was easily dispersed in the continuous phase, the microemulsion was termed oil-in-water.

Partition Coefficients Octanol/Buffer Partition Coefficient (P_{oct}): A known volume (25 ml) of 0.2 M phosphate buffer (pH 7.4) saturated with octanol containing an excess of drug was added to a known volume (25 ml) of octanol saturated with buffer. The mixture was shaken at room temperature

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until the drug was equilibrated between the two phases. The drug concentration was determined in the aqueous and organic phases after separation by centrifugation (9000×*g* for 10 min), and the octanol/buffer partition coefficient was calculated.

IPM/Buffer Partition Coefficient (P_{IPM}): The IPM/buffer partition coefficient of the drug was determined as described above for the octanol/buffer partition coefficient.

IPM/Buffer Partition Coefficient of the Drug in the Presence of the Co-emulsifier (P_{cos}): Phosphate buffer (11.7 ml, 0.2 M, pH 7.4) was added to a 50 ml centrifuge tube containing 2.2 ml of IPM and 2.7 ml of propylene glycol. An excess of the drug was added, then the tube was shaken until equilibrium was reached. The drug concentration in the aqueous and oleous phases was determined after centrifugation (9000×*g* for 10 min).

IPM/Buffer Partition Coefficients of the Drug in the Presence of the Co-emulsifier and Different Amounts of PEG 400: Different percentages of PEG 400 (10–50% v/v of the aqueous phase) were added to 2.2 ml of IPM, phosphate buffer and 2.7 ml of propylene glycol. The volume of the aqueous phase was maintained (11.7 ml) by substituting buffer with PEG 400. An excess of the drug was added to the mixture and shaken until equilibrium was reached between the two phases. After centrifugation, the concentration of the drug in both phases was determined.

The quantitation of nortriptyline HCl in the aqueous phase and oleous phase of the systems studied was performed spectrophotometrically by reading the absorbances of the drug dissolved in phosphate buffer 0.2 M, pH=7.4 and methanol, respectively, at a wavelength of 241 nm. The linearity of the analytical methods was assessed and r^2 values >0.99 were obtained for both methods. The precision was assessed as well by evaluating the repeatability and reproducibility obtaining relative standard deviations (RSD) of 0.197% and 0.374%, respectively, for the aqueous phase method, and of 0.253% and 0.421%, respectively, for the oleous phase method.

Permeability Experiments Permeability experiments were performed on solutions and microemulsions.

Solutions: a) Nortriptyline HCl in a phosphate buffer (0.2 M, pH 7.4) solution at a fixed concentration of 1 mg·ml⁻¹ due to the low aqueous solubility of the drug. b) Nortriptyline HCl dissolved in a phosphate buffer (0.2 M, pH 7.4)/PEG 400 (50/50 v/v) solution at a fixed concentration of 1 mg·ml⁻¹ in order to compare the results obtained with the ones found with the solution without PEG 400.

Microemulsions: c) Nortriptyline HCl alone at a concentration of 7 mg·ml⁻¹. d) Nortriptyline HCl at a fixed concentration (7 mg·ml⁻¹) and with increasing amounts of PEG 400 (10–50% v/v of the aqueous phase).

Diffusion apparatus: The release of nortriptyline HCl from both solutions and microemulsions was carried out in an apparatus consisting of a donor and a receptor compartment with a total available diffusion area of 2.06 cm². The receptor compartment was filled with 12.5 ml of a phosphate buffer (0.2 M, pH 7.4) with 8% (v/v) of a propylene glycol/PEG 400 mixture (1 : 1, v/v). One ml of the drug solutions or microemulsions were applied to the donor compartment and the donor chamber was closed from the atmosphere.

The receptor compartment was continuously stirred at 300 rpm and the whole system was placed in a thermoregulated waterjacket at 37 °C.

Two different types of membranes were used in these experiments, and they were chosen according to the work developed by Gasco *et al.*⁸⁾

– A hydrophilic cellulose membrane, Sigma D-9652, was soaked in phosphate buffer (0.2 M, pH 7.4) until swollen.

– A membrane, type MF VCWP 14250 (Millipore) impregnated with 1-decanol and 1-dodecanol (1 : 9, v:v) was used as a lipid barrier.

At appropriate times, an aliquot of 0.75 ml of the receptor fluid was sampled out and replaced by the same volume of fresh phosphate buffer (0.2 M, pH 7.4)/propylene glycol (8% v/v) mixture. The release of nortriptyline HCl from microemulsions and solutions through the lipophilic and hydrophilic membranes was followed for 24 h, and the concentration of the drug in the sample was determined spectrophotometrically using the buffer as a blank.

Calculation of Apparent Permeability Constants The permeability constants were determined by the following relationship (Eq. 1) which was applied over the linear portions of the release profiles obtained for each system assessed:

$$K_p = \frac{(C_2 - C_1)V}{(t_2 - t_1)C_0F} \quad (1)$$

where C_0 is the initial concentration of the drug in the donor compartment, C_2 and C_1 are the concentrations of the drug in the receptor compartment at

time t_2 and t_1 , respectively, F is the available area of membrane and V is the total receptor volume.

Since the drug had the same octanol/buffer partition coefficient value as prednisone, as seen in previous work,⁹⁾ the same mathematical model applied to the latter by Trotta *et al.*,⁹⁾ was applied here for nortriptyline HCl.

Results

(a) Partition Coefficients Table 1 shows the octanol/buffer (P_{oct}), isopropyl myristate/buffer (P_{IPM}), isopropyl myristate-propylene glycol-buffer (P_{cos}) partition coefficients of nortriptyline HCl. The partition coefficients were determined using the same amounts of IPM, propylene glycol and phosphate buffer (0.2 M, pH 7.4) as employed in the preparation of microemulsions.

Table 1 shows as well the isopropyl-myristate/propylene glycol/buffer partition coefficients of nortriptyline HCl in the presence of different concentrations of PEG 400 (10–50% v/v of the aqueous phase). The compositions were the same as those used for the preparation of microemulsions.

(b) Nortriptyline HCl in Aqueous Solutions: Permeability Constants The K_p of the drug dissolved in phosphate buffer (0.2 M, pH 7.4) and in phosphate buffer/PEG 400 (50/50, v/v) mixtures through either the hydrophilic or the lipophilic membrane are reported in Table 2. The data were obtained from the application of Equation 1.

(c) Permeability Constants of Nortriptyline HCl Diffused from Microemulsion Alone, and in the Presence Increasing Amounts of Polyethylene Glycol 400 (10–50% v/v of the Aqueous Phase) The K_p of the drug diffusing from microemulsions through the hydrophilic and lipophilic membranes, respectively, are shown in Table 3. The data were obtained as previously described (see Eq. 1), where C_0

Table 1. Partition Coefficients of Nortriptyline in Octanol/Phosphate Buffer pH=7.4 (P_{oct}); IPM/Phosphate Buffer pH=7.4 (P_{IPM}); IPM/Phosphate Buffer pH=7.4/Propylene Glycol (P_{cos}) and IPM/Phosphate buffer pH=7.4/Propylene Glycol/PEG 400 10–50% (v/v) ($P_{cos10-50}$) Systems

Qualitative system composition	Partition coefficients (P) ($n=3$)
Octanol/phosphate buffer (0.2 M, pH=7.4)	$P_{oct}=29.71 \pm 4.82$
IPM/phosphate buffer (0.2 M, pH=7.4)	$P_{IPM}=10.23 \pm 1.65$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol	$P_{cos}=5.77 \pm 0.64$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol/PEG 400 (10% v/v)	$P_{cos10}=0.67 \pm 0.08$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol/PEG 400 (20% v/v)	$P_{cos20}=0.24 \pm 0.03$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol/PEG 400 (30% v/v)	$P_{cos30}=0.18 \pm 0.03$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol/PEG 400 (40% v/v)	$P_{cos40}=0.011 \pm 0.01$
IPM/phosphate buffer (0.2 M, pH=7.4)/propylene glycol/PEG 400 (50% v/v)	$P_{cos50}=0.012 \pm 0.02$

Table 2. Permeability Constants of Nortriptyline through Lipophilic and Hydrophilic Membranes from Phosphate Buffer Solutions (0.2 M, pH=7.4) in the Absence and Presence of PEG 400 (50% v/v)

Phosphate buffer solution : PEG 400	$K_p \cdot 10^{-3} \text{ (cm} \cdot \text{h}^{-1}) \text{ (} n=3 \text{)}$	
	Lipophilic membrane	Hydrophilic membrane
100 : 0	5.76 ± 0.19	4.77 ± 0.27
50 : 50	2.75 ± 0.07	0.14 ± 0.01

Table 3. Permeability Constants of Nortriptyline through Lipophilic and Hydrophilic Membranes from Microemulsions in the Absence and Presence of PEG 400

Microemulsion : PEG 400	$K_p \cdot 10^{-3} \text{ (cm} \cdot \text{h}^{-1}) \text{ (n=3)}$	
	Lipophilic membrane	Hydrophilic membrane
100 : 0	1.36 ± 0.018	1.42 ± 0.006
90 : 10	1.79 ± 0.015	1.49 ± 0.010
80 : 20	2.59 ± 0.029	1.53 ± 0.009
70 : 30	5.32 ± 0.042	1.62 ± 0.013
60 : 40	7.66 ± 0.034	1.73 ± 0.017
50 : 50	7.80 ± 0.038	1.79 ± 0.021

Table 4. Permeability Constants (K_p) and Enhancement Ratios (ER) of Nortriptyline through the Lipophilic Membrane from Microemulsions in the Absence (Control) and Presence of PEG 400 at Different Concentrations (v/v)

Microemulsions	$K_p \cdot 10^{-3} \text{ (cm} \cdot \text{h}^{-1})$	ER
Control (without PEG 400)	1.36	1
10% PEG 400	1.79	1.32
20% PEG 400	2.59	1.90
30% PEG 400	5.32	3.91
40% PEG 400	7.66	5.63
50% PEG 400	7.80	5.73

was the total concentration of nortriptyline HCl in the total volume of microemulsion.

In order to normalize the permeability data for each microemulsion composition in respect to its control (microemulsion without PEG 400), enhancement ratios (ER) were calculated¹¹⁾ (Eq. 2):

$$ER = K_{p \text{ PEG 400}} / K_{p \text{ control}} \quad (2)$$

where $K_{p \text{ PEG 400}}$ is the permeability coefficient obtained for microemulsions with PEG 400 (10–50 % v/v of the aqueous phase), and $K_{p \text{ control}}$ is the permeability coefficient obtained for microemulsions without PEG 400.

Discussion

One consideration that should be made is that since microemulsion components present in the external phase (not considering the drug), and in particular propylene glycol and PEG 400, may diffuse through the membrane, a buffer containing an 8% (v/v) propylene glycol/PEG 400 mixture (1 : 1, v/v) was used as the acceptor medium in the permeation experiments in order to limit this diffusion.

The permeability constants of the drug dissolved in buffer alone and with PEG 400, (50 : 50, v/v), that is, the buffer with the maximum amount of PEG 400 added to the microemulsions, through a hydrophilic or lipophilic membrane, were measured to obtain reference data for the permeation studies with microemulsions. The differences found on the release rates obtained for both solutions could be attributed to the high solubility of the drug due to the presence of PEG 400 in the medium, which might diminish its diffusion towards the receptor compartment.

As can be seen in Fig. 1, the permeation coefficients for nortriptyline hydrochloride through the lipophilic and hydrophilic membranes from the systems studied were quite

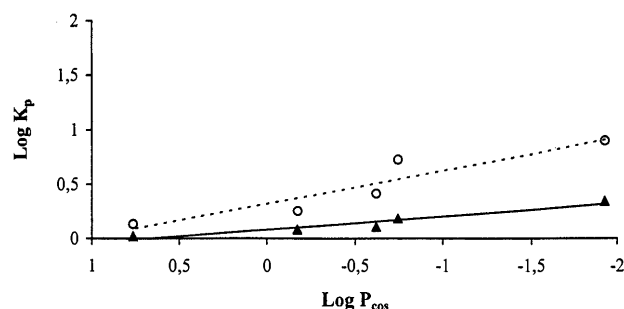


Fig. 1. Logarithm of Nortriptyline Permeation Rate Constants (K_p) through Lipophilic (---○---), and Hydrophilic (—▲—) Membranes Using Microemulsions with Different Amounts of PEG 400 versus the Logarithm of Nortriptyline Partition Coefficients (P_{cos})

linearly related to their IPM/buffer/propylene glycol and IPM/buffer/propylene glycol/PEG 400 (at the proportions described) partition coefficients. The results of the permeation studies presented in Tables 2 and 3 indicated that the *in vitro* release rates of the drug from microemulsions were lower than those from aqueous solutions only for systems without PEG 400.

As it is already known, drug release from microemulsions is ruled by two main processes: transfer of the drug from the dispersed to the continuous phase, and diffusion of the drug through the membrane, from the continuous phase to the sink solution. Only the drug dissolved in the continuous aqueous phase is initially able to permeate through the membranes. For this reason, drug release from microemulsions will be initially governed by the drug concentration in the external aqueous phase of such systems.

The permeability constants (K_p) of nortriptyline HCl from microemulsions through both hydrophilic and lipophilic membranes were measured considering the total concentration of the drug ($7 \text{ mg} \cdot \text{mL}^{-1}$) in the microemulsion as the initial concentration. The differences found in the release rates determined from solutions and from microemulsions could be attributed to the partition coefficients of the drug between the dispersed phase and the external aqueous phase of the microemulsions.

The real partition of the drug between the aqueous and the dispersed oil phase cannot be determined in microemulsions owing to the high stability of such dispersed systems.¹⁾ Therefore, the partition coefficients of the drug between oil, buffer and co-surfactant (P_{cos}) were determined and could be used as an approximation for the partition of the drug within the microemulsion.

The release of nortriptyline hydrochloride from microemulsions using fixed amounts of IPM, buffer, polysorbate 80 and propylene glycol with amounts of PEG 400, through both hydrophilic and lipophilic membranes, were studied in order to determine the role of PEG 400 in the release profiles of the drug. As can be seen in Fig. 2a, an increase in the percentage of PEG 400 added to the microemulsions led to an increase in the permeability of the drug through the lipophilic membrane. The maximum permeability value was observed with the 50% (v/v) proportion of PEG 400, with an enhancement factor of 5.73 (as can be seen in Table 4). This effect was not observed with the hydrophilic membrane (Fig. 2b), where there were not significant differences in the K_p calculated, probably due to the hydrophilic

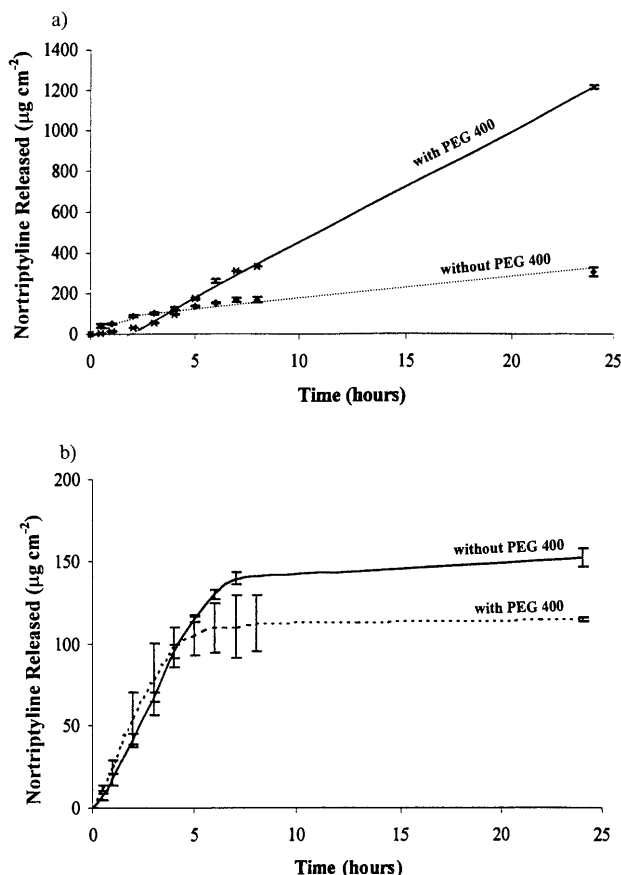


Fig. 2. a) Nortriptyline Released from Microemulsion (-----) and Microemulsion with 50% PEG (—) through Lipophilic Membrane. b) Nortriptyline Released from Microemulsion (—) and Microemulsion with 50% PEG (-----) through Hydrophilic Membrane

nature of the membrane, which might prevent the diffusion of the lipophilic drug from the external aqueous phase of the microemulsion towards the receptor compartment.

The partition coefficients of the drug between IPM, buffer, PG and PEG 400 at different percentages suggested that the effect observed on the release of the drug through the membranes could be due to PEG 400 helping in the partition of nortriptyline HCl from the internal oleous to the external aqueous phase of the microemulsions. Once the drug has reached this external phase, its diffusion across the different membranes studied will depend on the physico-chemical characteristics of the drug and the nature of the membrane. If the drug shows lipophilic behavior according to its P_{oct} value, such as for nortriptyline HCl, its permeability will be higher through the lipophilic membrane than through the hydrophilic one, which will act as a barrier that will delay the release of the drug, as can be seen in Table 3.

Another aim of this work was to investigate the reservoir effect of the microemulsion. In order to show this, the release of nortriptyline HCl from the different microemulsions studied was followed for 24 h and compared with the release of the drug from phosphate buffer solutions over the same period of time (Fig. 3). As can be seen in Fig. 2a, a pseudo-zero-order release through the lipophilic membrane was obtained from microemulsions with PEG 400. This behavior was more evident with the highest PEG 400 content, while, on the other hand, the release of nortriptyline HCl through the hydrophilic membrane from both solutions and microemul-

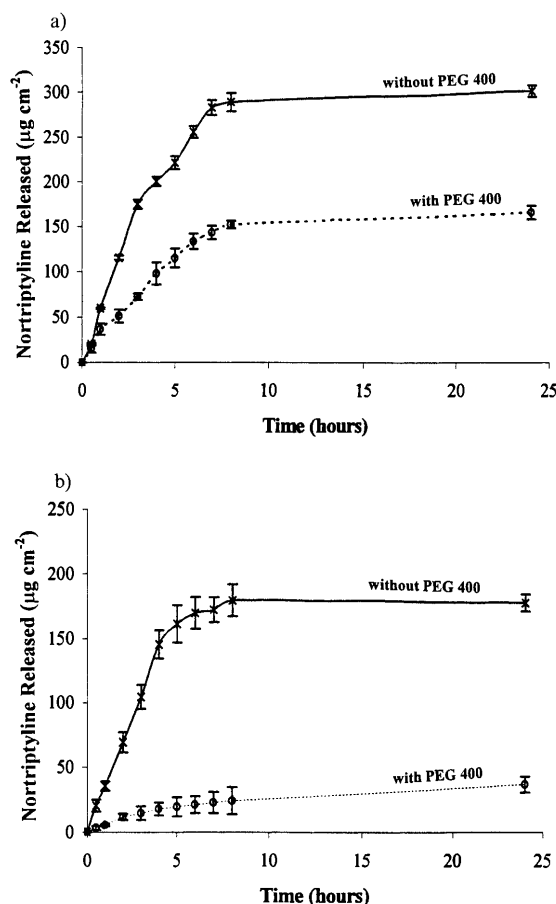


Fig. 3. a) Nortriptyline Released from Buffer Solution (—) and Buffer Solution with 50% PEG (v/v) (-----) through Lipophilic Membrane. b) Nortriptyline Released from Buffer Solution (—) and Buffer Solution with 50% PEG (v/v) (-----) through Hydrophilic Membrane

sions was dependent of the amount of PEG 400 added, and followed a first-order release profile, as seen in Figs. 3 and 2b, respectively. This could be due to the same explanation already mentioned and to the reservoir effect of the drug located in the inner oleous phase of the microemulsion, which permits a positive gradient of concentration towards the external phase of the dispersed system, facilitated by the presence of PEG 400, and hence to the receptor compartment.

It is interesting to observe the differences found in the release profiles of a drug from solutions depending upon the membranes used. Although in both cases a reduction of the K_p of the drug, as well as the maximum amount released, were clearly noted by the addition of PEG 400 to the solution (due to the solubility enhancement of the drug in the PEG 400/buffer solution that limited its diffusion across the membrane), enormous differences observed with the hydrophilic membrane could be attributed to the nature of the barrier itself, as mentioned above.

In a conclusion, the *in vitro* study suggests that microemulsions with PEG 400 proportions up to 50% (v/v), as a solubility modifier of nortriptyline hydrochloride, and hence as a diffusion modifier of such a drug, might be considered as a first approach for the transdermal formulation of nortriptyline hydrochloride, since it permits administration of the drug at a higher initial dosage (7 mg per ml of microemulsion) than the solution (1 mg · ml⁻¹). The release rate of the drug from such dispersed systems could be modulated by

varying the proportions of PEG 400 present in the formulation.

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References

- 1) Lawrence M. J., *Eur. J. Drug Metab. Pharmacokinet.*, **3**, 257—269 (1994).
- 2) Kriwet K., Muller-Goymann C. C., *Int. J. Pharm.*, **242**, 231—242 (1995).
- 3) Thevenin M. A., Grossiord J. L., Poelman M. C., *Int. J. Pharm.*, **137**, 177—186 (1996).
- 4) Ktistis G., Niopas I., *J. Pharm. Pharmacol.*, **50**, 413—418 (1998).
- 5) Grahramani P., Ellis S. W., Lennard M. S., Ramsay L. E., Tucker G. T., *Br. J. Clin. Pharmacol.*, **43**, 137—144 (1997).
- 6) Yue Q. Y., Zhong Z. H., Tybring G., Dalén P., Dahl M. L., Bertilsson L., Sjöqvist L., *Clin. Pharmacol. Ther.*, **64**, 384—390 (1998).
- 7) Sjöqvist F., "Neurochemical Pharmacology," ed. by Costa E., Raven Press, New York, 1989, pp. 303—318.
- 8) Gasco M. R., Carlotti M. E., Trotta M., *Int. J. Cosmetic Sci.*, **10**, 263—269 (1988).
- 9) Trotta M., Gasco M. R., Morel S., *J. Control. Rel.*, **10**, 237—243 (1989).
- 10) Constantinides P. P., *J. Control. Rel.*, **12**, 1561—1572 (1995).
- 11) Senel S., Hoogstraate A. J., Spies F., Cullander C., Verhoef J. C., Bos-van Geest A., Junginger H. E., Boddé H. E., *J. Control. Rel.*, **32**, 45—56 (1994).

Anti-cancer Activities of Pyrazolo[1,5-*a*]indole Derivatives¹⁾

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The structure activity relationship (SAR) in the anti-cancer activities of pyrazolo[1,5-*a*]indole derivatives was investigated, and the following conclusions were obtained: 1) *N*(1)-quaternarization is essential for anti-cancer activity, 2) the size and polarity of the 2-substituent is crucial for *in vitro* activity which allows further investigation in an *in vivo* test, 3) the effect of the 4-substituent on the activity is minor compared with the other two factors.

Key words pyrazolo[1,5-*a*]indole; anti-cancer screening; anti-cancer activity; structure activity relationship; hollow fiber test

We have been exploring anti-cancer agents and have reported that some quaternary salts of pyrazolo[1,5-*a*]indole derivatives, as represented with structure 2, have strong anti-cancer activities against cancer cells both in *in vitro* and *in vivo* tests.²⁾ These compounds also showed strong inhibitory activities against both DNA topoisomerase I and II, which could be the main course of the anti-cancer activities of our compounds. Although this series of compounds did have high cytotoxicity against cancer cells, they were also toxic to normal cells in an *in vivo* test. In order to reduce the side effects and to improve the activity and selectivity, we planned to investigate the following structure activity relationships (SAR): 1) effect of *N*(1)-quaternarization, 2) effect of the 2-substituent, 3) effect of the 4-substituent. Clarification of these points may allow us to propose the potential anti-can-

cer ability of our lead compound.

Chemistry For the above purposes we needed compounds 1–13 (Charts 1, 2). Among these, compounds 1, 2, 3, 6, and 9 were reported previously.²⁾ Compounds 7, 8, 10 were used to see effect 1) (Chart 1) and 11, 12, 13 to see effect 2) (Chart 2) were synthesized according to the pathway shown in Chart 3. 1-Adamantyl derivative 8 was prepared by the pathway (1).³⁾ When *o*-allylphenylhydrazine (14) was reacted with 1-formyladamantane in 60% acetic acid, hydrazone 15 (R=1-adamantyl), but also the polar cycloaddition product 16 (R=1-adamantyl), were obtained. The stereochemistry of 16 was deduced to be *trans* based on the chemical shift difference between C-2 and C-3a (Δ 3.0 ppm) in the ¹³C-NMR spectrum, which corresponds well with that of the *trans*-2-phenyl derivative (Δ 3.9 ppm).³⁾ Dehydrogenation of 16 (R=1-adamantyl) with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) gave 17 (R=1-adamantyl). *N*-Methylation of this product with methyl trifluoromethane sulfonate (MeOTf) yielded the salt 20 (R=1-adamantyl). Protons at position 4 (4-H) of 20 were acidic, and were thus gradually replaced with deuterium in deuterated methanol CD₃OD during ¹H-NMR measurement. This 4-H exchange reaction involves the isomerization of the 4*H*-isomer to the 1*H*-isomer of pyrazolo[1,5-*a*]indole.⁴⁾ The coupling of 20 (R=1-adamantyl)

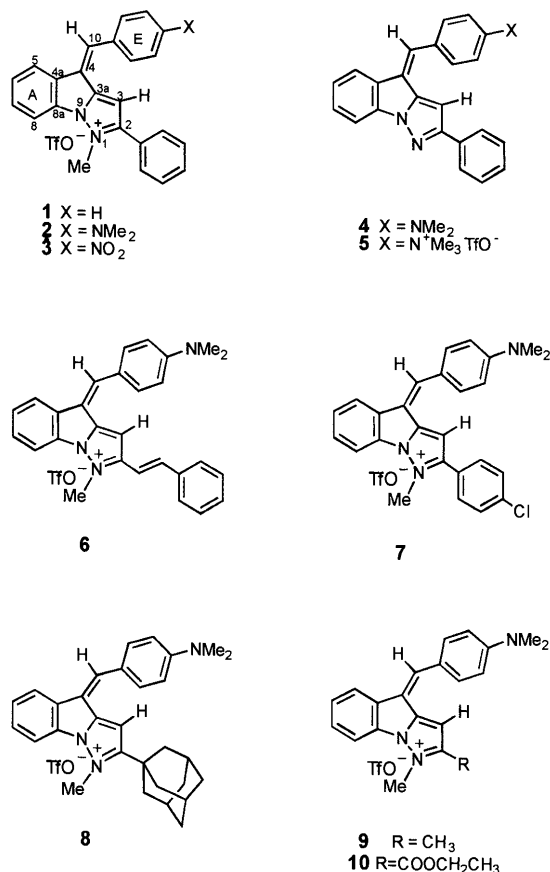


Chart 1

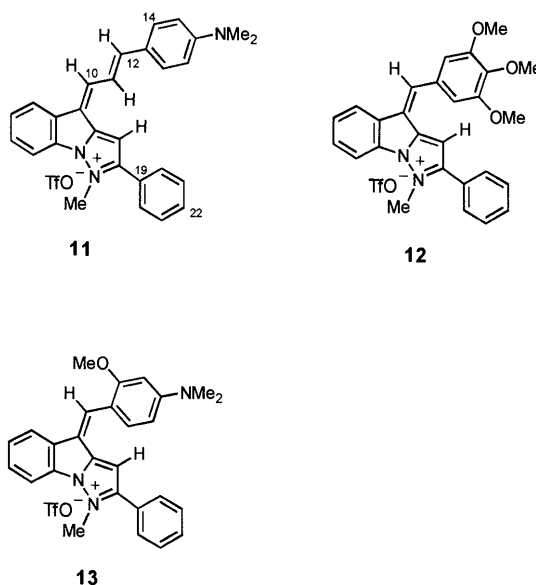


Chart 2

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with *p*-dimethylaminobenzaldehyde (**21**) ($X = \text{NMe}_2$ -*p*) (DMAB) was carried out in acetic acid by refluxing which involves the 1*H*-form as an intermediate to give the desired product **8** in good yield. The other products, **7**, **10**, **11**, **12**, and **13**, were prepared according to pathway (2) in Chart 3.²⁾ compounds **17** ($R = \text{C}_6\text{H}_4\text{-Cl-}p$)⁵⁾ and **17** ($R = \text{COOEt}$)⁶⁾ were methylated with MeOTf and coupled with aldehyde **21** to give **7** and **10**, respectively. 4-*H* of the salt **20** ($R = \text{COOEt}$) was also exchangeable with deuterium in CD_3OD . In the formation of **10**, the second product (15%), probably a decarboxylation product from the acid of **10**, was obtained. The major isomer of **10** was assigned to be a more stable *Z*-isomer, as in the case of **2**.¹⁾ Contamination of the *E*-isomer (7%) was detected by NMR spectra. Compounds **11**, **12** and **13** were prepared by coupling **20** ($R = \text{Ph}$) with the corresponding aldehydes, according to the route (2) in Chart 3. Contamination of the geometrical isomer was also detected in **11** and **13**. An attempt was made to prepare a 10-methyl derivative of **10**, but no coupling reaction was observed between **20** ($R = \text{Ph}$) and acetophenone.

Preparation of free bases **4** and **5** was carried out by the route (4) shown in Chart 4. Carbanion generated at position 4

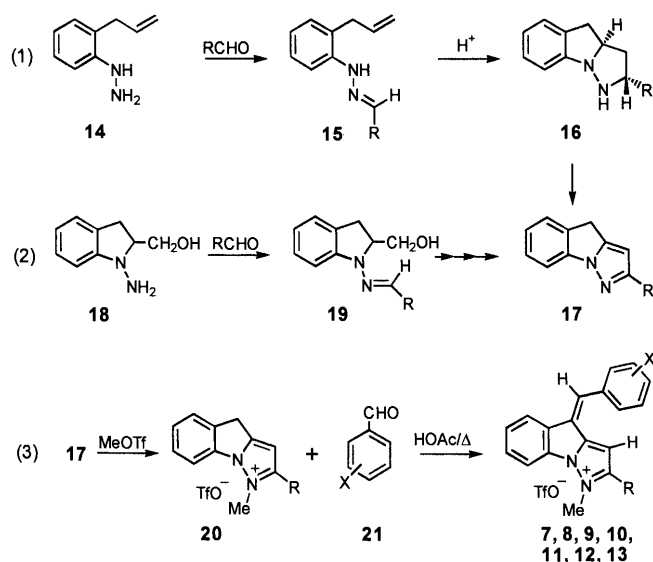


Chart 3

of 2-phenyl-4*H*-pyrazolo[1,5-*a*]indole (**17**) ($R = \text{Ph}$) was coupled with DMAB (**21**) ($X = \text{NMe}_2$ -*p*) at -78°C . The adduct **23** (61%) was a mixture of diastereomers (7:3), but one recrystallization (ether) yielded a pure isomer. Dehydration of **23** with methanesulfonyl chloride and triethylamine gave **4** in 78% yield as a mixture of geometrical isomers (7:3). Recrystallization of this product (methanol-isopropyl ether) gave isomerically pure **4** whose stereochemistry was assigned to be a more stable *Z*-configuration, as in the case of **2**. Methylation of crude **4** with MeOTf occurred at the dimethylamino group, and the salt **5** was obtained (64%), which was contaminated with a geometrical isomer (6%), even after preparative TLC purification. The trimethylammonio group of **5** was readily detected in the $^1\text{H-NMR}$ spectrum (δ 3.70 ppm, s, 9H). By this methylation, only trace amount of isomeric salt **2** was obtained.

Biological Activities *In vitro* anti-tumor screening was carried out at the National Cancer Institute in the U.S.A. (NCI, marked with NSC),⁷⁾ and in part at the Cancer Chemotherapy Center at the Japanese Foundation of Cancer Research (JCR, marked with JCI). The results are summarized in Table 1. MIG_MID is the Meangraph Midpoint. Assessments GI_{50} and LC_{50} are the concentration in moles per liter required for 50% growth inhibition and 50% lethality of cancer cells, respectively. TGI is the concentration in moles per liter required for total growth inhibition of cancer cells.

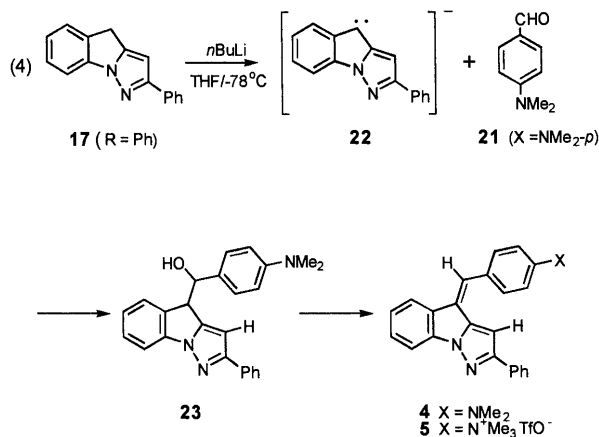


Chart 4

Table 1. MIG_MID of Log GI_{50} , Log TGI and Log LC_{50} ^{a)}

Entry	Code number	Structure	Log GI_{50}	Log TGI	Log LC_{50}
1	NSC 693575	1	-5.88	-5.18	-4.64
2	JCI 10341	2	-6.46	-5.82	-4.92
3	NSC 693571	2	-6.79	-5.70	-4.53
4	NSC 693574	3	-5.47	-4.51	-4.13
5	JCI 10340	4 ^{b)}	-4.12	-4.00	-4.00
6	JCI 10342	5 ^{b)}	-4.96	-4.46	-4.01
7	NSC 707773	6	-6.85	-6.08	-4.61
8	NSC 699479	7	-6.59	-5.83	-5.14
9	NSC 699475	8	-6.64	-5.88	-4.95
10	NSC 707772	9	-6.15	-5.30	-4.55
11	NSC 699478	10	-5.93	-4.87	-4.33
12	NSC 699477	11	-6.70	-5.73	-4.92
13	NSC 699474	12	-5.38	-4.80	-4.34
14	NSC 699476	13	-6.47	-5.69	-4.82

^{a)} Screen was carried out at National Cancer institute in U.S.A. (NSC) and Cancer Chemotherapy Center, Japanese Foundation of Cancer Research (JCI). MIG_MID is Meangraph Midpoint. GI_{50} and LC_{50} are the concentration in moles per liter required for 50% growth inhibition and 50% lethality. TGI is the concentration in moles per liter required for total growth inhibition. The values are an average of two or more screens. ^{b)} Single measurement.

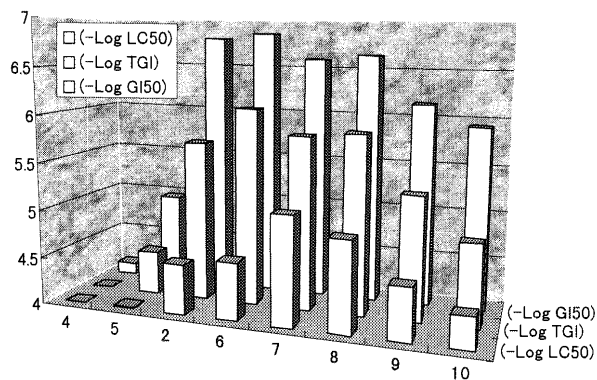


Fig. 1. Effect of *N*-Methylation and 2-Substituent
Plots of $-\text{Log GI}_{50}$, $-\text{Log TGI}$ and $-\text{Log LC}_{50}$ versus the selected compounds.

Although there is a small change in the cancer panels and cell lines between the two institutes, the differences in mean values observed with **2** were small, *i.e.*, 5.1% bigger for NSC in Log GI_{50} and 8.6% bigger for JCI in Log TC_{50} (entry 2, 3 in Table 1). Based on the values in Table 1, the effects of *N*-methylation and the 2-substituent are visualized in Fig. 1. Compounds **4** and **5** were selected to see the effect of *N*(1)-methylation, and **2** was placed as a reference for the comparison of activity.²⁾ To see these effects, the 4-substituent was fixed to be part of the *p*-dimethylaminobenzylidene group. The result of reference compound **2** (entry 2 in Table 1) was subjected to the COMPARE program at JCR, and good correlation with tetraphenylphosphonium chloride (r 0.781) and Rhodamine 123 (r 0.761) was obtained. This result may suggest that a part of the toxic activity of **2** may come from the activity on mitochondria. From Fig. 1, it is obvious that the quaternarization of *N*(1) is essential for anti-cancer activity (Log GI_{50}) since the free bases **4** and **5** lost most of their activity. Considering the Topliss scheme,⁸⁾ compound **7**, having a *p*-chlorophenyl group, was selected to demonstrate the effect of aromatic substitution at position 2. The 1-adamantyl group of **8** can provide information regarding the necessity of lipophilicity in the activity. The ester group in **10** increases the polarity of the compound and can also function to attach a side chain such as a DNA-binding unit at the C-2 position. Among these compounds, **7** and **8** showed comparable activity with **2** and **6** in the Log GI_{50} scale. In the Log LC_{50} scale, **7** and **8** were more active than **2**, which is a favorable indication for an anti-cancer agent. In contrast, the activity of **9** and **10** was small, thus indicating the necessity of size and lipophilicity of the 2-substituent in anti-cancer activity.

To see the effects of 4-substituents on anti-cancer activity, compounds **11**, **12**, and **13** were prepared in which the 2-substituent was fixed to be a phenyl group. Again, compound **2** was used as a reference. The effect of a vinylogous phenyl group, as in the case of **6**, was investigated with **11**. The effect of the substitution pattern of the aromatic ring of 4-substituents (E-ring) was checked with compounds **12** and **13**. Compound **13** was selected in analogy with a DNA topoisomerase inhibitor, Amsacrine (*m*-AMSA). The introduction of an *O*-substituent, *e.g.*, 4-acetoxyphenylbenzylidene derivative (replacement of NMe_2 in **2** with OAc), showed similar activity to **2** in the inhibition of DNA topoisomerase, but its *in vitro* activity was much weaker than **2**.⁹⁾ The effect of the *O*-substitution number at the E-ring was investigated with

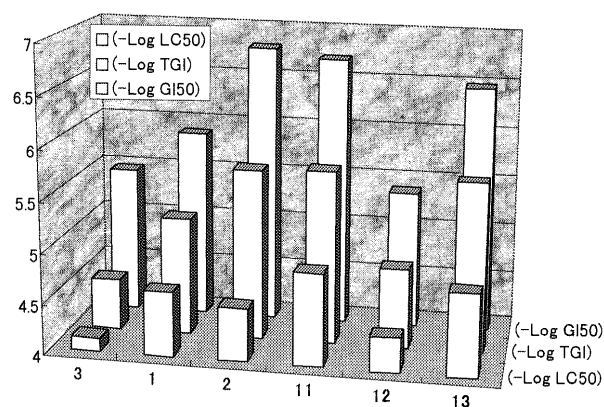


Fig. 2. Effect of 4-Substituent
Plots of $-\text{Log GI}_{50}$, $-\text{Log TGI}$ and $-\text{Log LC}_{50}$ versus the selected compounds.

compound **12**. Test results of these compounds plus those of **1**, **2** and **3**, which are also derivatives of the 4-substituent, are summarized in Fig. 2. The effectiveness of substitution of these three compounds in anti-cancer activity was **2** (4- NMe_2) > **1** (4-H) > **3** (4- NO_2), which was same as that reported for the cytotoxic activity of these compounds.²⁾ The activity of compound **11** was comparable with that of **2**, as in the case of **6**, and improvement of the activity in the Log LC_{50} scale was also observed. Thus, vinylogous lengthening of the phenyl substituent at either C-2 or C-4 does not affect *in vitro* activity.

Based on *in vitro* anti-cancer screening results, NCI selected compounds for a further test, an *in vivo* hollow fiber assay.¹⁰⁾ This system consists of twelve selected human tumor cell lines encased in hollow fibers which are implanted into mice. Six to eight days after administration of the test compound to the mice, both intraperitoneally (IP) and subcutaneously (SC), these fibers were collected, the cells removed, and growth inhibition measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). A score of 2 was given to each test in which there was a %T/C of 50 or less (tumor mass 50% or less than the control). The results for selected compounds are listed in Table 2. Compounds with a combined IP+SC score > 20, a SC score > 8 or a net cell kill of one or more cell lines was assessed as promising and referred for xenograft testing. Compound **1** was active against breast cancers and showed a total score of 20. Less than 14% of the compounds tested at the National Cancer Institute were reported to have a total score of more than 20.¹¹⁾ As the hollow fiber assay is not equivalent to drug assays against tumor implants, a high score does not fully predict an effective anti-cancer agent, and a typical anti-tumor agent only has a hollow fiber score of up to 5. High score compounds are too toxic and do not show activity against tumor implants. Conversely, all compounds showing high activity against tumor implants are active in the hollow fiber assay.¹²⁾ For compounds **1**, **2** and **3**, the order of effectiveness of the *in vivo* test (total score) was **1** > **3** > **2**, which differed from that of the *in vitro* test, *vide supra*. Compound **2** was most active in the *in vitro* test but least active in the *in vivo* test. No correlation was observed between *in vitro* and *in vivo* results.

Sensitivity of the cancer cell panel to the compounds (panel selectivity) was investigated by using *in vitro* results (Table 1). For this purpose, the MIG_MID of each cancer

Table 2. *In Vivo* Hollow Fiber Assay^{a)}

Entry	Structure	IP score	SC score	Total score	Cell kill
1	1	10	10	20	None
2	2	4	4	8	None
3	3	6	6	12	None
4	7	2	6	8	None
5	10	0	4	4	None
6	12	2	0	2	None
7	13	6	6	12	None

a) The Biological Testing Branch of the Developmental Therapeutics Program in National Cancer Institute in U.S.A. adopted a test. For this assay, human tumor cells were cultivated in polyvinylidene fluoride (PVDF) hollow fibers and a sample of each cell line was implanted into each of two physiologic compartments (intraperitoneal and subcutaneous) in mice. The fiber cultures were collected on the day following the last day of treatment. From this, the %T/C was calculated using the average optical density (MTT) of compound treated samples divided by the average optical density of the vehicle control. To simplify evaluation, a points system has been adopted. A value of 2 is assigned each compound dose which results in a 50% or greater reduction in viable cell mass. The intraperitoneal (IP) and subcutaneous (SC) samples are scored separately. Compounds with a combined IP+SC score >20, a SC score >8 or a net cell kill of one or more lines are referred for xenograft testing.

panel was calculated for each compound, and the results revealed that compounds **7** and **8** had high inhibitory activity against both colon cancer cells ($-\text{Log GI}_{50}$ 6.7, $-\text{Log TC}_{50}$ 5.9) and melanoma cancer cells ($-\text{Log GI}_{50}$ 6.6, $-\text{Log LC}_{50}$ 5.4). This analysis suggests that the size and hydrophobic character of the 2-substituent are quite important for effective anti-cancer activities.

In summary, we have obtained the following conclusions about the SAR of our compounds. 1) *N*(1)-quaternarization is essential for anti-cancer activity, 2) the size and polarity of the 2-substituent is crucial for *in vitro* activity, which allows further investigation in an *in vivo* test, and 3) the effect of the 4-substituent on activity is minor compared with the other two factors. Further studies on SAR based on these observations are now underway.

Experimental

Melting points (mp) were determined on Yanaco micro-melting point apparatus without correction. Infrared (IR) spectra (KBr pellet unless stated otherwise) were measured by Perkin-Elmer FT-IR 1720. ¹H- and ¹³C-Nuclear magnetic resonance (NMR) spectra were obtained at ambient temperature with JEOL JNM-FT 200 and JNM-ALPHA 400 in CDCl₃, unless otherwise specified, with tetramethylsilane as an internal standard. Mass spectra (MS) and high resolution MS (HR-MS) were recorded on a Hitachi RMU-7MG, and the figures in parentheses indicate the relative intensities. Anhydrous tetrahydrofuran (THF) was prepared by distillation in the presence of a ketyl radical and dry acetonitrile by distilling it with calcium hydride.

4-(4-Dimethylaminobenzyliden)-2-phenyl-4H-pyrazolo[1,5-a]indole (4) a) Carbanion was prepared by adding *n*-butyl lithium (1.08 M in hexane, 0.61 ml) to a solution of 2-phenyl-4H-pyrazolo [1,5-a] indole (**17**) (R=Ph) (119.4 mg, 0.51 mmol) in dry THF (10 ml) at -78°C under nitrogen for 15 min. A solution of DMAB (91.0 mg) in dry THF (1 ml) was introduced gradually, and the reaction was resumed for 30 min. Quenching with aqueous ammonium chloride, extraction with ether, washing (saturated brine) and drying (anhydrous sodium sulfate) of the extracts gave the crude product (289 mg), which was purified by column chromatography (silica gel, hexane–ethyl acetate 4 : 1). After the starting material (33.7 mg, 29% recovery), the adduct (118.4 mg, 61%) was eluted as a mixture of diastereomers (*ca.* 7 : 3 by ¹H-NMR analysis). Recrystallization from ether gave the pure isomer of **23** as yellow needles, mp $180\text{--}181^{\circ}\text{C}$. IR ν_{max} cm⁻¹: 3370, 1614, 1524, 1473, 1455, 1342, 1165, 1048, 813, 765, 750, 694. ¹H-NMR δ : 2.39 (1H, brs, OH), 2.97 (6H, s, NMe₂), 4.40 (1H, d, $J=8.6$ Hz, 4-H), 4.58 (1H, dd, $J=8.6$, 2.2 Hz, 10-H), 5.77 (1H, d, $J=0.9$ Hz, 3-H), 6.70 (2H, d, $J=8.8$ Hz, Ar-H), 7.14–7.43 (8H, m, Ar-H), 7.63 (1H, d, $J=7.8$ Hz, Ar-H), 7.73–7.78 (2H, m, Ar-H). HR-MS Calcd for C₂₅H₂₃N₃O: 381.1843. Found: 381.1841.

b) The above crude product (699.7 mg, 1.83 mmol) was treated with methanesulfonyl chloride (0.2 ml, 2.75 mmol), triethylamine (0.4 ml, 2.87 mmol) and 4-dimethylaminopyridine (25.8 mg, 0.22 mmol) in dry CH₂Cl₂ (7 ml) at 0°C for 30 min. The solution was poured into water and extracted with ether. The crude product thus obtained (746.7 mg) was a mixture of geometrical isomers (7 : 3) and was purified by column chromatography (sil-

ica gel, hexane–ethyl acetate 9 : 1). Pure isomer of **4** (444.2 mg, 67%) and **23** (179.8 mg, 26% recovery) were obtained. **4**, brown needle, mp $183\text{--}184^{\circ}\text{C}$ (ether). λ_{max} MeOH nm (log ϵ): 262 (4.53), 418 (4.48). IR ν_{max} cm⁻¹: 1597, 1526, 1469, 1368, 1171, 952, 762. ¹H-NMR δ : 3.09 (6H, s, NMe₂), 6.82 (2H, d, $J=8.9$ Hz, 3'', 5''-H), 7.03 (1H, s, 3-H), 7.21 (1H, t, $J=7.3$ Hz, 7-H), 7.33 (1H, t, $J=6.9$ Hz, 6-H), 7.37 (1H, t, $J=7.6$ Hz, 4'-H), 7.39 (1H, s, 10-H), 7.43 (2H, brt, $J=7.7$ Hz, 3', 5'-H), 7.68 (1H, d, $J=7.8$ Hz, 5-H), 7.72 (1H, d, $J=7.3$ Hz, 8-H), 7.73 (2H, d, $J=8.9$ Hz, 2'', 6''-H), 7.92 (2H, d, $J=7.3$ Hz, 2', 6'-H). ¹³C-NMR δ : 40.2 (NMe₂), 98.7 (C-3), 110.2 (C-5), 111.8 (C-3'', 5''), 120.0 (C-4), 120.1 (C-8), 123.5 (C-1''), 124.0 (C-7), 125.9 (C-2', 6'), 127.82 (C-6), 127.85 (C-4'), 128.3 (C-10), 128.6 (C-3', 5'), 131.0 (C-2'', 6''), 132.4 (C-4a), 133.8 (C-1'), 137.6 (C-8a), 142.9 (C-3a), 150.9 (C-4''), 155.9 (C-2). HR-MS Calcd for C₂₅H₂₁N₃: 363.1737; Found: 363.1768. Anal. Calcd for C₂₅H₂₁N₃: C, 82.68; H, 5.83; N, 11.57. Found: 82.47; H, 5.77; N, 11.54.

4-(4-Trimethylammoniobenzyliden)-2-phenyl-4H-pyrazolo[1,5-a]indole Trifluoromethanesulfonate (5) A solution of **4** (10.8 mg) in a mixture of CH₂Cl₂ and ether (0.5 ml, 0.5 ml) was alkylated with MeOTf (4 μ l) at room temperature (r.t.) overnight. The products were separated by preparative TLC (silica gel, CH₂Cl₂–MeOH 95 : 5), giving two products, **5** (10 mg, 64%) and **2** (0.4 mg), as well as **4** (2 mg). **5**, mp $189\text{--}191^{\circ}\text{C}$ (MeOH–isoPr₂O). ¹H-NMR δ (major isomer: 94%) (deuterated dimethylsulfoxide DMSO-*d*₆): 3.70 (9H, s, N⁺Me₃), 7.21 (1H, s, 3-H), 7.36 (1H, t, $J=7.6$ Hz, 7-H), 7.40 (1H, t, $J=7.3$ Hz, 6-H), 7.49 (2H, brt, $J=7.6$ Hz, 3', 5'-H), 7.54 (1H, t, $J=7.8$ Hz, 4'-H), 7.66 (1H, d, $J=7.8$ Hz, 5-H), 7.91 (1H, s, 10-H), 7.94 (2H, d, $J=7.3$ Hz, 2', 6'-H), 8.07 (1H, d, $J=7.3$ Hz, 8-H), 8.08 (2H, d, $J=8.9$ Hz, 2'', 6''-H), 8.17 (2H, d, $J=8.9$ Hz, 3'', 5''-H); δ (minor isomer: 6%) (DMSO-*d*₆): 3.66 (s, NMe₃), 7.27 (s, 3-H), 7.81 (s, 10-H). ¹³C-NMR δ (major isomer: 94%) (DMSO-*d*₆): 56.4 (N⁺Me₃), 100.3 (C-3), 110.0 (C-5), 121.3 (C-3'', 5''), 122.0 (C-8), 124.8 (C-4), 125.0 (C-7), 125.4 (C-2', 6'), 126.1 (C-10), 128.3 (C-6), 128.8 (C-3', 5'), 130.0 (C-4'), 130.4 (C-2'', 6''), 131.0 (C-1'), 132.6 (C-4a), 136.9 (C-8a), 137.3 (C-1''), 141.1 (C-3a), 146.9 (C-4''), 155.4 (C-2). Anal. Calcd for C₂₇H₂₄F₃N₃O₃S · 1/2H₂O: C, 60.40; H, 4.50; N, 7.77. Found: 60.40; H, 4.70; N, 7.83.

4-(4-Dimethylaminobenzyliden)-1-methyl-2-(4-chlorophenyl)-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (7) a) Free base **17** (R=C₆H₄–Cl-4)⁵⁾ (202 mg, 0.76 mmol) was methylated with MeOTf (0.17 ml, 1.5 mmol) in dry CH₂Cl₂ (10 ml) at r.t. for 7 d. Crystallization of the crude product from CH₂Cl₂–isoPr₂O gave crystals (271 mg, 83.1%), mp $138\text{--}141^{\circ}\text{C}$. Recrystallization from MeOH–ethyl acetate yielded pure **20** (R=C₆H₄–Cl-4) (147 mg, 45.1%), mp $147.0\text{--}148.5^{\circ}\text{C}$. IR ν_{max} cm⁻¹: 1603, 1550, 1515, 1466, 1265, 1162, 1030, 832, 754, 638. ¹H-NMR δ : 4.200 (2H, s, 4-H), 4.415 (3H, s, NMe), 6.885 (1H, s, 3-H), 7.409 (1H, td, $J=7.6$, 1.2 Hz, 6-H), *ca.* 7.5 (1H, t, $J=7$ Hz, 7-H), 7.527 (2H, AB type, $J=8.5$ Hz, Ar-H), 7.608 (2H, AB type, Ar-H), *ca.* 7.61 (1H, d, overlapping with AB type signals, 5-H), 7.879 (1H, d, $J=7.8$ Hz, 8-H). ¹³C-NMR δ : 29.90 (C-4), 37.44 (NMe), 104.26 (C-3), 112.77, 123.99, 127.15, 128.12, 129.12, 129.97 (2×C), 131.02 (2×C), 133.18, 137.05, 138.22, 150.38, 152.33. Anal. Calcd for C₁₈H₁₄ClF₃N₂O₃S: C, 50.18; H, 3.28; N, 6.50. Found: C, 50.30; H, 3.17; N, 6.47.

b) A coupling reaction of the above product (114 mg, 0.26 mmol) with DMAB (52 mg, 0.35 mmol) in acetic acid (20 ml) at refluxing temperature for 2 d, followed by column chromatography of the crude product (silica gel 10 g, CH₂Cl₂–EtOH 9 : 1), gave dark red crystals (134 mg, 90.1%). Recrystallization from MeOH–ethyl acetate (EtOAc) afforded **7** (80 mg, 53.8%),

mp 253.0–255.0 °C (dec). IR $\nu_{\max}$ cm^{-1} : 1621, 1573, 1530, 1360, 1296, 1260, 1156, 1030, 753, 735. $^1\text{H-NMR}$ δ (DMSO- d_6): 3.114 (6H, s, NMe₂), 4.428 (3H, s, NMe), 6.935 (2H, d, $J=8.6$ Hz, Ar-H), 7.548 (1H, s, 3-H), 7.54–7.59 (2H, m, Ar-H), 7.75–7.88 (6H, m, Ar-H), 8.13–8.26 (2H, m, Ar-H), 8.336 (1H, s, 10-H). $^{13}\text{C-NMR}$ δ (DMSO- d_6): 37.53 (NMe), 39.55 (NMe₂), 101.68, 112.12 (2×C), 112.63, 120.78, 121.19, 124.79, 126.78, 127.39, 129.34 (2×C), 131.45 (2×C), 131.62, 132.40, 133.47 (2×C), 136.15, 140.16, 141.91, 150.43, 155.57. *Anal.* Calcd for C₂₇H₂₃ClF₃N₂O₃S: C, 57.70; H, 4.12; N, 7.48. Found: C, 57.74; H, 3.82; N, 7.48.

4-(4-Dimethylaminobenzyliden)-1-methyl-2-(1-adamantyl)-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (8) a) 1-Formyladamantane (1.851 g, 11.3 mmol) and THF (50 ml) were added to a mixture of *o*-allylphenylhydrazine hydrochloride (14)·HCl (2.106 g, 11.4 mmol) in aqueous acetic acid (60% (v/v), 11.3 ml) buffered by adding sodium hydroxide (0.13 g, 13.6 mmol). The resulting solution was stirred for 1 d. Dilution with water (34 ml) and extraction with ether thrice gave the extracts which were washed (water once, aqueous sodium carbonate twice, saturated brine twice), dried and evaporated. Recrystallization of the product (3.26 g) from CH₂Cl₂-isoPr₂O gave *trans*-2-(1-adamantyl)-1,2,3,3a-tetrahydro-4H-pyrazolo[1,5-a]indole (16) (R=1-adamantyl) (1.0306 g, 31.1%), mp 151.0–153.0 °C. MS m/z : 294 (M⁺, 30.5), 159 (19.7), 158 (24.1), 135 (6.2), 132 (100), 131 (16.0), 117 (7.0). IR $\nu_{\max}$ cm^{-1} : 3287, 1605, 1471, 1448, 1252, 752, 716. $^1\text{H-NMR}$ δ : 1.44–1.76 (13H, m), 1.98 (3H, m), *ca.* 2.04 (1H, m), 2.64 (1H, t, $J=8.2$ Hz), 2.96 (1H, d, $J=15.9$ z), 3.19 (1H, dd, $J=8.1$, 15.9 Hz), 3.77–3.90 (2H, m), 6.87 (1H, dt, $J=1.2$, 7.3 Hz), 6.99–7.19 (3H, m). $^{13}\text{C-NMR}$ δ : 28.3 (3×C), 33.8, 34.6, 35.1, 37.2 (3×C), 39.5 (3×C), 65.5, 68.5, 114.7, 121.6, 124.6, 127.6, 128.9, 153.2.

b) The above product (604 mg, 2.05 mmol) was dehydrogenated with DDQ in THF (50 ml) at refluxing temperature for 7.5 h. DDQ was added in two portions to complete the oxidation (510+305=815 mg, 3.59 mmol). After evaporation, the residue was treated with aqueous 1 M sodium hydroxide solution and extracted with ether. The extracts gave, after washing and drying, the product (623 mg) which was purified by column chromatography (silica gel 50 g, CH₂Cl₂). The eluate (460 mg, 77%) was recrystallized from isoPr₂O to give 2-(1-adamantyl)-4H-pyrazolo[1,5-a]indole (17) (R=1-adamantyl) (194 mg, 33%), mp 105.0–105.5 °C (isoPr₂O). MS m/z : 290 (M⁺, 100), 247 (4.9), 233 (33.8), 196 (5.8). HR-MS Calcd. for C₂₀H₂₂N₂: 290.1781. Found: 290.1780. IR $\nu_{\max}$ cm^{-1} : 2903, 1627, 1547, 1478, 1403, 774, 740. $^1\text{H-NMR}$ δ : 1.80 (6H, br s), 2.04 (6H, br s), 2.07 (3H, br s), 3.79 (2H, s, 4-H₂), 6.13 (1H, s, 3-H), 7.11 (1H, dt, $J=1.0$, 7.6 Hz), 7.25–7.40 (2H, m), 7.60 (1H, d, $J=7.33$ Hz). $^{13}\text{C-NMR}$ δ : 28.3, 28.8 (3×C), 34.9, 37.0 (3×C), 42.9 (3×C), 96.7, 110.4, 123.7, 125.7, 127.9, 133.3, 140.9, 144.8, 167.7. *Anal.* Calcd for C₂₀H₂₂N₂: C, 82.72; H, 7.64; N, 9.6. Found: C, 82.78; H, 7.45; N, 9.66.

c) The above product (441 mg, 1.52 mmol) was reacted with MeOTf (0.45 ml, 3.98 mmol) in dry CH₂Cl₂ (20 ml) at r.t. for 6 d, and at refluxing temperature for 7 h. Crystallization of the evaporated residue from MeOH-isoPr₂O gave 2-(1-adamantyl)-1-methyl-4H-pyrazolo[1,5-a]indolium trifluoromethanesulfonate (20) (R=1-adamantyl) (552 mg, 80.0%), colorless fine needles, mp 273.0–275.5 °C (dec) (95% EtOH). IR $\nu_{\max}$ cm^{-1} : 1548, 1467, 1284, 1257, 1155, 1032, 767, 638. $^1\text{H-NMR}$ δ (CD₃OD): 1.899 (6H, m), 2.183 (3H, br s), 2.211 (6H, s, m), 4.244 (2H, s, 4-H), 4.657 (3H, s, NMe), 6.823 (1H, s, 3-H), 7.505 (1H, dt, $J=0.85$, 7.6 Hz), 7.603 (1H, t, $J=8.0$ Hz), 7.741 (1H, br d, $J=7.57$ Hz), 8.014 (1H, br d, $J=8.18$ Hz). $^{13}\text{C-NMR}$ δ (CD₃OD): 29.64, 30.31 (C-4), 36.58, 37.12, 39.87 (NMe), 41.09, 102.81, 114.17, 128.34, 128.77, 129.83, 135.37, 138.97, 151.47, 162.63. *These signals gradually disappeared after 50 h. *Anal.* Calcd for C₂₂H₂₅F₃N₂O₃S·1/2H₂O: C, 57.01; H, 5.44; N, 6.04. Found: C, 57.19; H, 5.57; N, 6.10.

d) A mixture of the above product (150 mg, 0.33 mmol) and DMAB (70 mg, 0.40 mmol) in acetic acid (30 ml) was refluxed for 2 d. After evaporation, the residue (240 mg) was purified by column chromatography (silica gel 10 g, CH₂Cl₂-EtOH 93:7) and the product 8 (146 mg, 75.5%) was obtained as dark red needles, mp 194–204 °C (MeOH). IR $\nu_{\max}$ cm^{-1} : 2910, 2855, 1583, 1532, 1380, 1278, 1258, 1160, 1030, 638. $^1\text{H-NMR}$ δ : 1.826 (6H, s), 2.123 (6H, s), 2.185 (3H, s), 3.157 (6H, s, NMe₂), 4.652 (3H, s, N⁺Me), 6.807 (1H, s, 3-H), 6.820 (2H, d, $J=8.5$ Hz), 7.31–7.51 (2H, m), 7.615 (2H, d, $J=8.5$ Hz), 7.81 (1H, br d, $J=7$ Hz), 7.826 (1H, br s), 8.01 (1H, br d, $J=7.5$ Hz). $^{13}\text{C-NMR}$ δ : 27.90 (3×C), 35.30, 35.93 (3×C), 39.75 (NMe), 40.02 (3×C), 40.16 (NMe₂), 99.22, 111.97 (2×C), 112.89, 113.79, 120.82, 121.02, 127.05, 128.24, 131.48, 133.18 (3×C), 139.85, 143.11, 152.88, 160.74. *Anal.* Calcd for C₃₁H₃₄F₃N₂O₃S·H₂O: C, 61.67; H, 5.68; N, 6.96. Found: C, 61.55; H, 5.77; N, 6.95.

4-(4-Dimethylaminobenzyliden)-1-methyl-2-ethoxycarbonyl-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (10) a) Ester 17 (R=

COOEt) (496 mg, 2.17 mmol) in dry CH₂Cl₂ (20 ml) was methylated by adding MeOTf in two portions (0.34 ml+0.20 ml=0.54 ml, 4.77 mmol) for 3+3 d at r.t. After another 3 d of reaction, the residue obtained by evaporation was crystallized from MeOH-isoPr₂O to give 20 (R=COOEt) (704 mg, 82.6%, mp 152.5–154.0 °C), mp 154.0–155.0 °C (MeOH-isoPr₂O), MS m/z : 256 (M⁺–136, 0.37%), 242 (M⁺–14, 30.0%), 228 (M⁺–CF₃SO₃H, 100), 214 (16.5), 200 (25.6), 183 (72.3), 169 (17.2), 155 (52.5), 130 (13.8), 128 (14.1). IR $\nu_{\max}$ cm^{-1} : 1749, 1491, 1477, 1451, 1282, 1261, 1238, 1163, 1032, 767, 637. $^1\text{H-NMR}$ δ (CD₃OD): 1.457 (3H, t, $J=7.20$ Hz, 11-H), 4.533 (2H, q, $J=7.20$ Hz, 10-H), 4.857 (3H, s, NMe), 7.484 (1H, s, 3-H), 7.626 (1H, dt, $J=1.10$, 7.57 Hz, 6-H), 7.689 (1H, ddd, $J=1.46$, 7.57, 8.19 Hz, 7-H), 7.829 (1H, ddd, $J=0.73$, 1.46, 7.57 Hz, 5-H), 8.177 (1H, ddd, $J=0.73$, 1.10, 8.19 Hz, 8-H). No 4-H was detected. $^{13}\text{C-NMR}$ δ (CD₃OD): 14.32 (C-11), 38.85 (NMe), 64.60 (C-10), 108.15 (C-3), 115.20 (C-8), 128.56 (C-5), 130.22 (C-7), 130.26 (C-6), 136.48 (C-4a), 138.36 (C-8a), 141.42 (C-2), 151.75 (C-3a), 158.65 (C-9). No C-4 was detected. *Anal.* Calcd for C₁₅H₁₅F₃N₂O₅S·H₂O: C, 46.16; H, 3.87; N, 7.18. Found: 45.85; H, 3.83; N, 7.19.

b) The above product (149 mg, 0.40 mmol) was coupled with DMAB (91 mg, 0.61 mmol) by refluxing in acetic acid (30 ml) for 14 h. Evaporation and chromatography of the residue on silica gel (10 g, CH₂Cl₂+7%, 10% EtOH) gave 10 (142 mg) and a by-product (26 mg) which has no carbonyl absorption in the IR spectrum. 10, a mixture of geometrical isomers, dark red needles, mp 182.0–183.0 °C (dec.) (MeOH), IR $\nu_{\max}$ cm^{-1} : 1730, 1577, 1527, 1263, 1160, 1031, 637. $^1\text{H-NMR}$ δ (major isomer): 1.457 (3H, t, $J=7.08$ Hz, OCH₂CH₃), 3.165 (6H, s, NMe₂), 4.526 (2H, q, $J=7.08$ Hz, OCH₂CH₃), 4.824 (2H, s, N⁺–Me), 6.816 (2H, d, $J=8.55$ Hz, Ar-H), 7.44–7.53 (2H, m, Ar-H), 7.502 (1H, s, 3-H), 7.655 (2H, d, $J=8.55$ Hz, Ar-H), 7.86 (1H, m, Ar-H), 7.87 (1H, s, 10-H), 8.02 (1H, m, Ar-H). δ (minor isomer, 30%): 4.787 (s, N⁺–Me). $^{13}\text{C-NMR}$ δ (major isomer): 14.07, 38.49, 40.23, 63.69, 105.19, 112.31 (2×C), 113.36, 121.10, 128.08, 128.19, 131.93, 132.55, 134.03 (2×C), 134.14, 139.33, 141.79, 142.48, 153.33, 157.27. δ (minor isomer): 38.37, 53.87, 105.39, 111.83, 113.27, 121.02, 121.08, 127.95, 128.15, 131.72, 132.61, 138.95, 141.83, 142.37, 153.39, 157.75. *Anal.* Calcd for C₂₄H₂₄F₃N₂O₅S: C, 55.06; H, 4.62; N, 8.03. Found: 54.99; H, 4.58; N, 7.99.

4-(4-Dimethylaminocinnamyliden)-1-methyl-2-phenyl-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (11) Reaction of 20 (R=Ph) (121 mg, 0.31 mmol) and 4-dimethylaminocinnamaldehyde (69 mg, 0.39 mmol) in refluxing acetic acid (25 ml) for 26 h gave the crude product (216 mg), which was chromatographed (silica gel 9 g, CH₂Cl₂+7% EtOH) to give 11 (143 mg) as dark needles, mp 138–142 °C (dec.) (MeOH). IR $\nu_{\max}$ cm^{-1} : 1571, 1527, 1515, 1461, 1357, 1260, 1149, 1030, 820, 962, 637. $^1\text{H-NMR}$ (DMSO- d_6) δ : 3.057 (6H, s, NMe₂), 4.434 (3H, s, N⁺–Me), 6.721 (2H, d, $J=9.0$ Hz, 15-H+17-H), 7.36–7.41 (1H, m, 12-H), 7.48–7.56 (3H, m, 6-H+7-H+11-H), 7.71–7.74 (5H, m, 21-H+22-H+23-H+14-H+18-H), 7.82–7.84 (2H, m, 20-H+24-H), 7.966 (1H, s, 3-H), 8.06–8.12 (3H, m, 5-H+8-H+10-H). δ (isomer, 7%): 3.081 (NMe₂), 4.388 (N⁺–Me). $^{13}\text{C-NMR}$ (DMSO- d_6) δ : 37.26 (N⁺–Me), 39.67 (NMe₂), 102.22, 111.70 (2×C), 112.54, 114.53, 118.85, 121.52, 123.02, 126.13, 126.68, 127.58, 129.30 (2×C), 129.51 (2×C), 130.28, 131.10 (2×C), 131.18, 133.13, 140.04, 142.43, 148.97, 151.93, 152.05. δ (minor isomer): 60.33, 72.27, 79.03, 111.80, 119.00, 122.21, 129.25, 129.39, 130.27. *Anal.* Calcd for C₂₉H₂₆F₃N₂O₅S·1/2H₂O: C, 61.91; H, 4.66; N, 7.47. Found: C, 61.93; H, 4.61; N, 7.51.

4-(3,4,5-Trimethoxybenzyliden)-1-methyl-2-phenyl-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (12) Salt 20 (R=Ph) (121 mg, 0.305 mmol) was coupled with 3,4,5-trimethoxybenzaldehyde in refluxing acetic acid (20 ml). Aldehyde was added portion-wise (70+85+71=226 mg) at the reaction time of 16 and 18 h, and the reaction was continued for 16 h. The product was purified by column chromatography (silica gel 9 g, CH₂Cl₂+6, 8, 10% EtOH) and the fraction (142 mg) was recrystallized to give 12 (93 mg) as yellow crystals, mp 194.0–195.5 °C (CH₂Cl₂-EtOAc). IR $\nu_{\max}$ cm^{-1} : 1632, 1579, 1507, 1470, 1332, 1279, 1261, 1152, 1129, 1032, 639. $^1\text{H-NMR}$ δ : 3.889 (6H, s, 2×OMe), 3.938 (3H, s, OMe), 4.470 (3H, s, NMe), 6.945 (1H, s, 3-H), 7.022 (2H, s, 2,6-H on E-ring), 7.405 (1H, t, $J=7.6$ Hz, 7-H), 7.497 (1H, t, $J=7.9$ Hz, 6-H), 7.58–7.61 (5H, m, Ar-H), 7.906 (1H, d, $J=8.06$ Hz, 5-H), 7.931 (1H, d, $J=7.81$ Hz, 8-H), 8.042 (1H, s, 10-H). δ (minor isomer, 10%): 3.845 (s), 3.954 (s), 6.970 (s). $^{13}\text{C-NMR}$ δ : 37.69 (NMe), 56.59 (3,5-OMe), 61.11 (4-OMe), 102.93 (C-3), 107.51 (C×2, 2,6-C on E-ring), 112.67 (C-5), 120.66, 122.20 (C-8), 125.53, 127.83 (C-7), 129.28, 129.52 (2×C, 3,5-C on D-ring), 129.70 (2×C, 2,6-C on D-ring), 129.86 (C-6), 130.54 (C-8a), 131.77 (C-4 on D-ring), 134.51 (C-4a), 139.05 (C-11), 140.93 (C-4 on E-ring), 143.96 (C-4), 153.45 (C-2), 153.66

(3,5-C on E-ring). δ (minor isomer): 56.44, 100.66, 107.75, 113.00, 119.17, 122.37, 125.24, 125.25, 129.66, 130.50, 131.68, 142.59. *Anal.* Calcd for $C_{28}H_{25}F_3N_2O_6S$: C, 58.53; H, 4.39; N, 4.87; O, 16.71. Found: C, 58.44; H, 4.31; N, 4.80.

4-(4-Dimethylamino-2-methoxybenzal)-1-methyl-2-phenyl-4H-pyrazolo[1,5-a]indolium Trifluoromethanesulfonate (13) Salt 20 (R=Ph) (137 mg, 0.346 mmol) and 2-methoxy-4-dimethylaminobenzaldehyde (83 mg, 0.463 mmol), which was prepared by Vilsmeier reaction of *N,N*-dimethyl-*m*-anisidine, were coupled in refluxing acetic acid (50 ml) for 30 h. Chromatography (silica gel 9 g, CH_2Cl_2 +8% EtOH) of the crude product (230 mg) gave a dark red fraction (153 mg) which was recrystallized from CH_2Cl_2 -isoPr₂O under ice-cooling to give dark red amorphous **13**, mp 121–127 °C (CH_2Cl_2 -EtOAc). IR ν_{max} cm^{-1} : 1621, 1579, 1536, 1364, 1279, 1257, 1031, 638. ¹H-NMR δ : 3.11 (6H, s, NMe₂), 3.89 (3H, s, N⁺Me), 4.40 (3H, s, OMe), 6.06 (1H, d, *J*=1.7 Hz, 3-H on E-ring), 6.48 (1H, dd, *J*=1.7, 8.4 Hz, 5-H on E-ring), 6.93 (1H, s, 3-H), 7.39 (2H, m, Ar-H), 7.5–7.7 (7H, m, Ar-H), 7.93 (1H, br d, *J*=7.6 Hz, 5-H), 8.12 (1H, s, 11-H). ¹³C-NMR δ : 37.7 (NMe), 40.3 (NMe₂), 55.7 (OMe), 93.4, 100.8, 105.7, 111.1, 111.9, 112.2, 120.7, 125.7, 126.8, 127.5, 129.4 (2×C), 129.7 (2×C), 131.5, 131.8, 131.9, 132.4, 135.4, 143.7, 152.7, 155.5, 161.4. *Anal.* Calcd for $C_{28}H_{26}F_3N_2O_4S$: C, 60.31; H, 4.70; N, 7.54. Found: C, 59.53; H, 4.54; N, 7.33.

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References and Notes

- 1) Studies on the Drugs against Refractory Diseases. Part 2. For part 1 see ref. 2.
- 2) Katayama H., Kawada Y., Kaneko K., Oshiyama T., Takatsu N., *Chem. Pharm. Bull.*, **47**, 48–53 (1999).
- 3) Katayama H., Takatsu N., Kitano H., Shimaya Y., *Chem. Pharm. Bull.*, **38**, 1129–1135 (1990).
- 4) Shen J.-K., Katayama H., *J. Chem. Soc. Perkin Trans. 1*, **1994**, 1871–1877.
- 5) Shen J.-K., Katayama H., Takatsu N., Shiro I., *J. Chem. Soc. Perkin Trans. 1*, **1993**, 2087–2097.
- 6) Katayama H., Sakurada M., Herath W. H., Takatsu N., Shen J.-K., *Chem. Pharm. Bull.*, **40**, 2267–2269 (1992).
- 7) a) Paull D. K., Shoemaker R. H., Hodes L., Monks A., Scudiero D. A., Rubinstein L., Plowman J., Boyd M. R., *J. Natl. Cancer Inst.*, **81**, 1088–1092 (1989); b) Alvarez M., Bobey R., Sandor V., Nishiyama K., Matsumoto Y., Paull K., Bates S., Fojo T., *Mol. Pharmacol.*, **54**, 802–814 (1998).
- 8) Patrick G. L., "An Introduction to Medicinal Chemistry," Oxford University Press, Oxford, 1995, p. 145.
- 9) Unreported results.
- 10) a) Plowman J., Dykes D. J., Hollingshead M., Simpson-Herren L., Alley M. C., Human Tumor Xenograft Models in NCI Drug Development, 1997, pp. 101–125, in "Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval," ed. by Teicher B., Humana Press, Inc., Totowa, NJ. b) Hollingshead M. G., Alley M. C., Camalier R. F., Abbot B. J., Mayo J. G., Malspeis L., Grever M. R., *Life Sci.*, **57**, 131–141 (1995).
- 11) See Fig. 1 in the report: Craig W. A., LeSueur B. W., Skibo E. B., *J. Med. Chem.*, **42**, 3324–3333 (1999).
- 12) Hollingshead M. G., Plowman J., Alley M., Mayo J., Sausville E., "Contributions to Oncology," Krager Verlag, Germany, 1999.

NO-Mediated Aromatic Nitration during Decomposition of Phenolic S-Nitrosothiols in Non-Aqueous Aerobic Medium

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Five novel S-nitrosothiol compounds (6–10) derived from L-cysteine were generated in solution and their decomposition rate was followed by UV spectroscopy. In acetonitrile, compounds 9 and 10 were the most stable of this series with a half-life of 24 h. The final organic decomposition products of the five S-nitrosothiols were also analysed. Derivatives 8, 9, and 10, possessing a phenolic hydroxyl group, afforded an unexpected decomposition pathway, with nitration of aromatic ring occurring in non-aqueous media. A mechanism involving a phenoxy radical seems to be implicated.

Key words S-nitrosothiol; phenol; nitration; phenoxy radical; peroxyntous acid

The endogenous free radical nitric oxide ($\cdot\text{NO}$), formed enzymatically from the amino acid L-arginine by nitric oxide synthase (NOS), is an important mediator in different physiological processes^{1,2)} such as cellular immunity,³⁾ inhibition of platelet aggregation⁴⁾ and neurotransmission.⁵⁾ This radical has an extremely short lifetime and it has been suggested that *in vivo* it is stabilized by reaction with a carrier molecule that prolongs its half-life and preserves its biological activity.^{6,7)} The most important class of $\cdot\text{NO}$ carrier molecules are those containing sulfhydryl (RSH) functional groups.⁸⁾ In biological systems, cysteine and glutathione are the most common members of this class. Their corresponding S-nitrosothiols may act as stores or pools of $\cdot\text{NO}$ and may also be the means by which $\cdot\text{NO}$ is transported around the body.^{9,10)} In contrast to beneficial activities, abnormally high levels of nitric oxide can be deleterious to the living cell. This cytotoxicity has been partially attributed to the formation of peroxyntite anion (ONOO^-), *via* reaction of $\cdot\text{NO}$ with the superoxide radical anion ($\text{O}_2^{\cdot-}$),¹¹⁾ which is able to oxidize a great diversity of biomolecules including proteins, DNA or lipids, and to nitrate and nitrosate phenolic compounds.

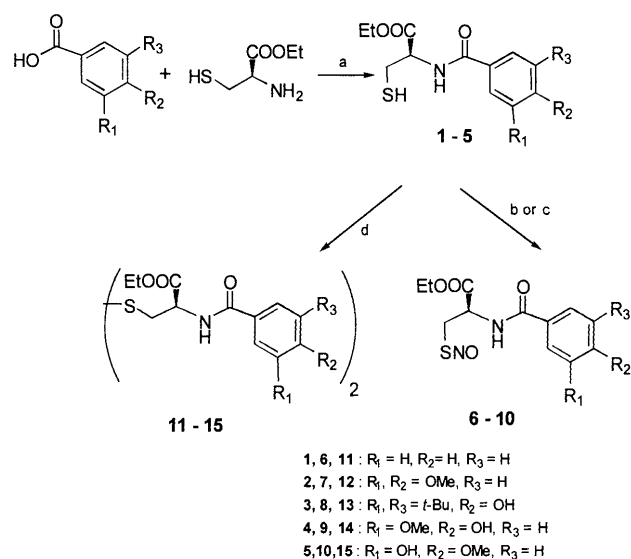
Our group has recently developed the concept of an original $\cdot\text{NO}$ carrier conjugated to antioxidant molecules¹²⁾ in order to associate two complementary activities: release of $\cdot\text{NO}$ and scavenging of free radicals ($\text{O}_2^{\cdot-}$) to subsequently reduce peroxyntite formation.

In view of the potential of S-nitrosothiols as NO-delivery compounds and phenols as antioxidant molecules,^{13,14)} we planned to synthesize novel nitrosothiols (Chart 1) by condensation of cysteine ethyl ester with various benzoic acid derivatives and examine their stability and decomposition pathways in aqueous and non-aqueous solution.

It is generally acknowledged that *in vitro* decomposition of S-nitrosothiol species gives nitric oxide and the corresponding disulphides, *via* homolytic cleavage of the S–N bond as the primary process.¹⁵⁾ This process is influenced by various factors such as metal ions,^{16–18)} light,¹⁹⁾ pH,¹⁵⁾ concentration, temperature,¹⁹⁾ and also inherent structure.¹⁵⁾ Consequently, stability studies of different S-nitrosothiols must be carried out under strictly identical conditions in order to be compared.

In this work, S-nitrosothiol derivatives 6–10 were prepared *in situ* from the corresponding thiols at an initial concentration of 50 mM. Their formation reached completion a few minutes after addition of 1 equivalent of *tert*-butyl nitrite (deep red solution). They were characterized in solution by UV-visible spectroscopy (at 320–360 nm and at 545 nm) and by $^1\text{H-NMR}$ (downfield shift of the CH_2S protons of the SNO function¹⁵⁾) and no trace of the starting thiol could be detected. Stability studies were then performed by UV spectroscopy, monitoring disappearance of the characteristic band at 545 nm, which correlated with the bleaching of the red solution. The results are expressed in terms of half-life ($t_{1/2}$) (Table 1).

These experiments showed that compounds 9 and 10 were the most stable of the series with a half-life of 24 h. It is interesting to note that derivative 7, which differs only by the absence of the free hydroxyl group, was much less stable than 9 or 10. Furthermore, it was also observed that compound 8, possessing a hindered hydroxyl group, showed a



a) $(\text{COCl})_2/\text{DMF}/\text{CH}_2\text{Cl}_2/\text{py}$; b) $t\text{-BuONO}/\text{CH}_3\text{CN}$; c) $\text{NaNO}_2/\text{H}_2\text{O}/\text{HCl}$; d) I_2/DMSO

Chart 1

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Table 1. Half-Life of *S*-Nitrosothiols

Compounds	$t_{1/2}$ SNO (50 mM/CH ₃ CN)
6	40 min
7	1 h 30 min
8	3 h
9	24 h ^{a)}
10	24 h ^{a)}

a) Half-life ($t_{1/2}$ confirmed by ¹H-NMR).

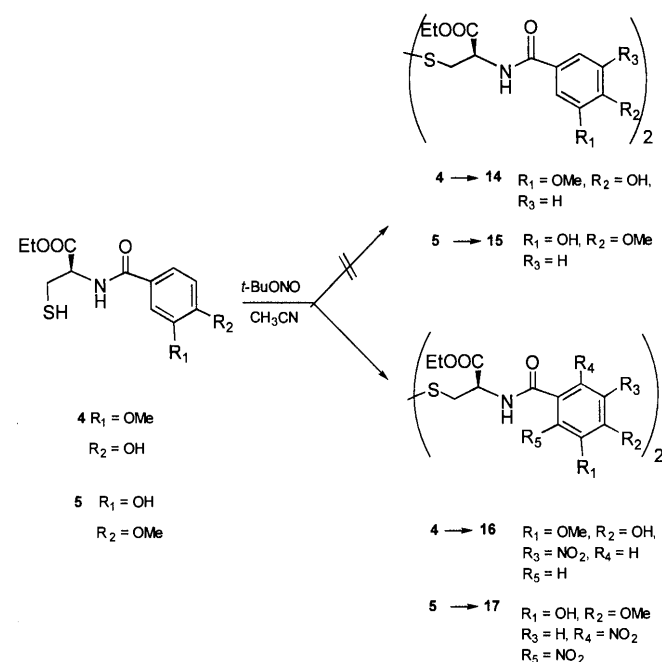


Chart 2

relatively fast decomposition in comparison with **9** and **10**. By EPR spectroscopy, the NO radical was indirectly detected from *S*-nitrosothiols **6**–**10**. All compounds in the presence of $[\text{Fe}(\text{II})(\text{MGD})_2]$ exhibited the characteristic triplet with a hyperfine splitting constant ($a^{\text{N}} = 12.85 \text{ G}$) attributed to the paramagnetic complex $[\text{Fe}(\text{II})(\text{MGD})_2\text{NO}]$.²⁰ The final organic decomposition product of each *S*-nitrosothiol was examined both in aqueous and non-aqueous media. It was found that derivatives **6** and **7** decompose (in organic and aqueous media) in a classical manner into the corresponding disulphides **11** and **12** as the sole final organic product. Their ¹H-NMR spectra were identical to those of the corresponding disulphide samples prepared by another route (I_2/DMSO). In contrast, formation of disulphide **14** was not observed after decomposition of *S*-nitrosothiol **9** in acetonitrile. Surprisingly, the final decomposition product obtained after addition of 1 eq of *tert*-butyl nitrite to thiol **4**, was nitro-phenol **16**, as evidenced by ¹H-, ¹³C-NMR and MS spectra (Chart 2).

When thiol **5** was treated with 1 eq of *tert*-butyl nitrite in acetonitrile, decomposition led to a mixture of *ortho* (minor) and *para* (major) (relative to the OH group) nitration products as evidenced by ¹H-NMR. After addition of an excess of *tert*-butyl nitrite both *ortho*- and *para*-adducts were converted to the same dinitro compound **17** (Chart 2).

In the case of **8**, which also possesses a phenolic hydroxyl group, the decomposition reaction gave a mixture of com-

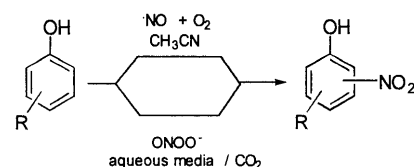


Chart 3

pounds which were unstable and could not be characterized. Based on our previous experiments, we suggest that these compounds may be the 2- and 4-(relative to the ketone) nitro-cyclohexadienone derivatives. This proposition is in agreement with previous work²¹⁾ showing that nitric oxide gas also reacts with 2,6-di-*tert*-butyl-4-methylphenol in cyclohexane to produce *C*-nitrosocyclohexadienone (in the absence of oxygen) as the major component of an unstable mixture. This reaction would occur *via* the formation of a phenoxy radical, which has been trapped and detected by EPR.

In order to verify if disulphides could be the substrate of this nitration step, we extended our investigation to the reaction of disulphides **14** and **15** with *tert*-BuONO in organic medium. In these conditions, the respective dinitrated disulphides **16** and **17** were formed, even though in this case the NO radical was generated from *tert*-BuONO itself and not *via* *S*-nitrosothiols. In fact, in this nucleophile free (RSH) experiment, *tert*-BuONO only release small amounts of NO and consequently a large excess of reagent was needed to reach completion. Thus, this result suggests that upon decomposition *S*-nitrosothiols firstly dimerise into disulphides which in turn give the corresponding nitro aromatic adducts. A nitroso-phenol derivative was not isolated in any case. Moreover, nitration reaction did not occur during decomposition of analogues **6** or **7** (in which there is no hydroxyl group available) under identical conditions.

Our results indicated that the reactivity of nitric oxide in aerobic acetonitrile towards phenols can be compared with that of peroxynitrite in aqueous/ CO_2 media,²²⁾ which also leads to formation of nitro-phenols (Chart 3). However, the mechanism by which this nitration reaction occurs is not clear.

Classical nitration reactions can implicate either non-radical (NO_2^+) or radical ($^\cdot\text{NO}_2$) mechanisms. In our case a mechanism involving NO_2^+ seems unlikely, since the nitration reaction did not occur with **6** and **7** (no free hydroxyl group). This result is more in accordance with a radical mechanism which requires an one-electron oxidation of the phenol by $^\cdot\text{NO}_2$ formed from oxidation of $^\cdot\text{NO}$, or by $^\cdot\text{NO}$ itself to give the phenoxy radical. Subsequent $^\cdot\text{NO}_2$ -nitration or $^\cdot\text{NO}/\text{O}_2$ nitrosation/oxidation would yield the nitrophenol. However, here the stoichiometry of the nitration reaction involves two $^\cdot\text{NO}$ molecules, leading to a mixture of final decomposition products (symmetric dinitrodisulphide, asymmetric mononitrodisulphide and disulphide) which was not observed in these experiments. Another mechanism that accounts for the data is that this nitration reaction in organic solution occurs *via* a process initially involving formation of nitrosyldioxy radical ($\text{ONOO}^\cdot$) (first intermediate in nitric oxide oxidation by dioxygen)²³⁾ which in turn generates the phenoxy radical and peroxynitrous acid (ONOOH).²⁴⁾ Since peroxynitrous acid belongs to the family of peroxo species with easily cleaved O–O bonds, it decomposes spontaneously giving the

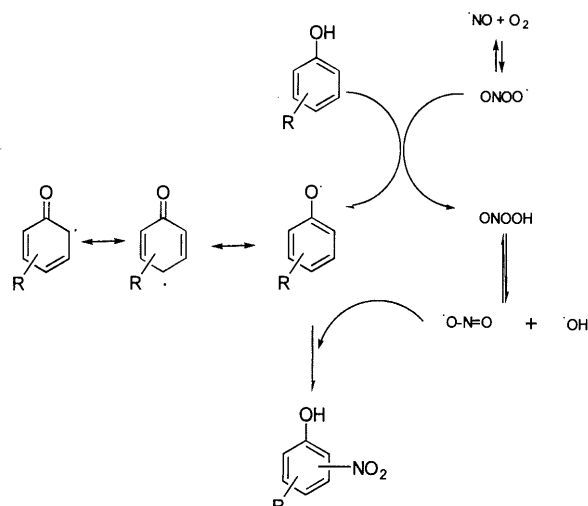


Chart 4

nitrogen dioxide radical which adds to the aromatic ring to produce nitro-phenols (Chart 4). It is worth noting that this decomposition pathway was not observed when thiols **3**, **4**, and **5** were treated either with NaNO_2 or with *tert*-butyl nitrite in aqueous media,²⁵ the corresponding disulphides being the only final organic decomposition products. Indeed, in aerobic aqueous media, nitric oxide reacts with dioxygen to give nitrite anion as the major product.

In contrast to peroxyxynitrite, it is known that nitric oxide in aerobic conditions is mainly a nitrosating agent¹⁹ (conversion of amines and thiols into nitrosamines and *S*-nitrosothiols, respectively), and to a lesser extent a nitrating agent. Here, we showed that NO generated from *S*-nitrosothiols in the presence of oxygen, can also behave as an efficient nitrating agent towards phenolic compounds when the reaction is performed in organic medium, whereas in aqueous solution it does not. As a consequence, we conclude that NO in combination with molecular oxygen could act as a source of nitrating agents near the lipophilic membrane, and thus could nitrate the tyrosine residues of membrane associated proteins. However, it should be noted that the reactions reported here take place at very high concentration (50 mM) compared to physiological NO concentrations, estimated to be in the nanomolar range under normal conditions, up to around $10\ \mu\text{M}$ near activated macrophages.

Experimental

The melting points were determined on a Büchi 510 and were not corrected. Optical rotations were measured using a Perkin-Elmer 241 with a 1 dm pathlength cell. Nuclear magnetic resonance spectra were recorded on Bruker instruments (250 or 400 MHz for ^1H). Electron paramagnetic resonance spectra were recorded on a Bruker ER 200D spectrometer at 9.71 GHz. Ultraviolet spectra were recorded with a UVIKON 931 spectrophotometer (Kontron). Elemental analysis and electrospray mass spectra were carried out at the Centre de Recherche Pierre Fabre (Castres, France). *L*-Cysteine ethyl ester hydrochloride, *tert*-butyl nitrite, iodine, DMSO, Mohr's salt and *N*-methyl-*D*-glucamine came from Aldrich. Oxalyl chloride was purchased from Lancaster and all carboxylic acids from Fluka. *N*-Methyl-*D*-glucamine dithiocarbamate (MGD) was synthesized from *N*-methyl-*D*-glucamine and carbon disulphide, and purified by recrystallization from 10% acetone in water as previously published.²⁶ DMF used for preparation of thiols was freshly distilled over CaH_2 . Analytical thin layer chromatography was carried out on 60 Å–17 μm silica plates from SDS Co. All purifications by flash chromatography were carried out with 60 Å silica from SDS Co.

Synthetic Procedures The thiols studied in this work (**1**–**5**) were prepared from the same starting material, cysteine ethyl ester hydrochloride, which was coupled to the corresponding aromatic acyl chloride in moderate yields.

General Procedure for Preparation of *N*-Aroylcysteine Derivatives

1–5 To a solution of carboxylic acid (18.4 mmol) in a mixture of dichloromethane (200 ml) and DMF (50 ml) was added dropwise a solution of oxalyl chloride (18.4 mmol) in dichloromethane (20 ml) at 5 °C. The resulting mixture was stirred for 30 min at room temperature and then added dropwise to a solution of *L*-cysteine ethyl ester hydrochloride (18.4 mmol) in dichloromethane (100 ml), pyridine (20 ml) and DMF (50 ml). The reaction mixture was stirred at room temperature for 20 h and then washed several times with hydrochloric acid (1 M). The organic layer was separated, dried over MgSO_4 , filtered and concentrated to dryness. The crude product was then purified by column chromatography (eluent: CH_2Cl_2).

N-Benzoyl-*L*-cysteine Ethyl Ester (**1**)²⁷: White solid (65%), mp 75 °C. $[\alpha]_D -33^\circ$ ($c=3.72$, MeOH). UV λ_{max} (CH_3CN) nm (ϵ): 246 (3545), 268 (2485). IR (KBr) cm^{-1} : 3280 (NH), 2560 (SH), 1730 (CO ester), 1630 (CO amide), 1600, 1580, 1515, 1490 (ar. C=C). $^1\text{H-NMR}$ (200 MHz; CDCl_3) δ : 1.33 (3H, t, $J=7.2$ Hz, CH_3), 1.36 (1H, t, $J=9.0$ Hz, SH), 3.16 (2H, m, CH_2S), 4.30 (2H, m ABX₃, $J=7.2$ Hz, CH_2O), 5.06 (1H, dt, $J=7.3$, 4.1 Hz, CH), 7.06 (1H, br d, $J=7.3$ Hz, NH), 7.48 (3H, m, 2×*m*-H and *p*-H) and 7.84 (2H, dd, $J=8.1$, 1.7 Hz, 2×*o*-H). $^{13}\text{C-NMR}$ (50.3 MHz; CDCl_3) δ : 14.3 (CH_3), 27.1 (CH_2S), 54.1 (CH), 62.2 (CH_2O), 127.2 (2×*m*-CH), 128.8 (2×*o*-CH), 132.1 (*p*-CH), 133.8 (C=O amide), 167.0 (CO amide), 170.3 (CO ester). MS (Cl/NH_3) m/z : 271 (MNH_4^+ , 45%), 254 (MH^+ , 100%).

N-(3,4-Dimethoxybenzoyl)-*L*-cysteine Ethyl Ester (**2**): White solid (45%). mp 117 °C. $[\alpha]_D -21^\circ$ ($c=5.32$, MeOH). UV λ_{max} (CH_3CN) nm (ϵ): 257 (15000), 288 (7270). IR (KBr) cm^{-1} : 3290 (NH), 2580 (SH), 1725 (CO ester), 1635 (CO amide), 1600, 1580, 1540, 1510 (ar. C=C). $^1\text{H-NMR}$ (250 MHz; CDCl_3) δ : 1.32 (3H, t, $J=7.1$ Hz, CH_3), 1.38 (1H, t, $J=8.8$ Hz, SH), 3.12 (2H, m, CH_2S), 3.91 (3H, s, CH_3O), 3.92 (3H, s, CH_3O), 4.28 (2H, m ABX₃, $J=7.1$ Hz, CH_2O), 5.02 (1H, dt, $J=7.0$, 4.1 Hz, CH), 6.87 (1H, d, $J=8.2$ Hz, $\text{CH-C(4)(OCH}_3\text{)}$), 7.00 (1H, br d, $J=7.0$ Hz, NH), 7.37 (1H, dd, $J=8.2$, 1.8 Hz, CH-C(CO amide)), 7.42 (1H, d, $J=1.8$ Hz, $\text{CH-C(3)(OCH}_3\text{)}$). $^{13}\text{C-NMR}$ (62.9 MHz; CDCl_3) δ : 14.2 (CH_3), 27.0 (CH_2S), 54.0 (CH), 56.1 (2× CH_3O), 62.1 (CH_2O), 110.3 ($\text{CH-C(4)(OCH}_3\text{)}$), 110.6 ($\text{CH-C(3)(OCH}_3\text{)}$), 119.8 (CH-C(CO amide)), 126.2 (C=O amide), 149.1 ($\text{C(3)(OCH}_3\text{)}$), 152.2 ($\text{C(4)(OCH}_3\text{)}$), 167.0 (CO amide), 170.3 (CO ester). Anal. Calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_5\text{S}$: C, 53.66; H, 6.11; N, 4.47. Found: C, 53.44; H, 5.86; N, 4.49.

N-(3,5-Di-*tert*-butyl-4-hydroxybenzoyl)-*L*-cysteine Ethyl Ester (**3**): White solid (57%). mp 164 °C. $[\alpha]_D -22^\circ$ ($c=2.81$, MeOH). UV λ_{max} (CH_3CN) nm (ϵ): 254 (13860), 312 (3220). IR (KBr) cm^{-1} : 3400 (OH), 3300 (NH), 2960 (C-H), 2560 (SH), 1725 (CO ester), 1630 (CO amide), 1600, 1535, 1435 (ar. C=C). $^1\text{H-NMR}$ (200 MHz; CDCl_3) δ : 1.32 (3H, t, $J=7.1$ Hz, CH_3), 1.36 (1H, t, $J=9.1$ Hz, SH), 1.45 (18H, s, 2×*t*-Bu), 3.13 (2H, m, CH_2S), 4.28 (2H, m ABX₃, $J=7.1$ Hz, CH_2O), 5.02 (1H, dt, $J=6.9$, 4.1 Hz, CH), 5.58 (1H, br s, OH), 6.91 (1H, br d, $J=6.9$ Hz, NH), 7.64 (2H, s, ArH). $^{13}\text{C-NMR}$ (50.3 MHz; CDCl_3) δ : 14.3 (CH_3), 27.1 (CH_2S), 30.2 (2× $\text{C(CH}_3\text{)}_3$), 34.5 (2× $\text{C(CH}_3\text{)}_3$), 54.0 (CH), 62.2 (CH_2O), 124.4 (2×ArCH), 124.9 (C=O amide), 136.1 (2× $\text{C(CH}_3\text{)}_3$), 157.3 (COH), 167.8 (CO amide), 170.6 (CO ester). Anal. Calcd for $\text{C}_{20}\text{H}_{31}\text{NO}_4\text{S}$: C, 62.96; H, 8.19; N, 3.67. Found: C, 62.88; H, 8.21; N, 3.70.

N-(4-Hydroxy-3-methoxybenzoyl)-*L*-cysteine Ethyl Ester (**4**): White solid (50%). mp 106 °C. $[\alpha]_D -23^\circ$ ($c=4.34$, MeOH). UV λ_{max} (CH_3CN) nm (ϵ): 254 (14400), 288 (6389). IR (KBr) cm^{-1} : 3350 (NH and OH), 2970 (C-H), 2560 (SH), 1735 (CO ester), 1620 (CO amide), 1600, 1580, 1500, 1430 (ar. C=C). $^1\text{H-NMR}$ (200 MHz; CDCl_3) δ : 1.30 (3H, t, $J=7.1$ Hz, CH_3), 1.37 (1H, t, $J=9.1$ Hz, SH), 3.09 (2H, m, CH_2S), 3.90 (3H, s, CH_3O), 4.26 (2H, m ABX₃, $J=7.1$ Hz, CH_2O), 5.00 (1H, dt, $J=7.0$, 3.8 Hz, CH), 6.21 (1H, br s, OH), 6.91 (1H, d, $J=8.4$ Hz, CH-C(OH)), 6.99 (1H, br d, $J=7.0$ Hz, NH), 7.30 (1H, dd, $J=8.4$, 1.8 Hz, CH-C(CO amide)), 7.41 (1H, d, $J=1.8$ Hz, $\text{CH-C(OCH}_3\text{)}$). $^{13}\text{C-NMR}$ (50.3 MHz; CDCl_3) δ : 14.3 (CH_3), 27.1 (CH_2S), 54.1 (CH), 56.2 (CH_3O), 62.2 (CH_2O), 110.5 ($\text{CH-C(OCH}_3\text{)}$), 114.1 (CH-C(OH)), 120.3 (CH-C(CO amide)), 125.9 (C=O amide), 146.7 (COH), 149.3 (C=OCH_3), 166.6 (CO amide), 170.4 (CO ester). Anal. Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_5\text{S}$: C, 52.16; H, 5.72; N, 4.68. Found: C, 52.27; H, 5.86; N, 4.67.

N-(3-Hydroxy-4-methoxybenzoyl)-*L*-cysteine Ethyl Ester (**5**): White solid (46%). mp 101 °C. $[\alpha]_D -22^\circ$ ($c=4.08$, MeOH). UV λ_{max} (CH_3CN) nm (ϵ): 254 (11000), 288 (6000). IR (KBr) cm^{-1} : 3100–3600 (NH and OH), 1710 (CO ester), 1630 (CO amide), 1580, 1500, 1450 (ar. C=C). $^1\text{H-NMR}$ (200 MHz; CDCl_3) δ : 1.28 (3H, t, $J=7.1$ Hz, CH_3), 1.36 (1H, t, $J=9.0$ Hz,

SH), 3.08 (2H, m, CH₂S), 3.89 (3H, s, CH₃O), 4.24 (2H, m ABX₃, $J = 7.1$ Hz, CH₂O), 4.99 (1H, dt, $J = 7.0, 3.7$ Hz, CH), 6.30 (1H, brs, OH), 6.84 (1H, d, $J = 8.4$ Hz, CH–C(OCH₃)), 6.99 (1H, br d, $J = 7.0$ Hz, NH), 7.36 (1H, dd, $J = 8.4, 2.2$ Hz, CH–C(CO amide)), 7.39 (1H, d, $J = 2.2$ Hz, CH–C(OH)). ¹³C-NMR (50.3 MHz; CDCl₃) δ : 14.3 (CH₃), 27.1 (CH₂S), 54.0 (CH), 56.2 (CH₃O), 62.2 (CH₂O), 110.2 (CH–C(OCH₃)), 113.5 (CH–C(OH)), 120.1 (CH–C(CO amide)), 126.9 (CCO amide), 145.7 (COH), 149.7 (COCH₃), 166.5 (CO amide), 170.4 (CO ester). *Anal.* Calcd for C₁₃H₁₇NO₅S: C, 52.16; H, 5.72; N, 4.68. Found: C, 51.89; H, 5.75; N, 4.64.

General Procedure for Preparation of *N*-Aroylcysteine Derivatives 11–15 To a suspension of thiol (0.35 mmol) in toluene (5 ml) was added DMSO (150 μ l) and a catalytic amount of iodine.²⁸ After a few minutes under vigorous stirring at room temperature, the reaction mixture was diluted with dichloromethane (30 ml) and washed with a saturated solution of sodium thiosulfate (20 ml) and brine. The organic layer was dried over MgSO₄, filtered, and then concentrated to dryness. All disulphides were obtained in quantitative yields.

N-Benzoyl-L-cystine Ethyl Ester (11): White solid. mp 182 °C. [α]_D –150° ($c = 3.57$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 225 (40 000). IR (KBr) cm^{–1}: 3330 (NH), 1750 (CO ester), 1635 (CO amide), 1605, 1580, 1525, 1490 (ar. C=C). ¹H-NMR (250 MHz; CDCl₃) δ : 1.29 (6H, t, $J = 7.0$ Hz, 2×CH₃), 3.36 (4H, d, $J = 5.2$ Hz, 2×CH₂S), 4.24 (4H, m ABX₃, $J = 7.0$ Hz, 2×CH₂O), 5.04 (2H, dt, $J = 7.3, 5.2$ Hz, 2×CH), 7.10 (2H, br d, $J = 7.3$ Hz, 2×NH), 7.45 (6H, m, 4×*m*-H and 2×*p*-H), 7.81 (4H, dd, $J = 8.4, 1.4$ Hz, 4×*o*-H). ¹³C-NMR (62.9 MHz; CDCl₃) δ : 14.1 (2×CH₃), 41.1 (2×CH₂S), 52.5 (2×CH), 62.2 (2×CH₂O), 127.2 (4×*m*-CH), 128.6 (4×*o*-CH), 131.9 (2×*p*-CH), 133.6 (2×CCO amide), 167.1 (2×CO amide), 170.4 (2×CO ester). FAB-MS m/z : 505 (MH⁺, 44%).

N-(3,4-Dimethoxybenzoyl)-L-cystine Ethyl Ester (12): White solid. mp 161 °C. [α]_D –75° ($c = 2.43$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 216 (48000), 259 (27000), 289 (17000). IR (KBr) cm^{–1}: 3320 (NH), 1750–1730 (CO esters), 1640 (CO amide), 1605, 1585, 1535, 1510, 1465 (ar. C=C). ¹H-NMR (300 MHz; CD₃COCD₃) δ : 1.25 (6H, t, $J = 7.1$ Hz, 2×CH₃), 3.25 (2H, dd ABX, $J = 13.9, 8.8$ Hz, 2×CHS), 3.40 (2H, dd ABX, $J = 13.9, 5.0$ Hz, 2×CHS), 3.83 (6H, s, 2×CH₃O), 3.85 (6H, s, 2×CH₃O), 4.19 (4H, q, $J = 7.1$ Hz, 2×CH₂O), 4.97 (2H, dt, $J = 6.9, 5.0$ Hz, 2×CH), 6.95 (2H, d, $J = 8.4$ Hz, 2×CH–C(4)(OCH₃)), 7.51 (4H, d, dd, $J = 8.4, 2.1$ Hz, 2×CH–C(3)(OCH₃) and 2×CH–C(CO amide)), 8.05 (2H, br d, $J = 6.9$ Hz, 2×NH). ¹³C-NMR (75.5 MHz; CD₃COCD₃) δ : 14.0 (2×CH₃), 40.3 (2×CH₂S), 53.0 (2×CH), 55.6 (4×CH₃O), 61.5 (2×CH₂O), 111.1 (2×CH–C(4)(OCH₃)), 111.3 (2×CH–C(3)(OCH₃)), 120.9 (2×CH–C(CO amide)), 126.7 (2×CCO amide), 149.4 (2×C(3)OCH₃), 152.7 (2×C(4)OCH₃), 166.8 (2×CO amide), 170.9 (2×CO ester). FAB-MS m/z : 625 (MH⁺, 4%).

N-(3,5-di-*tert*-Butyl-4-hydroxybenzoyl)-L-cystine Ethyl Ester (13): White solid. mp 220 °C. [α]_D –123° ($c = 4.38$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 213 (50000), 259 (30000). IR (KBr) cm^{–1}: 3625–3580 (OH), 3240 (NH), 2945 (CH) 1735, 1720 (CO esters), 1635, 1630 (CO amides), 1600, 1530, 1430 (ar. C=C). ¹H-NMR (250 MHz; CDCl₃) δ : 1.28 (6H, t, $J = 7.1$ Hz, 2×CH₃), 1.43 (36H, s, 4×*t*-Bu), 3.36 (4H, d, $J = 5.1$ Hz, 2×CH₂S), 4.22 (4H, m ABX₃, $J = 7.1$ Hz, 2×CH₂O), 5.02 (2H, dt, $J = 7.3, 5.1$ Hz, 2×CH), 5.57 (2H, brs, 2×OH), 6.98 (2H, br d, $J = 7.3$ Hz, 2×NH), 7.65 (4H, s, ArH). ¹³C-NMR (62.9 MHz; CDCl₃) δ : 14.1 (2×CH₃), 30.1 (4×C(CH₃)₃), 34.4 (4×C(CH₃)₃), 41.0 (2×CH₂S), 52.6 (2×CH), 62.0 (2×CH₂O), 124.5 (4×ArCH), 124.7 (2×CCO amide), 135.9 (4×C(*t*-Bu)), 157.2 (2×COH), 167.8 (2×CO amide), 170.6 (2×CO ester). *Anal.* Calcd for C₄₀H₆₀N₂O₈S₂: C, 63.13; H, 7.95; N, 3.68. Found: C, 63.01; H, 8.10; N, 3.64.

N-(4-Hydroxy-3-methoxybenzoyl)-L-cystine Ethyl Ester (14): White solid. mp 169 °C. [α]_D –134° ($c = 4.62$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 216 (50000), 259 (30000). IR (KBr) cm^{–1}: 3480–3400 (OH), 3300 (NH), 2980 (C–H), 1750, 1720 (CO esters), 1640 (CO amides), 1600, 1570, 1515, 1470 (ar. C=C). ¹H-NMR (250 MHz; CDCl₃) δ : 1.29 (6H, t, $J = 7.1$ Hz, 2×CH₃), 3.34 (4H, d, $J = 5.1$ Hz, 2×CH₂S), 3.90 (6H, s, 2×CH₃O), 4.23 (4H, m ABX₃, $J = 7.1$ Hz, 2×CH₂O), 5.01 (2H, dt, $J = 7.0, 5.1$ Hz, 2×CH), 5.97 (2H, brs, 2×OH), 6.88 (2H, d, $J = 8.4$ Hz, 2×CH–C(OH)), 7.01 (2H, br d, $J = 7.0$ Hz, 2×NH), 7.30 (2H, dd, $J = 8.4, 1.8$ Hz, 2×CH–C(CO amide)), 7.43 (2H, d, $J = 1.8$ Hz, 2×CH–C(OCH₃)). ¹³C-NMR (62.9 MHz; CDCl₃) δ : 14.1 (2×CH₃), 41.1 (2×CH₂S), 52.5 (2×CH), 56.1 (2×CH₃O), 62.1 (2×CH₂O), 110.4 (2×CH–C(OCH₃)), 114.0 (2×CH–C(OH)), 120.4 (2×CH–C(CO amide)), 125.7 (2×CCO amide), 146.6 (2×COH), 149.1 (2×COCH₃), 166.6 (2×CO amide), 170.6 (2×CO ester). *Anal.* Calcd for C₂₆H₃₂N₂O₁₀S₂: C, 52.34; H, 5.41; N, 4.69. Found: C, 52.46; H, 5.36; N, 4.52.

N-(3-Hydroxy-4-methoxybenzoyl)-L-cystine Ethyl Ester (15): White solid. mp 162 °C. [α]_D –141° ($c = 6.25$, MeOH). UV $\lambda_{\max}$ (MeOH) nm (ϵ): 216

(50000), 257 (30000), 293 (20000). IR (KBr) cm^{–1}: 3480–3410 (OH), 3310 (NH), 1750, 1730 (CO esters), 1630 (CO amide), 1590, 1530, 1510, 1460 (ar. C=C). ¹H-NMR (250 MHz; CDCl₃) δ : 1.29 (6H, t, $J = 7.1$ Hz, 2×CH₃), 3.34 (4H, m, 2×CH₂S), 3.93 (6H, s, 2×CH₃O), 4.24 (4H, q, $J = 7.1$ Hz, 2×CH₂O), 5.01 (2H, dt, $J = 7.0, 5.1$ Hz, 2×CH), 6.03 (2H, brs, 2×OH), 6.85 (2H, d, $J = 8.0$ Hz, 2×CH–C(OCH₃)), 6.98 (2H, br d, $J = 7.0$ Hz, 2×NH), 7.38 (4H, d and dd, $J = 8.0, 2.0$ Hz, 2×CH–C(CO amide) and 2×CH–C(OH)). ¹³C-NMR (62.9 MHz; CDCl₃) δ : 13.7 (2×CH₃), 40.7 (2×CH₂S), 51.9 (2×CH), 55.3 (2×CH₃O), 60.8 (2×CH₂O), 110.2 (2×CH–C(OCH₃)), 114.4 (2×CH–C(OH)), 119.0 (2×CH–C(CO amide)), 126.0 (2×CCO amide), 145.6 (2×COH), 150.1 (2×COCH₃), 166.5 (2×CO amide), 170.3 (2×CO ester). HRMS m/z : 596.1500 (Calcd for C₂₆H₃₂N₂O₁₀S₂: 596.1498).

General Procedure for Preparation of *S*-Nitrosothiols 6–10 Method a^{29–31}: Neat *tert*-butyl nitrite (1 mmol) was added dropwise to a solution of thiol (1 mmol) in acetonitrile (20 ml) or in water/MeOH (5:1) (20 ml) at room temperature. After complete addition of the reagent, the mixture turned red indicating formation of a SNO function.

Method b^{32,33}: A solution of sodium nitrite (1 mmol) in water (3 ml) was added to a solution of thiol (1 mmol), hydrochloric acid (1 M) (1.2 ml) and concentrated sulphuric acid (115 μ l) in methanol (5 ml) at room temperature. After complete addition of the reagent the reaction mixture turned red indicating formation of a SNO function.

N-Benzoyl-L-S-nitrosocysteine Ethyl Ester (6): UV $\lambda_{\max}$ (CH₃CN) nm (ϵ): 336 (900), 545 (16). ¹H-NMR (300 MHz; CD₃CN) δ : 1.26 (3H, t, $J = 7.1$ Hz, CH₃), 4.12–4.27 (4H, q and m, $J = 7.1$ Hz, CH₂O and CH₂S), 4.80 (1H, m, CH), 7.38 (1H, br d, $J = 5.3$ Hz, NH), 7.47 (3H, m, 2×*o*-H and *p*-H), 7.76 (2H, dd, $J = 8.4, 1.4$ Hz, 2×*m*-H).

N-(3,4-Dimethoxybenzoyl)-L-S-nitrosocysteine Ethyl Ester (7): UV $\lambda_{\max}$ (CH₃CN) nm (ϵ): 334 (930), 545 (20). ¹H-NMR (200 MHz; CD₃CN) δ : 1.24 (3H, t, $J = 7.1$ Hz, CH₃), 3.84, 3.85 (6H, 2×s, 2×OCH₃), 4.13–4.25 (4H, q and m, $J = 7.1$ Hz, CH₂O and CH₂S), 4.76 (1H, dt, $J = 7.0, 5.1$ Hz, CH), 6.97 (1H, d, $J = 8.4$ Hz, CH–C(4)(OCH₃)), 7.29 (1H, br d, $J = 7.0$ Hz, NH), 7.34 (2H, dd and d, $J = 8.4, 2.2$ Hz, CH–C(CO amide) and CH–C(3)(OCH₃)).

N-(3,5-Di-*tert*-Butyl-4-hydroxybenzoyl)-L-S-nitrosocysteine Ethyl Ester (8): UV $\lambda_{\max}$ (CH₃CN) nm (ϵ): 335 (880), 545 (20). ¹H-NMR (200 MHz; CD₃CN) δ : 1.24 (3H, t, $J = 7.1$ Hz, CH₃), 1.42 (18H, s, 2×*t*-Bu), 4.13–4.23 (4H, q and m, $J = 7.1$ Hz, CH₂O and CH₂S), 4.74 (1H, dt, $J = 8.0, 5.1$ Hz, CH), 5.95 (1H, brs, OH), 7.32 (1H, br d, $J = 8.0$ Hz, NH), 7.53 (2H, s, ArH).

N-(4-Hydroxy-3-methoxybenzoyl)-L-S-nitrosocysteine Ethyl Ester (9): UV $\lambda_{\max}$ (CH₃CN) nm (ϵ): 335 (900), 545 (19). ¹H-NMR (200 MHz; CDCl₃) δ : 1.25 (3H, t, $J = 7.1$ Hz, CH₃), 3.89 (3H, s, CH₃O), 4.12–4.23 (4H, q and m, $J = 7.1$ Hz, CH₂O and CH₂S), 4.77 (1H, dt, $J = 7.1, 5.1$ Hz, CH), 6.86 (1H, d, $J = 8.4$ Hz, CH–C(OH)), 7.27 (2H, dd and br d, $J = 8.4, 7.1, 2.0$ Hz, CH–C(CO amide) and NH), 7.34 (1H, d, $J = 2.0$ Hz, CH–C(OCH₃)).

N-(3-Hydroxy-4-methoxybenzoyl)-L-S-nitrosocysteine Ethyl Ester (10): UV $\lambda_{\max}$ (CH₃CN) nm (ϵ): 335 (1000), 545 (16). ¹H-NMR (200 MHz; CDCl₃) δ : 1.24 (3H, t, $J = 7.1$ Hz, CH₃), 3.89 (3H, s, CH₃O), 4.08–4.33 (4H, q and m, $J = 7.1$ Hz, CH₂O and CH₂S), 4.76 (1H, dt, $J = 7.0, 5.1$ Hz, CH), 6.97 (1H, d, $J = 8.4$ Hz, CH–C(OCH₃)), 7.20 (2H, d and br d, $J = 7.0, 2.2$ Hz, CH–C(OH) and NH), 7.27 (1H, dd, $J = 8.4, 2.2$ Hz, CH–C(CO amide)).

General Procedure for Preparation of Nitro Adducts To a solution of thiol (1 mmol) in acetonitrile (20 ml) was added dropwise neat *tert*-BuONO (1 mmol) at room temperature. The solution was stirred for 72 h and the solvent was evaporated under reduced pressure. For dinitro adduct 17 2 mmol of *tert*-BuONO were added.

N-(4-Hydroxy-5-methoxy-3-nitrobenzoyl)-L-cystine Ethyl Ester (16): Brown solid; IR (KBr) cm^{–1}: 3370–3280 (OH and NH), 2924 (CH), 1740 (CO esters), 1665 (CO amides), 1620, 1535, 1460 (ar. C=C), 1540, 1330 (NO₂). ¹H-NMR (400 MHz; CD₃COCD₃) δ : 1.25 (6H, t, $J = 6.9$ Hz, 2×CH₃), 3.22 (2H, dd ABX, $J = 13.9, 8.0$ Hz, 2×CHS), 3.40 (2H, dd ABX, $J = 13.9, 5.1$ Hz, 2×CHS), 3.96 (6H, s, 2×CH₃O), 4.18 (4H, q, $J = 6.9$ Hz, 2×CH₂O), 4.98 (2H, dt, $J = 8.0, 5.1$ Hz, 2×CH), 7.69 (2H, d, $J = 2.0$ Hz, 2×CH–C(OCH₃)), 8.13 (2H, d, $J = 2.0$ Hz, 2×CH–C(NO₂)), 8.45 (2H, br d, $J = 8.0$ Hz, 2×NH). ¹³C-NMR (50.3 MHz; CD₃COCD₃) δ : 13.2 (2×CH₃), 39.2 (2×CH₂S), 52.2 (2×CH), 55.9 (2×CH₃O), 60.8 (2×CH₂O), 116.1 (2×CH–C(OCH₃)), 116.3 (2×CH–C(NO₂)), 126.0 (2×CCO amide), 134.0 (2×CNO₂), 147.0 (2×COH), 149.5 (2×COCH₃), 164.3 (2×CO amide), 169.9 (2×CO ester). ESI⁺ (MeOH) m/z : 709.1 (MNa⁺, 28%), 687.1 (MH⁺, 100%). ESI[–] (MeOH) m/z : 685.3 (M–H)[–].

N-(3-Hydroxy-4-methoxy-2,6-dinitrobenzoyl)-L-cystine Ethyl Ester (17): Brown solid; ¹H-NMR (400 MHz; CD₃COCD₃) δ : 1.32 (6H, t, $J = 7.1$ Hz, 2×CH₃), 3.26 (4H, m, 2×CH₂S), 4.10 (6H, s, 2×OCH₃), 4.21 (4H, q, $J =$

7.1 Hz, $2\times\text{CH}_2\text{O}$), 4.93 (2H, dt, $J=7.9$, 6.0 Hz, $2\times\text{CH}$), 7.87 (2H, s, $2\times\text{Ar-H}$), 8.4 (2H, br d, $J=7.9$ Hz, $2\times\text{NH}$). ESI^+ (MeOH) m/z : 799.2 (MNa^+ , 100%) 777.2 (MH^+ , 90%).

UV Experiments To a solution of thiol (0.1 mmol) in acetonitrile (2 ml) was added neat *tert*-butyl nitrite (14 μl , 0.1 mmol) at room temperature, and the disappearance of the absorbance band at 545 nm was followed immediately.

NMR Experiments The spectrum of a solution of thiol (2.5×10^{-5} mol) in deuteriated acetonitrile (500 μl) was recorded. Then neat *tert*-butyl nitrite (14 μl , 0.1 mmol) was added at room temperature, and a new spectrum was recorded immediately. The same operation was done regularly.

EPR Experiments To a solution of thiol (2.9×10^{-5} mol) in DMF (580 μl) was added neat *tert*-butyl nitrite (4 μl , 2.9×10^{-5} mol) at room temperature. After an hour this solution (50 μl) was diluted in acetonitrile (450 μl) and the resultant solution (5 μl) was added to a mixture of acetonitrile (200 μl), an aqueous solution of Mohr's salt (10 μl , 5×10^{-7} mol) and an aqueous solution of MGD (50 μl , 2.5×10^{-6} mol). This reaction mixture was placed in a capillary of 50 μl , which was placed in the spectrometer cavity.

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References

- Radomski M. W., Palmer R. M. J., Moncada S., *Br. J. Pharmacol.*, **92**, 181—187 (1987).
- Furchgott R. F., Vanhoutte P. M., *EASEB J.*, **3**, 2007—2018 (1989).
- Lancaster J. R., Jr., Hibbs J. B., Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1223—1227 (1990).
- Marietta M., Facchinetti F., Neri I., Piccinini F., Volpe A., Torelli G., *Thrombosis Res.*, **88**, 229—235 (1997).
- Garthwaite J., *Trends in Neurosci.*, **14**, 60—67 (1991).
- Culotta E., Koshland D. E., Jr., *Science*, **258**, 1862—1865 (1992).
- Stamler J. S., Singel D. J., Loscalzo J., *Science*, **258**, 1898—1902 (1992).
- Stamler J. S., Jaraki O., Osborne J., Simon D. I., Keaney J., Vita J., Singel D., Valeri C. R., Loscalzo J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7674—7677 (1992).
- Oae S., Shinham K., *Org. Prep. Proc. Int.*, **15**, 167—198 (1983).
- Williams D. L. H., *Chem. Soc. Rev.*, **14**, 171—196 (1985).
- Beckman J. S., Koppenol W. H., *Am. J. Physiol. U.S.A.*, **271**, C1424—C1437 (1996).
- Petit C., Hoffmann P., Souchard J.-P., Labidalle S., *Phosphorus, Sulfur and Silicon*, **129**, 59—67 (1997).
- Jovanovic S. V., Steenken S., Tosic M., Marjanovic B., Simic M. G., *J. Am. Chem. Soc.*, **116**, 4846—4851 (1994).
- Rice-Evans C. A., Miller N. J., Paganga G., *Free Radic. Biol. Med.*, **20**, 933—956 (1996).
- Roy B., d'Hardemare A. M., Fontecave M., *J. Org. Chem.*, **59**, 7019—7026 (1994).
- McAninly J., Williams D. L. H., Askew S. C., Butler A. R., Russell C., *J. Chem. Soc., Chem. Commun.*, **1993**, 1758—1759.
- Askew S. C., Barnett D. J., McAninly J., Williams D. L. H., *J. Chem. Soc., Perkin Trans. 2*, **1995**, 741—745.
- Dicks A. P., Swift H. R., Williams D. L. H., Butler A. R., Al-Sa'doni H. H., Cox B. G., *J. Chem. Soc., Perkin Trans. 2*, **1996**, 481—487.
- Stamler J. S., Feelisch M., "Preparation and Detection of *S*-nitrosothiols" In "Methods in Nitric Oxide Research," ed. by Feelisch M., Stamler J. S., Wiley, New York, 1996, pp. 521—539.
- Komarov A., Mattson D., Jones M. M., Singh P. K., Lai C.-S., *Biochem. Biophys. Res. Commun.*, **195**, 1191—1198 (1993).
- Janzen E. G., Wilcox A. L., Manoharan V., *J. Org. Chem.*, **58**, 3597—3599 (1993).
- Goldstein S., Czapski G., Lind J., Merenyi G., *J. Biol. Chem.*, **275**, 3031—3036 (2000).
- Balavoine G. G. A., Geletii Y. V., Bejan D., *Nitric Oxide: Biol. Chem.*, **1**, 507—521 (1997).
- Daiber A., Mehl M., Ullrich V., *Nitric Oxide: Biol. Chem.*, **2**, 259—269 (1998).
- Oh S. M. N. Y. F., Williams L. H., *J. Chem. Soc., Perkin Trans. 2*, **1991**, 685—688.
- Shinobu L. A., Jones S. G., Jones M. M., *Acta Pharm. Toxicol.*, **54**, 189—194 (1984).
- Ruf M., Burth R., Weis K., Vahrenkamp H., *Chem. Ber.*, **129**, 1251—1257 (1996).
- Aida T., Akasaka T., Furukawa N., Oae S., *Bull. Chem. Soc. Jpn.*, **49**, 1441—1442 (1976).
- Crookes M. J., Williams D. L. H., *J. Chem. Soc., Perkin Trans. 2*, **1988**, 1339—1343.
- Patel H. M. S., Williams D. L. H., *J. Chem. Soc., Perkin Trans. 2*, **1989**, 339—341.
- Crookes M. J., Williams D. L. H., *J. Chem. Soc., Perkin Trans. 2*, **1989**, 759—763.
- Hart T. W., *Tetrahedron Lett.*, **26**, 2013—2016 (1985).
- Field L., Dils R. V., Ravichandran R., Lenhart P. G., Carnahan G. E., *J. Chem. Soc., Chem. Commun.*, **1978**, 249—250.

Study of the Melt Pelletization Process Focusing on the Micromeritic Property of Pellets

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Melt pelletization of lactose 450 M was carried out in an 8-l high shear mixer using PEG 3000 as the meltable binder. The pore size and size distribution of the melt pellets were determined using mercury intrusion porosimetry. The pore size distribution of melt pellets was found to be bimodal. With a higher binder concentration, post-melt impeller speed or longer post-melt processing time, the fraction of large pores in the agglomerates was reduced but the tendency of the agglomerates to develop sub-micron pores increased. The extent of formation of large pores was dependent on the interplay between the inter-particle distance of lactose particles and the contraction property of molten binder. High process temperature was associated with a greater amount of water loss from the melt agglomerates. The water vapor liberated from the lactose particles, was trapped in the molten PEG during the pelletization process. The formation of sub-micron pores was a result of escape of this water vapor on solidification of the molten PEG as well as agglomerate densification. The quantity of sub-micron pores produced was found to be related to the level of water loss. The melt agglomeration gave rise to large agglomerates when long post-melt processing time, high post-melt impeller speed or binder concentration was used.

Key words high shear mixer; melt pelletization; micromeritic property; pore

Methods for producing highly spherical pharmaceutical pellets include balling, extrusion-spheronization and rotary processing.¹⁾ These methods require the use of solvents. In recent years, melt pelletization has gained interest as an alternative to wet pelletization for producing high quality pellets and may be applied for water-sensitive drugs. The melt process has a major advantage of circumventing the use of volatile organic solvents commonly associated with processing water-sensitive materials.

Investigations into the production technique of melt pellets were often directed at the gross characteristics such as pellet size, size distribution, shape and porosity.^{2–8)} The influence of production variables such as processing time, impeller speed and binder concentration was evaluated through the characteristics of the resultant pellets. There had been no discussion on the internal structure of the melt pellets produced. This paper reports a study on the effects of production variables on the intra-pellet pore size distribution of melt pellets. It is hoped that with the information on the pore size distribution of the pellets, a better understanding of the formative process of melt pellets can be obtained.

Experimental

Materials Lactose (α -monohydrate lactose, Pharmatose 450 M, DMV, The Netherlands) was used as the bulk material with polyethylene glycol 3000 (PEG 3000, Hoechst, Germany) as the meltable binder. Lactose and PEG 3000 had mean particle sizes of 22 and 34 μ m, respectively (2600c with dry powder feeder, Malvern, U.K.). The melting point of PEG 3000 was 59.5–62.7 °C (capillary method, Gallenkamp, U.K.).

Preparation of Pellets A load of lactose monohydrate (1 kg) and PEG 3000 (expressed as the percentage weight of lactose) was used. The lactose and binder were first manually mixed for 5 min. The mixture was then transferred to and dry-mixed in an eight-litre high shear mixer (Pellmix PL 1/8, Niro, Denmark) at an impeller speed of 1300 rpm using curved-tip blades.²⁾ Outputs for impeller speed, jacket temperature and product temperature were recorded using a 3-pen recorder (LR92620, Linear, U.S.A.). The high impeller speed produced shear friction that increased the product temperature to the melting point of the binder. The time of onset of melting was read at an inflection point on the product temperature–time profile of the recorder tracing.⁸⁾ The post-melt processing time was the time taken from the point of inflection indicating the binder melting to the end of the run. Where re-

quired, the post-melt impeller speed was changed after two minutes of post-melt processing.

At the start of the run, the water jacket was maintained at 30 °C. One minute into the post-melt processing, it was increased to 80 °C to prevent adhesion of material onto the mixer wall. On completion of each run, the pellets were immediately collected, weighed and then spread out in thin layers on trays to cool.

The melt pellets were prepared using different binder concentrations and at various post-melt impeller speeds and processing times. The binder concentrations and processing conditions are shown in Table 1. The pellets were subjected to micromeritic testing, water content determination, size and surface morphology analysis.

Determination of Pore Size Distribution of Heat-Treated Lactose, Lactose-PEG 3000 Physical Mixture and Unheated Lactose A weighed amount of lactose was oven-heated at 105 °C for 21 min. A mixture of lactose and PEG 3000 (23% (w/w) of lactose quantity) was similarly treated. The heating time and temperature used were based on the estimated probable processing conditions encountered during the preparation of melt pellets. The micromeritic properties of heat-treated lactose, lactose-PEG 3000 physical mixture and unheated lactose were analysed using mercury intrusion porosimetry.

Investigation of Surface Morphology of PEG-Free Lactose Pellets A weighed amount of pellets was repeatedly washed with chloroform (analytical grade, Merck, Germany) to remove the PEG 3000. The end-point of washing was established by the absence of PEG 3000 in the washing liquid as determined by Near Infra-Red (NIR) at 2488 nm using a UV-VIS-NIR spectrophotometer (UV-3101PC, Shimadzu, Japan) as described by Wong *et al.*⁸⁾ The PEG-free lactose pellets were subjected to surface morphology analysis.

Determination of Specific Volume of Molten PEG 3000 An accurately weighed amount of PEG 3000 was placed in a 5 ml measuring glass and was heated to 60, 70, 80 and 90 °C. The volume occupied by the molten PEG was read at the respective temperature. The specific volume of the molten PEG was the quotient of volume of the liquified binder to the solid PEG mass. The effect of thermal expansion of measuring glass on the measurement of specific volume of molten PEG was corrected. Measurements were in duplicate and results were averaged.

Investigation of the Effect of Water Vapor on Pore Size Distribution of PEG 3000 Film An accurately weighed amount of PEG 3000 was melted and transferred onto a Teflon sheet to cool and form a film (thickness = 0.36 ± 0.09 mm). The solidified film was removed and dried to constant weight in a desiccator. Similar films were prepared with addition of water to the molten PEG before it was poured on the Teflon sheet. The dried PEG films were subjected to micromeritic testing.

Micromeritic Analysis The pore size distribution of pellets was determined using mercury intrusion porosimetry (Poresizer 9320, Micromeritics,

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U.S.A.). The operational principle of this process is based on the Washburn equation,

$$p \cdot r = -2 \cdot \gamma \cdot \cos \theta \quad (1)$$

where p is the applied pressure, r is the radius of pore into which mercury will intrude, γ is the surface tension of mercury and θ is the contact angle between the mercury and the pore wall. A 1.5 g sample of pellets was packed into a 6.131 ml penetrometer cell and filled with mercury after air within the cell was evacuated. Intrusion pressures between 0.5 to 2000 psia were used. Measurements were in duplicate and results were averaged.

The relationship between the specific intrusion volume of mercury and the intrusion pressure was analysed. The pore sizes corresponding to the specific intrusion volumes were calculated by assuming a circular pore opening, a surface tension for mercury of 485 dyn/cm and a contact angle between the mercury and the material of 130°. The plot of log differential specific intrusion volume against pore diameter was used to characterise the pore size distribution of pellets.

Water Content Determination The water content of pellets was determined by volumetric titration using a Karl-Fischer titrator (701 KF Titrino, Metrohm, Switzerland). Hydranal® Composite 2 (Riedel-de-Haën, Germany) was used as the titrant with a mixture of methanol (analytical grade, Merck, Germany) and formamide (A.C.S. grade, J. T. Baker, Germany) as the working solvent. A sample of about 100 mg was used for each determination. The sample was powdered pellets, previously ground in a grinder. Determinations were in duplicate and results were averaged.

Size Analysis The pellets from each run were divided into random samples using a spinning riffler (PT, Retsch, Germany). A sample of about 100 g was sized using a nest of sieves of aperture sizes 1, 1.4, 2, 2.8 and 4 mm, vibrated at an amplitude of 1 mm (EVS 1, Endecotts, U.K.) for 10 min. The sample collected in the receiving pan was subsequently transferred to another set of sieves of sizes 0.075, 0.125, 0.25, 0.355, 0.5 and 0.71 mm and vibrated for 20 min. The mean size of pellets was calculated as previously reported by Heng *et al.*³⁾

Surface Morphology Analysis The surface of the pellets was examined using scanning electron microscopy and stereomicroscopy. The pellets were fixed with a carbon paste onto studs, and the prepared studs were viewed directly under a scanning electron microscope (JSM 5200, Jeol, Japan). Representative sections were photographed to show the surface morphology of the pellets. The studs were also viewed under a stereomicroscope (SZH, Olympus, Japan) and representative samples were chosen to be photographed.

Results and Discussion

During melt pelletization, the agitation of the lactose-PEG 3000 powder mixture by impeller rotation generated the heat of friction and resulted in the melting of the solid binder particles. The molten binder acted as a granulating and binding liquid. The molten binder was responsible for agglomerating the solid lactose particles into highly spherical permanent entities. Using mercury intrusion porosimetry, the pore size distributions of lactose, heated lactose and the heated lactose-PEG 3000 physical mixture were determined. The mode of pore size distribution of the lactose particles ranged from approximately 1 to 7 μm (Fig. 1). The products formed after melt pelletization were found to be devoid of the pores associated with the constituent lactose particles (Figs. 2–4). This finding suggested that the molten binder had intruded into the crevices and pores of these particles. The molten binder filled both intra and inter-particle spaces of the lactose-based pellets. The melt pellet is basically an agglomerate of solid lactose particles containing solidified molten binder interspersed within and between the lactose particles. The melt pellets of lactose and PEG 3000 were found to exhibit a bimodal pore size distribution (Figs. 2–4), they showed two large populations with pores above 10 μm and at sub-micron level in size.

With short post-melt processing times, the melt pellets formed had a greater population of large pores (Fig. 2). These pores were the result of fissures on the pellets. These

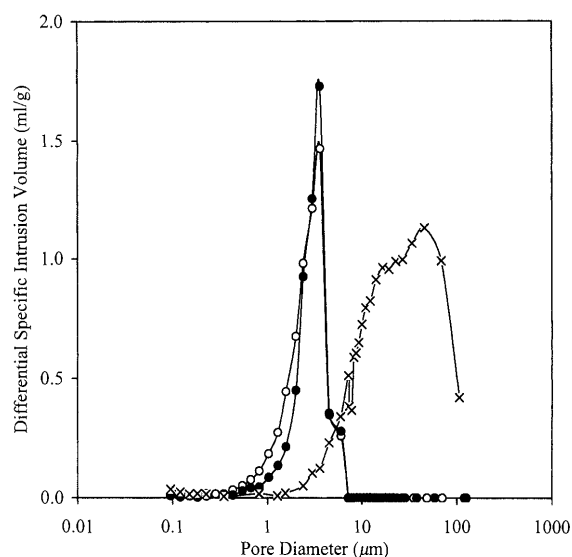


Fig. 1. Profiles of Pore Size Distribution of Lactose Powder (○), Heat-Treated Lactose Powder (●), and Lactose-PEG Physical Mixture after Heat Treatment (×)

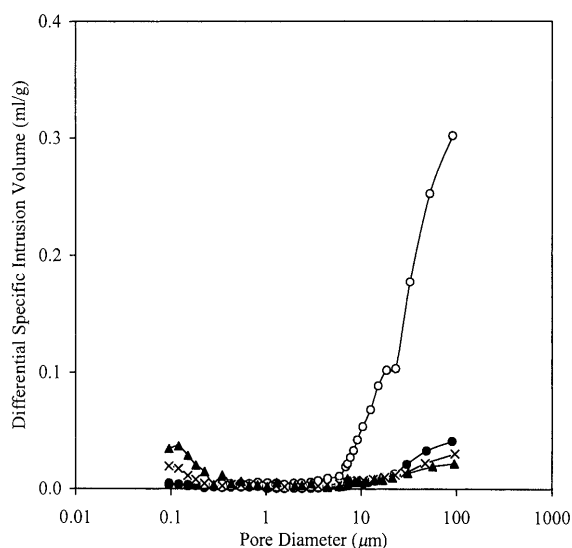


Fig. 2. Profiles of Pore Size Distribution of Pellets Prepared Using Post-Melt Processing Times of 5 (○), 13 (●), 21 (×), and 25 min (▲)

Post-melt impeller speed: 1150 rpm; PEG 3000 concentration: 23% (w/w).

fissures were the consequences of inter-particle voids not filled by the binder and loosely packed lactose-PEG masses. It is envisaged that the coalescence and growth of the agglomerates are accompanied by the molten binder reaching towards liquid saturation within the pellets. It was likely that during the pelletization process, the molten PEG was able to fill many of the inter-particle voids within each agglomerate. However, on completion of the pelletization process, cooling of pellets resulted in contraction of the volume of molten binder as the binder solidified. The contraction of binder volume on solidification in agglomerates with loosely packed lactose particles resulted in the production of a high population of fissures (Fig. 5(a)). When the volumetric contraction of molten binder on solidification was determined, the specific volume of the molten PEG was found to decrease from 0.97 ml/g at 80 °C and above to 0.96 and 0.95 ml/g when the binder temperature was reduced to 70 and 60 °C, respec-

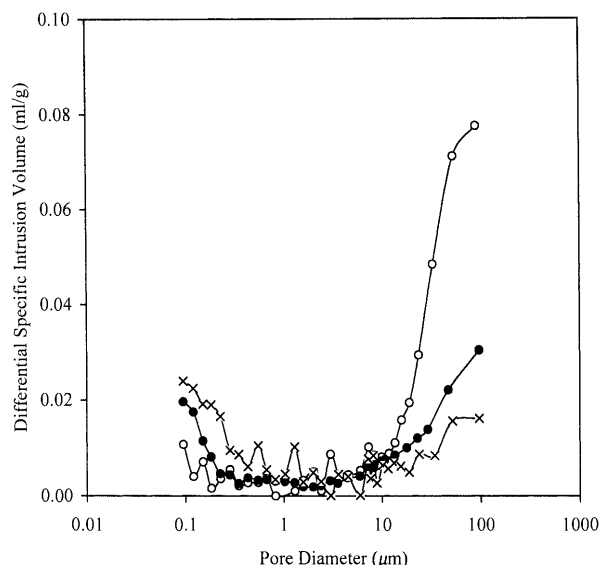


Fig. 3. Profiles of Pore Size Distribution of Pellets Prepared at Post-Melt Impeller Speeds of 850 (○), 1150 (●), and 1450 rpm (×)

Post-melt processing time: 21 min; PEG 3000 concentration: 23% (w/w).

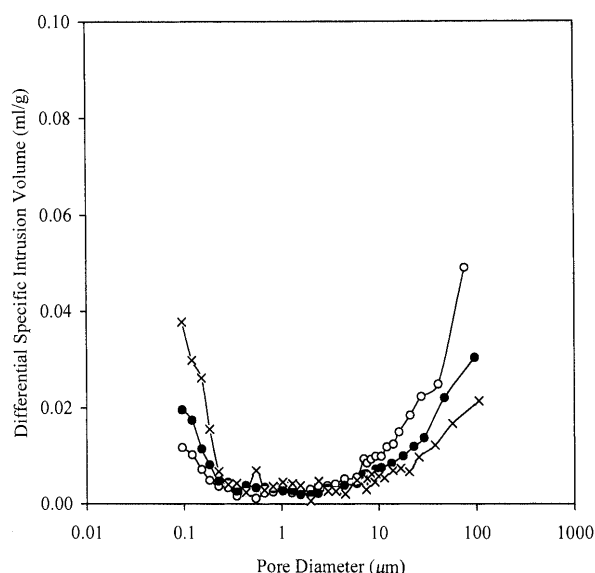


Fig. 4. Profiles of Pore Size Distribution of Pellets Prepared Using PEG 3000 Concentrations of 22 (○), 23 (●), and 24% (w/w) (×)

Post-melt processing time: 21 min; post-melt impeller speed: 1150 rpm.

tively. On cooling to 25 °C, the molten PEG solidified and showed a tendency to crack. Hence, on cooling, fissures were expected to form in pellets composed of loosely packed lactose particles.

By densification of the melt agglomerates, the population of large pores was decreased by the rearrangement of the lactose-PEG masses into the intra-agglomerate voids. The lactose-PEG masses were packed closer in agglomerates with longer post-melt processing time and, correspondingly, the amount of larger pores was gradually reduced. The densely packed lactose particles produced much smaller capillaries in the pellets and the surface tension of the molten binder in these small capillaries reduced the likelihood of formation of large pores as a result of binder contraction (Fig. 5(b)).

The fraction of large pores decreased with higher post-melt impeller speeds (Fig. 3) and binder concentrations (Fig.

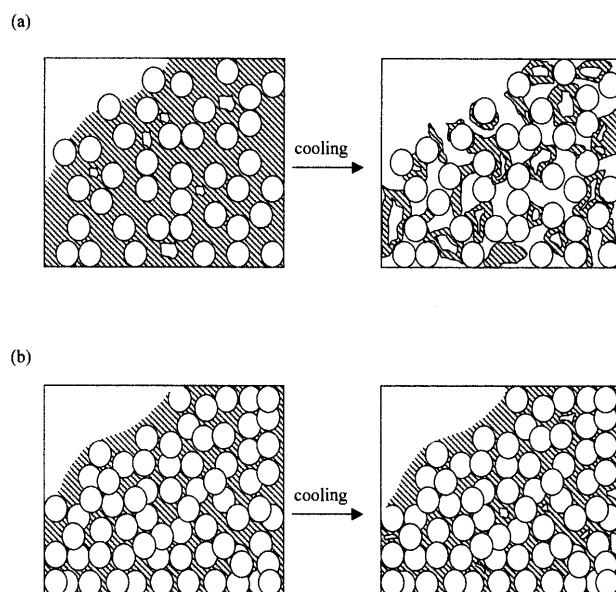


Fig. 5. Schematic Diagrams of Pellets Produced at (a) Initial and (b) Later Post-Melt Processing Phase with Respect to Volume Contraction of Molten PEG on Cooling

4). A high post-melt impeller speed increased the rate of rearrangement of the lactose-PEG masses in the agglomerates. With high binder concentrations, the increased amount of binder contributed to a greater availability of binder and produced more saturation of the voids present in the pellets.

The fraction of the sub-micron pores increased with longer post-melt processing time, higher post-melt impeller speed and binder concentration (Figs. 2–4). Heng *et al.* reported that there were two phases in the melt pelletization process.⁹⁾ In the initial phase, the agglomerates formed were smaller, irregularly shaped and porous. This could account for the large pores seen when a short post-melt processing time was used. In the later phase, the agglomerates were larger, denser and more spherical, and there were less intra-agglomerate voids available within which particles could rearrange. As the volume of large pores decreased with increased consolidation, a corresponding increase in the population of smaller pores including sub-micron pores was expected. The pore size distributions of melt pellets were found to be bimodal. The formation of the sub-micron pores was also likely to be associated with changes in the pellet constituents.

When lactose monohydrate powder was heated in an oven to 105 °C, the loss of the water of crystallization made the lactose particles more porous (Fig. 1). Nevertheless, these intra-particle lactose pores were obscured as seen with the heated physical mixture of lactose-PEG (Fig. 1) and the agglomerates (Figs. 2–4). The pores and crevices of lactose particles were filled with the molten PEG when PEG was melted.

The sub-micron pore fraction found in the melt pellets (Figs. 2–4) was absent in the heated lactose-PEG physical mixture (Fig. 1). These sub-micron pores present in melt pellets were likely to be formed by the loss of water vapor derived from the liberation of water of crystallization from the lactose monohydrate particles and by agglomerate densification. For the physical mixture of lactose and PEG, there was a remarkably high population of large pores. In loosely packed lactose-PEG mix, the conditions were not conducive

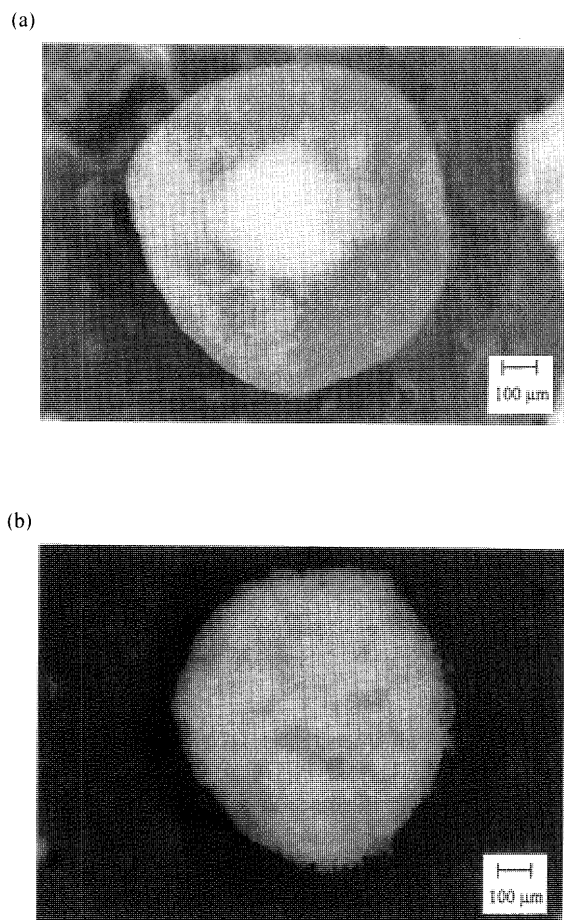


Fig. 6. Photographs of Cross Sectional View of (a) Lactose-PEG Pellets and (b) PEG-Free Lactose Pellets Prepared by Washing with Chloroform

Post-melt processing time: 21 min; post-melt impeller speed: 1150 rpm; PEG 3000 concentration: 23% (w/w).

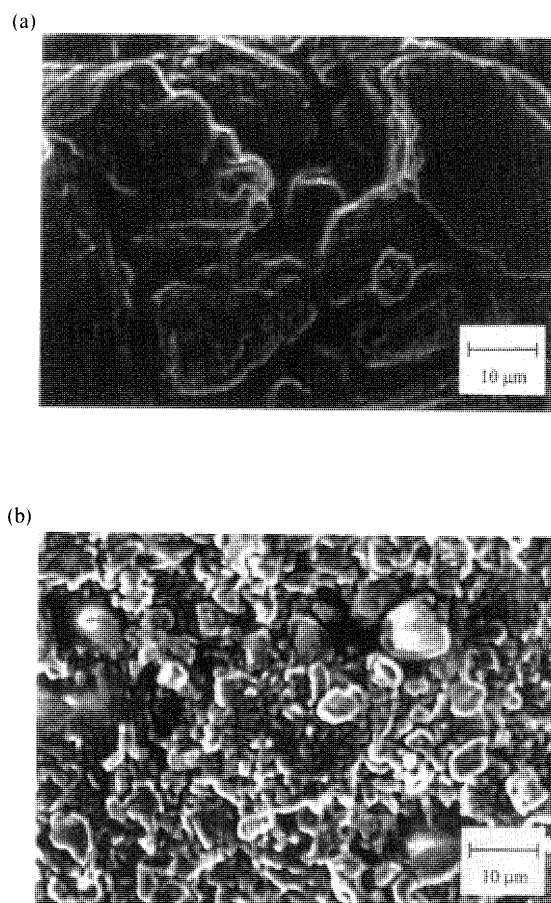


Fig. 7. Scanning Electron Photomicrographs of Pellets at (a) 5 and (b) 21 min of Post-Melt Processing Times

Post-melt impeller speed: 1150 rpm; PEG 3000 concentration: 23% (w/w).

to complete enveloping of the lactose particles with PEG. The very loosely packed lactose-PEG masses provided little barrier properties to the escaping crystallization water liberated, unlike the case of formed agglomerates. During pelletization, the densification process reduced the volume of the inter-particle agglomerate pores. The molten binder was squeezed away from the core towards the surfaces of the agglomerates (Fig. 6). The PEG mass was identified by the difference in appearance between the chloroform-treated (Fig. 6(b)) and untreated (Fig. 6(a)) pellets. There was a distinct PEG mass around the pellet surfaces as indicated in a cross sectional view of the pellet (Fig. 6). The densification process mobilized the molten binder to form a barrier nearer the surfaces of the agglomerates. The agglomerate surfaces appeared denser and no lactose particles were identified as the molten binder was squeezed out toward the surfaces by impact (Fig. 7).⁹⁾ In order to escape, the water vapor had to permeate through the PEG to the exterior of the agglomerates. However, puncture of the molten PEG barrier by the escaping water vapor was unlikely to be the mode of formation of sub-micron pores. PEG, being a molten liquid, would flow in the direction of stress and eliminate the pores by forming a continuous barrier. It was more likely that the escaping water vapor was first trapped within the molten PEG barrier. Upon solidification of the molten PEG, the excess water was "thrown out" of the PEG, forming water vesicles which upon

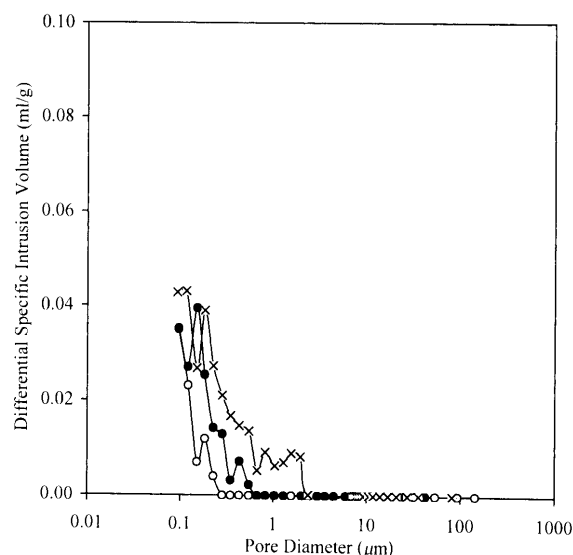


Fig. 8. Profiles of Pore Size Distribution of PEG 3000 Films Prepared without Addition of Water (○), with 8 (●), and 16% (w/w) (×) of Water Calculated as the Percentage of PEG 3000

drying contributed to the formation of sub-micron pores. The effect of water vapor on the size distribution of sub-micron pores is illustrated in Fig. 8. These pores were formed on the solidification of molten binder. Addition of water to the molten binder and followed by drying increased the fraction of sub-micron pores. The population of sub-micron pores

Table 1. Formulation and Processing Conditions Used in the Study of Pellet Pore Size Distribution, and the Mean Sizes of Pellets Formed under the Experimental Conditions

PEG 3000 concentration (% (w/w))	Post-melt impeller speed (rpm)	Post-melt processing time (min)	Mean pellet size (μm)
23	850	21	622.6
23	1150	21	937.8
23	1450	21	1175.8
22	1150	21	754.4
24	1150	21	1339.5
23	1150	5	453.3
23	1150	13	703.9
23	1150	25	1092.3

was higher with increase in the level of water to be evaporated by drying.

Melt pelletization was carried out in an enclosed, water-jacketed mixer bowl. The high speed shearing produced friction that heated up the product undergoing pelletization and a high product temperature resulted. There was evidence that more crystallization water was liberated from the lactose monohydrate with longer post-melt processing time, higher post-melt impeller speed and binder concentration by the presence of an increased amount of condensed water on the upper part of the mixer bowl. The water content determination by Karl-Fischer titration showed that the water content of the unheated lactose-PEG physical mixture was 4.66% (w/w). For pellets produced after post-melt processing of 21 min at 1150 and 1450 rpm, the water content was reduced to 4.48 and 4.11% (w/w), respectively. When the concentration of PEG 3000 used was raised from 23 to 24% (w/w), the water content was reduced to 4.28% (w/w), calculated by correction to the same binder level. The reduction of water content indicated that the likelihood of more water vesicles being formed was increased. This, in turn, was translated to an increased population of sub-micron pores when longer post-melt processing time, and higher post-melt impeller speed and binder concentration were used.

Heng *et al.* reported that a decrease in intra-pellet porosity of melt pellets was associated with an increase in pellet strength.²⁾ At longer post-melt processing time, and higher post-melt impeller speed and binder concentration, the decrease in the fraction of large pores of the agglomerates by densification is indicative of the reduced likelihood of breakdown and reformation of agglomerates. The agglomerates underwent longer periods of coalescence and, thereby, larger pellets were formed (Table 1). The pellets produced were larger at longer post-melt processing time, higher post-melt

impeller speed and binder concentration. The production of larger pellets was likely to be associated with the formation of a thicker barrier of molten PEG. This molten PEG barrier increased the surface plasticity of agglomerates and promoted the coalescence growth of pellets. A thicker barrier of molten PEG could also easily entrap the water vapor resulting in the subsequent larger population of sub-micron pores. This could explain the increase in the population of sub-micron pores with increase in pellet size.

Conclusion

The pore size distribution of melt pellets was bimodal and was affected by binder concentration, post-melt impeller speed and post-melt processing time. With a higher binder concentration, post-melt impeller speed or longer post-melt processing time, the fraction of large pores in agglomerate was reduced, thereby producing denser agglomerates. The extent of formation of large pores was dependent on the interplay between the inter-particle distance of lactose particles and the contraction property of molten binder. In contrast, the tendency of agglomerates to develop sub-micron pores increased with a higher binder concentration, post-melt impeller speed or longer post-melt processing time. The volume of sub-micron pores increased with a decrease in the population of large pores as a result of agglomerate densification. High process temperature was associated with a greater amount of water loss from the lactose monohydrate particles in the melt agglomerates. The water vapor was trapped in the molten PEG during the pelletization process. The sub-micron pores were also formed as a result of subsequent escape of this water vapor after solidification of the molten PEG. The amount of sub-micron pores produced was related to the level of water loss.

References

- 1) Ghebre-Sellassie I., *Pharmaceutical Pelletization Technology*, Vol. 37, Marcel Dekker, Inc., New York and Basel, 1989.
- 2) Heng P. W. S., Wan L. S. C., Wong T. W., *Pharm. Dev. Technol.*, **4**, 27–33 (1999).
- 3) Heng P. W. S., Wong T. W., Shu J. J., Wan L. S. C., *Chem. Pharm. Bull.*, **47**, 633–638 (1999).
- 4) Schaefer T., Holm P., Kristensen H. G., *Acta Pharm. Nord.*, **4**, 133–140 (1992).
- 5) Schaefer T., Taagegaard B., Thomsen L. J., Kristensen H. G., *Eur. J. Pharm. Sci.*, **1**, 125–131 (1993).
- 6) Thies R., Kleibudde P., *Int. J. Pharm.*, **188**, 131–143 (1999).
- 7) Thomsen L. J., Schaefer T., Sonnergaard J. M., Kristensen H. G., *Drug Dev. Ind. Pharm.*, **19**, 1867–1887 (1993).
- 8) Wong T. W., Wan L. S. C., Heng P. W. S., *Pharm. Dev. Technol.*, **4**, 449–456 (1999).
- 9) Heng P. W. S., Wong T. W., Chan L. W., *Chem. Pharm. Bull.*, **48**, 420–424 (2000).

Highly Potent and Orally Active Non-Peptide Arginine Vasopressin Antagonists for Both V_{1A} and V_2 Receptors: Synthesis and Pharmacological Properties of 4'-[(4,4-Difluoro-5-methylidene-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide Derivatives

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A series of compounds structurally related to 4'-[(4,4-difluoro-5-methylidene-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide were synthesized and evaluated for arginine vasopressin (AVP) antagonistic activity. Compounds with a (*Z*)-olefin geometry at the 5-position of benzoazepine possessed potent affinity for both the V_{1A} and V_2 receptors. Further study has shown that one of these derivatives, (*Z*)-4'-[(4,4-difluoro-5-[(4-dimethylaminopiperidino)carbonylmethylene]-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide monohydrochloride (29, YM-35471), exhibits exceptionally potent affinity for both of V_{1A} and V_2 receptors, even when administered orally. The synthesis and pharmacological properties of this compound are detailed in this paper.

Key words YM-35471; arginine vasopressin antagonist; difluorination; congestive heart failure

Arginine vasopressin (AVP) is a nona-peptide secreted by the posterior pituitary gland. AVP is known as an anti-diuretic hormone (ADH) and demonstrates potent anti-diuretic and vasoconstrictor activities. The physiological properties of the V_{1A} and V_2 receptors have been elucidated.¹⁾ The V_{1A} receptor is found in vascular smooth muscle, liver, platelets and renal mesangial cells. Stimulation of the receptor by AVP induces activation of phospholipase C to produce inositol-1,4,5-triphosphate and 1,2-diacyl glycerol. The V_2 receptor is found only in the kidney. Stimulation of V_2 receptor by AVP activates adenylate cyclase to increase the production of cyclic-AMP.²⁾ AVP, therefore, induces vascular contraction and cardiac suppression by binding to the V_{1A} receptor and acts as an anti-diuretic by binding to the V_2 receptor.

It has been suggested that there is a relationship between abnormal secretion of AVP and disorders such as congestive heart failure, hypertension or edema.³⁾ In particular, it has been reported that the plasma levels of AVP are significantly higher in patients with congestive heart failure than in those without.^{4,5)} Moreover, it is known that the life spans of patients with both congestive heart failure and hyponatremia are shorter than the life spans of patients with only congestive heart failure. It is also known that AVP plasma levels are higher in the former than in the latter.^{6,7)}

Considering the physiological properties of AVP and the pathological mechanisms of congestive heart failure, we set forth the following hypothesis. Preventing the effects of V_{1A} receptor activation would alleviate conditions that exacerbate heart failure, such as lung congestion and low cardiac output. Furthermore, blockage of the V_2 receptor would improve disorders caused by the retention of body fluid, such as edema and hyponatremia. Accordingly, compounds which act as antagonists of both V_{1A} and V_2 receptors are expected to be highly effective drugs for preventing exacerbation of heart failure.

To confirm our hypothesis, we have extensively studied the

synthesis of a variety of AVP receptor agonists.^{8–10)} Among the compounds having a methylidene group at the 5-position of the benzoazepine ring, (*E*)-4'-[5-(substituted methylidene)-2,3,4,5-tetrahydro-1H-1-benzazepin-1-carbonyl]benzanilide derivatives [(*E*)-*exo*-olefin isomers] were found to have potent affinities for both V_{1A} and V_2 receptors (Fig. 1).¹⁰⁾ Under acidic or alkaline conditions, however, the (*E*)-*exo*-olefin isomers were found to isomerize easily to compounds possessing lower affinities, 4'-[5-(substituted methyl)-2,3-dihydro-1H-1-benzazepin-1-carbonyl]benzanilide derivatives (*endo*-olefin isomers). This isomerization is likely to be caused by deprotonation at the 4-position of benzoazepine. We thought that introduction of substituents other than hydrogen to the 4-position would prevent isomerization. Difluorination was selected, because we thought 1) the fluorinated moiety would be stable in further synthetic reactions, and 2) the moiety would have little effect on the stereo-selectivity of the reaction to obtain desired product when introducing a methylidene group at the 5-position. It is well known that the carbon–fluorine bond is stable and has high bond energy, and also that a fluorine atom is closely hydrogen mimic with respect to steric requirements.^{11,12)} The synthesis and the structure–activity relationships of the difluorinated compounds are described below.

Chemistry

The synthetic pathways of the compounds listed in Tables 2–5 are shown in Charts 1 and 2. The key intermediate compounds, methyl [1-(4-aminobenzoyl)-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepin-5-ylidene]acetate ((*Z*)-olefin isomer: **9a**; (*E*)-olefin isomer: **9b**) were synthesized as shown in Chart 1. Fluorination of 1-(*p*-toluenesulfonyl)-1,2,3,4-tetrahydro-5H-1-benzazepin-5-one (**4**)¹³⁾ with *N*-fluorobenzenesulfonimide¹⁴⁾ gave a monofluorinated benzoazepine derivative (**5**). Compound **5** was reacted in the same procedures to give a difluorinated molecule (**6**) in a

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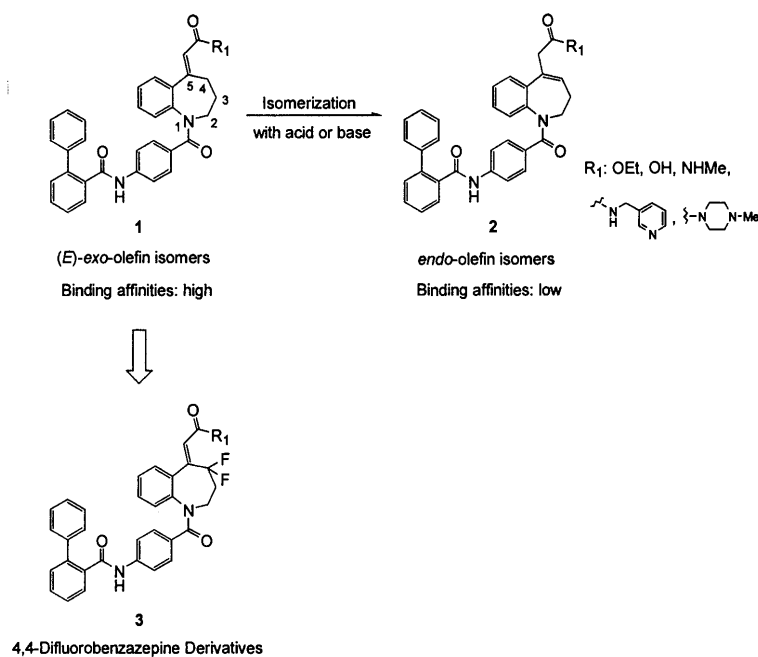


Fig. 1

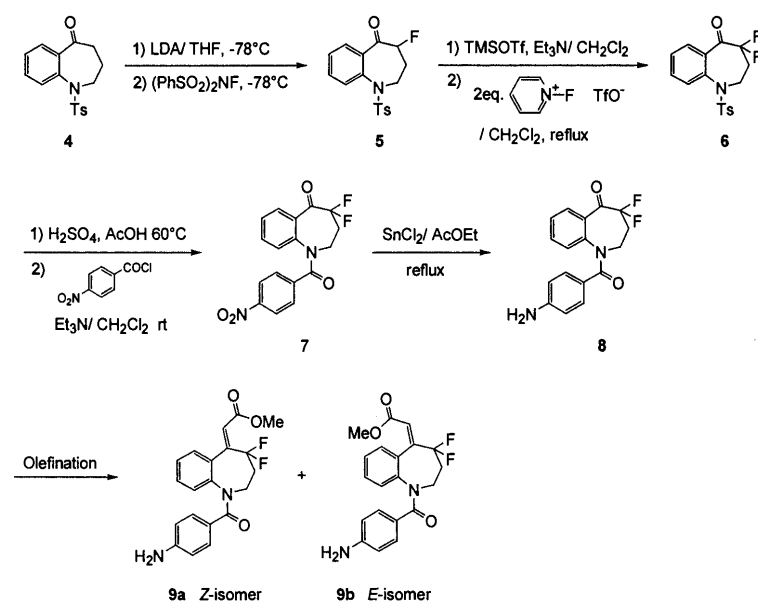


Chart 1

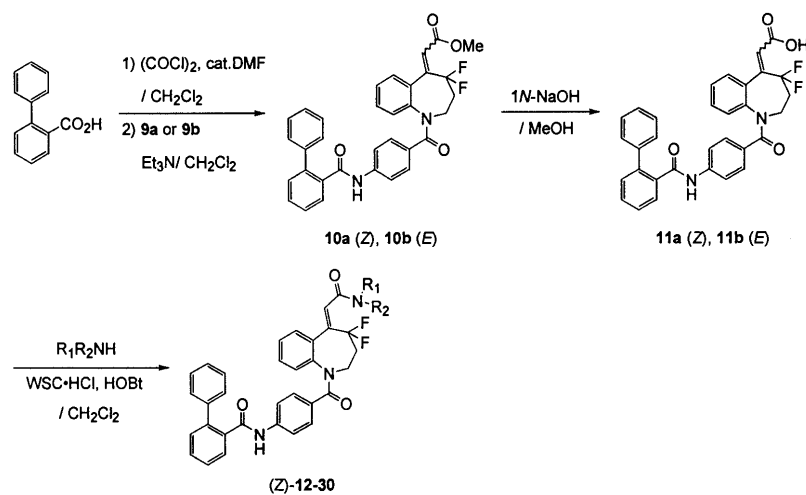


Chart 2

poor yield. Consequently, an Umemoto reaction¹⁵ was employed to prepare **6**. After the *p*-toluenesulfonyl group was removed, condensation of **6** with *p*-nitrobenzoyl chloride gave a nitrobenzoyl derivative (**7**). The nitro group of **7** was reduced with tin(II) chloride¹⁶ to obtain an aminobenzoyl derivative (**8**). A methylidene group was introduced at the 5-position of the benzoazepine ring of **8** by a Horner–Emmons¹⁷ or Peterson reaction¹⁸ to give the two isomers (**9a**, **9b**). These isomers were distinguished by nuclear Overhauser effect (NOE) in the NOE difference spectrum (Fig. 2). NOE spectrum interaction was observed between the hydrogen of the methylidene and the hydrogen at 6-position of the benzoazepine ring in **9a**, while no NOE was seen in **9b**. From these results, **9a** and **9b** were determined to be (*Z*)-isomer and (*E*)-isomer, respectively. At first, introduction of methylidene group of **8** was performed using triethyl phosphonoacetate to give a 1 : 1 mixture of **9a** and **9b** (Table 1; entry 1). This reaction was completed in a short time compared with that of unfluorinated compounds.¹⁰ This result suggested that introduction of fluorine atom caused electron deficiency of carbonyl-carbon at 5-position of the benzoazepine ring, so that the reactivity of ketone was increased. Then we studied introduction of a methylidene group for stereo selectivity. Consequently, we succeeded in obtaining **9a** and **9b** selectively under the conditions of entries 6 and 12,^{19–21} respectively. The pathways of synthesis of 4'-[(4,4-difluoro-5-methylidene-2,3,4,5-tetrahydro-1*H*-1-benzoazepin-1-yl)carbonyl]-2-phenylbenzanilide derivatives are shown in Chart 2. Benzanilide derivatives **10a** and **10b** were obtained by reacting *o*-phenyl benzoyl chloride with **9a** and **9b**, respectively. Each derivative was hydrolyzed to acetic acid derivative **11a** or **11b**, and then condensed with various amines using 1-ethyl-3-(dimethylaminopropyl)-carbodiimidehydrochloride (WSC.HCl) and 1-hydroxybenzotriazol (HOBt) to give target amide derivatives (**12–30**). No olefin

isomerization was occurred from **9a** (**9b**) to **12–30**. These were confirmed that introduction of fluorine atom at the 4-position of benzoazepine ring prevent isomerization of olefin at the 5-position.

Results and Discussion

Binding Affinities The methods for determining the *in vitro* AVP receptor binding affinities (rat liver for V_{1A} and rabbit kidney for V_2) have been described in a previous paper.⁸ The results of the binding assay of compounds are shown in Tables 2–5.

Compound **12a** was compared with the compound that have already been reported [(*E*)-*exo*-olefin isomer; **1a**]¹⁰ to examine the effect of difluorination (Table 2). The (*Z*)-olefin isomer (**12a**), like **1a**, showed potent affinities for both the V_{1A} and V_2 receptors. This might be due to the so-called “mimic effect” of fluorine atoms¹²; *i.e.*, fluorine mimics hydrogen with respect to steric requirements at enzyme receptor site.

The binding affinities of **12a** to receptors V_{1A} and V_2 were about 10-fold higher than those of the (*E*)-olefin isomer (**12b**). This was confirmed again that a *trans*-substituent group at the 5-position of benzoazepine, oriented to the opposite side of the benzene moiety, is important for achieving high binding affinities.¹⁰ Further synthetic study was focused on the (*Z*)-4,4-difluoro-5-methylidene benzoazepine derivatives that showed stability and high affinities.

Next, the structure–activity relationships of the (*Z*)-4,4-difluoro-5-methylidene benzoazepine derivatives are shown in Table 3. In cases where the substituents were ester (**10a**), acetic acid (**11a**) or amides (**12a**, **13–23**), the binding affinity potential was decreased in order of amide>ester>acetic acid. Among the alkylamide compounds (**14–18**), the methyl, ethyl and *n*-propyl substituted derivatives (**14–16**) exhibited potent affinities for both receptors. Introduction of a bulky substituent such as an isopropyl or benzyl group (**17**, **18**) was found to decrease binding affinities. The alkylamide compounds containing oxygen atoms (**19**, **23**) showed selectivity for the V_{1A} receptor. The compounds with amino groups (**20**, **12a**) showed potent affinities for both the V_{1A} and V_2 receptors. Secondary (**14–20**) and tertiary (**21–23**, **12a**) amides showed a slight difference in binding affinities.

The compounds with substituted alkyl groups (**15**, **16**) had potent affinities to both types of receptors; but were not very

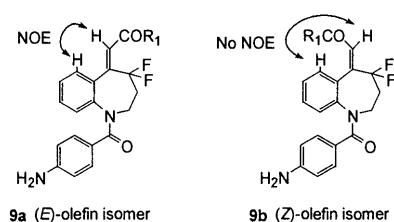


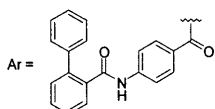
Fig. 2

Table 1. Olefination of Compound **8**

Entry	Reagent	Condition	Temp. (°C)	Ratio	
				9a	9b
1	(EtO) ₂ P(O)CH ₂ CO ₂ Et	NaH/THF	−40	1	1
2	(iso-PrO) ₂ P(O)CH ₂ CO ₂ Et	NaH/THF	−40	1	2
3	(CF ₃ CH ₂ O) ₂ P(O)CH ₂ CO ₂ Me	KN(TMS) ₂ , 18-crown-6/THF	−40	1.2	1
4	(MeO) ₂ P(O)CH ₂ CO ₂ Me	LiN(TMS) ₂ /THF	−40	1	1.3
5		KN(TMS) ₂ /THF	−40	1.4	1
6		NaH/THF	−40	3	1
7		NaH/THF	−40	1	1.4
8		NaH/THF–Toluene	−40	1.7	1
9		LiCl, DBU/CH ₃ CN	20	1	1.1
10	Ph ₃ P=CHCO ₂ Me	PhCO ₂ H/CH ₃ CN	100	2	1
11	TMSCH ₂ CO ₂ Et	LDA/THF	−78	2	1
12		LDA/THF, MgBr ₂	−78	1	15

Table 2. Receptor-Binding Affinities for Unfluorinated and Fluorinated Compounds

No.	Compounds	Structure	Binding affinity (pK_i)	
			$V_{1A}^a)$	$V_2^b)$
1a ^{c)}	(<i>E</i>)- <i>Exo</i> -olefin isomer		8.88	9.04
1b ^{c)}	<i>Endo</i> -olefin isomer		7.93	8.54
12a ^{d)}	(<i>Z</i>)-Olefin isomer		8.89	9.06
12b ^{d)}	(<i>E</i>)-Olefin isomer		7.70	8.12



a) pK_i of [^3H]vasopressin binding to rat liver membranes. b) pK_i of [^3H]vasopressin binding to rat kidney membranes. c) See reference 8. d) Caution: The meaning of *E* or *Z* is different from the compounds which fluorinated or unfluorinated.

soluble in water. The physicochemical property is undesirable for the use as intravenous drugs. On the other hand, compounds containing amino groups (**20**, **12a**) formed water-soluble salts upon treatment with hydrochloric acid. Further study was focused on compounds containing amino groups (Table 4).

The results revealed that both secondary (**20**, **24**, **25**) and tertiary (**12a**, **26**–**31**) amide derivatives exhibited potent affinities for both the V_{1A} and V_2 receptors, with the exception of the compound **28** which contained a bulky benzyl group. We discovered a series of compounds, such as 4-(*N,N*-dimethylamino)piperidine derivative **29** and homopiperazine derivatives (**30**, **31**), that were water-soluble and had potent affinities for both kinds of receptors.

Antagonist Activity and Oral Dosing The V_{1A} receptor antagonist activity was determined by measuring the inhibition of the AVP-induced diastolic blood pressure (DBP) response in pithed rats after intravenous (i.v.) administration. The dose of compound causing a 50% inhibition of the pressor response to AVP (ID_{50}) was calculated. The V_2 receptor antagonist activity was determined by measuring the change in the urine volume in dehydrated conscious rats after i.v. administration. The dose causing an increase in urine volume by 3 ml after compound dosing (ED_3) was determined. Activity for oral dosing was determined by measuring the change in the urine volume in dehydrated conscious rats after oral administration. Compounds or vehicle was administered orally and spontaneously voided urine was collected for 4 h period. The experimental methods of used to determine AVP

Table 3. Receptor-Binding Affinities for Ester, Acetic Acid and Amide Derivatives

No.	R_1	Binding affinity (pK_i)	
		$V_{1A}^a)$	$V_2^b)$
10a	OMe	8.74	7.48
11a	OH	7.82	7.49
13	NH ₂	9.60	8.59
14	NHMe	9.45	8.94
15	NHEt	9.72	9.16
16	NH <i>n</i> -Pr	9.23	9.24
17	NH <i>i</i> -Pr	9.44	8.08
18	NHBn	8.63	8.19
19		9.67	8.11
20		9.97	8.65
21		9.14	7.99
22		8.85	8.16
23		9.57	8.28
12a		8.89	9.06

a, b) See footnotes in Table 2.

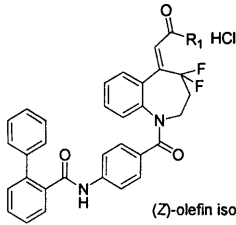
antagonist activity and effectiveness of oral dosing have been described in a previous paper.^{8,9)} The compounds (**20**, **29**–**31**) which showed potent binding affinities for both the V_{1A} and V_2 receptors, were selected and tested in an *in vivo* examination (Table 5). All compounds significantly suppressed the AVP-induced blood pressure increase and increased urine volume. No differences in antagonist activity were seen between these compounds, despite of their structural differences. Next, the effect on volume of urine excreted after oral administration of these compounds was determined. These compounds increased urine volume to 8 to 15 times that of untreated animals 4 h after administration. Compound **29** containing 4-(*N,N*-dimethylamino)piperidine group exhibited the highest oral activity of these compounds.

Conclusion

In order to prevent the isomerization of (*E*)-*exo*-olefin isomers to *endo*-olefin isomers, the 4-position of a benzodiazepine ring was difluorinated. Among 4,4-difluoro-5-methylidene benzodiazepine derivatives, (*Z*)-olefin isomers had activities similar to those of the unfluorinated compounds, (*E*)-*exo*-olefin isomers. We discovered a series of compounds possessing high binding affinities for both V_{1A} and V_2 receptors and potent AVP antagonist activity when administered orally. A compound, (*Z*)-4'-[4,4-difluoro-5-[(4-dimethylaminopiperidin-1-yl)carbonylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzodiazepin-1-yl]carbonyl-2-phenylbenzamide monohydrochloride (**29**, YM-35471), is currently in the stage of pre-clinical studies. YM-35471 is expected to be

applied clinically to diseases like heart failure which can be caused by abnormal secretion of AVP.

Table 4. Receptor-Binding Affinities for Amide Derivatives Containing Amino Groups



(Z)-olefin isomers

No.	R ₁	Binding affinity (pK _i)	
		V _{1A} ^{a)}	V ₂ ^{b)}
20		9.97	8.65
24		9.80	9.12
25		9.79	9.13
26		9.66	9.71
12a		8.89	9.06
27		8.89	8.52
28		7.97	7.48
29		10.10	9.58
30		10.08	9.37
31		10.50	9.26

a, b) See footnotes in Table 2.

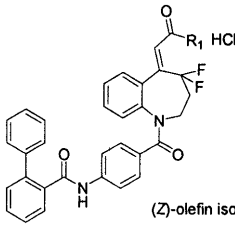
Experimental

¹H-NMR spectra were obtained on a JEOL JNM-EX90 or JNM-A500 spectrometer and the chemical shifts are expressed in δ (ppm) values with tetramethylsilane as an internal standard. Abbreviations of ¹H-NMR signal patterns are as follows: s, singlet; d, doublet; ddd, triple doublet; t, triplet; m, multiplet; br, broad peak. Mass spectra were obtained on a JEOL JMS-DX300 or Hitachi M-80 spectrometer. Elemental analysis was performed with a Yanaco MT-5. Melting points were determined on a Yanaco MP-500D micro melting point apparatus without correction. Column chromatography was performed using Merck KGaA Silica gel 60 (0.040–0.063 mm).

4-Fluoro-1-(p-toluenesulfonyl)-1,2,3,4-tetrahydro-5H-1-benzazepin-5-one (5) To a solution of diisopropylamine (2.56 g, 25.3 mmol) in tetrahydrofuran (THF) (150 ml) was added 1.6 M *n*-butyl lithium (14.9 ml, 23.8 mmol) at -78°C under an Ar atmosphere, and the mixture was stirred at 0°C for 30 min. A solution of 1-(p-toluenesulfonyl)-1,2,3,4-tetrahydro-5H-1-benzazepin-5-one¹³⁾ (**4**, 6.39 g, 20.3 mmol) in THF (60 ml) was added to the mixture at -78°C . After stirring at -78°C for 30 min, *N*-fluorobenzenesulfonimide (8.94 g, 28.3 mmol) in THF (90 ml) was added, and the mixture was stirred at 0°C for 1 h. To the mixture was added saturated aqueous NH₄Cl solution, and then concentrated *in vacuo*. The resulting mixture was extracted with ethyl acetate (AcOEt). The organic layer was washed with saturated aqueous Na₂S₂O₃ solution, saturated aqueous NaHCO₃ solution and brine, dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was chromatographed on silica gel, eluted with chloroform (CHCl₃) and recrystallized from ethanol (EtOH) to give 5.31 g (15.9 mmol, 78%) of **5** as a colorless powder, mp 111–113 $^{\circ}\text{C}$, ¹H-NMR (CDCl₃) δ : 2.13 (1H, m), 2.44 (3H, s), 2.69 (1H, m), 3.64 (1H, m), 4.30 (1H, m), 5.06 (1H, ddd, *J*=5.9, 9.8, 47.9 Hz), 7.31 (2H, d, *J*=8.3 Hz), 7.36 (1H, m), 7.53 (1H, m), 7.56 (1H, m), 7.64 (2H, d, *J*=8.3 Hz), 7.82 (1H, m). EI-MS *m/z*: 333 (*M*⁺). Anal. Calcd for C₁₇H₁₆FNO₃S: C, 61.25; H, 4.84; N, 4.20; F, 5.70; S, 9.62. Found: C, 61.07; H, 4.80; N, 4.16; F, 5.81; S, 10.01.

4,4-Difluoro-1-(p-toluenesulfonyl)-1,2,3,4-tetrahydro-5H-1-benzazepin-5-one (6) To an ice-cooled mixture of **5** (1.80 g, 5.40 mmol) and triethylamine (Et₃N) (4.52 ml, 32.4 mmol) in dichloromethane (CH₂Cl₂) (20 ml) was added trimethylsilyl trifluoromethanesulfonate (TMSOTf) (5.22 ml, 28.8 mmol). The mixture was stirred at room temperature for 1 h and then extracted with diethyl ether (Et₂O). The organic layer was washed with saturated aqueous NaHCO₃ solution, 1 M HCl, saturated aqueous NaHCO₃ solution and brine, dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was used for the next step without further purification. A mixture of the above residue and *N*-fluoropyridinium trifluoromethanesulfonate (2.67 g, 10.8 mmol) in dichloroethane (20 ml) was refluxed for 2 h. The mixture was diluted with CH₂Cl₂. The organic layer was washed with brine, dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was chro-

Table 5. Oral Activity and AVP-Antagonist Activities of Amide Derivatives Containing Amino Groups



(Z)-olefin isomers

No.	R ₁	Binding affinity (pK _i)		Antagonist activities		Oral activity
		V _{1A} ^{a)}	V ₂ ^{b)}	V _{1A} ^{c)} ID ₅₀ (mg/kg)	V ₂ ^{d)} ED ₃ (mg/kg)	UV ^{e,f)} (ml)
20		9.97	8.65	0.0032	0.037	4.59 ± 0.56
29		10.10	9.58	0.0027	0.050	8.93 ± 0.77
30		10.08	9.37	NT	NT	5.25 ± 2.84
31		10.50	9.26	0.0030	0.020	6.91 ± 0.46

a, b) See footnotes in Table 2. c) ID₅₀ represents the drug concentration (mg/kg) required to inhibit the AVP-induced pressor response in pithed rats by 50% by intravenous administration. d) ED₃ represents the drug concentration (mg/kg) required to increase urine volume by 3 ml during 3 h after intravenous administration of the drug to rats. e) UV values mean urine volume (ml) during 4 h after oral administration of the drug 3 mg/kg to rats and are expressed as mean ± SD. f) Urine volume for vehicle rats (0.5% MC): 0.59 ± 0.18 ml/0–4 h. NT; not tested.

matographed on a silica gel column using *n*-hexane–AcOEt (30 : 70) as eluent to give 1.78 g (5.07 mmol, 94%) of **6** as a colorless amorphous substance. ¹H-NMR (CDCl₃) δ: 2.41 (2H, m), 2.43 (3H, s), 4.09 (2H, m), 7.25 (2H, d, *J* = 8.0 Hz), 7.40 (1H, m), 7.48 (2H, d, *J* = 8.4 Hz), 7.55 (3H, m). EI-MS *m/z*: 351 (M⁺).

4,4-Difluoro-1-[4-(4-nitrobenzoyl)-2,3,4,5-tetrahydro-1H-1-benzazepine-5-one (7) To a solution of **6** (3.00 g, 8.54 mmol) in acetic acid (14 ml) was added concentrated sulfuric acid (7 ml), and the mixture was stirred at 60 °C for 14 h. After being cooled at 0 °C, the resulting mixture was made alkaline with 10% aqueous KOH solution and extracted with AcOEt. The organic layer was dried over anhydrous K₂CO₃ and concentrated *in vacuo*. The residue was used for the next step without further purification. To an ice-cooled mixture of the above residue and triethylamine (1.13 g, 11.1 mmol) in CH₂Cl₂ (20 ml) was added 4-nitrobenzoyl chloride (1.90 g, 10.2 mmol), and the mixture was stirred for 12 h at room temperature and then extracted with AcOEt. The organic layer was washed with 1 M HCl, 1 M NaOH and brine, dried over anhydrous MgSO₄ and concentrated *in vacuo*. The resulting residue was chromatographed on a silica gel column using CHCl₃ as eluent and recrystallized from AcOEt–*n*-hexane to give 2.23 g (6.64 mmol, 78%) of **7** as a colorless powder, mp 192–195 °C. ¹H-NMR (CDCl₃) δ: 2.73 (2H, m), 4.35 (2H, m), 6.62 (1H, m), 7.25 (1H, m), 7.33 (1H, m), 7.48 (2H, m), 8.00 (1H, m), 8.10 (2H, m). EI-MS *m/z*: 346 (M⁺). *Anal.* Calcd for C₁₇H₁₂F₂N₂O₄: C, 58.96; H, 3.49; N, 8.09; F, 10.97. Found: C, 59.00; H, 3.62; N, 7.93; F, 10.91.

1-(4-Aminobenzoyl)-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepine-5-one (8) To a solution of **7** 940 mg (2.71 mmol) in AcOEt (20 ml) was added SnCl₂ (3.07 g, 16.2 mmol) at room temperature, and the mixture was refluxed for 5 h. After being cooled at 0 °C, the resulting mixture was made alkaline with 1 M NaOH, and extracted with AcOEt. The organic layer was dried over anhydrous K₂CO₃ and concentrated *in vacuo*. The residue was recrystallized from CHCl₃–*n*-hexane to give 711 mg (2.25 mmol, 83%) of **8** as a colorless powder, mp 168–170 °C. ¹H-NMR (CDCl₃) δ: 1.59 (2H, m), 2.66 (2H, m), 3.88 (1H, m), 4.24 (1H, m), 6.45 (2H, m), 6.76 (1H, m), 7.17 (2H, m), 7.24 (2H, m), 7.95 (1H, m). EI-MS *m/z*: 316 (M⁺). *Anal.* Calcd for C₁₇H₁₄F₂N₂O₂·0.1H₂O: C, 64.19; H, 4.50; N, 8.81; F, 11.94. Found: C, 64.28; H, 4.62; N, 8.66; F, 11.93.

(Z)-Methyl [1-(4-Aminobenzoyl)-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetate (9a), (E)-Methyl [1-(4-Aminobenzoyl)-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetate (9b) To an ice-cooled suspension of 60% NaH (228 mg, 5.70 mmol) in THF (10 ml) was added trimethyl phosphonoacetate (1.11 g, 6.08 mmol), and the mixture was stirred at room temperature for 30 min. A solution of **8** (600 mg, 1.90 mmol) in THF (20 ml) was added to the mixture at –78 °C, and the temperature was raised slowly to 0 °C for 10 h. To the mixture was added saturated aqueous NH₄Cl solution, and then concentrated *in vacuo*. The resulting mixture was extracted with CHCl₃. The organic layer was dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was chromatographed on a silica gel column using *n*-hexane–AcOEt (45 : 55) as eluent to give 170 mg (0.457 mmol, 24%) of **9b** as a colorless amorphous substance. ¹H-NMR (CDCl₃) δ: 2.40 (2H, m), 3.18 (1H, m), 3.71 (3H, s), 3.78 (2H, m), 5.08 (1H, m), 6.42 (2H, d, *J* = 8.0 Hz), 6.59 (1H, s), 6.73 (1H, d, *J* = 7.3 Hz), 7.14 (1H, m), 7.20 (1H, t, *J* = 7.3 Hz), 7.29 (3H, m). EI-MS *m/z*: 372 (M⁺).

The further elution was given 536 mg (1.44 mmol, 76%) of **9a** as a colorless amorphous substance. ¹H-NMR (CDCl₃) δ: 2.50 (2H, m), 3.20 (1H, m), 3.78 (2H, m), 3.82 (3H, s), 5.05 (1H, m), 6.18 (1H, s), 6.38 (2H, m), 6.73 (1H, m), 6.97 (2H, m), 7.12 (1H, m), 7.23 (1H, m), 7.37 (1H, m). EI-MS *m/z*: 372 (M⁺).

(Z)-Methyl [4,4-Difluoro-1-[4-(2-phenylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetate (10a) To an ice-cooled mixture of *o*-phenylbenzoic acid (396 mg, 2.00 mmol) and catalytic *N,N*-dimethylformamide (DMF) in CH₂Cl₂ (10 ml) was added oxalyl chloride (0.422 g, 3.32 mmol). The mixture was stirred at room temperature for 3 h, diluted with benzene and concentrated *in vacuo* to give a colorless powder. To an ice-cooled mixture of **9a** (620 mg, 1.67 mmol) and pyridine (5 ml) in CH₂Cl₂ (10 ml) was added a solution of above colorless powder in CH₂Cl₂ (10 ml). The mixture was stirred at room temperature for 1 h, and then concentrated *in vacuo*. The residue was diluted with AcOEt. The organic layer was washed with saturated aqueous Na₂CO₃ solution, 1 M HCl and brine, dried over anhydrous MgSO₄ and concentrated *in vacuo*. The crude product was chromatographed on a silica gel column using *n*-hexane–AcOEt (6 : 1) as eluent to give 878 mg (1.59 mmol, 95%) of **10a** as a colorless amorphous substance. ¹H-NMR (CDCl₃) δ: 2.30–2.80 (2H, m), 3.21 (1H, m), 3.83 (3H, s), 5.03 (1H, m), 6.17 (1H, s), 6.66 (1H, m), 6.91 (3H, m), 7.01 (2H,

m), 7.09 (1H, t, *J* = 7.3 Hz), 7.24 (1H, t, *J* = 7.6 Hz), 7.33–7.49 (8H, m), 7.50–7.60 (1H, m), 7.83 (1H, d, *J* = 7.3 Hz). EI-MS *m/z*: 552 (M⁺).

(E)-Methyl [4,4-Difluoro-1-[4-(2-phenylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetate (10b) The title compound was prepared from **9b** (200 mg, 0.537 mmol) in the same manner as described in the synthesis of **10a** as a colorless amorphous substance (287 mg, 0.519 mmol, 96% yield). ¹H-NMR (CDCl₃) δ: 2.20–2.70 (2H, m), 3.30 (1H, m), 3.67 (3H, s), 4.98 (1H, m), 6.55–6.74 (2H, m), 6.85–7.70 (15H, m), 7.84 (1H, m). EI-MS *m/z*: 553 (M⁺ + 1).

(Z)-[4,4-Difluoro-1-[4-(2-phenylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetic Acid (11a) To an ice-cooled solution of **10a** (857 mg, 1.55 mmol) in methanol (MeOH) (10 ml) was added lithium hydroxide monohydrate (195 mg, 4.65 mmol) in water (2 ml). The mixture was stirred at room temperature for 7 h, and then concentrated *in vacuo*. The residue was dissolved in CHCl₃ and 1 M HCl, and extracted with CHCl₃. The organic layer was dried over anhydrous MgSO₄ and concentrated *in vacuo*. The residue was crystallized from *n*-hexane–Et₂O–AcOEt to give 633 mg (1.18 mmol, 76%) of **11a** as a colorless powder, mp 224–226 °C. ¹H-NMR (CDCl₃) δ: 2.41 (1H, m), 2.67 (1H, m), 3.24 (1H, m), 3.68 (1H, m), 5.00 (1H, m), 6.19 (1H, s), 6.67 (1H, m), 6.92 (2H, m), 6.98 (3H, m), 7.10 (1H, t, *J* = 7.6 Hz), 7.24 (1H, d, *J* = 7.3 Hz), 7.30–7.50 (8H, m), 7.53 (1H, m), 7.80 (1H, d, *J* = 7.8 Hz). EI-MS *m/z*: 538 (M⁺). *Anal.* Calcd for C₃₂H₂₄F₂N₂O₄·0.5H₂O: C, 70.19; H, 4.60; N, 5.12; F, 6.94. Found: C, 70.27; H, 4.83; N, 4.78; F, 6.72.

(E)-[4,4-Difluoro-1-[4-(2-phenylbenzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-1-benzazepine-5-ylidene]acetic Acid (11b) The title compound was prepared from **10b** (280 mg, 0.507 mmol) in the same manner as described in the synthesis of **11a** as a colorless powder (187 mg, 0.347 mmol, 68%), mp 139–141 °C. ¹H-NMR (CDCl₃) δ: 2.41 (2H, m), 3.10–3.50 (m, 2H), 4.99 (1H, m), 6.59 (1H, s), 6.67 (1H, m), 6.89 (2H, m), 7.08 (2H, m), 7.17 (1H, m), 7.24–7.48 (8H, m), 7.52 (1H, m), 7.73 (1H, m). EI-MS *m/z*: 538 (M⁺). *Anal.* Calcd for C₃₂H₂₄F₂N₂O₄·0.2H₂O: C, 70.89; H, 4.54; N, 5.17; F, 7.01. Found: C, 70.77; H, 4.63; N, 4.98; F, 6.93.

(Z)-4'-[(4,4-Difluoro-5-[(4-methyl-1-piperadiny]carbonylmethylene)-2,3,4,5-tetrahydro-1H-1-benzazepine-1-yl]carbonyl]-2-phenylbenzanilide Monohydrochloride (12a) To an ice-cooled mixture of **11a** (318 mg, 0.590 mmol) and 1-hydroxybenzotriazole hydrate (96 mg, 0.710 mmol) in CH₂Cl₂ (10 ml) and acetonitrile (CH₃CN) (10 ml) was added WSC.HCl (136 mg, 0.710 mmol) in CH₂Cl₂ (10 ml), and the mixture was stirred at room temperature for 1 h. After being cooled at 0 °C, *N*-methylpiperazine (0.079 ml, 0.712 mmol) was added, and the mixture was stirred at room temperature overnight. To the mixture was added 1 M NaOH and extracted with CHCl₃. The organic layer was dried over anhydrous K₂CO₃ and concentrated *in vacuo*. The residue was chromatographed on a silica gel column using CHCl₃–MeOH (95 : 5) as eluent to give 308 mg as a free amine. The resulting amine was diluted with MeOH, and the solution was cooled at 0 °C. To the solution, 4 M HCl/AcOEt was added and the mixture was concentrated *in vacuo*, and recrystallized from MeOH–Et₂O to give 185 mg (0.281 mmol, 48%) of **12a** as a colorless powder, mp 194–197 °C. ¹H-NMR (CDCl₃) δ: 2.42 (2H, m), 2.81 (3H, s), 2.82–3.28 (4H, m), 3.36–3.70 (3H, m), 4.09 (1H, m), 4.43 (1H, m), 4.86 (1H, m), 6.77 (1H, s), 6.82 (1H, m), 7.01 (2H, m), 7.20 (1H, t, *J* = 7.8 Hz), 7.25–7.41 (8H, m), 7.47 (2H, m), 7.56 (3H, m), 10.35 (1H, m), 11.13 (1H, m). EI-MS *m/z*: 621 (M⁺ + 1). *Anal.* Calcd for C₃₇H₃₄F₂N₄O₃·1HCl·0.5H₂O: C, 66.71; H, 5.45; N, 8.41; Cl, 5.32; F, 5.70. Found: C, 66.87; H, 5.28; N, 8.43; Cl, 5.23; F, 5.63.

(E)-4'-[(4,4-Difluoro-5-[(4-methyl-1-piperadiny]carbonylmethylene)-2,3,4,5-tetrahydro-1H-1-benzazepine-1-yl]carbonyl]-2-phenylbenzanilide Monohydrochloride (12b) The title compound was prepared from **11b** (160 mg, 0.297 mmol) in the same manner as described in the synthesis of **12a** as a colorless powder (90 mg, 0.137 mmol, 46% yield), mp 183–185 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.23–2.45 (2H, m), 2.70–3.25 (7H, m), 3.35–3.80 (3H, m), 4.08 (1H, m), 4.36 (1H, m), 4.91 (1H, m), 6.72 (1H, m), 6.97–7.59 (17H, m), 10.36 (1H, m), 10.72 (1H, m). EI-MS *m/z*: 621 (M⁺ + 1). *Anal.* Calcd for C₃₇H₃₄F₂N₄O₃·1HCl·2.9H₂O: C, 62.65; H, 5.80; N, 7.90; Cl, 5.00; F, 5.36. Found: C, 62.80; H, 5.58; N, 7.86; Cl, 5.12; F, 5.67.

(Z)-4'-[(5-Carbamoylmethylene-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepine-1-yl]carbonyl]-2-phenylbenzanilide (13) To an ice-cooled mixture of **11a** (539 mg, 1.00 mmol) and 1-hydroxybenzotriazole hydrate (149 mg, 1.10 mmol) in CH₂Cl₂ (10 ml) and acetonitrile (CH₃CN) (10 ml) was added WSC.HCl (211 mg, 1.10 mmol) in CH₂Cl₂ (10 ml), and the mixture was stirred at room temperature for 1 h. After being cooled at 0 °C, 28% NH₄OH (0.3 ml) was added, and the mixture was stirred at room temperature overnight to give a colorless crystal. The crystal was collected by filtration,

washed with H₂O and Et₂O, and then dried *in vacuo* to give 493 mg (0.917 mmol, 92%) of **13** as a colorless powder, mp >290 °C. ¹H-NMR (CDCl₃) δ: 2.37 (1H, br), 2.64 (1H, br), 3.06 (1H, br), 4.87 (1H, br), 6.46 (1H, s), 6.75 (1H, d, *J*=7.8 Hz), 7.78 (1H, s), 10.30 (1H, s). FAB-MS *m/z*: 538 (*M*⁺+1). *Anal.* Calcd for C₃₃H₂₅F₂N₃O₃·0.1H₂O: C, 71.23; H, 5.04; N, 7.79; F, 7.04. Found: C, 71.12; H, 4.92; N, 7.68; F, 6.89.

(Z)-4'-[(4,4-Difluoro-5-(*N*-methylcarbamoylmethylene)-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (14) The title compound was prepared from **11a** (500 mg, 0.928 mmol) and methylamine hydrochloride (126 mg, 1.87 mmol) in the same manner as described in the synthesis of **12a** as a colorless powder (228 mg, 0.413 mmol, 45% yield), mp 265–268 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.37 (1H, m), 2.66 (1H, m), 2.72 (1H, m), 3.06 (1H, m), 4.87 (1H, m), 5.76 (1H, s), 6.47 (1H, s), 6.75 (1H, m), 7.02 (2H, m), 7.14 (1H, t, *J*=7.1 Hz), 7.23–7.60 (13H, m), 8.22 (1H, m), 10.29 (1H, s). FAB-MS *m/z*: 552 (*M*⁺+1). *Anal.* Calcd for C₃₃H₂₇F₂N₃O₃·H₂O: C, 69.59; H, 5.13; N, 7.38; F, 6.67. Found: C, 69.84; H, 4.93; N, 7.56; F, 6.55.

(Z)-4'-[(4,4-Difluoro-5-(*N*-ethylcarbamoylmethylene)-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (15) The title compound was prepared from **11a** (500 mg, 0.928 mmol) and ethylamine hydrochloride (151 mg, 1.85 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from ethanol to give 421 mg (0.744 mmol, 80%) of **15** as a colorless powder, mp 264–266 °C. ¹H-NMR (DMSO-*d*₆) δ: 1.06 (3H, t, *J*=7.1 Hz), 2.35 (1H, m), 2.67 (1H, m), 3.15 (2H, m), 4.88 (1H, m), 6.47 (1H, s), 6.74 (1H, m), 7.02 (2H, m), 7.14 (1H, t, *J*=7.1 Hz), 7.24–7.58 (13H, m), 8.27 (1H, m), 10.28 (1H, s). FAB-MS *m/z*: 566 (*M*⁺+1). *Anal.* Calcd for C₃₄H₂₉F₂N₃O₃·0.6H₂O: C, 70.85; H, 5.28; N, 7.29; F, 6.59. Found: C, 70.76; H, 5.20; N, 7.26; F, 6.49.

(Z)-4'-[(4,4-Difluoro-5-(*N*-1-propylcarbamoylmethylene)-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (16) The title compound was prepared from **11a** (500 mg, 0.928 mmol) and 1-propylamine (110 mg, 1.86 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from chloroform-*n*-hexane to give 400 mg (0.690 mmol, 74%) of **16** as a colorless powder, mp 255–257 °C. ¹H-NMR (DMSO-*d*₆) δ: 0.89 (3H, t, *J*=7.1 Hz), 1.42 (2H, m), 2.36 (1H, m), 2.67 (1H, m), 3.09 (3H, m), 4.88 (1H, m), 6.48 (1H, s), 6.74 (1H, m), 7.02 (2H, m), 7.14 (1H, t, *J*=7.6 Hz), 7.25–7.59 (13H, m), 8.29 (1H, m), 10.29 (1H, s). FAB-MS *m/z*: 580 (*M*⁺+1). *Anal.* Calcd for C₃₅H₃₁F₂N₃O₃·0.6H₂O: C, 71.20; H, 5.50; N, 7.12; F, 6.44. Found: C, 71.06; H, 5.31; N, 7.24; F, 6.29.

(Z)-4'-[(4,4-Difluoro-5-(*N*-2-propylcarbamoylmethylene)-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (17) The title compound was prepared from **11a** (400 mg, 0.742 mmol) and 2-propylamine (66 mg, 1.12 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from CHCl₃-Et₂O to give 374 mg (0.645 mmol, 87%) of **17** as a colorless powder, mp 236–238 °C. ¹H-NMR (DMSO-*d*₆) δ: 1.11 (6H, d, *J*=6.7 Hz), 2.36 (1H, m), 2.64 (1H, m), 3.94 (1H, m), 4.88 (1H, m), 6.46 (1H, s), 6.74 (1H, m), 7.02 (2H, m), 7.13 (1H, t, *J*=7.3 Hz), 7.24–7.40 (8H, m), 7.42–7.58 (4H, m), 8.12 (1H, d, *J*=7.9 Hz), 10.28 (1H, s). FAB-MS *m/z*: 580 (*M*⁺+1). *Anal.* Calcd for C₃₅H₃₁F₂N₃O₃·1.2H₂O: C, 69.92; H, 5.60; N, 6.99; F, 6.32. Found: C, 69.74; H, 5.36; N, 6.95; F, 6.55.

(Z)-4'-[(5-(*N*-Benzylcarbamoylmethylene)-4,4-difluoro-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (18) The title compound was prepared from **11a** (539 mg, 1.00 mmol) and benzylamine (128 mg, 1.19 mmol) in the same manner as described in the synthesis of **12a** as a colorless powder (506 mg, 0.806 mmol, 81% yield), mp 189–192 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.85 (2H, s), 3.32 (1H, s), 4.37 (2H, d, *J*=5.9 Hz), 4.88 (1H, br), 6.56 (1H, s), 6.73 (1H, d, *J*=7.3 Hz), 7.02 (1H, d, *J*=8.3 Hz), 7.14 (1H, t, *J*=6.9 Hz), 7.22–7.58 (20H, m), 8.82 (1H, t, *J*=5.9 Hz), 10.28 (1H, s). FAB-MS *m/z*: 628 (*M*⁺+1). *Anal.* Calcd for C₄₀H₃₁F₂N₃O₃·0.25H₂O: C, 74.03; H, 5.73; N, 6.64; F, 6.01. Found: C, 74.03; H, 5.41; N, 6.38; F, 5.65.

(Z)-4'-[(4,4-Difluoro-5-[*N*-(2-methoxyethyl)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (19) The title compound was prepared from **11a** (500 mg, 0.928 mmol) and 2-methoxyethylamine (140 mg, 1.86 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from EtOH to give 420 mg (0.705 mmol, 76%) of **19** as a colorless powder, mp 230–231 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.37 (1H, m), 2.69 (1H, m), 3.03 (1H, m), 3.26 (3H, s), 3.28 (2H, m), 3.38 (2H, m), 4.89 (1H, m), 6.48 (1H, s), 6.74 (1H, m), 7.02 (1H, m), 7.13 (1H, t, *J*=7.8 Hz), 7.26–7.59 (13H, m), 8.45 (1H, t, *J*=5.6 Hz), 10.28 (1H, s). FAB-MS *m/z*: 596 (*M*⁺+1). *Anal.* Calcd for C₃₅H₃₁F₂N₃O₄·1H₂O: C, 68.50; H, 5.42; N, 6.85; F, 6.19. Found:

C, 68.61; H, 5.20; N, 7.05; F, 6.36.

(Z)-4'-[(4,4-Difluoro-5-[*N*-(2-dimethylaminoethyl)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide Hydrochloride (20) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N,N*-dimethylethylenediamine (59 mg, 0.674 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4*N* HCl/AcOEt solution, the residue was triturated with CHCl₃-diisopropylether (iso-Pr₂O) to give 200 mg (0.310 mmol, 67%) of **20** as a colorless amorphous substance. ¹H-NMR (DMSO-*d*₆) δ: 2.39 (1H, m), 2.65 (1H, m), 2.81 (6H, s), 3.09 (1H, m), 3.16 (2H, m), 3.49 (2H, m), 4.88 (1H, m), 6.53 (1H, s), 6.77 (1H, m), 7.02 (2H, m), 7.17 (1H, m), 7.24–7.41 (9H, m), 7.42–7.51 (2H, m), 7.52–7.60 (2H, m), 8.56–8.65 (1H, m), 9.99 (1H, m), 10.31 (1H, s). FAB-MS *m/z*: 609 (*M*⁺+1).

(Z)-4'-[(4,4-Difluoro-5-[(1-pyrrolidinyl)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (21) The title compound was prepared from **11a** (400 mg, 0.742 mmol) and pyrrolidine (79 mg, 1.11 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from dichloromethane-Et₂O-*n*-hexane to give 292 mg (0.494 mmol, 66%) of **21** as a colorless powder. mp 132–136 °C. ¹H-NMR (CDCl₃) δ: 1.60–2.03 (4H, m), 2.25–2.80 (2H, m), 3.20 (1H, m), 3.43–3.60 (4H, m), 4.99 (1H, m), 6.31 (1H, s), 6.65 (1H, m), 6.93 (3H, m), 6.98–7.12 (3H, m), 7.22 (1H, t, *J*=7.6 Hz), 7.31–7.47 (8H, m), 7.54 (1H, t, *J*=7.6 Hz), 7.84 (1H, d, *J*=7.3 Hz). FAB-MS *m/z*: 592 (*M*⁺+1). *Anal.* Calcd for C₃₆H₃₁F₂N₃O₃·1H₂O: C, 70.92; H, 5.46; N, 6.89; F, 6.23. Found: C, 70.96; H, 5.41; N, 6.95; F, 6.35.

(Z)-4'-[(4,4-Difluoro-5-[(1-piperidino)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (22) The title compound was prepared from **11a** (400 mg, 0.742 mmol) and piperidine (127 mg, 1.49 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from CHCl₃-Et₂O-*n*-hexane to give 289 mg (0.477 mmol, 64%) of **22** as a colorless amorphous substance. ¹H-NMR (DMSO-*d*₆) δ: 1.40–1.60 (6H, m), 1.73 (2H, m), 2.94–3.60 (5H, m), 4.85 (1H, m), 6.76 (1H, s), 6.80 (1H, m), 7.01 (2H, m), 7.17 (1H, t, *J*=7.3 Hz), 7.24–7.60 (13H, m), 10.31 (1H, s). FAB-MS *m/z*: 605 (*M*⁺+1).

(Z)-4'-[(4,4-Difluoro-5-[(1-morpholino)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide (23) The title compound was prepared from **11a** (400 mg, 0.742 mmol) and morpholine (97 mg, 1.11 mmol) in the same manner as described in the synthesis of **12a**. The residue was recrystallized from 2-propanol-Et₂O to give 324 mg (0.533 mmol, 72%) of **23** as a colorless powder, mp 161–165 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.40 (2H, m), 3.07 (1H, m), 3.42–3.60 (8H, m), 4.84 (1H, m), 6.76 (1H, s), 6.80 (1H, m), 7.01 (2H, m), 7.18 (1H, t, *J*=7.6 Hz), 7.27–7.60 (13H, m), 10.32 (1H, s). FAB-MS *m/z*: 608 (*M*⁺+1). *Anal.* Calcd for C₃₆H₃₁F₂N₃O₄·H₂O: C, 69.11; H, 5.58; N, 6.72; F, 6.07. Found: C, 69.43; H, 5.58; N, 6.52; F, 5.87.

(Z)-4'-[(4,4-Difluoro-5-[*N*-(3-dimethylaminopropyl)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide Hydrochloride (24) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N,N*-dimethylpropanediamine (72 mg, 0.707 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4*N* HCl/AcOEt solution, the residue was triturated with EtOH-Et₂O to give 245 mg (0.372 mmol, 80%) of **24** as a colorless amorphous substance. ¹H-NMR (DMSO-*d*₆) δ: 1.81–1.92 (2H, m), 2.39 (1H, m), 2.67 (1H, m), 2.73 (6H, s), 2.96–3.30 (5H, m), 4.88 (1H, m), 6.52 (1H, s), 6.76 (1H, m), 7.02 (2H, m), 7.12–7.41 (9H, m), 7.42–7.51 (3H, m), 7.52–7.60 (2H, m), 8.51 (1H, m), 10.32 (1H, s), 10.59 (1H, m). FAB-MS *m/z*: 623 (*M*⁺+1).

(Z)-4'-[(4,4-Difluoro-5-[*N*-(2-dimethylaminoethyl)-*N*-methylcarbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide Monohydrochloride (25) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N,N,N'*-trimethylethylenediamine (76 mg, 0.744 mmol) in the same manner as described in the synthesis of **12a** as a colorless powder (190 mg, 0.288 mmol, 62% yield), mp 157–160 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.41 (2H, m), 2.80 (6H, m), 3.08 (3H, s), 3.25 (2H, m), 3.55–4.00 (3H, m), 4.86 (1H, m), 6.79 (2H, m), 7.00 (2H, m), 7.19 (1H, m), 7.24–7.41 (8H, m), 7.46 (3H, m), 7.54 (5H, m), 10.34 (1H, s), 10.64 (1H, m). EI-MS *m/z*: 622 (*M*⁺). *Anal.* Calcd for C₃₇H₃₆F₂N₄O₃·1HCl·3H₂O: C, 62.31; H, 6.08; N, 7.86; Cl, 4.97; F, 5.33. Found: C, 62.34; H, 5.86; N, 7.66; Cl, 5.17; F, 5.63.

(Z)-4'-[(4,4-Difluoro-5-[(1-piperadino)carbamoylmethylene]-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl)carbonyl]-2-phenylbenzanilide Hydrochloride (26) The title compound was prepared from **11a** (94 mg, 0.175 mmol) and piperadine (90 mg, 1.04 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4*N*-HCl/AcOEt solution,

the residue was recrystallized from MeOH–Et₂O to give 70 mg (0.109 mmol, 62%) of **26** as a colorless amorphous substance. ¹H-NMR (CDCl₃) δ: 2.42 (2H, m), 3.02–3.22 (5H, m), 3.60–3.92 (4H, m), 4.87 (1H, m), 6.77 (1H, s), 6.82 (1H, m), 7.01 (2H, m), 7.20 (1H, m), 7.25–7.41 (8H, m), 7.47 (2H, m), 7.51–7.62 (3H, m), 9.30 (1H, m), 10.34 (1H, s). EI-MS *m/z*: 606 (M⁺).

(Z)-4'-([5-(4-Ethyl-1-piperadinyl)carbonylmethylene]-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl)-2-phenylbenzanilide Monohydrochloride (27) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N*-ethylpiperadine (79 mg, 0.693 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4 *N*-HCl/AcOEt solution, the residue was recrystallized from EtOH–Et₂O to give 221 mg (0.329 mmol, 48%) of **27** as a colorless powder, mp 189–191 °C. ¹H-NMR (DMSO-*d*₆) δ: 1.27 (3H, t, *J* = 7 Hz), 2.41 (2H, m), 2.78–3.30 (6H, m), 3.52 (2H, m), 3.64 (1H, m), 4.10 (1H, m), 4.46 (1H, m), 4.86 (1H, m), 6.77 (1H, s), 6.82 (1H, m), 7.01 (2H, m), 7.20 (1H, m), 7.24–7.41 (8H, m), 7.43–7.51 (2H, m), 7.52–7.58 (3H, m), 10.34 (1H, s), 11.15 (1H, m). FAB-MS *m/z*: 635 (M⁺ + 1). *Anal.* Calcd for C₃₈H₃₆F₂N₄O₃·HCl·0.2H₂O: C, 67.64; H, 5.59; N, 8.30; Cl, 5.25; F, 5.63. Found: C, 67.61; H, 5.56; N, 8.35; Cl, 5.41; F, 5.55.

(Z)-4'-([5-(4-Benzyl-1-piperadinyl)carbonylmethylene]-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl)-2-phenylbenzanilide Monohydrochloride (28) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N*-benzylpiperadine (98 mg, 0.558 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4 *N*-HCl/AcOEt solution, the residue was recrystallized from CH₃CN–Et₂O to give 200 mg (0.273 mmol, 59%) of **28** as a colorless powder, mp 164–169 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.41 (2H, m), 2.84–3.25 (4H, m), 3.30–3.46 (2H, m), 3.63 (1H, m), 4.02 (1H, m), 4.33–4.51 (3H, m), 4.87 (1H, m), 6.76 (1H, s), 6.81 (1H, m), 7.00 (2H, m), 7.19 (1H, m), 7.24–7.41 (8H, m), 7.42–7.77 (10H, m), 10.33 (1H, s), 11.19 (1H, m). FAB-MS *m/z*: 697 (M⁺ + 1). *Anal.* Calcd for C₄₃H₃₈F₂N₄O₃·HCl·1.5H₂O: C, 67.93; H, 5.57; N, 7.37; Cl, 4.66; F, 5.00. Found: C, 67.83; H, 5.40; N, 7.40; Cl, 4.36; F, 4.83.

(Z)-4'-([4,4-Difluoro-5-[(4-dimethylaminopiperidino)carbonylmethylene]-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl)-2-phenylbenzanilide Monohydrochloride (29) The title compound was prepared from **11a** (196 mg, 0.364 mmol) and 4-dimethylaminopiperidine (56 mg, 0.437 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4 *N*-HCl/AcOEt solution, the residue was recrystallized from CHCl₃–Et₂O to give 160 mg (0.233 mmol, 64%) of **29** as a colorless powder, mp 240 °C (dec.). ¹H-NMR (CDCl₃) δ: 1.39–1.80 (2H, m), 2.07 (2H, m), 2.41 (2H, m), 2.66 (1H, m), 2.72 (6H, s), 2.95–3.20 (2H, m), 3.43 (1H, m), 4.04 (1H, m), 4.52 (1H, m), 4.86 (1H, m), 6.78 (1H, s), 6.81 (1H, m), 7.01 (2H, m), 7.19 (1H, m), 7.26–7.43 (8H, m), 7.44–7.60 (5H, m), 10.35 (1H, s). EI-MS *m/z*: 649 (M⁺ + 1). *Anal.* Calcd for C₃₉H₃₈F₂N₄O₃·HCl: C, 68.36; H, 5.74; N, 8.18; Cl, 5.17; F, 5.55. Found: C, 68.44; H, 5.87; N, 8.09; Cl, 5.07; F, 5.41.

(Z)-4'-([5-[(1,4-Diazepin-1-yl)carbonylmethylene]-4,4-difluoro-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl)-2-phenylbenzanilide Monohydrochloride (30) The title compound was prepared from **11a** (220 mg, 0.408 mmol) and homopiperazine (205 mg, 2.05 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4 *N*-HCl/AcOEt solution, the residue was recrystallized from EtOH–Et₂O to give 220 mg (0.334 mmol, 82%) of **30** as a colorless powder, mp 196–201 °C. ¹H-NMR (DMSO-*d*₆) δ: 1.97–2.10 (2H, m), 2.41 (2H, m), 3.16 (5H, m), 3.50–3.92 (5H, m), 4.87 (1H, m), 6.76–6.86 (2H, m), 7.01 (2H, m), 7.20 (1H, m), 7.24–7.41 (8H, m), 7.43–7.61 (5H, m), 8.96 (1H, m), 10.34 (1H,

s). FAB-MS *m/z*: 621 (M⁺ + 1). *Anal.* Calcd for C₃₇H₃₄F₂N₄O₃·HCl·0.5H₂O: C, 66.71; H, 5.45; N, 8.41; Cl, 5.32; F, 5.70. Found: C, 66.62; H, 5.37; N, 8.41; Cl, 5.49; F, 5.64.

(Z)-4'-([4,4-Difluoro-5-[(4-methyl-1,4-diazepin-1-yl)carbonyl methyl-ene]-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)carbonyl)-2-phenylbenzanilide Monohydrochloride (31) The title compound was prepared from **11a** (250 mg, 0.464 mmol) and *N*-methylhomopiperazine (81 mg, 0.707 mmol) in the same manner as described in the synthesis of **12a**. After addition of 4 *N*-HCl/AcOEt solution, the residue was recrystallized from EtOH–Et₂O to give 222 mg (0.331 mmol, 71%) of **31** as a colorless powder, mp 216–218 °C. ¹H-NMR (DMSO-*d*₆) δ: 2.05–2.34 (2H, m), 2.35–2.55 (2H, m), 2.79 (3H, s), 2.92–4.08 (9H, m), 4.86 (1H, m), 6.74–6.89 (2H, m), 7.01 (2H, m), 7.19 (1H, m), 7.25–7.41 (8H, m), 7.42–7.52 (2H, m), 7.53–7.60 (3H, m), 10.35 (1H, s), 10.84 (1H, m). FAB-MS *m/z*: 635 (M⁺ + 1). *Anal.* Calcd for C₃₈H₃₆F₂N₄O₃·HCl·0.4H₂O: C, 67.28; H, 5.62; N, 8.26; Cl, 5.23; F, 5.60. Found: C, 67.27; H, 5.55; N, 8.32; Cl, 5.36; F, 5.59.

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References

- 1) Michel R. H., Kirk C. J., Billah M. M., *Biochem. Soc. Trans.*, **7**, 861–865 (1979).
- 2) Jard S., *Kidney Int., Supp.*, **26**, 38–42 (1988).
- 3) Yatsu T., Tomura T., Tahara A., Wada K., Tsukada J., Uchida W., Tanaka A., Takenaka T., *Eur. J. Pharmacol.*, **321**, 225–230 (1997).
- 4) Manning M., Sawyer W. H., *J. Lab. Clin. Med.*, **114**, 617–632 (1989).
- 5) Creager M. A., Facon D. P., Cutler S. S., Kohlmann O., Ryan T. J., Gavras H., *J. Am. Coll. Cardiol.*, **7**, 758–765 (1986).
- 6) Goldsmith S. R., Francis G. S., Cowly A. W., Jr., Goldenberg I. F., Cohn J. N., *J. Am. Coll. Cardiol.*, **8**, 779–783 (1986).
- 7) Lee W. H., Packer M., *Circulation*, **73**, 257–267 (1986).
- 8) Matsuhisa A., Tanaka A., Kikuchi K., Shimada Y., Yatsu T., Yanagisawa I., *Chem. Pharm. Bull.*, **45**, 1870–1874 (1997).
- 9) Matsuhisa A., Koshio H., Sakamoto K., Taniguchi N., Yatsu T., Tanaka A., *Chem. Pharm. Bull.*, **46**, 1566–1579 (1998).
- 10) Matsuhisa A., Kikuchi K., Sakamoto K., Yatsu T., Tanaka A., *Chem. Pharm. Bull.*, **47**, 329–339 (1999).
- 11) Bondi A., *J. Phys. Chem.*, **68**, 441–451 (1964).
- 12) Filler R., Kobayashi Y., "Biomedical aspects of fluorine chemistry," Kodansha, Tokyo, 1982, pp. 1–32.
- 13) Proctor G. R., *J. Chem. Soc.*, **1961**, 3989–3994.
- 14) Davis F. A., Han W., *Tetrahedron Lett.*, **32**, 1631–1634 (1991).
- 15) Umemoto T., Fukami S., Tomizawa G., Harasawa K., Kawada K., Tomita K., *J. Am. Chem. Soc.*, **112**, 8563–8575 (1990).
- 16) Bellamy F. D., Ou K., *Tetrahedron Lett.*, **25**, 839–842 (1984).
- 17) Wadsworth W. S. Jr., Emmons W. D., *J. Am. Chem. Soc.*, **83**, 1733–1738 (1961).
- 18) Ager D. J., *Org. React.*, **38**, 1–233 (1990).
- 19) Still W. C., Gennari C., *Tetrahedron Lett.*, **24**, 4405–4408 (1983).
- 20) Larcheveque M., Debel A., *J. Chem. Soc. Chem. Comm.*, **1981**, 877–878.
- 21) Bose A. K., Manhas M. S., Ramer R. M., *J. Chem. Soc. (C)*, **1969**, 2728–2730.

Molecular Orbital Studies on Pericyclic Reactions of Cinnamyl Xanthates in β -Cyclodextrin Cavities

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The solid β -cyclodextrin (β -CyD) complex of *O*-cinnamyl *S*-methyl dithiocarbonates (xanthate, **1a**), upon heating at 45 °C, underwent asymmetric [3,3]-sigmatropic rearrangement to give the optically *S*-(1-phenylallyl) *S*-methyl dithiocarbonate (**2a**) with 60% ee. Heating the β -CyD complex of **2a** at 120 °C caused extrusion of COS to give cinnamyl methyl sulfide (**3a**) in high yield. The reaction behavior and role of β -CyD are discussed based on molecular orbital calculation data.

Key words *O*-cinnamyl *S*-methyl dithiocarbonate; β -cyclodextrin; pericyclic reaction; *S*-(1-phenylallyl) *S*-methyl dithiocarbonate; PM3; asymmetric induction

Cyclodextrins (CyDs) consist of six or more α -1,4-linked D-glucose units forming truncated cone-shaped compounds. The inner cavity has a hydrophobic character, forming inclusion complexes with a wide variety of guest molecules. Therefore, CyDs have attracted much attention as host molecules in complexation and catalysis studies.¹⁾ In particular, reaction controls using the hydrophobic interior as a reaction field have been studied in recent years.²⁾ However, although some asymmetric reactions in solution have been attempted in the presence of CyDs, satisfactory optical yields have not yet been obtained.¹⁾

Allylic xanthates (**1**) underwent [3,3]-sigmatropic rearrangement to give the dithiolcarbonates (**2**)³⁾ which, on heating, extruded COS to give the allylically rearranged sulfides (**3**).^{4a)} In this connection, we have communicated a preliminary result of the asymmetric [3,3]-sigmatropic rearrangement of *O*-cinnamyl *S*-methyl xanthate (**1a**) in crystalline β -CyD complex as a chiral environment.⁵⁾

In this paper, we wish to report details of the asymmetric induction in the [3,3]-sigmatropic rearrangement of **1a** and rate enhancement of COS extrusion of the rearrangement product (**2a**) in β -CyD based on PM3^{6a)} calculation data.

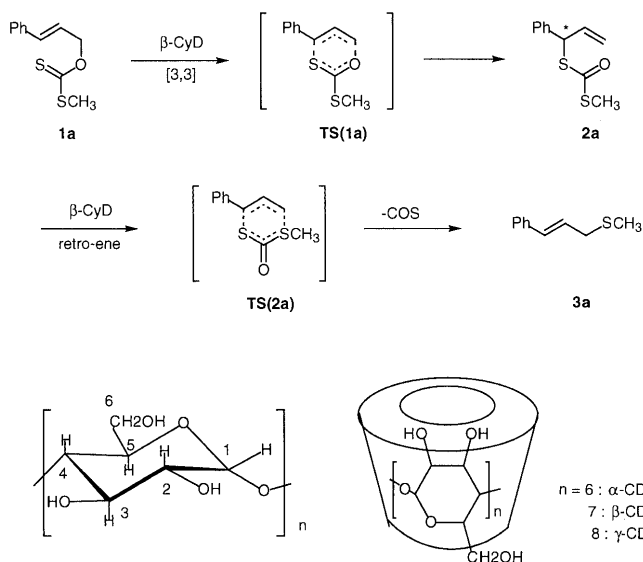


Chart 1

Results

Asymmetric Rearrangement of Cinnamyl Xanthates (1a—c) The complex of **1a** was prepared by the crystallization method from an aqueous saturated solution of β -CyD and was shown to be a 1 : 1 host guest complex by ¹H-NMR integration. The solid β -CyD complex of **1a** was heated at 80 °C for 6 h. The complex was dissolved in dimethyl sulfoxide (DMSO) and the guest was extracted with benzene to afford **2a** in 81% chemical yield. The product **2a** was enantiomerically enriched (46% ee). The extract from the complex of racemic **2a** did not show any optical activity, indicating that the observed enantioselectivity is not due to optical resolution. When the reaction was performed at 45 °C, an improvement in both yield (90%) and enantioselectivity (60% ee) was observed. The corresponding α or γ complex did not show sufficient enantioselectivity and *S*-alkyl groups bulkier than *S*-methyl showed low optical yields (see Table 1).

COS Extrusion Reaction of Dithiolcarbonates (2a—d) Next, the effect of CyD on the rate enhancement of the COS extrusion reaction was studied. Previously, we reported that the reaction was effectively accelerated by phenols which form hydrogen bonds between the dithiocarbonyl group and the hydroxy group of the phenols.^{4b)} When the 1 : 1 solid β -CyD complex of **2a** was heated at 120 °C for 6 h, cinnamyl methyl sulfide (**3a**) was formed as a single product in 81% yield (Table 2). In the case of the acetyl methyl derivative (**2d**), the extrusion of COS occurred smoothly to give the corresponding sulfide (**3d**) in 95% yield. The extrusion rate of **2a** based on the half-life of the complex was $2.0 \times 10^{-4} \text{ s}^{-1}$.

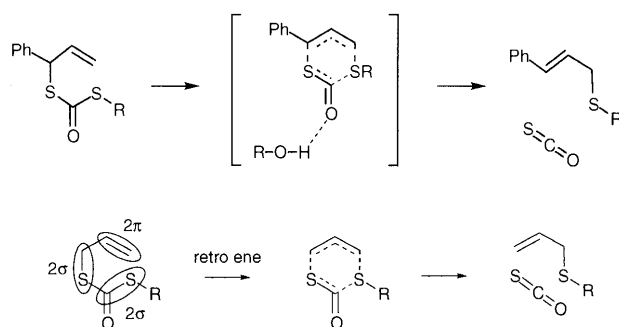


Chart 2

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while, in the absence of CyD, the rate was $1.18 \times 10^{-5} \text{ s}^{-1}$. The rate enhancement is comparable with that of *p*-nitrophenol catalyst ($1.23 \times 10^{-4} \text{ s}^{-1}$).^{4b)}

Molecular Orbital (MO) Calculations Semi-empirical MO calculations were run through the ANCHOR II interface using MOPAC6.0^{6a)} on a Fujitsu S-4/2 workstation or through the CS Chem3D Pro interface using MOPAC93 on a Power Macintosh G3 computer. Density functional theory (DFT) and Hartree-Fock (HF) computations⁷⁾ were carried out on a Scientists' Paradise Dragon AXP5A/433 computer or an HP Exemplar Technical Server V2250KS in the Kumamoto University Information Processing Center. The PM3-optimized structures were used as starting geometries for the HF and DFT calculations. The PM3 calculations of β -CyD and its inclusion compounds were carried out using a

locally modified version of the MOPAC package^{6a)} on a Dragon AXP433 computer.

Ground-State (GS) Structures of the Guests of Xanthate (1a) and Dithiolcarbonate (2a) First of all, conformational studies were undertaken on all the free guests. The PM3 calculation on **1a** showed the existence of two local minimum conformations. The methyl and methylene groups lie within the plane of the $-\text{O}(\text{C}=\text{S})\text{S}-$ moiety with *syn/anti* dispositions with respect to the $\text{C}=\text{S}$ bond.^{6b)} The *anti* conformation was calculated to be *ca.* $1.4 \text{ kcal mol}^{-1}$ more stable than the *syn* conformation. However, HF/6-31G* and DFT (B3LYP/6-31G*) calculations of **1a** gave an opposite prediction, the structural feature (*syn*) of which is in accordance with that observed in the crystal structures of analogous compounds.^{8a)}

The minimum-energy geometry of the allylicly rearranged product (**2a**) was also determined in a similar manner to that described above. The most stable conformation of **2a** is *syn*, in contrast to the PM3 optimized structure of **1a**. The structures calculated at 3-21G* and 6-31G* levels also have a *syn* conformation.^{8b)}

Transition Structure of [3,3]-Sigmatropic Rearrangement of Xanthate (1a) The transition structure strongly depends on the calculation levels. In the B3LYP/6-31G* transition-state (TS) structure, the bond distances to be created ($\text{S} \cdots \text{C}$) and broken ($\text{C} \cdots \text{O}$) were 2.808 and 2.057 Å, respectively, and those are very long in comparison with those of the PM3-calculated TS ($\text{C} \cdots \text{O}$: 2.094 Å; $\text{S} \cdots \text{C}$: 1.600 Å).

The energies calculated at the HF/3-21G* level are questionable. The rearranged product (**2a**) is estimated to be less stable ($1.53 \text{ kcal mol}^{-1}$ endothermic) than **1a** (Table 3). The HF/3-21G* calculations seem to overestimate the contribution of the resonance energy of the styrene moiety.⁹⁾ In contrast, the calculation at the B3LYP/6-31G* level is reasonable, indicating $3.67 \text{ kcal mol}^{-1}$ exothermic. The reaction barrier calculated by the B3LYP/6-31G* is $25.1 \text{ kcal mol}^{-1}$, which is close to the experimental value ($26.2 \text{ kcal mol}^{-1}$) in *n*-hexadecane. The PM3 reaction barrier ($25.0 \text{ kcal mol}^{-1}$) is also close to the observed value (see Table 3).

Table 1. [3,3]-Sigmatropic Rearrangement of *O*-Cinnamyl *S*-Alkyl Xanthates (**1a–c**) in the Crystalline CyD Complexes

R	CyD	Temp. (°C)	Time (h)	2 , Yield (%)	ee (%) ^{a)}
Me (1a)	β	80	6	81	46
	β	70	12	70	56
	β	60	48	60	58
	β	45	48	90	60
	β	35	120	82	51
	α	60	48	40	3 ^{b)}
	γ	60	48	87	12 ^{b)}
Et (1b)	β	45	48	41	14
PhCH ₂ (1c)	β	45	48	70	11

a) Determined by HPLC using a DAICEL CHILALCEL OJ column. b) See ref. 5a).

Table 2. COS Extrusion Reaction^{a)} of *S*-(1-Phenylallyl) *S*-Alkyl Dithiocarbonates (**2a–d**) in the Crystalline β -CyD Complexes

R	Product, Yield (%)
Me (2a)	3a , 81
Et (2b)	3b , 88
CH ₂ Ph (2c)	3c , 73
CH ₂ COMe (2d)	3d , 95

a) at 120 °C, 6 h.

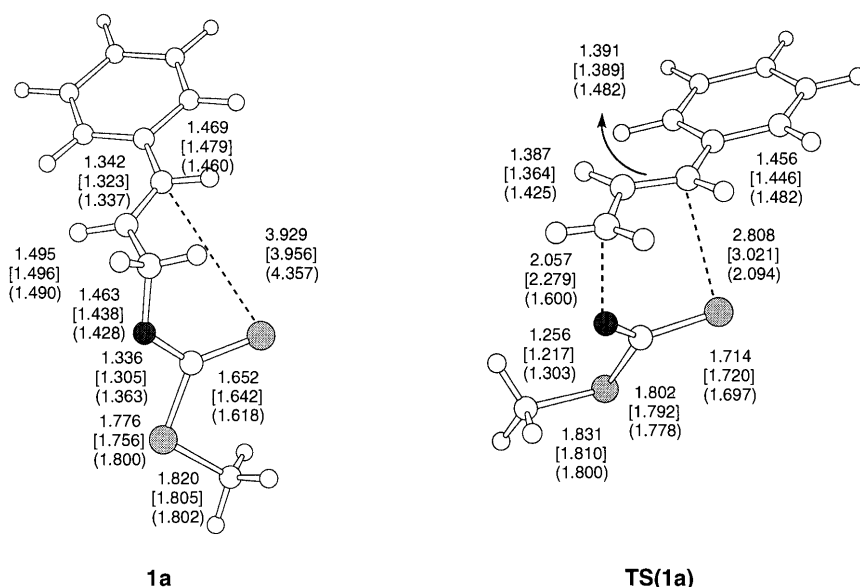
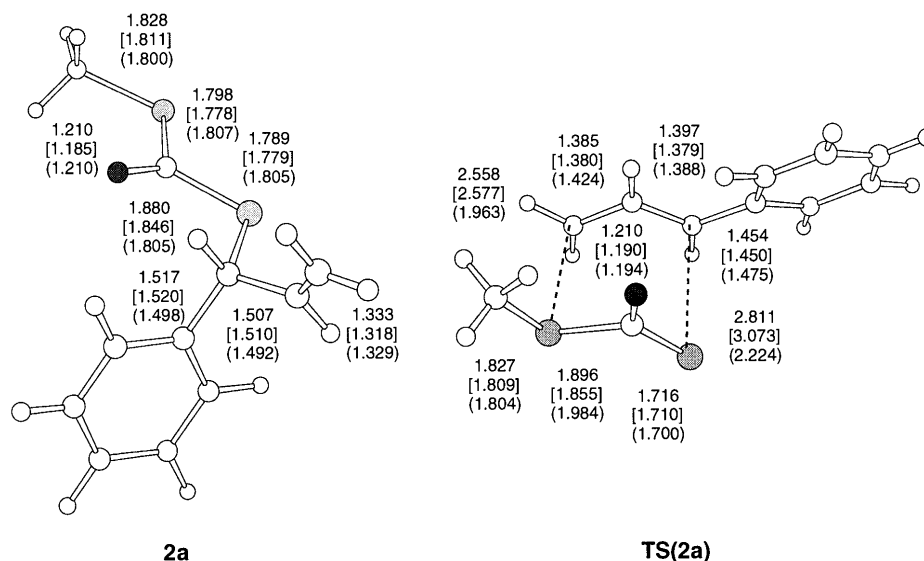


Fig. 1. B3LYP/6-31G*, HF/6-31G* and PM3 Optimized Structures of **1a** and TS(**1a**)

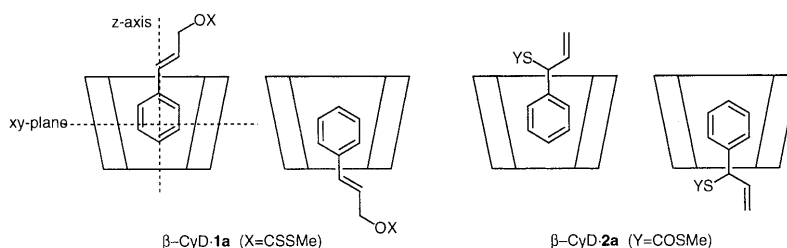
HF/6-31G* and PM3 calculated values in parentheses [] and (), respectively.

Fig. 2. B3LYP/6-31G*, HF/6-31G* and PM3 Optimized Structures of **2a** and TS(**2a**)

HF/6-31G* and PM3 calculated values in parentheses [] and (), respectively.

Table 3. Reaction Barriers and Heats of Formation for the [3,3]-Sigmatropic Rearrangement of **1a** and COS Extrusion of **2a**

Reaction calculated	PM3 ^{a)}	HF/3-21G* ^{b)}	HF/6-31G* ^{b)}	MP2//HF/6-31G* ^{b)}	B3LYP/6-31G* ^{b)}
Sigmatropy of 1a					
1a	41.9 ^{c)}	−1286.9366	−1293.3967	−1295.2128	−1297.9770 ^{d)}
TS	66.9 ^{c)}	−1286.8731	−1293.3386	−1295.1504	−1297.9370
2a	17.3	−1286.9318	−1293.4031	−1295.2287	−1297.9829
Reaction barrier	25.0	39.9 ^{a)}	36.5 ^{a)}	39.1 ^{a)}	25.1 ^{a)}
Heat of reaction	−24.6	1.53 ^{a)}	−3.97 ^{a)}	−9.97 ^{a)}	−3.67 ^{a)}
COS Extrusion of 2a					
TS	46.1		−1293.3290	−1295.1564	−1297.9383
3a +COS	7.8		−1293.4278	−1295.2441	−1298.0030
Reaction barrier	28.8		46.5 ^{a)}	45.4 ^{a)}	27.9 ^{a)}

a) kcal mol^{−1}, b) Hartree, 1Hartree=627.5 kcal mol^{−1}, c) *syn*, d) *anti*, E = −1297.9747.Fig. 3. Different Modes for the Entry of **1a** and **2a** into the Cavity of β -CyD

Transition Structure of COS Extrusion of Dithiolcarbonate (2a**)** The TS structures of the extrusion reaction show that the reaction proceeds through a six-membered nonionic cyclic $[2\pi+2\sigma+2\sigma]$ transition state.^{4a)} The B3LYP/6-31G* distances to be created and broken were 2.558 and 2.811 Å, respectively. The B3LYP/6-31G* reaction barrier is 27.9 kcal mol^{−1}.

PM3-Calculated Ground-State Structure of the Complexes To study the inclusion behavior, the energies and structures of the complexes were calculated. Theoretical treatments of the complexation process using molecular mechanics (MM) calculations have been reported.¹⁰⁾ However, there are few MM parameters involving sulfur functional groups in which electrostatic interactions have been esti-

mated. In this study, the PM3 MO method was used to investigate the complexation.^{6b)} As the number of doubly occupied levels is very large (261), a workstation equipped with a high-performance DEC α CPU was used.

The initial structure of β -CyD was constructed by connecting seven units of α -D-glucopyranose by α -1,4-bonding and this was fully minimized. For the starting structure of the guest (**1a**), the PM3 optimized *syn* conformation was used. The entry of the phenyl ring of the guests may occur through either the larger or smaller rim of the truncated cone of β -CyD, giving rise to two different orientations (Fig. 3).

Initial geometries of the complexes for different inclusion modes were located by docking the guests with the host. The β -CyD molecule was oriented so as to have almost seven

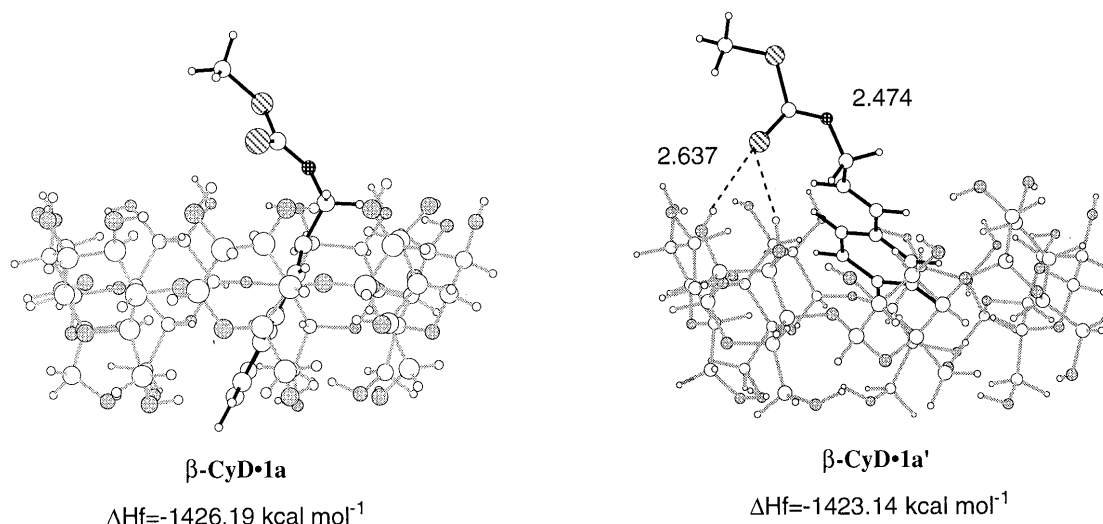


Fig. 4. GS Structures for the Nonhydrogen-Bonded and Hydrogen-Bonded Complexes of **1a** and β -CyD

Table 4. PM3 Calculated Heats of Formation (ΔH_f , kcal mol⁻¹) of the β -CyD Complexes of **1a**, TS(**1a**), **2a** and TS(**2a**)

Complex calculated	Orientation of the dithiocarbonyl group		[Guest + CyD]
	Larger rim side ^{a)}	Smaller rim side ^{b)}	
β -CyD 1a	-1426.2 (-21.0) ^{d)}	-1420.3 (-15.1) ^{d)}	-1405.2 [41.9 + (-1447.1)]
β -CyD· 1a ^{c)}	-1423.1 (-17.9) ^{d)}		
β -CyD·TS(1a) ^{c,ef)}	-1396.7 (-16.5) ^{d,g)}		-1380.2 [66.9 + (-1447.1)]
β -CyD· 2a ^{c)}	-1451.0 (-21.2) ^{d,h)}	-1443.4 (-13.6) ^{d)}	-1429.8 [17.3 + (-1447.1)]
β -CyD·TS(2a) ^{c)}	-1417.4 (-16.4) ^{d)}		-1401.0 [46.1 + (-1447.1)]

^{a)} The dithiocarbonyl moiety orients to the secondary hydroxy groups. ^{b)} The dithiocarbonyl moiety orients to the primary hydroxy groups. ^{c)} Hydrogen-bonded complex. ^{d)} Stabilization energy due to complexation in parenthesis. ^{e)} The lowest two eigenvalues of Hessian are -445.56 and -1.56. ^{f)} The AM1 calculation gave a similar TS structure. ^{g)} *R* form. The ΔH_f for *S* isomer [β -CyD-*S*-TS(**1a**)] is -1392.2 kcal mol⁻¹. ^{h)} *R* form. The ΔH_f for *S* isomer (β -CyD·*S*-**2a**) is -1446.7 kcal mol⁻¹.

glycoside oxygen atoms in the X-Y plane and the secondary hydroxy groups on the positive region of the Z axis. The guests were located by keeping the HC-Ph bond aligned with the C₇ symmetry axis (Z-axis) of β -CyD and several initial geometries were generated by moving the guest molecule along the Z axis from -4 to 4 Å distance to the XY plane. No restrictions were imposed on the optimization of the host and guest geometries. Energy minimization of the structures was performed until a gradient norm (GNORM) was less than about 0.1 kcal mol⁻¹. The structures^{6c)} and energies are shown in Fig. 4 and Table 4, respectively.

PM3-Calculated Transition Structures of the β -CyD Complex Next, the β -CyD complex (through the larger rim) of the cyclic TS [TS(**1a**)] of the [3,3]-sigmatropic rearrangement of **1a** was calculated by PM3. The crude TS geometry was obtained using the eigenvector following (EF) routine, keeping the C-O and C-S distances of the guest fixed (-CH₂··O 1.600 Å, =CHPh··S-CO 2.094 Å) (Table 4). Then, the obtained structure was fully optimized using the TS routine with the XYZ keyword. The TS geometry [β -CyD·TS(**1a**)] is shown in Fig. 5c.^{6c)} The vibrational frequency calculation indicated two imaginary frequencies for the stationary point (see Table 4 footnote e). However, the guest TS retains the authentic transition structure of TS(**1a**), in which the breaking C-O bond is slightly elongated (0.025 Å) compared with that of free TS(**1a**). Intrinsic reaction coordinate (IRC) calculation starting from the transition state confirms the presence of the shallow inclusion complex of **1a**

and β -CyD at the end of IRC. The rearrangement product has been obtained at the other end of IRC.

The β -CyD complex of the antipode (*S*) of TS(**1a**) is 4.5 kcal mol⁻¹ less stable than the β -CyD·TS(**1a**) (*R* form). The phenyl moiety of the *R* isomer was found to be more deeply inserted into the cavity compared with that of the *S* isomer.^{5b)}

Similarly, the complex of β -CyD and the cyclic transition state [TS(**2a**)] of the COS extrusion reaction of **2a** was calculated. The transition structure [β -CyD·TS(**2a**)] is shown in Fig. 6 (see also Table 4).

Discussion

The stabilization energy due to the formation of the inclusion compound was estimated as the energy difference ($\Delta\Delta H_f$) between $\Delta H_f(\text{guest} \cdot \beta\text{-CyD})$ and $\Delta H_f(\text{guest}) + \Delta H_f(\beta\text{-CyD})$. In the case of the β -CyD·**1a** complex, the values for the larger and smaller rim are 21.0 kcal mol⁻¹ and 15.1 kcal mol⁻¹, respectively. The difference [$\Delta H_f(\text{larger}) - \Delta H_f(\text{smaller})$] in heat of formation is 5.9 kcal mol⁻¹, suggesting that the entry of **1a** through the larger rim of β -CyD is favorable. Hereafter, we took no account of the complexation through the smaller rim.

The most stable GS structure of the complex [β -CyD·**1a**] has no hydrogen bonds between the thione sulfur and the secondary hydroxy groups of β -CyD, in which the phenyl ring is fixed by the H/H close contacts between the aromatic hydrogens and the inner >C-H protons of β -CyD. On the other

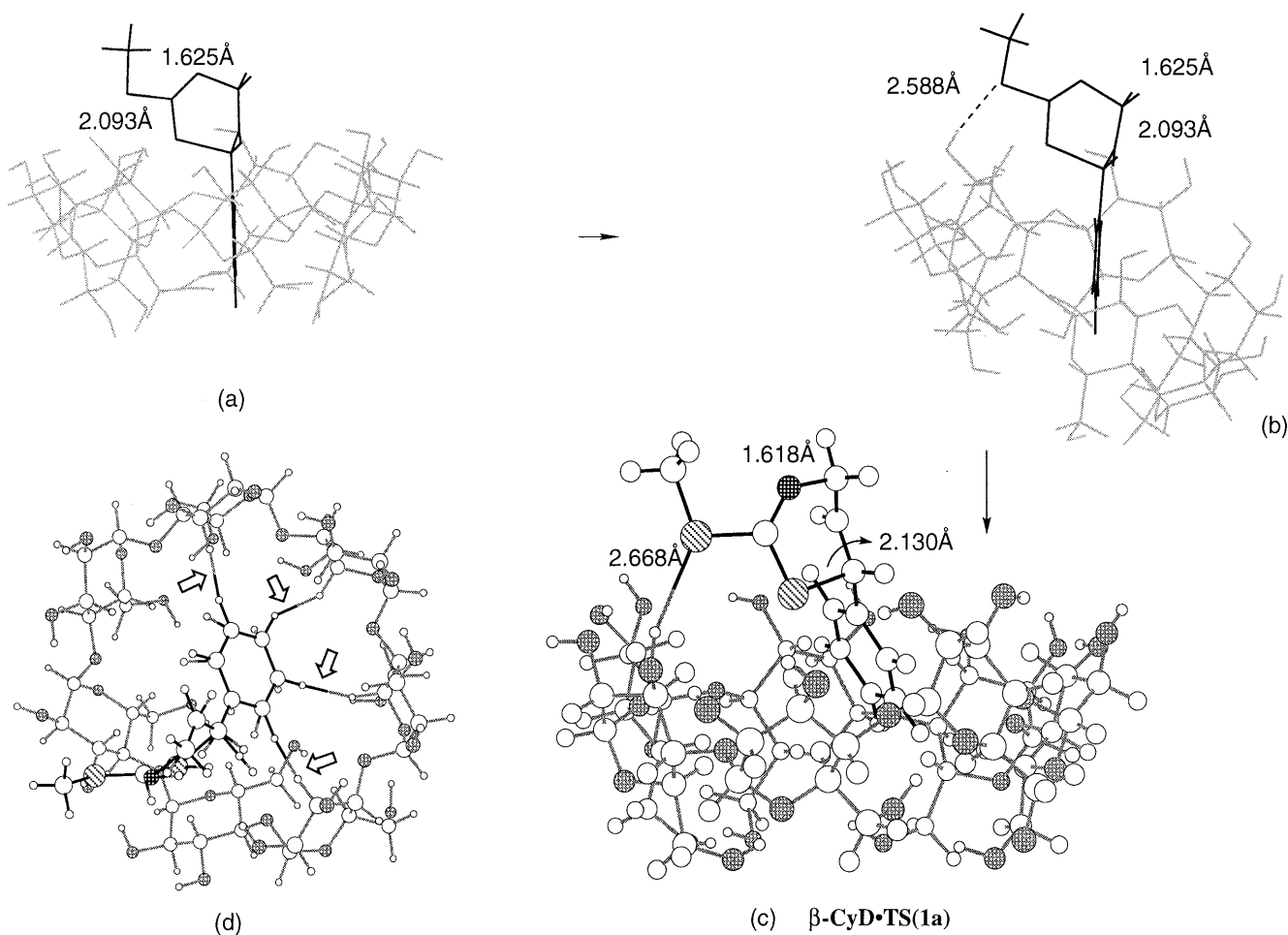


Fig. 5. Input GS Structure (a), Partially-Optimized TS (b) and Fully Optimized TS (c) Structures for $\beta\text{-CyD}\cdot\text{TS}(1a)$ Complex, (d) H/H Contacts in $\beta\text{-CyD}\cdot\text{TS}(1a)$ Complex

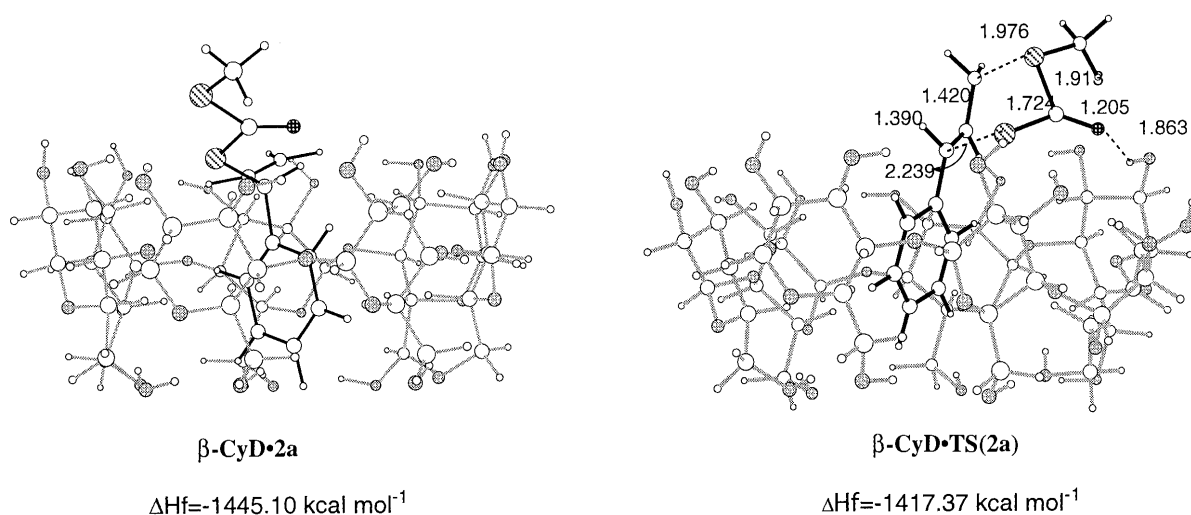


Fig. 6. GS Structure of **2a** and TS Structure for the Retro Ene Reaction of **2a**

hand, we obtained a hydrogen-bonded inclusion complex of **1a** and $\beta\text{-CyD}$ at the end of IRC starting from the [3,3]-sigmatropic TS [$\beta\text{-CyD}\cdot\text{TS}(1a)$]. The calculated structure of the complex [$\beta\text{-CyD}\cdot 1a'$] is depicted in Fig. 4. As can be seen in Fig. 4, the guest is shallowly inserted and the complex is about $3.0 \text{ kcal mol}^{-1}$ less stable than the deeply-inserted nonhydrogen-bonded complex, in which the thione sulfur forms hydrogen bonds with two secondary hydroxy

groups of $\beta\text{-CyD}$ (2.463, 2.708 Å). The degree of stabilization¹¹⁾ due to the hydrogen bonding is assumed to be *ca.* $6.3 \text{ kcal mol}^{-1}$ on the basis of a model calculation of the hydrogen-bond formation between *O*-allyl *S*-methyl xanthate and isopropyl alcohol (see Table 5), in which the hydrogen bond distance was calculated to be 2.57–2.62 Å. The HF and DFT calculations predicted a similar result.

The structures of the complex [$\beta\text{-CyD}\cdot\text{TS}(1a)$] of $\beta\text{-CyD}$

and the TS of **1a** are shown in Fig. 5c. The reaction center of the six-membered cyclic transition state of the guest is located entirely outside of the cavity and the sulfur atom of the methylthio group forms a hydrogen bond with one of the secondary hydroxy groups of the β -CyD edge. The DFT calculation (B3LYP/6-31+G**) indicates that the hydrogen bond energy of $>\text{S}\cdots\text{HOMe}$ is half that of $>\text{C}=\text{S}\cdots\text{HOMe}$.¹²⁾

A molecular modeling study of the deeply-inserted TS complexes made by docking, such that the six-membered cyclic reaction center is inserted into the middle of the cavity, indicated that such complexes are energetically unfavorable due to the presence of serious close contacts. During the structural optimization of a deeply-inserted TS complex [Fig. 5a], the reaction center got out of the β -CyD cavity and was transformed into the shallowly-inserted hydrogen-bonded TS complex, in which the phenyl moiety is fixed by the H/H close contacts (1.7—1.75 Å) between the aromatic hydrogens of the guest and the $>\text{C}-\text{H}$ hydrogens of β -CyD [see Fig. 5c, 5d].

The degree of stabilization due to the hydrogen bonding between the methylthio sulfur atom and a secondary hydroxy group of β -CyD is assumed to be *ca.* 1.1 kcal mol⁻¹ on the

basis of a model calculation of the hydrogen-bond formation of *O*-allyl *S*-methyl xanthate with isopropyl alcohol (see Table 5).

Inspection of the guest conformation of the complex [β -CyD·**1a**] indicates that the plane of the styrene moiety of the guest located at the center of the chiral environment is inclined against the pseudo-seven-fold axis of β -CyD (see Fig. 7A).¹³⁾ The two rooms divided by the plane involving the styrene moiety are not identical. This spatial discrepancy may affect enantioselectivity. As shown in Fig. 7, the thione sulfur would enantioselectively attack the inclined plane of the C=C bond from the side facing the wider opening site.

As described above, the hydrogen-bonded complex (β -CyD·**1a'**) is *ca.* 3 kcal mol⁻¹ less stable than the deeply-inserted complex (β -CyD·**1a**). The interreacting $=\text{S}\cdots\text{CH}(\text{Ph})=$ distance in β -CyD·**1a'** is *ca.* 1 Å longer than that of the deeply-inserted complex (β -CyD·**1a**). The hydrogen-bonded complex is considered to be formed only during the transformation process from the deeply-inserted complex (β -CyD·**1a**) to the TS [β -CyD·TS(**1a**)].

Previously, we reported the [3,3]-sigmatropic rearrangement rate of **1a** was moderately sensitive to solvent ionizing power compared with *O*-allyl *S*-methyl xanthate. Plots of the rearrangement rates of **1a** vs. Grunwald–Winstein *Y*-values showed an excellent linear relationship for alcohols,¹⁴⁾ and analysis of the rearrangement rates of **1a** by the Kamlet–Taft equation implied that solvent polarity and solvent hydrogen-bond donation ability (acidity) are important factors in the allylic thione-to-thiol rearrangement reactions.¹⁵⁾

Taking these facts into consideration, we can safely say that the TS of **1a** is anchored by the hydrogen bond between one of the secondary hydroxy groups of β -CyD and the methylthio sulfur atom.¹²⁾ However, the MO calculation of the complex of the TS of **1a** suggests that the [1,3]oxathiane ring is out of the cavity. This probably results in the reduced influence of the chiral environment upon the substrate, leading to moderate ee (*ca.* 60%) yields.

Next, the rate enhancement of the retro-ene type reaction^{4a)} of **2a** in β -CyD was investigated. In the complex (β -CyD·**2a**), the complexation through the larger rim is also favorable. The interacting distance between the sulfur atom of *S*-Me and the allyl γ carbon of **2a** (5.687 Å) is *ca.* 0.6 Å shorter than that of the relaxed GS structure (6.308 Å). Fig-

Table 5. Stabilization Energies due to Hydrogen Bonding between Alcohols and Xanthates

Method	Substrate	$\Delta\text{Hf}^{\text{a)}$ or $E^{\text{b)}$	$\Delta\Delta\text{Hf}$
PM3	Allyl xanthate	19.56 ^{a)}	
	IsoPrOH	-64.00 ^{a)}	
	Allyl xanthate+isoPrOH	-44.44 ^{a)}	
	Allyl xanthate-isoPrOH		
	Coordination to thioether	-45.56 ^{a)}	1.12
	Coordination to thione	-46.61 ^{a)}	2.17
	Two isoPrOH coordination	-114.55 ^{a)}	6.12
B3LYP/6-31G* (coordination to thione)	Dimethyl xanthate	-989.5243 ^{b)}	
	IsoPrOH	-194.3533 ^{b)}	
	Dimethyl xanthate+isoPrOH	-1183.8776 ^{b)}	
	Dimethyl xanthate-isoPrOH	-1183.8841 ^{b)}	4.1 ^{c)}
	Allyl xanthate	-1066.9158 ^{b)}	
	IsoPrOH	-194.3533 ^{b)}	
	Allyl xanthate-isoPrOH	-1261.2692 ^{b)}	
	Allyl xanthate-isoPrOH	-1261.2773 ^{b)}	5.1 ^{c)}

a) ΔHf : kcal mol⁻¹. b) *E*: Hartree. c) ΔE .

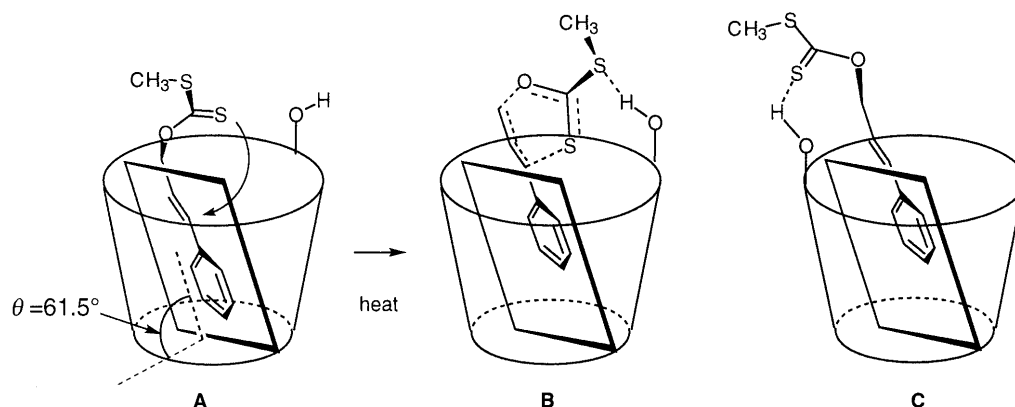


Fig. 7. Schematic Representation of the Asymmetric Induction

A: Deep inclusion complex of **1a** and β -CyD (nonhydrogen bonded). The angle [θ (Plane 1/Plane 2), deg.] between the best planes 1 and 2 is 61.5°, where Plane 1 is the best plane through the ether oxygen atoms connecting the sugar moieties, Plane 2 is that of the phenyl ring.

B: Inclusion complex of the TS of **2a** and β -CyD.

C: Shallow Inclusion complex of **1a** and β -CyD (IRC calculation product).

ure 6 shows that in the TS structure in β -CyD the interacting bond distances are essentially the same as those of the free TS. The carbonyl oxygen atom of the TS structure forms a hydrogen bond with one of the secondary hydroxy groups of β -CyD, very favorable for the extrusion of COS.^{4a)}

In summary, the [3,3]-sigmatropic rearrangement of **1** and the retro-ene type reaction of **2** in the solid β -CyD complex were investigated. Lowering the reaction temperature improved the enantioselectivity to 60% ee.¹⁶⁾ The PM3 calculations suggested that the phenyl rings of the guests (**1**, **2**) are included from the larger rim of the β -CyD host molecule and the TS structure are stabilized by a hydrogen bond with one of the hydroxy groups of β -CyD.

Experimental

IR Spectra were recorded with a Hitachi 270-30 spectrophotometer. ¹H-NMR Spectra were recorded with JEOL JNM-EX 270 (270 MHz) and GX-400 (400 MHz) spectrometers using tetramethylsilane (TMS) as an internal standard and chemical shifts are expressed in δ values. The coupling constants (*J*) are presented in Hz. High resolution mass spectra (HR-MS) were recorded with a JEOL JMS-DX303HF spectrometer. HPLC analyses were performed on a JASCO 880-PU chromatograph equipped with a JASCO 875-UV detector.

Materials β -CyD was supplied from Nippon Shokuhin Co. Ltd., and further purified by recrystallization from water. *O*-Cinnamyl *S*-alkyl xanthates (**1a**—**d**) were prepared by the procedure given in the previous paper.³⁾

Preparation of Crystalline β -CyD Inclusion Complexes Xanthate (**1a**, 135 mg, 0.6 mmol) was added to 100 ml saturated aqueous solution containing β -CyD (2.0 g). After stirring for 72 h at room temperature, the resulting white precipitates were collected by centrifugation, washed with Et₂O to remove **1a**, then dried under vacuum. The white crystalline powder was obtained as a 1 : 1 molar complex of **1a** with β -CyD. The amount of **1a** in the complex was determined by ¹H-NMR spectroscopy in DMSO-*d*₆. The ¹H-NMR spectra of the complexes prepared using an excess of β -CyD (>2 mol) indicated the presence of a 1 : 2 molar complex of **1a** with β -CyD.

The inclusion complexes of dithiolcarbonates (**2a**—**d**) with β -CyD were prepared in a similar manner.

Rearrangement of the Crystalline Inclusion Complex The solid 1 : 1 complex (100 mg) of **1a** with β -CyD was heated at 45 °C. After 48 h, the residue was dissolved in DMSO (4 ml), then stirred vigorously with benzene (30 ml) to extract the guests. The organic layer was separated, washed with water, and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure. The residue was purified by chromatography on silica-gel to afford the dithiolcarbonate (**2a**) as a colorless oil. The rearrangement product (**2a**) was identified by comparison of the spectral data with those of an authentic sample.³⁾

The enantiomeric excess of the dithiolcarbonate was determined by chiral HPLC analysis (eluent; *n*-hexane/ethanol, 8 : 2, flow rate; 1.0 ml min⁻¹; detection; UV 250 nm). **2a** t₁=11 min, t₂=27 min. **2b** t₁=9 min, t₂=17 min, **2c** t₁=16 min, t₂=26 min.

2d: IR (KBr) cm⁻¹: 1720 (>C=O), 1646 (ester SCOS); ¹H-NMR (270 MHz, CDCl₃): 2.26 (3H, s, CH₃), 3.81 (2H, s, SCH₂-CO), 5.23 (1H, ddd, *J*=10.2, 1.0, 1.0 Hz, CH=CH₂), 5.26 (1H, ddd, *J*=17.2, 1.0, 1.0, CH=CH₂), 5.41 (1H, d, *J*=6.9, >CHS-), 6.10 (1H, ddd, *J*=17.2, 10.2, 6.9, -CH=CH₂), 7.23—7.36 (5H, m, aromatic H); HR-MS/FAB Calcd for C₁₃H₁₄O₂S₂Na (M+Na⁺) 289.0278, Found 289.0333.

Sulfide Formation Reaction of the Crystalline Inclusion Complex The solid 1 : 1 complex (100 mg) of **2a** and β -CyD was heated at 120 °C. After 6 h, the residue was dissolved in DMSO (4 ml), then stirred vigorously with benzene (30 ml) to extract the reacted guests. The organic layer was separated, washed with water, and dried over anhydrous MgSO₄. The solvent was removed under reduced pressure and the residue was purified by chromatography on silica gel to give the sulfide (**3a**) as a colorless oil (81%). A similar procedure was followed for **2b**, **2c** and **2d**. The sulfides (**3a**—**d**) were identified by comparison of the spectral data with those of authentic samples.^{4b)}

3d: IR (KBr) cm⁻¹: 1708 (>C=O); ¹H-NMR (400 MHz, CDCl₃): 2.27 (3H, s, CH₃), 3.22 (2H, s, -SCH₂-CO), 3.29 (2H, dd, *J*=7.6, 1.3, =CHCH₂-S-), 6.12 (1H, dt, *J*=15.5, 7.6, PhCH=CH-), 6.47 (1H, d, *J*=16, PhCH=), 7.20—7.39 (5H, m, aromatic H); MS (*m/z*) 206 (M⁺). HR-MS/EI Calcd for C₁₂H₁₄OS (M⁺) 206.0765, Found 206.0737.

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References and Notes

- Bender M. L., Komiyama M., "Cyclodextrin Chemistry," Springer-Verlag, Berlin, 1978.
 - a) Breslow R., Guo T., *J. Am. Chem. Soc.*, **110**, 5613—5617 (1988); b) Tanaka Y., Sakuraba H., Nakanishi H., *J. Org. Chem.*, **55**, 564—567 (1990); c) Schneider H.-J., Sangwan N. K., *Angew. Chem. Int. Ed. Engl.*, **26**, 896—897 (1987); d) Pitchumani K., Velusamy P., Banu H. S., Srinivasan C., *Tetrahedron Lett.*, **36**, 1149—1152 (1995).
 - Harano K., Taguchi T., *Chem. Pharm. Bull.*, **20**, 2348—2356, 2357—2365 (1972); *Idem, ibid.*, **23**, 467—472 (1975).
 - a) Eto M., Nishimoto M., Kubota S., Matsuoka T., Harano K., *Tetrahedron Lett.*, **37**, 2445—2448 (1996); b) Harano K., Yamashiro S., Misaka K., Hisano T., *Chem. Pharm. Bull.*, **38**, 2956—2959 (1990). The reaction mechanism belongs to the category of a retro ene reaction and the rate enhancement due to the hydrogen bond is attributable to lowering of the LUMO energy level of the C—SR bond in the three-system interaction ($2\pi+2\sigma+2\sigma$), in which the C=C, MeS—CO and CH—S bonds are π -HOMO, σ -LUMO and σ -LUMO, respectively.
 - a) Harano K., Kiyonaga H., Hisano T., *Tetrahedron Lett.*, **32**, 7557—7558 (1991); b) The absolute configuration of the rearrangement product has not yet been determined. The NMR spectral information about the host-guest interaction has not yet been obtained because of the insolubility in D₂O.
 - a) Stewart J. J. P., A Semi-Empirical Molecular Orbital Program, QCPE 455 (1983); *idem, J. Comp. Chem.*, **10**, 209—220, 221—264 (1989). The program source written for the main frame computer systems was ported to the engineering workstation (Fujitsu S4/2, S4/10 and Scientists' Paradise Dragon AXP5A/433 computer). The main modifications are the time routine, input/output file system and enlargement of the array sizes. b) The AM1 calculation cannot reproduce the C=S distance of xanthates which is ca. 0.1 Å shorter than the crystallographic values. c) The coordinates of the calculated structures are available on request by E-mail.
 - Gaussian 94, Revision D.4, Frisch M. J., Trucks G. W., Schlegel H. B., Gill P. M. W., Johnson B. G., Robb M. A., Cheeseman J. R., Keith T., Petersson G. A., Montgomery J. A., Raghavachari K., Al-Laham M. A., Zakrzewski V. G., Ortiz J. V., Foresman J. B., Cioslowski J., Stefanov B. B., Nanayakkara A., Challacombe M., Peng C. Y., Ayala P. Y., Chen W., Wong M. W., Andres J. L., Replogle E. S., Gomperts R., Martin R. L., Fox D. J., Binkley J. S., Defrees D. J., Baker J., Stewart J. P., Head-Gordon M., Gonzalez C., Pople J. A., Gaussian, Inc., Pittsburgh PA, 1995.
 - a) Abrahamsson S., Innes M., *Acta Crystallogr. Sec. B*, **30**, 721—725 (1974); Dahlen B., *Acta Chem. Scand. Ser. A*, **31**, 407—411 (1977); b) X-Ray analysis of the [4+2]-cycloadduct of phenylcyclo and *S*-allyl *S*-methyl dithiocarbonate showed that the dithiol ester adopts the syn conformation, *Tetrahedron Lett.*, **41**, 4395—4400 (2000).
 - In the HF/3-21G* calculation, *S*-allyl *S*-methyl dithiocarbonate is 4.34 kcal mol⁻¹ more stable than *O*-allyl *S*-methyl xanthate (exothermic).
 - Jaime C., Redondo J., Sanches F. Virgili A., *J. Org. Chem.*, **55**, 4772—4776 (1990); Lipkowitz K. B., *ibid.*, **56**, 6357—6367 (1991); Fathallah M., Fotiadu F., Jaime C., *ibid.*, **59**, 1288—1293 (1994).
 - The value is of similar magnitude to the hydrogen-bond energy for the cytosine-thymine (C—T) base pair.
 - The calculated hydrogen-bond energies between dimethyl xanthate and methanol are as follows.
- | Method | >C=S··HOMe | >S··HOMe |
|----------------|-----------------------------|-----------------------------|
| PM3 | 2.62 kcal mol ⁻¹ | 1.21 kcal mol ⁻¹ |
| B3LYP/6-31+G** | 3.71 kcal mol ⁻¹ | 2.16 kcal mol ⁻¹ |
- The angles (θ , deg.) between the best planes A and B are as follows: θ (Plane 1/Plane 2), 61.5; θ (Plane 1/Plane 3), 64.0; θ (Plane 2/Plane 3), 11.7, where Plane 1 is the best plane through the ether oxygen atoms connecting the sugar moieties, Plane 2 is that of the phenyl ring and Plane 3 is that of the double bond.
 - a) Harano K., Kiyonaga H., Yokote C., Hisano T., *Chem. Pharm. Bull.*, **39**, 1952—1957 (1991); b) The hydrogen-bonding stabilization of the GS of xanthates seems to be small. In the visible absorption spectra of

- xanthates in alcohols, the blue shift of the C=S group of xanthates due to solvation of alcohols is very sensitive to the xanthate conformation (manuscript in preparation).
- 15) Eto M., Tajiri O., Nakagawa H., Harano K., *Tetrahedron*, **54**, 8009—8014 (1998).
- 16) The enhancement of ee at a lower temperature may be due to the suppression of mobility and thermal dissociation of the guest molecule.

Difference Spatial Distribution Function Analysis of Aqueous Solutions. IV.¹⁾ Hydration Structure Changes of Ethylene Glycol Solutions throughout Conformational Change Process from gGg' to tGg' Conformers

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Monte Carlo (MC) simulations were carried out for an infinitely dilute aqueous solution of two stable conformers (gGg' and tGg') and of three conformations between gGg' and tGg' conformers of ethylene glycol (EG) at 298 K. Based on the spatial distribution function (SDF) $g_{OO}(x, y, z)$, obtained from the MC simulation in the above conformations in liquid water, the high distribution of hydration water molecules could be divided into hydrogen acceptor (HA), hydrogen donor (HD), MIX (overlapped distribution of HA and HD), and hydrophobic hydration (HH) regions. The spatial orientations of hydrogen-bonded water molecules were found to be of a linear type with a triple-layer structure in the HA region and HA part (in the MIX region), and double-layer structures in the HD region and HD part (in the MIX region). In addition, it was apparent that the spatial orientations of these water molecules were of the linear type throughout the conformational change process from gGg' to tGg' conformers in liquid water. From the difference SDF (DSDF), $\Delta g_{OO}(x, y, z)$, between the SDFs of two conformations, we concluded that the distribution of hydration water molecules in the HA and HD parts of the MIX region are governed by the competition of internal hydrogen bonds between the hydrogen atom and two lone-pair electrons on the oxygen atom of an EG molecule.

Key words aqueous ethylene glycol solution; spatial distribution function; difference spatial distribution function; hydration structural change; conformational change

Theoretical investigations of the solvent effects on the physico-chemical properties of chemical substances and drugs are of growing importance in the study of chemical and biological reactions in solution. In order to clarify the structural geometries and physico-chemical properties of these molecules in aqueous solution, numerous researchers have carried out investigations by various molecular orbital (MO) methods and molecular mechanics.^{2–4)} The main approach of the MO method is reaction field theory,⁵⁾ in which solute molecules interact with a dielectric continuous medium of solvent in aqueous solution. In contrast, radial distribution function (RDF) results obtained from X-ray diffraction⁶⁾ and neutron scattering⁷⁾ methods have not suggested a homogeneous continuum structure of solvent and have shown that solvent effects cannot be reliably evaluated by the dielectric continuum model for solvent effects. Of course, molecular simulations⁸⁾ such as Monte Carlo (MC) and molecular dynamics (MD) simulations are entirely free from such approximations. However, these methods are too time-consuming to elucidate the hydration structure and solvent effect for a large solute molecule in aqueous solution.

Our studies in this series have therefore been carried out to address these problems regarding solvent effects and the calculation times required for MC simulations. First, our major goal was to develop a new method for the evaluation of solvent effects by considering the local structure of solvent water on the basis of the spatial distribution function⁹⁾ (SDF), $g_{OO}(x, y, z)$, as obtained from a MC simulation. Second, our approach was to establish the optimal construction method for SDF when applied to the hydration structure of a large molecular system by employing the SDFs of fundamental small molecular systems obtained from an MC simulation.

In general, it is considered that many chemical and biolog-

ical molecules exist in a state formed by internal and/or external hydrogen bonds in aqueous solution. In addition, based on the results of NMR measurements, it has been reported that the molecular conformations that make possible the formation of internal and/or external hydrogen bonds in apolar solvent differ significantly from the conformations in polar solvent, and that it is the polar solvent water that is primarily responsible for the induction of this change in molecular structure.¹⁰⁾ Therefore, we consider that it is very important to clarify the anisotropic hydration structures and solvent effects of such a molecule.

To succeed in our above approach, we adopted ethylene glycol (EG) as the simplest compound having two kinds of hydrogen bonds. The EG molecule is particularly hydrophilic due to its two hydroxyl groups, which are used as both hydrogen acceptor (HA) and hydrogen donor (HD) in hydrogen bonds with solvent water molecules. An interesting question arises regarding the hydration structure of various stable conformers based on the internal hydrogen bond between two adjacent hydroxyl groups and the external hydrogen bond with solvent water molecules. The EG molecule builds up a three-dimensional network of external hydrogen bonds with solvent water in aqueous EG solution, which suggests the existence of a variety of stable conformers based on the dihedral angle changes of the EG molecule. These conformers of EG are identified by the three O1–C1–C2–O2, H1–O1–C1–C2, and H2–O2–C2–C1 dihedral angles, and the central O1–C1–C2–O2 dihedral angle is denoted by a capital letter (e.g., gGg' and tGg' in Chart 1). The capital letter G indicates a conformation with a *gauche* position in the central dihedral angle. The symbols g, t, and g' represent *gauche* (+synclinal), *trans* (antiperiplanar), and *gauche'* (–cynclinal), as defined by Radom *et al.*, respectively.¹¹⁾

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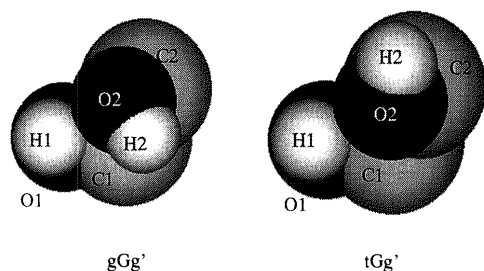


Chart 1. Two Conformers of Ethylene Glycol

Experimental studies on the conformational equilibrium of the EG molecule have been reported, *e.g.*, results measured by electron diffraction,¹²⁾ infrared (IR),¹³⁾ and microwave (MW)¹⁴⁾ spectroscopic methods. On the other hand, several researchers have theoretically investigated the conformational equilibrium of the EG molecule using various calculation methods.^{15–17)} Cramer and co-workers¹⁶⁾ have reported that the results at various levels of *ab initio* MO and semi-empirical MO calculations are consistent with experimental results. They have therefore concluded that populations (over roughly 95%) of *gauche* conformers (capital letter G) in the central dihedral angle for the equilibrium gas phase and aqueous solution at 298 K are much larger than those (within roughly 5%) of *trans* conformers. In addition, they have found that two *gauche* conformers (gGg' and tGg') with internal hydrogen bonds make up approximately 70–84% of the overall conformers in the gas phase and in aqueous solution. On the other hand, Nagy *et al.*¹⁷⁾ have investigated the hydration structures of gGg', tGg', g'Gg', and tTt conformers in aqueous solutions by means of the radial distribution function (RDF) obtained from a Monte Carlo (MC) simulation. However, the three-dimensional hydration structure and its change throughout the conformational change process in aqueous EG solution have not yet been characterized using an MC simulation.

In this investigation, we adopted the gGg' and tGg' conformers shown in Chart 1, and three conformations between gGg' and tGg' conformers. The respective five conformations in infinitely dilute aqueous solution are hereafter referred to as their respective conformations in liquid water.

This paper describes the following results for the SDF $g_{OO}(x, y, z)$, difference SDF (DSDF), $\Delta g_{OO}(x, y, z)$, proposed in our previous paper, and the binding energy (BE) of the five conformations in liquid water. First, the anisotropic hydration structures around an EG molecule were analyzed using the SDF $g_{OO}(x, y, z)$ obtained from an MC simulation for two conformers (gGg' and tGg') of EG in liquid water. Second, the difference in solvation energy between gGg' and tGg' was evaluated from the BE. Third, it was shown that the hydration structure changes of EG molecules throughout the conformational change process can be characterized by the DSDF $\Delta g_{OO}(x, y, z)$ between the SDFs for two conformations in liquid water.

Computational Procedure

Monte Carlo Simulation MC simulations⁸⁾ were carried out for all systems with one EG molecule and 215 water molecules in a cube with a cell length of 18.63 Å within the Metropolis scheme¹⁸⁾ in the NVT ensemble at 298 K. The molar volume obtained under the liquid water density of 1.0 g cm⁻³ was 18.015 cm³ mol⁻¹.

As described in our previous papers,¹⁾ SPC potential¹⁹⁾ and TIPS potential²⁰⁾ functions were employed to represent the intermolecular interactions for water, and for alcohol and ether, respectively. These potential functions were obtained from the sum of the Coulomb and Lennard–Jones terms as:

$$\Delta E = \sum_i \sum_j^A \sum_j^B (q_i q_j e^2 / r_{ij} + A_{ij} / r_{ij}^{12} - C_{ij} / r_{ij}^6)$$

The coefficients, A_{ij} and C_{ij} , were obtained from $\sqrt{A_{ii} \times A_{jj}}$ and $\sqrt{C_{ii} \times C_{jj}}$, respectively. The interatomic distance and bond angle values for water were $r(\text{OH}) = 1.0$ Å and $\angle\text{HOH} = 109.47^\circ$. These values for the EG molecule were $r(\text{OH}) = 0.945$ Å, $r(\text{CO}) = 1.430$ Å, $r(\text{CC}_\text{O}) = 1.512$ Å, $\angle\text{COH} = 108.5^\circ$, and $\angle\text{CCO} = 107.8^\circ$. The dihedral angles optimized by *ab initio* MO methods using MP2/6-31G** were $\angle\text{H2-O2-C2-C1} = 72.86^\circ$, $\angle\text{O1-C1-C2-O2} = 55.43^\circ$, and $\angle\text{H1-O1-C1-C2} = -42.19^\circ$ for the gGg' conformer and $\angle\text{H2-O2-C2-C1} = -165.79^\circ$, $\angle\text{O1-C1-C2-O2} = 60.04^\circ$, and $\angle\text{H1-O1-C1-C2} = -50.79^\circ$ for the tGg' conformer.^{15e)} The dihedral angles adopted in the MC simulation were $\angle\text{H2-O2-C2-C1} = 70^\circ$, $\angle\text{O1-C1-C2-O2} = 60^\circ$, and $\angle\text{H1-O1-C1-C2} = -50^\circ$ for the gGg' conformer and $\angle\text{H2-O2-C2-C1} = -170^\circ$, $\angle\text{O1-C1-C2-O2} = 60^\circ$, and $\angle\text{H1-O1-C1-C2} = -50^\circ$ for the tGg' conformer (Chart 1). The conformational change process from gGg' to tGg' conformers could easily be examined by this model. Further, in order to visualize the hydration structure changes throughout the conformational change process from the gGg' to tGg' conformers, three conformations for the dihedral angle, $\angle\text{H2-O2-C2-C1} = 100, 130$, and 160° , were calculated by the MC simulation. These geometries were fixed in the MC simulation. In the SDF calculation, the O1 atom, O1–C1 bond, and H1 atom lie on the origin of the coordinate, the *x*-axis, and the direction of *y*-axis, respectively. Therefore, three atoms, H1–O1–C1, lie on the *x*–*y* plane. After excluding the first 2500000 configurations, the subsequent 5000000 configurations were employed to obtain the statistical average in the MC simulations.

Difference Spatial Distribution Function Bosio *et al.*²¹⁾ have reported the temperature dependence of hydration structures using the difference radial distribution function (DRDF) for heavy water obtained from X-ray diffraction. They have described that the network structure of the hydration water decreases with rising temperature. In a previous paper,¹⁾ we proposed a new DSDF method for examining the effects of hydrophobic groups. This DSDF is obtained as the difference between the SDFs for two states calculated by the MC simulation. For example, the DSDF, $\Delta g_{OO}(x, y, z)$, for two conformations of EG molecules with the dihedral angle $\angle\text{H2-O2-C2-C1} = 130^\circ$ and 160° is determined as:

$$\Delta g_{OO}(x, y, z) = g_{OO}(x, y, z)_{160^\circ \text{ EG}} - g_{OO}(x, y, z)_{130^\circ \text{ EG}}$$

Binding Energy Decomposition The binding energy (BE)²²⁾ is obtained as the sum of all spatial cells of the coefficient products calculated from the volume and coordination number (CN), the SDF $g_{OO}(x, y, z)$, and the averaged potential energies $\langle E(x, y, z) \rangle$ between the solute and solvent molecules at a certain point in a cubic cell. Applying the linked-list clustering technique, the high-density distributions of the SDF $g_{OO}(x, y, z)$ for oxygen (solute)–oxygen (solvent) pairs appeared to fall in one of three different regions, that of a hydrogen acceptor (HA), a hydrogen donor (HD), or a hydrophobic hydration (HH). The BE are calculated for each region using the next equation. For example, the value of the BE for the HA region in alcohol solution is determined by:

$$\langle BE \rangle_{\text{HA}} = \sum_{x, y, z \in \text{HA}} \frac{N}{V} \times \Delta V \times \langle E(x, y, z) \rangle \times g_{OO}(x, y, z)$$

where N is the number of water molecules in the cube with a cell length of 18.63 Å, V is the volume of a cube, and ΔV is an infinitesimal volume. In addition, $\langle BE \rangle_{\text{Sum}}$ can be obtained as the sum of $\langle BE \rangle_x$ for the above three regions, HA, HD, and HH.

Results and Discussion

Hydration Structure The anisotropic hydration structures of water molecules around EG molecule could be visualized by SDF $g_{OO}(x, y, z)$ using the graphic display technique. With regard to the gGg' and tGg' conformers in liquid water, Fig. 1 shows the distributions of SDF $g_{OO}(x, y, z) = 2.1$ between solute and water molecules as obtained from the MC simulation at 298 K. As shown in Fig. 1, the high distrib-

gGg'

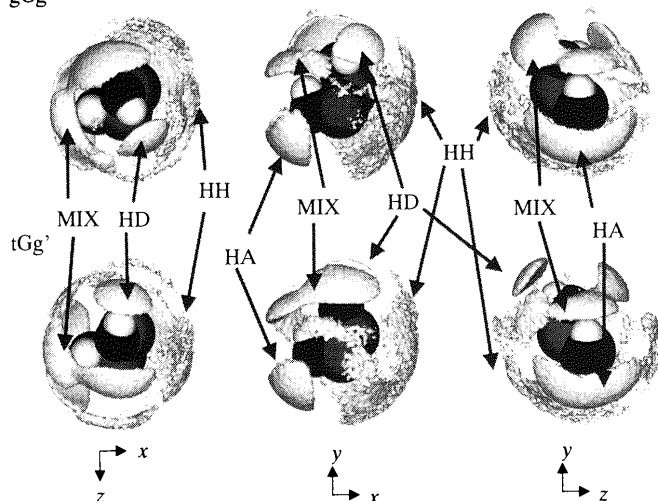


Fig. 1. Isosurfaces of Oxygen (Solute)-Oxygen (Water) SDF $g_{OO}(x, y, z) = 2.1$

Isosurfaces for top and bottom lines of the figure are shown for gGg' and tGg' conformers, respectively. Left, middle, and right columns are isosurfaces viewed down the y-axis, z-axis and x-axis, respectively.

Table 1. Results of Volume, CN, and Binding Energy Decomposition for gGg' and tGg' Conformers of Ethylene Glycol in Liquid Water in $g_{OO}(x, y, z) \geq 2.1$ Region at 298 K

	gGg'			tGg'		
	Volume	CN	$\langle BE \rangle_x$	Volume	CN	$\langle BE \rangle_x$
HA	7.2	0.92	-16.77	7.1	0.93	-17.81
HD	3.2	0.69	-12.85	3.4	0.62	-9.88
MIX + HH	23.1	2.25	-18.24	21.8	2.11	-14.91
Sum	33.5	3.86	-47.86	32.3	3.66	-42.60
Total			-113.59			-102.68

Units for volumes and $\langle BE \rangle_x$ are $\text{\AA}^3$ and kJ mol^{-1} , respectively. Subscript x on $\langle BE \rangle_x$ indicates HA, HD, MIX, Sum, and Total. $\langle BE \rangle_{\text{Total}}$ means the BE obtained from the overall cubic cell.

utions of SDF $g_{OO}(x, y, z)$ for the hydration water molecules around the EG molecule could be classified into the HA, HD, and HH regions; as the results for our previously reported alcohol solutions are also shown. A new overlapped distribution also appears in the area of the internal hydrogen bond of EG. This overlapped distribution composed of HA and HD regions is hereafter referred to as the MIX region. The distributions of the HA and HD regions for the two stable conformers, gGg' and tGg', are very similar to the SDF in all alcohol solutions reported previously. On the other hand, the ethylene portion of the molecule is surrounded by a distribution of hydration water molecules in the HH region, and the distribution area of this HH region is spread out in the vicinity of the distribution of the MIX region.

Applying the linked list clustering technique,²³⁾ the high distributions of SDF $g_{OO}(x, y, z) \geq 2.1$ for oxygen (solute)-oxygen (water) pairs fall into three different regions: HA, HD, and (MIX+HH) regions. Table 1 shows the volume, CN, and BE ($\langle BE \rangle_x$) for each region of SDF $g_{OO}(x, y, z) \geq 2.1$, and BE ($\langle BE \rangle_{\text{Total}}$) for all spatial regions in a cubic cell in aqueous EG solution at 298 K. It is considered that the results of this clustering technique characterize the hydration structures of the first hydration shell, because this SDF $g_{OO}(x, y, z) \geq 2.1$

is more than twice that for the bulk state ($g_{OO}(x, y, z) = 1.0$).

As shown in Table 1, in the HA region, the volume and CN values in the gGg' conformer in liquid water were $7.2 \text{ \AA}^3$ and 0.92, and these values in the tGg' conformer in liquid water were $7.1 \text{ \AA}^3$ and 0.93. The $\langle BE \rangle_{\text{HA}}$ ($-17.81 \text{ kJ mol}^{-1}$) in the latter was more stable than that ($-16.77 \text{ kJ mol}^{-1}$) in the former. In the HD region, the volume and CN values in gGg' conformer were $3.2 \text{ \AA}^3$ and 0.69, and those values in the tGg' conformer water were $3.4 \text{ \AA}^3$ and 0.62. The $\langle BE \rangle_{\text{HD}}$ ($-12.85 \text{ kJ mol}^{-1}$) in the former was considerably smaller than that ($-9.88 \text{ kJ mol}^{-1}$) in the latter. In the (MIX+HH) region, the volume and CN values in the gGg' conformer were $23.1 \text{ \AA}^3$ and 2.25, and those values in the tGg' conformer were $21.8 \text{ \AA}^3$ and 2.11. The $\langle BE \rangle_{(\text{MIX+HH})}$ ($-18.24 \text{ kJ mol}^{-1}$) in the former was appreciably smaller than that ($-14.91 \text{ kJ mol}^{-1}$) in the latter. These results indicate that the solvation energy increases with increasing CN values of hydration water molecules for each region.

From the $\langle BE \rangle_{\text{Sum}}$ and $\langle BE \rangle_{\text{Total}}$ values in Table 1, the energy differences in these values for two conformers, gGg' and tGg', in liquid water appear to have been 5.26 and 10.91 kJ mol^{-1} , respectively. These results indicate that solvation effect for gGg' conformer is greater than that for tGg'. In addition, our results support the results reported by Nagy *et al.*,¹⁷⁾ Hoofst *et al.*,²⁴⁾ and Cramer and co-workers.¹⁶⁾ It is apparent from these results that $\langle BE \rangle_{\text{HD}}$ and $\langle BE \rangle_{(\text{MIX+HH})}$ are the primary contributors to the solvation energy for gGg' conformer in liquid water.

Figure 2 shows contour maps of the BE on the x-y plane $z=0.075 \text{ \AA}$ in gGg' and tGg' conformers in liquid water. As can be clearly seen, the maps of the BE for the HA and HH regions in gGg' conformer are very similar to those for the same regions in tGg' conformer. In the HA region, the distributions of stable BE in gGg' and tGg' conformers are spread out in the vicinity centered at $x=-1.4 \text{ \AA}$ and $y=-2.3 \text{ \AA}$, and the negative value (-2.0 kJ mol^{-1}) in this field indicates the existence of a stable area. The distributions of stable BE (-2.0 kJ mol^{-1}) in the HH region are also spread out in the vicinity centered at $x=5.0 \text{ \AA}$ and $y=-1.0 \text{ \AA}$, although the distributions of unstable BE in two conformers are spread out on the side of the solute molecule. These contour maps for the HA and HH regions in two conformers are in agreement with those of alcohol solutions. In the other two distributions of stable BE in gGg' conformer, the distribution of stable BE is spread out in the vicinity centered at $x=-1.1 \text{ \AA}$ and $y=2.8 \text{ \AA}$, corresponding to the MIX region based on the distribution of HA and HD regions, because the distribution of stable BE in the vicinity centered at $x=3.2 \text{ \AA}$ and $y=4.1 \text{ \AA}$ is assigned to the HD region formed by hydrogen bonds between solvent water and the H2 atom of EG. In contrast, the widespread distribution in the tGg' conformer corresponds to the MIX region formed by the overlap of the HA and HD regions. Taken together, these contour maps also suggest that the MIX region is formed by the overlap of HA and HD regions relating to the O2 and H1 atoms of EG, respectively.

Figure 3 shows a superposition of oxygen-oxygen and oxygen-hydrogen SDFs, $g_{OO}(x, y, z)$ and $g_{OH}(x, y, z)$, in gGg' and tGg' conformers in liquid water. This figure shows that the hydration structures of the hydrogen-bonded water form a triple-layer structure in the HA region and HA part (in MIX), and a double-layer structure in the HD region and the HD

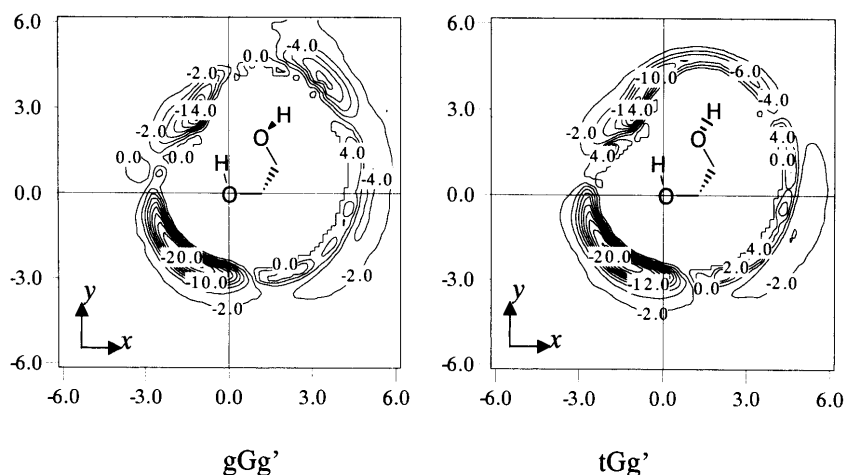


Fig. 2. Contour Maps of Binding Energies for gGg' and tGg' Conformers on the x - y Plane of $z=0.075$ Å at 298 K. Energy increment between two neighboring contour lines is 2 kJ mol^{-1} .

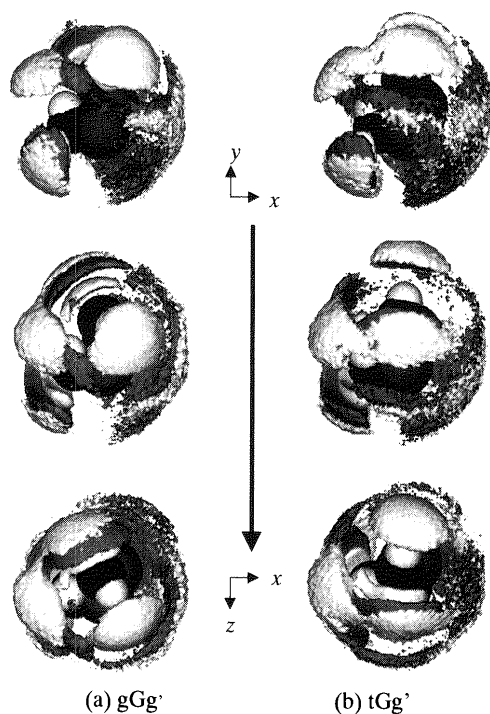


Fig. 3. Superposition Representations of Oxygen (Solute)-Oxygen (Water) and Oxygen (Solute)-Hydrogen (Water) SDF for (a) gGg' and (b) tGg' Conformers in Liquid Water at 298 K.

The dark area shows the oxygen-oxygen distribution at $g_{OO}(x,y,z)=2.1$, and the bright area shows the oxygen-hydrogen distribution at $g_{OH}(x,y,z)=1.6$. The top and bottom lines of the figure are superposition representations viewed down the z -axis and y -axis, respectively.

part (in MIX). Distribution peak distances between the H (or O) atom of solvent water molecules and the O (or H) atom of EG in five conformations are shown in Table 2. From Fig. 3 and Table 2, in the HA region, the maximum distributions of hydrogen, oxygen, and hydrogen atoms in hydration water molecules occur at distances of approximately 1.7, 2.8, and 3.3 Å from the O1 atom of gGg' and tGg' conformers, respectively. In the HD region, the maximum distributions of oxygen and hydrogen atoms in water molecules occur at distances of approximately 1.9 and 2.3–2.4 Å from the H2 atom of gGg' and tGg', respectively. Therefore, it is evident that the triple-layer and double-layer structures are composed

of each maximum distribution in the HA and HD regions, respectively.¹⁾ On the other hand, in the MIX region, the maximum distributions in the vicinity of the O2 atom of EG occur at distances of approximately 1.8 (hydrogen), 2.8 (oxygen), and 3.2–3.3 (hydrogen) Å from the O2 atom of gGg' and tGg'. Moreover, the maximum distributions in the vicinity of the H1 atom of EG also occur at distances of approximately 1.9–2.0 (oxygen) and 2.4 (hydrogen) Å from the H1 atom of gGg' and tGg'. From these distribution distances, the hydration structures in the MIX region also appear to have formed triple-layer and double-layer structures. From the water dimer structures optimized by our *ab initio* MO calculation,^{1a)} these spatial orientations of triple-layer and double-layer structures appear to be of all the linear type. Taken together, these findings clearly indicate that triple-layer and double-layer structures are composed of each maximum distribution in the HA region and HA part (in MIX) regions in the vicinity of the O2 atom, and the HD region and HD part (in MIX) regions in the vicinity of the H1 atom, respectively. Then, except in the case of the HH region, we can conclude that the spatial orientations of the hydration water molecules for all regions in the first hydration shell are of the linear type. Moreover, the isosurface of SDF and the superposition representation of the SDFs shown in Figs. 1 and 3 indicate that the distribution in the MIX region is in agreement with the overlapped distribution composed of the HA part relating to the O2 atom and the HD part relating to the H1 atom of the EG molecule.

Hydration Structure Changes throughout the Conformational Change Process from gGg' to tGg' Conformers

As mentioned above, the equilibrium populations of both gGg' and tGg' in all conformers are approximately 70–84% in aqueous solution.¹⁶⁾ Therefore, from the SDF and DSDF, we analyzed an anisotropic hydration structure change throughout the conformational change process from gGg' to tGg' conformers.

First, we determined the transition state and the conformational change pathway between gGg' to tGg' conformers of the EG molecule using the MNDO-AM1 method²⁵⁾ with eigenvector following²⁶⁾ and an intrinsic reaction coordinate,²⁷⁾ respectively. Table 3 shows three dihedral angles of the EG molecule at the pathway of the conformational

Table 2. Distribution Peak Distances between Two Atoms of Solvent Water Molecules and Five Conformations of Ethylene Glycol (EG) in Liquid Water

EG Water	O1			O2			H1		H2	
	H	O	H	H	O	H	O	H	O	H
gGg'	1.7	2.8	3.3	1.8	2.8	3.2	1.9	2.4	1.9	2.4
100°	1.7	2.8	3.3	1.8	2.9	3.3	2.0	2.4	1.9	2.3
130°	1.7	2.8	3.3	1.8	2.8	3.2	2.0	2.4	1.8	2.4
160°	1.7	2.8	3.3	1.9	2.8	3.3	2.0	2.2	1.9	2.2
tGg'	1.7	2.8	3.3	1.8	2.8	3.3	2.0	2.4	1.9	2.3

Units for all values are Å.

Table 3. Dihedral Angle Changes Accompanying Conformational Change from gGg' to tGg' Conformers of Ethylene Glycol

Reaction Coordinate	gGg'	-2	-1	TS	1	2	tGg'
H2-O2-C2-C1	73.2	81.7	108.9	133.0	143.4	166.4	175.4
O1-C1-C2-O2	62.6	62.6	62.4	62.7	63.0	63.8	64.2
H1-O1-C1-C2	-47.5	-48.9	-51.6	-53.5	-53.9	-55.2	-55.8

Transition state, TS, are found by MNDO-AM1 method with eigenvector following, and conformational change path are obtained from MNDO-AM1 method with intrinsic reaction coordinate. Units for all values are degree.

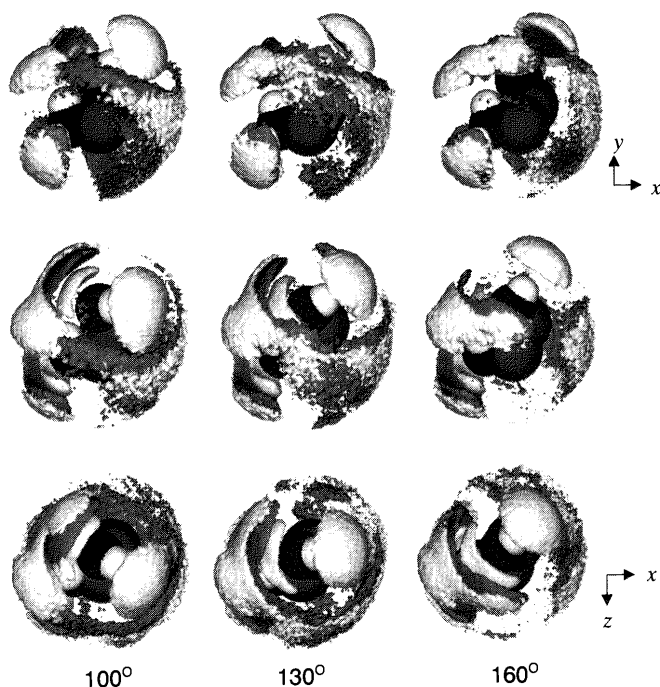


Fig. 4. Superposition Representations of Oxygen (Solute)-Oxygen (Water) and Oxygen (Solute)-Hydrogen (Water) SDF for 100°, 130°, and 160° Conformations in Liquid Water at 298 K

The dark area shows the oxygen-oxygen distribution at $g_{OO}(x,y,z)=2.1$, and the bright area shows the oxygen-hydrogen distribution at $g_{OH}(x,y,z)=1.6$. The top and bottom lines of the figure are superposition representations viewed down the z-axis and y-axis, respectively.

change. As shown in Table 3, it is apparent that the dihedral angle for each conformational change significantly with only $\angle H2-O2-C2-C1$, and $\angle O1-C1-C2-O2$ and $\angle H1-O1-C1-C2$ remaining unaltered. This result indicates that the conformational change from gGg' to tGg' can be expressed as the variation of the rotation angle around the H2 atom of EG.

Then, we proposed five conformations at 70 (gGg'), 100, 130, 160, and 190° (tGg') for the dihedral angles $\angle H2-O2-C2-C1$, and the hydration structure in each conformation in liquid water was analyzed by SDFs obtained from the MC

simulation at 298 K. Figure 4 shows a superposition of SDFs, $g_{OO}(x,y,z)$ and $g_{OH}(x,y,z)$, for each conformations. Moreover, the distances between the high distributions of oxygen (or hydrogen) atoms of solvent water and hydrogen (or oxygen) atoms of solute EG are shown in Table 2. As clearly seen in Fig. 4 and Table 2, the maximum distributions of hydrogen, oxygen, and hydrogen atoms in the hydration water molecules in the HA region occur at distances of approximately 1.7, 2.8, and 3.3 Å from the O1 atom of the EG molecule, respectively. In the HA part (in MIX) based on the O2 atom of EG, these distributions occur at distances of approximately 1.8–1.9, 2.8–2.9, and 3.2–3.3 Å, respectively. Therefore, it is evident that the triple-layer structure is composed of each maximum distribution in the HA region and HA part (in MIX region), and that their spatial orientations are of a linear type.

On the other hand, the maximum distributions of oxygen and hydrogen atoms in the hydration water molecules in the HD region occur at distances of approximately 1.8–1.9 and 2.2–2.4 Å from the H2 atom of EG, respectively. In the HD part (in MIX) based on the H1 atom of EG, these occur at distances of approximately 1.9–2.0 and 2.2–2.4 Å, respectively. Therefore, it is evident that the double-layer structure is composed of each maximum distribution in the HD region and HD part (in MIX), and that their spatial orientations are also of a linear type. Taken together, we can conclude that the hydration structure around EG continually holds both the triple-layer structure in the HA region and the double-layer structure in the HD region throughout the conformational change process from gGg' to tGg' conformers.

Except in the case of the HH region, Table 4 shows the volume, CN, and BE ($\langle BE \rangle_i$) in the HA, HD, and MIX regions for SDF $g_{OO}(x,y,z) \geq 2.5$ in EG in liquid water at 298 K. Compared with the SDF $g_{OO}(x,y,z) \geq 2.1$ in the (MIX+HH) region in Table 1 and SDF $g_{OO}(x,y,z) \geq 2.5$ in the MIX region in Table 4, the difference BE (-4.77 kJ mol⁻¹) between the BEs of the former and the latter for the gGg' conformer was approximately 30% that of the BE of the former, with 10, 5, and 15% contributions from the

Table 4. Results of Volume, CN, and Binding Energy Decomposition for Five Conformations of Ethylene Glycol in Liquid Water in $g_{OO}(x,y,z) \geq 2.5$ Region at 298 K

		gGg'				tGg'
		70°	100°	130°	160°	190°
HA	Volume	5.6	6.1	6.0	5.8	5.6
	CN	0.81	0.89	0.87	0.83	0.82
	$\langle BE \rangle_x$	-15.06	-17.06	-16.94	-16.47	-16.04
HD	Volume	2.6	2.8	2.7	2.8	2.8
	CN	0.65	0.59	0.58	0.57	0.58
	$\langle BE \rangle_x$	-12.29	-10.46	-9.76	-9.43	-9.35
MIX	Volume	7.1	6.0	5.8	4.9	6.0
	CN	1.06	0.89	0.77	0.70	0.93
	$\langle BE \rangle_x$	-13.47	-11.54	-8.98	-8.43	-10.92

Units for volumes and $\langle BE \rangle_x$ are $\text{\AA}^3$ and kJ mol^{-1} , respectively. Subscript x on $\langle BE \rangle_x$ indicates HA, HD, and MIX.

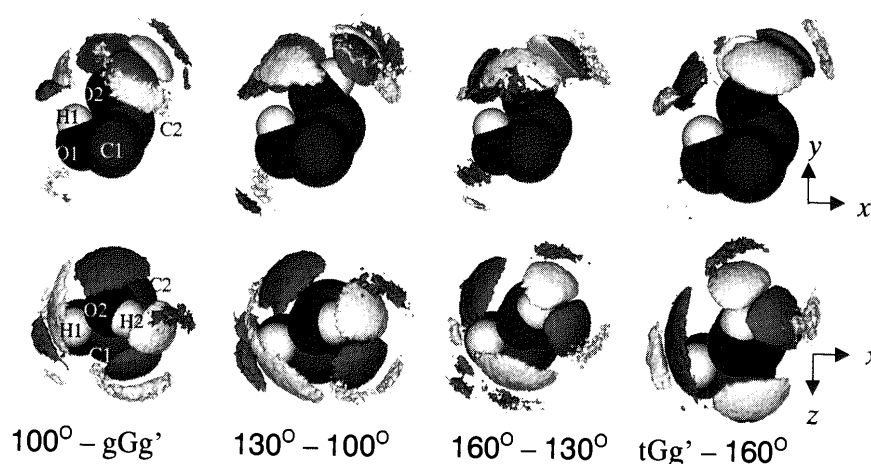


Fig. 5. Isosurfaces of Difference Oxygen (Solute)-Oxygen (Water) SDF between Two Conformations in Liquid Water

The top and bottom lines of the figure are isosurfaces viewed down the z-axis and y-axis, respectively. The bright area shows the increase of oxygen-oxygen distribution at $\Delta g_{OO}(x,y,z) = +1.0$, and the dark area shows the decrease of oxygen-oxygen distribution at $\Delta g_{OO}(x,y,z) = -1.0$.

HA, HD, and HH regions, respectively, contributions that remained constant throughout the conformational change process from gGg' to tGg'. Based on the above, it appears that the influence of the HH region connected with that of the MIX for the hydration structure may be less than that of the HA, HD, and MIX regions.

As seen clearly in Table 4, the volume and CN in the HA and HD regions didn't change as significantly as expected, but the BE in these regions varies appreciably with the conformational change. On the other hand, all of these values in the MIX region vary significantly with the conformational change. These results indicate that the hydration structure change is most strongly dependent on the MIX region relative to the HA and HH regions.

The origin of these effects can easily be determined using our novel DSDF method. Figure 5 shows an isosurface of DSDF $\Delta g_{OO}(x,y,z) = \pm 1.0$ obtained from SDFs of two conformations. As can clearly be seen, however, the high distribution area based on the H2 atom is appreciably shifted with the rotational transfer of the H2 atom. In addition, the high distribution areas of the hydration water molecules in the HD region relative to the H2 atom and in the HA region based on the O1 atom are practically unaltered.

If the conformational change pathway proceeds from gGg' to tGg', the high distribution of hydration water molecules in

the HA part (in MIX region) can be classified into two high distributions of solvent water with lone-pair electrons on the O2 atom with and without internal hydrogen bonds formed by the H1 atom. The former distribution increased as the conformational change proceeded from gGg' to tGg', while the latter decreased. These distribution changes could have been due to the internal hydrogen bond of the lone-pair electrons on the oxygen atom with the H1 atom, i.e., a disappearance in the former process and an appearance in the latter one. This result indicates that the distribution for the HA part (in MIX) decreases as the internal hydrogen bond formed by the H1 atom is enhanced and increases as this bond is weakened. To the contrary, however, it is apparent that the distribution for the HD part (in MIX), based on the behavior of the H1 atom, increase as the internal hydrogen bond formed by the H1 atom is enhanced. Considering the above, we have concluded that the hydration structure change is primarily governed by the contribution of the MIX region relative to that of the HA and HD regions

Conclusion

In the present paper, we have analyzed hydration structures and their changes throughout the conformational change process in aqueous EG solution using the SDF $g_{OO}(x,y,z)$ and our novel DSDF $\Delta g_{OO}(x,y,z)$ methods, respectively.

From the results of the SDF $g_{OO}(x, y, z)$ for five conformations of EG in liquid water at 298 K, it became apparent that the hydration water molecules for the first hydration shell are primarily distributed in the HA, HD, and MIX (an overlapped distribution of the HA and HD parts connected by an internal hydrogen bond) regions. These distributions show a triple-layer structure in the HA region and the HA part (in the MIX region), and a double-layer structure in the HD region and the HD part (in MIX region), and the spatial orientations of the hydration water molecules in these regions are obviously of a linear type. In addition, it became apparent that the spatial orientation of the hydration water molecules remains of a linear type throughout the conformational change process from gGg' to tGg' conformers.

In terms of the results of the DSDF $\Delta g_{OO}(x, y, z)$ obtained from the SDFs $g_{OO}(x, y, z)$ for the two conformations in liquid water, it was noted that the distribution of hydration water molecules in the HA and HD parts (in MIX region) are governed by the competition of internal hydrogen bonds between the H1 atom and the two lone-pair electrons on the O2 atom. More specifically, in the MIX region, the high distribution of hydration water molecules for the HA part related to the O2 atom decreases as the internal hydrogen bond formed by the H1 atom is enhanced, while that for the HD part increases as the hydrogen bond is enhanced.

In order to investigate in more detail the hydration structure around amino and carboxyl groups containing biologically important amino acid, we are presently examining MC simulations of amine and carboxylic acid solutions.

References

- 1) a) Hata T., Ono Y., *Chem. Pharm. Bull.*, **47**, 615—620 (1999); b) *Idem, ibid.*, **48**, 16—20 (2000); c) *Idem, ibid.*, **48**, 957—963 (2000).
- 2) Miertsu S., Scrocco E., Tomasi J., *Chem. Phys.*, **55**, 117—129 (1981).
- 3) Hoshi H., Sakurai M., Inoue Y., Chûjô R., *J. Chem. Phys.*, **87**, 1107—1115 (1987).
- 4) Klanmt A., Schüürmann G., *J. Chem. Soc., Perkin Trans. 2*, **1993**, 799—805.
- 5) Still W. C., Tempczyk A., Hawley R. C., Hendrickson T., *J. Am. Chem. Soc.*, **112**, 6127—6129 (1990).
- 6) Narten A. H., Danford M. D., Levy H. A., *Discuss. Faraday Soc.*, **43**, 97—107 (1967).
- 7) Soper A. K., Phillips M. G., *Chem. Phys.*, **107**, 47—60 (1986).
- 8) Allen M. P., Tildesley D. J., "Computer Simulation of Liquids," Oxford University Press, New York, 1987.
- 9) a) Svishchev I. M., Kusalik P. G., *J. Chem. Phys.*, **99**, 3049—3058 (1993); b) Kusalik P. G., Svishchev I. M., *Science*, **265**, 1219—1221 (1994); c) Svishchev I. M., Kusalik P. G., *J. Chem. Phys.*, **100**, 5165—5171 (1994); d) Liu Q., Brady J. W., *J. Am. Chem. Soc.*, **118**, 12276—12286 (1996); e) Laaksonen A., Kusalik P. G., Svishchev I. M., *J. Phys. Chem. A*, **101**, 5910—5918 (1997); f) Bergman D. L., Laaksonen L., Laaksonen A., *J. Mol. Graphics.*, **15**, 301—306 (1997); g) Pit- era J., Kollman P., *J. Mol. Graphics.*, **15**, 355—358 (1997).
- 10) a) Lossli H.-R., Kessler H., Oschkinat H., Weber H.-P., Petcher T. J., Widmer A., *Helv. Chim. Acta*, **68**, 692—704 (1985); b) Wegner R. M., France J., Bovermann G., Walliser L., Widmer A., Widmer H., *FEBS Lett.*, **340**, 255—259 (1994).
- 11) Radom L., Lathan W. A., Hehre W. J., Pople J. A., *J. Am. Chem. Soc.*, **95**, 693—698 (1973).
- 12) Bastiansen O., *Acta Chem. Scand.*, **3**, 415—421 (1949).
- 13) a) Frei H., Ha T.-K., Meyer R., Günthard H. H., *Chem. Phys.*, **25**, 271—298 (1977); b) Takeuchi H., Tasumi M., *ibid.*, **77**, 21—34 (1983).
- 14) a) Caminati W., *J. Mol. Spectrosc.*, **86**, 193—201 (1981); b) Caminati W., Corbelli G., *ibid.*, **90**, 572—578 (1981).
- 15) a) Ikuta S., Nomura O., *Chem. Phys. Lett.*, **154**, 71—76 (1989); b) Costa Cabral B. J., Albuquerque L. M. P. C., Silva Fernandes F. M. S., *Theor. Chim. Acta*, **78**, 271—280 (1991); c) Murcho M. A., DiPaola R. A., *J. Am. Chem. Soc.*, **114**, 10010—10018 (1992); d) Teppen B. J., Cao M., Frey R. F., van Alsenoy C., Miller D. M., Schafer L., *J. Mol. Struct. (Theochem.)*, **314**, 169—190 (1994); e) Reiling S., Brichmann J., Schlenkrich M., Bopp P. A., *J. Comput. Chem.*, **17**, 133—147 (1996); f) Costa Cabral B. J., *Int. J. Quantum Chem.*, 1651—1660 (1996).
- 16) Cramer C. J., Truhlar D. G., *J. Am. Chem. Soc.*, **116**, 3892—3900 (1994).
- 17) Nagy P. I., Dunn W. J., III, Alagona G., Ghio C., *J. Am. Chem. Soc.*, **113**, 6719—6729 (1991).
- 18) Metropolis N., Rosenbluth A. W., Rosenbluth M. N., Teller A. H., Teller E., *J. Chem. Phys.*, **21**, 1087—1092 (1953).
- 19) Berendsen H. J. C., Grigera J. R., Straatsma T. P., *J. Phys. Chem.*, **91**, 6269—6271 (1987).
- 20) Jorgensen W. L., *J. Am. Chem. Soc.*, **103**, 335—340 (1981).
- 21) Bosio L., Chen S.-H., Teixeira J., *Phys. Rev. A*, **27**, 1468—1475 (1983).
- 22) Ben-Naim, "Water and Aqueous Solutions, Introduction to a Molecular Theory," Chapter 5, Plenum Press, 1974.
- 23) Ōsawa E., Gotō H., Hata T., Deretey E., *J. Mol. Struct. (Theochem.)*, **398—399**, 229—236 (1987).
- 24) Hooft R. W. W., van Eijck B. P., Kroon J., *J. Chem. Phys.*, **97**, 3639—3646 (1992).
- 25) Dewar M. J. S., Zoebisch E. G., Healy E. F., Stewart J. J. P., *J. Am. Chem. Soc.*, **107**, 3902—3909 (1985).
- 26) Baker J., *J. Comp. Chem.*, **7**, 385—395 (1986).
- 27) Dieter K. M., Stewart J. J. P., *J. Mol. Struct. (Theochem)*, **163**, 143—149 (1988).

Studies on 8-Methoxyquinolones: Synthesis and Antibacterial Activity of 7-(3-Amino-4-substituted)pyrrolidinyl Derivatives

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A series of 8-methoxyquinolones bearing 3-amino-4-methylpyrrolidines or 3-amino-4-fluoromethylpyrrolidines at the C-7 position was synthesized and their physicochemical and biological properties were evaluated. All of the compounds synthesized showed more potent activity than LVFX (3) against both gram-positive and -negative bacteria. Increases in lipophilicity of these compounds had desirable effects on their potency of single intravenous toxicity and pharmacokinetic profiles in animals. Among the compounds synthesized, 1-fluorocyclopropyl derivatives 17 and 20, and 7-(*cis*-3-amino-4-fluoromethylpyrrolidinyl) derivative 19 (DC-756h) showed negative responses in the micronucleus test in mice while 1-cyclopropyl-7-(3-aminopyrrolidinyl) derivative 16 showed a positive response. These results suggested that the introduction of a fluorine atom into the 3-aminopyrrolidinyl substituent resulted in favorable influence on genetic toxicity as well as into the N-1 cyclopropyl substituent.

Key words 8-methoxyquinolone; 3-amino-4-fluoromethylpyrrolidine; micronuclei-formation; antibacterial activity; single intravenous toxicity; lipophilicity

The increased frequency of bacterial resistance has become a serious problem in the clinical practice of antimicrobial chemotherapy.¹⁾ In particular, incidence of resistant gram-positive organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA),²⁾ vancomycin-resistant enterococci (VRE),³⁾ and penicillin-resistant *Streptococcus pneumoniae* (PRSP)⁴⁾ is on the increase, and the development of effective drugs for infection caused by the resistant bacteria described above is urgently desired.

Since their introduction in the late 1970s, fluoroquinolone antibacterial agents have been one of the most clinically useful chemotherapeutic drugs.⁵⁾ Quinolones are anti-infective agents which selectively inhibit bacterial DNA gyrase⁶⁾ and DNA topoisomerase IV,⁷⁾ and are characterized by a broad spectrum of activity, high potency, and an excellent oral efficacy. In the investigation of structure–activity relationships (SARs) of quinolones, the introduction of a fluorine atom to the C-6 position of quinolone nucleus turned out to enhance antibacterial activity.⁸⁾ The quinolones available in clinical practice are categorized in these fluorinated quinolones such as norfloxacin (NFLX, 1),⁹⁾ ciprofloxacin (CPFX, 2),¹⁰⁾ levofloxacin (LVFX, 3),¹¹⁾ sparfloxacin (SPFX, 4),¹²⁾ and tosfloxacin (TFLX, 5).¹³⁾ As to basic substituents at the C-7 position, 3-aminopyrrolidines are more effective for the activity against gram-positive bacteria than piperazines.¹⁴⁾ Although numerous 6-fluoro-7-(3-aminopyrrolidinyl)-1-cyclopropylquinolones have been synthesized to improve activity against gram-positive bacteria, many 3-aminopyrrolidinyl derivatives showed positive responses in the micronuclei-formation test.

To research novel 7-(3-aminopyrrolidinyl)quinolones, we have been studying the influence of a fluorine atom at the N-1 cyclopropyl substituent on antibacterial activity and physicochemical properties,¹⁵⁾ and also reported that sitafloxacin (STFX, formerly DU-6859a, 6), optically active N1-*cis*-fluorocyclopropyl-C7-4'-spirocyclopropyl-3-aminopyrrolidinyl quinolone, had a potent activity against both gram-positive and -negative bacteria.¹⁶⁾ STFX (6) is a promising fluoroquinolone resulted in showing a negative response in the mi-

cronuclei-formation test by introduction of a fluorine atom into the N-1 cyclopropyl substituent,¹⁷⁾ and has been under clinical trial.

These results prompted us to study the effect of introducing a fluorine atom into 3-aminopyrrolidine substituents at the C-7 position to obtain novel 3-aminopyrrolidinyl quinolones with no micronuclei-formation activity.

Regarding the substituent at the C-8 position of quinolone nucleus, a methoxy group plays a significant role in reducing phototoxicity.¹⁸⁾ Therefore, we introduced 3-aminopyrrolidinyl substituents described above into the 8-methoxy quinolone nucleus to obtain novel compounds possessing a potent activity against gram-positive bacteria along with favorable safety profiles.

In this paper, we report the synthesis and SARs of a series of 8-methoxyquinolones bearing optically active 3-amino-4-fluoromethylpyrrolidines and other 3-aminopyrrolidines.

Chemistry

The synthesis of the desired chiral *cis*-4-fluoromethyl-3-aminopyrrolidine **13** is outlined in Chart 1. 4(*S*)-Hydroxymethylpyrrolidone **7**, prepared from itaconic acid and (*R*)-(+)-1-phenylethylamine according to the literature,¹⁹⁾ was treated with methanesulfonyl chloride (MsCl) in the presence of triethylamine (Et₃N) to afford a corresponding mesylate, which was converted to fluoromethyl derivative **8** by reaction with tetra-*n*-butylammonium fluoride (TBAF) in quantitative yield. This fluorination was also achieved directly by treatment of **7** with diethylaminosulfur trifluoride (DAST). Introduction of a hydroxy group to **8** using oxygen in the presence of lithium diisopropylamide (LDA) in tetrahydrofuran (THF) at –78 °C gave the 3-hydroxy derivative **9**. After treatment of **9** in a mixture of MsCl and Et₃N, the resulting mesylate was converted to azide **10** with sodium azide (NaN₃). Catalytic hydrogenation of **10** followed by protection of the resulting amine moiety with di-*tert*-butyl dicarbonate (Boc₂O) afforded *cis*-3-*tert*-butoxycarbonylamino-4-fluoromethylpyrrolidone **11**. Reduction of **11** with borane–THF (BH₃–THF)

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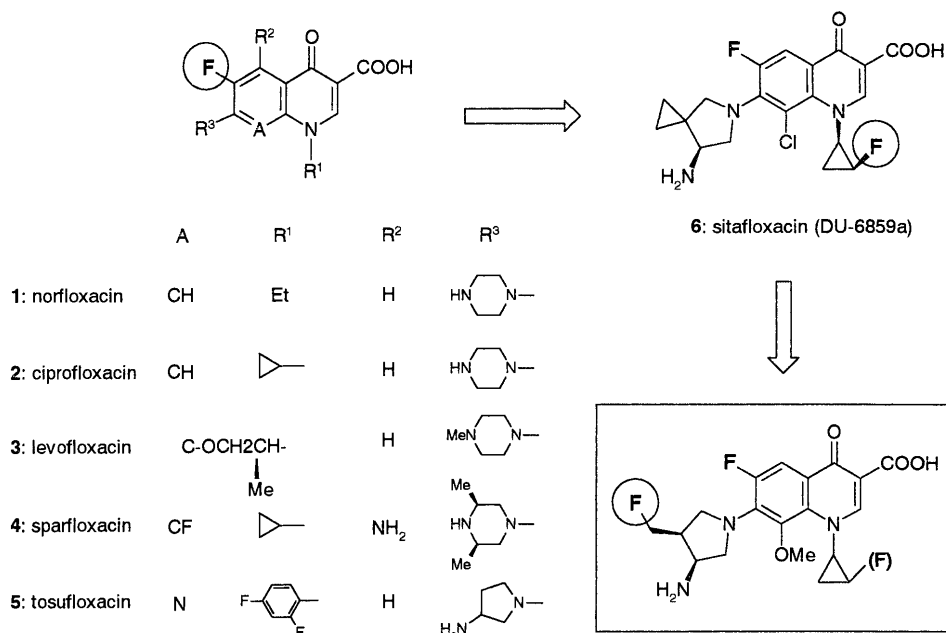


Fig. 1

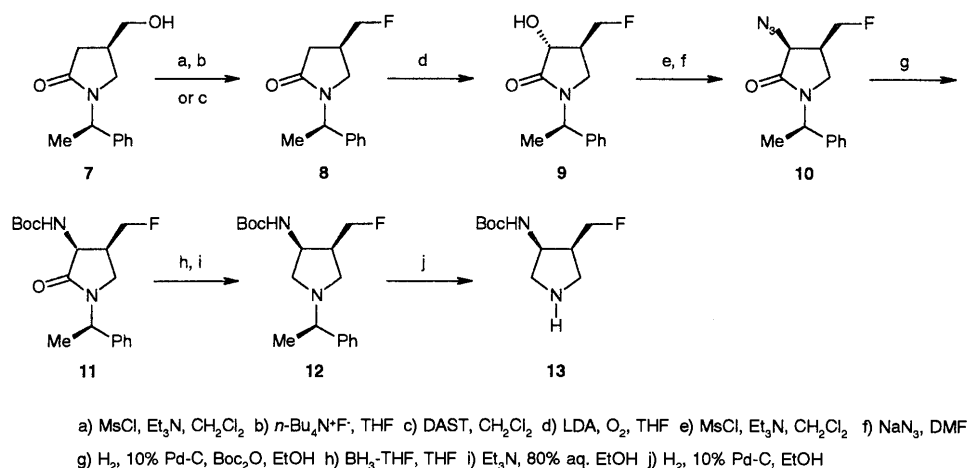


Chart 1

complex in THF followed by treatment Et₃N in aqueous ethanol (EtOH) yielded pyrrolidine **12**. Absolute configuration of C-3 and C-4 substituents on the pyrrolidine ring of **12** was determined to be *S* and *S* respectively, based on the X-ray analysis of **12** (Fig. 2). The N-1 phenylethyl group was removed by catalytic hydrogenation to afford target pyrrolidine **13**. The desired 7-substituted 8-methoxyquinolone derivatives **15**–**20** were obtained by treatment of BF₂-chelate **14** with various 3-aminopyrrolidines in dimethyl sulfoxide (DMSO) followed by dechelation with Et₃N in aqueous EtOH and successive deprotection of Boc group of the terminal amino substituent (Chart 2).

Results and Discussion

The activity of compounds **15**–**20** was tested against five representative gram-positive (*Staphylococcus aureus* 209P, *Staphylococcus epidermidis* 56556, *Streptococcus pyogenes* G36, *Streptococcus pneumoniae* J24, and *Enterococcus faecalis* ATCC19433) and three representative gram-negative (*Escherichia coli* NIHJ, *Klebsiella pneumoniae* Type 1, and

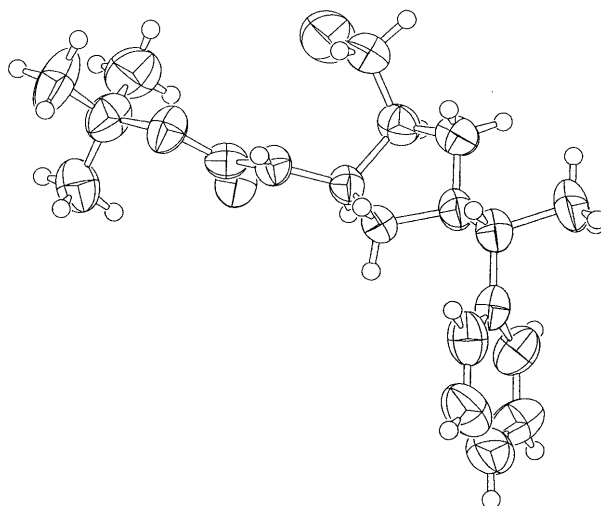


Fig. 2

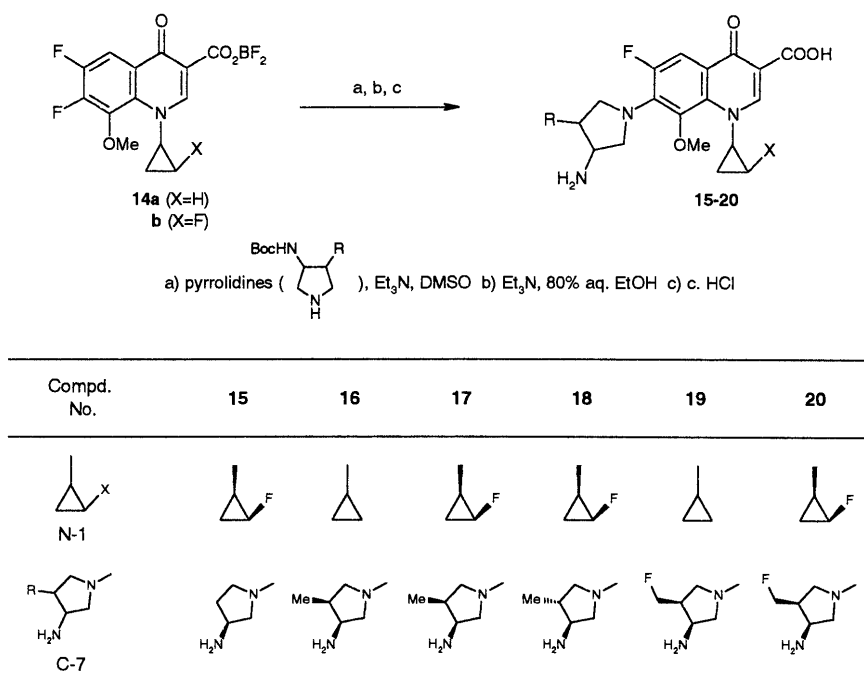


Chart 2

Table 1. *In Vitro* Antibacterial Activity of 7-(3-Aminopyrrolidinyl)-8-methoxyquinolones (MIC: $\mu\text{g/ml}$)^{a)}

Organism	15	16	17	18	19	20	LVFX (3)
<i>S. aureus</i> FDA 209P	0.013	0.013	0.013	0.013	0.013	0.013	0.20
<i>S. epidermidis</i> 56556	0.025	0.013	0.013	0.025	0.025	0.013	0.20
<i>S. pyogenes</i> G36	0.20	0.05	0.05	0.10	0.10	0.10	0.78
<i>S. pneumoniae</i> J24	0.05	0.025	0.025	0.05	0.025	0.025	0.78
<i>E. faecalis</i> ATCC19433	0.10	0.10	0.10	0.20	0.10	0.10	0.78
<i>E. coli</i> NIHJ	0.013	≤ 0.003	0.006	0.013	≤ 0.003	≤ 0.003	0.013
<i>K. pneumoniae</i> Type 1	0.10	0.025	0.025	0.05	0.025	0.05	0.10
<i>P. aeruginosa</i> PAO1	0.10	0.20	0.20	0.10	0.20	0.20	0.39

a) See Experimental section.

Pseudomonas aeruginosa PAO1) organisms. The minimum inhibitory concentrations (MIC, $\mu\text{g/ml}$) of these compounds compared with those of LVFX (3) are summarized in Table 1.

All of the compounds synthesized showed more potent activities against both gram-positive and -negative bacteria than LVFX (3), in particular exhibiting 4 to 32 times more potent activity against gram-positive organisms. Compound 17 having a methyl group at the C-4 position on the pyrrolidine ring was more active than non-alkylated derivative 15 against both gram-positive and -negative bacteria. This result suggests that alkylation at this position enhances antibacterial activity. In the configuration of the methyl group on the pyrrolidine ring, 3,4-*cis* derivative 17 showed approximately 2 times more potent activity than 3,4-*trans* isomer 18 against both gram-positive and -negative organisms. Introduction of a fluorine atom into the C-7 substituent had little effect on antibacterial activity (16 vs. 19, 17 vs. 20). Introduction of a fluorine into the cyclopropyl group at the N-1 position also had no significant effect on the antibacterial activities (16 vs. 17, 19 vs. 20).^{16a)}

In the next study, we tested the effect of the fluorine atom in the N-1 or C-7 substituent on physicochemical properties. These results are summarized in Table 2. Introduction of a

Table 2. Physicochemical Properties of 7-(3-Aminopyrrolidinyl)-8-methoxyquinolones 15–20

Compd. No.	15	16	17	18	19	20
$P'^a)$	1.3	34.5	8.7	8.0	39.6	9.6
Solubility ^{b)}	383	1112	833	582	932	186

a) Apparent partition coefficient, $\text{CHCl}_3/0.1\text{ M}$ phosphate buffer (pH 7.4). b) Solubility to water ($\mu\text{g/ml}$).

fluorine to cyclopropyl group at the N-1 position decreased lipophilicity of the compounds. Fluorocyclopropyl derivatives showed about a quarter of the apparent partition coefficients (P') compared with the corresponding cyclopropyl derivatives (16 vs. 17, 19 vs. 20). This result substantiated our previous report dealing with 8-chloroquinolones.^{16a)} On the other hand, 4-fluoromethylpyrrolidinyl derivatives showed slightly higher P' values than 4-methylpyrrolidinyl compounds (16 vs. 19, 17 vs. 20).

Although the lipophilicity of a series of the compounds had little influence on antibacterial activity, it had a considerable effect on their toxicity by a single intravenous injection in mice (Table 3). Non-substituted 3-aminopyrrolidinyl derivative 15 had the highest toxicity among the compounds tested, while 4-methyl or 4-fluoromethyl derivatives with

higher lipophilicity showed lower toxicity. Their toxic order (from the highest to the lowest) was **15** > **17** > **20** > **18** > **16** > **19**. This result was consistent with our previous report concerning pyridobenzoxazines.²⁰⁾ In the micronucleus test in the bone marrow of mice (Table 3), N-1 fluorocyclopropyl derivatives **17** and **20** did not induce micronuclei, while N-1 cyclopropyl derivative **16** showed such an effect. However, N-1 cyclopropyl derivative **19** bearing 3-amino-4-fluoromethylpyrrolidinyl substituent showed a negative response in this test. These results indicate that the introduction of a fluorine atom into the basic substituent at the C-7 position also contributes to the disappearance of actions of micronuclei-formation as well as into the N-1 cyclopropyl group.¹⁷⁾

The pharmacokinetic profiles of 4-fluoromethylpyrrolidinyl derivatives **19** and **20** after oral administration (20 mg/kg) to rats are given in Table 4. Compound **19** with more lipophilicity showed both higher peak plasma concentration ($C_{\max}$) and area under the time-concentration curve (AUC) than compound **20** with less lipophilicity. This result suggests that the more lipophilic compound has the advantage of absorption after oral administration.

In conclusion, we have synthesized optically active *cis*-3-amino-4-fluoromethylpyrrolidine successfully, and then introduced it to 1-cyclopropyl-8-methoxyquinolone substrate to prepare novel 7-(3-aminopyrrolidinyl)quinolones. The introduction of a fluorine atom to the 3-aminopyrrolidine moiety resulted in a favorable effect on their genetic toxicity, while the antibacterial activity was equipotent to those of corresponding non-fluorinated derivatives. We found that compound **19** showed a negative response in the micronu-

cleus test and exhibited well balanced and potent antibacterial activity, which was 4 to 8 times more active than SPFX (**4**) and TFLX (**5**) against gram-positive bacteria including MRSA, and 2 to 8 times more active than CPFX (**2**) and LVFX (**3**) against gram-negative bacteria except *P. aeruginosa* (Table 5). Compound **19** (DC-756h) showed a good pharmacokinetic profile in rats and was finally selected for clinical evaluation.

Experimental

All melting points were taken on a Yanagimoto micro melting point apparatus MP-500D and are uncorrected. Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on 400 MHz with a JEOL JNM-EX400 spectrometer. Chemical shifts are expressed in ppm (δ) with tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Elemental analyses are indicated only by the symbols of the elements; analytical results were within $\pm 0.4\%$ of the theoretical values unless otherwise noted. Optical rotations were measured at 589 nm with a Horiba SEPA-300 polarimeter. All organic solvent extracts were dried over anhydrous sodium sulfate. Column chromatography was carried out with Merck Silica gel 60 (230–400 mesh). Thin layer chromatography (TLC) was performed on Merck Silica gel 60 F254 TLC plates.

4(S)-Fluoromethyl-1-[N-1(R)-phenylethyl]-2-pyrrolidone (**8**) (i)

Table 4. Pharmacokinetic Parameters of Selected Compounds after Oral Administration to Rats (20 mg/kg)^{a)}

Compd. No.	$C_{\max}$ ($\mu\text{g/ml}$)	$AUC_{0-6\text{h}}$ ($\mu\text{g}\cdot\text{h/ml}$)	$P^{b)}$
19	3.1	6.9	39.6
20	1.8	2.2	9.6

a) See Experimental section. b) Apparent partition coefficient, $\text{CHCl}_3/0.1\text{ M}$ phosphate buffer (pH 7.4).

Table 3. Single Intravenous Toxicity in Mice and Micronucleus Test of Selected Compounds^{a)}

Compd. No.			15	16	17	18	19	20
Mortality	Dose (mg/kg)	150	2/2	2/5	2/2	3/5	1/5	2/3
		100	2/2	N.T.	1/5	N.T.	N.T.	1/5
		50	0/5	N.T.	N.T.	N.T.	N.T.	N.T.
Micronucleous Test	Dose (mg/kg)	100	N.T.	+	—	N.T.	—	—

a) See Experimental section. The mortality rate is expressed as number of animals dead/number of animals treated. The results of the micronucleus test are expressed as symbols (+: positive, -: negative). N.T.: Not tested.

Table 5. *In Vitro* Antibacterial Activity of **19** (DC-756h) and Reference Quinolones (MIC: $\mu\text{g/ml}$)^{a)}

Organism	19 (DC-756h)	CPFX (2)	LVFX (3)	SPFX (4)	TFLX (5)
<i>S. aureus</i> FDA 209P	0.013	0.10	0.20	0.05	0.05
<i>S. aureus</i> 870307 ^{b)}	0.39	>6.25	>6.25	3.13	0.78
<i>S. aureus</i> 891185 ^{b)}	0.78	>6.25	>6.25	6.25	>6.25
<i>S. epidermidis</i> 56556	0.025	0.20	0.20	0.05	0.10
<i>S. pyogenes</i> G36	0.10	1.56	0.78	0.78	0.39
<i>S. mitis</i> IID685	0.05	0.78	0.39	0.20	0.10
<i>S. pneumoniae</i> J24	0.025	0.39	0.78	0.10	0.20
<i>S. pneumoniae</i> 57664	0.05	0.39	0.78	0.20	0.20
<i>E. faecalis</i> ATCC19433	0.10	0.78	0.78	0.39	0.39
<i>E. coli</i> NIHJ	≤ 0.003	≤ 0.003	0.013	≤ 0.003	0.006
<i>E. coli</i> KL16	0.006	0.013	0.05	0.013	0.013
<i>K. pneumoniae</i> Type 1	0.025	0.025	0.10	0.05	0.05
<i>K. pneumoniae</i> Type 2	0.013	0.025	0.05	0.025	0.013
<i>S. marcescens</i> 10104	0.025	0.05	0.05	0.05	0.10
<i>P. aeruginosa</i> PAO1	0.20	0.05	0.39	0.39	0.10
<i>P. aeruginosa</i> 32234	0.20	0.05	0.20	0.20	0.10

a) See Experimental section. b) MRSA strains.

Method A: To a solution of 4-(*S*)-hydroxymethyl-1-[*N*-1(*R*)-phenylethyl]-2-pyrrolidone **7** (5.0 g, 22.80 mmol) and Et₃N (6.36 ml, 45.63 mmol) in dichloromethane (CH₂Cl₂) (100 ml) was added dropwise MsCl (2.65 ml, 34.24 mmol) at 0 °C, and then the mixture was stirred at the same temperature for 30 min. The reaction mixture was washed with 10% aqueous citric acid and brine. The organic layer was dried and concentrated to give a mesylate as slightly yellow oil, which was dissolved in THF (100 ml). To this mesylate solution was added TBAF [1.0 M solution in THF, 114 ml], and the mixture was refluxed for 1.5 h. After cooling, the reaction mixture was poured into 10% aqueous citric acid and concentrated under reduced pressure. The residue was extracted with CHCl₃ and the organic layer was separated, dried and concentrated. The resulting residue was chromatographed on silica gel using *n*-hexane–ethyl acetate (AcOEt) (1 : 3) as an eluent to afford **8** (5.05 g, quantitatively) as slightly yellow oil. NMR (CDCl₃) δ: 1.52 (3H, d, *J* = 7.33 Hz), 2.24–2.29 (1H, m), 2.52–2.63 (2H, m), 3.10 (1H, t, *J* = 9.76 Hz), 3.20 (1H, dd, *J* = 5.37, 9.76 Hz), 4.26–4.47 (2H, m), 5.50 (1H, q, *J* = 7.32 Hz), 7.26–7.36 (5H, m). [α]_D +176.7° (*c* = 0.958, CHCl₃).

(ii) Method B: To a solution of **7** (2.0 g, 9.12 mmol) in CH₂Cl₂ (50 ml) was added DAST (1.9 ml, 14.38 mmol) at –78 °C, and the mixture was stirred at the same temperature for 30 min and allowed to stand for 15 h at room temperature. The reaction mixture was washed with saturated aqueous NaHCO₃ and brine, dried and concentrated. The residue was chromatographed on silica gel using CHCl₃–methanol (MeOH) (97 : 3) as an eluent to afford **8** (1.11 g, 55%) as slightly yellow oil, which ¹H-NMR data agreed with that of **8** obtained by method A described above.

4(S)-Fluoromethyl-3(R)-hydroxy-1-[N-1(R)-phenylethyl]-2-pyrrolidone (9) A solution of diisopropylamine (1.65 ml, 11.75 mmol) in THF (20 ml) was cooled to –78 °C under a nitrogen atmosphere, treated dropwise with *n*-butyllithium [1.66 M solution in *n*-hexane, 7.08 ml, 11.75 mmol], and stirred for 5 min at 0 °C. To a solution of **8** (2.0 g, 9.04 mmol) in THF (20 ml) was added dropwise this LDA solution at –78 °C under a nitrogen atmosphere and the mixture was stirred for 15 min at the same temperature. After replacement of nitrogen to oxygen, the reaction mixture was stirred for 30 min at the same temperature under an oxygen atmosphere and quenched with 5% aqueous sodium thiosulfate. The solution was concentrated under reduced pressure and the resulting residue was extracted with AcOEt. The extract was washed with brine, dried and concentrated. The residue was chromatographed on silica gel using CHCl₃–methanol (MeOH) (97 : 3) as an eluent to afford **9** (1.57 g, 73%) as a colorless solid. mp 101–109 °C. NMR (CDCl₃) δ: 1.52 (3H, d, *J* = 7.32 Hz), 2.31–2.48 (1H, m), 3.05–3.10 (1H, m), 3.16–3.21 (1H, m), 4.29 (1H, d, *J* = 9.37 Hz), 4.53–4.67 (2H, m), 5.48 (1H, q, *J* = 7.33 Hz), 7.26–7.37 (5H, m). [α]_D +280.2° (*c* = 0.955, CHCl₃).

3(S)-Azide-4(S)-fluoromethyl-1-[N-1(R)-phenylethyl]-2-pyrrolidone (10) To a solution of **9** (2.61 g, 11.0 mmol) and Et₃N (3.07 ml, 22.02 mmol) in CH₂Cl₂ (40 ml) was added dropwise MsCl (1.28 ml, 16.54 mmol) at –10 °C, and then the mixture was stirred at the same temperature for 30 min. The reaction mixture was washed with 10% aqueous citric acid and brine. The organic layer was dried and concentrated to give mesylate as slightly yellow oil, which was dissolved in *N,N*-dimethylformamide (DMF) (80 ml). To this mesylate solution was added NaN₃ (2.86 g, 44.0 mmol), and the mixture was heated at 100 °C for 16 h. After cooling, the reaction mixture was diluted with water and extracted with AcOEt. The organic layer was separated, washed with brine, dried and concentrated. The residue was chromatographed on silica gel using *n*-hexane–AcOEt (3 : 1) as an eluent to afford **10** (1.81 g, 63%) as slightly yellow oil. NMR (CDCl₃) δ: 1.56 (3H, d, *J* = 7.32 Hz), 2.67–2.75 (1H, m), 3.02 (1H, dd, *J* = 7.32, 10.25 Hz), 3.23 (1H, dd, *J* = 4.39, 10.25 Hz), 4.27 (1H, d, *J* = 8.30 Hz), 4.38 (1H, ddd, *J* = 7.81, 9.28, 46.39 Hz), 4.59 (1H, ddd, *J* = 5.86, 9.28, 46.37 Hz), 5.48 (1H, q, *J* = 7.32 Hz), 7.26–7.37 (5H, m). [α]_D +32.3° (*c* = 0.896, CHCl₃).

3(S)-tert-Butoxycarbonylamino-4(S)-fluoromethyl-1-[N-1(R)-phenylethyl]-2-pyrrolidone (11) A mixture of **10** (1.81 g, 6.90 mmol), 50% aqueous–10% palladium on carbon (1.80 g) and Boc₂O (3.01 g, 13.79 mmol) in EtOH (100 ml) was shaken for 15 h under a hydrogen atmosphere (1.0 atm.) at room temperature. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel using *n*-hexane–AcOEt (2 : 1) as an eluent to afford **11** (1.68 g, 72%) as a colorless solid. mp 128–130 °C. NMR (CDCl₃) δ: 1.45 (9H, s), 1.55 (3H, d, *J* = 7.10 Hz), 2.80–2.96 (1H, m), 3.06 (1H, dd, *J* = 6.86, 10.53 Hz), 3.31 (1H, d, *J* = 10.53 Hz), 4.30–4.56 (3H, m), 5.10 (1H, br s), 5.49 (1H, q, *J* = 7.10 Hz), 7.25–7.37 (5H, m). [α]_D +158.2° (*c* = 1.052, CHCl₃).

3(S)-tert-Butoxycarbonylamino-4(S)-fluoromethyl-1-[N-1(R)-phenylethyl]pyrrolidine (12) To a solution of **11** (1.68 g, 4.99 mmol) in THF (60 ml) was added dropwise BH₃–THF complex [1 M solution in THF,

19.98 ml] at 0 °C, and then the mixture was stirred at room temperature for 18 h. After evaporation of THF, the residue was dissolved in 80% aqueous EtOH (40 ml) and refluxed in the presence of Et₃N (8.0 ml) for 2 h and then cooled. The solution was concentrated and dissolved in CHCl₃, which was washed with brine, dried and then evaporated to dryness. The residue was chromatographed on silica gel using *n*-hexane–AcOEt (3 : 1) as an eluent to afford **12** (1.54 g, 96%) as a colorless solid. mp 108–109 °C. NMR (CDCl₃) δ: 1.35 (3H, d, *J* = 6.59 Hz), 1.43 (9H, s), 2.35–2.82 (5H, m), 3.24 (1H, q, *J* = 6.59 Hz), 4.30–4.59 (3H, m), 4.85 (1H, d, *J* = 9.03 Hz), 7.20–7.35 (5H, m). [α]_D +37.1° (*c* = 1.030, CHCl₃).

3(S)-tert-Butoxycarbonylamino-4(S)-fluoromethylpyrrolidine (13) A mixture of **12** (484 mg, 1.50 mmol) and 50% aqueous–10% palladium on carbon (500 mg) in EtOH (50 ml) was shaken under a hydrogen atmosphere (1.0 atm.) at 50 °C for 15 h. The mixture was filtered, and the filtrate was concentrated under reduced pressure to give a crude **13** (328 mg, quantitatively) as an amorphous foam, which was used in the next reaction without further purification. NMR (CDCl₃) δ: 1.44 (9H, s), 1.69 (1H, br s), 2.45–2.53 (1H, m), 2.66 (1H, dd, *J* = 5.37, 10.74 Hz), 2.90–2.95 (1H, m), 3.18 (2H, dd, *J* = 7.81, 10.74 Hz), 4.18–4.27 (1H, m), 4.44–4.53 (1H, m), 4.56–4.65 (1H, m). [α]_D +28.6° (*c* = 1.035, CHCl₃).

7-[3(S)-Amino-4(S)-fluoromethyl-1-pyrrolidinyl]-6-fluoro-1-cyclopropyl-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (19) A solution of 1-cyclopropyl-6,7-difluoro-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic acid BF₂–chetele (**14a**, 8.31 g, 24.2 mmol), **13** (6.35 g, 29.3 mmol) and Et₃N (6.50 ml, 46.7 mmol) in DMSO (60 ml) was stirred at room temperature for 17 h. After removal of Et₃N under reduced pressure, water was added to the residue with ice-water cooling, and then the mixture was stirred at room temperature for 1 h. The precipitate was collected by filtration and washed with water, then was dissolved in 80% aqueous EtOH (500 ml). Et₃N (10 ml) was added to the solution and the mixture was refluxed for 4 h. After concentration, the residue was dissolved in CHCl₃, which was washed with 10% aqueous citric acid and brine, dried and then evaporated to dryness. The residue was dissolved in concentrated HCl (30 ml) with ice-water cooling and stirred for 5 min at room temperature. The mixture was adjusted to pH 11 with 20% aqueous NaOH with ice-water cooling, and then was neutralized with 10% aqueous HCl to pH 7.4. The precipitate was collected by filtration to give a crude **19**, which was recrystallized from EtOH–28% NH₄OH to give **19** (8.17 g, 86%) as slightly yellow solid. mp 247–255 °C (dec.). NMR (0.1 N NaOD) δ: 0.90–1.12 (4H, m), 2.73–2.78 (1H, m), 3.41–3.44 (1H, m), 3.58 (3H, s), 3.64–3.73 (3H, m), 3.90–3.96 (1H, m), 4.03–4.09 (1H, m), 4.66–4.82 (1H, m), 7.56 (1H, d, *J* = 14.65 Hz), 8.49 (1H, s). *Anal.* Calcd for C₁₉H₂₁F₂N₃O₄ · 1/2HCl: C, 55.44; H, 5.26; N, 10.21. Found: C, 55.59; H, 5.31; N, 10.18. [α]_D –92.0° (*c* = 0.275, 0.1 N NaOH).

An analogous procedure was used to obtain compounds **15**–**18** and **20** using corresponding 3-aminopyrrolidine derivatives prepared according to the literatures.²¹⁾

7-[3(S)-Amino-1-pyrrolidinyl]-6-fluoro-1-[2(S)-fluoro-1(R)-cyclopropyl]-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (15) **15** was prepared in 87% from **14b** as slightly yellow solid. mp 213–216 °C. NMR (0.1 N NaOD) δ: 1.46–1.71 (3H, m), 1.72–1.79 (1H, m), 2.16–2.22 (1H, m), 3.34–3.38 (1H, m), 3.54–3.60 (1H, m), 3.56 (3H, s), 3.61–3.73 (3H, m), 4.02–4.08 (1H, m), 4.83–4.90 (0.5H, m), 5.02–5.10 (0.5H, m), 7.66 (1H, d, *J* = 14.64 Hz), 8.47 (1H, s). *Anal.* Calcd for C₁₈H₁₉F₂N₃O₄: C, 56.99; H, 5.05; N, 11.08. Found: C, 56.73; H, 5.00; N, 11.08. [α]_D +92.4° (*c* = 0.552, 0.1 N NaOH).

7-[3(S)-Amino-4(S)-methyl-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (16) **16** was prepared in 61% from **14a** as colorless solid. mp 210–212 °C. NMR (0.1 N NaOD) δ: 0.79–1.14 (4H, m), 1.02 (3H, d, *J* = 6.84 Hz), 2.30–2.37 (1H, m), 3.31–3.35 (1H, m), 3.41–3.48 (2H, m), 3.50 (3H, s), 3.65 (1H, ddd, *J* = 1.96, 7.32, 10.25 Hz), 3.85 (1H, ddd, *J* = 2.44, 5.37, 10.35 Hz), 3.98–4.04 (1H, m), 7.58 (1H, d, *J* = 14.65 Hz), 8.42 (1H, s). *Anal.* Calcd for C₁₉H₂₂FN₃O₄ · 1/4H₂O: C, 60.07; H, 5.97; N, 11.06. Found: C, 60.10; H, 5.89; N, 11.01. [α]_D –111.4° (*c* = 0.972, 0.1 N NaOH).

7-[3(S)-Amino-4(S)-methyl-1-pyrrolidinyl]-6-fluoro-1-[2(S)-fluoro-1(R)-cyclopropyl]-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (17) **17** was prepared in 69% from **14b** as slightly yellow solid. mp 221–226 °C (dec.). NMR (0.1 N NaOD) δ: 1.09 (3H, d, *J* = 7.00 Hz), 1.48 (1H, ddd, *J* = 3.00, 6.00, 9.50, 27.00 Hz), 1.60 (1H, ddt, *J* = 6.00, 9.00, 17.50 Hz), 2.36–2.42 (1H, m), 3.36–3.53 (3H, m), 3.57 (3H, s), 3.73–3.78 (1H, m), 3.81–3.85 (1H, m), 4.04 (1H, dt, *J* = 5.50, 9.00 Hz), 4.91–5.11 (1H, m), 7.67 (1H, d, *J* = 14.50 Hz), 8.44 (1H, s). *Anal.* Calcd for C₁₉H₂₁F₂N₃O₄: C, 57.96; H, 5.38; N, 10.68. Found: C, 57.66; H, 5.36; N,

10.56. $[\alpha]_D -28.6^\circ$ ($c=0.620$, 0.1 N NaOH).

7-[3(S)-Amino-4(R)-methyl-1-pyrrolidinyl]-6-fluoro-1-[2(S)-fluoro-1(R)-cyclopropyl]-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (18) 18 was prepared in 86% from 14b as colorless solid. mp 269—271 °C (dec.). NMR (0.1 N NaOD) δ : 1.01 (3H, d, $J=6.60\text{ Hz}$), 1.46—1.64 (2H, m), 1.80—1.88 (1H, m), 2.89 (1H, dd, $J=8.06$, 15.38 Hz), 3.39—3.44 (2H, m), 3.46 (3H, s), 3.46—3.62 (2H, m), 3.94—3.99 (1H, m), 4.89—4.97 (0.5H, m), 7.57 (1H, d, $J=14.40\text{ Hz}$), 8.43 (1H, s). *Anal.* Calcd for $\text{C}_{19}\text{H}_{21}\text{F}_2\text{N}_3\text{O}_4 \cdot \text{H}_2\text{O}$: C, 55.47; H, 5.64; N, 10.21. Found: C, 55.31; H, 5.88; N, 10.01. $[\alpha]_D -28.0^\circ$ ($c=0.620$, 0.1 N NaOH).

7-[3(S)-Amino-4(S)-fluoromethyl-1-pyrrolidinyl]-6-fluoro-1-[2(S)-fluoro-1(R)-cyclopropyl]-1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic Acid (20) 20 was prepared in 70% from 14b as colorless solid. mp 232—238 °C. NMR (0.1 N NaOD) δ : 1.41—1.62 (2H, m), 2.62—2.79 (1H, m), 3.44—3.47 (1H, m), 3.57 (3H, s), 3.60—3.65 (1H, m), 3.70—3.73 (1H, m), 3.77—3.88 (2H, m), 4.00—4.05 (1H, m), 4.55—5.08 (3H, m), 7.66 (1H, d, $J=14.16\text{ Hz}$), 8.42 (1H, s). *Anal.* Calcd for $\text{C}_{19}\text{H}_{20}\text{F}_3\text{N}_3\text{O}_4 \cdot 1/4\text{H}_2\text{O}$: C, 54.87; H, 4.97; N, 10.10. Found: C, 54.78; H, 4.83; N, 10.00. $[\alpha]_D -13.2^\circ$ ($c=0.174$, 0.1 N NaOH).

In Vitro Antibacterial Activity The MICs of the compounds tested in this study were measured according to the two-fold micro broth dilution method using Mueller–Hinton broth (Difco Laboratories, Detroit, MI) with an inoculum size of approximately 10^5 cfu/ml . The MIC was defined as the lowest concentration which prevented visible bacterial growth after incubation at 37 °C for 18 h.

Determination of Apparent Partition Coefficients and Aqueous Solubility The apparent partition coefficients and aqueous solubility of the compounds tested in this study were measured according to the method reported previously.^{12,16a)}

Pharmacokinetic Studies The test compounds were administered in solution by oral gavage to groups of four animals each. Blood samples were obtained 0.25, 0.5, 1, 2, 4 and 6 h after dosing. Serum levels of the test compounds were determined by a microbiological assay. *Bacillus subtilis* ATCC 6051 was used as the test organism.

Single Intravenous Toxicity Test The test compounds were dissolved in 0.1 N NaOH in saline at different concentrations. The solution was administered intravenously to five week-old male ddY mice at a speed of 0.2 ml/min. The total volume of administration was adjusted to 10 ml/kg of body weight. The number of dead mice was counted 7 d after administration.

Micronucleus Test Six week-old male ddy mice were used in this test. The test compounds were dissolved in 0.1 N NaOH –saline and the solution was administered intravenously (100 mg/kg) in each group of five mice. Mice were sacrificed at 24 h after the treatment, and the smears of bone marrow cells were prepared and stained with acridine orange. For each animal, the numbers of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes (PCEs) and of PCEs per 1000 erythrocytes were counted under a fluorescent microscope. Statistical analysis was performed by the Kastenbaum and Bowman method.²²⁾

X-Ray Crystallographic Analysis of 12 12 was crystallized from $\text{MeOH-H}_2\text{O}$ to give a colorless plate of $\text{C}_{18}\text{H}_{27}\text{FN}_2\text{O}_2$ having approximate dimensions of $0.20 \times 0.25 \times 0.05\text{ mm}$. The lattice parameters and intensities were measured on a Rigaku AFC7R diffractometer with graphite monochromated $\text{CuK}\alpha$ radiation using ω – 2θ scan technique. The compound was crystallized in monoclinic space group $P2_1$ with cell dimensions $a=5.306(1)\text{ \AA}$, $b=12.091(2)\text{ \AA}$, $c=14.269(1)\text{ \AA}$, $\beta=92.74(1)^\circ$, and $V=914.4(2)\text{ \AA}^3$. For $Z=2$ and $\text{F.W.}=322.42$, the calculated density was 1.171 g/cm^3 . The structure was solved by the direct method with the program *sir92*.²³⁾ The final R value was 0.059. A perspective view of the molecule is shown in Fig. 2.

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References and Notes

- 1) a) Amyes S. G. B., Gemmell C. G., *J. Med. Microbiol.*, **36**, 4—29 (1992); b) Neu H. C., *Science*, **257**, 1064—1073 (1992).
- 2) Voss A., Doebbeling B. N., *Int. J. Antimicrob. Agents*, **1995**, 101—106.
- 3) Jones R. N., Sader H. S., Erwin M. E., Anderson S. C., *Diagn. Microbiol. Infect. Dis.*, **1995**, 85—93.
- 4) Lonks J. R., Medeiros A. A., *Med. Clin. North Am.*, **79**, 523—535 (1995).
- 5) a) Chu D. T. W., *Adv. Drug Res.*, **21**, 39—144 (1991); b) Asahina Y., Ishizaki T., Suzue S., *Prog. Drug Res.*, **38**, 57—106 (1992).
- 6) a) Gellert M., Mizuuchi K., O'Dea M. H., Nash H. A., *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3872—3876 (1976); b) Gootz T. D., Barrett J. F., Sutcliffe J. A., *Antimicrob. Agents Chemother.*, **34**, 8—12 (1990).
- 7) a) Hoshino K., Kitamura A., Morrissey I., Sato K., Kato J., Ikeda H., *Antimicrob. Agents Chemother.*, **38**, 2623—2627 (1994); b) Tanaka M., Onodera Y., Uchida Y., Sato K., Hayakawa I., *ibid.*, **41**, 2362—2366 (1997).
- 8) Domagala J. M., Hanna L. D., Heifetz C. L., Hutt M. P., Mich T. F., Sanchez J. P., Solomon M., *J. Med. Chem.*, **29**, 394—404 (1986).
- 9) Koga H., Itoh A., Maruyama S., Suzue S., Irikura T., *J. Med. Chem.*, **23**, 1358—1363 (1980).
- 10) Wise R., Andrews J. M., Edwards L. J., *Antimicrob. Agents Chemother.*, **23**, 559—564 (1983).
- 11) a) Atarashi S., Yokohama S., Yamazaki K., Sakano K., Imamura M., Hayakawa I., *Chem. Pharm. Bull.*, **35**, 1896—1902 (1987); b) Une T., Fujimoto T., Sato K., Osada Y., *Antimicrob. Agents Chemother.*, **32**, 1336—1340 (1988).
- 12) Miyamoto T., Matsumoto J., Chiba K., Egawa H., Shibamori K., Minamida A., Nishimura Y., Okada H., Kataoka M., Fujita M., Hirose T., Nakano J., *J. Med. Chem.*, **33**, 1645—1656 (1990).
- 13) Narita H., Konishi Y., Nitta J., Kitayama I., Miyajima M., Watanabe Y., Yotsuji A., Saikawa I., *Yakugaku Zasshi*, **106**, 802—807 (1986).
- 14) a) Egawa H., Miyamoto T., Minamida A., Nishimura Y., Okada H., Uno H., Matsumoto J., *J. Med. Chem.*, **27**, 1543—1548 (1984); b) Sanchez J. P., Domagala J. M., Hagen S. E., Heifetz C. L., Hutt M. P., Nichols J. B., Trehan A. K., *ibid.*, **31**, 983—991 (1988).
- 15) Atarashi S., Imamura M., Kimura Y., Yoshida A., Hayakawa I., *J. Med. Chem.*, **36**, 3444—3448 (1993).
- 16) a) Kimura Y., Atarashi S., Kawakami K., Sato K., Hayakawa I., *J. Med. Chem.*, **37**, 3344—3352 (1994); b) Sato K., Hoshino K., Tanaka M., Hayakawa I., Osada Y., *Antimicrob. Agents Chemother.*, **36**, 1491—1498 (1992).
- 17) Shimada H., Itoh S., *J. Toxicol. Environ. Health*, **47**, 115—123 (1996).
- 18) Domagala J. M., *J. Antimicrob. Chemother.*, **33**, 685—706 (1994).
- 19) Culbertson T. P., Domagala J. M., Nichols J. B., Priebe S., Skeean R. W., *J. Med. Chem.*, **30**, 1711—1715 (1987).
- 20) Kawakami K., Atarashi S., Kimura Y., Takemura M., Hayakawa I., *Chem. Pharm. Bull.*, **46**, 1710—1715 (1998).
- 21) a) Di Cesare P., Bouzard D., Essiz M., Jacquet J. P., Ledoussal B., Kiechel J. R., Remuzon P., Kessler R. E., Fung-Tomec J., Desirderio J., *J. Med. Chem.*, **35**, 4205—4213 (1992); b) Matsumoto J., Miyamoto T., Egawa H., Nakamura S., *Eur. Pat. Appl. EP242789* (1988) [*Chem. Abstr.*, **108**, 150325x (1988)]; c) Petersen U., Schenke T., Grohe K., Schriewer M., Haller I., Metzger K. G., Endermann R., Zeiler H. J., *Eur. Pat. Appl. EP326916* (1992) [*Chem. Abstr.*, **112**, 77163u (1988)].
- 22) Kastenbaum K., Bowman K., *Mutat. Res.*, **9**, 527—549 (1970).
- 23) Altomare A., Burla M. C., Camalli M., Cascarano M., Giacovazzo C., Guagliardi A., Polidori G., *J. Appl. Cryst.*, **27**, 435—435 (1994).

Medicinal Foodstuffs. XIX.¹⁾ Absolute Stereostructures of Canavalioside, a New *Ent*-Kaurane-Type Diterpene Glycoside, and Gladiatosides A₁, A₂, A₃, B₁, B₂, B₃, C₁, and C₂, New Acylated Flavonol Glycosides, from Sword Bean, the Seeds of *Canavalia gladiata*

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A new *ent*-kaurane-type glycoside, canavalioside, and eight new acylated flavonol glycosides, gladiatosides A₁, A₂, A₃, B₁, B₂, B₃, C₁, and C₂, were isolated from the seed of *Canavalia gladiata* together with robinin, kaempferol 3-*O*- β -D-galactopyranosyl-7-*O*- α -L-rhamnopyranoside, and kaikasaponin III. The absolute stereostructures of canavalioside and gladiatosides A₁, A₂, A₃, B₁, B₂, B₃, C₁, and C₂ were elucidated on the basis of chemical and physicochemical evidence.

Key words canavalioside; gladiatoside; *Canavalia gladiata*; *ent*-kaurane-type diterpene; acylated flavonol glycoside; sword bean

The Leguminosae plant *Canavalia* (*C.*) *gladiata* DC. (Sword bean, Japanese name “natamame”) has been cultivated as a vegetable in Asian countries. The seeds of *C. gladiata* (“刀豆” in Chinese) have been known to exhibit antiemetic and demulcent effects and to stop hiccups in Chinese traditional medicine. As chemical constituents of this plant, gibberellins,²⁾ an amino acid (canavanine),³⁾ and an enzyme (concanavalin A)⁴⁾ have been reported. In the course of our studies in search of bioactive constituents from medicinal foodstuffs,⁵⁾ we have characterized several triterpene and steroid saponins from Leguminous edible beans [e.g. fenu-greek (*Trigonella foenum-graecum*),⁶⁾ kidney bean (*Phaseolus vulgaris*),⁷⁾ hyacinth bean (*Dolichos lablab*),⁸⁾ and green peas (*Pisum sativum*)⁹⁾] and reported their inhibitory effect of histamine release and adjuvant activity.¹⁰⁾ In a continuing study, we have isolated a new *ent*-kaurane-type diterpene glycoside named canavalioside (**1**) and eight new acylated flavonol oligoglycosides termed gladiatosides A₁ (**3**), A₂ (**4**), A₃ (**5**), B₁ (**7**), B₂ (**8**), B₃ (**9**), C₁ (**11**), and C₂ (**12**). In this

paper, we describe the isolation and structure elucidation of these glycosides.¹¹⁾

The methanolic extract obtained from the seeds of *C. gladiata* cultivated in Japan was subjected to Diaion HP-20 column chromatography to remove the sugar and lipid components. The methanol-eluted fraction was separated by normal- and reversed-phase silica gel column chromatography and HPLC to afford canavalioside (**1**) and gladiatosides A₁ (**3**), A₂ (**4**), A₃ (**5**), B₁ (**7**), B₂ (**8**), B₃ (**9**), C₁ (**11**), and C₂ (**12**) together with robinin¹²⁾ (**14**), kaempferol 3-*O*- β -D-galactopyranosyl-7-*O*- α -L-rhamnopyranoside¹³⁾ (**15**), and kaikasaponin III¹⁴⁾ (**16**).

Canavalioside (**1**) was obtained as a white powder with negative optical rotation. The IR spectrum of **1** showed absorption bands at 3410, 1686, and 1073 cm⁻¹ assignable to glycosidic and carboxyl functions. In the positive-ion FAB-MS of **1**, quasimolecular ion peaks were observed at *m/z* 547 (M+H)⁺ and *m/z* 569 (M+Na)⁺, while the negative-ion FAB-MS of **1** showed a quasimolecular ion peak at *m/z* 545

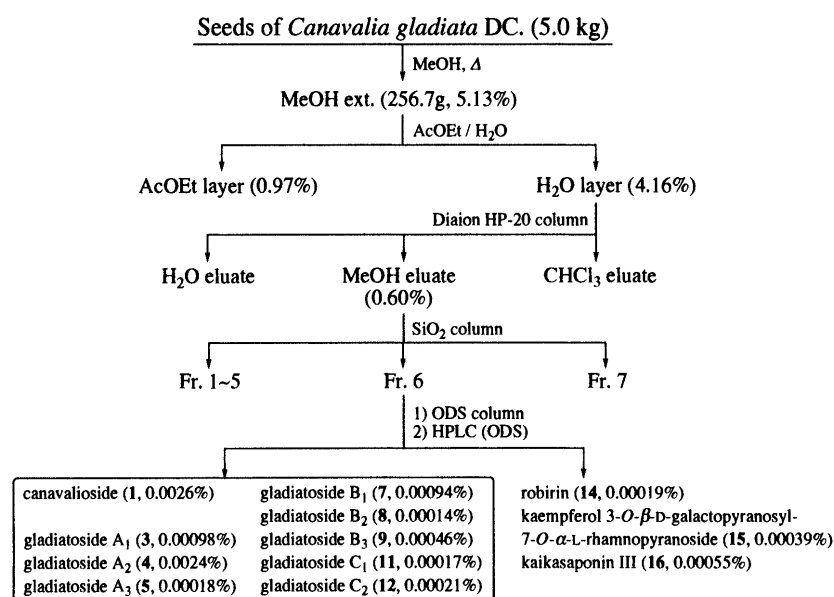


Chart 1

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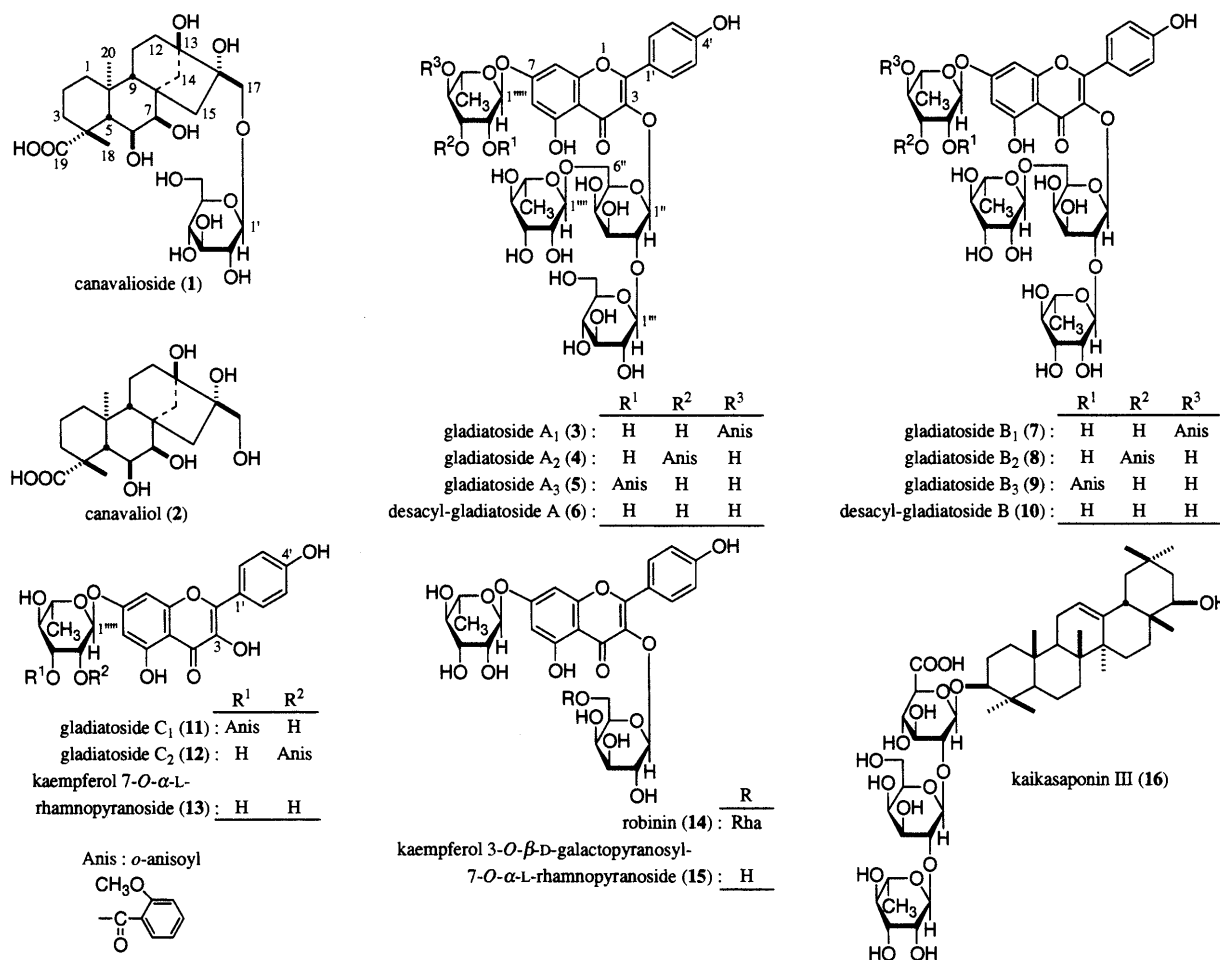


Chart 2

($M-H$)⁻. High-resolution MS analysis of the quasimolecular ion peak ($M+H$)⁺ revealed the molecular formula of **1** to be C₂₆H₄₂O₁₂. Methanolysis of **1** with 9% hydrogen chloride in dry methanol liberated methyl glucoside, which were identified by GLC analysis of trimethylsilyl derivatives. Acid hydrolysis of **1** with 5% aqueous H₂SO₄-1,4-dioxane (1:1) liberated D-glucose, which was identified by GLC analysis of the trimethylsilyl thiazolidine derivative.¹⁵ The ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 1) spectra of **1**, which were assigned by various NMR analytical methods,¹⁶ showed signals due to two tertiary methyls [δ 1.82, 1.23 (both s, 18, 20-H₃)], two methines bearing a hydroxyl group [δ 4.86 (dd, $J=2.0, 11.0$ Hz, 6-H), 4.05 (d, $J=2.0$ Hz, 7-H)], a hydroxymethylene [δ 4.45, 4.12 (both d, $J=11.1$ Hz, 17-H₂)], and a β -D-glucopyranosyl moiety [δ 5.04 (d, $J=7.9$ Hz, 1'-H)] together with a carboxyl and two quaternary carbons bearing a hydroxyl group. Enzymatic hydrolysis of **1** with naringinase liberated a new diterpene aglycone named canavaliol (**2**), whose molecular formula C₂₀H₃₂O₇ was determined from its positive-ion FAB-MS [m/z 407 ($M+Na$)⁺] and by high-resolution MS measurement. The planar structures of **1** and **2** were constructed on the basis of H-H COSY and HMBC experiments as shown in Fig. 1. Thus, the H-H COSY experiment for **1** and **2** indicated the presence of the partial structures in bold lines from C-1—C-3, from C-5—C-7, and from C-9—C-12. In the HMBC experiment, long-range correlations were observed between the following protons and carbons: 18-H₃ and 3, 4, 5, 18-C; 20-H₃ and 1, 5, 9,

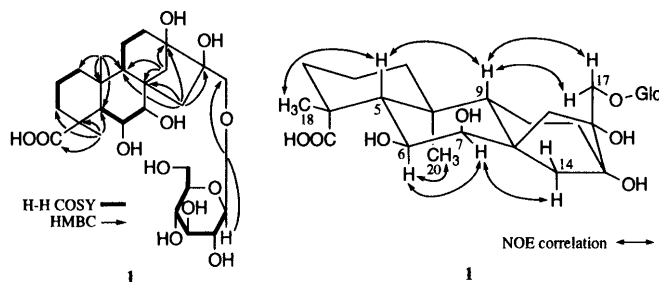


Fig. 1

10-C; 15-H₂ and 8, 9, 13, 16-C; 14-H₂ and 7, 9, 13-C, so that the connectivities of the quaternary carbons in **1** and **2** were clarified. By comparison of the ¹³C-NMR data for **1** with those for **2**, a glycosilation shift was observed around the 17-carbon of **1**. In addition, since the HMBC experiment for **1** showed a long-range correlation between the anomeric proton of the glucopyranosyl group and the 17-carbon, the β -D-glucopyranosyl moiety is linked to the 17-hydroxyl group of **2**. These findings and comparisons of the ¹H-NMR and ¹³C-NMR spectra of **1** with those of known *ent*-kaurane-type diterpene glycosides¹⁷ led us to formulate the 17-*O*- β -D-glucopyranosyl 6,7,13,16,17-pentahydroxy-19-kauranoic acid structure for **1**. The relative stereostructure of **1** was characterized by the difference nuclear Overhauser effect (NOE) experiment, which showed NOE correlations between the following protons: 5-H and 18-H₃, 9-H; 6-H and 7-H, 20-H₃;

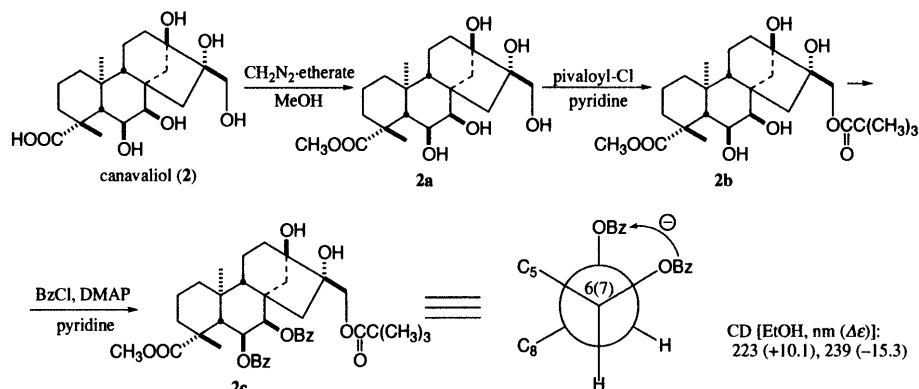


Fig. 2

Table 1. ^{13}C -NMR Data for Canavalioid (1) and Canavaliol (2)

	1	2		1
C-1	41.0	41.1	Glc-1'	105.6
C-2	19.7	19.8	Cle-2'	75.2
C-3	40.5	40.5	Cle-3'	78.4
C-4	44.5	44.6	Cle-4'	71.6
C-5	51.8	51.9	Cle-5'	78.4
C-6	71.8	72.0	Cle-6'	62.6
C-7	82.1	82.3		
C-8	46.2	46.2		
C-9	48.1	49.1		
C-10	40.9	41.0		
C-11	20.0	20.2		
C-12	35.0	34.9		
C-13	80.6	80.4		
C-14	43.3	43.6		
C-15	49.4	49.3		
C-16	77.6	78.2		
C-17	77.4	65.9		
C-18	33.1	33.2		
C-19	181.0	181.3		
C-20	16.9	17.0		

125 MHz, DMSO- d_6 .

7-H and 14 α -H; 17-H₂ and 9-H as shown in Fig. 1. In order to clarify the absolute stereostructure of **1**, the aglycone (**2**) examined by the circular dichroic exciton chirality method.¹⁸⁾ Namely, methylation of **2** with diazomethane (CH_2N_2)·etherate yielded the 19-methyl ester (**2a**), which was treated with pivaloyl chloride to give the 17-pivaloyl-19-methyl ester (**2b**). Benzoylation of **2b** with benzoyl chloride and pyridine in the presence of 4-dimethylaminopyridine (DMAP) furnished the 6,7-dibenzoate (**2c**). Since the circular dichroism (CD) spectrum of **2c** showed a negative Cotton effect at 239 nm, the 6- and 7-positions were determined to be *S* and *R* configurations, respectively (Fig. 2). On the basis of the above evidence, the absolute stereostructure of canavalioid (**1**) was elucidated.

Gladioside A₁ (**3**) was obtained as a yellow powder with a negative optical rotation. The IR spectrum of **3** showed absorption bands at 3432, 2940, 1655, 1601, and 1082 cm^{-1} ascribable to hydroxyl carbonyl, and ester functions, while its UV spectrum showed absorption maxima at 204 (log ϵ 4.6), 267 (4.2), and 350 (4.1) nm suggestive of a flavonol structure. The molecular formula $\text{C}_{47}\text{H}_{56}\text{O}_{26}$ was determined from the negative- and positive-ion FAB-MS [m/z 1035 ($\text{M}-\text{H}$)⁻, m/z 1059 ($\text{M}+\text{Na}$)⁺] and by high-resolution MS measure-

ment. Acid hydrolysis of **3** liberated D-galactose, L-rhamnose, and D-glucose with 5% H_2SO_4 -1,4-dioxane (1:1), which were identified by GLC analysis of trimethylsilyl thiazolidine derivatives. Alkaline hydrolysis of **3** with 10% potassium hydroxide-50% aqueous 1,4-dioxane liberated *o*-anisic acid and desacyl-gladioside A (**6**). The *o*-anisic acid was converted to the *p*-nitrobenzyl ester,¹⁹⁾ which was identified by HPLC analysis. The molecular formula $\text{C}_{39}\text{H}_{50}\text{O}_{24}$ of **6** was determined by the negative- and positive-ion FAB-MS [m/z 901 ($\text{M}-\text{H}$)⁻, m/z 925 ($\text{M}+\text{Na}$)⁺] and high-resolution MS measurement. The carbon and proton signals due to the aglycone moiety in the ^1H -NMR and ^{13}C -NMR spectra of **3** were superimposable to those of kaempferol 3,7-diglycosides.²⁰⁾ Namely, the ^1H -NMR (pyridine- d_5) and ^{13}C -NMR (Table 1) spectra of **6** showed signals due to a kaempferol moiety [δ 8.10 (d, $J=9.0$ Hz, 2', 6'-H), 6.90 (d, $J=9.0$ Hz, 3', 5'-H), 6.78 (d, $J=2.1$ Hz, 8-H), 6.43 (1H, d, $J=2.1$ Hz, 6-H)], a β -D-galactopyranosyl moiety [δ 5.60 (d, $J=7.7$ Hz, 1''-H)], a β -D-glucopyranosyl moiety [δ 4.57 (d, $J=7.7$ Hz, 1'''-H)], and two α -L-rhamnopyranosyl moieties [δ 5.54 (brs, 1''''-H), 4.37 (brs, 1''''-H), 1.14 (d, $J=6.0$ Hz, 6''''-H₃), 1.05 (d, $J=6.0$ Hz, 6''''-H₃)]. The bisdesmoside structure bonding to the 3- and 7-positions of kaempferol was identified by HMBC experiment. Thus, long-range correlations were observed between 1''-H and 3-C, between 1'''-H and 2''-C, between 1''''-H and 6''-C, and between 1''''-H and 7-C. On the basis of this evidence, the structure of **6** was elucidated as shown.

The ^1H -NMR (DMSO- d_6) and ^{13}C -NMR (Table 1) spectra of **3** showed signals assignable to an *o*-anisic acid moiety [δ 7.68 (dd, $J=1.6, 7.5$ Hz, anisoyl-3'-H), 7.54 (ddd-like, anisoyl-5'-H), 7.14 (dd-like, anisoyl-6'-H), 7.03 (ddd-like, anisoyl-4'-H), 3.81 (s, OMe)] together with a desacyl-gladioside A moiety [δ 5.13 (1H, t-like, 4''''-H)]. The position of the *o*-anisic acid moiety was determined by HMBC experiment, which showed a long-range correlation between the 4''''-H and the carbonyl carbon of *o*-anisic acid. Furthermore, an acylation shift was observed around the 4''''-position by a comparison of the ^{13}C -NMR data for **3** with those for **6**. Consequently, the structure of gladioside A₁ was determined to be 7-*O*-(4-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol 3-*O*-[β -D-glucopyranosyl(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -D-galactopyranoside (**3**).

Gladiosides A₂ (**4**) and A₃ (**5**) were found to have the same molecular formula $\text{C}_{47}\text{H}_{56}\text{O}_{26}$, which was elucidated from the quasimolecular ion peaks [m/z 1035 ($\text{M}-\text{H}$)⁻, 1059 ($\text{M}+\text{Na}$)⁺] in the negative- and positive-ion FAB-MS and by

Table 2. ^{13}C -NMR Data for Gladiatosides A₁ (**3**), A₂ (**4**), A₃ (**5**), B₁ (**7**), B₂ (**8**), B₃ (**9**), C₁ (**11**), and C₂ (**12**) and Desacyl-gladiatosides A (**6**) and B (**10**)

	3	4	5	6	7	8	9	10	11	12
C-2	156.4	156.4	156.4	156.3	156.9	156.9	156.9	156.9	147.6	147.6
-3	133.1	133.1	133.1	133.1	133.0	133.0	133.0	132.9	135.8	136.0
-4	177.6	177.6	177.6	177.6	177.4	177.4	177.4	177.4	176.1	176.1
-5	160.9	161.0	160.1	160.9	160.9	160.9	160.0	160.9	160.4	160.4
-6	99.4	99.4	99.4	99.3	99.4	99.3	99.4	99.3	98.8	98.9
-7	161.3	161.3	160.9	161.6	161.2	161.3	160.9	161.5	161.1	160.8
-8	94.6	94.7	94.6	94.5	94.7	94.8	94.7	94.6	94.5	94.5
-9	155.9	155.9	155.8	155.9	155.9	155.9	155.9	155.9	155.7	155.7
-10	105.6	105.7	105.7	105.5	105.7	105.7	105.8	105.6	104.8	104.9
-1'	120.5	120.3	120.0	120.6	120.6	120.3	120.0	120.6	121.5	121.5
-2', -6'	131.1	131.1	131.0	131.0	130.9	130.9	130.8	130.8	129.6	125.9
-3', -5'	115.2	115.2	115.2	115.2	115.1	115.1	115.0	115.1	115.4	115.4
-4'	160.1	160.1	158.9	160.1	160.0	160.0	158.9	160.0	159.4	160.3
3-Gal-1''	98.6	98.6	98.6	98.9	98.9	98.8	98.9	98.9		
-2''	80.1	80.0	80.1	80.1	75.0	75.0	75.0	75.0		
-3''	73.1	73.1	73.1	73.1	73.8	73.8	73.8	73.8		
-4''	69.8	69.8	69.8	69.8	68.5	68.5	70.1	68.6		
-5''	73.6	73.7	73.6	73.6	73.5	73.5	73.4	73.4		
-6''	64.9	64.9	64.8	64.9	65.2	65.1	65.1	65.1		
Glc or Rha-1'''	104.0	104.0	104.1	104.1	100.6	100.6	100.6	100.6		
-2'''	74.3	74.3	74.3	74.3	70.6	70.6	70.6	70.6		
-3'''	76.5	76.5	76.5	76.5	70.6	70.4	70.3	70.3		
-4'''	67.7	67.7	67.7	67.6	71.9	71.9	71.9	71.6		
-5'''	76.9	76.9	76.9	76.9	68.2	68.1	68.1	68.4		
-6'''	60.9	60.9	60.9	60.9	17.2	17.2	17.1	17.1		
Rha-1''''	99.9	99.9	99.8	99.8	100.0	100.0	100.0	100.0		
-2''''	70.6	70.6	70.6	70.6	70.5	70.4	70.6	70.5		
-3''''	70.3	70.3	70.3	70.3	70.3	70.2	70.3	70.3		
-4''''	71.9	71.9	71.8	71.9	71.9	71.9	71.8	71.9		
-5''''	68.1	68.1	68.1	68.1	68.1	68.2	68.2	68.1		
-6''''	17.8	17.8	17.8	17.7	17.8	17.8	17.8	17.8		
7-Rha-1'''''	98.2	98.4	95.3	98.5	98.1	98.3	95.3	98.5	98.3	95.3
-2'''''	69.9	67.4	71.9	69.8	69.9	67.3	72.0	69.8	67.3	71.9
-3'''''	67.9	73.9	68.4	70.2	67.9	73.9	68.4	70.0	73.9	68.4
-4'''''	73.7	70.2	72.0	72.5	73.7	70.2	71.9	72.5	70.2	72.0
-5'''''	67.7	68.8	70.1	68.4	67.7	68.8	68.5	68.2	68.8	70.1
-6'''''	17.5	17.7	17.8	17.8	17.4	17.7	17.8	17.8	17.8	17.8
Anis-1	165.1	164.6	164.4		165.1	164.6	164.4		164.6	164.4
-1'	120.7	120.3	120.6		120.7	120.3	120.6		120.4	119.3
-2'	158.1	158.5	158.8		158.1	158.5	158.8		158.5	158.9
-3'	130.4	131.1	131.3		130.4	131.1	131.3		131.1	131.3
-4'	120.0	119.8	119.3		120.0	119.8	119.3		119.8	120.0
-5'	133.4	133.5	134.0		133.3	133.4	134.0		133.4	134.0
-6'	112.6	112.6	112.8		112.6	112.6	112.8		112.6	112.8
-OCH ₃	55.8	55.8	55.9		55.8	55.8	55.9		55.8	55.9

100 MHz, DMSO-*d*₆.

high-resolution MS measurement. Alkaline hydrolysis of **4** and **5** furnished **6** and *o*-anisic acid, whereas acid hydrolysis of **4** and **5** liberated D-galactose, L-rhamnose, and D-glucose. The ^1H -NMR (dimethyl sulfoxide-*d*₆) and ^{13}C -NMR (Table 1) spectra of **4** and **5** showed signals due to *o*-anisic acid and desacyl-gladiatoside A moieties. The proton and carbon signals in the ^1H - and ^{13}C -NMR spectra of **4** and **5** were very similar to those of **3**, respectively, except for the signals due to the 7-*O*- α -L-rhamnopyranosyl moiety. In the HMBC experiment of **4** and **5**, long-range correlations were observed between the 3'''-proton and the carbonyl carbon of *o*-anisic acid in **4** and between the 2'''-proton and the carbonyl carbon of *o*-anisic acid in **5**. Consequently, the structures of gladiatosides A₂ and A₃ were determined as 7-*O*-(3-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol 3-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)]- β -D-galactopyranoside (**4**) and 7-*O*-(2-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol

3-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl-(1 $\rightarrow$ 6)]- β -D-galactopyranoside (**5**), respectively.

Gladiatosides B₁ (**7**), B₂ (**8**), and B₃ (**9**) were isolated as yellow powders. Since their IR and UV spectral data were similar to those of **3**, **4**, and **5**, their flavonol glycoside structures were deduced. Gladiatosides B₁ (**7**), B₂ (**8**), and B₃ (**9**) were found to have the same molecular formula C₄₇H₅₆O₂₅, which was determined from the quasimolecular ion peak in their negative- and positive-ion FAB-MS at *m/z* 1019 (M-H)⁻ and 1043 (M+Na)⁺ and by high-resolution MS measurement. Alkaline hydrolysis of **7**, **8**, and **9** liberated *o*-anisic acid and desacyl-gladiatoside B (**10**), while acid hydrolysis of **7**, **8**, and **9** liberated D-galactose and L-rhamnose. The ^1H -NMR (DMSO-*d*₆) and ^{13}C -NMR (Table 1) spectra of **10** showed the presence of a kaempferol moiety [δ 8.08 (d, *J*=9.0 Hz, 2', 6'-H), 6.87 (d, *J*=9.0 Hz, 3', 5'-H), 6.78 (d, *J*=2.1 Hz, 8-H), 6.43 (d, *J*=2.1 Hz, 6-H)], a β -D-galactopyra-

nosyl moiety [δ 5.57 (d, $J=7.7$ Hz, 1"-H)], and three α -L-rhamnopyranosyl moieties [δ 5.54 (brs, 1""-H), 5.05 (brs, 1""-H), 4.37 (brs, 1""-H), 1.13 (d, $J=6.0$ Hz, 6""-H₃), 1.05 (d, $J=6.0$ Hz, 6""-H₃), 0.79 (d, $J=6.2$ Hz, 6""-H₃)]. In the HMBC experiment of **10**, long-range correlations were observed between 1"-H and 3-C, between 1"-H and 2"-C, between 1""-H and 6"-C, and between 1""-H and 7-C. Consequently, the structure of desacyl-gladiatoside B (**10**) was elucidated as shown.

The $^1\text{H-NMR}$ (DMSO- d_6) and $^{13}\text{C-NMR}$ (Table 1) spectra of **7**, **8**, and **9** showed signals due to an *o*-anisoyl group in addition to a desacyl-gladiatoside A moiety. The proton and carbon signals in the $^1\text{H-}$ and $^{13}\text{C-NMR}$ spectra of **7**, **8**, and **9** were very similar to those of **3**, **4**, and **5**, respectively, except for the signals due to the 2"-*O*- α -L-rhamnopyranosyl moiety. By comparison of the $^{13}\text{C-NMR}$ data for **7**, **8**, and **9** with those for **10**, glycosidation shifts were observed around the 4""-, 3""-, and 2""-carbons, respectively. Furthermore, in the HMBC experiments for **7**, **8**, and **9**, long-range correlations were observed between the 4""-proton and the carbonyl carbon of *o*-anisic acid in **7**, between the 3""-proton and the carbonyl carbon of *o*-anisic acid in **8**, and between the 2""-proton and the carbonyl carbon of *o*-anisic acid in **9**. Consequently, the structures of gladiatosides B₁, B₂, and B₃ were determined as 7-*O*-(4-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol 3-*O*-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -D-galactopyranoside (**7**), 7-*O*-(3-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol 3-*O*-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -D-galactopyranoside (**8**), and 7-*O*-(2-*O*-*o*-anisoyl)- α -L-rhamnopyranosyl kaempferol 3-*O*-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2)][α -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -L-galactopyranoside (**9**).

Gladiatosides C₁ (**11**) and C₂ (**12**) were found to have the same molecular formula C₂₉H₂₆O₁₂, which was elucidated from the quasimolecular ion peaks [m/z 565 (M-H)⁻, 567 (M+H)⁺, 589 (M+Na)⁺] in the negative- and positive-ion FAB-MS and by high-resolution MS measurement. Alkaline hydrolysis of **11** and **12** furnished kaempferol 7-*O*- α -L-rhamnopyranoside (**13**)²¹ and *o*-anisic acid. The $^1\text{H-NMR}$ (DMSO- d_6) and $^{13}\text{C-NMR}$ (Table 2) spectra of **11** and **12** showed signals due to an *o*-anisic acid moiety and **13**. In the HMBC experiments for **11** and **12**, long-range correlations were observed between the 3""-proton and the carbonyl carbon of *o*-anisic acid in **11** and between the 2""-proton and the carbonyl carbon of *o*-anisic acid in **12**. Consequently, the structures of gladiatosides C₁ and C₂ were determined as kaempferol 7-*O*-(3-*O*-*o*-anisoyl)- α -L-rhamnopyranoside (**11**) and kaempferol 7-*O*-(2-*O*-*o*-anisoyl)- α -L-rhamnopyranoside (**12**), respectively.

Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l=5$ cm); UV spectra, Shimadzu UV-1200 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; CD spectra, J-720WI; $^1\text{H-NMR}$ spectra, JNM-LA500 (500 MHz) and Varian UNITY INOVA 400NB (400 MHz) spectrometer; $^{13}\text{C-NMR}$ spectra, JNM-LA500 (125 MHz) and Varian UNITY INOVA 400NB (100 MHz) spectrometers with tetramethylsilane as an internal standard; MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer and JMS-GC-MATE; HPLC, Shimadzu LC-10AS chromatograph.

The following experimental conditions were used for chromatography: normal-phase column chromatography; Silica gel BW-200 (Fuji Silysia

Chemical, Ltd., 150–350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); TLC, pre-coated TLC plates with Silica gel 60F₂₅₄ (Merck, 0.25 mm) (normal-phase) and Silica gel RP-18 60F₂₅₄ (Merck, 0.25 mm) (reversed-phase); HPTLC, pre-coated TLC plates with Silica gel RP-18 60WF_{254S} (Merck, 0.25 mm) (reversed-phase). Detection was performed by spraying with 1% Ce(SO₄)₂–10% aqueous H₂SO₄, followed by heating.

Isolation of Canavalioides (1) and Gladiatosides A₁ (3), A₂ (4), A₃ (5), B₁ (7), B₂ (8), B₃ (9), C₁ (11), and C₂ (12) from the Seeds of *C. gladiata*
The seeds of *C. gladiata* DC. (5.0 kg, cultivated in Hokkaido prefecture, Japan) was crush and extracted three times with MeOH under reflux. Evaporation of the solvent under reduced pressure provided the MeOH extract (257 g, 5.14%), and the MeOH extract (245 g) was partitioned into an AcOEt–H₂O (1:1, v/v) mixture. Removal of the solvent from the AcOEt-soluble and H₂O-soluble fractions under reduced pressure yielded the AcOEt extract (47 g, 0.94%) and H₂O extract (198 g, 3.96%). The H₂O extract (190 g) was subjected to Diaion HP-20 column chromatography [HP-20 (Nippon Rensou, 3 kg), H₂O→MeOH→CHCl₃] to give the H₂O eluate, MeOH eluate (27.3 g, 0.55%), and CHCl₃ eluate. Normal-phase silica gel column chromatography [BW-200 (Fuji Silysia Ltd., 820 g), CHCl₃–MeOH–H₂O (7:3:1, lower layer→65:35:10, lower layer→6:4:1, v/v)→MeOH] of the MeOH eluate (27.3 g) gave seven fractions. Fraction 6 (5.5 g) was separated by reversed-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Ltd., 160 g), MeOH–H₂O (40:60→60:40→80:20, v/v)→MeOH] to give four fractions. Fraction 6-1 (1.1 g) was further purified by reversed-phase silica gel column chromatography [Cosmosil 75C₁₈-OPN (Nakalai tesque, 30 g), H₂O→MeOH–H₂O (30:70, v/v)→MeOH] and HPLC [1) YMC-Pack Ph (YMC Co. Ltd., 250×20 mm i.d.), MeOH–H₂O (35:65, v/v); 2) YMC-Pack R&D-ODS-5-A (YMC Co. Ltd., 250×20 mm i.d.), MeOH–1% aq. trifluoroacetic acid (50:50, v/v); 3) COSMOSIL 5C₁₈ (250×20 mm i.d.), MeOH–H₂O (45:55, v/v)] to give canavalioides (**1**, 132.2 mg, 0.0026%), gladiatosides A₂ (**4**, 119.5 mg, 0.0024%) and B₂ (**8**, 67.5 mg, 0.0014%), robinin (**14**, 9.4 mg, 0.00019%), kaempferol 3-*O*- β -D-galactopyranosyl-7-*O*- α -L-rhamnopyranoside (**15**, 19.6 mg, 0.00039%), and tryptophan (164.1 mg, 0.0033%). Fraction 6-2 (534.4 mg) was purified by HPLC [YMC-Pack R&D-ODS-5-A, 1) CH₃CN–H₂O (25:75, v/v); 2) MeOH–H₂O (50:50, v/v)] to give gladiatosides A₁ (**3**, 48.9 mg, 0.00098%), A₃ (**5**, 8.8 mg, 0.00018%), B₁ (**7**, 47.0 mg, 0.00094%), B₃ (**9**, 23.0 mg, 0.00046%), C₁ (**11**, 8.3 mg, 0.00017%), and C₂ (**12**, 10.5 mg, 0.00021%). Fraction 6-3 (684.3 mg) was separated by HPLC [YMC-Pack R&D-ODS-5-A, MeOH–1% aq. AcOH (45:55, v/v)] to give kaikasaponin III (**16**, 27.3 mg, 0.00055%). The known compounds (**14**, **15**, **16**) were identified by comparison of their physical data ($[\alpha]_D$, IR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$) with reported values.^{11–13)}

Canavalioides (1): A white powder, $[\alpha]_D^{26} -43.0^\circ$ ($c=1.9$, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₂₆H₄₃O₁₂ (M+H)⁺: 547.2755. Found: 547.2769. IR (KBr): 3410, 2940, 1686, 1073 cm⁻¹. $^1\text{H-NMR}$ (pyridine- d_5 , 500 MHz) δ : 1.23, 1.82 (3H each, both s, 20, 18-H₃), 1.68 (1H, brd, $J=ca.$ 8 Hz, 9-H), 2.04 (1H, d, $J=14.7$ Hz, 15 β -H), 2.07 (1H, d, $J=10.1$ Hz, 14 β -H), 2.30 (1H, dd-like, 14 α -H), 2.59 (1H, d, $J=14.7$ Hz, 15 α -H), 2.34 (1H, d, $J=11.0$ Hz, 5-H), 3.89 (1H, m, 5'-H), 3.96 (1H, dd-like, 2'-H), 4.12, 4.45 (1H each, both d, $J=11.1$ Hz, 17-H₂), 4.05 (1H, d, $J=2.0$ Hz, 7-H), 4.12 (1H, dd, $J=5.2$, 11.9 Hz), 4.49 (1H, dd, $J=2.7$, 11.9 Hz) (6'-H₂), 4.16 (2H, m, 3', 4'-H), 4.86 (1H, dd, $J=2.0$, 11.0 Hz, 6-H), 5.04 (1H, d, $J=7.9$ Hz, 1'-H). $^{13}\text{C-NMR}$ (pyridine- d_5 , 125 MHz) δ_C : given in Table 1. Negative-ion FAB-MS: m/z 545 (M-H)⁻. Positive-ion FAB-MS: m/z 547 (M+H)⁺, 569 (M+Na)⁺.

Gladiatoside A₁ (3): A yellow powder, $[\alpha]_D^{25} -63.8^\circ$ ($c=0.2$, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₆Na (M+Na)⁺: 1059.2958. Found: 1059.2948. IR (KBr): 3432, 2940, 1655, 1601, 1082 cm⁻¹. UV [MeOH, nm (log ϵ)]: 204 (4.6), 267 (4.2), 350 (4.1). $^1\text{H-NMR}$ (DMSO- d_6 , 400 MHz) δ : 1.06 (3H, d, $J=6.2$ Hz, 6""-H₃), 1.14 (3H, d, $J=6.2$ Hz, 6""-H₃), 3.81 (3H, s, OMe), 4.36 (1H, br s, 1""-H), 4.58 (1H, d, $J=7.9$ Hz, 1""-H), 5.13 (1H, t-like, 4""-H), 5.61 (1H, d, $J=7.6$ Hz, 1"-H), 5.66 (1H, brs, 1""-H), 6.49 (1H, d, $J=2.1$ Hz, 6-H), 6.85 (1H, d, $J=2.1$ Hz, 8-H), 6.91 (2H, d, $J=9.0$ Hz, 3' 5'-H), 8.11 (2H, d, $J=9.0$ Hz, 2' 6'-H), 7.03 (1H, ddd-like, Anis-4'-H), 7.14 (1H, dd-like, Anis-6'-H), 7.54 (1H, ddd-like, Anis-5'-H), 7.68 (1H, dd, $J=1.6$, 7.5 Hz, Anis-3'-H), 12.64 (1H, br s, 5-OH). $^{13}\text{C-NMR}$ (DMSO- d_6 , 100 MHz) δ_C : given in Table 2. Negative-ion FAB-MS: m/z 1035 (M-H)⁻, 755 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₄)⁻, 285 (M-C₃₂H₄₇O₂₀)⁻. Positive-ion FAB-MS: m/z 1059 (M+Na)⁺.

Gladiatoside A₂ (4): A yellow powder, $[\alpha]_D^{25} -91.2^\circ$ ($c=0.2$, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₆Na (M+Na)⁺: 1059.2958. Found: 1059.2974. IR (KBr): 3432, 2924, 1655, 1601,

1080 cm⁻¹. UV [MeOH, nm (log ϵ): 206 (4.7), 233 (4.3), 268 (4.3), 352 (4.2). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 1.06 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 1.22 (3H, d, *J*=5.5 Hz, 6^{'''}-H₃), 3.84 (3H, s, OMe), 4.37 (1H, brs, 1^{'''}-H), 4.59 (1H, d, *J*=7.7 Hz, 1^{'''}-H), 5.15 (1H, dd-like, 3^{'''}-H), 5.62 (1H, d, *J*=7.5 Hz, 1^{'''}-H), 5.63 (1H, brs, 1^{'''}-H), 6.50 (1H, d, *J*=2.0 Hz, 6-H), 6.85 (1H, d, *J*=2.0 Hz, 8-H), 6.91 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.12 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.04 (1H, ddd-like, Anis-4'-H), 7.15 (1H, dd-like, Anis-6'-H), 7.55 (1H, ddd-like, Anis-5'-H), 7.84 (1H, dd, *J*=1.5, 7.7 Hz, Anis-3'-H), 12.65 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 1035 (M-H)⁻, 755 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₄)⁻, 285 (M-C₃₂H₄₇O₂₀)⁻. Positive-ion FAB-MS: *m/z* 1059 (M+Na)⁺.

Gladiatoside A₃ (5): A yellow powder. [α]_D²⁵ -76.3° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₆Na (M+Na)⁺: 1059.2958. Found: 1059.2944. IR (KBr): 3453, 2924, 1655, 1601, 1075 cm⁻¹. UV [MeOH, nm (log ϵ): 204 (4.7), 267 (4.2), 349 (4.1). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 1.05 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 1.21 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 3.85 (3H, s, OMe), 4.37 (1H, brs, 1^{'''}-H), 4.57 (1H, d, *J*=7.7 Hz, 1^{'''}-H), 5.29 (1H, dd, *J*=1.8, 3.3 Hz, 2^{'''}-H), 5.61 (1H, d, *J*=7.5 Hz, 1^{'''}-H), 5.78 (1H, brs, 1^{'''}-H), 6.51 (1H, d, *J*=2.2 Hz, 6-H), 6.88 (1H, d, *J*=2.2 Hz, 8-H), 6.89 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.11 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.07 (1H, ddd-like, Anis-4'-H), 7.18 (1H, dd-like, Anis-6'-H), 7.59 (1H, ddd-like, Anis-5'-H), 7.80 (1H, dd, *J*=1.8, 7.7 Hz, Anis-3'-H), 12.63 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 1035 (M-H)⁻, 755 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₄)⁻, 285 (M-C₃₂H₄₇O₂₀)⁻. Positive-ion FAB-MS: *m/z* 1059 (M+Na)⁺.

Gladiatoside B₁ (7): A yellow powder, [α]_D²⁵ -79.6° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₅Na (M+Na)⁺: 1043.3008. Found: 1043.3002. IR (KBr): 3453, 2940, 1655, 1601, 1086 cm⁻¹. UV [MeOH, nm (log ϵ): 204 (4.7), 267 (4.2). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 0.81 (3H, d, *J*=6.0 Hz, 6^{'''}-H₃), 1.06 (3H, d, *J*=6.0 Hz, 6^{'''}-H₃), 1.13 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 3.81 (3H, s, OMe), 4.38 (1H, brs, 1^{'''}-H), 5.07 (1H, brs, 1^{'''}-H), 5.12 (1H, t-like, 4^{'''}-H), 5.58 (1H, d, *J*=7.7 Hz, 1^{'''}-H), 5.66 (1H, brs, 1^{'''}-H), 6.51 (1H, d, *J*=2.0 Hz, 6-H), 6.85 (1H, d, *J*=2.0 Hz, 8-H), 6.88 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.10 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.03 (1H, ddd-like, Anis-4'-H), 7.14 (1H, dd-like, Anis-6'-H), 7.54 (1H, ddd, *J*=1.8, 7.4, 8.3 Hz, Anis-5'-H), 7.81 (1H, dd, *J*=1.8, 7.7 Hz, Anis-3'-H), 12.63 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 1019 (M-H)⁻, 739 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₃)⁻, 285 (M-C₃₂H₄₇O₁₉)⁻. Positive-ion FAB-MS: *m/z* 1043 (M+Na)⁺.

Gladiatoside B₂ (8): A yellow powder, [α]_D²⁵ -152.8° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₅Na (M+Na)⁺: 1043.3008. Found: 1043.3000. IR (KBr): 3453, 2932, 1653, 1601, 1088 cm⁻¹. UV [MeOH, nm (log ϵ): 206 (4.7), 234 (4.3), 267 (4.2), 349 (4.2). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 0.82 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 1.06 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 1.21 (3H, d, *J*=5.7 Hz, 6^{'''}-H₃), 3.84 (3H, s, OMe), 4.38 (1H, brs, 1^{'''}-H), 5.07 (1H, brs, 1^{'''}-H), 5.15 (1H, dd, *J*=3.3, 9.3 Hz, 3^{'''}-H), 5.59 (1H, d, *J*=7.7 Hz, 1^{'''}-H), 5.67 (1H, brs, 1^{'''}-H), 6.49 (1H, d, *J*=2.1 Hz, 6-H), 6.85 (1H, d, *J*=2.1 Hz, 8-H), 6.88 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.11 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.04 (1H, ddd-like, Anis-4'-H), 7.15 (1H, dd-like, Anis-6'-H), 7.55 (1H, ddd, *J*=1.8, 7.5, 8.6 Hz, Anis-5'-H), 7.84 (1H, dd, *J*=1.8, 7.5 Hz, Anis-3'-H), 12.62 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 1019 (M-H)⁻, 739 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₃)⁻, 285 (M-C₃₂H₄₇O₁₉)⁻. Positive-ion FAB-MS: *m/z* 1043 (M+Na)⁺.

Gladiatoside B₃ (9): A yellow powder, [α]_D²⁵ -107.9° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₄₇H₅₆O₂₅Na (M+Na)⁺: 1043.3000. Found: 1043.3008. IR (KBr): 3432, 2934, 1662, 1601, 1077 cm⁻¹. UV [MeOH, nm (log ϵ): 205 (4.8), 267 (4.3), 349 (4.2). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 0.80 (3H, d, *J*=6.2 Hz, 6^{'''}-H₃), 1.05 (3H, d, *J*=6.8 Hz, 6^{'''}-H₃), 1.20 (3H, d, *J*=6.0 Hz, 6^{'''}-H₃), 3.86 (3H, s, OMe), 4.38 (1H, brs, 1^{'''}-H), 5.07 (1H, brs, 1^{'''}-H), 5.30 (1H, dd-like, 2^{'''}-H), 5.58 (1H, d, *J*=7.7 Hz, 1^{'''}-H), 5.79 (1H, brs, 1^{'''}-H), 6.52 (1H, d, *J*=2.2 Hz, 6-H), 6.88 (1H, d, *J*=2.2 Hz, 8-H), 6.87 (2H, d, *J*=9.1 Hz, 3', 5'-H), 8.09 (2H, d, *J*=9.1 Hz, 2', 6'-H), 7.07 (1H, ddd-like, Anis-4'-H), 7.19 (1H, dd-like, Anis-6'-H), 7.59 (1H, ddd, *J*=1.8, 7.5, 8.6 Hz, Anis-5'-H), 7.81 (1H, dd, *J*=1.8, 7.5 Hz, Anis-3'-H), 12.65 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 1019 (M-H)⁻, 739 (M-C₁₄H₁₇O₆)⁻, 565 (M-C₁₈H₃₁O₁₃)⁻. Positive-ion FAB-MS: *m/z* 1043 (M+Na)⁺.

Gladiatoside C₁ (11): A yellow powder, [α]_D²⁵ -127.0° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₂₉H₂₇O₁₂ (M+H)⁺:

567.1503. Found: 567.1510. IR (KBr): 3453, 2940, 1655, 1599, 1090 cm⁻¹. UV [MeOH, nm (log ϵ): 206 (4.8), 267 (4.3). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 1.21 (3H, d, *J*=5.7 Hz, 6^{'''}-H₃), 3.84 (3H, s, OMe), 5.15 (1H, dd, *J*=3.3, 9.5 Hz, 3^{'''}-H), 5.63 (1H, brs, 1^{'''}-H), 6.48 (1H, d, *J*=2.1 Hz, 6-H), 6.88 (1H, d, *J*=2.1 Hz, 8-H), 6.94 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.10 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.04 (1H, ddd-like, Anis-4'-H), 7.15 (1H, dd-like, Anis-6'-H), 7.55 (1H, ddd, *J*=1.8, 7.4, 8.3 Hz, Anis-5'-H), 7.84 (1H, dd, *J*=1.8, 7.4 Hz, Anis-3'-H), 12.48 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 565 (M-H)⁻, 285 (M-C₁₄H₁₇O₆)⁻. Positive-ion FAB-MS: *m/z* 567 (M+H)⁺, 589 (M+Na)⁺.

Gladiatoside C₂ (12): A yellow powder, [α]_D²⁵ -32.6° (*c*=0.2, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₂₉H₂₇O₁₂ (M+H)⁺: 567.1503. Found: 567.1488. IR (KBr): 3453, 2940, 1655, 1599, 1089 cm⁻¹. UV [MeOH, nm (log ϵ): 206 (4.5), 266 (4.3). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 1.20 (3H, d, *J*=6.0 Hz, 6^{'''}-H₃), 3.86 (3H, s, OMe), 5.29 (1H, dd, *J*=1.8, 3.2 Hz, 2^{'''}-H), 5.79 (1H, brs, 1^{'''}-H), 6.50 (1H, d, *J*=2.2 Hz, 6-H), 6.91 (1H, d, *J*=2.2 Hz, 8-H), 6.93 (2H, d, *J*=9.0 Hz, 3', 5'-H), 8.09 (2H, d, *J*=9.0 Hz, 2', 6'-H), 7.07 (1H, ddd-like, Anis-4'-H), 7.18 (1H, dd-like, Anis-6'-H), 7.59 (1H, ddd, *J*=1.8, 7.7, 8.6 Hz, Anis-5'-H), 7.81 (1H, dd, *J*=1.8, 7.7 Hz, Anis-3'-H), 12.43 (1H, brs, 5-OH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ _C: given in Table 2. Negative-ion FAB-MS: *m/z* 565 (M-H)⁻, 285 (M-C₁₄H₁₇O₆)⁻. Positive-ion FAB-MS: *m/z* 567 (M+H)⁺, 589 (M+Na)⁺.

Methanolysis of Canavalioid (1) A solution of **1** (3 mg) in 9% HCl-dry MeOH (0.5 ml) was heated under reflux for 4 h. After cooling, the reaction solution was neutralized with Ag₂CO₃ powder and filtered. After removal of the solvent under reduced pressure, the residue was dissolved in pyridine (0.02 ml) and the solution was treated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (0.01 ml) for 1 h. The reaction solution was then subjected to GLC analysis to identify the trimethylsilyl (TMS) derivative of methyl glucoside. GLC conditions: column CBR1-M25-025 [0.25 mm (i.d.) $\times$ 25 m] capillary column; Injector temperature: 140 °C; Detector temperature: 280 °C; Column temperature: 140–240 °C, 5 °C/min; Initial time: 5 min; He flow rate: 15 ml/min; *t*_R: 21.3, 21.7 min.

Acid Hydrolysis of Canavalioid (1) A solution of **1** in 5% aq. H₂SO₄-1,4-dioxane (1 : 1, v/v, 1.0 ml) was stirred under reflux for 2 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH⁻ form) and the resin was removed by filtration. After removal of the solvent from the filtrate *in vacuo*, the residue was subjected to a Sep-Pak C18 cartridge column eluting with H₂O and MeOH. The H₂O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (3 mg) in pyridine (0.5 ml) at 60 °C for 1 h. After reaction, the solution was treated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (0.2 ml) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivative of D-glucose; GLC conditions: Supelco STBTM-1, 30 m $\times$ 0.25 mm (i.d.) capillary column, column temperature 230 °C, He flow rate 15 ml/min, *t*_R: 24.4 min.

Enzymatic Hydrolysis of Canavalioid (1) A solution of **1** (35 mg) in 0.1 M acetate buffer (pH 4.0, 3.0 ml) was treated with naringinase (Sigma Chemical Co., 30.0 mg) and the reaction mixture was stirred at 40 °C for 2 d. The reaction mixture was poured into EtOH and the whole was removed under reduced pressure. The residue (76 mg) was purified by reversed-phase [30 g, H₂O $\rightarrow$ MeOH] and normal-phase silica gel column chromatography [3.0 g, CHCl₃-MeOH-H₂O (10 : 3 : 1, lower layer, v/v)] to give canavaliol (**2**, 21.4 mg, 87.5%).

Canavaliol (**2**): A white powder, [α]_D²⁵ -30.5° (*c*=1.1, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₂₀H₃₂O₇Na (M+Na)⁺: 407.2046. Found: 407.2055. IR (KBr): 3410, 2934, 1697 cm⁻¹. ¹H-NMR (pyridine-*d*₅, 500 MHz) δ : 1.27, 1.83 (3H each, both s, 20, 18-H₃), 1.74 (1H, d, *J*=7.6 Hz, 9-H), 2.12 (1H, d, *J*=10.4 Hz, 14 β -H), 2.16 (1H, d, *J*=14.8 Hz, 15 β -H), 2.35 (1H, dd-like, 14 α -H), 2.37 (1H, d, *J*=10.7 Hz, 5-H), 2.67 (1H, d, *J*=14.8 Hz, 15 α -H), 4.10, 4.19 (1H each, both d, *J*=11.0 Hz, 17-H₂), 4.11 (1H, br d, 7-H), 4.89 (1H, dd, *J*=2.1, 10.7 Hz, 6-H). ¹³C-NMR (pyridine-*d*₅, 125 MHz) δ _C: given in Table 1. Positive-ion FAB-MS: *m/z* 407 (M+Na)⁺.

Methylation of Canavaliol (2) A solution of **2** (11.5 mg) in MeOH (1.0 ml) was treated with ethereal diazomethane (*ca.* 10 ml) until the yellow color persisted. The solution was stirred at room temperature (25 °C) for 30 min and the solvent was removed under reduced pressure to furnish a product. This product was purified by normal-phase silica gel column chromatography [1.0 g, CHCl₃-MeOH-H₂O (10 : 3 : 1, lower layer) to give **2a** (10.1 mg, 83.3%).

2a: A white powder. High-resolution EI-MS: Calcd for C₂₁H₃₄O₇ (M⁺): 398.2319. Found: 398.2304. ¹H-NMR (pyridine-*d*₅, 270 MHz) δ : 1.00, 1.72

(3H each, both s, 20, 18-H₃), 1.78 (1H, d, $J=7.4$ Hz, 9-H), 2.06 (1H, d, $J=11.0$ Hz, 14 β -H), 2.14 (1H, d, $J=14.7$ Hz, 15 β -H), 2.26 (1H, d, $J=11.0$ Hz, 5-H), 2.34 (1H, dd-like, 14 α -H), 2.64 (1H, d, $J=15.0$ Hz, 15 α -H), 3.65 (3H, s, OMe), 4.03 (1H, d, $J=2.0$ Hz, 7-H), 4.10, 4.20 (1H each, both d, $J=11.1$ Hz, 17-H₂), 4.70 (1H, dd, $J=2.0$, 11.0 Hz, 6-H). EI-MS (m/z , %, 20 eV): 398 (M⁺, 1), 380 (M⁺-H₂O, 2), 305 (100).

Selective Acylation of 2a A solution of **2a** in dry pyridine was treated with pivaloyl chloride (0.03 ml) and the mixture was stirred at room temperature (25 °C) under N₂ for 1 h. The reaction mixture was poured into ice-water and the whole was lyophilized. The residue (33.5 mg) was purified by normal-phase silica gel column chromatography [3.0 g, CHCl₃-MeOH (20:1, v/v)] to give **2b** (10.4 mg, 86.2%).

2b: A white powder. High-resolution positive-ion FAB-MS: Calcd for C₂₆H₄₂O₈Na (M+Na)⁺: 505.2777. Found: 505.2818. ¹H-NMR (pyridine-*d*₅, 270 MHz) δ : 0.97, 1.71 (3H each, both s, 20, 18-H₃), 1.27 (9H, s, Pv-H₉), 2.00 (1H, d, $J=6.9$ Hz, 9-H), 2.04 (1H, dd, $J=1.5$, 10.6 Hz, 14 β -H), 2.17 (1H, dd, $J=1.5$, 15.1 Hz, 15 β -H), 2.26 (1H, d, $J=10.9$ Hz, 5-H), 2.31 (1H, d, $J=10.6$ Hz, 14 α -H), 2.63 (1H, d, $J=15.1$ Hz, 15 α -H), 3.64 (3H, s, OMe), 4.01 (1H, d, $J=2.2$ Hz, 7-H), 4.60, 4.66 (1H each, both d, $J=11.4$ Hz, 17-H₂), 4.68 (1H, dd, $J=2.0$, 10.9 Hz, 6-H). Positive-ion FAB-MS: m/z 505 (M+Na)⁺.

Benzoylation of 2b A solution of **2b** (9.8 mg) in dry pyridine (1.0 ml) was treated with benzoyl chloride (0.05 ml) and DMAP (5 mg) and the mixture was stirred at 70 °C under N₂ for 2 h. The reaction mixture was poured into ice-water and the whole was lyophilized. The residue (117.7 mg) was purified by normal-phase silica gel column chromatography [10.0 g, *n*-hexane-AcOEt (1:1, v/v)] to give **2c** (2.1 mg, 15.2%).

2c: A white powder. High-resolution positive-ion FAB-MS: Calcd for C₄₀H₅₀O₁₀Na (M+Na)⁺: 713.3302. Found: 713.3303. UV [EtOH, nm (log ϵ): 230 (4.4). CD ($c=0.0003$, EtOH) [θ]_D²⁵: 223 (+10.1), 239 (-15.3). ¹H-NMR (CDCl₃, 270 MHz) δ : 1.04, 1.26 (3H each, all s, 20, 18-H₃), 1.13 (9H, s, Pv-H₉), 2.25 (1H, d, $J=11.6$ Hz, 5-H), 3.60 (3H, s, OMe), 4.11, 4.27 (1H each, both d, $J=11.6$ Hz, 17-H₂), 5.50 (1H, d, $J=2.1$ Hz, 7-H), 5.96 (1H, dd-like, 6-H), 7.32-8.00 (10H, m, Ph $\times$ 2). Positive-ion FAB-MS: m/z 713 (M+Na)⁺.

Acid Hydrolysis of Gladiatosides (3, 4, 5, 7, 8, 9) A solution of gladiatosides (**3**, **4**, **5**, **7**, **8**, **9**) in 5% aq. H₂SO₄-1,4-dioxane (1:1, v/v, 1.0 ml) was stirred under reflux for 2 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH⁻ form) and residue was removed by filtration. After removal of the solvent from the filtrate *in vacuo*, the residue was transferred to a Sep-Pak C18 cartridge with H₂O and MeOH. The H₂O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (3 mg) in pyridine (0.5 ml) at 60 °C for 1 h. After reaction, the solution was treated with *N*-O-bis(trimethylsilyl)trifluoroacetamide (0.2 ml) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-galactose (i) from **3**, **4**, **5**, **7**, **8**, **9**; L-rhamnose (ii) from **3**, **4**, **5**, **7**, **8**, **9**; D-glucose (iii) from **3**, **4**, **5**; GLC conditions: Supelco STBTM-1, 30 m $\times$ 0.25 mm (i.d.) capillary column, column temperature 230 °C, He flow rate 15 ml/min, t_R : i (13.8 min), ii (15.5 min), iii (24.2 min).

Alkaline Hydrolysis of Gladiatosides (3, 4, 5, 7, 8, 9, 11, 12) A solution of gladiatosides (**3**: 10.0 mg; **4**: 8.2 mg; **5**: 2.0 mg; **7**: 10.8 mg; **8**: 10.0 mg; **9**: 3.0 mg; **11**: 2.1 mg; **12**: 2.5 mg) in 50% aqueous dioxane (1 ml) was treated with 10% aqueous KOH (1 ml) and the whole was stirred at 37 °C for 1 h. After removal of the solvent from a part (0.1 ml) of the reaction mixture under reduced pressure, the residue was dissolved in dichloroethane (CH₂)₂Cl₂ (1 ml) and the solution was treated with *p*-nitrobenzyl-*N,N'*-diisopropylisourea (5 mg), then the whole was stirred at 80 °C for 1 h. The reaction solution was subjected to HPLC analysis to identify the *p*-nitrobenzyl esters of *o*-anisic acid. HPLC conditions: column, YMC-Pack ODS-A (YMC Co., Ltd.), 250 $\times$ 4.6 mm (i.d.); solvent, MeOH-H₂O (70:30, v/v); flow rate, 1.0 ml/min; t_R : 21.5 min.

The rest of the reaction mixture was neutralized with Dowex HCR W2 (H⁺ from) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a product, which was subjected to normal-phase silica gel column chromatography [1 g, CHCl₃-MeOH-H₂O (7:3:1, lower layer, v/v)] to give desacyl derivatives [desacyl-gladiatosides **A** (**6**, 8.0 mg, 90% from **3**; 6.5 mg, 91.5% from **4**; 1.4 mg, 82.4% from **5**); **B** (**10**, 8.1 mg, 81.8% from **7**; 8.4 mg, 96.6% from **8**; 2.3 mg, 85.5% from **9**); kaempferol 7-*O*- α -L-rhamnopyranoside (**13**, 1.4 mg, 87.5% from **11**; 1.6 mg, 84.2% from **12**)]. **13** was identified by physical data comparison with reported values.²¹⁾

Desacyl-gladiatoside **A** (**6**): A yellow powder, $[\alpha]_D^{25} -59.1^\circ$ ($c=0.2$, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₃₉H₅₀O₂₄Na

(M+Na)⁺: 925.2590. Found: 925.2580. IR (KBr): 3429, 2926, 1655, 1075 cm⁻¹. UV [MeOH, nm (log ϵ): 204 (4.6), 267 (4.4), 350 (4.3). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 1.05 (3H, d, $J=6.0$ Hz, 6'''-H₃), 1.14 (3H, d, $J=6.0$ Hz, 6'''-H₃), 4.37 (1H, brs, 1'''-H), 4.57 (1H, d, $J=7.7$ Hz, 1'''-H), 5.54 (1H, brs, 1'''-H), 5.60 (1H, d, $J=7.7$ Hz, 1''-H), 6.43 (1H, d, $J=2.1$ Hz, 6-H), 6.78 (1H, d, $J=2.1$ Hz, 8-H), 6.90 (2H, d, $J=9.0$ Hz, 3', 5'-H), 8.10 (2H, d, $J=9.0$ Hz, 2', 6'-H). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ_C : given in Table 2. Negative-ion FAB-MS: m/z 901 (M-H)⁻, 755 (M-C₆H₁₁O₄)⁻, 285 (M-C₂₄H₄₁O₁₈)⁻. Positive-ion FAB-MS: m/z 925 (M+Na)⁺.

Desacyl-gladiatoside **B** (**10**): A yellow powder, $[\alpha]_D^{25} -98.8^\circ$ ($c=0.2$, MeOH). High-resolution positive-ion FAB-MS: Calcd for C₃₉H₅₀O₂₃Na (M+Na)⁺: 909.2641. Found: 909.2626. IR (KBr): 3432, 2924, 1655, 1059 cm⁻¹. UV [MeOH, nm (log ϵ): 207 (4.8), 266 (4.7), 348 (4.6). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 0.79 (3H, d, $J=6.2$ Hz, 6'''-H₃), 1.05 (3H, d, $J=6.0$ Hz, 6'''-H₃), 1.13 (3H, d, $J=6.0$ Hz, 6'''-H₃), 4.37 (1H, brs, 1'''-H), 5.57 (1H, d, $J=7.7$ Hz, 1'''-H), 6.43 (1H, d, $J=2.1$ Hz, 6-H), 6.78 (1H, d, $J=2.1$ Hz, 8-H), 6.87 (2H, d, $J=9.0$ Hz, 3', 5'-H), 8.08 (2H, d, $J=9.0$ Hz, 2', 6'-H). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ_C : given in Table 2. Negative-ion FAB-MS: m/z 885 (M-H)⁻, 739 (M-C₆H₁₁O₄)⁻, 431 (M-C₁₈H₃₁O₁₃)⁻, 285 (M-C₂₄H₄₁O₁₇)⁻. Positive-ion FAB-MS: m/z 909 (M+Na)⁺, 887 (M+H)⁺.

References and Notes

- 1) Part XVIII: Murakami T., Kishi A., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, in press.
- 2) Tamura S., Takahashi N., Murofushi N., Yokota T., Kato J., Shiotani Y., *Planta*, **75**, 279-282 (1967) [CA **67**, 52929].
- 3) Schlueter M., Bords E., *Phytochemistry*, **11**, 3533-3534 (1972)
- 4) Akedo H., Mori Y., Tanigaki Y., Shinkai K., Morita K., *Biochim. Biophys. Acta*, **271**, 378-387 (1972).
- 5) a) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **368**, 237-243 (1999); b) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **65**, PL27-32 (1999); c) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Bioorg. Med. Chem.*, **7**, 323-327 (1999); d) Matsuda H., Li Y., Yamahara J., Yoshikawa M., *J. Pharmacol. Exp. Ther.*, **289**, 729-734 (1999); e) Matsuda H., Li Y., Yoshikawa M., *Eur. J. Pharmacol.*, **373**, 63-70 (1999); f) Matsuda H., Shimoda H., Yoshikawa M., *Bioorg. Med. Chem.*, **7**, 1445-1450 (1999); g) Matsuda H., Li Y., Yoshikawa M., *ibid.*, **7**, 1737-1741 (1999); h) Matsuda H., Shimoda H., Yamahara J., Yoshikawa M., *Biol. Pharm. Bull.*, **22**, 870-872 (1999); i) Matsuda H., Murakami T., Yashiro K., Yamahara J., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1725-1729 (1999); j) Matsuda H., Shimoda H., Uemura T., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **9**, 2647-2652 (1999); k) Murakami T., Matsuda H., Inadzuki M., Hirano K., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1717-1724 (1999); l) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **66**, PL41-46 (2000); m) Li Y., Wen S., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **387**, 339-344 (2000).
- 6) a) Yoshikawa M., Murakami T., Komatsu H., Murakami N., Yamahara J., Matsuda H., *Chem. Pharm. Bull.*, **45**, 81-87 (1997); b) Yoshikawa M., Murakami T., Komatsu H., Yamahara J., Matsuda H., *Heterocycles*, **47**, 397-405 (1998).
- 7) Yoshikawa M., Shimada H., Komatsu H., Sakurama T., Nishida N., Yamahara J., Shimoda H., Matsuda H., Tani T., *Chem. Pharm. Bull.*, **45**, 877-882 (1997).
- 8) a) Komatsu H., Murakami T., Matsuda H., Yoshikawa M., *Heterocycles*, **48**, 703-710 (1998); b) Yoshikawa M., Murakami T., Komatsu H., Matsuda H., *Chem. Pharm. Bull.*, **46**, 812-816 (1998).
- 9) Yoshikawa M., Kouno K., Murakami T., Yamahara J., Matsuda H., The 46th Annual Meeting of the Japanese Society of Pharmacognosy, Osaka, Nov. 1999, Abstract Paper, p. 79.
- 10) Oda K., Matsuda H., Murakami T., Katayama S., Ohgitani T., Yoshikawa M., *Biol. Chem.*, **381**, 67-74 (2000).
- 11) Murakami T., Kouno K., Matsuda H., Yoshikawa M., presented at the 43rd Symposium on the Chemistry of Terpenes, Essential Oils, and Aromatics, Oita, Nov. 1999, Abstract Paper, p. 285.
- 12) Ernest W., Hugo E. G., *Phytochemistry*, **16**, 1811-1816 (1977).
- 13) Bruce A. B., Nabel A. M. S., *Can. J. Bot.*, **50**, 2081-2083 (1972).
- 14) Kitagawa I., Taniyama T., Hong W. W., Hori K., Yoshikawa M., *Yakugaku Zasshi*, **108**, 538-546 (1988).
- 15) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **34**, 1843-1845 (1986).
- 16) The ¹H- and ¹³C-NMR spectra were assigned with the aid of homo-

- and hetero-correlation spectroscopy (^1H – ^1H , ^1H – ^{13}C COSY), distortionless Hartmann-Hahn spectroscopy (^1H – ^1H , ^1H – ^{13}C HOHAHA), and heteronuclear multiple bond correlation (HMBC) experiments.
- 17) Tanaka N., Murakami T., Saiki Y., Chen C. M., Iitaka Y., *Chem. Pharm. Bull.*, **29**, 663–666 (1981).
 - 18) Thomas A. W., Edward N. T., *J. Amer. Chem. Soc.* **89**, 3804–3809 (1967).
 - 19) Murakami T., Nakamura J., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1759–1764 (1999).
 - 20) Andersen W. K., Omar A. A., Christensen S. B., *Phytochemistry*, **26**, 291–294 (1987).
 - 21) Arisawa M., Minabe N., Saeki R., Takakuwa T., Nakaoki T., *Yakugaku Zasshi*, **91**, 522–524 (1971).

Chemical Modification of Oleanene Type Triterpenes and Their Inhibitory Activity against HIV-1 Protease Dimerization

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Oleanolic acid derivatives with different lengths of 3-*O*-acidic acyl chains were synthesized and evaluated for their inhibitory activity against HIV-1 protease. The lengths of the acidic chains were optimized to 6 and 8 carbons. Changing a 3-ester bond to an amide bond or dimerization of the triterpenes retained their inhibitory activity against HIV-1 protease. Introduction of an additional acidic chain to C-28 of oleanolic acid increased the inhibitory activity appreciably, though a derivative with only one acidic chain linked at C-28 also showed potent activity against HIV-1 protease.

The inhibitory mechanism was proved directly by size exclusion chromatography to be inhibition of dimerization of the enzyme polypeptides. The ester bonds of the triterpene derivatives were found to be stable to lipase under mild alkaline conditions.

Key words HIV-protease; triterpene; oleanolic acid; sophoradiol; inhibition

Being essential for the maturation of the AIDS virus, HIV protease (PR) has been regarded as one of the most promising targets for the development of anti-AIDS agents. Inhibitors of this enzyme such as saquinavir, zidovudine and didanosine have been used clinically as potent anti-AIDS drugs. However, their usage is limited by the emergence of drug resistant viral strains, severe side effects, drug-drug interactions, moderate bioavailability and short plasma half-life.¹⁾ Most of the drugs are of high cost, require frequent dosing and/or food restriction. A new generation of PR inhibitors with lower price, superior pharmacokinetics, fewer drug interactions, excellent tolerability and convenient dosing schedule are needed. For this purpose, we have conducted a search for HIV-1 PR inhibitors of a new structural-type or new inhibitory mechanism. In the present paper, we report on a series of non-peptide HIV-1 PR inhibitors with a novel mechanism of dimerization inhibition.

We previously reported that ursolic acid, oleanolic acid and betulinic acid as well as their dicarboxylic acid hemiesters showed potent inhibitory activity of HIV PR. Glutaric acid hemiesters of these triterpenes with the longest 3-acyl side chain showed the most potent activity,²⁾ which prompted us to synthesize longer 3-acyl derivatives of triterpenes to improve the activity. The present experiment was designed to find the optimized length of the 3-*O*-acyl chain,

and to investigate the effects of other substituents at position 3 and an additional acidic chain at other positions of the triterpene skeleton on their inhibitory activity against HIV-1 PR. In addition, the inhibitory mechanism and the stability of the triterpene derivatives were also investigated. For economic reasons, oleanolic acid, which is abundant in nature and is cheap in price was used as a starting material for the synthesis of most of the derivatives.

Results and Discussion

Chemistry 3-*O*-Acyl derivatives **11** and **14** were prepared by treatment of oleanolic acid with the corresponding acid anhydride; Compounds **7–10**, **12** and **13** were obtained by heating oleanolic acid and its methyl ester with the appropriate acid chloride followed by treatment with H₂O or, in the case of **12** and **13**, with MeOH.

Referring to the methods used by Sun *et al.* in the synthesis of betulin derivatives,³⁾ oleanolic acid and its methyl ester were oxidized with pyridinium chlorochromate (PCC) to give 3-oxo compounds, **15** and **16**. Treatment of the 3-oxo compounds with NH₂OH in pyridine yielded the hydroxyimino compounds **17** and **18**. Reduction of **18** with TiCl₃ and NaCNBH₃ yielded 3β- and 3α-amino compounds, **19** and **20**.

Treatment of **17** and **18** with adipoyl chloride yielded the

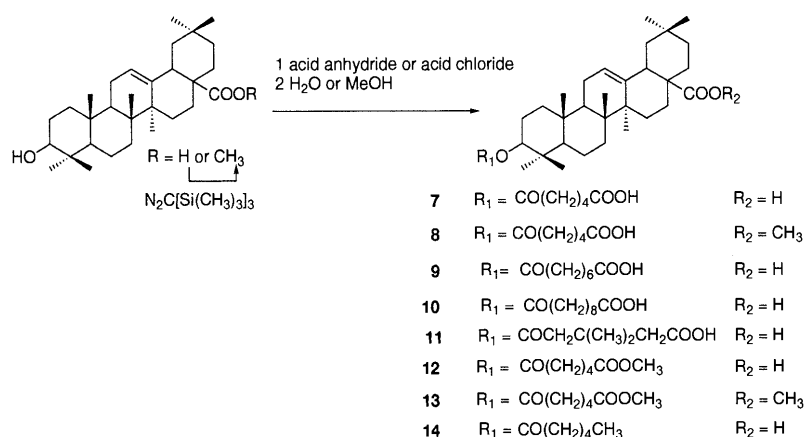


Chart 1. Synthesis of Oleanolic Acid Derivatives with Different Chain Lengths at C-3

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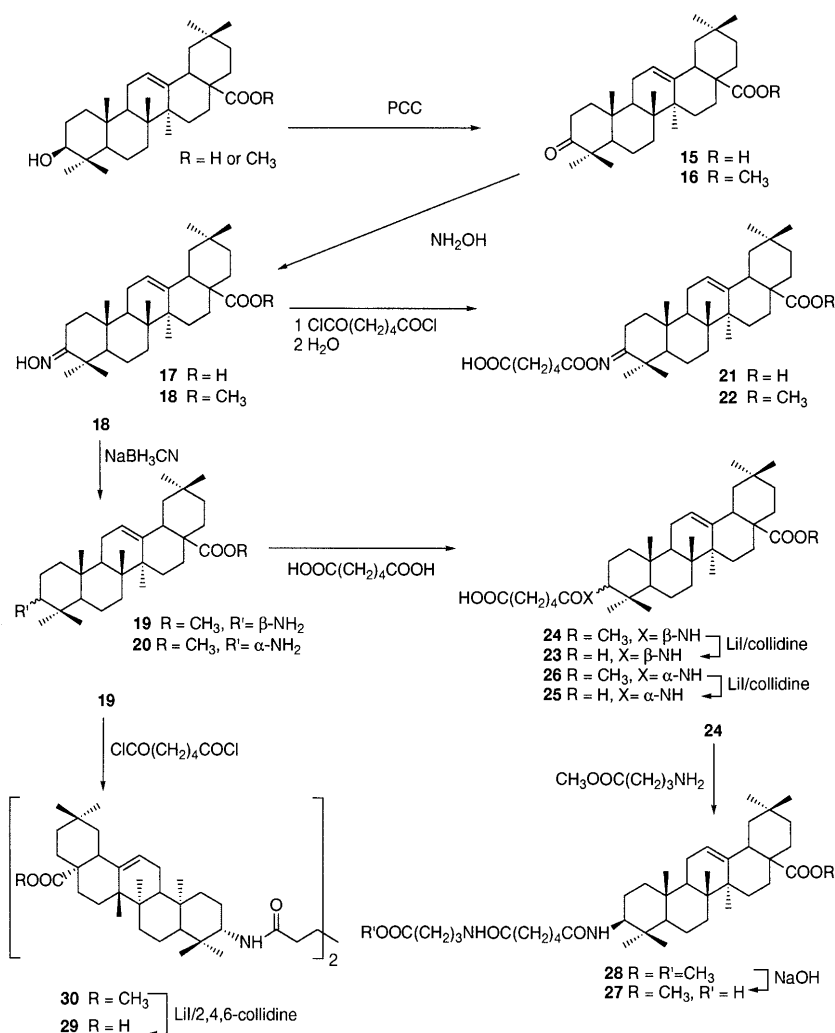


Chart 2. Synthesis of Oleanolic Acid Derivatives with Different Bond and Chain Natures at C-3

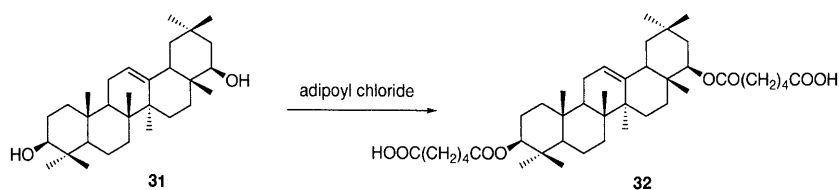


Chart 3. Synthesis of Oleanene Derivative with Two Acidic Chains at C-3 and C-22

respective acyl derivatives, **21** and **22**. Amides **24** and **26** were obtained by condensation of the amino compounds, **19** and **20**, with adipic acid in the presence of *N,N'*-dicyclohexyl carbodiimide (DCC). Hydrolysis of the sterically hindered 28-methyl ester proved to be very difficult. Several methods were tried and finally lithium iodide and 2,4,6-collidine was employed to halogenolyze⁴⁾ **24** and **26** to the corresponding 28-free acid compounds **23** and **25**. One more amide group was introduced to compound **24** to yield **28** by condensation of **24** with 4-amino-*n*-butyric acid methyl ester using a DCC-4-dimethylaminopyridine (DMAP) method. Saponification of **28** with aqueous sodium hydroxide in a methanol/THF mixture yielded compound **27** with a free carboxy group at the 3-acyl chain.

A dimeric compound, **30**, was obtained by treatment of **19** with adipoyl chloride. Halogenolysis of **30** yielded the 28-free acid dimeric compound **29**.

Treatment of sophoradiol (**31**) with adipoyl chloride afforded the diadipoylated compound **32**.

Referring to the method used by Evers *et al.* in the synthesis of betulinic acid derivatives,⁵⁾ oleanolic acid was first converted to its acetyl ester, which was then treated with oxalyl chloride to give acid chloride. Condensation of methyl 6-aminohexanoate with acetyl oleanolic acid chloride followed by saponification yielded the 28-acidic chain compound **33**. Treatment of **33** with adipoyl chloride afforded the 3, 28-diacidic chain compound **34**.

Structure and Activity Relationship The inhibitory activity of the synthesized triterpenes against HIV-1 PR was evaluated by quantitative analysis of the cleavage of a synthetic substrate by HPLC. Tables 1—3 show 50% inhibitory concentrations (IC_{50}) of the triterpene derivatives against HIV-1 PR.

As reported previously,²⁾ of the dicarboxylic acid

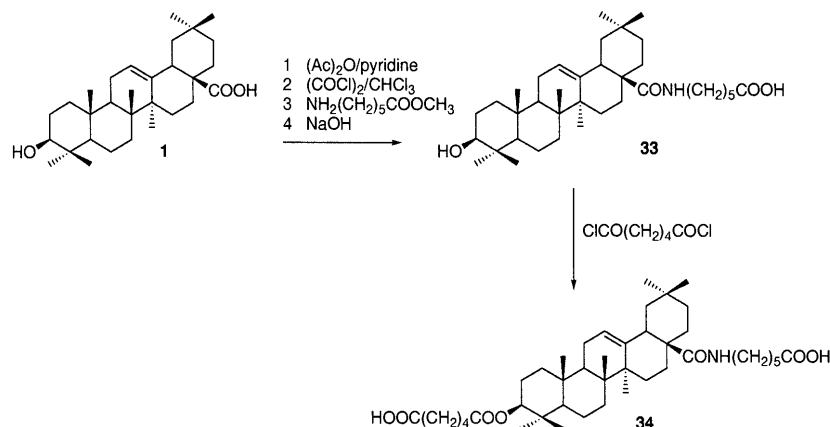


Chart 4. Synthesis of Oleanene Derivatives with Acidic Chain(s) at C-28 as Well as at Both C-3 and C-28

hemiesters of oleanolic acid, when the 3-acyl chains were within five carbons, the HIV-1 PR inhibitory activity increased as the lengths of the 3-acyl chains increased. The activity of glutaryl hemiester of oleanolic acid (IC_{50} 4 μM) was increased 2-fold as compared to oleanolic acid (IC_{50} 8 μM).

Lengthening the 3-acyl chain by one or three methylene units (**7**, **9**) led to increases in inhibitory activity, with an IC_{50} value of 3.0 μM for both compounds. Further extension of the acyl chain by two additional methylene units (**10**) led to slightly decreased inhibitory activity (IC_{50} 4.0 μM). Compounds with two methyl groups substituted at the acyl chain (**11**) showed similar activity as that of compounds with a straight acyl chain (**6**).

Methylation either of the 28- or of the 3-acyl carboxylic acid (**8**, **12**, respectively) decreased the inhibitory activity (IC_{50} 7.5 and 5.6 μM , respectively). Methylation of both carboxylic acid residues in the triterpene derivative led to complete loss of activity (**13**, $IC_{50} > 20$ μM).

Changing a 3-hydroxyl of oleanolic acid to an oxo or hydroxyimino group retained the inhibitory activity (**15**, **17**). Replacing a 3-hydroxyl of 28-methyl oleanolate to an oxo (**16**) or amino group (**19**, **20**) did not ameliorate the poor activity. However, 3-hydroxyimino-olean-12-en-28-oic acid methyl ester (**18**) exhibited two-fold increased activity as compared to methyl oleanolate.

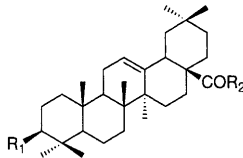
Introduction of an acidic chain to the 3-hydroxyimino and amino compounds significantly increased the inhibitory activity whether the 28-carboxy group was free or methylated (**22–26**, IC_{50} of 2–4 μM) (a 3-acylated hydroxyimino compound with a free carboxy group at position 28 (**21**) is an exception, which exhibited the same activity as that of the non-acylated compound (**17**)).

Introduction of a second amide group into the acyl chain of compound **24** was detrimental to the activity (**27**, IC_{50} = 6.0 μM). Its methylated derivative (**28**), like most of the other compounds without a free carboxy group in their structures, showed no activity at all.

The inhibitory activity of the dimeric compound was similar to that of the monomer. No inhibitory activity was observed in the 28-methylated dimer (**30**) without free carboxy groups in its structure. The dimeric compound (**29**) with two free carboxy groups in its structure showed inhibitory potency comparable to that of the monomer compound (**23**).

The above results indicated clearly that oleanolic acid with

Table 1. Anti-HIV-1 Protease Activity of Oleanolic Acid Derivatives with Various Chain Lengths at C-3



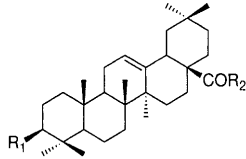
R_1	$R_2 = -OH$		$R_2 = -OCH_3$	
	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
$-OH$	1 ^{a)}	8	2 ^{a)}	20
$-OCOCOOH$	3 ^{a)}	20		
$-OCOCH_2COOH$	4 ^{a)}	8		
$-OCO(CH_2)_2COOH$	5 ^{a)}	4		
$-OCO(CH_2)_3COOH$	6 ^{a)}	4		
$-OCO(CH_2)_4COOH$	7	3.0	8	7.5
$-OCO(CH_2)_6COOH$	9	3.0		
$-OCO(CH_2)_8COOH$	10	4.0		
$-OCOCH_2C(CH_3)_2CH_2COOH$	11	3.8		
$-OCO(CH_2)_4COOCH_3$	12	5.6	13	>20
$-OCO(CH_2)_4CH_3$	14	>20		

a) Reported previously.²⁾

an acidic chain of six carbons linked at its position 3 exhibited potent inhibitory activity against HIV-1 PR. Consequently, we considered introducing an additional acidic chain to the oleanene skeleton. Sophoradiol (**31**, an aglycone of kaikasaponin III⁶⁾) is an oleanene type triterpene with two hydroxy groups at positions 3 and 22. The distance between these two hydroxy groups (12.321 Å) is quite similar to that of the 3-OH and 17-COOH in the structure of oleanolic acid (12.409 Å), as calculated with Chem 3D models. Acylation of **31** with adipoyl chloride yielded 3, 22-di-*O*-adipoyl-sophoradiol (**32**) which showed more than 8 fold more activity than the parent triterpene (**32** IC_{50} 2.3 vs. **31** IC_{50} 18.8 μM).

Encouraged by the positive result of the diadipoylated derivative (**32**), we tried to synthesize another oleanolic acid derivative, **34**, with two acidic chains linking at both positions 3 and 28. As expected, this compound demonstrated more potent inhibitory activity against HIV-1 PR, with an IC_{50} value of 1.7 μM . Compound **33**, with only one acidic

Table 2. Anti-HIV-1 Protease Activity of Oleanolic Acid Derivatives with Different Bond and Chain Natures at C-3



R ₁	R ₂ = -OH		R ₂ = -OCH ₃	
	Compound	IC ₅₀ (μM)	Compound	IC ₅₀ (μM)
β-OH	1 ^{a)}	8	2	20
=O	15	5.5	16	20
=NOH	17	5.5	18	9.5
β-NH			19	>20
α-NH			20	>20
=NOCO(CH ₂) ₄ COOH	21	5.5	22	4.0
β-NHCO(CH ₂) ₄ COOH	23	3.0	24	3.0
α-NHCO(CH ₂) ₄ COOH	25	2.1	26	3.5
β-NHCO(CH ₂) ₄ CONH(CH ₂) ₃ COOH			27	6.0
β-NHCO(CH ₂) ₄ CONH(CH ₂) ₃ COOCH ₃			28	>20
β-NHCO(CH ₂) ₄ CONH-β-oleanolic acid 28-R ₂	29	3.3	30	>20

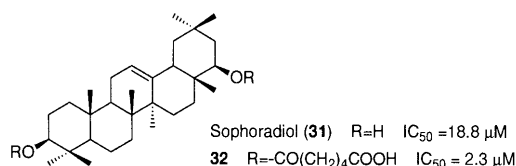
a) Reported previously.²⁾

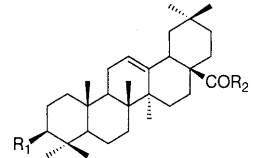
Fig. 1. Anti-HIV-1 Protease Activity of Oleanene Type Triterpenes with Hydroxyls or Acidic Chains at C-3 and C-22

chain linked at position **28**, was also obtained and tested for its activity. This **33** showed an inhibitory activity as potent as that of **34**, more than 4 times the potency of the parent triterpene, oleanolic acid. This can be explained by the highly steric hindrance of the 28-carboxylic acid in oleanolic acid hampering the hydrogen bond formation of oleanolic acid with enzyme, though the exact amino acid interacting is not clear at present. A 28-long acidic chain in compound **33** greatly reduced the steric hindrance, thus enabling this compound to form a hydrogen bond with an enzyme stronger than that of oleanolic acid.

A computer modeling study has predicted that esterification of the 3-OH of triterpene with dicarboxylic acids could improve their binding with HIV-1 PR.⁷⁾ The present results confirmed this presumption and showed that an acidic chain at position 28 could enhance the interaction with HIV-1 PR even more strongly than that at position 3 (**33** IC₅₀ 1.7 vs. **7** IC₅₀ 3.0 μM).

The above results demonstrated clearly that introduction of an acidic chain(s), especially that of six carbons, to the oleanane skeleton could increase the anti-HIV-1 PR activity significantly. To test the stability of the ester bond which links the acidic chain and the triterpene skeleton, two compounds, **7** and **14**, were incubated with lipase in a weak alkaline condition. No hydrolyzed products were detected in either compound even after 18 h incubation. Because the ester bond is known to be more stable under an acidic condition, these triterpene derivatives could be stable in the digestive

Table 3. Anti-HIV-1 Protease Activity of Oleanolic Acid Derivatives with Acidic Chain(s) at Either C-3 or C -28, as Well as at Both C-3 and C-28



	R ₁	R ₂	IC ₅₀ (μM)
1 ^{a)}	-OH	-OH	8
7	-OCO(CH ₂) ₄ COOH	-OH	3.0
33	-OH	-NH(CH ₂) ₅ COOH	1.7
34	-OCO(CH ₂) ₄ COOH	-NH(CH ₂) ₅ COOH	1.7

a) Reported previously.²⁾

system.

HIV-1 PR is known to be composed of two identical monomers, which assemble by noncovalent interactions to form the composite active site. This structural peculiarity has provided the possibility of a special inhibitory mechanism, *i.e.* a dimerization inhibition mechanism. A dimerization inhibitor of HIV-1 PR could dissociate the enzyme into the inactive monomer form and thus inhibit the enzyme's activity. Ursolic acid has been analyzed by kinetic study as a dimerization inhibitor of HIV-1 PR.⁷⁾ In the present experiment, the dissociation of HIV-1 PR by triterpene compound was monitored directly by size exclusion chromatography. According to an earlier study by Zutshi *et al.*,⁸⁾ the molecular weight of HIV-1 PR dimer was about 11000 by size exclusion of a Bachem HIV-1 PR of affinity purified grade. In the present study, two main proteins at retention times of 29.4 and 39.0 min were assigned to HIV-1 PR dimer and monomer, respectively, by interpolation of a standard protein curve (Fig. 2). After incubation with **7**, the peak of the HIV-1 PR monomer was dominant and that of the dimer had disappeared completely. On the other hand, after treatment with an

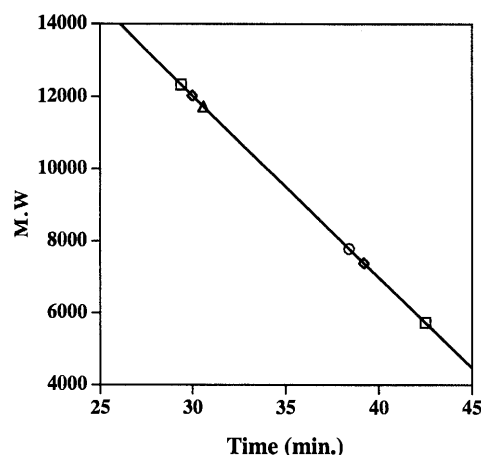


Fig. 2. Size Exclusion Chromatography of HIV-1 Protease (◇), and HIV-1 Protease with Compound 7 (○) and Acetyl Pepstatin (△)

A standard curve was generated using cytochrome C and insulin (□).

active site inhibitor, acetyl pepstatin, the dimer was dominant and the monomer had disappeared. The present experiment clearly demonstrated that the triterpene compound could dissociate the dimeric polypeptides of HIV-1 PR into a monomeric one, *i.e.* triterpene inhibited the activity of HIV-1 PR through the mechanism of dimerization inhibition.

The scaffold of triterpene compounds matches in its volume that of the backbone of a cyclic hexapeptide, and a computer docking study has revealed that some triterpenes could fit well into the hydrophobic interface site of the relaxed HIV-1 PR monomers.⁷⁾ Therefore, it is not surprising that structure modification of triterpene compounds could lead to HIV-1 PR inhibitors with the same inhibitory mechanism and similar inhibitory potency as some peptide compounds. Because the triterpene skeleton is more rigid and stable than peptide, it is expected that triterpene derivatives might be more specific to HIV-1 PR and have better pharmacokinetic properties.

In conclusion, we have presented here a number of triterpene derivatives with potent HIV-1 PR inhibitory activity based on a novel mechanism of dimerization inhibition. The potency of most of the derivatives synthesized in the present experiment was comparable to that of peptide compounds reported recently based on the same inhibition mechanism.^{8,9)} The most potent oleanolic acid derivatives (**33**, **34**) showed more than 4-fold increased activity against HIV-1 PR as compared to oleanolic acid (**33**, **34** IC_{50} 1.7, 1.7 μM vs. **1** IC_{50} 8 μM). A derivative of sophoradiol showed more than 8-fold more activity than its parent triterpene (**32** IC_{50} 2.3 μM vs. **31** IC_{50} 18.8 μM).

Experimental

General Experimental Procedures Optical rotations were measured with a Jasco DIP-360 automatic polarimeter. IR spectra were measured with a Jasco FT-IR-230 infrared spectrometer. ^1H - and ^{13}C -NMR spectra were measured with either a Varian Gemini 300 (^1H , 300 MHz; ^{13}C , 75 MHz), Varian Unity 500 (^1H , 500 MHz; ^{13}C , 125 MHz) or Jeol JNA-LA 400WB-FT (^1H , 400 MHz; ^{13}C , 100 MHz) NMR spectrometer, with chemical shifts being represented in ppm and TMS used as an internal standard. EIMS were measured with a Jeol JMS-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV. Electrospray ionization (ESI) MS was measured with a Perkin-Elmer SCIEX API-III biomolecular mass analyzer. Preparative HPLC was carried out on a Gilson instrument with a 231LX injector, a 119 UV/VIS detector and a TSK gel ODS-80T_M column (21.5×300 mm, Tosoh Co.).

HIV-1 PR assay kit was purchased from Bachem Bioscience. Sephadex G-50 (Particle sized: 20–80 μm) was purchased from Pharmacia Fine Chemicals AB Uppsala, Sweden. Cytochrome C (from bovine heart), insulin (from bovine pancreas), and lipase (type II, crude, from porcine pancreas) were purchased from Sigma Chemical Co.

General Procedure for Synthesis of Dicarboxylic Acid Esters (7–10, 12, 13) A solution of oleanolic acid (or methyl oleanolate for **8** and **13**, 0.10 mmol), and appropriate acid dichloride (10 equiv. mol) in anhydrous THF (50 ml) was heated for 3–4 h at 70 °C. The reaction mixture was poured into 100 ml of H₂O (or MeOH for **12** and **13**) and allowed to stand for 2 h. The mixture was extracted three times with 150 ml of CHCl₃. The CHCl₃ layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The residue was chromatographed on RP-18 and then a silica gel column to afford the respective dicarboxylic acid esters.

3-O-Adipoyloleanolic Acid (7) 50% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with MeOH/CHCl₃ (0–5%)], a white powder; $[\alpha]_D^{25} +58.5^\circ$ ($c=0.07$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.72 (3H, s), 0.80 (6H, s), 0.85 (3H, s), 0.88 (6H, s), 1.09 (3H, s) (7×CH₃), 1.58 (overlapped signals, H-3', 4'), 2.28 (4H, t-like, H-2', 5'), 2.77 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 4.44 (1H, t, $J=8.0$ Hz, H-3), 5.21 (1H, t-like, H-12); *Anal.* Calcd for C₃₆H₅₈O₆·H₂O: C, 71.72; H, 9.70. Found: C, 72.16; H, 9.23; MS (positive-ESI): 607 [M+Na]⁺ (50), 585 (M+1)⁺ (60); (negative-ESI): 583 (M–1)[–] (100), 421 (50).

3-O-Adipoyloleanolic Acid Methyl Ester (8) 55% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with acetone/hexane (5–20%)], a white powder; $[\alpha]_D^{25} +55.9^\circ$ ($c=0.56$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.72 (3H, s), 0.85 (6H, s), 0.90 (3H, s), 0.93 (6H, s), 1.13 (3H, s) (7×CH₃), 1.63 (overlapped signals, H-3', 4'), 2.36 (4H, t-like, H-2', 5'), 2.87 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 3.62 (3H, s, CH₃-28), 4.50 (1H, t, $J=7.8$ Hz, H-3), 5.28 (1H, t-like, H-12); *Anal.* Calcd for C₃₇H₅₈O₆: C, 74.21; H, 9.76. Found: C, 74.18; H, 9.75; MS (EI): 598 [M]⁺, 552, 538, 452, 262, 203 (base).

3-O-Suberoyloleanolic Acid (9) 45% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with MeOH/CHCl₃ (0–5%)], a white powder; $[\alpha]_D^{25} +59.6^\circ$ ($c=0.13$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.74 (3H, s), 0.86 (6H, s), 0.90 (3H, s), 0.93 (3H, s), 0.94 (3H, s), 1.13 (3H, s) (7×CH₃), 1.36 (overlapped signals, H-4', 5'), 1.61 (overlapped signals, H-3', 6'), 2.30 (2H, t, $J=7.1$ Hz, H-2'), 2.36 (2H, t, $J=7.1$ Hz, H-7'), 2.81 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 4.50 (1H, t, $J=8.0$ Hz, H-3), 5.27 (1H, t-like, H-12); *Anal.* Calcd for C₃₈H₆₀O₆·1.2H₂O: C, 71.93; H, 9.91. Found: C, 71.64; H, 9.45; MS (positive-ESI): 635 [M+Na]⁺ (75), 613 [M+1]⁺ (20), 439 (80); (negative-ESI): 611 [M–1][–] (100), 421 (30).

3-O-Sebacoyloleanolic Acid (10) 55% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with MeOH/CHCl₃ (0–5%)], a white powder; $[\alpha]_D^{25} +56.2^\circ$ ($c=0.79$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.75 (3H, s), 0.85 (3H, s), 0.86 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 0.94 (3H, s), 1.13 (3H, s) (7×CH₃), 1.31 (8H, s, H-4', 5', 6', 7'), 1.60 (overlapped signals, H-3', 8'), 2.29 (2H, t, $J=7.5$ Hz, H-2'), 2.34 (2H, t, $J=7.1$ Hz, H-9'), 2.81 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 4.50 (1H, t, $J=8.0$ Hz, H-3), 5.27 (1H, t-like, H-12); *Anal.* Calcd for C₄₀H₆₄O₆·H₂O: C, 72.91; H, 10.10. Found: C, 72.53; H, 9.76; MS (positive-ESI): 663 [M+Na]⁺ (80); (negative-ESI): 639 [M–1][–] (100), 421 (50).

3-O-(6'-O-Methyladipoyl)oleanolic Acid (12) 55% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with acetone/hexane (5–20%)], a white powder; $[\alpha]_D^{25} +60.8^\circ$ ($c=0.17$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.75 (3H, s), 0.83 (3H, s), 0.85 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 0.94 (3H, s), 1.13 (3H, s) (7×CH₃), 1.64 (overlapped signals, H-3', 4'), 2.32 (4H, q-like, H-2', 5'), 2.82 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 3.67 (3H, s, CH₃-6'), 4.50 (1H, t, $J=7.8$ Hz, H-3), 5.27 (1H, t-like, H-12); *Anal.* Calcd for C₃₇H₅₈O₆·0.5H₂O: C, 73.11; H, 9.78. Found: C, 72.87; H, 9.53; MS (EI): 598 [M]⁺, 552, 438, 351, 248 (base), 203.

3-O-(6'-O-Methyladipoyl)oleanolic Acid Methyl Ester (13) 60% yield [after chromatography on RP-18 with MeOH/H₂O (80–100%) and then on silica gel with acetone/*n*-hexane (5–20%)], a white powder; $[\alpha]_D^{25} +60.1^\circ$ ($c=0.84$, CHCl₃); ^1H -NMR (CDCl₃) δ 0.72 (3H, s), 0.85 (6H, s), 0.90 (3H, s), 0.93 (6H, s), 1.13 (3H, s) (7×CH₃), 1.64 (overlapped signals, H-3', 4'), 2.32 (4H, q-like, H-2', 5'), 2.85 (1H, dd, $J=13.5$, 3.4 Hz, H-18), 3.62 (3H, s, CH₃-28), 3.67 (3H, s, CH₃-6'), 4.49 (1H, t, $J=8.0$ Hz, H-3), 5.28 (1H, t-like, H-12); MS (EI): 612 [M]⁺, 552, 452, 349, 262 (base), 203 (base).

3-O-(3',3'-Dimethylglutaryl)oleanolic Acid (11) A solution of oleanolic acid (200 mg, 0.44 mmol), 4-dimethylaminopyridine (1000 mg, 8.19 mol; 200 mg, 1.64 mmol), and 3',3'-dimethylglutaryl anhydride (1000 mg, 7.04 mmol; 220 mg, 1.55 mmol) in anhydrous pyridine (10 ml) was refluxed for

36 h. The reaction mixture was concentrated under reduced pressure to dryness. The residue was chromatographed on an RP-18 column eluted with MeOH–H₂O to afford **11** as a white powder (150 mg, 57.1% yield): $[\alpha]_D^{25} +52.4^\circ$ ($c=0.97$, CHCl₃); ¹H-NMR (CDCl₃, 300 MHz) δ 0.72 (3H, s), 0.81 (3H, s), 0.87 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 0.95 (3H, s), 1.08 (3H, s), 1.11 (3H, s), 1.15 (3H, s) (9×CH₃), 2.28 (1H, d, $J=12.9$), 2.36 (1H, d, $J=15.9$ Hz), 2.54 (1H, d, $J=15.9$), 2.75 (1H, d, $J=12.9$ Hz) (H-2', 4'), 2.80 (1H, br d, $J=13.2$ Hz, H-18), 4.51 (1H, dd, $J=5.7$, 9.9 Hz, H-3), 5.27 (1H, t-like, H-12); *Anal.* Calcd for C₃₇H₅₈O₆·0.5H₂O: C, 73.11; H, 9.78. Found: C, 73.06; H, 9.63; MS (negative-ESI): 597 [M–1][–] (100), 455 (30).

3-O-Hexanoyloleonic Acid (14) A mixture of oleonic acid (30 mg, 0.066 mmol) and hexanoic anhydride (0.5 ml, 2.2 mmol) in 1 ml of pyridine was stirred overnight at room temperature. The mixture was concentrated to dryness and chromatographed on an RP-18 column eluted with 80–100% MeOH to afford **14** (11 mg, 30.2% in yield) as a white powder; ¹H-NMR (CDCl₃, 400 MHz) δ 0.81 (3H, s), 0.86 (3H, s), 0.90 (3H, s), 0.90 (3H, t, $J=7.5$ Hz), 0.93 (3H, s), 0.94 (3H, s), 1.13 (3H, s) (8×CH₃), 2.29 (2H, t, $J=7.5$ Hz, H-2'), 2.81 (1H, dd, $J=13.5$, 3.4 Hz, H-18), 4.50 (1H, t, $J=7.8$ Hz, H-3), 5.27 (1H, t-like, H-12).

3-Oxo-olean-12-en-28-oic Acid (15) To a solution of oleonic acid (1781 mg, 3.9 mmol) in acetone–CH₂Cl₂ (10 ml) was added PCC (2527 mg, 11.7 mmol). After stirring for 6 h at room temperature, the mixture was diluted with H₂O and extracted with CHCl₃. The CHCl₃ layer was concentrated to dryness and then subjected to silica gel column chromatography eluted with *n*-hexane–acetone (95:5) to give **15** as a colorless crystalline powder: (1016 mg, 57.3% in yield); $[\alpha]_D^{25} +73.6^\circ$ ($c=0.26$, CHCl₃); ¹H-NMR (CDCl₃, 400 MHz) δ 0.81 (3H, s), 0.91 (3H, s), 0.93 (3H, s), 1.03 (3H, s), 1.05 (3H, s), 1.08 (3H, s), 1.15 (3H, s) (7×CH₃), 2.39 (1H, ddd, $J=3.7$, 6.8, 16.0 Hz, H_a-2), 2.55 (1H, ddd, $J=7.6$, 11.2, 16.0 Hz, H_b-2), 2.84 (1H, dd, $J=13.9$, 3.9 Hz, H-18), 5.30 (1H, t, $J=3.4$ Hz, H-12).

3-Oxo-olean-12-en-28-oic Acid Methyl Ester (16) To a solution of methyl oleonolate (480 mg, 1 mmol) in CH₂Cl₂ (5 ml) was added PCC (320 mg, 1.5 mmol). After stirring for 1.5 h at room temperature, the mixture was diluted with Et₂O and filtered through a short Florisil column. The column was then washed with 300 ml of Et₂O. The combined Et₂O eluates were concentrated under vacuum to give **16** as a colorless crystalline powder: 99% yield; $[\alpha]_D^{25} +98.2^\circ$ ($c=0.10$, CHCl₃); ¹H-NMR (CDCl₃) δ 0.78 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 1.04 (6H, s), 1.09 (3H, s), 1.14 (3H, s) (7×CH₃), 2.36 (1H, ddd, $J=3.6$, 6.9, 15.4 Hz, H_a-2), 2.55 (1H, ddd, $J=7.1$, 11.3, 15.4 Hz, H_b-2), 2.87 (1H, dd, $J=13.7$, 3.5 Hz, H-18), 3.63 (3H, s, COOCH₃), 5.30 (1H, t-like, H-12); *Anal.* Calcd for C₃₁H₄₈O₃: C, 79.44; H, 10.32. Found: C, 79.92; H, 10.16; MS (EI): 468 [M]⁺, 408, 262, 203 (base).

3-Hydroxyimino-olean-12-en-28-oic Acid (17) A solution of **15** (841 mg, 1.9 mmol) and hydroxylamine hydrochloride (346 mg, 4.9 mmol) in pyridine (2 ml) was heated for 4 h at 50 °C. After cooling to room temperature, the reaction mixture was concentrated under vacuum to dryness and then purified with RP-18 (MeOH, 90–100%) to yield a white powder (445 mg, 49.7% yield): $[\alpha]_D^{25} +283.8^\circ$ ($c=0.10$, CHCl₃); ¹H-NMR (CDCl₃, 300 MHz) δ 0.82 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 1.02 (3H, s), 1.05 (3H, s), 1.13 (3H, s), 1.14 (3H, s) (7×CH₃), 2.17 (1H, ddd, $J=6.0$, 12.6, 15.3 Hz, H-2_a), 2.85 (1H, dd, $J=13.2$, 3.9 Hz, H-18), 3.05 (1H, ddd, $J=3.3$, 5.1, 15.3 Hz, H-2_b), 5.29 (1H, t-like, H-12); *Anal.* Calcd for C₃₀H₄₄O₃N·0.1H₂O: C, 76.42; H, 10.09; N, 2.97. Found: C, 76.17; H, 10.01; N, 2.84.

3-Hydroxyimino-olean-12-en-28-oic Acid Methyl Ester (18) A solution of **16** (450 mg, 0.96 mmol) and hydroxylamine hydrochloride (300 mg, 4.3 mmol) in pyridine (5 ml) was heated for 2 h at 50 °C. After cooling to room temperature, the reaction mixture was diluted with CH₂Cl₂ (75 ml) and washed with 10% HCl (3×50 ml). The CH₂Cl₂ layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The product was crystallized from diethyl ether and CH₂Cl₂ to yield a white powder (441 mg, 95%): $[\alpha]_D^{25} +26.2^\circ$ ($c=0.53$, CHCl₃); ¹H-NMR (CDCl₃) δ 0.76 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 1.03 (3H, s), 1.06 (3H, s), 1.11 (3H, s), 1.16 (3H, s) (7×CH₃), 2.15 (1H, ddd, $J=5.8$, 12.8, 15.4 Hz, H_a-2), 2.86 (1H, dd, $J=13.7$, 4.3 Hz, H-18), 3.08 (1H, ddd, $J=3.6$, 5.1, 15.4 Hz, H_b-2), 3.63 (3H, s, CH₃O-28), 5.29 (1H, t, $J=3.5$ Hz, H-12); *Anal.* Calcd for C₃₁H₄₉O₃N·0.2H₂O: C, 76.40; H, 10.22; N, 2.87. Found: C, 76.43; H, 10.11; N, 2.76; MS (EI): 483 [M]⁺, 262, 203 (base).

3β-Amino-olean-12-en-28-oic Acid Methyl Ester (19) and 3α-Amino-olean-12-en-28-oic Acid Methyl Ester (20) Sodium cyanoborohydride (0.58 g, 18 mmol) was added to a methanol solution of **18** (400 mg, 0.83 mmol) and ammonium acetate (0.74 g, 13.6 mmol) under argon atmosphere. The solution was chilled in ice water, and 15% aqueous titanium trichloride (2.4 ml, 2.8 mmol) was added dropwise over 20 min. The mixture was stirred at room temperature for 12 h and then adjusted with 2 N sodium hydroxide to

pH=10. The aqueous solution was extracted with CH₂Cl₂ (300 ml×2), and the organic layer was washed with distilled water and concentrated to dryness. The crude products were subjected to neutral Al₂O₃ chromatography, and eluted with MeOH/CHCl₃ (0–20%) to give **19** (310 mg) and **20** (60 mg).

Compound 19: A white powder, 79.8% yield; $[\alpha]_D^{25} +52.8^\circ$ ($c=1.00$, CHCl₃); ¹H-NMR (CDCl₃) δ 0.72 (3H, s), 0.73 (3H, s), 0.88 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 0.94 (3H, s), 1.13 (3H, s) (7×CH₃), 2.32 (1H, dd, $J=11.3$, 4.4 Hz, H-3), 2.85 (1H, dd, $J=13.2$, 4.1 Hz, H-18), 3.62 (3H, s, CH₃O-28), 5.28 (1H, t-like, H-12); *Anal.* Calcd for C₃₁H₅₁O₂N·H₂O: C, 76.34; H, 10.95; N, 2.98. Found: C, 76.12; H, 10.31; N, 2.78. MS (EI): 469 [M]⁺ (base), 454, 413, 262, 203 (base).

Compound 20: A white powder, 15.4% yield; $[\alpha]_D^{25} +58.1^\circ$ ($c=0.55$, CHCl₃); ¹H-NMR (CDCl₃) δ 0.72 (3H, s), 0.87 (3H, s), 0.89 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 0.93 (3H, s), 1.15 (3H, s) (7×CH₃), 2.60 (1H, brs, H-3), 2.85 (1H, dd, $J=13.8$, 4.1 Hz, H-18), 3.62 (3H, s, CH₃O-28), 5.28 (1H, t, $J=3.6$ Hz, H-12); *Anal.* Calcd for C₃₁H₅₁O₂N: C, 79.26; H, 10.94; N, 2.98. Found: C, 79.34; H, 10.83; N, 2.72. MS (EI): 469 [M]⁺ (base), 454, 413, 262, 203.

3-(N-Adipoylimino)olean-12-en-28-oic Acid (21) A solution of **17** (20 mg, 0.04 mmol) and adipoyl dichloride (100 μl, 0.55 mmol) in anhydrous THF (10 ml) was heated for 0.5 h at 60 °C. The reaction mixture was poured into 100 ml of H₂O, allowed to stand for 1 h and then concentrated under reduced pressure. The residue was chromatographed on RP-18 (H₂O–MeOH) and then silica gel (*n*-hexane–acetone 9:1–7:3) to afford a white powder (9.0 mg); $[\alpha]_D^{25} +41.3^\circ$ ($c=0.26$, CHCl₃); ¹H-NMR (CDCl₃, CD₃OD) δ 0.83 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 1.03 (3H, s), 1.14 (6H, s), 1.25 (3H, s) (7×CH₃), 1.58 (overlapped signals, H-3', 4'), 2.32 (2H, t, $J=6.6$ Hz, H-2'), 2.47 (2H, t, $J=6.6$ Hz, H-5'), 2.37 (1H, overlapped signals, H-2_a), 2.85 (1H, dd, $J=13.5$, 4.1 Hz, H-18), 2.91 (1H, dt, $J=3.3$, 15.3 Hz, H-2_b), 5.29 (1H, t-like, H-12); MS (negative-ESI): 596 (M–1)[–].

3-(N-Adipoylimino)olean-12-en-28-oic Acid Methyl Ester (22) A solution of **18** (18 mg, 0.04 mmol), adipic acid (54 mg, 0.37 mmol) and DCC (20 mg, 0.10 mmol) in anhydrous THF (2 ml) was stirred at room temperature for 3 h. The reaction mixture was concentrated to dryness and purified by ODS column chromatography, then eluted with MeOH–H₂O (1:1–100:0) to afford a white powder (22.0 mg, 97.3% yield); $[\alpha]_D^{25} +26.1^\circ$ ($c=0.50$, CHCl₃); ¹H-NMR (CDCl₃) δ 0.76 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 1.02 (3H, s), 1.13 (6H, s), 1.26 (3H, s) (7×CH₃), 2.39 (2H, t, $J=6.6$ Hz, H-2'), 2.46 (2H, t, $J=6.6$ Hz, H-5'), 2.30 (1H, m, H-2_a), 2.88 (1H, dd, $J=14.5$, 4.8 Hz, H-18), 2.91 (1H, dt, $J=3.3$, 15.3 Hz, H-2_b), 3.63 (3H, s, CH₃O-28), 5.29 (1H, t-like, H-12); MS (negative-ESI): 610 [M–1][–].

3β-(N-Adipoylamino)olean-12-en-28-oic Acid Methyl Ester (24): A solution of **19** (50 mg, 0.11 mmol), adipic acid (73 mg, 0.50 mmol) and DCC (30 mg, 0.15 mmol) in THF (10 ml) was stirred at room temperature for 4 h. The mixture was concentrated to dryness under vacuum, chromatographed on RP-18, then eluted with MeOH/H₂O (6:4–1:0) to obtain compound **24** as a white powder (54 mg, 84.5% in yield); $[\alpha]_D^{25} +45.6^\circ$ ($c=1.2$, CHCl₃); ¹H-NMR (CDCl₃, 500 MHz) δ 0.72 (3H, s), 0.77 (3H, s), 0.87 (3H, s), 0.89 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 1.13 (3H, s) (7×CH₃), 1.68 (overlapped signals, H-3', 4'), 2.22 (2H, t, $J=6.7$ Hz, H-2'), 2.37 (2H, t, $J=6.5$ Hz, H-5'), 2.85 (1H, dd, $J=14.0$, 4.5 Hz, H-18), 3.62 (3H, s, COOCH₃), 3.67 (1H, ddd, $J=4.5$, 9.2, 12.5 Hz, H-3), 5.28 (1H, t-like, H-12), 5.46 (1H, d, $J=9.2$ Hz, NH); *Anal.* Calcd for C₃₇H₅₉O₃N·H₂O: C, 72.16; H, 9.98; N, 2.34. Found: C, 72.63; H, 9.37; N, 2.09. MS (EI): 597 (M)⁺, 538, 336, 262, 203 (base).

3β-(N-Adipoylamino)olean-12-en-28-oic Acid (23) and 3α-(N-Adipoylamino)olean-12-en-28-oic Acid (25) Compound **24** or **26** (25 mg, 0.04 mmol) in 2.0 ml of freshly distilled collidine was heated under reflux in an atmosphere of argon with dehydride lithium iodide⁽¹⁰⁾ (50 mg) for 8 h. The reaction mixture was cooled, poured into water (5 ml), acidified to pH 4 with dilute hydrochloric acid, and extracted with ether. The ether layer was washed with 5% Na₂S₂O₃, dried over Na₂SO₄ and evaporated to give a solid, which was purified by SiO₂ column chromatography, eluted with CHCl₃–MeOH (100:0–90:10) and RP-18 column chromatography with MeOH–H₂O (50–80%) to give **23** (8 mg, yield 31.0%) and **25** (7 mg, yield 30.0%) as white powder.

Compound 23: ¹H-NMR δ 0.77 (3H, s), 0.78 (3H, s), 0.87 (3H, s), 0.90 (6H, s), 0.93 (3H, s), 1.15 (3H, s) (7×CH₃), 1.65 (overlapped signals, H-3', 4'), 2.21 (2H, t-like, H-2'), 2.33 (2H, t-like, H-5'), 2.83 (1H, dd, $J=14.0$, 4.5 Hz, H-18), 3.63 (1H, m, H-3), 5.27 (1H, t-like, H-12), 5.87 (1H, d, $J=8.1$ Hz, NH); *Anal.* Calcd for C₃₆H₅₇O₃N·H₂O: C, 71.84; H, 9.88; N, 2.33. Found: C, 71.36; H, 9.36; N, 2.25. MS (positive-ESI): 606 [M+Na]⁺ (90), 584 [M+1]⁺ (75); (negative-ESI): 582 [M–1][–] (100), 421 (45).

Compound **25**: $[\alpha]_D^{25} + 28.5^\circ$ ($c=0.41$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3 - CD_3OD 8:1) δ 0.79 (3H, s), 0.84 (3H, s), 0.91 (3H, s), 0.94 (3H, s), 0.95 (6H, s), 1.19 (3H, s) ($7\times\text{CH}_3$), 2.24 (2H, t, $J=6.8$ Hz, H-2'), 2.34 (2H, t, $J=6.8$ Hz, H-5'), 2.82 (1H, br d, $J=10.5$ Hz, H-18), 3.81 (1H, br s, H-3), 5.28 (1H, t-like, H-12); MS (negative-ESI): 582 $[\text{M}-1]^-$ (100), 421 (20).

3 α -(N-Adipoylamino)olean-12-en-28-oic Acid Methyl Ester (26) A solution of **20** (32 mg, 0.07 mmol), adipic acid (37 mg, 0.25 mmol) and DCC (30 mg, 0.15 mmol) in THF (5 ml) was stirred at room temperature for 3 h. The mixture was concentrated to dryness under vacuum and chromatographed on RP-18 with $\text{MeOH-H}_2\text{O}$ (6:4—1:0) to obtain compound **26** as a white powder (27 mg, 66.3% yield). $[\alpha]_D^{25} + 32.0^\circ$ ($c=0.40$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ 0.73 (3H, s), 0.84 (3H, s), 0.90 (3H, s), 0.93 (3H, s), 0.95 (6H, s), 1.18 (3H, s) ($7\times\text{CH}_3$), 1.68 (overlapped signals, H-3', 4'), 2.25 (2H, t, $J=6.8$ Hz, H-2'), 2.38 (2H, t, $J=6.2$ Hz, H-5'), 2.87 (1H, dd, $J=13.6$, 4.1 Hz, H-18), 3.61 (3H, s, COOCH_3), 3.84 (1H, br d, $J=9.5$, H-3), 5.29 (1H, t, $J=3.6$ Hz, H-12), 5.79 (1H, d, $J=9.5$ Hz, NH); *Anal.* Calcd for $\text{C}_{37}\text{H}_{59}\text{O}_5\text{N}$: C, 74.33; H, 9.95; N, 2.34. Found: C, 74.17; H, 9.91; N, 2.25. MS (EI): 597 $[\text{M}]^+$, 538, 336, 262, 203 (base).

3 β -[N-(3'-Carboxypropylamino)adipo-6'-yl]amino-olean-12-en-28-oic Acid Methyl Ester (27) To a solution of **28** (7.6 mg, 0.01 mmol) in a 1:1.5 mixture of CH_3OH and THF (2.5 ml) was added aqueous NaOH (4 N, 0.6 ml). After stirring for 5 h and concentrating in vacuo, the residue was suspended in distilled water. The suspension was acidified with 4 N HCl to pH 3. The mixture was passed through a PR-18 column, washed with H_2O and then MeOH to give compound **27** as a white powder: yield 67.3% (5.0 mg), $[\alpha]_D^{25} + 49.4^\circ$ ($c=0.11$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) δ 0.72 (3H, s), 0.78 (3H, s), 0.87 (3H, s), 0.89 (3H, s), 0.90 (3H, s), 0.92 (3H, s), 1.13 (3H, s) ($7\times\text{CH}_3$), 1.66 (overlapped signals, H-3', 4'), 1.87 (overlapped signals, H-2''), 2.25 (4H, br s, H-2', 3''), 2.41 (2H, t, $J=6.4$ Hz, H-5'), 2.87 (1H, dd, $J=14.1$, 4.1 Hz, H-18), 3.37 (2H, br s, H-1''), 3.62 (3H, s, COOCH_3), 3.62 (overlapped signal, H-3), 5.28 (1H, t, $J=3.2$ Hz, H-12), 5.66 (1H, d, $J=9.5$ Hz, NH), 6.48 (1H, t-like, NH); MS (negative-ESI): 681 $[\text{M}-1]^+$.

3 β -[N-(3'-Methoxycarbonylpropylamino)adipo-6'-yl]amino-olean-12-en-28-oic Acid Methyl Ester (28) A solution of **24** (43 mg, 0.072 mmol), 4-aminobutyric acid methyl ester hydrochloride (76.5 mg, 0.50 mmol), 4-dimethylamino-pyridine (13 mg, 0.09 mmol) and DCC (30 mg, 0.15 mmol) in THF (10 ml) was heated for 3 h at 70°C . The reaction mixture was cooled and acidified to pH 3 with diluted hydrochloric acid. After being concentrated to dryness, the mixture was chromatographed on RP-18 with $\text{MeOH-H}_2\text{O}$ (6:4—1:0) to obtain compound **28** as a white powder: yield 63.9% (32 mg), $[\alpha]_D^{25} + 43.6^\circ$ ($c=1.00$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3) δ 0.72 (3H, s), 0.77 (3H, s), 0.87 (3H, s), 0.90 (6H, s), 0.92 (3H, s), 1.13 (3H, s) ($7\times\text{CH}_3$), 1.66 (overlapped signals, H-3', 4'), 1.83 (overlapped signals, H-2''), 2.20 (4H, br s, H-2', 3''), 2.37 (2H, t, $J=7.0$ Hz, H-5'), 2.85 (1H, dd, $J=13.6$, 3.4 Hz, H-18), 3.29 (2H, dt, $J=6.7$, 6.0 Hz, H-1''), 3.62 (3H, s, $\text{CH}_3\text{O}-28$), 3.68 (3H, s, $\text{CH}_3\text{O}-4'$), 3.68 (overlapped signal, H-3), 5.28 (1H, t-like, H-12), 5.45 (1H, d, $J=9.9$ Hz, NH), 6.06 (1H, t-like, NH); MS (EI): 696 $[\text{M}]^+$ (40), 435 (95), 203 (base).

N¹,N⁶-Bis(28-carboxyolean-12-en-3-yl) Adipic Acid Diamide (29) Under an atmosphere of argon, compound **30** (10 mg, 0.01 mmol) in freshly distilled collidine (0.5 ml) was heated with dehydride lithium iodide (16 mg) under reflux for 9 h. The reaction mixture was cooled, poured into water (5 ml), acidified to pH 4 with dilute hydrochloric acid, and extracted with ether. The ether layer was extracted three times with 5% $\text{Na}_2\text{S}_2\text{O}_3$, dried over Na_2SO_4 and evaporated to give a solid, which was purified by a SiO_2 column with CHCl_3 -MeOH (98:2) to give **29** (3 mg) as a white powder, 30.0% yield. $^1\text{H-NMR}$ (CDCl_3 - CD_3OD 2:1): δ 0.77 (6H, s), 0.79 (6H, s), 0.87 (6H, s), 0.90 (12H, s), 0.93 (6H, s), 1.15 (6H, s) ($14\times\text{CH}_3$), 1.61 (overlapped signals, H-3', 4'), 2.20 (4H, t-like, H-2', 5'), 2.83 (2H, dd, $J=13.2$, 4.1 Hz, H-18, 18''), 3.66 (2H, overlapped with H_2O signal, H-3, 3''), 5.27 (2H, t-like, H-12, 12''); MS (negative-ESI): 1019 $[\text{M}-1]^-$ (3).

N¹,N⁶-Bis(28-methoxycarbonylolean-12-en-3-yl) Adipic Acid Diamide (30) Adipoyl chloride (18.3 μl , 0.1 mmol) was added to a solution of compound **19** (40 mg, 0.085 mmol) in THF (5 ml). The mixture was stirred at room temperature for 1.5 h and then partitioned with H_2O and CHCl_3 . The CHCl_3 layer was concentrated to dryness and subjected to neutral Al_2O_3 chromatography, with MeOH/CHCl_3 (0—10%) to give **30** (12 mg) as a white powder, 26.9% in yield. $[\alpha]_D^{25} + 45.9^\circ$ ($c=0.6$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3): δ 0.72 (6H, s), 0.76 (6H, s), 0.87 (6H, s), 0.89 (12H, s), 0.92 (6H, s), 1.13 (6H, s) ($14\times\text{CH}_3$), 1.66 (overlapped signals, H-3', 4'), 2.20 (4H, t-like, H-2', 5'), 2.85 (2H, dd, $J=13.2$, 4.1 Hz, H-18, 18''), 3.62 (6H, s, OCH_3-28 , 28''), 3.66 (2H, dt, $J=9.9$, 4.8 Hz, H-3, 3''), 5.28 (2H, t-like, H-12, 12''), 5.39 (2H, d, $J=9.9$ Hz, NH); *Anal.* Calcd for $\text{C}_{68}\text{H}_{108}\text{O}_6\text{N}_2\cdot 2\text{H}_2\text{O}$: C, 75.23; H, 10.40; N,

2.58. Found: C, 75.58; H, 10.15; N, 2.47. MS (positive-ESI): 1049 $[\text{M}+1]^+$ (10).

Olean-12-ene-3 β ,22 β -diol (Sophoradiol, 31) 3-O-[α -L-Rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-galactopyranosyl-(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-sophoradiol (kaikasaponin III, 150 mg)⁶ was refluxed with 7% HCl in H_2O -EtOH (1:1, 50 ml) for 4 h. The reaction mixture was partitioned between CHCl_3 and H_2O . The CHCl_3 layer was concentrated to dryness. The residue was chromatographed on an ODS column (H_2O -MeOH 1:1—0:1), and purified with HPLC (ODS column, 80—100% MeOH), and SiO_2 preparative TLC (benzene-EtOAc 9:1) to give sophoradiol (**31**, 15 mg, 20.9% yield). $[\alpha]_D^{24} + 77.5^\circ$ ($c=0.51$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3): δ 0.79 (3H, s), 0.88 (3H, s), 0.91 (3H, s), 0.95 (3H, s), 0.98 (3H, s), 1.00 (3H, s), 1.04 (3H, s), 1.12 (3H, s) ($8\times\text{CH}_3$), 2.10 (1H, br d, $J=11.2$ Hz, H-18), 3.22 (1H, dd, $J=5.1$, 11.0 Hz, H-3), 3.44 (1H, t, $J=5.1$ Hz, H-22), 5.25 (1H, t, $J=3.6$ Hz, H-12); MS (EI): 442 $[\text{M}]^+$ (15), 424 (15), 406 (8), 234 (100).

3,22-Di-O-adipoylsophoradiol (32) Compound **31** was treated with adipoyl chloride as described for **7** to yield **32** (5 mg, 45.3%). $[\alpha]_D^{24} + 57.8^\circ$ ($c=0.29$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3): δ 0.81 (3H, s), 0.87 (6H, s), 0.89 (3H, s), 0.97 (6H, s), 0.98 (3H, s), 1.15 (3H, s) ($8\times\text{CH}_3$), 2.19 (1H, br d, $J=10.5$ Hz, H-18), 2.35 (8H, m, H-2', 5', 2'', 5''), 4.51 (1H, t, $J=7.8$ Hz, H-3), 4.66 (1H, t-like, H-22), 5.26 (1H, t-like, H-12); MS (positive-ESI): 721 $[\text{M}+\text{Na}]^+$ (100); (negative-ESI): 697 $[\text{M}-1]^-$ (100), 587 (10), 569 (30).

N-[3 β -Hydroxyolean-12-en-28-oyl]-6-aminohexanoic Acid (33) To a solution of 3-O-acetyloleanolic acid²¹ (400 mg, 0.80 mmol) in CHCl_3 was added oxalyl chloride (0.3 ml, 3.42 mmol). The reaction mixture was stirred at room temperature for 20 h and the solvent was removed. Cyclohexane (20 ml) was added to the residue, and the mixture was evaporated to dryness. This was repeated twice to yield crude 3 β -acetoxyolean-12-en-28-oyl chloride.

To a suspension of 6-aminohexanoic acid (500 mg) in 20 ml of MeOH-benzene (5:1) was added 4 ml of 10% trimethylsilyldiazomethane (TMS-CHN_3) in hexane. The mixture was stirred at room temperature for 2 h, concentrated to dryness and partitioned with H_2O and CHCl_3 . The CHCl_3 layer was concentrated to dryness to yield methyl 6-aminohexanoate (150 mg).

A CH_2Cl_2 solution (50 ml) of the above prepared methyl 6-aminohexanoate (150 mg, 1.03 mmol) and crude 3 β -acetoxyolean-12-en-28-oyl chloride was stirred in the presence of triethylamine (0.3 ml, 2.15 mmol) at room temperature overnight. The reaction mixture was concentrated under vacuum and partitioned with H_2O and CHCl_3 . The CHCl_3 layer was concentrated to dryness and chromatographed over ODS with H_2O -MeOH. The MeOH eluate was further purified by SiO_2 column chromatography with hexane-AcOEt (9:1—8:1) to give methyl N-[3 β -acetoxyolean-12-en-28-oyl]-6-aminohexanoate as white powder (150 mg, 30.0% in yield). $[\alpha]_D^{25} + 37.4^\circ$ ($c=0.28$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3): δ 0.76 (3H, s), 0.86 (3H, s), 0.87 (3H, s), 0.91 (6H, s), 0.94 (3H, s), 1.17 (3H, s) ($7\times\text{CH}_3$), 2.05 (3H, s, CH_3CO), 2.31 (2H, t, $J=7.2$ Hz, H-5'), 2.50 (1H, br d, $J=12.6$ Hz, H-18), 2.99 (1H, m, H_A-1'), 3.36 (1H, m, H_B-1'), 3.67 (3H, s, 6'- OCH_3), 4.49 (1H, t, $J=7.8$ Hz, H-3), 5.37 (1H, t-like, H-12), 5.93 (1H, t-like, NH); MS (positive-ESI): 648 $[(\text{M}+\text{Na})^+]$, 90, 626 $[(\text{M}+1)^+]$, 100.

A solution of methyl N-[3 β -acetoxyolean-12-en-28-oyl]-6-aminohexanoate (130 mg, 0.21 mmol) and aqueous NaOH (4 N, 1.2 ml) in CH_3OH -THF (1:1.5, 5 ml) was stirred for 18 h at room temperature and then concentrated in vacuo. The residue was suspended in water, treated with 4 N HCl to pH 3 and extracted with CHCl_3 . The CHCl_3 layer was washed with H_2O to pH 6 and concentrated to dryness to give **33** (118 mg, 99.0% in yield). $[\alpha]_D^{23} + 47.2^\circ$ ($c=1.38$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3) δ 0.76 (3H, s), 0.79 (3H, s), 0.90 (9H, s), 0.99 (3H, s), 1.16 (3H, s) ($7\times\text{CH}_3$), 2.35 (2H, t, $J=7.5$ Hz, H-5'), 2.48 (1H, br d, $J=10.5$ Hz, H-18), 3.00 (1H, m, H_A-1'), 3.23 (1H, dd, $J=5.1$, 10.2 Hz, H-3), 3.36 (1H, m, H_B-1'), 5.38 (1H, t-like, H-12), 5.99 (1H, t-like, NH); MS (positive-ESI): 592 $[(\text{M}+\text{Na})^+]$, 100, 570 $[\text{M}+\text{H}]^+$ (100); *Anal.* Calcd for $\text{C}_{36}\text{H}_{59}\text{O}_4\text{N}\cdot 2\text{H}_2\text{O}$: C, 71.36; H, 10.48; N, 2.31. Found: C, 71.30; H, 9.78; N, 2.26. (negative-ESI): 568 $[\text{M}-\text{H}]^-$ (100).

N-[3 β -O-Adipoylolean-12-en-28-oyl]-6-aminohexanoic Acid (34) Compound **33** (50 mg, 0.09 mmol) was treated with adipoyl chloride as described for **7** to yield **34** (22 mg, 35.9%). $[\alpha]_D^{25} + 39.9^\circ$ ($c=0.69$, CHCl_3); $^1\text{H-NMR}$ (CDCl_3) δ 0.76 (3H, s), 0.86 (6H, s), 0.90 (6H, s), 0.94 (3H, s), 1.16 (3H, s) ($7\times\text{CH}_3$), 2.35 (6H, t, $J=7.2$ Hz, H-2', 5', 5''), 2.48 (1H, br d, $J=10.5$ Hz, H-18), 3.01 (1H, m, H_A-1'), 3.35 (1H, m, H_B-1'), 4.50 (1H, t, $J=7.8$ Hz, H-3), 5.38 (1H, t-like, H-12), 6.00 (1H, t-like, NH); MS (positive-ESI): 698 $[\text{M}+\text{H}]^+$ (100), 552 (100); (negative-ESI): 696 $[\text{M}-\text{H}]^-$ (100).

HIV-1 Protease Assay The same HIV-1 PR assay kit and method were used as described previously.²¹

Lipase Assay Four tubes, each containing 0.9 ml of a lipase-buffer solution (0.4 mg/ml, pH 7.7 at 37°C) and 75 μl of 1.2 mM compound **7** or **14**

(DMSO) were incubated at 37 °C for 1, 5, and 18 h, respectively. The mixture was then extracted with CHCl_3 (3×6 ml). The CHCl_3 layer was washed with 1 ml H_2O and then concentrated to dryness. The residues were co-chromatographed on silica gel TLC (CHCl_3 -acetone 20:1) with compounds **7** and **14**, and oleanolic acid. All the samples from 1, 5, and 18 h of incubation displayed only one spot, corresponding to the starting materials, **7** or **14**. Even a trace of the hydrolyzed product, oleanolic acid, could not be detected in any reaction mixture.

Size Exclusion Chromatography The chromatography was done at 4 °C using a column of Sephadex G-50, equilibrated with a buffer containing 20 mM phosphate, 1 mM DTT, and 1 mM EDTA at pH 5.5. A flow rate of 0.52 ml/min was maintained, and the eluant was monitored with a fluorospectrometer (for insulin at 302 nm, $\lambda_{\text{ex}} = 226$ nm; for PR at 365 nm, $\lambda_{\text{ex}} = 226$ nm) or a UV/VIS spectrometer for cytochrome C (413 nm). A standard curve was generated using insulin (M.W. 5733) and cytochrome C (M.W. 12327). Fifty microliters of 0.07 mg/ml HIV PR solution was loaded on the column, and the molecular weight of the eluent peaks was calculated from the standard curve. Similarly, 50 μl of 0.07 mg/ml HIV PR solution was preincubated with either 1 μl of a 5 mM solution of **7** (DMSO) or 1 μl of a 0.15 mM solution of acetylpepstatin (DMSO) for 2 h at room temperature (20 °C). This solution was loaded on the column and the molecular weight of the eluting species calculated.

References and Notes

- 1) Molla A., Granneman G. R., Sun E., Kempf D. J., *Antiviral Res.*, **39**, 1—23 (1998).
- 2) Ma C. M., Nakamura N., Miyashiro H., Hattori M., Shimotohno K., *Chem. Pharm. Bull.*, **47**, 141—145 (1999).
- 3) Sun I. C., Wang H. K., Kashiwada Y., Shen J. K., Cosentino L. M., Chen C. H., Yang L. M., Lee K. H., *J. Med. Chem.*, **41**, 4648—4657 (1998).
- 4) Elsinger F., Schreiber J., Eschenmoser A., *Hel. Chim. Acta*, **43**, 113—118 (1960).
- 5) Evers M., Poujade C., Soler F., Ribeill Y., James C., Lelievre Y., Gueguen J. C., Reisdorf D., Morize I., Pauwels R., Clercq E. D., Hénin Y., Bousseau A., Mayaux J. F., Pecq J. B. L., Dereu N., *J. Med. Chem.*, **39**, 1056—1068 (1996).
- 6) Ma C. M., Nakamura N., Hattori M., *Chem. Pharm. Bull.*, **46**, 982—987 (1998).
- 7) Quéré L., Wenger T., Schramm H. J., *Biochem. Biophys. Res. Comm.*, **227**, 484—488 (1996).
- 8) Zutshi R., Franciskovich J., Shultz M., Schweitzer B., Bishop P., Wilson M., Chmielewski J., *J. Am. Chem. Soc.*, **119**, 4841—4845 (1997).
- 9) Bouras A., Boggetto N., Benatalah Z., Rosny E. D., Sicsic S., Reboud-Ravaux M., *J. Med. Chem.*, **42**, 957—962 (1999).
- 10) Dehydride LiI was prepared from commercial $\text{LiI} \cdot 3\text{H}_2\text{O}$ by drying at 170 °C *in vacuo* in the presence of P_2O_5 for 4 h.

A Novel Class of Inhibitors for Human and Rat Steroid 5 α -Reductases: Synthesis and Biological Evaluation of Indoline and Aniline Derivatives. III^{1,2)}

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While searching for novel nonsteroidal inhibitors of human and rat prostatic 5 α -reductases, we found a new series of indoline and aniline derivatives that showed potent inhibitory activities for both enzymes. Among them, 3-chloro-4-[[1-(4-phenoxybenzyl)indolin-5-yl]oxy]benzoic acid (**2e**, YM-36117) showed a more potent inhibitory activity for the human enzyme than ONO-3805 with an IC₅₀ value of 5.3 nM and a reduced rat prostatic dihydrotestosterone (DHT) concentration by oral administration. The synthesis and the structure-activity relationships of these indoline and aniline derivatives are presented.

Key words 5 α -reductase inhibitor; benign prostatic hypertrophy; YM-36117

5 α -Reductase is an NADPH-dependent enzyme responsible for the conversion of testosterone to (DHT) which is an active agonist for androgen receptor. Benign prostatic hyperplasia (BPH), skin disorders such as acne, male pattern baldness and hirsutism are androgen-related disorders, associated with elevated levels of DHT.⁶⁾ As a consequence, a 5 α -reductase inhibitor is expected to provide a potential treatment for androgen-related disorders. Several 5 α -reductase inhibitors have been reported,^{7,8)} including both steroidal inhibitors, finasteride^{7a)} and epristeride,^{7b)} and a nonsteroidal inhibitor, ONO-3805^{8a)} (Chart 1). Following the discovery of these inhibitors, the existence of two different 5 α -reductase isozymes, called type 1 and type 2 5 α -reductases, have been reported in humans and rats.^{9–11)} The type 1 enzyme is normal in men with congenital 5 α -reductase deficiency and is expressed in skin tissue throughout the body that has an optimal pH of between 6 and 9. The type 2 enzyme is defective in men with congenital 5 α -reductase deficiency and is the dominant form of the enzyme in genital tissue, including

prostate that has an optimal pH of about 5.5. However, the physiological role of these isozymes has yet to be fully elucidated. Steroidal inhibitors, for example, finasteride, have been shown to be clinically effective for the treatment of BPH, however, the possibility of adverse effects due to their steroidal structures remains.¹²⁾ On the other hand, nonsteroidal inhibitors have not shown clinical efficacy for the treatment of BPH. Finasteride showed strong inhibitory activity (IC₅₀=4.1 nM)¹³⁾ for human prostatic 5 α -reductase, whereas ONO-3805 showed moderate activity (IC₅₀=538 nM)¹³⁾ which might not be sufficient to show clinical efficacy. We considered that a more potent nonsteroidal inhibitor of human prostatic 5 α -reductase than ONO-3805 could show clinical efficacy for the treatment of BPH and reduce the potential of side effects compared to steroidal inhibitors.

In our previous studies on novel human prostatic 5 α -reductase inhibitors, we found the 4-(1*H*-indol-5-yl)oxy benzoic acid derivative, YM-32906 (**1**), which showed potent inhibitory activity with an IC₅₀ value of 0.44 nM.²⁾ However,

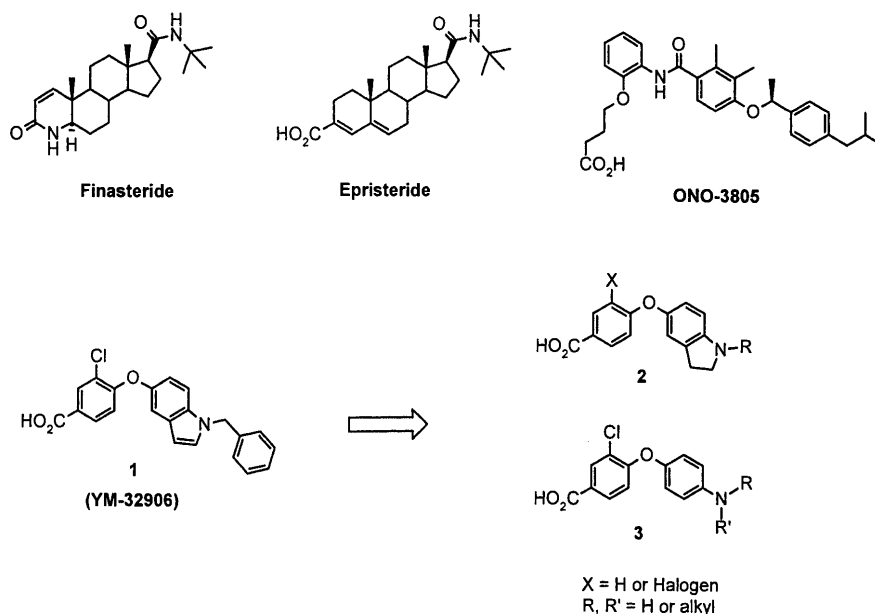


Chart 1

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these indole derivatives showed reduced inhibitory activity for rat prostatic 5α -reductase and were not sufficient to show inhibitory activity in a rat *in vivo* model.¹⁴ In this study, our attention focused on discovering novel benzoic acid derivatives having potent inhibitory activities for both human and rat prostatic 5α -reductases by the modification of the 1*H*-indol-5-yloxy group of **1**. The indolin-5-yloxy group and 4-aminophenoxy group as the substituent at the 4-position of the benzoic acid moiety were newly designed as described in Chart 1. We now report the synthesis and the structure-activity relationships of these benzoic acid derivatives **2** and **3**.

Chemistry The indoline derivatives (**2a**—**2j**) were prepared as described in Charts 2 and 3. Compound **4** was treated with diethyl pyrocarbonate and 4-dimethylaminopyridine (DMAP) to give compound **5**. The reduction of the indole ring and the debenzoylation were carried out by catalytic hydrogenation in the presence of Pd-C to afford compound **6**, which was alkylated with 3-chloro-4-fluorobenzonitrile in the presence of K_2CO_3 in *N,N*-dimethylformamide (DMF) and hydrolyzed with KOH to give the benzoic acid derivative **8**. The esterification of **8** was performed using H_2SO_4 in EtOH to afford compound **9**, which was alkylated with benzyl bromide or (1-bromoethyl)benzene and subsequent hydrolysis with NaOH to give compounds **2a** and **2b**. Compound **2c** was obtained by reductive alkylation of **8** in the presence of $NaBH(OAc)_3$ with 4-isopropylbenzaldehyde. Compound **11**, prepared using the same procedure for the preparation of **6** with di-*tert*-butyl pyrocarbonate, was alkylated with 4-fluorobenzonitrile derivatives followed by the

deprotection with CF_3CO_2H to give compound **12**. Compounds **2d**—**2j** were obtained by the *N*-alkylation of **12** with 2-(bromomethyl)naphthalene or aldehydes and subsequent hydrolysis with KOH.

The aniline derivatives (**3a**—**3h**) were prepared as described in Charts 4 and 5. The *O*-alkylation of the aminophenol derivatives **13** with 3-chloro-4-fluorobenzonitrile afforded **14**, which was converted to the benzoic acids **3a**, **3b** and **3h** in one or two steps as described in Chart 4. Compounds **3c**—**3g** were prepared as described in Chart 5. Compound **15** was alkylated with 1-fluoro-4-nitrobenzene followed by a catalytic hydrogenation in the presence of Raney-Ni to give **16**. Compound **3e** was obtained by the acylation with benzoyl chloride of **16** and subsequent hydrolysis with KOH. Compound **16** was alkylated with 2 eq of benzyl bromide followed by hydrolysis with NaOH to afford **3f**. The reductive alkylation of **16** was performed using aldehydes in the presence of $NaBH(OAc)_3$ to give **17**, which was methylated with formaldehyde in the presence of formic acid to afford **18**. Compounds **17** and **18** were converted to the benzoic acids **3c**, **3d** and **3g** by hydrolysis with NaOH.

Results and Discussion

The inhibitory activities of the indoline and aniline derivatives were evaluated on the basis of their ability to inhibit 5α -reductases in human prostate at pH 5.5 and rat prostate at pH 6.5, which are optimal for the activities of the type 2 enzymes. These results are described in Tables 1 and 2.

In our previous paper, we found that the indole derivative,

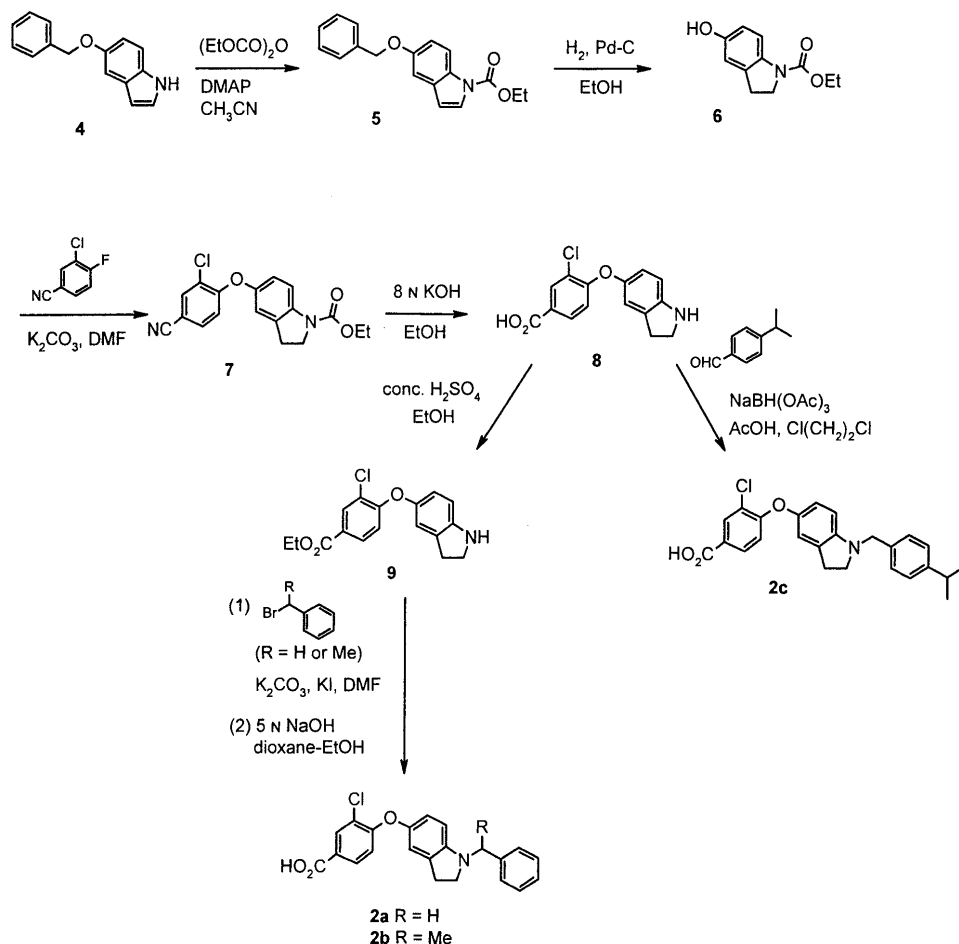


Chart 2

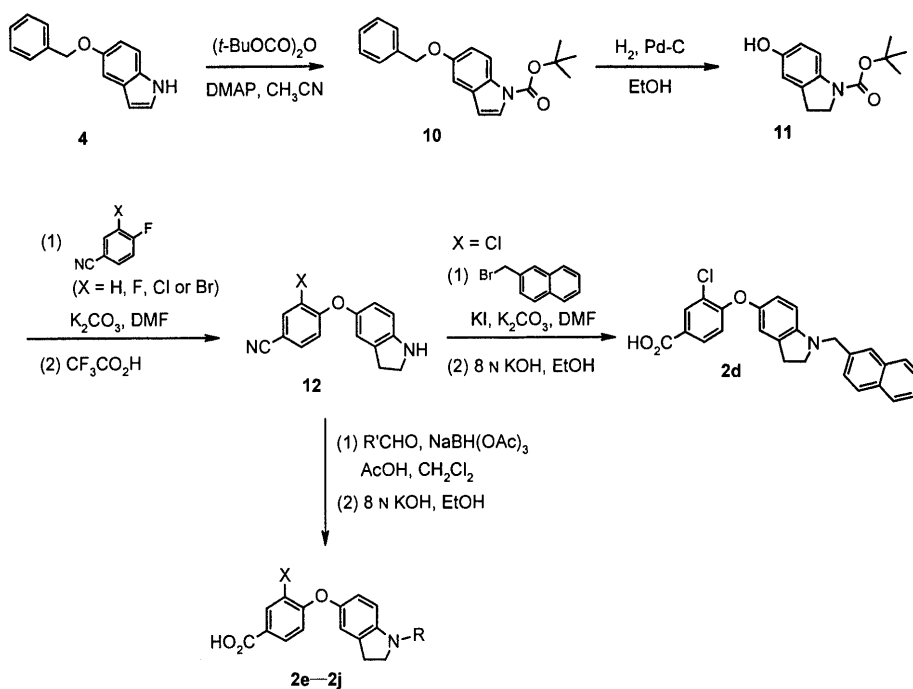


Chart 3

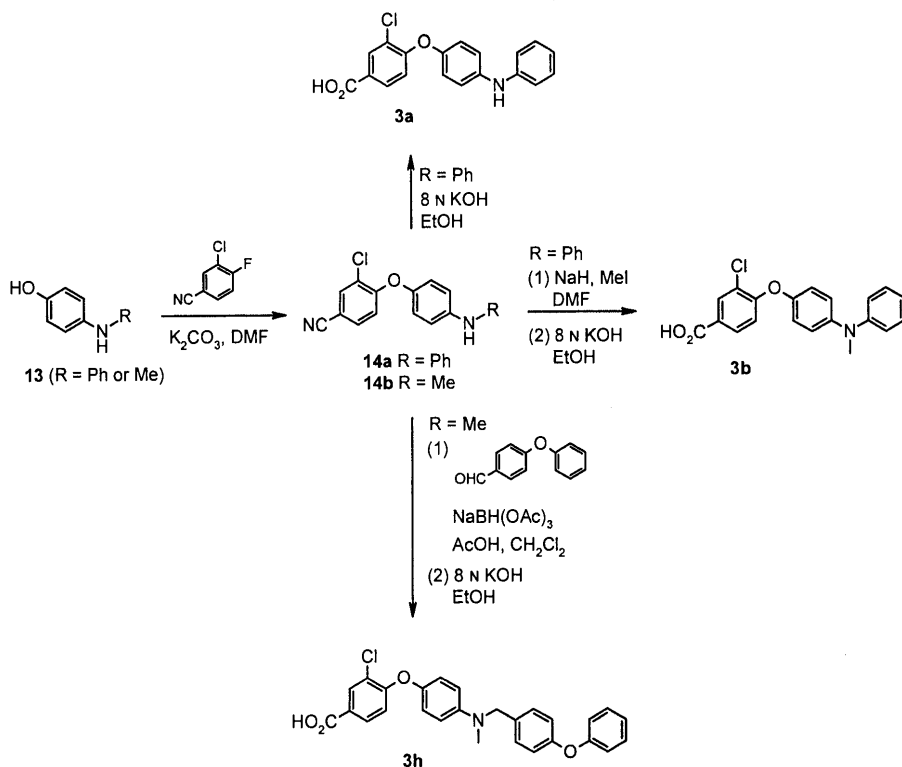


Chart 4

having a hydrophobic substituent such as the 4-phenoxybenzyl group at the 1-position of the indole ring, showed moderate inhibitory activity against the rat enzyme with an IC_{50} value of 73 nM.²⁾ However, this indole derivative was not sufficient to show inhibitory activity in a rat *in vivo* model. Initially, the effects of substituents at the 1-position of the indole ring were investigated as shown in Table 1. The benzyl derivative **2a**, the 1-phenylethyl derivative **2b**, the 4-isopropylbenzyl derivative **2c** and the naphthalen-2-ylmethyl

derivative **2d** showed potent inhibitory activities for the human enzyme with IC_{50} values below 10 nM but no inhibitory activities for the rat enzyme at 300 nM. On the other hand, the 4-phenoxybenzyl derivative **2e** retained its high potency for the human enzyme and showed moderate inhibitory activity for the rat enzyme with an IC_{50} value of 46 nM. Furthermore, the 4-benzyloxybenzyl derivative **2f** and the 4-(4-isopropylphenoxy)benzyl derivative **2g** resulted in a loss in activity for the rat enzyme, suggesting that the 4-phenoxy-

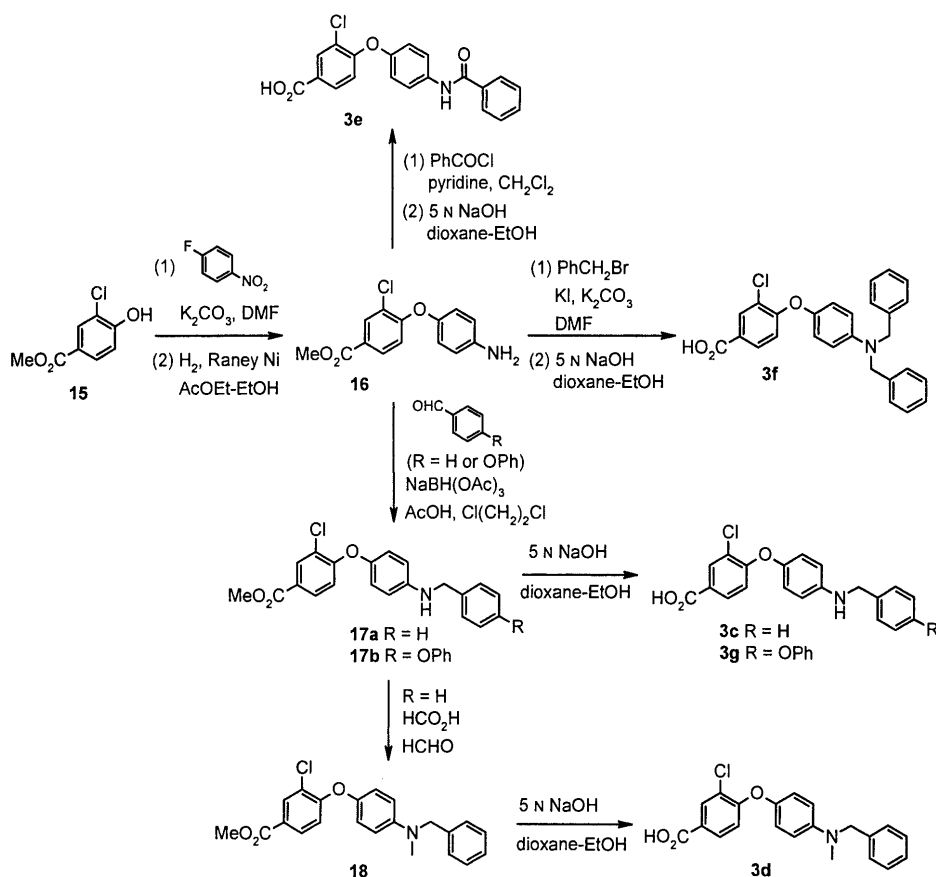


Chart 5

benzyl group plays an important role for showing potent inhibitory activity against the rat enzyme. Next, modification of the chloro group of compound **2e** was investigated. Compound **2h**, which has no substituent at the position, showed reduced activity, while the fluoro derivative **2i** and the bromo derivative **2j** retained potency for the rat enzyme. These findings suggested that a hydrophobic substituent at the 3-position of the benzoic acid moiety such as a halogen is favorable for the inhibitory activity against the rat enzyme.

Concerning the inhibitory activities of the aniline derivatives, the 4-anilinophenoxy derivatives (**3a**, **3b**) and 4-benzylaminophenoxy derivatives (**3c**, **3d**, **3f**) were selective human prostatic 5 α -reductase inhibitors as shown in Table 2. The *N*-methylated compounds **3b** and **3d** showed more potent inhibitory activity compared to the parent compounds **3a** and **3c**, while the 4-benzoylaminophenoxy derivative **3e** showed reduced potency and the 4-dibenzylaminophenoxy derivative **3f** resulted in a slight loss of potency compared to compound **3c**. These results suggested that a proton of the amino group is unfavorable and a hydrophobic and bulky substituent such as the dibenzylamino group is tolerant for the display of potent inhibitory activity against the human enzyme. On the other hand, the 4-[*N*-(4-phenoxybenzyl)amino]phenoxy derivatives (**3g**, **3h**) resulted in potent inhibitory activities for both the human and rat enzymes. Compound **3g** showed the most potent inhibitory activity against the rat enzyme in these aniline derivatives with an IC_{50} value of 5.5 nM. These results suggested that the 4-phenoxybenzyl group of these aniline derivatives is also effective for providing potent inhibitory activity against the rat enzyme as shown by the indo-

line derivatives.

In order to investigate *in vivo* inhibitory activity of these indoline and aniline derivatives, compounds **2e**, **2j**, **3g**, and **3h**, which showed potent inhibitory activities for the rat enzyme, were evaluated for their inhibitory activity of the DHT concentration in rat prostate by oral administration. These results are described in Table 3. In this model, ($\pm$)-ONO-3805 showed significant *in vivo* inhibitory activities at 10 mg/kg. The aniline derivative **3g**, having a potent *in vitro* inhibitory activity for the rat enzyme, did not improve the *in vivo* inhibitory activity compared to ($\pm$)-ONO-3805. On the other hand, the indoline derivative **2e**, having 18 times less potent *in vitro* inhibitory activity for the rat enzyme than ($\pm$)-ONO-3805, reduced prostatic DHT by 44% at a dose of 100 mg/kg. Since compound **2e** showed 100 times more potent *in vitro* inhibitory activity for the human enzyme compared to ($\pm$)-ONO-3805, there is a possibility to show clinical efficacy for the treatment of BPH.

Conclusions

In order to prepare a novel potent inhibitor against both human and rat prostatic 5 α -reductases, we modified the 1*H*-indol-5-yloxy group of YM-32906 to indolin-5-yloxy group or 4-aminophenoxy group. Consequently, we have identified a new series of indoline and aniline derivatives having a 4-phenoxybenzyl group as potent inhibitors for both human and rat prostatic 5 α -reductases. Particularly, compound **2e** (YM-36117) showed potent *in vivo* inhibitory activity on rat prostatic DHT. These derivatives have the possibility to display clinical efficacy for the treatment of BPH.

Table 1. Physicochemical Data and Inhibitory Activities of 5 α -Reductases in Human and Rat Prostate Homogenates for Indoline Derivatives

Compound No.	R	X	mp (°C)	Formula	Analysis (%)				IC ₅₀ (nM)	
					Calcd (Found)				Human	Rat
					C	H	N	Halogen		
2a		Cl	142—143	C ₂₂ H ₁₈ ClNO ₃	69.57 (69.63)	4.78 4.87	3.69 3.75	9.33 9.41)	1.8	>300
2b		Cl	123—125	C ₂₃ H ₂₀ ClNO ₃	70.14 (69.95)	5.12 5.12	3.56 3.65	9.00 9.03)	8.8	>300
2c		Cl	109—111	C ₂₅ H ₂₄ ClNO ₃	71.17 (71.24)	5.73 5.93	3.32 3.22	8.40 8.34)	2.2	>300
2d		Cl	182—183	C ₂₆ H ₂₀ ClNO ₃	72.64 (72.66)	4.69 4.63	3.26 3.27	8.25 8.17)	3.9	>300
2e (YM-36117)		Cl	164—165	C ₂₈ H ₂₂ ClNO ₄	71.26 (71.26)	4.70 4.69	2.97 2.99	7.51 7.40)	5.3	46
2f		Cl	116—118	C ₂₉ H ₂₄ ClNO ₄	71.68 (71.58)	4.98 4.96	2.88 2.73	7.30 7.29)	3.4	>300
2g		Cl	134—136	C ₃₁ H ₂₈ ClNO ₄	72.44 (72.41)	5.49 5.50	2.72 2.78	6.90 6.88)	11	>300
2h		H	142—144	C ₂₈ H ₂₃ NO ₄	76.87 (76.70)	5.30 5.37	3.20 3.17)		11	48% inhibition at 100 nM
2i		F	116—119	C ₂₈ H ₂₂ FNO ₄	73.84 (73.90)	4.87 4.83	3.08 3.07	4.17 4.17)	4.3	54
2j		Br	193—198	C ₂₈ H ₂₂ BrNO ₄	65.13 (64.99)	4.29 4.21	2.71 2.75	15.47 15.27)	6.4	19
1 (YM-32906) Finasteride (±)-ONO-3805									0.44 4.1 5.38	>300 13 2.6

Experimental

Melting points were taken on a Yanaco MP-3 melting point apparatus and are uncorrected. ¹H-nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JEOL FX-90, JNM-LA 300, JNM-LA 400, JNM-GX 400 or JNM-GX 500 spectrometer with tetramethylsilane as an internal standard. Mass spectra (MS) were recorded on a Hitachi M-80 (electron impact (EI)) or JEOL JMS-DX300 (FAB) mass spectrometer. Elemental analyses were performed with a Yanaco MT-5. Column chromatography was carried out on silica gel (Wakogel C-200). Unless otherwise noted, all reagents and solvents obtained from commercial suppliers were used without further purification. In general, the organic extract was dried over anhydrous Na₂SO₄ or MgSO₄, and the organic solvent was evaporated under reduced pressure. All nonaqueous reactions were performed in dry glassware under an atmosphere

of dry Ar.

Ethyl 5-Benzyloxy-1H-indole-1-carboxylate (5) A mixture of **4** (6.70 g, 30.0 mmol), diethyl pyrocarbonate (5.84 g, 36.0 mmol) and DMAP (370 mg, 3.03 mmol) in 30 ml of CH₃CN was stirred at room temperature for 15 h. After the reaction mixture was concentrated, the residue was extracted with AcOEt. The extract was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; hexane : AcOEt=95 : 5) to give **5** (5.38 g, 61%) as a colorless solid: ¹H-NMR (CDCl₃) δ: 1.45 (t, 3H, *J*=7 Hz), 4.46 (q, 2H, *J*=7 Hz), 5.10 (s, 2H), 6.50 (d, 1H, *J*=3 Hz), 7.02 (dd, 1H, *J*=9, 2 Hz), 7.10 (d, 1H, *J*=2 Hz), 7.3—7.4 (m, 3H), 7.45 (d, 2H, *J*=7 Hz), 7.58 (d, 1H, *J*=3 Hz), 8.05 (d, 1H, *J*=8 Hz); EI-MS *m/z* 295 (M⁺).

Ethyl 5-Hydroxyindoline-1-carboxylate (6) To a solution of **5** (5.38 g,

Table 2. Physicochemical Data and Inhibitory Activities of 5 α -Reductases in Human and Rat Homogenates for Aniline Derivatives

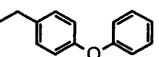
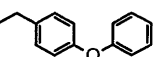
Compound No.	R ¹	R ²	mp (°C)	Formula	Analysis (%)				IC ₅₀ (nM)	
					Calcd (Found)				Human	Rat
					C	H	N	Cl		
3a		H	196—197	C ₁₉ H ₁₄ ClNO ₃	67.16 (67.04)	4.15 4.16	4.12 4.14	10.43 10.50)	14	>300
3b		Me	133—134	C ₂₀ H ₁₆ ClNO ₃	67.90 (67.86)	4.56 4.57	3.96 3.99	10.02 10.14)	3.3	>300
3c		H	167—168	C ₂₀ H ₁₆ ClNO ₃	67.90 (67.86)	4.56 4.60	3.96 3.94	10.02 10.12)	7.5	>300
3d		Me	154—155	C ₂₁ H ₁₈ ClNO ₃	68.57 (68.50)	4.93 4.85	3.81 3.80	9.64 9.74)	2.1	>300
3e		H	245—246	C ₂₀ H ₁₄ ClNO ₄	65.31 (65.09)	3.84 3.82	3.81 3.84	9.64 9.82)	100	>300
3f			183—184	C ₂₇ H ₂₂ ClNO ₃	73.05 (72.91)	5.00 5.11	3.16 3.12	7.99 7.99)	12	>300
3g		H	205—206	C ₂₆ H ₂₀ ClNO ₄	70.03 (69.87)	4.52 4.58	3.14 3.08	7.95 7.82)	10	5.5
3h		Me	177—178	C ₂₇ H ₂₂ ClNO ₄ ·1/4H ₂ O	69.83 (69.90)	4.88 5.01	3.02 3.10	7.63 7.87)	11	12

Table 3. Effects of Selected Indoline and Aniline Derivatives on Prostatic Content of DHT in Adult Male Rats

Compd. No.	<i>In vitro</i> IC ₅₀ (nM)		<i>In vivo</i> ^{a)}	
	Human	Rat	Dose (mg/kg, <i>p.o.</i>)	% Reduction of prostatic DHT
2e (YM-36117)	5.3	46	100	44 ± 6**
2j	6.4	19	100	20 ± 9*
3g	10	5.5	100	25 ± 10*
3h	11	12	100	36 ± 4**
(±)-ONO-3805	538	2.6	10	25 ± 10*
			30	65 ± 5**

a) *In vivo* effects of test compounds on reduction of DHT in rat prostate. Rat prostate was removed and homogenized 8 h after oral administration of test compounds. The concentrations of DHT in the homogenate were determined by radioimmunoassay. Results are the mean ± SE of five to seven separate assays. Statistically significant at **p* < 0.05 and ***p* < 0.01 compared with control value by Student's *t*-test.

18.2 mmol) in 80 ml of EtOH, 10% Pd-C (540 mg) was added. The flask was then placed in a hydrogen atmosphere and stirred at room temperature for 6 h. The catalyst was removed by filtration and the filtrate was concentrated to give **6** (3.72 g, 98%) as a colorless solid: ¹H-NMR (CDCl₃) δ: 1.33 (br s, 3H), 3.04 (t, 2H, *J*=8 Hz), 3.99 (br s, 2H), 4.2—4.4 (m, 2H), 6.04 (br s,

1H), 6.66 (d, 1H, *J*=9 Hz), 6.70 (s, 1H), 7.33 (br s, 0.3 H), 7.69 (br s, 0.7 H); EI-MS *m/z* 207 (M⁺).

Ethyl 5-(2-Chloro-4-cyanophenoxy)indoline-1-carboxylate (7) A mixture of **6** (3.69 g, 17.8 mmol), 3-chloro-4-fluorobenzonitrile (2.91 g, 18.7 mmol) and K₂CO₃ (3.69 g, 26.7 mmol) in 60 ml of DMF was stirred at 80 °C for 5 h. After dilution with AcOEt, the reaction mixture was washed with water and brine, dried and concentrated. The residue was washed with EtOH to give **7** (5.72 g, 94%) as a colorless solid: ¹H-NMR (CDCl₃) δ: 1.36 (br s, 3H), 3.14 (d, 2H, *J*=9 Hz), 4.0—4.2 (m, 2H), 4.29 (br s, 2H), 6.81 (d, 1H, *J*=8 Hz), 6.8—6.9 (m, 2H), 7.42 (dd, 1H, *J*=9, 2 Hz), 7.73 (d, 1H, *J*=2 Hz), 7.90 (br s, 1H); EI-MS *m/z* 342 (M⁺).

3-Chloro-4-(indolin-5-yloxy)benzoic Acid (8) To a solution of **7** (5.71 g, 15.08 mmol) in 50 ml of EtOH, 50 ml of 8*N* KOH aqueous solution was added, then the mixture was refluxed for 5 h. The reaction mixture was neutralized with 3*N* HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated. The residue was washed with EtOH to give **8** (3.56 g, 81%) as a gray solid: ¹H-NMR (DMSO-*d*₆) δ: 2.93 (t, 2H, *J*=8 Hz), 3.4—3.5 (m, 2H), 6.54 (d, 1H, *J*=8 Hz), 6.71 (dd, 1H, *J*=8, 2 Hz), 6.81 (d, 1H, *J*=9 Hz), 6.86 (d, 1H, *J*=2 Hz), 7.8—7.9 (m, 1H), 7.99 (d, 1H, *J*=2 Hz); EI-MS *m/z* 289 (M⁺).

Ethyl 3-Chloro-4-(indolin-5-yloxy)benzoate (9) To a solution of **8** (870 mg, 3.00 mmol) in 20 ml of EtOH, 2.0 ml of 36*N* H₂SO₄ was added, and the mixture was refluxed for 12 h. The reaction mixture was quenched with ice-water, neutralized with 5*N* NaOH aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; hexane : AcOEt=3 : 1) to give **9** (940 mg, 99%) as a colorless oil: ¹H-

NMR (CDCl₃) δ : 1.38 (t, 3H, $J=7$ Hz), 3.0—3.1 (m, 2H), 3.61 (t, 2H, $J=8$ Hz), 4.35 (q, 2H, $J=7$ Hz), 6.62 (d, 1H, $J=8$ Hz), 6.74 (dd, 1H, $J=8, 2$ Hz), 6.78 (d, 1H, $J=9$ Hz), 6.85 (s, 1H), 7.80 (dd, 1H, $J=9, 2$ Hz), 8.10 (d, 1H, $J=2$ Hz); EI-MS m/z 317 (M⁺).

4-[(1-Benzylindolin-5-yl)oxy]-3-chlorobenzoic Acid (2a) A mixture of **9** (620 mg, 1.95 mmol), benzyl bromide (370 mg, 2.16 mmol), KI (10 mg, 0.06 mmol) and K₂CO₃ (400 mg, 2.89 mmol) in 10 ml of DMF was stirred at room temperature for 2 h. After dilution with AcOEt, the reaction mixture was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; hexane: AcOEt=95:5) to give ethyl 4-[(1-benzylindolin-5-yl)oxy]-3-chlorobenzoate (590 mg, 74%) as a colorless oil. This intermediate (560 mg, 1.37 mmol) was dissolved in 9 ml of EtOH–dioxane (7:2), then 5 N NaOH aqueous solution (8 ml) was added, and the mixture was stirred at room temperature for 8 h. The reaction mixture was acidified with 3 N HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was recrystallized from EtOH to give **2a** (270 mg, 52%) as gray crystals: mp 142–143 °C; ¹H-NMR (DMSO-*d*₆) δ : 2.8—3.0 (m, 2H), 3.30 (t, 2H, $J=9$ Hz), 4.27 (s, 2H), 6.61 (d, 1H, $J=8$ Hz), 6.79 (dd, 1H, $J=9, 2$ Hz), 6.83 (d, 1H, $J=9$ Hz), 6.89 (s, 1H), 7.2—7.3 (m, 1H), 7.3—7.4 (m, 4H), 7.82 (dd, 1H, $J=9, 2$ Hz), 7.99 (d, 1H, $J=2$ Hz), 13.07 (brs, 1H); EI-MS m/z 379 (M⁺).

(±)-3-Chloro-4-[[1-(1-phenylethyl)indolin-5-yl]oxy]benzoic Acid (2b) Essentially the same procedure as described above for the preparation of **2a** afforded **2b** (50%) as gray crystals: mp 123–125 °C; ¹H-NMR (CDCl₃) δ : 1.55 (d, 3H, $J=7$ Hz), 2.95 (t, 2H, $J=8$ Hz), 3.3—3.5 (m, 2H), 4.67 (q, 2H, $J=7$ Hz), 6.30 (d, 1H, 8 Hz), 6.71 (dd, 1H, $J=8, 2$ Hz), 6.8—6.9 (m, 1H), 7.2—7.3 (m, 1H), 7.3—7.4 (m, 2H), 7.41 (d, 2H, $J=7$ Hz), 7.84 (dd, 1H, $J=9, 2$ Hz), 8.15 (d, 1H, $J=2$ Hz); EI-MS m/z 393 (M⁺).

3-Chloro-4-[[1-(4-isopropylbenzyl)indolin-5-yl]oxy]benzoic Acid (2c) A mixture of **8** (580 mg, 2.00 mmol), 4-isopropylbenzaldehyde (330 mg, 2.20 mmol), AcOH (120 mg, 2.00 mmol) and NaBH(OAc)₃ (640 mg, 3.00 mmol) in 20 ml of dichloroethane (20 ml) was stirred at room temperature for 4 h. The reaction mixture was quenched with water, neutralized with 1 N NaOH aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; CHCl₃: MeOH=95:5) to give **2c** (760 mg, 90%) as colorless crystals: mp 109–111 °C; ¹H-NMR (CDCl₃) δ : 1.25 (d, 6H, $J=7$ Hz), 2.8—3.0 (m, 3H), 3.35 (t, 2H, $J=8$ Hz), 4.22 (s, 2H), 6.49 (d, 1H, $J=9$ Hz), 6.7—6.9 (m, 3H), 7.21 (d, 2H, $J=8$ Hz), 7.29 (d, 2H, $J=8$ Hz), 7.8—7.9 (m, 1H), 8.17 (d, 1H, $J=2$ Hz); EI-MS m/z 421 (M⁺).

tert-Butyl 5-Benzyloxy-1H-indole-1-carboxylate (10) A mixture of **4** (2.23 g, 10.0 mmol), di-*tert*-butyl dicarbonate (2.62 g, 12.0 mmol) and DMAP (120 mg, 0.98 mmol) in 20 ml of CH₃CN was stirred at room temperature for 2 h. After dilution with AcOEt, the reaction mixture was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; hexane: AcOEt=97:3) to give **10** (2.86 g, 89%) as a colorless oil; ¹H-NMR (CDCl₃) δ : 1.65 (s, 9H), 5.10 (s, 2H), 6.48 (d, 1H, $J=4$ Hz), 6.9—7.1 (m, 1H), 7.09 (d, 1H, $J=2$ Hz), 7.31 (t, 1H, $J=7$ Hz), 7.3—7.4 (m, 2H), 7.45 (d, 2H, $J=7$ Hz), 7.56 (d, 1H, $J=3$ Hz), 8.03 (brs, 1H); EI-MS m/z 323 (M⁺).

tert-Butyl 5-Hydroxyindoline-1-carboxylate (11) Essentially the same procedure as described above for the preparation of **10** afforded **11** (96%) as a colorless solid; ¹H-NMR (CDCl₃) δ : 1.55 (s, 9H), 3.01 (t, 2H, $J=8$ Hz), 3.95 (brs, 2H), 5.77 (brs, 1H), 6.6—6.7 (m, 1H), 6.68 (s, 1H), 7.26 (brs, 0.5 H), 7.67 (brs, 0.5 H); EI-MS m/z 235 (M⁺).

3-Chloro-4-(indolin-5-yloxy)benzonitrile (12a) A mixture of **11** (5.51 g, 23.5 mmol), 3-chloro-4-fluorobenzonitrile (3.83 g, 24.6 mmol) and K₂CO₃ (4.86 g, 35.2 mmol) in 80 ml of DMF was stirred at 80 °C for 2 h. After dilution with AcOEt, the reaction mixture was washed with water and brine, dried and concentrated. This intermediate (9.24 g) was dissolved in 100 ml of trifluoroacetic acid, and the mixture was stirred at room temperature for 1 h and concentrated. The residue was neutralized with 1 N NaOH aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was recrystallized from EtOH to give **12a** (5.14 g, 81%) as a colorless solid; ¹H-NMR (CDCl₃) δ : 3.04 (t, 2H, $J=9$ Hz), 3.62 (t, 2H, $J=9$ Hz), 3.80 (brs, 1H), 6.62 (d, 1H, $J=8$ Hz), 6.73 (dd, 1H, $J=9, 2$ Hz), 6.80 (d, 1H, $J=9$ Hz), 6.84 (t, 1H, $J=1$ Hz), 7.40 (dd, 1H, $J=9, 2$ Hz), 7.70 (d, 1H, $J=2$ Hz); EI-MS m/z 270 (M⁺).

3-Chloro-4-[[1-(naphthalen-2-ylmethyl)indolin-5-yl]oxy]benzoic Acid (2d) A mixture of **12a** (540 mg, 2.00 mmol), 2-(bromomethyl)naphthalene (490 mg, 2.20 mmol), KI (10 mg, 0.06 mmol) and K₂CO₃ (410 mg, 3.00 mmol) in 10 ml of DMF was stirred at room temperature for 7 h. After dilution with AcOEt, the reaction mixture was washed with water and brine,

dried and concentrated. The residue was washed with EtOH to give 3-chloro-4-[[1-(naphthalen-2-ylmethyl)indolin-5-yl]oxy]benzonitrile (680 mg, 84%) as a brown solid. This intermediate (670 mg, 1.63 mmol) was dissolved in 5 ml of EtOH, then 5 ml of 8 N KOH aqueous solution was added, and the mixture was refluxed for 6 h. The reaction mixture was acidified with 3 N HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was washed from EtOH to give **2d** (530 mg, 76%) as pale yellow crystals: mp 182–183 °C; ¹H-NMR (CDCl₃) δ : 3.00 (t, 2H, $J=8$ Hz), 3.40 (t, 2H, $J=8$ Hz), 4.41 (s, 2H), 6.52 (d, 1H, $J=8$ Hz), 6.79 (dd, 1H, $J=8, 2$ Hz), 6.82 (d, 1H, $J=9$ Hz), 6.86 (d, 1H, $J=2$ Hz), 7.4–7.6 (m, 3H), 7.8—7.9 (m, 5H), 8.17 (d, 1H, $J=2$ Hz); EI-MS m/z 429 (M⁺).

3-Chloro-4-[[1-(4-phenoxybenzyl)indolin-5-yl]oxy]benzoic Acid (2e) A mixture of **12a** (540 mg, 2.00 mmol), 4-phenoxybenzaldehyde (440 mg, 2.20 mmol), AcOH (1.20 g, 20.0 mmol) and NaBH(OAc)₃ (640 mg, 3.00 mmol) in 20 ml of dichloromethane was stirred at room temperature for 1 h. The reaction mixture was quenched with water, neutralized with K₂CO₃ and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was recrystallized from EtOH to give 3-chloro-4-[[1-(4-phenoxybenzyl)indolin-5-yl]oxy]benzonitrile (740 mg, 82%) as a colorless solid. This intermediate (720 mg, 1.59 mmol) was dissolved in 5 ml of EtOH, then 5 ml of 8 N KOH aqueous solution was added, and the resulting mixture was refluxed for 7 h. The reaction mixture was acidified with 3 N HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was washed from EtOH to give **2e** (570 mg, 76%) as colorless crystals: mp 164–165 °C; ¹H-NMR (CDCl₃) δ : 2.97 (t, 2H, $J=8$ Hz), 3.36 (t, 2H, $J=8$ Hz), 4.22 (s, 2H), 6.49 (d, 1H, $J=9$ Hz), 6.78 (d, 1H, $J=2$ Hz), 6.80 (d, 1H, $J=2$ Hz), 6.83 (d, 1H, $J=9$ Hz), 6.99 (d, 2H, $J=8$ Hz), 7.01 (d, 2H, $J=9$ Hz), 7.10 (t, 1H, $J=7$ Hz), 7.3–7.4 (m, 4H), 7.85 (dd, 1H, $J=9, 2$ Hz), 8.17 (d, 1H, $J=2$ Hz); EI-MS m/z 471 (M⁺).

Essentially the same procedure as described above for the preparation of **2e** afforded **2f** (brown crystals, 58%), **2g** (colorless crystals, 63%), **2h** (pale brown crystals, 59%), **2i** (colorless crystals, 35%), and **2j** (colorless crystals, 44%).

4-(4-Anilinophenoxy)-3-chlorobenzonitrile (14a) Essentially the same procedure as described above for the preparation of **7** afforded **14a** (90%) as a pale yellow solid; ¹H-NMR (CDCl₃) δ : 6.85 (d, 1H, $J=9$ Hz), 6.9—7.0 (m, 3H), 7.0—7.2 (m, 4H), 7.2—7.3 (m, 2H), 7.43 (dd, 1H, $J=9, 2$ Hz), 7.72 (d, 1H, $J=2$ Hz); EI-MS m/z 320 (M⁺).

3-Chloro-4-[4-(*N*-methylamino)phenoxy]benzonitrile (14b) Essentially the same procedure as described above for the preparation of **7** afforded **14b** (58%) as a pale yellow solid; ¹H-NMR (DMSO-*d*₆) δ : 2.70 (d, 3H, $J=5$ Hz), 5.79 (q, 1H, $J=5$ Hz), 6.61 (d, 2H, $J=9$ Hz), 6.80 (d, 1H, $J=9$ Hz), 6.9—7.0 (m, 2H), 7.70 (dd, 1H, $J=9, 2$ Hz), 8.11 (d, 1H, $J=2$ Hz); EI-MS m/z 258 (M⁺).

4-(4-Anilinophenoxy)-3-chlorobenzoic Acid (3a) Essentially the same procedure as described above for the preparation of **8** afforded **3a** (25%) as pale brown crystals: mp 196–197 °C; ¹H-NMR (CDCl₃) δ : 6.87 (d, 1H, $J=9$ Hz), 6.95 (t, 1H, $J=7$ Hz), 7.00 (dd, 2H, $J=7, 2$ Hz), 7.06 (d, 2H, $J=8$ Hz), 7.1—7.2 (m, 2H), 7.28 (dd, 2H, $J=8, 7$ Hz), 7.89 (dd, 1H, $J=9, 2$ Hz), 8.20 (d, 1H, $J=2$ Hz); EI-MS m/z 339 (M⁺).

3-Chloro-4-[4-(*N*-methylanilino)phenoxy]benzoic Acid (3b) To a suspension of 60% NaH (130 mg, 3.30 mmol) in 5 ml of DMF, **14a** (960 mg, 3.00 mmol) was added, and stirred at room temperature for 1 h. Methyl iodide (510 mg, 3.60 mmol) was added and the mixture was stirred at room temperature for 22 h. After the addition of water, the mixture was extracted with AcOEt. The organic layer was washed with water and brine, dried and concentrated. The residue was purified by silica gel column chromatography (eluent; hexane: AcOEt=95:5) to give 3-chloro-4-[4-(*N*-methylanilino)phenoxy]benzonitrile (860 mg, 87%) as a colorless solid. This intermediate (820 mg, 2.45 mmol) was dissolved in 15 ml of EtOH, then 15 ml of 8 N KOH aqueous solution was added, and the mixture was refluxed for 6 h. The reaction mixture was acidified with 3 N HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was washed from EtOH to give **3b** (440 mg, 51%) as colorless crystals: mp 133–134 °C; ¹H-NMR (CDCl₃) δ : 3.32 (s, 3H), 6.88 (d, 1H, $J=9$ Hz), 6.9—7.1 (m, 7H), 7.29 (t, 2H, $J=8$ Hz), 7.90 (dd, 1H, $J=9, 2$ Hz), 8.20 (d, 1H, $J=2$ Hz); EI-MS m/z 353 (M⁺).

3-Chloro-4-[4-[*N*-methyl-*N*-(4-phenoxybenzyl)amino]phenoxy]benzoic Acid (3h) Essentially the same procedure as described above for the preparation of **2e** afforded **3h** (42%) as pale orange crystals: mp 177–178 °C; ¹H-NMR (CDCl₃) δ : 3.02 (s, 3H), 4.50 (s, 2H), 6.8—6.9 (m, 3H), 6.9—7.1 (m, 6H), 7.10 (t, 1H, $J=7$ Hz), 7.20 (d, 2H, $J=9$ Hz), 7.3—7.4 (m,

2H), 7.85 (dd, 1H, $J=9$, 2 Hz), 8.17 (d, 1H, $J=2$ Hz); EI-MS m/z 459 (M^+).

Methyl 4-(4-Aminophenoxy)-3-chlorobenzoate (16) A mixture of **15** (3.73 g, 20.0 mmol), 1-fluoro-4-nitrobenzene (2.82 g, 20.0 mmol) and K_2CO_3 (4.15 g, 30.0 mmol) in 50 ml of DMF was stirred at 70 °C for 1 d. After dilution with AcOEt, the organic layer was washed with water and brine, dried and concentrated. The residue was recrystallized from EtOH to give methyl 3-chloro-4-(4-nitrophenoxy)benzoate (4.87 g, 79%) as a pale yellow solid. This intermediate (4.84 g, 15.7 mmol) was dissolved in 40 ml of AcOEt, then a suspension of Raney-Ni (400 mg) in 2 ml of EtOH was added. The flask was then placed in a hydrogen atmosphere and stirred at room temperature for 5 h. The catalyst was removed by filtration and the filtrate was concentrated. The residue was recrystallized from EtOH to give **16** (3.19 g, 73%) as colorless crystals: ^1H -NMR ($CDCl_3$) δ : 3.68 (brs, 2H), 3.89 (s, 3H), 6.6—6.8 (m, 2H), 6.76 (d, 1H, $J=9$ Hz), 6.8—6.9 (m, 2H), 7.79 (dd, 1H, $J=9$, 2 Hz), 8.11 (d, 1H, $J=2$ Hz); EI-MS m/z 277 (M^+).

Methyl 4-[4-(Benzylamino)phenoxy]-3-chlorobenzoate (17a) Essentially the same procedure as described above for the preparation of **2c** afforded **17a** (77%) as colorless crystals: ^1H -NMR ($DMSO-d_6$) δ : 3.83 (s, 3H), 4.27 (d, 2H, $J=4$ Hz), 6.35 (brs, 1H), 6.65 (d, 2H, $J=9$ Hz), 6.79 (d, 1H, $J=9$ Hz), 6.89 (d, 2H, $J=9$ Hz), 7.24 (t, 1H, $J=7$ Hz), 7.34 (dd, 2H, $J=8$, 7 Hz), 7.38 (d, 2H, $J=7$ Hz), 7.81 (dd, 1H, $J=9$, 2 Hz), 8.00 (d, 1H, $J=2$ Hz); EI-MS m/z 367 (M^+).

Methyl 3-Chloro-4-[4-[N-(4-phenoxybenzyl)amino]phenoxy]benzoate (17b) Essentially the same procedure as described above for the preparation of **2c** afforded **17b** (100%) as a colorless oil; ^1H -NMR ($CDCl_3$) δ : 3.89 (s, 3H), 4.03 (brs, 1H), 4.30 (s, 2H), 6.6—6.7 (m, 2H), 6.76 (dd, 1H, $J=9$, 4 Hz), 6.9—7.1 (m, 6H), 7.10 (t, 1H, $J=8$ Hz), 7.3—7.4 (m, 4H), 7.78 (dd, 1H, $J=9$, 2 Hz), 8.10 (d, 1H, $J=2$ Hz); EI-MS m/z 459 (M^+).

Methyl 4-[4-(N-Benzyl-N-methylamino)phenoxy]-3-chlorobenzoate (18) A mixture of **17a** (350 mg, 0.95 mmol), formic acid (3 ml) and 35% formaldehyde aqueous solution (3 ml) was stirred at 100 °C for 1.5 h. After the addition of water, the reaction mixture was neutralized with K_2CO_3 and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was purified by silica gel column chromatography (eluent; hexane:AcOEt=9:1) to give **18** (310 mg, 85%) as colorless crystals; ^1H -NMR ($DMSO-d_6$) δ : 3.03 (s, 3H), 3.83 (s, 3H), 4.57 (s, 2H), 6.78 (d, 2H, $J=9$ Hz), 6.81 (d, 1H, $J=9$ Hz), 6.99 (d, 2H, $J=9$ Hz), 7.23 (d, 3H, $J=8$ Hz), 7.33 (dd, 2H, $J=8$, 7 Hz), 7.83 (d, 1H, $J=11$ Hz), 8.01 (d, 1H, $J=1$ Hz); EI-MS m/z 381 (M^+).

4-[4-(Benzoylamino)phenoxy]-3-chlorobenzoic Acid (3e) To a solution of **16** (830 mg, 3.00 mmol) in 8 ml of pyridine- CH_2Cl_2 (1:1), a solution of benzoyl chloride (440 mg, 3.13 mmol) in 4 ml of CH_2Cl_2 was added dropwise at 5 °C, then the mixture was stirred at room temperature for 5 h. After the addition of water, the mixture was extracted with AcOEt. The organic layer was washed with 1 N HCl aqueous solution, water and brine, dried and concentrated. The residue was washed with EtOH to give methyl 4-[4-(benzoylamino)phenoxy]-3-chlorobenzoate (900 mg, 79%) as a colorless solid. This intermediate (440 mg, 1.15 mmol) was dissolved in 7 ml of dioxane-EtOH (2:5), then 7 ml of 5 N NaOH aqueous solution was added, and the mixture was stirred at 50 °C for 2 h. The reaction mixture was acidified with 3 N HCl aqueous solution and extracted with AcOEt. The extract was washed with water and brine, dried and concentrated, and the residue was washed with EtOH to give **3e** (240 mg, 57%) as colorless crystals: mp 245—246 °C; ^1H -NMR ($CDCl_3$) δ : 6.88 (d, 1H, $J=9$ Hz), 7.0—7.1 (m, 2H), 7.4—7.6 (m, 3H), 7.8—7.9 (m, 3H), 7.9—8.0 (m, 2H), 8.11 (d, 1H, $J=2$ Hz), 9.94 (s, 1H); FAB-MS m/z 368 ($M^+ + H$).

3-Chloro-4-(4-dibenzylaminophenoxy)benzoic Acid (3f) A mixture of **16** (520 mg, 1.87 mmol), benzyl bromide (700 mg, 4.09 mmol), potassium iodide (30 mg, 0.18 mmol) and K_2CO_3 (780 mg, 5.64 mmol) in 10 ml of DMF was stirred at 80 °C for 8 h. After the addition of water, the reaction mixture was extracted with AcOEt. The organic layer was washed with water and brine, dried and concentrated, and the residue was purified by silica gel column chromatography (eluent; hexane:AcOEt=95:5) to give methyl 3-chloro-4-(4-dibenzylaminophenoxy)benzoate (710 mg, 83%) as a colorless oil. This intermediate (690 mg, 1.51 mmol) was hydrolyzed in the same manner as described for compound **3e** to give **3f** (530 mg, 79%) as colorless crystals: mp 183—184 °C; ^1H -NMR ($CDCl_3$) δ : 4.66 (s, 4H), 6.7—6.8 (m, 2H), 6.80 (d, 1H, $J=9$ Hz), 6.8—7.0 (m, 2H), 7.2—7.3 (m, 6H), 7.3—7.4 (m, 4H), 7.8—7.9 (m, 1H), 8.16 (d, 1H, $J=2$ Hz); EI-MS m/z 443 (M^+).

4-[4-(Benzylamino)phenoxy]-3-chlorobenzoic Acid (3c) Compound **17a** (350 mg, 0.95 mmol) was hydrolyzed in the same manner as described for compound **3e** to give **3c** (120 mg, 36%) as colorless crystals: mp 167—168 °C; ^1H -NMR ($DMSO-d_6$) δ : 4.27 (s, 2H), 6.36 (brs, 1H), 6.6—6.7 (m,

2H), 6.77 (d, 1H, $J=9$ Hz), 6.8—7.0 (m, 2H), 7.24 (t, 1H, $J=7$ Hz), 7.3—7.4 (m, 4H), 7.7—7.9 (m, 1H), 7.98 (d, 1H, $J=2$ Hz), 13.05 (brs, 1H); EI-MS m/z 353 (M^+).

3-Chloro-4-[4-[N-(4-phenoxybenzyl)amino]phenoxy]benzoic Acid (3g) Compound **17b** (470 mg, 1.02 mmol) was hydrolyzed in the same manner as described for compound **3e** to give **3g** (260 mg, 57%) as colorless crystals: mp 205—206 °C; ^1H -NMR ($CDCl_3$) δ : 4.31 (s, 2H), 6.67 (d, 2H, $J=9$ Hz), 6.79 (d, 1H, $J=9$ Hz), 6.93 (d, 2H, $J=9$ Hz), 7.0—7.1 (m, 4H), 7.11 (t, 1H, $J=7$ Hz), 7.3—7.4 (m, 4H), 7.84 (dd, 1H, $J=9$, 2 Hz), 8.16 (d, 1H, $J=2$ Hz); EI-MS m/z 445 (M^+).

4-[4-(N-Benzyl-N-methylamino)phenoxy]-3-chlorobenzoic Acid (3d) Compound **18** (280 mg, 0.73 mmol) was hydrolyzed in the same manner as described for compound **3e** to give **3d** (180 mg, 67%) as colorless crystals: mp 154—155 °C; ^1H -NMR ($DMSO-d_6$) δ : 3.02 (s, 3H), 4.57 (s, 2H), 6.7—6.9 (m, 3H), 6.9—7.0 (m, 2H), 7.24 (t, 3H, $J=4$ Hz), 7.33 (t, 2H, $J=7$ Hz), 7.8—7.9 (m, 1H), 7.99 (d, 1H, $J=2$ Hz), 13.08 (brs, 1H); EI-MS m/z 367 (M^+).

Authentic Materials The 5α -reductase inhibitors, finasteride^{7a)} and ($\pm$)-ONO-3805,^{8a)} were synthesized in our company according to the methods described in the literature.

Biological Methods. Preparation of 5α -Reductases from Rat Prostate and Human Prostate Male Wistar rats 8—12 weeks of age (Charles River Japan Inc., Atsugi, Japan) were sacrificed and the ventral prostates were removed. The prostatic tissues were rinsed with ice-cold saline and minced with scissors. Unless specified, all the following procedures were carried out at 4 °C. The minced tissues were homogenized with a Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland) in 3 tissue volumes of 20 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose and 0.1 mM dithiothreitol (DTT). The homogenate was centrifuged at 10000 $\times g$ for 10 min, and the resulting supernatant was centrifuged again at 140000 $\times g$ for 60 min. The pellets were resuspended in 40 mM sodium phosphate buffer, pH 6.5. The suspension was stored at -80 °C until use.

Human prostatic tissues from BPH patients who received a transurethral prostatectomy were kindly provided by Dr. T. Tahara at Yamato Hospital, Tokyo, Japan, and stored at -80 °C until preparation of the enzyme fractions. The frozen prostatic tissues were minced with scissors and homogenized with a Polytron homogenizer in 3 tissue volumes of 10 mM Tris-HCl buffer, pH 7.0, containing 0.33 M sucrose, 1 mM DTT and 1 μ M NADPH. The homogenate was centrifuged at 1000 $\times g$ for 5 min. The supernatant was centrifuged at 11000 $\times g$ for 20 min, and the resulting supernatant was centrifuged again at 140000 $\times g$ for 60 min. The resulting pellet was resuspended in 10 mM Tris-HCl buffer, pH 7.0, and stored at -80 °C until use.

5α -Reductase Assay in Vitro 5α -Reductase activities were assayed according to the method described by Liang *et al.*¹⁵⁾ with a minor modification. Briefly, the reaction solutions contained in a final volume of 0.5 ml: 1 μ M [4-¹⁴C] testosterone, 1 mM DTT, 50 mM NADPH, 50 mM buffer (Tris-HCl, pH 6.5, for the rat prostate enzyme; Tris-citrate, pH 5.0, for the human prostate enzyme) and the enzyme fractions. To identify the inhibitory effect of the drugs, the various concentrations of the test compounds were also added in 5 μ l DMSO (final conc. 1%). The reaction solutions in duplicate were incubated at 37 °C for 60 min, and the reaction was terminated by the addition of 2.0 ml cold AcOEt containing 10 μ g testosterone, 5α -DHT, 4-androstene-3,17-dione, 5α -androstan-3 α ,17 β -diol and 5α -androstan-3,17-dione as the standards. The organic phase was separated by centrifugation, evaporated under N_2 gas and resuspended with 40 μ l AcOEt. 20 μ l of AcOEt was spotted on the TLC plate and separated twice using AcOEt/cyclohexane (1:1) as the developing solvent. The steroid standards were located by UV (254 nm) and by spraying with a 1% $CeSO_4$ -10% H_2SO_4 solution followed by heating. The regions containing 5α -reduced metabolites (5α -DHT, 5α -androstan-3 α ,17 β -diol and 5α -androstan-3,17-dione) were cut from the TLC plate, soaked in 5 ml of Aquasol-2 and the radioactivity was counted by a scintillation counter. The IC_{50} values for 5α -reductase activity were obtained from the linear line drawn using the least-squares fitting method.

In Vivo Effects in Rats Male SD rats at 9 weeks of age (Charles River Japan Inc., Atsugi, Japan) were used to evaluate the effects of test compounds on the prostatic concentration of DHT. The test compounds or a vehicle were orally administered as an aqueous suspension with 0.5% methylcellulose. Eight hours after the dosing, the rat were sacrificed by chloroform anesthesia and the ventral prostate in the treated rats was removed and weighed. The ventral prostates were immediately placed in liquid nitrogen then stored at -80 °C until use.

The frozen prostatic tissues (wet weight 200—500 mg) were minced with scissors and homogenized using a Polytron homogenizer in 3 ml of ice-cold water. One milliliter of the homogenate was twice extracted using 3 ml of

ice-cold diethyl ether. The collected ether layer was concentrated under nitrogen gas and resuspended with 1.5 ml of assay buffer (50 mM Tris-HCl buffer, pH 8.0, containing 0.1% (w/v) gelatin). The DHT contents in the extract samples were determined by radioimmunoassay using testosterone/DHT [^3H] assay system (Amersham Pharmacia Biotech, Uppsala, Sweden). The DHT contents were calculated as pg/100 mg of prostatic wet weight.

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References and Notes

- 1) Igarashi S., Kimura T., Naito R., Hara H., Fujii M., Koutoku H., Oritani H., Mase T., *Chem. Pharm. Bull.*, **47**, 1073—1080 (1999).
- 2) Igarashi S., Inami H., Hara H., Fujii M., Koutoku H., Oritani H., Mase T., *Chem. Pharm. Bull.*, **48**, 382—388 (2000).
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- 6) a) Metcalf B. W., Levy M. A., Holt D. A., *Trends Pharmacol. Sci.*, **10**, 491—495 (1989); b) Adachi K., Kano M., *Biochem. Biophys. Res. Comm.*, **41**, 884—890 (1970); c) Books J. R., *J. Clin. Endocrinol. Metab.*, **74**, 391—405 (1986).
- 7) Recent studies of steroidal 5α -reductase inhibitors: a) Rasmusson G. H., Reynolds G. F., Steinberg N. G., Walton E., Patel G. F., Liang T., Cascieri M. A., Cheung A. H., Brooks J. R., Berman C., *J. Med. Chem.*, **29**, 2298—2315 (1986); b) Metcalf B. W., Holt D. A., Levy M. A., Erb J. M., Heaslip J. I., Brandt M., Oh H.-J., *Bioorg. Chem.*, **17**, 372—376 (1989); c) Holt D. A., Levy M. A., Ladd D. L., Oh H.-J., Erb J. M., Heaslip J. I., Brandt M., Metcalf B. W., *J. Med. Chem.*, **33**, 937—942 (1990); d) Holt D. A., Levy M. A., Oh H.-J., Erb J. M., Heaslip J. I., Brandt M., Lan-Hargest H.-Y., Metcalf B. W., *ibid.*, **33**, 943—950 (1990); e) Levy M. A., Metcalf B. W., Brandt M., Erb J. M., Oh H.-J., Heaslip J. I., Yen H.-K., Rozamus, L. W., Holt D. A., *Bioorg. Chem.*, **19**, 245—260 (1991); f) Bakshi R. K., Patel G. F., Rasmusson G. H., Baginsky W. F., Cimisi G., Ellsworth K., Chang B., Bull H., Tolman R. L., Harris G. S., *J. Med. Chem.*, **37**, 3871—3874 (1994); g) Li X., Singh S. M., Cote J., Laplante S., Veilleux R., Labrie F., *ibid.*, **38**, 1456—1461 (1995); h) Frye S. V., Haffner C. D., Maloney P. R., Hinters R. N., Dorsey G. F., Noe R. A., Unwalla R. J., Batchelor K. W., Bramson H. N., Stuart J. D., Schweiker S. L., Arnold J. V., Bickett D. M., Moss M. L., Tian G., Lee F. W., Tipping T. K., James M. K., Grizzle M. K., Long J. E., Croom D. K., *ibid.*, **38**, 2621—2627 (1995); i) Hausler A., Allegrini P. R., Biollaz M., Batzl C., Scheidegger E., Bhatnagar A. S., *J. Steroid Biochem. Mol. Biol.*, **57**, 187—195 (1996).
- 8) Recent studies of nonsteroidal 5α -reductase inhibitors: a) Nakai H., Terajima H., Arai Y., Eur. Patent Appl., EP 291245 [*Chem. Abstr.*, **110**, 212384t (1989)]; b) Jones C. D., Audia J. E., Lawhorn D. E., McQuaid L. A., Neubauer B. L., Pike A. J., Pennington P. A., Stamm N. B., Toomey R. E., Hirsch K. S., *J. Med. Chem.*, **36**, 421—423 (1993); c) Holt D. A., Yamashita D. S., Konialian-Beak A. L., Luengo J. I., Abell A. D., Bergsma D. J., Brandt M., Levy M. A., *ibid.*, **38**, 13—15 (1995); d) Blagg J., Ballard S. A., Cooper K., Finn P. W., Johnson P. S., MacIntyre F., Maw G. N., Spargo P. L., *Bioorg. Med. Chem. Lett.*, **6**, 1517—1522 (1996); e) Takami H., Koshimura H., Kishibayashi N., Ishii A., Nonaka H., Aoyama S., Kase H., Kumazawa T., *J. Med. Chem.*, **39**, 5047—5052 (1996); f) Kato M., Komada K., Namera A., Sakai Y., Okada S., Yamada A., Yokoyama K., Migita E., Minobe Y., Tani T., *Chem. Pharm. Bull.*, **45**, 1767—1776 (1997); g) Sawada K., Okada S., Golden P., Kayakiri N., Sawada Y., Hashimoto M., Tanaka H., *ibid.*, **47**, 481—491 (1999).
- 9) Andersson S., Russell D. W., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 3640—3644 (1990).
- 10) Moore R. J., Wilson J. D., *J. Biol. Chem.*, **251**, 5895—5900 (1976).
- 11) Andersson S., Berman D. M., Jenkins E. P., Russell D. W., *Nature (London)*, **354**, 159—161 (1991).
- 12) Rittmaster R. S., *N. Engl. J. Med.*, **330**, 120—125 (1994).
- 13) The inhibitory activities of finasteride and ($\pm$)-ONO-3805 for human prostatic 5α -reductase were evaluated in our company. The results are described in Table 1.
- 14) Giudici D., Briatico G., Cominato C., Zaccheo T., Iehle C., Nesi M., Panzeri A., di Salle E., *J. Steroid Biochem. Mol. Biol.*, **58**, 299—305 (1996).
- 15) Liang T., Cascieri M. A., Cheung A. H., Reynolds G. F., Rasmusson G. H., *Endocrinology*, **117**, 571—579 (1985).

Effects of *n*-Alkyltrimethylammonium on Skin Permeation of Benzoic Acid through Excised Guinea Pig Dorsal Skin

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Effects of cetyltrimethylammonium and two other *n*-alkyltrimethylammoniums on the permeation of benzoic acid through excised guinea pig dorsal skin were examined. Cetyltrimethylammonium markedly increased both the flux and permeability coefficient in the concentration range of 0.5 and 5 mM. However, 50 mM cetyltrimethylammonium decreased them. The presence of 2 mM cetyltrimethylammonium, which induced a maximal enhancement effect, also increased the fluxes of 4-methyl, 4-ethyl and 4-*n*-propyl substituents of benzoic acid, but the enhancement effects were less. Analysis of the free energy of transfer of the methylene group of the substituents from the aqueous phase to skin suggested that cetyltrimethylammonium made the skin relatively more hydrophilic. Electron spin resonance spectra analysis by a long-chain quaternary alkylammonium analog, 4-(*N,N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-15), showed that the spin label was present in a nearly solid state in both excised skin and its stratum corneum. This finding suggested the high affinity of the long-chain alkylammoniums to proteins in the stratum corneum and the involvement of the interaction between the cationic surfactants and the proteins in their improvement of the hydrophilic property of the skin, as well as their marked enhancement effects on the skin permeation of relatively hydrophilic drugs.

Key words *n*-alkyltrimethylammonium; skin permeation; absorption enhancer; benzoic acid; ESR

Surfactants have been determined to have enhancement effects on skin permeation of various drugs.^{1–3} Among them, long-chain alkylammoniums such as dodecyltrimethylammonium are known to show marked enhancement effects on skin absorption of drugs such as methyl nicotinamide.^{1,3} However, their enhancement mechanism is still unclear. In this work we first examined the characteristics of the enhancement effects of three *n*-alkyltrimethylammoniums, especially cetyltrimethylammonium, on *in vitro* skin permeation of benzoic acid and its 4-alkyl substituents through excised guinea pig dorsal skin. To reveal the mechanism of the enhancement effects, we examined changes in the relative lipophilicity of the skin after treatment with cetyltrimethylammonium by analyzing the free energy of transfer of the methylene group of the 4-alkyl substituents of benzoic acid from the aqueous phase to the skin.⁴

Since spin labeling is a good method for obtaining information on the interaction of chemicals with components in biological tissues and membranes,⁵ we also studied the mechanism of the effects of *n*-alkyltrimethylammoniums by observing the electron spin resonance (ESR) spectra of a long-chain quaternary ammonium analog, 4-(*N,N*-dimethyl-*N*-pentadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-15). CAT-15 and its analogs have been applied to measure membrane fluidity and analyze structural changes in lipopolysaccharides in biological membranes as spin labels.^{6,7}

Experimental

Materials Bromide salts of *n*-alkyltrimethylammoniums were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Benzoic acid, its 4-methyl, 4-ethyl and 4-*n*-propyl substituents were purchased from Wako Pure Chemical Industries (Osaka, Japan). CAT-15 was from Molecular Probes, Inc. (Junction City, OR, U.S.A.). All other reagents were purchased from Wako Pure Chemical Industries.

Skin Preparations Full thickness dorsal skin was excised from male guinea pigs and subcutaneous fat and other extraneous tissues were trimmed. The stratum corneum sheets were separated by trypsination from

the excised skin. The skin was placed stratum corneum side up, and floated on 0.2% trypsin in phosphate buffered saline (PBS) at 37 °C for 12 h.

Measurement of *in Vitro* Skin Penetration *In vitro* skin penetration of drugs was examined as described previously.⁸ The skin was mounted in a two-chamber diffusion cell with water jackets (37 °C). The available diffusion area was about 0.65 cm², and each half-cell volume was about 5.4 ml. The donor cells were filled with saline either in the presence or absence of *n*-alkyltrimethylammoniums, and the receiver cells with PBS (pH 7.4). The cells were stirred at 450 rpm by a magnetic stirrer during 12 h pretreatment of the skin. After washing both cells, a suspension of excess amounts of benzoic acid or its 4-alkyl substituents in saline either in the presence or absence of *n*-alkyltrimethylammoniums was added to the donor compartments unless otherwise mentioned, and the penetration experiment was started. One hundred fifty μ l of sample was taken from the receiver cells periodically over a maximum period of 8 h, then diluted with PBS twenty times or more and analyzed by UV absorbance at 224 nm for benzoic acid, at 235 nm for 4-methyl and 4-ethyl substituents and at 236 nm for the 4-*n*-propyl substituent. Solubilized components from skin and penetrated *n*-alkyltrimethylammoniums did not interfere with the UV absorbance.

Steady state fluxes of benzoic acid and its 4-alkyl substituents were obtained by the initial straight portions of the penetration curves. Apparent permeability coefficients were calculated by those and by their solubility, which was measured as described previously.⁸

Measurement of Critical Micelle Concentration (CMC) CMCs of alkyltrimethylammoniums were measured by observation of the spectrum change of bromophenol blue. Spectra of 0.017 mM bromophenol blue in saline were measured at 37 °C in the presence of various concentrations of alkyltrimethylammoniums; those concentrations which induced a change in the spectrum from that with a maximum absorbance at 592 nm to that with a maximum absorbance at 604 nm were determined.

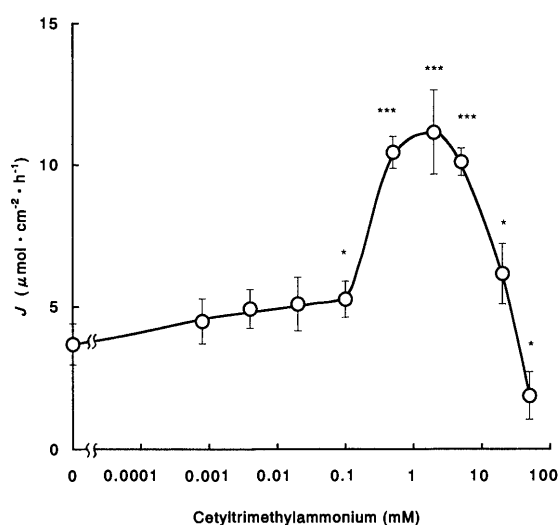
Measurement of Electron Resonance Spectra Both the epidermis side and dermis side of the skin were incubated with PBS at 37 °C for 2 h by placing the epidermis side upwards. After removing PBS from the epidermis side (area 0.65 cm²), 200 μ l 50 μ M CAT-15 in PBS was added and incubated for 2 h at 37 °C. Incubation of the stratum corneum with the spin label was carried out similarly. The spin label solution left on the epidermis or the stratum corneum was removed by washing with PBS. Slices of the spin-labeled skin samples were inserted into a quartz cell (Labotech, Tokyo), and ESR spectra were measured with a TE-200 (X-band) spectrometer (JEOL, Tokyo, Japan) with 100 kHz field modulation frequency and 0.2 mT modulation amplitude at an output power of 8 mW, as reported previously.⁸

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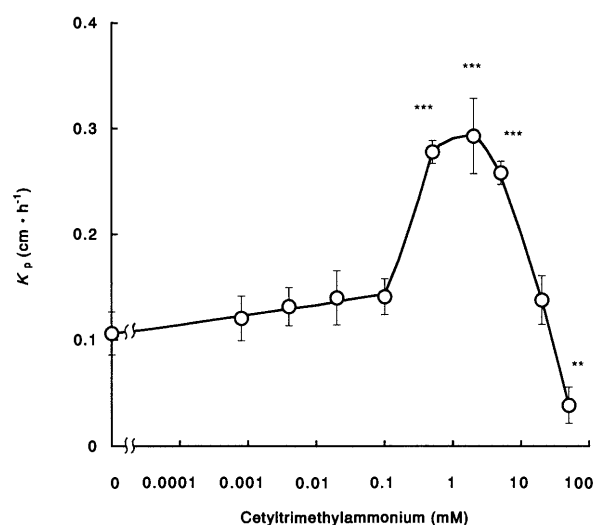
Table 1. Solubility (C_d), Flux (J) and Apparent Permeability Coefficients (K_p) of Benzoic Acid in the Presence of 2 mM *n*-Alkyltrimethylammoniums and Dodecylsulfate

Surfactant	C_d (mM)	J ($\mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$)	K_p ($\times 10^{-2} \text{cm} \cdot \text{h}^{-1}$)
None	34.7 ± 1.6	3.68 ± 0.71	10.6 ± 2.0
<i>n</i> -Dodecyltrimethylammonium	35.8 ± 0.7	$9.55 \pm 1.34^{***}$	$26.7 \pm 3.7^{***}$
<i>n</i> -Tetradecyltrimethylammonium	36.5 ± 0.3	$9.09 \pm 1.24^{***}$	$24.9 \pm 3.4^{***}$
Cetyltrimethylammonium	38.1 ± 2.1	$11.16 \pm 1.50^{***}$	$29.3 \pm 3.6^{***}$
Dodecylsulfate	35.4 ± 1.2	$7.15 \pm 0.95^{**}$	$20.2 \pm 2.6^{**}$

Data are means $\pm$ S.D. of four experiments. The significance of difference between values in the presence of the surfactant and those in their absence was determined by Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$.

Fig. 1. Concentration Dependence of Effects of Cetyltrimethylammonium on the Flux (J) of Benzoic Acid

Data are means $\pm$ S.D. of four experiments. The significance of difference between the flux of benzoic acid without the surfactant and that with the surfactant was determined by Student's *t*-test: * $p < 0.05$, *** $p < 0.001$.

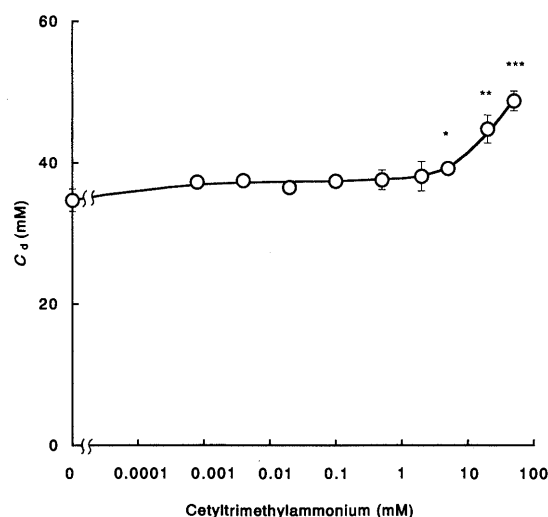
Fig. 2. Concentration Dependence of Effects of Cetyltrimethylammonium on the Apparent Permeability Coefficient (K_p) of Benzoic Acid

Apparent permeability coefficients were calculated using the solubility in the presence of the surfactant. Data are means $\pm$ S.D. of four experiments. The significance of difference between the permeability coefficient of benzoic acid without the surfactant and that with the surfactant was determined by Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$.

Results

Effects of Cetyltrimethylammonium and Other *n*-Alkyltrimethylammoniums on Skin Permeability of Benzoic Acid We first examined the effects of *n*-alkyltrimethylammoniums on the permeation of benzoic acid through guinea pig dorsal skin. As shown in Table 1, all three *n*-alkyltrimethylammoniums tested, *n*-dodecyltrimethylammonium, *n*-tetradecyltrimethylammonium and cetyltrimethylammonium, showed marked enhancement effects on the skin permeation of benzoic acid at 2 mM. The enhancement effects were larger than those of the anionic surfactant sodium dodecylsulfate (SDS). None of these surfactants induced any significant changes in the solubility of benzoic acid.

Observation of dose-dependent effects of cetyltrimethylammonium showed that the quaternary ammonium increased the flux of benzoic acid more than 2.5 times in the concentration range between 0.5 and 5 mM, as shown in Fig. 1. This concentration range was above the CMC of the surfactant (0.04 mM). The apparent permeability coefficient, which was calculated using the solubility of benzoic acid in the presence of cetyltrimethylammonium, was also increased more than 2.5 times in the same concentration range, as shown in Fig. 2. On the other hand, a high concentration (50 mM) of cetyltrimethylammonium decreased both the flux and the permeability coefficient. The solubility of benzoic acid

Fig. 3. Concentration Dependence of Effects of Cetyltrimethylammonium on the Solubility (C_d) of Benzoic Acid

Data are means $\pm$ S.D. of four experiments. The significance of difference between the solubility of benzoic acid without the surfactant and that with the surfactant was determined by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

increased at concentrations of more than 5 mM, as shown in Fig. 3.

Effects of Cetyltrimethylammonium on Skin Perme-

Table 2. Solubility (C_d), Flux (J) and Apparent Permeability Coefficients (K_p) of Benzoic Acid and Its 4-*n*-Alkyl Substituents in the Presence of 2 mM Cetyltrimethylammonium

Substituent	C_d (mM)	J ($\mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$)	K_p ($\times 10^{-2} \text{ cm} \cdot \text{h}^{-1}$)
None	35.8 $\pm$ 1.4 (34.7 $\pm$ 1.6)	10.7 $\pm$ 1.9*** (3.68 $\pm$ 0.71)	29.8 $\pm$ 5.1*** (10.6 $\pm$ 2.0)
Methyl	6.28 $\pm$ 0.33*** (3.80 $\pm$ 0.03)	1.85 $\pm$ 0.22*** (0.77 $\pm$ 0.22)	29.5 $\pm$ 3.2 (20.3 $\pm$ 5.8)
Ethyl	5.13 $\pm$ 0.50*** (3.34 $\pm$ 0.10)	1.62 $\pm$ 0.36 (1.16 $\pm$ 0.05)	31.5 $\pm$ 6.4 (34.8 $\pm$ 1.2)
<i>n</i> -Propyl	3.05 $\pm$ 0.07*** (0.70 $\pm$ 0.03)	0.87 $\pm$ 0.26* (0.44 $\pm$ 0.09)	28.6 $\pm$ 8.4** (62.7 $\pm$ 13.0)

Data are means $\pm$ S.D. of four experiments. Data in parentheses are those in the absence of cetyltrimethylammonium. The values of methyl, ethyl and *n*-propyl substituents in the absence of the surfactant were cited from Ref. 4. The significance of difference between values in the presence of the surfactant and those in its absence was determined by Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

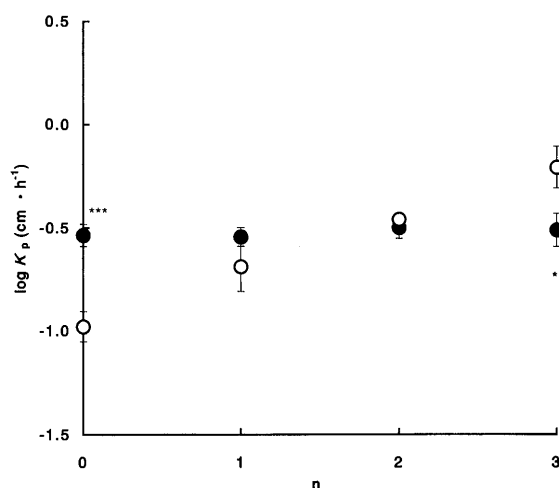


Fig. 4. Effects of 2 mM Cetyltrimethylammonium on Apparent Permeability Coefficients (K_p) of Benzoic Acid and Its 4-Alkyl Substituents through Guinea Pig Dorsal Skin

○, control; ●, with 2 mM cetyltrimethylammonium. *n* represents the number of carbon atoms of the 4-alkyl groups. Apparent permeability coefficients were calculated using the solubility in the presence of the surfactant. Data are means $\pm$ S.D. of four experiments. The significance of difference between the permeability coefficient of benzoic acid without the surfactant and that with the surfactant was determined by Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$.

ability of 4-Alkyl Substituents of Benzoic Acid We next examined the effects of 2 mM cetyltrimethylammonium on the skin permeation of 4-alkyl (methyl, ethyl, *n*-propyl) substituents of benzoic acid, in order to determine the effects of the cationic surfactant on permeants with different lipophilicity. The results are shown in Table 2. Fluxes of 4-methyl, 4-ethyl and 4-*n*-propyl substituents also increased compared with those in the absence of cetyltrimethylammonium. However, the enhancement effects were less than that on benzoic acid. Apparent permeability coefficients of the 4-alkyl substituents, which were calculated using the solubility in the presence of 2 mM cetyltrimethylammonium, were similar. As shown in Table 2 and Fig. 4, although the cationic surfactant induced about a 3-fold increase in the apparent permeability coefficient of benzoic acid, whose *n*-octanol/water partition coefficient is 1.87,⁹⁾ it did not induce any significant effect on the apparent permeability coefficients of 4-methyl and 4-ethyl substituents. In contrast, the cationic surfactant decreased that of the most lipophilic 4-*n*-propyl substituent.

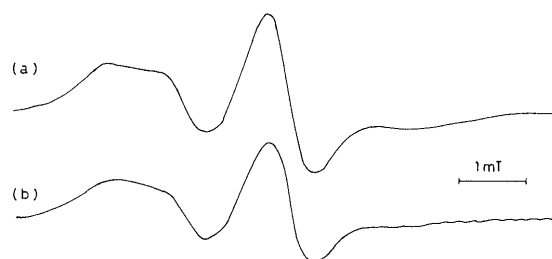


Fig. 5. ESR Spectra of CAT-15 in Excised Guinea Pig Dorsal Skin (a) and Its Stratum Corneum (b)

The solubility of 4-methyl, 4-ethyl and 4-*n*-propyl substituents of benzoic acid in the presence of 2 mM cetyltrimethylammonium, which are shown in Table 2, increased from those in the absence of the surfactant.

The free energy of transfer of the methylene group from the aqueous phase to skin, $\Delta(\Delta G)$, was calculated from the apparent permeability coefficients shown in Fig. 3, according to the following equation.^{4,10)}

$$\Delta(\Delta G) = -2.303 RT \Delta \log K_p \quad (1)$$

where *R* is the gas constant, *T* is the absolute temperature, and $\Delta \log K_p$ means the slope of $\log K_p$ vs. the carbon number plot shown in Fig. 4. The free energy of transfer in the absence of the surfactant was -1.50 kJ/mol, but its absolute value markedly decreased in the presence of 2 mM cetyltrimethylammonium ($\Delta(\Delta G) = -0.067$ kJ/mol). This finding is consistent with previous findings on the effects of other absorption enhancers such as *l*-menthol.⁴⁾

Analysis of ESR Spectra of CAT-15 We also examined the interaction of quaternary alkylammonium compounds with skin components using CAT-15 as a spin label. The broad signals in ESR spectra shown in Fig. 5 indicate that the spin labels were present in a nearly solid state in both excised skin and its stratum corneum. This finding was quite different from the previous findings on fatty acid spin labels such as 5-doxylstearic acid,^{8,11,12)} and 16-doxylstearic acid,¹³⁾ and from our findings on long-chain fatty acid methyl ester spin labels (data not shown).

Discussion

The present findings revealed that long-chain *n*-alkyltrimethylammoniums, such as cetyltrimethylammonium, have marked enhancement effects on the skin permeation of benzoic acid. The concentrations of alkyltrimethylammoniums which stimulated the permeation were above CMC (CMC values of *n*-dodecyltrimethylammonium, *n*-tetradecyltrimethylammonium and cetyltrimethylammonium were 1, 0.15 and 0.04 mM, respectively, in the experimental condition). The marked enhancement effects of the long-chain *n*-alkyltrimethylammoniums were consistent with those of other cationic surfactants such as dodecyltrimethylammonium.^{1,3)} On the other hand, a high concentration (50 mM) of cetyltrimethylammonium decreased the flux and permeability coefficient of benzoic acid, regardless of the increase in solubility. It has been reported that a high concentration of long-chain alkyltrimethylammoniums forms large rod-shaped micelles which have high viscosity.¹⁴⁾ This may decrease the partition or transfer rate of benzoic acid to the skin surface.

The present findings on the effects of cetyltrimethylammonium on the permeation of benzoic acid and its 4-alkyl substituents revealed a marked increase in the flux and permeability coefficient of relatively hydrophilic benzoic acid. Although the cationic surfactant also increased the fluxes of its 4-alkyl substituents, the enhancement effects were less. The cationic surfactant showed enhancement effects above CMC. The micelle formation may ease the penetration of the alkylammoniums to the stratum corneum. The apparent solubility of 4-alkyl substituents of benzoic acid, especially that of relatively lipophilic 4-*n*-propyl substituents, increased in the presence of 2 mM cetyltrimethylammonium. Therefore, most of the 4-*n*-propyl substituent molecules seem to have been present by being incorporated into the micelles or bound to their surface. Although the precise contribution of those molecules inside or on the surface of the micelles to the flux of the substituent is unclear, the molecules there also seem to at least partly permeate directly through the skin due to the interaction of the micelles with the stratum corneum mentioned above.

We analyzed the free energy of transfer of the methylene group of the 4-alkyl substituents of benzoic acid from the aqueous phase to the skin by using the apparent permeability coefficients which were obtained by assuming that free molecules of 4-alkyl substituents and the molecules inside or on the surface of the micelles equally partition into the skin surface. Our findings suggest that cetyltrimethylammonium made the skin relatively more hydrophilic compared to the medium. Although the energetic stability of the lipophilic substituents in the aqueous phase seems to have increased in the presence of the cationic surfactant, it is likely that the hydrophobicity of the skin itself was increased by the addition of the surfactant. Therefore, it is possible that this hydrophilic change in the skin may induce the largest enhancement effect on the flux of relatively hydrophilic benzoic acid, as well as increase its apparent permeability coefficient and decrease that of the lipophilic 4-*n*-propyl substituent.

ESR analysis suggested that long-chain quaternary alkylammonium cations were bound to proteins in the stratum corneum, probably keratin fiber. This is consistent with the suggestion that cationic surfactants penetrate and interact with proteins of the stratum corneum.¹⁵⁾ There is a possibility that the cationic surfactants alter the structure of keratin by uncoiling and extending its filaments.¹⁶⁾ Different from the cationic surfactants, long-chain fatty acids, which have been

reported to work as penetration enhancers,^{17,18)} seem to be present in the lipid lamella of the stratum corneum, as was also suggested by the ESR spectra of 5-doxyl stearic acid.⁸⁾ Like long-chain fatty acids, the cationic surfactants are also likely to interact with lipid lamella in the stratum corneum. Although the interaction with the lipid layer may also play a role, the interaction with proteins in the stratum corneum may be the main reason for the significant improvement of the hydrophilic property of the skin and their marked enhancement effects on the skin permeation of relatively hydrophilic drugs.

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References

- 1) Ashton P., Walters K. A., Brain K. R., Hadgraft J., *Int. J. Pharm.*, **87**, 261—264 (1992).
- 2) Walters K. A., Bialik W., Brain K. R., *Int. J. Cosmet. Sci.*, **15**, 260—270 (1993).
- 3) Ruddy S. B., "Percutaneous Penetration Enhancers," ed. by Smith E. W., Maibach H. I., CRC Press, Boca Raton, 1995, pp. 245—257.
- 4) Kitagawa S., Li H., *Chem. Pharm. Bull.*, **47**, 44—47 (1999).
- 5) Quan D., Maibach H. I., "Percutaneous Penetration Enhancers," ed. by Smith E. W., Maibach H. I., CRC Press, Boca Raton, 1995, pp. 427—439.
- 6) Coughlin R. T., Caldwell C. R., Haug A., McGroarty E. J., *Biochem. Biophys. Res. Comms.*, **100**, 1137—1142 (1981).
- 7) Kitagawa S., Kametani F., Tsuchiya K., Sakurai H., *Biochim. Biophys. Acta*, **1027**, 123—129 (1990).
- 8) Kitagawa S., Hosokai A., Kaseda Y., Yamamoto N., Kaneko Y., Matsuo E., *Int. J. Pharm.*, **161**, 115—122 (1998).
- 9) Leo A., Hansch C., Elkins D., *Chem. Rev.*, **71**, 525—616 (1971).
- 10) Itoh T., Xia J., Magavi R., Nishihata T., Rytting J. H., *Pharm. Res.*, **7**, 1042—1047 (1990).
- 11) Alonso A., Meirelles N. C., Yushmanov V. E., Tabak M., *J. Invest. Dermatol.*, **106**, 1058—1063 (1996).
- 12) Kawasaki Y., Quan D., Sakamoto K., Maibach H. I., *Dermatology*, **194**, 238—242 (1997).
- 13) Ogiso T., Iwaki M., Bechako K., Tsutsumi Y., *J. Pharm. Sci.*, **81**, 762—767 (1992).
- 14) Larsen J. W., Magid L. J., Payton V., *Tetrahedron Lett.*, **1973**, 2663—2666.
- 15) Ashton P., Walters K. A., Brain K. R., Hadgraft J., *Int. J. Pharm.*, **87**, 265—269 (1992).
- 16) Kushla G. P., Zatz J. L., *J. Pharm. Sci.*, **80**, 1079—1083 (1991).
- 17) Brain K. R., Walters K. A., *Drugs Pharm. Sci.*, **59**, 389—416 (1993).
- 18) Morimoto K., Haruta T., Tojima H., Takeuchi Y., *Drug Dev. Ind. Pharm.*, **21**, 1999—2012 (1995).

Screening Search for Organic Fluorophores: Syntheses and Fluorescence Properties of 3-Azolyl-7-diethylaminocoumarin Derivatives¹⁾

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In order to find a highly sensitive fluorophore, 3-azolyl-7-diethylaminocoumarin derivatives were synthesized. Both the absorption and fluorescence maxima of the coumarin-thiazole compounds showed red shifts with increases of the molar absorptivities and fluorescence intensities, in comparison with those of the corresponding coumarin-oxazole compounds. Among them, 3-(5-ethoxycarbonyl-1,3-thiazol-2-yl)-7-diethylamino-2H-chromen-2-one (3e) was one of the most promising candidates as a fluorophore accessible for analytical purposes in the fields of analytical and biological chemistry.

Key words fluorophore; diethylaminocoumarin; thiazole; oxazole; molar absorptivity; fluorescence intensity

Since fluorescence is highly sensitive to physicochemical environments, a variety of organic fluorescent compounds (fluorophores) have been widely used in many scientific fields, for example, as analytical tools such as fluorescent labeling reagents,²⁾ fluorescence probes,³⁾ fluorescence sensors,⁴⁾ and laser dyes.⁵⁾ However, the development of more highly sensitive fluorophores is still required.

In previous papers, we have shown that the introduction of a phenyl group at the 3-position of the coumarin ring causes bathochromic shifts in absorption and fluorescence maxima with increases in the molar absorptivity and fluorescence intensity. We developed 7-diethylamino-3-[4-(bromomethyl)phenyl]-2H-chromen-2-one (MPAC-Br)^{2a)} and 4-(7-diethylaminocoumarin-3-yl)benzoyl cyanide (DACB-CN)¹⁾ which are among the most sensitive and practically useful fluorescent derivatization reagents for carboxylic acids and for alcohols, respectively. In order to develop more highly sensitive fluorophores, the introduction of an azole in the place of the phenyl group at the 3-position of a coumarin ring was considered.

There are a few examples of studies⁶⁾ on 3-(benzazol-2-yl)coumarin derivatives, but little is known about the systematic study of 3-azolyl-substituted coumarins. This paper deals with the syntheses and spectroscopic properties of 3-azolyl-7-diethylaminocoumarins as screening in our search for fluorophores.

Results and Discussion

Synthesis of Coumarin-oxazole Derivatives (2a—g, 4a—c, 5) Twenty-eight compounds, [F]-azole-R (2—8) were synthesized, in which [F] represented 7-diethylamino-2-oxo-2H-chromen-3-yl (*i.e.* 7-diethylaminocoumarin-3-yl) (Charts 1 and 2). 2-[F]-Oxazole-R, 2a and 2c—f were synthesized from 7-diethylaminocoumarin-3-carboxamide (9) and the corresponding α -halocarbonyl compounds, respectively, according to a modified method of Liu *et al.*⁷⁾ 2-[F]-Oxazole-R, 2b and 2g were prepared by cyclization of the corresponding aroylaminomethyl ketones (10a, 10b) with phosphorus oxychloride, respectively, according to Heinze's method.⁸⁾ 5-[F]-Oxazole-R 4a was obtained from 7-diethylaminocoumarin-3-carbaldehyde (11) and tosylmethyl isocyanide in the presence of potassium carbonate in poor yield. 5-[F]-Oxazole-R 4b and 4c were synthesized from 3-

azidomethylcarbonylcoumarin (13) and benzoyl chloride or ethyl (chlorocarbonyl)formate, respectively. 4-(Coumarin-3-yl)-oxazole derivative 5 was prepared by condensation of a 3-bromoacetylcoumarin derivative (12) with benzamide in the presence of boron trifluoride etherate.

Synthesis of Coumarin-thiazole Derivatives (3a—h, 6a—c) 2-[F]-Thiazole-R 3a, 3b, and 3d—g were synthesized from 7-diethylaminocoumarin-3-carbothioamide (14) and the corresponding α -halocarbonyl compounds according to the modified methods of the oxazole synthesis described above. 2-[F]-Thiazole-R, 3c and 3h were prepared by condensation of the corresponding aroylaminomethyl ketones (10a, 10b) with phosphorus pentasulfide. 4-[F]-Thiazole-R, 6a—c, were prepared by condensation of 3-bromoacetylcoumarin derivative (12) with thioacetamide, ethyl thiooxamate, and thiobenzamide, respectively.

Synthesis of Coumarin-oxadiazole (7a—c) and Coumarin-thiadiazole Derivatives (8a—c) 2-[F]-Oxadiazole-R, 7a—c, were prepared by condensation of 7-diethylaminocoumarin-3-carbohydrazide (15) with ethyl orthoformate, trimethyl orthoacetate, and trimethyl orthobenzoate, respectively. Similarly, 2-[F]-thiadiazole-R, 8a—c, were obtained by condensation of coumarin-3-carbothiohydrazide (16) with ethyl orthoformate, trimethyl orthoacetate, and trimethyl orthobenzoate, respectively.

Absorption and Fluorescence Spectra Compound 1 was used as a standard sample for absorption and fluorescence spectra. The measurements of absorption and fluorescence spectra were carried out in ethanol solution at room temperature. Their spectroscopic properties, absorption maxima ($\lambda_{\max}$), molar absorptivities (ϵ), fluorescence maxima ($F\lambda_{\max}$), and the relative fluorescence intensities (RFI) are listed in Tables 1—3. The fluorescence sensitivity may be represented by the fluorescence intensity, that is, a peak-height at the fluorescence maximum. Unsubstituted [F]-azole-H, 2a, 3a, 4a, 7a, and 8a were defined as parent compounds (R=H) in 2-[F]-oxazole-R, 2-[F]-thiazole-R, 4-[F]-oxazole-R, 2-[F]-oxadiazole-R, and 2-[F]-thiadiazole-R series, respectively.

i) Influence of an Azole Introduction on the Spectroscopic Properties In comparison with the spectroscopic properties of the standard compound 1, both the absorption and fluorescence maxima ($\lambda_{\max}$ and $F\lambda_{\max}$) of all the [F]-

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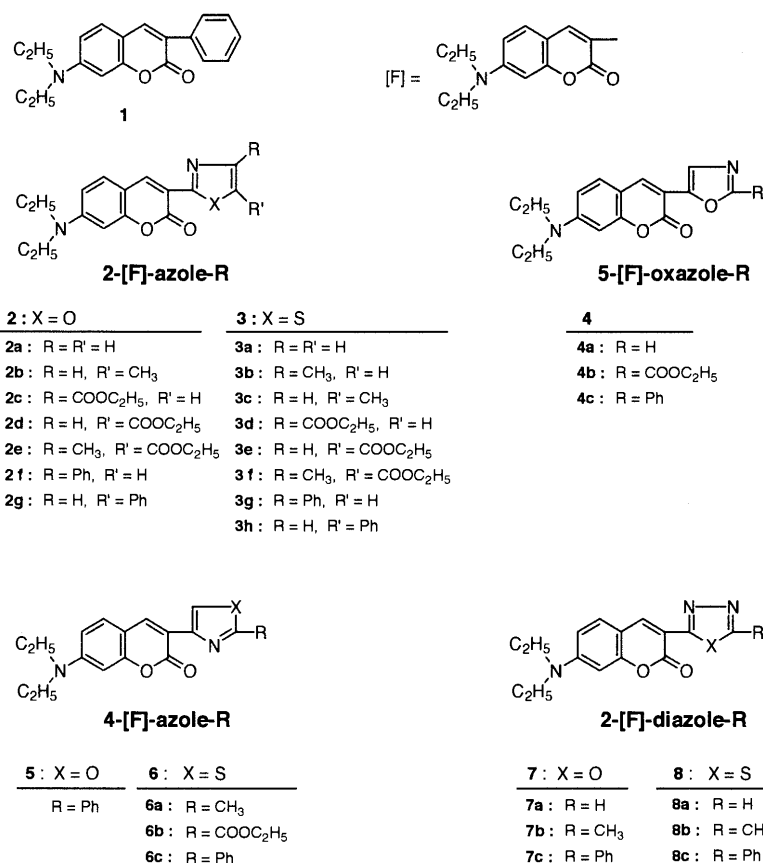


Chart 1

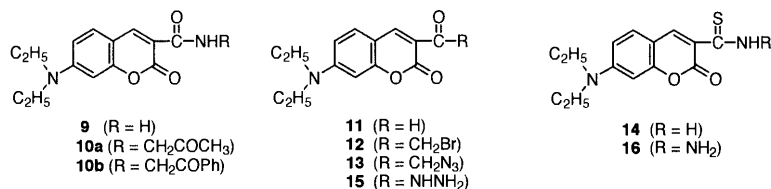


Chart 2

azole compounds (**2**–**8**) showed longer wavelength shifts, together with an increase in molar absorptivities (ϵ) (Tables 1–3). As for the relative fluorescence intensity (RFI), most of the azoles, except for 4-[F]-thiazoles (**6b**, **6c**) and 2-[F]-oxadiazoles (**7a**, **7b**), showed very similar or higher values than that of **1**.

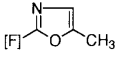
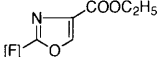
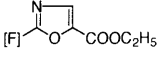
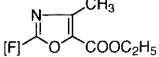
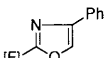
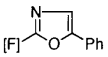
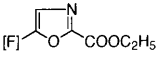
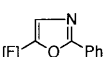
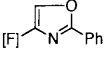
ii) Comparison of Spectroscopic Properties among [F]-Oxazoles, [F]-Thiazoles, and [F]-Diazoles Both the $\lambda_{\max}$'s and $F\lambda_{\max}$'s of 2-[F]-thiazole-R **3** shifted to longer wavelengths, accompanied by increases of the ϵ values and enhanced RFI's, compared with those of the corresponding 2-[F]-oxazole-R **2** (Table 1 and Table 2). Also, in comparison with [F]-diazole themselves, the $\lambda_{\max}$'s and the $F\lambda_{\max}$'s of **8** appeared at longer wavelengths, together with larger ϵ values and larger RFI's, compared with those of **7** (Table 3). In comparison among parent compounds themselves (2-[F]-azole-H; **2a**, **3a**, **7a**, and **8a**), the bathochromic and hypsochromic effects on the $\lambda_{\max}$ and ϵ value in the absorption spectra decreased in the order of **8a** > **3a** > **7a** > **2a**, but the values of the $F\lambda_{\max}$ and the RFI in the fluorescence spectra showed a tendency to decrease in the order of **3a** $\cong$ **8a** > **2a** $\cong$ **7a**. In the fluorescence spectra of diazoles, the values of the $F\lambda_{\max}$ of **7a**

and **8a** were similar to those of **2a** and **3a**, respectively. Furthermore, as for the RFI of 2-[F]-diazole-H (**7a**, **8a**), marked decreases were observed in comparison with those of the corresponding 2-[F]-azole-H (**2a**, **3a**).

Thus, the influence on the spectroscopic properties ($\lambda_{\max}$, $F\lambda_{\max}$, ϵ , RFI) of [F]-thiazoles was larger than those of [F]-oxazoles. This phenomenon may be explained in terms of the participation and delocalization of the lone pair of heteroatoms, predominantly *p* orbital electrons, on the basis of relative electronegativities of a sulfur and an oxygen atom. The extent of delocalization of the sulfur lone pair is larger than that of the oxygen one because of the higher nuclear charge of oxygen as compared with sulfur. Therefore, conjugation of the sulfur electrons with the four π electrons of the five-membered ring system is more complete than in the oxygen analogue. In addition, the low-lying *d* orbitals of sulfur play some role in the conjugation. As a result, thiazole compounds absorb at a longer wavelength than oxazole compounds. Such interpretation is put forward to explain the absorption spectra of five-membered heterocycles, in which thiophene absorbs at a longer wavelength than furan.⁹⁾

iii) Influence of the Positional Relation of [F] and a

Table 1. Spectral Properties of Coumarin-oxazole Derivatives (**2**, **4**, and **5**)

Compounds	Absorption ^{a)}		Fluorescence ^{b)}		
	$\lambda_{\max}$ (nm)	ϵ	Ex (nm)	F. $\lambda_{\max}$ (nm)	RFI ^{c)}
2a 	424	41100	424	481	1.02
2b 	425	42800	425	484	1.15
2c 	430	45200	430	483	0.96
2d 	443	48700	443	494	1.23
2e 	442	49700	442	493	1.49
2f 	428	43100	428	488	1.21
2g 	437	47300	437	498	1.32
4a 	422	40500	422	480	1.25
4b 	447	47900	447	504	1.45
4c 	433	46900	433	492	1.43
5 	411	38800	411	476	1.15
1 	398	34200	398	477	1.00

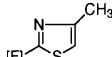
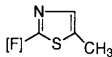
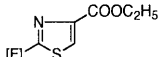
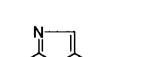
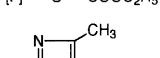
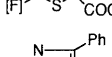
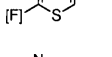
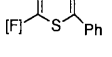
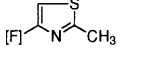
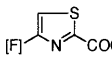
a) Concentration: 1.5×10^{-5} M. b) Concentration: 3.5×10^{-6} M. c) Relative fluorescence intensity: the fluorescence intensity of **1** is arbitrarily taken as 1.00.

Substituent (R) Connecting with an Azole Ring ([F]-azole-R) The influence of the connecting positions of two substituents ([F] and R) on an azole ring was investigated. The spectroscopic properties of four regioisomers of [F]-oxazole-Ph (positional isomers; **2f**, **2g**, **4c**, **5**) were compared (Table 4). The values of the $\lambda_{\max}$'s, the F. $\lambda_{\max}$'s, and the ϵ 's decreased in the order of 2-[F]-oxazole-5-Ph (**2g**) > 5-[F]-oxazole-2-Ph (**4c**) > 2-[F]-oxazole-4-Ph (**2f**) > 4-[F]-oxazole-2-Ph (**5**), but the order of the RFI's was **4c** > **2g** > **2f** > **5**. In [F]-thiazole-Ph, which is the sulfur analogue of the corresponding [F]-oxazole-Ph, a similar tendency was observed, in the order of **3h** > **3g** > **6c** for the $\lambda_{\max}$, the F. $\lambda_{\max}$, and the ϵ values, but in the order of **3g** = **3h** > **6c** for the RFI. Thus, in **2g**, **3h**, and **4c**, the presence of the conjugated system of a coumarin ring with a phenyl group through an azole ring was supported on the basis of their $\lambda_{\max}$'s appearing at longer wavelengths. However, both **5** and **6c** showed blue shifts in the $\lambda_{\max}$'s and F. $\lambda_{\max}$'s (**5**, $\lambda_{\max}$ 411 nm, F. $\lambda_{\max}$ 476 nm; **6c**, $\lambda_{\max}$ 412 nm, F. $\lambda_{\max}$ 477 nm), compared with compounds **2** and **3**. Also, in **6a**—**c** which contain the sulfur analogue of **5**, the $\lambda_{\max}$'s and F. $\lambda_{\max}$'s appeared at shorter wavelengths ($\lambda_{\max}$ 408—418 nm, F. $\lambda_{\max}$ 473—477 nm). Such blue shifts (hypsochromic effect) in the spectra of **5** and **6** seem to result from the conjugation of a coumarin ring with only a carbon—

carbon double bond moiety in an azole ring, not of the coumarin ring with a whole azole ring.

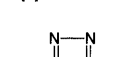
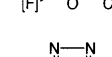
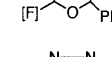
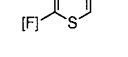
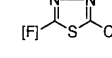
iv) Influence of Methyl, Ethoxycarbonyl, and Phenyl Substituents on an Azole Ring In order to determine the influence of each substituent, the spectroscopic properties of [F]-oxazole-R and [F]-thiazole-R (R = COOC₂H₅, Ph, CH₃) were compared with those of the corresponding parent compounds [F]-azole-H, respectively. In the substituent, the influence of 5-substituted compounds (2-[F]-thiazole-5-R; **3a**, **3c**, **3e**, **3h**), the values of the $\lambda_{\max}$'s and the ϵ 's decreased in the order of R = COOC₂H₅ > Ph > CH₃ > H, but on the F. $\lambda_{\max}$ in the order of R = COOC₂H₅ $\cong$ Ph > H $\cong$ CH₃. In 4-substituted compounds (2-[F]-thiazole-4-R), a similar tendency was observed. Thus, the ethoxycarbonyl substituent had the largest influence on the absorption and fluorescence properties. As for the methyl substituent ([F]-azole-5-CH₃; **2b**, **3c**, **7b**, and **8b**), the spectral values were either the same or slightly larger than those of the parent compounds (**2a**, **3a**, **7a**, **8a**), respectively. In addition, a methyl group at the 4-position of a thiazole ring had little influence on spectral properties. Also, in the cases of **2e** and **3f**, which have both an ethoxycarbonyl group and a methyl group, the values of the $\lambda_{\max}$, the F. $\lambda_{\max}$, and the ϵ were similar to those of **2d** and **3e**, having only an ethoxycarbonyl group, respectively. Thus, the introduction of

Table 2. Spectral Properties of Coumarin-thiazole Derivatives (**3** and **6**)

Compounds	Absorption ^{a)}		Fluorescence ^{b)}		
	$\lambda_{\max}$ (nm)	ϵ	Ex (nm)	F. $\lambda_{\max}$ (nm)	RFI ^{c)}
3a 	437	45100	437	495	1.42
3b 	437	45500	437	496	1.40
3c 	439	45500	439	493	1.40
3d 	449	50200	449	494	1.52
3e 	463	60100	463	512	1.93
3f 	463	59000	463	508	1.93
3g 	441	46400	441	498	1.47
3h 	453	54100	453	511	1.47
6a 	408	38900	408	475	1.16
6b 	418	39900	418	473	0.05
6c 	412	40100	412	477	0.72
1 	398	34200	398	477	1.00

a) Concentration: 1.5×10^{-5} M. b) Concentration: 3.5×10^{-6} M. c) Relative fluorescence intensity: the fluorescence intensity of **1** is arbitrarily taken as 1.00.

Table 3. Spectral Properties of Coumarin-diazole Derivatives (**7** and **8**)

Compounds	Absorption ^{a)}		Fluorescence ^{b)}		
	$\lambda_{\max}$ (nm)	ϵ	Ex (nm)	F. $\lambda_{\max}$ (nm)	RFI ^{c)}
7a 	429	44300	429	480	0.35
7b 	428	44300	428	481	0.47
7c 	437	50800	437	488	1.02
8a 	446	48600	446	494	1.01
8b 	446	48600	446	493	1.23
8c 	458	58500	458	502	1.86
1 	398	34200	398	477	1.00

a) Concentration: 1.5×10^{-5} M. b) Concentration: 3.5×10^{-6} M. c) Relative fluorescence intensity: the fluorescence intensity of **1** is arbitrarily taken as 1.00.

Table 4. Comparison of Spectral Properties among Four [F]-Oxazole-Ph

				
$\lambda_{\max}$ (nm)	437	433	428	411
ϵ	47300	46900	43100	38800
$F\lambda_{\max}$ (nm)	498	492	488	476
RFI	1.32	1.43	1.21	1.15

Table 5. Relative Quantum Yields of Selected Coumarin-thiazoles and Coumarin-oxazoles

Compounds	Excitation wavelengths (nm)	Relative quantum yields ^{a)}
1	398	1.59
2a	424	1.03
2b	430	1.17
2d	443	0.80
2g	430	1.09
3a	437	1.15
3c	445	1.12
3e	465	1.04
3h	445	1.02

a) The quantum yield of fluorescein in 0.1 N NaOH solution is arbitrarily taken as 1.00.

a methyl substituent on the azole ring had little influence on spectral properties in both the oxazole and thiazole series.

Next, the influence of the position of a substituent at the 4- or 5-position on an azole ring was examined. In 2-[F]-azole-COOC₂H₅, 5-substituted compounds (**2d**, **3e**) showed larger $\lambda_{\max}$ values, $F\lambda_{\max}$'s, and ϵ 's in comparison with 4-substituted compounds (**2c**, **3d**; 2-[F]-azole-4-COOC₂H₅). In comparisons between 4- and 5-substituted compounds themselves in the oxazole and thiazole series, the influence of an ethoxycarbonyl or a phenyl substituent in the 5-position was greater than that of the same substituent in the 4-position. Such influence of an ethoxycarbonyl group was also observed in the structure of **4b**, which is a positional isomer of **2d**.

In general, the introduction of an electron-withdrawing substituent at the 5-position on 2-[F]-azoles caused bathochromic shifts, with larger ϵ and enhanced RFI. This suggests the correlation of a push-pull system between a C7-electron donating group on a coumarin ring and a C5-electron-withdrawing group on an azole ring. Such a correlation may be explained analogously to that of a push-pull system between a C7-electron donating group and a C3-electron-withdrawing group on the coumarin ring.^{2a,10)}

v) Quantum Yields of Selected Coumarin-azoles

The quantum yields of selected coumarin-thiazoles and coumarin-oxazoles showed smaller values than that of **1** (Table 5). Compared with the parent compounds themselves, the quantum yield of **3a** showed a slightly larger value than that of **2a**. Other 2-[F]-thiazole-R (**3c**, **3e**, **3h**) also showed similar values to the corresponding 2-[F]-oxazole-R (**2b**, **2g**), except for **2d**. It was difficult to explain why the quantum yield of **2d** showed a lower value than those of the other 2-[F]-azole-R (**2b**, **2g** and **3c**, **3e**, **3h**).

In conclusion, among the coumarin-azoles synthesized in

this study, 2-[F]-thiazoles (**3**) were found to be most appropriate to fluorophore for fluorometric analysis, and the introduction of an electron-withdrawing substituent at the 5-position in this system would predominantly contribute to the enhancement of the relative fluorescence intensity of 7-diethylamino-3-thiazol-4-yl-chromen-2-one. Therefore, in the molecular design of strongly fluorescing coumarin-azoles, 2-[F]-thiazole-R **3** is one of the most promising candidates as a fluorophore from the viewpoint of larger molar absorptivity and stronger fluorescence intensity. Of these, **3e** showed the largest molar absorptivity and the highest fluorescence intensity. In addition, some of the coumarin-azoles synthesized in this study may serve as fluorophores for new application in various science fields, since they have excitation maxima at a longer wavelength region, being above 400 nm.

Experimental

All melting points were determined on a Yamato melting point apparatus (model MP-21) and are uncorrected. Infrared spectra (IR) were recorded on a JASCO FT/IR-300 spectrometer. Nuclear magnetic resonance spectra (NMR) were taken on JEOL JNM-LA-300 and JEOL JNM-EX-400 spectrometers. Mass spectra (MS) were measured with a Shimadzu GC MS-9100-MK gas chromatograph-mass spectrometer with a direct inlet system. Absorption and fluorescence spectra were measured with a Hitachi 288 dual-wavelength spectrophotometer, and a Hitachi F-4100 fluorescence spectrophotometer. Fluorescence quantum yields were measured with a Hitachi F-4500 fluorescence spectrometer equipped with an R928 photomultiplier (200–900 nm), and were determined according to the method of Parker and Rees,¹¹⁾ as reported previously,¹²⁾ in which fluorescein (uranine) in 0.1 N NaOH solution was used as a standard.¹³⁾

Materials and Methods Fluorescein sodium (uranine, JIS special grade reagent) and ethanol (for fluorometry, Uvasol) were purchased from Kanto Chemical Co., Inc.

7-Diethylamino-3-phenyl-2H-chromen-2-one (1) Compound **1** was synthesized according to the procedure described in a previous paper.^{2a)}

7-Diethylamino-2-oxo-2H-chromene-3-carboxamide (9) Compound **9** was obtained by ammonolysis of coumarin-3-carboxylic acid ester (ethyl 7-diethylamino-2-oxo-2H-chromene-3-carboxylate; 4.0 g, 13.8 mmol) with 25% NH₄OH (90 ml) in DMF (90 ml) at room temperature for 16 h. Yield, 82%, mp 218–219 °C (lit.,¹⁴⁾ mp 229–230 °C. ¹H-NMR (CDCl₃) δ : 1.24 (t, 6H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 3.46 (q, 4H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 5.76 (s, 1H, $-NH_2$), 6.50 (d, 1H, $J=2.4$ Hz, aromH), 6.64 (dd, 1H, $J=2.4$ Hz, 9.0 Hz, aromH), 7.42 (d, 1H, $J=9.0$ Hz, aromH), 8.55 (s, 1H, $-NH_2$), 8.71 (s, 1H, aromH). IR (Nujol) cm⁻¹: 1640, 1710. MS m/z : 260 (M⁺).

N-(2-Oxopropyl)-7-diethylamino-2-oxo-2H-chromene-3-carboxamide (10a) Isobutyl chloroformate (273 mg, 2.2 mmol) was added to a solution of coumarin-3-carboxylic acid (7-diethylamino-2-oxo-2H-chromene-3-carboxylic acid; 520 mg, 2.0 mmol) and triethylamine (404 mg, 4.0 mmol) in THF at room temperature, and stirred for several min. Powdered aminoacetone hydrochloride¹⁵⁾ was added to the mixture, and the reaction mixture was further stirred for 16 h. After evaporating THF *in vacuo*, the residue was chromatographed on silica gel using AcOEt as the eluent to give **10a**. Yellow needles (from EtOH), yield 56%, mp 186–188 °C. ¹H-NMR (CDCl₃) δ : 1.24 (t, 6H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 2.24 (s, 3H, $-CH_3$), 3.45 (q, 4H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 4.30 (d, 2H, $J=4.8$ Hz, $-CH_2-$), 6.50 (d, 1H, $J=2.4$ Hz, aromH), 6.64 (dd, 1H, $J=2.4$ Hz, 8.9 Hz, aromH), 7.42 (d, 1H, $J=8.9$ Hz, aromH), 8.67 (s, 1H, aromH). IR (Nujol) cm⁻¹: 1650, 1700, and 1730. MS m/z : 316 (M⁺).

N-(2-Oxo-2-phenylethyl)-7-diethylamino-2-oxo-2H-chromene-3-carboxamide (10b) Compound **10b** was prepared from coumarin-3-carboxylic acid (780 mg, 3.0 mmol) and aminoacetophenone hydrochloride (669 mg, 3.9 mmol) in a similar procedure (reaction temperature and time: room temperature, 16 h) to that carried out in the preparation of **10a**. Yellow prisms (from CHCl₃-hexane), yield 61%, mp 210–214 °C. ¹H-NMR (CDCl₃) δ : 1.25 (t, 6H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 3.46 (q, 4H, $J=7.2$ Hz, $-N(CH_2CH_3)_2$), 4.96 (d, 2H, $J=4.7$ Hz, $-CH_2-$), 6.53 (d, 1H, $J=2.3$ Hz, aromH), 6.65 (dd, 1H, $J=2.3$ Hz, 8.9 Hz, aromH), 7.47 (d, 1H, $J=8.9$ Hz, aromH), 7.5 (m, 3H, aromH), 8.0 (m, 2H, aromH), 8.72 (s, 1H, aromH). IR (Nujol) cm⁻¹: 1680, 1700, and 1750. MS m/z : 378 (M⁺).

7-Diethylamino-2-oxo-2H-chromene-3-carbaldehyde (11) Vilsmeier reagent was prepared from DMF (5 ml) and POCl₃ (1.53 g, 10 mmol) under

Table 6. Physical and Spectral Data of Coumarin-azole Derivatives (2—8)

Compd.	mp (°C)	Appearance (Solvent)	IR (Nujol) (cm ⁻¹)	MS (<i>m/z</i>) (M ⁺)	Formula	Analysis (%) Calcd (Found)			
						C	H	N	S
2a	142.5—144.0	Orange needles (AcOEt-hexane)	1720	284	C ₁₆ H ₁₆ N ₂ O ₃	67.59 (67.36)	5.67 (5.69)	9.74 (9.85)	
2b	136—138	Orange prisms (Benzene-hexane)	1730	298	C ₁₇ H ₁₈ N ₂ O ₃	68.44 (68.61)	6.08 (6.16)	9.39 (9.39)	
2c	126—128	Yellow prisms (AcOEt-hexane)	1720	356	C ₁₉ H ₂₀ N ₂ O ₅	64.02 (63.98)	5.66 (5.67)	7.86 (7.83)	
2d	154—155	Orange needles (AcOEt-hexane)	1740	356	C ₁₉ H ₂₀ N ₂ O ₅	64.02 (63.84)	5.66 (5.77)	7.86 (7.76)	
2e	181—184	Orange prisms (AcOEt-hexane)	1720 1740	370	C ₂₀ H ₂₂ N ₂ O ₅	64.84 (64.91)	5.99 (6.05)	7.57 (7.58)	
2f	158.5—160.0	Reddish orange needles (AcOEt-hexane)	1700	360	C ₂₂ H ₂₀ N ₂ O ₃	73.32 (73.06)	5.59 (5.59)	7.77 (7.54)	
2g	163—165	Reddish orange needles (CHCl ₃ -hexane)	1720	360	C ₂₂ H ₂₀ N ₂ O ₃	73.32 (73.09)	5.59 (5.49)	7.77 (7.67)	
3a	95—98	Orange prisms (AcOEt-hexane)	1700	300	C ₁₆ H ₁₆ N ₂ O ₂ S	63.98 (63.83)	5.37 (5.43)	9.33 (9.19)	10.65 (10.65)
3b	162—163	Yellow needles (CHCl ₃ -hexane)	1700	314	C ₁₇ H ₁₈ N ₂ O ₂ S	64.95 (65.14)	5.78 (5.86)	8.92 (8.83)	10.18 (10.33)
3c	173—175	Yellow prisms (EtOH)	1710	314	C ₁₇ H ₁₈ N ₂ O ₂ S	64.95 (64.84)	5.78 (5.77)	8.92 (8.81)	10.18 (10.15)
3d	157—158	Reddish orange prisms (CHCl ₃ -hexane)	1700	372	C ₁₉ H ₂₀ N ₂ O ₄ S	61.27 (61.11)	5.42 (5.46)	7.53 (7.51)	8.59 (8.63)
3e	215—217	Reddish orange needles (CHCl ₃ -hexane)	1700	372	C ₁₉ H ₂₀ N ₂ O ₄ S	61.27 (61.21)	5.42 (5.54)	7.53 (7.36)	8.59 (8.66)
3f	210—211	Orange needles (CHCl ₃ -hexane)	1700	386	C ₂₀ H ₂₂ N ₂ O ₄ S	62.16 (61.95)	5.74 (5.75)	7.25 (7.08)	8.28 (8.30)
3g	126—128 ^{a)}	Orange needles (CHCl ₃ -hexane)	1710	376	C ₂₂ H ₂₀ N ₂ O ₂ S	70.19 (70.26)	5.35 (5.39)	7.44 (7.26)	8.52 (8.65)
3h	200—202	Yellow prisms (AcOEt-hexane)	1720	376	C ₂₂ H ₂₀ N ₂ O ₂ S	70.19 (69.86)	5.35 (5.43)	7.44 (7.25)	8.52 (8.59)
4a	181—182	Yellow orange prisms (AcOEt-hexane)	1720	284	C ₁₆ H ₁₆ N ₂ O ₃	67.59 (67.73)	5.67 (5.51)	9.74 (9.76)	
4b	145—146	Orange prisms (AcOEt-hexane)	1710 1730	356	C ₁₉ H ₂₀ N ₂ O ₅	64.02 (64.06)	5.66 (5.74)	7.86 (7.90)	
4c	191—193	Yellow prisms (AcOEt-hexane)	1720	360	C ₂₂ H ₂₀ N ₂ O ₃	73.32 (73.44)	5.59 (5.46)	7.77 (7.90)	
5	173—174	Orange prisms (AcOEt-hexane)	1700	360	C ₂₂ H ₂₀ N ₂ O ₃	73.32 (73.44)	5.59 (5.46)	7.77 (7.68)	
6a	141.0—142.5	Yellow needles (AcOEt-hexane)	1700	314	C ₁₇ H ₁₈ N ₂ O ₂ S	64.95 (65.06)	5.78 (5.83)	8.92 (8.84)	10.18 (10.25)
6b	141—142	Yellow needles (AcOEt-hexane)	1710	372	C ₁₉ H ₂₀ N ₂ O ₄ S	61.27 (61.28)	5.42 (5.47)	7.53 (7.46)	8.59 (8.65)
6c	127—129 ^{b)}	Yellow needles (AcOEt-hexane)	1710	376	C ₂₂ H ₂₀ N ₂ O ₄ S	70.19 (70.35)	5.35 (5.49)	7.44 (7.30)	8.52 (8.58)
7a	192—194	Yellow needles (CHCl ₃ -hexane)	1720	285	C ₁₅ H ₁₅ N ₃ O ₃	63.15 (62.98)	5.30 (5.35)	14.73 (14.70)	
7b	121—123	Orange prisms (AcOEt-hexane)	1740	299	C ₁₆ H ₁₇ N ₃ O ₃	64.20 (64.27)	5.72 (5.89)	14.04 (13.97)	
7c	201—203	Yellow needles (CHCl ₃ -hexane)	1720	361	C ₂₁ H ₁₉ N ₃ O ₃	64.95 (64.84)	5.78 (5.77)	11.63 (11.61)	
8a	242—245	Orange prisms (AcOEt-hexane)	1700	301	C ₁₅ H ₁₅ N ₃ O ₂ S	59.78 (59.53)	5.02 (5.10)	13.95 (14.06)	10.62 (10.49)
8b	201—203	Yellow needles (CHCl ₃ -hexane)	1710	315	C ₁₆ H ₁₇ N ₃ O ₂ S	60.93 (60.91)	5.44 (5.54)	13.33 (13.48)	10.15 (9.95)
8c	262—263	Yellow needles (CHCl ₃ -hexane)	1700	377	C ₂₁ H ₁₉ N ₃ O ₂ S	66.82 (66.58)	5.08 (5.20)	11.14 (11.25)	8.48 (8.32)

a) **3g**, lit.,¹⁸⁾ mp 130—133 °C. b) **6c**, lit.,²⁰⁾ mp 130—133 °C.

cooling. The resulting reagent was added dropwise to a solution of 7-diethylaminocoumarin [7-diethylamino-chromen-2-one] (432 mg, 2 mmol) in DMF (2 ml), and the mixture was stirred at room temperature for 16 h. The reaction mixture was poured into a saturated NaHCO₃ solution, then extracted with AcOEt. The AcOEt extraction was washed with brine and dried over MgSO₄. After evaporation, the residue was purified by column chromatography on silica gel (AcOEt:hexane=2:1, v/v). Orange needles (from

CHCl₃-hexane), yield 49%, mp 159—160 °C (lit.,¹⁶⁾ mp 160—162 °C). ¹H-NMR (CDCl₃) δ: 1.26 (t, 6H, *J*=7.2 Hz, -N(CH₂CH₃)₂), 3.47 (q, 4H, *J*=7.2 Hz, -N(CH₂CH₃)₂), 6.49 (d, 1H, *J*=2.5 Hz, aromH), 6.64 (dd, 1H, *J*=2.5 Hz, 8.9 Hz, aromH), 7.42 (d, 1H, *J*=8.9 Hz, aromH), 8.25 (s, 1H, aromH), 10.13 (s, 1H, -CHO). IR (Nujol) cm⁻¹: 1680, 1710. MS *m/z*: 245 (M⁺).

3-(2-Bromoacetyl)-7-diethylamino-2H-chromen-2-one (12) A solu-

Table 7. ¹H-NMR Data of Coumarin-azole Derivatives (2–8)

Compounds	¹ H-NMR (CDCl ₃) δ (ppm)
2a	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.45 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.63 (dd, 1H, <i>J</i> = 2.4 Hz, 9.0 Hz, aromH), 7.25 (d, 1H, <i>J</i> = 0.7 Hz, aromH), 7.74 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 7.75 (d, 1H, <i>J</i> = 0.7 Hz, aromH), 8.39 (s, 1H, aromH)
2b	1.23 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 2.41 (d, 3H, <i>J</i> = 1.1 Hz, –CH ₃), 3.44 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.51 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.62 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 6.88 (q, 1H, <i>J</i> = 1.1 Hz, aromH), 7.37 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.34 (s, 1H, aromH)
2c	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.41 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.43 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.64 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.38 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.31 (s, 1H, aromH), 8.61 (s, 1H, aromH)
2d	1.25 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.41 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.42 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.64 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.40 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.88 (s, 1H, aromH), 8.49 (s, 1H, aromH)
2e	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.42 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 2.56 (s, 3H, –CH ₃), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.41 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.64 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.39 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.47 (s, 1H, aromH)
2f	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.45 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.54 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.62 (dd, 1H, <i>J</i> = 2.4 Hz, 9.1 Hz, aromH), 7.3 (m, 1H, aromH), 7.4 (m, 3H, aromH), 7.8 (m, 2H, aromH), 8.03 (s, 1H, aromH), 8.54 (s, 1H, aromH)
2g	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.45 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.53 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.64 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.3 (m, 3H, aromH), 7.40 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.48 (s, 1H, aromH), 7.7 (m, 2H, aromH), 8.40 (s, 1H, aromH)
3a	1.25 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.57 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.66 (dd, 1H, <i>J</i> = 2.4 Hz, 9.0 Hz, aromH), 7.40 (d, 1H, <i>J</i> = 3.3 Hz, aromH), 7.44 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 7.89 (d, 1H, <i>J</i> = 3.3 Hz, aromH), 8.72 (s, 1H, aromH)
3b	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 2.52 (d, 3H, <i>J</i> = 0.9 Hz, –CH ₃), 3.45 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.56 (d, 1H, <i>J</i> = 2.6 Hz, aromH), 6.65 (dd, 1H, <i>J</i> = 2.6 Hz, 9.0 Hz, aromH), 6.96 (d, 1H, <i>J</i> = 0.9 Hz, aromH), 7.43 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 8.69 (s, 1H, aromH)
3c	1.23 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 2.52 (d, 3H, <i>J</i> = 1.1 Hz, –CH ₃), 3.45 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.55 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.64 (dd, 1H, <i>J</i> = 2.4 Hz, 9.0 Hz, aromH), 7.41 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 7.53 (q, 1H, <i>J</i> = 1.1 Hz, aromH), 8.64 (s, 1H, aromH)
3d	1.25 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 1.44 (t, 3H, <i>J</i> = 7.3 Hz, –COOCH ₂ CH ₃), 3.46 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 4.45 (q, 2H, <i>J</i> = 7.3 Hz, –COOCH ₂ CH ₃), 6.56 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.67 (dd, 1H, <i>J</i> = 2.4 Hz, 8.8 Hz, aromH), 7.46 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 8.19 (s, 1H, aromH), 8.89 (s, 1H, aromH)
3e	1.26 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.39 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 3.47 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.38 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.56 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.68 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.45 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.45 (s, 1H, aromH), 8.78 (s, 1H, aromH)
3f	1.25 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.38 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 2.78 (s, 3H, –CH ₃), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.34 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.55 (d, 1H, <i>J</i> = 2.6 Hz, aromH), 6.67 (dd, 1H, <i>J</i> = 2.6 Hz, 9.0 Hz, aromH), 7.45 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 8.76 (s, 1H, aromH)
3g	1.25 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.46 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.59 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.66 (dd, 1H, <i>J</i> = 2.4 Hz, 8.8 Hz, aromH), 7.3 (m, 3H, aromH), 7.48 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 7.57 (s, 1H, aromH), 8.0 (m, 2H, aromH), 8.85 (s, 1H, aromH)
3h	1.25 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.58 (d, 1H, <i>J</i> = 2.5 Hz, aromH), 6.67 (dd, 1H, <i>J</i> = 2.5 Hz, 8.9 Hz, aromH), 7.3 (m, 3H, aromH), 7.45 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.6 (m, 2H, aromH), 8.06 (s, 1H, aromH), 8.72 (s, 1H, aromH)
4a	1.23 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.44 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.53 (d, 1H, <i>J</i> = 2.6 Hz, aromH), 6.63 (dd, 1H, <i>J</i> = 2.6 Hz, 8.8 Hz, aromH), 7.36 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 7.77 (s, 1H, aromH), 7.89 (s, 1H, aromH), 7.98 (s, 1H, aromH)
4b	1.24 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.47 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 3.46 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.50 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.53 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.65 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.40 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.93 (s, 1H, aromH), 8.20 (s, 1H, aromH)
4c	1.22 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.41 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.50 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.62 (dd, 1H, <i>J</i> = 2.4 Hz, 9.1 Hz, aromH), 7.36 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.5 (m, 3H, aromH), 7.85 (s, 1H, aromH), 7.99 (s, 1H, aromH), 8.0 (m, 2H, aromH)
5	1.23 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.43 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.55 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.63 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.41 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 7.5 (m, 3H, aromH), 8.1 (m, 2H, aromH), 8.44 (s, 2H, aromH)
6a	1.22 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 2.76 (s, 3H, –CH ₃), 3.42 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.61 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.38 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.12 (s, 1H, aromH), 8.56 (s, 1H, aromH)
6b	1.23 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 1.47 (t, 3H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 3.43 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 4.51 (q, 2H, <i>J</i> = 7.2 Hz, –COOCH ₂ CH ₃), 6.52 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.63 (dd, 1H, <i>J</i> = 2.4 Hz, 9.0 Hz, aromH), 7.42 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 8.57 (s, 1H, aromH), 8.74 (s, 1H, aromH)
6c	1.22 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.42 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.52 (d, 1H, <i>J</i> = 2.5 Hz, aromH), 6.62 (dd, 1H, <i>J</i> = 2.5 Hz, 8.8 Hz, aromH), 7.3 (m, 3H, aromH), 7.4 (m, 4H, aromH), 8.0 (m, 2H, aromH), 8.29 (s, 1H, aromH), 8.73 (s, 1H, aromH)
7a	1.26 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.47 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.53 (d, 1H, <i>J</i> = 2.5 Hz, aromH), 6.65 (dd, 1H, <i>J</i> = 2.5 Hz, 8.8 Hz, aromH), 7.41 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 8.49 (s, 1H, aromH), 8.52 (s, 1H, aromH)
7b	1.25 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 2.63 (s, 3H, –CH ₃), 3.46 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.51 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.65 (dd, 1H, <i>J</i> = 2.4 Hz, 8.8 Hz, aromH), 7.38 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 8.43 (s, 1H, aromH)
7c	1.25 (t, 6H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 3.47 (q, 4H, <i>J</i> = 7.3 Hz, –N(CH ₂ CH ₃) ₂), 6.53 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.65 (dd, 1H, <i>J</i> = 2.4 Hz, 8.8 Hz, aromH), 7.42 (d, 1H, <i>J</i> = 8.8 Hz, aromH), 7.5 (m, 3H, aromH), 8.2 (m, 2H, aromH), 8.52 (s, 1H, aromH)
8a	1.26 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.48 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.57 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.69 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.48 (d, 1H, <i>J</i> = 8.9 Hz, aromH), 8.92 (s, 1H, aromH), 9.14 (s, 1H, aromH)
8b	1.25 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 2.80 (s, 3H, –CH ₃), 3.47 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.55 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.67 (dd, 1H, <i>J</i> = 2.4 Hz, 9.0 Hz, aromH), 7.45 (d, 1H, <i>J</i> = 9.0 Hz, aromH), 8.83 (s, 1H, aromH)
8c	1.27 (t, 6H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 3.48 (q, 4H, <i>J</i> = 7.2 Hz, –N(CH ₂ CH ₃) ₂), 6.58 (d, 1H, <i>J</i> = 2.4 Hz, aromH), 6.71 (dd, 1H, <i>J</i> = 2.4 Hz, 8.9 Hz, aromH), 7.5 (m, 4H, aromH), 8.0 (m, 2H, aromH), 8.97 (s, 1H, aromH)

tion of 3-acetyl-7-diethylamino-2*H*-chromen-2-one¹⁴⁾ (5.2 g, 20 mmol) and tetra-*n*-butyl ammonium tribromide (19.3 g, 40 mmol) in CH₂Cl₂ (100 ml) was stirred at room temperature for 2 d. The resulting precipitates were

collected on a filter funnel and washed with AcOEt. Yellow prisms, yield 81%, mp 211–214 °C. IR (Nujol) cm^{–1}: 1710. MS *m/z*: 338 (M⁺). Product **12** was used in the next step without further purification.

3-(2-Azidoacetyl)-7-diethylamino-2H-chromen-2-one (13) One milliliter of an aqueous NaN_3 (488 mg, 7.5 mmol) and AcOH (0.43 ml, 7.5 mmol) were added to a solution of **12** (1.01 g, 3.0 mmol) in DMF (15 ml), and the reaction mixture was allowed to stand for 2 d at room temperature. The resulting precipitates were collected on a filter funnel. Orange needles, yield 62%, mp 172–175 °C. $^1\text{H-NMR}$ (CDCl_3) δ : 1.26 (t, 6H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 4.96 (s, 2H, $-\text{COCH}_2\text{N}_3$), 5.22 (q, 4H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 6.49 (d, 1H, $J=2.6$ Hz, aromH), 6.65 (dd, 1H, $J=2.6$ Hz, 8.8 Hz, aromH), 7.36 (d, 1H, $J=8.8$ Hz, aromH), 8.56 (s, 1H, aromH). IR (Nujol) cm^{-1} : 1670, 1710. MS m/z : 300 (M^+). Product **13** was used in the next step without further purification.

7-Diethylamino-2-oxo-2H-chromene-3-carbothioamide (14) A mixture of **9** (7.8 g, 30 mmol) and Lawesson's reagent (6.5 g, 16 mmol) in dioxane (170 ml) was refluxed for 18 h. After removing dioxane *in vacuo*, the residue was chromatographed on a silica gel column (eluent, CHCl_3) to give **14**. Yellow needles (from CHCl_3 -hexane), yield 71%, mp 216–218 °C. $^1\text{H-NMR}$ (CDCl_3) δ : 1.26 (t, 6H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 3.48 (q, 4H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 6.48 (d, 1H, $J=2.4$ Hz, aromH), 6.67 (dd, 1H, $J=2.4$ Hz, 9.0 Hz, aromH), 7.50 (d, 1H, $J=9.0$ Hz, aromH), 7.77 (s, 1H, $-\text{NH}_2$), 9.34 (s, 1H, aromH), 10.38 (s, 1H, $-\text{NH}_2$). IR (Nujol) cm^{-1} : 1690. MS m/z : 276 (M^+).

7-Diethylamino-2-oxo-2H-chromene-3-carbohydrazide (15) Compound **15** was prepared from ethyl 7-diethylaminocoumarin-3-carboxylate and hydrazine according to the modified procedure in the literature.¹⁷ Hydrazine monohydrate (3.0 ml, 60 mmol) was added to a solution of ethyl 7-diethylaminocoumarin-3-carboxylate (4.3 g, 15 mmol) in EtOH (40 ml), and the reaction mixture was stirred at room temperature for 12 min. After cooling in an ice bath for 15 min, the precipitates were collected on a filter funnel. Orange needles (from CHCl_3 -hexane), yield 50%, mp 160–165 °C. $^1\text{H-NMR}$ (CDCl_3) δ : 1.24 (t, 6H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 3.46 (q, 4H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 4.14 (s, 2H, $-\text{NHNH}_2$), 6.50 (d, 1H, $J=2.4$ Hz, aromH), 6.65 (dd, 1H, $J=2.4$ Hz, 9.0 Hz, aromH), 7.51 (d, 1H, $J=9.0$ Hz, aromH), 9.23 (s, 1H, aromH), 10.38 (s, 1H, $-\text{NHNH}_2$). IR (Nujol) cm^{-1} : 1700. MS m/z : 275 (M^+).

7-Diethylamino-2-oxo-2H-chromene-3-carbothiohydrazide (16) A solution of **15** (825 mg, 3.0 mmol) and Lawesson's reagent (606 mg, 1.5 mmol) in dioxane (170 ml) was refluxed for 23 h. After removing dioxane *in vacuo*, the residue was chromatographed on a silica gel column (eluent, hexane:AcOEt=2:1, v/v) to give **16**. Brownish needles (from CHCl_3 -hexane), yield 18%, mp 209–212 °C. $^1\text{H-NMR}$ (CDCl_3) δ : 1.25 (t, 6H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 3.47 (q, 4H, $J=7.2$ Hz, $-\text{N}(\text{CH}_2\text{CH}_3)_2$), 5.57 (s, 2H, $-\text{NHNH}_2$), 6.49 (d, 1H, $J=2.4$ Hz, aromH), 6.69 (dd, 1H, $J=2.4$ Hz, 9.0 Hz, aromH), 7.51 (d, 1H, $J=9.0$ Hz, aromH), 9.23 (s, 1H, aromH), 12.33 (s, 1H, $-\text{NHNH}_2$). IR (Nujol) cm^{-1} : 1680. MS m/z : 291 (M^+).

Preparation of 2a and 2c–2f: 7-Diethylamino-3-(1,3-oxazol-2-yl)-2H-chromen-2-one (2a) A solution of **9** (364 mg, 1.4 mmol) and bromoacetaldehyde (467 mg, 3.8 mmol) in DMF (10 ml) was refluxed for 2.5 h. After removing the solvent *in vacuo*, the residue was purified by column chromatography (hexane:AcOEt=1:1, v/v). Yield 23%. Similarly, **2c**, **2d**, **2e**, and **2f** were prepared from **9** and the corresponding ethyl bromopyruvate, ethyl 2-chloro-3-oxopropanoate, ethyl 2-chloroacetate, and bromoacetophenone in 14, 15, 49, and 9% yields, respectively.

Preparation of 2b and 2g: 7-Diethylamino-3-(5-methyl-1,3-oxazol-2-yl)-2H-chromen-2-one (2b) A mixture of **10a** (316 mg, 1 mmol) and POCl_3 (1.5 g, 10 mmol) was refluxed for 1 h. The reaction mixture was neutralized with a saturated aqueous NaHCO_3 solution, then extracted with CHCl_3 . The extract was washed with brine, dried over MgSO_4 , concentrated to dryness and the residue was purified by column chromatography using AcOEt-MeOH (9:1, v/v) as an eluent to give **2b**. Yield 72%. Similarly, **2g** was obtained from **10b**, in almost quantitative yield.

Preparation of 3a, 3b, and 3d–3g: 7-Diethylamino-3-(1,3-thiazol-2-yl)-2H-chromen-2-one (3a) A solution of coumarin-3-thioamide **14** (300 mg, 1.1 mmol) and bromoacetaldehyde (262 mg, 2.1 mmol) in DMF (10 ml) was stirred in the presence of K_2CO_3 (965 mg, 7.0 mmol) at room temperature for 7.5 h under an argon atmosphere. The reaction mixture was poured into brine, then extracted with AcOEt. The organic layer was washed with brine and dried over MgSO_4 . After evaporating the solvent, the residue was diluted with CHCl_3 (12 ml). To the resulting solution was added pyridinium *p*-toluenesulfonate (30 mg, 0.1 mmol), and the mixture was refluxed for 4.5 h. After removing the solvent *in vacuo*, the residue was purified by column chromatography (hexane:AcOEt=2:1, v/v). Yield 58%. Similarly, **3b**, **3d**, **3e**, **3f**, and **3g**¹⁸ were prepared from **14** and the corresponding bromoacetone, ethyl bromopyruvate, ethyl 2-chloro-3-oxopropanoate, ethyl 2-chloroacetate, and bromoacetophenone in 39, 68, 87, 14, and 93%

yields, respectively.

Preparation of 3c and 3h: 7-Diethylamino-3-(5-methyl-1,3-thiazol-2-yl)-2H-chromen-2-one (3c) A mixture of **10a** (95 mg, 0.3 mmol) and P_2S_5 (200 mg, 0.5 mmol) in CHCl_3 (6 ml) was refluxed for 2.5 h. After cooling, the reaction mixture was washed with brine and dried over MgSO_4 . After removing the solvent *in vacuo*, the residue was purified by recrystallization from EtOH to give yellow prisms. Yield 53%. Similarly, **3h** was obtained by cyclization of **10b** with P_2S_5 in 42% yield.

Preparation of 7-Diethylamino-3-(1,3-oxazol-5-yl)-2H-chromen-2-one (4a) Compound **4a** was prepared according to the modified method in the literature.¹⁹ A solution of 7-diethylaminocoumarin-3-carbaldehyde **11** (735 mg, 3 mmol) and [(4-methylphenyl)sulfonyl]-methaneisocyanide (585 mg, 3 mmol) in MeOH (50 ml) was refluxed in the presence of K_2CO_3 (414 mg, 3 mmol) for 5 h. After removing the solvent *in vacuo*, the residue was dissolved in AcOEt. The solution was washed brine and dried over MgSO_4 . After removing the solvent, the residue was purified by column chromatography (hexane:AcOEt=3:1, v/v). Yield 5%.

Preparation of 4b and 4c: Ethyl 5-(7-Diethylamino-2-oxo-2H-chromen-3-yl)-1,3-oxazole-2-carboxylate (4b) A mixture of azidoacetyl compound **13** (200 mg, 0.75 mmol), Ph_3P (263 mg, 1.0 mmol) and ethyl (chlorocarbonyl)formate (102 mg, 0.7 mmol) in THF (6 ml) was refluxed for 4 h. After removing the solvent, the residue was dissolved in CHCl_3 , washed with a saturated aqueous NaHCO_3 , brine, and dried over MgSO_4 . After evaporation, the residue was purified by column chromatography using CHCl_3 -EtOH (5:1, v/v) as an eluent to give **4b**. Yield 15%. Similarly, **4c** was prepared from azidoacetyl compound **13** and benzoyl chloride in 56% yield.

Preparation of 7-Diethylamino-3-(2-phenyl-1,3-oxazol-4-yl)-2H-chromen-2-one (5) A mixture of bromoacetyl compound **12** (838 mg, 2.0 mmol), benzamide (726 mg, 6.0 mmol), and $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.04 ml, 0.3 mmol) in DMF (8 ml) was heated at 130 °C for 24 h. After cooling, the reaction mixture was poured into a saturated aqueous NaHCO_3 solution, and was extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4 , and evaporated to dryness. The residue was purified by column chromatography using CHCl_3 -EtOH (5:1, v/v) as an eluent to give **5**. Yield 10%.

Preparation of 7-Diethylamino-3-(2-methyl-1,3-thiazol-4-yl)-2H-chromen-2-one (6a) To a solution of **12** (210 mg, 0.6 mmol) and thioacetamide (150 mg, 2.0 mmol) in DMF (3 ml) was added K_2CO_3 (138 mg, 1.0 mmol), and the mixture was stirred at room temperature for 3 d. After the reaction, the reaction mixture was poured into brine and extracted with AcOEt. The organic layer was washed with brine and dried over MgSO_4 . After evaporation, the residue was purified by column chromatography using hexane-AcOEt (3:1, v/v) as an eluent to give **6a**. Yield 58%.

Preparation of Ethyl 4-(7-Diethylamino-2-oxo-2H-chromen-3-yl)-1,3-thiazole-2-carboxylate (6b) A solution of **12** (36 mg, 0.3 mmol) and ethyl thiooxamate (ethyl 2-amino-2-thioacetate) (36 mg, 0.1 mmol) in DMF (1 ml) was heated at 120 °C for 2 h. After the reaction, the reaction mixture was poured into brine and extracted with AcOEt. The organic layer was washed with brine and dried over MgSO_4 . After evaporation, the residue was purified by column chromatography using hexane-AcOEt (3:1, v/v) as an eluent to give **6b**. Yield 71%.

Preparation of 7-Diethylamino-3-(2-phenyl-1,3-thiazol-4-yl)-2H-chromen-2-one (6c) Compound **6c** was prepared from **12** (328 mg, 1 mmol) and thiobenzamide (274 mg, 2 mmol) using the same procedure as above (reaction conditions: DMF 6 ml, 120 °C, 4 d). Yield 29% (lit.,²⁰ mp 130–133 °C).

Preparation of 7a, 7b and 7c: 7-Diethylamino-3-(1,3,4-oxadiazol-2-yl)-2H-chromen-2-one (7a) A mixture of 7-diethylaminocoumarin-3-carbohydrazide **15** (550 mg, 2 mmol) and $\text{HC}(\text{OC}_2\text{H}_5)_3$ (3.0 g, 20 mmol) was heated at 180 °C for 40 min. After removing excess $\text{HC}(\text{OC}_2\text{H}_5)_3$ *in vacuo*, the residue was dissolved in CHCl_3 (12 ml), then $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.02 ml, 0.16 mmol) was added to the solution, which was refluxed for 2 d. After cooling, the reaction mixture was treated with aqueous NaHCO_3 solution. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography (AcOEt). Yield 39%. Similarly, **7b** and **7c** were obtained from **15** and the corresponding $\text{CH}_3\text{C}(\text{OCH}_3)_3$ and $\text{PhC}(\text{OCH}_3)_3$ in 92 and 41% yields, respectively. In the case of **7b**, pyridinium *p*-toluenesulfonate in place of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ was used (refluxed for 24 h).

Preparation of 8a–8c: 7-Diethylamino-3-(1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (8a) A mixture of coumarin-3-carbothiohydrazide **16** (52 mg, 0.18 mmol) and $\text{HC}(\text{OC}_2\text{H}_5)_3$ (891 mg, 6.0 mmol) was heated at 160 °C for 2 h. After removing excess $\text{HC}(\text{OC}_2\text{H}_5)_3$ *in vacuo*, the residue was

purified by column chromatography (CHCl_3). Yield 79%. Similarly, **8b** and **8c** were prepared from **16** and the corresponding $\text{CH}_3\text{C}(\text{OCH}_3)_3$ and $\text{PhC}(\text{OCH}_3)_3$, in 55 and 41% yields, respectively.

The physical and analytical data of coumarin-azole compounds (**2**–**8**) are listed in Tables 6 and 7.

Fluorescence Quantum Yields of Selected Compounds Absorbances of the sample solution were kept below 0.2 at the excitation wavelength. Since the quantum yield of fluorescein is dependent on changes in excitation wavelengths,¹³ the relative quantum yields were taken as the ratio of [F]-azoles quantum yields to that of the fluorescein at each different excitation wavelength.

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References

- 1) Fluorescent Labeling Reagents. Part 3. Part 2: Takechi H., Goto Y., Machida M., *Chem. Pharm. Bull.*, **46**, 159–162 (1998).
- 2) a) Takechi H., Kamada S., Machida M., *Chem. Pharm. Bull.*, **44**, 793–799 (1996); b) Machida M., Machida M. I., Sekine T., Kanaoka Y., *ibid.*, **15**, 1678–1684 (1977).
- 3) a) Minta A., Kao J. P. Y., Tsien R. Y., *J. Biol. Chem.*, **264**, 8171–8178 (1989) and references cited therein; b) Grynkiewicz G., Poenie M., Tsien R. Y., *ibid.*, **260**, 3440–3450 (1985); c) Wang X., Krebs L. J., Al-Nuri M., Pudavar H. E., Ghosal S., Liebow C., Nagy A. A., Schally A. V., Prasad P. N., *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 11081–11084 (1999).
- 4) de Silva A. P., Gunaratne H. Q. N., Gunnlauugsson T., Huxley A. J. M., McCoy C. P., Rademacher J. T., Rice T. E., *Chem. Rev.*, **97**, 1515–1566 (1997).
- 5) Rettig W., *Angew. Chem. Int. Ed. Engl.*, **25**, 971–988 (1986).
- 6) a) Jones G. II, Jimenez J. A. C., *Tetrahedron Lett.*, **40**, 8551–8555 (1999); b) Jones G. II, Jackson W. R., Choi C.-Y., Bergmark W. R., *J. Phys. Chem.*, **89**, 294–300 (1985); c) Jones G. II, Griffin S. F., Choi C.-Y., Bergmark W. R., *J. Org. Chem.*, **49**, 2705–2708 (1984); d) Reynolds G. A., Drexhage K. H., *Opt. Commun.*, **13**, 222–225 (1975).
- 7) Liu P., Celatka C. A., Panek J. S., *Tetrahedron Lett.*, **38**, 5445–5448 (1997).
- 8) Heinze J., Baumgärtel H., *Chem. Ber.*, **103**, 1572–1577 (1970).
- 9) Jaffe H. H., Orchin M., "Theory and Applications of Ultraviolet Spectroscopy," John Wiley and Sons, Inc., New York, 1964, pp. 345–361.
- 10) Takadate A., Masuda T., Murata C., Shibuya M., Isobe A., *Chem. Pharm. Bull.*, **48**, 256–260 (2000) and references cited therein.
- 11) Parker C. A., Rees W. T., *Analyst*, **85**, 587–600 (1960).
- 12) Kanaoka Y., Machida M., Kokubun H., Sekine T., *Chem. Pharm. Bull.*, **16**, 1747–1753 (1968).
- 13) Dawson W. R., Windsor M. W., *J. Phys. Chem.*, **72**, 3251–3260 (1968).
- 14) Czerney P., Hartmann H., *J. Prakt. Chem.*, **324**, 21–28 (1982).
- 15) Hepworth J. D., "Organic Syntheses," Coll. Vol. 5, ed. by Baumgarten H. E., John Wiley and Sons, Inc., New York, 1973, pp. 27–29.
- 16) Komlev I. V., Tavrizova M. A., Khrolova O. R., Mikhailova T. A., *Zh. Obshch. Khim.*, **55**, 888–892 (1985) [*Chem. Abstr.*, **103**, 72603s (1985)].
- 17) Fujino H., Eguchi M., Goya S., *Yakugaku Zasshi*, **110**, 155–158 (1990).
- 18) Vamvakaris C., Patsch M., Mach W., Ger. Patent 2807761 (1979) [*Chem. Abstr.*, **92**, 41931x (1980)].
- 19) Anderson B. A., Becke L. M., Booher R. N., Flaugh M. E., Harn N. K., Kress T. J., Varie D. L., Wepsiec J. P., *J. Org. Chem.*, **62**, 8634–8639 (1997).
- 20) Hartmann H., Czerney P., Liebscher J., East Ger. Patent DD 204094 (1983) [*Chem. Abstr.*, **101**, 7145p (1984)].

Constituents of the Vietnamese Medicinal Plant *Orthosiphon stamineus*

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From the MeOH extract of the aerial part of Vietnamese *Orthosiphon stamineus*, five new isopimarane-type diterpenes [orthosiphols F–J (1–5)] and two new diterpenes [staminols A (6) and B (7)] with a novel carbon-framework, to which we proposed the name “staminane”, and three new highly-oxygenated staminane-type diterpenes [staminolactones A (8) and B (9) and norstaminol A (10)] were isolated. Moreover, staminolactone A (8) is 8,14-secostaminane-type and staminolactone B (9) is 13,14-secostaminane-type, while norstaminol A (10) is 14-norstaminane-type. Together with these new diterpenes, sixteen known compounds were also isolated and identified to be: 7,3',4'-tri-*O*-methylluteolin (11), eupatorin (12), sinensetin (13), 5-hydroxy-6,7,3',4'-tetramethoxyflavone (14), salvigenin (15), ladanein (16), tetramethylscutellarein (17), 6-hydroxy-5,7,4'-trimethoxyflavone (18), vomifoliol (19), aurantiamide acetate (20), rosmarinic acid (21), caffeic acid (22), oleanolic acid (23), ursolic acid (24), betulinic acid (25), and β -sitosterol (26). All the isolated compounds were tested for their cytotoxicity towards highly liver metastatic murine colon 26-L5 carcinoma cells, and the new diterpenes, except for 4, and flavonoids (11, 12, 16, 18) showed cytotoxicity with an ED₅₀ value between 10 and 90 μ g/ml.

Key words *Orthosiphon stamineus*; staminane-type diterpene; Vietnamese medicinal plant; isopimarane-type diterpene; cytotoxicity; colon 26-L5 carcinoma

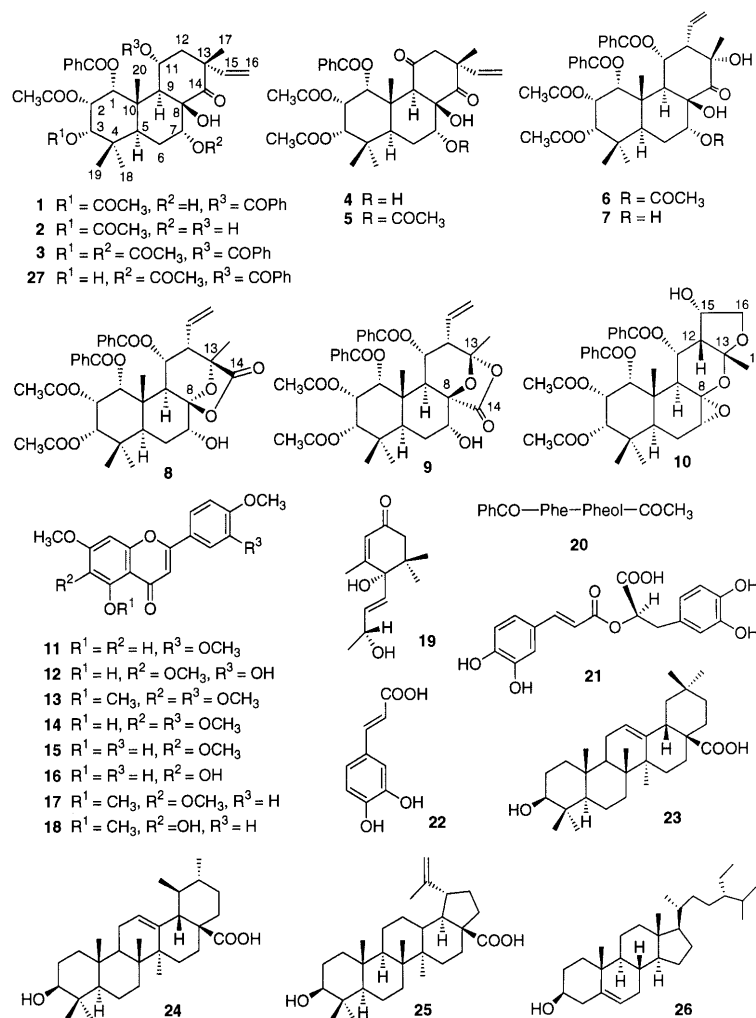
Orthosiphon (*O.*) *stamineus* BENTH. [syn.: *O. grandiflorus* BOLD., *O. spicatus* (THUNB.) BAK., *O. aristatus* (BL.) MIQ.; Lamiaceae] is a medicinal plant grown in Southeast Asia and is currently cultivated in Indonesia.¹⁾ In Indonesia, the leaves of this plant are used by the name of “Kumis kucing” as a diuretic, and to treat rheumatism, diabetes, hypertension, etc.,^{1a)} while in Vietnam its aerial part is used by the name of “Râu mèo” in treating urinary lithiasis, edema, eruptive fever, influenza, rheumatism, hepatitis, jaundice, and biliary lithiasis.^{1b)} From its popularity and demonstrated effectiveness, phytochemical and pharmacological studies have been conducted since the 1930's,^{2,3)} and highly-oxygenated isopimarane-type diterpenes, orthosiphols A–E,^{2a–c)} were reported, together with monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene, and myo-inositol. More recently, Shibuya *et al.* reported two isopimarane-type diterpenes,⁴⁾ two migrated pimarane-type diterpenes,⁵⁾ and a benzochromene⁴⁾ from the leaves of *O. aristatus* (= *O. stamineus*) in Indonesia. Recently, in the course of our study on Vietnamese medicinal plants,⁶⁾ we found that a MeOH extract from the aerial part of *O. stamineus* showed a cytotoxic activity against highly liver metastatic murine colon 26-L5 carcinoma cells.⁷⁾ Thus, we examined its chemical constituents and identified ten new diterpenes 1–10, including five (6–10) with a novel carbon-framework, together with sixteen known compounds. In this paper, we report the isolation and structural elucidation of the new diterpenes by spectroscopic techniques, together with their cytotoxic activities.⁸⁾

Isolation The dried aerial part of Vietnamese *O. stamineus* was extracted with refluxing MeOH, and the MeOH extract (ED₅₀ for cytotoxicity, 73.6 μ g/ml) was separated into hexane- (ED₅₀, >100 μ g/ml), CHCl₃- (ED₅₀, 76.2 μ g/ml), EtOAc- (ED₅₀, 82.4 μ g/ml), BuOH- (ED₅₀, >100 μ g/ml), and water-soluble (ED₅₀, >100 μ g/ml) fractions. The CHCl₃- and EtOAc-soluble fractions with cytotoxicity were separated, respectively, by a combination of silica-gel column chromatography and normal- and reversed-phase preparative TLC tech-

niques to give five new isopimarane-type diterpenes [orthosiphols F–J (1–5)], two new diterpenes [staminols A (6) and B (7)] with a novel carbon-framework, to which we proposed the name “staminane”, and three new highly-oxygenated staminane-type diterpenes [staminolactones A (8) and B (9), norstaminol A (10)] together with sixteen known compounds. The known compounds were identified by analyses of their spectroscopic data and comparison of the spectral data with those in the literature (11–19)^{2c,9)} or in our laboratory (20–26)^{6a,10)} to be: 7,3',4'-tri-*O*-methylluteolin (11), eupatorin (12), sinensetin (13), 5-hydroxy-6,7,3',4'-tetramethoxyflavone (14), salvigenin (15), ladanein (16), tetramethylscutellarein (17), 6-hydroxy-5,7,4'-trimethoxyflavone (18), vomifoliol (19), aurantiamide acetate (20), rosmarinic acid (21), caffeic acid (22), oleanolic acid (23), ursolic acid (24), betulinic acid (25), and β -sitosterol (26). The presence of 19 and 20 in *O. stamineus* is reported here for the first time.

Structures of Isopimarane-Type Diterpenes Orthosiphol F (1) was obtained as a colorless amorphous solid with an $[\alpha]_D^{25}$ value of -82.8° (CHCl₃). High-resolution FAB-MS (HR-FAB-MS) of 1 indicated the molecular formula C₃₈H₄₄O₁₁ (*m/z* 676), the same as that of orthosiphol A (27), and its IR spectrum showed absorption due to hydroxyl (3550, 3450 cm⁻¹) and ester carbonyl (1725 cm⁻¹) groups, similar to those of 27.⁶⁾ The ¹H- and ¹³C-NMR data (Tables 1, 2) also closely resembled those of 27,⁶⁾ but they were characterized by a downfield shift of H-3 (1, δ 4.99; 27, δ 3.49) and an upfield shift of H-7 (1, δ 4.22; 27, δ 5.43). Thus, orthosiphol F appeared to be 3-*O*-acetyl-7-*O*-deacetyl-orthosiphol A, which was confirmed by the ¹H–¹H shift correlation spectroscopy (COSY), heteronuclear shift correlation (HETCOR), and heteronuclear multiple bond correlation (HMBC) spectra (Fig. 1a, Table 2). Based on the nuclear Overhauser effects (NOEs) observed in the difference NOE experiment (Fig. 1c), the rings A and B were determined to have a chair conformation with the same configuration as 27, while the ring C was indicated, by the NOEs from H-9 to H-

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Chart 1. Structures of Constituents of Aerial Part of *Orthosiphon stamineus*

16 and from H₃-20 to H-11, to have a boat conformation with the vinyl and 11-*O*-benzoyl groups at the α -orientation. From these spectral data, orthosiphol F was concluded to be 3-*O*-acetyl-7-*O*-deacetylorthosiphol A (1).

Orthosiphol G (2), $[\alpha]_D^{25} -63.3^\circ$ (CHCl₃), and orthosiphol H (3), $[\alpha]_D^{25} -58.0^\circ$ (CHCl₃), were obtained as amorphous solids and displayed almost identical IR spectrum with that of 1. Their molecular formulas were determined by HR-FAB-MS to be C₃₁H₄₀O₁₀ (M.W. 572) and C₄₀H₄₆O₁₂ (M.W. 718), respectively, which suggested that 2 has one less benzoyl group and 3 has one more acetyl group than 1. The ¹H- and ¹³C-NMR data for 2 and 3 (Tables 1, 2) were also similar to each other and to those of 1, but those of 2 were characterized by the lack of signals of one of two benzoyl groups and those of 3 by presence of the signals of one more acetyl group. The locations of the debenzoylation in 2 and of the additional acetyl group in 3 were supposed to be at C-11 and at C-7, respectively, based on the upfield shift of H-11 in 2 (δ 4.43) and the downfield shift of H-7 in 3 (δ 5.45), compared to those of 1 (H-11, δ 5.64; H-7, δ 4.22). From these data, together with the analyses of the COSY, HETCOR, HMBC, and difference NOE spectra, orthosiphols G and H were determined to be 11-*O*-debenzoylorthosiphol F (2) and 3-*O*-acetylorthosiphol A (3), respectively.

Orthosiphol I (4), $[\alpha]_D^{25} -108.9^\circ$ (CHCl₃), and orthosiphol J (5), $[\alpha]_D^{25} -58.5^\circ$ (CHCl₃), were also obtained as amor-

phous solids. Their molecular formulas were determined by HR-FAB-MS to be C₃₁H₃₈O₁₀ (MW 570) and C₃₃H₄₀O₁₁ (MW 612), respectively. They showed ¹H- and ¹³C-NMR data similar to each other and to those of 1–3, and those of 5 showed the presence of one more acetyl group than 4 (Tables 1, 2). The ¹H- and ¹³C-NMR data for 4 were similar to those of orthosiphol G (2), but they were characterized by a lack of the signals of one (C-11) of two hydroxymethines in 2 and by the presence of a signal due to a ketone-carbonyl carbon. Thus, 4 was believed to have a carbonyl group instead of a hydroxyl group at C-11 in 2, and 5 was a 7-*O*-acetate of 4, which was confirmed by analyses of the COSY, heteronuclear multiple-quantum coherence (HMQC), and HMBC spectra. Based on these data and the results of difference NOE experiments, orthosiphols I and J were determined to be 11-dehydroxy-11-oxoorthosiphol G (4) and its 7-*O*-acetate (5), respectively.

Structures of Stamineane-Type Diterpenes Staminol A (6) was obtained as a colorless amorphous solid with an $[\alpha]_D^{25}$ value of -24.3° (CHCl₃), and its molecular formula was determined by HR-FAB-MS to be C₄₀H₄₆O₁₃ (MW 734). The IR spectrum of 6 showed the absorption of hydroxyl (3550, 3430, 3300 cm⁻¹), ester carbonyl (1725 cm⁻¹), and phenyl (1600, 1450 cm⁻¹) groups. The ¹H-NMR spectrum of 6 revealed signals due to four tertiary methyls, a vinyl, five oxygenated methines, and three aliphatic methines, together

Table 1. ^1H -NMR Data for Orthosiphols F—J (1—5) in CDCl_3 (J in Hz)

	1	2	3	4	5
1	5.29 d (3)	5.60 d (2.5)	5.16 d (2)	6.39 d (3)	6.35 d (3)
2	5.54 t (3)	5.52 dd (3.5, 2.5)	5.51 dd (4, 2)	5.61 t (3)	5.60 t (3)
3	4.99 d (3)	5.05 d (3.5)	4.99 d (4)	5.08 d (3)	5.06 d (3)
5	2.69 dd (13, 2.5)	2.58 m	2.48 dd (12.5, 3)	2.47 dd (13.5, 2.5)	2.26 m
6	2.03 ddd (14, 13, 2.5)	1.82 m	2.04 m	2.04 ddd (15, 13.5, 2.5)	2.24 m
	1.89 dt (14, 2.5)	1.95 m	2.02 m	1.84 dt (15, 2.5)	1.98 m
7	4.22 t (2.5)	4.14 brt (3)	5.45 t (3)	4.29 brt (2.5)	5.33 brt (2.2)
9	3.04 d (4.5)	2.60 d (5)	3.18 d (6.5)	3.49 s	3.52 s
11	5.64 ddd (4.5, 4, 2.5)	4.43 m	5.80 ddd (6.5, 5, 2)		
12	2.21 dd (15, 2.5)	2.35 dd (14.5, 5.5)	2.58 dd (15.5, 5)	2.66 d (18)	2.63 d (18.3)
	2.73 dd (15, 4)	1.73 dd (14.5, 4)	1.95 dd (15.5, 2)	2.76 d (18)	2.69 d (18.3)
15	5.77 dd (17.5, 11)	5.87 dd (17.5, 11)	5.67 dd (17, 11)	5.35 dd (17.5, 11)	5.28 dd (17.3, 10.7)
16	4.71 d (11)	4.69 d (11)	4.75 d (11)	4.16 d (11)	4.12 d (10.7)
	4.88 d (17.5)	4.87 d (17.5)	4.81 d (17)	4.67 d (17.5)	4.66 d (17.3)
17	1.26 s	1.23 s	1.13 s	1.14 s	1.11 s
18	0.99 s	1.00 s	0.90 s	0.98 s	0.90 s
19	1.12 s	1.13 s	1.14 s	1.12 s	1.12 s
20	1.47 s	1.45 s	1.52 s	1.42 s	1.44 s
1-OCOPh					
2',6'	7.59 dd (8, 1)	8.09 d (7.5)	7.70 dd (8, 1)	8.11 dd (8, 1)	8.11 d (7.3)
3',5'	7.21 t (8)	7.43 t (7.5)	7.32 t (8)	7.46 t (8)	7.43 t (7.3)
4'	7.44 dt (8, 1)	7.55 t (7.5)	7.54 tt (8, 1)	7.58 tt (8, 1)	7.58 t (7.3)
2-OCOCH ₃					
1''	1.82 s	1.97 s	1.84 s	1.95 s	1.95 s
3-OCOCH ₃					
1'''	1.50 s	1.66 s	1.44 s	1.77 s	1.76 s
7-OCOCH ₃					
1''''			2.19 s		2.06 s
11-OCOPh					
2''''',6'''''	7.49 dd (8, 1)		7.60 dd (8, 1)		
3''''',5'''''	6.99 t (8)		7.12 t (8)		
4'''''	7.31 dt (8, 1)		7.42 tt (8, 1)		

with those of two benzoyl and three acetyl groups (Table 3). Moreover, its ^{13}C -NMR spectrum (Table 4) indicated the presence of six (a ketone and five ester) carbonyl groups and four quaternary carbons, including two oxygenated ones. Analyses of these signals by the COSY and HETCOR spectra led to the partial structures depicted in Fig. 1b, which were connected based on the long-range correlations observed in the HMBC spectrum (Fig. 1b, Table 4). The stereostructure of **6** was elucidated from the J values of each proton and from the results of difference NOE experiments. The large J values for H-5, H-9, and H-11 ($J_{5,6\text{ax}}=13.5\text{ Hz}$; $J_{9,11}=11\text{ Hz}$) indicated their axial nature, while the small J values for H-7 and H-12 ($J_{6\text{eq},7}=3\text{ Hz}$; $J_{6\text{ax},7}=0\text{ Hz}$; $J_{11,12}=3.5\text{ Hz}$) suggested them to be equatorial. In the difference NOE experiments, the same NOEs were detected on rings A and B, while on ring C NOEs were observed from H₃-20 to H-11, from H-9 to H-15, and from H₃-17 to H-11 and H-12 (Fig. 1d). Thus, ring C was determined to have a chair conformation with the configuration indicated in Fig. 1d. From these data, the structure of staminol A was concluded to be **6**.

Staminol B (**7**), $[\alpha]_{\text{D}}^{25} -27.8^\circ$ (CHCl_3), showed the molecular formula $\text{C}_{38}\text{H}_{44}\text{O}_{12}$, $\text{C}_2\text{H}_2\text{O}$ less than that of **6**, in HR-FAB-MS. The ^1H - and ^{13}C -NMR data for **7** were similar to those of **6** (Tables 3, 4), but they showed the presence of only two acetyl groups instead of the three in **6**. Analysis of the COSY and HMQC spectra revealed an upfield shift of H-7 (δ 4.19), compared to that of **6** (δ 5.37), indicating that the free hydroxyl group should be located at C-7. Thus, the structure of staminol B was determined to be 7-*O*-deacetylstaminol A

(**7**).

Structures of Highly-Oxygenated Staminane-Type Diterpenes Staminolactones A (**8**) and B (**9**) were obtained as colorless amorphous solids, and their molecular formulas were determined to be the same ($\text{C}_{38}\text{H}_{42}\text{O}_{12}$, MW 690) by HR-FAB-MS. Their IR spectra were similar to each other and also to that of **7**, and showed the absorption of a hydroxyl group, an ester carbonyl, and a phenyl ring. They were, however, characterized by the presence of an absorption of γ -lactone carbonyl (**8**, 1800 cm^{-1} ; **9**, 1770 cm^{-1}). The ^1H - and ^{13}C -NMR spectra of **8** and **9** resembled and were similar to those of **7** (Tables 3, 4), and showed signals of a vinyl, eight methines including five oxygenated ones, a methylene, and four methyls, together with those of two benzoyl and two acetyl groups. On the other hand, the ^{13}C -NMR spectra of **8** and **9** were characterized by the presence of signals of a lactone carbonyl (**8**, δ 172.5; **9**, 176.4) and an ketal (**8**, δ 107.9; **9**, δ 110.9), and by the disappearance of the signals of a ketone (δ 214.1) and one of two oxygenated quaternary carbons (δ 77.2, 78.7) in **7**. These spectral data and the COSY and HMQC spectra suggested that **8** and **9** should be isomers on ring C, having the same rings A and B as **7**.

The long-range correlations of the tertiary methyls H₃-18, H₃-19 and H₃-20 in the HMBC spectra of **8** and **9** confirmed the structures of rings A and B (Table 4). On rings C and D, **8** and **9** showed different correlations which correspond to structural differences. In the case of **8**, the ketal carbon (δ 107.9) was correlated with H-7 and H-9, and the lactone carbonyl (δ 172.5) and the oxygenated quaternary carbon (δ

Table 2. ^{13}C -NMR Data for Orthosiphols F—J (1—5) in CDCl_3

	1		2		3		4		5
	δ	HMBC ^{a)}	δ	HMBC ^{a)}	δ	HMBC ^{a)}	δ	HMBC ^{a)}	δ
1	71.9	2, 3, 9, 20	74.4	3, 20	72.5	3, 9, 20	73.4	2, 3, 20	73.5
2	66.3	1, 3	66.8	1, 3	66.4	1, 3	65.9	1, 3	65.7
3	76.1	1, 2, 18, 19	76.2	1, 18, 19	76.1	18, 19	76.1	1, 2, 18, 19	75.9
4	37.5	3, 5, 18, 19	37.4	3, 5, 18, 19	37.2	3, 18, 19	37.2	3, 5, 18, 19	37.0
5	35.6	1, 3, 7, 18, 19, 20	35.2	1, 3, 18, 19, 20	36.7	1, 3, 18, 19, 20	34.8	1, 3, 9, 18, 19, 20	36.0
6	23.4	5	23.4		21.3		20.8		22.7
7	69.3	9	69.3		70.6		69.0		71.4
8	78.1	7, 9	78.1	9	75.8	9	78.0	9	76.3
9	42.5	7, 12	44.6	20	41.8	12, 20	51.0	20	51.7
10	44.0	1, 5, 9, 20	43.6	9, 20	43.7	1, 9, 11, 20	42.8	1, 5, 9, 20	42.8
11	68.9	9, 12	64.7	9	68.6	9, 12	205.7	9, 12	204.5
12	39.1	15, 17	43.9	9, 17	39.7	15, 17	47.1	17	47.0
13	47.9	12, 15, 16, 17	48.9	12, 15, 16	47.9	12, 15, 16	49.5	12, 15, 16	49.5
14	214.0	9, 12, 15, 17	213.5	12, 15, 17	208.8	12, 15, 17	211.1	12, 17	208.2
15	141.9	12, 16, 17	141.7	12, 16, 17	141.9	12, 16, 17	138.9	12, 16, 17	138.3
16	113.3		114.3		113.2		116.2		116.4
17	27.7	12, 15	26.1	12	26.4	12	25.1	12	25.0
18	27.9	3, 5, 19	28.0	19	27.9	3, 19	27.8	19	28.0
19	22.1	5, 18	22.5	18	22.3	18	22.1	18	22.1
20	17.2	5, 9	16.1	5, 9	16.5	5, 9	16.3	5, 9	16.1
1-OCOPh									
1'	130.3 ^{b)}	3', 5'	130.8	3', 5'	130.8	2', 6'	130.4	3', 5'	129.9
2',6'	129.7	3', 4', 5'	129.8	4'	129.6	4'	130.0	3', 5'	129.9
3',5'	128.0		128.3		127.8		128.2	2', 4', 6'	128.2
4'	132.4	2', 6'	132.8	2', 6'	132.7	2', 6'	132.9	2', 6'	133.1
CO	164.5	1, 2', 6'	166.1	1, 2', 6'	163.7	1, 2', 6'	164.3	1, 2', 6'	163.9
2-OCOCH ₃									
1''	170.1		170.6		170.1		170.1		169.9
CO	20.7	2, 1''	21.0	2, 1''	20.7	2, 1''	23.6	2, 1''	20.7
3-OCOCH ₃									
1'''	170.7		170.8		170.5		170.6		170.3
CO	20.5	3, 1'''	20.6	3, 1'''	20.3		23.6	3, 1'''	21.1
7-OCOCH ₃									
1'''					168.7				168.2
CO					20.9	7, 1'''			20.7
11-OCOPh									
1''''	130.2 ^{b)}	3''', 5''''			130.9	2''', 6''''			
2''',6''''	129.5	3''', 4''', 5''''			129.7	4''''			
3''',5''''	127.6				127.9				
4''''	132.1	2''', 6''''			132.3	2''', 6''''			
CO	165.8	11, 2''', 6''''			166.1	11, 2''', 6''''			

a) Long-range correlated protons observed in the HMBC spectra.

79.4) both were correlated with H₃-17 (δ 1.32) (Fig. 2a), while in the case of **9** a correlation was observed between the ketal carbon (δ 110.9) and H₃-17 (δ 1.59), and the oxygenated quaternary carbon (δ 78.9) was correlated with H-9 (δ 2.62) (Fig. 2b). Thus, the ketal and oxygenated quaternary carbons of **8** were concluded to be C-8 and C-13, respectively, and those of **9** were C-13 and C-8. Similarly, the location of two benzoyl groups was determined to be at C-1 and C-11 and that of two acetyl groups at C-2 and C-3, based on the long-range correlations of the ester carbonyl carbons (Table 4). Thus, the planar structures of staminolactones A and B were determined to be **8** and **9**, respectively.

The relative stereochemistries of **8** and **9** were elucidated based on analyses of the coupling constants (Table 3) and the rotating-frame Overhauser enhancement spectroscopy (ROESY) correlations (Figs. 2d, e). On the rings A and B, the coupling pattern and ROESY correlations, similar to those of **1**—**7**, were observed and indicated H-1, H-2, H-3, H-6_{ax}, H-7, H₃-19, and H₃-20 to be *cis* (β); H-5 and H-9 to be

cis (α); and the two groups to be *trans*. The coupling constant between H-9 and H-11 of **8** was large ($J=11$ Hz), while that of **9** was small (nearly zero), indicating that H-11 of **8** and **9** are axial and equatorial, respectively. On ring C, on the other hand, both **8** and **9** showed the same ROESY correlations, except for that between H₃-20 and H-12 of **9** (Figs. 2d, e). These data, together with consideration of the Dreiding stereomodel, indicated H-11 and H-12 to be β in both compounds; ring C of **8** to have a chair conformation (*i.e.*, lactone bridge has β -orientation); and that of **9** to have a boat conformation (*i.e.*, lactone bridge has α -orientation). Thus, the structures of staminolactones A and B were determined to be **8** and **9**, respectively.

Norstaminol A (**10**), $[\alpha]_{\text{D}}^{25} -38.0^\circ$ (CHCl_3), was isolated as a colorless amorphous solid. It gave a quasimolecular ion at m/z 701 ($\text{M}+\text{Na}$)⁺ and 679 ($\text{M}+\text{H}$)⁺ in FAB-MS and its molecular formula was determined by HR-FAB-MS to be $\text{C}_{37}\text{H}_{42}\text{O}_{12}$, one carbon less than **8** and **9**. The ^1H - and ^{13}C -NMR spectra of **10** were partially similar to those of **8** and **9**

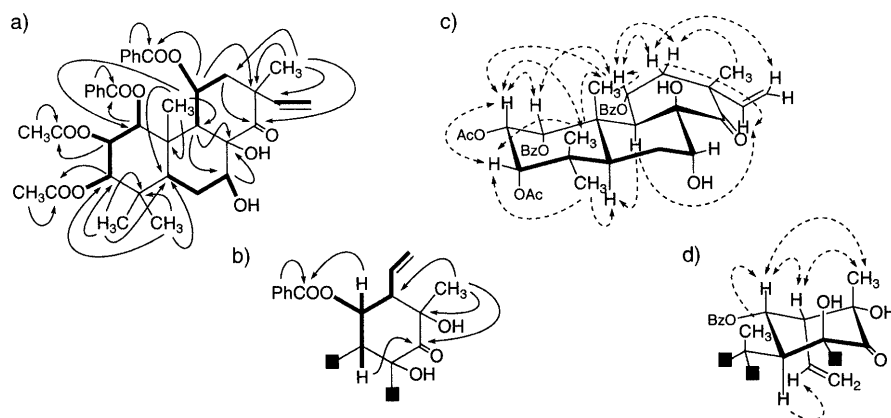


Fig. 1. Connectivities (Bold Line) Deduced by the COSY and HETCOR Spectra and Key Long-Range Correlations (Arrows, H→C) from the HMBC Spectrum of **1** (a) and **6** (b) and NOEs Observed in the Difference NOE Spectra of **1** (c) and **6** (d)

On rings A and B of **6**, the same long-range correlations and NOEs as those of **1** were observed.

Table 3. ^1H -NMR Data for Staminane-Type Diterpenes **6**–**10** in CDCl_3 (J in Hz)

	6	7	8	9	10
1	5.79 d (2)	5.69 brs	5.13 brs	5.57 brs	5.29 brs
2	5.38 dd (2, 3)	5.38 brs	5.34 brs	5.57 brs	5.50 brs
3	5.01 d (3)	4.99 d (2.9)	4.99 d (3.1)	5.08 brs	5.05 d (2.9)
5	2.63 dd (13.5, 2)	2.85 brd (12.2)	2.80 brd (13.8)	2.74 brd (11.7)	2.62 brd (13.3)
6	1.88 brt (13)	1.83 brt (12.2)	1.89 brt (13.8)	1.86 brt (13.9)	1.82 brt (13.3)
	2.08 brd (13)	1.99 brd (12.2)	2.02 brd (13.8)	1.93 brd (13.9)	1.91 brd (13.3)
7	5.37 d (3)	4.19 brs	4.00 brs	4.17 brs	3.80 brs
9	3.11 d (11)	3.10 d (10.2)	3.10 d (11)	2.62 s	2.74 d (3.4)
11	6.29 dd (11, 3.5)	6.21 dd (10.2, 3.1)	5.71 dd (11, 6.1)	5.53 d (5.2)	5.58 t (3.4)
12	2.96 dd (9.7, 3.5)	3.05 dd (9.7, 3.1)	2.79 dd (10.5, 6.1)	2.78 dd (10.2, 5.2)	2.41 t (3.4)
15	5.15 dt (17, 9.7)	5.14 dt (16.6, 9.7)	5.56 dt (17.3, 10.5)	5.47 dt (16.9, 10.2)	4.59 brs
16	4.51 dd (9.7, 1.5)	4.58 d (9.7)	5.03 d (10.5)	5.19 d (10.2)	3.81 brd (10.4)
	4.83 dd (17, 1.5)	4.86 d (16.6)	4.88 d (17.3)	5.30 d (16.9)	3.69 brd (10.4)
17	1.68 s	1.67 s	1.32 s	1.59 s	1.66 s
18	0.91 s	1.09 s	1.03 s	1.04 s	1.04 s
19	1.09 s	1.00 s	1.11 s	1.15 s	1.12 s
20	1.40 s	1.38 s	1.40 s	1.49 s	1.33 s
7-OH				4.40 s	
1-OCOPh					
2',6'	8.13 dd (8, 1)	8.04 d (7.6)	8.06 d (7.3)	7.86 d (7.6)	7.68 d (7.3)
3',5'	7.42 t (8)	7.42 d (7.6)	7.52 t (7.3)	7.10 t (7.6)	7.29 t (7.3)
4'	7.58 tt (8, 1)	7.48 t (7.6)	7.63 t (7.3)	7.21 t (7.6)	7.53 t (7.3)
2-OCOCH ₃					
1''	1.93 s	1.98 s	1.75 s	1.99 s	1.82 s
3-OCOCH ₃					
1'''	1.60 s	1.59 s	1.57 s	1.74 s	1.70 s
7-OCOCH ₃					
1''''	2.19 s				
11-OCOPh					
2''''',6'''''	8.26 dd (8, 1)	8.16 d (7.5)	7.72 d (7.6)	7.52 d (7.3)	7.58 d (7.6)
3''''',5'''''	7.48 t (8)	7.42 d (7.5)	7.33 t (7.6)	7.18 t (7.3)	7.07 t (7.6)
4'''''	7.61 tt (8, 1)	7.55 t (7.5)	7.53 t (7.6)	7.37 t (7.3)	7.41 t (7.6)

(Tables 3, 4), but they were characterized by a lack of the signals of a vinyl group, a lactone carbonyl, and an oxygenated quaternary carbon, and by the presence of signals of an oxymethylene, an oxymethine, and an additional ketal carbon. These data were analyzed by the COSY, HMQC, and HMBC spectra and suggested the planar structure. The ketal carbon at δ 104.8 was located at C-13 based on the long-range correlations with H₃-17 and H-15, while the other ketal carbon (δ 94.9) was assigned as C-8 on the basis of the correlations with H-6, H-7, and H-9 (Fig. 2c, Table 4). Moreover, the former ketal carbon (C-13) showed a correlation

with H-16, indicating the presence of a furan ring. Finally, the ketal carbons (C-8, C-13) would be connected through an oxygen to construct an epoxide ring at C-7 and C-8, because the molecular formula of **10** ($\text{C}_{37}\text{H}_{42}\text{O}_{12}$) indicated that there is no other atom. The locations of two benzoyl and two acetyl groups were also determined by analyses of the HMBC spectrum to be at C-1 and C-11 and at C-2 and C-3, respectively (Table 4). The configuration at the chiral centers in rings A and B was determined to be the same as that in **8** and **9** on the basis of the coupling constants and the ROESY correlations (Fig. 2f) of each proton. On the configuration of ring C,

Table 4. ^{13}C -NMR Data for Staminane-Type Diterpenes **6**–**10** in CDCl_3

	6		7		8		9		10	
	δ	HMBC ^{a)}	δ	δ	δ	HMBC ^{a)}	δ	HMBC ^{a)}	δ	HMBC ^{a)}
1	74.4	3, 20	74.0	73.8	3, 20		71.8	3, 9, 20	71.2	3, 9, 20
2	66.4	1, 3	66.5	66.6	1, 3		66.1	1, 3	66.0	1, 3
3	76.1	1, 18, 19	76.1	75.8	1, 18, 19		76.0	1, 2, 18, 19	75.8	1, 18, 19
4	37.3	5, 18, 19	37.3	37.2	5, 18, 19		37.4	5, 18, 19	37.3	3, 5, 18, 19
5	35.8	1, 3, 18, 19, 20	34.3	34.7	1, 3, 7, 18, 19, 20		34.6	1, 3, 18, 19, 20	35.1	1, 3, 6, 7, 18, 19, 20
6	21.0		22.6	24.5			24.3	5, 7-OH	24.4	5
7	70.3	9	69.4	69.7			67.9		69.7	
8	77.4		78.7	107.9	7, 9		78.9	9, 11	94.9	6, 7, 9
9	40.7	20	40.6	39.8	11, 12, 20		42.5	11, 12, 20	45.3	20
10	43.5	5, 9, 20	43.7	42.8	1, 9, 5, 20		43.0	5, 9, 20	41.7	1, 5, 6, 9, 11, 20
11	70.2	9	70.4	68.4	9, 12		66.9	9	64.6	9
12	54.3	9, 16, 17	54.7	48.6	16, 17		50.2	9, 11, 15, 16, 17	43.8	16, 17
13	76.8	17	77.2	79.4	17		110.9	12, 17	104.8	15, 16, 17
14	209.0	9, 17	214.1	172.5	17		176.4			
15	131.2	11	144.7	129.7			131.0		72.8	11, 16
16	121.1		121.2	122.9	12		122.4	12, 15	72.8	15
17	29.1		28.2	18.2			22.1	12	21.4	
18	28.3	5, 19	28.1	28.2	5, 19		27.8	19	27.9	19
19	22.3	18	22.3	22.7	18		22.2	3, 5, 18	22.4	5, 18
20	15.5	5, 9	15.7	14.1	5, 9		17.3	1, 5, 9	15.8	5, 9
1-OCOPh										
1'	128.5	2', 6'	130.7 ^{b)}	129.7	2', 6'		129.4	2', 6'	129.7	2', 6'
2',6'	129.5	4'	129.7	129.7	4'		129.3	4'	129.9	4'
3',5'	128.3		128.4	128.4			127.9	2', 6'	128.1	2', 6'
4'	133.0	2', 6'	132.7	132.9	2', 6'		132.6	2', 6'	132.7	2', 6'
CO	164.0	1, 2', 6'	164.2	163.3	1, 2', 6'		164.5	1, 2', 6'	164.6	1, 2', 6'
2-OCOCH ₃										
1''	169.9		170.2	170.1			170.6 ^{b)}		169.9	
CO	20.7	2, 1''	20.8	20.7	2, 1''		20.7 ^{c)}	2, 1''	20.7	2, 1''
3-OCOCH ₃										
1'''	170.5		170.8	170.6			170.5 ^{b)}		170.6	
CO	20.5	3, 1'''	20.5	20.5	3, 1'''		20.9 ^{c)}	3	20.8	3, 1'''
7-OCOCH ₃										
1'''	169.3									
CO	21.2	7, 1'''								
11-OCOPh										
1''''	128.5	2''', 6''''	130.3 ^{b)}	130.6	2''', 6''''		128.5	2''', 6''''	129.9	2''', 6''''
2''',6''''	130.6	4''''	130.5	130.6	4''''		129.4	4''''	129.3	4''''
3''',5''''	128.3		128.2	127.6			128.0		128.0	2''', 6''''
4''''	133.3	2''', 6''''	133.2	132.9	2''', 6''''		132.6	2''', 6''''	132.6	2''', 6''''
CO	166.8	11, 2''', 6''''	166.7	166.2	11, 2''', 6''''		163.1	11, 2''', 6''''	165.5	11, 2''', 6''''

a) Long-range correlated protons observed in the HMBC spectra. b, c) Assignments may be interchanged in each column.

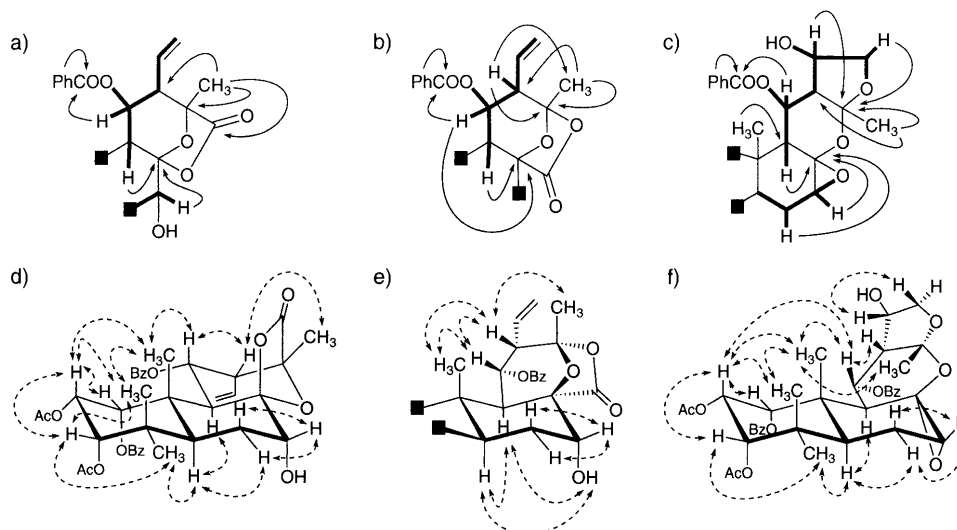


Fig. 2. Connectivities (Bold Line) Deduced by the COSY and HMQC Spectra and Key Long-Range Correlations (Arrows, H→C) on Ring C from the HMBC Spectra of **8**–**10** (a–c) and ROESY Correlations Observed in the ROESY Spectra (Mixing time, 0.5 s) of **8**–**10** (d–f)

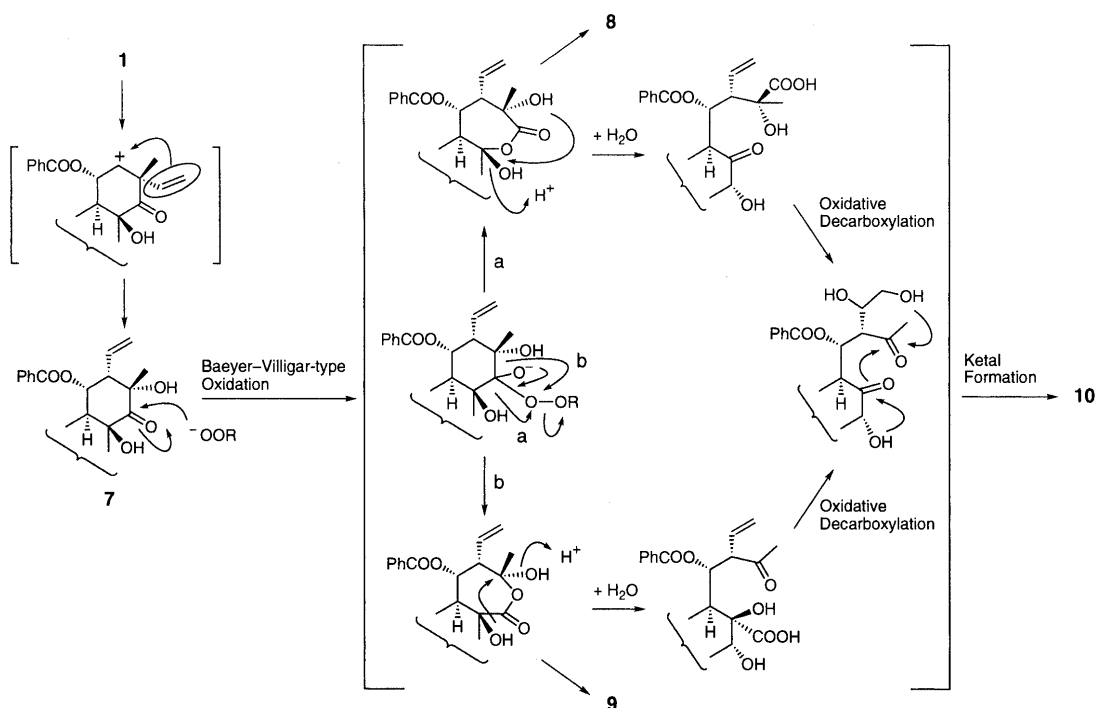


Chart 2. Possible Biogenetic Pathway of Novel Diterpenes 7–10

on the other hand, the ROESY correlations of H-11 indicated H-11, H-12, H₃-17, and H₃-20 should have a β -orientation, and the correlations of H-12 with H-15 and H-16 β and the broad singlet nature of H-15 suggested that H-15 also has a β -orientation and that ring C has a boat conformation. Finally, the configuration of the epoxy ring was concluded to be α based on the ROESY correlation between H₃-17 and H₃-20. Thus, the structure of norstaminol A was concluded to be represented by the structure formula 10.

Biogenetic Consideration In this paper, we have reported the isolation and structures of ten new diterpenes; orthosiphols F–J (1–5), staminols A (6) and B (7), staminolactones A (8) and B (9), and norstaminol A (10). Among them, 1–5 are isopimarane-type diterpenes, while others (6–10) have a novel carbon-framework, to which we proposed the name “staminane”. Moreover, staminolactone A (8) is 8,14-secostaminane-type and staminolactone B (9) is 13,14-secostaminane-type, while norstaminol A (10) is 14-norstaminane-type. The co-existence of a staminane-type (6–10) with isopimarane-type (1–5) suggests that the former is biosynthesized from the latter through the migration of the vinyl group from C-13 α to C-12 α (Chart 2). Then, staminolactones A (8) and B (9) would be biosynthesized from a staminane-type diterpene such as staminol B (7) through a Baeyer-Villiger-type oxidation¹¹; migration of C-8 leads to 8 (route a) and migration of C-13 leads to 9 (route b). At last, further oxidation of 8 or 9 with oxidative decarboxylation would lead to norstaminol A (10).

Cytotoxic Activity All the isolated compounds were tested for their cytotoxicity towards highly liver metastatic murine colon 26-L5 carcinoma cells. The new diterpenes, except for 4, showed weak cytotoxicity with an ED₅₀ value between 50 and 90 μ g/ml, while on flavonoids 7,3',4'-tri-*O*-methylfluteolin (11), eupatorin (12), ladanein (16), and 6-hydroxy-5,7,4'-trimethoxyflavone (18) showed cytotoxic activi-

ties (ED₅₀: 11, 87.7 μ g/ml; 12, 11.3 μ g/ml; 16, 50.4 μ g/ml; 18, 56.5 μ g/ml). Three flavonoids, except for 11, contained a free hydroxyl group in a molecule and their cytotoxicity was stronger than that of 11. This would indicate that the presence of free hydroxyl group in a molecule would enhance their antioxidative activity and lead to enhanced cytotoxicity. This tendency was also observed in previous cases.¹² These compounds would contribute to the cytotoxic activity of the MeOH extract of *O. stamineus*.

Experimental

Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in CHCl₃ solutions. FAB-MS measurements were performed on a JEOL JMS-700T spectrometer with *m*-nitrobenzylalcohol (NBA) or glycerol as a matrix. NMR spectra were taken on a JEOL JNM-GX400 spectrometer with tetramethylsilane (TMS) as the internal standard, and chemical shifts are expressed in δ values. Column chromatography was performed with silica-gel (Fuji Silysia BW-820MH), while TLC and preparative TLC were carried out on pre-coated Merck Kieselgel 60F₂₅₄ (0.25 or 0.50 mm) or RP-18F₂₅₄ (0.25 mm) plates.

Extraction and Isolation The aerial part of *Orthosiphon stamineus* Benth. was purchased at the local market at Ho Chi Minh City, Vietnam in 1997. The voucher sample (TMPW No. 18623) is preserved in the Museum for Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University. The air dried aerial part of *O. stamineus* (4 kg) was extracted with MeOH (60 l, reflux, 3 h, $\times 2$), and the MeOH extract (150 g) was suspended on water and extracted successively with hexane, CHCl₃, EtOAc, and BuOH to yield hexane- (15.8 g), CHCl₃- (47.3 g), EtOAc- (11.5 g), and BuOH-soluble (14 g) fractions, respectively.

Treatment of CHCl₃-Soluble Fraction CHCl₃-soluble fraction (47.3 g) was chromatographed over silica gel with an EtOAc-hexane solvent system to give five fractions (fr. 1, 4.2 g; fr. 2, 3.6 g; fr. 3, 23.3 g; fr. 4, 9.4 g; fr. 5, 2.7 g).

Fraction 1 afforded oleanolic acid (23, 4 mg) and β -sitosterol (26, 9 mg), while fr. 2 gave 7,3',4'-tri-*O*-methylfluteolin (11, 4.5 mg) and betulinic acid (25, 6 mg).

Fraction 3 [hexane-EtOAc (7:3) eluate, 8.2 g] was rechromatographed over silica gel with a benzene-acetone solvent system to afford four sub-fractions [fr. 3-1: benzene-acetone (95:5) eluate, 1.94 g; fr. 3-2: benzene-acetone (92.5:7.5) eluate, 1.87 g; fr. 3-3: benzene-acetone (90:10) eluate,

2.07 g; fr. 3-4, benzene-acetone (80:20) eluate, 0.93 g]. Subfraction 3-1 was separated by preparative TLC with hexane-acetone (3:1) to give orthosiphon H (**3**, 9 mg), norstaminol A (**10**, 30 mg), 5-hydroxy-6,7,3',4'-tetramethoxyflavone (**14**, 3 mg), salvigenin (**15**, 7 mg), and tetramethylscutellarein (**17**, 3 mg). Subfraction 3-2 was separated by preparative TLC with hexane-acetone (3:2) to yield orthosiphon F (**1**, 20 mg), **3** (228 mg), and aurantiamide acetate (**20**, 7.5 mg), together with crude orthosiphon J (**5**), which was purified by reversed-phase preparative TLC with H₂O-MeOH (1:9) to give pure **5** (3 mg). Subfractions 3-3 and 3-4 were separately subjected to preparative TLC with hexane-acetone (1:1), and **1** (100 mg) and orthosiphon G (**2**, 5 mg) were obtained from subfraction 3-3; **1** (22 mg), staminol A (**6**, 26 mg), and 6-hydroxy-5,7,4'-trimethoxyflavone (**18**, 3 mg) were from subfraction 3-4.

Fraction 4 [hexane-EtOAc (7:4) eluate, 7.0 g] was also rechromatographed over silica-gel with a benzene-acetone solvent system to give four subfractions [fr. 4-1: benzene-acetone (95:5) eluate, 0.2 g; fr. 4-2: benzene-acetone (92.5:7.5) eluate, 0.5 g; fr. 4-3: benzene-acetone (90:10) eluate, 1.9 g; fr. 4-4: benzene-acetone (80:20) eluate, 3.4 g]. Each subfraction was separated by preparative TLC with hexane-acetone (1:1) to give the following compounds: fr. 4-1: staminolactone A (**8**, 7.8 mg), staminolactone B (**9**, 18 mg), **14** (16 mg); fr. 4-2: **1** (54 mg), eupatorin (**12**, 24 mg), ladanein (**16**, 24 mg); fr. 4-3: orthosiphon I (**4**, 7 mg), **12** (13 mg), **16** (13 mg); fr. 4-4: staminol B (**7**, 14 mg), **12** (13 mg), sinensetin (**13**, 75 mg).

Orthosiphon F (**1**): Colorless amorphous solid, $[\alpha]_D^{25} -82.8^\circ$ ($c=2.10$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3450, 1725, 1455, 1370, 1280, 1110, 1050. FAB-MS m/z : 699 (M+Na)⁺, 677 (M+H)⁺. HR-FAB-MS m/z : 677.2958 [Calcd for C₃₈H₄₅O₁₁: 677.2962 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 1 and 2.

Orthosiphon G (**2**): Colorless amorphous solid, $[\alpha]_D^{25} -63.3^\circ$ ($c=0.47$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3400, 1720, 1455, 1370, 1280, 1115, 1045. FAB-MS m/z : 595 (M+Na)⁺, 573 (M+H)⁺. HR-FAB-MS m/z : 573.2668 [Calcd for C₃₁H₄₁O₁₀: 573.2700 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 1 and 2.

Orthosiphon H (**3**): Colorless amorphous solid, $[\alpha]_D^{25} -58.0^\circ$ ($c=0.63$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3400, 1725, 1455, 1370, 1280, 1240, 1110, 1045. FAB-MS m/z : 741 (M+Na)⁺, 719 (M+H)⁺. HR-FAB-MS m/z : 719.3051 [Calcd for C₄₀H₄₇O₁₂: 719.3067 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 1 and 2.

Orthosiphon I (**4**): Colorless amorphous solid, $[\alpha]_D^{25} -108.9^\circ$ ($c=0.55$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3400, 1725, 1455, 1375, 1270, 1110, 1045. FAB-MS m/z : 593 (M+Na)⁺, 571 (M+H)⁺. HR-FAB-MS m/z : 571.2568 [Calcd for C₃₁H₃₉O₁₀: 571.2543 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 1 and 2.

Orthosiphon J (**5**): Colorless amorphous solid, $[\alpha]_D^{25} -58.5^\circ$ ($c=0.24$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 1725, 1460, 1370, 1270, 1230, 1110, 1050. FAB-MS m/z : 612 (M+H)⁺. HR-FAB-MS m/z : 613.2646 [Calcd for C₃₃H₄₁O₁₁: 613.2648 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 1 and 2.

Staminol A (**6**): Colorless amorphous solid, $[\alpha]_D^{25} -24.3^\circ$ ($c=0.51$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3430, 3300, 1725, 1600, 1450, 1370, 1200—1270, 1090, 1070, 1040. FAB-MS m/z : 757 (M+Na)⁺, 735 (M+H)⁺. HR-FAB-MS m/z : 735.2970 [Calcd for C₄₀H₄₇O₁₃: 735.3017 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 3 and 4.

Staminol B (**7**): Colorless amorphous solid, $[\alpha]_D^{25} -27.8^\circ$ ($c=0.69$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 3430, 3300, 1725, 1600, 1450, 1200—1270, 1090, 1070, 1040. FAB-MS m/z : 715 (M+Na)⁺, 693 (M+H)⁺. HR-FAB-MS m/z : 715.2740 [Calcd for C₃₈H₄₄O₁₂Na: 715.2730 (M+Na)⁺]. ¹H- and ¹³C-NMR: Tables 3 and 4.

Staminolactone A (**8**): Colorless amorphous solid, $[\alpha]_D^{25} -97.2^\circ$ ($c=0.067$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3570, 1800, 1730, 1600, 1455, 1370, 1270, 1200—1240. FAB-MS m/z : 713 (M+Na)⁺, 691 (M+H)⁺. HR-FAB-MS m/z : 691.2731 [Calcd for C₃₈H₄₃O₁₂: 691.2754 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 3 and 4.

Staminolactone B (**9**): Colorless amorphous solid, $[\alpha]_D^{25} -98.9^\circ$ ($c=0.12$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3500, 1770, 1730, 1600, 1455, 1390, 1370, 1270, 1200—1240. FAB-MS m/z : 713 (M+Na)⁺, 691 (M+H)⁺. HR-FAB-MS m/z : 691.2746 [Calcd for C₃₈H₄₃O₁₂: 691.2754 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 3 and 4.

Norstaminol A (**10**): Colorless amorphous solid, $[\alpha]_D^{25} -38.0^\circ$ ($c=0.41$, CHCl₃). IR $\nu_{\max}$ (CHCl₃) cm⁻¹: 3550, 1725, 1600, 1455, 1370, 1280, 1200—1240, 1110. FAB-MS m/z : 701 (M+Na)⁺, 679 (M+H)⁺. HR-FAB-MS m/z : 679.2788 [Calcd for C₃₇H₄₃O₁₂: 679.2754 (M+H)⁺]. ¹H- and ¹³C-NMR: Tables 3 and 4.

Treatment of EtOAc-soluble Fraction EtOAc-soluble fraction (11.5 g) was chromatographed over silica-gel with a hexane-EtOAc solvent system

to give three subfractions (fr. 4-1, 2 g; fr. 4-2, 1.5 g; fr. 4-3, 2.4 g). Fraction 4-1 was separated by preparative TLC with hexane-EtOAc (3:1) to afford **11** (3 mg) and **15** (11 mg). Fraction 4-2 gave **1** (223 mg), **3** (12.7 mg), **6** (33 mg), **12** (10 mg), **14** (3 mg), and **16** (3 mg) by preparative TLC with acetone-benzene (5:95). On the other hand, fr. 4-3 yielded vomifolol (**19**, 7.5 mg), rosmarinic acid (**21**, 10 mg), caffeic acid (**22**, 4 mg), and ursolic acid (**24**, 5 mg) by preparative TLC with benzene-acetone-AcOH (70:29:1).

Cytotoxic Assay A cytotoxic assay was done using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assays as described previously,¹³⁾ with slight modification. In brief, exponentially growing cells were harvested and 100 μ l of cell suspension containing 2000 cells was plated in 96-well plates (Falcon, Becton Dickinson, NJ, U.S.A.). After 24 h incubation at 37 °C under 5% CO₂, the cells were treated with varying concentrations of test specimens in 100 μ l medium and incubated for 3 d under the same conditions. At 3 h after adding an MTT solution, UV absorption of the formazan formed was measured at 590 nm using a Perkin-Elmer HTS-7000 plate reader. Test specimens were dissolved in dimethyl sulfoxide (DMSO) and then diluted by the medium. DMSO less than 0.25% in the test solution had no effect on the all. 5-Fluorouracil (5-FU) was used as a positive control, and ED₅₀ values were calculated from the mean values of data from three wells. The following compounds showed cytotoxicity with an ED₅₀ value less than 100 μ g/ml: **1**, 51.6 μ g/ml; **2**, 89.7 μ g/ml; **3**, 56.7 μ g/ml; **5**, 48.2 μ g/ml; **6**, 61.7 μ g/ml; **7**, 78.9 μ g/ml; **8**, 68.5 μ g/ml; **9**, 79.8 μ g/ml; **10**, 56.1 μ g/ml; **11**, 87.7 μ g/ml; **12**, 11.3 μ g/ml; **16**, 50.4 μ g/ml; **18**, 56.5 μ g/ml; **20**, 70.1 μ g/ml; **21**, 53.3 μ g/ml; **22**, 13.5 μ g/ml; **25**, 75.4 μ g/ml; **5-FU**, 0.24 μ g/ml.

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References and Notes

- 1) a) P. T. Eisai Indonesia (ed.), "Medicinal Herb Index in Indonesia (Second Edition)," P. T. Eisai Indonesia, Indonesia, 1995, p. 263; b) Tran K. (ed.), "Medicinal Plants in Viet Nam," WHO Regional Office for the Western Pacific Manila and Institute of Material Medica Hanoi, Science and Technology Publishing House, Hanoi, 1990, p. 271; c) Hartke K., Mutschler E. (eds.), "DAB-9 Kommentar," Vol. 3, Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, Govi-Verlag GmbH, Frankfurt, 1986, pp. 2607—2609; d) Wichtl M., "Teedrogen," ed. by Wichtl M., Wissenschaftliche Verlagsgesellschaft GmbH, Stuttgart, 1984, pp. 244—246; e) Wagner H., "Pharmazeutische Biologie: Drogen und ihre Inhaltsstoffe," 2nd Ed., Gustav Fischer Verlag, Stuttgart, 1982, p. 49; f) Hegnauer R., "Chemotaxonomie der Pflanzen," Vol. 4, Birkhäuser Verlag, Basel, Stuttgart, 1966, p. 314.
- 2) Phytochemical studies: a) Takeda Y., Matsumoto T., Terao H., Shingu T., Futatsushii Y., Nohara T., Kajimoto T., *Phytochemistry*, **33**, 411—415 (1993); b) Masuda T., Masuda K., Shiragami S., Jitoe A., Nakatani N., *Tetrahedron*, **48**, 6787—6792 (1992); c) Sumaryono W., Proksch P., Wray V., Witte L., Hartmann T., *Planta Med.*, **57**, 176—180 (1991); d) Guerin J. C., Reveillere H. P., Ducrey P., Toupet L., *J. Nat. Prod.*, **52**, 171—175 (1989); e) Malterud K. E., Hanche-Olsen I. M., Smith-Kiekkand I., *Planta Med.*, **55**, 569—570 (1989); f) Wollenweber E., Mann K., *ibid.*, **51**, 459—460 (1985); and references cited therein.
- 3) Pharmacological studies: Beaux D., Fleurentin J., Mortier F., *Phytother. Res.*, **13**, 222—225 (1999); Lyckander I. M., Malterud K. E., *Prostaglandins Leukot. Essent. Fatty Acids*, **54**, 239—246 (1996); Engler J., Harnischfeger G., *Planta Med.*, **58**, 237—238 (1992); Doan D. D., Nguyen N. H., Doan H. K., Nguyen T. L., Phan T. S., Van Dau N., Grabe M., Johansson R., Lindgren G., Stjernstrom N. E., *J. Ethnopharmacol.*, **36**, 225—231 (1992); Nirdnoy M., Muangman V., *J. Med. Assoc. Thai.*, **74**, 318—321 (1991); Casadebaig-Lafon J., Jacob M., Cassanas G., Marion C., Puech A., *Pharm. Acta Helv.*, **64**, 220—224 (1989).
- 4) Shibuya H., Bohgaki T., Matsubara T., Watarai M., Ohashi K., Kitagawa I., *Chem. Pharm. Bull.*, **47**, 695—698 (1999).
- 5) Ohashi K., Bohgami T., Matsubara T., Shibuya H., *Chem. Pharm. Bull.*, **48**, 433—435 (2000); Shibuya H., Bohgami T., Ohashi K., *ibid.*, **47**, 911—912 (1999). In these papers, the authors called the carbon-framework which we had named "staminane" (ref. 8a)) as "migrated pimarane".
- 6) a) Banskota A. H., Tezuka Y., Tran K. Q., Tanaka K., Saiki I., Kadota S., *Chem. Pharm. Bull.*, **48**, 496—504 (2000); b) *Idem*, *J. Nat. Prod.*,

- 63, 57—64 (2000); c) Banskota A. H., Tezuka Y., Adnyana I. K., Xiong Q., Hase K., Tran K. Q., Tanaka K., Saiki I., Kadota S., *Biol. Pharm. Bull.*, **23**, 456—460 (2000); d) Adnyana I. K., Tezuka Y., Banskota A. H., Xiong Q., Tran K. Q., Kadota S., *J. Nat. Prod.*, **63**, 496—500 (2000); e) Adnyana I. K., Tezuka Y., Awale S., Banskota A. H., Tran K. Q., Kadota S., *Chem. Pharm. Bull.*, **48**, 1114—1120 (2000); f) Banskota A. H., Tezuka Y., Phung L. K., Tran K. Q., Saiki I., Miwa Y., Taga T., Kadota S., *Bioorg. Med. Chem. Lett.*, **8**, 3519—3524 (1998).
- 7) Ohnishi Y., Sakamoto T., Fujii H., Kimura F., Murata J., Tazawa K., Fujimaki M., Sato Y., Kondo M., Une Y., Uchino J., Saiki I., *Tumor Biol.*, **18**, 113—122 (1997).
- 8) The preliminary reports: a) Stampoulis P., Tezuka Y., Banskota A. H., Tran K. Q., Saiki I., Kadota S., *Tetrahedron Lett.*, **40**, 4239—4242 (1999); b) *Idem*, *Org. Lett.*, **1**, 1367—1370 (1999).
- 9) Das B., Chakravarty A. K., *Phytochemistry*, **33**, 493—496 (1993); Van der Westhuizen J. H., Ferreira D., Roux D., *J. Chem. Soc. Perkin Trans. I*, **1980**, 1003—1006; Barberán F. A., Hernández L., Ferreres F., Tomás F., *Planta Med.*, **51**, 452—454 (1985); Takasugi M., Anetai M., Katsui N., Masamune T., *Chem. Lett.*, **1973**, 245—248.
- 10) Zhao W., Guo Y., Tezuka Y., Kikuchi T., *Zhongguo Zhongyao Zazhi (China Journal of Chinese Materia Medica)*, **23**, 41 (1998); Tezuka Y., Kasimu R., Li J. X., Basnet P., Tanaka K., Namba T., Kadota S., *Chem. Pharm. Bull.*, **46**, 107—112 (1998); Fan W., Tezuka Y., Komatsu K., Namba T., Kadota S., *Biol. Pharm. Bull.*, **22**, 157—161 (1999); Kikuchi T., Matsuda S., Kadota S., Sakai Y., Namba T., Watanabe K., Dissanayake D. M. R. B., *Chem. Pharm. Bull.*, **32**, 3906—3911 (1984).
- 11) Recent reports of Baeyer–Villiger-type oxidation in the biosynthesis of natural products: Prado L., Fernandez E., Weissbach U., Blanco G., Quiros L. M., Brana A. F., Mendez C., Rohr J., Salas J. A., *Chem. Biol.*, **6**, 19—30 (1999); Wright J. L. C., Hu T., McLachlan J. L., Needham J., Walter J. A., *J. Am. Chem. Soc.*, **118**, 8757—8758 (1996); Watanabe C. M. H., Townsend C. A., *J. Org. Chem.*, **61**, 1990—1993 (1996); Amegadzie A. K., Ayer W. A., Sigler L., *Can. J. Chem.*, **73**, 2119—2125 (1995); Bockholt H., Udvarnoki G., Rohr J., Mocek U., Beale J. M., Floss H. G., *J. Org. Chem.*, **59**, 2064—2069 (1994).
- 12) Banskota A. H., Tezuka Y., Midorikawa K., Matsushige K., Kadota S., *J. Nat. Prod.*, **63**, 1277—1279 (2000); Woerdenbag H. J., Merfort I., Pareiter C. M., Schmidt T. J., Willuhn G., Van Uden W., Pras N., Kampinga H. H., Konings A. W. T., *Planta Med.*, **60**, 434—437 (1994).
- 13) Banskota A. H., Tezuka Y., Prasain J. K., Matsushige K., Saiki I., Kadota S., *J. Nat. Prod.*, **61**, 896—900 (1998).

Bioactive Saponins and Glycosides. XVII.¹⁾ Inhibitory Effect on Gastric Emptying and Accelerating Effect on Gastrointestinal Transit of Tea Saponins: Structures of Assamsaponins F, G, H, I, and J from the Seeds and Leaves of the Tea Plant

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Following the investigation of assamsaponins A, B, C, D, and E, four new saponins termed assamsaponins F, G, H, and I were isolated from the seeds of the tea plant (*Camellia sinensis* L. var. *assamica* PIERRE), while assamsaponin J was isolated from its leaves. The structures of assamsaponins F–J were elucidated on the basis of chemical and physicochemical evidence and found to be 16,22-*O*-diacetyl-21-*O*-angeloyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid, 21-*O*-angeloyl-22-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid, 21-*O*-angeloyl-28-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid, 21-*O*-tigloyl-28-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid, and 16,21-*O*-diacetyl-22-*O*-cinnamoyltheasapogenol B 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-rhamnopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid, respectively.

The saponin mixture from the seeds of the tea plant was found to exhibit an inhibitory effect on gastric emptying and an accelerating effect on gastrointestinal transit in mice. Theasaponin E₁, the principle saponin of the tea plant, showed potent activity, while theasaponin E₂ showed none, so that the position of the acyl groups in the sapogenin moiety is important from a pharmacological point of view.

Key words assamsaponin; *Camellia sinensis* var. *assamica*; tea plant; oleanene-type triterpene oligoglycoside; gastric emptying inhibitor; gastrointestinal transit accelerator

In the course of our studies of the bioactive saponins and glycosides from medicinal foodstuffs and natural medicines,²⁾ we isolated two triterpene saponins, theasaponins E₁ (15) and E₂ (16), from the seeds of the Japanese tea plant [*Camellia* (*C.*) *sinensis* or *Thea sinensis*] and characterized their structures and antisweet activity.³⁾ In a continuing study of the tea plant, the saponin mixture from the seeds of the tea plant (*C. sinensis* L. var. *assamica* PIERRE) cultivated in Sri Lanka was found to have a potent protective effect on gastric mucosal lesions induced by ethanol in rats. From the saponin mixture, we have isolated nine acylated polyhydroxy-oleanene-type triterpene saponins called assamsaponins A (8), B (11), C (12), D (13), E (14), F (1), G (2), H (3), and I (4) together with three known saponins, camelliasaponin B₁ (9)⁴⁾ and theasaponins E₁ (15)³⁾ and E₂ (16).³⁾ In the preceding paper,⁵⁾ we reported the structures of assamsaponins A (8), B (11), C (12), D (13), and E (14) and the inhibitory effect of 15 and 16 on ethanol-induced gastric lesions in rats. We recently found that the saponin mixture from the seeds of *C. sinensis* var. *assamica* exhibited an inhibitory effect on gastric emptying and an accelerating effect on gastrointestinal transit in mice. In addition, we isolated a new saponin called assamsaponin J (6) from the leaves of *C. sinensis* var. *assamica*. This paper deals with the structural elucidation of five new acylated polyhydroxyoleanene-type triterpene saponins, assamsaponins F (1), G (2), H (3), I (4), and J (6) from the seeds and leaves of *C. sinensis* var. *assamica*. We also describe the effects of the saponin mixture and theasaponins E₁ (15) and E₂ (16), saponins common to both vari-

eties of tea plant, on gastric emptying and gastrointestinal transit.

Structures of Assamsaponins F (1), G (2), H (3), and I (4) from the Seeds of *C. sinensis* var. *assamica* Assamsaponin F (1) was obtained as colorless fine crystals from CHCl₃–MeOH with a mp 209.8–210.3 °C. The IR spectrum of 1 showed absorption bands at 1721 and 1649 cm^{–1} assignable to carbonyl and α,β -unsaturated ester functions, and broad bands at 3432, 1078, and 1046 cm^{–1} suggestive of an oligoglycoside structure. In the negative- and positive-ion FAB-MS of 1, quasimolecular ion peaks were observed at *m/z* 1301 (M–H)[–] and *m/z* 1325 (M+Na)⁺, and high-resolution MS analysis revealed the molecular formula of 1 to be C₆₂H₉₄O₂₉. Furthermore, fragment ion peaks at *m/z* 1139 (M–C₆H₁₁O₅)[–] and *m/z* 1007 (M–C₁₁H₁₉O₉)[–], which were presumed to be derived by cleavage of the glycosidic linkages at the 2''- and 3'-positions (*vide infra*), were observed in the negative-ion FAB-MS of 1. Alkaline hydrolysis of 10% aqueous potassium hydroxide–50% aqueous dioxane (1:1, v/v) provided desacyl-assamsaponin F (5), angelic acid and acetic acid. The organic acids were converted to the *p*-nitrobenzyl ester derivatives,⁶⁾ which were identified by HPLC.

On acid hydrolysis with 5% aqueous sulfuric acid (H₂SO₄)–1,4-dioxane (1:1, v/v), desacyl-assamsaponin F (5) liberated D-glucuronic acid, D-galactose, L-arabinose, and D-glucose, which were identified by GLC⁷⁾ of their trimethylsilyl thiazolidine derivatives. The molecular formula of 5, C₅₃H₈₄O₂₆, was also determined from negative- and positive-ion FAB-MS [*m/z* 1135 (M–H)[–], 973 (M–C₆H₁₁O₅)[–], 841

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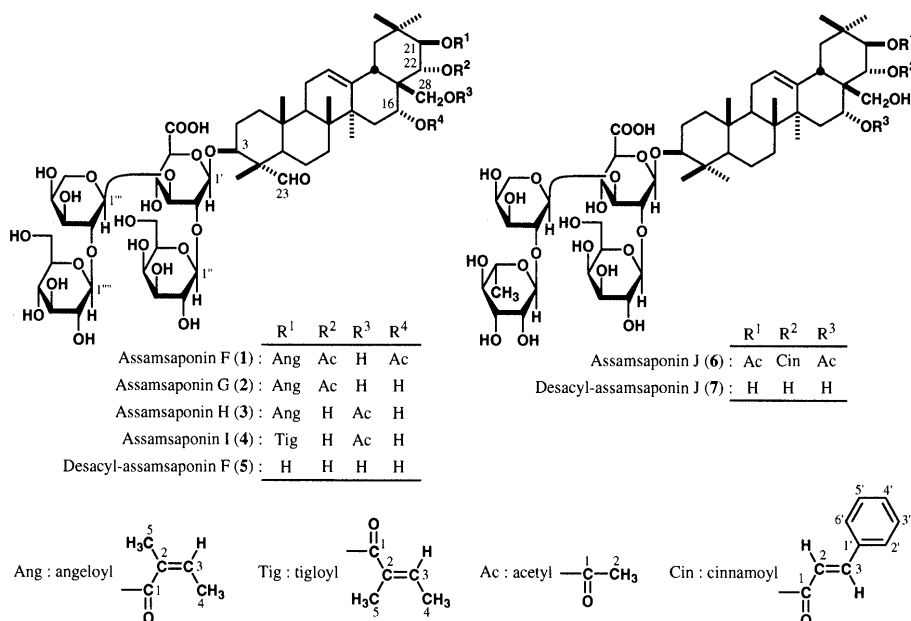


Chart 1

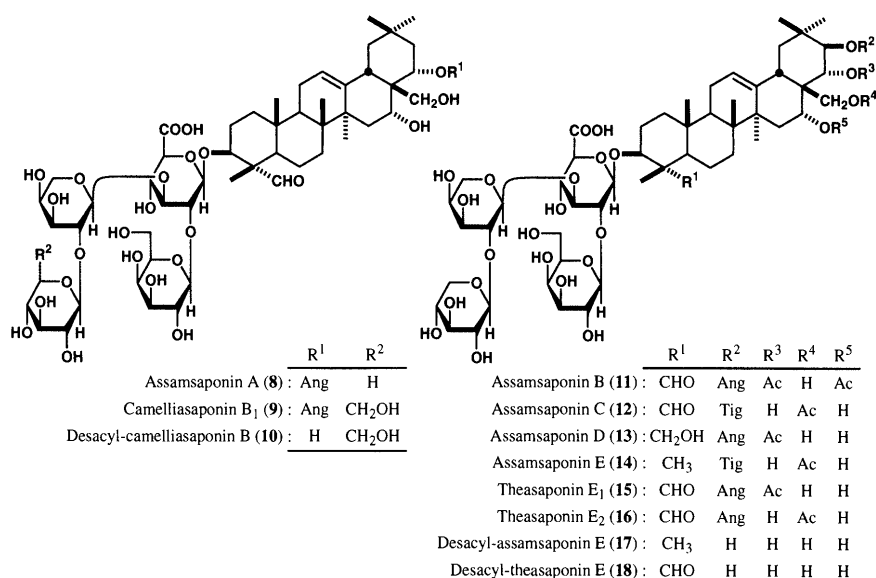


Chart 2

($M-C_{11}H_{19}O_9$)⁻; m/z 1159 ($M+Na$)⁺] and by high-resolution MS measurement. The proton and carbon signals assignable to the sapogenin moiety in the ¹H-NMR and ¹³C-NMR spectra of **5** were superimposable on those of desacyl-theasaponin E (**18**),³ while the proton and carbon signals due to the oligoglycoside moiety in **5** were very similar to those of desacyl-camelliasaponin B (**10**).⁴ The ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 1) spectra of **5**, which were assigned by various NMR analytical methods,⁸ showed signals due to a theasapogenol E moiety [δ 0.82, 0.85, 1.30, 1.34, 1.43, 1.78 (all s, 25, 26, 29, 30, 24, 27-H₃), 2.68 (dd-like, 18-H), 3.61, 3.87 (both m, 28-H₂), 4.03 (m, 3-H), 4.55 (m, 22-H), 4.72 (d, $J=9.7$ Hz, 21-H), 4.96 (br s, 16-H), 5.36 (br s, 12-H), 9.86 (s, 23-H)], a β -D-glucopyranosiduronic acid moiety [δ 4.85 (d, $J=6.9$ Hz, GlcA-1-H)], a β -D-galactopyranosyl moiety [δ 5.61 (d-like, Gal-1-H)], an α -L-arabinopyranosyl moiety [δ 5.75 (d, $J=5.3$ Hz, Ara-1-H)], and a β -D-glucopyranosyl moiety [δ 5.09 (d, $J=5.3$ Hz, Glc-1-H)]. The tetrasaccharide

structure bonding to the 3-position of the theasapogenol E moiety was identified by HMBC experiments. Thus, long-range correlations were observed between the 1'-proton of the glucuronic acid moiety and the 3-carbon of the theasapogenol E moiety, between the 1"-proton of the galactopyranosyl moiety and the 2'-carbon of the glucuronic acid moiety, between the 1'''-proton of the arabinopyranosyl moiety and 3'-carbon of glucuronic acid moiety, and between the 1''''-proton of the glucopyranosyl moiety and the 2'''-carbon of the arabinopyranosyl moiety. On the basis of this evidence, the structure of desacyl-assamsaponin F (**5**) was elucidated as shown.

The ¹H-NMR (pyridine-*d*₅) and ¹³C-NMR (Table 1) spectra⁸ of **1** showed signals assignable to an angeloyl group [δ 1.98 (s, Ang-5-H₃), 2.05 (d, $J=7.6$ Hz, Ang-4-H₃), 5.98 (dq-like, Ang-3-H)], together with the desacyl-assamsaponin F moiety [δ 5.58 (brs, 16-H), 5.85 (d, $J=10.3$ Hz, 21-H), 6.09

(d, $J=10.3$ Hz, 22-H)]. The positions of the angeloyl and acetyl groups of **1** were determined by an HMBC experiment on **1**, which showed long-range correlations between the 16- and 22-protons of the theasapogenol E moiety and two carbonyl carbons of acetyl groups and between the 21-proton of the theasapogenol E moiety and the carbonyl carbon of the angeloyl group. Finally, comparison of the ^1H - and ^{13}C -NMR data from **1** with those from **5** revealed acylation shifts around the 16-, 21-, and 22-positions of the theasapogenol E moiety. Consequently, the structure of assamsaponin F was determined to be 16,22-*O*-diacetyl-21-*O*-angeloyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid (**1**).

Assamsaponins G (**2**), H (**3**), and I (**4**) were isolated as colorless fine crystals from CHCl_3 -MeOH. In the negative- and positive-ion FAB-MS of **2**, **3**, and **4**, quasimolecular ion peaks were observed at m/z 1259 ($\text{M}-\text{H}$) $^-$ and m/z 1283 ($\text{M}+\text{Na}$) $^+$ and high-resolution MS analysis revealed the common molecular formula of **2**, **3**, and **4** to be $\text{C}_{60}\text{H}_{92}\text{O}_{28}$. Furthermore, fragment ion peaks at m/z 1097 ($\text{M}-\text{C}_6\text{H}_{11}\text{O}_5$) $^-$ and 965 ($\text{M}-\text{C}_{11}\text{H}_{19}\text{O}_9$) $^-$, which were thought to be derived by cleavage of the glycosidic linkage at the 2''- and 3'-positions (*vide infra*), were observed in the negative-ion FAB-MS of **2**, **3**, and **4**. Alkaline hydrolysis of 10% aqueous potassium hydroxide-50% aqueous dioxane (1:1, v/v) provided desacyl-assamsaponin F (**5**) and organic acids (acetic acid and angelic acid from **2** and **3**, and acetic acid and tiglic acid from **4**). The organic acids were converted to *p*-nitrobenzyl ester derivatives,⁶⁾ which were identified by HPLC.

The ^1H -NMR (pyridine- d_5) and ^{13}C -NMR (Table 1) spectra⁸⁾ of **2** showed signals assignable to the angeloyl moiety [δ 2.02 (s, Ang-5- H_3), 2.07 (d, $J=5.6$ Hz, Ang-4- H_3), 6.00 (dq-like, Ang-3-H)] and the acetyl moiety [δ 1.94 (Ac-2- H_3)] together with the desacyl-assamsaponin F moiety [δ 3.03 (dd-like, 18-H), 3.37, 3.59 (both d-like, 28- H_2), 4.02 (m, 3-H), 4.43 (brs, 16-H), 5.39 (brs, 12-H), 6.14 (d, $J=10.4$ Hz, 22-H), 6.56 (d, $J=10.4$ Hz, 21-H), 9.90 (s, 23-H)]. The positions of the angeloyl and acetyl groups of **2** were determined by HMBC, which showed long-range correlations between the 21-protons of the theasapogenol E moiety and the carbonyl carbon of the angeloyl group and between the 22-proton of the theasapogenol E moiety and the carbonyl carbon of the acetyl group. Consequently, the structure of assamsaponin G was determined to be 21-*O*-angeloyl-22-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid (**2**).

The ^1H -NMR (pyridine- d_5) and ^{13}C -NMR (Table 1) spectra⁸⁾ of **3** and **4** showed signals due to two acyl groups (acetyl and angeloyl groups for **3**, acetyl and tigloyl groups for **4**) and the 21,28-di-*O*-acylated structure of the desacyl-assamsaponin F moiety [**3**: δ 4.21 (m, 28- H_2), 6.42 (d, $J=10.2$ Hz, 21-H); **4**: δ 4.26 (m, 28- H_2), 6.38 (d, $J=10.0$ Hz, 21-H)]. The proton and carbon signals in the ^1H - and ^{13}C -NMR data from **3** and **4** were very similar to those of assamsaponin G (**2**), except for the signals due to the acyl groups of **3** and **4**. Long-range correlations were observed between the 21-proton and the carbonyl carbon of the angeloyl or tigloyl group and between the 28-protons and the carbonyl carbon of the acetyl

Table 1. ^{13}C -NMR Data for Assamsaponins F (**1**), G (**2**), H (**3**), I (**4**), and J (**6**) and Desacyl-assamsaponins F (**5**) and J (**7**)

	1	2	3	4	6	5	7
C- 1	38.2	38.2	38.3	38.3	38.8	38.3	38.9
2	25.3	25.2	25.3	25.3	26.4	25.2	26.5
3	84.4	84.2	84.3	84.3	89.9	84.2	89.9
4	55.0	55.1	55.1	55.1	39.5	55.1	39.7
5	48.5	48.4	48.5	48.5	55.8	48.5	55.9
6	20.5	20.4	20.4	20.4	18.3	20.4	18.5
7	32.4	32.5	32.5	32.5	33.1	32.5	33.3
8	40.3	40.3	40.4	40.4	40.1	40.3	40.1
9	46.8	46.8	46.8	46.9	46.9	46.9	47.1
10	36.0	36.1	36.0	36.1	36.8	36.1	36.9
11	23.7	23.8	23.9	23.9	23.8	23.8	23.9
12	124.9	124.0	122.7	122.7	125.3	122.7	122.9
13	141.0	142.9	142.8	142.8	140.9	144.0	144.0
14	41.2	41.7	41.9	41.9	41.2	42.1	42.1
15	31.0	34.5	34.6	34.6	31.2	34.2	34.4
16	71.3	68.1	67.7	67.7	71.7	68.4	67.9
17	46.9	48.0	47.1	47.1	47.2	47.3	47.3
18	39.6	40.2	40.6	40.6	39.7	41.2	41.3
19	47.2	47.2	47.4	47.4	47.3	48.2	48.3
20	36.1	36.3	36.1	36.3	35.9	36.3	36.4
21	78.5	78.9	81.4	81.7	78.8	78.7	78.8
22	73.4	74.5	71.4	71.5	73.3	77.4	77.4
23	210.1	209.9	209.8	209.9	28.0	209.7	28.2
24	11.1	11.0	11.1	11.1	16.7	11.0	16.9
25	15.8	15.8	15.8	15.8	15.6	15.8	15.7
26	16.8	16.9	17.0	17.0	16.8	16.9	17.0
27	27.0	27.4	27.4	27.4	27.0	27.4	27.4
28	63.9	64.0	66.5	66.5	63.4	68.5	68.6
29	29.4	29.5	29.8	29.8	29.3	30.5	30.6
30	19.7	20.2	20.2	20.1	19.4	19.4	19.4
GlcA-1'	104.1	104.0	104.0	104.0	105.5	101.7	105.4
2'	78.4	78.4	78.5	78.4	79.3	78.4	79.4
3'	84.4	84.4	84.5	84.5	82.8	84.5	83.0
4'	71.0	71.0	71.0	71.0	71.2	71.0	71.3
5'	77.2	77.2	77.3	77.2	77.3	77.2	77.6
6'	171.8	171.9	171.8	171.8	172.1	171.8	172.2
Gal-1''	103.6	103.5	103.6	103.6	103.7	103.6	103.8
2''	73.7	73.7	73.7	73.7	73.5	73.7	73.4
3''	75.1	75.1	75.2	75.1	75.2	75.2	75.2
4''	70.3	70.3	70.3	70.3	70.0	70.5	70.0
5''	76.6	76.6	76.6	76.6	76.8	76.6	76.8
6''	62.2	62.1	62.2	62.1	62.5	62.1	62.5
Ara-1'''	101.7	101.6	101.7	101.7	101.1	104.0	101.2
2'''	81.3	81.3	81.3	81.2	76.9	81.3	77.0
3'''	72.5	72.5	72.4	72.4	73.6	72.4	73.6
4'''	67.7	67.7	67.6	67.6	69.0	67.6	68.8
5'''	64.9	64.9	64.9	64.9	65.6	64.9	65.7
Glc or Rha-1'''	106.0	105.9	106.0	106.0	102.3	106.0	102.3
2'''	75.9	75.8	75.9	75.8	72.5	75.9	72.5
3'''	78.3	78.4	78.4	78.4	72.6	78.4	72.7
4'''	71.6	71.6	71.6	71.6	74.0	71.6	74.0
5'''	78.4	78.4	78.4	78.4	70.1	78.5	70.2
6'''	62.7	62.7	62.8	62.7	18.2	62.7	18.3
16-Ac-1	169.8				170.3		
2	22.0				22.1		
21-Ang, Tig, or Ac-1	167.8	167.9	168.7	168.6	171.1		
2	128.5	129.0	129.6	129.9	20.8		
3	138.0	137.1	136.0	136.3			
4	15.9	15.9	15.9	14.1			
5	20.9	20.9	21.0	12.5			
22-Ac or Cin-1	170.5	171.1			166.4		
2	20.8	20.9			118.6		
3					145.5		
1'					135.1		
2', 6'					128.6		
3', 5'					129.2		
4'					130.6		
28-Ac-1			170.6	170.7			
2			20.9	20.7			

500 MHz, pyridine- d_5 .

group in the HMBC experiment on **3** and **4**. In addition, acylation shifts were observed around the 21- and 28-positions in the ^{13}C -NMR data from **3** and **4**. Consequently, the structures of assamsaponins H and I were determined to be 21-*O*-angeloyl-28-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid (**3**) and 21-*O*-tigloyl-28-*O*-acetyltheasapogenol E 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)][β -D-glucopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid (**4**), respectively.

Isolation and Structural Elucidation of Assamsaponin J (6) from the Leaves The methanolic extract obtained from the leaves of the tea plant, *C. sinensis* var. *assamica* cultivated in Sri Lanka, was partitioned between an ethyl acetate and water mixture. In order to remove the polyphenols, the aqueous phase was treated with polyvinylpyrrolidone and filtered. The filtrate was extracted with 1-butanol and the 1-butanol extract was separated by normal-phase and reversed-phase silica gel column chromatography and finally HPLC to give assamsaponin J (**6**, 0.0012%). Assamsaponin J (**6**) was also isolated as colorless fine crystals of mp 230.2–232.0 °C. In the IR spectrum of **6**, absorption bands were observed at 3453, 1718, and 1653 cm^{-1} due to hydroxyl and carbonyl functions. The negative- and positive-ion FAB-MS of **6** showed quasimolecular ion peaks at m/z 1319 ($\text{M}-\text{H}^-$) and m/z 1343 ($\text{M}+\text{Na}^+$) and the molecular formula $\text{C}_{53}\text{H}_{86}\text{O}_{24}$ was determined by high-resolution MS measurement. The alkaline hydrolysis of **6** liberated desacyl-assamsaponin J (**7**) and two organic acids, cinnamic acid and acetic acid, which were identified by HPLC of their *p*-nitrobenzyl esters.⁶⁾ Acid hydrolysis of **7** liberated D-glucuronic acid, D-galactose, L-arabinose, and L-rhamnose, which were identified by GLC analysis. The proton and carbon signals in the ^1H -NMR and ^{13}C -NMR spectra of **7** resembled those of desacyl-assamsaponin E (**17**)⁵⁾ except for the signals due to the terminal α -L-rhamnopyranosyl moiety of **7**. The ^1H -NMR (pyridine- d_5) and ^{13}C -NMR (Table 1) spectra⁸⁾ of **7** showed signals due to a theasapogenol B moiety [δ 0.83, 0.89, 1.14, 1.26, 1.31, 1.35, 1.83 (all s, 25, 26, 24, 23, 29, 30, 27- H_3), 1.52 (d, $J=5.8$ Hz, Rha-6- H_3), 2.72 (dd-like, 18-H), 3.27 (dd-like, 3-H), 3.68, 3.99 (both d, $J=10.7$ Hz, 28- H_2), 4.55 (m, 22-H), 4.72 (d, $J=9.8$ Hz, 21-H), 5.37 (br s, 12-H)] and a tetrasaccharide moiety [δ 1.52 (d, $J=5.8$ Hz, Rha-6- H_3), 4.96 (d, $J=7.3$ Hz, GlcA-1-H), 5.62 (d, $J=7.6$ Hz, Gal-1-H), 5.98 (br s, Rha-1-H), 6.04 (d, $J=5.5$ Hz, Ara-1-H)]. Furthermore, the HMBC of **7** showed long-range correlations between the following protons and carbons: the 1'-proton of the glucopyranosiduronic acid moiety and the 3-carbon of theasapogenol B; the 1''-proton of the galactopyranosyl moiety and the 2'-carbon of the glucopyranosiduronic acid moiety; the 1'''-proton of the arabinopyranosyl moiety and the 3'-carbon of the glucopyranosiduronic acid moiety; the 1''''-proton of the rhamnopyranosyl moiety and the 2'''-carbon of the arabinopyranosyl moiety. On the basis of these findings, the structure of desacyl-assamsaponin J (**7**) was determined as shown.

The ^1H -NMR (pyridine- d_5) and ^{13}C -NMR (Table 1) spectra⁸⁾ of **6** showed the presence of a cinnamoyl group [δ 6.73 (d, $J=16.2$ Hz, Cin-2-H), 7.32 (m, Cin-3', 4', 5'-H), 7.64 (m, Cin-2', 6'-H), 8.04 (d, $J=16.2$ Hz, Cin-3-H)] and two acetyl groups [δ 2.04, 2.56 (both s, Ac-2, Ac-2'- H_3)] together with

Table 2. Effects of the Saponin Mixture and Theasaponins E_1 (**15**) and E_2 (**16**) from *C. sinensis* var. *assamica* on Gastric Emptying (GE) and Gastrointestinal Transit (GIT)

Treatment	Dose (mg/kg, <i>p.o.</i>)	N	GE (%)	GIT (%)
Control	—	10	88.5 $\pm$ 1.8	45.2 $\pm$ 2.7
Saponin Mixture	50	10	83.2 $\pm$ 3.2	50.2 $\pm$ 2.4
	100	10	75.0 $\pm$ 3.7**	73.5 $\pm$ 1.0**
	200	10	53.3 $\pm$ 2.0**	75.1 $\pm$ 1.8**
Control	—	7	90.9 $\pm$ 1.4	44.8 $\pm$ 1.6
Theasaponin E_1 (15)	25	7	89.5 $\pm$ 3.0	46.4 $\pm$ 1.5
	50	7	76.5 $\pm$ 2.8**	49.8 $\pm$ 4.3
	100	7	59.2 $\pm$ 4.0**	60.0 $\pm$ 3.4*
Theasaponin E_2 (16)	25	7	95.2 $\pm$ 0.6	52.0 $\pm$ 5.0
	50	7	90.7 $\pm$ 1.9	53.1 $\pm$ 5.1
	100	7	94.7 $\pm$ 1.8	47.6 $\pm$ 3.9

Values represent the mean $\pm$ S.E.M. Significantly different from the control group, * $p<0.05$, ** $p<0.01$.

a desacyl-assamsaponin J moiety [δ 3.07 (dd, $J=4.0$, 14.4 Hz, 18-H), 3.24 (dd, $J=5.2$, 11.6 Hz, 3-H), 3.48, 3.62 (both d, $J=9.2$ Hz, 28- H_2), 45.43 (br s, 16-H), 5.87 (d, $J=10.4$ Hz, 21-H), 6.27 (d, $J=10.4$ Hz, 22-H)]. The positions of the cinnamoyl and acetyl groups in **6** were determined by HMBC, which showed long-range correlations between the 16,21-protons of the theasapogenol B moiety and the two carbonyl carbons of the acetyl groups and between the 22-proton of the theasapogenol B moiety and the carbonyl carbon of the cinnamoyl group. Finally, comparison of the ^1H - and ^{13}C -NMR data from **6** with those from **7** revealed acylation shifts around the 16, 21, and 22-positions of the theasapogenol B moiety of **7**. Consequently, the structure of assamsaponin J was determined to be 16,21-di-*O*-acetyl-22-*O*-cinnamoyltheasapogenol B 3-*O*-[β -D-galactopyranosyl(1 $\rightarrow$ 2)]-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2)- α -L-arabinopyranosyl(1 $\rightarrow$ 3)]- β -D-glucopyranosiduronic acid (**6**).

As shown in Table 2, the saponin mixture obtained from the seeds of *C. sinensis* var. *assamica* was found to exhibit an inhibitory effect on gastric emptying and an accelerating effect on gastrointestinal transit in mice. It is noteworthy that no drug having both gastric emptying inhibitory activity and gastrointestinal transit accelerating activity has been identified. These activities of the saponin fraction may be beneficial for the prevention and treatment of the inhibition of gastrointestinal transit such as affects the ileus in clinical situations. Next, the effects of theasaponins E_1 (**15**) and E_2 (**16**), isolated as common constituents from the seeds of *C. sinensis* var. *assamica* as well as Japanese *C. sinensis*, on gastric emptying and gastrointestinal transit were examined. Theasaponin E_1 (**15**) was found to inhibit gastric emptying and accelerate gastrointestinal transit. On the other hand, **16** showed no such activities. These findings indicate that the position of the acyl groups is important for pharmacological activity.

Experimental

The instruments used to obtain physical data and the experimental conditions for chromatography were the same as described in our previous paper.¹⁾

Isolation of Assamsaponins F (1), G (2), H (3), and I (4) from the Seeds of *C. sinensis* var. *assamica* Assamsaponins F (**1**), G (**2**), H (**3**), and I (**4**) were isolated from the fresh seeds of *C. sinensis* var. *assamica* cultivated in Nuwara Eliya, Sri Lanka, as described earlier.⁵⁾

Assamsaponin F (**1**): Colorless fine crystals from CHCl_3 -MeOH, mp 209.8–210.3 °C, $[\alpha]_D^{26} +8.8^\circ$ ($c=0.1$, MeOH). IR (KBr): 3432, 1721, 1649, 1078 cm^{-1} . High-resolution positive-ion FAB-MS: Calcd for $\text{C}_{62}\text{H}_{94}\text{O}_{29}\text{Na}$

(M+Na)⁺: 1325.5778. Found: 1325.5798. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.74, 0.78, 1.07, 1.26, 1.43, 1.45, 1.98, 2.03, 2.51 (3H each, all s, 26, 25, 30, 29, 27, 24, Ang-5, Ac-2, Ac-2'-H₃), 2.05 (3H, d, *J*=7.6 Hz, Ang-4-H₃), 2.98 (1H, dd-like, 18-H), 3.45, 3.57 (1H each, both d, *J*=13.4 Hz, 28-H₂), 3.99 (1H, dd, *J*=2.8, 9.8 Hz, 3-H), 4.82 (1H, d, *J*=7.3 Hz, GlcA-1-H), 5.10 (1H, d, *J*=7.4 Hz, Glc-1-H), 5.39 (1H, brs, 12-H), 5.58 (1H, brs, 16-H), 5.61 (1H, d, *J*=7.7 Hz, Gal-1-H), 5.77 (1H, d, *J*=5.1 Hz, Ara-1-H), 5.85 (1H, d, *J*=10.3 Hz, 21-H), 5.98 (1H, dq-like, Ang-3-H), 6.09 (1H, d, *J*=10.3 Hz, 22-H), 9.94 (1H, s, 23-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1301 (M-H)⁻, 1139 (M-C₆H₁₁O₅)⁻, 1007 (M-C₁₁H₁₉O₉)⁻. Positive-ion FAB-MS: *m/z* 1325 (M+Na)⁺.

Assamsaponin G (2): Colorless fine crystals from CHCl₃-MeOH, mp 187.7–188.5 °C, [α]_D²⁰ +14.9° (*c*=0.1, MeOH). IR (KBr): 3454, 1719, 1655, 1076 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₆₀H₉₂O₂₈Na (M+Na)⁺: 1283.5673. Found: 1283.5686. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.82, 0.84, 1.08, 1.30, 1.44, 1.77, 1.94, 2.02 (3H each, all s, 25, 26, 29, 30, 24, 27, Ac-2, Ang-5-H₃), 2.07 (3H, d, *J*=5.6 Hz, And-4-H₃), 3.03 (1H, dd-like, 18-H), 3.37, 3.59 (1H each, both d-like, 28-H₂), 4.02 (1H, m, 3-H), 4.43 (1H, brs, 16-H), 4.86 (1H, d, *J*=7.3 Hz, GlcA-1-H), 5.11 (1H, d, *J*=7.0 Hz, Glc-1-H), 5.39 (1H, brs, 12-H), 5.61 (1H, d-like, Gal-1-H), 5.75 (1H, d, *J*=5.2 Hz, Ara-1-H), 6.00 (1H, dq-like, Ang-3-H), 6.14 (1H, d, *J*=10.4 Hz, 22-H), 6.56 (1H, d, *J*=10.4 Hz, 21-H), 9.90 (1H, s, 23-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1259 (M-H)⁻, 1097 (M-C₆H₁₁O₅)⁻, 965 (M-C₁₁H₁₉O₉)⁻. Positive-ion FAB-MS: *m/z* 1283 (M+Na)⁺.

Assamsaponin H (3): Colorless fine crystals from CHCl₃-MeOH, mp 206.3–207.6 °C, [α]_D²⁰ +41.0° (*c*=0.1, MeOH). IR (KBr): 3432, 1719, 1655, 1078 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₆₀H₉₂O₂₈Na (M+Na)⁺: 1283.5673. Found: 1283.5685. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.83, 0.95, 1.11, 1.29, 1.44, 1.76, 1.98, 1.99 (3H each, all s, 25, 26, 29, 30, 24, 27, Ang-5, Ac-2-H₃), 2.04 (3H, d, *J*=7.4 Hz, Ang-4-H₃), 2.81 (1H, dd-like, 18-H), 4.04 (1H, dd, *J*=4.6, 11.6 Hz, 3-H), 4.21 (2H, m, 28-H₂), 4.44 (1H, d-like, 22-H), 4.69 (1H, brs, 16-H), 4.85 (1H, d, *J*=7.3 Hz, GlcA-1-H), 5.09 (1H, d, *J*=7.3 Hz, Glc-1-H), 5.43 (1H, brs, 12-H), 5.62 (1H, d, *J*=7.6 Hz, Gal-1-H), 5.77 (1H, d, *J*=5.3 Hz, Ara-1-H), 5.90 (1H, dq-like, Ang-3-H), 6.42 (1H, d, *J*=10.2 Hz, 21-H), 9.90 (1H, s, 23-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1259 (M-H)⁻, 1097 (M-C₆H₁₁O₅)⁻, 965 (M-C₁₁H₁₉O₉)⁻. Positive-ion FAB-MS: *m/z* 1283 (M+Na)⁺.

Assamsaponin I (4): Colorless fine crystals from CHCl₃-MeOH, mp 208.2–209.5 °C, [α]_D²⁰ +35.6° (*c*=0.1, MeOH). IR (KBr): 3432, 1719, 1655, 1078 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₆₀H₉₂O₂₈Na (M+Na)⁺: 1283.5673. Found: 1283.5653. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.83, 0.95, 1.10, 1.31, 1.43, 1.76, 1.86, 2.03 (3H each, all s, 25, 26, 29, 30, 24, 27, Tig-5, Ac-2-H₃), 1.61 (3H, d, *J*=7.2 Hz, Tig-4-H₃), 2.83 (1H, dd-like, 18-H), 4.04 (1H, m, 3-H), 4.26 (2H, m, 28-H₂), 4.47 (1H, m, 22-H), 4.70 (1H, brs, 16-H), 4.85 (1H, d, *J*=7.2 Hz, GlcA-1-H), 5.09 (1H, d, *J*=6.5 Hz, Glc-1-H), 5.50 (1H, brs, 12-H), 5.60 (1H, d, *J*=7.2 Hz, Gal-1-H), 5.76 (1H, d, *J*=4.5 Hz, Ara-1-H), 6.38 (1H, d, *J*=10.0 Hz, 21-H), 7.00 (1H, dq-like, Tig-3-H), 9.89 (1H, s, 23-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1259 (M-H)⁻, 1097 (M-C₆H₁₁O₅)⁻, 965 (M-C₁₁H₁₉O₉)⁻. Positive-ion FAB-MS: *m/z* 1283 (M+Na)⁺.

Isolation of Assamsaponins J (6) from the Leaves of *C. sinensis* var. *assamica* The leaves of *C. sinensis* L. var. *assamica* PIERRE (4.1 kg, cultivated in Nuwara Eliya, Sri Lanka) were cut and extracted three times with MeOH under reflux. Evaporation of the solvent under reduced pressure provided the MeOH extract (1316 g, 32.1%). Part of it (418 g) was partitioned in an AcOEt-H₂O (1:1, v/v) mixture. The aqueous layer was suspended in water and treated with polyvinylpyrrolidone (Nacalai Tesque) to remove the polyphenols. After filtration, the filtrate was extracted with *n*-BuOH. Removal of the solvent from the AcOEt-soluble, *n*-BuOH-soluble, and H₂O-soluble fractions under reduced pressure yielded an AcOEt extract (235 g, 5.7%), an *n*-BuOH extract (73 g, 1.8%), and an H₂O phase. The *n*-BuOH extract (62 g) was subjected to normal-phase silica gel column chromatography [1 kg, CHCl₃-MeOH-H₂O (7:3:1, lower layer→7:3:0.5, v/v)→MeOH] to give eight fractions [Fr. 1 (9.4 g), 2 (20.5 g), 3 (3.8 g), 4 (4.5 g), 5 (2.7 g), 6 (5.8 g), 7 (1.7 g), 8 (7.5 g)]. Reversed-phase silica gel column chromatography [114 g, MeOH-H₂O (50:50→80:20, v/v)→MeOH] of fraction 3 (3.8 g) gave three fractions [Fr. 3-1 (1.8 g), 2 (494 mg), 3 (1.4 g)]. Fraction 3-2 (480 mg) was purified by HPLC [CH₃CN-1% aq. AcOH (45:55, v/v)] to give assamsaponin J (6, 49 mg, 0.0012%).

Assamsaponin J (6): Colorless fine crystals from CHCl₃-MeOH, mp

230.2–232.0 °C, [α]_D²⁵ -32.2° (*c*=0.1, MeOH). IR (KBr): 3453, 1718, 1653, 1075 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₆₆H₉₆O₂₉Na (M+Na)⁺: 1343.6037. Found: 1343.6047. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.77, 0.78, 1.07, 1.15, 1.27, 1.28, 1.50, 2.04, 2.56 (3H each, all s, 26, 25, 29, 24, 23, 30, 27, Ac-2, Ac-2'-H₃), 1.51 (3H, d, *J*=6.4 Hz, Rha-6-H₃), 3.07 (1H, dd, *J*=4.0, 14.4 Hz, 18-H), 3.24 (1H, dd, *J*=5.2, 11.6 Hz, 3-H), 3.48, 3.62 (1H each, both d, *J*=9.2 Hz, 28-H₂), 4.93 (1H, d, *J*=7.6 Hz, GlcA-1-H), 5.43 (1H, brs, 16-H), 5.99 (1H, brs, Rha-1-H), 5.63 (1H, d, *J*=7.6 Hz, Gal-1-H), 5.71 (1H, brs, 12-H), 5.87 (1H, d, *J*=10.4 Hz, 21-H), 6.04 (1H, d, *J*=5.8 Hz, Ara-1-H), 6.27 (1H, d, *J*=10.4 Hz, 22-H), 6.73 (1H, d, *J*=16.2 Hz, Cin-2-H), 7.32 (3H, m, Cin-3', 4', 5'-H), 7.64 (2H, m, Cin-2', 6'-H), 8.04 (1H, d, *J*=16.2 Hz, Cin-3-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1319 (M-H)⁻. Positive-ion FAB-MS: *m/z* 1343 (M+Na)⁺, 1365 (M+2Na-H)⁺.

Alkaline Hydrolysis of Assamsaponins F (1), G (2), H (3), and I (4)

A solution of assamsaponins (1, 2: 5 mg; 3: 30 mg; 4: 20 mg) in 50% aqueous dioxane (2 ml) was treated with 10% aqueous KOH (2 ml) and stirred at 37 °C for 1 h. After removal of the solvent from an aliquot (0.1 ml) of the reaction mixture under reduced pressure, the residue was dissolved in (CH₂)Cl₂ (2 ml) and the solution was treated with *p*-nitrobenzyl-*N,N'*-diisopropylisourea (10 mg), then stirred at 80 °C for 1 h. The reaction solution was subjected to HPLC analysis to identify the *p*-nitrobenzyl esters of angelic acid (a) from 1, 2, and 3, tiglic acid (b) from 4, and acetic acid (c) from 1, 2, 3, and 4. HPLC conditions: column, YMC-Pack ODS-A (YMC Co., Ltd.), 250×4.6 mm (i.d.); solvent, MeOH-H₂O (70:30, v/v); flow rate, 1.0 ml/min; *t*_R: a: 18.6 min; b: 17.1 min; c: 8.2 min.

The rest of the reaction mixture was neutralized with Dowex HCR W2 (H⁺ from) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a product, which was subjected to normal-phase silica gel column chromatography [3 g, CHCl₃-MeOH-H₂O (6:4:1, v/v)] to give desacyl-assamsaponin F (5, 3 mg from 1; 16 mg from 2; 17 mg from 3; 4 mg from 4).

Desacyl-assamsaponin F (5): Colorless fine crystals from CHCl₃-MeOH, mp 200.2–201.0 °C, [α]_D²⁰ +36.0° (*c*=0.1, MeOH). IR (KBr): 3453, 1719, 1647, 1078 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₅₃H₈₄O₂₆Na (M+Na)⁺: 1159.5149. Found: 1159.5192. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.82, 0.85, 1.30, 1.34, 1.43, 1.78 (3H each, all s, 25, 26, 29, 30, 24, 27-H₃), 2.68 (1H, dd-like, 18-H), 3.61, 3.87 (1H each, both m, 28-H₂), 4.03 (1H, m, 3-H), 4.55 (1H, m, 22-H), 4.72 (1H, d, *J*=9.7 Hz, 21-H), 4.85 (1H, d, *J*=6.9 Hz, GlcA-1-H), 4.96 (1H, brs, 16-H), 5.09 (1H, d, *J*=5.3 Hz, Glc-1-H), 5.36 (1H, brs, 12-H), 5.61 (1H, d-like, Gal-1-H), 5.75 (1H, d, *J*=5.3 Hz, Ara-1-H), 9.86 (1H, s, 23-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS: *m/z* 1135 (M-H)⁻, 973 (M-C₆H₁₁O₅)⁻, 841 (M-C₁₁H₁₉O₉)⁻. Positive-ion FAB-MS: *m/z* 1159 (M+Na)⁺.

Alkaline Hydrolysis of Assamsaponins J (6) A solution of assamsaponins (6, 11 mg) in 50% aqueous dioxane (2 ml) was treated with 10% aqueous KOH (2 ml) and stirred at 37 °C for 1 h. After removal of the solvent from an aliquot (0.1 ml) of the reaction mixture under reduced pressure, the residue was dissolved in (CH₂)Cl₂ (2 ml) and the solution was treated with *p*-nitrobenzyl-*N,N'*-diisopropylisourea (10 mg), then stirred at 80 °C for 1 h. The reaction solution was subjected to HPLC analysis to identify the *p*-nitrobenzyl ester of acetic acid from 6. HPLC conditions: column, YMC-Pack ODS-A (YMC Co., Ltd.), 250×4.6 mm (i.d.); solvent, MeOH-H₂O (70:30, v/v); flow rate, 1.0 ml/min; *t*_R: 8.3 min.

The rest of the reaction mixture was neutralized with Dowex HCR W2 (H⁺ from) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a product, which was subjected to normal-phase silica gel column chromatography [3 g, CHCl₃-MeOH-H₂O (6:4:1, v/v)] to give desacyl-assamsaponin J (7, 6 mg) and cinnamic acid (1 mg). Cinnamic acid was found to be identical to an authentic sample following TLC and ¹H-NMR (CDCl₃) comparisons.

Desacyl-assamsaponin J (7): Colorless fine crystals from CHCl₃-MeOH, mp 228.3–239.7 °C, [α]_D²⁰ -6.8° (*c*=0.1, MeOH). IR (KBr): 3453, 1638, 1078 cm⁻¹. High-resolution positive-ion FAB-MS: Calcd for C₅₃H₈₆O₂₄Na (M+Na)⁺: 1129.5407. Found: 1129.5422. ¹H-NMR (500 MHz, pyridine-*d*₅) δ: 0.83, 0.89, 1.14, 1.26, 1.31, 1.35, 1.83 (3H each, all s, 25, 26, 24, 23, 29, 30, 27-H₃), 1.52 (3H, d, *J*=5.8 Hz, Rha-6-H₃), 2.72 (1H, dd-like, 18-H), 3.27 (1H, dd-like, 3-H), 3.68, 3.99 (1H each, both d, *J*=10.7 Hz, 28-H₂), 4.55 (1H, m, 22-H), 4.72 (1H, d, *J*=9.8 Hz, 21-H), 4.96 (1H, d, *J*=7.3 Hz, GlcA-1-H), 5.00 (1H, brs, 16-H), 5.37 (1H, brs, 12-H), 5.62 (1H, d, *J*=7.6 Hz, Gal-1-H), 5.98 (1H, brs, Rha-1-H), 6.04 (1H, d, *J*=5.5 Hz, Ara-1-H). ¹³C-NMR (125 MHz, pyridine-*d*₅) δ_C: given in Table 1. Negative-ion FAB-MS:

m/z 1105 ($M-H$)⁻, 943 ($M-C_6H_{11}O_5$)⁻. Positive-ion FAB-MS: m/z 1129 ($M+Na$)⁺.

Acid Hydrolysis of Desacyl-assamsaponins F (5) and J (7) A solution of desacyl-assamsaponins (5, 7, 2 mg each) in 5% aq. H_2SO_4 -1,4-dioxane (1:1, v/v, 1 ml) was heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resin was filtered. After removal of the solvent under reduced pressure, the residue was passed through a Sep-Pak C18 cartridge using H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (0.01 ml) in pyridine (0.02 ml) at 60 °C for 1 h. After reaction, the solution was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (0.01 ml) at 60 °C for 1 h. The supernatant was then subjected to GLC to identify the derivatives of D-glucuronic acid (i), D-galactose (ii), and L-arabinose (iii) from 5 and 7; and D-glucose (iv) from 5; L-rhamnose from 7. GLC conditions: column, SupelcoTM-1, 0.25 mm (i.d.) $\times$ 30 m; column temperature, 230 °C; t_R , i: 26.5 min; ii: 25.6 min; iii: 15.1 min; iv: 24.4 min; v: 12.2 min.

Measurement of Gastric Emptying Gastric emptying was determined by a modification of the phenol red method.⁹⁾ A solution of 1.5% carboxymethyl cellulose sodium salt (CMC-Na), 40% glucose, milk [milk powder: water (w/w)=1:3], or 60% ethanol containing 0.05% phenol red as a marker was given intragastrically (0.5 ml/mouse) to conscious mice. Thirty minutes later, mice were sacrificed by cervical dislocation. The abdominal cavity was opened, and the gastroesophageal junction and pylorus were clamped, then the stomach was removed, weighed and placed in 14 ml 0.1 N of NaOH and homogenized. The suspension was allowed to settle for 1 h at room temperature and 5 ml of the supernatant was added to 0.5 ml of 20% trichloroacetic acid (w/v) and then centrifuged at 3000 rpm for 20 min. The supernatant was mixed with 4 ml of 0.5 N NaOH, and the amount of phenol red was determined from the absorbance at 560 nm (Beckman, DU530). Phenol red recovered from animals sacrificed immediately after the administration of a test meal was used as a standard (0% emptying). The gastric emptying (%) during the 30 min period was calculated from the following equation: Gastric emptying (%)=(1-amount of test sample/amount of standard) $\times$ 100. The test sample was given orally by means of a metal orogastric tube 30 min prior to the administration of the test meal.

Measurement of Gastrointestinal Transit A charcoal meal containing a solution of 1.5% CMC-Na and 5% charcoal as a marker was given intragastrically (0.2 ml/mouse) to conscious mice. Twenty minutes later, mice were sacrificed by cervical dislocation. The abdominal cavity was opened, and the gastrointestinal tract was removed. The distance traveled by the marker was the length of the small intestine from the pylorus to the caecum. Test samples were given orally by means of a metal orogastric tube 60 min prior to the administration of the charcoal meal.

Statistics Values are expressed as means $\pm$ S.E.M. One-way analysis of variance following Dunnett's test¹⁰⁾ for multiple comparison analysis were

used for statistical analysis. Probability (*p*) values less than 0.05 were considered significant.

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References and Notes

- 1) Part XVI: Yoshikawa M., Murakami T., Ohminami H., Matsuda H., *Chem. Pharm. Bull.*, **48**, 1045—1050 (2000).
- 2) a) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **368**, 237—243 (1999); b) Matsuda H., Li Y., Yoshikawa M., *ibid.*, **373**, 63—70 (1999); c) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Bioorg. Med. Chem.*, **7**, 323—327 (1999); d) Matsuda H., Li Y., Yamahara J., Yoshikawa M., *J. Pharmacol. Exp. Ther.*, **289**, 729—734 (1999); e) Yoshikawa M., Murakami T., Hirano K., Matsuda H., Yamahara J., Ohtani K., Kasai R., Yamasaki K., *Heterocycles*, **49**, 93—96 (1999); f) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **65**, PL27—PL32 (1999); g) Murakami T., Matsuda H., Inadzuki M., Hirano K., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1717—1724 (1999); h) Matsuda H., Murakami T., Ikebata A., Yamahara J., Yoshikawa M., *ibid.*, **47**, 1744—1748 (1999); i) Li Y., Matsuda H., Wen S., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **387**, 337—342 (2000); j) Li Y., Matsuda H., Yamahara J., Yoshikawa M., *ibid.*, **329**, 71—77 (2000); k) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **66**, 2233—2238 (2000).
- 3) Kitagawa I., Hori K., Motozawa T., Murakami T., Yoshikawa M., *Chem. Pharm. Bull.*, **46**, 1901—1906 (1998).
- 4) Yoshikawa M., Murakami T., Yoshizumi S., Murakami N., Yamahara J., Matsuda H., *Chem. Pharm. Bull.*, **44**, 1899—1907 (1996).
- 5) Murakami T., Nakamura J., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **47**, 1759—1764 (1999).
- 6) Yoshikawa K., Nakagawa M., Yamamoto R., Arihara S., Matsuura K., *Chem. Pharm. Bull.*, **40**, 1779—1782 (1992).
- 7) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **34**, 1843—1845 (1986).
- 8) The ^1H - and ^{13}C -NMR spectra of 1, 2, 3, 4, 5, 6, and 7 were assigned on the basis of homo- and hetero-correlation spectroscopy (^1H - ^1H , ^1H - ^{13}C COSY), homo- and heteronuclear Hartmann-Hahn spectroscopy (^1H - ^1H , ^1H - ^{13}C HOHAHA) and heteronuclear multiple bond correlation (HMBC) experiments.
- 9) a) Taché Y., Maeda M., Hagiwara M., Turkelson C. M., *Am. J. Physiol.*, **253**, G241—G245 (1986); b) Barquist E., Bonaz B., Martinez V., Rivier J., Ziner M. J., Taché Y., *ibid.*, **270**, R888—R894 (1996).
- 10) a) Dunnett C. W., *J. Am. Statist. Assoc.*, **75**, 789—795 (1980); b) *Idem*, *ibid.*, **75**, 796—800 (1980).

A New Type of Stilbene-Related Secondary Metabolite, Idenburgene, from *Cryptocarya idenburgensis*

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A new type of natural product, idenburgene (1), was isolated from *Cryptocarya idenburgensis*, and its unique structure was elucidated. Four known compounds, 3-hydroxy-5-methoxystilbene (2), 2',6'-dihydroxy-4'-methoxydihydrochalcone (3), stigmast-4-ene-3-one, and β -sitosterol were also isolated and identified.

Key words *Cryptocarya idenburgensis*; Lauraceae; idenburgene; stilbene; 3-hydroxy-5-methoxystilbene; 2',6'-dihydroxy-4'-methoxydihydrochalcone

The genus *Cryptocarya* belonging to the Lauraceae family is known as a rich source of secondary metabolites such as flavonoids, pyrones, lignans, terpenoids and alkaloids. Some compounds obtained from this genus show antitumor activity: cryptocaryone, goniothalamine, kamahar lactone, and kurzilactone which were respectively isolated from *C. bourdillonii*¹⁾ and *C. laevigata*,²⁾ *C. calounera*,³⁾ *C. kamahar*,⁴⁾ and *C. kurzii*.⁵⁾ During our chemical study of this genus,^{2,4,6–8)} *Cryptocarya idenburgensis* ALLEN., which is a big tree growing in the forests of Irian Jaya Island, Indonesia, at altitude ca. 1400 m, was examined because the species has not been previously investigated chemically. According to Kosterman,⁹⁾ the synonym for this species is *Cryptocarya gonioclada* KAN. & HAT.

A new compound, idenburgene (1), was obtained as a yellow viscous solid from the CHCl₃ extract of the stem bark of *Cryptocarya idenburgensis*. The HR-FAB/MS spectrum displayed an M⁺ ion at *m/z* 442.2126 corresponding to a molecular formula C₂₉H₃₀O₄. The ¹³C-NMR spectrum exhibited the presence of 29 carbons including a carbonyl group (δ 174.2). The partial structures in 1 were analyzed from the spectroscopic data. The first partial structure, A, is a stilbene moiety. In the ¹H-NMR spectrum, the signals appeared at δ_{H} 7.03 (d, *J*=16.1 Hz, H-7) and 7.05 (d, *J*=16.1 Hz, H-8) and were assigned to a pair of *trans*-olefinic protons. The signals at δ_{H} 6.62 (H-2, d, *J*=1.6 Hz) and 6.66 (H-6, d, *J*=1.6 Hz) were apparently two *meta* coupled aromatic protons. The ¹H-NMR also showed the presence of a methoxy group and a hydroxy group at the benzene ring, which resonated at δ_{H} 3.88 (s) and 5.20 (s), respectively. The signals at δ_{H} 7.26 (H-4', m), 7.35 (H-3' and H-5', m) and 7.50 (H-2' and H-6', br d, *J*=7.6 Hz) indicated the presence of a monosubstituent benzene group. The HMBC spectrum showed a correlation between an olefinic proton at H-8 and aromatic carbons at δ_{C} 126.5 (C-2' and C-6') and also between H-7 and aromatic carbons at δ_{C} 102.0 (C-6) and 107.9 (C-2). The ¹H- and ¹³C-NMR data of this stilbene moiety in 1 were very similar to those of 2, which was isolated together with 1, except for the data at the C-4 position. From the above observations, the presence of a 3-hydroxy-5-methoxystilbene moiety in 1 was suggested.

Additionally, the presence of methylene protons was observed at δ_{H} 1.59 (H-3'', m) and 1.67 (H-3'', m) that were coupled to two other methylenes at δ_{H} 1.90 (H-4'', m) and

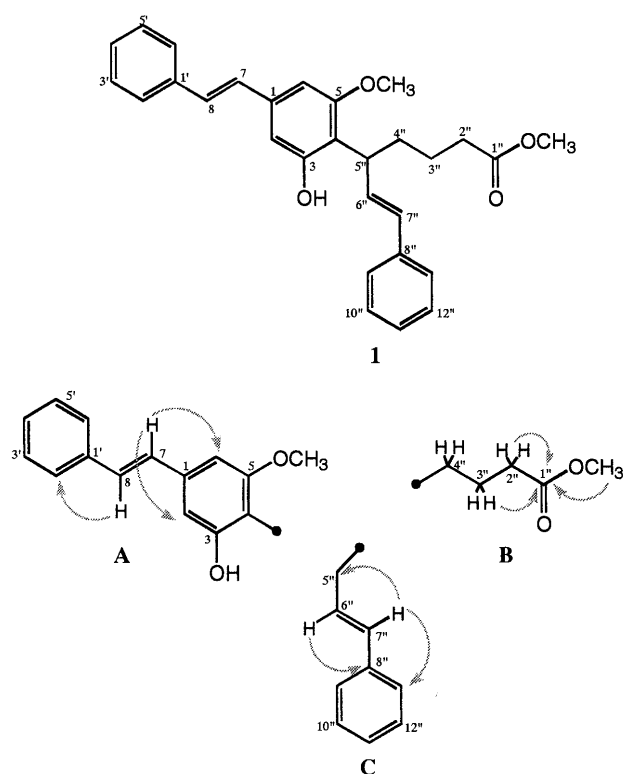
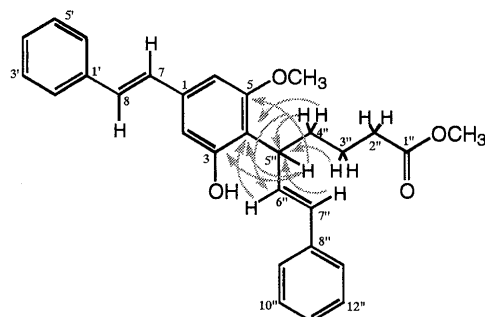
2.35 (H-2'', m). The HMBC spectrum showed correlations between a carbonyl carbon at δ_{C} 174.2 (C-1'') and the protons at H-2'', H-3'', and a methoxy group (δ 3.65), which confirmed the presence of an *n*-butyric acid methyl ester moiety in 1 as a second partial structure, B.

The existence of another pair of *trans*-olefinic protons was identified from the signals at δ_{H} 6.52 (H-7'', dd, *J*=1.5, 16.1 Hz) and 6.63 (H-6'', br d, *J*=16.1 Hz), which were coupled with each other. Furthermore, the presence of the second monosubstituent benzene group was revealed from ¹H-NMR, which displayed signals at δ_{H} 7.19 (H-11'', br dd, *J*=7.3, 7.3 Hz), 7.25 (H-10'' and H-12'', m) and 7.36 (H-9'' and H-13'', m). The cross peaks in the HMBC spectrum exhibited a correlation between the proton at H-7'' and carbons at δ_{C} 126.2 (C-9'' and C-13'') and H-6'' with C-8'' (δ_{C} 137.3) confirming that the *trans*-olefin group was connected with the benzene ring to form a styrene structural moiety. The ¹H–¹H correlation between the olefinic proton (H-6'') and the methine proton at δ_{H} 4.21 (H-5'') in the COSY spectrum as well as the connectivity between H-7'' and C-5'' in the HMBC spectrum enabled us to construct the third partial structure C in 1 as a phenylpropanoid unit (Ph–CH=CH–CH–).

As we clarified the basic structures of three units (A, B and C) in 1 (Fig. 1), we next constructed the full structure using the HMBC spectrum (Fig. 2), as follows. Both the protons at H-3'' and H-4'' correlated to C-5'', which indicated the attachment of the terminal carbon of an *n*-butyric acid moiety to C-5'' in a phenylpropanoid moiety. A correlation appearing between protons at H-5'' and carbons at δ_{C} 117.2 (C-4), 158.6 (C-5) and 155.1 (C-3) confirmed that the C-4 position the stilbene moiety was also attached to C-5''. Further, cross peaks between H-4'' and C-4, between H-4'' and C-6'' (δ 132.3), and also between H-6'' and C-4, confirmed the connecting position described above. The proposed structure 1 was strongly supported by the EI-MS data, which showed the existence of a molecular ion peak at *m/z* 442 (28%) and the loss of an aliphatic ester molecule (C₅H₉O₂), which appeared at *m/z* 341 as the base peak. The CD spectrum of 1 revealed a cotton effect due to a chiral center, but its absolute configuration is unknown at this stage.

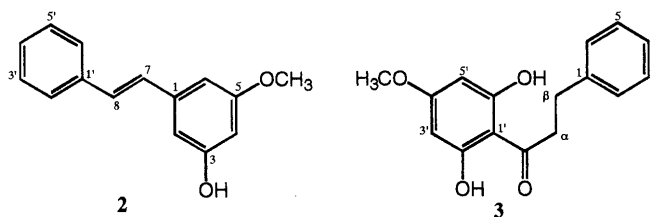
We are interested in understanding the biosynthetic construction of this unique molecule (1). The structure of 1 can be dissected at the bond between C-4 and C-5'' into two

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Fig. 1. Partial Structures of **1** and Their Selected HMBCFig. 2. Selected HMBC between Partial Structures in **1**

parts, a stilbene unit and the remaining half. It is quite obvious that the stilbene part is biosynthesized through condensation of a cinnamoyl-CoA with three units of malonate followed by ring closure. Intensive enzymatic studies have been done on so called CHS-super families, the enzymes that catalyze formation of chalcones, stilbenes and other polyketides in higher plants.¹⁰ Obviously similar enzymes are in operation in this plant to form the stilbene part of **1**. More interesting is the biosynthetic origin of the other half, the C-1''—C-13'' moiety of **1**. It is likely that this part again is formed from a cinnamoyl-CoA and malonate-derived C₂ units. This time two units of malonate are involved in the chain elongation to form the C₆—C₃—C₄ backbone and no cyclization takes place here. Similar co-occurrence of a linear-type polyketide with chalcone and stilbene class of cyclized molecules is observed in *Hydrangea macrophylla* var. *thunbergii*.¹¹ Ebizuka *et al.* succeeded in isolating a new member of polyketide synthase (PKS) that catalyzes only chain elongation without ring cyclization.

The known compounds, 3-hydroxy-5-methoxystilbene (**2**),^{12,13} 2',6'-dihydroxy-4'-methoxydihydrochalcone (**3**),^{14,15}

Table 1. ¹H- and ¹³C-NMR Spectral Data of Idenburgene (**1**)

Position	δ _H	δ _C
1		137.2*
2	6.62 (1H, d, J=1.6 Hz)	107.9
3		155.1
3-OH	5.20 (1H, s)	
4		117.2
5		158.6
5-OMe	3.88 (3H, s)	55.8
6	6.66 (1H, d, J=1.6 Hz)	102.0
7	7.03 (1H, d, J=16.1 Hz)	128.3
8	7.05 (1H, d, J=16.1 Hz)	128.7
1'		137.1*
2'	7.50 (1H, br d, J=7.6 Hz)	126.5
3'	7.35 (1H, m)	128.6
4'	7.26 (1H, m)	127.6
5'	7.35 (1H, m)	128.6
6'	7.50 (1H, br d, J=7.6 Hz)	126.5
1''		174.2
1''-OMe	3.65 (3H, s)	51.5
2''	2.35 (2H, m)	33.9
3''	1.59 (1H, m)	23.1
	1.67 (1H, m)	
4''	1.90 (2H, m)	31.9
5''	4.21 (1H, ddd, J=6.7, 6.7, 6.9 Hz)	37.3
6''	6.63 (1H, brd, J=16.1 Hz)	132.3
7''	6.52 (1H, dd, J=1.5, 16.1 Hz)	129.9
8''		137.3
9''	7.36 (1H, m)	126.2
10''	7.25 (1H, m)	128.5
11''	7.19 (1H, br dd, J=7.3, 7.3 Hz)	127.2
12''	7.25 (1H, m)	128.5
13''	7.36 (1H, m)	126.2

* Assignment may be interchanged.

stigmast-3-ene-4-one,¹⁶ and β-sitosterol^{17,18} were identified by comparing their melting points and spectroscopic data with those described in the literature.

Compound **3** was assayed using a brine shrimp (*Artemia salina* LEACH) lethality test^{19,20} to determine its bioactivity following the microwells method.²¹ The brine shrimp lethality test has been widely used as a general bioassay tool before applying other bioassays. The result of brine shrimp lethality test of **3** exhibited positive activity (LC₅₀ 7.26 μg/ml). Compound **3** has also been isolated from *Piper aduncum* (Piperaceae) and it showed cytotoxic activity on the KB cell of nasopharyngeal carcinoma cells (ED₅₀>10 μg/ml) and antimicrobial activity against *Bacillus subtilis* and *Micrococcus luteus*.¹⁵

Stilbene-type compounds are rare in the Lauraceae family and only one result from *Lindera reflexa*²² has been published. Compounds **1** and **2** are the first examples to be isolated from *Cryptocarya* genus.

Experimental

¹H- and ¹³C-NMR spectra were recorded on a JEOL JNM GSX500A. Proton spectra were measured at 500 MHz and carbon spectra at 125 MHz (TMS as an internal standard). UV spectra were recorded on a JASCO V-560 model UV spectrometer. IR spectra were measured on a JASCO FT/IR-230 IR spectrometer. Mass spectra were obtained by use of a JEOL JMS-AM20 (EIMS) or a JEOL JMS-HX-110A (FABMS, HR-FABMS) mass spectrometer. CD spectra were taken on a JASCO J-720WI instrument. For liquid chromatography, a silica gel prepacked column (Kusano CPS-HS-221-5) was used. Column chromatography was carried out by use of Merck Silica gel 60 [70–230 mesh (for open chromatography) or 230–400 mesh (for flash chromatography)]. For TLC Merck Silica gel 60 F₂₅₄ plates (0.25 mm thick) were used.

Plant Material Stem bark of *Cryptocarya idenburgensis* ALLEN. was collected from Passvale village, Kecamatan Kurulu, Kabupaten Jaya Wijaya, Irian Jaya Island, Indonesia. A herbarium specimen was deposited at the Herbarium Bogorienses, Center of Biological Research and Development, National Institute of Science, Bogor, Indonesia.

Extraction and Isolation The dried-milled stem bark of *C. idenburgensis* (2.5 kg) was percolated with *n*-hexane, then with MeOH to give the *n*-hexane extract (3.43 gr) and MeOH extract (206 g). The MeOH extract was then partitioned with CHCl₃ to afford a CHCl₃ extract (41.0 g). Separation of the CHCl₃ extract used column chromatography on silica gel with *n*-hexane, EtOAc, and then subjected it to silica gel flash column chromatography, MPLC and followed by preparative TLC to give a new compound idenburgene as a yellow viscous solid (**1**, 10.7 mg), 3-hydroxy-4-methoxystilbene (**2**, 47.5 mg), and 2',6'-dihydroxy-4'-methoxydihydrochalcone (**3**, 36.4 mg). On the other hand, the *n*-hexane extract was separated using silica gel chromatography with solvent *n*-hexane–benzene–CHCl₃–methanol. Purification of two major fractions by recrystallization yielded stigmast-4-ene-3-one (80.3 mg) and β -sitosterol (18.6 mg).

Idenburgene (**1**): Yellowish viscous solid. *R*_f value 0.63 (SiO₂, solvent system: 50% EtOAc/*n*-hexane). ¹H-NMR see Table 1. ¹³C-NMR see Table 1. IR (KBr) cm⁻¹: 742, 782, 1049, 1112, 1213, 1725, 2931, and 3019. UV λ_{max} (MeOH) nm (log ϵ): 203 (3.77), 241 (3.53), 254 sh (3.47), 308 (3.45), 318 (3.43), 330 sh (3.26). CD (MeOH, *c* 0.54 $\times 10^{-3}$, 18 °C) nm ($\Delta\epsilon$): 205.6 (+1.9), 212.6 (0), 220.8 (−3.0), 235.4 (−0.7), 242.2 (0), 265.6 (−0.5), 272.6 (0), 298.6 (+1.3). EI-MS *m/z*: 442 (*M*⁺, 28%), 341 (100%). HR-FABMS (NBA) *m/z*: 442.2126 *M*⁺ (Calcd for C₂₉H₃₀O₄: 442.2145).

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References

- Govindachari T. R., Parthasarathy P. C., Desai H. K., Shanbhag M. N., *Tetrahedron*, **29**, 3091–3094 (1973).
- Achmad S. A., Efendi Ghisalberty E. L., White A. H., Hakim E. H., Juliawaty L. D., Makmur L., *ITB Proceedings*, **24** (2/3), 9–16 (1991).
- Hlubucek J. R., Robertson A. V., *Aust. J. Chem.*, **20**, 2199–2206 (1967).
- Achmad S. A., Kasuma S., Hakim E. H., Makmur L., Juliawaty L. D., (unpublished results).
- Fu X., Sevenet T., Remy F., Pais M., *Phytochemistry*, **33**, 1272–1274 (1993).
- Achmad S. A., Azminah E., Ghisalberty E. L., Hakim E. H., Makmur L., White A. H., *Aust. J. Chem.*, **45**, 445–450 (1992).
- Achmad S. A., Hakim E. H., Juliawaty L. D., Makmur L., Syah Y. M., *Rep. Asahi Glass Found.*, **1994**, 691–695.
- Juliawaty L. D., Kitajima M., Takayama H., Achmad S. A., Aimi N., *Phytochemistry*, **54**, 989–993 (2000).
- Kosterman A. J. G. H., “Material for A Revision of Lauraceae I,” Reinwardt, Herbarium Bogoriense, Bogor, Indonesia, 1968.
- Schroder J., *Trends Plant Sci.*, **2**, 373–378 (1997).
- Akiyama T., Shibuya M., Liu H., Ebizuka T., *Eur. J. Biochem.*, **263**, 834–839 (1999).
- Rowe J. W., Bower C. L., Wagner E. R., *Phytochemistry*, **8**, 235–241 (1969).
- Asakawa Y., *Bull. Chem. Soc. Jpn.*, **44**, 2761–2766 (1971).
- Burke B., Nair M., *Phytochemistry*, **25**, 1427–1430 (1986).
- Orjala J., Wright A. D., Behrends H., Folkers G., Sticher O., *J. Nat. Prod.*, **57**, 18–26 (1994).
- Hayashi S., Okude T., Shimizu A., Matsuura T., *Chem. Pharm. Bull.*, **17**, 163–167 (1969).
- Rubinstein I., Goad L. J., Clague A. D. H., Mulheirn L. J., *Phytochemistry*, **15**, 195–200 (1976).
- Yano K., Akihisa T., Kawaguchi R., Tamura T., Matsumoto T., *Phytochemistry*, **31**, 1741–1746 (1992).
- Meyer B. N., Ferrigni N. R., Putnam J. E., Jacobsen L. B., Nichols D. E., Mc Laughlin J. L., *Planta Medica*, **45**, 31–34 (1982).
- Mc Laughlin J. L., “Methods in Plant Biochemistry,” Vol. 2, ed. by Dey P. M., Harborne J., Academic Press, London, New York, 1991, pp. 1–32.
- Solis P. N., Wright C. W., Anderson M., Gupta M. P., Phillipson J. D., *Planta Medica*, **59**, 250–252 (1993).
- Zhang J., Fang Q., *Planta Medica*, **60**, 190–191 (1994).

[2-(ω -Phenylalkyl)phenoxy]alkylamines III: Synthesis and Selective Serotonin-2 Receptor Binding (2)

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A series of [2-(2-phenylethyl)phenoxy]ethylpyrrolidine derivatives were synthesized, and their affinity for serotonin-2 (5-HT₂) and dopamine-2 (D₂) receptors was examined. Among them, compound 17, (2*R*,4*R*)-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl-1-methylpyrrolidine hydrochloride, showed high 5-HT₂ receptor affinity *in vitro*. This compound was a more potent inhibitor of *ex vivo* 5-HT-induced platelet aggregation than compound 3, which was previously shown to be more potent than ketanserin (1) and sarpogrelate (2a). However, compound 17 produced gastric irritation in rats. Therefore, we carried out a further derivatization of 17, and compound 45 (R-102444), a lauryl ester prodrug of compound 17, was found to be a promising candidate as an antithrombotic agent. Oral administration of R-102444 produced a marked inhibition of 5-HT-induced *ex vivo* platelet aggregation, and R-102444 did not cause any gastric irritation. The antiaggregatory effects of R-102444 were more potent than those of sarpogrelate (2a) and its active metabolite, M-1 (2b). In addition, R-102444 exhibited more potent antithrombotic effects than sarpogrelate in a rat photochemically-induced thrombosis model.

Key words serotonin-2 (5-HT₂) receptor; antiplatelet; R-102444; antagonist; [2-(2-phenylethyl)phenoxy]alkylamines

Platelet activation and vasoconstriction at the site of vascular injury play an important role in hemostasis and thrombosis.²⁾ Platelets are activated by a variety of substances, including adenosine diphosphate (ADP), thromboxane A₂ (TXA₂), and serotonin (5-HT).^{3–5)} Among them, 5-HT stored in the electrondense granules of platelets is released during platelet activation, and accelerates platelet aggregation.⁶⁾ 5-HT is also a potent vasoconstrictor, and thus, released 5-HT constricts vascular vessels which accelerates thrombus formation.⁷⁾ Since these responses of platelets and vascular muscles to 5-HT are mediated by the 5-HT₂ receptors on these tissues, 5-HT₂ antagonists are expected to be useful in the treatment of thrombosis.⁸⁾

Ketanserin (1), a 5-HT₂ receptor antagonist, inhibits 5-HT-induced platelet aggregation and vasoconstriction.⁹⁾ However, this compound produces an antihypertensive action *via* its adrenergic (α_1) receptor antagonistic activity.^{10,11)} Sarpogrelate (2a), a more selective 5-HT₂ antagonist, has been recently developed, and is now clinically available for the treatment of peripheral arterial occlusive disease in Japan.^{12,13)}

We previously reported that [2-(2-phenylethyl)phenoxy]alkylamine derivatives with a pyrrolidine or piperidine substructure show high 5-HT₂ but low D₂ receptor affinity.¹⁴⁾ Among them, compound 3 was the most potent and selective to 5-HT₂ receptors in the binding assays. This compound inhibited the 5-HT-induced vasoconstriction *in vitro* and platelet aggregation both *in vitro* and *ex vivo*. However, this compound was irritant to the rat stomach. Thus, we examined modifications on the pyrrolidine moiety in compound 3. A hydroxylation at the 4-position of the pyrrolidine ring in compound 3 successfully produced compound 17. Some of the acyl derivatives of 17 exhibited an *ex vivo* antithrombotic effect in rats. Furthermore, lauryl ester 45 (R-102444) and palmitoyl ester 46 did not cause any gastric irritation. In this paper, we describe the synthesis and structure–activity relationships (SAR) of a series of these compounds. We also re-

port a promising candidate, R-102444, as a prodrug-type 5-HT₂ antagonist in the cardiovascular system. Furthermore, the biological activities of R-102444 with those of sarpogrelate (2a) and its active metabolite, M-1 (2b) are compared.

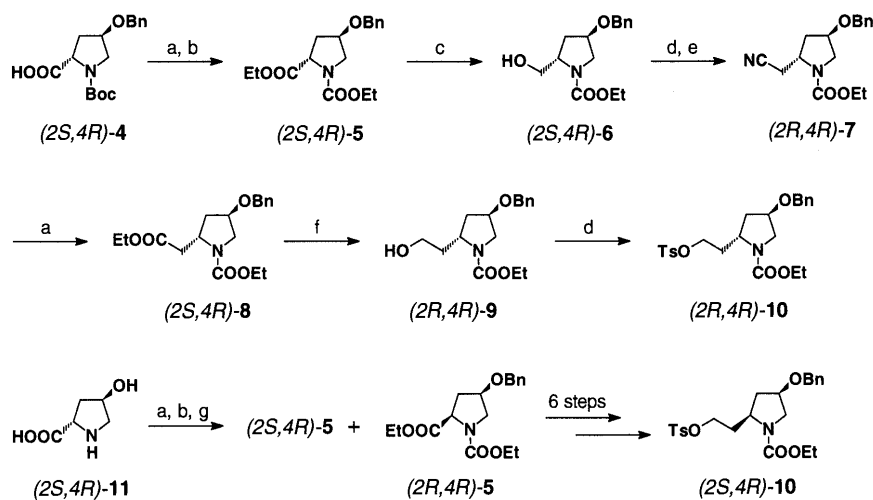
Chemistry

The syntheses of the optically active pyrrolidine intermediates, (2*R*,4*R*)-10 and its isomer (2*S*,4*R*)-10, are outlined in Chart 1. The tosylate, (2*R*,4*R*)-10, was prepared in eight steps starting from the commercially available (2*S*,4*R*)-4-benzyloxy-1-*tert*-butoxycarbonylproline (2*S*,4*R*)-4. The simultaneous esterification and deprotection of (2*S*,4*R*)-4, followed by re-protection by ethyl chloroformate gave a carbamate (2*S*,4*R*)-5. The carbamate was reduced with lithium borohydride (LiBH₄) to give an alcohol (2*S*,4*R*)-6. The alcohol was converted to (2*R*,4*R*)-7 *via* a tosylate, and the following acidic ethanolysis of the resulting nitrile group gave an ester (2*S*,4*R*)-8. The ester was reduced with lithium aluminum hydride (LiAlH₄) under cooled conditions to give an alcohol (2*R*,4*R*)-9, which was then converted to the tosylate (2*R*,4*R*)-10. A diastereoisomer (2*S*,4*R*)-10 was prepared in the same manner, from the carbamate (2*R*,4*R*)-5, which was derived from the commercially available *trans*-4-hydroxy-L-proline (2*S*,4*R*)-11, in three steps.

The synthetic routes of the compounds (15–20) listed in Table 3 are shown in Chart 2. The alkylation of phenols 12^{13,15)} with the tosylate (2*R*,4*R*)-10 provided carbamates 13, which were then hydrogenated to compounds 14. The carbamate group of 14 was converted into an *N*-methyl group with LiAlH₄ to give 4-hydroxypyrrolidine derivatives 15–20.

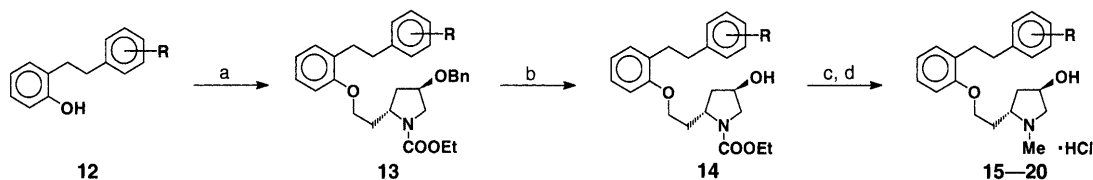
The synthetic pathways of three diastereomers of compound 17, namely compound 22, 25 and 27, are illustrated in Chart 3. The (2*R*,4*S*) isomer, 22, was prepared by means of the Mitsunobu inversion¹⁶⁾ using 14a and benzoic acid, followed by reduction of the resulting ester 21 with LiAlH₄. The (2*S*,4*R*) isomer, 25, was synthesized from the phenol 12a and

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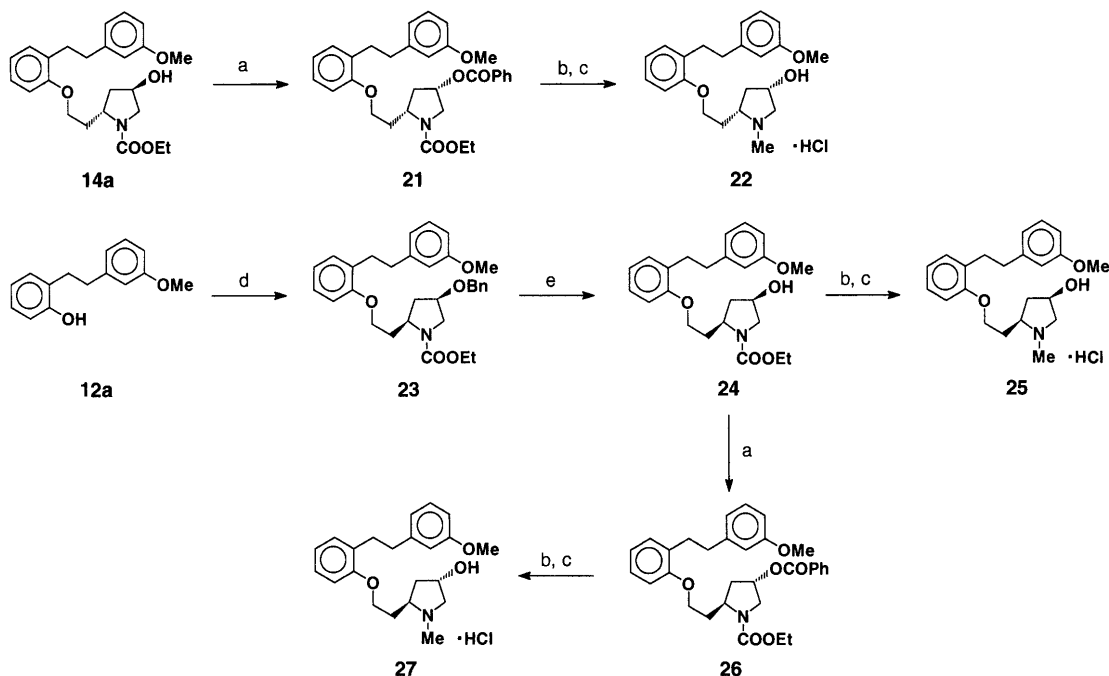
a) HCl (gas) / EtOH; b) ClCOOEt, Et₃N / acetone-H₂O; c) LiBH₄ / THF; d) Ts₂O, Et₃N / CH₂Cl₂; e) NaCN / DMF; f) LiAlH₄ / THF; g) BnBr, NaH, Bu₄NI / THF

Chart 1



a) (2*R*,4*R*)-10, *tert*-BuOK / DMA; b) H₂, Pd-C / EtOH; c) LiAlH₄ / THF; d) HCl / AcOEt or dioxane.

Chart 2



a) benzoic acid, PPh₃, DEAD / CH₂Cl₂; b) LiAlH₄ / THF; c) HCl / AcOEt or dioxane; d) (2*S*,4*R*)-10, *tert*-BuOK / DMA; e) H₂, Pd-C / EtOH.

Chart 3

(2*S*,4*R*)-10 in the same manner as in the synthesis of 17. The (2*S*,4*S*) isomer, 27, was prepared from 24 similarly to the preparation of 22. Each of the diastereomers was confirmed respectively by X-ray crystallographic analysis (Fig. 2).

Modifications at the 4-position of the pyrrolidine ring were achieved in the manner as shown in Chart 4. Oxopyrrolidine derivative 28 was prepared by Swern oxidation¹⁷⁾ of 17. Reaction of 28 with hydroxylamine hydrochloride gave (*Z*)-

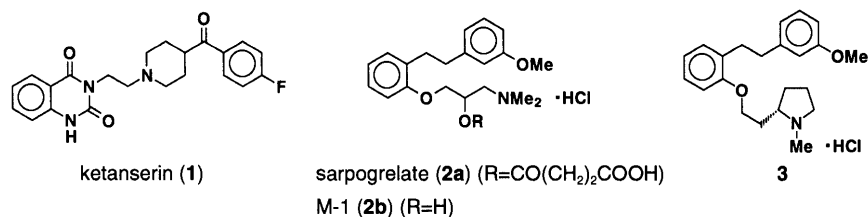
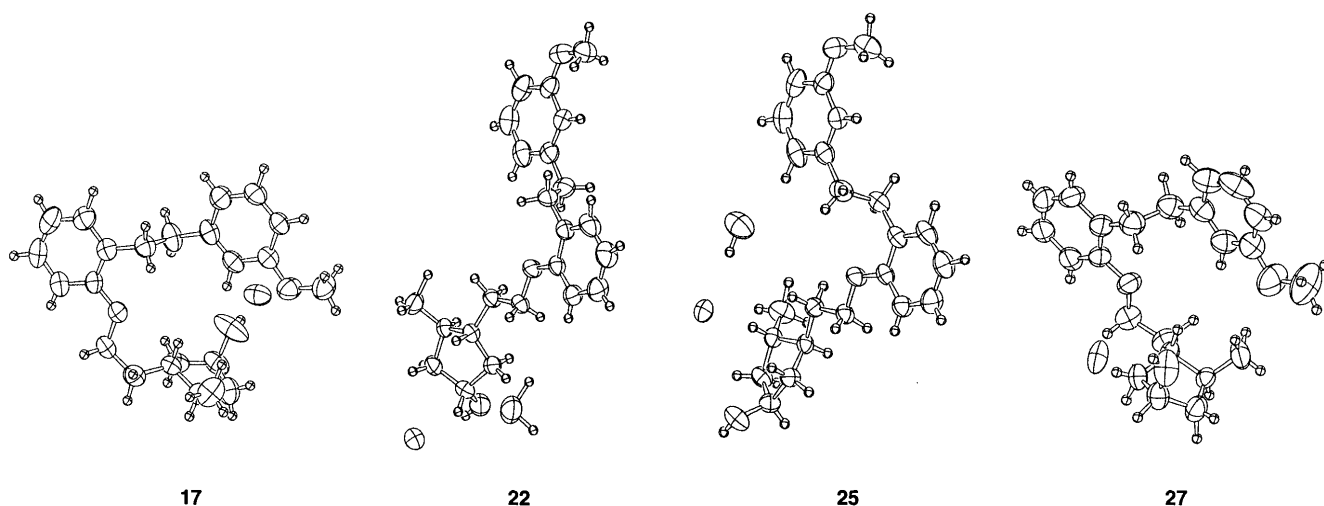
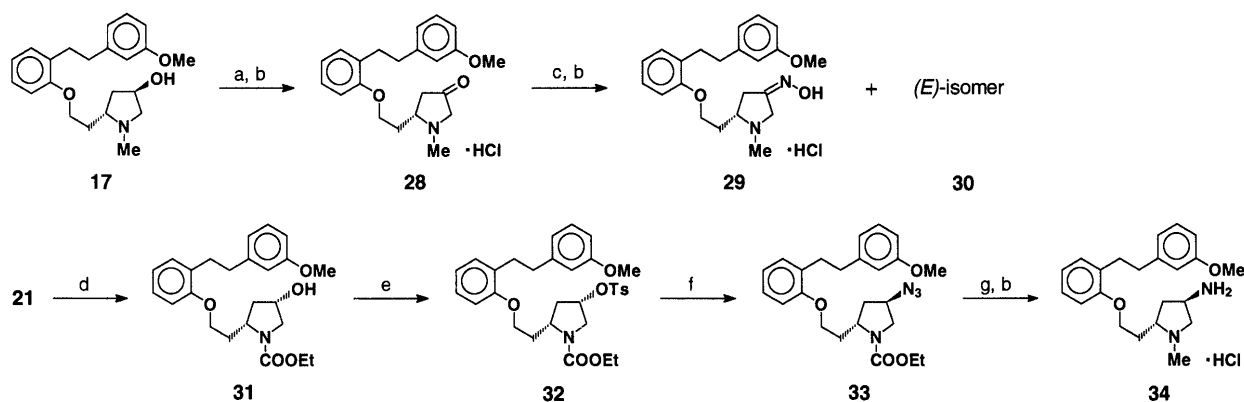


Fig. 1

Fig. 2. ORTEP Drawing of Molecules **17**, **22**, **25**, and **27**

a) DMSO, $(\text{COCl})_2$, Et_3N / CH_2Cl_2 ; b) HCl / AcOEt or dioxane; c) hydroxylamine hydrochloride, AcONa / MeOH ; d) LiBH_4 / THF ; e) Ts_2O , Et_3N / CH_2Cl_2 ; f) NaN_3 / DMF ; g) LiAlH_4 / THF .

Chart 4

oxime **29** and (*E*)-oxime **30**, which were separated by silica gel column chromatography.¹⁸⁾ Aminopyrrolidine derivative **34** was synthesized as described below. Reduction of **21** with LiBH_4 gave an alcohol **31**, which was then converted to a tosylate **32**. The $\text{S}_{\text{N}}2$ reaction of the tosylate **32** with sodium azide yielded an inverse azide **33**, which was then reduced with LiAlH_4 to give the desired product **34**.

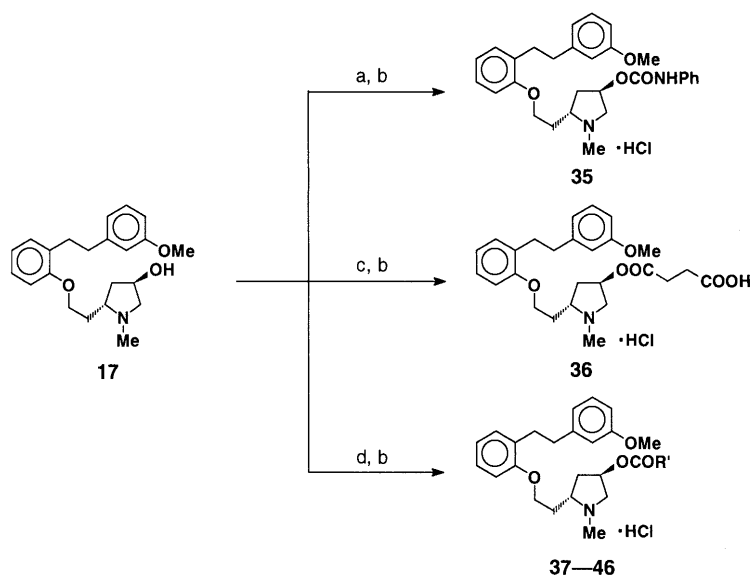
The syntheses of the prodrug derivatives are outlined in Chart 5. Carbamate **35** was obtained from the treatment of **17** with phenyl isocyanate. Carbonates **37** and **38**, and esters (**36**, **39–46**) were prepared by the acylation of **17** with the corresponding acid anhydride or acyl chloride.

Results and Discussion

In the previous paper, we reported that (*S*)-2-[2-[2-(3-

methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine hydrochloride **3** has high affinity and specificity for 5-HT₂ receptors in the binding assays. Indeed, compound **3** was a potent inhibitor of *in vitro* 5-HT-induced vasoconstriction and of both *in vitro* and *ex vivo* platelet aggregation. However, oral administration of this compound caused gastric irritation in rats. Therefore, further modifications were made on the pyrrolidine moiety.

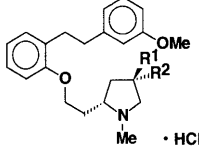
Although 5-HT₂ receptors are distributed both in the brain and peripheral tissues, 5-HT₂ receptor antagonists, as an antithrombotic agent, are expected to act solely on platelets and arterial walls. It is well known that lipophilic compounds are inclined to cross the blood brain barrier (BBB) more than hydrophilic compounds.¹⁹⁾ We reported that the D₂ receptor affinity depends on the lipophilicity around the amino moiety



a) phenyl isocyanate, Et₃N / CH₂Cl₂; b) HCl / AcOEt or dioxane; c) succinic anhydride / acetone; d) (R'CO)₂O or R'COCl / pyridine

Chart 5

Table 1. Affinity of Compound **3** and Other Pyrrolidine Derivatives, Substituted at the 4-Position of the Pyrrolidine Ring for 5-HT₂ and D₂ Receptors



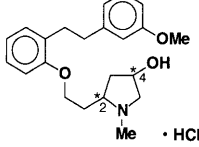
Compd.	R ¹	R ²	IC ₅₀ (nm) ^{a)}		Ratio ^{b)} D ₂ /5-HT ₂
			5-HT ₂	D ₂	
3	H	H	1.8	1100	610
34	NH ₂	H	4.7	>5000	ND ^{c)}
28		=O	49	>5000	ND ^{c)}
29		=NOH (Z)	42	>5000	ND ^{c)}
30		=NOH (E)	24	>5000	ND ^{c)}
17	OH	H	2.2	2400	1100
Sarpogrelate (2a)			150	>5000	ND ^{c)}
M-1 (2b)			16	>5000	ND ^{c)}
Ketanserin (1)			5.3	940	180

a) Interaction of the compounds with rat brain 5-HT₂ and D₂ receptors was determined by conventional binding assay using [³H]ketanserin and [³H]raclopride. b) Ratio: the IC₅₀ values for D₂ vs. 5-HT₂ receptors. c) Not determined.

in a series of compounds.^{14,20)} Thus, we hypothesized that the selectivity in peripheral tissues depended on the hydrophilicity of the compounds, and we accordingly designed polar and/or hydrophilic modifications (amino (**34**), oxo (**28**), hydroxyimino (**29**, **30**), and hydroxy (**17**) derivatives) at the 4-position of the pyrrolidine ring. Binding assays of these compounds for 5-HT₂ and D₂ receptors showed that the modifications resulted in a reduction of the D₂ receptor binding (Table 1). All compounds shown in Table 1 exhibited low D₂ affinity with IC₅₀ values of above 2400 nm. In addition, there was a small amount of ¹⁴C-labeled **17** which was distributed in the brain after an intravenous administration to rats (data not shown).

In a 5-HT₂ receptor binding assay, compound **34** with an amino group exhibited a slightly lower affinity than **3**. An oxo compound (**28**) and hydroxyimino compounds (**29**, **30**)

Table 2. Affinity of Compound **17** and Its Optical Isomers for 5-HT₂ Receptors



Compd.	Config.	IC ₅₀ (nm) ^{a)}
17	(2R,4R)	2.2
22	(2R,4S)	36
25	(2S,4R)	64
27	(2S,4S)	51

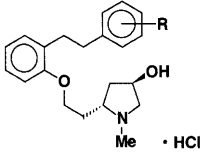
a) Interaction of the compounds with rat brain 5-HT₂ was determined by conventional binding assay using [³H]ketanserin.

had significantly (thirteen-fold) less affinity than **3**. However, compound **17**, in which a hydroxy group was introduced, showed a similar high affinity for 5-HT₂ receptors, but lower D₂ receptor affinity compared to **3**. Among these compounds, **17** exhibited the largest D₂/5-HT₂ ratio (1100).

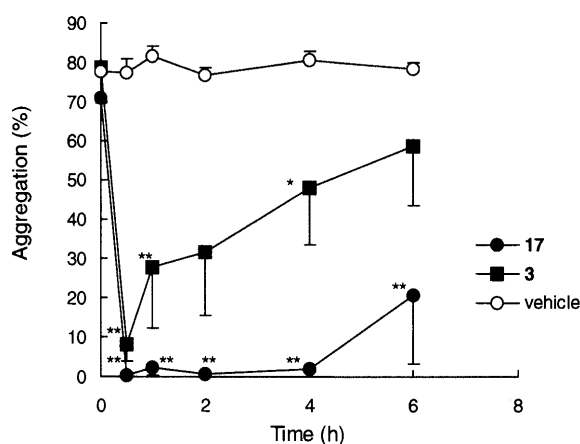
Compound **17**, with high affinity and specificity to 5-HT₂ receptors, has two asymmetric carbon atoms at the 2- and 4-position of the pyrrolidine ring. Therefore, the other three optical isomers were prepared and their binding affinity for 5-HT₂ receptors were examined (Table 2). It was revealed that the compound with (2R, 4R) configuration, **17**, is the biologically active isomer. Compound **17** exhibited the highest affinity with an IC₅₀ value of 2.2 nm, while the affinity of the other three optical isomers remarkably decreased by more than fifteen-fold compared with **17**. The configuration at the 2- and 4-position of the pyrrolidine ring was found to be important for high 5-HT₂ receptor affinity, which is consistent with the previous results of compound **3** and its enantiomer.¹⁴⁾

The effect of substituents on the ω-phenyl group is summarized in Table 3. The methoxy-substituted compound at the 3-position (**17**) of the ω-phenyl ring showed higher 5-

Table 3. Affinity of Compound **17** and Its Derivatives for 5-HT₂ Receptors

		
Compd.	R	IC ₅₀ (nM) ^{a)}
15	H	5.5
16	2-OMe	3.8
17	3-OMe	2.2
18	4-OMe	28
19	3,5-diOMe	5.2
20	3-OH	12

a) Interaction of the compounds with rat brain 5-HT₂ was determined by conventional binding assay using [³H]ketanserin.

Fig. 3. *Ex Vivo* Antiplatelet Effects of Compound **3** and **17** in Cats

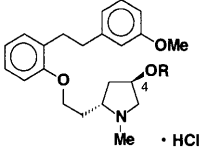
Data are represented as the mean $\pm$ S.E.M. ($n=4-6$). * $p < 0.05$, ** $p < 0.01$ vs. vehicle (Dunnett's test).

HT₂ receptor affinity than those substituted at the 2- or 4-position (**16** or **18**). The 3,5-dimethoxy-substituted compound, **19**, exhibited slightly weaker activity than **17**. This result shows that the introduction of a bulky substituent decreases the affinity for 5-HT₂ receptors, which is consistent with that of (*RS*)-2-(2-pyrrolidinylethyl) compounds in the previous paper.¹⁴⁾ The de-methylation of the methoxy group of **17**, in the case of compound **20**, decreased the 5-HT₂ receptor affinity. In fact, a hydrophilic modification at the 3-position on the ω -phenyl ring resulted in the reduction of 5-HT₂ receptor binding affinity.

Our previous study¹⁴⁾ demonstrated that the *ex vivo* effects of compound **3** on platelet aggregation were more potent than those of ketanserin and sarpogrelate. In the present study, the inhibitory effects of compound **17** on 5-HT-induced platelet aggregation in cats were compared to those of compound **3** (Fig. 3). A single bolus administration of **17** (100 μ g/kg, i.v.) produced a complete inhibition of platelet aggregation at 0.5 h postdose, which continued up to 4 h postdose. A marked inhibition (72%) was still evident even at 6 h. As shown in Fig. 3, compound **17** was more potent than compound **3**. These results suggest that **17** is a potent antiplatelet agent with a long duration of action *in vivo*.

The addition of **17** alone to platelet-rich plasma (PRP) did not cause any platelet aggregation up to 1 mM, as well as compound **3**. This compound also did not cause vasocon-

Table 4. 5-HT₂ Receptor Affinity and *Ex Vivo* Antiplatelet Effects of Compound **17** and Its Prodrug Derivatives

		
Compd.	R	<i>In vitro</i> ^{a)} IC ₅₀ (nM) <i>Ex vivo</i> ^{b)} inhibition (%) ^{c)}
17	H	2.2 74.0 $\pm$ 6.0 **
35	CONHPh	7.2 12.9 $\pm$ 7.1
36	CO(CH ₂) ₂ COOH	2.2 71.9 $\pm$ 8.2 **
37	COOEt	3.0 NT ^{d)}
38	COOPh	2.7 NT ^{d)}
39	COPh	8.1 NT ^{d)}
40	COCH ₃	0.9 78.6 $\pm$ 7.2 **
41	CO(CH ₂) ₂ CH ₃	2.2 74.3 $\pm$ 3.7 **
42	CO(CH ₂) ₃ CH ₃	23 70.7 $\pm$ 4.6 **
43	CO(CH ₂) ₆ CH ₃	43 70.2 $\pm$ 7.3 **
44	CO(CH ₂) ₈ CH ₃	170 82.2 $\pm$ 5.9 **
45 (R-102444)	CO(CH ₂) ₁₀ CH ₃	240 74.9 $\pm$ 2.4 **
46	CO(CH ₂) ₁₄ CH ₃	310 79.2 $\pm$ 6.2 **
Sarpogrelate (2a)		21.3 $\pm$ 7.5 ^{e)}
		57.3 $\pm$ 5.9 ** ^{f)}
M-1 (2b)		35.6 $\pm$ 3.2 ** ^{e)}

a) Interaction of the compounds with rat brain 5-HT₂ was determined by conventional binding assay using [³H]ketanserin. b) Inhibitory effects (%) of test compounds (1 mg/kg, *p.o.*) on 5-HT-induced platelet aggregation in rats. Platelet aggregation was measured 1 h after dosing. c) Data are represented as the mean $\pm$ S.E.M. ($n=6$). * $p < 0.05$, ** $p < 0.01$ vs. vehicle (Dunnett's test). d) Not tested. e) Inhibitory effects (%) of sarpogrelate or M-1 (10 mg/kg, *p.o.*) on 5-HT-induced platelet aggregation in rats. f) Inhibitory effects (%) of sarpogrelate (100 mg/kg, *p.o.*) on 5-HT-induced platelet aggregation in rats.

striction, but inhibited 5-HT-induced vasoconstriction with an IC₅₀ value of 1.8 nM. These results suggest that compound **17** is a 5-HT₂ receptor antagonist, and not an agonist.

The receptor binding specificity of **17** was further examined for other receptors (α_1 , β , 5-HT₁, 5-HT₃). Compound **17** exhibited low affinity with IC₅₀ values (nM) of 310 for α_1 , 3700 for 5-HT₁, and over 5000 for β and 5-HT₃, respectively. This indicates that **17** is highly selective for 5-HT₂ receptors.

The *ex vivo* effects of test compounds (**17**, sarpogrelate, M-1) on 5-HT-induced platelet aggregation were examined in rats (Table 4). Oral administration of **17** (1 mg/kg) resulted in a marked inhibition (74%) of platelet aggregation at 1 h postdose. Sarpogrelate (10 and 100 mg/kg, *p.o.*) produced only moderate inhibition (21% and 57%, respectively), suggesting its weak potency. In addition, M-1 was found to be less potent than **17**. These results suggest that compound **17** exhibits more potent *ex vivo* antiaggregatory activity compared to that of sarpogrelate and M-1.

However, toxicology studies showed that compound **17** is an irritant to the rat stomach as much as compound **3**. The rat stomach has been shown to have 5-HT receptors including 5-HT_{2A},²¹⁾ 5-HT_{2B},²²⁾ and 5-HT_{2C}.²³⁾ Therefore, we hypothesized that the irritation is, at least in part, related to the 5-HT₂ receptor action. Thus, the prodrug derivatives (**35**–**46**), in which the hydroxy group at the 4-position of the pyrrolidine ring in **17** is protected, were prepared and examined *in vitro* for their 5-HT₂ receptor binding and *ex vivo* antiplatelet effects (Table 4). Carbamate **35** was slightly less potent than **17** *in vitro*, and **35** hardly inhibited platelet aggregation. Succinyl derivative **36** was as potent as **17** both *in vitro* and *ex*

Table 5. Physical Data for [2-(2-Phenylethyl)phenoxy]ethylpyrrolidine Derivatives

Compd. ^{a)}	Formula	Yield (%) ^{b)}	mp (°C)	[α] _D (MeOH)	Analysis (%) Calcd (Found)			
					C	H	N	Cl
15	C ₂₁ H ₂₇ NO ₂ ·HCl	50	113—114	−13° (c=1.11)	69.69 (69.69)	7.80 (8.01)	3.87 (3.97)	9.80 (9.90)
16	C ₂₂ H ₂₉ NO ₃ ·HCl·0.35H ₂ O	69	136—137	−11° (c=0.99)	66.35 (66.28)	7.77 (7.77)	3.52 (3.48)	8.90 (8.96)
17	C ₂₂ H ₂₉ NO ₃ ·HCl	69	100—102	−12° (c=1.06)	67.42 (67.25)	7.72 (7.77)	3.57 (3.53)	9.05 (9.18)
18	C ₂₂ H ₂₉ NO ₃ ·HCl	60	111—112	−11° (c=1.19)	67.42 (67.03)	7.72 (7.60)	3.57 (3.55)	9.05 (9.18)
19	C ₂₃ H ₃₁ NO ₄ ·HCl	76	134—136	−12° (c=1.15)	65.47 (65.39)	7.64 (7.60)	3.32 (3.32)	8.40 (8.17)
20	C ₂₁ H ₂₇ NO ₃ ·HCl·0.50H ₂ O	64	49—50	−15° (c=1.27)	65.19 (65.26)	7.55 (7.52)	3.62 (3.57)	9.16 (9.01)
22	C ₂₂ H ₂₉ NO ₃ ·HCl·0.50H ₂ O	66	63—65	−24° (c=1.14)	65.90 (65.99)	7.79 (7.86)	3.49 (3.57)	8.84 (8.85)
25	C ₂₂ H ₂₉ NO ₃ ·HCl·0.30H ₂ O	62	75—76	+24° (c=1.00)	66.50 (66.52)	7.76 (7.78)	3.53 (3.65)	8.92 (8.64)
27	C ₂₂ H ₂₉ NO ₃ ·HCl	69	102—103	+12° (c=1.12)	67.42 (67.25)	7.72 (7.94)	3.57 (3.64)	9.05 (9.18)
28	C ₂₂ H ₂₇ NO ₃ ·HCl	66	109—110	−72° (c=1.05)	67.77 (67.42)	7.24 (7.29)	3.59 (3.65)	9.09 (8.97)
29	C ₂₂ H ₂₈ N ₂ O ₃ ·HCl·0.10H ₂ O	24	164—167	−53° (c=0.95)	64.97 (64.70)	7.24 (7.26)	6.89 (6.99)	8.72 (9.12)
30	C ₂₂ H ₂₈ N ₂ O ₃ ·HCl·0.15H ₂ O	27	161—163	−52° (c=1.08)	64.82 (64.61)	7.24 (7.03)	6.87 (6.97)	8.70 (9.05)
34	C ₂₂ H ₃₀ N ₂ O ₄ ·2HCl	52	160—163	−11° (c=1.00)	61.82 (61.62)	7.55 (7.48)	6.55 (6.60)	16.59 (16.32)
35	C ₂₉ H ₃₄ N ₂ O ₄ ·HCl·0.80H ₂ O	83	Oil	+2.0° (c=1.32)	66.29 (66.27)	7.02 (7.21)	5.33 (5.20)	6.75 (6.79)
36	C ₂₆ H ₃₃ NO ₆ ·HCl·0.75H ₂ O	69	Oil	+1.2° (c=0.93)	61.77 (61.77)	7.08 (7.04)	2.77 (2.64)	7.01 (7.18)
37	C ₂₃ H ₃₃ NO ₅ ·HCl	90	94—95	−5.9° (c=1.18)	64.71 (64.77)	7.39 (6.99)	3.02 (2.97)	7.64 (7.65)
38	C ₂₉ H ₃₃ NO ₅ ·HCl	66	125—129	−6.6° (c=1.11)	68.03 (67.76)	6.69 (6.43)	2.74 (2.70)	6.92 (7.05)
39	C ₂₉ H ₃₃ NO ₄ ·HCl	77	100—101	+10° (c=1.09)	70.22 (69.93)	6.91 (6.91)	2.82 (2.77)	7.15 (6.84)
40	C ₂₄ H ₃₁ NO ₄ ·HCl·0.35H ₂ O	63	67—68	−3.8° (c=1.30)	65.47 (65.46)	7.49 (7.56)	3.18 (3.21)	8.05 (8.06)
41	C ₂₈ H ₃₉ NO ₄ ·HCl	59	87—88	−3.2° (c=1.11)	68.62 (68.31)	8.23 (8.38)	2.86 (2.94)	7.23 (7.00)
42	C ₂₉ H ₄₁ NO ₄ ·HCl	66	93—94	−2.6° (c=1.00)	69.10 (69.23)	8.40 (8.32)	2.78 (2.76)	7.03 (7.11)
43	C ₃₀ H ₄₃ NO ₄ ·HCl	84	86—88	−2.6° (c=1.01)	69.54 (69.44)	8.56 (8.70)	2.70 (2.75)	6.84 (6.77)
44	C ₃₂ H ₄₇ NO ₄ ·HCl	38	58—60	−2.0° (c=1.14)	70.37 (70.44)	8.86 (9.10)	2.56 (2.65)	6.49 (6.40)
45 (R-102444)	C ₃₄ H ₅₁ NO ₄ ·HCl	73	73—74	−2.1° (c=1.19)	71.12 (70.87)	9.13 (9.08)	2.44 (2.48)	6.17 (6.13)
46	C ₃₈ H ₅₉ NO ₄ ·HCl·0.25H ₂ O	86	Waxen Oil	−2.2° (c=1.06)	71.89 (71.84)	9.61 (9.54)	2.21 (2.21)	5.58 (5.68)

a) Compound **3** was reported in ref. 14. b) Yield not optimized.

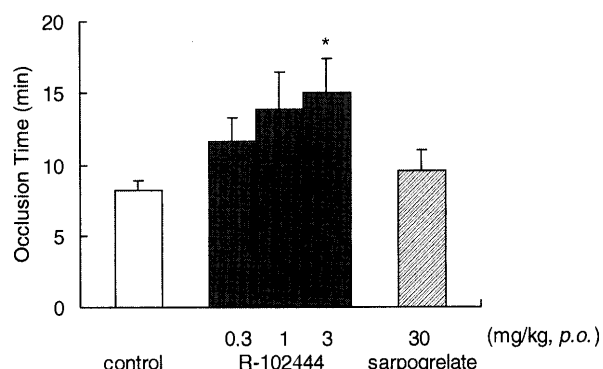


Fig. 4. Inhibitory Effects of R-102444 and Sargogrelate on the Occlusion Time of the Rat Femoral Artery

Data are represented as the mean ± S.E.M. ($n=8-12$). * $p<0.05$ vs. the control group (Dunnett's test).

vivo. Thus, *ex vivo* inhibitory effects of **35** and **36** were those of the compounds themselves; they did not function as pro-drug derivatives. Carbonates **37** and **38** were also as potent as **17** for their 5-HT₂ receptor affinity. Benzoate **39**, acetate **40**, and hexanoic ester **41**, as the acyl moiety, also showed high affinity ($IC_{50}<10$ nm) *in vitro*. Since these seven compounds (**35**—**41**) with high affinity *in vitro* have a relatively small substituent at the 4-position of the pyrrolidine ring, the introduction of a more bulky substituent is presumably required to diminish the *in vitro* activity. Thus, we introduced higher carboxylic acids to the 4-position of the pyrrolidine ring. As expected, the introduction of higher carboxylic acids resulted in a reduction of the 5-HT₂ receptor affinity. The introduction of higher carboxylic acids, lauryl ester **45** (R-102444) and palmitoyl ester **46**, showed low affinity with IC_{50} values

larger than 240 nm, indicating that these compounds are inactive *in vitro*. In contrast, these compounds orally administered to rats inhibited *ex vivo* platelet aggregation as potentially as **17**. This result suggests that they were hydrolyzed at the acyl ester linkage after oral administration, and this newly formed **17** potentially inhibited platelet aggregation. Furthermore, R-102444 and compound **46** did not cause gastric irritation in rats. R-102444 is obtained as a crystal form with a melting point of 73—74 °C, and compound **46**, as a waxen oil (Table 5). From the viewpoint of physical properties, R-102444 was selected for further study.

Figure 4 shows the antithrombotic effects of R-102444 and sargogrelate in a rat photochemically-induced thrombosis model.²⁴⁾ In the control group (0.5% gum tragacanth-treatment), the femoral artery was completely occluded within 15 min (8.18 ± 0.69 min, $n=12$) after the initiation of photochemical reaction (rose bengal injection). Pretreatment of R-102444 (0.3—3 mg/kg, *p.o.*) prolonged the time required for thrombotic occlusion of the artery in a dose-dependent manner. Statistically significant ($p<0.05$) prolongation was observed in the R-102444 (3 mg/kg)-treated group. In contrast, sargogrelate even at 30 mg/kg produced only a moderate prolongation, which was not statistically significant ($p>0.05$). These results indicated that the antithrombotic effect of R-102444 is more potent than that of sargogrelate.

In conclusion, we found that 4-hydroxypyrrolidine derivative, **17** with (2*R*, 4*R*) configuration, showed a similar high affinity for 5-HT₂ receptors, but lower D₂ receptor affinity compared to compound **3**. This compound was also efficacious in inhibiting 5-HT-induced *in vitro* vasoconstriction and *ex vivo* platelet aggregation. Although compound **17** was irritant to the rat stomach, its prodrug, R-102444 with potent

Table 6. Physical Data for [2-(2-Phenylethyl)phenoxy]ethylpyrrolidine Derivatives

Compd. ^{a)}	¹ H-NMR δ (CDCl ₃)
15	1.98—2.16 (1H, m), 2.31—2.60 (2H, m), 2.36 (1H, dd, $J=5.8$, 14 Hz), 2.77—3.08 (1H, m), 2.84 (3H, s), 2.88 (4H, s), 3.75—4.23 (4H, m), 4.52—4.64 (1H, m), 6.83 (1H, d, $J=8.1$ Hz), 6.91 (1H, t, $J=7.3$ Hz), 7.08—7.35 (7H, m)
16	2.00—2.15 (1H, m), 2.28—2.63 (2H, m), 2.32 (1H, dd, $J=5.8$, 14 Hz), 2.79—3.04 (1H, m), 2.84 (3H, s), 2.87 (4H, s), 3.73—4.24 (4H, m), 3.79 (3H, s), 4.51—4.62 (1H, m), 6.77—6.96 (4H, m), 7.05—7.24 (4H, m)
17	2.02—2.14 (1H, m), 2.28—2.46 (1H, m), 2.34 (1H, dd, $J=5.6$, 14 Hz), 2.48—2.60 (1H, m), 2.76—2.95 (4H, m), 2.86 (3H, s), 2.99 (1H, d, $J=12$ Hz), 3.77 (3H, s), 3.82—4.22 (4H, m), 4.54—4.63 (1H, m), 6.70—6.80 (3H, m), 6.82 (1H, d, $J=8.2$ Hz), 6.91 (1H, t, $J=7.4$ Hz), 7.11—7.23 (3H, m)
18	2.00—2.17 (1H, m), 2.27—2.63 (2H, m), 2.33 (1H, dd, $J=5.8$, 14 Hz), 2.72—3.07 (5H, m), 2.85 (3H, s), 3.73—4.26 (4H, m), 3.78 (3H, s), 4.54—4.66 (1H, m), 6.78—6.96 (4H, m), 7.03—7.22 (4H, m)
19	2.04—2.18 (1H, m), 2.33 (1H, dd, $J=5.9$, 14 Hz), 2.36—2.49 (1H, m), 2.51—2.66 (1H, m), 2.72—3.05 (5H, m), 2.89 (3H, s), 3.76 (6H, s), 3.83—4.25 (4H, m), 4.56—4.65 (1H, m), 6.28—6.36 (3H, m), 6.83 (1H, d, $J=8.0$ Hz), 6.92 (1H, t, $J=7.3$ Hz), 7.12—7.24 (2H, m)
20	2.01—2.17 (1H, m), 2.23—2.55 (3H, m), 2.67—2.94 (4H, m), 2.87 (3H, s), 3.12 (1H, d, $J=13$ Hz), 3.76—4.08 (4H, m), 4.53—4.65 (1H, m), 6.62 (1H, d, $J=7.4$ Hz), 6.69 (1H, dd, $J=1.7$, 7.9 Hz), 6.75 (1H, d, $J=7.9$ Hz), 6.88—6.95 (2H, m), 7.06 (1H, t, $J=7.9$ Hz), 7.11—7.21 (2H, m)
22	2.08—2.22 (1H, m), 2.46—2.59 (2H, m), 2.63—2.96 (6H, m), 2.78 (3H, s), 3.21—3.36 (1H, m), 3.77 (3H, s), 3.79—4.00 (2H, m), 4.15—4.25 (1H, m), 4.44—4.55 (1H, m), 6.64—6.78 (3H, m), 6.83 (1H, d, $J=7.3$ Hz), 6.94 (1H, t, $J=6.9$ Hz), 7.13—7.25 (3H, m)
25	2.10—2.23 (1H, m), 2.48—2.62 (2H, m), 2.65—2.97 (6H, m), 2.79 (3H, s), 3.20—3.38 (1H, m), 3.77 (3H, s), 3.80—4.00 (1H, m), 4.14—4.26 (1H, m), 4.44—4.58 (1H, m), 6.64—6.78 (3H, m), 6.83 (1H, d, $J=8.6$ Hz), 6.94 (1H, t, $J=7.6$ Hz), 7.14—7.25 (3H, m)
27	2.05—2.21 (1H, m), 2.33 (1H, dd, $J=5.3$, 14 Hz), 2.36—2.69 (2H, m), 2.77—3.09 (5H, m), 2.88 (3H, s), 3.79 (3H, s), 3.81—4.28 (4H, m), 4.55—4.67 (1H, m), 6.70—6.82 (3H, m), 6.84 (1H, d, $J=8.6$ Hz), 6.93 (1H, t, $J=7.9$ Hz), 7.13—7.26 (3H, m)
28	2.53—3.02 (8H, m), 2.89 (3H, s), 3.11—3.28 (1H, m), 3.53—3.71 (1H, m), 3.75 (3H, s), 3.83—3.95 (1H, m), 4.08—4.24 (2H, m), 6.62—6.76 (3H, m), 6.80 (1H, d, $J=8.6$ Hz), 6.96 (1H, t, $J=7.9$ Hz), 7.12—7.25 (3H, m)
29 ^{b)}	2.13—2.32 (1H, m), 2.46—3.12 (7H, m), 2.90 (3H, s), 3.62—4.24 (4H, m), 3.71 (3H, s), 4.31 (1H, d, $J=16$ Hz), 6.70—6.82 (3H, m), 6.87 (1H, t, $J=7.2$ Hz), 6.97 (1H, d, $J=8.2$ Hz), 7.10—7.23 (3H, m)
30 ^{b)}	2.13—2.32 (1H, m), 2.46—3.12 (7H, m), 2.90 (3H, s), 3.64—4.20 (4H, m), 3.71 (3H, s), 4.32 (1H, d, $J=16$ Hz), 6.70—6.83 (3H, m), 6.87 (1H, t, $J=7.4$ Hz), 6.97 (1H, d, $J=8.0$ Hz), 7.10—7.24 (3H, m)
34 ^{b)}	2.07—2.32 (2H, m), 2.35—2.49 (2H, m), 2.72—2.98 (4H, m), 2.91 (3H, s), 3.12—3.28 (1H, m), 3.72 (3H, s), 3.83—4.10 (5H, m), 6.71—6.90 (4H, m), 6.96 (1H, d, $J=7.3$ Hz), 7.10—7.25 (3H, m)
35	2.28—2.43 (1H, m), 2.46—2.73 (3H, m), 2.78—2.99 (4H, m), 2.88 (3H, s), 3.06 (1H, d, $J=13$ Hz), 3.72—4.07 (2H, m), 3.76 (3H, s), 4.25—4.46 (2H, m), 5.35—5.45 (1H, m), 6.73—6.90 (4H, m), 6.94 (1H, t, $J=7.0$ Hz), 7.07 (1H, t, $J=7.2$ Hz), 7.14—7.32 (7H, m)
36	2.32—2.65 (8H, m), 2.78—2.94 (4H, m), 2.88 (3H, s), 3.00 (1H, d, $J=14$ Hz), 3.69—3.81 (1H, m), 3.77 (3H, s), 3.94—4.04 (1H, m), 4.17—4.26 (1H, m), 4.32 (1H, dd, $J=4.8$, 14 Hz), 5.26—5.33 (1H, m), 6.70—6.79 (3H, m), 6.84 (1H, d, $J=7.7$ Hz), 6.93 (1H, t, $J=7.7$ Hz), 7.13—7.23 (3H, m)
37	1.26 (3H, t, $J=7.2$ Hz), 2.27—2.42 (1H, m), 2.45—2.62 (3H, m), 2.76—2.96 (4H, m), 2.83 (3H, s), 3.00 (1H, d, $J=13$ Hz), 3.61—3.82 (1H, m), 3.73 (3H, s), 3.92—4.05 (1H, m), 4.10—4.26 (1H, m), 4.17 (2H, q, $J=7.2$ Hz), 4.35 (1H, dd, $J=5.4$, 13 Hz), 5.23—5.34 (1H, m), 6.68—6.80 (3H, m), 6.83 (1H, d, $J=8.3$ Hz), 6.93 (1H, t, $J=7.7$ Hz), 7.12—7.25 (3H, m)
38	2.35—2.50 (1H, m), 2.53—2.67 (3H, m), 2.78—2.99 (4H, m), 2.86 (3H, s), 3.10 (1H, d, $J=13$ Hz), 3.67—3.86 (1H, m), 3.74 (3H, s), 3.94—4.08 (1H, m), 4.15—4.29 (1H, m), 4.35—4.49 (1H, m), 5.34—5.44 (1H, m), 6.70—6.82 (3H, m), 6.85 (1H, d, $J=8.1$ Hz), 6.95 (1H, t, $J=7.2$ Hz), 7.09 (2H, d, $J=7.9$ Hz), 7.13—7.31 (4H, m), 7.37 (2H, t, $J=7.9$ Hz)
39	2.40—2.71 (4H, m), 2.75—2.95 (4H, m), 2.90 (3H, s), 3.09 (1H, d, $J=13$ Hz), 3.70 (3H, s), 3.76—3.93 (1H, m), 3.96—4.12 (1H, m), 4.21—4.34 (1H, m), 4.47 (1H, dd, $J=5.6$, 13 Hz), 5.53—5.62 (1H, m), 6.63—6.74 (3H, m), 6.86 (1H, d, $J=8.1$ Hz), 6.92 (1H, t, $J=7.1$ Hz), 7.08—7.25 (3H, m), 7.33 (2H, t, $J=7.5$ Hz), 7.54 (1H, t, $J=7.5$ Hz), 7.87 (2H, d, $J=7.5$ Hz)
40	1.93 (3H, s), 2.26—2.78 (4H, m), 2.76—3.00 (5H, m), 2.84 (3H, s), 3.63—3.82 (1H, m), 3.78 (3H, s), 3.93—4.06 (1H, m), 4.19—4.40 (2H, m), 5.27—5.37 (1H, m), 6.70—6.82 (3H, m), 6.85 (1H, d, $J=7.9$ Hz), 6.94 (1H, t, $J=7.3$ Hz), 7.13—7.26 (3H, m)
41	0.84 (3H, t, $J=6.9$ Hz), 1.10—1.33 (4H, m), 1.42—1.58 (2H, m), 2.14 (2H, t, $J=7.6$ Hz), 2.27—2.48 (2H, m), 2.51—2.70 (2H, m), 2.75—3.00 (5H, m), 2.85 (3H, s), 3.65—3.83 (1H, m), 3.77 (3H, s), 3.93—4.06 (1H, m), 4.19—4.30 (1H, m), 4.35 (1H, dd, $J=5.7$, 13 Hz), 5.29—5.37 (1H, m), 6.70—6.81 (3H, m), 6.85 (1H, d, $J=8.1$ Hz), 6.94 (1H, t, $J=7.3$ Hz), 7.13—7.26 (3H, m)
42	0.85 (3H, t, $J=6.8$ Hz), 1.10—1.33 (6H, m), 1.37—1.58 (2H, m), 2.14 (2H, t, $J=7.5$ Hz), 2.25—2.48 (2H, m), 2.50—2.67 (2H, m), 2.76—2.97 (5H, m), 2.85 (3H, s), 3.65—3.82 (1H, m), 3.77 (3H, s), 3.93—4.05 (1H, m), 4.19—4.30 (1H, m), 4.35 (1H, dd, $J=5.5$, 13 Hz), 5.28—5.39 (1H, m), 6.70—6.81 (3H, m), 6.85 (1H, d, $J=7.9$ Hz), 6.94 (1H, t, $J=7.4$ Hz), 7.13—7.25 (3H, m)
43	0.86 (3H, t, $J=6.8$ Hz), 1.11—1.34 (8H, m), 1.42—1.58 (2H, m), 2.14 (2H, t, $J=7.6$ Hz), 2.26—2.48 (2H, m), 2.50—2.67 (2H, m), 2.76—2.99 (5H, m), 2.85 (3H, s), 3.65—3.84 (1H, m), 3.77 (3H, s), 3.92—4.05 (1H, m), 4.18—4.30 (1H, m), 4.34 (1H, dd, $J=5.4$, 13 Hz), 5.30—5.38 (1H, m), 6.70—6.81 (3H, m), 6.85 (1H, d, $J=7.9$ Hz), 6.94 (1H, t, $J=7.5$ Hz), 7.13—7.26 (3H, m)
44	0.87 (3H, t, $J=6.7$ Hz), 1.10—1.35 (12H, m), 1.41—1.57 (2H, m), 2.14 (2H, t, $J=7.6$ Hz), 2.27—2.48 (2H, m), 2.50—2.68 (2H, m), 2.75—2.99 (5H, m), 2.85 (3H, s), 3.64—3.83 (1H, m), 3.77 (3H, s), 3.93—4.05 (1H, m), 4.18—4.30 (1H, m), 4.35 (1H, dd, $J=5.5$, 13 Hz), 5.29—5.38 (1H, m), 6.69—6.80 (3H, m), 6.85 (1H, d, $J=8.1$ Hz), 6.94 (1H, t, $J=7.4$ Hz), 7.12—7.25 (3H, m)
45 (R-102444)	0.88 (3H, t, $J=6.8$ Hz), 1.13—1.35 (16H, m), 1.43—1.57 (2H, m), 2.13 (2H, t, $J=7.6$ Hz), 2.27—2.46 (2H, m), 2.50—2.68 (2H, m), 2.76—2.96 (5H, m), 2.85 (3H, s), 3.65—3.83 (1H, m), 3.77 (3H, s), 3.94—4.05 (1H, m), 4.20—4.31 (1H, m), 4.34 (1H, dd, $J=5.3$, 13 Hz), 5.29—5.38 (1H, m), 6.70—6.80 (3H, m), 6.85 (1H, d, $J=8.2$ Hz), 6.94 (1H, t, $J=7.4$ Hz), 7.13—7.25 (3H, m)
46	0.88 (3H, t, $J=6.6$ Hz), 1.11—1.38 (24H, m), 1.41—1.57 (2H, m), 2.13 (2H, t, $J=7.6$ Hz), 2.24—2.48 (2H, m), 2.51—2.67 (2H, m), 2.75—2.97 (5H, m), 2.85 (3H, s), 3.63—3.82 (1H, m), 3.77 (3H, s), 3.92—4.07 (1H, m), 4.20—4.43 (2H, m), 5.28—5.40 (1H, m), 6.69—6.81 (3H, m), 6.85 (1H, d, $J=7.9$ Hz), 6.93 (1H, t, $J=7.6$ Hz), 7.12—7.28 (3H, m)

a) Compound 3 was reported in ref. 14. b) In DMSO-*d*₆.

ex vivo antiaggregatory effects, produced no gastric irritation. Moreover, R-102444 exhibited antithrombotic effect more potently than sarpogrelate in the photochemically-induced thrombosis model. R-102444 is currently under fur-

ther evaluation in pharmacology and toxicology studies, and these data will be reported elsewhere.

Experimental

Melting points were determined with a Yanagimoto micro melting point apparatus and were uncorrected. ^1H - and ^{13}C -NMR spectra were obtained on a JEOL EX270 spectrometer and were reported as δ values relative to Me $_4\text{Si}$ as the internal standard. Abbreviations of the ^1H -NMR peak patterns are as follows: br=broad, s=singlet, d=doublet, t=triplet, q=quartet, and m=multiplet. Optical rotations were measured on a Perkin-Elmer 141 spectrometer. Merck Silica gel 60 (230–400 mesh) was used in the column chromatography. Tetrahydrofuran, *N,N*-dimethylformamide, *N,N*-dimethylacetamide, and dimethylsulfoxide are abbreviated as THF, DMF, DMA and DMSO, respectively.

(2*S*,4*R*)-4-Benzoyloxy-1,2-diethoxycarbonylpyrrolidine (2*S*,4*R*)-5 To a solution of (2*S*,4*R*)-4-benzoyloxy-1-*tert*-butoxycarbonylproline (2*S*,4*R*)-4 (15.0 g, 46.7 mmol) in EtOH (50 ml) was bubbled hydrogen chloride for 2 h. The resulting mixture was concentrated to give (2*S*,4*R*)-4-benzoyloxy-2-ethoxycarbonylpyrrolidine as a colorless oil. To a solution of this oil in acetone (80 ml) and H $_2$ O (80 ml) was added triethylamine (13.0 ml, 93.3 mmol) and the mixture was stirred at 0 °C for 10 min. Then ethyl chloroformate (4.89 ml, 51.4 mmol) was added to the solution. After stirring at room temperature for 3 h, the resulting mixture was concentrated and extracted with EtOAc. Then the extract was washed with brine. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=13/7) to give (2*S*,4*R*)-5 (13.4 g, 41.7 mmol, 89%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.17–1.31 (6H, m), 2.03–2.14 (1H, m), 2.36–2.48 (1H, m), 3.59–3.80 (2H, m), 4.04–4.24 (5H, m), 4.37–4.56 (3H, m), 7.24–7.38 (5H, m). $[\alpha]_D^{25} = -53^\circ$ ($c = 1.19$, MeOH).

(2*S*,4*R*)-4-Benzoyloxy-1-ethoxycarbonyl-2-hydroxymethylpyrrolidine (2*S*,4*R*)-6 A solution of (2*S*,4*R*)-4-benzoyloxy-1,2-diethoxycarbonylpyrrolidine (2*S*,4*R*)-5 (10.2 g, 31.7 mmol) in THF (30 ml) was added dropwise to a suspension of LiBH $_4$ (2.07 g, 95.2 mmol) in THF (70 ml) at 0 °C. The mixture was stirred at room temperature for 7 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=2/3) to give (2*S*,4*R*)-6 (8.05 g, 28.8 mmol, 91%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.27 (3H, t, $J = 7.3$ Hz), 1.58–1.72 (1H, m), 2.14–2.28 (1H, m), 3.43 (1H, dd, $J = 4.3$, 12 Hz), 3.51–3.65 (1H, m), 3.68–3.83 (2H, m), 4.01–4.24 (2H, m), 4.15 (2H, q, $J = 7.3$ Hz), 4.51 (2H, s), 4.67–4.78 (1H, m), 7.22–7.41 (5H, m). $[\alpha]_D^{25} = -48^\circ$ ($c = 2.17$, MeOH).

(2*R*,4*R*)-4-Benzoyloxy-2-cyanomethyl-1-ethoxycarbonylpyrrolidine (2*R*,4*R*)-7 To a solution of (2*S*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-hydroxymethylpyrrolidine (2*S*,4*R*)-6 (8.00 g, 28.6 mmol) and *p*-toluenesulfonic anhydride (Ts $_2$ O) (10.28 g, 31.5 mmol) in CH $_2$ Cl $_2$ (100 ml) was added triethylamine (4.39 ml, 31.5 mmol) at 0 °C and the mixture was stirred at room temperature for 2.5 h. The resulting mixture was diluted with CH $_2$ Cl $_2$ and washed with H $_2$ O and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=13/7) to give (2*S*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-(*p*-toluenesulfonyloxy)methylpyrrolidine (11.67 g, 26.9 mmol, 94%) as an oil. To a solution of this oil (11.53 g, 26.6 mmol) in DMF (80 ml) was added NaCN (1.30 g, 26.5 mmol) and the mixture was stirred at 80 °C for 2 h. The resulting mixture was diluted with EtOAc and washed with H $_2$ O and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=4/1) to give (2*R*,4*R*)-7 (7.14 g, 24.8 mmol, 93%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.27 (3H, t, $J = 7.1$ Hz), 1.95–2.12 (1H, m), 2.32–2.48 (1H, m), 2.70 (1H, dd, $J = 2.8$, 17 Hz), 2.83, 3.15 (1H, dd, $J = 6.0$, 17 Hz), 3.51 (1H, dd, $J = 4.2$, 12 Hz), 3.74, 3.93 (1H, d, $J = 12$ Hz), 4.07–4.27 (2H, m), 4.14 (2H, q, $J = 7.1$ Hz), 4.51 (2H, s), 7.23–7.41 (5H, m). $[\alpha]_D^{25} = -72^\circ$ ($c = 1.94$, MeOH).

(2*S*,4*R*)-4-Benzoyloxy-1-ethoxycarbonyl-2-(ethoxycarbonylmethyl)pyrrolidine (2*S*,4*R*)-8 To a solution of (2*R*,4*R*)-4-benzoyloxy-2-cyanomethyl-1-ethoxycarbonylpyrrolidine (2*R*,4*R*)-7 (11.63 g, 40.3 mmol) in EtOH (20 ml) was bubbled hydrogen chloride for 2 h. The resulting mixture was concentrated *in vacuo*. The residue was diluted with EtOAc and washed with NaHCO $_3$ solution and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=3/1) to give (2*S*,4*R*)-8 (11.93 g, 35.6 mmol, 88%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.25 (6H, t, $J = 7.3$ Hz), 1.85–2.02 (1H, m), 2.28–2.53 (2H, m), 2.82–3.12 (1H, m), 3.37–3.50 (1H, m), 3.59–3.78 (1H, m), 4.03–4.37 (2H, m), 4.12 (4H, q, $J = 7.3$ Hz), 4.50 (2H, d, $J = 4.6$ Hz), 7.21–7.40 (5H, m). $[\alpha]_D^{25} = -45^\circ$ ($c = 1.30$, MeOH).

(2*R*,4*R*)-4-Benzoyloxy-1-ethoxycarbonyl-2-(2-hydroxyethyl)pyrrolidine (2*R*,4*R*)-9 A solution of (2*S*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-(ethoxy-

carbonylmethyl)pyrrolidine (2*S*,4*R*)-8 (11.90 g, 35.5 mmol) in THF (50 ml) was added dropwise to a suspension of LiAlH $_4$ (1.35 g, 35.5 mmol) in THF (200 ml), vigorously stirring at -10°C . After stirring at -10°C for 30 min, Na $_2$ SO $_4$ decahydrate was added slowly to the resulting suspension and the slurry was stirred for 1 h continuously. The insoluble material was filtered away and the filtrate was concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=1/3) to give (2*R*,4*R*)-9 (8.86 g, 30.2 mmol, 85%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.27 (3H, t, $J = 6.9$ Hz), 1.45–1.61 (1H, m), 1.66–1.93 (2H, m), 2.14–2.29 (1H, m), 3.43 (1H, dd, $J = 5.6$, 12 Hz), 3.54–3.71 (3H, m), 3.84–3.96 (1H, m), 4.07–4.36 (2H, m), 4.15 (2H, q, $J = 6.9$ Hz), 4.50 (2H, s), 7.25–7.42 (5H, m). $[\alpha]_D^{25} = -36^\circ$ ($c = 1.77$, MeOH).

(2*R*,4*R*)-4-Benzoyloxy-1-ethoxycarbonyl-2-[2-(*p*-toluenesulfonyloxy)ethyl]pyrrolidine (2*R*,4*R*)-10 To a solution of (2*R*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-(2-hydroxyethyl)pyrrolidine (2*R*,4*R*)-9 (8.83 g, 30.1 mmol) and Ts $_2$ O (10.81 g, 33.1 mmol) in CH $_2$ Cl $_2$ (300 ml) was added triethylamine (4.61 ml, 33.1 mmol) at 0 °C and the mixture was stirred at room temperature for 2.5 h. The resulting mixture was diluted with CH $_2$ Cl $_2$ and washed with H $_2$ O and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=13/7) to give (2*R*,4*R*)-10 (10.78 g, 24.1 mmol, 80%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.22 (3H, t, $J = 6.9$ Hz), 1.71–1.90 (2H, m), 2.03–2.34 (2H, m), 2.44 (3H, s), 3.24–3.42 (1H, m), 3.57–3.90 (1H, m), 3.92–4.19 (6H, m), 4.48 (2H, s), 7.24–7.41 (7H, m), 7.78 (2H, d, $J = 7.9$ Hz). $[\alpha]_D^{25} = -22^\circ$ ($c = 1.18$, MeOH).

(2*R*,4*R*)-4-Benzoyloxy-1-ethoxycarbonyl-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethylpyrrolidine (13a: R=3-OMe) To a solution of 2-[2-(3-methoxyphenyl)ethyl]phenol **12a** (R=3-OMe) (500 mg, 2.19 mmol) in DMA (13 ml) was added *tert*-BuOK (270 mg, 2.41 mmol) and the mixture was stirred at 0 °C for 10 min. Then a solution of (2*R*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-[2-(*p*-toluenesulfonyloxy)ethyl]pyrrolidine (2*R*,4*R*)-10 (1.19 g, 2.66 mmol) in DMA (7 ml) was added and the whole was stirred at room temperature for 4 h. The resulting suspension was diluted with EtOAc and washed with H $_2$ O and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=4/1–3/1) to give **13a** (881 mg, 1.75 mmol, 80%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.10–1.34 (3H, m), 1.76–2.07 (2H, m), 2.23–2.57 (2H, m), 2.79–2.98 (4H, m), 3.43 (1H, dd, $J = 4.6$, 12 Hz), 3.55–4.28 (7H, m), 3.75 (3H, s), 4.45 (2H, s), 6.68–6.92 (5H, m), 7.06–7.37 (8H, m). $[\alpha]_D^{25} = -1.3^\circ$ ($c = 1.20$, MeOH).

(2*R*,4*R*)-1-Ethoxycarbonyl-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethylpyrrolidine (14a: R=3-OMe) A solution of (2*R*,4*R*)-4-benzoyloxy-1-ethoxycarbonyl-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethylpyrrolidine **13a** (R=3-OMe) (853 mg, 1.69 mmol) in EtOH (6 ml) was hydrogenated over 5% Pd–C (84 mg) at 60 °C for 7 h with stirring. The catalyst was filtered away, and the filtrate was concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=1/1) to give **14a** (654 mg, 1.58 mmol, 93%) as a colorless oil. ^1H -NMR (CDCl $_3$) δ : 1.11–1.33 (3H, m), 1.72–2.25 (3H, m), 2.29–2.58 (1H, m), 2.80–2.98 (4H, m), 3.46 (1H, dd, $J = 4.6$, 12 Hz), 3.50–3.75 (1H, m), 3.78 (3H, s), 3.95–4.29 (5H, m), 4.37–4.48 (1H, m), 6.70–6.93 (5H, m), 7.08–7.28 (3H, m). $[\alpha]_D^{25} = -12^\circ$ ($c = 0.73$, MeOH).

(2*R*,4*R*)-4-Hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl-1-methylpyrrolidine Hydrochloride (17: R=3-OMe) A solution of (2*R*,4*R*)-1-ethoxycarbonyl-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethylpyrrolidine **14a** (R=3-OMe) (640 mg, 1.55 mmol) in THF (20 ml) was added dropwise to a suspension of LiAlH $_4$ (176 mg, 4.64 mmol) in THF (10 ml), vigorously stirring at room temperature. The mixture was refluxed for 1 h and then cooled. To the resulting suspension was slowly added Na $_2$ SO $_4$ decahydrate and the slurry was stirred for 2 h continuously. The insoluble material was filtered away and the filtrate was concentrated. The resulting residue was chromatographed on a silica gel column (CH $_2$ Cl $_2$ /MeOH=9/1) to give (2*R*,4*R*)-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl-1-methylpyrrolidine (523 mg, 1.47 mmol, 95%) as a colorless oil. This oil was dissolved in dioxane (5 ml) and was treated with 4 *N* HCl in dioxane (1.10 ml, 4.40 mmol). The mixture was stirred at room temperature for 10 min, then concentrated. The oily residue was dissolved in a mixture of EtOAc/CH $_2$ Cl $_2$ =20/1, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **17** (420 mg, 1.07 mmol, 73%).

Similarly, other 4-hydroxypyrrolidine derivatives, **15**, **16**, **18**–**20**, were prepared by the alkylation of phenols **12**^{13,15} with the tosylate (2*R*,4*R*)-10, followed by catalytic hydrogenation and reduction with LiAlH $_4$.

(2*R*,4*S*)-4-Benzoyloxy-1-ethoxycarbonyl-2-[2-[2-(3-methoxyphenyl)-

ethyl]phenoxy]ethyl]pyrrolidine (21) Diethyl azodicarboxylate (DEAD) (1.54 ml, 9.80 mmol) was added to a solution of (2*R*,4*R*)-1-ethoxycarbonyl-4-hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine **14a** (*R*=3-OMe) (2.70 g, 6.53 mmol), benzoic acid (1.20 g, 9.83 mmol) and triphenylphosphine (2.57 g, 9.80 mmol) in CH₂Cl₂ (30 ml) at 0 °C and the mixture was stirred at room temperature for 1.5 h. The solvent was removed and the resulting residue was chromatographed on a silica gel column (hexane/EtOAc=3/1) to give **21** (3.20 g, 6.18 mmol, 95%) as a colorless oil. ¹H-NMR (CDCl₃) δ: 1.06–1.34 (3H, m), 2.06–2.65 (4H, m), 2.78–2.97 (4H, m), 3.59–3.71 (1H, m), 3.74 (3H, s), 3.79–4.21 (5H, m), 4.23–4.35 (1H, m), 5.48–5.62 (1H, m), 6.66–6.91 (5H, m), 7.04–7.20 (3H, m), 7.31–7.46 (2H, m), 7.54 (1H, t, *J*=7.3 Hz), 7.99 (2H, d, *J*=7.9 Hz). [α]_D –3.7° (*c*=1.08, MeOH).

(2*R*,4*S*)-4-Hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine Hydrochloride (22) A solution of (2*R*,4*S*)-4-benzoyloxy-1-ethoxycarbonyl-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine **21** (1.07 g, 2.07 mmol) in THF (7 ml) was added dropwise to a suspension of LiAlH₄ (234 mg, 6.17 mmol) in THF (13 ml), vigorously stirring at room temperature. The mixture was refluxed for 1 h and then cooled. To the resulting suspension was slowly added Na₂SO₄ decahydrate and the slurry was stirred for 2 h continuously. The insoluble material was filtered away and the filtrate was concentrated. The resulting residue was chromatographed on a silica gel column (CH₂Cl₂/MeOH=17/3) to give (2*R*,4*S*)-4-hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (662 mg, 1.86 mmol, 90%) as a colorless oil. This oil (638 mg, 1.79 mmol) was dissolved in dioxane (5 ml) and was treated with 4*N* HCl in dioxane (1.35 ml, 5.40 mmol). The mixture was stirred at room temperature for 10 min, then concentrated. The oily residue was dissolved in a mixture of hexane/EtOAc=1/10, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **22** (510 mg, 1.30 mmol, 73%).

Similarly, the other two optical isomers of compound **17** were synthesized. The (2*S*,4*R*) isomer, **25**, was synthesized from the phenol **12a** and (2*S*,4*R*)-**10**, in the same manner as in the synthesis of **17**. The (2*S*,4*S*) isomer, **27**, was prepared from **24** similarly to the preparation of **22**. The tosylate, (2*S*,4*R*)-**10**, was provided in the same manner as in the synthesis of (2*R*,4*R*)-**10** from the carbamate (2*R*,4*R*)-**5**, which was synthesized as described below.

(2*R*,4*R*)-4-Benzoyloxy-1,2-diethoxycarbonylpyrrolidine ((2*R*,4*R*)-5) To a solution of (2*S*,4*R*)-4-hydroxyproline (2*S*,4*R*)-**11** (53.23 g, 406 mmol) in EtOH (250 ml) was bubbled hydrogen chloride for 3 h. The resulting mixture was concentrated to deposit colorless crystals. The crystals were collected by filtration to give (2*S*,4*R*)-2-ethoxycarbonyl-4-hydroxypyrrolidine hydrochloride (78.04 g, 399 mmol, 98%). To a solution of the crystals (10.0 g, 51.1 mmol) in acetone (80 ml) and H₂O (80 ml) was added triethylamine (15.0 ml, 108 mmol) and the mixture was stirred at 0 °C for 10 min. Then ethyl chloroformate (5.35 ml, 56.2 mmol) was added to the solution. After stirring at room temperature for 3 h, the resulting mixture was concentrated and extracted with EtOAc, and the extract was washed with brine. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=1/3) to give (2*S*,4*R*)-1,2-diethoxycarbonyl-4-hydroxypyrrolidine (11.3 g, 48.9 mmol, 96%) as a colorless oil. To a solution of this oil (10.6 g, 45.8 mmol) in THF (70 ml) was added NaH (2.41 g, 55.2 mmol, as a 55% w/w dispersion in mineral oil) and the mixture was stirred at 0 °C for 15 min. To the resulting solution was added benzyl bromide (6.57 ml, 55.2 mmol) and tetrabutylammonium iodide (Bu₄NI) (340 mg, 0.92 mmol) and the mixture was stirred at room temperature for 3 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=7/3) to give (2*S*,4*R*)-**5** (less polar: 6.64 g, 20.7 mmol, 45%) and (2*R*,4*R*)-**5** (more polar: 4.56 g, 14.2 mmol, 31%) as a colorless oil, respectively. ¹H-NMR (CDCl₃) δ: 1.15–1.32 (6H, m), 2.22–2.43 (2H, m), 3.55–3.74 (2H, m), 4.02–4.23 (5H, m), 4.35–4.53 (1H, m), 4.47 (2H, s), 7.22–7.39 (5H, m). [α]_D +34° (*c*=1.18, MeOH).

(*R*)-2-[2-[2-[2-(3-Methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-oxopyrrolidine Hydrochloride (28) To a solution of DMSO (0.52 g, 6.66 mmol) in CH₂Cl₂ (10 ml) was added oxalyl chloride (0.58 ml, 6.65 mmol) and the mixture was stirred at –70 °C for 5 min. To the resulting solution was added a solution of (2*R*,4*R*)-4-hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (1.57 g, 4.42 mmol) in CH₂Cl₂ (5 ml) and the mixture was stirred at –70 °C for 1 h. Then triethylamine (1.85 ml, 13.3 mmol) was added to the mixture. After stirring at –70 °C for 1.5 h, the resulting solution was diluted with CH₂Cl₂ and washed with H₂O and

brine successively. The organic layer was dried and concentrated, and the resulting residue was chromatographed on a silica gel column (hexane/EtOAc=1/1) to give (*R*)-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-oxopyrrolidine (1.30 g, 3.68 mmol, 83%) as an oil. This oil (485 mg, 1.37 mmol) was dissolved in dioxane (7 ml) and was treated with 4*N* HCl in dioxane (1.03 ml, 4.12 mmol). The mixture was stirred at room temperature for 1 h, then concentrated. The oily residue was dissolved in a mixture of ether/EtOAc/MeOH=10/10/1, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **28** (421 mg, 1.08 mmol, 79%).

(*S*)-(4*Z*)-Hydroxyimino-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine Hydrochloride (29) and (*S*)-(4*E*)-Hydroxyimino-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine Hydrochloride (30) To a solution of (*R*)-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-oxopyrrolidine (797 mg, 2.25 mmol) and hydroxylamine hydrochloride (178 mg, 2.56 mmol) in MeOH (5 ml) was added sodium acetate (AcONa) (407 mg, 4.96 mmol), and the mixture was stirred at 0 °C for 2.5 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (MeOH/CH₂Cl₂/EtOAc=1/9/10) to give (*Z*)-oxime (less polar: 409 mg, 1.11 mmol, 49%) and (*E*)-oxime (more polar: 298 mg, 0.81 mmol, 36%) as a colorless solid, respectively. (*Z*)-oxime: ¹³C-NMR (CDCl₃) δ: 32.2, 32.5, 35.8, 36.5, 40.0, 55.1, 56.2, 63.0, 65.0, 111.0, 111.1, 114.3, 120.6, 120.9, 127.3, 129.2, 130.1, 130.2, 144.0, 156.6, 159.6, 160.4. (*E*)-oxime: ¹³C-NMR (CDCl₃) δ: 32.4 (2C), 33.2, 36.5, 40.1, 55.1, 58.7, 62.9, 64.9, 111.0, 111.1, 114.4, 120.6, 120.9, 127.2, 129.2, 130.0, 130.2, 144.0, 156.6, 159.6, 160.2.

(*Z*)-Oxime **29**: This oil (346 mg, 0.94 mmol) was dissolved in dioxane (5 ml) and was treated with 4*N* HCl in dioxane (0.70 ml, 2.80 mmol). The resulting solution was concentrated. The oily residue was dissolved in a mixture of MeOH/CH₂Cl₂/EtOAc=1/10/10, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **29** (188 mg, 0.46 mmol, 49%).

(*E*)-Oxime **30**: This oil (243 mg, 0.66 mmol) was dissolved in dioxane (5 ml) and was treated with 4*N* HCl in dioxane (0.49 ml, 1.96 mmol). The resulting solution was concentrated. The oily residue was dissolved in a mixture of CH₂Cl₂/EtOAc=1/1, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **30** (201 mg, 0.50 mmol, 75%).

(2*R*,4*S*)-1-Ethoxycarbonyl-4-hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine (31) A solution of (2*R*,4*S*)-4-benzoyloxy-1-ethoxycarbonyl-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine **21** (3.15 g, 6.09 mmol) in THF (20 ml) was added dropwise to a suspension of LiBH₄ (398 mg, 18.3 mmol) in THF (10 ml), vigorously stirring at 0 °C. After stirring at room temperature for 18.5 h, LiBH₄ (265 mg, 12.2 mmol) was added, and the resulting mixture was stirred at 40 °C for 8 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=1/3) to give **31** (2.49 g, 6.02 mmol, 99%) as a colorless oil. ¹H-NMR (CDCl₃) δ: 1.06–1.33 (3H, m), 1.93–2.30 (3H, m), 2.36–2.62 (1H, m), 2.81–2.98 (4H, m), 3.33–3.44 (1H, m), 3.55–3.85 (1H, m), 3.78 (3H, s), 3.95–4.23 (5H, m), 4.40–4.51 (1H, m), 6.70–6.93 (5H, m), 7.09–7.24 (3H, m). [α]_D +14° (*c*=1.22, MeOH).

(2*R*,4*S*)-1-Ethoxycarbonyl-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-4-(*p*-toluenesulfonyloxy)pyrrolidine (32) To a solution of (2*R*,4*S*)-1-ethoxycarbonyl-4-hydroxy-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine **31** (2.44 g, 5.90 mmol) and Ts₂O (2.38 g, 7.07 mmol) in CH₂Cl₂ (50 ml) was added triethylamine (0.99 ml, 7.10 mmol) at 0 °C and the mixture was stirred at room temperature for 3 h. Then Ts₂O (995 mg, 2.96 mmol) and triethylamine (0.41 ml, 2.94 mmol) was added, and the resulting mixture was stirred continuously overnight at room temperature. The reaction mixture was diluted with CH₂Cl₂ and washed with H₂O and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=13/7) to give **32** (2.99 g, 5.27 mmol, 83%) as a colorless oil. ¹H-NMR (CDCl₃) δ: 1.00–1.34 (3H, m), 1.90–2.06 (1H, m), 2.09–2.52 (3H, m), 2.42 (3H, s), 2.76–2.98 (4H, m), 3.40–3.82 (2H, m), 3.76 (3H, s), 3.87–4.28 (5H, m), 5.02–5.11 (1H, m), 6.68–6.92 (5H, m), 7.05–7.23 (3H, m), 7.31 (2H, d, *J*=7.9 Hz), 7.76 (2H, d, *J*=7.9 Hz). [α]_D +4.9° (*c*=0.98, MeOH).

(2*S*,4*R*)-4-Azido-1-ethoxycarbonyl-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]pyrrolidine (33) To a solution of (2*R*,4*S*)-1-ethoxycarbonyl-2-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-4-(*p*-toluenesul-

fonyloxy)pyrrolidine **32** (1.00 g, 1.76 mmol) in DMF (20 ml) was added sodium azide (344 mg, 5.29 mmol) and the mixture was stirred at room temperature for 1 h and then 80 °C for 3 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (hexane/EtOAc=7/3) to give **33** (709 mg, 1.62 mmol, 92%) as a colorless oil. ¹H-NMR (CDCl₃) δ: 1.10–1.35 (3H, m), 1.81–2.01 (1H, m), 2.03–2.16 (1H, m), 2.18–2.55 (2H, m), 2.80–2.98 (4H, m), 3.49 (1H, dd, *J*=5.3, 12 Hz), 3.54–3.82 (1H, m), 3.78 (3H, s), 3.95–4.24 (6H, m), 6.70–6.92 (5H, m), 7.07–7.27 (3H, m). [α]_D²⁰ = -12° (*c*=1.23, MeOH).

(2S,4R)-4-Amino-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine Hydrochloride (34) A solution of (2S,4R)-4-azido-1-ethoxycarbonyl-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethylpyrrolidine **33** (681 mg, 1.55 mmol) in THF (7 ml) was added dropwise to a suspension of LiAlH₄ (177 mg, 4.66 mmol) in THF (13 ml), vigorously stirring at room temperature. After stirring at room temperature for 2 h, Na₂SO₄ decahydrate was added slowly to the resulting suspension and the slurry was stirred for 2 h continuously. The insoluble material was filtered away and the filtrate was concentrated. The resulting residue was chromatographed on a silica gel column (CH₂Cl₂/MeOH=13/7) to give (2S,4R)-4-amino-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (310 mg, 0.87 mmol, 56%) as a colorless oil. This oil (291 mg, 0.82 mmol) was dissolved in dioxane (5 ml) and was treated with 4N HCl in dioxane (0.61 ml, 2.44 mmol). The mixture was stirred at room temperature for 10 min, then concentrated. The oily residue was dissolved in a mixture of CH₂Cl₂/EtOAc=1/10, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **34** (325 mg, 0.76 mmol, 93%).

(2R,4R)-2-[2-[2-(3-Methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-phenylcarbamoyloxypyrrolidine Hydrochloride (35) To a solution of (2R,4R)-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (500 mg, 1.41 mmol) and phenyl isocyanate (0.23 ml, 2.12 mmol) in CH₂Cl₂ (8 ml) was added triethylamine (0.22 ml, 1.58 mmol) at 0 °C and the mixture was stirred at room temperature for 6 h. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (CH₂Cl₂/MeOH=4/1) to give (2R,4R)-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-phenylcarbamoyloxypyrrolidine (578 mg, 1.22 mmol, 87%) as a colorless oil. This oil (543 mg, 1.14 mmol) was dissolved in dioxane (5 ml) and was treated with 4N HCl in dioxane (0.86 ml, 3.44 mmol). The resulting solution was concentrated to give **35** (564 mg, 1.10 mmol, 96%) as an oil.

(2R,4R)-2-[2-[2-(3-Methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-succinyloxypyrrolidine Hydrochloride (36) A solution of (2R,4R)-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (500 mg, 1.41 mmol) and succinic anhydride (141 mg, 1.41 mmol) in acetone (8 ml) was refluxed for 4 h. The reaction mixture was cooled and then concentrated. The resulting residue was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried and evaporated. The resulting residue was chromatographed on a silica gel column (CH₂Cl₂/MeOH=7/3) to give (2R,4R)-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-4-succinyloxypyrrolidine (477 mg, 1.05 mmol, 75%) as a colorless oil. This oil (460 mg, 1.01 mmol) was dissolved in dioxane (7 ml) and was treated with 4N HCl in dioxane (0.76 ml, 3.04 mmol). The resulting solution was concentrated to give **36** (462 mg, 0.94 mmol, 93%) as an oil.

(2R,4R)-4-Lauryloxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine Hydrochloride (45 (R-102444)) To a solution of (2R,4R)-4-hydroxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (388 mg, 1.09 mmol) and lauric anhydride (543 mg, 1.42 mmol) in pyridine (5 ml) was added 4-dimethylaminopyridine (DMAP) (40 mg, 0.33 mmol) and the mixture was stirred at room temperature for 4 h. The resulting solution was diluted with EtOAc and washed with 1N HCl and brine successively. The organic layer was dried and concentrated. The resulting residue was chromatographed on a silica gel column (EtOAc) to give (2R,4R)-4-lauryloxy-2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methylpyrrolidine (549 mg, 1.02 mmol, 94%) as a colorless oil. This oil (535 mg, 0.99 mmol) was dissolved in dioxane (6 ml) and was treated with 4N HCl in dioxane (0.75 ml, 3.00 mmol). The resulting solution was concentrated. The oily residue was dissolved in a mixture of ether/EtOAc=10/1, and the solution was allowed to stand at room temperature to give colorless crystals. The crystals were collected by filtration to give **45** (R-102444) (446 mg, 0.78 mmol, 78%).

Similarly, carbonates, **37**, **38**, and other esters, **39–44**, **46**, were prepared

by the acylation of **17** with the corresponding acid anhydride or acyl chloride.

5-HT₂ Receptor Binding Assay The 5-HT₂ receptor binding assay of Laysen *et al.*²⁵ was employed with some modifications. It was performed using the 5-HT₂ antagonist, [³H]ketanserin, as the ³H-ligand, and the cortex as reported in ref. 20.

D₂ Receptor Binding Assay The D₂ receptor binding assay of Köhler *et al.*²⁶ was employed with some modifications. It was performed using the D₂ antagonist, [³H]raclopride, as the ³H-ligand, and the striatum as reported in ref. 20.

α₁ Receptor Binding Assay The α₁ receptor binding assay of Green-grass and Bremner²⁷ was employed with modifications. It was performed using the α₁ antagonist, [³H]prazosin, as the ³H-ligand, and the cortex as reported in ref. 14.

β Receptor Binding Assay The β receptor binding assay of U'Prichard *et al.*²⁸ was employed with modifications. It was performed using the β antagonist, [³H]dihydroalprenolol (DHA), as the ³H-ligand, and the cortex as reported in ref. 14.

5-HT₁ Receptor Binding Assay The 5-HT₁ receptor binding assay of Middlemiss²⁹ was employed with modifications. It was performed using [³H] 5-HT as the ³H-ligand, and the cortex as reported in ref. 14.

5-HT₃ Receptor Binding Assay The 5-HT₃ receptor binding assay of Kilpatrick *et al.*³⁰ was employed with modifications. It was performed using the 5-HT₃ antagonist, [³H]GR65630, as the ³H-ligand and the cortex as reported in ref. 14.

5-HT-Induced Vasoconstriction Experiment The method involving contractions of the rat caudal arteries of Van Nueten *et al.*³¹ was employed with some modifications as reported in ref. 20.

5-HT-Induced PRP Aggregation Preparation of platelets and measurements of platelet aggregation was reported in ref. 14.

Ex Vivo Platelet Aggregation Groups of 5 male Sprague-Dawley rats (Japan SLC) were used in the experiments. Test compound suspended in 0.5% gum tragacanth solution was orally administered to rats in a volume of 1 ml/kg body weight, and, 1 h later, blood was collected from the abdominal aorta of rats under pentobarbital-anesthesia into a syringe containing 3.8% sodium citrate as an anticoagulant. The blood was centrifuged (3000 rpm, 15 min), and platelet-poor plasma (PPP) was obtained. Platelet counts in PRP were adjusted by the method of Born.³² using a platelet aggregometer (PAM-6C, Mebanix). To PRP (0.24 ml) preincubated at 37 °C for 2 min was added 0.01 ml of ADP (final concentration: 1 μM) alone and in combination with 5-HT (final concentration: 10 μM), and platelet aggregation was monitored for 5 min. Aggregation responses were quantified as the area under the curve (AUC) of change in light transmission according to the method of Kil-lam and Cohen.³³ The potentiation to 5-HT was calculated as the AUC measured after the ADP-induced aggregation subtracted from the AUC measured after the (5-HT+ADP)-induced aggregation. The antiaggregatory effect of the test compound was expressed as inhibition (%), which compared the potentiated AUC in the test compound-treated group to that in the control (0.5% gum tragacanth solution) group.

Photochemically-Induced Thrombosis Model in Femoral Artery A photochemically-induced thrombosis model was used according to the method established by Matsuno *et al.*²⁴ After anesthetizing male Sprague-Dawley rats (210–250 g) with pentobarbital (40 mg/kg, i.p.), a 5 mm segment of the left femoral artery distal to the inguinal ligament was separated carefully and a pulse Doppler flow probe (LMS) was attached to the vessel for monitoring the blood flow. The right femoral artery was cannulated with a polyethylene tube for monitoring the blood pressure. After establishing the baseline blood flow, the femoral artery positioned about 5 mm proximal to the flow probe was irradiated with a green light (540 nm) using a Xenon lamp (Hamamatsu Photonics, Japan). Two min after irradiation, rose bengal (20 mg/kg, Wako) was injected into the right femoral vein. Arterial blood flow was continuously monitored for a maximum of 30 min on a thermal array recorder (Nihon Koden). Time to occlusion indicated by complete cessation of blood flow indicated thrombogenesis. If complete occlusion was not observed within 30 min after injection of rose bengal, the time to occlusion was taken to be 30 min. Test agents were orally administered to rats 1 h before rose bengal injection.

X-Ray Crystallographic Analysis The reflection data were collected on a Rigaku AFC-7R diffractometer with graphite-monochromated CuKα radiation (λ=1.5418 Å). The structures were solved by direct methods using the SIR92 program³⁴ for **17** and **22**, and by heavy atom methods using the DIRDIF92 PATTY program³⁵ for **25** and **27**. The structures were then refined by the full-matrix least-squares procedure with anisotropic temperature factors for the non-hydrogen atoms and isotropic temperature factors for the

hydrogen atoms. Crystal data for **17**: $C_{22}H_{29}NO_3 \cdot HCl$; $M=391.94$; orthorhombic, space group $P2_12_12_1$, $a=13.4470$ (9) Å, $b=21.317$ (1) Å, $c=7.7412$ (9) Å; $V=2219.0$ (3) Å³, $D_c=1.173$ g/cm³, $Z=4$, $R=0.031$, $R_w=0.045$ for 1588 reflections with $I>3\sigma(I)$. Crystal data for **22**: $C_{22}H_{29}NO_3 \cdot HCl \cdot H_2O$; $M=409.05$; monoclinic, space group $C2$, $a=13.170$ (2) Å, $b=7.464$ (1) Å, $c=22.622$ (1) Å, $\beta=94.400$ (7)°; $V=2217.3$ (4) Å³, $D_c=1.228$ g/cm³, $Z=4$, $R=0.031$, $R_w=0.045$ for 1895 reflections with $I>3\sigma(I)$. Crystal data for **25**: $C_{22}H_{29}NO_3 \cdot HCl \cdot H_2O$; $M=409.05$; monoclinic, space group $C2$, $a=13.162$ (1) Å, $b=7.4656$ (7) Å, $c=22.6077$ (8) Å, $\beta=94.441$ (4)°; $V=2214.8$ (3) Å³, $D_c=1.229$ g/cm³, $Z=4$, $R=0.028$, $R_w=0.042$ for 1906 reflections with $I>3\sigma(I)$. Crystal data for **27**: $C_{22}H_{29}NO_3 \cdot HCl$; $M=391.94$; orthorhombic, space group $P2_12_12_1$, $a=10.785$ (1) Å, $b=29.036$ (2) Å, $c=7.138$ (2) Å; $V=2235.3$ (6) Å³, $D_c=1.165$ g/cm³, $Z=4$, $R=0.048$, $R_w=0.070$ for 1387 reflections with $I>3\sigma(I)$.

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References and Notes

- 1) Present address: Patent Department, Sankyo Co., Ltd.
- 2) Ross R., *N. Engl. J. Med.*, **314**, 488—500 (1986).
- 3) De Clerck F., Herman A. G., *Fed. Proc.*, **42**, 228—232 (1983).
- 4) Mallarkey G., Smith G. M., *Br. J. Pharmacol.*, **84**, 425—430 (1985).
- 5) Panak E., Maffrand J. P., Picard-Fraire C., Vallee E., Blanchard J., Roncucci R., *Haemostasis*, **13**, suppl. 1, 1—54 (1983).
- 6) White J. G., *Scand. J. Haematol.*, **7**, 145—151 (1970).
- 7) Van Nueten J. M., Janssens W. J., Vanhoutte P. M., Raven Press, New York, **1985**, pp 95—103.
- 8) Van Nueten J. M., *Fed. Proc.*, **42**, 223—227 (1983).
- 9) De Cree J., Leempoels J., Demon B., Roels V., Verhaegen H., *Agents Actions*, **16**, 313—317 (1985).
- 10) Fozard J. R., *J. Cardiovasc. Pharmacol.*, **4**, 829—838 (1982).
- 11) Cohen M. L., Fuller R. W., Kurz K. D., *Hypertension*, **5**, 676—681 (1983).
- 12) Hara H., Osakabe M., Kitajima A., Tamao Y., Kikumoto R., *Thromb. Haemost.*, **65**, 415—420 (1991).
- 13) Kikumoto R., Hara H., Ninomiya K., Osakabe M., Sugano M., Fukami H., Tamao Y., *J. Med. Chem.*, **33**, 1818—1823 (1990).
- 14) Tanaka N., Goto R., Ito R., Hayakawa M., Sugidachi A., Ogawa T., Asai F., Fujimoto K., *Chem. Pharm. Bull.*, **48**, 245—255 (2000).
- 15) Fujimoto K., Tanaka N., Asai F., Ito T., Koike H., Eur. Pat. EP600717 (1994) [*Chem. Abstr.*, **123**, 169510k (1995)].
- 16) Mitsunobu O., *Synthesis*, **1981**, 1—28.
- 17) Mancuso A. J., Huang S. L., Swern D., *J. Org. Chem.*, **43**, 2480—2482 (1978).
- 18) The ¹³C-NMR of **29** revealed a downfield methylene resonance (35.8 ppm) at 3-position of the pyrrolidine ring compared to the corresponding methylene resonance (33.2 ppm) of **30**. The ¹³C-NMR of **29** also revealed a upfield methylene resonance (56.2 ppm) at 5-position compared to the corresponding methylene resonance (58.7 ppm) of **30**. According to these data, we determined that compound **29** is (Z)-oxime and compound **30** is (E)-oxime, respectively.
- 19) Brodie B. B., Kurz H., Schanker L. S., *J. Pharmacol. Exp. Ther.*, **130**, 20—25 (1960).
- 20) Tanaka N., Goto R., Ito R., Hayakawa M., Ogawa T., Fujimoto K., *Chem. Pharm. Bull.*, **46**, 639—646 (1998).
- 21) Gregg C. N., Osborne R. H., *Br. J. Pharmacol.*, **85**, 312P (1985).
- 22) Baxter G. S., Murphy O. E., Blackburn T. P., *Br. J. Pharmacol.*, **112**, 323—331 (1994).
- 23) Buchheit K. H., Engel G., Hagenbach A., Hoyer D., Kalkman H. O., Seiler M. P., *Br. J. Pharmacol.*, **88**, 367P (1986).
- 24) Matsuno H., Uematsu T., Nagashima S., Nakashima M., *J. Pharmacol. Meth.*, **25**, 303—317 (1991).
- 25) Leysen J. E., Niemegeers C. J. E., Van Nueten J. M., Laduron P. M., *Mol. Pharmacology*, **21**, 301—314 (1982).
- 26) Köhler C., Hall H., Ögren S., Gawell L., *Biochem. Pharmacol.*, **34**, 2251—2259 (1985).
- 27) Greengrass P., Bremner R., *Eur. J. Pharmacol.*, **55**, 323—326 (1979).
- 28) U'Prichard D. C., Bylund D. B., Snyder S. H., *J. Biol. Chem.*, **253**, 5090—5102 (1978).
- 29) Middlemiss D. N., *Eur. J. Pharmacol.*, **101**, 289—293 (1984).
- 30) Kilpatrick G. J., Jones B. J., Tyers M. B., *Nature (London)*, **330**, 746—748 (1987).
- 31) Van Nueten J. M., Janssen P. A. J., Van Beek J., Xhonneux R., Verbeuren T. J., Vanhoutte P. M., *J. Pharmacol. Exp. Ther.*, **218**, 217—230 (1981).
- 32) Born G. V. R., *Nature (London)*, **194**, 927—929 (1962).
- 33) Killam A. L., Cohen M. L., *Thrombo. Res.*, **64**, 331—340 (1991).
- 34) Altomare A., Cascarano G., Giacobozzo C., Guagliardi A., Burla M. C., Polidori G., Camalli M., *J. Appl. Cryst.*, **27**, 435 (1994).
- 35) Beurskens P. T., Admiraal G., Beurskens G., Bosman W. P., Garcia-Granda S., Gould R. O., Smits J. M. M., Smykalla C., The DIRDIF program system, Technical Report of the Crystallography Laboratory, University of Nijmegen, The Netherlands (1992).
- 36) Present address: Research Institute, Sankyo Co., Ltd.
- 37) Present address: Biomedical Research Laboratories, Sankyo Co., Ltd.

Amino Acids and Peptides. XXXV. Facile Preparation of *p*-Nitroanilide Analogs by the Solid-Phase Method

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p-Nitroanilides of amino acids and peptides are widely used as the chromogenic substrates for the determination of the activity of proteolytic enzymes. However, the preparation of a *p*-nitroanilide is not easy, in part due to the low nucleophilicity of the amino group of *p*-nitroaniline. A facile preparation of *p*-nitroanilide analog by the solid-phase method was investigated. 5-Amino-2-nitrobenzoic acid (Anb^{5,2}) was used instead of *p*-nitroaniline (pNA) for preparation of *p*-nitroanilide analogs. Anb^{5,2} was introduced on a *p*-methylbenzhydrylamine resin without protection of the amino group of Anb^{5,2} by the 2-(*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) method in the presence of *p*-dimethylaminopyridine. The coupling reaction of a *N*^α-,*N*^ε-protected arginine with a Anb^{5,2}-resin was difficult to achieve by common coupling methods (such as the carbodiimide and diphenylphosphoryl azide methods), but the phosphoryl chloride method was relatively successful. Synthetic benzoyl-Arg-Anb^{5,2}-NH₂ and benzoyl-Arg-pNA were hydrolyzed by trypsin and the both reaction mixtures exhibited same spectroscopic characteristics. H-D-Val-Leu-Arg-Anb^{5,2}-NH₂, an analog of human urine kallikrein substrate, was readily prepared by the solid-phase method. H-Arg-Anb^{5,2}-OH and H-D-Val-Leu-Arg-Anb^{5,2}-OH were also synthesized on a Wang resin by the solid-phase method. The aqueous solubility of these free-carboxyl materials was better than those of the corresponding amide analogs. 4-Amino-3-nitrobenzoic acid (Anb^{4,3}) was also introduced on the *p*-methylbenzhydrylamine resin, but the resulting H-Anb^{4,3}-resin did not react with *N*^α-,*N*^ε-protected arginine by any of the coupling methods.

Key words *p*-nitroanilide; solid-phase peptide synthesis; 5-amino-2-nitrobenzoic acid; phosphoryl chloride method; enzyme substrate

Several *p*-nitroanilide¹⁾ derivatives are useful enzyme substrates, but their preparation is problematic. Since the amino group of *p*-nitroaniline (pNA) exhibits poor nucleophilicity by virtue of the electron-withdrawing effect of the nitro group, preparation of a *p*-nitroanilide by common coupling methods [such as the dicyclohexylcarbodiimide (DCC) method,²⁾ the azide method³⁾ and the active ester method⁴⁾] is difficult. A synthetic intermediate of a *p*-nitroanilide often exhibits poor solubility in organic solvents and is problematic with regard to purification of a *p*-nitroanilide during synthesis in solution. Extraction of a synthetic *p*-nitroanilide from a reaction mixture and purification of a *p*-nitroanilide by recrystallization with organic solvents are often difficult. Furthermore, the poor aqueous solubility of a *p*-nitroanilide is not always appropriate for biochemical applications and dimethylsulfoxide or dimethylformamide (DMF) is often used to dissolve the *p*-nitroanilide. A pNA derivative which has hydrophilic side chain (such as a carboxyl group) on the phenyl ring may be a promising approach toward improving aqueous solubility. We investigated a facile preparation of *p*-nitroanilide analogs by the solid-phase method using 5-amino-2-nitrobenzoic acid (Anb^{5,2} or H-Anb^{5,2}-OH) and 4-amino-3-nitrobenzoic acid (Anb^{4,3} or H-Anb^{4,3}-OH) as reactants, instead of *p*-nitroaniline for preparation of *p*-nitroanilide analogs.

Burdick *et al.*⁵⁾ reported solid-phase synthesis of a *p*-nitroanilide using *p*-diaminobenzene as a linker to the resin. They prepared a peptide with *p*-aminoanilide by the solid-phase method and oxidized it to the corresponding *p*-nitroanilide. Since this procedure requires an oxidation reaction as the final step, anilides containing methionine, cysteine and tryptophan could not be prepared without interference

from side reactions.

Results and Discussion

Initially, a Anb^{5,2} analog was prepared. Benzoyl-Arg-Anb^{5,2} amide (Bz-Arg-Anb^{5,2}-NH₂) was prepared with reference to the trypsin substrate, Bz-Arg-pNA.⁶⁾ Since the amino group of Anb^{5,2} exhibits poor nucleophilicity due to the effect of the nitro group, Anb^{5,2} was introduced on a *p*-methylbenzhydrylamine resin (MB resin)⁷⁾ without protection of the amino group. The reaction did not proceed by the diphenylphosphoryl azide (DPPA) method⁸⁾ and the diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) method.⁹⁾ The coupling reaction was not successful with the 2-(*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/HOBt method.¹⁰⁾ Although TBTU was reported to offer advantages over more traditional coupling reagents [such as DCC and benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP)¹¹⁾], the coupling reaction by the TBTU/HOBt method did not proceed, even at 40 °C. The coupling reaction was successful by the TBTU method in a presence of *p*-dimethylaminopyridine (DMAP). The resulting Anb^{5,2}-MB resin was treated with hydrogen fluoride (HF) and the crude product (H-Anb^{5,2}-NH₂) was analyzed by HPLC. One major peak

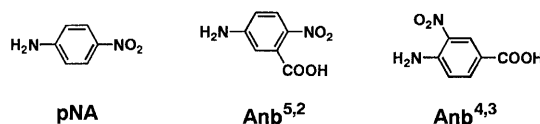


Fig. 1. Structures of *p*-Nitroaniline (pNA), 5-Amino-2-nitrobenzoic Acid (Anb^{5,2}) and 4-Amino-3-nitrobenzoic Acid (Anb^{4,3})

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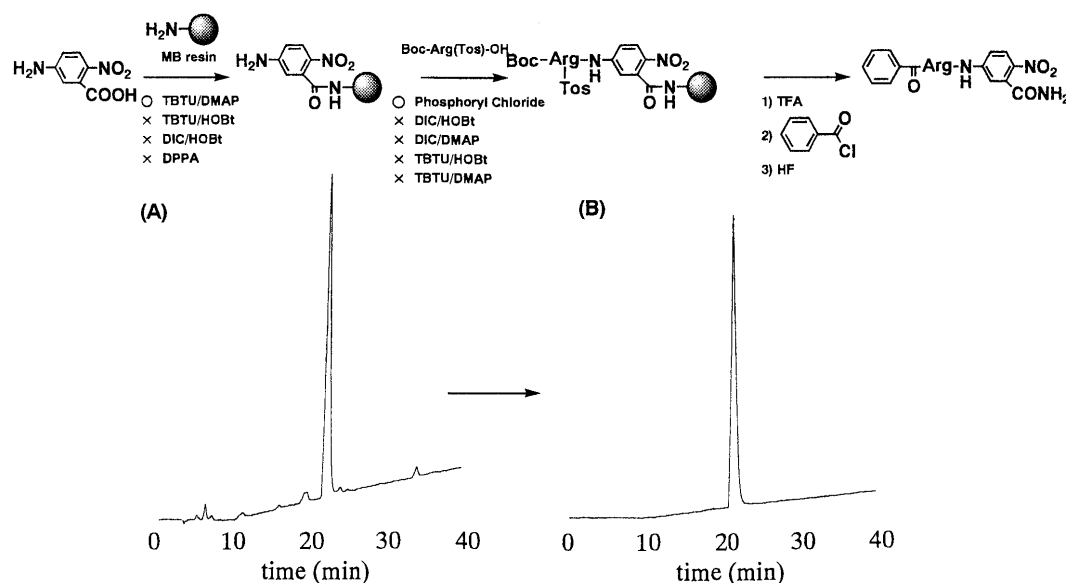


Fig. 2. Synthetic Scheme for Bz-Arg-Anb^{5,2}-NH₂, and HPLC Profiles of Crude (A) and Purified (B) Bz-Arg-Anb^{5,2}-NH₂. HPLC: DAISOPAK column (4.6×250 mm). Eluent, CH₃CN/H₂O (1 ml/min, 10/90—50/50, 40 min).

was observed and it contained a compound with a molecular weight (determined by mass spectroscopy) identical to that of the 5-amino-2-nitrobenzoic acid amide. The results of HPLC and analysis by mass spectroscopy proved that the coupling reaction did not occur on the amino group of Anb^{5,2}. *N*^α-*tert*-butyloxycarbonyl-*N*^α-tosyl-arginine (Boc-Arg(Tos)-OH)¹² coupling was attempted with the Anb^{5,2}-MB resin by the DIC/HOBt, DIC/DMPAP, TBTU/HOBt and TBTU/DMPAP methods. Since all the above reactions did not proceed, the phosphoryl chloride method¹³ (which was reported as an effective coupling method to prepare a *p*-nitroanilide) was examined. The reaction was successful and the resulting Boc-Arg(Tos)-Anb^{5,2}-MB resin was treated with trifluoroacetic acid (TFA) to remove the Boc group, followed by treatment with benzoyl chloride (Bz-Cl). The whole resin was treated with HF to remove Bz-Arg-Anb^{5,2}-NH₂. The HPLC profiles of the crude and the purified Anb^{5,2} analogs are shown in Fig. 2. Bz-Arg-Anb^{5,2}-NH₂ and Bz-Arg-pNA were treated with trypsin and the spectroscopic profile of the reaction mixtures is shown in Fig. 3. Both were hydrolyzed completely within 5 min at 37 °C and their absorption spectra are almost identical when compared before and after hydrolysis. The absorption maxima of both reaction mixtures are 313 and 380 nm before and after hydrolysis. These results proved that Anb^{5,2} analogs are suitable alternatives to *p*-nitroanilides as chromogenic substrates.

Preparation of Bz-Arg-Anb^{4,3}-NH₂ was also examined. Anb^{4,3} was introduced to the MB resin by various methods. Although Anb^{5,2} could not be introduced by the TBTU/HOBt method, Anb^{4,3} could be introduced by the same method. Furthermore, Anb^{4,3} could be introduced by the DIC/HOBt method, but the DPPA method was not successful. Thus the reactivity of carboxyl groups of Anb^{4,3} and Anb^{5,2} are different. The effect of nitro groups at *para* and *meta* positions to the carboxy group may be responsible for this difference in reactivity. The resulting Anb^{4,3}-MB resin was used for coupling with Boc-Arg(Tos)-OH by the DIC/HOBt, TBTU/HOBt, TBTU/DMPAP, mixed anhydride (pivaloyl chloride¹⁴)

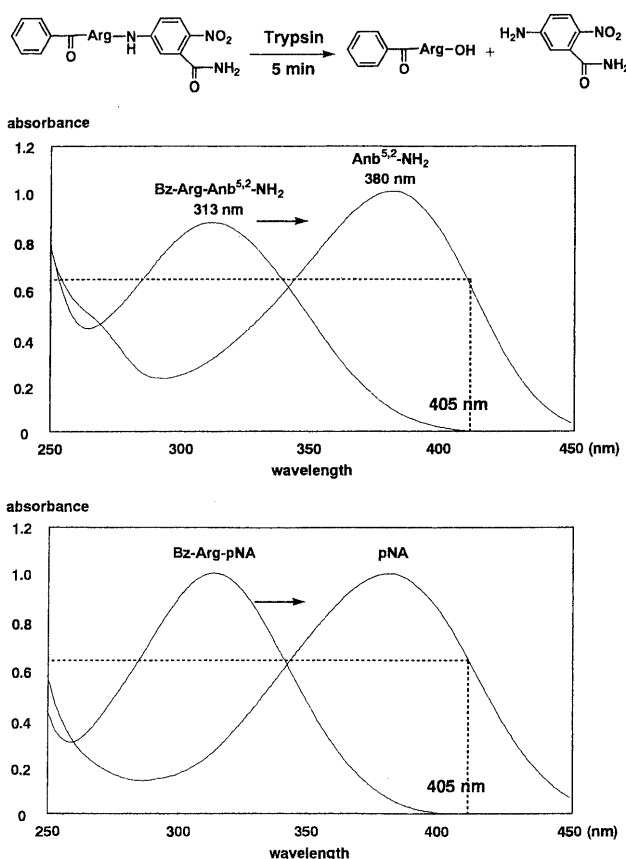
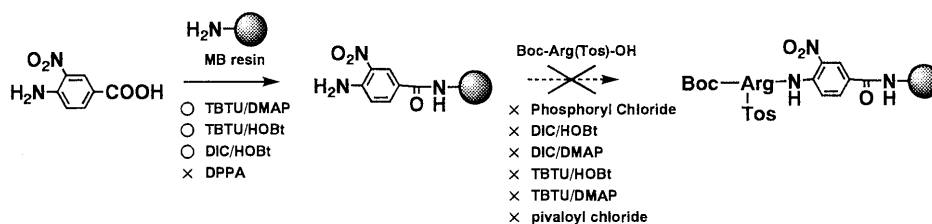
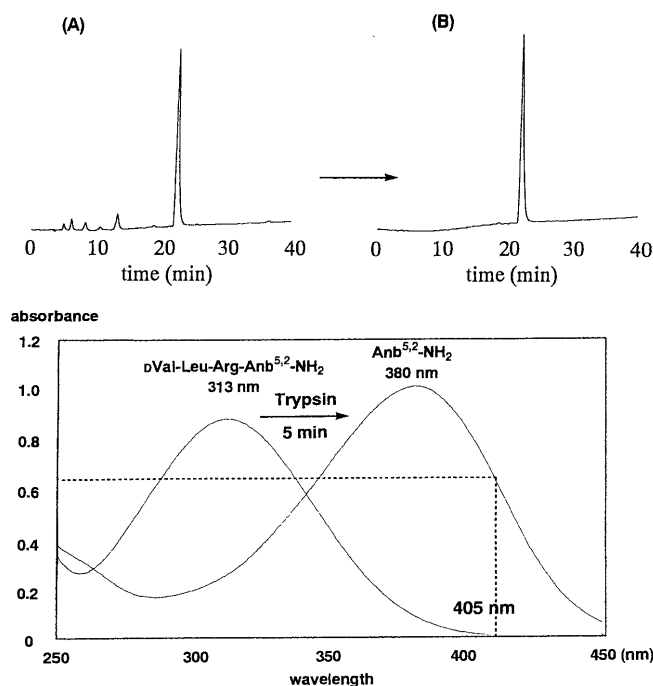


Fig. 3. Absorption Spectra from Trypsin-Mediated Hydrolysis of Bz-Arg-Anb^{5,2}.

and phosphoryl chloride methods. All the above reactions did not proceed. Although Noda *et al.*^{14b} reported the pivaloyl chloride method as a suitable method to prepare a *p*-nitroanilide, the reaction was unsuccessful. Thus, formation of an amide bond between the carboxyl group of Arg and an amino group of Anb^{4,3}-MB resin was not achieved.

Next, an Anb^{5,2} analog of H-D-Val-Leu-Arg-pNA,¹⁵

Fig. 4. Synthetic Scheme for Arg-Anb^{4.3}Fig. 5. HPLC Profile of Crude (A) and Purified (B) H-D-Val-Leu-Arg-Anb^{5.2}-NH₂, and Absorption Spectra from Trypsin-Mediated Hydrolysis of H-D-Val-Leu-Arg-Anb^{5.2}-NH₂

which is a substrate of human urine kallikrein was prepared. Anb^{5.2} was introduced on the MB resin by the TBTU/DMAP method and Boc-Arg(Tos)-OH was introduced on the Anb^{5.2}-MB resin by the phosphoryl chloride method in the same manner as described above. Successive introduction of Boc-Leu-OH and Boc-D-Val-OH on the H-Arg-Anb^{5.2}-MB resin was readily achieved by the DIC/HOBt method. The synthetic peptide-MB resin (H-D-Val-Leu-Arg(Tos)-Anb^{5.2}-MB resin) was treated with HF, followed by HPLC purification. The product, H-D-Val-Leu-Arg-Anb^{5.2}-NH₂, was hydrolyzed with trypsin. In solution, the product had the same absorption spectrum as that of *p*-nitroanilide (H-D-Val-Leu-Arg-pNA) before and after treatment with trypsin.

In general, *p*-nitroanilides exhibit poor solubility in aqueous media and an amide compound is less soluble than a free carboxyl compound. To examine solubility in aqueous media, a free carboxyl analog, H-D-Val-Leu-Arg-Anb^{5.2}-OH, was also prepared by the fluorenylmethyloxycarbonyl (Fmoc) strategy. Anb^{5.2} was introduced on a Wang resin (*p*-benzyloxybenzyl alcohol resin, WA resin) by the TBTU/DMAP method. The resulting Anb^{5.2}-WA resin was coupled with Fmoc-Arg(Pmc)-OH (Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonyl)¹⁶⁾ by the phosphoryl chloride method to give Fmoc-Arg(Pmc)-WA resin. Fmoc-Arg(Pmc)-OH could not be introduced on the Anb^{5.2}-WA resin by any of the other

coupling methods. Fmoc-Leu-OH and Fmoc-D-Val-OH were readily introduced on the resin by the DIC/HOBt method. The synthetic peptide-resin, Fmoc-D-Val-Leu-Arg-Anb^{5.2}-WA resin, was treated with 20% piperidine/dimethylformamide, followed by TFA treatment to give H-D-Val-Leu-Arg-Anb^{5.2}-OH. The product has a free carboxyl group and exhibited better water-solubility, when compared to the amide analog, H-D-Val-Leu-Arg-Anb^{5.2}-NH₂. Additionally, Bz-Arg-Anb^{5.2}-OH was prepared by the solid-phase method and its solubility was compared with that of Bz-Arg-Anb^{5.2}-NH₂. The solubility of the free carboxyl compound in water was much better than that of the amide compound. In general, solubility of pNA derivatives in water is poor and a mixture of DMF (or dimethylsulfoxide) and water is often used as a solvent for an enzyme reaction with a *p*-nitroanilide derivative as a substrate. We dissolved Bz-Arg-pNA and Bz-Arg-Anb^{5.2}-NH₂ in DMF and the solution was diluted with a buffer when we hydrolyzed them with trypsin. Since Bz-Arg-Anb^{5.2}-OH and H-D-Val-Leu-Anb^{5.2}-OH were soluble in water, DMF was not used when assessing trypsin-mediated hydrolysis. Since hydrolysis of free carboxyl Anb^{5.2} analogs by trypsin was complete within 5 min, the rate of hydrolysis of the free carboxyl analogs is similar to that of the amide analogs. A free carboxyl Anb^{5.2} analog has advantages over a pNA analog due to the ease of preparation by the solid-phase method and superior aqueous solubility.

Preparation of Fmoc-Arg(Pmc)-Anb^{4.3}-WA resin was also examined. Anb^{4.3}-WA resin was prepared by the TBTU/DMAP method. The resulting Anb^{4.3}-WA resin was used for coupling with Fmoc-Arg(Pmc)-OH by the DIC/HOBt, TBTU/HOBt, TBTU/DMAP, mixed anhydride (pivaloyl chloride) and phosphoryl chloride methods. All the above reactions did not proceed. Furthermore, the fluoride method¹⁷⁾ which was reported as a rapid coupling reaction was also examined. Fmoc-Arg(Pmc)-OH was reacted with cyanuric fluoride, but the fluoride, Fmoc-Arg(Pmc)-F, could not be obtained. The fluoride might have been unstable by formation of a lactam by intra-molecular cyclization. The molecular weight of the product, measured by high resolution mass spectroscopy, was 664.2674 (C₃₅H₄₀N₄O₆S), which corresponded to the molecular weight of the lactam.

As described above, Burdick *et al.*⁵⁾ reported solid-phase synthesis of a *p*-nitroanilide using *p*-diaminobenzene as a linker to a resin. Their procedure contains interesting chemical reactions, but requires phosgene as a reagent. Furthermore, their procedure has oxidation reaction at the final step in order to convert the amino group to a nitro group. Peptides containing methionine, cysteine and tryptophan could not be prepared without complications. Our synthetic procedure is a relatively effective solid-phase method and our synthetic *p*-

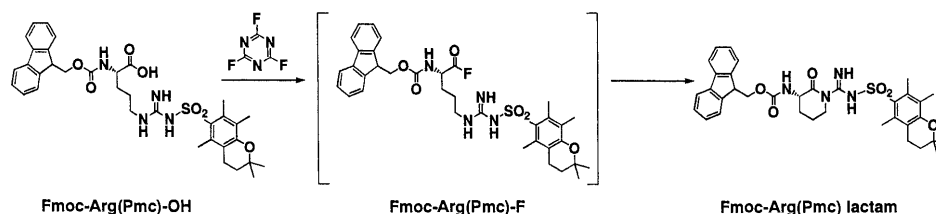


Fig. 6. Formation of Fmoc-Arg(Pmc) Lactam by the Reaction of Fmoc-Arg(Pmc)-OH and Cyanuric Fluoride

nitroanilide analogs, which have free carboxyl groups, have the advantage over *p*-nitroanilides due to enhanced solubility in aqueous media.

Experimental

Resins, amino acid derivatives and coupling reagents were purchased from Watanabe Chemical Industries, Ltd. Bz-Arg-pNA and D-Val-Leu-Arg-pNA were purchased from Sigma-Aldrich Japan. Amino acid compositions of acid hydrolysates were determined with a Waters Pico-TAG amino acid analyzer and RP-HPLC was performed using a Waters 600 with a DAISOPAK column and gradient systems of CH₃CN/water containing 0.05% TFA. TOF-mass spectra were obtained from a SHIMADZU/KRATOS KOMPACT MALDI IV spectrometer. High resolution mass spectra were obtained from a Hitachi M-4100 spectrometer. Rotations were measured with a Jasco DIP-360 polarimeter. Absorption spectra were measured with a Hitachi U-3000 spectrophotometer.

Anb^{5,2}-MB Resin MB resin hydrochloride (amino group content: 0.68 meq/g, 2 g, 1.32 mmol) was washed with 5% *N*-methylmorpholine (NMM)/DMF and DMF successively. A DMF solution (9 ml) containing Anb^{5,2} (729 mg, 4 mmol), TBTU (1.28 g, 4 mmol), *N,N*-diisopropylethylamine (DIEA, 1.39 ml, 8 mmol) and DMAP (326 mg, 2.67 mmol) was added to the resin and the mixture was stirred for 3 h. This procedure was repeated 3 times. The resin was washed with DMF and a mixture of dichloromethane (DCM) and MeOH successively. The resin was negative by the Kaiser test (ninhydrin test).¹⁸ Yield 2.21 g.

Introduction of Anb^{5,2} on the MB resin was also examined by the DPPA method at room temperature, by the DIC/HOBt method at room temperature, by the TBTU/HOBt method at room temperature and by the TBTU/HOBt method at 40 °C. Anb^{5,2}-MB resin could not be prepared by these methods.

Anb^{4,3}-MB Resin a) By the DIC/HOBt Method: A DMF solution (6 ml) of Anb^{4,3} (91 mg, 0.5 mmol), HOBt (68 mg, 0.5 mmol) and DIC (78 μ l, 0.5 mmol) was stirred at 0 °C for 10 min. Then the solution was added to the MB resin [prepared from 250 mg (0.17 meq.) of the hydrochloride by neutralization with NMM] and the whole mixture was stirred for 2 h. This procedure was repeated 3 times and the resin was washed with DMF and a mixture of DCM and MeOH successively. The resin was negative by the Kaiser test (ninhydrin test). Yield 271 mg.

b) By the TBTU/HOBt Method: A DMF solution (6 ml) of Anb^{4,3} (364 mg, 2 mmol), HOBt (270 mg, 2 mmol), DIEA (0.7 ml, 4 mmol) and TBTU (640 mg, 2 mmol) was added to swollen MB resin [prepared from 1 g of the hydrochloride (0.68 meq)] and the whole mixture was stirred at room temperature for 2 h. This procedure was repeated 3 times. The resin was washed with DMF and DCM/MeOH successively. The resin was negative by the Kaiser test (ninhydrin test). Yield 1.12 g.

c) By the TBTU/DMAP Method: Anb^{4,3} (729 mg, 4 mmol) was introduced on MB resin (2 g of the hydrochloride, 1.32 meq) in the same manner described for preparation of Anb^{5,2}-MB resin. Yield 2.25 g.

d) By the DPPA Method: Anb^{4,3} (91 mg, 0.5 mmol) was used to react with MB resin (0.17 meq) by the DPPA method at 6 °C. The reaction mixture was not Kaiser test (ninhydrin test) negative after multiple trials.

HF Treatment of Anb^{5,2}-MB Resin H-Anb^{5,2}-MB resin (100 mg) was treated with 5% anisole/HF (10 ml) at 0 °C for 3 h. HF was removed *in vacuo* and the residue was washed with ether 5 times. The residue was extracted with MeOH and the MeOH was removed *in vacuo* to give a yellow solid. Yield 16 mg. TOF-MS *m/z*: 182.4 (*M*⁺ + 1).

HF Treatment of Anb^{4,3}-MB Resin H-Anb^{4,3}-MB resin (100 mg) was treated with HF in the same manner described above. Yield 16 mg. TOF-MS *m/z*: 182.4 (*M*⁺ + 1).

Introduction of Boc-Arg(Tos)-OH on the Anb^{5,2}-MB Resin A pyridine solution (20 ml) of Boc-Arg(Tos)-OH (2.14 g, 5 mmol) was added to

swollen Anb^{5,2}-MB resin (1 g, 0.61 meq.) and the whole was cooled to -15 °C. POCl₃ (450 μ l, 5 mmol) was added and the mixture was stirred at the same temperature for 20 min. The mixture was stirred for 30 min at room temperature and then stirred at 40 °C for 6 h. The resin was washed with DMF and MeOH, and dried. Yield 1.2 g (96%). Arg-content was measured after acid hydrolysis: Arg-content, 0.43 mmol/g.

Introduction of Boc-Arg(Tos)-OH on the Anb^{4,3}-MB Resin This procedure was attempted using the DIC/HOBt, DIC/DMAP, TBTU/HOBt, TBTU/DMAP and mixed anhydride (the pivaloyl chloride) methods. All the reactions did not proceed. The reaction did not proceed with the phosphoryl chloride method at 60 °C. Arg was not detected in an acid hydrolysate of the product obtained from these reactions.

Bz-Arg-Anb^{5,2}-NH₂ Boc-Arg(Tos)-Anb^{5,2}-MB resin (400 mg, 0.43 meq) was treated with a mixture of TFA/DCM/anisole (50 : 45 : 5, 10 ml) for 1 h and the resin was washed with DCM, 0.5 *N* NMM/DMF and DMF, successively. The resulting H-Arg(Tos)-Anb^{5,2}-MB resin was reacted with benzoyl chloride (100 μ l, 0.86 mmol) and DIEA (224 μ l, 1.29 mmol) in DMF (10 ml) at room temperature for 1 h. The resin was washed with DMF and DCM/MeOH (1/1) and dried. Yield 412 mg. The resin was treated with 5% anisole/HF (10 ml) at 0 °C for 1 h. HF was removed *in vacuo* and the residue was dried in a desiccator. After washing with ether, the residue was extracted with 5% AcOH and the extract was lyophilized to give 58 mg of a fluffy powder. The product was purified by HPLC. Yield 31 mg. [α]_D²⁴ +44.7° (*c*=1.0, MeOH), TOF-MS *m/z*: 443.2 (*M*⁺ + 1). UV $\lambda_{\max}$ (1% DMF/0.05 *M* Tris-HCl-0.1 *M* NaCl, pH 8.1) nm (log ϵ): 313 (4.02). Recovery of Arg in an acid hydrolysate, 88%.

H-D-Val-Leu-Arg-Anb^{5,2}-NH₂ The peptide was prepared on the H-Arg-Anb^{5,2}-MB resin by the Boc strategy according to the procedure reported previously.¹⁹ Briefly, Boc groups were removed by 50% TFA/DCM and the TFA salt was neutralized with 1 *M* NMM/DMF. Boc-Leu-OH and Boc-D-Val-OH were introduced on the H-Arg(Tos)-Anb^{5,2}-MB resin by the DIC/HOBt method in stepwise manner. The synthetic H-D-Val-Leu-Arg-Anb^{5,2}-MB resin (1 g) was treated with HF and the final product was purified by HPLC. Yield 101 mg, [α]_D²² -53.7° (*c*=1.0, MeOH). UV $\lambda_{\max}$ (1% DMF/0.05 *M* Tris-HCl-0.1 *M* NaCl, pH 8.1) nm (log ϵ): 313 (4.02). Amino acid ratios in an acid hydrolysate: Val 0.90, Leu 1.00, Arg 1.00 (average recovery 99%).

Introduction of Anb^{5,2} on WA Resin A DMF solution (10 ml) of DIEA (1.04 ml, 6 mmol), H-Anb^{5,2}-OH (546 mg, 3 mmol), TBTU (963 mg, 3 mmol) and DMAP (191 mg, 1.56 mmol) was added to the WA resin (2 g, 1.56 meq) and the mixture was stirred at room temperature for 12 h. The resin was washed with DMF and the same coupling reaction was repeated once more. The resin was washed with DMF and a mixture of MeOH/DCM successively, and dried. Yield 2.27 g.

Introduction of Anb^{4,3} on WA Resin H-Anb^{4,3}-OH (546 mg, 3 mmol) was introduced on WA resin (2 g, 1.56 meq) by the TBTU/DMAP method in the same way described above. Yield 2.30 g.

Introduction of Fmoc-Arg(Pmc)-OH on the Anb^{5,2}-WA Resin Introduction of Fmoc-Arg(Pmc)-OH (2 g, 3 mmol) on the Anb^{5,2}-WA resin (1 g, 0.69 meq) was performed by the phosphoryl chloride method in the same way described in the introduction section for Boc-Arg(Tos)-OH on the Anb^{5,2}-MB resin. Yield 1.28 g (89%). Arg content, 0.34 mmol/g. Additionally, the DIC/HOBt, DIC/DMAP, TBTU/HOBt, TBTU/DMAP, and mixed anhydride (pivaloyl chloride) methods were tested. After the reactions, resins were hydrolyzed and Arg content in the hydrolysates were analyzed. Arg was not detected in these hydrolysates, *i.e.*, the introduction of Fmoc-Arg(Pmc)-OH on the Anb^{5,2}-WA resin was unsuccessful by these methods.

Introduction of Fmoc-Arg(Pmc)-OH on the Anb^{4,3}-WA resin This procedure was attempted in the same way described in the introduction section for Boc-Arg(Tos)-OH on the Anb^{4,3}-MB resin. The introduction of Fmoc-Arg(Pmc)-OH on the Anb^{4,3}-WA resin was unsuccessful even by the phosphoryl chloride method at 60 °C.

Fmoc-Arg(Pmc) Lactam Since Fmoc-Arg(Pmc)-OH could not be introduced on the Anb^{4,3}-WA resin, the fluoride method was examined. Preparation of Fmoc-Arg(Pmc)-F was examined according to the method reported by Carpino *et al.*¹⁷⁾ The product was purified by silica gel column chromatography using ethyl acetate (AcOEt) as an eluent. High resolution mass spectroscopy of the product indicated a M.W. of 664.2674 (C₃₀H₄₀N₄O₆S), which corresponded to the M.W. of the lactam. mp 124 °C, $[\alpha]_D^{24} -14.3^\circ$ ($c=1.0$, MeOH). Anal. Calcd for C₃₀H₄₀N₄O₆S·AcOEt: C, 65.34; H, 6.75; N, 7.82. Found: C, 65.31; H, 6.72; N, 7.77.

Bz-Arg-Anb^{5,2}-OH Fmoc-Arg(Pmc)-Anb^{5,2}-WA resin (500 mg, 0.17 meq) was treated with 20% piperidine/DMF for 30 min to remove the Fmoc group and washed with DMF. Benzoyl chloride (79 μ l, 0.68 mmol), DIEA (142 μ l, 0.82 mmol) was added to the resin in DMF (10 ml) and allowed to react at room temperature for 1 h. The resin was washed with DMF and 50% DCM/MeOH, and dried. Yield 443 mg. The resin was treated with a mixture of TFA/*m*-cresol/thioanisole (96:2:2, 20 ml) at room temperature for 3 h. TFA was removed *in vacuo* and the residue was washed with ether and dried. The residue was extracted with 5% AcOH and the aqueous layer was washed with ether, followed by lyophilization. The product was purified by HPLC. Yield 23 mg. $[\alpha]_D^{24} +87.7^\circ$ ($c=1.0$, DMF). TOF-MS m/z : 444.2 (M⁺+1). UV λ_{max} (0.05 M Tris-HCl-0.1 M NaCl, pH 8.1) nm (log ϵ): 313 (4.01). Recovery of Arg in an acid hydrolysate 87%.

H-D-Val-Leu-Arg-Anb^{5,2}-OH This peptide was prepared by Fmoc strategy. The Fmoc group was removed with 20% piperidine/DMF and coupling reactions were performed by the DIC/HOBt method in DMF. Fmoc-Leu-OH and Fmoc-D-Val-OH were introduced on H-Arg-Anb^{5,2}-WA resin in stepwise manner. Synthetic H-D-Val-Leu-Arg-Anb^{5,2}-WA resin (500 mg) was treated with a mixture of TFA/*m*-cresol/thioanisole (96:2:2, 30 ml) at room temperature for 3 h. TFA was removed *in vacuo* and ether was added to the residue. The residue was washed with ether 5 times and extracted with 5% AcOH. The extract was washed with ether and lyophilized. Yield 81 mg. The product was purified by HPLC. Yield 62 mg. Fluffy powder. $[\alpha]_D^{24} -44.7^\circ$ ($c=1.0$, MeOH), TOF-MS m/z : 551.3 (M⁺+1). UV λ_{max} (0.05 M Tris-HCl-0.1 M NaCl, pH 8.1) nm (log ϵ): 313 (4.01). Amino acid ratios in an acid hydrolysate: Val 0.99, Leu 1.00, Arg 0.93 (average recovery 96%).

Trypsin Hydrolysis of the Synthetic Substrates 10 mM Bz-Arg-Anb^{5,2}-NH₂ solution in a mixture of 30% DMF/0.05 M Tris-HCl-0.1 M NaCl was prepared. The 10 mM Bz-Arg-Anb^{5,2}-NH₂ solution (30 μ l) and 2 nM Trypsin solution/0.9% NaCl (30 μ l) and 0.05 M Tris-HCl-0.1 M NaCl (3 ml) were combined and the mixture was incubated at 36 °C. Spectra obtained from the reaction mixture were obtained at various time intervals. The absorption maximum of Bz-Arg-Anb^{5,2}-NH₂ at 313 nm shifted to 380 nm within 5 min.

Other synthetic substrates (Bz-Arg-Anb^{5,2}-OH, H-D-Val-Leu-Anb^{5,2}-NH₂ and H-D-Val-Leu-Anb^{5,2}-OH) were hydrolyzed with trypsin in the

same manner. In the case of Bz-Arg-Anb^{5,2}-OH and H-D-Val-Leu-Anb^{5,2}-OH, DMF was not used since the both were soluble in water. The absorption spectra of these enzymatic reaction mixtures exhibited the same absorption maximum at 313 nm and 380 nm, before and after hydrolysis, respectively.

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References

- 1) Lottenberg R., Christensen U., Jacson C. M., Coleman P. L., "Methods in Enzymology," Vol. 80, ed. by Lorand L., Academic Press, New York, 1981, pp. 341—361.
- 2) Sheehan J. C., Hess G. P., *J. Am. Chem. Soc.*, **77**, 1067—1068 (1955).
- 3) Curtius T., *Chem. Ber.*, **35**, 3226—3228 (1904).
- 4) Bodanszky M., du Vigneaud V., *J. Am. Chem. Soc.*, **81**, 5688—5691 (1959).
- 5) Burdick D. J., Struble M. E., Burnie P., *Tetrahedron Lett.*, **34**, 2589—2592 (1993).
- 6) Gravett P. S., Viljoen C. C., Oosthuizen M. M., *Int. J. Biochem.*, **23**, 1085—1099 (1991).
- 7) Matsueda G. R., Stewart J. M., *Peptide*, **2**, 45—50 (1981).
- 8) Shioiri T., Ninomiya K., Yamada S., *J. Am. Chem. Soc.*, **94**, 6203—6205 (1972).
- 9) a) Tartar A., Gesquiere J.-C., *J. Org. Chem.*, **44**, 5000—5002 (1979); b) Konig W., Geiger R., *Chem. Ber.*, **103**, 788—798 (1970).
- 10) Knorr R., Trzeciak A., Bannwarth W., Gillesen D., *Tetrahedron Lett.*, **30**, 1927—1930 (1989).
- 11) Castro B., Dormoy J. R., Evin G., Selve C., *Tetrahedron Lett.*, **1975**, 1219—1222.
- 12) Yamashiro D., Black J., Li C. H., *J. Am. Chem. Soc.*, **94**, 2855—2859 (1972).
- 13) Rijkers D. T. S., Adams H. P. H. M., Hemker H. C., Tesser G. I., *Tetrahedron*, **51**, 11235—11250 (1995).
- 14) a) Zoral M., *Collect. Czech. Chem. Commun.*, **27**, 1273—1277 (1962); b) Noda K., Oda M., Sato M., Yoshida N., *Int. J. Peptide Protein Res.*, **36**, 197—200 (1990).
- 15) Amundsen E., Putter J., Friberger P., Knos M., Larsbraten M., Claeson G., *Adv. Exp. Med. Biol.*, **120**, 83—95 (1979).
- 16) Ramage R., Green J., *Tetrahedron Lett.*, **28**, 2287—2290 (1987).
- 17) Carpino L. A., Sadat-Aalae D., Chao H. G., DeSelms R. H., *J. Am. Chem. Soc.*, **112**, 9651—9652 (1990).
- 18) Kaiser E., Colescott R. L., Bossinger C. D., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).
- 19) Maeda M., Izuno Y., Kawasaki K., Kaneda Y., Mu Y., Tsutsumi Y., Nakagawa S., Mayumi T., *Chem. Pharm. Bull.*, **46**, 347—350 (1998).

An Alternative Base-Pairing of Catechol-Bearing Nucleosides by Borate Formation

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A chelator-type β -C-nucleoside having a catechol ligand as the nucleobase was found to form a stable 2 : 1 complex with trimethyl borate, which was characterized by ^1H -NMR and electrospray ionization time-of-flight (ESI-TOF) mass spectroscopies. Both phosphoramidite and phosphotriester derivatives of this nucleoside were also prepared as synthetic precursors for DNA oligomer syntheses.

Key words DNA; base-pairing; borane complex; C-nucleoside

In recent years, a large number of nonnatural analogues of DNA nucleosides have been synthesized as probes or regulators of DNA as well as drugs.¹⁾ Especially, most studies in this field have been focused on modification of hydrogen bonding patterns, because hydrogen bonding plays a central role in the high selective recognition in DNA base pair identification.

Recently,²⁾ we have reported an alternative base pairing of artificial nucleosides in which hydrogen-bonded base pairing is replaced by metal-induced base pairing, thereby creating a novel hybridization motif in DNA duplexes (Chart 1). This approach has been expected to provide a wide range of applications to functionalized molecules based on its use as an alternative base pair along with the naturally occurring base pairs, AT and GC. Natural nucleobases form pairs with slightly propeller twisted planar motifs. In contrast, our strategy based on coordination geometry would provide not only a planar geometry but also structurally unique characters that could be introduced into a DNA duplex or triplex motif. Our recent report on a palladium(II)-assisted base pair of *o*-phenylenediamine-bearing β -C-nucleoside **1** showed its planar coordination geometry.²⁾ Herein we present an alternative base pairing of catechol-bearing β -C-nucleoside **3** mediated by borate formation where two catechol moieties may possibly be at right angles to each other on the tetrahedral boron(III) center.^{3,4)} Incorporation of this base pair into DNA strands could induce structural change of a DNA double strand (bending of a duplex or hydrogen bond scission of neighboring base pairs) and subsequent biological events. Such a geometrical control of DNA base pairing would be applicable to molecular biology, medicinal chemistry, and supramolecular chemistry.

Catechol-bearing β -C-nucleoside **3**, which was synthesized from a 2'-deoxyribose derivative **4** and *O*-protected catechol **5** via Friedel–Crafts coupling reaction and subsequent deprotection,^{2b)} was treated with half amount of trimethyl borate and triethylamine in DMSO-*d*₆ at room temperature to obtain a 2 : 1 complex **7** between nucleoside **3** and a boron ion (Chart 2). The isotopically resolved ESI-TOF mass spectrum of this solution in the negative mode provided clear evidence for the 2 : 1 complexation between **3** and a boron ion. The enlarged plot of the signal at *m/z* 459.15 [**7**–Et₃NH⁺][–] gave excellent agreement with the theoretical isotopic distribution, indicating the boron-assisted base pairing with **3** (Fig.

1). 500 MHz ^1H -NMR resonances of the aromatic protons of the complex **7** in DMSO-*d*₆ were upfield-shifted from those of the nucleoside **3**. The signals for hydroxy protons of the catechol moiety disappeared upon complexation whereas those for the ribose moiety remained unchanged (Fig. 2). These results indicate that the phenoxy groups coordinated to the boron(III) center in the deprotonated form. Although there are two possible diastereomeric structures for the complex **7** arising from chiral centers on both the D-ribose skeleton and the boron ion, we observed only one set of proton signals for the complex **7** in the spectrum. These results establish that the borate complex **7** is stable in solution.

Chart 3 shows the synthetic routes for compounds **11** and **12** to incorporate a catechol-type β -C-nucleoside as a “chelator-nucleoside” building block into a DNA strand by means of a phosphotriester or a phosphoramidite approach. The hydroxy group at the 5'-position of **6** was tritylated (4,4'-dimethoxytrityl) and then the TBDMS protecting groups at the phenoxy groups of the catechol moiety were replaced by acetyl groups to obtain **10** as a common intermediate for both approaches. These groups were expected to be readily removed by treatment with aqueous ammonium hydroxide solution after DNA oligomer synthesis.⁵⁾ Compound **11** was synthesized from **10** in 90% yield by the phosphotriester approach. Compound **10** was also converted into cyanoethyl

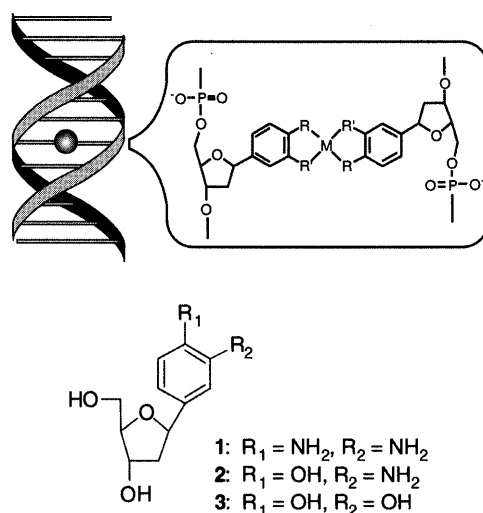


Chart 1

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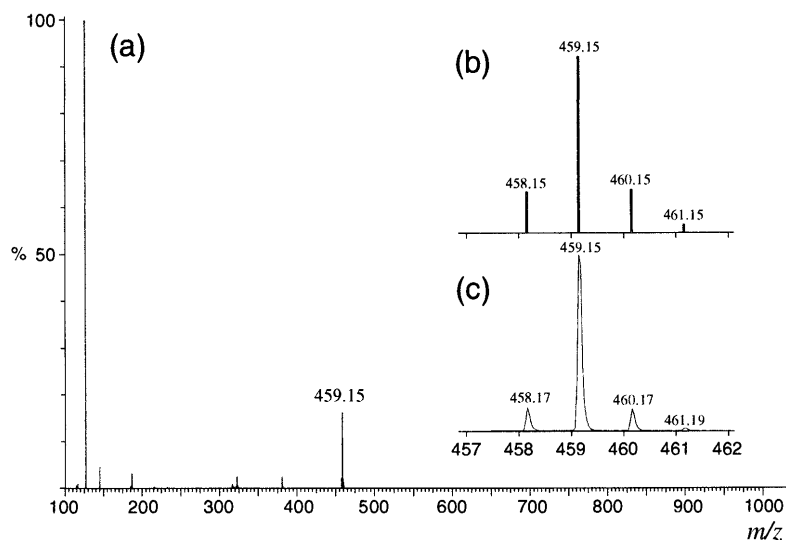


Fig. 1. ESI-TOF Mass Spectrum of Complex **7** in the Negative Mode

(a) m/z 100–1000, (b) the theoretical isotopic distribution, and (c) the experimental isotopic distribution at m/z 459.15 ($[7-\text{Et}_3\text{NH}]^+$ Calcd 459.15).

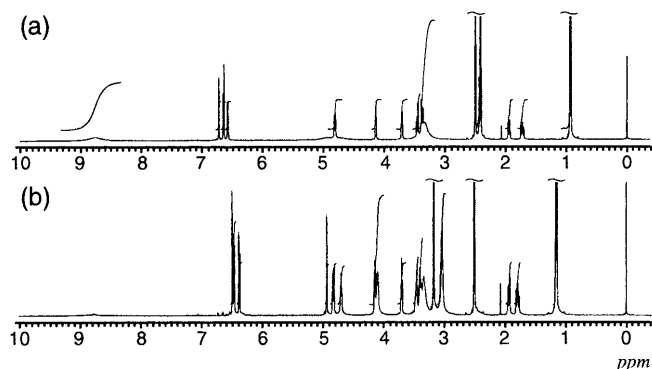


Fig. 2. 500 MHz ^1H -NMR Spectra of (a) C-Nucleoside **3** and (b) **3**-Borate (2 : 1) Complex, **7**, in $\text{DMSO}-d_6$

$[3]=44$ mM. $[\text{B}(\text{OMe})_3]=(a) 0$ and (b) 22 mM. $[\text{Et}_3\text{N}]=22$ mM.

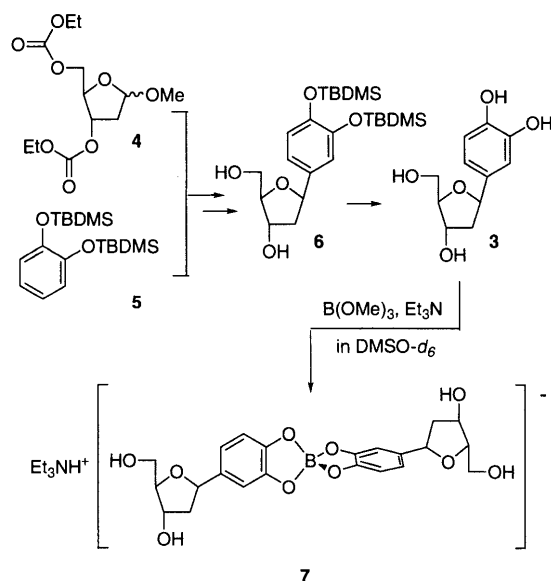


Chart 2. A Schematic Representation of a Synthetic Route for Catechol-nucleoside **3** and Its Borane Complex

phosphoramidite **12** in 47% yield, which can be used in a standard automated DNA synthesizer.

The present work demonstrates a novel base pairing mode assisted by borate formation, providing an alternative DNA base pair. Site-specific incorporation of this novel base pair into oligo-DNA is now underway.

Experimental

General Information All reactions were carried out in oven dried glassware under argon atmosphere with commercial dehydrated solvents (Wako). 4-(1,2-Dideoxy- β -D-ribofuranos-1-yl)catechol was prepared according to previously published procedures.^{4b)} 4,4'-Dimethoxytrityl chloride (DMTr-Cl), 4-chlorophenyl dichlorophosphate, *N*-methylimidazol, and 3-hydroxypropionitrile were purchased from TCI. 1 M *n*-Bu₄NF in THF and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite were purchased from Aldrich. All other reagents were purchased from Wako and were used without further purification. Column chromatography was performed using Wakogel C-300 gel (Wako).

^1H -NMR spectra referenced to TMS were recorded on a JEOL lambda 500 (500 MHz) or a Bruker DRX500 (500 MHz) spectrometer. ^{31}P -NMR spectrum was recorded on a JEOL lambda 500 (202.35 MHz) spectrometer. The spectrum was referenced to external 10 mM phosphoric acid in D_2O . Chemical shifts (δ) are reported in ppm; multiplicities are indicated by: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets of doublets), m (multiplet), br (broad). Coupling constants, J , are reported in Hz. Electrospray ionization time-of-flight (ESI-TOF) mass spectra were recorded on a Micromass LCT spectrometer.

Synthesis of Bis-*O,O'*-*tert*-butyldimethylsilyl-4-[1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)- β -D-ribofuranos-1-yl]catechol (8**)** To a solution of bis-*O,O'*-*tert*-butyldimethylsilyl-4-(1,2-dideoxy- β -D-ribofuranos-1-yl)catechol, **6**, (0.43 g, 0.95 mmol) in dry pyridine (4 ml) was added DMTr-Cl (0.39 g, 1.15 mmol) in 6 portions at 0 °C and stirred for 2 h at room temperature. Additional DMTr-Cl (80 mg, 0.24 mmol) was added and further stirred for 1 h. After the reaction was quenched with methanol (2 ml), the mixture was poured into ice-water (100 ml), and extracted with AcOEt three times. The combined organic layer was dried over anhydrous MgSO_4 and concentrated. The mixture was purified by silica gel column chromatography with *n*-hexane-ether (2 : 1) to obtain 0.53 g (74%) of **8** as a colorless solid. ^1H -NMR (CDCl_3): δ 7.19–7.46 (10.3H, m, including residual CHCl_3), 6.75–6.83 (7H, m), 5.02 (1H, dd, $J=5.5, 10.1$ Hz), 4.41 (1H, m), 4.01 (1H, ddd, $J=2.5, 4.2, 6.5$ Hz), 3.78 (6H, s), 3.37 (1H, dd, $J=4.6, 9.8$ Hz), 3.20 (1H, dd, $J=6.4, 9.4$ Hz), 2.17 (1H, ddd, $J=2.0, 5.6, 13.1$ Hz), 1.97 (1H, ddd, $J=6.1, 10.0, 13.1$ Hz), 1.84 (1H, d, $J=3.4$ Hz, D_2O exchangeable), 0.97 (9H, s), 0.94 (9H, s), 0.17 (3H, s), 0.13 (3H, s), 0.13 (3H, s).

Synthesis of 4-[1,2-Dideoxy-5-*O*-(4,4'-dimethoxytrityl)- β -D-ribofuranos-1-yl]catechol (9**)** To a solution of **8** (0.59 g, 0.78 mmol) in 14 ml dry

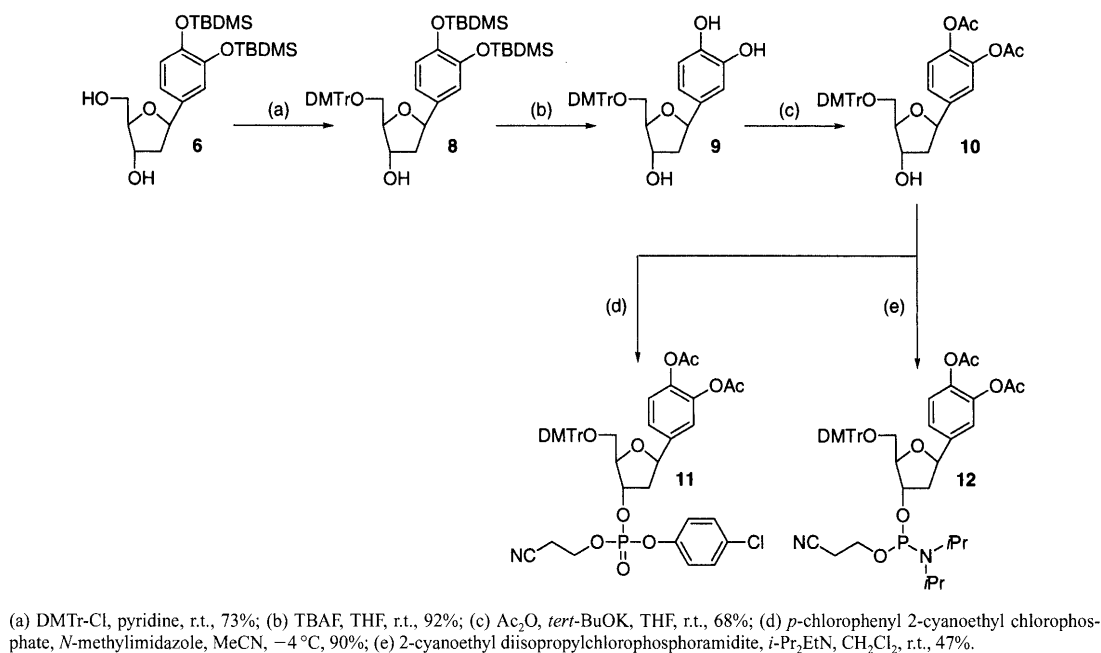


Chart 3

THF was added dropwise 1 M *n*-Bu₄NF in THF (1.59 ml, 1.59 mmol). After 10 min, the reaction was quenched by an aqueous solution of 10% NaHCO₃ (1.3 ml). The mixture was concentrated and purified by silica gel chromatography with CHCl₃-CH₃OH (20 : 1) to obtain 0.38 g of **9** (92%) as a colorless solid. ¹H-NMR (CDCl₃): δ 7.45–7.47 (2H, m), 7.33–7.37 (4H, m), 7.20–7.30 (7H, m, including residual CHCl₃), 5.05 (1H, dd, *J*=5.6, 10.0 Hz), 4.42 (1H, m), 4.03 (1H, ddd, *J*=2.7, 4.8, 4.8 Hz), 3.79 (6H, s), 3.35 (1H, dd, *J*=4.4, 9.6 Hz), 3.28 (1H, dd, *J*=5.1, 9.8 Hz), 2.18 (1H, ddd, *J*=2.0, 5.5, 13.2 Hz), 2.03 (1H, ddd, *J*=6.1, 10.0, 13.1 Hz).

Synthesis of Di-*O,O'*-acetyl-4-[1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-β-*D*-ribofuranos-1-yl]catechol (10) To a solution of **9** (0.23 g, 0.44 mmol) in 7 ml THF was added potassium *tert*-butoxide (50 mg, 0.45 mmol) and a THF solution (0.4 ml) of acetic anhydride (42 μl, 0.45 mmol). Thirty minutes later, additional potassium *tert*-butoxide (50 mg, 0.45 mmol) and a THF solution (0.4 ml) of acetic anhydride (42 μl, 0.45 mmol) were added and stirred for 30 min. The reaction mixture was evaporated and the resulting residue was chromatographed on silica gel with CHCl₃-CH₃OH (40 : 1) to obtain 0.18 g (67%) of **10** as an oil. ¹H-NMR (CDCl₃): δ 7.44–7.46 (2H, m), 7.31–7.36 (4H, m), 7.19–7.29 (6.4H, m, including residual CHCl₃), 7.12–6.13 (1H, m), 6.81–6.84 (4H, m), 5.15 (1H, dd, *J*=5.6, 10.0 Hz), 4.40 (1H, m), 4.04 (1H, ddd, *J*=2.7, 4.8, 4.8 Hz), 3.79 (6H, s), 3.33 (1H, dd, *J*=4.5, 9.9 Hz), 3.26 (1H, dd, *J*=5.2, 9.9 Hz), 2.22–2.27 (7H, m), 2.03 (1H, ddd, *J*=6.1, 10.0, 13.1 Hz), 1.83 (1H, d, *J*=3.2 Hz, D₂O exchangeable).

Synthesis of Di-*O,O'*-acetyl-4-[1,2-dideoxy-3-*O*-(4-chlorophenoxy)(2-cyanoethoxy)phosphinyl]-5-*O*-(4,4'-dimethoxytrityl)-β-*D*-ribofuranos-1-yl]catechol (11) 4-Chlorophenyl dichlorophosphate (0.31 ml, 1.9 mmol) was dissolved in 0.6 ml dry CH₃CN and *N*-methylimidazole (0.19 ml, 2.4 mmol) was added dropwise into the solution at -37 °C. Then to the mixture was added dropwise 3-hydroxypropionitrile (0.16 ml, 2.3 mmol) in 1 ml dry CH₃CN at -25 °C, and the reaction mixture was stirred for 1.5 h at -9 °C (solution A).

To the solution of di-*O,O'*-acetyl-4-[1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-β-*D*-ribofuranos-1-yl]catechol (0.56 g, 0.92 mmol) in dry CH₃CN (2.7 ml) was added *N*-methylimidazole (0.27 ml, 3.4 mmol) and the mixture was stirred for 30 min at -25 °C. To the reaction mixture was added dropwise solution A and stirred for 1 h at -4 °C. Then the mixture was poured into ice water (100 ml), extracted with AcOEt (100 ml×3), and dried over anhydrous MgSO₄. The residue was purified by silica gel column chromatography with CHCl₃ to obtain 0.71 g (91%) of **11** as a colorless foam consisting of a 1 : 1 mixture of diastereoisomers. ¹H-NMR (CDCl₃): δ 7.41–7.43 (2H, m), 7.20–7.33 (17H, m, including residual CHCl₃), 7.08–7.16 (3H, m), 6.80–6.83 (4H, m), 5.12–5.15 (1.5H, m), 5.06 (0.5H, dd, *J*=4.8, 10.9 Hz), 4.24–4.31 (3H, m), 3.78 (3H, s), 3.78 (3H, s), 3.24–3.35 (2H, m), 2.69 (1H, t, *J*=6.2 Hz), 2.64 (1H, t, *J*=6.2 Hz), 2.54 (0.5H, ddd, *J*=not detected, 5.0, 13.8 Hz), 2.45 (0.5H, ddd, *J*=not detected, 5.6, 13.9 Hz), 2.28 (3H, m),

2.22 (3H, m), 2.12–2.17 (1H, m).

Synthesis of Di-*O,O'*-acetyl-4-[1,2-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-β-*D*-ribofuranos-1-yl]catechol-3'-*O*-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (12) To a solution of **10** (0.11 g, 0.18 mmol) and *N,N*-diisopropylethylamine (122 μl, 0.70 mmol) in 2 ml dry pyridine was added dropwise 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (78 μl, 0.35 mmol). After 10 min, the reaction was quenched with CH₃OH (0.6 ml), and the mixture was dissolved in AcOEt (100 ml), washed with saturated aqueous NaHCO₃ (100 ml×3), brine (50 ml×3), dried over anhydrous MgSO₄, and concentrated. The mixture was purified by silica gel column chromatography with *n*-hexane-ethyl acetate (2 : 1) (including 5% pyridine) to afford 0.13 g (89%) of **12** as a colorless oil consisting of a 1 : 1 mixture of diastereoisomers. ¹H-NMR (CDCl₃): δ 7.45–7.48 (2H, m), 7.33–7.37 (4H, m), 7.25–7.29 (4.6H, m, including residual CHCl₃), 7.18–7.22 (1H, m), 7.12–7.15 (1H, m), 6.80–6.84 (4H, m), 5.12–5.16 (1H, m), 4.48–4.51 (1H, m), 4.21–4.22 (1H, m), 3.79 (3H, s), 3.78 (3H, s), 3.54–3.77 (2H, m), 3.21–3.33 (2H, m), 2.61 (1H, t, *J*=6.5 Hz), 2.45 (1H, t, *J*=6.5 Hz), 2.40–2.44 (0.5H, m), 2.31–2.34 (0.5H, m), 2.22 (0.5H, s), 2.21 (0.5H, s), 2.00–2.06 (1H, m), 1.18 (3H, d, *J*=6.6 Hz), 1.17 (3H, d, *J*=6.6 Hz), 1.15 (3H, d, *J*=6.9 Hz), 1.07 (3H, d, *J*=6.9 Hz). ³¹P-NMR (CDCl₃): δ 148.2, 148.0.

¹H-NMR Measurement of 4-(1,2-Dideoxy-β-*D*-ribofuranos-1-yl)catechol (3) ¹H-NMR (4-(1,2-dideoxy-β-*D*-ribofuranos-1-yl)catechol (22 μmol) and triethyl amine (11 μmol) in 0.5 ml DMSO-*d*₆): δ 8.76 (2H, br), 6.72 (1H, d, *J*=1.8 Hz), 6.64 (1H, d, *J*=8.0 Hz), 6.57 (1H, dd, *J*=1.8, 8.1 Hz), 4.80 (1H, dd, *J*=5.3, 10.4 Hz), 4.13 (1H, m), 3.71 (1H, ddd, *J*=1.8, 5.5, 5.5 Hz), 3.45 (1H, dd, *J*=5.1, 11.2 Hz), 3.37 (1H, dd, *J*=6.1, 11.2 Hz), 2.42 (5H, q, *J*=7.1 Hz), 1.95 (1H, ddd, *J*=1.2, 5.3, 12.7 Hz), 1.72 (1H, ddd, *J*=5.7, 10.6, 12.7 Hz), 0.93 (7H, t, *J*=7.1 Hz).

¹H-NMR Measurement of 4-(1,2-Dideoxy-β-*D*-ribofuranos-1-yl)catechol Boron (2 : 1) Complex (7) ¹H-NMR (4-(1,2-dideoxy-β-*D*-ribofuranos-1-yl)catechol (22 μmol), trimethyl borate (11 μmol) and triethyl amine (11 μmol) in 0.5 ml DMSO-*d*₆): δ 6.48 (1H, br), 6.44–6.46 (1H, m), 6.35–6.67 (1H, m), 4.92 (1H, d, *J*=3.8 Hz, 3'-OH), 4.82 (1H, dd, *J*=5.2, 10.4 Hz), 4.69 (1H, br, 5'-OH), 4.13 (1H, br), 4.09 (1H, br), 3.69 (1H, ddd, *J*=1.9, 5.5, 5.5 Hz), 3.43–3.48 (1H, m), 3.37–3.41 (1H, m), 3.17 (4.6H, s), 3.04 (3.4H, br), 3H, 1.93 (1H, ddd, *J*=1.3, 5.3, 12.8 Hz), 1.78 (1H, ddd, *J*=5.7, 10.5, 12.7 Hz), 1.15 (4.7H, t, *J*=7.2 Hz). ESI-TOF MS: *m/z*=459.15 as [7-Et₃NH⁺]⁻¹ (Calcd 459.15).

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References and a Note

- 1) a) McMinn P. L., Ogawa A. K., Wu Y., Liu J., Schultz P. G., Romesberg F. E., *J. Am. Chem. Soc.*, **121**, 11585—11586 (1999); b) Morales J., Kool E. T., *ibid.*, **122**, 1001—1007 (2000); c) Ren R. X.-F., Chaudhuri N. C., Paris P. L., Rumney S., IV, Kool E. T., *ibid.*, **118**, 7671—7678 (1996) and references cited therein.
- 2) a) Tanaka K., Shionoya M., *J. Org. Chem.*, **64**, 5002—5003 (1999); b) Tanaka K., Tasaka M., Cao H., Shionoya M., *Eur. J. Pharm. Sci.*, in press; c) Shionoya M., Tanaka K., *Bull. Chem. Soc. Jpn.*, **73**, 1945—1954 (2000).
- 3) Bockstahl F., Graf E., Hosseini M. W., Suhr D., De Cian A., Fischer J., *Tetrahedron Lett.*, **38**, 7539—7542 (1997).
- 4) Shinkai S., “Chemosensors of Ion and Molecular Recognition,” ed. by Desvergne J. P., Czarnic A. W., Kluwer Academic Publishers, Dordrecht, 1997, pp. 37—59.
- 5) The acetyl groups of the catechol moiety could be removed quantitatively in aqueous ammonium hydroxide solution at room temperature to obtain **3**.

Optically Active *N*-Acetyldopamine Dimer of the Crude Drug “Zentai,” the Cast-off Shell of the Cicada, *Cryptotympana* sp.

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Two optically active *N*-acetyldopamine dimers together with four phenolic monomers were isolated from the crude drug “Zentai,” a cast-off shell of the cicada of *Cryptotympana* sp. (Cicadidae). The former two were 2-(3',4'-dihydroxyphenyl)-1,4-benzodioxane derivatives carrying substituents at the 3 and 6 (or 7) positions, which are known to be components of sclerotized insect cuticles. Their structures including absolute configurations were determined on the basis of NMR and circular dichroism (CD) spectroscopic data.

Key words *Cryptotympana* sp.; *N*-acetyldopamine dimer; 1,4-benzodioxane; cicada; cuticle; Cicadidae

The traditional Chinese crude drug, “Zentai,” is a cast-off shell of the cicada of *Cryptotympana* sp. or *Platylomia* sp. (Cicadidae). This drug has been used as an antifebrile, a spasmolytic and an antiphlogistic. In addition, this was found to exhibit a setative and sympathetic ganglionic blocking activities.¹⁾ But there is no report of components, except for the presence of chitin and amino acids.¹⁾

We examined the constituents of this drug, and isolated two 1,4-benzodioxane derivatives corresponding to *N*-acetyldopamine dimers (**5**, **6**) together with four phenolic monomers (**1**–**4**). The former two are known as the components of sclerotized insect cuticles, and they have already been obtained from the femurs of locusts and beetles by Anderson and Roepstorff.²⁾ They did not report, however, whether these compounds were optical active, and therefore, the absolute configuration as well as determination of the positional isomers remains unclear. Quite recently, Fujita and co-workers have also isolated four optically inactive 1,4-benzodioxane analogues from the cockroach belonging to Epilampridae.³⁾

Because two 1,4-benzodioxane derivatives isolated in this study are optical active, we determined their structures including the absolute configurations. The structures of other phenolic monomers were also characterized.

Isolation of Compounds 1–6 The MeOH extract of a cast-off shell of the cicada was treated with ether to give ether-soluble and insoluble portions. The latter gave, on concentration, a fraction, which was shaken with *n*-BuOH–AcOEt and H₂O. As described in the experimental section, the *n*-BuOH–AcOEt soluble fraction was subjected to HPLC using a recycling mode to afford compounds, **1**–**6**.

Structures of Phenolic Monomers 1–4 Compounds **1** and **2** were identified respectively as 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid by comparison of their spectral data with those of authentic samples.

Compound **3** exhibited the molecular ion peak at *m/z* 195, while compound **4** showed the molecular ion peak at *m/z* 209, in their electron impact mass spectra (EI-MS). Their ¹H- and ¹³C-NMR spectroscopic analyses revealed that **3** and **4** are *N*-acetyldopamine and 2-oxo-*N*-acetyldopamine, respectively (Fig. 1).

Structures of *N*-Acetyldopamine Dimers, **5 and **6**** The ¹H-NMR spectrum of **5** (FAB-MS *m/z*: 387 [M+H]⁺, [α]_D²⁰ +51.7°) showed two ABC-type signals in the aromatic re-

gion (δ 6.5–7.0), and signals ascribable to two methylenes (δ 2.63 and 3.26) and two methines (δ 4.75, d, *J*=7.3 Hz and δ 5.61, dd, *J*=7.3, 9.3 Hz) together with four signals due to OH and/or NH protons. Its ¹³C-NMR spectrum exhibited twelve signals in the aromatic region in addition to two signals each of methine, methylene and carbonyl carbons. These observations together with the ¹H–¹H and ¹H–¹³C chemical shift correlation spectroscopy (COSY) and two dimensional nuclear Overhauser spectroscopy (NOESY) spectral data revealed that **5** consists of unit A (3,4-disubstituted phenyl group linked to a *N*-acetylamino-2-ethyl group) and unit B (dihydroxyphenyl group and two oxymethine carbons) (Fig. 2). The ¹H-detected heteronuclear multiple bond connectivity (HMBC) spectrum of **5** showed clear correlation peaks as depicted in figure 2; however, no significant correlation between the A and B units was observed.

Treatment of **5** with diazomethane gave two compounds, **5a** and **5b**, in the ratio of 8:1. In their positive ion FAB-MS, both of them exhibited the quasi-molecular ion peak at *m/z* 415, which was 28 mass units more than that of **5**. Interestingly, unlike the starting material (**5**), both compounds showed specific rotation, [α]_D²⁰ < ±0.05° (*c*=1.0, MeOH), and gave no CD maximum, indicating that they were racemates.

The ¹H-NMR spectra of **5a** and **5b** were quite similar to each other, but the methine proton signals of the former were observed at δ 4.84 (d, *J*=7.3 Hz) and δ 5.65 (dd, *J*=7.3,

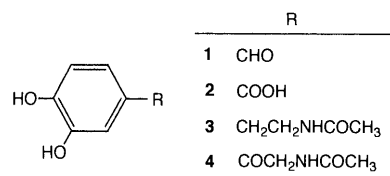


Fig. 1. Structures of **1**–**4**

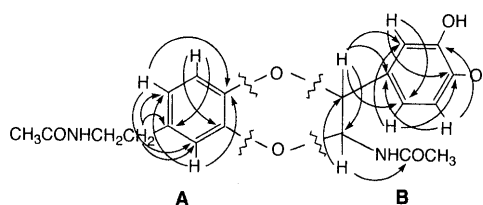


Fig. 2. HMBC Correlation

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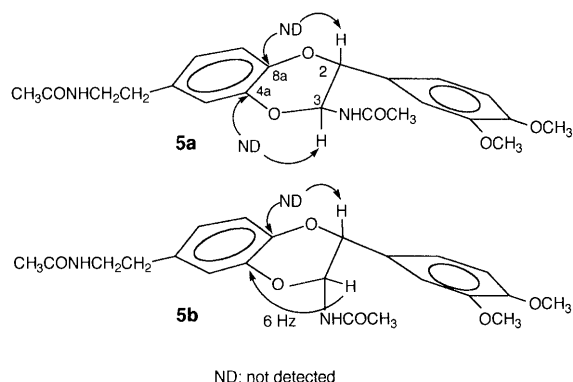


Fig. 3. HMBC Correlation of H-2/C-8a and H-3/C-4a of **5a** and **5b**

9.7 Hz), while those of the latter appeared at δ 5.22 (d, $J=2.0$ Hz) and δ 5.98 (dd, $J=2.0, 10.1$ Hz). These findings showed that they are racemates of 1,2-disubstituted *cis* and *trans* stereoisomers in the dioxane ring, which were formed under the methylation condition.

The HMBC spectrum of **5a** gave, similar to that of **5**, no diagnostic cross peak between the A and B units; in contrast, that of **5b** showed a clear long-range correlation peak between H-3 (δ 5.98) and C-4a (δ 140.6) which connects A with B, thereby constructing a 1,4-benzodioxane framework.

The $^3J_{\text{CH}}$ coupling constants are related to the dihedral angle between carbon and proton, as known from the Karplus-Conroy relation for $^3J_{\text{HH}}$.⁴⁾ In view of the above HMBC spectral observations, it was proven that the H-2 and H-3 bonds in **5a**, and the H-2 bond in **5b** stand vertically on the 1,4-benzodioxane ring ($^3J_{\text{CH}} < 1$ Hz), while the H-3 bond of **5b** is located almost in the 1,4-benzodioxane plane ($^3J_{\text{CH}} = 6$ Hz)⁵⁾ (Fig. 3).

Detailed analyses of the HMBC and NOESY spectra of **5a** and **5b** demonstrated that the *N*-acetylamino-2-ethyl group is located at the 6 position, and the two methoxy groups are located at the 3' and 4' positions in the phenyl group. Based on the coupling constant ($J_{2\text{H},3\text{H}} = 7.3$ Hz) and the above HMBC spectral observations of **5a**, the stereochemistry of the 2 and 3 positions was concluded to be *trans* (H-2 axial, H-3 axial) form. On the other hand, compound **5b** has the *cis* (H-2 axial, H-3 equatorial) form, that is, the 3-epimer of **5a**.

As the parent compound **5** showed the same coupling constant ($J_{2\text{H},3\text{H}} = 7.3$ Hz) as that of **5a**, it was concluded that the 2-phenyl and 3-acetylamino groups of **5** have a 2,3-*trans* (H-2 axial, H-3 axial) configuration.

The ^1H - and ^{13}C -NMR spectra of **6** (m/z 387 $[\text{M}+\text{H}]^+$, $[\alpha]_{\text{D}} +28.2^\circ$) were quite similar to those of **5**, suggesting that **6** is a positional isomer of **5**.

Treatment of **6** with diazomethane gave two racemates, **6a** and **6b**, in the ratio of 8 : 1. The NMR spectral data including the HMBC correlations of **6a** and **6b** were almost identical with those of the respective **5a** and **5b**. The HMBC and NOESY spectral analyses of **6a** and **6b** revealed that they differ only in the position of the substituted group, the *N*-acetylamino-2-ethyl group, placed at the 7-position instead of the 6-position in **5a** and **5b**.

Determination of the Absolute Configuration In 1985, Arnoldi and Merlini obtained (2*R*,3*S*)- and (2*S*,3*S*)-3-methyl-2-phenyl-1,4-benzodioxanes by stereospecific synthesis, and their absolute stereochemistry was determined by the CD

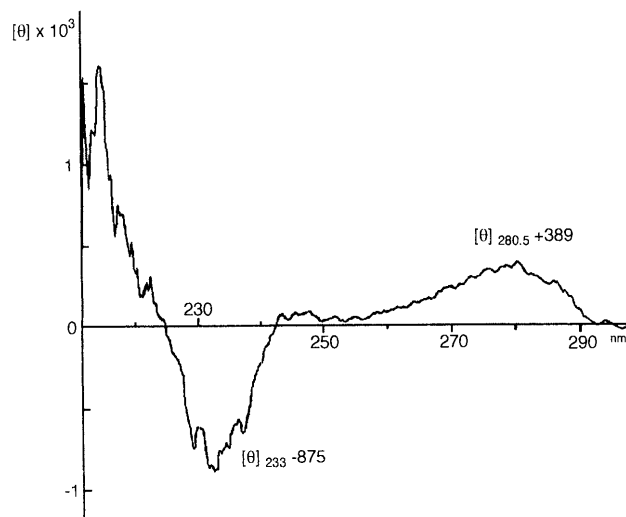


Fig. 4. CD Spectrum of **5**

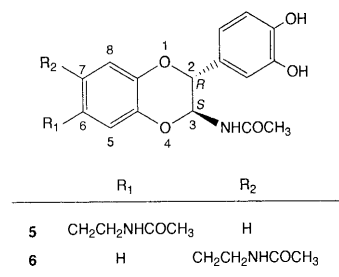


Fig. 5. Structures of **5** and **6**

spectral data.⁶⁾ In addition, Quaglia *et al.* synthesized other optically active 1,4-benzodioxane derivatives, and their CD curves were presented.⁷⁾ These reports showed that the CD curves of the 1,4-benzodioxanes having the phenyl group linked directly with the 2-upper equatorial position (2*S* form) exhibited a maximum at *ca.* 230 nm (a positive sign in the range of 225—240 nm), and a negative Cotton effect in the range of 270—290 nm. While those having the phenyl group attached at the 2-lower equatorial position (2*R* form) displayed almost the mirror image. The CD spectrum of **5** showed a negative sign in the range of 225—240 nm, and a positive sign in the range of 270—290 nm (Fig. 4), and this spectrum was quite similar to those of the 1,4-benzodioxanes having the phenyl group attached directly at the 2-lower equatorial position. From the results obtained above, it was clear that the 3',4'-dihydroxyphenyl group in **5** was located at the 2-lower equatorial position, and thus the structure of **5** was defined as (2*R*,3*S*)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(*N*-acetyl-2"-aminoethyl)-1,4-benzodioxane.

Compound **6** gave an almost identical CD curve as that of **5**, showing the same chiral center (2*R*,3*S*) as that of **5**, and hence **6** was assigned the structure (2*R*,3*S*)-2-(3',4'-dihydroxyphenyl)-3-acetylamino-7-(*N*-acetyl-2"-aminoethyl)-1,4-benzodioxane.⁸⁾ Figure 5 depicts their structures.

To further confirm the above conclusion, we attempted to prepare a suitable derivative for X-ray crystallographic analysis; however, an optical active crystal could not be obtained due to racemization. The mechanism of easy racemization of these dimers is as yet unclear.

Two 1,4-benzodioxane derivatives isolated from animal

Table 1. ¹H-NMR Spectral Data (DMSO-*d*₆)

¹ H	5	5a	5b	6	6a	6b
2	4.75 (d, 7.3)	4.84 (d, 7.3)	5.22 (d, 2.0)	4.72 (d, 7.3)	4.86 (d, 7.3)	5.24 (d, 2.0)
3	5.61 (dd, 7.3, 9.3)	5.65 (dd, 7.3, 9.7)	5.98 (dd, 2.0, 10.1)	5.56 (dd, 7.3, 9.5)	5.64 (dd, 7.3, 9.7)	5.97 (dd, 2.0, 10.1)
5	6.76 (d, 2.0)	6.77 (d, 2.0)	6.78 (d, 2.0)	6.72 (d, 8.6)	6.84 (d, 8.1)	6.84 (d, 8.1)
6	—	—	—	6.68 (dd, 2.0, 8.6)	6.73 (dd, 2.0, 8.1)	6.77 (dd, 2.0, 8.1)
7	6.70 (dd, 2.0, 8.0)	6.71 (dd, 2.0, 8.3)	6.75 (dd, 2.0, 8.1)	—	—	—
8	6.74 (d, 8.0)	6.88 (d, 8.3)	6.96 (d, 8.1)	6.71 (d, 2.0)	6.82 (d, 2.0)	6.90 (d, 2.0)
2'	6.81 (d, 2.0)	7.04 (d, 2.0)	7.23 (d, 2.0)	6.78 (d, 2.0)	7.05 (d, 2.0)	7.23 (d, 2.0)
5'	6.71 (d, 8.0)	6.96 (d, 8.1)	6.95 (d, 8.5)	6.78 (d, 8.4)	6.96 (d, 8.3)	6.96 (d, 8.5)
6'	6.86 (dd, 2.0, 8.0)	6.97 (dd, 2.0, 8.1)	7.07 (dd, 2.0, 8.5)	6.82 (dd, 2.0, 8.4)	6.98 (dd, 2.0, 8.3)	7.07 (dd, 2.0, 8.5)
1''	2.63 (t, 8.4)	2.61 (t, 7.4)	2.63 (t, 7.4)	2.60 (t, 8.5)	2.62 (t, 7.4)	2.64 (t, 7.4)
2''	3.26 (td, 5.2, 8.4)	3.22 (td, 5.4, 7.4)	3.22 (td, 5.4, 7.4)	3.21 (td, 5.2, 8.4)	3.23 (td, 5.4, 7.4)	3.23 (td, 5.4, 7.4)
CH ₃	1.80 (s), 1.81 (s)	1.78 (s), 1.79 (s)	1.77 (s), 1.79 (s)	1.80 (s), 1.81 (s)	1.78 (s), 1.79 (s)	1.76 (s), 1.79 (s)
OCH ₃	—	3.75 (s), 3.76 (s)	3.76 (s), 3.77 (s)	—	3.75 (s), 3.76 (s)	3.76 (s), 3.77 (s)

δ in ppm from TMS (splitting patterns and coupling constants); *J* in Hz are given in parentheses.

Table 2. ¹³C-NMR Spectral Data (DMSO-*d*₆)

¹³ C	5	5a	5b	6 ^{a)}	6a	6b
2	76.1	76.0	75.8	78.3	76.1	75.6
3	76.7	76.6	75.1	78.3	76.6	74.9
5	116.6	116.6	117.3	118.0	116.5	117.1
6	133.0	133.2	133.7	123.2	121.9	122.3
7	121.5	121.5	121.4	134.2	132.8	132.7
8	116.4	116.5	117.2	118.2	116.7	117.3
4a	141.9	141.0	140.6	142.2	140.5	139.1
8a	141.1	141.9	141.5	144.3	142.4	142.8
1'	126.9	128.3	128.0	128.9	128.3	127.9
2'	114.8	111.3	110.8	115.6	111.3	110.7
3'	145.0	148.4	148.5	146.5	148.4	148.4
4'	145.7	149.0	148.7	147.1	149.0	148.7
5'	115.2	111.4	111.3	116.2	111.4	111.2
6'	118.9	120.0	119.3	120.6	120.0	119.2
1''	34.4	34.3	34.4	35.8	34.4	34.4
2''	40.1	40.2	40.3	42.2	40.2	40.2
CH ₃	22.5	22.5	22.3	22.6	22.5	22.2
	22.5	22.5	22.6	22.6	22.5	22.5
OCH ₃	—	55.4	55.4	—	55.4	55.4
	—	55.5	55.6	—	55.5	55.6
CO	169.0	169.0	169.1	173.3	169.4	169.0
	169.0	169.4	170.3	173.3	169.4	170.2

a) Compound 6 was dissolved in CD₃OD. δ in ppm from TMS.

sources in the present study are, unlike those so far reported, the first examples of optically active dimers, which consist of 2 units of the *N*-acetyldopamine. Recently some 1,4-benzodioxanes with a high affinity for 5-HT receptor subtypes have been shown to have neuroleptic activity, suggesting that they are predictive tools for novel anxiolytic agents.⁹⁾ This information leads us to suppose that the sedative activity of the drug, "Zentai," is probably due to the *N*-acetyldopamine dimers.

Experimental

The NMR spectra were recorded on a JEOL JNM GX-400 instrument at 400 MHz (¹H) and 100 MHz (¹³C) at a probe temperature of 35 °C using tetramethylsilane (TMS) as an internal reference. The abbreviations used are as follows: s, singlet; brs, broad singlet; d, doublet; t, triplet; dd, double doublet; td, triple doublet; m, multiplet. MS were acquired on a JEOL JMS DX-300 spectrometer (EI-MS: ionization voltage, 30 eV; accelerating voltage 3—10 kV; positive ion FAB-MS: accelerating voltage 3 kV; matrix, glycerol; collision gas, Xe). CD spectra were recorded on a J-725 (JASCO) in MeOH (2 mm). Optical rotations were measured at 25 °C with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel HPTLC with Al sheets (Merck Art. 5556). Spots were visualized with 5% H₂SO₄ in MeOH (by

heating). Column chromatography was carried out on Merck Silica gel (230—400 mesh, Art. 9385). Preparative HPLC was conducted over Inertsil Prep ODS-3 and Inertsil Ph-3 (10 μm, 20×250 mm, GL Sciences) on a JASCO 880-PU equipped with a JASCO 830-RI. Recycling HPLC was carried out on a JASCO 880-PU equipped with a JASCO preparative recycle valve.

Isolation of Compounds 1—6 The crude drug (1 kg), "Zentai," a cast-off shell of the cicada of *Cryptotympana* sp. purchased from Tochimoto Tenkaido Co. was extracted with CHCl₃—MeOH (1 : 1, 5 l). The extract was concentrated to give a brown powder (18.4 g). This was treated with ether to give a dissolved fraction (1.1 g) and an insoluble fraction (15.2 g). The latter was shaken with CHCl₃—MeOH—H₂O (1 : 1 : 1, 900 ml) and the upper layer was collected. After removal of the solvent, the residue (4.6 g) was shaken with AcOEt—*n*-BuOH—H₂O (1 : 1 : 1, 300 ml) and the upper layer was collected. This was concentrated to give a fraction (fr. 1, 3.4 g). Fr. 1 was subjected to preparative HPLC on an Inertsil Prep ODS-3 (size, 20×250 mm: 10 μm) using 20% CH₃CN as an eluent to give three fractions, fr. 1—fr. 3. Fraction 1 was further separated by HPLC on an Inertsil Ph-3 (size, 20×250 mm; 10 μm) using 18% CH₃CN as an eluent to give four fractions, fr. 4—fr. 7. Each fraction was subjected to HPLC (Inertsil Ph-3) in a recycling mode using 12% CH₃CN as an eluent to give 1 (30 mg, from fr. 7), 2 (10 mg, from fr. 4), 3 (11 mg, from fr. 6) and 4 (19 mg, from fr. 5). Fraction 2 was subjected to HPLC (Inertsil ODS-3) in a recycling mode using 25% CH₃CN as an eluent to give 5 (83 mg) and 6 (68 mg). Compounds 1—2 were identified respectively as 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid by comparison of their spectral data with those of authentic samples (Wako Pure Chemical Industries, Ltd.).

***N*-Acetyldopamine (3)** Yellow powder, mp 153—160 °C. EI-MS *m/z*: 195 (M)⁺. ¹H-NMR (DMSO-*d*₆, 400 MHz) δ: 1.80 (3H, s, CH₃), 6.57 (d, *J*=2.0 Hz, H-2), 6.63 (d, *J*=8.3 Hz, H-5), 6.43 (dd, *J*=2.0, 8.3 Hz, H-6), 3.15 (2H, td, *J*=5.3, 7.3 Hz, H-1'), 2.50 (2H, t, *J*=7.3 Hz, H-2'), 7.86 (t, *J*=5.3 Hz, NH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ: 22.5 (CH₃), 34.6 (C-2'), 40.5 (C-1'), 115.3 (C-2), 115.8 (C-5), 119.0 (C-6), 130.1 (C-1), 143.4 (C-3), 144.9 (C-4), 168.9 (OAc).

2-Oxo-*N*-acetyldopamine (4) Yellow crystal, mp 168—175 °C. EI-MS *m/z*: 209 (M)⁺. ¹H-NMR (DMSO-*d*₆, 400 MHz) δ: 1.91 (3H, s, CH₃), 7.35 (d, *J*=1.8 Hz, H-2), 6.83 (d, *J*=8.3 Hz, H-5), 7.38 (dd, *J*=1.8, 8.3 Hz, H-6), 4.46 (2H, d, *J*=5.2 Hz, H-2'), 7.94 (t, *J*=5.2 Hz, NH). ¹³C-NMR (DMSO-*d*₆, 100 MHz) δ: 22.3 (CH₃), 45.1 (C-2'), 114.6 (C-2), 115.1 (C-5), 120.9 (C-6), 126.9 (C-1), 145.2 (C-3), 150.8 (C-4), 169.4 (OAc), 193.2 (C-1').

(2*R*,3*S*)-2-(3',4'-Dihydroxyphenyl)-3-acetyl-6-(*N*-acetyl-2'-aminoethyl)-1,4-benzodioxane (5) Yellow powder, mp 118—126 °C. [α]_D²⁵ +51.7° (*c*=1.0, MeOH). Positive ion FAB-MS *m/z*: 387 (M+H)⁺. ¹H- and ¹³C-NMR (DMSO-*d*₆, 400 MHz) δ: see Tables 1 and 2.

(2*R*,3*S*)-2-(3',4'-Dihydroxyphenyl)-3-acetyl-7-(*N*-acetyl-2'-aminoethyl)-1,4-benzodioxane (6) Yellow powder, mp 115—120 °C. [α]_D²⁵ +28.2° (*c*=1.0, MeOH). CD [θ]₂₈₀ +400, [θ]₂₃₀ -905. Positive ion FAB-MS *m/z*: 387 (M+H)⁺. ¹H- and ¹³C-NMR (DMSO-*d*₆, 400 MHz) δ: see Tables 1 and 2.

Methylation of 5 and 6 Each compound (5 and 6, 50 mg each) was dissolved in MeOH (5 ml). Diazomethane in ether was added to this solution, until the spot due to the starting material disappeared by TLC. After removal of the solvent under a nitrogen stream, the residue was subjected to HPLC

on an Inertsil ODS-3 (size, 20×250 mm; 10 μ m) using 33% CH₃CN as an eluent to give **5a** (36.1 mg) and **5b** (4.2 mg) from **5**, and **6a** (37.6 mg) and **6b** (4.8 mg) from **6**. NMR spectral data for these four compounds are shown in Tables 1 and 2.

Acknowledgments We are indebted to Dr. Takashi Takakuwa of JASCO Corporation for measurement of the CD spectra. We thank Professor Tetsuro Fujita of this university for his valuable discussion. Thanks also due to Dr. Masatoshi Nishi and Mr. Shoji Inoue of this university for measurements of the NMR spectra and MS. The present study was financially supported by a Grant-in-Aids for Scientific Research (No. 10672013) from the Ministry of Education, Science, Sports and Culture.

References and Notes

- 1) Okuda T. (ed.), "Encyclopedia of Natural Medicine," Hirokawa Publishing Co., Tokyo, 1986, p. 244.
- 2) a) Andersen S. O., Jacobsen J. P., Roepstorff P., *Tetrahedron*, **36**, 3249—3252 (1980); b) Andersen S. O., Roepstorff P., *Insect Biochem.*, **11**, 25—31 (1981).
- 3) Minami Y., Oumori K., Muranaka Y., Yamada S., Yamawaki I., Kaneda M., Kimura H., Hironaka Y., Fujita T., Jpn. Kokai Tokkyo Koho JP 07242543 [95242543] [*Chem. Abstr.*, **124**, 4, p. 574 (1996)].
- 4) Breitmaier E., Voelter W., "Carbon-13 NMR Spectroscopy," VCH Verlagsgesellschaft, Weinheim, 1987, p. 143.
- 5) These ¹H-¹³C long-range (J_{CCH}) coupling constants were directly obtained by the J_{CCH} measurement using NOE method.
- 6) Arnoldi A., Merlini L., *J. Chem. Soc., Perkin Trans. I*, **1985**, 2555—2557 (1985).
- 7) Quaglia W., Pignini M., Tayebati S. K., Piergentili A., Giannella M., Leonardi A., Taddei C., Melchiorre C., *J. Med. Chem.*, **39**, 2253—2258 (1996).
- 8) According to the nomenclature rules in the index of Chemical Abstracts or Ring Index, the 2 and 3 positions are reversed, and compound **6** is (2*S*,3*R*)-2-acetylamino-3-(3',4'-dihydroxyphenyl)-6-(*N*-acetyl-2"-aminoethyl)-1,4-benzodioxane.
- 9) Hibert M. F., Gittos M. W., Middlemiss D. N., Mir A. K., Fozard J. R., *J. Med. Chem.*, **31**, 1087—1093 (1988).

Total Synthesis of Prostaglandin $F_{2\alpha}$ Using Nickel-Catalyzed Stereoselective Cyclization of 1,3-Diene and Tethered Aldehyde *via* Transmetalation of Nickelacycle with Diisobutylaluminum Acetylacetonate

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Total synthesis of prostaglandin $F_{2\alpha}$ utilizing a nickel(0)-catalyzed cyclization of 1,3-diene and tethered aldehyde was achieved. The cyclization proceeded *via* a transmetalation of nickelacycle with diisobutylaluminum acetylacetonate ($\text{Bu}_2\text{-ALAC}$). Thus, the reaction of 19, having a side chain corresponding to the α -chain in $\text{PGF}_{2\alpha}$ with $\text{Ni}(\text{cod})_2$ (10 mol %), PPh_3 (20 mol %), and 1,3-cyclohexadiene (25 mol %) in the presence of $\text{Bu}_2\text{-ALAC}$ (1.5 eq) proceeded stereoselectively to give the cyclized product 26 in 54% yield. During the cyclization of 19, the *Z*-olefin at C-5 in the side chain completely retained its geometry, and the four contiguous chiral carbon centers in $\text{PGF}_{2\alpha}$ were stereoselectively constructed. Transformation of the key intermediate 19 into $\text{PGF}_{2\alpha}$ was successfully achieved.

Key words nickel; cyclization; nickelacycle; transmetalation; 1,3-diene; aldehyde

The nickel-promoted intramolecular oligomerization of 1,3-dienes and tethered multiple bonds is a useful methodology for the stereoselective construction of cyclic compounds.^{1,2)} In a recent study, we succeeded in developing a nickel-promoted cyclization of ω -formyl-1,3-dienes.³⁾ The reaction of ω -formyl-1,3-diene **1** using a stoichiometric amount of low-valent nickel complex **2**, generated by reduction of $\text{Ni}(\text{acac})_2$ with DIBAL-H in the presence of PPh_3 , afforded the cyclized products **3-I** and **3-T** in a stereoselective manner with respect to the stereochemistry of the substituents on the cycloalkane ring (Eq. 1, Chart 1).^{3a)} It was also found that the cyclized product **3-T**, having a terminal olefin in the side chain, was produced predominantly under the same reaction conditions in the presence of 1,3-cyclohexadiene (1,3-CHD) as an additive in the reaction mixture (Eq. 2).^{3b,3g)} The reaction course of this cyclization can be accounted for by two possible mechanisms. In one mechanism, a nickel hydride complex plays a key role and the cyclization proceeds *via* a π -allylnickel intermediate. In the other mechanism, a zerovalent nickel complex is the active species and the cyclization proceeds *via* nickelacycle intermediates.^{3g)} These mechanistic considerations led us to find two nickel(0)-catalyzed cyclizations of ω -formyl-1,3-dienes, which are depicted as the reactions of Type I and Type II in Chart 1. In the former reaction (Type I), a nickel(II) hydride complex is initially formed by the oxidative addition of trialkylsilane to a zero-valent nickel complex. The nickel(II) hydride complex reacts with substrate **1** to produce π -allylnickel intermediate **4**. The π -allyl moiety in **4** is reacted with tethered aldehyde to give the cyclized product **3'-I**, having an internal olefin in the side chain, through reductive elimination. On the other hand, a nickelacycle intermediate is initially generated by the reaction of a zero-valent nickel complex with substrate **1** in the latter reaction (Type II). Then the cyclized product **3-T** having a terminal olefin in the side chain is produced *via* transmetalation of a nickelacycle intermediate with diisobutylaluminum acetylacetonate ($\text{Bu}_2\text{-ALAC}$) such as intermediate **5**. These cyclizations are quite interesting and synthetically useful since they are comple-

mentary to each other for the regio-chemistry of olefin in the side chain to afford the cyclized product **3'-I** or **3-T** in a stereoselective manner using the same catalyst system, $\text{Ni}(\text{cod})_2\text{-PPh}_3$, only depending upon the difference in the additive, Et_3SiH or $\text{Bu}_2\text{-ALAC}$. These unique properties encouraged us to apply these cyclizations to the synthesis of natural products.^{3c,3d)} Herein, we describe an application of the reaction of Type II to the synthesis of prostaglandin $F_{2\alpha}$.⁴⁾

Results and Discussion

Plan for the Synthesis of Prostaglandin $F_{2\alpha}$ Using Nickel-Catalyzed Intramolecular Cyclization *via* Transmetalation of Nickelacycle with $\text{Bu}_2\text{-ALAC}$ For several decades, prostaglandins (PGs) have attracted the interest of synthetic organic chemists as targets for total synthesis, and

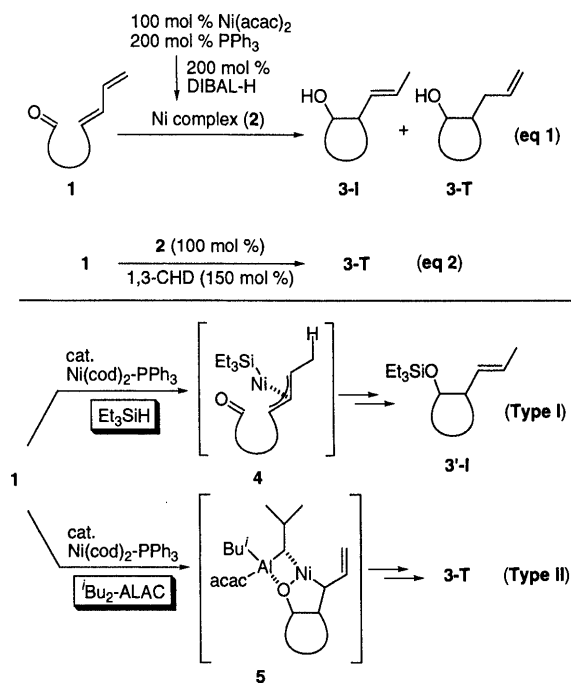


Chart 1

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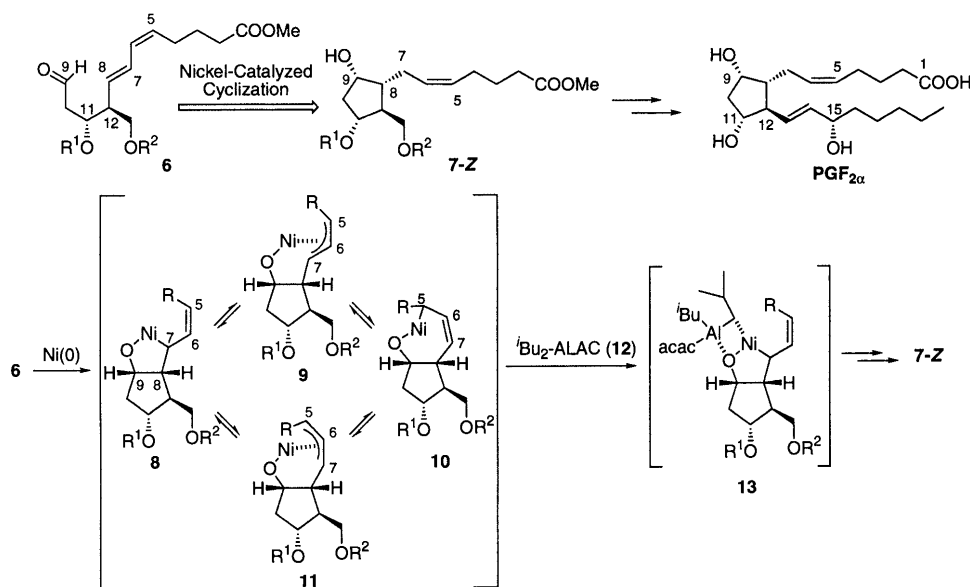


Chart 2

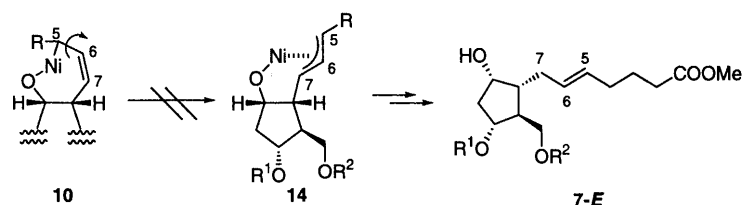


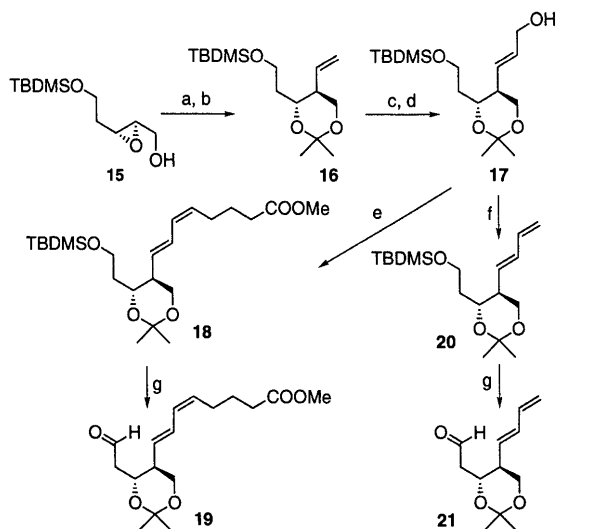
Fig. 1

various efficient methods for their syntheses have been reported.⁵⁾ We planned to apply the above-mentioned nickel(0)-catalyzed cyclization to the synthesis of $\text{PGF}_{2\alpha}$ as shown in Chart 2. According to the mechanism described above, the nickelacycle intermediates **8**–**11** would be generated by the reaction of the substrate **6** and a zero-valent nickel complex. Thus, regio- and stereoselective C–C bond formation would be expected to occur between C-8 and C-9 in **6**.⁶⁾ In general, a *syn*- π -allylmetal complex such as **14** (Fig. 1) should be more stable than an *anti*-complex such as **9** or **11**, and a rearrangement from an *anti*- π -allyl complex to a *syn*- π -allyl complex would occur *via* a σ -allylmetal complex in a linear π -allylmetal system.⁷⁾ In this cyclization, however, the π - σ - π rearrangement to **14** is restricted due to its cyclic form, as shown in Fig. 1. Thus, it was expected that the *Z*-olefin at C-5 in **6** would retain its geometry during the cyclization to give the cyclopentanoid **7-Z** in a stereoselective manner, and it was thought that the cyclized product **7-Z** would be readily transformed into $\text{PGF}_{2\alpha}$ (Chart 2).

Synthesis of Substrates for Cyclization The regiospecific ring-opening reaction of **15**⁸⁾ was accomplished by treatment with vinylmagnesium bromide (5 eq) in the presence of CuCN (0.5 eq)⁹⁾ to give the desired 1,3-diol in 70% yield,¹⁰⁾ and this was converted into acetonide **16**. Ozonolysis of **16** and successive reaction of the resulting crude aldehyde with (carbomethoxymethylene)triphenylphosphorane gave α,β -unsaturated ester, which was treated with DIBAL-H to produce allyl alcohol **17**. After oxidation of **17** with PCC reagent, the resulting aldehyde was reacted with the Wittig reagent generated from (4-carboxybutyl)triphenylphosphonium bromide and sodium methylsulfinylmethylide in

DMSO ,¹¹⁾ and then treated with diazomethane to give the desired (5*Z*,7*E*)-dodecadienoic acid derivative **18** (66% from **17**) along with the (5*E*,7*E*)-isomer (14%), which were easily separated by silica gel column chromatography. The substrate **19** for the cyclization was obtained in 80% yield (2 steps) by deprotection of the TBDMS group in **18** followed by oxidation with Dess–Martin reagent. The substrate **21** was also synthesized from allyl alcohol **17** for a model study of the cyclization. Thus, the aldehyde, derived from **17** by PCC oxidation, was condensed with the Wittig reagent generated from methyl triphenylphosphonium bromide and BuLi to give 1,3-diene **20**. The substrate **21** was obtained from **20** (84%, 2 steps) by procedures similar to those used for the synthesis of **19** from **18** (Chart 3).

Model Study of Cyclization Initially, the cyclization of **21** was investigated as a model study of cyclization. To a stirred solution of $\text{Ni}(\text{cod})_2$ (5 mol %), PPh_3 (10 mol %), and $i\text{Bu}_2\text{-ALAC}$ (**12**) (1.5 eq) in toluene was added a toluene solution of **21** at 0 °C, and the mixture was stirred at the same temperature for 2 h. We were very pleased to find that the cyclized product **22** was obtained in 91% yield as a single isomer (Chart 4). The stereochemistry of **22** was unambiguously determined by its NOESY spectrum, as shown in Fig. 2, which indicated that the four contiguous chiral carbon centers in **22** were stereoselectively produced with the same absolute configuration as those in $\text{PGF}_{2\alpha}$. Furthermore, conversion of **22** into the Corey lactone **24** or **25** was also achieved, as shown in Chart 5. Ozonolysis of **22** followed by reductive work-up gave the lactol **23**. Oxidation of **23** with PCC followed by treatment with DOWEX 50^w produced Corey lactone **24**, which was converted into **25**. The spectral data and



(a) $\text{CH}_2=\text{CHMgBr}$, CuCN , $\text{Et}_2\text{O-THF}$, $-12\text{ }^\circ\text{C}$, 70%. (b) 2,2-dimethoxypropane, PPTS, r.t., 88%. (c) (1) O_3 , CH_2Cl_2 , $-78\text{ }^\circ\text{C}$; (2) $\text{Ph}_3\text{P=CHCO}_2\text{Me}$, benzene, $65\text{ }^\circ\text{C}$, 93% (2 steps). (d) DIBAL-H , toluene, $-78\text{ }^\circ\text{C}$, 95%. (e) (1) PCC , MS 4A, NaOAc , CH_2Cl_2 , $0\text{ }^\circ\text{C}$; (2) $\text{BrPh}_3\text{PC}_4\text{H}_8\text{COOH}$, NaH , DMSO , r.t.; (3) CH_2N_2 , $\text{MeOH-Et}_2\text{O}$, 66% (3 steps). (f) (1) PCC , MS 4A, CH_2Cl_2 , $0\text{ }^\circ\text{C}$; (2) Ph_3PMeBr , BuLi , THF , $-78\text{ }^\circ\text{C}$ – $0\text{ }^\circ\text{C}$, 84% (2 steps). (g) (1) TBAF , THF , r.t.; (2) Dess-Martin reagent, CH_2Cl_2 , $0\text{ }^\circ\text{C}$, **19** from **18**: 80%, **21** from **20**: 84% (2 steps).

Chart 3

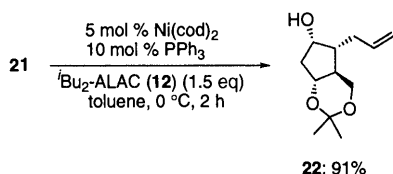
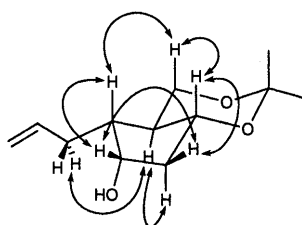
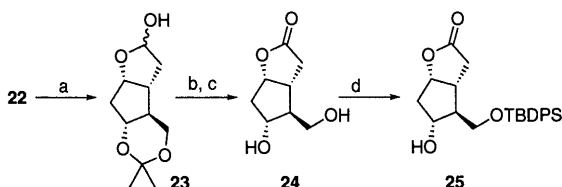


Chart 4

Fig. 2. NOESY Correlations of **22**

(a) (1) O_3 , CH_2Cl_2 , $-78\text{ }^\circ\text{C}$; (2) PPh_3 , $-78\text{ }^\circ\text{C}$ –r.t., 78% (2 steps). (b) PCC , MS 4A, NaOAc , CH_2Cl_2 , $0\text{ }^\circ\text{C}$, 80%. (c) $\text{DOWEX } 50^{\text{W}}$, $\text{THF-H}_2\text{O}$, r.t., 100%. (d) TBDPSCI , imidazole, DMF , $0\text{ }^\circ\text{C}$, 90%.

Chart 5

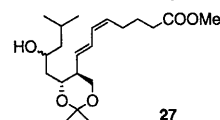
the $[\alpha]_D$ value of **24** and **25** were completely identical with those previously reported.¹²⁾

Study for the Cyclization of 19 The cyclization of the substrate **19**, having a side chain corresponding to the α -chain in $\text{PGF}_{2\alpha}$, was examined, and the results are summarized in Table 1. As mentioned above, we have already shown

Table 1. Cyclization of **19** Using Ni(cod)_2 and PPh_3 in the Presence of $t\text{Bu}_2\text{-ALAC (12)}$

Run	Ni complex (mol %)	1,3-CHD (mol %)	$t\text{Bu}_2\text{-ALAC}$ (eq)	Time (h)	Yield (%)
1	Ni(cod)_2 (100) PPh_3 (200)	150	2.2	15	51
2	Ni(cod)_2 (10) PPh_3 (20)	—	3.0	15	31
3 ^{a)}	Ni(cod)_2 (10) PPh_3 (20)	25	1.5	15	40
4 ^{b)}	Ni(cod)_2 (10) PPh_3 (20)	25	1.5	6	54

a) The by-product **27** was also obtained in 25% yield.

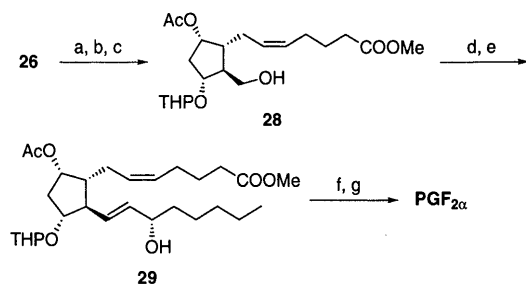


b) $t\text{Bu}_2\text{-ALAC}$ was added slowly to the reaction mixture over a period of about 2 h.

that the addition of 1,3-cyclohexadiene (1,3-CHD) in nickel(0)-promoted cyclization of ω -formyl-1,3-dienes remarkably affected the regiochemistry of olefin on the side chain of the cyclized product.^{3b,3g)} Thus, the reaction of **19** was initially carried out using a stoichiometric amount of nickel(0) complex and $t\text{Bu}_2\text{-ALAC (12)}$ in THF in the presence of 1,3-CHD (run 1). As a result, the cyclized product **26** was obtained in 51% yield as a single isomer. As expected, the ω -side chain of **26** had a *Z*-geometry, and the four contiguous chiral carbon centers in $\text{PGF}_{2\alpha}$ were stereoselectively constructed from **19**.¹³⁾ Next, we tried a catalytic reaction. In the catalytic cyclization, it was expected that the regio- and stereochemistry of the cyclized product would be controlled in the absence of 1,3-CHD because the coordination of the 1,3-diene moiety in the substrate would play the same role of additive 1,3-diene.^{3g)} However, cyclization using 10 mol% Ni(cod)_2 , 20 mol% PPh_3 , and 3.0 eq of **12** in the absence of 1,3-CHD gave the desired product **26** in only 31% yield along with some regio- and/or geometrical isomers with respect to the olefin at the side chain (run 2). Since a *s-cis* conformation of 1,3-diene moiety would be necessary for strong coordination to the central nickel metal,^{3b,3g)} it was thought that this result was caused by the structure of the 1,3-diene moiety in **19**. Thus, a *s-cis* conformation in **19** would be more unfavorable than a *s-trans* one due to the steric repulsion, which resulted in low stereoselectivity in the cyclization (Chart 6). A catalytic cyclization of **19** in the presence of 1,3-CHD was therefore examined, and the desired product **26** was obtained in 40% yield along with **27** in 25% yield (Table 1, run 3). We were pleased to find that the formation of **27** could be reduced by slow addition of **12** to the reaction mixture (over 2 h), and the yield of **26** was finally improved up to 54% (run 4). It is remarkable that compound **26**, having four chiral carbon centers and the α -chain in $\text{PGF}_{2\alpha}$, was stereoselectively produced from the linear diene **19** by the Ni(0) -catalyzed cyclization *via* a transmetalation process of nickelacy-



Chart 6



(a) Ac_2O , Pyridine, CH_2Cl_2 , r.t., 95%. (b) (1) DOWEX 50^W, MeOH, 50 °C; (2) $^t\text{BuPh}_2\text{SiCl}$, Et_3N , CH_2Cl_2 , 90% (2 steps). (c) (1) DHP, PPTS, CH_2Cl_2 , r.t.; (2) TBAF, THF, r.t., 100% (2 steps). (d) (1) PCC, MS 4A, NaOAc , CH_2Cl_2 , 0 °C; (2) $(\text{MeO})_2\text{POCH}_2\text{CO}(\text{CH}_2)_4\text{CH}_3$, NaH, THF, r.t., 76% (2 steps). (e) (*S*)-binaphthol, LiAlH_4 , EtOH, THF, -100 °C ~ -78 °C, 92%. (f) $\text{AcOH-H}_2\text{O-THF}$, 40 °C, 93%. (g) 1N NaOH, MeOH-THF, r.t., 95%.

Chart 7

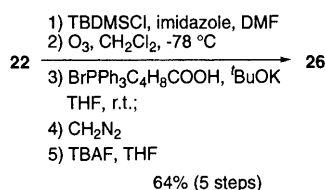


Chart 8

cle with $^t\text{Bu}_2\text{-ALAC}$ (**12**).

Total Synthesis of $\text{PGF}_{2\alpha}$ The cyclized product **26** was transformed into $\text{PGF}_{2\alpha}$ according to a procedure similar to that previously reported (Chart 7).¹⁴ After manipulation of the protecting groups in **26**, introduction of a ω -chain followed by stereoselective reduction with (*S*)-BINAL- H^{15} provided **29** in good yield. Finally, **29** was successfully converted into $\text{PGF}_{2\alpha}$ in the naturally occurring form.¹⁶

Conclusion

In summary, we have demonstrated that nickel(0)-catalyzed cyclization of 1,3-diene and tethered aldehyde *via* a transmetalation of nickelacycle with $^t\text{Bu}_2\text{-ALAC}$ (**12**) could be used to construct cyclopentanoids, and the total synthesis of $\text{PGF}_{2\alpha}$ was successfully achieved. A unique characteristic of the synthesis described herein is the regio- and stereoselective formation of the key intermediate **26** from the simple linear diene **19** in a one-pot reaction. The present results provide a basis for the development of new methods for the synthesis of cyclopentanoids.

Experimental

General All manipulations were performed under an argon atmosphere unless otherwise mentioned. All solvents and reagents were purified when necessary using standard procedures. Column chromatography was performed on silica gel 60 (Merck, 70–230 mesh), and flash chromatography was performed on silica gel 60 (Merck, 230–400 mesh) using the indicated

solvent. Melting points are uncorrected. ^1H - and ^{13}C - NMR spectra were recorded at 270, 400, or 500 MHz and at 67.5, 100, 125 MHz, respectively. Infrared spectra were recorded on a Perkin-Elmer FTIR 1605 spectrometer. Mass spectra were measured on JEOL DX-303 and JEOL HX-110 mass spectrometer.

(2*R*,3*S*)-5-*tert*-Butyldimethylsilyloxy-2,3-epoxypentan-1-ol (15**)** To a stirred suspension of activated molecular sieves 4A (powdered, 1.84 g) in CH_2Cl_2 (50 ml) were added successively a solution of D-(-)-diethyl tartrate (352 mg, 1.70 mmol) in CH_2Cl_2 (5 ml), $\text{Ti}(\text{O-iso Pr})_4$ (0.38 ml, 1.27 mmol), and *tert*-butyl hydroperoxide (5.0 M in decane, 8.5 ml, 43 mmol) at -20 °C, and the resulting mixture was stirred at the same temperature for 30 min. To the mixture was added a solution of 5-*tert*-butyldimethylsilyloxy-2-propen-1-ol (4.6 g, 21.2 mmol) in CH_2Cl_2 (30 ml) at -20 °C, and the mixture was stirred at the same temperature for 20 h. To the mixture was added 10% aqueous solution of citric acid monohydrate (15 ml), and the resulting mixture was stirred at 0 °C for 20 min. The mixture was filtered through Celite, and the filtrate was treated with 30% NaOH aq. (saturated with sodium chloride, 15 ml) at 0 °C for 1 h in order to hydrolyze the tartrate ester. The mixture was extracted with Et_2O , and the organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=2:1) to afford epoxide **15** (4.67 g, 94%, 91% ee) as a colorless oil. $[\alpha]_D^{25} +29.0^\circ$ ($c=1.26$, CHCl_3). IR (neat) cm^{-1} : 3429, 2957, 2937, 2856, 1470, 1461. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 0.06 (6 H, s), 0.89 (9 H, s), 1.62 (1 H, dd, $J=12.5$, 7.4 Hz), 1.68–1.89 (2H, m), 2.98 (1H, m), 3.09 (1H, m), 3.63 (1H, ddd, $J=12.5$, 7.4, 4.4 Hz), 3.76 (2H, dd, $J=6.7$, 5.4 Hz), 3.93 (1H, ddd, $J=12.5$, 5.5, 2.6 Hz). MS m/z : 199 ($\text{M}^+ - \text{MeOH-H}$), 189, 157, 145, 75. Anal. Calcd for $\text{C}_{11}\text{H}_{24}\text{O}_3\text{Si}$: C, 56.85; H, 10.41. Found: C, 56.77; H, 10.37. The enantiomeric purity of **15** was determined to be 91% ee on $^1\text{H-NMR}$ spectrum of the corresponding MTPA ester.

(2*S*,3*R*)-5-*tert*-Butyldimethylsilyloxy-2-ethenylpentan-1,3-diol To a suspension of CuCN (1.09 g, 12.2 mmol) in Et_2O (150 ml) was added vinylmagnesium bromide (1.1 M in THF, 109 ml, 122 mmol) at -78 °C, and the mixture was stirred at -30 °C for 1 h. To the resulting mixture was added a solution of **15** (5.68 g, 24.4 mmol) in Et_2O (35 ml), and the mixture was stirred at -12 °C for 8 h. The reaction mixture was poured into basic sat. NH_4Cl aq (that had been basified to pH 8 by addition of conc. NH_4OH), and the mixture was stirred until a clear aqueous phase was separated. The aqueous layer was extracted with ethyl acetate, and the combined organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=2:1) to afford the 1,3-diol (4.40 g, 70%) as an oil. $[\alpha]_D^{25} -3.7^\circ$ ($c=1.10$, CHCl_3). IR (neat) cm^{-1} : 3405, 2960, 2927, 2855, 1743, 1728, 1476. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 0.03 (6H, s), 0.84 (9H, s), 1.54–1.78 (2H, m), 2.28 (1H, m), 3.23 (1H, dd, $J=6.8$, 4.6 Hz), 3.57–3.92 (5H, m), 4.18 (1H, d, $J=2.4$ Hz), 5.08 (1H, d, $J=10.4$ Hz), 5.09 (1H, d, $J=17.1$ Hz), 5.55 (1H, ddd, $J=17.1$, 10.4, 9.0 Hz). MS m/z : 261 ($\text{M}^+ + \text{H}$), 203, 189, 185. Anal. Calcd for $\text{C}_{13}\text{H}_{28}\text{O}_3\text{Si}$: C, 59.95; H, 10.84. Found: C, 59.68; H, 10.46.

(4*R*,5*S*)-4-(2-*tert*-Butyldimethylsilyloxyethyl)-2,2-dimethyl-1,3-dioxo-5-ethenylcyclohexane (16**)** To a solution of the above 1,3-diol in CHCl_3 (50 ml) were added 2,2-dimethoxy propane (8.0 ml, 64.8 mmol) and pyridinium *p*-toluenesulfonate (100 mg, 0.39 mmol), and the mixture was stirred at room temperature for 12 h. To the mixture was added sat. NaHCO_3 aq., and the aqueous layer was extracted with Et_2O . The organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=20:1) to afford **16** (3.43 g, 88%) as a colorless oil. $[\alpha]_D^{25} +32.0^\circ$ ($c=1.18$, CHCl_3). IR (neat) cm^{-1} : 2994, 2953, 2928, 2856, 1638, 1471, 1463. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 0.04 (6H, s), 0.89 (9H, s), 1.38 (3H, s), 1.45 (3H, s), 1.48 (1 H, m), 1.85 (1H, m), 2.25 (1H, m), 3.60–3.74 (4H, m), 3.85 (1H, td, $J=9.7$, 2.5 Hz), 5.13 (1H, d, $J=10.2$ Hz), 5.15 (1H, d, $J=17.4$ Hz), 5.48 (1H, ddd, $J=17.4$, 10.2, 8.6 Hz). MS m/z : 285 ($\text{M}^+ - \text{Me}$), 227, 213, 185, 75. Anal. Calcd for $\text{C}_{16}\text{H}_{32}\text{O}_3\text{Si}$: C, 63.95; H, 10.73. Found: C, 63.71; H, 10.66.

Methyl (2*E*)-2-[(1*S*,2*R*)-2-*tert*-Butyldimethylsilyloxyethyl-4,4-dimethyl-3,5-dioxacyclohexyl]-2-propenoate A solution of **16** (2.95 g, 9.81 mmol) in CH_2Cl_2 (50 ml) was cooled to -78 °C and treated with a stream of O_3/O_2 at -78 °C until the colorless solution had been converted to a blue one. The reaction mixture was treated with a stream of dry O_2 at -78 °C to remove an excess amount of O_3 . To the reaction mixture was added a solution of PPh_3 (3.0 g, 11.8 mmol) in CH_2Cl_2 (5 ml) at -78 °C, and the mixture was allowed to stir at room temperature for 12 h. After removal of the solvent, the residue was filtered through a short column of silica gel (hexane/ Et_2O =10:1) in order to remove triphenylphosphine oxide giving the crude aldehyde. To a solution of the crude aldehyde in benzene (47 ml) was added

Ph₃PCHCO₂Me (3.94 g, 11.7 mmol), and the mixture was stirred at 65 °C for 7 h. After removal of the solvent, the residue was purified by silica gel column chromatography (hexane/ethyl acetate=15/1) to afford the ester (3.27 g, 93% from **16**) as a colorless oil. [α]_D²⁰ +27.3° (*c*=1.02, CHCl₃). IR (neat) cm⁻¹: 2994, 2953, 2391, 2958, 1728, 1656, 1472, 1463. ¹H-NMR (270 MHz, CDCl₃) δ : 0.04 (6H, s), 0.89 (9H, s), 1.39 (3H, s), 1.46 (3H, s), 1.50 (1H, m), 1.75 (1H, m), 2.45 (1H, m), 3.59—3.78 (4H, m), 3.74 (3H, s), 3.94 (1H, dd, *J*=9.6, 9.6, 2.4 Hz), 5.93 (1H, d, *J*=15.8 Hz), 6.68 (1H, dd, *J*=15.8, 9.3 Hz). MS *m/z*: 357 (M⁺-H), 343, 301, 285, 243, 131. Anal. Calcd for C₁₈H₃₄O₃Si: C, 60.30; H, 9.56. Found: C, 60.34; H, 9.46.

(2E)-3-[(1S,2R)-2-(2-tert-Butyldimethylsilyloxyethyl)-4,4-dimethyl-3,5-dioxacyclohexyl]-2-propen-1-ol (17) To a solution of the above ester (3.27 g, 9.12 mmol) in toluene (46 ml) was added DIBAL-H (0.93 M in hexane, 23 ml, 21.4 mmol) at -78 °C, and the mixture was stirred at -78 °C for 1 h. To the mixture was added methanol (2 ml) followed by the addition of saturated aqueous solution of Rochell salt (45 ml), and the mixture was allowed to stir at room temperature. The mixture was extracted with Et₂O, and the organic layer was washed with sat. NH₄Cl aq. and brine, dried with Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=3:1) to afford **17** (2.86 g, 95%) as a colorless oil. [α]_D²⁰ +29.6° (*c*=1.17, CHCl₃). IR (neat) cm⁻¹: 3426, 2993, 2954, 2930, 2850, 1472, 1461. ¹H-NMR (270 MHz, CDCl₃) δ : 0.04 (6H, s), 0.89 (9H, s), 1.33 (1 H, t, *J*=5.8 Hz), 1.38 (3H, s), 1.44 (3H, s), 1.49 (1H, m), 1.83 (1H, m), 2.28 (1H, m), 3.60—4.76 (4H, m), 3.84 (1H, ddd, *J*=9.9, 9.9, 2.3 Hz), 4.11 (2H, dd, *J*=5.8, 5.5 Hz), 5.39 (1H, dd, *J*=15.6, 8.8 Hz), 5.57 (1H, dt, *J*=15.6, 5.5 Hz). MS *m/z*: 315 (M⁺-Me), 273 (M⁺-tert-Bu), 215, 75. Anal. Calcd for C₁₇H₃₄O₄Si: C, 61.77; H, 10.37. Found: C, 61.58; H, 10.21.

Methyl (5Z,7E)-8-[(1S,2R)-2-(2-tert-Butyldimethylsilyloxyethyl)-4,4-dimethyl-3,5-dioxacyclohexyl]-5,7-octadienoate (18) To a solution of **17** (440 mg, 1.33 mmol) in CH₂Cl₂ (13 ml) were added sequentially molecular sieves 4A (2.0 g), anhydrous NaOAc (273 mg, 3.33 mmol), and PCC (574 mg, 3.29 mmol) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was diluted with Et₂O and filtered through a short column of Florisil. The filtrate was evaporated to provide the crude aldehyde (392 mg). A suspension of NaH (60% dispersion in mineral oil, 200 mg, 4.98 mmol) in DMSO (4 ml) was stirred at 75 °C for 1 h. To the resulting mixture was added a solution of 4-carboxybutyltriphenylphosphonium bromide (1.08 g, 2.42 ml) in DMSO (4 ml) at room temperature, and the mixture was stirred for 15 min. To the resulting red solution was added a solution of the crude aldehyde in DMSO (4 ml) at room temperature, and the mixture was stirred for 1 h. The reaction mixture was poured into ice-water, and the solution was acidified to pH 3 by the addition of cooled 1 N HCl. The aqueous layer was extracted with Et₂O, and the organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was dissolved in methanol, and the mixture was treated with an ethereal solution of diazomethane at 0 °C. After an excess of diazomethane was decomposed by the addition of acetic acid, the mixture was diluted with Et₂O and washed with sat. NaHCO₃ aq. and brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (chloroform/hexane/ethyl acetate=50:50:2) to afford **18** (374 mg, 66%) as a colorless oil along with (5E,7E)-isomer (90 mg, 14%). [α]_D²² +7.7° (*c*=1.09, CHCl₃). IR (neat) cm⁻¹: 2994, 2953, 2929, 2857, 1740, 1654, 1459, 1437. ¹H-NMR (270 MHz, CDCl₃) δ : 0.04 (6H, s), 0.89 (9H, s), 1.45 (3H, s), 1.38 (3H, s), 1.50 (1H, m), 1.72 (2H, t, *J*=7.5, 7.3 Hz), 1.82 (1H, m), 2.21 (2H, dt, *J*=7.8, 7.5 Hz), 2.27 (1H, m), 2.32 (2H, t, *J*=7.5 Hz), 3.59—3.77 (4H, m), 3.67 (3H, s), 3.84 (1H, ddd, *J*=11.9, 11.9, 2.4 Hz), 5.32 (1H, dd, *J*=15.1, 9.1 Hz), 5.34 (1H, dt, *J*=7.8 Hz), 5.95 (1H, dd, *J*=10.7, 10.7 Hz), 6.36 (1H, dd, *J*=15.1, 11.2 Hz). MS *m/z*: 411 (M⁺-Me), 369 (M⁺-tert-Bu), 351, 311 (M⁺-tert-BuMe₂Si), 281, 219, 180. Anal. Calcd for C₂₃H₄₂O₅Si: C, 64.75; H, 9.92. Found: C, 64.58; H, 10.01.

Methyl (5Z,7E)-8-[(1S,2R)-4,4-Dimethyl-3,5-dioxo-2-(2-hydroxyethyl)-cyclohexyl]-5,7-octadienoate To a solution of **18** (280 mg, 0.66 mmol) in THF (6 ml) was added tetrabutylammonium fluoride (1.0 M in THF, 0.98 ml, 0.98 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. To the mixture was added sat. NH₄Cl aq., and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=2:1) to afford the alcohol (196 mg, 95%) as a colorless oil. [α]_D²⁴ +27.8° (*c*=1.02, CHCl₃). IR (neat) cm⁻¹: 3472, 2995, 2947, 2865, 1738, 1655, 1460, 1437. ¹H-NMR (270 MHz, CDCl₃) δ : 1.41 (3 H, s), 1.49 (3 H, s), 1.58—1.75 (2 H, m), 1.72 (2 H, t, *J*=7.3, 7.2 Hz), 1.86 (1H, m), 2.21 (2H, dt, *J*=7.7, 7.2 Hz), 2.33 (2H, t, *J*=7.4 Hz), 2.38 (1H, m), 2.53 (1H, br s), 3.67 (3H, s), 3.72 (1H, d, *J*=8.2 Hz),

3.70—3.80 (2H, m), 3.98 (1H, ddd, *J*=10.4, 8.8, 2.8 Hz), 5.29 (1H, dd, *J*=15.2, 9.1 Hz), 5.36 (1H, dt, *J*=10.7, 7.7 Hz), 5.94 (1H, dd, *J*=10.5, 9.1 Hz), 6.39 (1H, dd, *J*=15.2, 11.1 Hz). MS *m/z*: 311 (M⁺-H), 297 (M⁺-Me), 237, 180, 106. Anal. Calcd for C₁₇H₂₈O₅: C, 65.36; H, 9.03. Found: C, 65.45; H, 8.95.

Methyl (5Z,7E)-8-[(1S,2R)-4,4-Dimethyl-3,5-dioxo-2-(2-oxoethyl)-cyclohexyl]-5,7-octadienoate (19) To a solution of the above alcohol (70 mg, 0.22 mmol) in CH₂Cl₂ (5 ml) was added Dess–Martin reagent (144 mg, 0.34 mmol) at 0 °C, and the mixture was stirred at the same temperature for 2 h. The reaction mixture was diluted with Et₂O, and filtered. To the filtrate was added a mixture of sat. NaHCO₃ aq. and 10% Na₂S₂O₃ aq. (1:1), and the mixture was stirred at 0 °C for 15 min. The resulting mixture was extracted with Et₂O, and the organic layer was washed with sat. NaHCO₃ aq. and brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=7:1) to afford **19** (59 mg, 84%) as a colorless oil. [α]_D²⁷ +9.7° (*c*=1.12, CHCl₃). IR (neat) cm⁻¹: 2995, 2950, 2862, 2731, 1738, 1729, 1654, 1459, 1433. ¹H-NMR (270 MHz, CDCl₃) δ : 1.31 (3H, s), 1.43 (3H, s), 1.66 (2H, t, *J*=7.5, 7.5 Hz), 2.14 (2H, dt, *J*=7.6, 7.5 Hz), 2.28 (1H, m), 2.26 (2H, t, *J*=7.5 Hz), 2.44 (1H, ddd, *J*=16.3, 7.8, 2.5 Hz), 2.52 (1H, ddd, *J*=16.3, 4.1, 1.7 Hz), 3.60 (3H, s), 3.68 (2H, d, *J*=8.2 Hz), 4.18 (1H, ddd, *J*=10.4, 7.8, 4.1 Hz), 5.21 (1H, dd, *J*=15.2, 9.3 Hz), 5.32 (1H, dt, *J*=10.3, 7.6 Hz), 5.87 (1H, dd, *J*=11.1, 10.3 Hz), 6.33 (1H, dd, *J*=15.2, 11.1 Hz), 9.69 (1H, dd, *J*=2.5, 1.7 Hz). MS *m/z*: 310 (M⁺), 295 (M⁺-Me), 252, 235, 180, 106. Anal. Calcd for C₁₇H₂₆O₅: C, 65.78; H, 8.44. Found: C 65.98, H, 8.21.

(4R,5S)-4-{5-[(1E)-1,3-Butadienyl]-4-(2-tert-butyldimethylsilyloxyethyl)-2,2-dimethyl-1,3-dioxacyclohexane (20) To a solution of **17** (1.50 g, 4.5 mmol) in CH₂Cl₂ (50 ml) were added sequentially molecular sieves 4A (7.0 g), anhydrous NaOAc (744 mg, 9.1 mmol), and PCC (1.96 g, 9.1 mmol) at 0 °C, and the mixture was stirred at the same temperature for 1 h. The reaction mixture was diluted with Et₂O, and filtered through a pad of Florisil. The filtrate was evaporated to provide a crude aldehyde (1.36 g). To a suspension of methyltriphenylphosphonium bromide (1.93 mg, 5.4 mmol) in THF (18 ml) was added BuLi (1.64 M in hexane, 3.3 ml, 5.4 mmol) at 0 °C, and the mixture was stirred at 0 °C for 15 min. To the mixture was added a solution of the above crude aldehyde (1.36 g) in THF (10 ml) at -78 °C. The mixture was warmed to 0 °C over 2 h, and then stirred at the same temperature for 1 h. To the reaction mixture was added sat. NH₄Cl aq., and the aqueous layer was extracted with Et₂O. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=35:1) to afford **20** (1.25 g, 84% from **17**) as a colorless oil. [α]_D²⁰ +19.6° (*c*=1.10, CHCl₃). IR (neat) cm⁻¹: 2994, 2956, 2929, 2856, 1654, 1603, 1471, 1461. ¹H-NMR (270 MHz, CDCl₃) δ : 0.04 (6H, s), 0.89 (9H, s), 1.38 (3H, s), 1.45 (3H, s), 1.40—1.57 (1H, m), 1.77—1.90 (1H, m), 2.20—2.36 (1H, m), 3.60—3.75 (4 H, m), 3.80—3.90 (1H, m), 5.04 (1H, dd, *J*=10.2, 1.9 Hz), 5.16 (1H, dd, *J*=16.7, 1.9 Hz), 5.36 (1H, dd, *J*=15.0, 9.0 Hz), 6.15 (1H, dd, *J*=15.0, 10.4 Hz), 6.27 (1H, dd, *J*=16.7, 10.2, 10.2 Hz). MS *m/z*: 311 (M⁺-Me), 253, 211, 80. Anal. Calcd for C₁₈H₃₄O₃Si: C, 66.21; H, 10.49. Found: C, 66.08; H, 10.41.

2-{(1R,6S)-6-[(1E)-1,3-Butadienyl]-3,3-dimethyl-2,4-dioxacyclohexyl}ethanol To a solution of **20** (365 mg, 1.11 mmol) in THF (6 ml) was added tetrabutylammonium fluoride (1.0 M in THF, 1.7 ml, 1.7 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. To the mixture was added sat. NH₄Cl aq., and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=2.5:1) to afford the alcohol (235 mg, 100%) as a colorless oil. [α]_D²¹ +47.7° (*c*=1.18, CHCl₃). IR (neat) cm⁻¹: 3434, 2994, 2942, 2864, 1659, 1603, 1455, 1418. ¹H-NMR (270 MHz, CDCl₃) δ : 1.40 (3H, s), 1.49 (3H, s), 1.59—1.75 (1H, m), 1.81—1.93 (1H, m), 2.31—2.47 (1H, m), 2.54 (1H, br s), 3.65—3.84 (4H, m), 3.92 (1H, ddd, *J*=9.7, 8.1, 2.6 Hz), 5.07 (1H, dd, *J*=10.2, 1.9 Hz), 5.18 (1H, dd, *J*=16.6, 1.9 Hz), 5.33 (1H, dd, *J*=14.7, 9.1 Hz), 6.16 (1H, ddd, *J*=14.7, 10.3, 1.8 Hz), 6.26 (1H, ddd, *J*=16.6, 10.2, 10.2 Hz). MS *m/z*: 212 (M⁺), 197, 154, 137, 43. Anal. Calcd for C₁₂H₂₀O₃: C, 67.89; H, 9.50. Found: C, 67.64; H, 9.55.

2-{(1R,6S)-6-[(1E)-1,3-Butadienyl]-3,3-dimethyl-2,4-dioxacyclohexyl}ethanal (21) To a solution of the above alcohol (230 mg, 1.08 mmol) in CH₂Cl₂ (11 ml) was added Dess–Martin reagent (690 mg, 1.62 mmol) at 0 °C, and the mixture was stirred at the same temperature for 1 h. The reaction mixture was diluted with Et₂O, and filtered. A mixture of sat. NaHCO₃ aq. and 10% Na₂S₂O₃ aq. (1:1) was added to the filtrate, and the mixture was stirred at 0 °C for 15 min. The mixture was extracted with Et₂O, and the organic layer was washed with sat. NaHCO₃ aq. and brine, dried

over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/ethyl acetate=7:1) to afford **21** (192 mg, 84%) as a colorless oil. $[\alpha]_D^{24} + 30.7^\circ$ ($c=1.14$, CHCl_3). IR (neat) cm^{-1} : 3089, 2993, 2939, 2860, 2727, 1726, 1602, 1460. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 1.38 (3H, s), 1.50 (3H, s), 2.35 (1H, m), 2.45—2.64 (2H, m), 3.75 (2H, d, $J=4.7$ Hz), 4.25 (1H, m), 5.09 (1H, d, $J=10.4$ Hz), 5.19 (1H, d, $J=15.6$ Hz), 5.32 (1H, dd, $J=14.7$, 9.2 Hz), 6.17 (1H, dd, $J=14.7$, 10.4 Hz), 6.24 (1H, ddd, $J=15.6$, 10.4, 10.4 Hz), 9.76 (1H, t, $J=2.4$ Hz). MS m/z : 195 ($\text{M}^+ - \text{Me}$), 167, 149, 122, 80. Anal. Calcd for $\text{C}_{12}\text{H}_{18}\text{O}_3$: C, 68.55; H, 8.63. Found: C, 68.42; H, 8.79.

(1R,6S,7R,8S)-3,3-Dimethyl-2,4-dioxo-7-(2-propenyl)bicyclo[4.3.0]nonan-8-ol (22) (Cyclization of **21**). $\text{Ni}(\text{cod})_2$ (3.5 mg, 0.013 mmol) and PPh_3 (6.6 mg, 0.026 mmol) were dissolved in degassed-toluene (0.33 ml), and the mixture was stirred at 0°C for 20 min. To the mixture was added $^i\text{Bu}_2\text{-ALAC}$ (1.0 M in toluene, 0.38 ml, 0.38 mmol), and the mixture was stirred at room temperature for 15 min. To the mixture was added a solution of **21** (53 mg, 0.25 mmol) in degassed-toluene (6.3 ml) at 0°C , and the mixture was stirred at 0°C for 2 h. The reaction mixture was hydrolyzed with sat. NH_4Cl aqueous solution, and the aqueous layer was extracted with ether. After usual work-up, the residue was purified by silica gel column chromatography (hexane/ AcOEt =2:1) to afford **22** (48 mg, 91%) as a colorless solid, which was recrystallized from hexane/ether (mp $64\text{--}69^\circ\text{C}$). $[\alpha]_D^{20} + 56.3^\circ$ ($c=1.16$, CHCl_3). IR (CHCl_3) cm^{-1} : 3422, 3073, 2944, 2938, 2880, 1639, 1438. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 1.43 (3H, s), 1.45 (3H, s), 1.46—1.63 (2H, m), 1.65—1.80 (1H, m), 1.82 (1H, br d, $J=4.2$ Hz), 2.12—2.26 (1H, m), 2.27—2.40 (1H, m), 2.53 (1H, ddd, $J=13.0$, 7.4, 7.4 Hz), 3.55 (1H, ddd, $J=10.7$, 7.4, 7.4 Hz), 3.71 (1H, dd, $J=10.7$, 10.7 Hz), 4.02 (1H, dd, $J=10.4$, 4.2 Hz), 4.24—4.34 (1H, m), 5.04 (1H, d, $J=10.2$ Hz), 5.14 (1H, d, $J=17.2$ Hz), 5.84 (1H, dddd, $J=17.2$, 10.2, 7.1, 6.3 Hz). $^{13}\text{C-NMR}$ (67.5 MHz, CDCl_3) δ : 19.5, 29.7, 31.6, 39.8, 42.4, 44.6, 65.9, 69.8, 72.7, 99.5, 115.9, 137.4. MS m/z : 213 ($\text{M}^+ + \text{H}$), 197, 171, 155, 137, 59. Anal. Calcd for $\text{C}_{12}\text{H}_{20}\text{O}_3$: C, 67.89; H, 9.50. Found: C, 67.65; H, 9.53.

Transformation of 22 into 25. (1S,2S,6R,8S)-10,10-Dimethyl-5,9,11-trioxatricyclo[6.4.0.0^{2,6}]dodecan-4-ol (23) A solution of **22** (140 mg, 0.66 mmol) in CH_2Cl_2 (10 ml) was cooled to -78°C and treated with a stream of O_3/O_2 at -78°C until the colorless solution had been converted to a blue one. The reaction mixture was treated with a stream of dry O_2 to remove an excess amount of O_3 . To the reaction mixture was added a solution of PPh_3 (345 mg, 1.31 mmol), and the mixture was allowed to stir at ambient temperature for 15 h. After removal of the solvent, the residue was purified by silica gel column chromatography (hexane/ AcOEt =1:1) to give **23** (110 mg, 78%). $[\alpha]_D^{24} + 14.5^\circ$ ($c=1.07$, CHCl_3). IR (CHCl_3) cm^{-1} : 3882, 2980, 2906, 2967. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 1.42 (3H, br s), 1.46 (3H, br s), 1.49—1.71 (1H, m), 1.77—2.07 (3H, m), 2.10—2.32 (1H, m), 2.36—2.52 (1H, m), 3.17—3.30 (1H, m), 3.44—3.80 (2H, m), 3.98—4.10 (1H, m), 4.55—4.70 (1H, m), 5.60—5.71 (1H, m). MS m/z : 215 ($\text{M}^+ + \text{H}$), 199, 59. Anal. Calcd for $\text{C}_{11}\text{H}_{18}\text{O}_4$: C, 61.67; H, 8.46. Found: C, 61.38; H, 8.32.

(1S,2S,6R,8S)-10,10-Dimethyl-5,9,11-trioxatricyclo[6.4.0.0^{2,6}]dodecan-4-one To the solution of **23** (90 mg, 0.42 mmol) in CH_2Cl_2 (5.0 ml) were added MS 4A (540 mg), AcONa (70 mg, 0.84 mmol), and PCC (181 mg, 0.84 mmol) at 0°C , and the mixture was stirred at 0°C for 2 h. The mixture was diluted with ether and filtered through a filtration paper. The filtrate was concentrated, and the residue was purified by silica gel column chromatography (hexane/ AcOEt =1:1) to give the lactone (72 mg, 80%). $[\alpha]_D^{20} - 2.6^\circ$ ($c=1.10$, CHCl_3). IR (CHCl_3) cm^{-1} : 3002, 2947, 2887, 1786, 1447, 1430. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 1.43 (3H, s), 1.46 (3H, s), 1.65 (1H, ddd, $J=11.0$, 11.0, 4.6 Hz), 1.82 (1H, ddd, $J=13.0$, 11.0, 4.5 Hz), 2.30—2.47 (2H, m), 2.50—2.66 (1H, m), 2.69 (1H, dd, $J=18.2$, 9.2 Hz), 3.67 (1H, ddd, $J=11.0$, 11.0, 6.9 Hz), 3.79 (1H, dd, $J=11.0$, 4.5 Hz), 4.05 (1H, dd, $J=11.0$, 4.5 Hz), 4.90 (1H, ddd, $J=7.2$, 7.2, 4.6 Hz). MS m/z : 213 ($\text{M}^+ + \text{H}$), 197, 155, 54. Anal. Calcd for $\text{C}_{11}\text{H}_{16}\text{O}_4$: C, 62.25; H, 7.60. Found: C, 62.43; H, 7.51.

(3aR,4S,5R,6aS)-Hexahydro-5-hydroxy-4-(hydroxymethyl)-2H-cyclopenta[b]furan-2-one (24) The above lactone (28 mg, 0.134 mmol) was dissolved in THF (2.0 ml) and H_2O (0.2 ml), and Dowex 50 $\times$ W8 (5 mg) was added to the mixture. The mixture was stirred at room temperature for 3 h. After the resin was removed by filtration, and the filtrate was concentrated. The residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$ =10:1) to give **24** (23 mg, 100%), whose spectral data were completely identical with those previously reported.^{12a)}

(3aR,4S,5R,6aS)-Hexahydro-5-hydroxy-4-[(tert-butyl)diphenylsilyloxy)methyl]-2H-cyclopenta[b]furan-2-one (25) To a solution of **24** (50 mg, 0.29 mmol) in DMF (2.0 ml) was added imidazole (30 mg, 0.44 mmol) and *tert*-butyldiphenylchlorosilane (0.09 ml, 0.35 mmol) at 0°C , and the

mixture was stirred at 0°C for 2 h. After usual work-up, the residue was purified by silica gel column chromatography (hexane/ AcOEt 3:2) to give **25** (107 mg, 90%), whose spectral data were completely identical with those previously reported.^{12b)}

Methyl (5Z)-7-[(1R,6S,7R,8S)-3,3-Dimethyl-2,4-dioxo-8-hydroxybicyclo[4.3.0]nonan-7-yl]-5-heptenoate (26) (Cyclization of **19**). $\text{Ni}(\text{cod})_2$ (4.8 mg, 0.017 mmol) and PPh_3 (9.1 mg, 0.034) were dissolved in degassed-THF (4.3 ml), and the mixture was stirred at 0°C for 20 min. To the mixture were added 1,3-CHD (0.004 ml, 0.04 mmol) and a solution of **19** (54 mg, 0.173 mmol) in degassed-THF (4.3 ml) at 0°C , and then $^i\text{Bu}_2\text{-ALAC}$ (0.25 M in toluene, 1.0 ml, 0.25 mmol) was slowly added to the mixture over a period of about 2 h at 25°C . The mixture was stirred at 25°C for 6 h. To the mixture was added sat. potassium sodium tartrate aqueous solution, and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with brine, and dried over Na_2SO_4 . After removal of the solvent, the residue was purified by silica gel column chromatography (hexane/ AcOEt =2:1) to give **26** (30 mg, 54%). $[\alpha]_D^{27} + 41.1^\circ$ ($c=1.07$, CHCl_3). IR (neat) cm^{-1} : 3469, 2990, 2944, 2864, 1735, 1652. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 1.43 (3H, s), 1.45 (3H, s), 1.45—1.69 (2H, m), 1.66—1.77 (3H, m), 1.78 (1H, br s), 2.07—2.18 (3H, m), 2.30 (1H, m), 2.32 (2H, t, $J=7.3$ Hz), 2.53 (1H, ddd, $J=13.1$, 7.5, 7.4 Hz), 3.54 (1H, ddd, $J=10.5$, 10.5, 7.5 Hz), 3.67 (3H, s), 3.72 (1H, dd, $J=10.7$, 10.7 Hz), 4.01 (1H, dd, $J=10.7$, 4.4 Hz), 4.25 (1H, ddd, $J=6.8$, 6.8, 4.4 Hz), 5.36—5.43 (2H, m). $^{13}\text{C-NMR}$ (67.5 MHz, CDCl_3) δ : 19.5, 24.6, 25.1, 26.6, 29.7, 33.3, 39.9, 43.3, 44.6, 51.4, 65.9, 69.7, 127.7, 99.4, 128.8, 129.9, 173.9. MS m/z : 312 (M^+), 297 ($\text{M}^+ - \text{Me}$), 279, 187, 43. Anal. Calcd for $\text{C}_{17}\text{H}_{28}\text{O}_5$: C, 65.36; H, 9.03. Found: C, 65.59; H, 9.28.

Transformation of 22 into 26¹³⁾. (1R,6S,7R,8S)-8-tert-Butyldimethylsilyloxy-3,3-dimethyl-2,4-dioxo-7-(2-propenyl)-bicyclo[4.3.0]nonane To a solution of **22** (71 mg, 0.334 mmol) in DMF (2.0 ml) were added imidazole (45 mg, 0.68 mmol) and *tert*-butyldimethylsilyl chloride (76 mg, 0.50 mmol) at room temperature, and the mixture was stirred for 7 h. After the usual workup, the residue was purified by silica gel column chromatography (hexane/ethyl acetate=20:1) to afford the silyl ether (95 mg, 87%) as a colorless oil. $[\alpha]_D^{31} + 58.7^\circ$ ($c=1.29$, CHCl_3). IR (neat) cm^{-1} : 2991, 2956, 2923, 2890, 2855, 1734, 1636, 1472, 1462. $^1\text{H-NMR}$ (270 MHz, CDCl_3) δ : 0.02 (3H, s), 0.03 (3H, s), 0.88 (9H, s), 1.42 (3H, s), 1.44 (3H, s), 1.42—1.58 (2H, m), 1.75 (1H, m), 2.09 (1H, m), 2.30 (1H, m), 2.41 (1H, ddd, $J=12.6$, 7.4, 7.1 Hz), 3.52 (1H, ddd, $J=10.7$, 10.5, 7.4 Hz), 3.68 (1H, dd, $J=10.8$, 10.8 Hz), 4.05 (1H, dd, $J=10.8$, 4.4 Hz), 4.20 (1H, ddd, $J=7.1$, 6.7, 4.3 Hz), 4.94 (1H, d, $J=10.1$ Hz), 5.03 (1H, d, $J=17.4$ Hz), 5.77 (1H, dddd, $J=17.4$, 10.1, 7.9, 5.9 Hz). MS m/z : 325 ($\text{M}^+ - \text{H}$), 311 ($\text{M}^+ - \text{Me}$), 269 ($\text{M}^+ - \text{tert-Bu}$), 251, 211, 75. Anal. Calcd for $\text{C}_{18}\text{H}_{34}\text{O}_3\text{Si}$: C, 66.21; H, 10.49. Found: C, 66.14; H, 10.78.

Methyl (5Z)-7-[(1R,6S,7R,8R)-8-Acetoxy-3,3-dimethyl-2,4-dioxabicyclo[4.3.0]nonan-7-yl]-5-heptenoate A solution of the above silyl ether (44 mg, 0.134 mmol) in CH_2Cl_2 (3 ml) was cooled to -78°C and treated with a stream of O_3/O_2 at -78°C until the colorless solution had been converted to a blue one. The reaction mixture was treated with a stream of dry O_2 to remove an excess amount of O_3 . To the reaction mixture was added a solution of PPh_3 (42 mg, 0.160 mmol) in CH_2Cl_2 (2 ml), and the mixture was allowed to stir at ambient temperature for 12 h. After removal of the solvent, the residue was filtered through a short column of silica gel (hexane/ Et_2O =10:1) in order to remove triphenylphosphine oxide giving the crude aldehyde. A suspension of potassium *tert*-butoxide (57 mg, 0.511 mmol) and 4-carboxybutyltriphenyl-phosphonium bromide (113 mg, 0.255 mmol) in THF (1.5 ml) was stirred at room temperature for 1 h. To the mixture was added a solution of the crude aldehyde (42 mg) in THF (1.5 ml), and the mixture was stirred for 15 min. After being cooled to 0°C , the reaction mixture was diluted with Et_2O , and the solution was acidified (*ca.* pH 3) by the addition of cooled 1 N HCl aq., and the aqueous layer was extracted with Et_2O . The organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was dissolved in methanol, and the mixture was treated with ethereal solution of diazomethane at 0°C . After the usual workup, the residue was purified by silica gel column chromatography (hexane/ethyl acetate=12:1) to afford the methyl ester (45.6 mg, 80%, 3 steps) as a colorless oil. $[\alpha]_D^{24} + 46.1^\circ$ ($c=1.00$, CHCl_3). IR (neat) cm^{-1} : 2991, 2956, 2923, 2892, 2852, 1740, 1474, 1462, 1436. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 0.03 (6H, s), 0.88 (9H, s), 1.42 (3H, s), 1.42 (1H, m), 1.43 (3H, s), 1.52 (1H, ddd, $J=12.5$, 10.5, 4.4 Hz), 1.64—1.77 (3H, m), 2.03—2.10 (2H, m), 2.11—2.22 (2H, m), 2.31 (2H, t, $J=7.5$ Hz), 2.41 (1H, ddd, $J=12.5$, 7.5, 7.5 Hz), 3.50 (1H, ddd, $J=10.5$, 10.5, 7.4 Hz), 3.67 (3H, s), 3.68 (1H, dd, $J=11.0$, 10.7 Hz), 4.01 (1H, dd, $J=10.7$, 4.4 Hz), 4.19 (1H, ddd, $J=6.7$, 6.7, 4.2 Hz), 5.28—5.40 (2H, m). MS m/z : 426 (M^+), 411 ($\text{M}^+ - \text{Me}$), 395 ($\text{M}^+ - \text{OMe}$),

369 (M^+ —*tert*-Bu). *Anal.* Calcd for $C_{23}H_{42}O_5Si$: C, 64.75; H, 9.92. Found: C, 64.91; H, 9.61.

To a solution of the above methyl ester (40 mg, 0.093 mmol) in THF (1.5 ml) was added tetrabutylammonium fluoride (1.0 M in THF, 0.14 ml, 0.14 mmol) at 0 °C, and the mixture was stirred at room temperature for 3 h. After the usual workup, the residue was purified by silica gel column chromatography (hexane/ethyl acetate=2:1) to afford the corresponding alcohol (27 mg, 92%) as a colorless oil, whose spectral data were completely identical with those of **26** derived from **19**.

Synthesis of PGF_{2α} from 26. **Methyl (5Z)-7-[(1R,6S,7R,8R)-8-acetoxy-3,3-dimethyl-2,4-dioxabicyclo[4.3.0]nonan-7-yl]-5-heptenoate** To a solution of **26** (115 mg, 0.368 mmol) in CH_2Cl_2 (3.5 ml) were added sequentially pyridine (0.25 ml, 2.21 mmol), DMAP (2 mg, 0.016 mmol), and acetic anhydride (0.11 ml, 1.10 mmol) at 0 °C, and the mixture was stirred at room temperature for 2 h. To the mixture was added sat. $NaHCO_3$ aq., and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with sat. $NaHCO_3$ aq. and brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt=4:1) to afford the acetate (124 mg, 95%) as a colorless oil. $[\alpha]_D^{28} +44.3^\circ$ ($c=1.04$, $CHCl_3$). IR (neat) cm^{-1} : 2992, 2943, 2898, 2863, 1737, 1457, 1436. ^1H -NMR (270 MHz, $CDCl_3$) δ : 1.43 (3H, s), 1.45 (3H, s), 1.46—1.82 (6H, m), 2.01—2.19 (3H, m), 2.05 (3H, s), 2.31 (2H, t, $J=7.4$ Hz), 2.59 (1H, ddd, $J=13.4$, 7.7, 7.4 Hz), 3.59 (1H, ddd, $J=10.3$, 10.3, 7.7 Hz), 3.67 (3H, s), 3.73 (1H, dd, $J=10.8$, 10.8 Hz), 4.04 (1H, dd, $J=10.8$, 4.1 Hz), 5.18 (1H, ddd, $J=6.7$, 6.7, 4.2 Hz), 5.23—5.42 (2H, m). MS m/z : 355 (M^+ +H), 339 (M^+ —Me), 323 (M^+ —OMe), 312 (M^+ —Ac), 296, 279, 266, 43. *Anal.* Calcd for $C_{30}H_{48}O_6$: C, 64.39; H, 8.53. Found: C, 64.72; H, 8.90.

Methyl (5Z)-7-[(1R,2S,3R,5R)-5-Acetoxy-2-(*tert*-butyldiphenylsilyloxy-methyl)-3-hydroxycyclopentyl]-5-heptenoate To a solution of the above acetate (124 mg, 0.349 mmol) in methanol (3.5 ml) was added Dowex 50 $\times$ 8 (6 mg), and the mixture was stirred at 50 °C for 30 min. After the mixture was cooled to room temperature, the resin was removed by filtration. The filtrate was evaporated to obtain the crude diol (108 mg), which was dissolved in CH_2Cl_2 (3.5 ml), and to the mixture were added sequentially Et_3N (0.11 ml, 1.06 mmol), DMAP (2 mg, 0.016 mmol), and *tert*-Butyldiphenylsilyl chloride (0.20 ml, 0.77 mmol) at room temperature. After the mixture was stirred at the same temperature for 36 h, sat. $NaHCO_3$ aq. was added to the solution, and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt=2:1) to afford the TBDPS ether (173 mg, 90%, 2 steps) as a colorless oil. $[\alpha]_D^{27} +25.6^\circ$ ($c=1.08$, $CHCl_3$). IR (neat) cm^{-1} : 3466, 3075, 3010, 2930, 2685, 1736, 1652, 1588, 1475, 1426. ^1H -NMR (270 MHz, $CDCl_3$) δ : 1.06 (9H, s), 1.62 (2H, tt, $J=7.5$, 7.4 Hz), 1.70—1.86 (2H, m), 1.87—2.18 (5H, m), 2.05 (3H, s), 2.19—2.40 (2H, m), 2.25 (2H, t, $J=7.5$ Hz), 3.59 (1H, dd, $J=10.1$, 6.9 Hz), 3.63 (3H, s), 3.89 (1H, d, $J=10.1$, 4.1 Hz), 4.21 (1H, m), 5.15 (1H, ddd, $J=5.2$, 5.2, 2.4 Hz), 5.18—5.34 (2H, m), 7.35—7.49 (6H, m), 7.62—7.69 (4H, m). MS m/z : 521 (M^+ —OMe), 496, 435, 199. *Anal.* Calcd for $C_{37}H_{44}O_6Si$: C, 69.53; H, 8.02. Found: C, 69.76; H, 7.85.

Methyl (5Z)-7-[(1R,2S,3R,5R)-5-Acetoxy-2-(*tert*-butyldiphenylsilyloxy-methyl)-3-(2-oxacyclohexyl)oxy-cyclopentyl]-5-heptenoate To a solution of the above TBDPS ether (155 mg, 0.280 mmol) in CH_2Cl_2 (1.5 ml) were added 2,3-dihydropyran (0.10 ml, 1.12 mmol) and pyridinium *p*-toluenesulfonate (3.5 mg, 0.014 mmol) at room temperature, and the mixture was stirred for 12 h. To the solution was added sat. $NaHCO_3$ aq., and the aqueous layer was extracted with Et_2O , and the organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt=5:1) to afford the THP ether (178 mg, 100%) as a colorless oil. $[\alpha]_D^{29} +13.9^\circ$ ($c=1.20$, $CHCl_3$). IR (neat) cm^{-1} : 3063, 2939, 2856, 1737, 1557, 1472, 1428. ^1H -NMR (270 MHz, $CDCl_3$) δ : 1.05 (9H, s), 1.40—2.38 (16H, m), 2.03 (3H, s), 2.26 (2H, t, $J=7.7$ Hz), 3.39 (1H, m), 3.60—3.93 (3H, m), 3.65 (3H, s), 4.10 (1/2H, m), 4.30 (1/2H, m), 4.47 (1/2H, m), 4.54 (1/2H, m), 5.08 (1H, m), 5.25—5.40 (2H, m), 7.32—7.47 (6H, m), 7.61—7.70 (4H, m). MS m/z : 605 (M^+ —OMe), 579 (M^+ —*tert*-Bu), 552, 521, 495, 435, 85. *Anal.* Calcd for $C_{37}H_{52}O_7Si$: C, 69.78; H, 8.23. Found: C, 69.97; H, 8.14.

Methyl (5Z)-7-[(1R,2S,3R,5S)-5-Acetoxy-2-hydroxymethyl-3-[(2-oxacyclohexyl)oxy]cyclopentyl]-5-heptenoate (28) A solution of the above THP ether (175 mg, 0.274 mmol) in THF (3 ml) was treated with tetrabutylammonium fluoride (1.0 M in THF, 0.41 ml, 0.41 mmol) at 0 °C for 16 h. After the usual workup, the residue was purified by silica gel column chromatography (hexane/AcOEt 1:1) to afford **28** (110 mg, 100%) as a colorless oil. $[\alpha]_D^{28} +31.9^\circ$ ($c=1.05$, $CHCl_3$). IR (neat) cm^{-1} : 3648, 2944, 2867, 1736, 1654, 1453, 1437. ^1H -NMR (270 MHz, $CDCl_3$) δ : 1.26—2.45 (17H, m),

2.06 (3H, s), 2.32 (2H, t, $J=6.4$ Hz), 3.44—3.69 (2H, m), 3.68 (3H, s), 3.74—4.18 (3H, m), 4.57 (1/2H, m), 4.72 (1/2H, m), 5.06 (1H, m), 5.31—5.46 (2H, m). MS m/z : 398 (M^+), 367 (M^+ —OMe), 314, 283, 43. *Anal.* Calcd for $C_{21}H_{34}O_7$: C, 63.30; H, 8.60. Found: C, 63.19; H, 8.32.

Methyl (5Z)-7-[(1R,2R,3R,5R)-5-Acetoxy-3-(2-oxacyclohexyl)oxy-2-[(1E)-3-oxo-1-octenyl]cyclopentyl]-5-heptenoate To a solution of **28** (50 mg, 0.125 mmol) in CH_2Cl_2 (2.5 ml) were added sequentially MS 4A (280 mg), anhydrous NaOAc (31 mg, 0.38 mmol), and PCC (82 mg, 0.38 mmol) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was diluted with Et_2O , and filtered through a short column of Florisil. The filtrate was evaporated to afford the crude aldehyde. To a suspension of NaH (60% dispersion in mineral oil, 7.5 mg, 0.188 mmol) in THF (1.0 ml) was added a solution of dimethyl 2-oxoheptylphosphonate (42 mg, 0.188 mmol) in THF (1.0 ml) at room temperature. After the mixture was stirred for 5 min, a solution of the crude aldehyde in THF (1.0 ml) was added to the mixture, and the mixture was stirred for 1 h. To the reaction mixture was added sat. NH_4Cl aq., and the aqueous layer was extracted with Et_2O . The organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt=4:1) to afford the α,β -unsaturated ketone (47 mg, 76%) as a colorless oil. $[\alpha]_D^{28} +41.8^\circ$ ($c=1.00$, $CHCl_3$). IR (neat) cm^{-1} : 2946, 2867, 1737, 1699, 1672, 1629, 1454, 1436. ^1H -NMR (270 MHz, $CDCl_3$) δ : 0.89 (3H, t, $J=6.4$ Hz), 1.23—1.40 (4H, m), 1.40—1.90 (12H, m), 1.90—2.30 (4H, m), 2.06 (3H, s), 2.28 (2H, t, $J=7.5$ Hz), 2.36—2.80 (2H, m), 2.54 (2H, t, $J=9.0$ Hz), 3.43 (1H, m), 3.66 (3H, s), 3.77 (1H, m), 4.02 (1.2H, m), 4.10 (1/2H, m), 4.55 (1H, m), 5.09 (1H, m), 5.23—5.42 (2H, m), 6.21 (1/2H, d, $J=15.8$ Hz), 6.25 (1/2H, d, $J=15.8$ Hz), 6.70 (1/2H, dd, $J=15.8$, 9.1 Hz), 6.73 (1/2H, dd, $J=15.8$, 8.8 Hz). MS m/z : 492 (M^+), 461 (M^+ —OMe), 408, 348, 207. *Anal.* Calcd for $C_{28}H_{44}O_7$: C, 68.27; H, 9.00. Found: C, 68.16; H, 9.26.

Methyl (5Z)-7-[(1R,2R,3R,5S)-5-Acetoxy-2-[(1E,3S)-3-hydroxy-1-octenyl]-3-[(2-oxacyclohexyl)oxy]cyclopentyl]-5-heptenoate (29) To a solution of $LiAlH_4$ (1.0 M in THF, 0.41 ml, 0.41 mmol) were added ethanol (1.0 M in THF, 0.41 ml, 0.41 mmol) and (*S*)-binaphthol (118 mg, 0.41 mmol) in THF (1.5 ml) at 0 °C. After the mixture was stirred at room temperature for 1 h, a solution of the above α,β -unsaturated ketone (58 mg, 0.118 mmol) in THF (1.5 ml) was added to the resulting solution of (*S*)-BINAL-H at -100 °C, and the mixture was stirred at the same temperature for 1 h. The solution was allowed to warm to -78 °C and stirred for 1 h. To the mixture were added successively methanol and sat. NH_4Cl aq., and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with sat. NH_4Cl aq. and brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (hexane/AcOEt=2:1) to afford **29** (54 mg, 92%) as a colorless oil. $[\alpha]_D^{29} +37.8^\circ$ ($c=1.05$, $CHCl_3$). IR (neat) cm^{-1} : 3467, 2932, 2860, 1737, 1656, 1452, 1437. ^1H -NMR (270 MHz, $CDCl_3$) δ : 0.90 (3H, t, $J=6.7$ Hz), 1.20—1.90 (21H, m), 1.90—2.18 (5H, m), 2.06 (3H, s), 2.30 (2H, t, $J=7.4$ Hz), 2.33—2.60 (2H, m), 3.45 (1H, m), 3.78—4.06 (2H, m), 4.13 (1H, m), 4.60 (1/2H, m), 4.68 (1/2H, m), 5.08 (1H, m), 5.28—5.42 (2H, m), 5.49—5.74 (2H, m). MS m/z : 476 (M^+ — H_2O), 463 (M^+ —OMe), 410, 332, 191, 67. *Anal.* Calcd for $C_{28}H_{46}O_7$: C, 68.00; H, 9.37. Found: C, 68.22; H, 9.45.

Methyl (5Z)-7-[(1R,2R,3R,5R)-5-Acetoxy-3-hydroxy-2-[(1E,3S)-3-hydroxy-1-octenyl]cyclopentyl]-5-heptenoate A solution of **29** (54 mg, 0.109 mmol) in $AcOH-H_2O-THF$ (19:11:3, 1.5 ml) was stirred at 40 °C for 2.5 h, and the solution was diluted with water, and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (chloroform/methanol=20:1) to afford the diol (42 mg, 93%) as colorless crystals. $[\alpha]_D^{25} +36.2^\circ$ ($c=1.00$, $CHCl_3$). IR ($CHCl_3$) cm^{-1} : 3415, 3017, 2955, 2931, 2859, 1731, 1654, 1636, 1437. ^1H -NMR (270 MHz, $CDCl_3$) δ : 0.88 (3H, t, $J=6.6$ Hz), 1.17—1.72 (12H, m), 1.90—2.15 (5H, m), 2.05 (3H, s), 2.29 (2H, t, $J=7.5$ Hz), 2.32 (1H, m), 2.52 (1H, ddd, $J=15.1$, 8.8, 6.1 Hz), 2.68 (1H, br s), 3.67 (3H, s), 3.88 (1H, m), 4.08 (1H, m), 5.10 (1H, ddd, $J=5.9$, 5.9, 2.1 Hz), 5.25—5.41 (2H, m), 5.44 (1H, dd, $J=15.3$, 8.8 Hz), 5.61 (1H, dd, $J=15.3$, 7.1 Hz). MS m/z : 410 (M^+), 392, 368, 332, 43. *Anal.* Calcd for $C_{23}H_{38}O_6$: C, 67.29; H, 9.33. Found: C, 67.38; H, 9.43.

Prostaglandin F_{2α} To a solution of the above diol (42 mg, 0.085 mmol) in methanol (1.0 ml) and THF (0.5 ml) was added 1.0 M NaOH aq. (0.26 ml, 0.26 mmol) at room temperature, and the solution was stirred for 2 h. To the solution was added 1.0 M HCl aq. (0.3 ml, 0.3 mmol) at 0 °C, and the aqueous layer was extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 , and concentrated. The residue was purified by silica gel column chromatography (chloroform/methanol/acetic acid=100:10:1) to afford PGF_{2α} (29 mg, 95%), whose spectral data were com-

pletely identical with those previously reported.¹⁶⁾ $[\alpha]_D^{29} + 22.7^\circ$ ($c = 1.59$, THF). IR (neat) cm^{-1} : 3365, 959, 2930, 2860, 1729, 1705, 1577. $^1\text{H-NMR}$ (270 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD} = ca. 10/1$) δ : 0.88 (3H, t, $J = 6.6$ Hz), 0.88 (3H, t, $J = 6.6$ Hz), 1.20–1.78 (12H, m), 1.88–2.37 (9H, m), 3.90 (1H, m), 4.02 (1H, m), 4.14 (1H, m), 5.29–5.50 (3H, m), 5.52 (1H, dd, $J = 15.2, 6.6$ Hz). $^{13}\text{C-NMR}$ (67.5 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD} = ca. 10/1$) δ : 13.8, 22.4, 24.8, 25.0, 25.0, 26.3, 31.5, 33.8, 36.7, 42.2, 50.0, 55.0, 72.0, 72.8, 77.2, 128.9, 129.5, 132.7, 134.9, 178.3. MS m/z : 354 (M^+), 336 ($\text{M}^+ - \text{H}_2\text{O}$), 318 ($\text{M}^+ - 2\text{H}_2\text{O}$), 300 ($\text{M}^+ - 3\text{H}_2\text{O}$), 264, 43.

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References and Notes

- For reviews, see: a) Fischer K., Jonas K., Misbach P., Stabba R., Wilke G., *Angew. Chem., Int. Ed. Engl.*, **12**, 943–953 (1973); b) Heimback P., *Angew. Chem., Int. Ed. Engl.*, **12**, 975–989 (1973); c) Wilke G., *Angew. Chem. Int. Ed. Engl.*, **27**, 185–206 (1988).
- For [4+4] cycloadditions of bis-1,3-dienes, see: a) Wender P. A., Tebbe M. J., *Synthesis* **1991**, 1089–1094; b) Wender P. A., Ihle N. C., Correia, C. R. D., *J. Am. Chem. Soc.*, **110**, 5904–5906 (1988); c) Wender P. A., Ihle N. C., *Tetrahedron Lett.*, **28**, 2451–2454 (1987); d) Wender P. A., Snapper M. L., *ibid.*, **28**, 2221–2224 (1987); e) Wender P. A., Ihle N. C., *J. Am. Chem. Soc.*, **108**, 4678–4679 (1986). For [4+2] cycloadditions of 1,3-diene with tethered alkyne, see: f) Wender P. A., Smith T. E., *Tetrahedron*, **54**, 1255–1275 (1998); g) Wender P. A., Smith T. E., *J. Org. Chem.*, **61**, 824–825 (1996); h) Wender P. A., Smith T. E., *ibid.*, **60**, 2962–2963 (1995); i) Wender P. A., Jenkins T. E., *J. Am. Chem. Soc.*, **111**, 6432–6434 (1989). For cyclization of 1,3-diene with tethered allene, see: j) Wender P. A., Jenkins T. E., Suzuki S., *J. Am. Chem. Soc.*, **117**, 1843–1844 (1995). For other examples, see: k) Tamao K., Kobayashi K., Ito Y., *Synlett.* **1992**, 539–546; l) Tamao K., Kobayashi K., Ito Y., *J. Synth. Org. Chem. Jpn.*, **48**, 381–388 (1990); m) Montgomery J., Oblinger E., Savchenko A. V., *J. Am. Chem. Soc.*, **119**, 4911–4920 (1997).
- a) Sato Y., Takimoto M., Hayashi K., Katsuhara T., Takagi K., Mori M., *J. Am. Chem. Soc.*, **116**, 9771–9772 (1994); b) Sato Y., Takimoto M., Mori M., *Tetrahedron Lett.*, **37**, 887–890 (1996); c) Sato Y., Saito N., Mori M., *ibid.*, **38**, 3931–3934 (1997); d) *Idem*, *Tetrahedron*, **54**, 1153–1168 (1998); e) Sato Y., Takanashi T., Hoshiba M., Mori M., *Tetrahedron Lett.*, **39**, 5579–5582 (1998); f) Sato Y., Takanashi T., Mori M., *Organometallics*, **18**, 4891–4893 (1999); g) Sato Y., Takimoto M., Mori M., *J. Am. Chem. Soc.*, **122**, 1624–1634 (2000). h) Sato Y., Saito N., Mori M., *ibid.*, **122**, 2371–2373 (2000).
- The total synthesis of $\text{PGF}_{2\alpha}$ via the cyclization of 1,3-diene and tethered aldehyde using a stoichiometric amount of nickel complex has previously been communicated; Sato Y., Takimoto M., Mori M., *Synlett*, **1997**, 734–736.
- a) Corey E. J., Cheng X.-M., “The Logic of Chemical Synthesis,” Wiley, New York, 1989, pp. 249–309 and references cited therein; b) Mitra A., “The Synthesis of Prostaglandins,” Wiley, New York, 1977; c) Bindra J. S., Bindra R., “Prostaglandin Synthesis,” Academic Press, New York, 1977.
- All numbering used in Scheme 2 indicates the positions of the carbons in the eventual $\text{PGF}_{2\alpha}$.
- Collman J. P., Hegedus L. S., Norton J. R., Finke R. G., “Principles and Applications of Organotransition Metal Chemistry,” University Science Books, Mill Valley, California, 1987, p. 175.
- The epoxy-alcohol **15** was obtained in 94% yield (91% ee) by Sharpless epoxidation of 6-(*tert*-butyldimethylsiloxy)-2-pentenol using $\text{Ti}(\text{O}^i\text{Pr})_4$ (6 mol %), (–)-diethyl tartrate (8 mol %), and $t\text{BuOOH}$ (2.0 equiv) in the presence of MS 4A in CH_2Cl_2 at -20°C .
- Tius M. A., Fauq A. H., *J. Org. Chem.*, **48**, 4131–4132 (1983).
- In this reaction, the 1,2-diol, formed by nucleophilic attack of vinyl cuprate at the C-3 position in **15**, was also obtained in 17% yield, which was easily separated from the desired 1,3-diol by silica gel column chromatography.
- Corey E. J., Weinshenker N. M., Schaaf T. K., Huber W., *J. Am. Chem. Soc.*, **91**, 5675–5677 (1969).
- a) Corey E. J., Shirahama H., Yamamoto H., Terashima S., Venkateswarlu A., Schaaf T. K., *J. Am. Chem. Soc.*, **93**, 1490–1491 (1971); b) Collington E. W., Wallis C. J., Waterhouse I., *Tetrahedron Lett.*, **24**, 3125–3128 (1983).
- To confirm the stereochemistry of **26**, **22** was converted into **26** as shown in Chart 8. All of the spectral data of the product derived from **22** were completely identical with those obtained by the cyclization of **19**.
- Schaaf T. K., Corey E. J., *J. Org. Chem.*, **37**, 2921–2922 (1972).
- a) Noyori R., Tomino I., Tanimoto Y., Nishizawa M., *J. Am. Chem. Soc.*, **106**, 6709–6716 (1984). b) Noyori R., Tomino I., Yamada M., Nishizawa M., *ibid.*, **106**, 6717–6725 (1984).
- The synthesized $\text{PGF}_{2\alpha}$ showed $[\alpha]_D^{29} + 22.7^\circ$ ($c = 1.59$, THF), which agreed with the reported $[\alpha]_D^{25} + 23.5^\circ$ ($c = 1.00$, THF),¹⁷⁾ and its $^{13}\text{C-NMR}$ spectrum was identical to those reported elsewhere.¹⁸⁾ The spectral data of $\text{PGF}_{2\alpha}$ methyl ester, obtained by treatment of synthetic $\text{PGF}_{2\alpha}$ with diazomethane, were also completely identical to those reported previously.¹⁹⁾
- Corey E. J., Schaaf T. K., Huber W., Koelliker U., Weinshenker N. M., *J. Am. Chem. Soc.*, **92**, 397–398 (1970).
- Lukacs G., Piriou F., Gero S. D., Van Dorp D. A., *Tetrahedron Lett.* **1973**, 515–518.
- Suzuki M., Yanagisawa A., Noyori R., *J. Am. Chem. Soc.*, **110**, 4718–4726 (1988).

Studies on the Synthesis of Myriaporones: Stereoselective Synthesis of the C5–C13 Fragment Starting from D-Glucose *via* Regioselective Reductive Opening of Methoxybenzylidene Acetal

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A stereoselective synthesis is described of the C5–C13 fragment (4) of myriaporone 4 (1) starting from D-glucose by a coupling of the C5–C9 aldehyde (5), prepared using a regioselective reductive ring-opening of methoxybenzylidene acetal, with the C10–C13 iodoolefin (6).

Key words stereoselective synthesis; marine product; protecting group; benzylidene acetal

Myriaporone 4 (1), a cytotoxic marine product (IC_{50} =100 ng/ml vs. L-1210), was isolated as an equilibrium mixture with myriaporone 3 (2) from a bryozoan, *Myriapora truncata*, by Rinehart in 1995.¹⁾ The synthesis of 1 is of great interest because of its structural similarity to the C10–C23 part of the marine sponge macrolide, tedanolide (3),²⁾ the synthesis of which is currently under study by several research groups.³⁾ Recently, we reported the synthesis of the C13–C23 part of 3 starting from (*R*)- and (*S*)-3-hydroxy-2-methylpropionates,^{3f)} and this synthetic methodology can be applied to the synthesis of 1. However, we are now investigating an alternative approach by the use of an intermediate (8) in the synthetic work of erythronolide A,⁴⁾ and this report presents a synthesis of the C5–C13 fragment (4) of 1,⁵⁾ corresponding to the C13–C21 part of 3, starting from D-glucose (9)⁶⁾ *via* the regioselective reductive opening of methoxybenzylidene (MP) acetals.⁷⁾

A retrosynthetic analysis of 1 is shown in Chart 1. The key intermediate is 4, from which 1 will be derived *via* elongation of the C1–C4 and C14–C15 units and epoxidation of the C10–C11 double bond, and 4 was planned to be synthesized by coupling between the C5–C9 aldehyde (5) and the known C10–C13 iodoalkene (6).^{3f,8)} The synthesis of 5 would be achieved starting from 9 *via* 8, and attention was paid to the construction of the C6 chiral center and the regioselective opening of the MP acetal of 11 (see Chart 2).

During the course of synthetic work on erythronolide A, the olefin (8) was already synthesized starting from 9 *via* the secondary alcohol (12).⁴⁾ A stereoselective hydroboration of 8 was presumed to be a good way to give 5. However, it was required to improve the conversion of 10,⁴⁾ readily available from 9, into 12, since the overall yield of three steps (tosylation, epoxidation, MPMONa treatment) was only 39%, which is too low to complete the total synthesis of 1.

Direct and selective protection of the primary alcohol of

10 was first examined by treatment with 4-methoxybenzyl chloride (MPMCl) and silver oxide (Ag_2O),⁹⁾ but only a poor result to give 12 in 32% yield was obtained. Quite recently, we reported a facile reductive opening of MP acetals with tri-*n*-butyltin hydride (Bu_3SnH) in the presence of magnesium bromide ($MgBr_2$).⁷⁾ This method is characterized by the reverse regioselectivity to the usual selectivity of reduction mainly with diisobutylaluminum hydride (DIBALH). When a

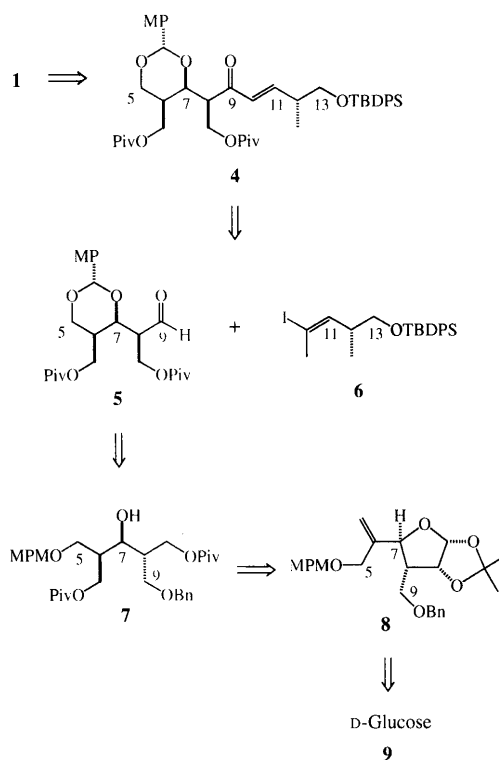


Chart 1

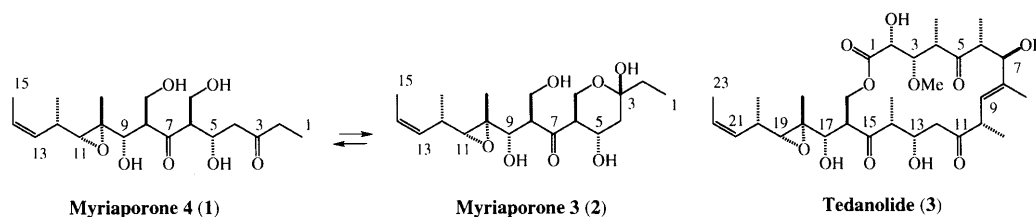


Fig. 1

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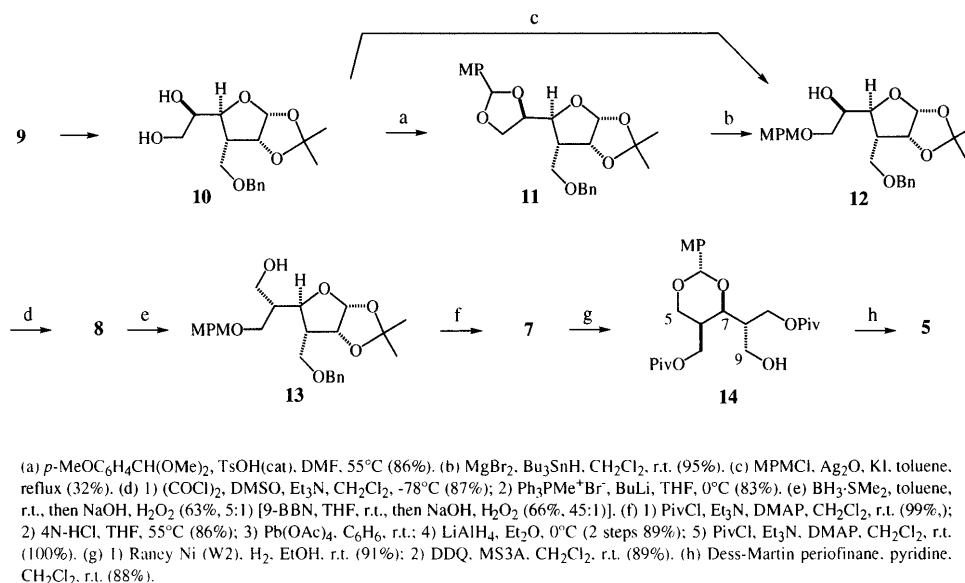


Chart 2

5 : 4 mixture of MP acetals (**11**), readily converted from **10** in the usual way, was treated with Bu₃SnH and MgBr₂ in dichloromethane (CH₂Cl₂) at room temperature, complete regioselective reduction proceeded quite smoothly to give the expected **12** in 95% yield.⁷⁾ Swern oxidation of **12** followed by Wittig methylenation easily gave **8**. Hydroboration of **8** with a borane dimethyl sulfide complex (BH₃·Me₂S) in toluene gave a 5 : 1 mixture of the expected alcohol (**13**) and its C6 isomer. The reaction with 9-borabicyclo[3.3.1]nonane (9-BBN) took place slowly and **13** was isolated with excellent stereoselectivity (45 : 1).¹⁰⁾ Although its selectivity was not so good, BH₃·Me₂S was useful in practical syntheses. After protection of the primary alcohol of crude **13** with a pivaloyl (Piv) group, selective hydrolysis of the acetonide was carefully examined under several acidic conditions, and a good result was obtained when the pivaloate was warmed at 50 °C with 4 N HCl in THF.¹¹⁾ The resulting diol was cleaved with lead tetraacetate [Pb(OAc)₄], and the lithium aluminum hydride (LiAlH₄) reduction gave the triol, whose two primary alcohols were protected with PivCl to give a 5 : 1 mixture of **7** and its C6 isomer (**7a**), which were readily separated by silica gel chromatography. Selective hydrogenolysis of the benzyl (Bn) group over Raney nickel (Ni) proceeded very smoothly without any detectable cleavage of the MPM group.¹²⁾ Oxidation of the resulting diol with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in the presence of molecular sieves (MS) gave the MP acetal (**14**)¹³⁾ as a single product, whose primary alcohol was oxidized with Dess–Martin periodinane¹⁴⁾ to give the C5–C9 aldehyde (**5**), which was immediately used in the next reaction without purification. The overall yield of 13 steps from **10** to **5** was 20%. The configuration at C6 was substantiated as follows; hydrogenolysis of **7**, after conversion to a MP acetal, over palladium charcoal (Pd-C) gave the triol, which was treated with acetic anhydride (Ac₂O) to give the meso triacetate (**15**).

Excess **6**^{3f)} was first lithiated with *tert*-butyllithium (*t*-BuLi) and allowed to react with crude **5**. The coupling reaction proceeded smoothly at -78 °C, and, after reprotection of the primary alcohols with PivCl, gave a 2 : 1 mixture of C9 alcohols (**16**), which was finally oxidized with Dess–Martin

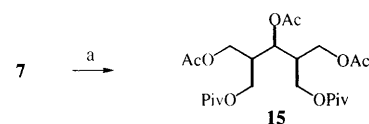


Chart 3

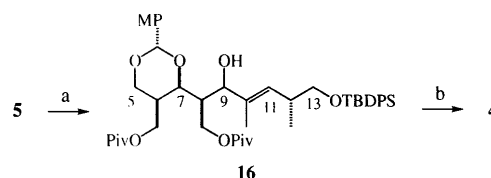


Chart 4

periodinane¹⁴⁾ to the title compound (**4**). The results presented here, though in small-scale experiments, may possibly lead to the total synthesis of **1**.

Experimental

3-C-Benzylloxymethyl-3-deoxy-1,2-O-isopropylidene-5,6-O-(4-methoxybenzylidene)-α-D-allofuranose (11) *p*-Anisaldehyde dimethyl acetal (3.8 ml, 22.50 mmol) and *p*-toluenesulfonic acid (TsOH·H₂O) (643 mg, 3.38 mmol) were added to a solution of **10**⁴⁾ [¹H-NMR (500 MHz, CDCl₃) δ: 7.35–7.24 (5H, m), 5.75 (1H, d, *J*=3.7 Hz), 4.66 (1H, dd, *J*=4.0, 3.7 Hz), 4.59 (1H, d, *J*=11.6 Hz), 4.50 (1H, d, *J*=11.6 Hz), 3.92 (1H, t, *J*=8.7 Hz), 3.87 (1H, dd, *J*=9.0, 3.7 Hz), 3.77 (1H, dd, *J*=11.6, 3.7 Hz), 3.77 (1H, dd, *J*=11.6, 3.1 Hz), 3.67 (1H, d, *J*=5.6 Hz), 3.65 (1H, d, *J*=9.0 Hz), 3.54 (1H, m), 2.10–2.18 (1H, m), 1.46 (3H, s), 1.26 (3H, s)]. ¹³C-NMR (125 MHz, CDCl₃) δ: 136.5, 128.6, 128.2, 128.0, 112.2, 104.5, 82.4, 81.8, 73.8, 67.4, 64.2, 49.1, 26.6, 26.2] (3.65 g, 11.25 mmol) in DMF (100 ml) at 55 °C. After 4 h, the reaction was quenched with saturated aqueous NH₄Cl, and the mixture was extracted with EtOAc (100 ml×5). The extract was washed with saturated aqueous NH₄Cl (20 ml×2) and brine (20 ml), then dried over Na₂SO₄, concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–Et₂O (3 : 2), to give a 5 : 4 mixture of **11** as a colorless oil (4.28 g, 86%). ¹H-NMR (500 MHz, CDCl₃) δ: 7.36–7.25 (5H, m), 7.31 (8/9H, d, *J*=8.5 Hz), 7.30 (10/9H, d, *J*=8.5 Hz), 6.88 (8/9H, d,

$J=8.5$ Hz), 6.86 (10/9H, d, $J=8.5$ Hz), 5.85 (4/9H, s), 5.82 (4/9H, d, $J=3.4$ Hz), 5.79 (5/9H, d, $J=3.4$ Hz), 5.69 (5/9H, s), 4.77 (5/9H, dd, $J=4.3$, 3.4 Hz), 4.75 (4/9H, dd, $J=4.3$, 3.4 Hz), 4.52 (1H, s), 4.49 (1H, d, $J=2.4$ Hz), 4.27 (4/9H, dd, $J=12.2$, 6.7 Hz), 4.21 (5/9H, dd, $J=12.2$, 6.7 Hz), 4.18 (1H, dd, $J=6.7$, 4.9 Hz), 4.11 (1H, dd, $J=8.5$, 4.9 Hz), 4.07 (1H, ddd, $J=8.5$, 6.7, 4.9 Hz), 3.95 (5/9H, dd, $J=8.5$, 6.7 Hz), 3.91 (4/9H, dd, $J=8.5$, 6.7 Hz), 3.80 (12/9H, s), 3.79 (15/9H, s), 3.73 (1H, m), 2.27 (5/9H, m), 2.15 (4/9H, m), 1.51 (12/9H, s), 1.50 (15/9H, s), 1.33 (3H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 160.4, 160.2, 138.3, 138.1, 128.3, 128.2, 128.1, 127.8, 127.6, 127.5, 127.5, 127.4, 113.6, 112.2, 112.1, 105.2, 105.1, 104.3, 103.9, 81.2, 81.0, 80.1, 79.6, 77.9, 73.3, 73.2, 68.0, 67.2, 66.1, 66.0, 55.2, 48.7, 48.2, 26.8, 26.7, 26.4, 26.3. IR (neat) cm^{-1} : 2935, 1615, 1516, 1455, 1077, 753. FAB-MS m/z (%): 443 ($\text{M}^+ + 1$, 28), 441 (21), 385 (23), 277 (11), 186 (9.1), 185 (100), 179 (17), 137 (38), 135 (59), 121 (40), 93 (100), 91 (100), 75 (29). HR-MS Calcd for $\text{C}_{23}\text{H}_{31}\text{O}_7$ ($\text{M}^+ + 1$): 443.2070. Found: 443.2058.

3-C-Benzoyloxymethyl-3-deoxy-1,2-O-isopropylidene-6-O-(4-methoxybenzyl)- α -D-allofuranose (12) a) To a stirred solution of **11** (1.33 g, 3.01 mmol) in CH_2Cl_2 (15 ml) was added $n\text{-Bu}_3\text{SnH}$ (1.8 ml, 9.03 mmol) and MgBr_2 (1.11 g, 6.02 mmol) at room temperature under argon. After 1 h, the reaction was quenched with saturated aqueous NaHCO_3 , and the mixture was extracted with EtOAc (100 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (10 ml), H_2O (10 ml) and brine (10 ml), dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with n -hexane– EtOAc (3:2), to give **12** as a colorless oil (1.28 g, 95%). $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.35–7.26 (5H, m), 7.25 (2H, d, $J=8.5$ Hz), 6.84 (2H, d, $J=8.5$ Hz), 5.74 (1H, d, $J=3.7$ Hz), 4.69 (1H, t, $J=4.3$ Hz), 4.58 (1H, d, $J=11.6$ Hz), 4.49 (1H, d, $J=11.6$ Hz), 4.48 (2H, dd, $J=11.6$, 9.5 Hz), 3.93 (1H, dd, $J=9.5$, 7.6 Hz), 3.83 (1H, dd, $J=9.5$, 5.2 Hz), 3.78 (3H, s), 3.73–3.66 (3H, m), 3.51 (1H, dd, $J=9.5$, 6.6 Hz), 2.22–2.16 (1H, m), 1.48 (3H, s), 1.29 (3H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 159.1, 137.1, 130.3, 129.4, 128.5, 127.9, 127.9, 113.7, 112.0, 104.5, 81.9, 81.0, 73.6, 73.0, 72.6, 71.2, 67.1, 55.2, 48.9, 26.7, 26.4.

b) A suspension of **10** (289 mg, 0.82 mmol), Ag_2O (475 mg, 2.05 mmol), KI (68 mg, 0.41 mmol) and MPM chloride (0.17 ml, 1.23 mmol) in toluene (8 ml) was heated under reflux for 15 h. After the reaction mixture was passed through a florisil pad (Et_2O), the filtrate was concentrated *in vacuo*, and the residue was chromatographed as described above to give **12** (115 mg, 32%) and recovered **10** (179 mg, 62%).

3-C-Benzoyloxymethyl-3,5,6-trideoxy-1,2-O-isopropylidene-5-C-(4-methoxybenzyloxymethyl)- α -D-ribo-hex-5-enofuranose (8) DMSO (2.02 ml, 28.4 mmol) was added to a stirred solution of $(\text{COCl})_2$ (1.24 ml, 14.2 mmol) in CH_2Cl_2 (30 ml) at -78°C under argon. After 15 min, a solution of **12** (3.16 g, 3.19 mmol) in CH_2Cl_2 (30 ml) was added, and the stirring was continued for 1 h. After dropwise addition of Et_3N (6.96 ml, 49.7 mmol), the solution was allowed to warm to 0°C over 1.5 h, and the reaction was quenched with saturated aqueous NH_4Cl . The mixture was extracted with EtOAc (100 ml $\times$ 3). The extract was washed with saturated aqueous NH_4Cl (20 ml $\times$ 3), H_2O (20 ml $\times$ 2) and brine (20 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with n -hexane– EtOAc (7:3), to give 3-C-benzoyloxymethyl-3-deoxy-1,2-O-isopropylidene-6-O-(4-methoxybenzyl)- α -D-ribo-hexofuranos-5-ulose as a colorless oil (2.73 g, 87%). $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.32–7.22 (5H, m), 7.23 (2H, d, $J=8.5$ Hz), 6.84 (2H, d, $J=8.5$ Hz), 5.84 (1H, d, $J=3.4$ Hz), 4.72 (1H, t, $J=4.0$ Hz), 4.48 (2H, d, $J=1.8$ Hz), 4.45 (2H, d, $J=11.0$ Hz), 4.33 (2H, d, $J=5.2$ Hz), 4.25 (1H, d, $J=10.7$ Hz), 3.77 (3H, s), 3.79–3.75 (1H, m), 3.67 (2H, dd, $J=9.3$, 5.7 Hz), 2.39–2.33 (1H, m), 1.46 (3H, s), 1.31 (3H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 205.7, 159.4, 138.0, 129.6, 129.2, 128.3, 127.6, 127.6, 113.8, 112.6, 105.8, 81.5, 80.3, 73.3, 72.8, 72.0, 65.6, 55.2, 48.2, 26.8, 26.3; IR (neat) cm^{-1} : 2935, 1732, 1612, 1513, 1455, 1373, 1246, 1116.

A 1.53 M solution of $n\text{-BuLi}$ in hexane (8.9 ml, 13.5 mmol) was added to a stirred suspension of $\text{Ph}_3\text{PCH}_3^+\text{Br}^-$ (4.85 g, 13.5 mmol) in THF (40 ml) at 0°C under argon. After 30 min, a solution of the ketone (2.73 g, 6.17 mmol) in THF (20 ml) was added dropwise. After 6 h, the reaction was quenched with H_2O , and the mixture was extracted with EtOAc (100 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (20 ml $\times$ 3), H_2O (20 ml $\times$ 2) and brine (20 ml), dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with n -hexane– EtOAc (3:1), to give **8** as a colorless oil (2.27 g, 83%). $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.34–7.26 (5H, m), 7.24 (2H, d, $J=8.5$ Hz), 6.85 (2H, d, $J=8.5$ Hz), 5.81 (1H, d, $J=3.7$ Hz), 5.31 (2H, d, $J=1.2$ Hz), 5.22 (1H, brs), 4.75 (1H, t, $J=4.0$ Hz), 4.52 (1H, d, $J=11.9$ Hz), 4.48 (1H, d, $J=11.9$ Hz), 4.45 (1H, d, $J=11.6$ Hz), 4.38 (1H, d, $J=11.6$ Hz), 4.32 (1H, d, $J=10.4$ Hz), 4.12 (1H, d, $J=12.8$ Hz), 3.98 (1H, d, $J=12.8$ Hz), 7.80–3.74 (1H, m), 3.77 (3H, s),

3.47 (1H, dd, $J=9.3$, 4.8 Hz), 2.46–2.40 (1H, m), 1.52 (3H, s), 1.35 (3H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 159.0, 142.4, 138.2, 130.2, 129.2, 128.1, 127.4, 127.4, 116.3, 113.6, 111.5, 104.8, 80.8, 80.5, 73.1, 71.7, 69.0, 65.8, 55.1, 48.2, 26.6, 26.3.

3-C-Benzoyloxymethyl-3,5-dideoxy-1,2-O-isopropylidene-6-O-(4-methoxybenzyl)-5-C-hydroxymethyl- β -L-talofuranose (13) a) A 1.0 M solution of $\text{BH}_3\cdot\text{Me}_2\text{S}$ in toluene (12.3 ml, 12.33 mmol) was added to a stirred solution of **8** (2.17 g, 4.93 mmol) in toluene (30 ml) at room temperature under argon. After 20 min, the reaction mixture was cooled to 0°C , and quenched with H_2O . After addition of 3 N NaOH (30 ml) and 30% H_2O_2 (25 ml), vigorous stirring was continued for 30 min, and the mixture was extracted with ether (100 ml $\times$ 3). The extract was washed with saturated aqueous NH_4Cl (20 ml $\times$ 3) and brine (20 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with n -hexane– EtOAc (3:2), to give a 5:1 mixture of **13** and its C6 isomer as a colorless oil (1.43 g, 63%). $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.34–7.26 (5H, m), 7.23 (10/6H, d, $J=8.6$ Hz), 7.22 (2/6H, d, $J=8.6$ Hz), 6.86 (10/6H, d, $J=8.6$ Hz), 6.85 (2/6H, d, $J=8.6$ Hz), 5.76 (5/6H, d, $J=3.7$ Hz), 5.73 (1/6H, d, $J=3.7$ Hz), 4.71 (5/6H, t, $J=4.3$ Hz), 4.69 (1/6H, t, $J=4.3$ Hz), 4.52 (1H, d, $J=12.2$ Hz), 4.50 (1H, d, $J=12.2$ Hz), 4.43 (1H, d, $J=11.2$ Hz), 4.42 (1H, d, $J=11.2$ Hz), 4.09 (5/6H, dd, $J=10.5$, 2.8 Hz), 4.06 (1/6H, dd, $J=10.5$, 3.4 Hz), 3.83–3.75 (2H, m), 3.79 (15/6H, s), 3.77 (3/6H, s), 3.68 (1/6H, dd, $J=9.5$, 5.5 Hz), 3.66 (5/6H, dd, $J=9.2$, 7.6 Hz), 3.60 (1/6H, dd, $J=9.5$, 6.7 Hz), 3.59 (5/6H, dd, $J=9.5$, 6.1 Hz), 3.54 (1/6H, dd, $J=9.5$, 5.5 Hz), 3.48 (5/6H, dd, $J=9.5$, 6.4 Hz), 2.71 (5/6H, dd, $J=7.6$, 3.7 Hz), 2.63 (1/6H, dd, $J=7.6$, 4.0 Hz), 2.42–2.35 (1H, m), 2.13–2.07 (1H, m), 1.48 (3H, s), 1.31 (3H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 159.1, 138.1 (1/6C), 137.9 (5/6C), 130.2 (5/6C), 130.1 (1/6C), 129.2 (5/6C), 129.1 (1/6C), 128.3 (5/6C), 128.2 (1/6C), 127.6, 127.5 (5/6C), 127.4 (1/6C), 113.7, 111.8 (5/6C), 111.7 (1/6C), 104.6 (1/6C), 104.4 (5/6C), 80.9 (5/6C), 80.8 (1/6C), 80.1 (5/6C), 80.0 (1/6C), 73.3 (5/6C), 73.2 (1/6C), 72.9, 70.4 (5/6C), 68.4 (1/6C), 66.4 (5/6C), 66.3 (1/6C), 64.4 (1/6C), 61.2 (5/6C), 55.2, 46.4 (5/6C), 46.3 (1/6C), 42.5 (5/6C), 42.4 (1/6C), 26.6 (1/6C), 26.5 (5/6C), 26.3 (1/6C), 26.2 (5/6C). IR (neat) cm^{-1} : 3476, 2934, 1612, 1512, 1454, 1371, 1303, 1249.

b) A 0.5 M solution of 9-BBN in THF (0.36 ml, 0.18 mmol) was added dropwise to a stirred solution of **8** (40.1 mg, 0.09 mmol) in THF (0.5 ml) at room temperature under argon. After 24 h, the reaction was quenched with H_2O , then 4 N KOH (0.2 ml) and 30% H_2O_2 (0.2 ml) were added, and the mixture was stirred vigorously for 30 min. After concentration *in vacuo*, the residue was extracted with Et_2O (10 ml $\times$ 3). The extract was washed with saturated aqueous NH_4Cl (2 ml $\times$ 3) and brine (2 ml), dried over MgSO_4 , concentrated *in vacuo*, and chromatographed as described above to give a 45:1 mixture of **13** and its C6 isomer (27.4 mg, 66%).

(2S,3S,4R)-1-Benzoyloxy-2,4-bis(2,2-dimethylpropanoyloxymethyl)-3-hydroxy-5-(4-methoxybenzyloxy)pentane (7) and **(2S,3S,4S)-1-Benzoyloxy-2,4-bis(2,2-dimethylpropanoyloxymethyl)-3-hydroxy-5-(4-methoxybenzyloxy)pentane (7a)** Et_3N (1.15 ml, 8.20 mmol), DMAP (40 mg, 0.33 mmol) and PivCl (404 μl , 3.28 mmol) were added to a stirred solution of the 5:1 mixture of **13** and its C6 isomer (752 mg, 1.64 mmol) in CH_2Cl_2 (16 ml) at 0°C under argon. After 3 h at room temperature, the reaction was quenched with cold saturated aqueous NH_4Cl , and the mixture was extracted with EtOAc (50 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (10 ml $\times$ 2) and brine (10 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with n -hexane– EtOAc (3:1), to give 5:1 mixture of 3-O-benzoyloxymethyl-3,5-dideoxy-1,2-O-isopropylidene-6-O-(4-methoxybenzyl)-5-C-(2,2-dimethylpropanoyloxymethyl)- β -L-talofuranose and its C6 isomer as a colorless oil (884 mg, 99%). $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.32–7.24 (5H, m), 7.22 (10/6H, d, $J=8.5$ Hz), 7.21 (2/6H, d, $J=8.5$ Hz), 6.85 (10/6H, d, $J=8.5$ Hz), 6.84 (2/6H, d, $J=8.5$ Hz), 5.74 (5/6H, d, $J=3.7$ Hz), 5.72 (1/6H, d, $J=3.7$ Hz), 4.71 (5/6H, t, $J=4.3$ Hz), 4.69 (1/6H, t, $J=4.3$ Hz), 4.52 (1H, d, $J=12.2$ Hz), 4.49 (1H, d, $J=12.2$ Hz), 4.41 (1H, d, $J=12.2$ Hz), 4.39 (1H, d, $J=12.2$ Hz), 4.17 (1H, dd, $J=5.8$, 2.7 Hz), 4.10 (5/6H, dd, $J=10.4$, 2.7 Hz), 4.05 (1/6H, dd, $J=10.5$, 2.7 Hz), 3.78 (15/6H, s), 3.76 (3/6H, s), 3.75 (5/6H, dd, $J=9.8$, 6.1 Hz), 3.64 (1/6H, dd, $J=9.8$, 6.1 Hz), 3.55–3.51 (2H, m), 3.50 (5/6H, dd, $J=9.5$, 6.1 Hz), 3.40 (1/6H, dd, $J=9.5$, 6.1 Hz), 2.47–2.43 (1/6H, m), 2.43–2.37 (5/6H, m), 2.33–2.29 (1/6H, m), 2.28–2.22 (5/6H, m), 1.48 (15/6H, s), 1.47 (3/6H, s), 1.32 (3H, s), 1.16 (9/6H, s), 1.15 (45/6H, s). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 178.2, 159.0, 138.2, 130.2 (5/6C), 130.1 (1/6C), 129.2 (5/6C), 129.1 (1/6C), 128.3 (5/6C), 128.2 (1/6C), 127.6, 127.5 (1/6C), 127.4 (5/6C), 113.7 (5/6C), 111.5 (1/6C), 104.6 (1/6C), 104.5 (5/6C), 80.9 (5/6C), 78.1 (5/6C), 78.0 (1/6C), 77.2 (1/6C), 73.1 (5/6C), 73.2 (1/6C), 72.6 (5/6C), 72.5 (1/6C), 68.6 (5/6C), 66.4 (1/6C), 63.3, 61.4 (5/6C), 60.3 (1/6C), 55.1, 46.3 (5/6C), 46.2 (1/6C), 40.5 (1/6C), 40.1 (1/6C), 38.6

(1/6C), 38.5 (5/6C), 27.0 (5/6C), 26.9 (1/6C), 26.6 (5/6C), 26.5 (1/6C), 26.4 (1/6C), 26.3 (5/6C). IR (neat) cm^{-1} : 2975, 1728, 1612, 1513, 1480, 1455, 1370. FAB-MS m/z (%): 543 ($M^+ + 1$, 12), 485 (20), 461 (22), 369 (64), 365 (13), 278 (26), 277 (100), 275 (26), 241 (16), 187 (28), 186 (100), 185 (100), 165 (23), 149 (21), 137 (38), 135 (40), 122 (88), 121 (100), 107 (39), 94 (56), 93 (100), 91 (100), 75 (100). HR-MS Calcd for $\text{C}_{35}\text{H}_{43}\text{O}_5$ ($M^+ + 1$): 543.3111. Found: 543.2942.

4 N HCl (0.60 ml) was added to a stirred solution of the pivaloate (139 mg, 0.26 mmol) in THF (3 ml) at 55 °C. After 6 h, the reaction mixture was neutralized with saturated aqueous NaHCO_3 , and extracted with CH_2Cl_2 (50 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2) and brine (10 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (3:2), to give a 5:1 mixture of diols as a colorless oil (111 mg, 86%).

$\text{Pb}(\text{OAc})_4$ (106 mg, 0.24 mmol) was added to a stirred solution of the diol (111 mg, 0.22 mmol) in benzene (2 ml) at room temperature. After 10 min, the reaction mixture was filtered with florisil, and the filtrate, after concentration, was used for the next step without purification.

LiAlH_4 (16.7 mg, 0.44 mmol) was added to a stirred solution of the aldehyde (110 mg, 0.22 mmol) in ether (2 ml) at 0 °C under argon. After 20 min, the reaction was quenched with saturated aqueous NH_4Cl . The mixture was diluted with ether (2 ml), vigorously stirred for 10 min, and extracted with CHCl_3 (50 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2) and brine (10 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (2:3), to give a 5:1 mixture of (2*R*,3*S*,4*S*)-1-benzyloxy-2,4-dihydroxy-methyl-3-hydroxy-5-(4-methoxybenzyloxy)pentane and its C6 isomer as a colorless oil (76.8 mg, 89%). FAB-MS m/z (%): 391 ($M^+ + 1$, 27), 281 (12), 241 (15), 233 (10), 211 (20), 165 (11), 154 (14), 151 (14), 137 (100), 122 (100), 121 (100), 107 (44), 91 (100). HR-MS (FAB) m/z Calcd for $\text{C}_{22}\text{H}_{31}\text{O}_6$: 391.2121 ($M^+ + \text{H}$). Found: 391.2133.

Et_3N (168 μl , 1.20 mmol), DMAP (18.3 mg, 0.15 mmol) and PivCl (81 μl , 0.66 mmol) were added to a stirred solution of the triol (116 mg, 0.30 mmol) in CH_2Cl_2 (3 ml) at 0 °C under argon. After 1 h at room temperature, the reaction was quenched with MeOH, and the mixture was extracted with ether (30 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl , H_2O and brine, then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (6:1), to give **7** (139 mg, 84%) and **7a** (27 mg, 16%) as colorless oils. **7**: ^1H -NMR (500 MHz, CDCl_3) δ : 7.34–7.26 (5H, m), 7.21 (2H, d, $J=8.9$ Hz), 6.85 (2H, d, $J=8.9$ Hz), 4.48 (2H, s), 4.41 (2H, s), 4.36 (2H, dt, $J=12.5$, 4.6 Hz), 4.29 (1H, dd, $J=7.3$, 4.9 Hz), 4.26 (1H, dd, $J=7.3$, 4.9 Hz), 3.95–3.91 (1H, m), 3.79 (3H, s), 3.61 (1H, dd, $J=9.3$, 4.6 Hz), 3.58 (1H, dd, $J=9.3$, 4.6 Hz), 3.55 (1H, dd, $J=9.5$, 4.6 Hz), 3.52 (1H, dd, $J=9.5$, 4.6 Hz), 3.22 (1H, d, $J=3.1$ Hz), 2.17–2.10 (2H, m), 1.15 (18H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 178.5, 159.2, 137.9, 129.9, 129.3, 129.2, 128.4, 127.7, 127.5, 113.8, 113.7, 73.4, 73.0, 71.0, 69.1, 68.9, 61.8, 55.2, 41.0, 40.9, 38.7, 27.2, 27.1, 27.1; IR (neat) cm^{-1} : 3359, 2868, 1728. FAB-MS m/z (%): 559 ($M^+ + 1$, 76), 558 (M^+ , 12), 451 (11), 289 (11), 228 (9.5), 138 (47), 107 (59), 91 (100). HR-MS (FAB) Calcd for $\text{C}_{32}\text{H}_{46}\text{O}_8$: 559.3271 ($M^+ + \text{H}$). Found: 559.3250. **7a**: ^1H -NMR (500 MHz, CDCl_3) δ : 7.35–7.26 (5H, m), 7.21 (2H, d, $J=8.9$ Hz), 6.85 (2H, d, $J=8.9$ Hz), 4.65 (1H, dd, $J=12.8$, 0.9 Hz), 4.58 (1H, dd, $J=12.8$, 0.9 Hz), 4.47 (2H, d, $J=2.7$ Hz), 4.40 (2H, d, $J=0.9$ Hz), 4.13–4.06 (4H, m), 3.80 (3H, s), 3.44–3.38 (2H, m), 3.09–3.02 (1H, m), 1.59 (1H, s), 1.18 (9H, s), 1.15 (9H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 178.3, 178.0, 159.1, 138.0, 134.7, 130.1, 129.8, 129.1, 128.4, 128.3, 127.7, 127.6, 113.7, 72.8, 72.4, 69.8, 66.0, 65.6, 64.5, 55.2, 38.8, 38.7, 37.9, 27.2, 27.1.

(2*S*,3*S*,4*R*)-2,4-Bis(2,2-dimethylpropanoyloxymethyl)-3,5-[(*S*)-4-methoxybenzylidenedioxy]pentan-1-ol (14) To a stirred solution of **7** (41.4 mg, 74.1 μmol) in EtOH (1 ml) was added Raney-Ni (W2) at room temperature under H_2 , and stirring was continued for 26 h. After filtration, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (3:2), to give (2*S*,3*S*,4*R*)-2,4-bis(2,2-dimethylpropanoyloxymethyl)-5-(4-methoxybenzyloxy)pentane-1,3-diol as a colorless oil (31.5 mg, 91%). ^1H -NMR (500 MHz, CDCl_3) δ : 7.23 (2H, d, $J=8.5$ Hz), 6.87 (2H, d, $J=8.5$ Hz), 4.46 (1H, d, $J=7.9$ Hz), 4.43 (1H, d, $J=6.7$ Hz), 4.36 (1H, ddd, $J=11.3$, 8.9, 4.3 Hz), 4.29 (1H, dd, $J=11.3$, 7.0 Hz), 3.95–3.92 (1H, m), 3.80 (3H, s), 3.71–3.69 (2H, m), 3.62 (1H, dd, $J=9.2$, 4.3 Hz), 3.55 (1H, dd, $J=9.2$, 4.3 Hz), 3.44 (1H, d, $J=2.7$ Hz), 2.63 (1H, dd, $J=6.1$, 5.8 Hz), 2.18–2.13 (1H, m), 2.02–1.97 (1H, m), 1.21 (9H, s), 1.02 (9H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 179.5, 178.8, 129.7, 129.4, 129.3, 113.9, 113.8, 73.1, 71.6, 69.4, 61.7, 61.5, 55.2, 42.6, 40.9, 38.8, 38.7, 27.2, 27.1. FAB-MS m/z (%): 469 ($M^+ + 1$, 12), 461 (11), 369 (33), 277 (100), 186 (84), 185 (100), 165 (14), 149 (17),

122 (26), 121 (100), 94 (62), 93 (100), 91 (48), 75 (100). HR-MS Calcd for $\text{C}_{25}\text{H}_{41}\text{O}_8$ ($M^+ + 1$): 469.2954. Found: 469.2808.

MS **3A** (10 mg) and DDQ (12 mg, 51.6 μmol) was added to a stirred solution of the diol (22 mg, 46.9 μmol) in CH_2Cl_2 (1 ml) at room temperature under argon. After 4 h, the reaction was quenched with saturated aqueous NaHCO_3 , and the mixture was extracted with ether (10 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (10 ml $\times$ 2) and brine (10 ml), then dried over Na_2SO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (3:2), to give **14** as a colorless oil (19.5 mg, 89%). ^1H -NMR (500 MHz, CDCl_3) δ : 7.39 (2H, d, $J=8.5$ Hz), 6.89 (2H, d, $J=8.5$ Hz), 5.49 (1H, s), 4.52 (1H, dd, $J=11.0$, 5.0 Hz), 4.47 (1H, dd, $J=11.3$, 7.9 Hz), 4.41 (1H, dd, $J=11.0$, 9.5 Hz), 4.33 (1H, dd, $J=11.3$, 3.4 Hz), 4.11 (1H, dd, $J=10.7$, 2.1 Hz), 4.05 (1H, dd, $J=11.0$, 7.0 Hz), 4.01 (1H, dd, $J=10.7$, 1.0 Hz), 3.80 (3H, s), 3.81 (1H, m), 3.75 (1H, dd, $J=11.0$, 4.6 Hz), 2.54 (1H, brs), 2.17–2.11 (1H, m), 2.02–2.00 (1H, m), 1.21 (9H, s), 1.20 (9H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 179.4, 178.4, 159.7, 130.4, 127.0, 113.3, 101.9, 76.3, 68.4, 61.2, 60.8, 57.8, 55.0, 41.7, 38.7, 38.5, 34.3, 27.0, 26.9.

(2*S*,3*R*,4*R*)-2,4-Bis(2,2-dimethylpropanoyloxymethyl)-3,5-[(*S*)-4-methoxybenzylidenedioxy]pentanal (5) A solution of **14** (21.9 mg, 46.9 μmol) in CH_2Cl_2 (0.5 ml) was added to a mixture of pyridine (9.5 μl , 117 μmol) and Dess–Martin periodinane (39.8 mg, 93.9 μmol) in CH_2Cl_2 (0.5 ml) at room temperature under argon. After 20 min, the mixture was diluted with ether (1 ml), and quenched with saturated aqueous NaHCO_3 (1 ml) and saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$ (1 ml). The mixture was extracted with ether (10 ml), and washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (5 ml $\times$ 2) and brine (10 ml), then dried over Na_2SO_4 . The extract was concentrated *in vacuo*, and the residue was chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (7:3), to give **5** as a colorless oil (19.2 mg, 88%) [^1H -NMR δ : 9.82 (1H, s)], which was immediately used to the next reaction.

(2*R*,3*S*,4*S*)-2,4-Bis(2,2-dimethylpropanoyloxymethyl)-1,3,5-triaceptoxypentane (15) To a stirred solution of **7** (22 mg, 46.9 μmol) in CH_2Cl_2 (1 ml) was added MS **3A** (10 mg) and DDQ (12 mg, 51.6 μmol) at room temperature under argon. After 4 h, the reaction was quenched with saturated aqueous NaHCO_3 . The mixture was diluted with ether (1 ml), extracted with ether (10 ml $\times$ 2), and washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (10 ml $\times$ 2) and brine (10 ml), then dried over Na_2SO_4 . The extract was concentrated *in vacuo*, and the residue was chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (3:2), to give an acetal as a colorless oil (19.5 mg, 89%). ^1H -NMR (500 MHz, CDCl_3) δ : 7.38 (2H, d, $J=8.5$ Hz), 7.35–7.26 (5H, m), 6.88 (2H, d, $J=8.5$ Hz), 5.47 (1H, s), 4.49 (2H, d, $J=4.6$ Hz), 4.47 (1H, dd, $J=5.1$, 2.1 Hz), 4.40 (1H, dd, $J=11.3$, 9.2 Hz), 4.33 (1H, dd, $J=10.7$, 3.4 Hz), 4.30 (1H, dd, $J=11.3$, 1.0 Hz), 4.27 (1H, dd, $J=10.7$, 7.9 Hz), 4.10 (1H, dd, $J=10.7$, 1.8 Hz), 3.96 (1H, dd, $J=11.6$, 1.8 Hz), 3.80 (3H, s), 3.64 (1H, dd, $J=9.8$, 4.3 Hz), 3.61 (1H, dd, $J=9.8$, 3.7 Hz), 2.28–2.22 (1H, m), 1.89–1.84 (1H, m), 1.19 (9H, s), 1.13 (9H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 178.4, 178.1, 159.9, 138.0, 130.6, 128.4, 127.7, 127.2, 114.2, 113.6, 113.5, 102.1, 76.9, 73.3, 73.1, 68.5, 65.6, 61.7, 61.0, 55.3, 40.1, 38.8, 34.6, 27.2. FAB-MS m/z (%): 556 (M^+ , 43), 449 (8.8), 188 (5.4), 165 (8.5), 148 (4.9), 134 (6.4), 121 (24), 107 (18), 91 (100). HR-MS (FAB) m/z Calcd for $\text{C}_{32}\text{H}_{46}\text{O}_8$: 557.3115 ($M^+ + \text{H}$). Found: 557.3089.

To a stirred solution of the acetal (20 mg, 35.9 μmol) in EtOH (1 ml) was added 10% Pd/C at room temperature under H_2 . The stirring was continued for 2 h. After careful filtration through a celite layer, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on a silica gel column, eluting with EtOAc, to give a triol as a colorless oil (12.5 mg, 100%).

To a stirred solution of the triol (10 mg, 28.7 μmol) in CH_2Cl_2 (0.5 ml) was added DMAP (7.0 mg, 57.4 μmol) and Ac_2O (8.9 μl , 94.7 μmol) at room temperature. After 10 min, the reaction was quenched with saturated aqueous NH_4Cl , and then the mixture was extracted with ether (20 ml) and washed with H_2O (2 ml $\times$ 2) and brine (2 ml), then dried over MgSO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane–EtOAc (2:3), to give **15** as a colorless oil (13.6 mg, 100%). ^1H -NMR (500 MHz, CDCl_3) δ : 5.31 (1H, t, $J=6.1$ Hz), 4.23 (2H, dd, $J=11.6$, 4.6 Hz), 4.14–4.12 (4H, m), 4.03 (2H, dd, $J=11.6$, 6.7 Hz), 2.46–2.41 (2H, m), 2.07 (9H, s), 1.20 (18H, s). ^{13}C -NMR (125 MHz, CDCl_3) δ : 178.2, 170.6, 169.8, 68.8, 61.9, 60.7, 39.2, 38.8, 27.1, 20.7, 20.6.

(2*R*,3*E*,6*S*,7*R*,8*R*)-6,8-Bis(2,2-dimethylpropanoyloxymethyl)-1-[1,1-(dimethylethyl)diphenylsilyloxy]-2,4-dimethyl-7,9-[(*S*)-4-methoxybenzylidenedioxy]-3-nonen-5-one (4) A 1.64 M solution of *tert*-BuLi in pentane (90.9 μl , 149 μmol) was added to a stirred solution of **6** (33.3 mg, 71.6 μmol) in ether (0.5 ml) at -78 °C under argon. The mixture was stirred for 1

h at -78°C and for 1 h at room temperature, then cooled again to -78°C , and a solution of **5** (12.9 mg, $27.8\text{ }\mu\text{mol}$) in ether (0.5 ml) was added dropwise. The stirring was continued for 1 h at -78°C , then the reaction was quenched with saturated aqueous NH_4Cl , and the mixture was extracted with ether (10 ml). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (5 ml $\times$ 2) and brine (10 ml), then dried over Na_2SO_4 , and concentrated *in vacuo*. The residue (26.2 mg) was dissolved in CH_2Cl_2 , and to this stirred solution were added Et_3N (23.1 μl , 0.17 mmol), DMAP (2.5 mg, $20.6\text{ }\mu\text{mol}$) and PivCl (10.2 μl , $82.5\text{ }\mu\text{mol}$) at 0°C . After 1 h at room temperature, the reaction was quenched with saturated aqueous NH_4Cl , and the mixture was extracted with ether (10 ml $\times$ 2). The extract was washed with saturated aqueous NH_4Cl and brine, dried over Na_2SO_4 , and concentrated *in vacuo* to give a 2 : 1 mixture of crude alcohols (**16**) (17.7 mg, 79%).

A solution of the alcohols (**16**) (9 mg, $11.2\text{ }\mu\text{mol}$) in CH_2Cl_2 (0.5 ml) was added to a mixture of pyridine (1.8 μl , $22.4\text{ }\mu\text{mol}$) and Dess–Martin periodinane (5.2 mg, $12.3\text{ }\mu\text{mol}$) in CH_2Cl_2 (0.5 ml) at room temperature under argon. After 1 h, the mixture was diluted with ether (1 ml), and quenched with saturated aqueous NaHCO_3 (1 ml) and saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$ (1 ml). The mixture was extracted with ether (10 ml). The extract was washed with saturated aqueous NH_4Cl (5 ml $\times$ 2), H_2O (5 ml $\times$ 2) and brine (10 ml $\times$ 1), then dried over Na_2SO_4 , concentrated *in vacuo*, and chromatographed on a silica gel column, eluting with *n*-hexane– EtOAc (4 : 1), to give **4** as a colorless oil (9 mg, 100%). $[\alpha]_{\text{D}}^{20} +24.9^{\circ}$ ($c=1.0$, CHCl_3). $^1\text{H-NMR}$ (500 MHz, C_6D_6) δ : 7.81–7.78 (5H, m), 7.54 (2H, d, $J=8.5\text{ Hz}$), 7.31–7.22 (5H, m), 6.79 (2H, d, $J=8.5\text{ Hz}$), 6.49 (1H, d, $J=9.3\text{ Hz}$), 5.39 (1H, s), 4.66 (1H, dd, $J=10.8, 7.3\text{ Hz}$), 4.64 (1H, dd, $J=10.8, 5.5\text{ Hz}$), 4.59 (1H, dd, $J=10.8, 3.7\text{ Hz}$), 4.44 (1H, dd, $J=10.2, 2.3\text{ Hz}$), 4.43 (1H, dd, $J=10.2, 3.7\text{ Hz}$), 4.26 (1H, dd, $J=11.6, 1.0\text{ Hz}$), 3.80 (1H, ddd, $J=10.2, 7.3, 3.7\text{ Hz}$), 3.71 (1H, dd, $J=9.8, 5.5\text{ Hz}$), 3.64 (1H, dd, $J=9.8, 7.3\text{ Hz}$), 3.57 (1H, d, $J=11.6\text{ Hz}$), 3.24 (3H, s), 2.79–2.73 (1H, m), 1.89 (3H, s), 1.86–1.82 (1H, m), 1.20 (9H, s), 1.12 (9H, s), 1.04 (3H, d, $J=6.7\text{ Hz}$), 1.00 (9H, s). $^{13}\text{C-NMR}$ (125 MHz, C_6D_6) δ : 200.0, 177.5, 177.3, 160.5, 145.3, 138.5, 136.0, 135.9, 131.4, 130.1, 130.0, 128.3, 128.2, 127.8, 113.8, 102.5, 77.7, 67.9, 67.5, 64.3, 60.6, 54.7, 46.4, 38.8, 38.6, 37.1, 35.1, 27.3, 27.1, 27.0, 19.5, 16.6, 12.0. FAB-MS m/z (%): 801 (M^++1 , 3.0), 743 (1.1), 563 (2.3), 553 (1.7), 369 (9.3), 365 (3.4), 337 (2.4), 307 (2.3), 283 (6.3), 278 (4.1), 277 (3.5), 275 (5.3), 259 (2.4), 239 (5.0), 223 (4.1), 215 (4.3), 207 (4.6), 199 (8.7), 197 (10), 186 (24), 185 (100), 183 (11), 167 (6.4), 165 (6.6), 137 (6.6), 135 (16), 121 (6.2), 115 (7.8), 94 (10), 93 (100), 75 (53). HR-MS Calcd for $\text{C}_{47}\text{H}_{65}\text{O}_9\text{Si}$ (M^++1): 801.4396. Found: 801.4393.

References and Notes

- 1) a) Rinehart K., Tachibana K., *J. Nat. Prod.*, **58**, 344–358 (1995); b) Rinehart K., Kenneth L., Cheng J.-F., Lee J.-S., U.S. Patent US 5514708, 7 May 1996 [*Chem. Abstr.*, **125**, 5896f(1996)].
- 2) Schmitz F. J., Gunasekera S. P., Yalamanchili G., Hossain M. B., van der Helm D., *J. Am. Chem. Soc.*, **106**, 7251–7252 (1984).
- 3) a) Yonemitsu O., *Yuki Gosei Kagaku Kyokai Shi*, **52**, 946–955 (1994); b) Matsushima T., Horita K., Nakajima N., Yonemitsu O., *Tetrahedron Lett.*, **37**, 385–388 (1996); c) Matsushima T., Mori M., Nakajima N., Maeda H., Uenishi J., Yonemitsu O., *Chem. Pharm. Bull.*, **46**, 1335–1336 (1998); d) Matsushima T., Mori M., Zheng B.-Z., Maeda H., Nakajima N., Uenishi J., Yonemitsu O., *ibid.*, **47**, 308–321 (1999); e) Matsushima T., Zheng B.-Z., Maeda H., Nakajima N., Uenishi J., Yonemitsu O., *Synlett*, **1999**, 780–782; f) Zheng B.-Z., Maeda H., Mori M., Kusaka S., Yonemitsu O., Matsushima T., Nakajima N., Uenishi J., *Chem. Pharm. Bull.*, **47**, 1288–1296 (1999); g) Taylor R. E., Ciavarrri J. P., Hearn B. R., *Tetrahedron Lett.*, **39**, 9361–9364 (1998); h) Liu J.-F., Abiko A., Pei Z., Buske D. C., Masamune S., *ibid.*, **39**, 1873–1876 (1998); i) Roush W. R., Lane G. C., *Org. Lett.*, **1**, 95–98 (1999); j) Jung M. E., Karama U., Marquez R., *J. Org. Chem.*, **64**, 663–665 (1999); k) Jung M. E., Marquez R., *Tetrahedron Lett.*, **40**, 3129–3132 (1999); l) Smith A. B., III, Lodies S. A., *Org. Lett.*, **1**, 1249–1252 (1999); m) Matsushima T., Nakajima N., Zheng B.-Z., Yonemitsu O., *Chem. Pharm. Bull.*, **48**, 855–860 (2000); n) Jung M. E., Marquez R., *Org. Lett.*, **2**, 1669–1672 (2000).
- 4) Oikawa Y., Nishi T., Yonemitsu O., *J. Chem. Soc. Perkin Trans. 1*, **1985**, 19–26.
- 5) Unless otherwise noted, numbering is based on that of **1**.
- 6) In this synthesis, five out of six carbons in D-glucose (**9**) were incorporated in the title compound (**4**).
- 7) Zheng B.-Z., Yamauchi M., Dei H., Kusaka S., Matsui K., Yonemitsu O., *Tetrahedron Lett.*, **41**, 6441–6445 (2000).
- 8) Panek J. S., Hu T., *J. Org. Chem.*, **62**, 4912–4913 (1997).
- 9) Bouzide A., Sauve G., *Tetrahedron Lett.*, **38**, 5945–5948 (1997).
- 10) $\text{BH}_3\cdot\text{THF}$ also gave a good result (82% yield, 11 : 1 selectivity), but the reproducibility was poor.
- 11) The TBDPS protecting group instead of the pivaloyl group was rather labile under acidic conditions.
- 12) Oikawa Y., Tanaka T., Horita K., Yonemitsu O., *Tetrahedron Lett.*, **25**, 5397–5400 (1984).
- 13) Oikawa Y., Yoshioka T., Yonemitsu O., *Tetrahedron Lett.*, **23**, 889–892 (1982).
- 14) Dess D. B., Martin J. C., *J. Org. Chem.*, **48**, 4155–4156 (1983).

A New Cyanogenic Glycoside from *Sorbaria sorbifolia* var. *stellipila*

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A new cyanogenic glycoside, sutherlandin-5-*trans*-*p*-coumarate was isolated along with a known cardiospermin-5-(4-hydroxy) benzoate from the aerial parts of *Sorbaria sorbifolia* (L.) A. Br. var. *stellipila* MAX. (Rosaceae). The structure of the new compound was established based on spectral evidence.

Key words cyanogenic glycoside; sutherlandin-5-*trans*-*p*-coumarate; *Sorbaria sorbifolia* var. *stellipila*; Rosaceae

The stem bark of *Sorbaria sorbifolia* (L.) A. Br. var. *stellipila* MAX. (Rosaceae) has been used as a traditional Chinese drug for the treatment of bone fracture and bruise.¹⁾ This plant grows in the mountainous areas of Kangwondo, Korea.²⁾ In a previous study of *S. sorbifolia* var. *stellipila*, we reported the isolation and characterization of two cytotoxic constituents, cucurbitacins D and F,³⁾ and two lignans.⁴⁾ In continuation of our phytochemical studies of this plant, we have reinvestigated the extracts from the aerial parts of the plant, collected in Kangwondo. Previous chemical studies on this genus, several leucine-derived cyanogenic glycosides, cardiospermin-5-(4-hydroxy) benzoate, heterodendrin, and cardiospermin-5-*trans*-*p*-coumarate were reported from *S. arborea*.⁵⁾ In this paper, we report the isolation and structural elucidation of a known (**1**) and a new (**2**) cyanogenic glycoside.

Repeated column chromatography of the methanol extract yielded two cyanogenic glycosides. The structure of cardiospermin-5-(4-hydroxy) benzoate (**1**) was established by its positive-ion high resolution FAB-MS, ¹H-, ¹³C-, 2D NMR, and a heteronuclear multiple bond correlation (HMBC) experiment (Fig. 1), together with comparison of the NMR spectral data with that reported in the literature.⁵⁾

Compound **2** was assigned the molecular formula C₂₀H₂₃NO₉ (*m/z*, 422.1457, [M⁺+H]) by its positive-ion high resolution FAB-MS. The IR spectrum of **2** revealed the presence of hydroxyl (3350 cm⁻¹), nitrile (2235 cm⁻¹), and carbonyl groups (1710 cm⁻¹). The signals assigned to the sugar moiety indicated the presence of a glucose unit. In the ¹H- and ¹³C-NMR spectra, the anomeric signal was observed at δ_H 4.33 (d, *J*=7.8 Hz) and δ_C 104.3 as a β-glucopyranoside. The ¹H-NMR data showed two doublets centered at δ 7.49 and 6.81 (each 2H, *J*=8.8 Hz), assigned to the A₂B₂ system of the aromatic protons, and another two doublets at δ 7.69 and 6.41 (each 1H, *J*=16.1 Hz), which indicates a *trans*-*p*-coumaroyl moiety.⁶⁾ The ¹H- and ¹³C-NMR spectral data and HMBC experiment showed that the remaining signals were 3-oxymethyl-2-butenenitrile moiety. In the HMBC spectrum, the proton signal of H-4 (δ 4.70 and 4.60) showed ¹H–¹³C long-range correlations with anomeric carbon (δ 104.3) and C-2 (δ 97.6). The carbon signal C-1' (δ 168.1) of the cinnamoyl moiety correlated with H-5 (δ 5.00) and H-3' (δ 7.69). Finally, the whole structure was established as that of **2**. The ¹³C-NMR data of 4-(β-D-glucopyranosyloxy)-3-(oxymethyl)-2-butenenitrile moiety were nearly identical with sutherlandin reported in the literature.⁷⁾ Comparison of the NMR data of **2** with those reported compounds sutherlandin⁷⁾ and its isomer,⁸⁾ confirmed the stereochemistry about

the double bond (*E*) of butenenitrile moiety. In conclusion, the structure of **2** was determined to be 4-(β-D-glucopyranosyloxy)-3-(4-hydroxycinnamoyloxymethyl)-2-(*E*)-butenenitrile (sutherlandin-5-*trans*-*p*-coumarate).

Experimental

General Procedures IR spectrum was obtained on a JASCO FT/IR 410 spectrometer and UV spectrum was measured in MeOH on a Shimadzu UV-1601 UV-Visible spectrophotometer. ¹H- and ¹³C-NMR spectra were recorded on JEOL JMN-EX 400 and Bruker AMX-500 NMR spectrometers. HR-FAB-MS were recorded on a Tandem MS instrument.

Plant Material *Sorbaria sorbifolia* var. *stellipila* was collected in June 1997 on the Odaesan, Kangwondo, Korea. A voucher specimen is deposited in the Herbarium of College of Pharmacy, Woosuk University (WSU-97-002).

Extraction and Isolation The air-dried plant material (2 kg) was finely powdered and extracted at room temperature with MeOH for 2 weeks. The resultant MeOH extract (70 g), followed by the successive solvent partition gave CH₂Cl₂ (30 g), ethylacetate (5 g), and water soluble fractions. Ethylacetate soluble fraction was chromatographed (CC) over Sephadex LH-20 (60 g) with MeOH (150 ml) to afford four fractions (fr. 1: yield 0.8 g; fr. 2: 0.3 g; fr. 3: 1.2 g; fr. 4: 0.6 g). Rechromatography of fr. 2 over silica gel (20 g, 230–400 mesh) using *n*-hexane–EtOAc–MeOH (5 : 15 : 2, 1.2 l) to afford two fractions. Secondary fraction (220 mg) was purified on a reversed-phase Lobar-A column (Merck) using MeOH : H₂O (1 : 1) to yield **1** (50 mg) and **2** (15 mg), respectively.

Cardiospermin-5-(4-hydroxy) benzoate (**1**): HR-FAB-MS (*m*-nitrobenzyl alcohol as matrix) *m/z*: 396.1303 [M⁺+1, Calcd for C₁₈H₂₂NO₉ 396.1295]. ¹³C-NMR (CD₃OD) δ 167.6 (C-1'), 163.9 (C-5'), 139.1 (C-3), 133.1 (C-3', 7'), 121.7 (C-2'), 119.5 (C-4), 117.4 (C-1), 116.3 (C-4', 6'), 102.1 (C-1''), 78.5 (C-5''), 77.8 (C-3''), 74.7 (C-2''), 71.4 (C-4''), 67.6 (C-2), 64.4 (C-5), 62.7 (C-6''). ¹H-NMR data were in good agreement with those reported in the literature,³⁾ while ¹³C-NMR data have not yet been reported. * Chemical shift values in the same column are interchangeable.

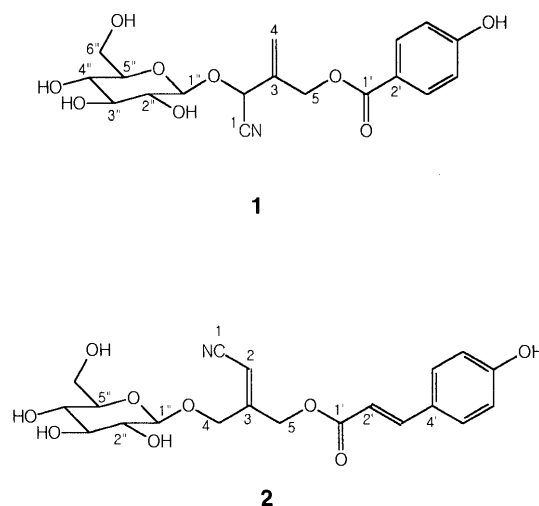


Fig. 1

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Table 1. NMR Data for Compound **2** (CD₃OD)

C	DEPT ^{a)}	¹³ C ^{a)}	¹ H ^{b)}	HMBC (¹ H→ ¹³ C) ^{c)}
1	C	116.5	—	—
2	CH	97.6	5.73 (1H, s)	4-C, 5-C
3	C	159.8	—	—
4	CH ₂	68.4	4.70 (1H, d, <i>J</i> =13.2 Hz) 4.60 (1H, d, <i>J</i> =13.2 Hz)	2-C, 3-C, 5-C, 1''-C 2-C, 3-C, 5-C, 1''-C
5	CH ₂	64.4	5.00 (2H, s)	2-C, 3-C, 1'-C
1'	C	168.1	—	—
2'	CH	114.2	6.41 (1H, d, <i>J</i> =16.1 Hz)	4'-C, 1'-C
3'	CH	147.6	7.69 (1H, d, <i>J</i> =16.1 Hz)	1'-C, 5'-C, 9'-C
4'	C	127.0	—	—
5'	CH	131.4	7.49 (1H, d, <i>J</i> =8.8 Hz)	3'-C, 7'-C
6'	CH	116.9	6.81 (1H, d, <i>J</i> =8.8 Hz)	4'-C
7'	C	161.6	—	—
8'	CH	116.9	6.81 (1H, d, <i>J</i> =8.8 Hz)	4'-C
9'	CH	131.4	7.49 (1H, d, <i>J</i> =8.8 Hz)	3'-C, 7'-C
1''	CH	104.3	4.33 (1H, d, <i>J</i> =7.8 Hz)	4-C
2''	CH	74.9	—	—
3''	CH	78.0*	—	—
4''	CH	71.4	—	—
5''	CH	78.2*	—	—
6''	CH ₂	62.5	—	—

a) 100 MHz, CD₃OD. b) 400 MHz, CD₃OD. c) 500 MHz, CD₃OD. *Chemical shift values in the same column are interchangeable.

Sutherlandin-5-*trans*-*p*-coumarate (**2**): white needle crystals, $[\alpha]_D^{18} +27.8^\circ$ (*c*=0.09, MeOH), mp 88—89 °C, IR (Nujol) $\nu_{\max}$ cm⁻¹: 3350, 2235, 1710. UV (MeOH) $\lambda_{\max}$ nm: 315, 211. HR-FAB-MS (*m*-nitrobenzyl alcohol as matrix) *m/z*: 422.1457 [*M*⁺+1, Calcd for C₂₀H₂₄NO₉ 422.1451]. ¹H- and ¹³C-NMR see Table 1.

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References and Notes

- 1) "The Dictionary of Chinese Drugs," Vol. 3, Shanghai Science and Technology Press and Shougakukan, Tokyo, 1985, p. 1813.
- 2) Lee T. B., "Illustrated Flora of Korea," Hyang Moon Sa, Seoul, 1989, p. 426.
- 3) Kim D. K., Choi S. H., Lee J. O., Ryu S. Y., Park D. K., Shin D. H., Jung J. H., Pyo S. K., Lee K. R., Zee O. P., *Arch. Pharm. Res.*, **20**, 85—87 (1997).
- 4) Kim D. K., Eun J. S., Lim J. P., Lee K. R., Zee O. P., *Yakhak Hoeji*, **43**, 285—288 (1999).
- 5) Nahrstedt A., Scheid H., *Phytochemistry*, **20**, 1309—1310 (1981).
- 6) Takeda Y., Masuda T., Honda G., Takaishi Y., Ito M., Ashurmetov O. A., Khodzhimatov O. K., Otsuka H., *Chem. Pharm. Bull.*, **47**, 1338—1340 (1999).
- 7) Swenson W. K., Dunn J. E., Conn E. E., *Phytochemistry*, **26**, 1835—1836 (1987).
- 8) Braekman J. C., Daloze D., Pasteels J. M., *Biochem. System. Ecol.*, **10**, 355—364 (1982).

Phenylpropanoids from *Umbilicus pendulinus*

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Phytochemical investigation of the leaves of *Umbilicus pendulinus* afforded in addition to 2-*O*-caffeoyl malate, isoquercitrin and *Z*-venusol, the new isomer *E*-venusol. Special NMR experiments were carried out to elucidate the configuration of the two latter compounds.

Key words *Umbilicus*; Crassulaceae; phenylpropanoid; venusol; 2-*O*-caffeoyl malate; isoquercitrin

Umbilicus pendulinus DC. (Crassulaceae) is a robust perennial herb widespread in western and southern Europe. Species from this calcifuge genus grow generally on rocks, walls and more rarely on trees. Only four studies concerning isolation or characterization of secondary metabolites have been carried out on the genus. Some authors mentioned the presence of betaines¹⁾ and leucodelphinidin.²⁾ Others studies led to the isolation of *Z*-venusol, a phenylpropanoid glycoside.^{3,4)} Our preliminary investigations of HPLC chromatograms obtained from crude extract of *Umbilicus* leaves showed the presence of compounds with UV spectra close to *Z*-venusol. In the present paper, we wish to describe the isolation and the structural elucidation from the lyophilised leaves of *Umbilicus pendulinus* of a *O*-glucosylflavonol and 3 phenylpropanoids among which *E*-venusol is described for the first time.

Results and Discussion

Lyophilised leaves of *Umbilicus pendulinus* were directly extracted by a mixture of CH₂Cl₂–MeOH (1 : 1). The crude extract was partitioned against solvents of increasing polarity. The EtOAc layer was fractionated by a combination of gel filtration and chromatographic techniques including C₁₈,

Diol bonded silica and polyamide CC. This led to the isolation of *Z*- and *E*-venusol (**1**, **2**), 2-*O*-caffeoyl malate **3** and isoquercitrin **4**. Compound **3** was first isolated from *Phaseolus vulgaris*.⁵⁾ Compound **4** is of common occurrence in the plant kingdom.⁶⁾ Compounds **3** and **4** have been identified by comparison of their spectral data with the literature.^{7–8)}

In the course of the isolation of **1**, we noticed that prolonged exposure to MeOH led to a major compound **5** in mixture with **1** as an impurity. Analysis of the ¹H-NMR spectrum (Table 1) of **5** indicates the presence of a *p*-disubstituted aromatic ring (δ 6.75, H-6/8, d, J =8.7 Hz; δ 7.72, H-5/9, d, J =8.7 Hz), an anomeric sugar signal (δ 5.04, H-1', d, J =7.5 Hz), an OMe group (δ 3.73, 3H, s) and an olefinic proton (H-3) as a singlet at 6.77 ppm. The ¹³C-NMR spectrum of **5** (Table 1) displays characteristic signals for a *O*-glucose moiety. On the HMBC spectrum of **5**, the OMe at 3.73 ppm is found to be strongly correlated with the carbon signal at 164.9 ppm (C-1), suggesting the presence of a COOMe function. In addition, a ³ J correlation between H-3 and the aromatic carbons C-5 and C-9 demonstrate that the latter proton is localized near the aromatic ring. Furthermore, H-3 is correlated (² J) to the quaternary C at 139.5 ppm (C-2). The chemical shift of C-2, together with the presence of a ³ J correlation

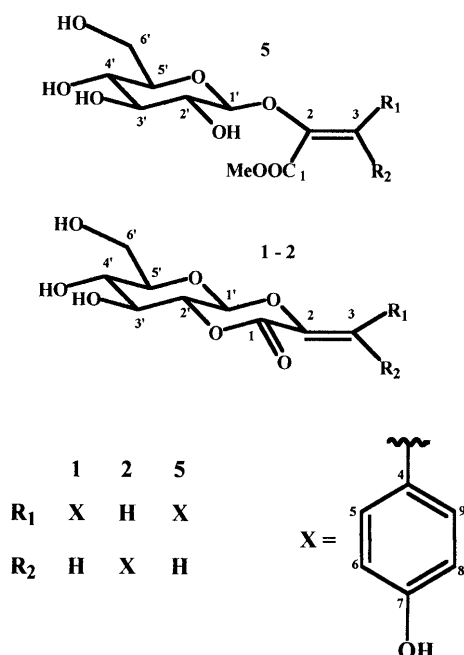


Table 1. NMR Spectral Data for **5** in DMSO-*d*₆ (¹³C: 125 MHz, ¹H: 500 MHz, δ ppm, J Hz)

Position	5		
	¹ H	¹³ C	HMBC
1		164.94	
2		139.52	
3	6.77 s	123.70	C-1; C-2; C-5/9
4		125.01	
5/9	7.72 d (8.7)	133.04	C-3; C-7; C-5/9
6/8	6.75 d (8.7)	116.11	C-4; C-7; C-6/8
7		159.16	
OMe	3.73 s	52.75	C-1
Glucose			
1'	5.04 d (7.5)	101.42	C-2
2'	3.17–3.24 m	74.94	
3'	3.17–3.24 m	77.42	C-4'; C-2'
4'	3.10 m	70.73	
5'	3.06 m	78.44	
6'a	3.58 m	61.55	
6'b	us ^{a)}		

a) Under solvent.

Table 2. NMR Spectral Data for **1** and **2** in Acetone- d_6 (^{13}C : 125 MHz, ^1H : 500 MHz, δ ppm, J Hz)

Position	1			2		
	^1H	^{13}C	HMBC	^1H	^{13}C	HMBC
1		160.91			160.30	
2		136.97			136.00	
3	6.79 s	119.13	C-1; C-2; C-5/9	6.67 s	124.08	C-1; C-2; C-4; C-5/9
4		125.91			124.08	
5/9	7.71 d (8.8)	133.28	C-3; C-7; C-5/9 C-6/8	7.55 d (8.8)	132.29	C-3; C-7; C-5/9; C-4
6/8	6.89 d (8.8)	116.38	C-4; C-7; C-6/8	6.81 d (8.8)	115.23	C-4; C-7; C-6/8
7		159.33			158.30	
Glucose						
1'	5.23 d (7.8)	95.87	C-2'	5.10 d (7.8)	95.80	C-2'
2'	4.08 dd (9.4; 7.8)	79.64	C-1'; C-3'	4.03 dd (9.6; 7.8)	78.76	C-1'; C-3'
3'	3.82 m	74.49		3.82 m	73.80	C-4'
4'	ca. 3.60 m	70.98		ca. 3.58 m	70.66	C-5'
5'	ca. 3.63 m	79.50		ca. 3.58 m	78.76	C-4'
6'a	3.97 dd (12; 14)	62.06		3.90 m	61.67	
6'b	3.82 m			3.78 m		

with H-1' indicates that this carbon is linked to the glucosyl moiety through an osidic bond. This data suggest a modified cinnamoyl unit in which C-2 of the olefinic bond bears an oxygen atom. Thus compound **5** is identified as methyl lespedezate.

Both *Z*- and *E*-methyl lespedezate have been synthesized by Shigemori *et al.*⁹⁾ The ^1H -NMR data for the two isomers (CD_3OD) and a few related compounds indicates that H-3 is more deshielded in the case of the *Z*-isomer than that of the *E*-isomer.⁹⁾ Similar examination of the proton spectra ($\text{DMSO}-d_6$) identifies **5** as *Z*-methyl lespedezate (H-3: $\delta=6.77$ ppm).

FAB-MS analyses of **1** and **2** demonstrate that they have the same molecular ion $[\text{M}+\text{H}]^+$ at m/z 325. Therefore **1** and **2** are isomers each other, differing from **5** by 32 mu. ^1H -NMR spectra of **1** and **2** are close to those of **5**, except for the absence of the OMe signal and a significant deshielding of the glucose H-2' (approximately +0.9 ppm). This suggests that position 2' of glucose is esterified. This is confirmed i) by the 4.70 ppm deshielding of C-2' in **1** (acetone- d_6) as compared to **5** ($\text{DMSO}-d_6$), and ii) by the 5.55 ppm shielding of C-1' in **1** as compared to **5**. A similar pattern is observed between **5** and **2**. Therefore, compounds **1** and **2** are two isomers of venusol, probably differing by the configuration of the double bond. Visualization of *Z*- and *E*-venusol by molecular modeling indicates that the main difference between the two compounds is the distance between H-1' of the sugar moiety and H-5/9 of the aromatic ring (*Z*-venusol: 0.341 nm and *E*-venusol: 0.453 nm). Thus to assess the configuration of **1** and **2**, Noediff experiments were carried out. Irradiation of H-5/9 in compound **1** leads to a 1.2% increase of the H-1' signal. A similar experiment on **2** does not show any effect, suggesting that **1** and **2** are *Z*- and *E*-venusols, respectively. Further confirmation is obtained after measurement of the ^3J heteronuclear coupling constant between H-3 and C-1 with 1D long range ^1H - ^{13}C correlation experiments. The *Z*-isomer **1** displays a constant of 10 Hz while a 4 Hz constant is obtained in the case of the *E*-isomer **2**.

Z-venusol has been already characterised by Proliac *et al.*³⁾ On the contrary, *E*-venusol is reported here for the first time in the plant Kingdom. Natural coexistence of *E* and *Z* isomers of cinnamoyl derivatives has already been reported in

the past.^{10,11)} However light-induced *trans/cis* isomerization of *Z*-venusol cannot be excluded as the source of *E* isomer. Furthermore, we noticed that *Z*-methyl lespedezate can easily originate from methanolic solution of *Z*-venusol. Therefore the natural origin of the above mentioned compounds was checked by performing the complete extraction-purification processes in the dark and in the absence of MeOH. HPLC analyses of the resulting fractions showed the natural occurrence of *E*-venusol only.

Experimental

General Methods FAB-MS were obtained in the positive ion mode using thioglycerol as matrix. Molecular modeling of *Z*- and *E*-venusol used Chemsite[®] software. ^1H - and ^{13}C -NMR spectra were measured at 500 and 125 MHz respectively on a BRUKER DRX500 spectrometer. Noediff experiments used the standard BRUKER noemult pulse sequence. The 1D long range ^1H - ^{13}C correlation experiments used a home made sequence based on a classical 2D gradient HSQC and incorporating a Dante-Z pulse for the selection of the ^{13}C signal.¹²⁾ Column chromatography was performed over Sephadex LH-20[®] (Pharmacia). MPLC was carried out on Büchi 460×36, 460×15 and 230×15 mm columns over Polyamide CC6 (Macherey-Nagel), Silica gel 60 RP-18 (40–63 μm , Merck) or Lichroprep[®] Diol (40–63 μm , Merck). The following solvent combinations were used for chromatographic separation: solvent 1 (CHCl_3 to iso-PrOH gradient); solvent 2 (H_2O –EtOH–TFA, gradient from 90/10/0.1 to 80/20/0.1); solvent 3 (H_2O –EtOH–TFA, gradient from 90/10/0.1 to 50/50/0.1); solvent 4 (gradient of MeOH in CHCl_3); solvent 5 (H_2O –MeOH–TFA 20 : 80 : 0.1); solvent 6 (gradient of EtOH in toluene); solvent 7 (H_2O –MeOH–TFA 75 : 25 : 0.1).

Plant material *Umbilicus pendulinus* leaves were collected during winter 1996 at St Michel and Courzieu (Loire and Rhône, France). A voucher specimen has been deposited at Texinfine Laboratories.

Extraction and Isolation of **1, **2**, and **4**** Dried lyophilized and powdered leaves (5 kg) were extracted with a mixture of CH_2Cl_2 –MeOH (1 : 1). After filtration and removal of the solvent, the residue (700 g) was partitioned according to the previous method modified as follows:¹³⁾ 20 g of the total crude extract were dissolved in MeOH (800 ml) and hexane (800 ml) plus H_2O (88 ml) were added. The two phases were separated and the hypophase was extracted twice with hexane (2×800 ml). After addition of 248 ml H_2O the hypophase was extracted again with hexane (800 ml). The last hypophase was concentrated to one third of its original volume, diluted with H_2O (400 ml) and extracted with CHCl_3 (2×800 ml). The aqueous phase was finally partitioned against 2×800 ml of EtOAc. The total EtOAc extract (2×44 g) was fractionated into six fractions (1–6) by MPLC on Diol (solvent 1).

Fraction 5 (5 g) was submitted to MPLC on C_{18} (solvent 2) to give fraction 5a (pure **1**, 900 mg) and 5b (223 mg). MPLC fractionation of fraction 5b on Diol using solvent 3 gave **2** (12 mg). **4** (4 mg) was isolated from fraction 6 (2.8 g) by two successive MPLC on C_{18} (solvent 3) and Polyamide (MeOH).

Extraction and Isolation of 3 Following the same process as described above, another EtOAc extract (4 g) from 1 kg of dried lyophilized leaves was produced and subjected to successive gel filtration on Sephadex LH-20 using EtOAc and MeOH. Fractions containing **3** were submitted successively to two successive MPLC on Diol (solvent 4) and C₁₈ (solvent 5). Final purification was achieved by HPLC (250×10 mm, Lichrospher® ODS 5 µm, solvent 5, 5 ml/min) to give pure **3** (3.8 mg).

HPLC Demonstration of the Natural Occurrence of 1—2 All steps were performed in the dark and in the absence of MeOH. Dried material (50 g) was extracted (2 l) by a mixture of CH₂Cl₂–EtOH (1 : 1) for 48 h. After filtration and evaporation, the resulting crude extract was dissolved in toluene and chromatographed by MPLC on Polyamide using solvent 6. From the twelve fractions obtained, aliquots were analysed by HPLC (250×4.6 mm, Lichrospher® ODS 5 µm, solvent 7, 1 ml/min) for their content in compounds **1—2**.

Compound **1**: UV λ_{max}nm: 323; ¹H- and ¹³C-NMR: Table 2; FAB-MS (rel. int.): *m/z*: 325 [M+H]⁺ (41).

Compound **2**: UV λ_{max}nm: 326; ¹H- and ¹³C-NMR: Table 2; FAB-MS (rel. int.): *m/z*: 325 [M+H]⁺ (16).

Compound **5**: UV λ_{max}nm: 300; ¹H- and ¹³C-NMR: Table 1; FAB-MS (rel. int.): *m/z*: 379 [M+Na]⁺ (23), 357 [MH]⁺ (14), 195 [M–Glc+H₂O+H]⁺ (55).

References

- 1) Adrian-Romero M., Wilson S. J., Blunden G., Yang M. H., Carabot-Cuervo A., Bashir A. K., *Biochem. Syst. Ecol.*, **26**, 535–543 (1998).
- 2) Combier H., Thèse de docteur-ingénieur, Lyon (1968).
- 3) Proliac A., Desage M., Favre-Bonvin J., *Tetrahedron Lett.*, **22**, 3583–3584 (1981).
- 4) Pagani F., *Boll. Chim. Farmaceutico*, **129**, 279–280 (1990).
- 5) Scarpati M. L., Oriente G., *Gazz. Chim. Ital.*, **90**, 212 (1960).
- 6) Harborne J. B., Grayer R., "Flavonoids and Insects in the Flavonoids Advances in Research Since 1986," ed. by J. B. Harborne, Chapman & Hall, 1994, p. 598.
- 7) Wang S., Ghisalberti E. L., *J. Nat. Prod.*, **61**, 508–510 (1998).
- 8) Seto T., Yasuda I., Akiyama K., *Chem. Pharm. Bull.*, **40**, 2080–2082 (1992).
- 9) Shigemori H., Sakai N., Miyoshi E., Shizuri Y., Yamamura S., *Tetrahedron*, **46**, 383–394 (1990).
- 10) Larsen L. M., Olsen O., Plöger A., Sorensen H., *Phytochemistry*, **22**, 219–222 (1983).
- 11) Skrzypek Z., Wysokinska H., Swiatek L., Wroblewski A. E., *J. Nat. Prod.*, **62**, 127–129 (1999).
- 12) Parella T., *Magn. Reson. Chem.*, **34**, 329 (1996).
- 13) Haugan J. A., Aakermann T., Liaaen-Jensen S., *Methods Enzymol.*, **213**, 231–245 (1992).

1) Adrian-Romero M., Wilson S. J., Blunden G., Yang M. H., Carabot-

A Novel β -Glucuronidase Inhibiting Triterpenoid from *Paeonia emodi*

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A novel β -glucuronidase inhibiting triterpene (1) has been isolated from the chloroform fraction of *Paeonia emodi*. Its structure has been established as 1 β ,3 β ,5 α ,23,24-pentahydroxy-30-norolean-12,20(29)-dien-28-oic acid on the basis of spectroscopic analysis, including high resolution mass spectroscopy, one and two-dimensional NMR techniques. Oleanolic acid, betulinic acid, ethyl gallate, methyl grevillate and 1,5-dihydroxy-3-methyl-anthraquinone are also reported for the first time from *Paeonia emodi*.

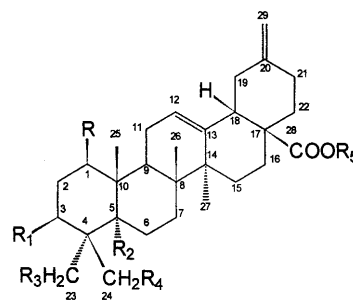
Key words *Paeonia emodi*; Paeoniaceae; triterpenoid; enzyme inhibition

The genus *Paeonia* (Paeoniaceae) comprises herbs and shrubs distributed in India, Pakistan and Afghanistan. Its various species are used as anticoagulant, emmenagogue, antipyretic and in the treatment of scarlet fever, haemorrhage, bilious, obstructions, dropsy, epilepsy and hysteria.^{1–3} *Paeonia emodi* WALL. occurs in northern areas of Pakistan at a high altitude. Previously five monoterpene glycosides have been reported from this plant.^{4–5} During a search for new bioactive compounds from indigenous medicinal plants, we isolated a novel β -glucuronidase inhibiting triterpenoid (1) besides oleanolic acid, betulinic acid, ethyl gallate, methyl grevillate and 1,5-dihydroxy-3-methylanthraquinone from the chloroform soluble fraction.

Results and Discussion

The IR spectrum of 1 displayed absorptions for hydroxyl groups (3450 cm⁻¹), acid carbonyl (1688 cm⁻¹), double bond (1655 cm⁻¹) and exocyclic double bond (880 cm⁻¹).⁶ The high-resolution (HR)-FAB-MS provided [M+H]⁺ peak at *m/z* 505.3155 indicating the molecular formula C₂₉H₄₄O₇. Acetylation of 1 provided tetra-acetate 1a, which still showed the hydroxyl absorption in IR spectrum confirming the presence of one or more tertiary hydroxyls. Methylation afforded the monomethyl ester 1b confirming the presence of one carboxyl group. In EIMS, the peaks at *m/z* 232 and 187 could be assigned to the fragments of the D/E rings formed by retro-Diels–Alder fragmentation of the C-ring of the β -amyrin Δ^{12} -skeleton.⁷ These fragment ions were 16 mass units less than the corresponding fragment of hederagenin indicating the presence of an exomethylene group in rings D/E of 1. In addition the ¹³C-NMR spectrum showed 29 carbon signals which also suggested the nor skeleton. Further, the low field carbon signals at δ 149.8 (s) and 107.0 (t) were assigned to the exomethylene C-20 and C-29 respectively, by comparison with the chemical shifts of akebonic acid.⁸ The location of the exomethylene was confirmed by heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple-bond correlation (HMBC) experiments which showed ²*J* coupling of exomethylene protons at δ 4.57 to C-20 (δ 149.8) and ³*J* interaction to C-19 (δ 42.7) and C-21 (δ 39.0). Further HMBC spectrum showed ²*J* coupling of H-18 proton at δ 2.74 to C-19 (δ 42.7), C-13 (δ 144.9) and C-17 (δ 47.6) and ³*J* coupling to C-20 (δ 149.8), C-12 (δ 123.8), C-14 (δ 43.0), C-28 (δ 182.6), C-16 (δ 24.4) and C-22 (δ 31.0). The ¹H-NMR spectrum of 1 closely resembled those of akebonic

acid and 3 β ,23-dihydroxy-30-norolean-12,20(29)-dien-28-oic acid⁹ revealing the same substitution pattern in rings C, D and E. The spectrum included signals for three methyl singlets at δ 0.88, 1.09 and 1.19. The absence of two methyl signals and the appearance of four protons AB doublets at δ 3.26, 3.50, 3.42 and 3.60 which were shifted downfield to δ 3.83, 4.07, 3.98 and 4.19 in the corresponding acetate 1a implied that these positions are substituted with hydroxymethyl groups. Their locations were confirmed by HMQC and HMBC experiments, which revealed ²*J* correlations of the oxymethylene protons to C-4 (δ 43.9) and ³*J* correlations to C-5 (δ 76.1) and C-3 (δ 78.4). The downfield signals at δ 5.28 (1H) and 4.57 (2H) were due to the olefinic protons (H-12) and (2H-29) respectively. The molecular formula of 1 displayed seven oxygen atoms in the molecule, four of which were due to oxymethyl and carboxylic groups. The mass fragmentation pattern suggested the location of the carboxylic moiety at C-17 which was confirmed by the HMBC experiment. Acetylation of 1 to the corresponding tetra-acetyl derivative 1a showed that two out of three remaining oxygens are accounted by secondary hydroxyls, providing evidence for one tertiary hydroxyl. It was supported by the presence of the corresponding carbon signals at δ 71.6 (d), 78.4 (d) and 76.1 (s) in the ¹³C-NMR spectrum. As the mass fragmentation pattern of 1 indicated that all of the hydroxyl groups are present in rings A and/or B, their positions at C-1, C-3 and C-5 were conclusively established through HMQC and HMBC experiments which showed ²*J* interactions of the methyl protons at δ 1.09 (Me-25) to C-10 (δ 37.5) and ³*J* interactions to C-1 (δ 71.6), C-5 (δ 76.1) and C-9 (δ 48.8) while the oxymethylene protons showed ²*J* correlation to C-4



- 1 R = R₁ = R₂ = R₃ = R₄ = OH, R₅ = H
 1a R = R₁ = R₃ = R₄ = OAc, R₂ = OH, R₅ = H
 1b R = R₁ = R₃ = R₄ = OAc, R₂ = OH, R₅ = CH₃

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(δ 43.9) and ^3J correlations to C-3 (δ 78.4) and C-5 (δ 76.1). The remaining problem was to assign the configurations at C-1 and C-3. The $^1\text{H-NMR}$ spectrum showed two double doublets at δ 3.58 ($J_{aa}=9.5$ and $J_{ac}=5.5$ Hz) and δ 3.35 ($J_{aa}=9.5$ and $J_{ac}=5.0$ Hz) for H-1 and H-3 allowing us to assign β and equatorial configurations for the hydroxyl groups. The β -configuration of the secondary hydroxyl groups was also supported by the appreciable downfield shift of 0.11 ppm suffered by the Me-25 when the $^1\text{H-NMR}$ spectrum of **1** was repeated in pyridine- d_5 instead of $\text{CDCl}_3 + \text{CD}_3\text{OD}$. The α -configuration was assigned to OH-5 on the basis of biogenetic grounds and experimental results. Re-recording of the $^1\text{H-NMR}$ spectrum of **1a** in pyridine- d_5 did not show an anisotropic effect of OH-5 on Me-25, ruling out its β -configuration. Further confirmation of the *trans*-fusion of the A/B ring system was provided by the $^{13}\text{C-NMR}$ spectrum. The presence of a β -hydroxy function at C-5 causes an upfield shift of Me-25 by about δ 8.0–9.62 due to *gauche*-interaction.^[10] However, no such upfield shift of Me-25 was observed. Therefore, the structure of compound **1** was elucidated as 1 β ,3 β ,5 α ,23,24-pentahydroxy-30-norolean-12,20(29)-dien-28-oic acid. Compound **1** is one of the rare examples of oxygenation at both C-23 and C-24 following earlier isolation of belleric acid from *Terminalia berlerica* Roxb.^[11] and also the first naturally occurring triterpene with a hydroxyl function at C-5.

The inhibitory activity of **1** against β -glucuronidase was determined by the method described in the experimental section. The IC_{50} value of **1** was found to be $0.16 \text{ mM} \pm 0.003$ ($n=5$). β -Glucuronidase, a glycohydrolase, has been found in animals, plants and bacteria.^[12] This enzyme catalyzes the hydrolysis of β -glucuronides produced in the body as well as in plants, such as benzo- $[\alpha]$ -pyrene and glycyrrhizin, respectively.

Experimental

General Optical rotations were measured on a JASCO DIP-360 polarimeter. IR spectrum was recorded on 460 Shimadzu spectrometer. EIMS and HR-FAB-MS were recorded on JMS-HX-110 with a data system and on JMS-DA 500 mass spectrometers. The $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, HMQC and HMBC spectra were recorded on Bruker spectrometers operating at 500 MHz for ^1H and 125.7 MHz for $^{13}\text{C-NMR}$ respectively. The chemical shift values are reported in ppm (δ) units and the coupling constants (J) are in Hz. For TLC pre-coated aluminium sheets, silica gel 60 F₂₅₄ (20 $\times$ 20 cm 0.2 mm thick; E-Merck) and for column flash silica (230–400 mesh) were used. Visualization of the TLC plates was achieved under UV at 254 and 366 nm and by spraying with ceric sulphate reagent solution (with heating). For enzyme inhibition assay, all chemicals used and β -glucuronidase (from bovine liver) were purchased from Sigma Co. Limited.

Plant Material The roots of *Paeonia emodi* (10 kg) were collected from Swat (Pakistan) in July, 1997 and identified by Mr. Habib Ahmed, Plant Taxonomist Post Graduate College Swat where a voucher specimen is deposited.

Extraction and Isolation The roots of *Paeonia emodi* were shade dried ground and extracted thrice with ethanol. The combined ethanolic extract was partitioned between hexane and MeOH. The MeOH fraction was further partitioned with CHCl_3 and then with EtOAc. The CHCl_3 fraction (30 g) was subjected to column chromatography over flash silica eluting with hexane– CHCl_3 , CHCl_3 , CHCl_3 –MeOH and MeOH in increasing order of polarity. The fractions obtained from column eluting with hexane– CHCl_3 (2.5:7.5) were a mixture of three components. These fractions were combined and subjected to preparative TLC (CHCl_3 :hexane; 78:22) to afford ethyl gallate (30 mg), methyl grevillate (21 mg) and 1,5-dihydroxy-3-methylanthraquinone (23 mg), respectively. The fractions which eluted with CHCl_3 –MeOH (9.5:0.5) were a mixture of two components which could be resolved through preparative TLC using solvent system (CHCl_3 :MeOH; 98:02) to obtain betulinic acid (25 mg) and oleanolic acid (19 mg), respectively. The identity of all these compounds was confirmed through compari-

son of physical and spectral data with those reported in the literature.^[13–17]

The fractions which were obtained from the column through elution with CHCl_3 –MeOH (80:20) were combined and rechromatographed over flash silica eluting with CHCl_3 –MeOH in increasing order of polarity. The fractions obtained in CHCl_3 –MeOH (78:22) were subjected to preparative TLC (CHCl_3 :MeOH:H₂O; 82.0:17.8:0.2) to afford the pure compound **1** (32 mg).

1 β ,3 β ,5 α ,23,24-Pentahydroxy-30-norolean-12,20(29)-dien-28-oic acid (1): Obtained as a white amorphous powder. $[\alpha]_D^{28} +98.2^\circ$ ($c=0.058$, MeOH+ CHCl_3). IR (KBr) $\nu_{\text{max}} \text{ cm}^{-1}$: 3450, 2500–3500, 1688, 1655, 880. $^1\text{H-NMR}$ (500 MHz, $\text{CD}_3\text{OD}+\text{CDCl}_3$) δ : 0.88 (3H, s, Me-26), 1.09 (3H, s, Me-25), 1.19 (3H, s, Me-27), 1.63 (1H, m, H-9), 2.74 (1H, dd, $J=13.5$, 4.0 Hz, H-18), 3.26 and 3.50 (2H, ABd, $J=11.0$ Hz, H₂-23), 3.35 (1H, dd, $J=9.5$, 5.0 Hz, H-3), 3.42 and 3.60 (2H, ABd, $J=10.5$ Hz, H₂-24), 3.58 (1H, dd, $J=9.5$, 5.5 Hz, H-1), 4.57 (2H, s, H-29), 5.28 (1H, t, $J=3.5$ Hz, H-12). $^{13}\text{C-NMR}$ (125.7 MHz, $\text{CD}_3\text{OD}+\text{CDCl}_3$) δ : 15.7 (C-25), 18.1 (C-26), 24.3 (C-11), 24.4 (C-16), 26.4 (C-27), 26.8 (C-6), 28.7 (C-15), 31.0 (C-22), 33.1 (C-7), 37.5 (C-10), 39.0 (C-21), 39.1 (C-2), 40.3 (C-8), 42.7 (C-19), 43.0 (C-14), 43.9 (C-4), 47.6 (C-17), 48.0 (C-18), 48.8 (C-9), 64.0 (C-24), 66.6 (C-23), 71.6 (C-1), 76.1 (C-5), 78.4 (C-3), 107.0 (C-29), 123.8 (C-12), 144.9 (C-13), 149.8 (C-20), 182.6 (C-28); HR-FAB-MS $[\text{M}+\text{H}]^+$ m/z 505.3155 (Calcd for $\text{C}_{29}\text{H}_{45}\text{O}_7$: 505.3152). EIMS m/z (rel. int. %): 458 (5), 427 (22), 270 (3), 232 (84), 219 (14), 204 (19), 187 (100), 153 (17), 107 (16), 69 (28), 55 (46).

Acetylation of (1) Compound **1** (16.0 mg) was acetylated with Ac_2O (2 ml) in pyridine (2 ml) at room temperature for 24 h. Usual workup provided the corresponding acetyl derivative **1a**. Amorphous solid. $[\alpha]_D^{28} +55.4^\circ$ ($c=0.062$ MeOH); IR (KBr) $\nu_{\text{max}} \text{ cm}^{-1}$: 3410, 1720, 1690, 1655, 880. $^1\text{H-NMR}$ (500 MHz, CDCl_3+2 drops CD_3OD) δ : 0.81 (3H, s, Me-26), 1.11 (3H, s, Me-25), 1.15 (3H, s, Me-27), 2.03 (6H, s, $2\times\text{OAc}$), 2.06 (6H, s, $2\times\text{OAc}$), 2.75 (1H, dd, $J=13.5$, 4 Hz, H-18), 3.83 and 4.07 (2H, AB d, $J=11.0$ Hz, H₂-23), 3.91 (1H, dd, $J=9.5$, 5.0 Hz, H-3), 3.98 and 4.19 (2H, ABd, $J=11.0$ Hz, H₂-24), 4.18 (1H, dd, $J=9.5$, 5.6 Hz, H-1), 4.62 (2H, s, H-29), 5.28 (1H, t, $J=3.6$ Hz, H-12). HR-FAB-MS $[\text{M}+\text{H}]^+$ m/z : 673.3580 (Calcd for $\text{C}_{37}\text{H}_{53}\text{O}_{11}$: 673.3587). EIMS m/z (rel. int. %): 432 (6), 387 (18), 232 (81), 187 (100).

Methylation of (1a) To an ethereal solution of **1a** (12 mg) freshly prepared CH_2N_2 was added in excess and kept at room temperature overnight. Usual workup of the reaction mixture afforded **1b**, which crystallized in white plates from concentrated methanolic solution in the cold: mp 241–243 °C. $^1\text{H-NMR}$ (400 MHz, CDCl_3+2 drops CD_3OD) δ : 0.88 (3H, s, Me-26), 1.08 (3H, s, Me-25), 1.19 (3H, s, Me-27), 2.02 (6H, s, $2\times\text{OAc}$), 2.06 (6H, s, $2\times\text{OAc}$), 2.74 (1H, dd, $J=13.5$, 4.0 Hz, H-18), 3.12 (3H, s, OCH_3), 3.84 and 4.09 (2H, AB d, $J=10.5$ Hz, H₂-23), 3.96 (1H, dd, $J=10$, 5.5 Hz, H-3), 4.01 and 4.19 (2H, AB d, $J=11.0$ Hz, H₂-24), 4.20 (1H, dd, $J=9.5$, 5.0 Hz, H-1), 4.57 (2H, s, H-29), 5.28 (1H, t, $J=3.5$ Hz, H-12). HR-FAB-MS $[\text{M}+\text{H}]^+$ m/z 687.3745 (Calcd for $\text{C}_{38}\text{H}_{55}\text{O}_{11}$: 687.3743). EIMS (rel. int. %) m/z : 446 (33), 246 (32), 187 (100).

Assay of β -Glucuronidase Inhibition Enzyme inhibitory activity of **1** against β -glucuronidase was determined according to Kawasaki *et al.*^[18] with some modification. The reaction mixture contained 101 μl of 0.1 M acetate buffer (pH 5.0), 50 μl of 0.1 mM *p*-nitrophenyl- β -D-glucuronide and 20 units of enzyme. After incubation for 30 min at 37 °C, the reaction was interrupted by the addition of 39.0 μl 0.2 M sodium carbonate. The increase of absorbance with the release of *p*-nitrophenol from the *p*-nitrophenyl- β -D-glucuronide was measured at 405 nm by means of spectramax 340 spectrophotometer. Glucosaccharo-lactone (1:4) was used as a standard inhibitor for positive control. The experiment was repeated five times for measuring means and S.D. The inhibitory activity (%) was calculated according to the following formula: $(E-S)/E \times 100$ where E is the activity of enzyme without test material and S is the activity of enzyme with test material.

References

- Nasir E., Ali S. I., "Flora of West Pakistan," Vol. 121, Department of Botany, University of Karachi, 1978, p. 2.
- Watt G., "Dictionary of the Economic Products of India," Vol. VI, M.B.C.M.I.E., Cosmo Publication, 1992, p. 4.
- Perry L. M., Metzger J., "Medicinal Plants of East and Southeast Asia," The MIT Press Cambridge, London, 1980, p. 336.
- Kaneda M., Iitaka Y., Shibata S., *Tetrahedron*, **28**, 4309–4317 (1972).
- Muhammad P., Ahmed S., Nawaz H. R., Ullah N., Malik A., *Z. Naturforsch.*, **54b**, 544–548 (1999).
- Siddiqui S., Begum S., Siddiqui B. S., Hafeez F., *J. Nat. Prod.*, **52**, 57–62 (1989).

- 7) Budzikiewicz H., Wilson J. M., Djerassi C., *J. Am. Chem. Soc.*, **85**, 3688—3699 (1963).
- 8) Ikuta A., Itokawa H., *Phytochemistry*, **25**, 1625—1628 (1986).
- 9) Ikuta A., Itokawa H., *Phytochemistry*, **27**, 2813—2815 (1988).
- 10) Fajardo V., Podesta F., Shamma M., Freyer A. J., *J. Nat. Prod.*, **54**, 554—563 (1991).
- 11) Nandy A. K., Podder G., Sahu N. P., Mahato S. B., *Phytochemistry*, **28**, 2769—2772 (1989).
- 12) Fishman W. H., "Methods of Enzymatic Analysis," 2nd Ed., ed. by Bergmeyer H. V., Academic Press: New York, 1974, p. 929.
- 13) Imre S., Oztunc A., Buyuktimkin N., *Phytochemistry*, **13**, 681—682 (1974).
- 14) Sheu. S. Y., Hsu F. L., Lin Y. C., *Phytochemistry*, **31**, 2465—2468 (1992).
- 15) Ara I., Siddiqui B. S., Faizi S., Siddiqui S., *Phytochemistry*, **28**, 1177—1180 (1989).
- 16) Betancor C., Freire R., Gonzalez A. G., Salazar J. A., Pascard C., Prange T., *Phytochemistry*, **19**, 1989—1993 (1980).
- 17) Sholichin M., Yamasaki K., Kasai R., Tanaka O., *Chem. Pharm. Bull.*, **28**, 1006—1008 (1980).
- 18) Kawasaki M., Hayashi T., Arisawa M., Morita N., Berganza L. H., *Phytochemistry*, **27**, 3709—3711 (1988).

A Novel Bisnorditerpenelactone from *Mikania hirsutissima*

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A novel bisnorkaurenic acid-type diterpenelactone, named mikania lactone (1), was isolated along with five known kaurenic acid-type diterpenes (2–6) from the aerial parts of *Mikania hirsutissima* DC (Compositae). The structure of the new bisnorditerpene was determined by spectroscopic means.

Key words *Mikania hirsutissima*; Compositae; bisnorditerpenelactone; *ent*-kaurene

Mikania hirsutissima is a medicinal plant which has been used as a folk medicine for treatment of diarrhea, rheumatism and gout in Brazil.¹⁾ In the course of our studies on biologically active substances from South American medicinal plants, we reported the isolation and structural elucidation of two new norhumulene-type sesquiterpenes together with nine known compounds, seven kaurenic acid-type diterpenes, a coumarin and a flavone, from the aerial parts of *M. hirsutissima*.²⁾ Further studies on the constituents of this plant led to the isolation of a novel bisnorditerpenelactone, named mikania lactone (1), along with five known *ent*-kaurenic acid-type diterpenes (2),³⁾ (3),⁴⁾ (4),⁵⁾ (5),⁶⁾ (6)⁷⁾ from *M. hirsutissima*.

Results and Discussion

Compound 1 was obtained as colorless crystals. mp 256–259 °C, $[\alpha]_D^{25} -72.5^\circ$ ($c=1.0$, MeOH). Its IR spectrum showed absorption bands at 3290, 1726, 1754 cm^{-1} , suggesting the presence of a carboxyl group and a γ -lactone ring. The ^1H - and ^{13}C -NMR spectra, including DEPT (Distortionless Enhancement by Polarization Transfer), indicated the presence of two tertiary methyls, eight methylenes, three methines, three quaternary carbons and two carboxyl carbons (Table 1). The EI-MS spectrum of 1 did not show the ion peak corresponding to the molecular formula presumed from its ^{13}C - and ^1H -NMR spectral data. Treatment of 1 with excess diazomethane gave a monomethyl ester⁸⁾ which exhibited a molecular ion peak at m/z 320.1986 (M^+) corresponding to the molecular formula $\text{C}_{19}\text{H}_{28}\text{O}_4$. The ^1H - ^1H COSY spectrum of 1 suggested the presence of the partial structures [A], [B] and [C].

The connectivities of these partial structures and the functional groups were investigated by analysis of the HMBC spectrum of 1. As shown in Fig. 2, the methyl proton signal at δ 1.28 showed cross peaks with carbon signals at δ 43.6 (quaternary carbon), 183.4 (COOH), and 55.6 in the partial structure [B] and δ 37.4 in the partial structure [A]. The other methyl proton signal at δ 0.93 exhibited long range correlations with the carbon signals at δ 39.7 (quaternary carbon), 40.1 in [A], 55.6 in [B] and 50.7 in [C]. Further-

more, long range correlations were observed between the following proton and carbon signals: δ 2.02 in [B], 1.59, 2.35, 2.71 in [C] and δ 88.8 (oxygen-bearing quaternary carbon); δ 1.62, 2.35 in [C] and δ 179.0 (lactone carbonyl carbon). Thus, the plane structure of mikania lactone was confirmed as 1a shown in Fig. 2. Finally, the stereostructure of 1 was determined by difference NOE experiments on 1. Irradiation of the methyl proton signal at δ 1.28 (H-16) produced significant enhancement of the H-5 and H-6 β (δ 2.02) signals, while irradiation of the methyl proton signal at δ 0.93 (H-18) enhanced the signals due to H-2 α (δ 1.51), H-6 α (δ 1.87), H-12 α (δ 1.62) and H-14 α (δ 2.35). Therefore, the relative stereostructure of mikania lactone was represented by structure 1b. Compound 1 and kaurenic acid,⁹⁾ a major component of this plant, showed inhibitory activity (7–10% at a concentration of 10 $\mu\text{g}/\text{ml}$) on the proliferation of peripheral blood lymphocytes,¹⁰⁾ while 2, 3 and 6 slightly increased (2–3% at 10 $\mu\text{g}/\text{ml}$) the proliferation. The details of the biological activities of the isolated compounds from *M. hirsutissima* will be published elsewhere.

Experimental

The plant material was purchased from LABORATORIO FARMAERVAS LTDA. in Sao Paulo, Brazil and a voucher specimen was deposited in the herbarium of the College of Pharmacy, Nihon University. The ^1H - and ^{13}C -NMR spectra were measured on a JEOL GSX-400 spectrometer in CDCl_3 containing TMS as internal standard. The MS spectra were recorded on a Hitachi RMU-6M instrument. Optical rotation was measured on a JASCO DIP-370 polarimeter.

Isolation of Compounds (1–6) The aerial part of dried *M. hirsutissima* (1.9 kg) was extracted with ethanol (2.0 l $\times$ 3) under ultrasonication. The alcoholic extract was concentrated under reduced pressure to give 91.8 g oily material which was then chromatographed on an HP-20 resin (Nippon Rensui[®]) column eluted successively with stepwise gradients of MeOH– H_2O [2:3 (2.0 l), 7:3 (2.0 l)], MeOH (3.0 l) and then acetone (2.0 l). The MeOH fraction (47.6 g) was chromatographed on a silica gel column eluted successively with stepwise gradients of hexane–EtOAc [10:1 (3.6 l), 5:1 (3.0 l), 1:1 (6.0 l)], EtOAc (1.0 l) and then MeOH (2.0 l). The EtOAc fraction (15.1 g) was chromatographed on a silica gel column eluted successively with hexane:EtOAc=7:1, 4:1, 2:1, 1:1 and then EtOAc. The hexane:EtOAc=1:1 and EtOAc fractions were combined and then concentrated under reduced pressure to leave an oily material which was separated by HPLC (Kaseisorb LC 60-5, 20 $\times$ 250 mm, flow rate; 16.0 ml/min) to af-

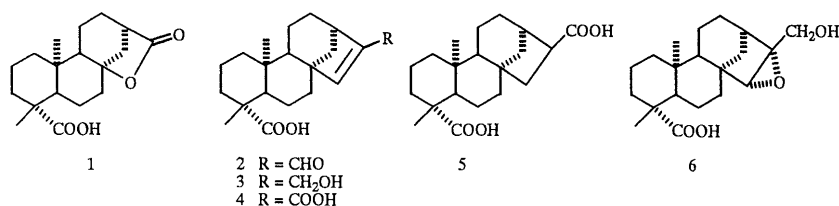


Fig. 1

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Table 1. ^1H - (400 MHz) and ^{13}C -NMR (100 MHz) Data for **1**

Position	^1H -NMR (δ , CDCl_3)	^{13}C -NMR (δ , CDCl_3)
1	0.89 m, 0.98 m	40.1
2	1.51 br d (14.7) 1.83 m	18.9
3	1.05 dt (4.4, 13.2) 2.19 d (13.2)	37.4
4		43.6
5	1.18 m	55.6
6	1.87 m 2.02 m	20.4
7	1.85 m 1.93 m	38.7
8		88.8
9	1.59 m	50.7
10		39.7
11	1.79	18.3
12	1.62 m 1.97 m	26.1
13	2.71 s	40.6
14	1.95 m 2.35 d (11.7)	38.9
15		179.0
16	1.28 s	28.8
17		183.4
18	0.93 s	15.1

The numbers in parentheses are J values in Hz.

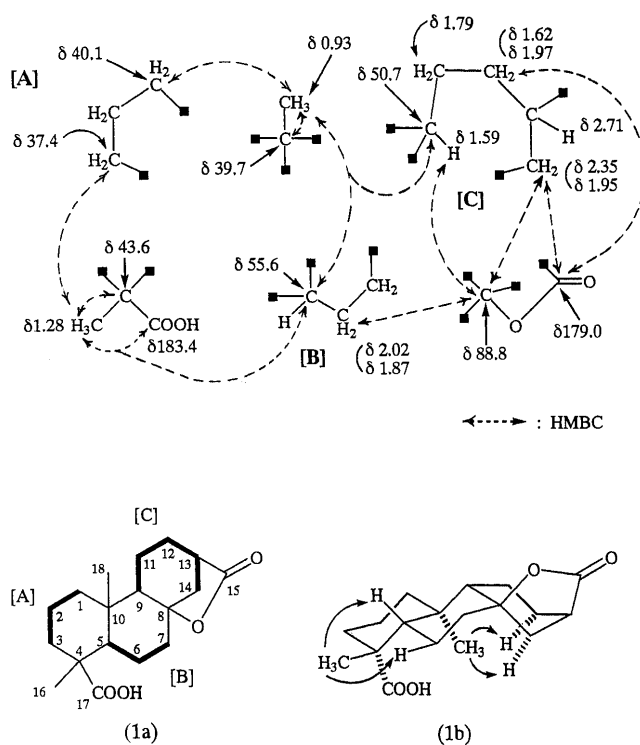


Fig. 2

ford two fractions (fr. A, retention time: 16–20.0 min, fr. B, retention time: 20–36 min). Fraction A was purified by HPLC (Senshu Pak silica 4251N, 10×250 mm, hexane–acetone=5:1, flow rate: 3.0 ml/min) to give **1** (16.4 mg, retention time: 23.0 min) and **6** (284.0 mg, retention time: 38.0 min). Fraction B was purified by HPLC (Senshu Pak PEGASIL ODS, 10×250 mm, CH_3CN – H_2O =55:45, flow rate: 3.0 ml/min) to give **2** (477.0 mg, retention time: 21.0 min), **3** (102.0 mg, retention time: 18.0 min), **4** (14.3 mg, retention time: 11.0 min) and **5** (8.0 mg, retention time: 13.0 min).

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References and Notes

- 1) Hashimoto G., "Illustrated Cyclopedia of Brazilian Medicinal Plants," Aboc-Sha, Japan, 1996, p. 313.
- 2) Ohkoshi E., Makino M., Fujimoto Y., *Chem. Pharm. Bull.*, **47**, 1436–1438 (1999).
- 3) Bohlmann F., Suding H., Cuatrecasas J., Robinson H., King R. M., *Phytochemistry*, **19**, 2399–2403 (1980).
- 4) Yahara S., Ishida M., Yamasaki K., Tanaka O., Mihashi S., *Chem. Pharm. Bull.*, **22**, 1629–1631 (1974).
- 5) Gao F., Wang H., Mabry T. J., *Phytochemistry*, **26**, 779–781 (1987).
- 6) Gao F., Miski M., Gage D. A., Norris J. A., Mabry T. J., *J. Nat. Prod.*, **48**, 489–490 (1987).
- 7) Herz W., Kulanthaivel P., Watanabe K., *Phytochemistry*, **22**, 2021–2025 (1983).
- 8) Colorless needles, mp 168–170°, ^{13}C -NMR (125 MHz, CDCl_3) δ : 15.0 (C-18), 18.3 (C-11), 19.0 (C-2), 20.6 (C-6), 26.1 (C-12), 28.7 (C-16), 37.7 (C-3), 38.8 (C-7), 38.9 (C-14), 39.5 (C-10), 40.2 (C-1), 40.6 (C-13), 43.7 (C-4), 50.7 (C-9), 51.4 (OCH_3), 55.7 (C-5), 88.8 (C-9), 177.5 (C-17), 178.4 (C-15).
- 9) See ref. 2 and references cited therein.
- 10) Hayakawa S., Nagai N., Kanaeda T., Karasaki-Suzuki M., Ishii M., Chishima F., Satoh K., *American Journal of Reproductive Immunology*, **41**, 320–329 (1999).

Synthesis and Cytotoxic Activity of a Series of Diacetylenic Compounds Related to Falcarindiol¹⁾

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The synthesis of a series of diacetylenic compounds related to the natural product falcarindiol has been carried out. Unsymmetrical diacetylenes were prepared by a modification of the Cadiot-Chodkiewicz coupling reaction, while a Glaser coupling was used to prepare symmetrical diacetylenes. These compounds have been tested for *in vitro* cytotoxic activity against Hep-G2, and H-4-II-E cell lines. Diacetylenes with additional unsaturation at C-1, 2, appended with hydroxyl groups at C-3 and C-8, or with long hydrophobic chains, exhibited IC₅₀ values in the micromolar range.

Key words antineoplastic; cytotoxin; diyne; natural product

Diacetylenic phytochemicals, most notably from members of the Araliaceae,²⁾ have been shown to exhibit *in vitro* cytotoxic activity against various tumor cell lines.^{3–6)} Even though these materials show cytotoxic activity against malignant cells, they tend to be less toxic to normal cells.^{3,5)} While this class of compounds represents potential new antineoplastic agents, studies on them have been hampered by the difficulty in isolating them, their inherent instability, and low yields in their syntheses. In this work, we report the preparation of a series of diacetylenic compounds related to the above natural products and an examination of their *in vitro* cytotoxic activities against Hep-G2 (human hepatocellular carcinoma) and H-4-II-E (rat hepatoma) cell lines.

Unsymmetrical diacetylenes **1a–f** were prepared by a modification of the Cadiot-Chodkiewicz coupling reaction of an alkynyl bromide with a terminal alkyne (Chart 1).^{7–9)} A competing side reaction in this coupling is the formation of the symmetrical diacetylene. In order to minimize this competing reaction, both the free cuprous ion concentration and the alkynyl bromide concentration were kept low. The alkynyl bromides, **3**, were prepared from the corresponding acetylene by reaction with hypobromite (95–100% yield). Propargyl alcohols were best prepared by reaction of lithium acetylide ethylenediamine complex with the appropriate aldehyde in a THF/DMSO solvent mixture.

A number of symmetrical diacetylenes were prepared by coupling of the corresponding terminal acetylenes using the

Zal'kind modification of the Glaser coupling (Chart 3).¹⁰⁾ Thus, 2,7-dimethyl-3,5-octanediyn-2,7-diol (**5a**) and 2,4-hexanediyn-1,6-diol (**5b**) were synthesized by this technique. Other symmetrical couplings using this method failed, apparently due to air sensitivity of the final products. Alternatively, symmetrical coupling has been achieved using the corresponding alkynyl bromides (Chart 3). Experimental details for these syntheses are available from the author of correspondence.

In vitro cytotoxic activities have been carried out on the diacetylenes using Hep-G2,¹¹⁾ and H-4-II-E¹²⁾ cell lines. The MTS spectrophotometric technique¹³⁾ was utilized to determine cell viability. IC₅₀ values were determined using the Reed-Muench method.¹⁴⁾ The results are summarized in Table 1. It has already been reported that the diyne moiety is primarily responsible for the biological activity of these materials.^{15,16)} The results of this present study suggest that di-

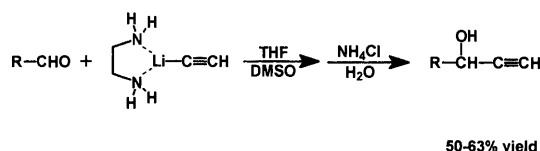
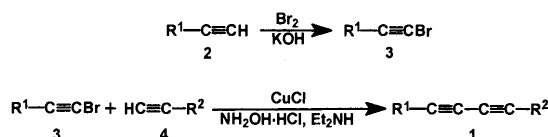
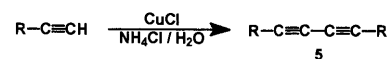


Chart 2. Preparation of Propargylic Alcohols

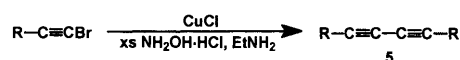


	R ¹	R ²	% yield
a:	CH ₂ =CH-CH(OH)-	-(CH ₂) ₈ -CH ₃	24
b:	CH ₂ =CH-CH(OH)-	-CH(OH)-(CH ₂) ₈ -CH ₃	77
c:	CH ₂ =CH-CH(OH)-	-CH(OH)-CH=C(CH ₃) ₂ -(CH ₂) ₂ -CH=C(CH ₃) ₂	18
d:	CH ₃ CH ₂ -CH(OH)-	-(CH ₂) ₈ -CH ₃	25
e:	HOCH ₂ -	-(CH ₂) ₈ -CH ₃	25
f:	HOCH ₂ CH ₂ -	-(CH ₂) ₈ -CH ₃	8

Chart 1. Preparation of Unsymmetrical Diacetylenes



- a: R = (CH₃)₂C(OH)- 78% yield
b: R = HOCH₂- 23% yield



- c: R = CH₂=CH-CH(OH)- 65% yield
d: R = CH₃CH₂CH(OH)- 84% yield
e: R = CH₃(CH₂)₈CH(OH)- 5% yield

Chart 3. Preparation of Symmetrical Diacetylenes

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Table 1. Cytotoxic Activities of Diacetylenes

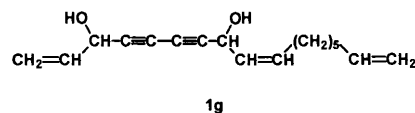
Diacetylene	Cytotoxicity (IC ₅₀ , μ M)	
	Hep-G2	H-4-II-E
1a	32.5 $\pm$ 1.4	4.15 $\pm$ 0.01
1b	0.95 $\pm$ 0.07	1.96 $\pm$ 0.06
1c	4.34 $\pm$ 0.15	11.6 $\pm$ 0.2
1d	309 $\pm$ 35	—
1e	59.3 $\pm$ 3.9	73.1 $\pm$ 3.1
1f	42.8 $\pm$ 3.6	27.6 $\pm$ 1.0
1g^{a)}	35.8 $\pm$ 0.3	149.0 $\pm$ 0.4
5a	>750	—
5b	806 $\pm$ 26	181 $\pm$ 13
5c	10.3 $\pm$ 1.0	41.5 $\pm$ 2.0
5d	247 $\pm$ 23	—
5e	12.8 $\pm$ 1.2	40.2 $\pm$ 1.4
5f	956 $\pm$ 146	—
Tingenone ^{b)}	1.91 $\pm$ 0.09	2.74 $\pm$ 0.40

a) Ref. 5. b) Tingenone was used as a positive control (ref. 24).

acetylenes in which R¹ is an allylic alcohol group and R² is a long hydrophobic chain are essential for high cytotoxicity. Thus, **1a**, **1b**, and **1c** are the most active in this study. The importance of the unsaturation at C-1, 2 is reflected in the relative cytotoxicities of **1a** and **1d**. Bernart *et al.*⁶⁾ have reported a decrease in cytotoxicity with saturation at C-1, 2. Symmetrical diacetylenes with two allylic alcohol moieties (**5c**) or two long hydrophobic chains (**5e**) show intermediate cytotoxicities. Fujimoto and co-workers¹⁷⁾ had observed that shorter chain lengths decrease the cytotoxic activity in diacetylenic compounds, and our results confirm this observation. Thus, cytotoxicities for symmetrical diacetylenes drops off dramatically as chain length shortens; **5b**<**5d**<**5e**. Unsaturation in the hydrophobic (R²) chain seems to attenuate cytotoxic activity; dehydrofalcariindiol (the natural product, **1g**) is less toxic than **1b**. Park *et al.*¹⁸⁾ and Bernart *et al.*⁶⁾ have reported that unsaturation at C-16, 17 decreases cytotoxicity.

To our knowledge, the mechanism of cytotoxic activity of this class of compounds is not well understood. Because the compounds are lipophilic, they would be expected to associate strongly with membrane lipids,¹⁹⁾ potentially leading to disorganization of membrane systems.²⁾ Phototoxicity studies of polyacetylenes suggest that these materials may act as photosensitizers in lipid peroxidation, and this may be responsible for the biological activity.^{20,21)} Antioxidant activity of polyacetylenes^{22,23)} indicate free radical intermediates of these compounds may also be important. Further investigations into the mechanism of activity of these materials are currently underway in our laboratories.

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References and Notes

- 1) Taken in part from the MS thesis (Chemistry, 1998) of Xuyuan Gu, Department of Chemistry, University of Alabama in Huntsville. This work was presented at the 14th Annual Rocky Mountain Regional meeting of the American Chemical Society, March 15–18, 1998, Tucson, Arizona: Gu X., Wells E. B., Setzer W. N., Synthesis and cytotoxic activity of diacetylene compounds related to falcariindiol. Paper number O5.
- 2) Hansen L., Boll P. M., *Phytochemistry*, **25**, 285–293 (1986).
- 3) Matsunaga H., Katano M., Yamamoto H., Fujito H., Mori M., Takata K., Meyer C. A., *Chem. Pharm. Bull.*, **38**, 3480–3482 (1990).
- 4) Fujimoto Y., Satoh M., Takeuchi N., Kirisawa M., *Chem. Pharm. Bull.*, **39**, 521–523 (1991).
- 5) Setzer W. N., Green T. J., Whitaker K. W., Moriarity D. M., Yancey C. A., Lawton R. O., Bates R. B., *Planta Med.*, **61**, 470–471 (1995).
- 6) Bernart M. W., Cardellina J. H., Balaschak M. S., Alexander M. R., Shoemaker R. H., Boyd M. R., *J. Nat. Prod.*, **59**, 748–753 (1996).
- 7) Cadiot P., Chodkiewicz W., "Chemistry of Acetylenes," ed. by Viehe H. G., Marcel Dekker, New York, 1969, pp 597–648.
- 8) Bew R. E., Cambie R. C., Jones E. R. H., Lowe G., *J. Chem. Soc. (C)*, **1966**, 135–138.
- 9) Jones E. R. H., Lowe G., Shannon P. V. R., *J. Chem. Soc. (C)*, **1966**, 139–144.
- 10) Zal'kind Y. S., Aizikovich M. A., *J. Gen. Chem. (USSR)*, **7**, 227–233 (1937).
- 11) Knowles B. B., Howe C. C., Aden D. P., *Science*, **209**, 497–499 (1980).
- 12) Pitot H. C., Peraino C., Morse P. A., Potter V. R., *Natl. Cancer Inst. Monogr.*, **13**, 229–245 (1964).
- 13) Promega Technical Bulletin #245. CellTiter 96[®] AQueous one solution cell proliferation assay. Promega Corp.: Madison, WI, 1996.
- 14) Ipsen J., Feigle P., "Bancroft's Introduction to Biostatistics," 2nd Ed. Harper & Row, NY, 1970, Chapter 15.
- 15) Saita T., Matsunaga H., Yamamoto H., Nagumo F., Fujito H., Mori M., Katano M., *Biol. Pharm. Bull.*, **17**, 798–802 (1994).
- 16) Hu C., Chang J., Lee K., *J. Nat. Prod.*, **53**, 932–935 (1990).
- 17) Fujimoto Y., Wang H., Satoh M., Takeuchi N., *Phytochemistry*, **35**, 1255–1257 (1994).
- 18) Park S. Y., Kim J., *Yakhak Hoechi*, **39**, 681–688 (1995).
- 19) Muir A. D., Cole A. L. J., Walker J. R. L., *Planta Med.*, **44**, 129–133 (1982).
- 20) Hudson J. B., *Antiviral Res.*, **12**, 55–74 (1989).
- 21) Ebermann R., Alth G., Kreitner M., Kubin A., *J. Photochem. Photobiol. B*, **36**, 95–97 (1996).
- 22) Utrilla M. P., Navarro M. C., Jimenez J., Montilla M. P., Martin A., *J. Nat. Prod.*, **58**, 1749–1752 (1995).
- 23) Cavin A., Poterat O., Wolfender J. L., Hostettmann K., Dyatmyko W., *J. Nat. Prod.*, **61**, 1497–1501 (1998).
- 24) Setzer W. N., Hopper A. L., Moriarity D. M., Lehrman G. K., Niekamp K. L., Morcomb S. M., Bates R. B., McClure K. J., Stessman C. C., Haber W. A., *Planta Med.*, **64**, 583 (1998).

Synthesis and Antitumor Activity of Benzimidazolyl-1,3,5-triazine and Benzimidazolylpyrimidine Derivatives

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Triamino-substituted 1,3,5-triazine and pyrimidine derivatives were synthesized and tested for antitumor activities using some human cancer cell lines and murine leukemia cell lines. All the compounds having benzimidazolyl and morpholino groups as substituents on the 1,3,5-triazine ring showed antitumor activity. Pyrimidine derivatives having the same groups as substituents also showed antitumor activity. Among them, the compounds having 1-benzimidazolyl, morpholino and *cis*-2,3-dimethylmorpholino groups as substituents on the 1,3,5-triazine ring or pyrimidine ring exhibited the most potent antitumor activity, and these compounds exhibited no or very weak aromatase inhibitory activity. In contrast, the compounds having imidazolyl group instead of benzimidazolyl group as a substituent on the 1,3,5-triazine ring showed a potent aromatase inhibitory activity.

Key words 1,3,5-triazine; pyrimidine; antitumor activity; P388; MCF-7

We have recently reported the discovery of 1,3,5-triazine derivatives bearing both direct cytotoxicity and selective aromatase inhibitory activity.¹⁻⁴⁾ In the study of 1,3,5-triazine derivatives, 2-(1-imidazolyl)-4,6-dimorpholino-1,3,5-triazine (**1**) showed significant aromatase inhibitory activity and cytotoxicity against various human breast cancer cell lines, such as MCF-7 and a murine leukemia cell line, P388 leukemia.²⁾ Moreover, compound **1** showed the antitumor activity on 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors, due mainly to its direct cytotoxicity.⁴⁾ The cytotoxic activity of compound **1** was greater than that of hydroxymethylpentamethylmelamine (HMPMM). HMPMM is the hydroxylated metabolite and a major active form of hexamethylmelamine (HMM) (Fig. 1).^{5,6)} HMM, a 1,3,5-triazine derivative, has been clinically used as an antitumor agent against lung, ovarian and breast cancers.⁷⁻⁹⁾

Aromatase inhibitors might be applicable as antitumor agents for estrogen-dependent breast cancer in postmenopausal women in whom, the major pathway of estrogen biosynthesis is the peripheral conversion of androstenedione secreted from the adrenal to estrone. This conversion is catalyzed by aromatase which is a cytochrome P450-dependent monooxygenase.¹⁰⁾ However, the use of aromatase inhibitors as antitumor agents in patients other than postmenopausal women might not be approved, because this activity would certainly be accompanied by side effects relating to the suppression of estrogen production.

Therefore, we attempted to synthesize compounds having weaker aromatase inhibitory activity and more potent cytotoxicity than compound **1** for more suitable antitumor agents. In our previous study, the compounds having a 2-position substituted imidazolyl group or a 4,5-position substituted imidazolyl group in the 1,3,5-triazine derivatives did not inhibit aromatase.¹⁾ Our efforts to discover novel antitumor agents were primarily focused on the synthesis of compounds by converting the imidazolyl groups of **1** into various heterocycle groups including the substituted imidazolyl groups such as 4,5-dimethylimidazolyl group and benzimidazolyl group. Among the synthesized compounds, compound **4a** having a

benzimidazolyl group showed weaker aromatase inhibitory activity and more potent cytotoxicity. Several 2-benzimidazolyl-1,3,5-triazine or pyrimidine derivatives were therefore synthesized and their pharmacological activities were examined.

Chemistry The desired 1,3,5-triazine derivatives **4** were prepared by nucleophilic substitution reaction with azole or aliphatic amines as shown in Chart 1.

Compound **2**¹⁾ was treated with benzimidazole in the presence of potassium carbonate in *N,N*-dimethylformamide (DMF) at room temperature to afford compound **3**, followed by treatment with amines to afford compound **4**. The desired pyrimidine derivatives **7** were prepared as shown in Chart 2 employing the similar reaction as for the synthesis of **4**.

In the case of the pyrimidine derivatives, compound **7** was synthesized not from dichloromorpholinopyrimidine but from benzimidazolyl-dichloropyrimidine **5**. The desired precursor **5** was easily obtained by recrystallization from the reaction mixture of trichloropyrimidine with benzimidazole, where the reaction mixture mainly consisted of two isomers, 2-(1-benzimidazolyl)-4,6-dichloropyrimidine (**5**) and 4-(1-benzimidazolyl)-2,6-dichloropyrimidine.

Compound **7** was obtained in poor yield when the same method as for the synthesis of **4** was employed, due to the low activity of monochloropyrimidine **6** for nucleophilic substitution. To obtain pyrimidine **7** in high yield, the reaction of **6** with amine should be carried out with a large excess of amine at a high reaction temperature. DMF was inappropriate as a reaction solvent. The reason is that dimethylamine,

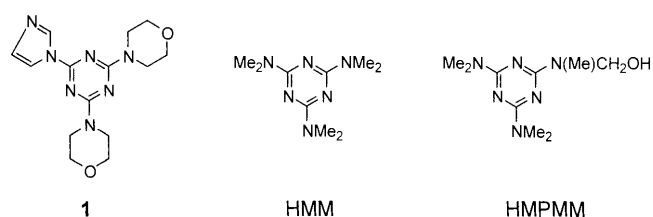


Fig. 1

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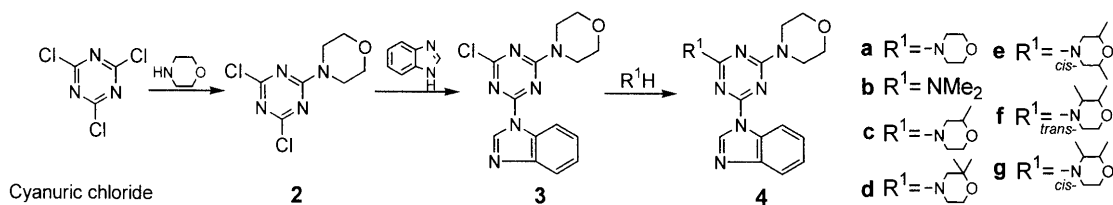


Chart 1

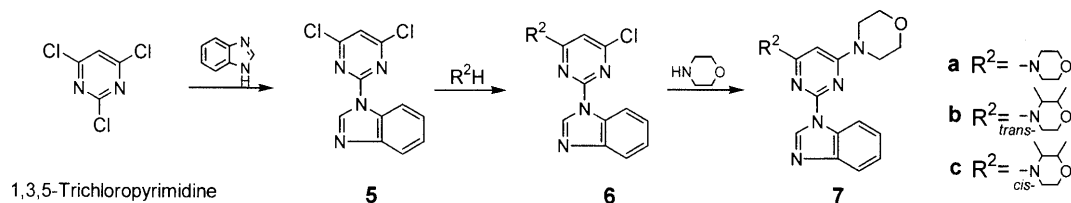


Chart 2

Table 1. Antitumor Activities of 1,3,5-Triazine and Pyrimidine Derivatives toward Cancer Cell Lines

Compd.	GI ₅₀ (μM)						Aromatase Inhibition % (μM)
	MCF-7	A549	WiDr	B16F10	L1210	P388	
4a	3.2	7.0	4.2	6.0	4.1	2.8	8 (10)
4b	11	>25	11	15	11	10	
4c	4.8	>10	6.8	7.4	8.5	3.7	
4d	2.2	6.5	2.3	7.3	3.6	1.8	
4e	2.9	>10	5.5	>10	6.8	4.2	11 (20)
4f	1.9	6.8	3.5	6.4	4.3	2.8	
4g	1.4	4.1	2.6	4.6	2.4	1.5	
7a	2.3	>5.0	2.3	>5.0	2.4	1.3	
7b	2.2	5.4	2.7	5.2	2.1	1.7	17 (100)
7c	1.1	3.1	1.7	2.5	0.92	0.70	
1	17	>50	21	46	23	18	
HMPMM	>100	83	67	75	52	15	

Aromatase inhibitory activity was measured as described in our previous paper.¹⁾ No data in table: not determined.

which is produced by decomposition of DMF at a reaction temperature higher than 80 °C, reacts with 6 to give an undesirable 2-(1-benzimidazolyl)-4-dimethylamino-6-morpholinopyrimidine.

On the other hand, the use of morpholine as a reaction solvent and a nucleophile is suitable in the synthesis of 7, because morpholine is commercially available and the undesirable product is not produced in the reaction of 6 to 7. Therefore, 5 was first treated with morpholines at room temperature to afford 6, followed by treatment with morpholine at the optimal temperature of 80 °C (instead of room temperature as employed for the synthesis of 4) to afford 7.

Pharmacological Results and Discussion The compounds listed in Table 1 were evaluated for cytotoxicity against some human cancer and murine leukemia cell lines and aromatase inhibitory activity. Replacing one morpholino group of 4a with a dimethylamino group 4b resulted in a reduction in cytotoxicity. This result suggests that the morpholino group in these compounds was important for potent antitumor activity, similar to the fact that this group was the most effective substituent for aromatase inhibitory activity of 1,3,5-triazine derivatives.¹⁾ The introduction of methyl group 4c or *cis*-2,6-dimethyl group 4e at the 2- or 2,6-position on one morpholine ring of 4a exhibited comparable or slightly less cytotoxic activity than 4a. The introduction of dimethyl

groups at the 2,2- or 2,3-position on one morpholine ring as in compounds 4d, 4f and 4g slightly increased cytotoxicity.

Furthermore, pyrimidine derivatives 7a, 7b and 7c also showed slightly increased cytotoxicity. The cytotoxicity of 7c was the most potent and its activity was 10–20 fold greater in all cancer cell lines tested than that of the parent compound 1. Compound 4a having benzimidazolyl group, which prevents access of the imidazole ring to the active site of the enzyme due to condensed phenyl group with imidazole, showed little aromatase inhibitory activity. Moreover, compound 7c which has benzimidazolyl and a substituted morpholino group showed slightly or no aromatase inhibitory activity. The reason may be that these bulky groups more strongly prevent access of the imidazole ring to the active site of the enzyme. In order to understand whether or not these compounds have potent antitumor activity, we examined their effects *in vivo* using nude mice. The results will be reported elsewhere.

Experimental

Chemistry Melting points were determined on a Yamato melting point apparatus Model Mp-21 and were uncorrected. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded on a JEOL SX102 mass spectrometer. ¹H-NMR spectra were taken at 270 MHz with a JEOL JMN-RX270 spectrometer with tetramethylsilane as an internal standard. Column chromatography was carried out on silica gel (Wakogel C-200). TLC was performed using aluminum sheets precoated with silica gel (Kieselgel 60

F₂₅₄ layer thickness 0.2 mm; Merck). Compound **2** was prepared as reported.¹⁾ Morpholines were prepared according to the literature,^{11–14)} except morpholine and *cis*-2,6-dimethylmorpholine which were purchased from Tokyo Kasei Kogyo Co., Ltd.

2-(1-Benzimidazolyl)-4-chloro-6-morpholino-1,3,5-triazine (3) Benzimidazole (62.1 g, 526 mmol) and K₂CO₃ (207.3 g, 1.50 mol) were added to a solution of 2,4-dichloro-6-morpholino-1,3,5-triazine (**2**) (123.6 g, 526 mmol) in DMF (1500 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h, then poured into water, and the resulting precipitate was collected. The precipitate was washed with a small amount of acetone, and dried to give **3** (114.7 g) in 69% yield. Colorless powder, mp 197–199 °C. ¹H-NMR (CDCl₃) δ: 3.7–4.1 (8H, m), 7.3–7.5 (2H, m), 7.8–7.9 (1H, m), 8.3–8.5 (1H, m), 8.96 (1H, s). MS *m/z*: 316 (M⁺). This was used for the next reaction without further purification.

General Procedures for Preparation of Compound 4 Synthesis of 2-(1-benzimidazolyl)-4-(*trans*-2,3-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4f**): *trans*-2,3-Dimethylmorpholine hydrochloride (13.8 g, 91.0 mmol) and K₂CO₃ (55.3 g, 400 mmol) were added to a solution of 2-(1-benzimidazolyl)-4-chloro-6-morpholino-1,3,5-triazine (**3**) (26.0 g, 82.1 mmol) in DMF (500 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h, poured into water, and then extracted with ethyl acetate (AcOEt). The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **4f** (27.6 g) as a diastereomixture in 85% yield. Colorless powder, mp 166–168 °C. ¹H-NMR (CDCl₃) δ: 1.1–1.3 (6H, m), 3.1–3.4 (1H, m), 3.5–4.1 (11H, m), 4.3–4.8 (2H, m), 7.3–7.5 (2H, m), 7.81 (1H, dd, *J*=2, 6 Hz), 8.36 (1H, dd, *J*=2, 6 Hz), 8.97 (1H, d, *J*=2 Hz). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2065.

Compounds **4a–4e** and **4g** were obtained using a similar procedure to that described for **4f**.

2-(1-Benzimidazolyl)-4,6-dimorpholino-1,3,5-triazine (**4a**): 98% yield. Colorless powder, mp 222–224 °C. ¹H-NMR (CDCl₃) δ: 3.8–4.0 (16H, m), 7.3–7.5 (2H, m), 7.8–7.9 (1H, m), 8.3–8.5 (1H, m), 8.97 (1H, s). HRMS *m/z*: Calcd for C₁₆H₁₉N₇O₂: 367.1757. Found: 367.1760.

2-(1-Benzimidazolyl)-4-dimethylamino-6-morpholino-1,3,5-triazine (**4b**): 97% yield. Colorless powder, mp 147–148 °C. ¹H-NMR (CDCl₃) δ: 3.20 (3H, s), 3.29 (3H, s), 3.7–4.0 (8H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 9.00 (1H, s). HRMS *m/z*: Calcd for C₁₆H₁₉N₇O: 325.1651. Found: 325.1646.

2-(1-Benzimidazolyl)-4-(2-methylmorpholino)-6-morpholino-1,3,5-triazine (**4c**): 87% yield. Colorless powder, mp 159–161 °C. ¹H-NMR (CDCl₃) δ: 1.30 (3H, d, *J*=6 Hz), 2.7–2.9 (1H, m), 3.0–3.3 (1H, m), 3.5–4.1 (11H, m), 4.5–4.7 (2H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.38 (1H, dd, *J*=2, 6 Hz), 9.14 (1H, s). HRMS *m/z*: Calcd for C₁₉H₂₃N₇O₂: 381.1913. Found: 381.1906.

2-(1-Benzimidazolyl)-4-(2,2-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4d**): 88% yield. Colorless powder, mp 172–173 °C. ¹H-NMR (CDCl₃) δ: 1.28 (6H, s), 3.4–4.0 (14H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.38 (1H, dd, *J*=2, 6 Hz), 8.97 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2065.

2-(1-Benzimidazolyl)-4-(*cis*-2,6-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4e**): 70% yield. Colorless powder, mp 195–197 °C. ¹H-NMR (CDCl₃) δ: 1.30 (6H, br s), 2.5–2.8 (2H, m), 3.6–3.8 (2H, m), 3.8–4.0 (8H, m), 4.5–4.7 (2H, m), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.36 (1H, dd, *J*=2, 7 Hz), 8.97 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2062.

2-(1-Benzimidazolyl)-4-(*cis*-2,3-dimethylmorpholino)-6-morpholino-1,3,5-triazine (**4g**): 88% yield. Colorless powder, mp 172–174 °C. ¹H-NMR (CDCl₃) δ: 1.35 (3H, d, *J*=7 Hz), 1.40 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.7–4.0 (11H, m), 4.4–4.7 (2H, m), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 6 Hz), 8.37 (1H, dd, *J*=2, 6 Hz), 8.98 (1H, s). HRMS *m/z*: Calcd for C₂₀H₂₅N₇O₂: 395.2070. Found: 395.2085.

2-(1-Benzimidazolyl)-4,6-dichloropyrimidine (5) Benzimidazole (59.1 g, 500 mmol) and K₂CO₃ (138.2 g, 1.00 mol) were added to a solution of 2,4,6-trichloropyrimidine (91.7 g, 500 mmol) in DMF (800 ml) at room temperature. The reaction mixture was stirred at room temperature for 17 h, and then poured into water. The resulting precipitate was collected and washed with a small amount of acetone. The precipitate was recrystallized from methanol to give **5** (70.0 g) in 53% yield. Colorless powder, mp 171–173 °C. ¹H-NMR (CDCl₃) δ: 7.26 (1H, s), 7.3–7.5 (2H, m), 7.82 (1H, dd, *J*=2, 7 Hz), 8.48 (1H, dd, *J*=2, 7 Hz), 8.99 (1H, s). MS *m/z*: 265 (M⁺). This was used for the next reaction without further purification.

General Procedures for Preparation of Monochloropyrimidine 6

Synthesis of 2-(1-benzimidazolyl)-4-chloro-6-(*trans*-2,3-dimethylmorpholino)pyrimidine (**6b**): *trans*-2,3-Dimethylmorpholine hydrochloride (15.2 g, 100 mmol) and K₂CO₃ (27.6 g, 200 mmol) were added to a solution of 2-(1-benzimidazolyl)-4,6-dichloropyrimidine (**5**) (18.0 g, 67.9 mmol) in DMF (300 ml) at room temperature. The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was poured into water, and then extracted with AcOEt. The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **6b** (21.3 g) in 91% yield. Colorless powder, mp 88–92 °C. ¹H-NMR (CDCl₃) δ: 1.26 (6H, d, *J*=7 Hz), 3.2–3.4 (1H, m), 3.6–3.9 (4H, m), 4.0–4.2 (1H, m), 6.38 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 8.97 (1H, s). MS *m/z*: 343 (M⁺). Compounds **6a** and **6c** were obtained using a similar procedure to that described for **6b**.

2-(1-Benzimidazolyl)-4-chloro-6-morpholinopyrimidine (**6a**): 91% yield. Colorless powder, mp 191–193 °C. ¹H-NMR (CDCl₃) δ: 3.7–3.9 (8H, m), 6.39 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.42 (1H, dd, *J*=2, 7 Hz), 8.95 (1H, s). MS *m/z*: 315 (M⁺).

2-(1-Benzimidazolyl)-4-chloro-6-(*cis*-2,3-dimethylmorpholino)pyrimidine (**6c**): 77% yield. Colorless powder, mp 117–119 °C. ¹H-NMR (CDCl₃) δ: 1.38 (3H, d, *J*=7 Hz), 1.42 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.7–4.2 (5H, m), 6.38 (1H, s), 7.3–7.5 (2H, m), 7.83 (1H, dd, *J*=2, 7 Hz), 8.44 (1H, dd, *J*=2, 7 Hz), 8.95 (1H, s). MS *m/z*: 343 (M⁺). These were used for the next reaction without further purification.

General Procedures for Preparation of Compound 7 Synthesis of 2-(1-benzimidazolyl)-4-(*trans*-2,3-dimethylmorpholino)-6-morpholinopyrimidine (**7b**): 2-(1-Benzimidazolyl)-4-chloro-6-(*trans*-2,3-dimethylmorpholino)pyrimidine (**6b**) (7.22 g, 21.0 mmol) was stirred in morpholine (52.0 ml) at 80 °C for 2 h. The reaction mixture was poured into water, and then extracted with AcOEt. The extract was washed with brine, dried over MgSO₄, and evaporated *in vacuo*. The residue was purified by silica gel column chromatography with hexane–AcOEt (1 : 1) to give **7b** (7.78 g) as a diastereomixture in 94% yield. Colorless powder, mp 177–180 °C. ¹H-NMR (CDCl₃) δ: 1.20 (3H, d, *J*=7 Hz), 1.24 (3H, d, *J*=7 Hz), 3.1–3.4 (1H, m), 3.6–4.1 (13H, m), 5.44 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₂₁H₂₆N₆O₂: 394.2117. Found: 394.2119.

Compounds **7a** and **7c** were obtained using a similar procedure to that described for **7b**.

2-(1-Benzimidazolyl)-4,6-dimorpholinopyrimidine (**7a**): 82% yield. Colorless powder, mp 297–299 °C. ¹H-NMR (CDCl₃) δ: 3.6–3.9 (16H, m), 5.48 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₁₉H₂₂N₆O₂: 366.1804. Found: 366.1823.

2-(1-Benzimidazolyl)-4-(*cis*-2,3-dimethylmorpholino)-6-morpholinopyrimidine (**7c**): 86% yield. Colorless powder, mp 170–172 °C. ¹H-NMR (CDCl₃) δ: 1.37 (3H, d, *J*=7 Hz), 1.39 (3H, d, *J*=7 Hz), 3.3–3.5 (1H, m), 3.6–4.2 (13H, m), 5.43 (1H, s), 7.2–7.4 (2H, m), 7.8–7.9 (1H, m), 8.3–8.4 (1H, m), 8.96 (1H, s). HRMS *m/z*: Calcd for C₂₁H₂₆N₆O₂: 394.2117. Found: 394.2135.

Cell Lines The cytotoxic activity was analyzed using human MCF-7, breast, A549 lung, WiDr, and colon cancer cell lines and murine P388 leukemia, L1210 leukemia, and B16F10 melanoma cell lines. MCF-7 was kindly supplied by Dr. T. Kubota, Department of Surgery, School of Medicine, Keio University, Tokyo. A549 and WiDr were purchased from Dainippon Pharmaceutical Co., Ltd. P388 and L1210 were generously provided by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, and B16F10 by the Cancer Center, Faculty of Medicine, Kyushu University, Fukuoka.

Cytotoxic Activity Test RPMI1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 25 mM HEPES (Dojin Chemical Laboratory, Ltd.) and 100 μg/ml kanamycin (Dainippon Pharmaceutical Co., Ltd.) was used as the assay medium. Cells were incubated at 37 °C in a humidified atmosphere of 95% air–5% CO₂. MCF-7, A549, WiDr, and B16F10 cells in exponential growth phase were harvested by trypsinization using Trypsin-EDTA (GIBCO BRL) and were inoculated into 96 well-microculture plates (TC MICRO WELL 96F, NUNC) at the density of 4 × 10³, 3 × 10³, 2 × 10³, or 10³ cells per well in 100 μl assay medium. After 1 day incubation, 100 μl of the assay medium containing various concentrations of sample compounds dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) were added to each well, and cells were incubated for another 3 d. For P388 and L1210 cell cultures the ascitic fluid was removed from mice 6 or 7 d after intraperitoneal transplantation of P388 and L1210 cells. Cells were separated by centrifugation at 800 × *g* for a few minutes and were inoculated into 25 cm² tissue culture flasks (TC

FLASK 25CM2, NUNC) at 2×10^6 cells per flask in 5 ml assay medium supplemented with $10 \mu\text{M}$ 2-hydroxyethyl disulfide (Aldrich Chemical Co.). After 1 d incubation, P388 and L1210 cells were harvested and reinoculated into 96 well-microculture plates at 4×10^3 and 2×10^3 cells, respectively, per well in 100 μl assay medium. The assay medium supplemented with $10 \mu\text{M}$ 2-hydroxyethyl disulfide, and 100 μl of assay medium containing various concentrations of sample compounds dissolved in DMSO were added to culture wells. Cells were incubated for another 3 d.

XTT Assay¹⁵⁾ Cytotoxic activity was determined by the XTT assay. Briefly, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-(phenylaminocarbonyl)-2H-tetrazolium hydroxide (XTT, Sigma) was prepared at 1 mg/ml in phenol red-free RPMI1640 medium (GIBCO BRL) supplemented with 25 mM HEPES and 100 $\mu\text{g}/\text{ml}$ kanamycin. Phenazine methosulfate (PMS, Sigma) was prepared at 0.765 mg/ml in phenol red-free RPMI1640 medium (GIBCO BRL) supplemented with 25 mM HEPES and 100 $\mu\text{g}/\text{ml}$ kanamycin. Twenty-five microliters of PMS solution (0.765 mg/ml) was added per 5 ml of XTT solution (1 mg/ml). Fifty microliters of this mixture (final concentrations, 50 μg of XTT and 0.19 μg PMS per well) was added to each well and incubated for 4 h. The plates were agitated on a mechanical plate shaker and absorption-spectra of formazan were measured using a microplate spectrophotometer (MTP-32, CORONA) at 450 nm. The percentage of cell growth inhibition was calculated as follows: percent of cell growth inhibition = $100 \times [1 - (T - T_0)/(C - T_0)]$, where C stands for the mean optical density at 450 nm (OD450) of the control group, T for the mean OD450 of treated group and T0 for the mean OD450 at time zero (the time at which samples were added). Means were obtained by 4 determinations. Growth inhibition of 50% (GI_{50}) value was read on the dose-response curve.

Aromatase Inhibitory Activity The inhibitory activity was measured by the method reported in our previous paper.¹⁾

References

- 1) Matsuno T., Kato M., Tsuchida Y., Takahashi M., Terada S., *Chem. Pharm. Bull.*, **45**, 291—296 (1997).
- 2) Yaguchi S., Izumisawa Y., Sato M., Nakagane T., Koshimizu I., Sakita K., Kato M., Yoshioka K., Sakato M., Kawashima S., *Biol. Pharm. Bull.*, **20**, 698—700 (1997).
- 3) Takahashi M., Kayo T., Karakida T., Nakagawa S., Kato M., Matsuno T., Koide Y., Sato M., Kawashima S., *Endocrine Res.*, **23**, 1—13 (1997).
- 4) Iino Y., Karakida T., Sugamata N., Andoh T., Takei H., Takahashi M., Yaguchi S., Matsuno T., Sato M., Kawashima S., Morishita Y., *Anti-cancer Res.*, **18**, 171—176 (1998).
- 5) Rutty C. J., Connors T. A., *Biochem. Pharm.*, **26**, 2385—2391 (1977).
- 6) Gescher A., D'Incalci M., Fanelli R., Farina P., *Life Sci.*, **26**, 147—154 (1980).
- 7) Wilson W. L., Bisel H. F., Cole D., Rochlin D., Ramirez G., Madden R., *Cancer*, **25**, 567—570 (1970).
- 8) Legha S. S., Slavik M., Carter S. K., *Cancer*, **38**, 27—35 (1970).
- 9) Foster B. J., Harding B. J., Leyland-Jones B., Hoth D., *Cancer Treat. Rev.*, **113**, 197—217 (1986).
- 10) a) Thompson E. A. Jr., Siiteri P. K., *J. Biol. Chem.*, **249**, 5364—5372 (1974); b) Henderson D., Norbistrath D., Kerb U., *J. Steroid Biochem.*, **24**, 303—306 (1986).
- 11) Cottle D. L., Jeitsch A. E., Stout T. H., Walters D. R., *J. Org. Chem.*, **11**, 286—291 (1946).
- 12) Vargha L., Kasztreiner E., Borsy J., Farkas L., Kuszmann J., Dumbovich B., *Biochem. Pharm.*, **11**, 639—649 (1962).
- 13) Nilsson B., Hernestam S., *Org. Magn. Reson.*, **11**, 116—122 (1978).
- 14) Anderson A., Boyd A. C., Byford A., Campbell A. C., Gemmell D. K., Hamilton N. M., Hill D. R., Hill-Venning C., Lambert J. J., Maidment M. S., May V., Marshall R. J., Peter J. A., Rees D. C., Stevenson D., Sundaram H., *J. Med. Chem.*, **40**, 1668—1681 (1997).
- 15) Dominic A. S., Robert H. S., Kenneth D. P., Anne M., Siobhan T., Thomas H. N., Michael J. C., Donna S., Michael R. B., *Cancer Res.*, **48**, 4827—4833 (1988).

Dissolution Profiles of Principal Ingredients in Kampo Medicinal Powders by High-Performance Liquid Chromatography

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We describe here a method using HPLC for the simultaneous determination of albiflorin, paeoniflorin, glycyrrhizin and six flavanone glycosides (liquiritin, liquiritin apioside, naringin, neohesperidin, hesperidin and narirutin) in the Kampo medicines, Shigyaku-san and Haino-san. All nine components were separated in less than 40 min by linear gradient elution using a mobile phase containing aqueous phosphoric acid and acetonitrile. The dissolution of these components from powders of Shigyaku-san in aqueous solution at pH 1.80, 4.08 and 6.89 was examined. All of the components except glycyrrhizin were dissolved entirely within 5 min regardless of pH. Dissolution of glycyrrhizin was dependent on the pH of the aqueous solution, and increased with increasing pH.

Key words Kampo medicine; dissolution; albiflorin; paeoniflorin; glycyrrhizin; flavanone glycoside

Most Kampo medicines are usually prescribed as decoctions, but powdered formulations of crude drugs are also used. These different dosage forms have been developed on the basis of clinical experience. Components of the decoction present in solution or suspension in water are believed to be well dispersed in the alimentary canal. Dissolution of components in powders is related to their absorption in the alimentary canal. However, there have been few studies of dissolution of components in such powders with gastric and intestinal juice, and it is important to clarify the relationship between dosage form and dissolution.

Shigyaku-san and Haino-san are used for the treatment of cholecystitis, cholelithiasis and gastric ulcer, and acute purulent dermatitis, respectively.¹⁾ These formulations consist of peony root and immature orange, and also include licorice root and bupleurum root, and platycodon root, respectively. Peony root and licorice root contain albiflorin (**1**) and paeoniflorin (**2**), and liquiritin (**3**), liquiritin apioside (**4**) and glycyrrhizin (**5**) as characteristic components, respectively. Immature orange from the immature fruit of *Citrus aurantium* L. var. *daidai* MAKINO and *C. natsudaidai* HAYATA, contains naringin (**6**) and neohesperidin (**7**) as major components, both of which are flavanone neohesperidosides. Narirutin (**8**) and hesperidin (**9**), both flavanone rutinosides, are present as minor components in the immature fruit of *C. aurantium* L. var. *daidai* MAKINO and *C. natsudaidai* HAYATA.²⁾

Shigyaku-san and Haino-san have the name “san” indicating that they are powders (SS-P and HS-P, respectively), and they have been traditionally used as powdery dosage forms of crude drugs. They are often administered in the form of decoctions prepared by boiling crude drugs, which are called Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D), respectively.

The determination of **2**, **6** and **7** in methanol extract of HS-P was accomplished using a dual wavelength TLC-scanner.³⁾ In a recent study using HPLC, **2**, **5**, **9** and saikosaponin a in SS-D were determined by an ion-pair technique.⁴⁾ Since Kampo medicines are combinations of crude drugs, it is important for both quality control and scientific evaluation of the formulations to analyze more of the characteristic components specific for crude drugs. In the present study, we ex-

amined dissolution profiles of nine marker substances in SS-P by HPLC.

Experimental

Materials Commercial powders of SS-P and HS-P were obtained from Uchida Wakanyaku. The cut crude drugs were purchased from Tochimoto-Tenkaido. Compounds **1**, **2**, **3**, **5** and **9** were purchased from Wako Pure Chemical Industries (Wako). Compounds **6**, **7** and **8** were purchased from Funakoshi. Compound **4** was kindly provided by Prof. M. Kobayashi of Osaka University. HPLC grade acetonitrile and analytical reagent grade phosphoric acid (85%) were purchased from Wako. Water was purified by a Millipore Milli-Q water system.

Preparation of Decoction Daily dosage of SS-D (immature orange 2 g, peony root 4 g, licorice root 1.5 g, bupleurum root 5 g)¹⁾ and HS-D (immature orange 3 g, peony root 3 g, platycodon root 1 g)¹⁾ were prepared as follows: daily dosage of cut crude drugs was boiled with 500 ml water on an electric heater for 40 min, halving the original volume. The decoction was filtered through a colander while hot, and after cooling the volume was adjusted to 250 ml with water. Aliquots of the adjusted decoction were diluted two-fold (SS-D) or four-fold (HS-D) with water, and the mixtures were filtered through a Sartorius syringe filter (Minisart-RC 15, 0.45 μ m) and analyzed.

Instrumentation and HPLC Analysis The HPLC apparatus was a Hewlett-Packard system composed of an HP1100 Series Binary Pump and an HP1100 Series Autosampler (set at 5 μ l). The detection wavelength was set at 230 nm for determination and in the range of 200 to 500 nm for validation of peak purity. An HP1100 Series 3D ChemStation was used for data acquisition and integration. Separation was achieved on a Wako Wakosil-II 5C18 AR reversed-phase semi-micro column (particle size 5 μ m, 150 $\times$ 2.0 mm i.d.). The mobile phase consisted of a mixture of (A) water containing 200 μ l $\cdot$ l⁻¹ (ca. 0.017%) phosphoric acid and (B) acetonitrile (linear gradient program: 0–10 min, 9% B; 15 min, 13% B; 17 min, 18% B; 27 min, 23% B; 32 min, 46% B; 40 min, 46% B), and was degassed in an ultrasonic bath prior to use. A re-equilibration period of 15 min was used between individual runs. Chromatography was performed at 45 $^{\circ}$ C with a flow rate of 0.2 ml $\cdot$ min⁻¹. Chromatographic peaks were identified and purity was estimated with an HP1100 Series Photodiode Array Detector.

Standard Curve Preparation Compounds **1**–**9** were dissolved in 10% methanol to give concentrations within the range 0.125 to 4986 μ g $\cdot$ ml⁻¹. The standard curves were prepared using the peak areas of ten different concentrations in triplicate assays.

Interference Trial and Peak Purity Variant decoctions (blank decoction), in which one crude drug in each Kampo medicine was excluded, were also prepared by the procedures described above. The purity of the peaks was checked by diode-array detection. The UV spectra of peaks at three different points (upslope, top and downslope) were compared with those of authentic specimens.

Recovery A mixture of **1**–**9** dissolved in methanol was used as a standard solution. The test solutions were prepared by addition of SS-D, HS-D

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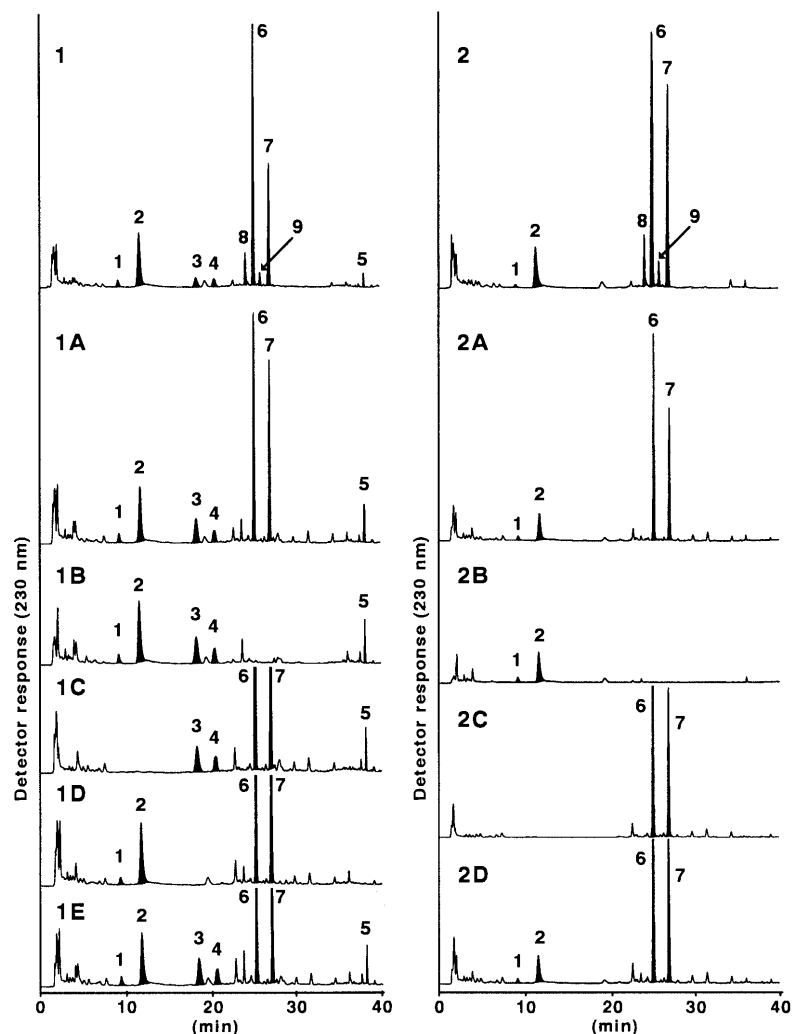


Fig. 1. Chromatograms of Shigyaku-san and Haino-san and Their Blank Decoctions

1 = extract of commercial SS-P, 1A = SS-D, 1B = 1A without immature orange, 1C = 1A without peony root, 1D = 1A without licorice root, 1E = 1A without bupleurum root, 2 = extract of commercial HS-P, 2A = HS-D, 2B = 2A without immature orange, 2C = 2A without peony root, 2D = 2A without platycodon root. For peak numbers, see text. Commercial powders (2 g) of SS-P and HS-P were refluxed for 60 min with 50 ml of water.

or water (0.9 ml) to standard solution (0.1 ml), followed by 2-, 4-, 8- and 16-fold dilution with SS-D-methanol (9:1), HS-D-methanol (9:1) or water-methanol (9:1). All test solutions were filtered through a Minisart-RC 15 filter and subjected to HPLC. The recoveries were determined from the slope ratios of regression equations with/without the decoction.

Dissolution of Marker Substances from Powders SS-P was prepared by mixing with powdered crude drugs (80 mesh). A daily dosage of SS-P (immature orange 2 g, peony root 4 g, licorice root 1.5 g, bupleurum root 5 g)¹⁾ was shaken with 250 ml of three aqueous solutions (pH 1.80: 34.5 mM NaCl, 84 mM HCl, pH 4.08: 0.2 M acetate buffer, pH 6.89: 50 mM KH₂PO₄, 23.6 mM NaOH) on a water bath shaker (120 times $\cdot$ min⁻¹) at 37 °C. After 5, 60, 120 and 240 min, the aqueous solution (1 ml) was filtered with a Millex-GP filter. The filtrate was diluted two-fold with water, and the mixture was analyzed.

Results and Discussion

To select the best detection conditions, the UV spectra of marker substances were recorded by three-dimensional chromatography. The spectra of flavanone glycosides 3, 4, 6, 7, 8 and 9 exhibited major absorption maxima in the regions of 210 to 230 and 280 nm. Absorption maxima of 1 and 2, and 5 were observed around 230 and 250 nm, respectively. While their maxima belonged to three distinct UV regions, at 200 to 230, 250 and 280 nm, the optimum monitoring wavelength for quantification was fixed at 230 nm, where the appreciable

absorbance shown by these marker substances secured a suitable analytical response.

The retention times (and capacity factors, k') were 9.12 (1, k' =4.44), 11.50 (2, k' =5.87), 18.14 (3, k' =9.83), 20.28 (4, k' =11.11), 24.19 (8, k' =13.42), 25.20 (6, k' =14.09), 26.01 (9, k' =14.58), 27.04 (7, k' =15.19) and 38.07 min (5, k' =21.73). The calibration curves of 1—9 were linear in the ranges of 0.51 to 526, 4.83 to 10388, 2.58 to 1399, 0.34 to 1490, 2.32 to 5003, 4.21 to 618, 0.52 to 558, 0.49 to 319 and 0.21 to 502 μ M, respectively. The standard curve showed good linearity, as indicated by linear least-squares regression analysis, and the method permitted determination of these components in Kampo medicines over a wide range of concentrations. The detection limits (signal-to-noise ratio = 3) of 1—9 were 1.4, 2.1, 0.5, 0.5, 0.4, 0.1, 0.2, 0.1 and 0.2 pmol per injection (5 μ l), respectively.

Figure 1 shows typical chromatograms obtained from commercial powders, decoctions and blank decoctions of Shigyaku-san and Haino-san. Nine marker components were clearly separated without any prepurification, and determined within 40 min without any pre-cleaning of the column. Distinct elution profiles of 1—9 were obtained without any interfering peaks from the decoctions, prepared by excluding

immature orange, peony root, licorice root, bupleurum root or platycodon root from SS-D and HS-D. The commercial SS-P and HS-P showed peaks of **8** and **9**, which were attributed to different sources of immature orange. Saikosaponin d (t_R : 42.10 min, k' : 24.13) and b2 (t_R : 39.03 min, k' : 22.30) contained in bupleurum root were not detected in the present decoctions. Synephrine contained in citrus plants was eluted

within 2 min, and neoeriocitrin (t_R : 22.65 min, k' : 12.50), a flavanone neohesperidoside in immature orange, could not be separated from peaks of the component of peony root. A peak of isoliquiritin (t_R : 27.83 min, k' : 15.61) in licorice root overlapped with peaks of other components of licorice root.

The within-day and the day-to-day precision of the method for detection of each marker substance were evaluated using SS-D and HS-D. The within-day precision was examined with ten replicate assays per day and the day-to-day precision by assays on five different days. As shown in Table 1, the within-day and the day-to-day RSDs were 0.19 to 1.43% and 0.26 to 1.35%, respectively. Recovery was investigated using SS-D and HS-D spiked with a standard mixture of the marker substances described above. The slopes of regression equations of standards and the recoveries by this method are shown in Table 2. The recoveries of marker substances were 99.0 to 103%.

Kampo medicines are generally administered in the form of decoctions prepared by boiling crude drugs, but SS-P and HS-P are traditionally used as powdered dosage forms of crude drugs. To examine dissolution of components in the powders, we analyzed **1**–**7** in SS-P dissolved in aqueous solutions at pH 1.80, 4.08 and 6.89 for 5, 60, 120 and 240 min. With the exception of **5**, marker components were dissolved entirely within 5 min regardless of the pH. Table 3 shows the quantitative results between SS-D and the aqueous solution of SS-P at different pH at the first sampling time point for 5

Table 1. Within-Day and Day-to-Day Relative Standard Deviations (RSD) for Marker Substances in Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D)

Marker substance	RSD (%)	
	Within-day ^{a)}	Day-to-day ^{b)}
Shigyaku-san-ryo (SS-D)		
Albiflorin (1)	0.56	0.79
Paeoniflorin (2)	0.68	0.57
Liquiritin (3)	0.73	0.59
Liquiritin apioside (4)	0.60	0.63
Naringin (6)	0.41	0.48
Neohesperidin (7)	0.36	0.49
Glycyrrhizin (5)	0.76	0.26
Haino-san-ryo (HS-D)		
Albiflorin (1)	1.43	1.35
Paeoniflorin (2)	0.95	0.81
Naringin (6)	0.19	0.27
Neohesperidin (7)	0.20	0.45

a) $n = 10$. b) $n = 5$.

Table 2. Slopes of Regression Equations of Marker Substances with and without Shigyaku-san-ryo (SS-D) and Haino-san-ryo (HS-D)

Marker substance	Initial amount of decoction (μM)	Slope of standard solution ^{a)}		Recovery ^{b)} (%)
		With decoction	Without decoction	
Shigyaku-san-ryo (SS-D)				
Albiflorin (1)	44.9	10.22	9.906	103
Paeoniflorin (2)	348	8.826	8.520	104
Liquiritin (3)	126	16.10	16.08	100
Liquiritin apioside (4)	65.4	13.05	12.60	104
Naringin (6)	279	20.23	19.83	102
Neohesperidin (7)	172	22.80	22.05	103
Glycyrrhizin (5)	183	3.765	3.652	103
Haino-san-ryo (HS-D)				
Albiflorin (1)	20.6	10.19	9.906	103
Paeoniflorin (2)	148	8.846	8.520	104
Naringin (6)	191	19.63	19.83	99.0
Neohesperidin (7)	120	22.11	22.05	100

a) Concentration of standards (μM): SS-D: **1** = 115, **2** = 923, **3** = 237, **4** = 127, **6** = 226, **7** = 215, **5** = 322; HS-D: **1** = 81, **2** = 672, **6** = 291, **7** = 215. b) Values are means of five experiments.

Table 3. Contents of Marker Substances in Decoction and Dissolution of Shigyaku-san

Marker substance	Contents (mg/g crude drug)			
	Shigyaku-san-ryo (SS-D)	Shigyaku-san (SS-P)		
		pH 1.80	pH 4.08	pH 6.89
Albiflorin (1)	2.38 $\pm$ 0.34	2.20 $\pm$ 0.05	2.15 $\pm$ 0.10	2.00 $\pm$ 0.18
Paeoniflorin (2)	22.1 $\pm$ 1.15	28.8 $\pm$ 0.40	29.1 $\pm$ 0.43	29.6 $\pm$ 0.89
Liquiritin (3)	17.5 $\pm$ 0.97	18.0 $\pm$ 0.23	19.5 $\pm$ 0.17	21.3 $\pm$ 0.85
Liquiritin apioside (4)	12.5 $\pm$ 0.54	12.9 $\pm$ 0.28	14.2 $\pm$ 0.24	14.8 $\pm$ 0.26
Naringin (6)	59.4 $\pm$ 5.46	74.1 $\pm$ 1.78	77.9 $\pm$ 0.94	80.8 $\pm$ 1.82
Neohesperidin (7)	37.3 $\pm$ 3.70	47.0 $\pm$ 1.19	49.8 $\pm$ 0.60	52.0 $\pm$ 1.30
Glycyrrhizin (5)	46.7 $\pm$ 1.69	3.82 $\pm$ 0.91	21.3 $\pm$ 0.14	71.9 $\pm$ 2.20

Results are means $\pm$ standard deviations from five experiments.

min. The concentrations of **1**–**4**, **6** and **7** in the aqueous solution were 84 to 139% of those in SS-D. The dissolution amounts of **5** at pH 1.08 and 4.08 were 8 and 46% of that in SS-D, respectively, but the amount at pH 6.89 was significantly higher (154%). The solubility of **5** was thought to be enhanced by increase in the pH in aqueous solution because **5** has a glucuronic acid moiety. Compound **5** used for the treatment of gastric ulcers⁵ has been reported to be hydrolyzed to the active metabolic glycyrrhetic acid (**5a**) by the action of intestinal bacteria in the large intestine.⁶ Thus, the presence of a considerable amount of **5** in the aqueous solution at pH 6.89 may enhance the efficacy due to **5a** obtained by the microbial metabolism of **5** dissolved in intestinal juice.

Although **3** was stable in aqueous solution at pH 1.80 for at least 4 h, the quantities of **3** at pH 4.08 and 6.89 were decreased to 63 and 53%, respectively, during a period of about 240 min. Consequently, a new peak (t_R : 28.12 min, k' : 15.76) corresponding to liquiritigenin (**3a**), an aglycone of **3**, appeared associated with a corresponding decrease in the peak of **3**. Since the hydrolysis of **3** to **3a** at pH 4.08 and 6.89 was inhibited by heating at 90 °C for 10 min and by dilution with methanol (not shown), the reduction of **3** might be mediated by enzymes.

An effective HPLC method using a semi-micro reversed-phase column was developed to analyze Kampo medicines containing **1**–**9**. The concentrations of components dissolved from SS-P with the exception of **5** were similar to those in SS-D. Dissolution of **5** was dependent on the pH of the aqueous solution, and increased with increasing pH. These findings suggested the possible availability of **5** by oral administration in the form of SS-P, because the dissolution of **5** would be significantly increased with the elevated pH in the large intestine.

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References and Notes

- 1) Supervision by the Pharmaceutical and Supply Bureau of Health and Welfare Ministry of Japan (ed.), *Ippan-yo Kampo Shoho No Tebiki*, Yakugyo Jihosha, Tokyo, 1976.
- 2) Hosoda K., Noguchi M., *Yakugaku Zasshi*, **108**, 1008–1011 (1988).
- 3) Saito K., Konoshima M., Kano Y., *Shoyakugaku Zasshi*, **39**, 126–130 (1985).
- 4) Ueda J., Ohsawa K., *Natural Medicines*, **51**, 186–189 (1997).
- 5) Takagi K., Okabe S., Sasaki R., *J. Pharmacol.*, **19**, 418–426 (1969).
- 6) Hattori M., Sakamoto T., Kobashi K., Namba T., *Planta Medica*, **48**, 38–42 (1983).

Inhibitory Effects of 2'-Hydroxychalcones on Rat Lens Aldose Reductase and Rat Platelet Aggregation

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Inhibitory effects of synthetic 2'-hydroxychalcone derivatives on rat lens aldose reductase (RLAR) and on platelet aggregation were investigated for the prevention or the treatment of chronic diabetic complications. 5'-chloro-4,2'-dihydroxychalcone (8) and 5'-chloro-3,2'-dihydroxychalcone (27) exhibited a potent inhibitory effect on rat platelet aggregation induced by ADP (IC_{50} =0.10 and 0.06 mg/ml, respectively) and collagen (IC_{50} =44 and 16 μ g/ml, respectively) but showed relatively weak inhibitory activities on RLAR.

Key words 5'-chloro-3,2'-dihydroxychalcone; rat lens aldose reductase; platelet aggregation; diabetic complication

The therapeutic potential of aldose reductase inhibitors for the prevention of the secondary complications of diabetes have been extensively reported.¹⁾ On the other hand, hyperaggregability of platelets in diabetic patients has also been reported as a cause of chronic diabetic complications.^{2–4)} So it seems likely that normalization of aggregability of platelets in diabetic patients may be beneficial for the prevention or the treatment of chronic diabetic complications. Aida *et al.* reported that isoliquiritigenin (**2**, 4,2',4'-trihydroxychalcone), purified from *Glycyrrhizae radix*, inhibited RLAR⁵⁾ and human platelet aggregation.⁶⁾

In a previous paper, we synthesized twenty-four derivatives of 2'-hydroxychalcone and their inhibitory effects on RLAR and antioxidant effects have been tested.⁷⁾ Some chalcone derivatives synthesized were found to possess AR inhibition and antioxidant activities *in vitro* as well as inhibition in the accumulation of sorbitol in tissue *in vivo*, in which 3,4,2',4'-tetrahydroxychalcone (**18**, butein) was the most promising compound for the prevention or treatment of diabetic complications.

This paper describes the inhibitory effects of previously synthesized (A and B series)⁷⁾ and newly synthesized chalcones (C series) on RLAR and on rat platelet aggregation. Among these compounds tested only a few were of some importance for further investigations.

Results and Discussion

Table 2 shows the inhibitory activities of 2'-hydroxychalcones (A, B and C series) tested on RLAR with DL-glyceraldehyde as a substrate and rat platelet aggregation induced by 2.5 μ M ADP and 10.0 μ g/ml collagen. Aspirin (As), epalrestat (Ep) and isoliquiritigenin (Iso, **2** in Table 1) are also included for comparison.

As far as RLAR inhibitory activities are concerned, **11** and **22** of series B exhibited more potent inhibitory effects comparable to Iso, used as positive control. Compound **33** and **36** were most potent in series C, however they were less potent than Iso. The other compounds in series C did not show any significant activity.

The 2',4'-dihydroxychalcone derivatives **10–24** (B series), having various functional groups instead of the 4-hydroxy group, showed a potent inhibitory activity on RLAR but possessed a weak anti-platelet activity against ADP

and/or collagen except some derivatives. IC_{50} values in Table 1 show that the effect of compound tested on collagen-induced platelet aggregation is more sensitive than that on ADP-induced platelet aggregation in rat platelet-rich-plasma (PRP).

As far as inhibition of platelet aggregation is concerned, 5'-chloro-4,2'-dihydroxychalcone (**8**) and 5'-chloro-3,2'-dihydroxychalcone (**27**) exhibited a strong inhibitory effect comparable with Iso, used as positive control.

Figure 1 shows the effects of **27**, Iso, Ep and As on the platelet aggregation rate induced by 2.5 μ M of ADP (Fig. 1a) and 10.0 μ g/ml of collagen (Fig. 1b). In Fig. 1a, a representative aggregometer tracing representing the effect of compounds on ADP-induced platelet aggregation in rat PRP is shown. Compound **27**, Iso and As inhibited the rate of platelet aggregation by 67.3, 41.5 and 30.7% at 75 μ g/ml, respectively. Ep, an aldose reductase inhibitor,⁸⁾ did not show a significant inhibitory effect at 75 μ g/ml. A representative aggregometer tracing representing the effect of compounds on collagen-induced platelet aggregation in rat PRP is shown in Fig. 1b. Compound **27**, Iso and As inhibited the rate of platelet aggregation by 52.1, 41.5, and 21.3% at 20 μ g/ml, respectively. Ep did not show a significant inhibitory effect at 20 μ g/ml.

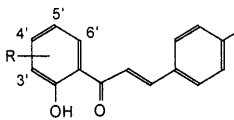
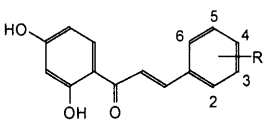
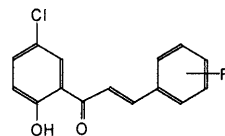
It has been reported that the inhibitory effect of Iso on platelet aggregation *in vitro* and *in vivo* was comparable to that of As and the mechanism by which Iso inhibits platelet aggregation can be explained by its significant effects on the arachidonic acid cascade.⁶⁾ These findings may indicate that Iso elicits an anti-platelet action by inhibiting not only cyclooxygenase but also lipoxygenase or peroxidase activity in platelets. Compound **27** was more effective than Iso to inhibit the platelet aggregation induced by ADP and collagen *in vitro*. Further study of the structure-activity relationships on synthetic compounds is in progress.

Experimental

General Experimental Procedures Melting points (mp) were measured by a Mitamura-Riken melting point apparatus and are uncorrected. Proton (¹H-) and carbon (¹³C-) nuclear magnetic resonance spectra were determined in dimethyl sulfoxide-*d*₆ and acetone-*d*₆ on a Varian Gemini 2000 spectrometer at 300 and 75 MHz, respectively. Infrared (IR) spectra were measured by JASCO FT/IR-5000. High resolution mass spectra (HRMS) were taken on a JEOL JMS-AX500WA mass spectrometer. Mass spectra (MS) were taken on a HP 5989B mass spectrometer. Biological activities

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Table 1. Inhibitory Effect of 2'-Hydroxychalcones on Rat Lens Aldose Reductase (RLAR) and Rat Platelet Aggregation

								
		1—9 (A)		10—24 (B)		25—38 (C)		
Compd. No.	R	RLAR Inhibitory effect ^{a)}		Rat platelet aggregation Inhibitory effect				
		% Inhibition at 10 μ M	IC ₅₀ (μ M)	Conc. (mg/ml)	ADP (%)	ADP (IC ₅₀) (mg/ml)	Collagen (%)	Collagen (IC ₅₀) (mg/ml)
A								
1	H	32.0	4.25	1	34.5		96.5	0.31
2	4'-OH (Iso)	93.2	0.40	0.3	60.2	0.12	83.5 ^{b)}	0.021
3	5'-OH	34.0	5.39	1	32.6		86.2	0.59
4	4'-OCH ₃	48.0	5.24	1	21.1		98.3	0.28
5	5'-OCH ₃	56.7	3.18	1	30.8		87.8	0.42
6	6'-OCH ₃	49.4	3.27	1	60.5	0.81	95.4	0.15
7	5'-CH ₃	35.9	4.38	1	33.5		95.8	0.21
8	5'-Cl	70.8	1.01	0.15	76.5	0.10	84.5	0.044
9	5'-Br	59.6	2.15	1	78.6	0.45	97.5	0.12
B								
10	H	89.9	1.01	1	45.8		74.3	0.69
11	2-OH	96.0	0.38	1	35.2		20.9	
12	4-OCH ₃	88.9	0.93	1	44.8		88.9	0.62
13	4-Br	87.9	1.12	1	20.5		65.4	0.94
14	4-NO ₂	74.4	3.21	1	−12.3		10.7	
15	4-CH ₃	92.1	0.81	1	−15.5		−13.5	
16	4-N(CH ₃) ₂	89.0	1.15	1	8.7		20.5	
17	3,4-diOCH ₃	93.6	1.05	1	30.7		54.9	0.98
18	3,4-diOH	96.2	0.45	1	85.1	0.41	97.6	0.24
19	3,4-diCl	83.4	1.21	1	45.8		78.6	0.62
20	3-F, 4-OCH ₃	84.8	0.91	1	44.7		46.9	
21	2,4-diCH ₃	92.6	0.40	1	25.9		50.7	1.02
22	2,4-diOH	97.8	0.16	1	78.7	0.43	95.4	0.32
23	2,5-diOH	96.2	0.58	1	60.5	0.85	87.6	0.43
24	2,3,4-triOH	93.6	0.75	1	10.5		12.4	
C								
25	H	44.4	11.5	0.3	68.1	0.23	95.3	0.21
26	2-OH	81.4	1.25	1	38.0		93.3	0.28
27	3-OH	58.7	10.5	0.15	92.5	0.06	89.7 ^{b)}	0.016
28	3-CH ₃	57.7	10.2	1	32.6		82.6	0.61
29	4-CH ₃	60.5	9.8	1	28.5		75.8	0.64
30	4-OCH ₃	27.8	14.8	0.3	53.7	0.28	86.8	0.25
31	4-Cl	32.4	13.7	1	53.8	0.94	94.3	0.39
32	2,4-diCH ₃	58.4	7.8	1	−20.5		−17.5	
33	2,4-diOH	89.6	0.85	1	69.6	0.72	81.7	0.65
34	3-F, 4-OCH ₃	41.8	10.9	1	52.8	0.98	95.7	0.31
35	3,4-diOCH ₃	44.9	11.8	1	50.8	1.02	72.9	0.69
36	3,4-diOH	91.0	0.55	1	55.4	0.94	89.5	0.28
37	2,3,4-triOCH ₃	57.9	8.2	1	56.0	0.91	92.6	0.29
38	2,3,4-triOH	77.0	2.37	0.3	65.4	0.23	90.2	0.21
Epalrestat (Ep)		99.8	0.08	0.3	71.3	0.19	93.3	0.14
Aspirin (As)		—	—	0.3	77.2	0.14	73.2 ^{b)}	0.026

Stimulatory activity is expressed by a minus sign (—). a) Data of series A and B on rat lens aldose reductase are cited from ref. 7. b) Inhibition percent at 0.05 mg/ml.

were measured on a Hitachi U-3210 spectrophotometer and a platelet aggregometer (Chrono-Log, M-500US). Thin-layer chromatographic separations were performed with silica gel 60 F₂₅₄ plates (Merck Art. 5715) and silica gel 60 (Merck Art. 7734; 70—230 mesh), respectively.

Chemical 2'-Hydroxychalcones (Series A and B) tested were synthesized by the method described in our previous paper.⁷⁾ In the case of synthesis of compounds possessing 2',4'- or 3,4-dihydroxyl group, the hydroxyl group of the component was protected with methoxymethyl (MOM) using chloro methylmethyl ether.⁹⁾ The source of other materials were as follows: DL-glyceraldehyde (Sigma Chemical Co., Ltd.), β -NADPH (β -nicotinamide adenine dinucleotide phosphate tetrasodium, Sigma Chemical Co., Ltd.), ADP, Collagen purchased from Aldrich Inc. (U.S.A.).

General Procedure for 5'-Chloro-2'-hydroxychalcone Derivatives (Series C). 5'-Chloro-2'-hydroxychalcone (**25**) To a mixture of 5'-chloro-2'-hydroxyacetophenone (0.19 g, 1.39 mmol) and 50% aqueous potassium hydroxide solution (5.3 ml, as KOH: 2.64 g, 47.0 mmol) in 20 ml of 95% ethanol was added benzaldehyde (0.29 g, 1.39 mmol). After stirring for 5 h at room temperature, the reaction was quenched with distilled water. The resulting solution was acidified with 1 N HCl, and diluted with ethyl acetate. The organic phase was washed with brine, dried over anhydrous magnesium sulfate and concentrated. The crude product, 5'-chloro-2'-hydroxychalcone (**25**), was purified by column chromatography (silica gel, eluted with 20% ethyl acetate in hexane) giving yellow needles. TLC, *R_f* 0.53 (5:3 Hexane/EtOAc). Physical and spectral data for compounds **25—38** are

Table 2. Physical and Spectral Data for Compounds 25–38

Compd No.	Yield (%)	mp (°C)	IR (cm ⁻¹)	¹ H-NMR (300 MHz, acetone- <i>d</i> ₆)	¹³ C-NMR (75 MHz, acetone- <i>d</i> ₆)	Formula	MS <i>m/z</i> (rel. int.)	HRMS Calcd (Found)
25	56.8	162—164	3437, 1645	12.84 (s, 1H), 8.32 (d, <i>J</i> =2.4 Hz, 1H), 8.13 (d, <i>J</i> =15.3 Hz, 1H), 7.93 (d, <i>J</i> =15.3 Hz, 1H), 7.92 (m, 2H), 7.48—7.50 (m, 3H), 7.60 (dd, <i>J</i> =2.4, 9.0 Hz, 1H), 7.02 (d, <i>J</i> =9.0 Hz, 1H) 12.96 (s, 1H), 8.22 (d, <i>J</i> =2.1 Hz, 1H), 8.38 (d, <i>J</i> =15.3 Hz, 1H), 8.06 (d, <i>J</i> =15.3 Hz, 1H), 7.20 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 7.31 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 7.5—7.8 (m, 2H), 6.96 (d, <i>J</i> =8.7 Hz, 1H), 7.02 (dd, <i>J</i> =2.1, 8.7 Hz, 1H)	124.1 (C-1'), 164.0 (C-2'), 120.7 (C-3'), 135.5 (C-4'), 129.8 (C-5'), 132.0 (C-6'), 206.1 (C=O), 121.0 (C-α), 147.1 (C-β), 137.0 (C-1), 129.8 (C-2), 140.1 (C-3), 120.8 (C-4), 130.4 (C-4'), 120.7 (C-6) 126.4 (C-1'), 142.6 (C-2'), 117.1 (C-3'), 136.3 (C-4'), 126.9 (C-5'), 130.3 (C-6'), 206.2 (C=O), 121.1 (C-α), 136.8 (C-β), 120.9 (C-1), 136.3 (C-2), 120.1 (C-3), 130.1 (C-4), 120.8 (C-5), 127.6 (C-6)	C ₁₅ H ₁₁ O ₃ Cl	258 (M ⁺ , 74), 181 (52), 154 (59), 103 (63), 77 (100)	258.0448 (258.0529)
26	27.7	104—106	3234, 1676	12.96 (s, 1H), 8.22 (d, <i>J</i> =2.1 Hz, 1H), 8.38 (d, <i>J</i> =15.3 Hz, 1H), 8.06 (d, <i>J</i> =15.3 Hz, 1H), 7.20 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 7.31 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 7.5—7.8 (m, 2H), 6.96 (d, <i>J</i> =8.7 Hz, 1H), 7.02 (dd, <i>J</i> =2.1, 8.7 Hz, 1H)	124.1 (C-1'), 164.0 (C-2'), 120.7 (C-3'), 135.5 (C-4'), 129.8 (C-5'), 132.0 (C-6'), 206.1 (C=O), 121.0 (C-α), 147.1 (C-β), 137.0 (C-1), 129.8 (C-2), 140.1 (C-3), 120.8 (C-4), 130.4 (C-4'), 120.7 (C-6) 126.4 (C-1'), 142.6 (C-2'), 117.1 (C-3'), 136.3 (C-4'), 126.9 (C-5'), 130.3 (C-6'), 206.2 (C=O), 121.1 (C-α), 136.8 (C-β), 120.9 (C-1), 136.3 (C-2), 120.1 (C-3), 130.1 (C-4), 120.8 (C-5), 127.6 (C-6)	C ₁₅ H ₁₁ O ₃ Cl	274 (M ⁺ , 23), 58 (100), 155 (19), 99 (12)	274.0397 (274.0432)
27	34.8	149—150	3344, 1641	12.86 (s, 1H), 8.28 (d, <i>J</i> =2.7 Hz, 1H), 7.99 (d, <i>J</i> =15.3 Hz, 1H), 7.86 (d, <i>J</i> =15.3 Hz, 1H), 8.63 (s, 1H) 7.01 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 7.2—7.4 (m, 2H), 7.53 (dd, <i>J</i> =2.4 Hz, 9.0, 1H), 6.96 (m, 1H) 12.73 (s, 1H), 7.86 (d, <i>J</i> =2.4 Hz, 1H), 7.91 (d, <i>J</i> =15.3 Hz, 1H), 7.54 (d, <i>J</i> =15.3 Hz, 1H), 7.45 (d, <i>J</i> =8.7 Hz, 1H), 7.2—7.3 (m, 3H), 7.48 (d, <i>J</i> =8.7 Hz, 1H), 6.97 (d, <i>J</i> =8.7 Hz, 1H), 2.41 (s, 3H) 12.79 (s, 1H), 7.83 (d, <i>J</i> =2.4 Hz, 1H), 7.90 (d, <i>J</i> =15.3 Hz, 1H), 7.49 (d, <i>J</i> =15.3 Hz, 1H), 7.56 (d, <i>J</i> =8.7 Hz, 2H), 7.24 (d, <i>J</i> =8.7 Hz, 2H), 6.95 (d, <i>J</i> =8.7 Hz, 1H), 7.40 (dd, <i>J</i> =2.4, 8.7 Hz, 1H), 2.38 (s, 3H)	123.4 (C-1'), 158.0 (C-2'), 118.6 (C-3'), 136.3 (C-4'), 129.7 (C-5'), 130.2 (C-6'), 205.6 (C=O), 121.3 (C-α), 146.6 (C-β), 136.2 (C-1), 115.7 (C-2), 163.0 (C-3), 120.3 (C-4), 121.0 (C-5), 120.1 (C-6) 127.4 (C-19), 163.0 (C-29), 120.8 (C-39), 137.0 (C-49), 127.4 (C-59), 132.8 (C-69), 206.1 (C=O), 124.0 (C-α), 147.4 (C-β), 135.4 (C-1), 129.7 (C-2), 139.5 (C-3), 130.3 (C-4), 130.5 (C-5), 124.0 (C-6), 21.2 (CH ₃) 126.3 (C-1'), 147.2 (C-2'), 120.1 (C-3'), 136.4 (C-4'), 127.3 (C-5'), 132.8 (C-6'), 206.1 (C=O), 121.1 (C-α), 136.9 (C-β), 130.5 (C-1), 130.0 (C-2), 6, 130.3 (C-3, 5), 136.4 (C-4), 21.5 (CH ₃)	C ₁₅ H ₁₁ O ₃ Cl	274 (M ⁺ , 82), 181 (70), 155 (95), 147 (18), 120 (100), 91 (84)	274.0397 (274.0454)
28	38.7	84—86	3452, 1641	12.73 (s, 1H), 7.86 (d, <i>J</i> =2.4 Hz, 1H), 7.91 (d, <i>J</i> =15.3 Hz, 1H), 7.54 (d, <i>J</i> =15.3 Hz, 1H), 7.45 (d, <i>J</i> =8.7 Hz, 1H), 7.2—7.3 (m, 3H), 7.48 (d, <i>J</i> =8.7 Hz, 1H), 6.97 (d, <i>J</i> =8.7 Hz, 1H), 2.41 (s, 3H) 12.79 (s, 1H), 7.83 (d, <i>J</i> =2.4 Hz, 1H), 7.90 (d, <i>J</i> =15.3 Hz, 1H), 7.49 (d, <i>J</i> =15.3 Hz, 1H), 7.56 (d, <i>J</i> =8.7 Hz, 2H), 7.24 (d, <i>J</i> =8.7 Hz, 2H), 6.95 (d, <i>J</i> =8.7 Hz, 1H), 7.40 (dd, <i>J</i> =2.4, 8.7 Hz, 1H), 2.38 (s, 3H)	123.4 (C-1'), 158.0 (C-2'), 118.6 (C-3'), 136.3 (C-4'), 129.7 (C-5'), 130.2 (C-6'), 205.6 (C=O), 121.3 (C-α), 146.6 (C-β), 136.2 (C-1), 115.7 (C-2), 163.0 (C-3), 120.3 (C-4), 121.0 (C-5), 120.1 (C-6) 127.4 (C-19), 163.0 (C-29), 120.8 (C-39), 137.0 (C-49), 127.4 (C-59), 132.8 (C-69), 206.1 (C=O), 124.0 (C-α), 147.4 (C-β), 135.4 (C-1), 129.7 (C-2), 139.5 (C-3), 130.3 (C-4), 130.5 (C-5), 124.0 (C-6), 21.2 (CH ₃) 126.3 (C-1'), 147.2 (C-2'), 120.1 (C-3'), 136.4 (C-4'), 127.3 (C-5'), 132.8 (C-6'), 206.1 (C=O), 121.1 (C-α), 136.9 (C-β), 130.5 (C-1), 130.0 (C-2), 6, 130.3 (C-3, 5), 136.4 (C-4), 21.5 (CH ₃)	C ₁₆ H ₁₃ O ₂ Cl	272 (M ⁺ , 98), 181 (58), 154 (45), 145 (12), 127 (28), 115 (100)	272.0604 (272.0649)
29	49.3	126—128	3447, 1641	12.79 (s, 1H), 7.83 (d, <i>J</i> =2.4 Hz, 1H), 7.90 (d, <i>J</i> =15.3 Hz, 1H), 7.49 (d, <i>J</i> =15.3 Hz, 1H), 7.56 (d, <i>J</i> =8.7 Hz, 2H), 7.24 (d, <i>J</i> =8.7 Hz, 2H), 6.95 (d, <i>J</i> =8.7 Hz, 1H), 7.40 (dd, <i>J</i> =2.4, 8.7 Hz, 1H), 2.38 (s, 3H)	130.1 (C-1'), 163.0 (C-2'), 118.3 (C-3'), 136.7 (C-4'), 132.2 (C-5'), 132.4 (C-6'), 206.2 (C=O), 123.3 (C-α), 142.8 (C-β), 127.2 (C-1), 127.2 (C-2,6), 127.2 (C-3,5), 161.2 (C-4), 56.6 (OCH ₃) 129.9 (C-1'), 145.5 (C-2'), 120.8 (C-3'), 137.2 (C-4'), 130.4 (C-5'), 131.6 (C-6'), 206.1 (C=O), 120.7 (C-α), 147.2 (C-β), 134.0 (C-1), 129.9 (C-2,6), 131.5 (C-3, 5), 134.4 (C-4)	C ₁₆ H ₁₃ O ₂ Cl	288 (M ⁺ , 28), 162 (4), 135 (100), 107 (21), 77 (35), 57 (49)	288.0553 (288.0572)
31	70.6	74—76	3449, 1645	12.72 (s, 1H), 7.85 (d, <i>J</i> =2.1 Hz, 1H), 7.88 (d, <i>J</i> =15.3 Hz, 1H), 7.80 (d, <i>J</i> =15.3 Hz, 1H), 7.53 (d, <i>J</i> =8.7 Hz, 2H), 7.44 (d, <i>J</i> =8.7 Hz, 2H), 7.26 (d, <i>J</i> =8.7 Hz, 1H), 7.01 (dd, <i>J</i> =2.1, 8.7 Hz, 1H) 12.94 (s, 1H), 8.26 (d, <i>J</i> =2.7 Hz, 1H), 8.24 (d, <i>J</i> =15.6 Hz, 1H), 7.91 (d, <i>J</i> =15.6 Hz, 1H), 7.54 (dd, <i>J</i> =2.7, 9.0 Hz, 1H), 7.08 (d, <i>J</i> =8.4 Hz, 1H), 6.98 (d, <i>J</i> =9.0 Hz, 1H), 7.05 (d, <i>J</i> =9.0 Hz, 1H), 2.45 (s, 3H), 2.37 (s, 3H)	130.3 (C-2), 128.9 (C-3), 132.5 (C-4), 128.1 (C-5), 120.8 (C-6), 21.4 (CH ₃), 19.6 (CH ₃) 126.4 (C-1'), 163.0 (C-2'), 117.1 (C-3'), 136.2 (C-4'), 129.8 (C-5'), 131.4 (C-6'), 206.3 (C=O), 121.2 (C-α), 142.4 (C-β), 116.2 (C-1), 163.4 (C-2), 102.9 (C-3), 162.4 (C-4), 109.4 (C-5), 130.0 (C-6) 128.5 (C-1'), 156.6 (C-2'), 116.1 (C-3'), 136.9 (C-4'), 126.9 (C-5'), 130.3 (C-6'), 206.1 (C=O), 123.3 (C-α), 146.1 (C-β), 128.6 (C-1), 114.4 (C-2), 146.2 (C-3), 148.2 (C-4), 115.9 (C-5), 122.8 (C-6), 56.6 (OCH ₃)	C ₁₅ H ₁₀ O ₂ Cl ₂	292 (M ⁺ , 61), 181 (41), 165 (22), 154 (100), 138 (51), 101 (48)	292.0058 (292.0076)
32	68.3	155—156	3390, 1639	12.94 (s, 1H), 8.26 (d, <i>J</i> =2.7 Hz, 1H), 8.24 (d, <i>J</i> =15.6 Hz, 1H), 7.91 (d, <i>J</i> =15.6 Hz, 1H), 7.54 (dd, <i>J</i> =2.7, 9.0 Hz, 1H), 7.08 (d, <i>J</i> =8.4 Hz, 1H), 6.98 (d, <i>J</i> =9.0 Hz, 1H), 7.05 (d, <i>J</i> =9.0 Hz, 1H), 2.45 (s, 3H), 2.37 (s, 3H)	128.0 (C-1'), 163.0 (C-2'), 120.4 (C-3'), 136.9 (C-4'), 128.1 (C-5'), 130.3 (C-6'), 206.1 (C=O), 128.1 (C-α), 144.2 (C-β), 132.5 (C-1), 130.3 (C-2), 128.9 (C-3), 132.5 (C-4), 128.1 (C-5), 120.8 (C-6), 21.4 (CH ₃), 19.6 (CH ₃) 126.4 (C-1'), 163.0 (C-2'), 117.1 (C-3'), 136.2 (C-4'), 129.8 (C-5'), 131.4 (C-6'), 206.3 (C=O), 121.2 (C-α), 142.4 (C-β), 116.2 (C-1), 163.4 (C-2), 102.9 (C-3), 162.4 (C-4), 109.4 (C-5), 130.0 (C-6) 128.5 (C-1'), 156.6 (C-2'), 116.1 (C-3'), 136.9 (C-4'), 126.9 (C-5'), 130.3 (C-6'), 206.1 (C=O), 123.3 (C-α), 146.1 (C-β), 128.6 (C-1), 114.4 (C-2), 146.2 (C-3), 148.2 (C-4), 115.9 (C-5), 122.8 (C-6), 56.6 (OCH ₃)	C ₁₇ H ₁₅ O ₂ Cl	286 (M ⁺ , 61), 181 (19), 155 (30), 132 (80), 127 (33), 115 (65)	286.0761 (286.0785)
33	57.3	118—120	3343, 1624	12.84 (s, 1H), 7.65 (br s, 1H), 8.17 (d, <i>J</i> =15.6 Hz, 1H), 7.39 (d, <i>J</i> =15.6 Hz, 1H), 7.38 (br s, 1H), 7.12 (d, <i>J</i> =8.7 Hz, 1H), 6.96 (d, <i>J</i> =8.7 Hz, 1H), 6.86 (d, <i>J</i> =9.0 Hz, 1H), 6.24 (d, <i>J</i> =9.0 Hz, 1H) 12.90 (s, 1H), 8.31 (d, <i>J</i> =2.4 Hz, 1H), 8.01 (d, <i>J</i> =15.3 Hz, 1H), 7.91 (d, <i>J</i> =15.6 Hz, 1H), 7.84 (d, <i>J</i> =2.1 Hz, 1H), 7.54 (dd, <i>J</i> =2.4, 9.0 Hz, 1H), 7.66 (br d, <i>J</i> =8.7 Hz, 1H), 6.99 (d, <i>J</i> =9.0 Hz, 1H), 7.23 (d, <i>J</i> =8.7 Hz, 1H), 3.96 (s, 3H)	C ₁₅ H ₁₁ O ₄ Cl	290 (M ⁺ , 31), 172 (100), 163 (11), 144 (12)	290.0346 (290.0366)	
34	67.3	118—120	3447, 1641	12.88 (br s, 1H), 7.83 (br s, 1H), 7.87 (d, <i>J</i> =15.3 Hz, 1H), 7.37 (d, <i>J</i> =15.3 Hz, 1H), 7.25 (d, <i>J</i> =8.7 Hz, 1H), 7.40 (d, <i>J</i> =8.7 Hz, 1H), 6.94 (d, <i>J</i> =9.0 Hz, 1H), 7.14 (br s, 1H), 6.90 (d, <i>J</i> =9.0, 1H), 3.94 (s, 3H), 3.90 (s, 3H)	C ₁₆ H ₁₂ O ₂ FCl	306 (M ⁺ , 40), 181 (10), 152 (100), 139 (26), 107 (22), 83 (11)	306.0459 (306.0428)	
35	64.3	98—100	3462, 1635	12.87 (s, 1H), 8.26 (br s, 1H), 8.07 (d, <i>J</i> =15.3 Hz, 1H), 7.91 (d, <i>J</i> =15.3 Hz, 1H), 7.11 (s, 1H), 7.54 (d, <i>J</i> =8.7 Hz, 1H), 7.90 (br d, <i>J</i> =8.7 Hz, 1H), 7.01 (d, <i>J</i> =9.0 Hz, 1H), 7.11 (d, <i>J</i> =9.0 Hz, 1H) 12.89 (s, 1H), 7.83 (d, <i>J</i> =2.1 Hz, 1H), 8.11 (d, <i>J</i> =15.3 Hz, 1H), 7.59 (d, <i>J</i> =15.3 Hz, 1H), 7.24 (d, <i>J</i> =8.7 Hz, 1H), 7.41 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 6.96 (d, <i>J</i> =8.4 Hz, 1H), 6.73 (d, <i>J</i> =8.4 Hz, 1H), 3.88 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H)	C ₁₇ H ₁₅ O ₄ Cl	318 (M ⁺ , 88), 164 (100), 151 (43), 99 (12)	318.0659 (318.0667)	
36	74.0	158—160	3449, 1633	12.87 (s, 1H), 8.26 (br s, 1H), 8.07 (d, <i>J</i> =15.3 Hz, 1H), 7.91 (d, <i>J</i> =15.3 Hz, 1H), 7.11 (s, 1H), 7.54 (d, <i>J</i> =8.7 Hz, 1H), 7.90 (br d, <i>J</i> =8.7 Hz, 1H), 7.01 (d, <i>J</i> =9.0 Hz, 1H), 7.11 (d, <i>J</i> =9.0 Hz, 1H) 12.89 (s, 1H), 7.83 (d, <i>J</i> =2.1 Hz, 1H), 8.11 (d, <i>J</i> =15.3 Hz, 1H), 7.59 (d, <i>J</i> =15.3 Hz, 1H), 7.24 (d, <i>J</i> =8.7 Hz, 1H), 7.41 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 6.96 (d, <i>J</i> =8.4 Hz, 1H), 6.73 (d, <i>J</i> =8.4 Hz, 1H), 3.88 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H)	C ₁₅ H ₁₁ O ₄ Cl	290 (M ⁺ , 68), 155 (49), 137 (100), 99 (22)	290.0346 (290.0352)	
37	69.9	112—114	3443, 1639	13.19 (s, 1H), 8.15 (d, <i>J</i> =2.1 Hz, 1H), 8.33 (d, <i>J</i> =15.3 Hz, 1H), 7.92 (d, <i>J</i> =15.3 Hz, 1H), 7.37 (d, <i>J</i> =8.7 Hz, 1H), 7.52 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 6.97 (d, <i>J</i> =8.4 Hz, 1H), 6.51 (d, <i>J</i> =8.4 Hz, 1H)	C ₁₈ H ₁₇ O ₃ Cl	348 (M ⁺ , 19), 317 (100), 194 (4), 179 (11), 155 (12), 92 (6)	348.0765 (348.0778)	
38	74.1	289—295	3495, 1685	13.19 (s, 1H), 8.15 (d, <i>J</i> =2.1 Hz, 1H), 8.33 (d, <i>J</i> =15.3 Hz, 1H), 7.92 (d, <i>J</i> =15.3 Hz, 1H), 7.37 (d, <i>J</i> =8.7 Hz, 1H), 7.52 (dd, <i>J</i> =2.1, 8.7 Hz, 1H), 6.97 (d, <i>J</i> =8.4 Hz, 1H), 6.51 (d, <i>J</i> =8.4 Hz, 1H)	C ₁₅ H ₁₁ O ₃ Cl	306 (M ⁺ , 12), 296 (100), 179 (14), 55 (10), 92 (15)	306.0295 (306.0256)	

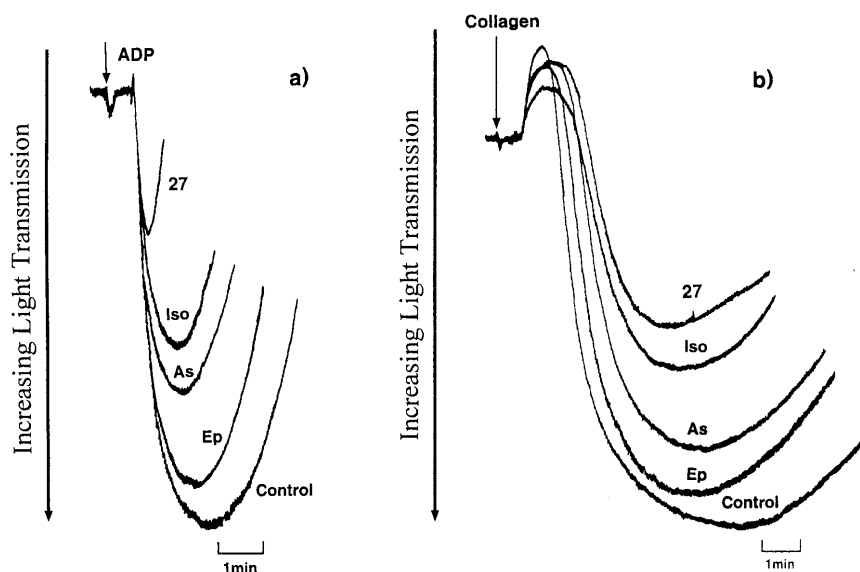


Fig. 1. Effect of 5'-Chloro-3,2'-dihydroxychalcone (**27**), Isoliquiritigenin, Epalrestat and Aspirin on Rat Platelet Aggregation Induced by 2.5 μ M of ADP (a) and 10.0 μ g/ml of Collagen (b)

Platelets were stimulated at the time indicated by the arrows. The concentration of the drugs was 75 μ g/ml on the a) and 20 μ g/ml on the b). Control, Ep, As, Iso and **27** indicate vehicle only, epalrestat, aspirin, isoliquiritigenin and 5'-chloro-3,2'-dihydroxychalcone, respectively. AU; Arbitrary Unit.

shown in Table 2.

Biological Testing. Preparation of RLAR Crude RLAR was prepared as follows: rat lenses were removed from Sprague-Dawley rats weighing 250–280 g and frozen until use. The supernatant fraction of the rat lens homogenate was prepared according to Hayman and Kinoshita¹⁰ and then partially purified according to Inagaki *et al.*¹¹ The partially purified material was separated into 1.0 ml aliquots and stored at -40°C .

Measurements of RLAR Activity RLAR activities were assayed spectrophotometrically by measuring the decrease in absorption of NADPH at 340 nm over a 4 min period with DL-glyceraldehyde as a substrate.¹² Each 1.0 ml cuvette contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2), 0.3 mM NADPH with or without 10 mM substrate and inhibitor. The concentration of inhibitors giving 50% inhibition of enzyme activity (IC_{50}) was calculated from the least-squares regression line of the logarithmic concentrations plotted against the remaining activity.

Platelet Aggregation Fresh blood, obtained from Sprague-Dawley male rats weighing 250–290 g, containing 0.22% sodium citrate was centrifuged at 1200 rpm for 10 min at room temperature to obtain a supernatant of platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was prepared by re-centrifugation of the remaining blood at 3000 rpm for 15 min. Each compound was dissolved in DMSO and 5 μ l of each was added to 485 μ l PRP (5×10^5 platelets/ μ l). Adenosine 5'-diphosphate (ADP) and collagen were used as inducers at final concentrations of 2.5 μ M and 10.0 μ g/ml, respectively. Platelet aggregation was determined by the turbidimetric method using a platelet aggregometer.¹³ A water soluble sample was dissolved in saline and 5 μ l of this was added to 485 μ l PRP, while an insoluble sample in saline was dissolved in DMSO, and 5 μ l of this was added to 485 μ l PRP. After incubation at 37°C for 1 min with stirring, platelet aggregation was initiated by addition of 10 μ l inducer. Saline or DMSO was used as a control. The degree of anti-platelet aggregation (%) was calculated from the following equation; the ratio of anti-platelet aggregation potency of the

control)] $\times 100$.

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References

- 1) Constantino L., Rastelli G., Vianello P., Cignarella G., Barlocco D., *Med. Res. Rev.*, **19**, 3–23 (1999).
- 2) Zatz R., Brenner B. M., *Am. J. Med.*, **80**, 443–453 (1986).
- 3) Brownlee M., Cerami A., *Ann. Rev. Biochem.*, **50**, 385–432 (1981).
- 4) Colwell J. A., Winocour P. D., Halushka P. V., *Diabetes*, **32** (Suppl. 2), 14–19 (1983).
- 5) Aida K., Tawata M., Shindo H., Onaya T., Sasaki H., Yamaguchi T., Chin M., Mitsuhashi H., *Planta Med.*, **57**, 254–258 (1990).
- 6) Tawata M., Aida K., Noguchi T., Ozaki Y., Kume S., Sasaki H., Chin M., Onaya T., *Euro. J. Pharm.*, **212**, 87–92 (1992).
- 7) Lim S. S., Jung S. H., Ji J., Shin K. H., Keum S. R., *Pharm. Res.*, submitted (2000).
- 8) Terashima H., Hama K., Yamamoto R., Tsuboshima M., Kikkawa R., Hatanaka I., Shigeta Y., *J. Pharmacol. Exp. Ther.*, **229**, 226–229 (1984).
- 9) Rall G. J. H., Oberholzer M. E., Ferreira D., Roux D. G., *Tetrahedron Lett.*, **13**, 1033–1036 (1976).
- 10) Hayman S., Kinoshita J. H., *J. Biol. Chem.*, **240**, 877–882 (1965).
- 11) Inagaki K., Miwa I., Okuda J., *Arch. Biochem. Biophys.*, **216**, 337–344 (1982).
- 12) Sato S., Kador P. F., *Biochem. Pharmacol.*, **40**, 1033–1042 (1990).
- 13) Born G. V. R., *Nature (London)*, **194**, 927–929 (1962).

Enantiomeric Separation by Cyclodextrin Modified Capillary Zone Electrophoresis (CD-CZE) of Quaternary Tetrahydroprotoberberine Alkaloids

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A cyclodextrin modified capillary zone electrophoresis (CD-CZE) for the enantiomeric separation of tetrahydroprotoberberine *N*-metho salts was established. The resolution was optimized by changing the concentration of the electrolyte solution, hydroxypropyl- β -CD (0.02 M, 0.07 M, or 0.14 M) or dimethyl- β -CD (0.05 M or 0.15 M) in phosphate buffer (pH 2.5 or 3) containing 10% acetonitrile with an applied voltage of 20 kV. This method was applied toward the enantioselective bio-conversion of quaternary tetrahydroprotoberberine *N*-metho salts in cultured cells of *Corydalis* species.

Key words CD-CZE; quaternary tetrahydroprotoberberine *N*-metho salt; enantiomeric separation

Most protoberberine alkaloids exist in nature either as tetrahydroprotoberberines or as quaternary protoberberine salts. A large number of tetrahydroprotoberberines substituted in rings A and D have shown promising tranquilizing properties.¹⁾ In many cases, pharmacological and toxicological effects of racemic compounds are related to only one of the enantiomers. It is therefore important to develop an enantiomeric separation method to determine the ratio of enantiomers and purity of these alkaloids. Racemic tetrahydroprotoberberine alkaloids are usually resolved by fractional recrystallization of diastereomeric salts,²⁾ by paper and cellulose column chromatography,³⁾ or by HPLC.⁴⁾

There are naturally occurring tetrahydroprotoberberine α - and β -*N*-metho salts with the *cis*-B/C and *trans*-B/C fused systems, respectively. Tetrahydroprotoberberine *N*-metho salts are important intermediates in the biosynthetic process of tetrahydroprotoberberine type compounds to protopine type compounds.^{5,6)} The enantiomeric separation of both types of *N*-metho salts is also required for bio-synthetic study.

Separation of enantiomers by capillary zone electrophoresis (CZE) with CD as the chiral selector was just introduced by Fanali⁷⁾ and is one of the successful techniques for enantiomeric separation by CE.^{8,9)} CD is an electrically neutral compound and hence it migrates at the electroosmotic velocity in CZE. When a charged solute is included in CD, the inclusion complex thus formed has a charge identical with that of the free solute but an increased molecular mass, and hence a lower electrophoretic mobility than the free solute. In an enantiomeric separation, free enantiomers have identical electrophoretic mobilities and the included enantiomers also probably have the same mobilities. Therefore, the separation principle of CD-CZE for enantiomeric separation is the difference in inclusion-complex formation constants between a pair of enantiomers and CD. The more strongly included enantiomer has a lower effective mobility.⁸⁾

We describe here the application of a simple and rapid method for separating enantiomers of several quaternary tetrahydroprotoberberine *N*-metho salts. Complete separation of racemic mixtures in their enantiomers was obtained by using hydroxypropyl- β -CD or dimethyl- β -CD as a chiral additive in the phosphate buffer.

Experimental

Reagents and Materials Sodium dihydrogenphosphate, sodium tetraborate, phosphoric acid, γ -CD, and hydroxypropyl- β -CD (HP- β -CD) were purchased from Wako (Osaka, Japan), α -CD, β -CD and di-*o*-methyl- β -CD (DM- β -CD) from Nacalai Tesque (Kyoto, Japan), and boric acid from Fluka (Buchs, Switzerland). Acetonitrile, methanol, and water were of HPLC grade. ($\pm$)-Tetrahydroberberine *N*-methochloride (**1**), ($\pm$)-tetrahydropalmatine *N*-methochloride (**3**), ($\pm$)-thalictrocin *N*-methochloride (**4**), ($\pm$)-epiopiocarpine *N*-methochloride (**5**), ($\pm$)-opiocarpine *N*-methochloride (**6**), ($\pm$)-corydaline *N*-methochloride (**7**), and ($\pm$)-mesocorydaline *N*-methochloride (**8**), were obtained according to the procedure described in previous papers.^{5,10)} ($\pm$)-Tetrahydroprotoberberine *N*-methochloride (**2**) was prepared from tetrahydroprotoberberine.¹¹⁾

Apparatus CD-CZE was carried out on a Beckman (Fullerton, CA, U.S.A.) P/ACE SYSTEM 5000 apparatus equipped with an UV detector set at 254 nm. CZE was performed at 20 °C in a fused-silica capillary (570 mm $\times$ 75 μ m i.d.; 500 mm effective length) (Beckman) applying a potential of 20 kV. The sampling time was 5 s (hydrodynamic mode; 3.5 kPa).

Procedure The separation solutions were prepared by dissolving CDs in a 0.05 M (0.07 M, 0.12 M, or 0.15 M) phosphate buffer prepared by mixing 0.05 M (0.07 M, 0.12 M, or 0.15 M) sodium dihydrogenphosphate solution with appropriate volumes of 0.05 M (0.07 M, 0.12 M, or 0.15 M) phosphoric acid (pH 2.5 or 3) or borate buffer (boric acid solution with appropriate volumes of sodium tetraborate solution) containing 10% acetonitrile.

A sample solution of each alkaloid was prepared by dissolving a compound in the separation solution at a concentration of ca. 10 ppm.

Results and Discussion

Separation of Enantiomers Enantiomeric separation by CZE was achieved through the addition of CDs to the BGE. The main parameters affecting enantiomeric separations with CD-based electrolytes are thought to be the nature and concentration of CD and the buffer pH. Secondary parameters are ionic strength, presence of an organic solvent, and electroosmotic flow modifiers. The quaternary alkaloids in *Coptidis rhizoma* have been well separated by a CZE technique with the use of 85% 0.1 M sodium acetate solution and 15% methanol.¹²⁾ However, analysis under a similar condition carried out with CDs failed in the enantiomeric separation of ($\pm$)-tetrahydroberberine *N*-methochloride (**1**). Several electrolyte systems have been used for electrophoretic experiments to separate quaternary alkaloids.^{13–16)} An analysis using phosphate and borate buffers in the concentration range of 0.01–0.1 M and pH values between 2 and 9 while maintaining constant voltage suggested that good results would be obtained with 0.05 M phosphate buffer at pH 2.5.

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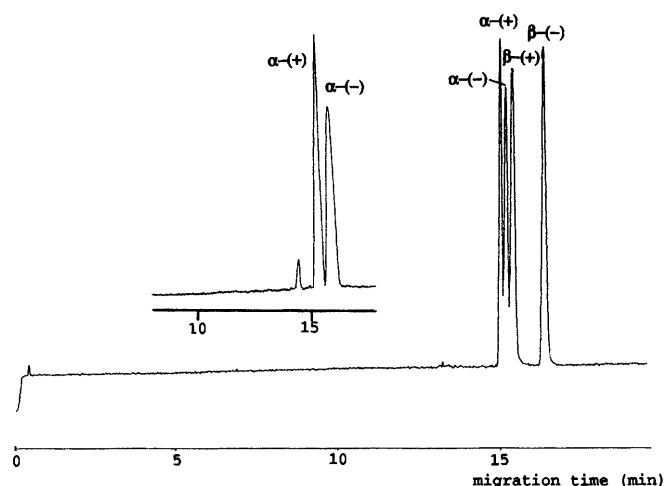


Fig. 1. Electropherogram Showing the Enantiomeric Separation of (±)-Tetrahydroberberine α - and β -N-Methochloride Using 0.05 M Phosphate Buffer (pH 2.5) Containing 0.02 M HP- β -CD and 10% Acetonitrile

Chiral recognition depends on the type of CD employed. Several interactions are involved when CDs are used as chiral selectors, e.g. hydrophobic interaction with the cavity, hydrogen bonding between hydroxyl groups on the rim of CD's and the analytes. The cavity diameter and hydrophobic nature of the internal portion of CDs probably influenced the effective or differential inclusion-complex formation of the enantiomers, leading to differential migration and successful enantiomeric separation. The difference in the stability of the diastereomeric inclusion-complexes permits good enantiomeric resolution. The influence of three neutral CDs (γ -, HP- β -, and DM- β -CD) on the enantiomeric resolution of racemic *N*-metho salts was investigated. CD concentrations were 0.005–0.2 M. The cavity size of γ -CD seems to be most suitable for chiral recognition of tetrahydroberberine alkaloids, judging from a study of CD-modified micellar electrokinetic capillary chromatography,¹⁷⁾ but separation of the enantiomers by CZE with γ -CD failed in tetrahydropyprotoberberine *N*-metho salts. In **1**, the best result in our experiments was obtained using 0.05 M phosphate buffer (pH 2.5) containing 0.02 M HP- β -CD and 10% acetonitrile (Fig. 1). The organic solvents acetonitrile and methanol made a good resolution: the migration time when acetonitrile was added was found to be faster than when methanol was added in our experiments. The resolution values (R_s : $R_s = 2(t_{(-)} - t_{(+)}) / (w_{(+)} + w_{(-)})$) of **1** were 1.25 (10% acetonitrile), 1.24 (10% methanol), and 0.81 (0% organic solvent). The migration times of enantiomers of eight alkaloids (**1**–**8**), the optimum separation conditions, and R_s are shown in Table 1.

Application to the Metabolism of Tetrahydropyprotoberberine *N*-Metho Salts in Cell Cultures It has been demonstrated that (–)-form of tetrahydroberberine α -*N*-methochloride having the *trans*-B/C fused system is transformed by cultured cells of the *Corydalis* species.^{5,6)}

The enantiomeric separation of quaternary metho salts by CD-CZE method was examined as an application to metabolic studies of quaternary protoberberine *N*-metho salts. Feeding experiments with **1** and (±)-thalictrovine *N*-methochloride (**4**) were carried out in cultured cells of *Corydalis pallida* and *C. platycarpa*, respectively. After incubation, the medium and cells were extracted according to the

Table 1. Migration Times, Conditions, and Resolution Values (R_s) of Tetrahydropyprotoberberine *N*-Methochlorides

Compound	Condition	Migration time (min)							
1	A, a	α -(+)	15.02	α -(−)	15.21	β -(+)	15.52	β -(−)	16.48
	Rs	α : 0.88				β : 2.24			
2	B, b	α -(+)	36.37	α -(−)	36.79	β -(+)	34.36	β -(−)	35.82
	Rs			2.55				1.03	
3	B, c	α -(+)	24.49	α -(−)	25.42	β -(+)	25.08	β -(−)	26.16
	Rs			1.92				2.04	
4^{a)}	C, d	α -(+)	31.24	α -(−)	31.76	β	30.39	β	32.24
	Rs			0.99				2.33	
5^{a)}	A, a	α -(+)	12.61	α -(−)	12.97	β	14.06	β	14.55
	Rs			1.82				1.92	
6^{a)}	D, e	α	52.78	α	53.22	β	58.30	β	60.66
	Rs			0.79				1.42	
7^{a)}	A, c	α	16.74	α	17.28	β	17.93	β	18.76
	Rs			1.09				1.59	
8^{a,b)}	D, f	α	39.17	α	41.45				
	Rs			2.37					

Conditions: Phosphate buffer (pH 2.5): A, 0.05 M; B, 0.15 M; C, 0.07 M; D, 0.12 M. CD: a, 0.02 M HP- β -CD; b, 0.14 M HP- β -CD; c, 0.05 M DM- β -CD; d, 0.07 M HP- β -CD; e, 0.15 M DM- β -CD (pH 3); f, 0.15 M HP- β -CD containing 10% acetonitrile. a) (+)- or (–)-form could not be determined in α -*N*-metho salts **6**–**8** and β -*N*-metho salts **4**–**7**. b) α -*N*-metho salt only.

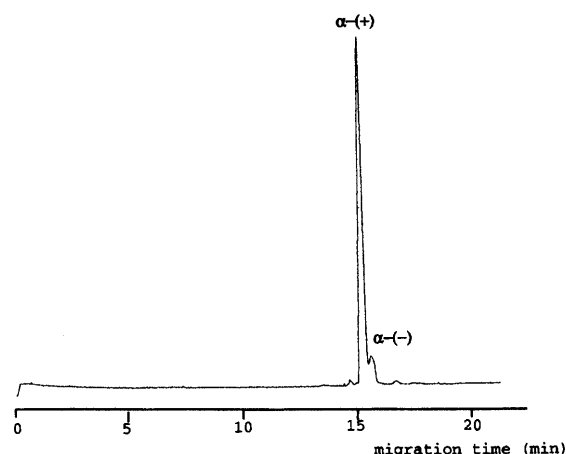


Fig. 2. Electropherogram Showing the Separation of (+)- and (–)-Tetrahydroberberine α -N-Methochlorides in the Chloroform Extract of *Corydalis pallida*

procedure in Iwasa and Takao's method.⁶⁾ The alkaloid extract soluble in chloroform was subjected to CD-CZE of optimized conditions of electrolyte for **1** and **4**. Figure 2 shows a capillary electropherogram of the chloroform extract of *C. pallida* (feeding of (±)-tetrahydroberberine *N*-metho salt). As can be seen, only the (–)-form of **1** is transformed by the cultured cells of *C. pallida*. The same result was obtained for **4**.

The reproducibility of this method, expressed as relative standard deviation (RSD), calculated on the basis of peak area over three replicate injections was between 0.33 and 3.80% and the migration time repeatability was 0.15–2.39%. RSD of the migration times and peak areas is shown in Table 2.

The CD-CZE described here has been proved to be a useful technique for enantiomeric separation of the (±)-tetrahydropyprotoberberine *N*-metho salts using HP- β -CD and DM- β -CD, since it is easy, simple, and reproducible.

Table 2. Repeatability of Migration Times and Peak Areas of Tetrahydroprotoberberin *N*-Methochlorides

Compound	RSD (%) (<i>n</i> =3)			
	α	α	β	β
1 MT	0.18	0.23	0.68	0.64
PA	3.15*	0.37*	1.55	1.29
2 MT	0.55	0.53	0.55	0.27
PA	2.21	1.09	0.73	2.53
3 MT	0.45	0.50	0.46	0.47
PA	0.91	1.65	3.79	2.88
4 MT	0.25	0.24	0.21	0.23
PA	2.04*	2.21*	1.94	0.97
5 MT	0.83	1.21	1.36	1.40
PA	2.37	2.30	1.94	0.33
6 MT	0.80	0.84	0.92	0.82
PA	0.65*	0.75*	1.50	1.35
7 MT	0.64	0.62	0.67	0.67
PA	1.29	1.54	0.41	0.52
8 MT	0.33	0.39	—	—
PA	3.80	1.38	—	—

MT, migration time; PA, peak area. Order of the compounds is the same as in Table 1. * When the peaks were not totally resolved, RSDs calculated from the peak area which the perpendicular dropped for peaks and the baseline drawn to each minimum valley were similar.

References

- 1) Roussel-UCLAF Patents, *Chem. Abstr.*, **69**, 59475m (1968).
- 2) Yamahara J., Konoshima T., Sakakibara Y., Ishiguro M., Sawada T., Fujimura H., *Chem. Pharm. Bull.*, **24**, 1909—1912 (1976).
- 3) Tomita M., Sugamoto M., *Yakugaku Zasshi*, **82**, 1141—1144 (1962).
- 4) Tagahara K., Koyama J., Okatani T., Suzuta Y., *Chem. Pharm. Bull.*, **34**, 5166—5168 (1986).
- 5) Iwasa K., Kamigauchi M., Takao N., Cushman M., *J. Nat. Prod.*, **56**, 2053—2067 (1993).
- 6) Iwasa K., Takao N., *Phytochem.*, **21**, 611—614 (1982).
- 7) Fanali S., *J. Chromatogr.*, **474**, 441—446 (1989).
- 8) Terabe S., Otsuka K., Nishi H., *J. Chromatogr. A*, **666**, 295—319 (1994).
- 9) Fanali S., *J. Chromatogr. A*, **875**, 89—122 (2000). Reviews of applications of chiral capillary electrophoresis, *J. Chromatogr. A*, **875**, 1—293 (2000).
- 10) Iwasa K., Sugiura M., Takao N., *J. Org. Chem.*, **47**, 4275—4280, (1982).
- 11) Tani C., Takao N., Tagahara K., *Yakugaku Zasshi*, **93**, 197—201 (1973).
- 12) Liu Y.-M., Sheu S.-J., *J. Chromatogr.*, **623**, 196—199 (1992).
- 13) Chen Y.-R., Wen K.-C., Her G.-R., *J. Chromatogr. A*, **866**, 273—280 (2000).
- 14) Akada Y., Ishii N., *Bunseki Kagaku*, **46**, 491—494 (1997).
- 15) Sheu S.-J., Lu C.-F., *J. High Resolut. Chromatogr.*, **19**, 409—412 (1996).
- 16) Lee M.-C., Sheu S.-J., *J. Liq. Chrom. & Rel. Technol.*, **20**(1), 63—78 (1997).
- 17) Koyama J., Toyokuni I., Kino A., Tagahara K., *Phytochem. Anal.*, **10**, 175—180 (1999).

Structural Features of an Anti-Diabetic Polysaccharide (TAP) from *Tremella aurantia*

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The structure of an anti-diabetic polysaccharide (TAP) obtained from the fruiting bodies of *Tremella aurantia* was investigated by methylation analysis, Smith degradation, partial acid hydrolysis, ¹³C-NMR spectrometry, and enzymatic digestion. The results suggested that TAP was composed of (1→3)-linked α -D-mannopyranosyl residues as a backbone, some of which were substituted at position 2 with (1→3)-linked β -D-xylopyranose side chains and with β -D-glucopyranuronic acid at position 4 linked to terminal α -D-mannopyranose.

Key words *Tremella aurantia*; polysaccharide; structure; anti-diabetes

We previously reported that an acidic polysaccharide (TAP) isolated from the fruiting bodies of *Tremella aurantia* was composed of mannose, xylose, glucuronic acid, and glucose (molar ratio, 4:2:1:0.3), and contained *O*-acetyl groups (2.2%) in the molecule, and exhibited a significant hypoglycemic activity in diabetic mouse models following intraperitoneal administration and suppressed hyperglycemia by continuous oral administration.¹⁾ Furthermore, we reported that TAP was effective towards the activity of a key hepatic enzyme and plasma cholesterol levels.²⁾

In the present study, the structural features of TAP were investigated by a combination of chemical, enzymatic, and spectroscopic methods.

A TAP and its carboxyl-reduced polysaccharide (TAP-R), in which the carboxyl groups of glucuronic acid residues were reduced with a water-soluble carbodiimide and sodium borohydride, were fully methylated and the products hydrolyzed with acid; this converted sugars into partially methylated alditol acetates.³⁾ The results of GC and GC-MS spectrometry are shown in Table 1. Since the 2,3,6-tri-*O*-methyl-D-glucose derivative peak was only present in trace amounts in TAP, and was greatly increased in its carboxyl-reduced product TAP-R, the methylated sugar should be derived from (1→4)-linked glucuronic acid residues. The identification of 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-mannose, and 2,3,4,6-tetra-*O*-methyl-D-glucose indicates that the non-reducing terminal residues were D-xylopyranose, D-mannopyranose, and small amounts

of glucose. The identification of 4,6-di-*O*-methyl-D-mannose shows the presence of branching (1→2,3)-linked D-mannopyranosyl residues.

The de-*O*-acetylated polysaccharide (TAP-D) obtained by alkali treatment and its carboxyl-reduced polysaccharide (TAP-D-R) were subsequently oxidized with periodate and reduced with sodium borohydride to give the polyalcohols. In the hydrolysate of the polyalcohols (the first Smith degradation products), the molar ratio of xylose was reduced by half and the glucuronic acid disappeared, and the first Smith degradation product of TAP-D-R yielded erythritol (derived from 1,4-linked hexose), in addition to mannose, xylose and a trace amount of glucose. Furthermore, the partially degraded polysaccharide obtained by mild-acid hydrolysis of the polyalcohol was again oxidized, reduced, and mildly hydrolyzed to give the second controlled Smith degradation product (TAP-D-2CS). TAP-D-2CS was found to be only composed of (1→3)-linked α -D-mannopyranose by methylation analysis and IR spectrum (820 cm⁻¹). These results suggest the presence of side chains consisting of (1→3)-linked xylose residues attached to terminal xylose residues, and a core of (1→3)-linked α -D-mannopyranosyl residues.

The ¹³C-NMR spectra of TAP and TAP-R in dimethylsulfoxide (DMSO)-*d*₆ (Fig. 1) were compared with data in the literature.^{4,5,6)} The lower field signals at 171.6 and 170.5 ppm were assigned to the carbonyl of glucuronic acid and *O*-acetyl groups. The signals at 104.4, 102.9, and 100.3 ppm in the anomeric region could be attributed to C-1 of β -D-xylose,

Table 1. GC and GC-MS of Alditol Acetate Derived from Methylated Product of Carboxyl-Reduced TAP (TAP-R)

Methylated sugar (as alditol acetate)	T ^{a)}		Primary mass fragments (<i>m/z</i>)	Molar ratio	Mode of linkage
	Column A	Column B			
2,3,4-Me ₃ -Xyl	0.62	0.64	117, 161	2	[Xylp]1→
2,3,4,6-Me ₄ -Man	0.99	1.00	45, 117, 161, 205	2	[Manp]1→
2,3,4,6-Me ₄ -Glc	1.00	1.00	45, 117, 161, 205	0.3	[Glc]1→
2,4-Me ₂ -Xyl	1.08	1.34	117, 233	2	→3[Xylp]1→
2,4,6-Me ₃ -Man	1.84	2.17	45, 117, 161, 233	3	→3[Manp]1→
2,3,6-Me ₃ -Glc ^{b)}	2.18	2.49	45, 117, 233	2	→4[Glc]1→
4,6-Me ₂ -Man	2.81	3.51	45, 161, 261	4	→2,3[Manp]1→

a) Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Column A: DB 225 (capillary column), column B: 3% ECNSS-M. b) This was a slight amount in methylation analysis of native polysaccharide (TAP).

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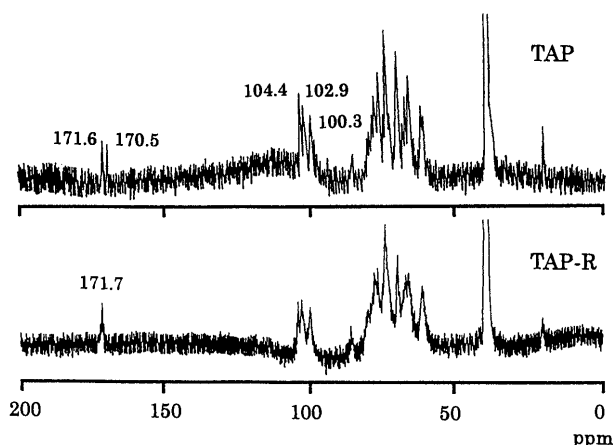


Fig. 1. ^{13}C -NMR Spectra of TAP and TAP-R in $\text{DMSO}-d_6$

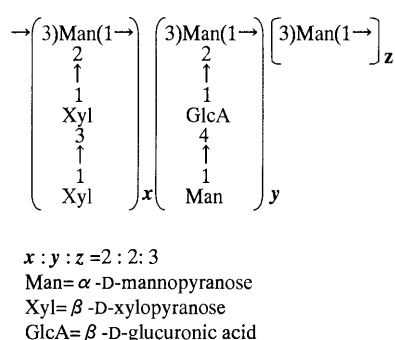


Fig. 2. A Possible Repeating Unit of TAP

α -D-glucuronic acid, and α -D-mannose, respectively.

The degraded polysaccharide (TAP-PH) prepared by partial acid hydrolysis showed a higher $[\alpha]_D + 12^\circ$ in water than the native polysaccharide (TAP) ($[\alpha]_D - 7^\circ$), and had half the amount of xylose, although the amounts of the other component sugars were not reduced. Consequently, it may be assumed that xylose exists as a β -D-linked sugar side chain.

We attempted to attack the polymer with an exo-type enzyme, α -mannosidase from jack bean. The de-*O*-acetylated and carboxyl-reduced glucuronic acid (TAP-D-R) derivative of TAP was used to facilitate the enzymatic digestion, then the removed sugars were analyzed by HPLC as *p*-aminobenzoic ethyl ester (ABEE)-derivatives. Mannose was identified as the removed sugar. It could thus be postulated that α -D-mannose exists as the non-reducing terminal residues of the side chains.

From the foregoing results, TAP seems to be constructed with a (1 $\rightarrow$ 3)-linked α -D-mannopyranosyl backbone, some of which is substituted at the position 2 with (1 $\rightarrow$ 3)-linked β -D-xylopyranose side chains and with β -D-glucopyranosyluronic acid at position 4 linked to terminal α -D-mannopyranose, as shown in Fig. 2. The non-reducing terminal α -D-mannopyranosyl residues may contribute to the potent hypoglycemic activity of TAP in diabetic mouse models. TAP an acidic polysaccharide obtained from the fruiting bodies of *T. aurantia*, was shown to have a similar structural unit, except for the terminal mannanopyranosyl residues, to the polysaccharides of *T. fuciformis*,⁷⁾ *Auricularia auricula-judae*,⁸⁾ *T. mesenterica*⁹⁾ belonging to the same Heterobasidiae, and capsular polysaccharides of *Cryptococcus neoformans*⁶⁾ showing

a certain chemotaxonomic relationship to *Tremella* species.⁷⁾

Experimental

Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded on a JASCO A-102 spectrometer. GC was carried out a Shimadzu GC-4CM apparatus and a Shimadzu GC-15A instrument, equipped with a hydrogen-flame ionization detector. GC-MS was conducted using a Hewlett-Packard Model 5890II combined GC-MS spectrometry system equipped with a fused-silica capillary column (0.25 cm $\times$ 30 m, film thickness 0.25 μm) of DB 225. ^{13}C -NMR spectroscopy was performed on a JEOL FT-NMR EX-400 spectrometer in $\text{DMSO}-d_6$ (50 mg/0.5 ml) at room temperature. The HPLC analysis was carried out using a Shimadzu LC-9A HPLC system equipped with a JASCO FP-920 fluorescence detector.

De-*O*-acetylation The polysaccharide was stirred in 0.1 M sodium methoxide-methanol solution for 8 h. The product collected by centrifugation was dissolved in water, dialyzed against water, and lyophilized to give the de-*O*-acetylated polysaccharide (TAP-D) in 95% yield, which showed no acetyl absorption band (1730 cm^{-1}) in the IR spectrum.

Carboxyl Reduction Carboxyl-reduction of glucuronic acid in the polysaccharide was performed according to the method of Taylor *et al.*,¹⁰⁾ using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and sodium borohydride as previously described.¹¹⁾ The reduction process was repeated 5 times until the signal at 171.6 ppm in the ^{13}C -NMR spectrum disappeared. Carboxyl-reduced polysaccharide (TAP-R) and de-*O*-acetyl and carboxyl-reduced polysaccharide (TAP-D-R) were obtained in 86 and 81% yields from TAP, respectively.

Methylation Analysis The polysaccharide was methylated 3 times by the Hakomori method as previously described.¹²⁾ The fully methylated polysaccharide was heated successively with 90% formic acid for 8 h at 100°C and 2 M trifluoroacetic acid for 8 h at 100°C. The partially methylated sugars were converted into alditol acetate derivatives. The resulting partially methylated alditol acetates were analyzed by GC (3% ECNSS-M column at 175°C and DB 225 capillary column at 180°C) and GC-MS spectrometry, as previously described.¹³⁾

Smith Degradation Each polysaccharide (TAP-D and TAP-D-R) was oxidized with 25 mM sodium periodate at 4°C for 2 d, and reduced with sodium borohydride, as previously described,¹³⁾ to give the polyalcohols in 59% and 60% yields, respectively. A part of the polyalcohol was mildly hydrolyzed with 0.1 M acetic acid at pH 2.0 adjusted with 0.1 M HCl for 35 min at 100°C, and further oxidized similarly as above, then reduced to give the second polyalcohol. Each polyalcohol was hydrolyzed with 2 M sulfuric acid for 6 h at 100°C, and the hydrolysates (the first and second Smith degradation products: TAP-D-2CS) were analyzed by GC as previously described.

Partial Acid Hydrolysis The polysaccharide (TAP) was partially hydrolyzed with 0.1 M HCl for 6 h at 100°C and the neutralized solution dialyzed against water using cellulose tubing (Viscose Corp.). The non-dialyzable solution was lyophilized to give degraded polysaccharide (TAP-PH) in 73% yield.

Enzymatic Digestion and Sugar Analysis by HPLC After a solution of TAP-D-R (100 μg) in 50 mM acetate buffer (pH 5.0, 100 μl) was incubated with α -mannosidase from jack bean (0.5 mg, 9 units, Sigma) for 24 h at 37°C, the reaction mixture was heated in a boiling water bath for 3 min, and centrifuged. Sugars were analyzed by HPLC as ABEE-derivatives, according to the method of Yasuno *et al.*¹⁴⁾ The supernatant (20 μl) was reacted with ABEE reagent (40 μl , Honen Corp.) for 1 h at 80°C, and the solution was washed with chloroform. The aqueous layer was injected into a Honepak C18 column (75 $\times$ 4.6 mm, Honen Corp.) at a flow rate of 1.0 ml/min at 30°C, and detected by fluorescence monitoring (excitation 305 nm, emission 360 nm) with 0.2 M potassium borate buffer (pH 8.9) containing 7% acetonitrile as eluent. The ABEE-converted mannose was detected at 10.6 min.

References

- 1) Kiho T., Morimoto H., Sakushima M., Usui S., Ukai S., *Biol. Pharm. Bull.*, **18**, 1627–1629 (1995).
- 2) Kiho T., Morimoto H., Kobayashi T., Usui S., Ukai S., Aizawa K., Inakuma T., *Biosci. Biotech. Biochem.*, **62**, 417–419 (2000).
- 3) Björndall H., Hellerqvist C. G., Lindberg B., Svensson S., *Angew. Chem. Int., Ed. Engl.*, **9**, 610–619 (1970).
- 4) Gorin P. A. J., Barreto-Berger E., Travassos L. R., *Carbohydr. Res.*, **95**, 205–218 (1981).
- 5) Kogan G., Pavliak V., Masler L., *Carbohydr. Res.*, **172**, 243–253

- (1988).
- 6) Bacon B. E., Cherniak R., Kwon-Cheng K. J., Jacobson E. S., *Carbohydr. Res.*, **283**, 95—110 (1996).
 - 7) a) Ukai S., Hirose K., Kiho T., Hara C., *Chem. Pharm. Bull.*, **25**, 338—344 (1977); Ukai S., Kiho T., Hara C., *ibid.*, **26**, 3871—3876 (1978). b) Gao Q., Seljelid R., Chen H., Jiang R., *Carbohydr. Res.*, **288**, 135—142 (1996).
 - 8) Ukai S., Morisaki S., Goto M., Kiho T., Hara C., Hirose K., *Chem. Pharm. Bull.*, **30**, 635—643 (1982); Sone Y., Kakuta M., Misaki A., *Agric. Biol. Chem.*, **42**, 417—425 (1978).
 - 9) Fraser C. G., Jennings H. J., Moyna P., *Can. J. Biochem.*, **51**, 219—224 (1973).
 - 10) Taylor R. L., Shively J. E., Conrad H. E., *Methods Carbohydr. Chem.*, **VII**, 149—151 (1976).
 - 11) Kiho T., Tsujimura Y., Sakushima M., Usui S., Ukai S., *Yakugaku Zasshi*, **114**, 308—315 (1994).
 - 12) Kiho T., Ito M., Nagai K., Hara C., Ukai S., *Chem. Pharm. Bull.*, **37**, 2770—2772 (1989).
 - 13) Kiho T., Itahashi S., Sakushima M., Matsunaga T., Usui S., Ukai S., Mori H., Sakamoto Y., Ishiguro Y., *Biol. Pharm. Bull.*, **20**, 118—121 (1997).
 - 14) Yasuno S., Murata T., Kokubo K., Yamaguchi T., Kamei M., *Biosci. Biotech. Biochem.*, **61**, 1944—1946 (1997); Yasuno S., Kokubo K., Kamei M., *ibid.*, **63**, 1353—1359 (1999).

A New Caffeoyl Quinic Acid from *Aster scaber* and Its Inhibitory Activity against Human Immunodeficiency Virus-1 (HIV-1) Integrase

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The phytochemical study of the aerial parts of *Aster scaber* THUNB. (Asteraceae) yielded a new caffeoyl quinic acid, (–) 3,5-dicafeoyl-muco-quinic acid (**2**) and three known compounds, (–) 3,5-dicafeoyl quinic acid (**1**), (–) 4,5-dicafeoyl quinic acid (**3**), (–) 5-caffeoyl quinic acid (**4**). The structures were established by high resolution spectroscopic methods. The antiviral effects against HIV-1 integrase of the compounds was evaluated. (–) 3,5-Dicafeoyl-muco-quinic acid (**2**) exhibited potent antiviral activity with an IC_{50} value of $7.0 \pm 1.3 \mu\text{g/ml}$.

Keywords *Aster scaber*; Asteraceae; caffeoyl quinic acid; (–) 3,5-dicafeoyl-muco-quinic acid; HIV-1 integrase

Aster scaber THUNB. (Asteraceae) is widespread and cultivated as a culinary vegetable in Korea.¹⁾ *Aster* species has also been used in traditional Chinese medicine for treatment of bruises, snakebite, headache and dizziness.²⁾ A review of the literature of *A. scaber* shows that triterpene glycosides^{3,4)} and volatile compounds⁵⁾ were reported. In the course of our phytochemical studies on *Aster* species, we isolated a new caffeoyl quinic acid (**2**), together with three known caffeoyl quinic acids (**1**, **3**, **4**) from *A. scaber* cultivated in Korea. Quinic acid derivatives exhibited diverse biological activities, especially antiviral^{6,7)} and antihepatotoxic activities.⁸⁾ Most quinic acids are mono-, di-, or triesters of gallic, caffeic, and ferulic acids and are of several positional and stereochemical isomers.^{9,10)} Because of the structural similarities and complexities of quinic acid derivatives, the assignments of NMR spectra have not been unambiguous. Very recently, Pauli *et al.*¹¹⁾ tried to assign the ¹H-NMR spectra of seven natural quinic acids through high resolution NMR techniques.

This paper deals with the isolation and structure determination of a new (**2**) and three known quinic acid derivatives (**1**, **3**, **4**), along with their inhibitory activity against human immunodeficiency virus type 1 (HIV-1) integrase.

Three known compounds, 3,5-dicafeoyl quinic acid (**1**), 4,5-dicafeoyl quinic acid (**3**) and 5-caffeoyl quinic acid (chlorogenic acid) (**4**) were characterized by comparing their physical and spectroscopic data with those reported in the literatures.^{12,13)}

Compound **2** was obtained as a yellowish gum ($[\alpha]_D^{25} -153.8^\circ$) and its molecular formula was determined to be $C_{25}H_{24}O_{12}$ by HRFABMS (m/z 517.1349, $[M+H]^+$). Its IR spectrum displayed absorption bands at 3300 and 1690 cm^{-1} , indicating the presence of hydroxy and ester groups. The ¹H-NMR spectrum of **2** showed very broad and distorted signals (see Experimental) and, therefore, their coupling constants were analyzed through DQF-¹H-¹H-COSY experiment. The ¹H-NMR spectrum showed signals by two methylenes at δ 1.96 (2H, m), 2.11 (1H, m) and 2.14 (1H, m), three oxygenated protons at δ 3.83 (1H, br s), 5.13 (1H, br s), and 5.19 (1H, br s), and five hydroxy protons at δ 5.30 (1H, br s), 9.18 (2H, br s) and 9.57 (2H, br s). The ¹³C-NMR spectrum showed two methylene carbons at δ 35.9 and 37.0, four oxy-

genated carbons at δ 68.8, 71.8, 72.0 and 73.7, and a carbonyl carbon signal at δ 176.5. In addition, ¹H- and ¹³C-NMR spectra of **2** indicated the presence of two *trans*-caffeoyl groups.¹⁰⁾ The above spectral data were consistent with those of dicafeoyl quinic acid derivatives.^{12,13)} The relationship of two caffeoyls and quinic acid was established by the analysis of ¹H-¹H COSY, HMQC and HMBC spectra. In HMBC spectrum, long-range connectivities were observed between H-3 and H-5 protons (δ 5.13 and 5.19) in quinic acid moiety and the caffeoyl ester carbons (δ 166.7 and 167.2). Thus, we speculated the structure of **2** to be 3,5-dicafeoyl quinic acid; however, NMR data and HPLC retention time of **2** were quite different from those of known 3,5-dicafeoyl quinic acid (**1**). The retention time of **2** in HPLC analysis was found to be greater than that of **1** (**2**: 35.7 min, **1**: 33.9 min) and when its ¹H-NMR spectrum of the quinic acid region was compared with those of **1**, H-3 and H-5 of **2** were shifted upfield, while H-4 was shifted downfield. Their coupling constants also showed differences (**2**: $J_{3,4}=11$ Hz and $J_{4,5}=16$ Hz; **1**: $J_{3,4}=8$ Hz and $J_{4,5}=15$ Hz). The large coupling constants between $J_{2ax,3}$ (14 Hz) and $J_{3,4}$ (11 Hz), and between $J_{4,5}$ (16 Hz) and $J_{5,6ax}$ (15 Hz) indicated the diaxial

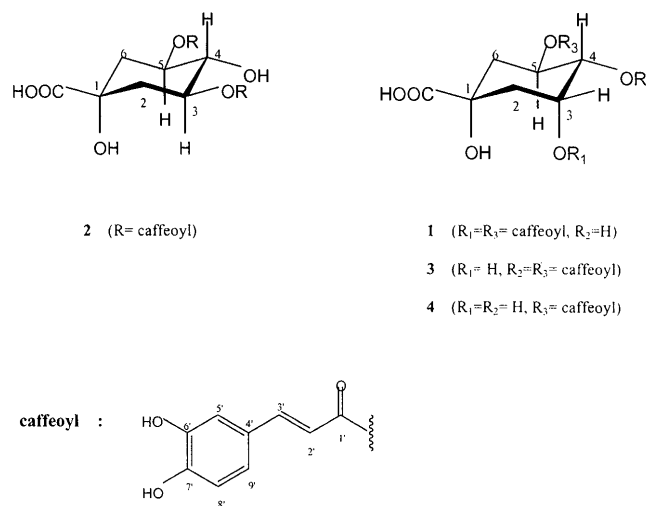
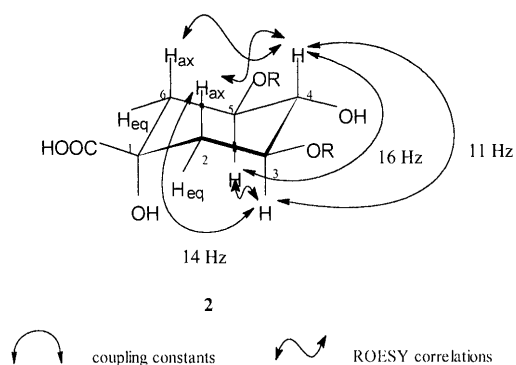


Fig. 1. Structures of Compounds **1**–**4**

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Fig. 2. Selected Coupling Constants and ROESY Correlations of **2**Table 1. HIV-1 Integrase Inhibitory Activities of Compounds **1**–**4**

Compound	IC ₅₀ (μg/ml) ^a	Compound	IC ₅₀ (μg/ml) ^a
1	16.9±2.0	3	14.5±1.7
2	7.0±1.3	4	ca. 97.0

^a) IC₅₀ values with standard deviations are from at least three independent experiments.

relations of H-3 and H-4, and H-4 and H-5, respectively. Usually, the J values in the substituted cyclohexanes are expected to have over 10 Hz for axial–axial orientation.¹⁴ Further, the ROESY spectrum exhibited the correlations of H-4, H-2_{ax} and H-6_{ax}, and of H-3 and H-5 (Fig. 2), implying that the configurations of H-3, H-4 and H-5 were axial, axial and axial, respectively. The configuration of OH group at C-1 was established to be axial by comparison of ¹H and ¹³C chemical shifts values of the known 3-caffeoyl-muco-quinic acid, 3,5-dicafeoylquinic acid and the other quinic acid derivatives.^{8,10,11,13,15,16} Based on the evidences and comparison of NMR data of **1** and **2**, the structure of **2** was determined to be (–) 3,5-dicafeoyl-muco-quinic acid. A muco-quinic acid derivative has been isolated from *Asimina triloba*.¹⁰ Although the ³ J values and ROESY data are in agreement with the proposed chair conformation, comparatively large axial–equatorial $J_{5,6eq}$ (10 Hz) value indicates that the conformation could be deviated from a proposed chair form and seems to be consistent with a skewed or boat conformation.¹⁰ Therefore, unambiguous determination of the absolute stereochemistry must be further studied, for example, by X-ray crystallography.

The anti-HIV-1 integrase activities of compounds **1**–**4** were investigated.¹⁷ As shown in Table 1, compound **2** showed most the potent inhibitory activity against HIV-1 integrase (IC₅₀ value of 7.0±1.3 μg/ml).

Experimental

NMR: in DMSO-*d*₆, Bruker AMX 500 and Varian UNITY INOVA 500 spectrometers. IR: in Nujol, Nicolet model 205 FT-IR spectrophotometer. MS: VG70-VSEQ mass spectrometer. Prep. HPLC: JAI LC-908 model with refractive index detector, UV detector and Econosil C18 10u column (250mm×22mm). Anal. HPLC: Gilson 306 dual pump, 811C dynamic mixer, 112 UV/VIS detector (280nm) and Microsorb-MV column (5mm×200mm, 5 μm), eluent,¹⁵ CH₃CN and 5% acetic acid (1:49, v/v) for 10min, the linear gradient from 1:49 to 3:7 for 40min, and 3:7 for 10min; flow rate 1.0ml/min. Column chromatography: silica gel (Merck, 70–230 mesh and 230–400 mesh). TLC: Merck precoated Si gel F₂₅₄ plates and RP-18 F₂₅₄ plates. LPLC: Merck Lichroprep Lobar®-A Si 60 (240×10mm).

Extraction and Isolation The *Aster scaber* cultivated was purchased in ChukRyung Mt., Kyungi-Do, Korea in September 1997. A voucher specimen (SKK-98-001) is deposited in the College of Pharmacy at SungKyunKwan University. The dried and chopped aerial parts of *A. scaber* (2.1 kg) were extracted with MeOH two times at room temperature and once at 50 °C for 5 h. The resultant MeOH extract (200 g) was separated by successive solvent partitioning, gave methylene chloride (CH₂Cl₂, 28 g), ethylacetate (EtOAc, 17 g) and *n*-butanol (BuOH, 33 g) soluble fractions. The EtOAc soluble fraction (17 g) was chromatographed on a silica gel column using EtOAc:MeOH:H₂O (9:2:0.5) to give six subfractions (E1–E6). The subfraction E4 was chromatographed on Sephadex LH-20 to give a mixture, which was separated with HPLC (35% MeOH) to afford yellowish gum **1** (95 mg) and **2** (75 mg). The subfraction E5 was chromatographed on Sephadex LH-20 and purified with HPLC (35% MeOH) to give yellowish gum **3** (98 mg).

The BuOH soluble fraction (33 g) was chromatographed on Diaion HP-20 column using H₂O and MeOH to give two subfractions (B1 and B2). The subfraction B1 was chromatographed on Sephadex LH-20 (MeOH) to give a mixture. The mixture was purified with HPLC (35% MeOH) to afford yellowish gum **4** (230 mg).

(–) 3,5-Dicafeoyl quinic acid (**1**): Yellowish gum. [α]_D –183.4° (c =0.51, MeOH). FAB-MS m/z (rel. int.): 517 ([M+H]⁺, 100). ¹H-NMR (500 MHz, DMSO-*d*₆) δ : 1.86 (1H, br m, H-2_{eq}), 1.91 (2H, br m, H-6), 2.13 (1H, br d, J =12.5 Hz, H-2_{ax}), 3.73 (1H, br d, J =9.3 Hz, H-4), 5.21 (1H, br s, H-3), 5.30 (1H, br dd, J =15.5, 8.9 Hz, H-5), 6.25–6.22 (each 1H, d, J =15.5 Hz, H-2'), 6.76–6.75 (each 1H, d, J =8.0 Hz, H-8'), 6.96 (2H, d, J =8.0 Hz, H-9'), 7.06 (2H, s, H-5'), 7.46–7.45 (each 1H, d, J =15.5 Hz, H-3'). Coupling constants in DQF-¹H-¹H-COSY (Hz): ² $J_{2ax,2eq}$ =15, ² $J_{6ax,6eq}$ =14, ³ $J_{2ax,3}$ =9, ³ $J_{2eq,3}$ =6, ³ $J_{3,4}$ =8, ³ $J_{4,5}$ =15, ³ $J_{5,6ax}$ =17, ³ $J_{5,6eq}$ =8. ¹³C-NMR (125 MHz, DMSO-*d*₆) δ : 37.1 (C-2), 40.1 (C-6), 71.8 (C-4), 72.0 (C-5), 73.8 (C-3), 75.5 (C-1), 115.9–115.7 (C-5'), 116.3–116.3 (C-8'), 117.0–116.9 (C-2'), 122.4–122.0 (C-9'), 126.8–126.7 (C-4'), 145.8–145.5 (C-3'), 146.8–146.8 (C-6'), 149.5–149.4 (C-7'), 167.5–167.3 (C-1'), 178.9 (COOH).

(–) 3,5-Dicafeoyl-muco-quinic acid (**2**): Yellowish gum. [α]_D –153.8° (c =0.78, MeOH). UV λ_{max} (EtOH) nm (log ϵ): 328 (4.52), 300sh (4.40), 244 (4.28), 219 (4.43). IR (nujol) cm^{–1}: 3300, 1690, 1600. HR-FAB-MS m/z : 517.1349 ([M+H]⁺, Calcd for C₂₅H₂₅O₁₂: 517.1346). ¹H-NMR (500 MHz, DMSO-*d*₆) δ : 1.96 (2H, m, H-2_{eq}, H-6_{eq}), 2.11 (1H, m, H-2_{ax}), 2.14 (1H, m, H-6_{ax}), 3.83 (1H, br s, H-4), 5.13 (1H, br s, H-3), 5.19 (1H, br s, H-5), 5.30 (1H, br s, 4-OH), 6.23–6.14 (each 1H, d, J =16.0 Hz, H-2'), 6.77–6.73 (each 1H, d, J =8.0 Hz, H-8'), 6.99–6.98 (each 1H, br d, J =8.0 Hz, H-9'), 7.04 (2H, d, J =2.5 Hz, H-5'), 7.46–7.43 (each 1H, d, J =16.0 Hz, H-3'), 9.18 (2H, br s, caffeoyl OH), 9.57 (2H, br s, caffeoyl OH). Coupling constants in DQF-¹H-¹H-COSY (Hz): ² $J_{2ax,2eq}$ =13, ² $J_{6ax,6eq}$ =12, ³ $J_{2ax,3}$ =14, ³ $J_{3,4}$ =11, ³ $J_{4,5}$ =16, ³ $J_{5,6ax}$ =15, ³ $J_{5,6eq}$ =10. ¹³C-NMR (125 MHz, DMSO-*d*₆) δ : 35.9 (t, C-2), 37.0 (t, C-6), 68.8 (d, C-4), 71.8 (d, C-5), 72.0 (d, C-3), 73.7 (s, C-1), 115.3–115.3 (d, C-5'), 116.0–115.9 (d, C-8'), 117.0–116.9 (d, C-2'), 122.5–122.2 (d, C-9'), 126.8–126.7 (s, C-4'), 146.2–145.8 (d, C-3'), 146.7–146.7 (s, C-6'), 149.5–149.4 (s, C-7'), 167.2–166.7 (s, C-1'), 176.5 (s, COOH).

(–) 4,5-Dicafeoyl quinic acid (**3**): Yellowish gum. [α]_D –257.8° (c =0.46, MeOH). FAB-MS, ¹H- and ¹³C-NMR data were in good agreement with those in previous papers.^{12,13,16}

(–) 5-Caffeoyl quinic acid (**4**): Yellowish gum. [α]_D –24.8° (c =0.49, MeOH). FAB-MS, ¹H- and ¹³C-NMR data were in good agreement with those in previous papers.^{12,13,16}

Bioassay of HIV-1 Integrase Inhibitory Activity¹⁷ A standard reaction assay of endonucleolytic activity was carried out in the presence of potential inhibitor containing 0.1 pmol of duplex oligonucleotide substrate and 15 pmol of HIV-1 integrase. Inhibitors of drugs were dissolved in 100% DMSO and added to the reaction mixture; the final mixture was 5% DMSO. Reaction products were visualized by autoradiography of the wet gel. IC₅₀ values were calculated by scanning bands on Kodak-5 film (Image Master VDS, Pharmacia Biotech., Piscataway, NJ, U.S.A.).

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References

- Lee T. B., Illustrated Flora of Korea, HyangMunSa, Seoul, 1989, p. 760.
- Kim C. M., Sin M. K., An T. K., Lee K. S., Dictionary of Chinese

- Herbs, JungDam Publisher, Seoul, 1997, p. 1431.
- 3) Nagao T., Okabe H., *Chem. Pharm. Bull.*, **40**, 886—888 (1992).
 - 4) Nagao T., Tanaka R., Iwase Y., Okabe H., *Chem. Pharm. Bull.*, **41**, 659—665 (1993).
 - 5) Chung T. Y., Eiserich J. P., Shibamoto T., *J. Agric. Food Chem.*, **41**, 1693—1697 (1993).
 - 6) Gesa M., Anthony D. W., Gabriele M. K., *Planta Medica*, **65**, 493—506 (1999).
 - 7) Mahmood N., Piacente S., Burke A., Khan A., Pizza C., *Antiviral Chemistry & Chemotherapy*, **8**, 70—74 (1997).
 - 8) Basnet P., Matsushige K., Hase K., Kadota S., Namba T., *Biol. Pharm. Bull.*, **19**, 655—657 (1996).
 - 9) Eliel E. L., Ramirez M. B., *Tetrahedron: Asymmetry*, **8**, 3551—3554 (1997).
 - 10) Haribal M., Feeny P., Lester C. C., *Phytochemistry*, **49**, 103—108 (1998).
 - 11) Pauli G. F., Poetsch F., Nahrstedt A., *Phytochem. Anal.*, **9**, 177—185 (1998).
 - 12) Tatefuji T., Izumi N., Ohta T., Arai S., Ikeda M., Kurimoto M., *Biol. Pharm. Bull.*, **19**, 966—970 (1996).
 - 13) Cheminat A., Zawatzky R., Becker H., Brouillard R., *Phytochemistry*, **27**, 2787—2794 (1988).
 - 14) Atta-ur-Rahman, *Nuclear Magnetic Resonance*, Springer-Verlag, New York, 1986, p. 74.
 - 15) Murata M., Okada H., Homma S., *Biosci. Biotech. Biochem.*, **59**, 1887—1890 (1995).
 - 16) Lin L. C., Kuo Y. C., Chou C. J., *J. Nat. Prod.*, **62**, 405—408 (1999).
 - 17) Kim H. J., Woo E. R., Shin C. G., Park H. K., *J. Nat. Prod.*, **61**, 145—148 (1998).

Reductive Dimerization of Alkylidenemalonates Using Samarium(II) Diiodide and ^1H -NMR Behavior of the Dimers, 2,3-Diaryl-1,1,4,4-butanetetracarboxylates

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Alkylidenemalonates were readily dimerized in the presence of SmI_2 to give 2,3-disubstituted 1,1,4,4-butanetetracarboxylates as mixtures of *meso* and racemic isomers in moderate to good yields. The structure of the less polar isomer of tetraethyl 2,3-diphenyl-1,1,4,4-butanetetracarboxylate was determined by X-ray crystallographic analysis to be the *meso* form. Characteristic ^1H -NMR behavior of the *meso* and racemic isomers is also discussed.

Key words samarium diiodide; alkylidenemalonate; reductive dimerization; 1,1,4,4-butanetetracarboxylate; X-ray crystallographic analysis; MOPAC calculation

Kagan and co-workers reported the first use of samarium diiodide (SmI_2) in synthetic organic chemistry¹⁾ in 1977, and recently it has become very important as a one-electron reducing agent because of its easy handling and wide utility.²⁾ Many synthetic applications of this reagent have been developed, and we have also reported several new reactions.³⁾ In the literature, dimerization of various substrates such as acid chlorides, ketones or aldehydes, α,β -unsaturated esters or amides, imines, and isocyanates by SmI_2 have been reported.³⁾ We previously reported reductive dimerization of 2-substituted-1,1-cyclopropanedicarboxylates with SmI_2 , giving 3,4-disubstituted 1,1,6,6-hexanetetracarboxylates in refluxing tetrahydrofuran (THF).^{3a)} The current paper deals with the reductive dimerization of alkylidenemalonates (**1a–l**) using SmI_2 and the characteristic ^1H -NMR behavior of the *meso* and racemic dimers (**2a–j**).

Diethyl benzylidenemalonate (**1a**, $\text{R}=\text{Ph}$) was treated with four equivalents of SmI_2 in THF at room temperature for 1 h.⁴⁾ Silica gel column chromatography of the product mixture afforded two compounds [**2a-I** (less polar) and **2a-II** (polar)].⁵⁾ The common formula $\text{C}_{28}\text{H}_{34}\text{O}_8$ for **2a-I** and **2a-II** was obtained from the electron impact ionization high-resolution MS (HRMS), and indicates that **2a-I** and **2a-II** are diastereomeric dimers of **1a**. The stereochemistry of the less polar dimer (**2a-I**) was clarified to be *meso* by X-ray crystallographic analysis as shown in Fig. 1,⁶⁾ therefore, the polar dimer (**2a-II**) was concluded to be the racemate. The stereochemistries of these products were also confirmed by HPLC analysis on chiral stationary phases.^{3a,7)} Thus, under common HPLC conditions [Column: CHIRALCEL OD, 4.6 mm i.d. $\times$ 25 cm (DAICEL Chemical Industries), eluent: hexane/2-propanol=90/10 by volume, flow: 0.5 ml/min, detection: UV 254 nm], the *meso* compound (**2a-I**) showed only a single

peak ($t_R=9.5$ min) whereas the racemic compound (**2a-II**) showed two peaks ($t_R=9.8$ and 11.4 min) with approximately the same magnitude.

Characteristic differences in the ^1H -NMR spectra of **2a-I** and **2a-II** were observed. In the *meso* form (**2a-I**), the methyl proton signals of the ethoxy groups were observed at 0.91 (6H) and 1.11 ppm (6H) ($\Delta 0.2$ ppm).⁸⁾ These signals are at somewhat higher magnetic field compared to ordinarily observed positions (1.3–1.4 ppm). The upfield shift can be explained from the X-ray stereostructure, in which all ethoxy groups are located in the shielded area of the benzene rings, as shown in Fig. 1.

On the other hand, in the racemate (**2a-II**), the methyl proton signals of the ethoxy groups were observed at 0.79 (6H) and 1.42 ppm (6H) ($\Delta 0.63$ ppm).⁸⁾ In this case, one of the ethoxy protons seems to be shifted to higher magnetic field from the ordinarily observed position, and the others are located in the ordinary position. These results suggest that one of the two ethoxy groups in the racemic compound (**2a-II**) is shielded by the benzene rings, while the other is not effected by the benzene rings. In order to confirm this assumption, we performed a semiempirical molecular orbital calculation for **2a-II**. The most stable structure for **2a-II** was calculated by the MOPAC PM3 method,⁹⁾ and the obtained structure is shown in Fig. 2. The calculated result is highly consistent with the above-mentioned assumption.

To examine the generality of the present dimerization, several alkylidenemalonates (**1b–l**) were prepared and subjected to the dimerization conditions to give the corresponding diastereomeric mixtures of **2b–l** (see Experimental).

The diastereomers of **2b–g** were easily separated by silica gel column chromatography or preparative TLC (PTLC) to the less polar compound (I) and the polar compound (II).

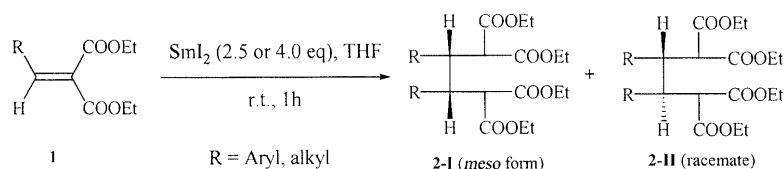


Chart 1

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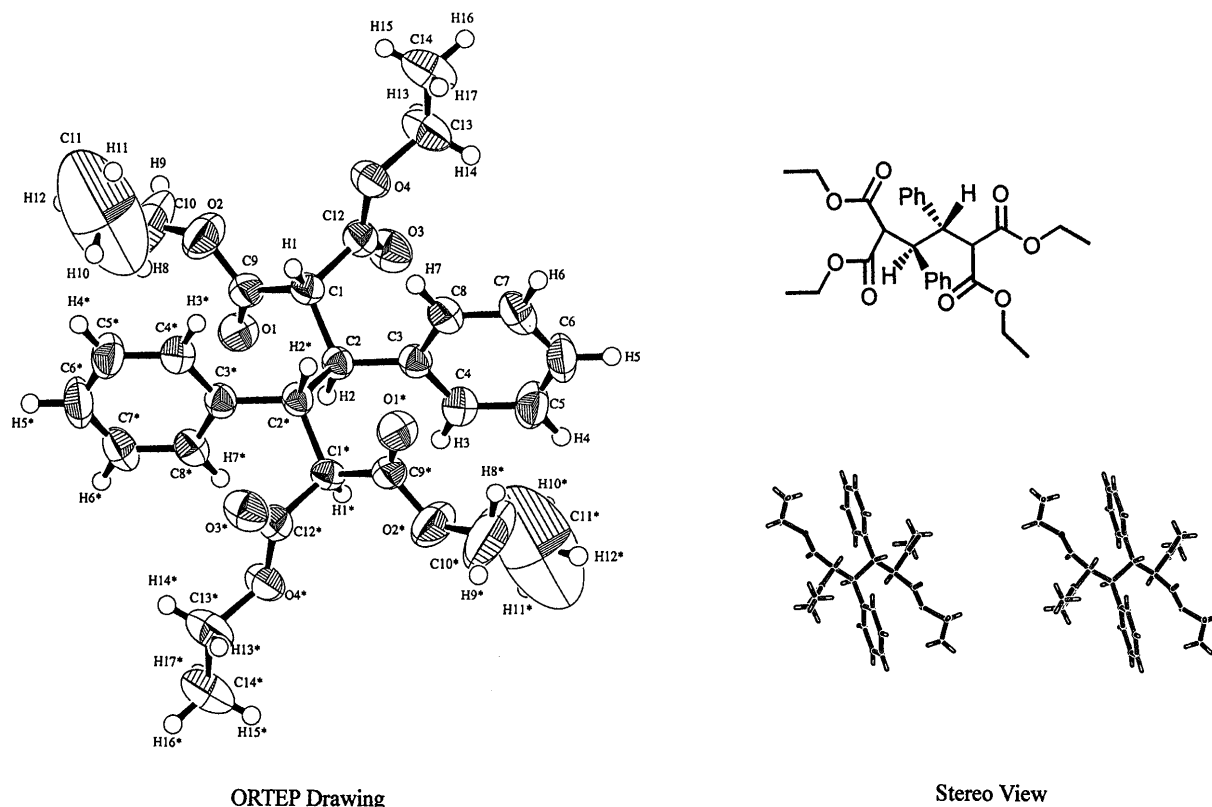


Fig. 1. X-Ray Structure of **2a-I** (Less Polar Compound; *meso* Form)

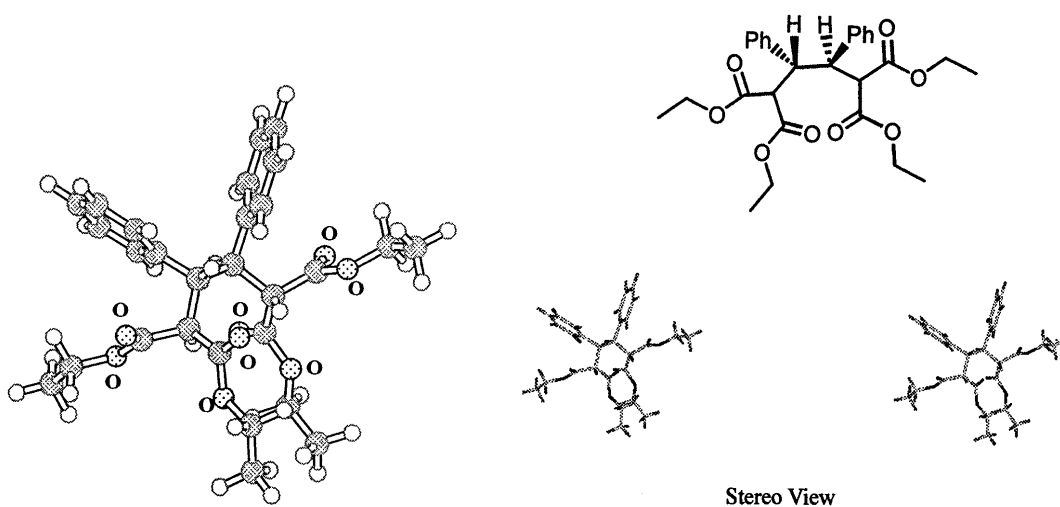


Fig. 2. The Structure of **2a-II** (Polar Compound; Racemic Form) Obtained by MOPAC (PM3) Calculation

Stereochemistries of these compounds were determined on the basis of analogy in $^1\text{H-NMR}$ spectra and HPLC analysis on chiral stationary phases with **2a-I** and **2a-II**. Diastereomers of the products (**2h—l**) could not be separated by column chromatography, but the diastereomeric ratios of **2h—j** were easily estimated on the basis of their $^1\text{H-NMR}$ spectra. On the other hand, the diastereomeric ratios of **2k** and **2l**, which had aliphatic substituents on the 2- and 3-positions of butanetetracarboxylate, could not be estimated on the basis of their $^1\text{H-NMR}$ spectra because of complicated signal patterns.

In conclusion, we found that alkylidenemalonates (**1a—l**) were dimerized to *meso* and racemate mixtures of 2,3-disubstituted 1,1,4,4-butanetetracarboxylates (**2a—l**) in the pres-

ence of SmI_2 in THF, and the dimeric products (**2a—j**) having aromatic substituents on the 2- and 3-positions of butanetetracarboxylate showed characteristic patterns in their $^1\text{H-NMR}$ spectra. These results should be useful to determine the stereochemistry of 2,3-diaryl-1,1,4,4-butanetetracarboxylic acids and related compounds.

Experimental

Melting points were measured with a Yanaco MP micro-melting point apparatus and are uncorrected. $^1\text{H-NMR}$ spectra were measured on a Varian XL-300 (300 MHz) or a JEOL EX-270 (270 MHz) in CDCl_3 with tetramethylsilane as an internal standard and chemical shifts are reported in ppm. IR spectra were taken with a Shimadzu IR-435 spectrophotometer in CHCl_3 . Shimadzu GCMS-QP 1000 and JEOL JMS-SX 102A QQ spectrometers were used for low-resolution MS and HRMS except for **2k** and **2l**. FAB-MS were measured with a JEOL JMS-SX 102A QQ spectrometer. Silica gel

chromatography [Silica gel 60 (grade 7734, 60–230 mesh, Merck)] and PTLC [Silica gel 60 PF₂₅₄ (Nacalai Tesque Inc.)] were used. HPLC analyses were carried out on a Shimadzu HPLC instrument at 25 °C. As a chiral stationary phase, a CHIRALCELL OD column [4.6 mm i.d. × 25 cm (DAICEL Chemical Industries)] was used. The eluent ratio (hexane:2-propanol, volumes), flow rate (ml/min), and retention time(s) (*t_R*, min) are shown in parentheses. A UV detector (wavelength, 254 nm) was used for detection of peaks.

Reductive Dimerization of Diethyl Benzylidenemalonate (1a) Using SmI₂ (General Procedure) To a suspension of samarium metal (360 mg, 2.40 mmol) in THF (0.5 ml) was added dropwise a solution of 1,2-diiodoethane (564 mg, 2.00 mmol) in THF (1.5 ml) at room temperature under an N₂ atmosphere, and the mixture was stirred for 1 h. A solution of **1a** (124 mg, 0.50 mmol) in THF (1 ml) was added dropwise to the blue suspension of SmI₂ at room temperature and the whole was stirred for an additional 1 h. After acidification with 3% HCl under ice-cooling, the mixture was extracted with diethyl ether (10 ml × 3). The combined organic layers were washed successively with water, saturated Na₂S₂O₃ solution, water, and brine followed by drying over MgSO₄ and evaporated under reduced pressure. The crude residue was chromatographed on silica gel (ethyl acetate: *n*-hexane = 1:5) to give a less polar compound (**2a-I**, 35 mg, 28%) and a polar compound (**2a-II**, 36 mg, 29%).

Tetraethyl meso-2,3-Diphenylbutane-1,1,4,4-tetracarboxylate (2a-I): Colorless prisms (from hexane), mp 88.0–90.0 °C. ¹H-NMR δ: 0.91 (6H, t, *J* = 7.1 Hz), 1.11 (6H, t, *J* = 7.2 Hz), 3.67 (2H, dd, *J* = 2.2, 6.0 Hz), 3.82 (4H, q, *J* = 7.1 Hz), 3.89 (2H, dq, *J* = 10.7, 7.2 Hz), 3.93 (2H, dq, *J* = 10.7, 7.2 Hz), 4.09 (2H, dd, *J* = 2.3, 6.0 Hz), 7.15–7.35 (10H, m). IR cm⁻¹: 1741, 1722. MS *m/z*: 498 (0.5, M⁺), 135 (100). HRMS *m/z*: 498.2249 (Calcd for C₂₈H₃₄O₈: 498.2253). Anal. Calcd for C₂₈H₃₄O₈: C, 67.45; H, 6.87. Found: C, 67.86; H, 7.05. HPLC (90:10; 0.5; 9.5).

Tetraethyl rac-2,3-Diphenylbutane-1,1,4,4-tetracarboxylate (2a-II): Colorless oil. ¹H-NMR δ: 0.79 (6H, t, *J* = 7.1 Hz), 1.42 (6H, t, *J* = 7.1 Hz), 3.74 (2H, dq, *J* = 10.8, 7.1 Hz), 3.77 (2H, d, *J* = 11.9 Hz), 3.81 (2H, dq, *J* = 10.8, 7.1 Hz), 3.90 (2H, d, *J* = 11.9 Hz), 4.37 (2H, dq, *J* = 10.8, 7.1 Hz), 4.45 (2H, dq, *J* = 10.8, 7.1 Hz), 7.10–7.40 (10H, m). IR cm⁻¹: 1747, 1723. MS *m/z*: 498 (6.3, M⁺), 338 (100). HRMS *m/z*: 498.2247 (Calcd for C₂₈H₃₄O₈: 498.2253). HPLC (90:10; 0.5; 9.8, 11.4).

Compounds (**2b–I**) were prepared by the above-mentioned procedure. Two and half equivalents of SmI₂ were used except for the cases of **1b**, **1i**, and **1j** (4.0 eq).

Tetraethyl meso-2,3-Bis(4-methylphenyl)butane-1,1,4,4-tetracarboxylate (2b-I): Yield; 43%. Colorless needles (from hexane), mp 109.0–110.0 °C. ¹H-NMR δ: 0.95 (6H, t, *J* = 7.1 Hz), 1.10 (6H, t, *J* = 7.1 Hz), 2.30 (6H, s), 3.64 (2H, dd, *J* = 2.2, 5.8 Hz), 3.81 (2H, dq, *J* = 10.7, 7.1 Hz), 3.85 (2H, dq, *J* = 10.7, 7.1 Hz), 3.88 (2H, dq, *J* = 10.7, 7.1 Hz), 3.93 (2H, dq, *J* = 10.7, 7.1 Hz), 4.02 (2H, dd, *J* = 2.2, 5.7 Hz), 7.11 (4H, d, *J* = 8.1 Hz), 7.16 (4H, d, *J* = 8.1 Hz). IR cm⁻¹: 1740, 1722. MS *m/z*: 526 (0.3, M⁺), 149 (100). Anal. Calcd for C₃₀H₃₈O₈: C, 68.42; H, 7.27. Found: C, 68.33; H, 7.25. HPLC (95:5; 0.5; 12.3).

Tetraethyl rac-2,3-Bis(4-methylphenyl)butane-1,1,4,4-tetracarboxylate (2b-II): Yield; 37%. Colorless crystals (from hexane), mp 106.5–107.5 °C. ¹H-NMR δ: 0.81 (6H, t, *J* = 7.1 Hz), 1.41 (6H, t, *J* = 7.1 Hz), 2.29 (6H, s), 3.68 (2H, d, *J* = 11.8 Hz), 3.75 (2H, dq, *J* = 10.7, 7.1 Hz), 3.81 (2H, dq, *J* = 10.7, 7.1 Hz), 3.84 (2H, d, *J* = 11.8 Hz), 4.35 (2H, dq, *J* = 10.8, 7.1 Hz), 4.43 (2H, dq, *J* = 10.7, 7.1 Hz), 6.90–7.05 (8H, m). IR cm⁻¹: 1750, 1724. MS *m/z*: 526 (0.5, M⁺), 149 (100). HRMS *m/z*: 526.2569 (Calcd for C₃₀H₃₈O₈: 526.2566). HPLC (95:5; 0.5; 9.8, 13.4).

Tetraethyl meso-2,3-Bis(4-ethylphenyl)butane-1,1,4,4-tetracarboxylate (2c-I): Yield; 49%. Colorless needles (from hexane), mp 93.0–94.5 °C. ¹H-NMR δ: 0.91 (6H, t, *J* = 7.2 Hz), 1.08 (6H, t, *J* = 7.1 Hz), 1.19 (6H, t, *J* = 7.5 Hz), 2.59 (4H, q, *J* = 7.5 Hz), 3.65 (2H, dd, *J* = 2.0, 5.7 Hz), 3.84 (4H, dq, *J* = 10.5, 7.1 Hz), 3.89 (4H, dq, *J* = 10.5, 7.1 Hz), 4.04 (2H, dd, *J* = 2.1, 5.6 Hz), 7.08 (4H, d, *J* = 7.9 Hz), 7.21 (4H, d, *J* = 8.1 Hz). IR cm⁻¹: 1739, 1720. MS *m/z*: 554 (0.4, M⁺), 163 (100). Anal. Calcd for C₃₂H₄₂O₈: C, 69.29; H, 7.63. Found: C, 69.43; H, 7.63. HPLC (95:5; 1.0; 5.2).

Tetraethyl rac-2,3-Bis(4-ethylphenyl)butane-1,1,4,4-tetracarboxylate (2c-II): Yield; 39%. Colorless oil. ¹H-NMR δ: 0.77 (6H, t, *J* = 7.2 Hz), 1.20 (6H, t, *J* = 7.6 Hz), 1.41 (6H, t, *J* = 7.1 Hz), 2.59 (4H, q, *J* = 7.6 Hz), 3.69 (2H, d, *J* = 11.9 Hz), 3.74 (4H, dq, *J* = 10.6, 7.1 Hz), 3.81 (4H, dq, *J* = 10.6, 7.1 Hz), 4.35 (4H, dq, *J* = 10.7, 7.1 Hz), 4.41 (4H, dq, *J* = 10.7, 7.1 Hz), 6.2–6.8 (4H, br), 6.98 (4H, d, *J* = 6.1 Hz). IR cm⁻¹: 1747, 1721. MS *m/z*: 554 (1.6, M⁺), 394 (100). HRMS *m/z*: 554.2887 (Calcd for C₃₂H₄₂O₈: 554.2879). HPLC (95:5; 1.0; 5.4, 10.4).

Tetraethyl meso-2,3-Bis(4-isopropylphenyl)butane-1,1,4,4-tetracarboxylate

(2d-I): Yield; 40%. Colorless needles (from hexane), mp 103.5–105.0 °C. ¹H-NMR δ: 0.87 (6H, t, *J* = 7.1 Hz), 1.05 (6H, t, *J* = 7.1 Hz), 1.21 (12H, d, *J* = 6.9 Hz), 2.85 (2H, septet, *J* = 7.5 Hz), 3.65 (2H, dd, *J* = 2.1, 5.7 Hz), 3.78 (4H, dq, *J* = 10.6, 7.1 Hz), 3.86 (4H, dq, *J* = 10.7, 7.2 Hz), 4.04 (2H, dd, *J* = 2.2, 5.6 Hz), 7.10 (4H, d, *J* = 8.3 Hz), 7.24 (4H, d, *J* = 8.3 Hz). IR cm⁻¹: 1739, 1720. MS *m/z*: 582 (0.4, M⁺), 422 (100). Anal. Calcd for C₃₄H₄₆O₈: C, 70.08; H, 7.96. Found: C, 70.03; H, 8.08. HPLC (90:10; 0.5; 6.9).

Tetraethyl rac-2,3-Bis(4-isopropylphenyl)butane-1,1,4,4-tetracarboxylate (2d-II): Yield; 40%. Colorless needles (from hexane), mp 98.0–100.5 °C. ¹H-NMR δ: 0.72 (6H, t, *J* = 7.1 Hz), 1.207 (6H, t, *J* = 6.9 Hz), 1.214 (6H, d, *J* = 6.9 Hz), 1.41 (6H, t, *J* = 7.1 Hz), 2.84 (2H, septet, *J* = 6.9 Hz), 3.70 (2H, d, *J* = 11.1 Hz), 3.74 (4H, dq, *J* = 11.2, 7.1 Hz), 3.81 (4H, dq, *J* = 10.9, 7.1 Hz), 3.87 (2H, d, *J* = 11.6 Hz), 4.35 (4H, dq, *J* = 10.7, 7.1 Hz), 4.44 (4H, dq, *J* = 10.7, 7.1 Hz), 6.3–6.9 (4H, br), 7.01 (4H, d, *J* = 7.4 Hz). IR cm⁻¹: 1745, 1720. MS *m/z*: 582 (1.6, M⁺), 177 (100). Anal. Calcd for C₃₄H₄₆O₈: C, 70.08; H, 7.96. Found: C, 70.16; H, 8.14. HPLC (90:10; 0.5; 7.8, 12.5).

Tetraethyl meso-2,3-Bis(4-tert-butylphenyl)butane-1,1,4,4-tetracarboxylate (2e-I): Yield; 45%. Colorless prisms (from hexane), mp 120.0–122.0 °C. ¹H-NMR δ: 0.86 (6H, t, *J* = 7.1 Hz), 1.03 (6H, t, *J* = 7.2 Hz), 1.28 (18H, s), 3.65 (2H, dd, *J* = 2.1, 5.6 Hz), 3.76 (4H, dq, *J* = 10.8, 7.1 Hz), 3.84 (4H, dq, *J* = 10.7, 7.1 Hz), 4.04 (2H, dd, *J* = 2.1, 5.6 Hz), 7.26 (8H, s). IR cm⁻¹: 1740, 1719. MS *m/z*: 610 (0.6, M⁺), 450 (100). Anal. Calcd for C₃₆H₅₀O₈: C, 70.79; H, 8.25. Found: C, 70.89; H, 8.42. HPLC (90:10; 0.5; 7.3).

Tetraethyl rac-2,3-Bis(4-tert-butylphenyl)butane-1,1,4,4-tetracarboxylate (2e-II): Yield; 38%. Colorless needles (from hexane), mp 118.0–119.5 °C. ¹H-NMR δ: 0.69 (6H, t, *J* = 7.1 Hz), 1.28 (18H, s), 1.42 (6H, t, *J* = 7.1 Hz), 3.70 (2H, d, *J* = 11.9 Hz), 3.73 (4H, dq, *J* = 10.8, 7.1 Hz), 3.80 (4H, dq, *J* = 10.8, 7.1 Hz), 3.88 (2H, d, *J* = 11.5 Hz), 4.36 (4H, dq, *J* = 10.8, 7.1 Hz), 4.44 (4H, dq, *J* = 10.8, 7.1 Hz), 6.3–7.0 (4H, br), 7.16 (4H, d, *J* = 8.1 Hz). IR cm⁻¹: 1747, 1720. MS *m/z*: 610 (1.5, M⁺), 450 (100). Anal. Calcd for C₃₆H₅₀O₈: C, 70.79; H, 8.25. Found: C, 70.76; H, 8.41. HPLC (90:10; 0.5; 7.8, 11.8).

Tetraethyl meso-2,3-Bis(4-isobutylphenyl)butane-1,1,4,4-tetracarboxylate (2f-I): Yield; 47%. Colorless prisms (from hexane), mp 78.0–81.5 °C. ¹H-NMR δ: 0.87 (12H, d, *J* = 6.8 Hz), 0.91 (6H, t, *J* = 7.1 Hz), 1.09 (6H, t, *J* = 7.1 Hz), 1.82 (2H, nonet, *J* = 6.7 Hz), 2.42 (4H, d, *J* = 7.3 Hz), 3.65 (2H, dd, *J* = 2.1, 5.9 Hz), 3.81 (4H, q, *J* = 7.1 Hz), 3.89 (4H, q, *J* = 7.1 Hz), 4.05 (2H, dd, *J* = 2.1, 5.9 Hz), 7.10 (4H, d, *J* = 8.1 Hz), 7.19 (4H, d, *J* = 8.1 Hz). IR cm⁻¹: 1740, 1719. MS *m/z*: 610 (0.4, M⁺), 450 (100). Anal. Calcd for C₃₆H₅₀O₈: C, 70.79; H, 8.25. Found: C, 71.05; H, 8.28. HPLC (90:10; 0.5; 7.5).

Tetraethyl rac-2,3-Bis(4-isobutylphenyl)butane-1,1,4,4-tetracarboxylate (2f-II): Yield; 37%. Colorless oil. ¹H-NMR δ: 0.77 (6H, t, *J* = 7.1 Hz), 0.84 (6H, d, *J* = 6.4 Hz), 0.85 (6H, d, *J* = 6.4 Hz), 1.42 (6H, t, *J* = 7.2 Hz), 1.80 (2H, nonet, *J* = 6.7 Hz), 2.37 (2H, dd, *J* = 7.2, 18.5 Hz), 2.44 (2H, dd, *J* = 7.2, 18.5 Hz), 3.74 (2H, d, *J* = 11.9 Hz), 3.74 (2H, dq, *J* = 10.8, 7.1 Hz), 3.81 (2H, dq, *J* = 10.7, 7.1 Hz), 3.81 (2H, d, *J* = 11.9 Hz), 4.35 (2H, dq, *J* = 10.7, 7.1 Hz), 4.44 (2H, dq, *J* = 10.9, 7.1 Hz), 6.0–7.2 (8H, m). IR cm⁻¹: 1738, 1721. MS *m/z*: 610 (1.7, M⁺), 450 (100). HRMS *m/z*: 610.3499 (Calcd for C₃₆H₅₀O₈: 610.3505). HPLC (90:10; 0.5; 7.5, 10.8).

Tetraethyl meso-2,3-Bis(4-biphenyl)butane-1,1,4,4-tetracarboxylate (2g-I): Yield; 20%. Colorless prisms (from PhH–hexane), mp 160.5–162.0 °C. ¹H-NMR δ: 0.94 (6H, t, *J* = 7.1 Hz), 1.10 (6H, t, *J* = 7.1 Hz), 3.75 (2H, dd, *J* = 2.1, 5.8 Hz), 3.86 (4H, q, *J* = 7.1 Hz), 3.94 (4H, dq, *J* = 3.5, 7.1 Hz), 4.18 (2H, dd, *J* = 2.2, 5.8 Hz), 7.30–7.65 (18H, m). IR cm⁻¹: 1740, 1721. MS *m/z*: 650 (1.3, M⁺), 325 (100). Anal. Calcd for C₄₀H₄₂O₈: C, 73.83; H, 6.51. Found: C, 73.88; H, 6.56. HPLC (90:10; 0.5; 12.5).

Tetraethyl rac-2,3-Bis(4-biphenyl)butane-1,1,4,4-tetracarboxylate (2g-II): Yield; 35%. Colorless needles (from PhH–hexane), mp 144.0–147.5 °C. ¹H-NMR δ: 0.82 (6H, t, *J* = 7.1 Hz), 1.45 (6H, t, *J* = 7.1 Hz), 3.82 (2H, d, *J* = 11.7 Hz), 3.70–3.95 (4H, m), 3.99 (2H, d, *J* = 11.7 Hz), 4.40 (2H, dq, *J* = 10.7, 7.1 Hz), 4.48 (2H, dq, *J* = 10.7, 7.1 Hz), 6.0–7.2 (2H, br), 7.3–7.6 (16H, m). IR cm⁻¹: 1746, 1722. MS *m/z*: 650 (3.3, M⁺), 490 (100). Anal. Calcd for C₄₀H₄₂O₈: C, 73.83; H, 6.51. Found: C, 73.53; H, 6.52. HPLC (90:10; 0.5; 11.4, 24.0).

Mixture of Tetraethyl 2,3-Bis(4-methoxycarbonylphenyl)butane-1,1,4,4-tetracarboxylate (2h): Yield; 67%. *meso* form:racemate = 7:6. Colorless needles (from AcOEt), mp 167.0–170.0 °C. ¹H-NMR δ: 0.84 (2.8H, t, *J* = 7.0 Hz), 0.93 (3.2H, t, *J* = 7.0 Hz), 1.15 (3.2H, t, *J* = 7.1 Hz), 1.45 (2.8H, t, *J* = 7.0 Hz), 3.65 (0.9H, d, *J* = 7.4 Hz), 3.91 (6H, s), 3.00–4.20 (8.4H, m), 4.19 (0.9H, d, *J* = 6.9 Hz), 4.38 (0.9H, dq, *J* = 10.8, 7.1 Hz), 4.46 (0.9H, dq, *J* = 10.8, 7.2 Hz), 6.0–7.2 (1.8H, br), 7.33 (2.2H, d, *J* = 8.1 Hz), 7.86 (1.8H, br-s), 7.95 (2.2H, d, *J* = 8.1 Hz). IR cm⁻¹: 1740, 1719. MS *m/z*: 614 (1.9, M⁺), 193 (100). Anal. Calcd for C₃₂H₃₈O₁₂: C, 62.53; H, 6.23. Found: C,

62.62; H, 6.27.

Mixture of Tetraethyl 2,3-Bis(4-methoxyphenyl)butane-1,1,4,4-tetracarboxylate (**2i**): Yield; 77%. *meso* form:racemate=3:2. Colorless prisms (from AcOEt-hexane), mp 111.5–112.0 °C. ¹H-NMR δ: 0.84 (2.4H, t, *J*=7.1 Hz), 0.97 (3.6H, t, *J*=7.1 Hz), 1.11 (3.6H, t, *J*=7.1 Hz), 1.41 (2.4H, t, *J*=7.1 Hz), 3.60–4.05 (10H, m), 3.78 (6H, s), 4.36 (0.8H, dq, *J*=10.6, 7.1 Hz), 4.43 (0.8H, dq, *J*=10.6, 7.1 Hz), 6.65–6.75 (4.8H, m), 6.80 (1.6H, d, *J*=8.8 Hz), 7.22 (1.6H, d, *J*=8.8 Hz). IR cm⁻¹: 1745, 1722. MS *m/z*: 558 (1.0, M⁺), 279 (100). *Anal.* Calcd for C₃₀H₃₈O₁₀: C, 64.50; H, 6.86. Found: C, 64.01; H, 6.86.

Mixture of Tetraethyl 2,3-Bis(3,4-methylenedioxyphenyl)butane-1,1,4,4-tetracarboxylate (**2j**): Yield; 77%. *meso* form:racemate=3:2. Colorless needles (from CHCl₃-hexane), mp 165.5–166.5 °C. ¹H-NMR δ: 0.88 (2.4H, t, *J*=7.1 Hz), 1.03 (3.6H, t, *J*=7.2 Hz), 1.14 (3.6H, t, *J*=7.2 Hz), 1.41 (2.4H, t, *J*=7.2 Hz), 3.50–4.10 (10.4H, m), 4.30–4.50 (1.6H, m), 5.9–6.0 (2.4H, m), 6.6–6.8 (3.2H, m), 6.93 (2.4H, s). IR cm⁻¹: 1745, 1723. MS *m/z*: 556 (6.8, M⁺), 175 (100). *Anal.* Calcd for C₃₀H₃₄O₁₂: C, 61.65; H, 5.90. Found: C, 61.43; H, 5.84.

Mixture of Tetraethyl 2,3-dimethylbutane-1,1,4,4-tetracarboxylate (**2k**): Yield; 69%. Unknown ratio. Colorless oil. ¹H-NMR δ: 0.90 (6H, d, *J*=6.8 Hz), 1.07 (6H, d, *J*=6.6 Hz), 1.26 (6H, t, *J*=7.1 Hz), 1.27 (6H, t, *J*=7.1 Hz), 1.28 (6H, t, *J*=7.1 Hz), 1.29 (6H, t, *J*=7.1 Hz), 2.30–2.45 (4H, m), 3.33 (2H, d, *J*=9.7 Hz), 3.48 (2H, d, *J*=6.4 Hz), 4.19 (4H, q, *J*=7.1 Hz), 4.20 (4H, q, *J*=7.1 Hz), 4.20 (4H, q, *J*=7.1 Hz), 4.23 (4H, q, *J*=7.1 Hz). IR cm⁻¹: 1740, 1722. FAB-MS *m/z*: 375.2026 (Calcd for C₁₈H₃₀O₈+H; 375.2019).

Mixture of Tetraethyl 2,3-Diisopropylbutane-1,1,4,4-tetracarboxylate (**2l**): Yield; 55%. Unknown ratio. Colorless oil. ¹H-NMR δ: 0.83, 0.93, 0.96, 1.01 (total 12H, each d, *J*=6.6 Hz), 1.271, 1.276 (total 12H, each t, *J*=7.2 Hz), 2.00–2.20 (2H, m), 2.30–2.45, 2.60–2.70 (total 2H, m), 3.56, 3.79 (total 2H, each d, *J*=4.8, 3.9 Hz), 4.10–4.30 (12H, m). IR cm⁻¹: 1730, 1720. FAB-MS *m/z*: 431.2649 (Calcd for C₂₂H₃₈O₈+H; 431.2645).

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References and Notes

- 1) a) Namy J. L., Girard P., Kagan H. B., *Nouv. J. Chim.*, **1**, 5–7 (1977); b) Girard P., Namy J. L., Kagan H. B., *J. Am. Chem. Soc.*, **102**, 2693–2698 (1980).
- 2) For reviews: Molander G. A., Harris C. R., *Tetrahedron*, **54**, 3321–3354 (1998); Kunishima M., Tani S., *Yuki Gosei Kagaku Kyokai Shi*, **57**, 127–135 (1999); Krief A., Laval A.-M., *Chem. Rev.*, **99**, 745–777 (1999) and references cited therein.
- 3) a) Yamashita M., Okuyama K., Ohhara T., Kawasaki I., Michihiro Y., Sakamaki K., Ito S., Ohta S., *Chem. Pharm. Bull.*, **47**, 1439–1443 (1999); b) References cited in ref. 3a).
- 4) The dimerization reaction requires more than 2.0 eq of SmI₂.
- 5) Jiang and Zhang reported the reductive coupling of diethyl benzylidene-malonate in a SmI₂-hexamethylphosphoramide (HMPA)-THF-*tert*-BuOH system but did not discuss the stereochemistries of the products. [Jiang H.-J., Zhang Y.-M., *Youji Huaxue*, **17**, 242–246 (1997), CA, **127**: 81215q]. On the other hand, Inanaga *et al.* reported that α,β-unsaturated esters were dimerized to hexanedioic acid ester derivatives (48–90%) by treatment with SmI₂ in a HMPA-THF system in the presence of a proton source, and that in the absence of HMPA, slow reduction of the double bond or no dimerization reaction was observed [Inanaga J., Handa Y., Tabuchi T., Otsubo K., Yamaguchi M., Hanamoto T., *Tetrahedron Lett.*, **32**, 6557–6558 (1991)]. In the present case, it was found that HMPA and a proton source were not always necessary for the coupling.
- 6) C₂₈H₃₄O₈; FW 498.57; monoclinic; C2/c (# 15); *a*=23.156 (3), *b*=5.696 (3), *c*=22.890 (2) Å, β=115.709(9)°; *V*=2720 (1) Å³; *Z*=4; *D*_{calc}=1.217 g/cm³; λ(CuKα)=1.54178 Å; μ(CuKα)=7.33 cm⁻¹; *F*(000)=1064.00; *T*=296 K; *R*=0.074 (*R*_w=0.137) for 1616 observations (*I*>3.00σ(*I*)). Detailed X-ray crystallographic data are available from the Cambridge Crystallographic Data Centre.
- 7) Pappas S. P., Pappas B. C., Okamoto Y., Sakamoto H., *J. Org. Chem.*, **53**, 4404 (1988).
- 8) Elinson *et al.* observed similar tendencies in CH₃O-signals [3.37 and 3.43 ppm (Δ0.06 ppm) for the *meso* compound; 3.30 and 3.94 ppm (Δ0.64 ppm) for the racemic compound] of the diastereomers of tetramethyl 2,3-diphenylbutane-1,1,4,4-tetracarboxylate, which were prepared by a different method [Elinson M. N., Feducovich S. K., Znkharenikov A. A., Ugrak B. I., Nikishin G. I., Lindeman S. V., Struchkov J. T., *Tetrahedron*, **51**, 5035–5046 (1995)].
- 9) A structure generated by the Monte Carlo method in the CONF-SEARCH program was used as the initial one for MOPAC calculation (MOL-MOLIS/CRYST version 2.4, Daikin Industries Ltd., Tokyo, Japan). MOPAC calculation: a) Stewart J. J. P., *J. Am. Chem. Soc.*, **107**, 3902–3909 (1985); b) *idem.*, *J. Comp. Chem.*, **10**, 209–220 (1989); c) *idem.*, *ibid.*, **10**, 221–264 (1989); d) The present PM3 calculation was performed using MOPAC ver. 6.0 (QCPE No. 445) [*idem.*, *QCPE Bull.*, **10**, 86 (1990)].

Glycosidic Constituents from *in Vitro* *Anoectochilus formosanus*

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The glycosidic constituents of whole plants of *Anoectochilus formosanus* propagated by tissue culture were investigated. A new compound, 2-(β -D-glucopyranosyloxymethyl)-5-hydroxymethylfuran, along with the known compounds, 3-(*R*)-3- β -D-glucopyranosyloxybutanolide (kinsenocide), 3-(*R*)-3- β -D-glucopyranosyloxy-4-hydroxybutanoic acid, 1-*O*-isopropyl- β -D-glucopyranoside, (*R*)-(+)-3,4-dihydroxybutanoic acid γ -lactone, 4-(β -D-glucopyranosyloxy)benzyl alcohol, (6*R*,9*S*)-9-hydroxy-megastigma-4,7-dien-3-one-9-*O*- β -D-glucopyranoside, and corchoionoside C were isolated.

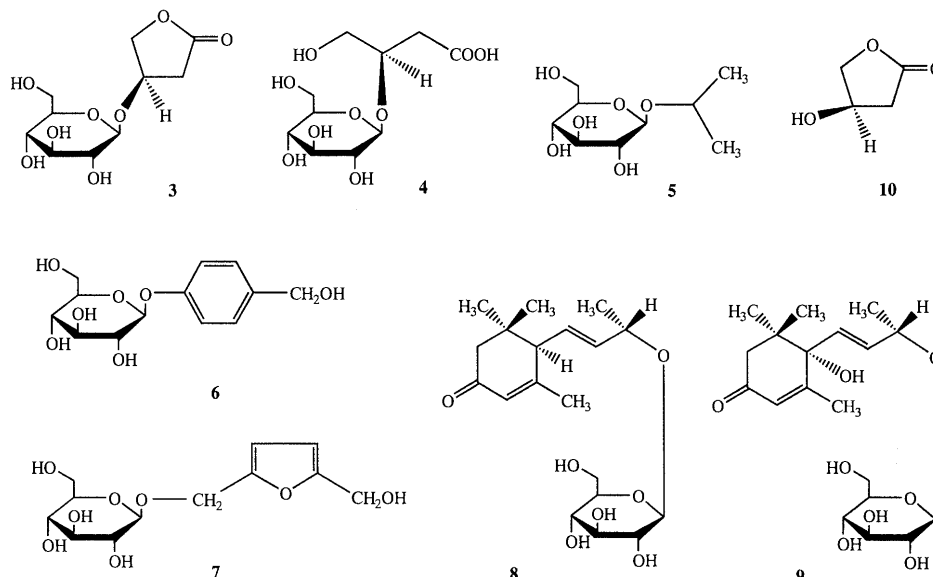
Key words *Anoectochilus formosanus*; tissue culture; glycoside

Anoectochilus formosanus HAYATA is an orchidaceous perennial herb distributed only in Taiwan (China) and Okinawa (Japan). Its whole plant has been used since ancient times as a folk medicine for the treatment of fever, pleurodynia, snake-bite, lung disease, and underdeveloped children in Taiwan.¹⁾ Recently, this herbal drug was also used to cure hypertension, diabetes mellitus, consumption and nephritis, etc.²⁾ Since the natural sources of *A. formosanus* have been largely depleted, we started to investigate the propagation of this species by tissue culture techniques, and worked on its chemical components and pharmacological profiles.^{3–6)} In the present paper, we report here the further isolation and characterization of glycosidic constituents from the *in vitro* whole plants of cultured *A. formosanus*.

The dried whole plants cultured were extracted with MeOH at room temperature, and the concentrate was suspended in H₂O and partitioned successively with CHCl₃ and *n*-BuOH. The residues obtained from the H₂O layer and the *n*-BuOH layer were separately subjected to normal-phase and reversed-phase silica gel column chromatography, then preparative TLC to give compounds 1–10. The structures of the known compounds were identified as glucose (1), sucrose (2), 3-(*R*)-3- β -D-glucopyranosyloxybutanolide (3),^{3,5,7)} 3-(*R*)-3- β -D-glucopyranosyloxy-4-hydroxybutanoic acid (4),^{3,5)} 1-

O-isopropyl- β -D-glucopyranoside (5),³⁾ (*R*)-(+)-3,4-dihydroxybutanoic acid γ -lactone (10),⁷⁾ 4-(β -D-glucopyranosyloxy)benzyl alcohol (6),⁸⁾ (6*R*,9*S*)-9-hydroxy-megastigma-4,7-dien-3-one-9-*O*- β -D-glucopyranoside (8),⁹⁾ and corchoionoside C (9),¹⁰⁾ by comparing their spectral data with those previously reported.

Compound 7 gave a molecular ion at m/z 290 [M]⁺ in EI mass spectrometry, and high-resolution EI-MS revealed the molecular formula C₁₂H₁₈O₈ ([M]⁺ 290.0997, Calcd 290.1002). In the ¹H- and ¹³C-NMR spectra of 7, the signals at δ_C 57.5 [CH₂, δ_H 4.48 (2H, s)], δ_C 63.7 [CH₂, δ_H 4.63 (1H, d, J =12.8 Hz), 4.79 (1H, d, J =12.8 Hz)], δ_C 109.2 [CH, δ_H 6.25 (1H, d, J =3.3 Hz)], δ_C 111.7 [CH, δ_H 6.36 (1H, d, J =3.3 Hz)], δ_C 152.4 [C], and δ_C 156.5 [C] revealed the presence of a 2,5-dihydroxymethylfuran group. On complete acidic hydrolysis, 7 gave D-glucose. The absolute configuration of the sugar was defined by GC analysis using the Hara method.¹¹⁾ In the ¹H- and ¹³C-NMR spectra, anomeric signals were observed at δ_H 4.34 (1H, d, J =7.9 Hz) and δ_C 102.9, as a β -linkage of D-glucose. The NOESY spectrum of 7 showed a cross peak between δ_H 4.34 (H-1' of Glc) and δ_H 4.63 (H-6b), indicating that glucose unit is linked to the 5-hydroxymethyl of the furan ring (Fig. 1). In the correlation spectroscopy *via* long-range coupling (COLOC) spectrum of 7



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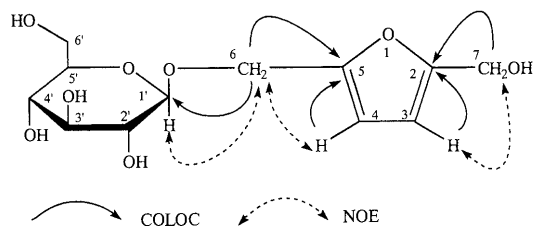


Fig. 1. NOE Correlations in NOESY and C-H Long-Range Correlations in COLOC Spectra of **7**

(Fig. 1), the long-range correlation was observed from the δ_{H} 4.63 (H-6b) and 4.79 (H-6a) to the δ_{C} 102.9 (C-1 of Glc), supporting the above deduction. Thus, **7** was determined to be 2-(β -D-glucopyranosyloxymethyl)-5-hydroxymethylfuran.

The new furanoid glycoside (**7**) was assayed for several pharmacological activities such as the inhibition of α -glucosidase, and interaction with rat adipocytes,¹² however, no activity was found. Compound **10** was only isolated from the dried plant, suggesting that **10** is produced as an artificial product, because **10** was not found in the fresh plants of *A. formosanus* and *A. koshunensis*. This is one reason the fresh plants are widely used as the Chinese folk remedy.

Experimental

General Procedures ^1H - and ^{13}C -NMR were measured in CD_3OD or pyridine- d_5 on a JEOL 270 instrument, using TMS as an internal standard. Analytical GC was carried out on a Hitachi G-3000 gas chromatograph. Melting points were determined with a YAZAWA BY-2 apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1010 digital polarimeter.

Micropropagation System of *A. formosanus* The ripened fruits of *A. formosanus* (collected in Taiwan province) were sterilized with 1% (v/v) of NaOCl containing 0.05% Tween 20 for 15 min, then with 70% (v/v) EtOH for 30 s, and washed with sterilized H_2O 3 times. The fruits were dissected longitudinally and seeds were cultured on 1/2 MS medium¹³ supplemented with 1 mg/l 6-benzylaminopurine (BA) and 2 g/l peptone. The seeds were germinated at 25 °C under 1500 lux for 16 h per day by fluorescent propagation light. After 30 d the seeds germinated and formed protocorm. Afterwards the seedlings, 6–8 mm in length, were transferred to 1/2 MS liquid medium to produce the multiple shoot complex. They were cultured in 1/2 MS liquid medium supplemented with 0.3 mg/l BA and 0.03 mg/l α -naphthaleneacetic acid (NAA) under shaking at 30 rpm. After 2 months they were transferred to 1/2 MS liquid medium without growth regulator and shaken for 4 more months. The regenerated plantlets were transplanted into a soil mixture for 6–8 months to grow until they reached 15–18 cm in height and 2.8–3.3 g in weight per plantlet. A voucher specimen has been deposited at the Herbarium of the Medicinal Plant Garden of Pharmaceutical Sciences, Kyushu University.

Extraction and Isolation The dried whole plants of cultured *A. formosanus* (2.3 kg) were powdered and percolated with MeOH at room temperature, and the concentrate (695 g) was partitioned successively between CHCl_3 and H_2O , *n*-BuOH and H_2O . The residue obtained from the H_2O -soluble portion (576 g) was subjected to an ODS (Cosmosil 75C₁₈-OPN) column eluted with H_2O to give four fractions. A small part of the fr. 1 (0.1 g), and a small part of the fr. 2 (0.1 g) were chromatographed on silica gel

(CHCl_3 -MeOH- H_2O , 6:4:1) to afford sucrose (23 mg) and glucose (38 mg), respectively. A part of the fr. 3 (1 g) was chromatographed on silica gel eluted with a gradient of CHCl_3 -EtOH (8:3—7:5) to give **3** (780 mg). Purification of fr. 3 (80 mg) by preparative TLC (Kieselgel 60 F₂₅₄, 1 mm, Merck) with CHCl_3 -MeOH- H_2O (6:4:1) gave **4** (17 mg). The fr. 4 (1.8 g) was purified by silica gel chromatography (CHCl_3 -MeOH- H_2O , 7:3:0.1) to give **5** (970 mg) and **10** (12 mg). The residue obtained from the *n*-BuOH portion (39 g) was subjected to Diaion HP-20 (H_2O -MeOH, 90:10—0:100), Cosmosil 75C₁₈-OPN (MeOH- H_2O , 20:80—95:5) and silica gel (CHCl_3 -MeOH- H_2O , 7:3:0.5) column chromatographies to provide compounds **6** (73 mg), **7** (59 mg), **8** (22 mg), and **9** (18 mg).

2-(β -D-Glucopyranosyloxymethyl)-5-hydroxymethylfuran (7**)** A colorless amorphous powder. $[\alpha]_{\text{D}}^{20} -73.5^\circ$ ($c=1.3$, MeOH). EI-MS m/z 290 $[\text{M}]^+$; HR-EI-MS m/z 290.0997 $[\text{M}]^+$, Calcd for $\text{C}_{12}\text{H}_{18}\text{O}_8$; 290.1002. ^1H -NMR (CD_3OD) δ : 6.36 (1H, d, $J=3.3$ Hz, H-4), 6.25 (1H, d, $J=3.3$ Hz, H-3), 4.79 (1H, d, $J=12.8$ Hz, H-6a), 4.63 (1H, d, $J=12.8$ Hz, H-6b), 4.48 (2H, s, H₂-7), 4.34 (1H, d, $J=7.9$ Hz, H-1'), 3.87 (1H, dd, $J=11.9$, 2.1 Hz, H-6'), 3.67 (1H, dd, $J=11.9$, 5.5 Hz, H-6'), 3.34 (1H, m, H-3'), 3.31 (1H, m, H-4'), 3.26 (1H, m, H-5'), 3.19 (1H, dd, $J=7.9$, 9.0 Hz, H-2'). ^{13}C -NMR (CD_3OD) δ : 156.5 (C-2), 109.2 (C-3), 111.7 (C-4), 152.4 (C-5), 63.7 (C-6), 57.5 (C-7), 102.9 (C-1'), 75.1 (C-2'), 78.1 (C-3'), 71.7 (C-4'), 78.1 (C-5'), 62.9 (C-6').

Acidic Hydrolysis of **7** Compound **7** (3 mg) was hydrolyzed with 1 M H_2SO_4 (1 ml) under 95 °C for 1 h. The reaction mixture was diluted with H_2O and extracted with CHCl_3 . The aqueous layer was neutralized with $\text{Ba}(\text{OH})_2$, then filtered. The filtrate was concentrated, and the residue was purified by Sephadex LH-20 column chromatography (MeOH) to afford a sugar fraction. The sugar sample was subjected to GC analysis after conversion into the TMSi ether of methyl thiazolidine-4 (*R*)-carboxylate derivatives according to Hara *et al.*¹¹ (condition: GL Sciences OV-17, 50 m $\times$ 0.25 mm i.d.; isothermal 220 °C; He at 1.5 g cm⁻²; t_{R} (min): 27.08 (D-glucose).

References

- Sun Z. C., "The Herbs," Harvest Farm Magazine, Taipei, 1987, pp. 57–63.
- Liang W. L., Chen R. C., Chiang Y. J., Su C. H., Yang L. L., Yen K. Y., *Formosan Sci.*, **43**, 47–58 (1990).
- Du X.-M., Yoshizawa T., Shoyama Y., *Phytochemistry*, **49**, 1925–1928 (1998).
- Du X.-M., Sun N.-Y., Shoyama Y., *Phytochemistry*, **53**, 997–1000 (2000).
- Du X.-M., Sun N.-Y., Chen Y., Irino N., Shoyama Y., *Biol. Pharm. Bull.*, **23**, 731–734 (2000).
- Du X.-M., Sun N.-Y., Sugiura M., Yoshizawa T., Irino N., Hayashi J., Shoyama Y., The 12th Symposium on the Development and Application of Naturally Occurring Drug Materials Abstracts, Hiroshima, July 1999, pp. 83–86.
- Ito A., Yasumoto K., Kasai R., Yamasaki K., *Phytochemistry*, **36**, 1465–1467 (1994).
- Taguchi H., Yoshioka I., Yamasaki K., Kim L., *Chem. Pharm. Bull.*, **29**, 55–62 (1981).
- Pabst A., Barron D., Sémon E., Schreier P., *Phytochemistry*, **31**, 1649–1652 (1992).
- Yoshikawa M., Shimada H., Saka M., Yoshizumi S., *Chem. Pharm. Bull.*, **45**, 464–469 (1997).
- Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **35**, 501–506 (1987).
- Rodbell M., *J. Biol. Chem.*, **239**, 375–380 (1964).
- Murashige T., Skoog F., *Physiol. Plant*, **15**, 473 (1962).

Isolation of a Potent Anti-MRSA Sesquiterpenoid Quinone from *Ulmus davidiana* var. *japonica*

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A highly potent anti-MRSA sesquiterpenoid has been isolated from *Ulmus davidiana* var. *japonica*, which has been traditionally used to treat infectious diseases in Korea. This naturally occurring antibiotic was identified as mansonone F (1). This compound has been found to be highly active specifically against MRSA and showed an MIC range of 0.39–3.13 µg/ml which is comparable to that of vancomycin.

Key words anti-MRSA sesquiterpenoid; *Ulmus davidiana* var. *japonica*; mansonone F

During the last two decades, the increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) has had an enormous impact on infection control policies.¹⁾ Few, if any classes of glycopeptides, have played a greater role in the severe infection or allergy to β -lactam-derived antibiotics than vancomycin analogues.²⁾ However, even though they are widely used, the structural complexity and toxic side effects of these antibiotics have prompted increased efforts to find and investigate new and effective antibiotics.³⁾

In continuation of our extensive studies on a new anti-MRSA natural alternative to vancomycin, we have recently evaluated the anti-MRSA activities of the components isolated from the root bark of *Ulmus davidiana* var. *japonica*, which has traditionally been used to treat infectious diseases in Korea.⁴⁾ We herewith report the isolation of an important antibacterial component specifically against MRSA strains from the plant.

Experimental

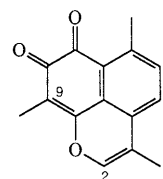
Extraction and Isolation The root bark of *U. davidiana* Planch. var. *japonica* NAKAI were collected in Korea in July 1996 and identified by one (H.H.) of the authors. The dried root bark (2 kg) of this plant was extracted three times with MeOH (8 l) under reflux. The MeOH extract (166 g) in water suspension was partitioned successively with diethyl ether and *n*-BuOH. The residue (17 g) from the ether fraction was chromatographed on a silica gel column using a mixture of *n*-hexane and ethyl acetate (2:3) to afford seven fractions. The fourth fraction (3 g), which showed the highest antibacterial activity (MIC 25 µg/ml) against MRSA, was further chromatographed on a silica gel column using a mixture of CHCl₃ and MeOH (50:1) to give the pure antibacterial component (18 mg) as a characteristic dark purple solid.

Antibacterial Activity Assays The antibacterial activity tests of the extract and mansonone F from this species were carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) procedure⁵⁾ and MICs were determined by a two-fold serial agar dilution method. The media used for preculture and MIC determination were Mueller–Hinton broth (Difco Laboratories, Detroit, MI, U.S.A.) and Mueller–Hinton agar (Difco), respectively. Overnight cultures were diluted with sterile saline solution to the final bacterial density of 10⁷ cfu/ml and each bacterial suspension was inoculated with a replicator (Microplanter, Sakuma, Tokyo, Japan) onto a series of Mueller–Hinton agar plates containing each antibacterial agent. The MIC was defined as the lowest concentration of an antibacterial agent that inhibited the development of visible bacterial growth after incubation for 18 h at 37 °C.

Results and Discussion

The fourth fraction among the diethyl ether-soluble portion of an aqueous MeOH extract of *U. davidiana* var. *japonica* bark showed outstanding antibacterial activity against Gram-positive bacterial strains compared to the other fractions. An active compound (1) was isolated from the fourth fraction using silica gel column chromatography. Its IR spectrum exhibited absorption bands at 1685 cm⁻¹ (C=O), 1600 (olefinic C=C) and 1580 (aromatic C=C). The EI-mass spectrum of 1 showed a characteristic peak of *ortho*-naphthoquinone due to [M+2]⁺ at *m/z* 242. The ¹H-NMR spectrum suggested the compound to be mansonone F (1), showing signals due to three methyl groups (3H each, s at δ 1.92, 2.06, and 2.65). In addition to these signals, a singlet aromatic proton of H-2 (1H, s at δ 7.02) and two doublet aromatic protons of H-4 (1H, d, *J*=8.4 Hz at δ 7.41) and H-5 (1H, d, *J*=8.4 Hz at δ 7.35) were observed. Based on these data together with the ¹³C-NMR spectrum and mp (219–222 °C), the active compound was identified as 1 by comparison of its spectral data with those reported.^{6,7)}

Among the constituents isolated from this plant, 1 was known to be a phytoalexin which is accumulated in the heartwood of the genus *Ulmus* in response to infections of plant pathogens.^{8–10)} This fact indicates the probability of the antibacterial activity of 1. As expected, a component which has shown exceptionally potent antibacterial activity was identified as 1 of a known sesquiterpenoid quinone. This compound was selected after initial antibacterial activity tests of all the isolated constituents against the Gram-positive and Gram-negative bacterial strains. The other components ex-



1

Fig. 1. Chemical Structure of Mansonone F (1)

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Table 1. Antibacterial Activities of **1** against Gram-Positive and Gram-Negative Bacterial Strains^{a)}

Standard strains	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i> SG 511	1.56
<i>S. aureus</i> Giorgio	1.56
<i>S. aureus</i> 209 P	1.56
<i>S. aureus</i> 285	0.78
<i>S. aureus</i> 503	0.78
<i>S. aureus</i> Smith	0.78
<i>Micrococcus luteus</i> ATCC 9341	0.2
<i>Bacillus subtilis</i> ATCC 6633	0.78
<i>Escherichia coli</i> TEM 02	>50
<i>Pseudomonas aeruginosa</i> ATCC 6027	>50
<i>Klebsiella pneumoniae</i> ATCC 10031	>50
<i>Proteus mirabilis</i> ATCC 14273	>50
<i>Enterobacter cloacae</i> 1321 E	>50
<i>Citrobacter freundii</i> ATCC 9090	>50
<i>Serratia marcescens</i> 6093	>50

a) Only seven representative MICs among the 29 MICs of mansonone F against the Gram-negative strains tested were presented.

Table 2. Antibacterial Activities of **1** against 19 MRSAs^{a)}

Antibacterial agents	MIC ($\mu\text{g/ml}$)		
	Range	MIC ₅₀	MIC ₉₀
Mansonone F	0.39—3.13	1.56	1.56
Vancomycin	0.39—1.56	0.78	0.78

a) The clinical isolates were obtained from Seoul National University Hospital in Seoul, Korea.¹¹⁾

cept **1** showed low or no activity against all the bacterial strains tested.

Table 1 summarizes the minimal inhibitory concentrations (MICs) of **1** against eight Gram-positive bacterial strains and 29 Gram-negative bacterial strains which are routinely used for evaluation of the efficacy of potential antibacterial substances. Mansonone F specifically showed potent activity against all the Gram-positive bacterial strains tested. How-

ever, it showed relatively low or no activity against the Gram-negative bacterial strains.

Because **1** showed excellent activity against all of six *Staphylococcus aureus* strains, its effectiveness against MRSA was also intensively evaluated. Its MICs against 19 MRSA strains were examined. The ranges of MIC, MIC₅₀ and MIC₉₀ for **1** and vancomycin as a positive control are listed in Table 2. All the strains of MRSA were susceptible to mansonone F and vancomycin. In particular, the antibacterial activity of mansonone F against MRSA was comparable to that of vancomycin.

This structurally simple and unique tricyclic *ortho*-naphthoquinone obviously offers a novel and promising lead as a new anti-MRSA drug prospect.

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References

- 1) Keane C. T., "Methicillin-Resistant *Staphylococcus aureus*. Methicillin-Resistant *Staphylococcus aureus* (MRSA): An Overview," ed. by Cafferkey M. I., Marcel Dekker, Inc., New York, 1992, pp. 1—9.
- 2) Farber B. F., Moellering R. C., *Antimicrob. Agents Chemother.*, **23**, 138—141 (1983).
- 3) Service R. E., *Science*, **270**, 724—727 (1995).
- 4) Lee S. J., "Korean Folk Medicine," Seoul National University, Seoul, 1966, p. 39.
- 5) National Committee for Clinical Laboratory Standards, "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically," 3rd ed., Approved Standard M7-A3. NCCLS, Villanova, PA, U.S.A., 1993.
- 6) Bettolo G. B. M., Casinovi C. G., Galeffi C., *Tetrahedron Lett.*, **1965**, 4857—4864.
- 7) Thomson R. H., "Naturally Occurring Quinones," Academic Press, London, 1971, pp. 198—366.
- 8) Overeem J. C., Elgersma D. M., *Phytochemistry*, **9**, 1949—1952 (1970).
- 9) Dumas M. T., Strunz G. M., Hubbs S. M., Jeng R. S., *Experimentia*, **39**, 1089—1090 (1983).
- 10) Duchesne L. C., Jeng R. S., Hubbs M., *Can. J. Bot.*, **63**, 678—680 (1984).
- 11) Choi K. H., Hong J. S., Kim S. K., Lee D. K., Yoon S. J., Choi E. C., *J. Antimicrob. Chemother.*, **39**, 509—514 (1997).

Effects of Application Voltage and Cathode and Anode Positions at Electroporation on the *in Vitro* Permeation of Benzoic Acid through Hairless Rat Skin¹⁾

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The enhancing effect of electroporation on the *in vitro* skin permeation of benzoate was evaluated. Needle and ring electrodes made of Ag/AgCl were connected to an electrical power source, which produced exponentially decaying pulses. The needle electrode was kept in contact with the skin surface, and the ring electrode was positioned either on or under the skin. The electrical pulse was applied to abdominal hairless rat skin at 150–600 V every minute from 4 to 6 h during the 10-h permeation experiment. Skin permeation of benzoate was promoted by electroporation and the effect was increased by application of a higher voltage. No immediate recovery to the control flux, however, was observed for high voltage groups after turning off the voltage application. When the cathode and anode were separated by the skin membrane by setting in the epidermal and dermal sides, respectively, an iontophoretic effect may also play a role in benzoate flux. These results indicated that the drug permeation by electroporation is the result of passive diffusion and an iontophoretic effect as well as the electroporation effect.

Key words electroporation; skin permeation; benzoic acid; electrode position; application voltage

Improvement in skin permeation rate of drugs is necessary to effectively use the skin surface as a site for administration of many drugs. There are two methods for enhancement of skin permeation. The first is to increase thermodynamic activity or electrochemical potential of a drug in the formulation; for example chemical modification to a prodrug with higher thermodynamic activity than the parent drug and application of iontophoresis to weak acid or basic drugs. The other is related to enlargement or creation of permeation pathways in the skin barrier, the stratum corneum; use of chemical enhancers may be included in this category. In addition, electroporation is a good example of this latter mechanism, because it causes pore formation in biological membranes.²⁾ Electroporation has been widely used for introducing DNA and RNA into cells and biological tissues, since the original model was developed by Neuman *et al.*³⁾ and Zimmermann *et al.*⁴⁾ Recently, this electroporation technology was utilized to increase transdermal drug delivery.^{5–8)} We also reported the effect of electroporation on the skin permeation of mannitol, a nonionized model material.⁹⁾ In the report we compared the effect by electroporation with iontophoresis. In the present study, sodium benzoate was used as a model ionized material. The effect of applied potential was determined as a continuation of the mannitol study. The possibility of the effect of iontophoresis was also evaluated on the skin permeation of benzoate at electroporation.

Experimental

Materials Sodium benzoate was obtained from Wako Pure Chemicals (Osaka, Japan). Other reagents were of analytical grade and were used without further purification.

Needle type Ag/AgCl electrodes (1.0 mm in diameter and 3 cm in length) and ring type Ag/AgCl electrodes (0.04 mm in thickness) were prepared by silver plate (Murata Yohaku, Tokyo, Japan) in our laboratory. The tip of the needle electrode was bent to prevent damage of the skin barrier.

Skin Permeation Experiment Male hairless rats (WBN/ILA Ht), 210–230 g, were supplied either by Life Science Research Center, Josai University (Sakado, Saitama, Japan), or Ishikawa Experimental Laboratory

(Fukaya, Saitama, Japan). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the abdominal skin was excised. After trimming, the fresh skin piece was mounted in a vertical Franz type diffusion cell with an effective diffusion area of 3.14 cm², with a water jacket connected to a water bath maintained at 37 °C.¹⁰⁾ Sodium benzoate at a concentration of 30 mg/ml in physiological saline (3.0 ml) was applied to skin, with just saline (17.0 ml) on the dermal side. The permeation experiment was performed for 10 h. The receiver side was stirred at 1200 rpm with a star head-magnetic bar and a magnetic stirrer (Multistirrer, Scinics, Tokyo). Skin permeation of benzoic acid was followed by periodic sampling of 0.5 ml from the receiver solution, and then the same volume of fresh saline was added to keep the volume constant. The drug concentration in each sample was assayed by HPLC to determine the flux through the skin at each sampling period. The animal experiments were performed in accordance with the guidelines of Life Science Research Center, Josai University.

One needle electrode was always set on the skin surface. A ring electrode was positioned either on the skin surface or the dermal side. The needle electrode was either the cathode or anode with the ring electrode acting as the respective opposite terminal. The diffusion cell and the position and size of the electrodes and are shown schematically in Fig. 1. These electrodes were connected to a Gene Pulser[®] (Bio-Rad, Hercules, CA, U.S.A.), commonly used for electroporating bacterial and other cell membranes. The power source delivers exponentially decaying pulses. Capacity of the electroporation apparatus was set at 1 μ F, and 120 pulses of 150, 300, 450 or 600 V were generated for 2 h (1 pulse/min).

Assay Each sample was mixed with the same volume of acetonitrile containing 10 μ g/ml *p*-ethyl benzoic acid as an internal standard. After centrifugation, the mixed solution was injected into an HPLC apparatus composed of a pump (LC-10AS, Shimadzu, Kyoto, Japan), UV detector (SPD-10A, Shimadzu), integrator (C-R5A, Shimadzu), system controller (SCL-10A, Shimadzu), auto injector (SIL-10AXL, Shimadzu) and a reverse phase column (Lichrospher[®] 100 RP-18(e), 4 mm $\times$ 250 mm, Kanto Kagaku, Tokyo). Flow rate was 1.0 ml/min, mobile phase was acetonitrile:0.05 M phosphate buffer (1 : 1) and UV wavelength was 230 nm.

Results

Figure 2 shows the time course of benzoate flux through excised hairless rat skin. Electroporation was applied to the skin for 4 to 6 h. Figure 2a shows the results obtained with the needle electrode as the cathode and ring electrode as the anode, and Fig. 2b shows those under the reverse conditions. An almost constant (steady-state) flux was observed from

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about 2 h without voltage application (control). When the voltage (150–600 V) was applied every minute, flux increased almost linearly with time in both cases (Figs. 2a and b). After turning off the voltage application, however, the flux was not recovered to the control value especially following application at 450 or 600 V. In contrast, the flux showed a tendency to return to the control value after electroporation at a low voltage (150 V). The maximum flux was observed from 5.67 to 6 h. The profiles in Fig. 2a were similar to those in Fig. 2b.

The ratio of the maximum flux (each group/control group) was then plotted against voltage difference from the needle to ring electrode (Fig. 3). Regardless of whether the needle electrode was the cathode or anode, the flux increased with increases in the voltage applied. The increase was almost completely linear when the needle electrode was used as the anode, whereas a slightly convex curve was obtained when the needle electrode was the cathode. Each flux was the sum of the electrically assisted transport and passive diffusion.

The ring electrode was set under the skin, whereas the needle electrode was applied to the skin surface. Figure 4 shows the time course of benzoate flux through excised hairless rat skin. Similarly to the experiments shown in Fig. 2, 150 to 600 V was applied using the needle electrode as the cathode and ring electrode as the anode (Fig. 4a) or *vice versa* (Fig. 4b). The permeation fluxes during electroporation shown in Fig. 4b were similar to those shown in Figs. 2a and b. In contrast, the fluxes during electroporation in Fig. 4a were higher than the others, when the skin permeation was compared among the same application voltage.

The ratio of the maximum flux was again plotted against voltage difference from the needle to ring electrode (Fig. 5). The flux profile when the needle electrode was used as the anode was almost the same as that shown in Fig. 3. Interest-

ingly, a higher flux was observed when the needle electrode was set as the cathode.

Discussion

Electroporation is a technique to promote gene introduction into cells and biological tissues.^{3,4)} The effect of electroporation was reported to be reversible.¹¹⁾ In the present skin permeation study, however, the effect of electroporation on the skin permeation of benzoate was not reversible, especially at high voltages. Voltage application probably causes the formation of tiny pores in the primary skin barrier, the stratum corneum. Ceramides, primary lipids, are tightly and rigidly packed in the stratum corneum compared to lecithin in other biological membranes. Thus, it may be very difficult for pores produced in the stratum corneum by electroporation to immediately close again, which may explain the observed irreversibility of the electroporation effect.

The enhancement ratio at the application of 600 V was calculated to 3- to 3.5-fold as compared to the control (Fig. 3). Electroporation enlarges the permeation pathway in the skin barrier to enhance the skin permeation of drugs. When electroporation is applied to skin, the resulting flux may be due to the sum of electrically assisted flux and original passive diffusion, the former of which contains passive diffusion through newly created pore pathway. Therefore, the original pathway for the passive diffusion may be decreased with the pore creation by electroporation.

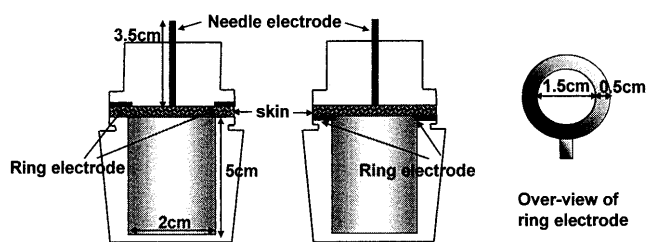


Fig. 1. Schematic Illustration of the Diffusion Cell and the Position and Size of the Electrodes

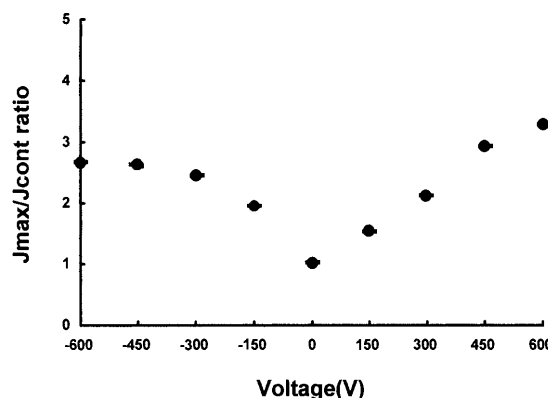


Fig. 3. Relationship between Enhancement Ratio and Electroporation Application Voltage with Both Electrodes on the Skin Surface

$J_{\max}$ is the maximum flux observed from 5.67 to 6 h, and J_{cont} is steady-state flux without of voltage application. Each point represents the mean $\pm$ S.E. of three experiments.

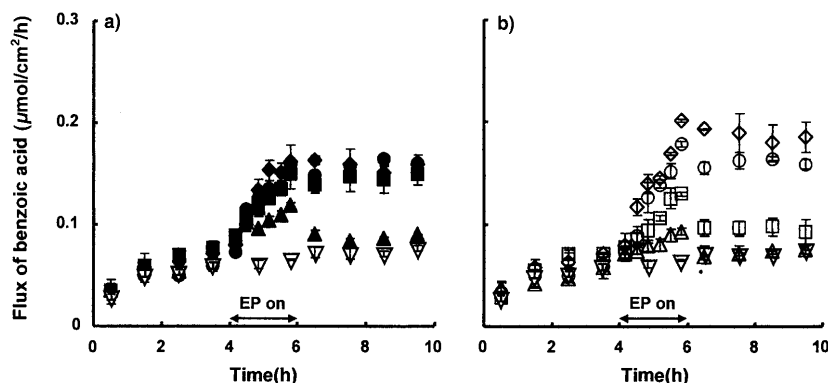


Fig. 2. Effect of Application Voltage on the Skin Permeation of Benzoate with Both Electrodes on the Skin Surface

∇ , 0 V (control); $\blacktriangle$ and $\triangle$, 150 V; $\blacksquare$ and $\square$, 300 V; $\bullet$ and $\circ$, 450 V; $\blacklozenge$ and $\lozenge$, 600 V. Closed and open symbols represent that needle electrode was cathode (a) and anode (b), respectively. Each point represents the mean $\pm$ S.E. of three experiments.

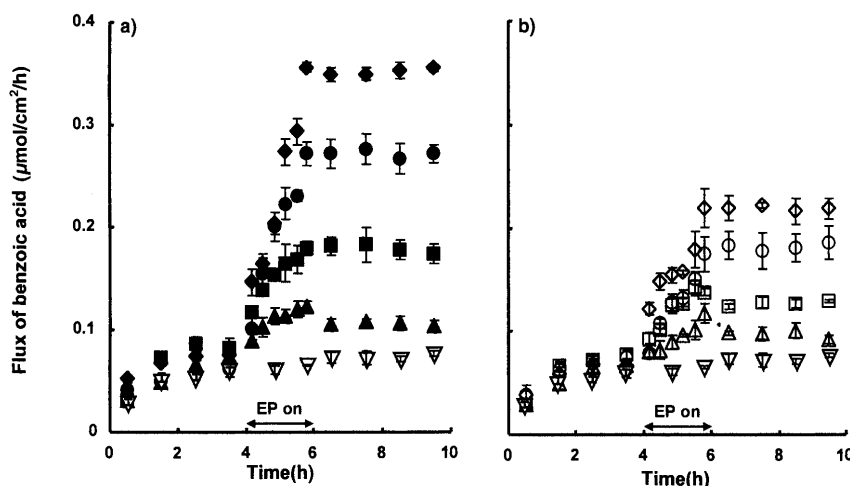


Fig. 4. Effect of Application Voltage on the Skin Permeation of Benzoate with the Two Electrodes Separated by the Skin

Symbols are the same as in Fig. 2. Each point represents the mean $\pm$ S.E. of three experiments.

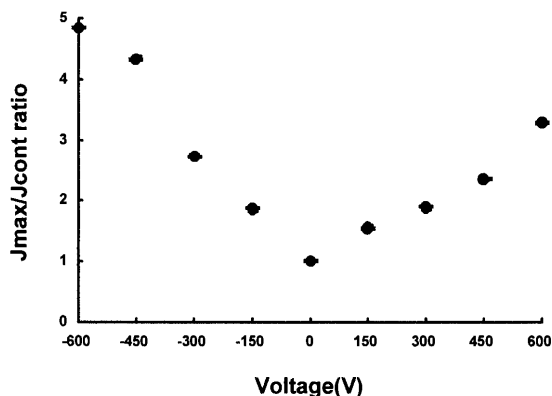


Fig. 5. Relationship between Enhancement Ratio and Electroporation Application Voltage with the Two Electrodes Separated by the Skin

Each point represents the mean $\pm$ S.E. of three experiments.

Benzoic acid is an anionic drug, so there may also be an iontophoretic effect when the cathode is applied to the donor (stratum corneum) side with the anode in the receiver (dermis) side. Thus, the electrically assisted flux may contain that by the iontophoretic effect. The differences in flux between Figs. 2a, 2b, 4b and Fig. 4a may have been due to the iontophoretic effect. This was lower than the inherent electroporation effect under the present conditions. However, flux-decreasing effect by iontophoresis was not observed when anode was applied to the donor side with the cathode in the receiver (see Figs. 2b and 4b), probably because the primary mechanism of benzoate permeation through skin without electroporation is passive diffusion of its unionized form, not of ionized form.

In conclusion, the *in vitro* skin permeation of benzoate was promoted by electroporation and the effect was increased by application of higher voltage. No immediate recovery to the control level, however, was observed following application of a high voltage. When the cathode and anode were separated by the skin membrane by setting in the epidermal and dermal sides, respectively, an iontophoretic effect may also be involved in benzoate flux. Drug permeation during electroporation is a result of passive diffusion and the iontophoretic effect as well as the electroporation effect.

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References and Notes

- 1) Part of this work was presented at the Millennial World Congress of Pharmaceutical Sciences, San Francisco, April 2000.
- 2) Rosenheck K., Lindner P., Pecht I., *J. Membrane Biol.*, **20**, 1—12 (1975).
- 3) Neumann E., Rosenheck K., *J. Membrane Biol.*, **10**, 279—290 (1972).
- 4) Zimmermann U., Schulz J., Pilwat G., *Biophys. J.*, **13**, 1005—1013 (1973).
- 5) Jadoul A., Lecounturier N., Mesens J., Caers W., Preat V., *J. Controlled Release*, **54**, 265—272 (1998).
- 6) Lombry C., Dujardin N., Preat V., *Pharm. Res.*, **17**, 32—37 (2000).
- 7) Sharma A., Kara M., Smith F. R., Krishnan T. R., *J. Pharm. Sci.*, **89**, 528—544 (2000).
- 8) Chang S., Hofmann G. A., Zhange L., Deffos L. J., Banga A. K., *J. Controlled Release*, **66**, 127—133 (2000).
- 9) Mori K., Watanabe T., Hasegawa T., Sato S., Sugibayashi K., Morimoto Y., *Drug Delivery System*, **14**, 485—490 (1999).
- 10) Yukawa J., Sugibayashi K., Morimoto Y., *Yakuzaigaku*, **49**, 254—262 (1989).
- 11) Donald C. C., Thomas S. R., *Biophys. J.*, **58**, 1—12 (1990).

Preparation of 2,2'-Dihydroxytriphenylmethanes Using Metal Phenolates with Aromatic Aldehydes

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The reactions of oxophilic metal phenolates having electron-donating substituents (1a—c) with aromatic aldehydes (2, 3) were selectively condensed at the *ortho*-position of the starting phenol to afford 2,2'-dihydroxytriphenylmethanes (8—11). This method was also applicable to the preparation of bisphenols (12—17) starting with naphthaldehydes (4, 5) and pyridinecarboxaldehydes (6, 7), but in low yields. In the case of these aldehydes (4—7), satisfactory yields could be obtained by sonication.

Key words triphenylmethane; bisarylation; bisphenol; sonication; Lewis acid

Triphenylmethane-type phenol derivatives have been known to exhibit antioxidant properties.^{1,2)} Recently, interesting biological aspects of triphenylmethane-type phenols regarding antitumor activity³⁾ and inhibition activity for histidine protein kinases⁴⁾ have also been reported.

The acid-catalyzed condensation reaction of phenols with aldehydes has been used in the synthesis of such bisphenols and has shown them to be nonregiospecific.⁵⁾ Casnati *et al.* have reported that aryloxymagnesium bromide reacts with aromatic aldehydes to afford 2,2'-dihydroxytriphenylmethanes by means of a *C-ortho*-regiospecific attack of an aromatic aldehyde at an ambident phenoxide ion.^{6,7)} Furthermore, oxophilic metal phenolates⁸⁾ or naphtholates⁹⁾ are shown to undergo selective *C-ortho*-condensation with aromatic aldehydes, such as phthalaldehydes, to produce bisarylation products.

In this paper, we wish to report the conventional synthesis of triphenylmethane-type phenols using oxophilic metal phenolates.

Results and Discussion

To begin with, we examined the reactions of substituted bromomagnesium phenolates with various aromatic aldehydes, adopting the procedure of Cornia *et al.*¹⁰⁾ with some minor modifications (see, Method A in Experimental). Thus, the bromomagnesium salt of 3,4-methylenedioxyphenol (**1a**) was allowed to react with aromatic aldehydes (**2**, **3**), giving *C-ortho*-bisarylation products, 2,2'-dihydroxytriphenylmethanes (**8**, **9**) in excellent yields (82 and 70%, respectively) (see, entries 1 and 2 in Table 1). The application of 3,5-dimethoxyphenol (**1b**) in this method (see, entries 3 and 4) also gave *C-ortho*-bisarylation products (**10**, **11**) in satisfactory yields (92 and 81%, respectively). In these reactions with **1a**, we detected a small amount of diarylmethane (**18**) in addition to the formation of these triarylmethanes.¹¹⁾ The origin of a methylene group in the diarylmethane (**18**) is obviously related to the aldehyde functionality of the starting material. The formation of diarylmethanes in the course of thermal degradation of triarylated products is observed on mass spectroscopy. Thus, compounds (**8**, **9**) gave diagnostically important fragment ion peaks corresponding to the dearylated structure **18**.

To apply this arylation procedure (Method A), we further investigated the reactions of naphthaldehydes (**4**, **5**) and

pyridinecarboxaldehydes (**6**, **7**). The reactions with phenolates of **1a** and **1b** afforded the expected new 2,2'-dihydroxytriarylmethanes (**12**, **13**, **15**, **16**, **17**) and resulted in fairly good yields by the use of sonication¹²⁾ (entries 5—9 in Table 1).

The reaction of 4-methoxyphenolate (**1c**) with 2-naphthaldehyde (**5**) in dichloromethane did not afford the desired product; however, the use of sonication or the replacement of the solvent by benzene gave the desired bisarylation product (**14**), together with a trace amount of 2,2'-ethylenedibisphenol derivative (**19**) and 2-naphthalenemethanol¹³⁾ (see, entries 10 and 11 in Table 1).

The reaction of bromomagnesium phenolate (**1d**) being the most electron-deficient of the phenols (**1a—d**) with aldehydes (**2** or **3**) didn't give the desired condensation products. The tendency for electron-rich bromomagnesium phenolates to enhance the reactivity of the system and of the electron-donating substituents on aromatic aldehydes to decrease the reactivity of these reactions has been reported.⁶⁾ It is noteworthy that the aldehyde (**3**) having a typical electron-donating substituent (-OMe) reacted with phenolates (**1a**, **1b**) to give the corresponding title compounds (**9**, **11**) in a *C-ortho*-regiospecific manner.

The lack of reactivity of bromomagnesium phenolate (**1d**) with aldehyde **2** or **3** prompted us to use Lewis acid as a catalyst. The effect of Lewis acid has already been mentioned in the bisarylation of aldehydes using titanium naphtholates.⁹⁾ Attempts to adopt this procedure (Method B in Experimental) into the bisarylation reaction of **2** or **3** using **1d** were unsuccessful.¹⁴⁾

Furthermore, we tried to examine the reaction of the least reactive phenol **1d** with **3** in the presence of boron trifluoride etherate (BF₃·OEt₂) as an alternative conventional Lewis acid (Method C). Although the reaction of **1a** with **3** resulted

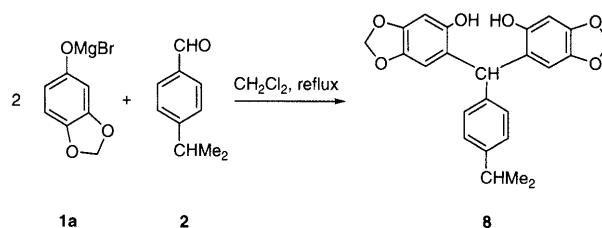


Chart 1

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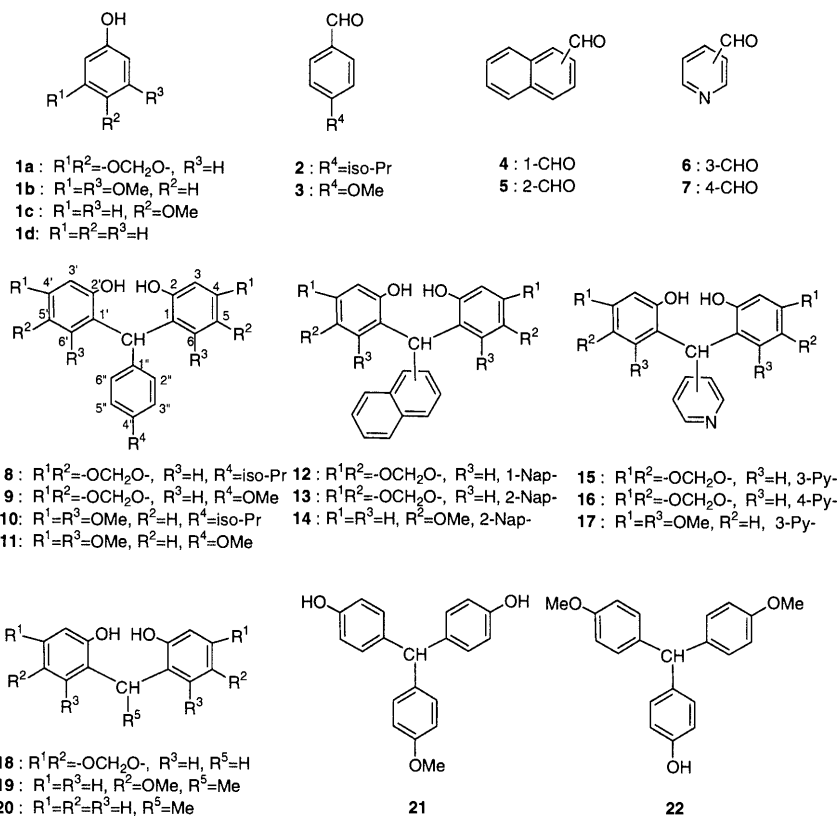


Chart 2

Table 1. Reaction between Phenolates and Aldehydes

Entry	Phenol	Aldehyde	Method ^{a)}	Conditions	Product (Yield %)
1	1a	2	A	Reflux, CH ₂ Cl ₂ , 19 h	8 (82), 18 (15)
2	1a	3	A	Reflux, CH ₂ Cl ₂ , 19 h	9 (70), 18 ^{b)}
3	1b	2	A	Reflux, CH ₂ Cl ₂ , 5 h	10 (92)
4	1b	3	A	r.t., CH ₂ Cl ₂ , 23 h	11 (81)
5	1a	4	A	Reflux, CH ₂ Cl ₂ , 22 h, sonication	12 (75), 18 ^{b)}
6	1a	5	A	Reflux, CH ₂ Cl ₂ , 25 h, sonication	13 (82), 18 ^{b)}
7	1a	6	A	Reflux, CH ₂ Cl ₂ , 19 h, sonication	15 (59)
8	1a	7	A	Reflux, CH ₂ Cl ₂ , 19 h, sonication	16 (53)
9	1b	6	A	Reflux, CH ₂ Cl ₂ , 19 h, sonication	17 (45)
10	1c	5	A	Reflux, CH ₂ Cl ₂ , 19 h, sonication	14 (13), 19 (2) ^{c)}
11	1c	5	A	Reflux, benzene, 23 h	14 (19), 19 (4) ^{c)}
12	1a	3	B	0 °C→r.t., CH ₂ Cl ₂ , 16 h	9 (61)
13	1a	3	C	r.t., benzene, 1 h	9 (99)

a) Method A: ArOMgBr. Method B: TiCl₄(OⁱPr)₂. Method C: BF₃·OEt₂. b) A trace amount of the dearylated compound (**18**) was detected. c) In addition to these compounds, 2-naphthalenemethanol was also isolated in 16% (entry 10) and 20% yield (entry 11), respectively.

in the formation of bisarylation product **9** in almost quantitative yield (entry 13), the reaction of **1d** with **3** by this method gave bisphenol **21** (37%) in addition to monophenol **22** (16%). Thus, this reaction was found to proceed in a *C*-*para*-regiospecific manner.^{1,15)}

In summary, the bisarylation of aromatic aldehyde having electron-donating substituents (such as an alkyl or alkoxy group) by phenolates described above is suitable for electron-rich phenols and provides a convenient regiospecific route to 2,2'-dihydroxytriarylmethanes.¹⁶⁾

Experimental

Melting points were determined by a micro melting point apparatus (Yanagimoto MP-S3) without correction. IR spectra were measured with a Shimadzu FTIR-8100 IR spectrophotometer. The absorption bands attrib-

able to OH, CH, aromatic C=C, and C-O bonds were observed at 3200—3500, 2835—2990, 1505—1635, and 1150—1250 cm⁻¹, respectively. Low- and high-resolution mass spectra (MS and HR-MS) were taken with a JEOL JMS HX-110 double-focusing model equipped with a FAB ion source interfaced with a JEOL JMA-DA 7000 data system. A methanol or ethyl acetate solution of the sample was mixed with *m*-nitrobenzylalcohol and subjected to FAB mass spectrometry. ¹H-(500 MHz) and ¹³C-(125 MHz) NMR spectra were obtained on a JEOL JNM A-500. Chemical shifts were expressed in δ ppm downfield from an internal tetramethylsilane (TMS) signal (0 ppm) for ¹H-NMR and the carbon signal of the corresponding solvent (CDCl₃ (77.0 ppm), DMSO-*d*₆ (39.5 ppm) and CD₃OD (49.0 ppm)) for ¹³C-NMR, respectively. The signal assignments were confirmed with the aid of two-dimensional NMR spectral techniques (¹H-¹H COSY, ¹H-¹³C COSY, and long-range ¹H-¹³C COSY spectra). Microanalyses were performed with a Yanaco MT-6 CHN coder. Routine monitoring of reactions was carried out using precoated Kieselgel 60 F₂₅₄ plates (E. Merck). Flash column chromatography was performed using a silica gel (Fuji Silysia FL-60D) with a UV detec-

Table 2. Analytical and MS Spectroscopic Data for 2,2'-Dihydroxytriarylmethanes (**8**—**22**)^{a)}

No.	mp (°C)	Formula	Analysis (%)			MS <i>m/z</i>	HR positive ion FAB-MS <i>m/z</i> Calcd (Found)
			C	H	N		
8	73—78	C ₂₄ H ₂₂ O ₆ ·0.5H ₂ O	69.39 (69.27)	5.58 5.57	0 (0)	C ₂₄ H ₂₂ O ₆ M ⁺	406.1416 (406.1408)
9	96—98	C ₂₂ H ₁₈ O ₇ ·0.5H ₂ O	65.51 (65.51)	4.75 5.03	0 (0)	C ₂₂ H ₁₈ O ₇ M ⁺	394.1053 (394.1059)
10	64—66	C ₂₆ H ₃₀ O ₆	71.21 (71.22)	6.90 7.03	0 (0)	C ₂₆ H ₃₀ O ₆ M ⁺	438.2042 (438.2062)
11	60—63	C ₂₄ H ₂₆ O ₇ ·0.2H ₂ O	67.03 (66.83)	6.19 6.30	0 (0)	C ₂₄ H ₂₆ O ₇ Na [M+Na] ⁺	449.1576 (449.1576)
12	90—94	C ₂₅ H ₁₈ O ₆	72.46 (72.20)	4.38 4.53	0 (0)	C ₂₅ H ₁₈ O ₆ M ⁺	414.1103 (414.1100)
13	52—55	C ₂₅ H ₁₈ O ₆ ·H ₂ O	69.44 (69.67)	4.66 4.95	0 (0)	C ₂₅ H ₁₈ O ₆ M ⁺	414.1103 (414.1091)
14	47—49	C ₂₅ H ₂₂ O ₄ ·0.8H ₂ O	74.91 (74.79)	5.93 6.18	0 (0)	C ₂₅ H ₂₂ O ₄ M ⁺	386.1518 (386.1517)
15	245—250	C ₂₀ H ₁₅ NO ₆ ·0.3H ₂ O	64.79 (64.92)	4.24 4.40	3.78 (3.76)	C ₂₀ H ₁₅ NO ₆ [M+H] ⁺	366.0978 (366.0977)
16	292—296	C ₂₀ H ₁₅ NO ₆	65.75 (65.52)	4.14 4.34	3.83 (3.79)	C ₂₀ H ₁₅ NO ₆ [M+H] ⁺	366.0978 (366.0979)
17	235—242	C ₂₂ H ₂₃ NO ₆	66.49 (66.22)	5.83 5.99	3.52 (3.45)	C ₂₂ H ₂₃ NO ₆ [M+H] ⁺	398.1603 (398.1609)
18	183—184 (decn.)	C ₁₅ H ₁₂ O ₆	62.50 (62.28)	4.20 4.33	0 (0)	C ₁₅ H ₁₂ O ₆ M ⁺	288.0633 (288.0634)
19	106—113	C ₁₆ H ₁₈ O ₄	70.06 (70.24)	6.61 6.77	0 (0)	C ₁₆ H ₁₈ O ₄ M ⁺	274.1205 (274.1202)
20	oil	C ₁₄ H ₁₄ O ₂ ·0.1H ₂ O	77.83 (77.95)	6.62 6.66	0 (0)	C ₁₄ H ₁₄ O ₂ M ⁺	214.0994 (214.0966)
21	129—130	C ₂₀ H ₁₈ O ₃ ·0.1H ₂ O	77.95 (77.85)	5.95 6.07	0 (0)	C ₂₀ H ₁₈ O ₃ M ⁺	306.1256 (306.1257)
22	oil	C ₂₁ H ₂₀ O ₃ ·0.2H ₂ O	77.85 (77.73)	6.35 6.40	0 (0)	C ₂₁ H ₂₀ O ₃ M ⁺	320.1412 (320.1414)

a) The products (**18**, **19**, **20**) listed in the Table have exceptional structures of diarylalkanes (see Chart 2).

Table 3-1. ¹³C-NMR Data for 2,2'-Dihydroxytriarylmethanes (**8**—**15**)

C No.	8 ^{a)}	9 ^{b)}	10 ^{a)}	11 ^{a)}	12 ^{c)}	13 ^{a)}	14 ^{a)}	15 ^{c)}
1, 1'	121.43	150.44	108.73	108.72	121.57	121.21	130.20	120.84
2, 2'	147.97	124.53	157.33 (or 157.36)	157.27	148.99	148.32	147.49	149.21
3, 3'	98.87	98.68	95.89	95.86	97.63	98.91	116.48	97.63
4, 4'	141.71	147.36	160.23	160.19	145.53	146.81	112.63	145.70
5, 5'	146.77	141.65	92.37	92.28	139.29	141.61	153.91	139.41
6, 6'	109.12	110.45	157.36 (or 157.33)	157.34	109.01	109.19	116.99	108.88
—CH<	43.30	43.48	34.00	33.59	38.50	43.42	44.80	40.40
OCH ₂ O	100.99 101.04	101.87			100.42	101.03		100.50
OCH ₃			55.32 [on 4,4'] 56.08 [on 6,6']	55.28 [on 4,4'] 56.05 [on 6,6']			55.61	
1''	138.50	137.71	137.10	131.69	140.97	127.24	127.49	
2''	128.98	131.06	126.63	127.75	125.01	139.42	138.64	149.93
3''	126.74	114.44	126.03	113.44	125.19	127.82 (or 127.87)	127.83	139.79
4''	147.47	159.26	146.34	157.75	126.37	128.16	128.29	135.87
5''	126.74	114.44	126.03	113.44	128.39	127.51	127.56	123.06
6''	128.98	131.06	126.63	127.75	125.73	125.72	127.94	146.71
7''					125.25	126.02	126.05 (or 125.80)	
8''					123.89	127.87 (or 127.82)	125.80 (or 126.05)	
4a''					133.51	132.34	132.47	
8a''					131.42	133.40	133.46	
4''-CH(CH ₃)	23.89		24.03					
4''-CH(CH ₃)	33.63		33.61					
4''-OCH ₃		55.70		55.23				

a) Measured in CDCl₃. b) Measured in CD₃OD. c) Measured in DMSO-*d*₆.

Table 3-2. ^{13}C -NMR Data for 2,2'-Dihydroxytriarylmethanes (**16**–**22**)^d

C No.	16 ^c	17 ^c	18 ^c	19 ^a	20 ^a	21 ^b	22 ^a
1, 1'	120.22	108.63	119.01	132.56	131.42	137.30	136.93
2, 2'	149.34	156.11	149.10	146.46	152.37	131.24	130.40
3, 3'	97.59	94.35	97.47	116.49	115.87	115.87	115.08
4, 4'	145.80	159.36	145.35	111.90	127.45	156.52	153.89
5, 5'	139.42	90.94	139.54	154.27	121.53	115.87	115.08
6, 6'	108.87	159.28	109.34	113.41	126.17	131.24	130.40
–CH<	42.12	32.08	28.87	30.39	29.88	55.68	54.39
						(or 55.74)	
OCH ₂ O	100.53		100.29				
OCH ₃		54.83 (on 6)		55.73		55.74	55.24
		55.79 (on 4)				(or 55.68)	
1''			d)	d)	d)	138.62	136.82
2''	149.18	148.41				131.22	130.20
3''	124.00	138.91				114.52	113.68
4''	153.48	134.31				159.35	157.93
5''	124.00	122.68				114.52	113.68
6''	149.18	145.79				131.22	130.20
CH ₃				19.49	19.58		

a) Measured in CDCl₃. b) Measured in CD₃OD. c) Measured in DMSO-*d*₆. d) Since the compounds (**18**–**20**) have exceptional structures of diarylmethanes (see Chart 2), these have no aromatic substituent.

tor. Sonicated reactions were carried out with a Branson Model B-8200 ultrasonic cleaner (43 KHz). Commercially available starting materials were used without further purification. The following abbreviations were used for 1-naphthyl (1-Nap-), 2-naphthyl (2-Nap-), 3-pyridyl (3-Py-), and 4-pyridyl (4-Py-).

Synthesis of 2,2'-Dihydroxytriarylmethanes (8–17) (Method A) Under a nitrogen stream, a solution of the selected phenol (**1**) (20 mmol) in Et₂O (50 ml) was added dropwise to a solution of 3 M EtMgBr (6.7 ml, 20 mmol) in Et₂O (40 ml) with stirring at room temperature, and the mixture was kept for 20 min, then Et₂O was removed under a vacuum. After the addition of CH₂Cl₂ (300 ml) to the residue, a solution of the selected aldehyde (**2**–**7**) (5 mmol) in CH₂Cl₂ (100 ml) was added with stirring. The resulting mixture was refluxed with or without sonication (see Table 1). The reaction was quenched with saturated aqueous NH₄Cl (100 ml), and the mixture was extracted with EtOAc (3×100 ml). The combined extracts were dried (MgSO₄) and concentrated under reduced pressure. The desired compounds (**8**–**14**) and byproducts (**18**–**20**) were purified by flash chromatography (*n*-hexane–EtOAc as solvent) of the residue. In the case of **15**–**17**, the residue was purified by recrystallization from methanol. The structures of the products were determined by elemental analysis and spectroscopic methods. The yields and physical data are summarized in Tables 1–3.

Synthesis of 2,2'-Dihydroxy-4"-methoxy-4,5: 4',5'-dimethylenedioxytriphenylmethane (9) (Method B) A solution of 3,4-methylenedioxyphenol (**1a**) (343 mg, 2.48 mmol) in CH₂Cl₂ (25 ml) was added to a suspension of TiCl₄ (260 mg, 1.37 mmol), Ti(O^{*i*}Pr)₄ (390 mg, 1.37 mmol) and 4 Å molecular sieves (330 mg) in CH₂Cl₂ (25 ml) at 0 °C under nitrogen. A solution of 4-methoxybenzaldehyde (**3**) (150 mg, 1.10 mmol) in CH₂Cl₂ (25 ml) was added to the above mixture at 0 °C, then the resulting mixture was allowed to stand at ambient temperature, and stirring was continued for 16 h. The reaction mixture was quenched with saturated aqueous NH₄Cl (7 ml) and portioned between CH₂Cl₂ (200 ml) and water (100 ml). The aqueous layer was extracted with CH₂Cl₂ (2×200 ml), the combined layer was dried (MgSO₄) and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (*n*-hexane–EtOAc as solvent) to yield **9** as a pale yellow solid.

(Method C) A solution of 3,4-methylenedioxyphenol (**1a**) (2.76 g, 20 mmol) and 4-methoxybenzaldehyde (**3**) (0.68 g, 5.0 mmol) in absolute benzene (10 ml) was degassed for 20 min, then BF₃·OEt₂ (0.185 g, 1.5 mmol)

was injected. The reaction mixture was stirred for 1 h at room temperature, then diluted with CH₂Cl₂ (50 ml) and immediately washed with 0.1 N aqueous NaOH (50 ml). The organic layer was washed with water and dried (MgSO₄). After evaporation of the solvent under reduced pressure, flash chromatography (*n*-hexane–EtOAc as solvent) afforded the desired product **9**.

By this method, the reaction of phenol (**1d**) and 4-methoxybenzaldehyde (**3**) gave 4"-methoxy-4,4'-dihydroxytriphenylmethane (**21**) and 4"-hydroxy-4,4'-dimethoxytriphenylmethane (**22**) in 37% and 16% yields, respectively. The isolated products (**21** and **22**) were attributable to condensation at the *para*-position of phenol (**1d**). It is vague in this reaction whether the two methoxy substituents in product **22** occurred by the reaction of 2 moles of aldehyde (**3**) with 1 mole of phenol (**1d**) or during the subsequent methylation of **21**.

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References and Notes

- 1) Sato T., Kise H., *Yukagaku*, **37**, 166–170 (1988).
- 2) Asakura K., Matsumura S., Yoshikawa S., *Yukagaku*, **37**, 265–270 (1988).
- 3) Yamato M., Hashigaki K., Yasumoto Y., Sakai J., Luduena R. F., Banerjee A., Tsukagoshi S., Tashiro T., Tsuruo T., *J. Med. Chem.*, **30**, 1897–1900 (1987).
- 4) Hilliard J. J., Goldschmidt R. M., Licata L., Baum E. Z., Bush K., *Antimicrob. Agents Chemother.*, **43**, 1693–1699 (1999).
- 5) a) Olah G. A. (ed.), "Friedel-Crafts and Related Reactions," Vol. 2, Part 1, Interscience, London, 1964, p. 597; b) Kozlov N. S., Klein A. G., Sukhodolova V. P., *Uch. Zap., Yarosl. Gos. Pedagog. Inst.*, **151**, 54–60 (1975).
- 6) Casiraghi G., Casnati G., Cornia M., Sartori G., Ungaro R., *J. Chem. Soc. Perkin Trans. 1*, **1974**, 2077–2079.
- 7) Casnati G., Casiraghi G., Pochini A., Sartori G., Ungaro R., *Pure & Appl. Chem.*, **55**, 1677–1688 (1983).
- 8) Sartori G., Bigi F., Maggi R., Pastorio A., Porta C., Bonfanti G., *J. Chem. Soc. Perkin Trans. 1*, **1994**, 1879–1882.
- 9) Brimble M. A., Oppen E., *Synth. Commun.*, **27**, 989–1007 (1997).
- 10) Cornia M., Rassu G., Casiraghi G., *Carbohydr. Res.*, **198**, 368–374 (1990).
- 11) In the reaction of 2,3-dimethylphenol with [Fe(DMF)₃Cl₂][FeCl₄] complex, the formation of similar bisphenol-type compounds were observed. See, Khan N. U., Manchanda V., Parveen N., *Indian J. Chem.*, **36B**, 916–917 (1997).
- 12) Without sonication, the yield was quite low (*ca.* 2%) in the case of **1a** and **6**. Sonication during the reaction may contribute to easily reaching the transition state from reactants to products. The same effect on this type of condensation has been observed. See, Lorimer J. P., Mason T. J., *Chem. Soc. Rev.*, **16**, 239–274, 275–311 (1987).
- 13) When the solvent dichloromethane was replaced by benzene for the reaction of **1d** with **3**, only 2,2'-ethylidenebisphenol (**20**, 6%) was obtained, and the desired bisarylation product could not be isolated. The ethylidene functionality in compound (**20**) may be attributable to the ethylmagnesium bromide.
- 14) When the more electron-rich phenolate **1a** was applied to the reaction with 4-methoxybenzaldehyde (**3**), the desired bisarylated condensation product (**9**) could be isolated in 61% yield (entry 12 in Table 1).
- 15) Driver J. E., Sousa J. B., *J. Chem. Soc.*, **1954**, 985–989.
- 16) The procedure (Method A) is also applicable to the reactions of aliphatic aldehydes with electron-rich phenolates as starting materials to give the corresponding C-*ortho*-bisarylation products. These results will be reported elsewhere.

Synthesis of Isoquinoline Derivatives by Diels–Alder Reactions of 2(1*H*)-Pyridones Having an Electron-withdrawing Group with Methoxy-1,3-butadienes

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Diels–Alder (DA) reactions of 2(1*H*)-pyridones having an electron-withdrawing group at the 4-position with 2,3-dimethoxy- and 2-methoxy-1,3-butadienes gave isoquinoline derivatives. Furthermore, an isoquinoline alkaloid (6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone) was synthesized by elimination of hydrogen cyanide and dehydrogenation of the DA-adduct having a cyano group at the 4a-position.

Key words Diels–Alder reaction; 2(1*H*)-pyridone; dimethoxy-1,3-butadiene; isoquinoline derivative; electron-withdrawing group

2(1*H*)-Pyridones are classified as aromatic heterocycles, nevertheless there are many reports^{1,2)} for Diels–Alder (DA) reactions of 2(1*H*)-pyridones acting as a diene. However, publications of the reactions of 2(1*H*)-pyridones acting as a dienophile are a few.^{3*a–e*)} Previously, we have reported a novel synthetic methodology of isoquinoline derivatives by the reactions of 1-alkyl-2(1*H*)-pyridones having an electron-withdrawing group at the 4-position with several kinds of alkylated 1,3-butadienes.^{3*a–c*)} Herein, we have focused our continuing efforts on obtaining isoquinoline derivatives having methoxy groups, which are commonly found in many isoquinoline alkaloids. In the present paper, we wish to report DA reactions of 2(1*H*)-pyridones bearing an electron-withdrawing group at the 4-position acting as a dienophile with methoxy-1,3-butadienes and its application to an alternative synthesis of an isoquinoline alkaloid (6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone).⁴⁾

First, we tried DA reactions of four kinds of 2(1*H*)-pyridones (**1–4**) having a cyano, a methoxycarbonyl, an acetyl, and a benzoyl group, respectively, at the 4-position with 2,3-dimethoxy-1,3-butadiene (**6**). The reactions of **1–4** with **6** were carried out under the reaction conditions (170 or 180 °C and 5 or 6 d) as shown in Table 1, and the corresponding *cis*-adducts (**9–12**) were obtained stereoselectively in 52%, 33%, 58%, and 18% yields, respectively. Isomerization of **10** using LDA as a base at –78 °C proceeded to give the *trans*-adduct (**13**) in 62% yield (Chart 1 and Table 1). The stereochemistries of the ring juncture in **9–13** were confirmed by examination of the ¹H-NMR spectra. Generally, the signals due to H-8a in the *cis*-adducts appear at 2.84–2.97 ppm, and those in the *trans*-adducts are located at 2.00–2.80 ppm^{3*a–c*)} (Chart 2). The corresponding signals of **9–12** appeared at 3.03–3.22 ppm in their ¹H-NMR spectra, respectively. Therefore, the stereochemistries of the ring juncture in **9–12** were confirmed as *cis*. On the other hand, **13** was deduced as a *trans* form, since the signal due to H-8a in the ¹H-NMR spectrum was observed at 2.70 ppm. In addition, DA reactions of **1** and **2** with 2-methoxy-1,3-butadiene (**7**) were performed at 160 °C for 4 d and the *cis*-adducts (**14, 15**) were produced regio- and stereoselectively in 30% and 26% yields (Chart 1 and Table 1). The position of the methoxy group in **14** and **15** was examined as follows. The ¹H–¹H correlation spectroscopy experiments for **14** and **15** did not confirm a

correlation between the methylene protons at C-8 and the olefinic proton in their cyclohexene moiety. Based on these data, the methoxy group in **14** and **15** is located at C-7. Moreover, as the signals due to H-8a in the ¹H-NMR spectra of **14** and **15** were observed at 3.06 and 3.20 ppm, the *cis*-stereochemistries of the ring juncture in them were confirmed.

Furthermore, we examined DA reactions of *N*-unsubstituted 4-methoxycarbonyl-2(1*H*)-pyridone (**5**) with the butadienes [**6** and 2,3-dimethyl-1,3-butadiene (**8**)]. As the results, **5** reacted with **8** at 180 °C for 6 d and the desired DA products were obtained in 52% yield as a mixture of the *cis* (**16**) (28%) and *trans* (**18**) (24%) forms. However, the reaction of **5** with **6** under the above reaction conditions did not give a satisfactory result [5% yield as a mixture of the *cis* (**17**) (3%) and *trans* (**19**) (2%) forms] (Chart 1 and Table 1). The stereochemistries of the ring juncture in **16–19** were confirmed by deriving the corresponding *N*-methylated adducts from them as follows. Methylations of **16–19** with methyl iodide and KF-alumina^{3*d*)} formed the corresponding *N*-methylated adducts, which were identified as *cis*-4a,5,8,8a-tetrahydro-4a-methoxycarbonyl-2,6,7-trimethyl-1(2*H*)-isoquinolone (**20**),^{3*a,b*)} **10**, *trans*-4a,5,8,8a-tetrahydro-4a-methoxy-carbonyl-2,6,7-trimethyl-1(2*H*)-isoquinolone (**21**),^{3*a,b*)} and **13** by their spectral analyses, respectively. Consequently, the stereochemistries of the ring juncture in **16** and **17** are *cis*, and those in **18** and **19** are *trans*. Generally, DA adduct is a *cis* form according to the well-known Alder–Stein rule (*cis* principle). Considering the rule, it might be deduced that the *cis*-adducts (**16, 17**) were isomerized by heating to give the *trans*-adducts (**18, 19**) during the course of the reaction, since the *cis*-adduct [*cis*-4a-cyano-4a,5,8,8a-tetrahydro-2,6,7-trimethyl-1(2*H*)-isoquinolone (**22**)] obtained by the reaction of **1** with **8** was isomerized to the *trans*-adduct (**23**) under heating, but the reverse isomerization did not occur in our previous report (Chart 3).^{3*a,b*)} Next, we investigated the reactive difference between the alkylbutadiene (**8**) and the methoxybutadienes (**6, 7**) to the 2(1*H*)-pyridones (**1–5**) from the yields of the adducts of the present work and our previous papers,^{3*a–c*)} in which the reactions of **1–4** with **8** afforded the corresponding adducts in high yields (72–99%). Accordingly, it was proved that the reactivity of **8** was higher than that of **6** and **7**. The structures of **9–19** were de-

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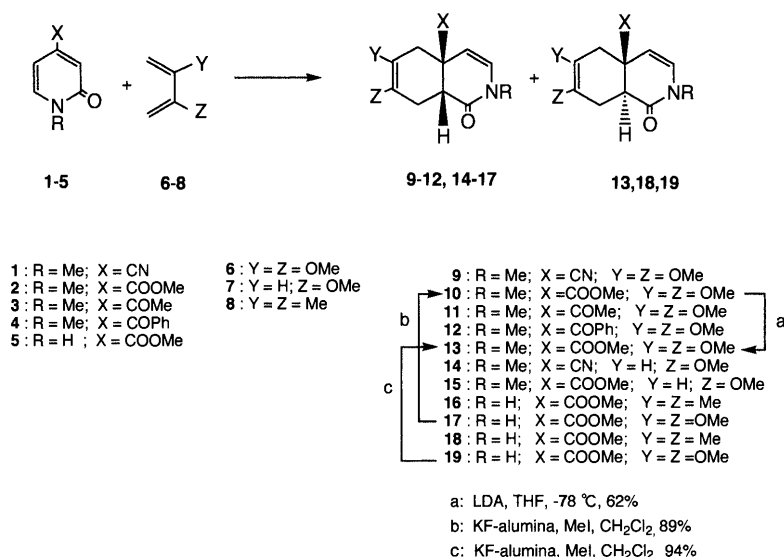


Chart 1

Table 1. DA Reactions of 1—5 with 6—8

Pyridone	Diene	Temp. (°C)	Time (d)	Adduct	Yield (%)	Adduct	Yield (%)
1	6	170	5	9	52		
2	6	180	5	10	33		
3	6	170	6	11	58		
4	6	170	5	12	18		
1	7	160	4	14	30		
2	7	160	4	15	26		
5	8	180	6	16	28	18	24
5	6	180	6	17	3	19	2

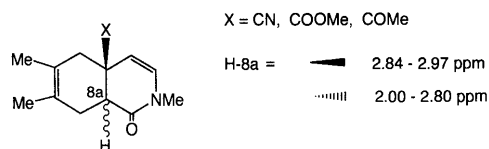


Chart 2

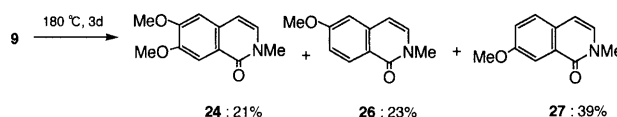


Chart 4

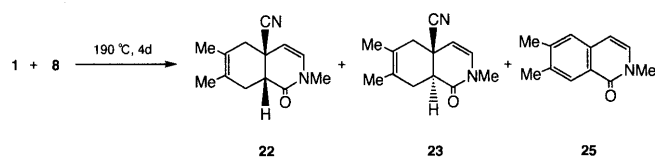


Chart 3

same pathway as the above aromatization in 21% yield with 6-methoxy- and 7-methoxy-2-methyl-1(2*H*)-isoquinolones [26⁵] (23%) and 27⁶] (39%)] formed by eliminations of hydrogen cyanide and methanol from 9 (Chart 4).

In conclusion, we have developed an effective synthetic methodology for preparing methoxyisoquinoline derivatives by DA reactions of 2(1*H*)-pyridones acting as a dienophile. In addition, this methodology was applied to the synthesis of an isoquinoline alkaloid (24) and the desired 24 was readily synthesized in two steps from 1.

Experimental

Melting points were determined on a Yanaco melting point apparatus and are uncorrected. IR spectra were measured with a Perkin Elmer FT-IR 1725X spectrophotometer. MS and high-resolution MS (HR-MS) were taken on a JEOL JMS-DX303/JMA-DA5000 spectrometer. ¹H-NMR spectra were recorded on a JNM-GSX400, a JNM-EX270, and a JEOLJNM-PMX60 spectrometers with TMS as an internal standard. The coupling patterns are indicated as follows: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; m, multiplet; br, broad. Preparative TLC was carried out on precoated silica gel 60F₂₅₄ TLC plate (2 mm), Merck. Chromatography was performed on Merck Kiesegel 60 (230—400 mesh).

General Procedure for DA Reactions of 1—4 with 6 A mixture of 1

terminated by ¹H-NMR, ¹³C-NMR, IR, and MS spectral analyses.

Finally, we tried the synthesis of an isoquinoline alkaloid [6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone (24)⁴] by our methodology. Our previous report^{3a,b} showed that heating of 1 with 8 gave a mixture of 22 (31%), the *trans* isomer [23 (7.5%)], and the aromatized compound [2,6,7-trimethyl-1(2*H*)-isoquinolone (25) (17.6%)], which resulted by elimination of hydrogen cyanide and dehydrogenation during the course of the reaction (Chart 3). Considering that 25 was obtained, aromatization of 9 was carried out in *o*-xylene at 180 °C for 3 d. Thus, the alkaloid (24) was produced by the

(1.61 g, 12 mmol) and **6** (6.84 g, 60 mmol) was heated in a sealed tube under the conditions as shown in Table 1. The reaction mixture was chromatographed on a column of silica gel. The solvent of the first fraction eluted with acetone was evaporated. The residue was purified by preparative TLC over silica gel with ether-hexane (1:1) to give *cis*-4a,5,8,8a-tetrahydro-6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone (**9**). The reactions of **2**—**4** and **6** were performed in the same manner as described above to give *cis*-4a,5,8,8a-tetrahydro-6,7-dimethoxy-4a-methoxycarbonyl-2-methyl-1(2*H*)-isoquinolone (**10**), *cis*-4a-acetyl-4a,5,8,8a-tetrahydro-6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone (**11**), and *cis*-4a-benzoyl-4a,5,8,8a-tetrahydro-6,7-dimethoxy-2-methyl-1(2*H*)-isoquinolone (**12**), respectively. The solvents of the corresponding second fractions eluted with acetone were evaporated to recover **1** (38%), **2** (42%), **3** (4%), and **4** (58%), respectively. The yields of **9**—**12** are shown in Table 1.

9: Pale brown oil. IR (film) cm^{-1} : 2241, 1683. $^1\text{H-NMR}$ (CDCl_3) δ : 2.17—2.6 (4H, m), 3.03 (1H, m), 3.11 (3H, s), 3.61 (3H, s), 3.67 (3H, s), 5.32 (1H, d, $J=7.7$ Hz), 6.17 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 22.62, 33.14, 34.46, 35.20, 42.55, 57.48, 57.23, 58.08, 106.45, 121.10, 132.10, 132.92, 136.98, 166.98. MS m/z : 248 (M^+), 134 ($\text{M}^+ - \text{C}_6\text{H}_{10}\text{O}_2$). HR-MS Calcd for $\text{C}_{13}\text{H}_{16}\text{N}_2\text{O}_3$: 248.1161. Found: 248.1147.

10: Pale brown oil. IR (film) cm^{-1} : 1734, 1678. $^1\text{H-NMR}$ (CDCl_3) δ : 2.27 (1H, d, $J=16.5$ Hz), 2.40 (1H, dd, $J=7.0$, 16.7 Hz), 2.69 (1H, d, $J=16.5$ Hz), 2.88 (1H, dd, $J=2.2$, 16.7 Hz), 3.08 (3H, s), 3.16 (1H, m), 3.61 (3H, s), 3.67 (3H, s), 3.74 (3H, s), 5.07 (1H, d, $J=7.7$ Hz), 6.09 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 22.98, 31.33, 34.13, 41.34, 45.66, 52.74, 57.23, 57.50, 108.79, 131.14, 134.30, 136.71, 169.82, 173.51. MS m/z : 281 (M^+), 167 ($\text{M}^+ - \text{C}_6\text{H}_{10}\text{O}_2$). HR-MS Calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_5$: 281.1263. Found: 281.1248.

11: Pale brown oil. IR (film) cm^{-1} : 1706, 1675. $^1\text{H-NMR}$ (CDCl_3) δ : 2.21 (3H, s), 2.24—2.58 (4H, m), 3.03 (1H, d, $J=2.2$ Hz), 3.05 (3H, s), 3.59 (3H, s), 3.62 (3H, s), 4.96 (1H, dd, $J=1.1$, 7.7 Hz), 6.14 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 23.77, 24.48, 31.61, 33.85, 40.60, 50.91, 57.12, 57.95, 107.33, 132.39, 134.02, 137.23, 170.47, 205.45. MS m/z : 265 (M^+), 151 ($\text{M}^+ - \text{C}_6\text{H}_{10}\text{O}_2$), 114 ($\text{C}_6\text{H}_{10}\text{O}_2^+$). HR-MS Calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_4$: 265.1314. Found: 265.1312.

12: Pale brown oil. IR (film) cm^{-1} : 1682, 1650. $^1\text{H-NMR}$ (CDCl_3) δ : 2.26 (1H, dd, $J=6.6$, 16.5 Hz), 2.43 (1H, d, $J=16.9$ Hz), 2.60 (1H, dd, $J=7.0$, 16.5 Hz), 2.67 (1H, d, $J=16.9$ Hz), 3.06 (3H, s), 3.22 (1H, dd, $J=6.6$, 7.0 Hz), 3.56 (3H, s), 3.59 (3H, s), 5.33 (1H, d, $J=7.3$ Hz), 6.19 (1H, d, $J=7.3$ Hz), 7.41—7.54 (3H, m), 7.89—7.91 (2H, m). $^{13}\text{C-NMR}$ (CDCl_3) δ : 23.43, 32.55, 33.87, 42.25, 50.39, 57.24, 57.87, 108.17, 128.29, 128.39, 128.52, 129.12, 132.10, 132.42, 134.58, 135.48, 136.77, 170.54, 199.33. MS m/z : 327 (M^+), 213 ($\text{M}^+ - \text{C}_6\text{H}_{10}\text{O}_2$). HR-MS Calcd for $\text{C}_{19}\text{H}_{21}\text{NO}_4$: 327.1471. Found: 327.1438.

General Procedure for DA Reactions of 1 and 2 with 7 A mixture of **1** (0.8 g, 6 mmol), **7** (2.52 g, 30 mmol), and mesitylene (3 ml) was heated in a sealed tube at 160 °C for 4 d. The reaction mixture was chromatographed on a column of silica gel. The solvent of the first fraction eluted with acetone was evaporated. The residue was purified by preparative TLC over silica gel with ether-hexane (1:1) to give *cis*-4a-cyano-4a,5,8,8a-tetrahydro-7-methoxy-2-methyl-1(2*H*)-isoquinolone (**14**). The reaction of **2** and **7** was performed in the same manner as described above to give *cis*-4a,5,8,8a-tetrahydro-7-methoxy-4a-methoxy-carbonyl-2-methyl-1(2*H*)-isoquinolone (**15**). The solvents of the respective second fractions were evaporated to recover **1** (47%) and **2** (45%). The yields of **14** and **15** are shown in Table 1.

14: Colorless needles (hexane), mp 87—89 °C. IR (KBr) cm^{-1} : 2220, 1674. $^1\text{H-NMR}$ (CDCl_3) δ : 2.33 (1H, d, $J=17.6$ Hz), 2.50—2.59 (2H, m), 2.95 (1H, d, $J=17.6$ Hz), 3.06 (1H, d, $J=5.1$ Hz), 3.10 (3H, s), 3.53 (3H, s), 4.51 (1H, brs), 5.33 (1H, d, $J=7.7$ Hz), 6.14 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 24.47, 30.98, 34.32, 34.46, 52.58, 54.23, 107.24, 121.73, 131.73, 132.11, 153.71, 167.39. MS m/z : 218 (M^+), 134 ($\text{M}^+ - \text{C}_5\text{H}_8\text{O}$). HR-MS Calcd for $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_3$: 218.1055. Found: 218.1064.

15: Pale yellow oil. IR (film) cm^{-1} : 1730, 1670. $^1\text{H-NMR}$ (CDCl_3) δ : 2.22 (1H, dd, $J=3.3$, 15.0 Hz), 2.33—2.40 (1H, m), 2.61 (1H, dd, $J=4.8$, 15.0 Hz), 2.75 (1H, dd, $J=1.4$, 4.7, 17.2 Hz), 3.07 (3H, s), 3.20 (1H, $J=4.7$, 7.0 Hz), 3.48 (3H, s), 3.73 (3H, s), 4.50 (1H, dd, $J=3.3$, 4.8 Hz), 5.07 (1H, d, $J=7.7$ Hz), 6.06 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 25.14, 29.60, 34.08, 41.43, 45.00, 52.63, 54.03, 89.62, 109.71, 130.88, 153.08, 170.29, 174.21. MS m/z : 251 (M^+), 167 ($\text{M}^+ - \text{C}_5\text{H}_8\text{O}$). HR-MS Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_4$: 251.1158. Found: 251.1151.

General Procedure for DA Reactions of 5 with 6 and 8 A mixture of **5** (0.6 g, 5 mmol), **6** (2.85 g, 25 mmol), and *o*-xylene (3 ml) was heated in a sealed tube at 180 °C for 6 d. The reaction mixture was chromatographed on a column of silica gel. The solvent of the first fraction eluted with acetone was evaporated and the residue was purified by preparative TLC over silica

gel with ether to give *cis*-4a,5,8,8a-tetrahydro-6,7-dimethoxy-4a-methoxy-carbonyl-1(2*H*)-isoquinolone (**17**) and *trans*-4a,5,8,8a-tetrahydro-6,7-dimethoxy-4a-methoxycarbonyl-1(2*H*)-isoquinolone (**19**). The second fraction recovered **5** (61%). The reaction of **5** and **8** was performed in the same manner as described above to give *cis*-4a,5,8,8a-tetrahydro-4a-methoxycarbonyl-6,7-dimethyl-1(2*H*)-isoquinolone (**16**) and *trans*-4a,5,8,8a-tetrahydro-4a-methoxycarbonyl-6,7-dimethyl-1(2*H*)-isoquinolone (**18**). The second fraction recovered **5** (20%). The yields of **16**—**19** are shown in Table 1.

16: Colorless prisms (CH_2Cl_2 -hexane), mp 137—139 °C. IR (KBr) cm^{-1} : 1737.5, 1681. $^1\text{H-NMR}$ (CDCl_3) δ : 1.62 (6H, brs), 2.17—2.50 (4H, m), 3.05 (1H, m), 3.72 (3H, s), 4.97 (1H, d, $J=7.7$ Hz), 6.12 (1H, dd, $J=4.8$, 7.7 Hz), 7.54 (1H, brs). $^{13}\text{C-NMR}$ (CDCl_3) δ : 18.56, 18.96, 29.27, 41.19, 45.09, 52.41, 109.02, 122.29, 124.21, 125.16, 172.86, 174.18. MS m/z : 235 (M^+), 154 ($\text{M}^+ - \text{C}_6\text{H}_{10}$). HR-MS Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_3$: 235.1208. Found: 235.1200.

17: Pale yellow oil. IR (film) cm^{-1} : 3286, 3135, 1733, 1697, 1654. $^1\text{H-NMR}$ (CDCl_3) δ : 2.36—2.93 (4H, m), 3.08 (1H, m), 3.57 (3H, s), 3.60 (3H, s), 3.77 (3H, s), 5.07 (1H, d, $J=8.0$ Hz), 6.2 (1H, dd, $J=4.0$, 8.0 Hz), 7.89 (1H, brs). MS m/z : 267 (M^+), 114 ($\text{C}_6\text{H}_{10}\text{O}_2^+$). HR-MS Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_5$: 267.1107. Found: 267.1085.

18: Colorless columns (CH_2Cl_2 -hexane), mp 173—175 °C. IR (KBr) cm^{-1} : 1732, 1687. $^1\text{H-NMR}$ (CDCl_3) δ : 1.64 (6H, brs), 2.16—2.65 (4H, m), 2.70 (1H, m), 3.64 (3H, s), 4.90 (1H, dd, $J=1.1$, 7.7 Hz), 6.18 (1H, dd, $J=4.8$, 7.7 Hz), 7.41 (1H, brs). $^{13}\text{C-NMR}$ (CDCl_3) δ : 18.76, 18.86, 29.58, 40.36, 43.00, 45.79, 108.16, 123.36, 124.87, 126.23, 172.01, 172.65. MS m/z : 235 (M^+), 176 ($\text{M}^+ - \text{COOCH}_3$). HR-MS Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_3$: 235.1208. Found: 235.1192.

19: Pale yellow oil. IR (film) cm^{-1} : 3286, 3135, 1729, 1696, 1651. $^1\text{H-NMR}$ (CDCl_3) δ : 2.31—3.08 (5H, m), 3.57 (6H, s), 3.65 (3H, s), 4.88 (1H, d, $J=8.0$ Hz), 6.17 (1H, dd, $J=6.0$, 8.0 Hz), 7.31 (1H, brs). MS m/z : 267 (M^+), 208 ($\text{M}^+ - \text{COOCH}_3$). HR-MS Calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_5$: 267.1107. Found: 267.1124.

Methylations of 16—19 MeI (0.01 ml, 0.164 mmol) was added to a suspension of acetonitrile (7 ml), **17** (29 mg, 0.109 mmol), and $\text{KF} \cdot \text{Al}_2\text{O}_3$ (16 mg, 0.273 mmol) under a nitrogen atmosphere. The suspension was stirred at room temperature for 2 d. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was purified by preparative TLC over silica gel with ether to give **10** (27 mg, 89%). Methylation of **16** (61 mg, 0.26 mmol), **18** (15 mg, 0.057 mmol) and **19** (70 mg, 0.298 mmol) was worked up in a similar manner as described above to give **20** (64 mg, 86%), **21** (64 mg, 86%),^{3a)} and *trans*-4a,5,8,8a-tetrahydro-6,7-dimethoxy-4a-methoxycarbonyl-2-methyl-1(2*H*)-isoquinolone [**13** (15 mg, 94%)], respectively.

13: Pale brown oil. IR (film) cm^{-1} : 1729, 1681. $^1\text{H-NMR}$ (CDCl_3) δ : 2.32 (1H, m), 2.57—2.73 (2H, m), 2.70 (1H, m), 2.90 (1H, d, $J=15.4$ Hz), 3.08 (3H, s), 3.59 (6H, s), 3.66 (3H, s), 4.92 (1H, d, $J=7.7$ Hz), 6.12 (1H, d, $J=7.7$ Hz). $^{13}\text{C-NMR}$ (CDCl_3) δ : 22.95, 31.23, 34.20, 41.34, 45.66, 52.67, 57.20, 57.50, 108.81, 131.15, 134.40, 136.71, 169.90, 173.71. MS m/z : 281 (M^+), 222 ($\text{M}^+ - \text{COOCH}_3$). HR-MS Calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_5$: 281.1263. Found: 281.1248.

Isomerization of 10 with LDA A 1.6 M hexane solution of *n*-butyllithium (2.5 ml, 4 mmol) was added at −30 °C to a cooled solution of diisopropylamine (0.56 ml, 4 mmol) in THF (3 ml). After being stirred at the same temperature for 15 min, the mixture was cooled to −78 °C. A solution of **10** (562 mg, 2 mmol) in THF (2 ml) was added to the cooled solution, and after 5 min, the mixture was stirred at −78 °C for 80 min. To the reaction mixture, MeOH (0.5 ml) was added, and after 5 min, the mixture was treated with saturated aqueous NH_4Cl (1 ml). The mixture was stirred while warming to room temperature, and poured into 10% HCl (20 ml). The acidic mixture was extracted with benzene. The benzene extract was washed with saturated aqueous NaCl (10 ml), dried over Na_2SO_4 , and evaporated. The residue was chromatographed on a column of silica gel. The solvent of the fraction eluted with hexane-acetone (4:1) was evaporated to give **13** (349 mg, 62%).

Heating of 9 A solution of **9** (340 mg, 1.37 mmol) in *o*-xylene (2 ml) was heated in a sealed tube at 180 °C for 3 d. The reaction mixture was concentrated *in vacuo* and the residue was purified by preparative TLC over silica gel with ether to give **24** ($\text{Rf}=0.08$, 62 mg, 21%),⁴⁾ **26** ($\text{Rf}=0.21$, 60 mg, 23%),⁵⁾ and **27** ($\text{Rf}=0.25$, 100 mg, 39%).⁶⁾

References

- a) Sheinin E. B., Wright G. E., Bell C. L., Bauer L., *J. Heterocycl. Chem.*, **5**, 859—862 (1968); b) Tomisawa H., Hongo H., *Tetrahedron Lett.*, **29**, 2465—2468 (1969); c) Heep U., *ibid.*, **31**, 77—80 (1975); d) Somekawa K., Watanabe T., Kumamoto S., *Nippon Kagaku Kaishi*,

- 1978, 412—417; e) Mariano P. S., Huseman P. L., Beamer R. L., *Tetrahedron*, **34**, 2617—2626 (1978); f) Gisby G. P., Royall S. E., Sammes P. G., *J. Chem. Soc. Chem. Commun.*, **1979**, 501—502; g) Tomisawa H., Hongo H., Kato H., Fujita R., *Heterocycles*, **16**, 1947—1950 (1981); h) Gisby G. P., Royall S. E., Sammes P. G., *J. Chem. Soc. Perkin Trans. 1*, **1982**, 169—173; i) Kuzuya M., Noguchi A., Mano E., Okuda T., *Bull. Chem. Soc. Jpn.*, **58**, 1149—1155 (1985); j) Posner G. H., Switzer C., *J. Org. Chem.*, **52**, 1644—1646 (1987); k) Tomisawa H., Nakano H., Hongo H., *Heterocycles*, **30**, 359—362 (1990); l) Nakano H., Tomisawa H., Hongo H., *J. Chem. Soc. Chem. Commun.*, **1990**, 1775—1776; m) Posner G. H., Vinader V., Afarinkia K., *J. Org. Chem.*, **57**, 4088—4097 (1992); n) Nakano H., Saito Y., Hongo H., *Chem. Pharm. Bull.*, **40**, 2876—2878 (1992); o) Nakano H., Hongo H., *Heterocycles*, **35**, 37—40 (1993).
- 2) Review for Diels–Alder reactions of 2(1*H*)-pyridones; Afarinkia K., Vinader V., Nelson T. D., Posner G. H., *Tetrahedron*, **48**, 9111—9171 (1992).
- 3) a) Kato H., Fujita R., Hongo H., Tomisawa H., *Heterocycles*, **12**, 1 (1979); b) Tomisawa H., Kato H., Fujita R., Hongo H., *Chem. Pharm. Bull.*, **27**, 810—812 (1979); c) Tomisawa H., Fujita R., Kato H., Hayasaka K., Kamimura T., Hongo H., *ibid.*, **36**, 1882—1885 (1988); d) Nakano H., Date T., Okamura K., Tomisawa H., Hongo H., *ibid.*, **39**, 2471—2473 (1991); e) Hongo H., Nakano H., Matsuzaki H., Okuyama Y., *Yakugaku Zasshi*, **118**, 105—109 (1998).
- 4) a) Decker H., Pscher R., *Ber.*, **37**, 3396—3404 (1904); b) Pscher R., *ibid.*, **37**, 1926—1942 (1904); c) Belgaonkar V. H., Usgaonkar R. N., *J. Heterocycl. Chem.*, **15**, 257—261 (1978); d) Abdizhabbarova S., Maekh S. Kh., Yunusov S. Yu., Yagudaev M. R., Kurbakov D., *Khim. Prir. Soedin.*, **1978**, 472—474; e) Wu W. N., Beal J. L., Doskkotch R. W., *J. Nat. Prod.*, **43**, 372—381 (1980); f) Kunitomo J., Murakami Y., Oshikata M., Shingu T., Lu S. T., Chen I. S., Akasu M., *Yakugaku Zasshi*, **101**, 431—436 (1981); g) Ruchirawat S., *Heterocycles*, **6**, 1724—1729 (1977).
- 5) a) Cuvas J. C., Patil P., Snieckus V., *Tetrahedron Lett.*, **30**, 5841—5844 (1989); b) Kondo H., Ishiwata S., *Ber.*, **70**, 2427—2437 (1937).
- 6) a) Ajao J. F., Bird C. W., *J. Heterocycl. Chem.*, **22**, 329—331 (1985); b) Belgaonkar V. H., Usgaonkar R. N., *J. Chem. Soc. Perkin Trans. 1*, **1977**, 702—706.

Clerodane-type Diterpenoids from the Japanese Liverwort *Jungermannia infusca* (MITT.) STEPH.

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Three new clerodane-type diterpenoids have been isolated from the Japanese liverwort *Jungermannia infusca* (MITT.) STEPH., together with previously known compounds, nine clerodane- and four labdane-type diterpenoids, and δ -tocopherol. The structures of the new compounds were confirmed by 2D NMR experiments and X-ray crystallographic analysis.

Key words *Jungermannia infusca*; liverwort; clerodane-type; labdane-type; infuscolide A; diterpenoid

A number of liverworts (Hepaticae) have characteristic fragrant odors and an intense hot and bitter taste. Generally, liverworts are not damaged by insects, snails, slugs and other small animals. Furthermore, some liverworts cause potent allergic contact dermatitis. Most of the Hepaticae possess cellular oil bodies which are very important markers for the classification of liverworts.^{1–3)} We are continuing our studies of the chemical constituents of Hepaticae by determining the structures and biological activity of the isolated compounds and investigating the chemosystematics of Hepaticae. It has been shown that most liverworts contain lipophilic mono-, sesqui- and diterpenoids and phenolic compounds which are present in the oil bodies and the biological activity of liverwort is due to these substances.⁴⁾

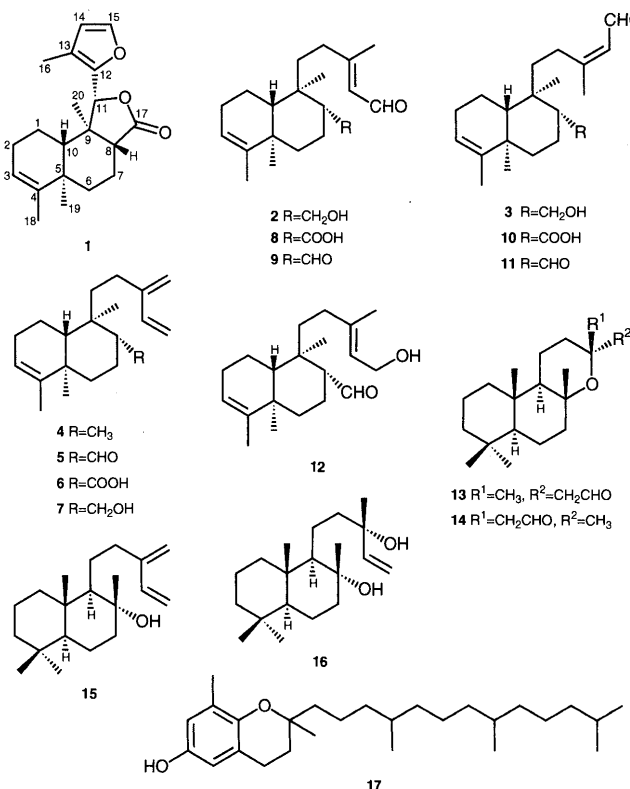
As a part of the search for novel skeletal terpenoids, aromatic compounds and biologically active compounds in liverworts, we are focussing on the *Jungermannia* L. belonging to the Jungermanniaceae (Jungermanniales) because many of them have a very characteristic fragrance and/or potent bitter taste. There are 41 *Jungermannia* species in Japan. Among them, *J. infusca* is not only a morphologically but also a chemically interesting species because it is polymorphic and its chemical constituents are very dependent on the collection site.⁵⁾ In our previous paper,⁶⁾ we reported the isolation and structure characterization of several sesqui- and diterpenoids of *J. infusca*. In order to confirm the chemo-type of this species, we collected it in Ehime prefecture and reinvestigated its chemical constituents. We were able to isolate three new clerodane-type diterpenoids (**1**–**3**), along with their related clerodane- (**4**–**12**) and labdane-type diterpenoids (**13**–**16**) and δ -tocopherol. Here we wish to report the structure determination of the new compounds and the chemosystematics of the present species.

Three new clerodane-type diterpenoids, infuscolide A (**1**), 17-hydroxy-3,13*E*-clerodadien-15-al (**2**) and 17-hydroxy-3,13*Z*-clerodadien-15-al (**3**), were isolated from the ether extract of *J. infusca*, together with known clerodane-types: cleroda-3,13(16),14-triene (**4**),⁷⁾ cleroda-3,13(16),14-trien-17-al (**5**),⁸⁾ infuscaic acid (**6**) which possesses superoxide anion radical release inhibitory activity,⁹⁾ cleroda-3,13(16),14-trien-17-ol (**7**),⁹⁾ 15-oxo-3,13*E*-clerodadien-17-oic acid (**8**),^{8,10)} cleroda-3,13*E*-diene-15,17-dial (**9**),⁸⁾ 15-oxo-3,13*Z*-clerodadien-17-oic acid (**10**),^{8,10)} cleroda-3,13*Z*-diene-15,17-dial (**11**)⁸⁾ and 15-hydroxy-3,13*E*-clerodadien-17-al (**12**),⁹⁾ labdane-types: gomeraldehyde (**13**),⁹⁾ *epi*-gomeraldehyde

(**14**),⁹⁾ isoabienol (**15**)¹¹⁾ and 13-*epi*-sclareol (**16**),¹²⁾ and δ -tocopherol (**17**).¹³⁾

The IR spectrum of **1** showed the presence of a γ -lactone (1780 cm^{−1}). The molecular formula was found to be C₂₀H₃₆O₃ (obs. *m/z* 314.1870 [M]⁺) by high-resolution electron impact mass spectrometry (HR-EIMS), indicating eight degrees of unsaturation. The ¹H- and ¹³C-NMR (Tables 1 and 2) spectra of **1** showed the presence of a trisubstituted double bond, two methines and carbonyl carbon of the lactone ring, and two methines and two quaternary carbons of an α,β -disubstituted furan ring, together with two tertiary methyls, two olefinic methyls, four methylenes, two methines and two quaternary carbons. The gross structure of **1** was suggested to be a *trans*-clerodane-type diterpenoid by comparison of the above spectral data with those of compounds **4**–**12**.

The ¹H–¹H correlated spectroscopy (¹H–¹H COSY) and the heteronuclear multiple quantum coherence (HMQC) of **1** showed two partial structures, (i) –C=CH–CH₂–CH₂–CH–



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and (ii) $-\text{CH}_2-\text{CH}_2-\text{CH}-$. Moreover, long-range $^1\text{H}-^{13}\text{C}$ correlations were observed in the heteronuclear multiple bond correlation (HMBC) experiment as shown in Fig. 1. Subsequently, the phase sensitive nuclear Overhauser enhancement

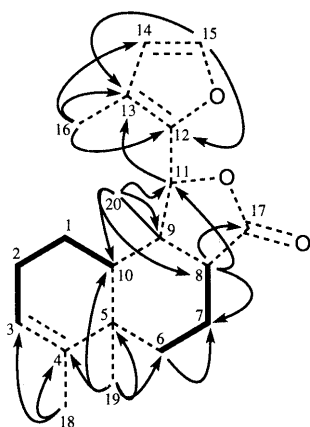


Fig. 1. $^1\text{H}-^1\text{H}$ Correlations (Bold Lines) and Long-Range $^1\text{H}-^{13}\text{C}$ Correlations (Arrows) of **1**

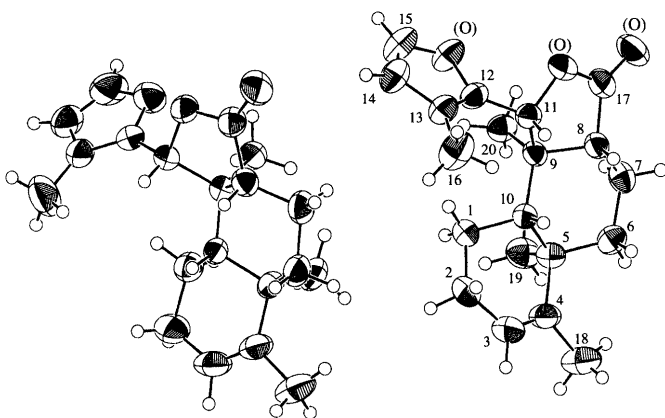


Fig. 2. The ORTEP Drawing of **1**

Table 1. ^1H -NMR Data of **1**–**3** (600 MHz, C_6D_6)

H	1 *	2	3
1	1.48 m, α 1.25 m, β	1.25–1.56 2H, m	1.25–1.43 2H, m
2	1.96–2.02 2H, m	1.88 m	1.91 m
3	5.16 br s	1.97 br d (16.2)	1.97 br d (16.2)
6	1.94 dt (13.2, 3.3) [†] α 1.29 ddd (13.2, 13.2, 4.1) β	5.20 br s 1.07 ddd (14.3, 14.3, 4.9)	5.20 br s 1.08 ddd (12.4, 12.4, 3.3)
7	1.69 dddd (12.9, 12.9, 12.9, 3.3) α 1.96–2.02 m, β	1.64–1.68 m 1.20–1.23 m	1.62–1.70 m 1.20 m
8	2.30 dd (12.9, 3.0)	1.64–1.68 m	1.62–1.70 m
10	1.75 dd (12.9, 2.2)	1.25–1.56 m	1.25–1.43 m
11	4.96 s	1.20–1.23 m	1.23 br d (11.8)
12		1.25–1.56 2H, m	1.25–1.43 2H, m
14	6.24 d (1.9)	1.63 m	1.89 ddd (13.2, 13.2, 5.2)
15	7.36 d (1.9)	1.80 ddd (12.4, 12.4, 5.2)	2.26 ddd (12.4, 12.4, 4.7)
16	2.07 s	5.91 dd (8.0, 1.1)	5.80 dd (7.4, 1.4)
17		9.91 d (8.0)	9.96 d (7.4)
18	1.62 dd (3.6, 2.2)	1.59 s	1.48 d (1.4)
19	1.03 s	3.00 t (10.2)	3.01 dd (10.4, 6.9)
20	1.14 s	3.41 dd (10.2, 4.4)	3.42 dd (10.4, 5.2)
		1.59 d (1.1)	1.59 sext. like
		0.96 s	0.95 s
		0.60 s	0.58 s

* Measured in CDCl_3 , [†] Coupling constants (J in Hz) are given in parentheses.

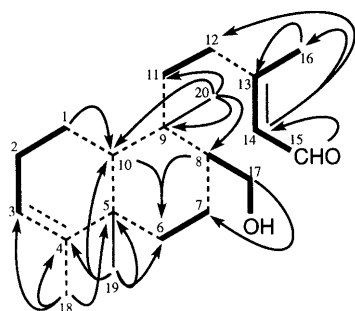
and exchange spectroscopy (NOESY) spectrum of **1** showed NOEs between (i) H-11 and H-8, H-10, (ii) H-10 and H-8, H-6 β , (iii) H-19 and H-1 α , H-6 α , H-7 α , H-20, and (iv) H-20 and H-1 α , H-7 α , H-19, respectively. Conclusive evidence of the structure of **1** was obtained by X-ray crystallographic analysis as shown in Fig. 2. Thus, the relative stereochemistry of infuscolide A was assigned as 12,15-epoxy-3,12,14-clerodatrien-17,11-olide (**1**). The circular dichroism (CD) spectrum of **1** indicated first positive (296 nm), second negative (240 nm) and third positive (226 nm) Cotton effects, however, the absolute configuration of **1** was not obtained by this analysis.

The EIMS of **2** showed m/z 304 $[\text{M}]^+$, and its molecular formula was found to be $\text{C}_{20}\text{H}_{32}\text{O}_2$ by HR-EIMS. The IR spectrum confirmed the presence of a hydroxyl (3420 cm^{-1}) and a carbonyl group (1670 cm^{-1}). Its ^1H -NMR (Table 1) showed the signals of a formyl proton, a methylene proton bearing a hydroxyl group, two olefinic protons and two tertiary methyls and two olefinic methyls. The ^{13}C -NMR (Table 2) and distortionless enhancement by polarization transfer (DEPT) spectra suggested the presence of two trisubstituted double bonds, a formyl carbon, a methylene bearing a hydroxyl group, together with four methyls, six methylenes, two methines and two quaternary carbons, respectively. These NMR spectra resembled those of *trans*-clerodanes **4**–**12**. The $^1\text{H}-^1\text{H}$ and long-range $^1\text{H}-^{13}\text{C}$ correlations of **2** were confirmed by the $^1\text{H}-^1\text{H}$ COSY, HMQC and HMBC spectra as shown in Fig. 3. On the basis of the above spectral evidence, the structure of **2** was suggested to be a *trans*-clerodane-type diterpenoid with a hydroxyl and a formyl group. In addition, the NOESY spectrum showed NOEs between (i) H-15 and H-16, (ii) H-12 and H-14, and (iii) H-20 and H-19, H-17. From these results, the geometry of the 13,14-double bond is *E*. Thus, the structure of **2** was established as 17-hydroxy-3,13*E*-clerodadien-15-al.

The IR spectrum of **3** showed the presence of a hydroxyl

Table 2. ^{13}C -NMR Data of **1**—**3** (150 MHz, C_6D_6)

C	1 *	2	3 †
1	19.2	18.0	18.16
2	26.0	27.0	27.1
3	120.6	120.9	120.9
4	143.0	143.9	144.0
5	38.0	38.17‡	38.2
6	37.1	36.6	36.7
7	16.6	22.3	22.6
8	54.4	44.3	44.4
9	49.1	38.23‡	38.7
10	51.0	46.7	46.6
11	83.3	36.3	38.0
12	143.9	34.2	26.8
13	120.9	163.1	163.8
14	113.1	127.5	127.7
15	142.4	189.9	189.9
16	10.2	17.2	24.8
17	175.1	64.0	64.3
18	18.3	18.2	18.24
19	21.5	20.0	20.0
20	11.4	19.3	19.1

* Measured in CDCl_3 . † Measured by 100 MHz. ‡ May be interchanged.Fig. 3. ^1H — ^1H Correlations (Bold Lines) and Long-Range ^1H — ^{13}C Correlations (Arrow) of **2**

(3325 cm^{-1}) and an unsaturated carbonyl group (1670 cm^{-1}). The molecular formula of **3**, $\text{C}_{20}\text{H}_{32}\text{O}_2$ (m/z 304.2380 $[\text{M}]^+$), was confirmed by HR-EIMS. The ^1H - and ^{13}C -NMR spectra (Tables 1 and 2) were almost similar to those of **2**, indicating that compound **3** was a *trans*-clerodane-type diterpenoid. Furthermore, detailed analysis of ^1H — ^1H COSY, HMQC and HMBC spectra showed that compound **3** was the geometrical isomer of **2**. The NOESY spectrum of **3** showed NOEs between (i) H-16 and H-14, (ii) H-15 and H-12, (iii) H-20 and H-19, H-17, respectively. Thus, the structure of **3** was confirmed as 17-hydroxy-3,13Z-clerodadien-15-al.

The absolute configuration of **1**—**3** could be the same as that of **8** which was established by X-ray crystallographic analysis of the carbamate derived from **8**,⁷⁾ coexisting in the same species.

At present, four chemo-types of *J. infusca*; [I] *ent*-kaurane-type, [II] clerodane- and labdane-type, [III] bis(bibenzyl)-type and [IV] cuparane- and diterpene-type, are known.^{3,6)} The present *J. infusca*, which contains clerodane- and labdane-type diterpenoids as its main constituents, is classified as type [II].

Experimental

Melting points were measured on a Yanagimoto micromelting points apparatus without correction. Optical rotations were measured on a Jasco DIP-1000 polarimeter with CHCl_3 . IR spectra were recorded on a Jasco FT/IR-

5300 infrared spectrophotometer. The ^1H - and ^{13}C -NMR spectra were measured on Varian Unity-600 (^1H ; 600 MHz, ^{13}C ; 150 MHz) and Jeol Eclipse-400 (^1H ; 400 MHz, ^{13}C ; 100 MHz) instruments. Chemical shift values expressed in δ (ppm) downfield from tetramethylsilane as an internal standard (^1H -NMR), and δ 77.03 (ppm) from CDCl_3 and δ 128.0 (ppm) from C_6D_6 as a standard (^{13}C -NMR). Mass spectra were obtained on a Jeol JMS AX-500 instrument. X-ray crystallographic analysis was carried out on a Mac Science DIP-2020 instrument. TLC was carried out using Silica gel 60F₂₅₄ plates (Merck). Column chromatography was performed on Silica gel 60 (Merck, 230—400 and 35—70 mesh), reverse phase silica gel (Cosmosil 140C₁₈, Nacalai), Sephadex™ LH-20 (Amersham Pharmacia Biotech, sol. CH_2Cl_2 -MeOH 1:1) and Lobar® (LiChroprep, Merck) columns. TLC plates were examined under UV (254 nm) light and by spraying with 10% H_2SO_4 or Godin reagent,¹⁴⁾ followed by heating.

Plant Material *Jungermannia infusca* (MITT.) STEPH. (9509501) was collected in Higashikawadani, Ehime pref. Japan. Dr. M. Mizutani (The Hattori Botanical Laboratory, Miyazaki, Japan) identified this species and a voucher specimen was deposited at the Institute of Pharmacognosy, Tokushima Bunri University.

Extraction and Isolation The ether extract (4.5 g) of *J. infusca* was divided into nine fractions by column chromatography (CC) on silica gel (35—70 mesh) using an *n*-hexane-EtOAc gradient solvent system. Cleroda-3,13(16),14-triene (**4**, 30 mg) was isolated from fr. 1. fr. 2 was chromatographed on silica gel (230—400 mesh, *n*-hexane-EtOAc 19:1) and Sephadex™ LH-20 to give clerod-3,13(16),14-trien-17-al (**5**, 30 mg). Fraction 3 was divided into five fractions (3-1—3-5) by CC on Sephadex™ LH-20 and silica gel (230—400 mesh, *n*-hexane-EtOAc 9:1). Gomerdehyde (**13**, 163 mg) and isoabienol (**15**, 391 mg) were isolated from fr. 3-2 and fr. 3-4, respectively. *epi*-Gomerdehyde (**14**, 37 mg) was purified by preparative HPLC (NUCLEOSIL 50-5, *n*-hexane-EtOAc 9:1) of fr. 3-3. Fraction 3-5 was also purified by preparative HPLC (NUCLEOSIL 50-5, *n*-hexane-EtOAc 9:1) to give δ -tocopherol (**17**, 16 mg) ($[\alpha]_D^{19} +1.0^\circ$ $c=1.53$; lit.¹⁵⁾ $[\alpha]_D^{25} +3.4^\circ$ $c=15.48$, EtOH. $[\alpha]_D^{25} +1.1^\circ$ $c=10.86$, benzene).

Fraction 4 was divided into three fractions (4-1—4-3) by CC on Sephadex™ LH-20 and reverse phase silica gel (Cosmosil 140C₁₈, CH_3CN). Fraction 4-1 was subjected to preparative HPLC (NUCLEOSIL 50-5, *n*-hexane-EtOAc 4:1) to give infuscolide A (**1**, 3 mg) and cleroda-3,13Z-diene-15,17-dial (**11**, 4 mg). Fraction 4-2 was methylated, and rechromatographed on silica gel (230—400 mesh, *n*-hexane-EtOAc 4:1) to give the methyl ester (33 mg) of infuscaic acid (**6**) and cleroda-3,13(16),14-trien-17-ol (**7**, 34 mg). Fraction 5 was rechromatographed on Sephadex™ LH-20 and silica gel (230—400 mesh, CH_2Cl_2 -Et₂O 97:3) to give infuscaic acid (**6**, 306 mg) and cleroda-3,13E-diene-15,17-dial (**9**, 25 mg).

Fraction 7 was chromatographed on Sephadex™ LH-20 and reverse phase silica gel (Cosmosil 140C₁₈, CH_3CN) to yield 15-oxo-3,13Z-clerodadien-17-oic acid (**10**, 86 mg) and a mixture of diterpenes. The diterpene fraction was rechromatographed on silica gel (230—400 mesh, CH_2Cl_2 -EtOAc 19:1) to give 17-hydroxy-3,13E-clerodadien-15-al (**2**, 5 mg), 17-hydroxy-3,13Z-clerodadien-15-al (**3**, 3 mg) and 15-hydroxy-3,13E-clerodadien-17-al (**12**, 3 mg). Fraction 8 was purified by CC on Sephadex™ LH-20 and Lobar® (RP-8 40—63 μm , CH_3CN) to give 13-*epi*-sclareol (**16**, 147 mg) and 15-oxo-3,13E-clerodadien-17-oic acid (**8**, 223 mg).

Infuscolide A (**1**): mp 130—132 °C. $[\alpha]_D^{23} -90.9^\circ$ ($c=1.22$). FT-IR cm^{-1} : 1780 (lactone). UV (MeOH) λ_{max} (log ϵ): 222 nm (3.73) ($c=3.4 \times 10^{-4}$). CD (MeOH): $\Delta\epsilon_{296\text{nm}} +0.82$, $\Delta\epsilon_{240\text{nm}} -0.17$, $\Delta\epsilon_{226\text{nm}} +0.25$ ($c=3.4 \times 10^{-4}$). ^1H - and ^{13}C -NMR: Tables 1 and 2. HR-EIMS: obs. 314.1870 $\text{C}_{20}\text{H}_{26}\text{O}_3$ requires 314.1882. EIMS m/z (int.): 314 $[\text{M}]^+$ (26), 176 (73), 161 (100), 147 (12), 133 (32), 121 (28), 111 (53), 95 (19), 81 (15), 69 (10), 55 (12), 44 (27).

X-ray crystallographic analysis of **1**: All diagrams were prepared and calculations were performed using maXus (MacScience, Japan). Data collection: DIP Image plate. Data reduction: maXus. Program used to solve the structure: maXus SIR92. Program used to refine the structure: maXus. Crystal data: $\text{C}_{20}\text{H}_{26}\text{O}_3$, M.W.=314, monoclinic, $P2_1$, $a=0.377(0)$ Å, $b=10.021(0)$ Å, $c=17.15800(10)$ Å, $V=1774.199951(0)$ Å³, $Z=4$, $\text{MoK}\alpha$ ($\lambda=0.71073$ Å), $D_x=1.530$ Mg/cm³, $\theta=1$ —20°, $\mu=0.77$ mm⁻¹, 2823 reflections, 426 parameters, $R=0.056$, $R_w=0.141$, $S=2.325$, only coordinates of H atoms refined, Extinction correction: no atomic scattering factors.¹⁶⁾

17-Hydroxy-3,13E-clerodadien-15-al (**2**): $[\alpha]_D^{20} -94.5^\circ$ ($c=0.44$). FT-IR cm^{-1} : 3418 (OH), 1670 (C=O). ^1H - and ^{13}C -NMR: Tables 1 and 2. HR-EIMS: obs. 304.2430 $\text{C}_{20}\text{H}_{32}\text{O}_2$ requires 304.2402. EIMS m/z (int.): 304 $[\text{M}]^+$ (11), 302 (16), 286 (24), 252 (19), 205 (33), 187 (41), 145 (33), 119 (60), 107 (55), 95 (100), 81 (52), 69 (31), 55 (35), 45 (6).

17-Hydroxy-3,13Z-clerodadien-15-al (**3**): $[\alpha]_D^{14} -152.9^\circ$ ($c=0.48$). FT-IR cm^{-1} : 3325 (OH), 1670 (C=O). ^1H - and ^{13}C -NMR: Tables 1 and 2. HR-

EIMS obs. m/z 304.2380 $C_{20}H_{32}O_2$ requires 304.2402. EIMS m/z (int.): 304 $[M]^+$ (24), 271 (6), 253 (9), 241 (5), 205 (52), 187 (20), 159 (19), 145 (28), 119 (41), 107 (51), 95 (100), 81 (36), 67 (18), 55 (22), 41 (21).

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References and Notes

- 1) Asakawa Y., "Progress in the Chemistry of Organic Natural Products," Vol. 42, ed. by Herz W., Grisebach H., Kirby G. W., Springer, Vienna, 1982, pp. 1—285.
- 2) Asakawa Y., "Progress in the Chemistry of Organic Natural Products," Vol. 65, ed. by Herz W., Grisebach H., Kirby G. W., Moore R. E., Steiglich W., Tamm Ch., Springer, Vienna, 1995, pp. 1—562.
- 3) Asakawa Y., *Heterocycles*, **46**, 795—848 (1997).
- 4) Asakawa Y., "Phytochemicals in Human Health Protection, Nutrition, and Defense," ed. by Romeo, Kluwer Academic/Plenum Publishers, New York, 1999, pp. 319—342.
- 5) Nagashima F., Asakawa, Y., "Recent Research Developments in Phytochemistry," Vol. 2, Part II, ed. by Pandalai S. G., Research Signpost, India, 1998, pp. 327—382.
- 6) Nagashima F., Suzuki M., Takaoka S., Asakawa Y., *Tetrahedron*, **55**, 9117—9132 (1999).
- 7) Nagashima F., Takaoka S., Asakawa Y., *Phytochemistry*, **49**, 601—608 (1998).
- 8) Toyota M., Nagashima F., Asakawa Y., *Phytochemistry*, **28**, 3415—3419 (1989).
- 9) Toyota M., Nagashima F., Asakawa Y., *Phytochemistry*, **28**, 2507—2509 (1989).
- 10) Bohlmann F., Zdero C., King R. M., Robinson H., *Phytochemistry*, **20**, 1657—1663 (1981).
- 11) Chirkova M. A., Gorbunova A. E., Lisina A. I., Pentegova V. A., *Khim. Priro. Soedin.*, **2**, 99—104 (1966).
- 12) Torrenegra R., Pedrozo J., Robles J., Waibel R., Achenbach H., *Phytochemistry*, **31**, 2415—2418 (1992).
- 13) Matsuo M., Urano S., *Tetrahedron*, **32**, 229—231 (1976).
- 14) Godin P., *Nature* (London), **174**, 134 (1954).
- 15) Stern M. H., Robenson C. D., Weisler L., Baxter J. G., *J. Am. Chem. Soc.*, **69**, 869—874 (1947).
- 16) Waasmaier D., Kirfel A., *Acta Cryst.*, **A51**, 416—431 (1995).

Application of Rh-Catalyzed Cyclization to the Formation of a Chiral Quaternary Carbon

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Rh-Catalyzed cyclization was applied to the formation of a chiral quaternary carbon. It has become clear that the Rh-complex can discriminate between isopropenyl and 2-isopentenyl (or isopentyl) substituents, and the cyclization afforded 3,3,4-trisubstituted cyclopentanones with a chiral quaternary carbon in a stereoselective manner. The cyclization of 4-pentenals **6a, b by an achiral neutral $\text{Rh}(\text{PPh}_3)_3\text{Cl}$ afforded 3,3,4-*cis*-trisubstituted cyclopentanones ($\pm$)-**7a, b** in 86–96%, and the cyclization by a cationic $\text{Rh}[(R)\text{-BINAP}]\text{ClO}_4$ afforded 3,3,4-*trans*-trisubstituted cyclopentanones (–)-**8a, b** of 82–86% ee in 88–98% yields. The mechanism of stereoselection by Rh-complexes is also discussed.**

Key words Rhodium-catalyzed cyclization; quaternary carbon; cyclopentanone; enantioselective; diastereoselective

Catalytic asymmetric process to produce a chiral quaternary carbon is especially fascinating to organic chemists because both the formation of the chiral quaternary carbons and the catalytic asymmetric reactions using transition metals are interesting.¹⁾ Recently, we reported that cyclization using Rh-complex²⁾ could be applicable for the concurrent induction of two chiral centers from symmetrical 4-pentenals³⁾ and, furthermore, the reaction could be used to produce a chiral quaternary carbon.⁴⁾ Here, we wish to report the Rh-catalyzed cyclization of 4-pentenals⁵⁾ bearing a quaternary carbon, which is composed of diisopropenyl, formylmethyl, and 2-isopentenyl (or isopentyl) groups. For enantio- and diastereoselective cyclization, the Rh-complex and BINAP ligand need to discriminate between isopropenyl and 2-isopentenyl substituents. The structure of these two substituents seems to be very similar. When the substituent at the quaternary carbon is a 2-isopentenyl group, the substrate **6a** is a 5-pentenal as well as a 4-pentenal.⁶⁾

¹H–¹H-NMR spectrum. A correlation between the methyl proton signals δ : 0.89 (3H, d) at the C(4)-position and the methyl signals δ : 1.74 (3H, brs) of the isopropenyl group was observed. Cyclization by a neutral $\text{Rh}[(R)\text{-BINAP}]\text{Cl}$ was unsuccessful, due to the low catalytic activity of the neutral $\text{Rh}[(R)\text{-BINAP}]\text{Cl}$. The cyclization by a cationic $\text{Rh}[(R)\text{-BINAP}]\text{ClO}_4$ proceeded smoothly to produce *trans*-(–)-**8a** in 98% yield. In the NOESY ¹H–¹H-NMR spectrum of **8a**, a correlation between the methylene signals δ : 2.10 (1H, dd) and 2.32 (1H, dd) of the 2-isopentenyl group and the methyl signals δ : 1.07 (3H, d) at the C(4)-position was observed. The ratio of diastereomers was 2 (*cis*-**7a**) to 98 (*trans*-**8a**). The reaction required only 5 mol% of Rh-complex, and the starting material **6a** disappeared after 2 h at room temperature. The cyclization of **6a** by the cationic $\text{Rh}[(S)\text{-BINAP}]\text{ClO}_4$ afforded an enantiomer; (+)-**8a** in 96% yield. The cyclization of **6b** by the Rh-complex gave similar results to those of **6a**. The selectivity of *cis* and *trans* was a little lower

Results and Discussion

Preparation of 4-Pentenals Acetylacetone **1** was converted into ester **2** by alkylation with methyl bromoacetate in 92% yield. The ester **2** was again alkylated with 2-isopentenyl bromide to afford β -keto ester **3a** bearing a quaternary carbon in 71% yield. Olefination of the 1,3-dicarbonyl function in **3a** with Nysted reagent⁷⁾ gave diene **4a** in 74% yield. Moreover, hydrogenation of the olefin function in **3a**, followed by olefination, afforded diene **4b** in 60% overall yield. The dienes **4a, b** were converted into the corresponding aldehydes **6a, b** by reduction with LiAlH_4 and subsequent oxidation with PCC (**6a**: 73% from **4a**, and **6b**: 62% from **4b**), respectively (Chart 1).

Rh-Catalyzed Cyclization The results of cyclization by Rh-complexes are summarized in Table 1. The cyclization of **6a** by an achiral $\text{Rh}(\text{PPh}_3)_3\text{Cl}$ afforded 3,3,4-*cis*-trisubstituted cyclopentanones in 96% yield, as a major product. The ratio of *cis* and *trans* was determined to be 91 (*cis*-**7a**) to 9 (*trans*-**8a**), based on the ratio of methyl proton signals at δ : 1.07 (3H, d, $J=6.9$ Hz) and δ : 0.89 (3H, d, $J=6.9$ Hz) in the ¹H-NMR spectrum. The relative configuration of **7a** was unambiguously determined to be 3,4-*cis* from the NOESY

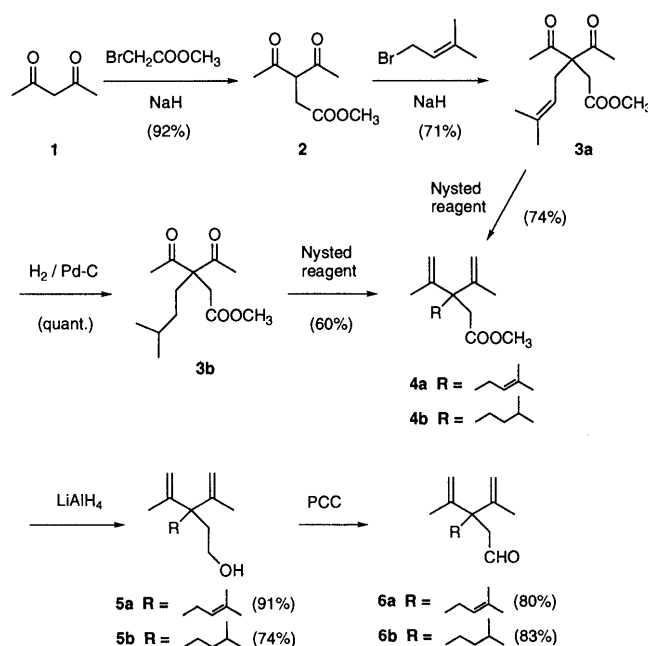
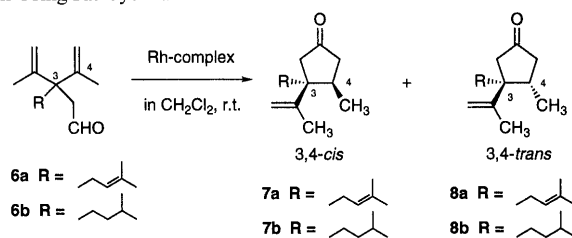


Chart 1

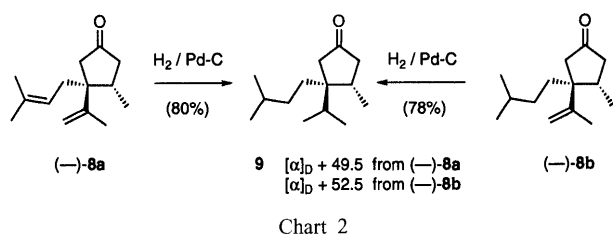
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Table 1. Formation of a Quaternary Carbon Using Rh-Cyclization



Entry	Substrate	Rh-complex (eq)	Reaction time (h)	Isolated yield (%) ^{a)}	cis/trans	Opt. purity of major product (% ee)	[α] _D
1	6a	Rh(PPh ₃) ₃ Cl (0.5)	24	96	91/9	—	—
2	6a	Rh[(<i>R</i>)-BINAP]ClO ₄ (0.05)	2	98	2/98	86	−16.2
3	6a	Rh[(<i>S</i>)-BINAP]ClO ₄ (0.05)	2	96	2/98	82	+16.2
4	6b	Rh(PPh ₃) ₃ Cl (0.5)	24	86	88/12	—	—
5	6b	Rh[(<i>R</i>)-BINAP]ClO ₄ (0.05)	2	88	4/96	85	−81.9
6	6b	Rh[(<i>S</i>)-BINAP]ClO ₄ (0.05)	2	90	3/97	86	+83.1

a) Products **7** and **8** were obtained as inseparable mixtures of *cis* and *trans*.



than that of **6a**, both with the Rh(PPh₃)₃Cl and the cationic Rh[BINAP]ClO₄, as shown by entries 4–6 in Table 1.

The enantiomeric excess of (−)-**8a, b** and (+)-**8a, b** was determined by the ¹H-NMR spectra, after conversion of the ketone into (*R,R*)-2,3-butanediol acetal. For example, the (*R,R*)-2,3-butanediol acetal derived from (−)-**8a** showed olefinic proton signals at δ: 4.77 (br s) and 4.74 (br s) in the ratio of 2 to 28, while that of (+)-**8a** showed δ: 4.77 (br s) and 4.74 (br s) in the ratio of 30 to 3 in the ¹H-NMR spectra. The ¹³C-NMR spectra of the (*R,R*)-2,3-butanediol acetals derived from (−)- and (+)-**8b** also supported the enantiomeric excess. The hydrogenation of (−)-**8a**, which was cyclized by the cationic Rh[(*R*)-BINAP]ClO₄ gave cyclopentanone **9** in 80% yield, and the hydrogenation of (−)-**8b** also afforded the same product **9** in 78% yield. The specific rotation of **9** prepared from (−)-**8a** showed [α]_D²⁶ +49.5°, and that of **9** from (−)-**8b** also showed [α]_D²⁸ +52.5°, as shown in Chart 2. This result means that no difference of enantioselection exists between the cyclization of **6a** and **6b**.

Plausible Mechanism for Stereoselection The stereoselectivity in the Rh-catalyzed cyclization could be explained by the plausible acyl–hydride intermediates.^{3,4)} The cyclization of **6** by Rh(PPh₃)₃Cl afforded 3,3,4-*cis*-trisubstituted cyclopentanone (**±**)-**7**. The *cis*-product **7** would be obtained by cyclization through a favorable intermediate (i). Considering the plausible acyl–hydride intermediates, the intermediate (ii), which would produce 3,3,4-*trans*-trisubstituted cyclopentanone **8**, seems to be less stable than intermediate (i) because the unfavorable steric repulsion between the isopropenyl group and the Rh metal would be stronger than that between the 2-isopentenyl group and the Rh metal (Fig. 1).

Cyclization by the cationic Rh[(*R*)-BINAP]ClO₄ afforded

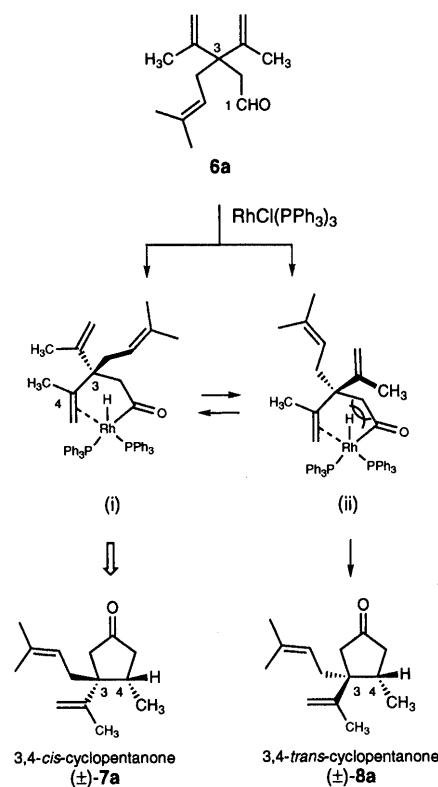


Fig. 1. Plausible Acyl–Hydride Rhodium Intermediate for the Rh(PPh₃)₃Cl-Catalyzed Cyclization

3,3,4-*trans*-trisubstituted cyclopentanone (−)-**8**. To produce the 3,3,4-*trans*-trisubstituted cyclopentanone **8**, the reaction needs to proceed not by way of the favorable intermediate (iv), but by the unfavorable intermediate (iii) where the repulsion between the isopropenyl group and the Rh metal exists. We have already reported that cyclization by the cationic Rh-complex proceeded by way of the unfavorable acyl–hydride intermediate to give the 3,3,4-*trans*-trisubstituted cyclopentanone. The cyclization of **6a, b** by the cationic Rh[(*R*)-BINAP]ClO₄ seems to proceed by way of the unfavorable intermediate (iii) where two repulsions exist. One is the repulsion between the methyl group at the C(4)-position

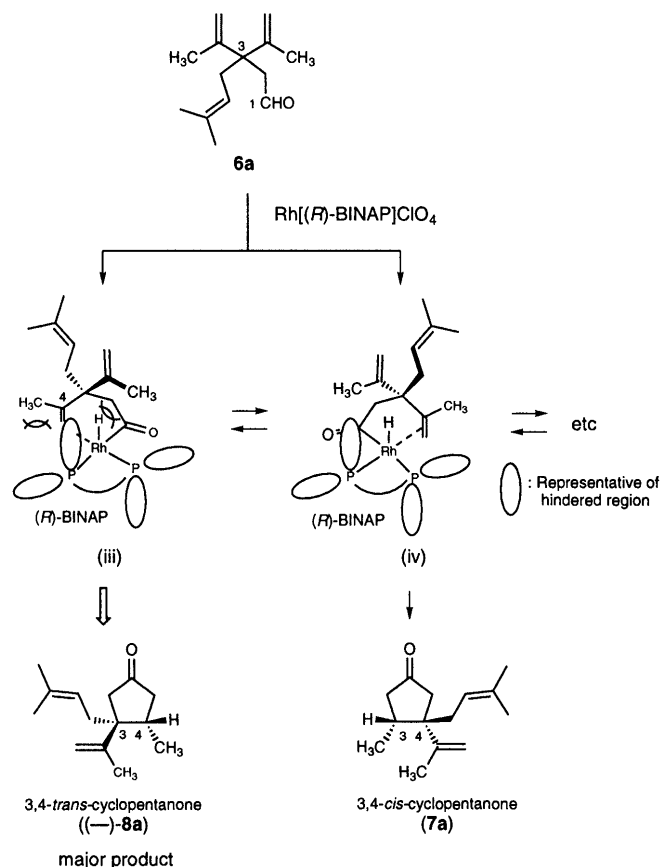


Fig. 2. Plausible Acyl-Hydride Rhodium Intermediate for the Cationic Rh-Catalyzed Cyclization

and the (*R*)-BINAP ligand, and the other is between the isopropenyl function and the Rh metal. Cyclization by way of intermediate (iii) would afford (3*S*,4*S*)-(-)-**8a**, as shown in Fig. 2.

Conclusion

The Rh-catalyzed cyclization was used to obtain cyclopentanones with a chiral quaternary carbon. It has become clear that the neutral $\text{Rh}(\text{PPh}_3)_3\text{Cl}$ could discriminate between 2-isopentenyl and isopropenyl substituents, and the cyclization afforded ($\pm$)-3,3,4-*cis*-trisubstituted cyclopentanones **7a**, **b**, stereoselectively. Also, it has been shown that the cationic $\text{Rh}[(R)\text{-BINAP}]\text{ClO}_4$ could discriminate between similar substituents, such as 2-isopentenyl and isopropenyl, and cyclization gives the optically active (-)-3,3,4-*trans*-trisubstituted cyclopentanones **8a**, **b** in a diastereo- and enantioselective manner.

Experimental

General Methods THF was purchased from Kanto Chemical Co., and used without distillation. Benzene and CH_2Cl_2 were distilled from P_2O_5 . (*R*)- and (*S*)-BINAP were purchased from Kanto Chemical Co. Inc. ^1H -NMR spectra were determined at 60, 270 or 500 MHz. Infrared spectra were recorded on a JASCO A-100 spectrometer. EI-MS, FAB-MS, and HRMS spectra were obtained on a JEOL JMS 610H, DX300, or SX102 spectrometer. General procedures used for syntheses followed those in previous reports.²⁻⁴⁾

3-Methoxycarbonylmethyl-2,4-pentanedione (2) A solution of acetylacetone (5.00 g, 50 mmol) in THF (20 ml) was added to a stirred suspension of NaH (2.40 g, 60%) in THF (60 ml) at 0°C, and the whole was stirred at room temperature for 10 min. Methyl bromoacetate (9.18 g, 60 mmol) in THF (20 ml) was added to the solution at 0°C, and stirred overnight at room temperature. The solution was diluted with brine, extracted with ether,

washed with brine, and dried over MgSO_4 . After removal of the solvent, the residue was purified by column chromatography on silica gel (30% EtOAc in hexane) to give **2** (7.90 g, 92%) as a colorless oil: IR (neat) cm^{-1} : 1730, 1600. ^1H -NMR (60 MHz, CDCl_3) δ : 4.13 (1H, t, $J=7$ Hz), 3.72 (3H, s), 2.89 (2H, d, $J=7$ Hz), 2.22 (6H, s). EI-MS m/z : 172 (M^+).

Methyl 3,3-Diacetyl-6-methyl-5-heptenoate (3a) Compound **3a** was prepared from **2** in a similar manner to that described for the preparation of **2**: 71% yield; a colorless oil; IR (neat) cm^{-1} : 1735, 1700. ^1H -NMR (270 MHz, CDCl_3) δ : 4.78 (1H, tm, $J=7.6$ Hz), 3.65 (3H, s), 2.98 (2H, s), 2.76 (2H, brd, $J=7.6$ Hz), 2.16 (6H, s), 1.68 (3H, brs), 1.58 (3H, s). FAB-MS m/z : 241 ($\text{M}^+ + \text{H}$).

Methyl 3,3-Diacetyl-6-methylheptanoate (3b) A suspension of **3a** (1.00 g, 4.2 mmol) and Pd-C (10%, 1.00 g) in MeOH (50 ml) and THF (10 ml) was stirred under an H_2 atmosphere for 2 h. The Pd catalyst was filtered off, and the solution was evaporated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel. The fraction eluted with 10% EtOAc in hexane afforded **3b** (1.00 g, quant.) as a colorless oil: IR (neat) cm^{-1} : 1720, 1700. ^1H -NMR (270 MHz, CDCl_3) δ : 3.64 (3H, s), 2.96 (2H, s), 2.14 (6H, s), 1.98 (2H, m), 1.51 (1H, m), 0.93 (2H, m), 0.87 (6H, d, $J=6.6$ Hz). FAB-MS m/z : 243 ($\text{M}^+ + \text{H}$).

Methyl 3,3-Diisopropenyl-6-methyl-5-heptenoate (4a) A solution of **3a** (6.00 g, 25 mmol) in THF (100 ml) was added dropwise to a vigorously stirred suspension of Nysted reagent (20% suspension in THF, 148 g) in THF (100 ml) at -78°C , and stirred for 15 min. Then, TiCl_4 (5.6 ml) was added dropwise to the stirred mixture at -78°C , and then the whole was warmed to room temperature, and stirred for a further 30 min. The mixture was diluted with water, and extracted with EtOAc. The extract was washed with 5% aqueous NaHCO_3 , brine, and dried over MgSO_4 . After removal of the solvent, the oily residue was purified by column chromatography on silica gel (1% EtOAc in hexane) to give **4a** (4.40 g, 74%) as a colorless oil: IR (neat) cm^{-1} : 1735, 1635. ^1H -NMR (270 MHz, CDCl_3) δ : 4.96 (2H, s), 4.95 (1H, m), 4.79 (2H, s), 3.58 (3H, s), 2.58 (2H, s), 2.48 (2H, brd, $J=6.9$ Hz), 1.70 (3H, brs), 1.66 (3H, brs), 1.62 (6H, brs). FAB(+)-HRMS m/z : 237.1851 (Calcd for $\text{C}_{15}\text{H}_{25}\text{O}_2$ ($\text{M}^+ + \text{H}$): 237.1854).

Methyl 3,3-Diisopropenyl-6-methylheptanoate (4b) Compound **4b** was prepared from **3b** in a similar manner to that described for the preparation of **4a**: 60% yield; a colorless oil; IR (neat) cm^{-1} : 1740, 1635. ^1H -NMR (270 MHz, CDCl_3) δ : 4.95 (2H, m), 4.77 (2H, brs), 3.59 (3H, s), 2.61 (2H, s), 1.68 (2H, m), 1.60 (6H, brs), 1.52 (1H, m), 1.05 (2H, m), 0.90 (6H, d, $J=6.9$ Hz). FAB(+)-HRMS m/z : 239.2006 (Calcd for $\text{C}_{15}\text{H}_{27}\text{O}_2$ ($\text{M}^+ + \text{H}$): 239.2011).

3,3-Diisopropenyl-6-methyl-5-hepten-1-ol (5a) A solution of **4a** (5.90 g, 25 mmol) in ether (50 ml) was added dropwise to a stirred suspension of LiAlH_4 (950 mg, 25 mmol) in ether (150 ml) at room temperature, and stirred for 5 h. The reaction was quenched with EtOAc and H_2O , then filtered through celite, and the filtrate dried over MgSO_4 . Removal of the solvent afforded an oily residue, which was purified by column chromatography on silica gel (4% EtOAc in hexane) to give **5a** (4.70 g, 91%) as a colorless oil: IR (neat) cm^{-1} : 3320 (br), 1635. ^1H -NMR (270 MHz, CDCl_3) δ : 4.97 (2H, m), 4.94 (1H, m), 4.86 (2H, brs), 3.52 (2H, t, $J=7.4$ Hz), 2.25 (2H, brd, $J=6.6$ Hz), 1.83 (2H, t, $J=7.4$ Hz), 1.69 (3H, brs), 1.63 (3H, brs), 1.58 (6H, brs), 1.42 (1H, br). FAB-MS m/z : 231 ($\text{M}^+ + \text{Na}$), 207 ($\text{M}^+ - \text{H}$).

3,3-Diisopropenyl-6-methyl-1-heptanol (5b) Compound **5b** was prepared from **4b** in a similar manner to that described for the preparation of **5a**: 74% yield; a colorless oil; IR (neat) cm^{-1} : 3320 (br), 1630. ^1H -NMR (270 MHz, CDCl_3) δ : 4.96 (2H, m), 4.85 (2H, brs), 3.54 (2H, t, $J=7.4$ Hz), 1.86 (2H, t, $J=7.4$ Hz), 1.57 (6H, brs), 1.41—1.60 (4H, m), 0.97 (2H, m), 0.89 (6H, d, $J=6.6$ Hz). EI-MS m/z : 210 (M^+ , 3), 195 (44), 165 (24), 151 (22), 139 (100).

3,3-Diisopropenyl-6-methyl-5-heptenal (6a) A mixture of **5a** (1.04 g, 5.00 mmol), PCC (1.10 g, 5.10 mmol), and NaOAc (100 mg, 1.20 mmol) in CH_2Cl_2 (30 ml) was stirred at room temperature for 3 h. The mixture was diluted with ether, and filtered through florisil to remove chromate. The filtrate was concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel. The fraction eluted with 2% ether in pentane afforded **6a** (824 mg, 80%) as a colorless oil: IR (neat) cm^{-1} : 1720, 1635. ^1H -NMR (270 MHz, CDCl_3) δ : 9.59 (1H, t, $J=3.0$ Hz), 5.04 (2H, brs), 4.96 (1H, dm, $J=7.0$ Hz), 4.86 (2H, brs), 2.49 (2H, d, $J=3.0$ Hz), 2.41 (2H, brd, $J=7.0$ Hz), 1.70 (3H, brs), 1.63 (6H, brs), 1.62 (3H, brs). FAB(+)-HRMS m/z : 207.1751 (Calcd for $\text{C}_{14}\text{H}_{23}\text{O}_1$ ($\text{M}^+ + \text{H}$): 207.1749).

3,3-Diisopropenyl-6-methylheptanal (6b) Compound **6b** was prepared from **5b** in a similar manner to that described for the preparation of **6a**: 83% yield; a colorless oil; IR (neat) cm^{-1} : 1720, 1630. ^1H -NMR (270 MHz, CDCl_3) δ : 9.58 (1H, t, $J=3.0$ Hz), 5.03 (2H, m), 4.84 (2H, brs), 2.54 (2H, d,

$J=3.0$ Hz), 1.65 (2H, m), 1.61 (6H, brs), 1.50 (1H, m), 1.01 (2H, m), 0.88 (6H, d, $J=6.6$ Hz). EI-MS m/z : 208 (M^+ , 2), 193 (4), 190 (6), 175 (5), 168 (23), 151 (35), 137 (100). HRMS m/z : 208.1830 (Calcd for $C_{14}H_{24}O$ (M^+): 208.1827).

(3*R*,4*SR*)-(±)-3-Isopropenyl-3-(3-methyl-2-butenyl)-4-methylcyclopentanone (7a) A solution of **6a** (103 mg, 0.50 mmol) in CH_2Cl_2 (2 ml) was added dropwise to a stirred solution of $Rh(PPh_3)_3Cl$ (231 mg, 0.25 mmol) in CH_2Cl_2 (3 ml), and the solution was stirred overnight at room temperature. Removal of the solvent afforded a residue, which was dissolved in ether (30 ml), and the precipitated Rh-complex was filtered off. After removal of ether, the oily residue was purified by column chromatography on silica gel to afford (±)-**7a** (99 mg, 96%, *cis/trans*=91/9) as a colorless oil: IR (neat) cm^{-1} : 1740, 1640. ^1H -NMR (270 MHz, $CDCl_3$) δ : 4.94 (1H, brs), 4.89 (1H, m), 4.61 (1H, brs), 2.62 (1H, dd, $J=7.9$, 18.8 Hz), 2.41 (1H, d, $J=17.8$ Hz), 2.20–2.32 (2H, m), 1.95–2.13 (3H, m), 1.74 (3H, brs), 1.67 (3H, brs), 1.54 (3H, brs), 0.89 (3H, d, $J=6.9$ Hz). ^{13}C -NMR (125.7 MHz, $CDCl_3$) δ : 218.9, 146.0, 134.0, 119.6, 112.7, 52.2, 45.1, 44.5, 37.5, 34.8, 25.9, 19.5, 18.0, 17.5. EI-MS m/z : 206 (M^+ , 6), 163 (11), 150 (6), 138 (18), 123 (16), 109 (26), 95 (31), 69 (100). HRMS m/z : 206.1666 (Calcd for $C_{14}H_{22}O$ (M^+): 206.1671).

(3*R*,4*SR*)-(±)-3-Isomyl-3-isopropenyl-4-methylcyclopentanone (7b) Cyclization of **6b** by $Rh(PPh_3)_3Cl$ afforded **7b** in 86% yield (*cis/trans*=88/12): a colorless oil; IR (neat) cm^{-1} : 1745, 1640. ^1H -NMR (270 MHz, $CDCl_3$) δ : 4.92 (1H, brs), 4.61 (1H, brs), 2.58 (1H, dd, $J=7.9$, 19.0 Hz), 2.47 (1H, d, $J=17.8$ Hz), 2.25 (1H, m), 2.11 (1H, d, $J=19.0$ Hz), 1.98 (1H, dm, $J=17.8$ Hz), 1.71 (3H, brs), 1.60 (1H, m), 1.44 (1H, m), 1.24 (1H, m), 0.90–0.99 (2H, m), 0.93 (3H, d, $J=6.6$ Hz), 0.83–0.90 (6H, m). ^{13}C -NMR (125.7 MHz, $CDCl_3$) δ : 218.9, 146.1, 112.7, 51.8, 44.9, 44.5, 38.5, 34.4, 33.5, 28.4, 22.7, 22.4, 19.4, 17.5. EI-MS m/z : 208 (M^+ , 2), 152 (4), 138 (21), 123 (23), 95 (16), 82 (100). HRMS m/z : 208.1827 (Calcd for $C_{14}H_{24}O$ (M^+): 208.1827).

(3*S*,4*S*)-(–)-3-Isopropenyl-3-(3-methyl-2-butenyl)-4-methylcyclopentanone (–)-(8a**)⁸** A solution of $Rh[(NBD)(R)-BINAP]ClO_4$ (23 mg, 0.025 mmol) in CH_2Cl_2 (4 ml) was stirred under an H_2 atmosphere at room temperature for 2 h. Then, Ar gas was bubbled into the solution for 15 min. This bright red solution of $Rh[(R)-BINAP]ClO_4$ was used for the cyclization without isolation. A solution of **6a** (103 mg, 0.50 mmol) in CH_2Cl_2 (3 ml) was added dropwise to the stirred solution of $Rh[(R)-BINAP]ClO_4$ under an Ar atmosphere. After being stirred at room temperature for 2 h, the solution was concentrated *in vacuo* to leave a residue. The residue was dissolved in ether (20 ml), and the precipitated Rh-complex was filtered off. After removal of the solvent, the residue was purified by column chromatography on silica gel to give (–)-**8a** (101 mg, 98%, *cis/trans*=2/98) as a colorless oil: $[\alpha]_D^{20} -16.17^\circ$ ($c=1.45$, $CHCl_3$). IR (neat) cm^{-1} : 1735, 1630. ^1H -NMR (270 MHz, $CDCl_3$) δ : 4.92–5.02 (2H, m), 4.74 (1H, brs), 2.50 (1H, dd, $J=8.5$, 19.0 Hz), 2.38–2.45 (2H, m), 2.31 (1H, dd, $J=5.9$, 15.2 Hz), 2.22 (1H, d, $J=18.3$ Hz), 2.07 (1H, dd, $J=7.9$, 15.2 Hz), 1.93 (1H, dd, $J=6.5$, 19.0 Hz), 1.77 (3H, brs), 1.67 (3H, brs), 1.58 (3H, brs), 1.07 (3H, d, $J=6.9$ Hz). ^{13}C -NMR (125.7 MHz, $CDCl_3$) δ : 218.6, 147.3, 133.4, 120.3, 112.5, 50.5, 47.6, 44.9, 36.6, 30.7, 25.9, 20.3, 18.0, 15.6. EI-MS m/z : 206 (M^+ , 18), 163 (27), 137 (25), 123 (18), 109 (35), 95 (37), 69 (100). HRMS m/z : 206.1669 (Calcd for $C_{14}H_{22}O$ (M^+): 206.1671).

(3*R*,4*R*)-(+)-3-Isopropenyl-3-(3-methyl-2-butenyl)-4-methylcyclopentanone (+)-(8a**)** Cyclization of **6a** by $Rh[(S)-BINAP]ClO_4$ afforded (+)-**8a** in 96% yield (*cis/trans*=2/98). $[\alpha]_D^{20} +16.23^\circ$ ($c=1.13$, $CHCl_3$).

Determination of Enantiomeric Excess of 8a The enantiomeric excesses of **8a** were determined from the ^1H -NMR spectra of the acetals derived from (*R,R*)-2,3-butanediol. A mixture of cyclopentanone **8a** (15 mg, 0.07 mmol), (*R,R*)-butanediol (20 mg, 0.22 mmol), and *p*-TsOH- H_2O (5 mg) in benzene (20 ml) was refluxed for 3 h in a Dean-Stark apparatus. After being cooled to room temperature, the solution was washed with 5% aqueous $NaHCO_3$, brine, and dried over $MgSO_4$. After removal of the solvent, the residue was briefly purified by column chromatography on silica gel to give the crude acetal. The ^1H -NMR spectrum of the butanediol acetal of (–)-**8a** cyclized by $Rh[(R)-BINAP]ClO_4$ showed olefinic proton signals at δ 4.77 (brs) and 4.74 (brs) in the ratio of 2 to 28, while that from (+)-**8a** cyclized by $Rh[(S)-BINAP]ClO_4$ showed olefinic signals at δ 4.77 (brs) and 4.74

(brs) in the ratio of 30 to 3.

(3*S*,4*S*)-(–)-3-Isoamyl-3-isopropenyl-4-methylcyclopentanone (–)-(8b**)** Cyclization of **6b** by $Rh[(R)-BINAP]ClO_4$ afforded (–)-**8b** in 88% yield (*cis/trans*=4/96). $[\alpha]_D^{25} -81.92^\circ$ ($c=1.43$, $CHCl_3$). IR (neat) cm^{-1} : 1745, 1635. ^1H -NMR (270 MHz, $CDCl_3$) δ : 4.92 (1H, brs), 4.68 (1H, brs), 2.45–2.55 (2H, m), 2.38 (1H, m), 2.16 (1H, dd, $J=1.2$, 18.3 Hz), 1.85 (1H, dm, $J=18.3$ Hz), 1.75 (3H, brs), 1.61 (1H, td, $J=6.6$, 15.2 Hz), 1.47 (1H, m), 1.32 (1H, td, $J=6.6$, 15.2 Hz), 1.04 (3H, d, $J=6.9$ Hz), 0.90–1.01 (2H, m), 0.88 (3H, d, $J=6.6$ Hz), 0.86 (3H, d, $J=6.6$ Hz). ^{13}C -NMR (125.7 MHz, $CDCl_3$) δ : 218.6, 147.5, 112.6, 50.4, 46.7, 45.0, 36.1, 34.1, 30.9, 28.6, 22.7, 22.5, 19.9, 16.2. EI-MS m/z : 208 (M^+ , 3), 165 (3), 152 (6), 138 (22), 123 (28), 110 (9), 95 (14), 82 (100). HRMS m/z : 208.1835 (Calcd for $C_{14}H_{24}O$ (M^+): 208.1827).

(3*R*,4*R*)-(+)-3-Isoamyl-3-isopropenyl-4-methylcyclopentanone (+)-(8b**)** Cyclization of **6b** by $Rh[(S)-BINAP]ClO_4$ afforded (+)-**8b** in 90% yield (*cis/trans*=3/97). $[\alpha]_D^{25} +83.13^\circ$ ($c=1.15$, $CHCl_3$).

Determination of Enantiomeric Excess of 8b The ^1H -NMR spectrum of the (*R,R*)-2,3-butanediol acetal of (–)-**8b** cyclized by $Rh[(R)-BINAP]ClO_4$ showed olefinic proton signals at δ 4.78 (brs) and 4.76 (brs) in the ratio of 2 to 24, while that from (+)-**8b** cyclized by $Rh[(S)-BINAP]ClO_4$ showed olefinic signals at δ 4.78 (brs) and 4.76 (brs) in the ratio of 27 to 2. The enantiomeric excesses were also supported by the ^{13}C -NMR spectra. (*R,R*)-2,3-Butanediol acetal of (–)-**8b**: ^{13}C -NMR (125.7 MHz, $CDCl_3$, major peaks) δ : 148.9, 115.4, 110.9, 78.3, 78.0, 51.3, 47.1, 45.7, 39.7, 34.0, 29.2, 28.8, 22.8, 22.7, 20.8, 17.2, 17.1, 15.0. (*R,R*)-2,3-Butanediol acetal of (+)-**8b**: ^{13}C -NMR (125.7 MHz, $CDCl_3$, major peaks) δ : 148.8, 115.7, 111.1, 78.2, 77.9, 51.4, 46.8, 45.5, 39.2, 34.0, 30.4, 28.8, 22.7, 22.7, 20.6, 17.3, 17.2, 15.3.

(3*S*,4*S*)-3-Isoamyl-3-isopropyl-4-methylcyclopentanone (9) A suspension of (–)-**8a** (41.4 mg, 0.20 mmol) and 10% Pd-C (80 mg) in MeOH (6 ml) was stirred under an H_2 atmosphere for 2 h. The Pd catalyst was filtered off, and the filtrate was concentrated *in vacuo* to leave an oily residue, which was purified by column chromatography on silica gel. The fraction eluted with 2% EtOAc in hexane afforded **9** (34 mg, 80%) as a colorless oil: $[\alpha]_D^{26} +49.51^\circ$ ($c=1.15$, $CHCl_3$). IR (neat) cm^{-1} : 1730. ^1H -NMR (270 MHz, $CDCl_3$) δ : 1.93–2.49 (4H, m), 1.81 (1H, m), 1.11–1.59 (4H, m), 1.07 (3H, d, $J=6.9$ Hz), 0.95–1.00 (2H, m), 0.87–0.94 (12H, m). EI-MS m/z : 210 (M^+ , 10), 195 (6), 167 (18), 139 (19), 111 (19), 97 (31), 84 (40), 69 (100). HRMS m/z : 210.1980 (Calcd for $C_{14}H_{26}O$ (M^+): 210.1984). Compound (–)-**8b** was also converted into **9** (78%) in the similar conditions: $[\alpha]_D^{28} +52.52^\circ$ ($c=1.30$, $CHCl_3$).

References and Notes

- Corey E. J., Guzman-Perez A., *Angew. Chem. Int. Ed.*, **37**, 388–401 (1998).
- a) Sakai K., Ide J., Oda O., Nakamura N., *Tetrahedron Lett.*, **13**, 1287–1290 (1972); b) Taura Y., Tanaka M., Wu X.-M., Funakoshi K., Sakai K., *Tetrahedron*, **47**, 4879–4888 (1991); c) K., J. Sakai, *Synth. Org. Chem. (Jpn)*, **51**, 733–743 (1993); d) Fujio M., Tanaka M., Wu X. M., Funakoshi K., Sakai K., Suemune H., *Chem. Lett.*, **1998**, 881–882.
- Wu X.-M., Funakoshi K., Sakai K., *Tetrahedron Lett.*, **34**, 5927–5930 (1993).
- Tanaka M., Imai M., Fujio M., Sakamoto E., Takahashi M., Eto-Kato Y., Wu X.-M., Funakoshi K., Sakai K., Suemune H., *J. Org. Chem.*, **5806**–5816 (2000).
- a) Ducray P., Rousseau B., Mioskowski C., *J. Org. Chem.*, **64**, 3800–3801 (1999); b) Bosnich B., *Acc. Chem. Res.*, **31**, 667–674 (1998); c) Barnhart R. W., McMorran D. A., Bosnich B., *Chem. Commun.*, **1997**, 589–590.
- Koga I., Funakoshi K., Matsuda A., Sakai K., *Tetrahedron Asym.*, **4**, 1857–1868 (1993).
- Nysted reagent is commercially available from Aldrich Co.: Matsubara S., Sugihara M., Utimoto K., *Synlett*, **1998**, 313–315.
- The absolute configuration of products was assumed based on the plausible intermediates and the previous results of Rh-catalyzed cyclizations.

Two Novel Actinidine-Type Monoterpene Alkaloids from *Incarvillea delavayi*

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Two new actinidine-type monoterpene alkaloids, delavayines B (1) and C (2), were isolated from the MeOH extract of the aerial parts of *Incarvillea delavayi*, a close species of which, *I. sinensis*, is used as an analgesic for rheumatic pain in China, and the structures have been elucidated on the basis of spectroscopic evidence.

Key words *Incarvillea delavayi*; actinidine-type monoterpene alkaloid; delavayine B; delavayine C

We previously reported several new monoterpene alkaloids from the Chinese medical plant *Incarvillea sinensis*,^{1–5)} including incarvillateine, which showed antinociceptive and anti-inflammatory activity.^{6,7)} Subsequently, we obtained a related antinociceptive monoterpene alkaloid from *Incarvillea delavayi* Bureau & Franchet.⁸⁾ Here, we report the isolation and structure determination of two additional new actinidine-type monoterpene alkaloids, delavayines B (1) and C (2) from *I. delavayi*.

The isolation of the monoterpene alkaloids 1 and 2 was performed by extracting the dried aerial parts of *I. delavayi* with MeOH, followed by fractionation of the crude extract by a variety of column chromatographic procedures (Diaion HP-20, Chromatorex and Si gel) to afford 1 (0.00080%, dry weight) and 2 (0.0011%, dry wt).

Compound 1 showed an $[M+H]^+$ ion peak at m/z 356 in the positive-mode FAB-MS, and its molecular formula was established as $C_{21}H_{25}NO_4$ by HR-FAB-MS. The ^{13}C -NMR signals of 1 were resembled to those of an actinidine-type alkaloid⁹⁾ [δ 139.2 (d), 140.9 (d), 133.0 (s), 161.8 (s), 148.8 (s), 31.1 (t), 33.7 (t), 37.8 (d), 19.2 (q)] and a 2-substituted dihydrocinnamate group [δ 136.6 (s), 128.5 (d), 128.7 (d), 126.9 (s), 41.0 (t), 78.6 (d), 167.5 (s)]. The HMBC data confirmed the actinidine skeleton and allowed the placement of the dihydrocinnamate group at N-2 [connectivities between the C-8' carbon (δ 78.6 d) and the H-1, H-3, H-7a' and H-7b' protons (δ 8.42 s, 8.48 s, 3.37 m and 4.04 m, respectively)]. The two methoxy groups (δ_H 3.17 and δ_H 3.29 correlated with C-10 (δ 98.5), indicating the presence of a dimethyl acetal functionality (Fig. 1). Therefore, the structure

of delavayine B was established as 1, although the configurations at C-8, 8' were not determined. The MS and NMR data indicated that 1 was isolated as a zwitterion, as shown.¹⁰⁾

The ^1H - and ^{13}C -NMR spectra of 2 were similar to those of 1 for the actinidine portion of the molecule, but indicated a different substituent at N-2 [δ_H 2.55 (2H, m), 3.00 (1H, m), 3.17 (1H, m) and 5.20 (2H, m); δ_C 174.7 (s), 31.8 (t), 27.8 (t) and 60.7 (t)]. These signals were assignable to a γ -substituted butyrate moiety. The HMBC spectrum showed correlations between the H-4' and C-1, C-3, C-3' and C-2' signals; thus, the location of the butyrate side chain was assigned to N-2. Therefore, the structure of 2 was established, although again the stereochemistry at C-8 was not determined.

Investigation of the potential pharmacological activity of these compounds is now progress.

Experimental

General Procedures Optical rotations were taken with a JASCO DIP-360 polarimeter. The ^1H - and ^{13}C -NMR were recorded in pyridine- d_5 solution on JEOL JNM-GX-400 and JEOL α -500 spectrometers, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. ^{13}C -NMR assignments were determined from HMQC and HMBC spectra. FAB-MS and HR-FAB-MS were recorded on a JEOL DX-303HF mass spectrometer. Column chromatography was carried out on silica gel 60 (spherical, 40–100 mesh, Kanto Chemicals), Chromatorex Chromatography Silica gel (DM-1020, 100–200 mesh, FUJI Silylia) and Diaion HP-20 (Mitsubishi Chemicals). TLC was performed on precoated Kieselgel 60 F₂₅₄ (0.2 mm, Merck), and spots and bands were viewed by UV light (254 and 366 nm). TLC solvent system was $CHCl_3$ -MeOH- H_2O (7:3:0.5).

Plant Material The bulbs of *Incarvillea delavayi* Bureau & Franchet (Bignoniaceae) were purchased from Heiwa-en Ltd., Tenri, Japan. The plants were grown at the Botanical Garden of the Faculty of Pharmaceutical Sciences, Kumamoto University, and collected in June 1998. A voucher specimen is kept at the same garden.

Extraction and Separation Dried aerial parts of *Incarvillea delavayi* (1.5 kg) were extracted with MeOH at room temperature. The MeOH extract was concentrated under reduced pressure to a syrup (yield 128 g), and was partitioned with 80% MeOH (MeOH- H_2O 8:2) and n -hexane. The 80% MeOH-sol. part was repeatedly chromatographed over Diaion HP-20 column with H_2O -MeOH (10:0–0:10), silica gel column with $CHCl_3$ -

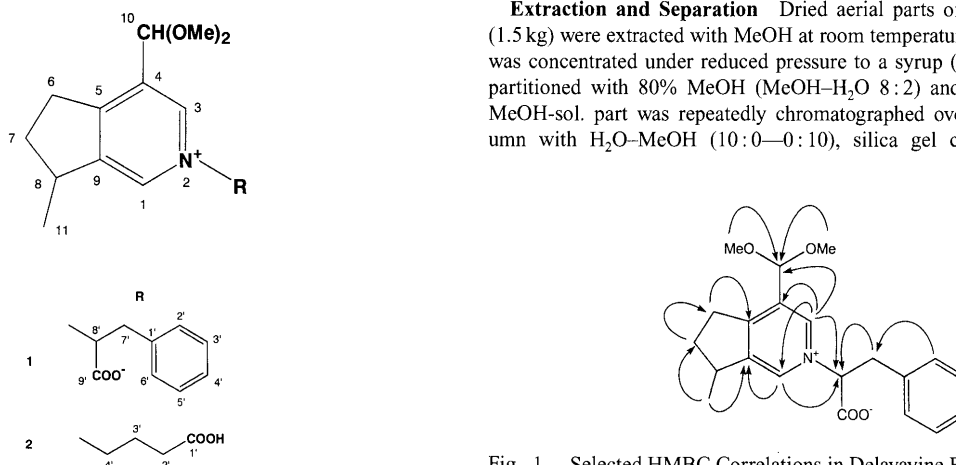


Fig. 1. Selected HMBC Correlations in Delavayine B (1)

MeOH-H₂O (30:1:0 to 6:4:1) and chromatorex column with CHCl₃-MeOH-NH₃ soln. (9:1:0.1 to 6:4:1) to afford compounds delavayine B (1, 12 mg) and C (2, 17 mg).

Compound 1: A off-white powder, $[\alpha]_D^{24} -6.5^\circ$ ($c=1.8$, pyridine). Positive FAB-MS m/z : 356 $[M+H]^+$, negative FAB-MS m/z : 354 $[M-H]^-$. HR-FAB-MS m/z : 356.1861 $[M+H]^+$ (Calcd for C₂₁H₂₅NO₄: 356.1862). ¹H-NMR (pyridine-*d*₅) δ : 1.25 (3H, d, $J=8.0$ Hz, H-11), 1.73 (1H, m, H-7a), 2.45 (1H, m, H-7b), 3.00 (1H, m, H-6a), 3.13 (1H, m, H-6b), 3.17 (3H, s, OMe), 3.29 (3H, s, OMe), 3.33 (1H, m, H-8), 3.37 (1H, m, H-7'a), 4.04 (1H, m, H-7'b), 5.29 (1H, br d, $J=4.7$ Hz, H-8'), 5.43 (1H, s, H-10), 7.01 (2H, d, $J=7.0$ Hz, H-2'), 7.12 (2H, t, $J=7.0$ Hz, H-3'), 7.15 (1H, t, $J=7.0$ Hz, H-4'), 8.42 (1H, s, H-1), 8.48 (1H, s, H-3). ¹³C-NMR (pyridine-*d*₅) δ : 19.2 (C-11), 31.1 (C-6), 33.7 (C-7), 37.8 (C-8), 41.0 (C-7'), 52.4 (OMe), 53.1 (OMe), 78.6 (C-8'), 98.5 (C-10), 126.9 (C-4'), 128.5 (C-2', 6'), 128.7 (C-3', 5'), 133.0 (C-4), 136.6 (C-1'), 139.2 (C-1), 140.9 (C-3), 148.8 (C-9), 161.8 (C-5), 167.5 (C-9').

Compound 2: A off-white powder, $[\alpha]_D^{24} -72.3^\circ$ ($c=0.5$, MeOH). ¹H-NMR (pyridine-*d*₅) δ : 1.37 (3H, d, $J=7.0$ Hz, H-11), 1.56 (1H, m, H-7a), 2.26 (1H, m, H-7b), 2.55 (2H, m, H-3'), 2.73 (2H, m, H-6), 3.00–3.17 (2H, m, H-2'), 3.36 (3H, s, OMe), 3.38 (1H, m, H-8), 3.40 (3H, s, OMe), 5.20 (2H, m, H-4'), 5.72 (1H, s, H-10), 9.28 (1H, s, H-3), 9.80 (1H, s, H-1). ¹³C-NMR (pyridine-*d*₅) δ : 19.3 (C-11), 27.8 (C-3'), 31.6 (C-6), 31.8 (C-2'), 34.0 (C-7), 38.4 (C-8), 53.5 (OMe), 54.0 (OMe), 60.7 (C-4'), 99.9 (C-10), 134.8 (C-4), 141.1 (C-3), 141.5 (C-1), 150.5 (C-9), 163.0 (C-5), 174.7 (C-1').

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References

- 1) Chi Y., Hashimoto F., Yan W., Nohara T., *Phytochemistry*, **39**, 1485–1487 (1995).
- 2) Chi Y., Hashimoto F., Yan W., Nohara T., *Phytochemistry*, **40**, 353–354 (1995).
- 3) Chi Y., Hashimoto F., Yan W., Nohara T., *Phytochemistry*, **46**, 763–769 (1997).
- 4) Chi Y., Hashimoto F., Yan W., Nohara T., Yamashita M., Marubayashi N., *Chem. Pharm. Bull.*, **45**, 495–498 (1997).
- 5) Nakamura M., Chi Y., Kinjo J., Yan W., Nohara T., *Phytochemistry*, **51**, 595–597 (1999).
- 6) Nakamura M., Chi Y., Yan W., Nakasugi Y., Yoshizawa T., Irino N., Hashimoto F., Kinjo J., Nohara T., Sakurada S., *J. Nat. Prod.*, **62**, 1293–1294 (1999).
- 7) Chi Y., Hashimoto F., Nohara T., Nakamura M., Yoshizawa T., Yamashita M., Marubayashi N., “38th Symposium on the Chemistry of Natural Products,” Sendai, Japan, October 14, 1996, Symposium Papers, pp. 43–48.
- 8) Nakamura M., Kido K., Kinjo J., Nohara T., *Phytochemistry*, **53**, 253–256 (2000).
- 9) Shiao M., Chia W., Peng C., Shen C., *J. Org. Chem.*, **58**, 3162–3164 (1993).
- 10) Kinjo J., Matsumoto K., Inoue M., Takeshita T., Nohara T., *Chem. Pharm. Bull.*, **39**, 116–119 (1991).

Preparation and Characterization of Acrylic Hydrogels Neutralized by Basic Amino Acids

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Basic amino acids were used as neutralizers in the gelation of Eudispert[®] as an acrylic hydrogel. Arginine and lysine successfully neutralized Eudispert[®], as did sodium hydroxide, and formed hydrogels. A gentle rise of pH was observed as the dosage of the base increased when arginine and lysine were used, in contrast to the sharp rise of pH observed when sodium hydroxide was used. The rank of viscosity of the prepared hydrogels was as follows: lysine > arginine > NaOH. The release rate of model drugs (salicylic acid, theophylline, and bovine insulin) from the prepared hydrogels ranked as follows: NaOH > lysine > arginine, the sustained-release profile being observed with arginine. The rate of diffusion of the model drug from the hydrogel was inversely proportional to the molecular weight of the cationized neutralizer used. It is concluded that the strategy of neutralization of acidic polymers by basic amino acids has advantage with the respect to both the sustained-release characteristics of the gel and the biocompatibility of the basic amino acids themselves.

Key words basic amino acid; acrylic polymer; lysine; arginine; neutralizer; salicylic acid; theophylline; bovine insulin; hydrogel

Recently, gel formulation has been given attention in various fields. A gel can be defined as an apparently solid, often jelly-like material formed from a colloidal solution on standing. If the solvent used to prepare the gel is organic, the prepared gel is called as an organogel, while if water is used as a vehicle, the prepared product is called a hydrogel.¹⁾ Hydrogels in particular are not only used in formulation development for oral,^{2,3)} percutaneous^{4,5)} and rectal administration,^{6,7)} but also in various other fields.⁸⁾ Eudispert[®]⁹⁾ is a block-copolymer composed of methacrylic acid, and methacrylic acid methylester in the ratio of 2 : 1, and used as a coating material or enteric polymer. It forms the hydrogel in neutral or weak alkaline, but prepared gel gradually eroded into large amount of neutral or weak alkaline since the carboxy group is contained in the intramolecule. We have previously reported on this acrylic-type of hydrogel made from Eudispert[®], and examined its *in vitro* drug-release characteristics.¹⁰⁾ *In vivo* evaluations such as rectal retention, have also been carried out, and various advantages of this system have been demonstrated.⁶⁾ Sodium hydroxide has been used widely for the neutralization of acidic polymers. Sodium hydroxide may not be the best choice for patients with circulatory system diseases such as hypertension, however, because the sodium ion is discharged from the prepared hydrogel. We therefore decided to study the use of basic amino acids as neutralizers, as these acids are natural components of the human body, and biocompatible. In the present study, we set out to prepare Eudispert[®] hydrogels neutralized by basic amino acids such as arginine, lysine, and histidine, and to compare the fundamental physicochemical properties (*e.g.*, apparent viscosity, neutralization curve), and the release behavior of model drugs from these hydrogels with hydrogel neutralized by sodium hydroxide. As model drugs, salicylic acid and theophylline as a small molecular weight drug (M.W.=138, 180 respectively) and bovine insulin as a large molecular weight (M.W.=5733) were used.

Experimental

Materials Eudispert hv[®] was donated by Higuchi Inc., (Tokyo, Japan). Arginine, lysine, and histidine were provided by Otsuka Pharmaceutical Industry Co. Ltd., (Tokushima, Japan). The sodium hydroxide was from Nakalai Tesque Co., Kyoto, Japan. Salicylic acid, theophylline, and bovine insulin (grade V) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of special reagent grade.

The Preparation of Eudispert[®] Hydrogel Using Various Neutralizers Eudispert hv was added to purified water, and agitation was continued for 10 min. Various quantities of neutralizers were then added, and agitation continued. For de-aeration, prepared hydrogel was left overnight and the pH of the prepared gel was measured in the morning. The weight of each hydrogel batch was 20 g, the Eudispert[®] concentration was 7%, and the concentration of the various neutralizers was 1.0 mol/l. The pH measurements were performed using a pH meter (F-8DP, HORIBA, Tokyo, Japan).

The Measurement of Apparent Viscosity of Eudispert[®] Hydrogel Prepared by Various Neutralizers For the hydrogels prepared using basic amino acids as neutralizers, the apparent viscosity was measured at pH 6, 7, and 9. In the case of hydrogel neutralized by sodium hydroxide, measurements were made at pH 6, 7, 9, and 13. The viscosity measurements were performed using rotor No. 4 of the B-type viscosity meter (type BM, Tokyo Akeisoku Co. Ltd., Tokyo, Japan).

Preparation of Eudispert[®] Hydrogels Containing Model Drugs and Release Test Mixtures of purified water and model drugs (salicylic acid, theophylline and bovine insulin) were agitated thoroughly. Eudispert[®] was added to the mixtures which were then agitated again. Neutralizers were added and gelation was allowed to proceed gradually. The gel was left overnight at room temperature for de-aeration. The neutralizer concentration was also adjusted to 1.0 mol/l, Eudispert[®] concentration to 7.0% (w/w) of the whole gel and drug loading to 2.5% (w/w), respectively. The pH of hydrogel formulation was adjusted to 7.0.

The release test of the hydrogels prepared was performed as described in the Japanese Pharmacopeia JPXIII using the rotation basket method. A medium of pH 7.4 phosphoric acid buffer solution (500 ml) was employed and the test was carried out at 37 °C at 50 rpm. A known quantity of hydrogel preparation was gently injected into the basket using a syringe, and the test was started immediately. The sampling was carried out at 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, and 180 min. Five ml of the sample medium was taken, centrifuged (3000 rpm for 10 min) and the concentration of the model drug in the supernatant was analyzed. For the determination of salicylic acid and theophylline concentrations, absorbance was measured at 295 nm and 270 nm, respectively. The concentration of bovine insulin in the supernatant was determined using the HPLC method: 100 μ l was injected onto a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R6A) and a reversed phase column (Cosmosil 5C18-AR, 4.6 $\times$ 150 mm, Nakalai Tesque Co., Ltd.,

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Kyoto). The following mobile phase system was used: A, 0.1% trifluoroacetic acid (TFA) in H₂O; B, 0.1% TFA in acetonitrile. A linear gradient was used: phase B from 30% to 50% (10 min). The flow rate was 1.5 ml/min and the wavelength was set at 220 nm.

Results and Discussion

Arginine and lysine were successfully used to neutralize Eudispert[®], as was sodium hydroxide. Histidine had to be excluded from the study as 1.0 mol/l solution of histidine could not be prepared due to its low solubility. Amino acid solubilities in 100 g of water reported in the literature¹¹⁾ are arginine 15 g, histidine 4.2 g, lysine 111.5 g. These data agreed with our experimental findings.

Figure 1 shows the neutralization curves for sodium hydroxide, arginine, and lysine. When sodium hydroxide was used as the neutralizer, the pH of the neutralized polymer solution rapidly increased to over pH 12. This sharp rise of pH seems to be due to the strong alkaline characteristics of sodium hydroxide. On the contrary, a gentle rise of pH was observed as the dosage of the base increased when arginine and lysine were used for neutralization. This phenomenon seems to be due to the weak basic characteristics of arginine and lysine, and the pH of the gel did not exceed pH 10 even on further addition of bases.

The apparent viscosities of hydrogels prepared with the three neutralizers were measured. Figure 2 shows the relationship between the pH of the prepared hydrogel and the observed viscosity. When sodium hydroxide was used as a neutralizer, as the pH of prepared hydrogel increased from 6 to 9, the viscosity increased. However, the viscosity of the prepared hydrogel with pH 13 was less than that of hydrogel with pH 9. The reason for this decrease in pH seems to be dilution of the hydrogel or erosion of the polymer itself by sodium hydroxide. In other words, the gelation was almost complete at pH 9, and addition of excess base caused dilution of the prepared gel structure, and thereby decreased the viscosity. In the case of arginine, the viscosity data at pH 6 are not shown, since the gelation was not yet complete (the gel was not formed in acidic condition), and the sample solution was not uniform. When the pH of the prepared gel increased from 7 to 9, a uniform hydrogel was obtained, with a greater viscosity than those of the hydrogels neutralized using sodium hydroxide. The most viscous sample was prepared when lysine was used as a neutralizer. When the pH of the mixture of Eudispert[®] and lysine was 6, its viscosity was significantly greater than that of hydrogel neutralized by sodium hydroxide. As the pH of the hydrogel increased from 7 to 9, the viscosity rose to 100 000 cps, the maximum range for measurement. Its apparent viscosity was significantly greater than those of hydrogels neutralized by either sodium hydroxide or arginine.

The viscosities of hydrogels prepared using the various neutralizers clearly differed. The viscosity was the greatest for hydrogel prepared using lysine followed, in descending order, by arginine and sodium hydroxide.

Release Study of Hydrogel Containing Model Drugs

Figure 3 shows the drug-release profiles of the hydrogels prepared using the various neutralizers. The drug release rates from three Eudispert[®] hydrogels was different, and was ranked in the following order: NaOH>lysine>arginine for all three model drugs. The molecular weights of the neutralizers are arginine 174.2, lysine 146.2, NaOH 40.0, respec-

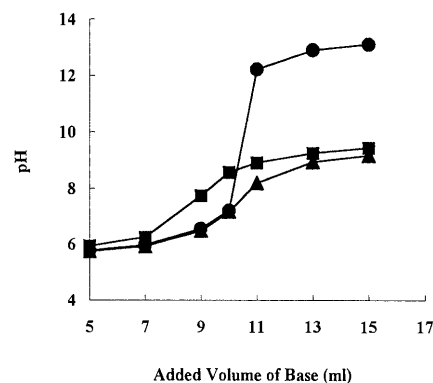


Fig. 1. Neutralization Curves of Eudispert hv[®] Using Sodium Hydroxide (●), Arginine (▲), and Lysine (■)

Data was the average of two experiments.

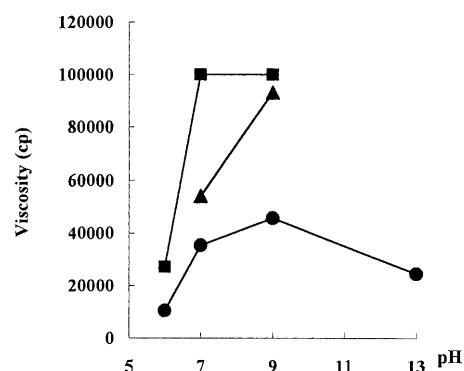


Fig. 2. Viscosity Curves of Eudispert hv[®] Neutralized by Sodium Hydroxide (●), Arginine (▲), and Lysine (■)

Data was the average of two experiments.

tively, and are thus ranked in the following order: arginine>lysine>NaOH.

The three-dimensional network structure which is assumed to be formed in Eudispert[®] hydrogel. There seems to be covalent bonds, ionic bonds, hydrogen bonds and intermolecular interactions in the gel structure, although we could not evaluate how much each bond or interaction contributed in the present Eudispert[®] hydrogel network structure. In general, the three-dimensional network structure controls the diffusion of water or any ionized molecule in hydrogel.¹²⁾ Therefore, it is likely that the three-dimensional structure of a gel containing a basic amino acid ion is tighter than a gel containing sodium ions, since a cationized basic amino acid is more bulky than a sodium ion. It was therefore considered that the diffusion of the drug from the hydrogel would be inversely proportional to the molecular weight of the cationized neutralizer. The present experimental results seem to agree with the above speculation. However, the quantitative relationship between the apparent hydrogel viscosity and release rates of the prepared gel could not be determined adequately. While the hydrogel made using lysine as a neutralizer was more viscous than that made using arginine, the gel neutralized by arginine showed the sustained-release profile for all model drugs used.

During the dissolution test, we observed that the erosion rate of Eudispert[®] gel neutralized by arginine was slower than that of gel neutralized by lysine (detail data not shown). Even though we did not determine precise erosion rates of

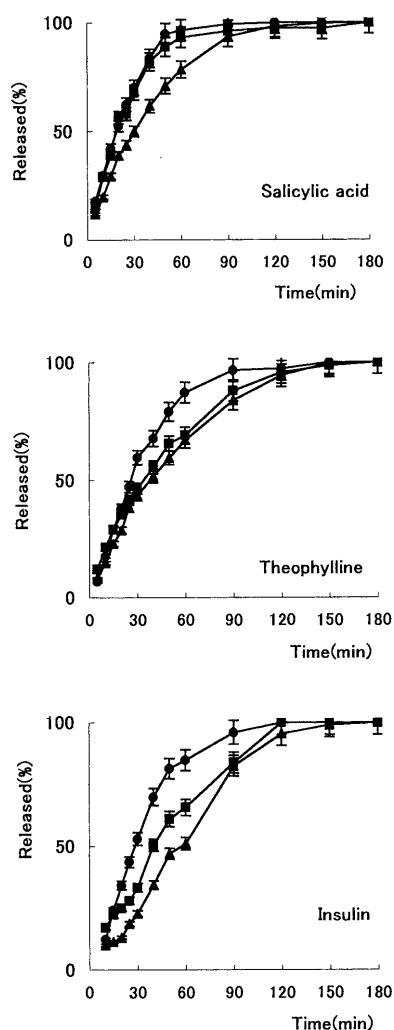


Fig. 3. Salicylic Acid, Theophylline, and Insulin Release (%) from Eudispert hv[®] Hydrogel Neutralized by Sodium Hydroxide (●), Arginine (▲), and Lysine (■)

Bars represent standard deviation of 3–4 experiments.

hydrogel in buffer medium, this phenomenon might be one reason for the better sustained-release characteristics of hydrogel made using arginine.

Other issues such as viscoelasticity or water content in prepared hydrogels have still to be resolved both *in vitro* and *in vivo*. However, in the present study, the strategy of neutral-

ization of acidic polymers by basic amino acids has been shown to be advantageous due to the sustained-release characteristics of the gels and the biocompatibility of the basic amino.¹³⁾ Recently, a new type of poly(amino acid)-glutaraldehyde hydrogel has been prepared.¹⁴⁾ This type of gel may be degraded by *in vivo* protease. Elisseeff *et al.*, have also reported on a hydrogel in which semi-interpenetrating networks of varying proportions of poly(ethylene oxide)-dimethacrylate and poly(ethylene oxide) and primary bovine articular chondrocytes were implanted in athymic mice.⁴⁾ Thus, amino acids and acrylic polymers may be suitable materials for development as carriers for drug delivery.

The present study indicates the possibility of neutralizing giant molecules using basic drugs in a hydrogel system. The concept demonstrated in the present study also suggests that basic drugs may be used as neutralizers for acidic polymers, or may be embedded in them.

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References

- 1) Chemical Soc. of Japan edition, "Polymer Handbook," Soc. of Polymer Sci., Japan edition, Asakura Co. Ltd., p. 210.
- 2) Machida Y., Tokumura T., Nagai T., *Drug Des. Delivery*, **1**, 179–186 (1987).
- 3) Yang L., Eshraghi J., Fasshihi R., *J. Control. Release*, **57**, 215–222 (1999).
- 4) Elisseeff J., Anseth K., Sims D., McIntosh W., Randolph M., Yaremchuk M., Langer R., *Plast. Reconstr. Surg.*, **104**, 1014–1022 (1999).
- 5) Sudo J., Iwase H., Terui J., Kakuno K., Soyama M., Takayama K., Nagai T., *Eur. J. Pharm. Sci.*, **7**, 67–71 (1998).
- 6) Umejima H., Kikuchi A., Kim N.-S., Uchida T., Goto G., *J. Pharm. Sci.*, **84**, 199–202 (1995).
- 7) Uchida T., Sakakibara S., Toida Y., Nagareya N., Yasuda N., Matsuyama K., *Pharm. Pharmacol. Commun.*, **5**, 523–527 (1999).
- 8) Belay A., Collins A., Ruzgas T., *J. Pharm. Biomed. Anal.*, **19**, 93–105 (1999).
- 9) Rohm Pharm. Information Sheet, "Eudispert".
- 10) Uchida T., Sekiya N., Toida Y., Yasuda N., Tazuya K., Nishikata M., Matsuyama K., *Chem. Pharm. Bull.*, **47**, 1655–1658 (1999).
- 11) Chemical Soc. of Japan edition, "kagakubinran," Maruzen Co. Ltd., 1993, pp. 431–434.
- 12) Peppas N. A., Modelling of drug diffusion through swellable polymeric systems, *J. Membrane Sci.*, **7**, 241–253 (1980).
- 13) Quong D., Yeo J. N., Neufeld R. J., *J. Microencapsul.*, **16**, 73–82 (1999).
- 14) Ohkawa K., Kitsuki T., Amaike M., Saitoh H., Yamamoto H., *Biomaterials*, **19**, 1855–1860 (1998).

Catalytic Activity of Anion-Exchange Resins Modified with Metal-Porphine in Oxidative Reactions of Phenols

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Anion-exchange resins modified with metal-porphine (M-P_r) have been investigated to develop a solid catalyst in the oxidative reaction of phenols by O₂ in air. Co-P_r, which is easily prepared and separable from the reaction mixture, has been proved to accelerate the oxidative reaction of phenols such as 3,5-di-*tert*-butyl-4-hydroxyanisole. The resulting main oxidative products were identified to be quinones by using the GC-MS method.

Key words oxidation catalyst; immobilized metal-porphine; oxidation of phenols

Metal complexes of porphines¹⁾ and salens^{1,2)} have been studied as oxidation catalysts in the oxidative reaction of phenols. Recent studies on metal-phthalocyanine functionalized silicas³⁾ and metal-salens encapsulated in zeolite^{4,5)} have provided noteworthy approaches to the development of a solid catalyst. Our previous studies on the catalytic activities of ion-exchange resins modified with metal-porphine (M-P_r) have revealed that Co- and Mn-P_r exhibit potent activities in oxidative reactions of uric acid and ascorbic acid by O₂ in air.^{6–10)} We have continuously studied the catalytic activity of M-P_r in oxidative reactions of phenols. In the present paper, we report that Co-P_r accelerates the oxidative reaction of phenols to a quinones as a solid catalyst.

Experimental

The H₂-porphines shown in Fig. 1 were converted into metal-complexes (M-P, M=Co³⁺, Mn³⁺, Fe³⁺, and Cu²⁺) by the methods described in the literatures.^{11,12)} M-P_r, in which 50 μmole of M-P was modified on 1.0 g of dry Amberlite IRA 900 (24–42 mesh) or dry Dowex MSC-1 (20–50 mesh), was prepared according to the simple method reported previously.^{6–8)}

Commercially available 3,5-di-*tert*-butyl-4-hydroxyanisole (DBHA), 2,6-di-*tert*-butyl-4-methylphenol (DBMP), and 2,6-di-*tert*-butylphenol (DBP) were purified by recrystallization. Authentic quinones used as standards were obtained from commercial sources and prepared by the methods published in the literature (see Notes).

GC-MS was measured by the electron-impact (EI) ionization method on a VG-70 SE mass spectrometer equipped with a Hewlett Packard 5896 GC instrument.

For evaluation of the oxidative catalytic activity, M-P_r (100 mg) was added to a methanol solution of phenols (5 mM, 10.0 ml), and the mixture was incubated at 35 °C for 1 h. The unchanged phenols and generated products in the supernatant, which was easily separable from M-P_r, were determined by the GC method using DBMP as an internal standard. For check of repeated uses, incubation was carried out for 2 h per use.

Results and Discussion

Figure 2 represents the time course of DBHA level in the

reaction mixture, which shows that most of the DBHA was consumed after 2-h incubation with Co-TSPP_r. Figure 3 represents a GC chart of the reaction mixture after 3-h incubation with Co-TSPP_r. As seen from Fig. 3, an extremely small peak of DBHA at 9.9 min and a very strong peak attributed to a main product at 7.2 min were observed. When the MS of the peak at 7.2 min was measured, a medium peak at 220 amu, which may be attributed to the ion of [2,6-*tert*-butyl-1,4-benzoquinone (*t*BBQ)]⁺, was observed. In addition, the MS fragment pattern and the retention time of the main product agreed with those obtained from a commercially available *t*BBQ. We therefore identified the product as *t*BBQ, which has been reported to be one oxidative product of DBP.¹⁾ Furthermore, since no decrease of DBHA was ob-

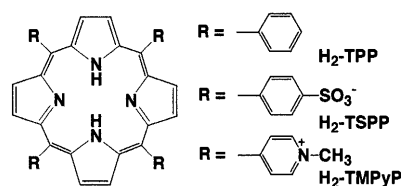


Fig. 1. Structures of H₂-Porphines

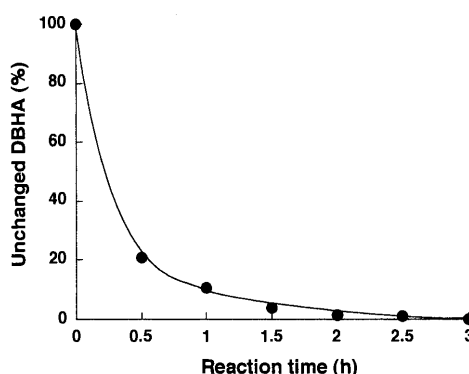


Fig. 2. Time Course of DBHA Level during Treatment with Co-TSPP_r. GC Conditions: an OV-1 column (0.5 mm×50 m), 150→250 °C (8 °C/min).

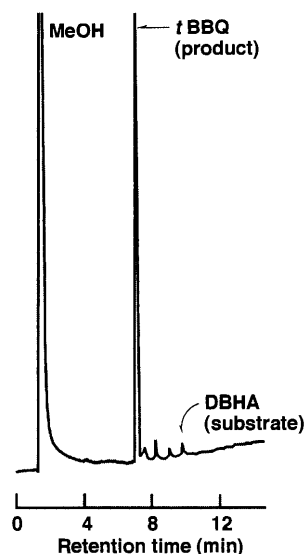


Fig. 3. GC of the Reaction Mixture Incubated for 3.0 h at 35 °C. GC Conditions: an OV-1 column (0.5 mm×50 m), 150→250 °C (8 °C/min).

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Table 1. Unchanged DBHA (%) after treatment with M-P_r^{a)}

M=	Co ³⁺	Mn ³⁺	Fe ³⁺	Cu ²⁺
M-TSPP _r	18	85	99	98
M-TMPyP _r	99	100	96	100

a) Incubated for 1.0 h at 35 °C in methanol.

served under an N₂-atmosphere, we confirmed that DBHA was mainly oxidized to *t*BBQ by O₂ in air.

Table 1 shows the unchanged DBHA (%) in the reaction mixture after treatment for 1 h with the individual modified resins. Co³⁺-TSPP_r resulted in the largest change of DBHA among the modified resins tested. When H₂-TSPP_r was used, 90% of DBHA remained unchanged even after 4-h treatment under the same conditions, which strongly suggests that the reaction is catalyzed by cobalt-ion in Co-TSPP_r. When Fe³⁺- and Cu²⁺-TSPP_r were used, almost 100% of DBHA remained unchanged, as seen from Table 1, suggesting that the central metal ions in the porphine ring are essential for the oxidation reaction of DBHA. It is of interest that M-TMPyP_r exhibited no notable activity. These results indicated that the oxidative activity of M-P_r depends not only on the central metal ion but also on side chains of the porphine ring. In the present study, since Co-TSPP_r showed the highest activity among the M-P_r tested, we decided to examine the activities of Co-TSPP_r in detail.

To confirm that Co-TSPP_r acts as a catalyst in the oxidative reaction of DBHA, its activity in the repeated uses was determined. After 10 time uses, Co-TSPP_r still had sufficient catalytic activity. This means that Co-TSPP could catalyze at least 100-fold moles of DBHA, since the molar ratio of Co-TSPP on the resin to DBHA in the initial reaction mixture was 1 : 10. Thus we concluded that Co-TSPP_r serves as a catalyst in the oxidative reaction of phenols. Furthermore, we compared the rate constants of phenol disappearance (*k*) catalyzed by Co-TSPP_r in methanol to those in CH₃CN used in previous studies.¹⁾ As a result, the *k*-value for DBHA in methanol was more than three times higher than that in CH₃CN. This result suggests that methanol is superior to CH₃CN as a solvent for Co-TSPP_r.

In addition to DBHA, we have investigated the catalytic activity of Co-TSPP_r on DBMP and DBP by using methods similar to those described above. As a result, 85% of

DBP was oxidized after 24-h incubation with Co-TSPP_r. The oxidative products included *t*BBQ (equivalent to 68% of DBP) and 3,3',5,5'-tetra-*tert*-butyldiphenquinone¹³⁾ (15% of DBP). This result is of interest from the view point of immobilization of the catalytic activity of Co-porphine, because the oxidative reaction of DBP was not catalyzed by Co-TPP (see Fig. 1) solution in CH₃CN after 24-h incubation.¹⁾ In the case of DBMP, although 100% of DBMP was converted into two main oxidative products, we could identify only 2,6-di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one¹⁴⁾ (equivalent to 57% of DBMP).

In conclusion, Co-TSPP_r exhibited the catalytic activity in the oxidative reactions of phenols such as DBHA, and is expected to be not only a practically applicable solid catalyst but also a model of diphenol oxidases active on catecholamines such as epinephrine and dopamine.

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References and Notes

- 1) Frsotin-Rio M., Pujol D., Bied-Charreton C., Perree-Fauvet M., Gaudemer A., *J. Chem. Soc., Perkin Trans. I*, **1984**, 1971–1979.
- 2) Nishinaga A., Tomiya H., Nishizawa K., Mtatoura T., *J. Chem. Soc., Dalton*, **1981**, 1504–1514.
- 3) Sorokin A. B., Tuel A., *Catalysis Today*, **57**, 45–59 (2000).
- 4) Jacob C. R., Varkey S. P., Ratnasamy P., *Appl. Catalysis A*, **168**, 353–364 (1998).
- 5) Jacob C. R., Varkey S. P., Ratnasamy P., *Micro. Meso. Material.*, **22**, 465–474 (1998).
- 6) Saito Y., Mifune M., Kawaguchi T., Tanaka Y., Chikuma M., Tanaka H., *Chem. Pharm. Bull.*, **34**, 2885–2889 (1986).
- 7) Saito Y., Mifune M., Nakashima S., Tanaka Y., Chikuma M., Tanaka H., *Chem. Pharm. Bull.*, **34**, 5016–5019 (1986).
- 8) Mifune M., Odo J., Iwado A., Saito Y., *Talanta*, **38**, 779–783 (1991).
- 9) Iwado A., Mifune M., Harada R., Akizawa, H., Motohashi N., Saito Y., *Inorg. Chim. Acta*, **283**, 44–50 (1998).
- 10) Iwado A., Mifune M., Hazawa T., Mukuno T., Oda J., Motohashi N., Saito Y., *Anal. Sci.*, **15**, 841–846 (1999).
- 11) Pasternack R. F., Francesconi L., Raff D., Spiro E., *Inorg. Chem.*, **12**, 2606–2611 (1973).
- 12) Harriman A., Porter G., *J. Chem. Soc., Faraday Trans. II*, **75**, 1532–1542 (1979).
- 13) Authentic compound, which was prepared by the method reported in *J. Am. Chem. Soc.*, **81**, 6335 (1959) by H. D. Kaesz *et al.*, was identified by using IR ¹H-NMR, MS, and element analysis.
- 14) Authentic compound, which was prepared by the method reported in *Bull. Chem. Soc. Jpn.*, **48**, 1683 (1975) by A. Nishinaga *et al.*, was identified by the methods of ¹H-NMR, IR, and element analysis.

First Total Synthesis of Mytiloxanthin

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The first total synthesis of mytiloxanthin 2 was accomplished via the cyclopentyl ketone 7 prepared by stereoselective rearrangement of the epoxide 4a.

Key words mytiloxanthin; stereoselective rearrangement; cyclopentyl compound; conjugated enolic β -diketone; total synthesis

Recently, we found¹⁾ that the acyclic-tetrasubstituted olefinic compounds and cyclopentyl ketones were derived by Lewis acid-promoted stereoselective rearrangement of epoxy compounds as shown in Chart 1. Then, the first biomimetic total synthesis of crassostreaxanthin B 1 (Fig. 1) with a novel acyclic-tetrasubstituted olefinic end group was accomplished²⁾ applying this rearrangement reaction.

Mytiloxanthin 2 (Fig. 1), containing a unique cyclopentyl enolic β -diketone group conjugated to the polyene chain, was first isolated from *Mytilus californianus* by Scheer³⁾ in 1940, and the structure and synthesis of the 9Z-isomer were reported by Weedon's group.⁴⁾ Its absolute configuration was determined by Maoka and Fujiwara⁵⁾ in 1996. The cyclopentyl end group of mytiloxanthin 2 is biogenetically believed⁴⁾ to arise from a pinacollic rearrangement of the epoxy end group of 5,6-epoxy carotenoids.⁶⁾ Here, we describe the biomimetic total synthesis of mytiloxanthin 2 by the application of the same rearrangement.

Among several epoxides (Chart 1) previously investigated,^{1b)} epoxide 3 (Chart 2) with the acetoxy ethane group at the C-6 position provided the cyclopentyl compound by treatment with $\text{BF}_3 \cdot \text{OEt}_2$. However, this reaction proceeded very slowly and resulted in low-yield formation of the C-5 diastereomer 5 instead of the desired compound 6. To obtain 6 effectively, we explored other Lewis acids involving aminium salts. As a result, treatment of epoxide 3 with tris(4-bromophenyl)aminium hexachloroantimonate⁷⁾ was found to afford 6 in reasonable yield (69%). Thus we planned the biomimetic synthesis of 2 using a rearrangement of epoxide 4 with *tert*-butyldimethylsilyl (TBS) ether as a protecting

group through the C_{15} -aldehyde 8 possessing an enolic β -diketone moiety as shown in Chart 2.

Epoxides 4a and b were derived from the previously prepared optically active aldehyde 9²⁾ by reduction with NaBH_4 , followed by acetylation and subsequent epoxidation with *m*-chloroperbenzoic acid (*m*-CPBA) (4a: 26% from 9; 4b: 46% from 9) as shown in Chart 3. The *anti*(α)-epoxide 4a was then treated with aminium salt⁷⁾ to give the stereoselective rearranged product, cyclopentyl ketone 7, in 63% yield, from which construction of an enolic β -diketone moiety was carried out as follows. Reduction of the ketone 7 with NaBH_4 and protection of the resulting hydroxy group with *p*-methoxybenzyl (PMBM) chloride⁸⁾ gave compound 11, which was converted to aldehyde 12 by reduction with LiAlH_4 and subsequent oxidation with *o*-iodoxybenzoic acid (IBX).⁹⁾ The C_4 -unit was then introduced into 12 by reaction with vinyl bromide 13¹⁰⁾ in the presence of *tert*-BuLi. The resulting alcohol was oxidized with IBX to yield the ketone 14, which after deprotection of the PMBM group with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)^{8b)} was transformed by dimethyl sulfoxide (DMSO) oxidation into the enolic β -diketone 16 (10% from 7: 8 steps).

Unfortunately, direct conversion of 16 into the C_{15} - β -diketone-aldehyde 8a (Chart 2) by deprotection of the allylic TBS group and subsequent oxidation of the resulting alcohol using several reagents (IBX, MnO_2 , etc.) was unsuccessful, leading to the formation of complicated products, probably due to the instability of the β -diketone moiety. Thus after protection of the diketone moiety in 16 by an acetyl group, the resulting acetate 17 was transformed into the C_{15} -aldehyde 8b.¹¹⁾ Partial deprotection of the allylic TBS group in 17 with tetrabutylammonium fluoride (TBAF) followed by oxidation with IBX and subsequent deprotection of the another TBS group with HF afforded 8b (43% from 16: 4 steps).

Next, the C_{15} -aldehyde 8b was allowed to react with the C_{10} -phosphonium salt 18¹²⁾ in the presence of KOH as a base to give condensed products which were subjected to acid hydrolysis to afford an isomeric mixture of C_{25} -apocarotenals 19 (92% from 8b). Finally, this mixture 19 was condensed with the acetylenic Wittig salt (C_{15}) 20¹³⁾ in the presence of KOH to give the condensed products (30%), repeated purification of which by preparative HPLC led to all-*E* mytiloxan-

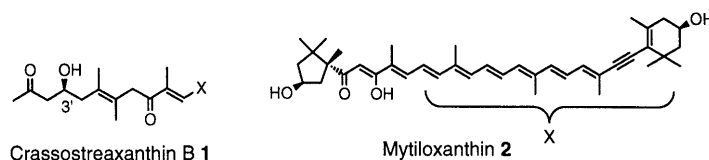


Fig. 1

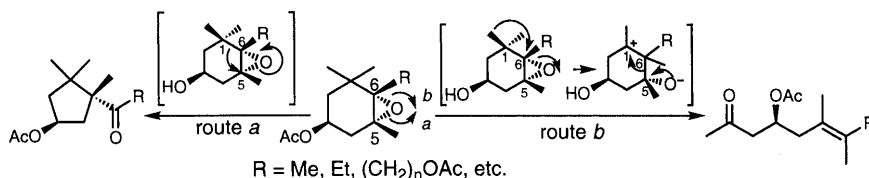


Chart 1

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thin **2** accompanied by 9Z, 9Z,11Z and unidentified isomers (all-*E*:9Z:9Z,11Z~1:1:1). The spectral data (IR, UV-VIS, $^1\text{H-NMR}$, MS, and CD [circular dichroism]) of synthetic mytiloxanthin¹⁴ were in good agreement with those of a natural specimen.⁵ This is the first total synthesis of optically active all-*E* mytiloxanthin.

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References and Notes

- 1) a) Yamano Y., Tode C., Ito M., *J. Chem. Soc., Perkin Trans. 1*, **1996**, 1337—1339; b) *Idem, ibid.*, **1998**, 2569—2581.
- 2) Tode C., Yamano Y., Ito M., *J. Chem. Soc., Perkin Trans. 1*, **1999**, 1625—1626.
- 3) Scheer B. T., *J. Biol. Chem.*, **136**, 275—299 (1940).
- 4) a) Chopra A. K., Moss G. P., Weedon B. C. L., *J. Chem. Soc., Chem.*

- Commun.*, **1977**, 467—468; b) Chopra A. K., Khare A., Moss G. P., Weedon B. C. L., *J. Chem. Soc., Perkin Trans. 1*, **1988**, 1383—1388; c) Weedon B. C. L., *Pure Appl. Chem.*, **35**, 113—130 (1973).
- 5) Maoka T., Fujiwara Y., *J. Jpn. Oil Chem. Soc.*, **45**, 667—670 (1996).
- 6) We have employed the numbering system used for carotenoids.
- 7) Lopez L., Mele G., Fiandanese V., Cardellicchio C., Nacci A., *Tetrahedron*, **50**, 9097—9106 (1994).
- 8) a) Benneche T., Atrande P., Undheim K., *Synthesis*, **1983**, 762—763; b) Kozikowski A. P., Wu J.-P., *Tetrahedron Lett.*, **28**, 5125—5128 (1987).
- 9) Frigerio M., Santagostino M., Sputore S., Palmisano G., *J. Org. Chem.*, **60**, 7272—7276 (1995).
- 10) a) Schlosser M., Hammer E., *Helv. Chim. Acta*, **57**, 2547—2550 (1974); b) de Lera A. R., Iglesias B., Rodríguez J., Álvarez R., López S., Villanueva X., Padrós E., *J. Am. Chem. Soc.*, **117**, 8220—8231 (1995).
- 11) Compound **8b**: $^1\text{H-NMR}$ (CDCl_3) δ : 0.84 (3H, s), 1.19 (3H, s), 1.35 (3H, s), 1.46 (1H, dd, $J=14.5$, 3 Hz), 1.70 (1H, dd, $J=14$, 5 Hz), 1.96 (1H, dd, $J=14$, 8 Hz), 2.31 (3H, s), 2.33 (3H, s), 2.85 (1H, dd, $J=14.5$, 8.5 Hz), 4.47 (1H, m), 6.37 (1H, d, $J=7.5$ Hz), 6.60 (1H, s), 10.17 (1H, d, $J=7.5$ Hz). IR (CHCl_3) cm^{-1} : 3611, 3476, 1773, 1674, 1588. UV λ_{max} (EtOH) nm: 283. HRMS m/z : 308.1648 (calcd. for $\text{C}_{17}\text{H}_{24}\text{O}_5$: 308.1625). $[\alpha]_D^{26} -10.0^\circ$ ($c=0.40$ in CHCl_3).
- 12) Bernhard K., Kienzle F., Mayer H., Müller R. K., *Helv. Chim. Acta*, **63**, 1473—1490 (1980).
- 13) Widmer E., Soukup M., Zell R., Broger E., Wagner H. P., Imfeld M., *Helv. Chim. Acta*, **73**, 861—867 (1990).
- 14) all-*E* Mytiloxanthin: $^1\text{H-NMR}$ (CDCl_3) δ : 0.85 (3H, s), 1.14 (3H, s), 1.19 (3H, s), 1.20 (3H, s), 1.35 (3H, s), 1.45 (1H, t), 1.55 (1H, m), 1.71 (1H, dd), 1.83 (1H, m), 1.92 (3H, s), 1.98 (6H, s), 1.99 (3H, s), 2.01 (3H, s), 2.07 (1H, m), 2.09 (1H, dd), 2.43 (1H, dd), 2.88 (1H, dd), 4.00 (1H, m), 4.52 (1H, m), 5.86 (1H, s), 6.28 (1H, d), 6.36 (1H, d), 6.38 (1H, d), 6.46 (1H, d), 6.55 (1H, dd), 6.60 (1H, dd), 6.64 (1H, dd), 6.65 (1H, d), 6.71 (1H, dd), 7.23 (1H, d). UV-VIS λ_{max} (EtOH) nm: 470. HRMS m/z : 598.4016 (calcd. for $\text{C}_{40}\text{H}_{54}\text{O}_4$: 598.4025).

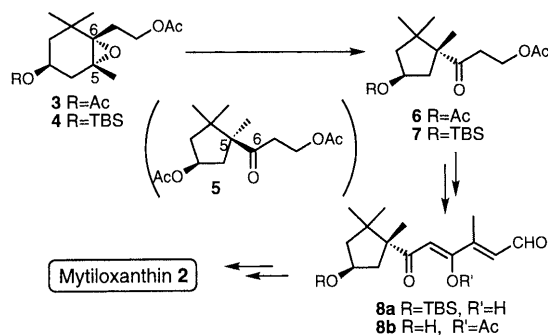
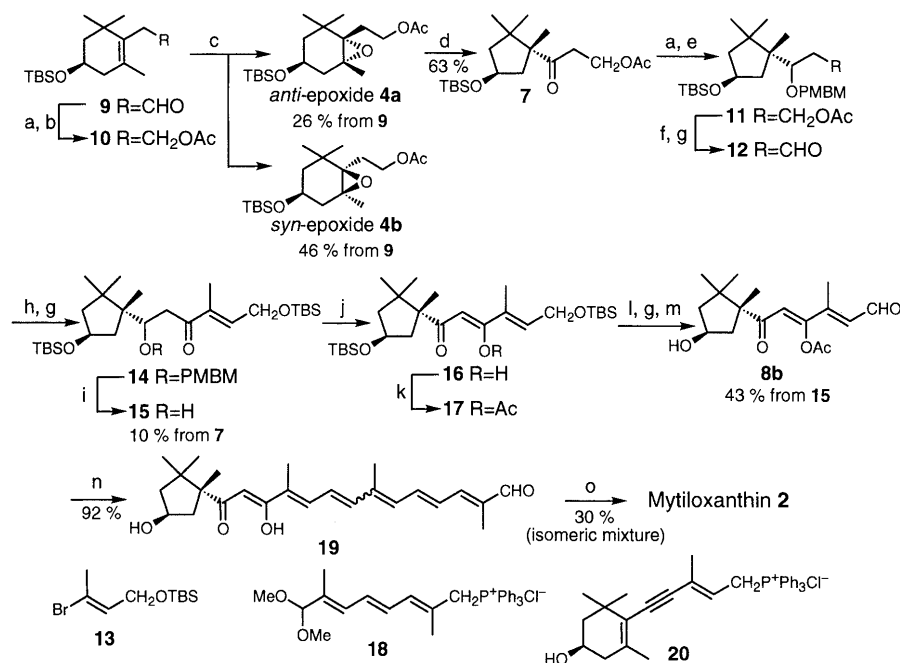


Chart 2



- a) NaBH_4 / MeOH; b) Ac_2O , Py; c) *m*-CPBA / CH_2Cl_2 ; d) $(p\text{-BrC}_6\text{H}_4)_3\text{N}^+\text{SbCl}_6^-$ / CH_2Cl_2 ; e) PMBMCl, $(i\text{-Pr})_2\text{EtN}$ / CH_2Cl_2 ; f) LiAlH_4 / Et_2O ; g) IBX / DMSO; h) **13**, $t\text{-BuLi}$ / Et_2O ; i) DDQ / CH_2Cl_2 - H_2O (18 : 1); j) DMSO, Ac_2O ; k) Ac_2O , DMAP, Et_3N / CH_2Cl_2 ; l) TBAF / THF; m) HF / CH_3CN -THF (9 : 1); n) **18**, KOH / $i\text{-PrOH}$ then H^+ ; o) **20**, KOH / $i\text{-PrOH}$

Chart 3

Three-Dimensional Structure–Activity Relationship Analysis between Motilin and Motilide Using Conformational Analysis and a Novel Molecular Superposing Method

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Motilide, an erythromycin derivative, has been shown to equal activity to that of motilin as an agonist at the motilin receptor. However, there is little information on the three-dimensional (3D) structure–activity relationship between these two molecules, largely because they have quite different structures. In this study, we applied a rational computational procedure consisting of conformational analysis and a novel superposing method to investigate the 3D structure–activity relationship between motilide and motilin. We propose common 3D structural features between these molecules, which may be important for their similar activity.

Key words motilide; motilin; agonist; pharmacophore; molecular dynamics; alignment

The contractions of gastrointestinal smooth muscles are stimulated by motilin, a linear peptide hormone with 22 amino acids residues.^{1–3} Recently, a receptor for motilin has been identified in the human gastrointestinal system.⁴ It has also been found that erythromycin A (EM-A) and its derivatives work as agonists at the motilin receptor site.^{5,6} Therefore these agonists were termed “motilide(s),” meaning a motilin-like macrolide.⁷ From the perspective of structure–activity relationships, it is interesting that both motilin and motilide act as agonists of the motilin receptor, given that they have quite different chemical structures. They must possess some common three-dimensional (3D) structural features relative to their biological activity. To date, several studies on synthetic fragments of motilin have appeared.^{8–11} It has been reported that four hydrophobic residues, Phe1, Val2, Ile4, and Tyr7, are most essential for its activity.^{9–11} Recently, the 3D structure of motilin in SDS micelles, *i.e.*, in a membrane-like environment, was determined using NMR spectroscopy with high resolution.¹² This structure can be considered the best potential candidate for the active, *i.e.*, receptor-bound, conformation. In this structure, Phe1 and Val2, near the N-terminus, are in almost extended conformation. Phe1, Val2, and Ile4 then form a large hydrophobic region, and Tyr7 forms another small one. The spatial disposition of these two hydrophobic regions is assumed to be essential for the activity of motilin. The entire hydrophobic region of motilin, residues 1–9, is at nearly a right angle against the axis of the clearly observable helical part in the center of the molecule. Chemical modification studies of motilide have been reported.^{13,14} However, little information is available in the current literature on the structure–activity relationships

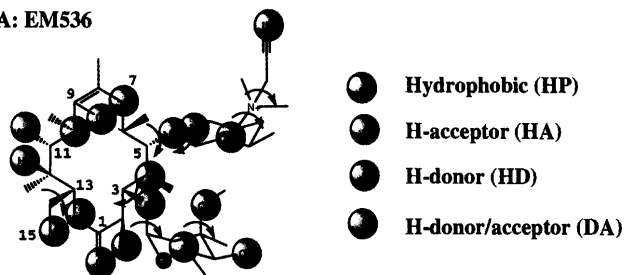
between motilin and motilide.

In this study, we performed the following computational calculations to investigate the 3D structure–activity relationships between motilin and motilide. First, conformational analysis was performed to obtain a number of energetically stable conformations of EM536, one of the motilides. Because the activity of EM536 is as potent as that of motilin,⁷ EM536 is thought to have hydrophobic regions corresponding to the Phe1, Val2, Ile4, and Tyr7 of motilin. As our point of departure, we employed the CAChe package (CAChe Scientific, Oxford Molecular Group Inc., Beaverton, OR, U.S.A.) to prepare structure files for EM536, based on the X-ray analysis of EM523, which has same cyclic structures as EM536 (personal communication). Then the Conformational Analyzer with Molecular Dynamics And Sampling (CAMDAS) program,¹⁵ developed in our laboratory, was employed. This program generates the energetically stable conformations of a target molecule by performing the high-temperature molecular dynamics (MD) calculation and sampling conformations along the MD trajectory. CAMDAS then clusters similar conformations based upon values of dihedral angles defined before calculation. A total of 7 dihedral angles used to cluster similar conformations is indicated by arrows in Fig. 1A. The dihedral angles involved in a 14-membered ring and two sugar rings were constrained in MD calculations to retain their cyclic structures that were derived from X-ray analysis. The constraint energy term was quadratic, and the force constants were 100 kcal mol^{−1} rad^{−2}. We finally obtained a total of 714 distinct conformations for EM536 using CAMDAS.

Next, the alignment between the NMR structure of motilin and a total of 714 distinct conformations for EM536 were carried out using the novel molecular overlay program “SUPERPOSE,”¹⁶ assuming that the NMR structure in SDS micelles of motilin is its active conformation. SUPERPOSE, recently developed in our laboratory, enables us to obtain reliable alignment between molecules for 3D quantitative structure–activity relationship analysis (3D-QSAR). The SUPERPOSE program superposes two molecules based on their respective physicochemical properties by treating a pseudo-molecule consisting of functional atoms instead of a real molecule. The properties of the functional atoms are divided into four types, comprising hydrophobic atoms (HP), hydrogen-bonding donors (HD), hydrogen-bonding acceptors (HA), and hydrogen-bonding donors/acceptors (DA), each of which is represented as a sphere or a few spheres with a given radius depending on the chemical properties. In order to perform the superposition of two molecules systematically, first the center of mass of each molecule is translated to the origin of coordinates, and then the circumscribed rectangular boxes are calculated. The molecule with a large box volume is fixed, and then the center of mass for the molecule with a small box volume is translated and rotated. The range of translation is the maximum distance that the small box can translate inside the large box. The translational increment is 1 Å and the center of mass is translated on the body-centered cubic lattice points in the circumscribed rectangular box of large volume. The rotation is performed on each of the lattice points using three Eulerian angles. The ranges of the three Eulerian angles are 0 ≤ ϕ, ψ ≤ 350°, and 0 ≤ θ ≤ 180° and the

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A: EM536



B: motilin

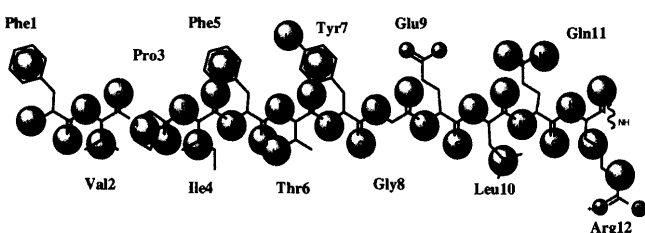


Fig. 1. Chemical Structures and Functional Atoms of EM536 (A) and Motilin (B)

The large circles represent 1 Å and the small circles represent 0.5 Å with respect to the property radii of the functional atoms. The dihedral angles used to cluster similar conformations are indicated by round arrows (A).

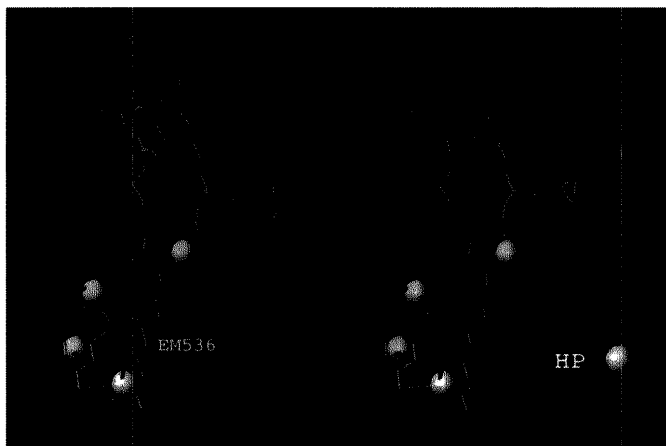
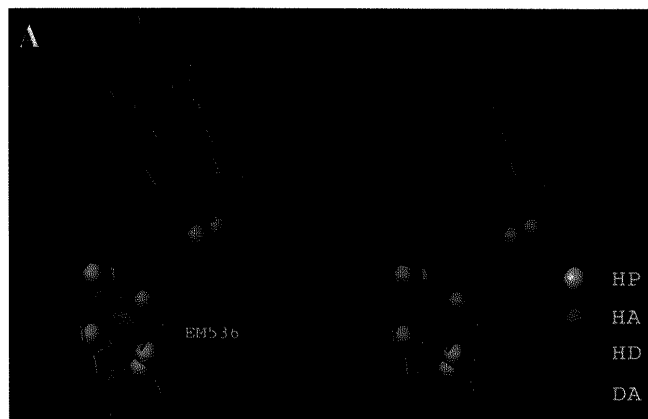


Fig. 2. Stereopairs of the Resulting Alignment between the NMR Structure in SDS Micelles of Motilin (Green) and the Selected Conformation of EM536 (Yellow)

Phe1, Val2, Ile4, and Tyr7 of motilin are displayed in red. The four HP atoms located on the propargyl group, the amino sugar ring, C₇, and C₁₅ in EM536 are represented by white spheres.

rotational increment is 10°.

Compared with other superposition techniques such as DISCO,¹⁷⁾ SUPERPOSE has the advantage that there is no need to estimate in advance a corresponding functional group for the superposition between two molecules. Therefore we can easily obtain alignment between two molecules, even though they have quite different chemical structures as in the case of motilin and EM536. According to the criteria in our previously published paper, the functional atoms for EM536 and motilin can be defined as shown in Figs. 1A and B, respectively. The functional atoms of motilin are defined only in the region of Phe1-Arg12, because the fragment of Phe1-Arg12 has been shown to have activity almost identical to that of the wild form.⁸⁾ After calculations using SUPERPOSE, we obtained a total of 714 different alignments between motilin and EM536. At that point, we needed to deter-



B

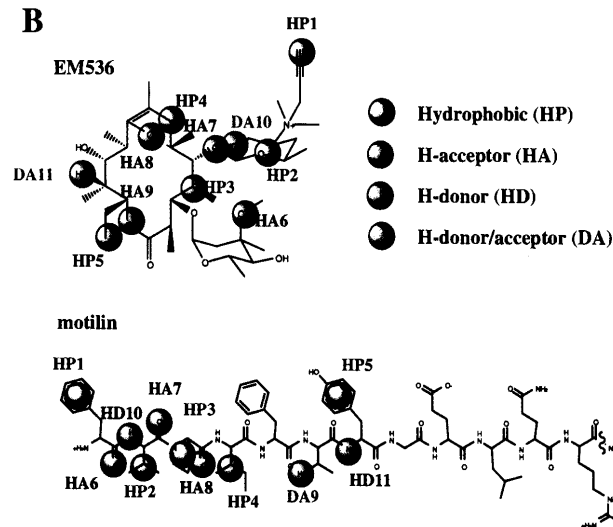


Fig. 3. Stereopairs of the Resulting Alignment with All of the Functional Atoms Superposed between Motilin and EM536 (A)

Chemical Structures with All of the Functional Atoms Superposed between Motilin and EM536 (B)

Superposed atoms with the same numbers (B). HA9, DA10, and DA11 atoms for EM536 are superposed on DA9, HD10, and HD11 atoms for motilin, respectively.

mine the best alignment among these 714. As described earlier, Phe1, Val2, Ile4, and Tyr7 are the most essential for the activity of motilin. Therefore we can use this information to determine the best alignment between motilin and EM536, i.e., we have selected the alignment where all four HP atoms corresponding to Phe1, Val2, Ile4, and Tyr7 of motilin are superposed on the HP atoms of EM536. We found only one alignment satisfying this constraint.

The selected conformation for EM536 has a difference in energy value of about 9 kcal mol⁻¹ from the most stable conformation obtained from the conformational analysis. Figure 2 shows the resulting alignment between motilin and EM536. In this alignment, the four HP atoms located on the propargyl group, the amino sugar ring, C₇, and C₁₅ in EM536 are superposed on those corresponding to Phe1, Val2, Ile4, and Tyr7 of motilin, respectively (Fig. 2). Studies on mutants of motilin have shown the importance of an aromatic side chain in position 1 for activity.¹⁰⁾ Studies on the activity of motilides have also shown that a propargyl or an allyl substituent on the quaternary ammonium group is important for high activity.⁷⁾ These experimental results seem to indicate the importance of the π electron for activity in each region.

Therefore the correspondence between Phe1 of motilin and the propargyl group of EM536 could be considered reasonable. In addition, the activity of motilin mutant F1A, in which Phe1 is replaced by an alanine residue, was reduced below 1% of that of the wild form,¹⁰ and the replacement of the propargyl group with a methyl one in EM536 also caused a reduction in potency of more than two log units.⁷ This coincidence suggests a correspondence between the Phe1 of motilin and the propargyl group of EM536. The HP atom located in the 6,9-hemiacetal region of EM536 has been considered to be a pharmacophore corresponding to the Ile4 of motilin. This is consistent with experimental results indicating that the 6,9-hemiacetal region may be important for the activity of EM536.¹³ Based on the findings described above, we have concluded that the resulting alignment in this study is reliable. Therefore the selected conformation for EM536 could be considered to be its active conformation. In addition, the resulting alignment may suggest other pharmacophores for both motilin and EM536, which may be important for their activity as agonists of the motilin receptor (Fig. 3). For example, the ethyl group at C₁₃ in EM536 is assumed to be important for its activity, because the HP atom for this group is presumed to correspond to the one for the Tyr7 of motilin. Because motilin and EM536 exhibit several common relative spatial dispositions with respect to HA, HD, and DA atoms (Fig. 3A), the property of being a hydrogen-bonding acceptor or donor may contribute to the ability to bind with the motilin receptor. To obtain more information on the interaction mode between motilides and the motilin receptor, a 3D-QSAR study is currently under way in our laboratory.

Acknowledgments We thank Professor A. Gräslund for generously providing us with the NMR structure in SDS micelles of motilin.

References and Notes

- 1) Brown J. C., Cook M. A., Dryburgh J. R., *Can. J. Biochem.*, **51**, 533—537 (1973).
- 2) Itoh Z., Honda R., Hiwatashi S., Takeuchi I., Aizawa R., Takayanagi R., Couch E. F., *Scand. J. Gastroenterol.*, Suppl., **11**, 93—110 (1976).
- 3) The amino acid sequence of motilin: Phe-Val-Pro-Ile-Phe-Thr-Tyr-Gly-Glu-Leu-Gln-Arg-Met-Gln-Glu-Lys-Glu-Arg-Asn-Lys-Gly-Gln.
- 4) Feighner S. D., Tan C. P., McKee K. K., Palyha O. C., Hreniuk D. L., Pong S.-S., Austin C. P., Figueroa D., MacNeil D., Cascieri M. A., Nargund R., Bakshi R., Abramovitz M., Stocco R., Kargman S., O'Neil G., Van Der Ploeg L. H., Evans J., Patchett A. A., Smith R. G., Howard A. D., *Science*, **284**, 2184—2188 (1999).
- 5) Itoh Z., Suzuki M., Nakaya M., Inoue M., Mitsuhashi S., *Antimicrob. Agents Chemother.*, **26**, 863—869 (1984).
- 6) Kondo Y., Torii K., Omura S., Itoh Z., *Biochem. Biophys. Res. Commun.*, **150**, 877—882 (1988).
- 7) Omura S., Tsuzuki K., Sunazuka T., Marui S., Toyoda H., Inatomi N., Itoh Z., *J. Med. Chem.*, **30**, 1941—1943 (1987).
- 8) Poitras P., Gagnon D., St-Pierre S., *Biochem. Biophys. Res. Commun.*, **183**, 36—40 (1992).
- 9) Peeters T. L., Macielag M. J., Depoortere I., Konteatis Z. D., Florance J. R., Lessor R. A., Galdes A., *Peptides*, **13**, 1103—1107 (1992).
- 10) Miller P., Gagnon D., Dickner M., Aubin P., St-Pierre S., Politras P., *Peptides*, **16**, 11—18 (1995).
- 11) Haramura M., Tsuzuki K., Okamachi A., Yogo K., Ikuta M., Kozono T., Takanashi H., Murayama E., *Chem. Pharm. Bull.*, **47**, 1555—1559 (1999).
- 12) Jarvet J., Zdunek J., Damberg P., Gräslund A., *Biochemistry*, **36**, 8153—8163 (1997).
- 13) Tsuzuki K., Sunazuka T., Marui S., Toyoda H., Omura S., *Chem. Pharm. Bull.*, **37**, 2687—2700 (1989).
- 14) Sunazuka T., Tsuzuki K., Marui S., Toyoda H., Omura S., Inatomi N., Itoh Z., *Chem. Pharm. Bull.*, **37**, 2701—2709 (1989).
- 15) Tsujishita H., Hirono S., *J. Comput.-Aided Mol. Design*, **11**, 305—315 (1997).
- 16) Iwase K., Hirono S., *J. Comput.-Aided Mol. Design*, **13**, 499—512 (1999).
- 17) Martin Y. C., Bures M. G., Danaher E. A., DeLazzer J., Lico I., Pavlik A., *J. Comput.-Aided Mol. Design*, **7**, 83—102 (1993).

Aldol Reaction of Enol Esters Catalyzed by Cationic Species Paired with Tetrakis(pentafluorophenyl)borate

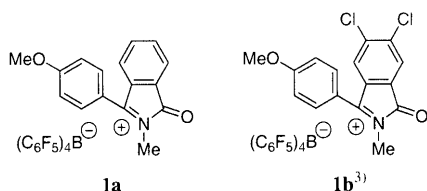
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The crossed aldol reaction of enol esters, which are weak carbon nucleophiles, with aldehydes was effectively carried out under mild conditions by using a catalytic amount of several cationic species paired with tetrakis(pentafluorophenyl)borate.

Key words catalytic aldol reaction; enol ester; tetrakis(pentafluorophenyl)borate; cationic species

The carbon-carbon bond forming reaction is successfully carried out by treating weakly nucleophilic carbon species with carbonyl compounds using a Lewis acid. For example, the aldol reaction of silyl enol ethers with carbonyl compounds promoted by Lewis acids has been employed in the construction of a wide variety of carbon skeletons.¹⁾ Recently, it was reported^{1a)} that a catalytic aldol reaction of aldehydes with silyl enol ethers proceeded smoothly to afford aldols using carbocationic species (**1a**) paired with tetrakis(pentafluorophenyl)borate which is known to be a very effective noncoordinating anion of which the counteranion exists as a naked one.²⁾



On the other hand, the aldol reaction using enol esters, which are less reactive nucleophiles, is not as common compared with that using silyl enol ethers. One of the most common examples is carried out under basic conditions by generating lithium enolates from enol esters with methyllithium and its subsequent addition to carbonyl compounds.⁴⁾ On the other hand, few reports have been published to date concerning the aldol reaction under acidic or neutral conditions using enol esters, which are readily available and act as weak carbon nucleophiles.⁵⁾

It was reported in 1974 that enol acetate reacts with various acetals or benzaldehyde to afford the corresponding aldol-type addition products in good yields using stoichio-

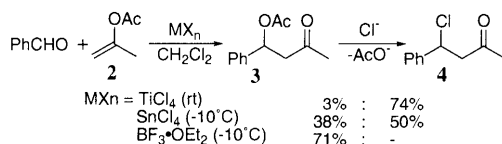


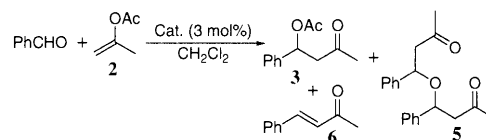
Chart 1

metric amounts of Lewis acids such as TiCl_4 , AlCl_3 , SnCl_4 , ZnCl_2 , and $\text{BF}_3 \cdot \text{OEt}_2$ (Chart 1).^{5a)} In most of the above reactions, a remarkable by-product, β -chloroketone **4**, is formed by subsequent nucleophilic replacement of initially formed aldol acetate **3** with chloride ion originating from the Lewis acids.

It was thought that if the Lewis acid paired with borate that had no nucleophilicity was employed in the aldol reaction of aldehydes and enol esters, the above-mentioned subsequent nucleophilic substitution by their own anionic species would not take place and the yield of the aldol acylate would thus increase. Therefore the aldol reaction between aldehydes and various enol esters using a catalytic amount of several cationic species paired with tetrakis(pentafluorophenyl)borate was attempted.

First, the catalytic aldol reaction of isopropenyl acetate (**2**) with benzaldehyde was examined using several cationic species paired with the borate mentioned above (see Table 1). A typical experimental procedure is described for the reaction of isopropenyl acetate (**2**) with benzaldehyde: to a suspension of $\text{AgB}(\text{C}_6\text{F}_5)_4$ (0.0075 mmol) in CH_2Cl_2 (0.5 ml) at room temperature TMSCl (0.0075 mmol) was added and the resulting suspension was stirred for 1 h at room temperature and then cooled to 0°C . To this suspension, a solution of benzaldehyde (0.25 mmol) and **2** (0.75 mmol) in CH_2Cl_2 (1.5 ml) was added dropwise and after the reaction was monitored using thin-layer chromatography, saturated aqueous sodium bicarbonate and CH_2Cl_2 were added at the same temperature, and the organic layer was extracted, washed with

Table 1



Entry	Cat.	Temp./ $^\circ\text{C}$	Time/h	Yield/%		
				3	5	6
1	TMSOTf	-78	1	10	48	0
2	TMSOTf	0	Inst.	73	8	5
3	TMSOTf	r.t.	Inst.	81	2	2
4	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	-78	1	29	32	0
5	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	-45	1	56	25	0
6	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	-28	1	84	4	2
7	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	-15	0.5	85	—	2
8	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	0	Inst.	91	—	0
9	$\text{TMSB}(\text{C}_6\text{F}_5)_4$ (7)	r.t.	Inst.	80	—	2
10	$\text{AgB}(\text{C}_6\text{F}_5)_4$	r.t.	0.5	0	0	0
11	TMSCl	r.t.	0.5	0	0	0
12	$\text{SnClB}(\text{C}_6\text{F}_5)_4$	0	1	84	13	1
13	$\text{Sn}[\text{B}(\text{C}_6\text{F}_5)_4]_2$	0	0.5	75	12	—
14	$\text{SnCl}_3\text{B}(\text{C}_6\text{F}_5)_4$ (8)	0	Inst.	94	3	1
15	$\text{TiClB}(\text{C}_6\text{F}_5)_4$	0	1	80	13	2
16	$\text{Ti}[\text{B}(\text{C}_6\text{F}_5)_4]_2$	0	0.5	83	10	1
17	$\text{TiCl}_3\text{B}(\text{C}_6\text{F}_5)_4$ (9)	0	Inst.	92	3	1
18	$(i\text{PrO})_2\text{TiClB}(\text{C}_6\text{F}_5)_4$	0 ^{a)}	2 ^{a)}	71	16	3
19	1a ^{b)}	r.t.	1.5	72	9	2
20	1a	r.t.	1	91	5	0
21	$\text{TrB}(\text{C}_6\text{F}_5)_4$ (10)	r.t.	1	82	—	5

a) The reaction was carried out at 0°C for 1 h then at room temperature for 1 h. b) 1 mol% of catalyst was used.

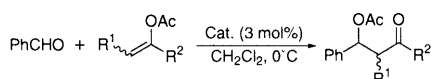
brine, and evaporated. The resulting crude product was submitted to silica-gel chromatography to afford the corresponding aldol acetate (91%) as a pale yellow oil (Table 1, Entry 8). Other metal tetrakis(pentafluorophenyl)borates were prepared *in situ* in a manner similar to that described above using the corresponding metal chlorides instead of TMSCl.

It is significant that the ethereal by-product **5** was obtained at lower temperature (below -45°C , Entries 1, 4, and 5), and thus the yield of the aldol acetate **3** was low. On the other hand, the corresponding aldols were obtained in high yields when the reaction was carried out at 0°C using 3 mol% of TMS-, Sn(IV)- or Ti(IV) tetrakis(pentafluorophenyl)borate,

or **1a** as a catalyst (Entries 8, 14, 17, and 20, respectively). In the above reactions, neither silver tetrakis(pentafluorophenyl)borate⁶ nor chlorotrimethylsilane alone exhibited any catalytic activity (Entries 10 and 11). Next, the scope and limitations of this reaction were investigated using combinations of various enol acetates⁷ and aldehydes.

Each cationic species smoothly catalyzed the reaction of the enol ester derived from acetophenone to give the corresponding aldol acetate in high yield (Table 2, Entries 1–5), while the reactions of the α -substituted enol acetates were very weak. The structure of the aldehyde influenced the reactivity of the present reaction: that is, aliphatic aldehydes did not afford any aldol adducts, and even if aromatic aldehyde was used, only the electron-withdrawing group on the aromatic ring afforded the aldol adduct (Table 3). In order to prepare a new catalyst that promotes various aldol reactions, a carbocationic species **1b** was prepared based on the consideration that the carbocationic character would be enhanced by introducing electron-withdrawing atoms onto the aromatic

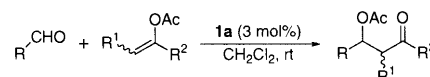
Table 2



Entry	Enol ester	Cat.	Time/h	Yield/%
1		7	Inst.	83
2		8	Inst.	98
3		9	Inst.	97
4 ^{a)}		1a	0.5	92
5 ^{a)}		10	1	73
6		7	1	Trace
7		8	1	Trace
8		9	1	Trace
9 ^{a)}		1a	39	19
10 ^{a)}		1b	5	48
11		7	1	Trace
12		8	1	Trace
13		9	1	Trace
14 ^{a)}		1a	48	25
15 ^{a)}		1b	48	38
16 ^{a)}		10	5	66

a) The reaction was carried out at room temperature.

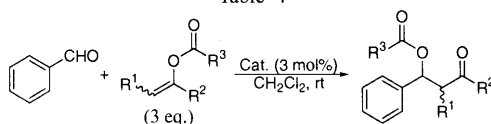
Table 3

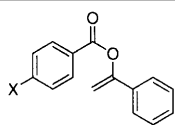
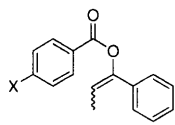
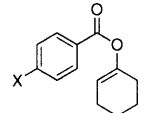


Entry	R	Enol ester	Time/h	Yield/%
1	4-ClPh	2	1.2	72
2 ^{a)}		2	Inst.	88
3		11	1.2	84
4 ^{a)}	4-MeOPh	11	Inst.	96
5		2	15	Trace
6		11	15	0
7	PhCH ₂ CH ₂ –	2	15	0
8	PhCH=CH–	11	15	0
9		11	22.5	0

a) 3 mol% of **1b** was used as a catalyst instead of **1a**.

Table 4



Entry	Enol ester	Yield/% ^{a)}				
		Cat.	1a	1b	7	8
1		X = OMe	85 (1)	80 (inst.)	91 (inst.) ^{b)}	96 (inst.) ^{b)}
2		X = H	84 (1)	94 (inst.)	99 (inst.) ^{b)}	99 (inst.) ^{b)}
3		X = CF ₃	21 (1)	61 (1)	71 (inst.) ^{b)}	72 (inst.) ^{b)}
4		X = OMe	Trace (3)	62 (3)	76 (1)	78 (1)
5		X = OMe	93 (5) ^{c)}	95 (1) ^{c)}	36 (1) ^{b)}	32 (1) ^{b)}
6		X = H	41 (48) ^{c)}	82 (3) ^{c)}	Trace (1) ^{b,d)}	Trace (1) ^{b,d)}
7		X = CF ₃	0 (48) ^{c)}	0 (48) ^{c)}	Trace (1) ^{b,d)}	Trace (1) ^{b,d)}

a) The values in parentheses indicate the reaction time (h). b) The reaction was carried out at 0°C . c) 10 mol% of catalyst was used. d) When the reaction was carried out at room temperature using 10 mol% of each catalyst, the reaction proceeded smoothly to give the corresponding adducts and the yield was 83%, 88%, and 88%, respectively, for catalysts **7**, **8**, and **9**.

ring of the catalyst **1a**. Expectedly, carbocationic species **1b** with two chlorine atoms in its aromatic ring was proved to be a Lewis acid catalyst much stronger than **1a** (Table 2, Entries 10 and 15; Table 3, Entries 2 and 4).

To examine the effect of the acyl moiety on the reactivity of the enol ester, several substituted enol benzoates⁷⁾ were employed in the present reaction (Table 4). As shown in Table 4, the yields of the corresponding aldol acylates increased by introducing an electron-donating group into the benzoyl moiety (see Entries 1—3 and 5—7). The more electron-deficient cationic species **1b** gave the adducts in higher yields compared with **1a** (see Entries 1—6).

It is noted that the catalytic crossed-aldol reaction of enol esters, which are weak carbon nucleophiles, with aldehydes was carried out effectively using several cationic species paired with tetrakis(pentafluorophenyl)borate such as TMSB(C₆F₅)₄, SnCl₃B(C₆F₅)₄, TiCl₃B(C₆F₅)₄, **1a**, and **1b**. In addition, the structure-activity relationship of the cationic species was examined using **1a** and **1b**, and it was determined that the more electron-deficient cationic species **1b** had higher catalytic activity than **1a** in the present reaction.

References and Notes

- 1) a) Mukaiyama T., Yanagisawa M., Iida D., Hachiya I., *Chem. Lett.*, **2000**, 606—607; b) Chan T.-H., "Comprehensive Organic Synthesis," Vol. 2, ed. by Trost B. M., Fleming I., Pergamon Press, Oxford, 1991, pp. 595—628; c) Gennari C., "Comprehensive Organic Synthesis," Vol. 2, ed. by Trost B. M., Fleming I., Pergamon Press, Oxford, 1991, pp. 629—660.
- 2) a) Piers W. E., Chivers T., *Chem. Soc. Rev.*, **26**, 345—354 (1997); b) Fujiki K., Ikeda S., Kobayashi H., Mori A., Nagira A., Nie J., Sonoda T., Yagupolskii Y., *Chem. Lett.*, **2000**, 62—63.
- 3) Prepared from 4,5-dichlorophthalic anhydride by the method described in Ref. 1a.
- 4) House H. O., Crumrine D. S., Teranishi A. Y., Olmstead H. D., *J. Am. Chem. Soc.*, **95**, 3310—3324 (1973).
- 5) a) Mukaiyama T., Izawa T., Saigo K., *Chem. Lett.*, **1974**, 323—326; b) Mukaiyama T., Hanna J., Inoue T., Sato T., *Chem. Lett.*, **1974**, 381—382; c) Izawa T., Mukaiyama T., *Chem. Lett.*, **1974**, 1189—1192; d) Izawa T., Mukaiyama T., *Chem. Lett.*, **1975**, 161—164; e) Masuyama Y., Kobayashi Y., Yanagi R., Kurusu Y., *Chem. Lett.*, **1992**, 2039—2042; f) Masuyama Y., Sakai T., Kato T., Kurusu Y., *Bull. Chem. Soc. Jpn.*, **67**, 2265—2272 (1994); g) Fujii H., Watanabe N., Hayashi M., Oguni N., *Syn. Comm.*, **24**, 3249—3254 (1994); h) Hayashi M., Inoue T., Miyamoto Y., Oguni N., *Tetrahedron*, **50**, 4385—4398 (1994); i) Hayashi M., Tanaka K., Oguni N., *Tetrahed. Asymm.*, **6**, 1833—1836 (1995); j) Oguni N., Tanaka K., Ishida H., *Synlett*, **1998**, 601—602; k) Mukaiyama T., Shibata J., Shimamura T., Shiina I., *Chem. Lett.*, **1999**, 951—952; l) Yanagisawa A., Matsumoto Y., Asakawa K., Yamamoto H., *J. Am. Chem. Soc.*, **121**, 892—893 (1999).
- 6) Prepared from commercially available lithium tetrakis(pentafluorophenyl)borate by the method described in the literature: Hayashi Y., Rohde J. J., Corey E. J., *J. Am. Chem. Soc.*, **118**, 5502—5503 (1996).
- 7) Ito H., Ishizuka T., Tateiwa J., Hosomi A., *Tetrahedron Lett.*, **39**, 6295—6298 (1998).

Chemiluminescence-HPLC for the Assay of Guanidino Compounds

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A chemiluminescence-HPLC system for the assay of serum guanidino compounds was established. In this study, we found that lucigenin added to the reaction mixture containing guanidino compounds and alkaline-ninhydrin reagent resulted in strong chemiluminescence. The lucigenin chemiluminescence was not derived from fluorescent derivatives of guanidino compounds or ninhydrin. The interaction of guanidino compounds and ninhydrin under alkaline conditions produced reactive oxygen species. We applied this phenomenon to the microanalysis of guanidino compounds.

Key words guanidino compound; chemiluminescence; ninhydrin; lucigenin; HPLC; chronic renal failure

Guanidino compounds are uremic toxins that accumulate in the blood of chronic renal failure patients. Guanidino compounds are candidates for markers of renal dysfunction since they are more toxic than creatinine (CTN) or blood urea nitrogen (BUN).^{1,2)} We recently found that uremic patients capable of excreting urine even on hemodialysis have markedly lower serum guanidinosuccinic acid levels compared with anuric patients.^{3,4)} HPLC coupled with fluorometry has been used to determine guanidino compound. However, the detailed investigation of guanidino compounds for the elucidation of their behavior requires higher sensitivity, since serum concentrations of guanidino compounds are far lower than those of CTN or BUN.

We previously developed fluorometry of guanidino compounds using an alkaline-ninhydrin reagent.^{3–7)} In this study, we found that lucigenin added to the reaction mixture containing guanidino compounds and alkaline-ninhydrin reagent resulted in strong chemiluminescence, whereas no luminescence under alkaline conditions was detected with either guanidino compounds or ninhydrin alone. This phenomenon was also seen in the microanalysis of guanidino compounds.

Figure 1A shows the serial changes in fluorescence intensities after mixing guanidinoacetic acid (GAA) with alkaline-ninhydrin reagent. The reaction mixture contained ninhydrin 0.4 mM, GAA 4 mM, and sodium hydroxide 400 mM in a water–2-propanol mixture (94:6 v/v%). The fluorescence intensity increased gradually after 2.5 min, and plateaued after 15 min.

Figure 1B shows lucigenin chemiluminescence intensities from an aliquot of reaction mixture during reaction between GAA and alkaline-ninhydrin reagent. The reaction mixture contained ninhydrin 0.2 mM, GAA 2 mM, lucigenin 0.065 mM, and sodium hydroxide 200 mM in a water–2-propanol mixture (97:3 v/v%). The reaction mixture during reaction be-

tween GAA and alkaline-ninhydrin reagent was used in Fig. 1A, lucigenin solution was mixed in a cuvette. The lucigenin chemiluminescence was measured using a spectrofluorometer at 465 nm, and the lamp was turned off during measurement. Although the fluorescence intensity was weak at 2.5 min (Fig. 1A), the chemiluminescence intensity from the reaction mixture was maximum. These findings indicate that lucigenin chemiluminescence was mainly due to the reactive oxygen species from the interaction of the guanidino group of GAA with the diketone of *o*-carboxyphenylglyoxal changed from ninhydrin under alkaline conditions, not from fluorescent derivatives of the GAA and ninhydrin.

We applied this phenomenon to the microanalysis of guanidino compounds. Chemiluminescence analysis is a sensitive method because there is no background light source. The lucigenin has high chemiluminescence efficiency and does not require a catalyst, unlike luminol, and therefore lucigenin was used for chemiluminescence-HPLC of guanidino compounds.

The HPLC consisted of an HP 1100 liquid chromatograph (Yokogawa Analytical Systems Inc., Tokyo, Japan) with three isocratic pumps (Model G1310A Iso Pump, Yokogawa), an autosampler (Model G1313A ALS, Yokogawa), and an A/D converter (Model HP 35900E Dual Channel Interface, Yokogawa). Detection was performed with a chemiluminescence detector (Model CLD-10A, Shimadzu Seisakusho Co., Kyoto, Japan). The host computer was a Vectra XM series 3 16/90 personal computer (Yokogawa) equipped with Chem Station chromatographic software (Yokogawa).

The eluent was a mixture of sodium dihydrogenphosphate 5 mM solution (pH 4.6) containing 1-octanesulfonic acid sodium salt 5 mM and 2-propanol (85:15 v/v%). It was delivered from pump A at a flow rate of 1.0 ml/min. A 5 μ l sample was injected by the autosampler. Guanidino compounds that formed ion pairs with 1-octanesulfonic acid were separated on the column (Senshu Pak PEGASIL ODS, 150 $\times$ 4.6 mm i.d., particle size 5 μ m). The eluate from the separation column was mixed with reagent solution (ninhydrin 5 mM and lucigenin 0.098 mM in a water–2-propanol mixture, 98:2 v/v%) and sodium hydroxide 500 mM solution delivered from pumps B and C at a flow rate of 0.5 ml/min. The mixture was

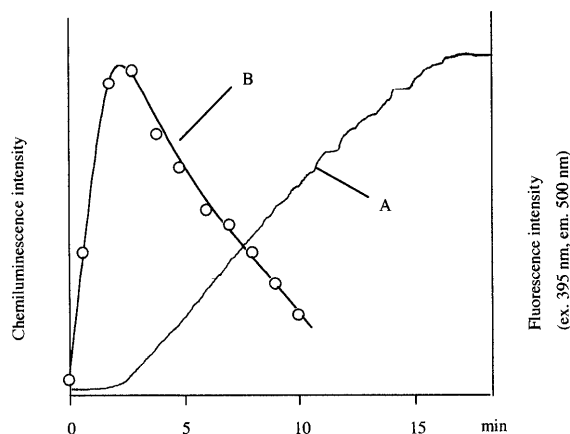


Fig. 1. Serial Changes in Fluorescence Intensities after Mixing Guanidinoacetic Acid with Alkaline-Ninhydrin Reagent (A), and Lucigenin Chemiluminescence Intensities from an Aliquot of Reaction Mixture during the Reaction between Guanidinoacetic Acid and Alkaline-Ninhydrin Reagent (B)

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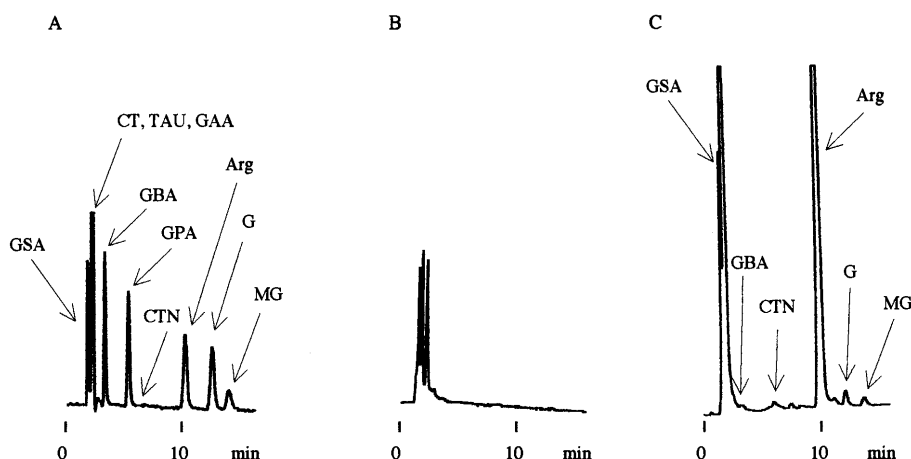


Fig. 2. Chromatogram of a Standard Mixture of Guanidino Compounds (GAA, GPA, and MG: 50 pmol/Injection; CT, GBA, GSA, and TAU: 100 pmol/Injection; Arg and G: 200 pmol/Injection; CTN: 500 pmol/Injection) (A), Chromatograms of a CRF Patient Serum before (B) and after (C) Addition of Ninhydrin 5 mM to the Mobile Phase

reacted in a coil (5 m $\times$ 0.5 mm i.d.), placed in a detector at 25 °C, and the chemiluminescence was monitored with the chemiluminescence detector.

Figure 2A shows a chromatogram of a guanidino compound standard mixture containing arginine (Arg), creatine (CT), CTN, guanidine (G), GAA, guanidinobutyric acid (GBA), guanidinopropionic acid (GPA), guanidosuccinic acid (GSA), methylguanidine (MG), and taurocyamine (TAU). GSA, GBA, GPA, CTN, Arg, G, and MG were clearly separated.

Figures 2B and 2C show chromatograms of a chronic renal failure (CRF) patient serum before (Fig. 2B) and after (Fig. 2C) the addition of ninhydrin 5 mM to the mobile phase. The sample solution was obtained by deproteinization of serum according to the method reported by Kubo and coworkers.⁷⁾ Guanidino compounds were not detected by the chemiluminescence detector (Fig. 2B), but some unknown peaks were detected because lucigenin reacts with reductants or oxidizing agents under alkaline conditions. In our procedure, lucigenin chemiluminescence of GSA, CTN, Arg, G, and MG, which are found in serum of CRF patients, was detected following the addition of ninhydrin to the mobile phase (Fig. 2C). The detection limits (S/N=2) were as follows: GSA and GBA, 10 pmol/injection; GPA, 5 pmol/injection; CTN, 2 nmol/injection; Arg and G, 40 pmol/injection; and MG, 25 pmol/injection. This chemiluminescence-HPLC system has

sensitivity similar to HPLC coupled with fluorometry using an alkaline-ninhydrin reagent as reported by Kobayashi *et al.*⁷⁾

This method is specific for the guanidino group, not amine. On the other hand, this chemiluminescence-HPLC system is an easy and simple one, because the reaction of alkaline-ninhydrin reagent with guanidino compounds generates lucigenin chemiluminescence immediately, and the lucigenin chemiluminescence reaction proceeds at room temperature, whereas the fluorescence reaction used in the conventional detection of guanidino compounds requires heating of the reaction mixture.

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References and Notes

- 1) Nagase S., Aoyagi K., Sakamoto M., Narita M., Tojo S., *Nephrol. Dial. Transplant.*, **3**, 790–794 (1988).
- 2) Ikezaki N., *Jpn. J. Nephrol.*, **32**, 283–290 (1990).
- 3) Nohara Y., Hanai T., Suzuki J., Matsumoto G., Iinuma F., Kubo H., Kinoshita T., *Chem. Pharm. Bull.*, **46**, 1844–1845 (1998).
- 4) Nohara Y., Hanai T., Suzuki J., Matsumoto G., Iinuma F., Kubo H., Kinoshita T., Watanabe M., *Biol. Pharm. Bull.*, **23**, 1015–1020 (2000).
- 5) Hiraga Y., Kinoshita T., *J. Chromatogr.*, **226**, 43–51 (1981).
- 6) Hiraga Y., Kinoshita T., *J. Chromatogr.*, **342**, 269–275 (1985).
- 7) Kobayashi Y., Kubo H., Kinoshita T., *J. Chromatogr.*, **400**, 113–121 (1987).

Quantitative Evaluation of the Bitterness of Commercial Medicines Using a Taste Sensor

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The bitterness of 11 commercial medicines was evaluated both by a multichannel taste sensor and in human gustatory sensation tests with 15 volunteers. For basic drugs with amino groups in the molecule, such as quinine, there was a comparatively strong relative response electric potential (mV) of channels 1 or 2, those containing negatively charged membranes and the bitterness determined by human gustatory sensation tests. The suppression of the bitterness of quinine by sucrose and aspartame could be quantified using the artificial taste sensor and the results concurred with those from gustatory sensation tests. The usefulness of the sensor was thus confirmed for this type of compound.

Anionic drugs, such as diclofenac sodium or salicylic acid gave rise in a negative response electric potential in channels 5 or 6, those containing positively charged membrane, seemed to be useful information even though their tastes are being sour rather than bitter.

For drugs with both an amino (cationic) group and carboxylic acid (anionic) group in the molecule, such as theophylline, caffeine, and metronidazole, the relative response electric potential (mV) of channels containing negatively charged membranes was not increased, even though bitterness was observed in human gustatory sensation tests. Therefore, a different design of membrane component is required for more general evaluation of the bitterness of various medicines.

Key words taste sensor; bitterness; human gustatory sensation; quinine; sucrose; aspartame; caffeine

Medicines with a bitter taste are sometimes hard for patients to swallow, and easily give rise to noncompliance and thus decreased therapeutic efficacy. The evaluation of the bitterness of medicines is therefore an important factor in drug design. Taste seems to be have five components, namely, sourness, saltiness, sweetness, bitterness, and umami.

A multichannel taste sensor, an electric 'tongue' with global selectivity, comprises several kinds of lipid/polymer membranes which transform information about substances producing taste into electrical signals.^{1–3)} These signals are analysed by the computer and the sensor output has been shown to produce different patterns for groups of chemical substances with similar tastes. Using this sensor, the tastes of foodstuffs such as beer,⁴⁾ coffee,⁵⁾ milk,⁶⁾ sake,⁷⁾ rice, soybean paste and vegetables can be expressed quantitatively. Even though a taste sensor was just applied to quinine by Takagi *et al.*,⁸⁾ there have been no systematic trials using the sensor to evaluate the taste of medicines. In the present study, we eval-

uated the bitterness of 11 commercial medicines (basic or acidic drugs) using both a multichannel taste sensor and human gustatory sensation tests with 15 volunteers.

The measurement of electric potential was performed using the taste-sensing system SA402 of Anritsu Co., Ltd., Japan (Fig. 1). The electrode set was attached to a mechanically controlled robot arm. The detecting sensor part of the equipment consists of eight electrodes made of lipid/polymer membranes (channels). The lipids used in the present study are listed in Table 1. Each lipid was mixed in a test tube containing poly(vinyl chloride) and dioctylphenylphosphonate as plasticizer, dissolved in tetrahydrofuran in a test tube, and dried on a glass plate at a temperature of 30°C to form a transparent thin film (almost 200 μ m thick). Each electrode was made of an Ag wire whose surface was plated with Ag/AgCl, with an internal cavity filled with 3 M KCl solution. The difference between the electric potential of the working electrode and the reference electrode was measured by means of a high input impedance amplifier connected to a computer.

Samples consisting of 1 mM solutions of the following drugs were used in the study: quinine hydrochloride (taken as a standard of bitterness), trimebutine maleate, dibucaine hydrochloride, metronidazole, betamethasone-21-phosphate, salicylic acid, benzoic acid, diclofenac sodium, theophylline, acetaminophene, and anhydrous caffeine, purchased from Sigma Co. Ltd., U.S.A. Fresh 30 mM KCl solution containing 0.3 mM tartaric acid was used as a reference sample and also to rinse the electrodes after every measurement. The electric potential of the sample was measured as the difference be-

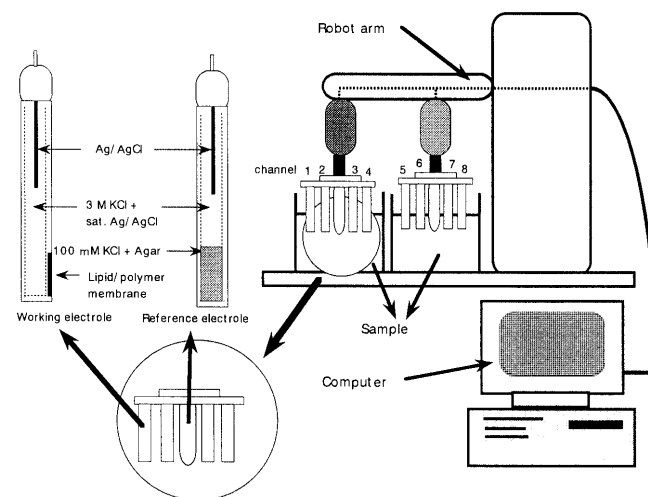


Fig. 1. Taste-Sensing System

Table 1. Lipids Used for the Membranes

Channel	Lipid component
1	Phosphoric acid di- <i>n</i> -decyl ester, dioctyl phenyl-phosphonate
2 ^{a)}	Phosphoric acid di- <i>n</i> -decyl ester, 2-nitrophenyl octyl ether
3	Hexadecanoic acid, dioctyl phenyl-phosphonate
4	Dioctyl phenyl-phosphonate
5, 6	Tetradodecylammoniumbromide, dioctyl phenyl-phosphonate
7, 8	Tetradodecylammoniumbromide, 2-nitrophenyl octyl ether

^{a)} The membrane used for channel 2 was more sensitive than that used for channel 1.

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tween the potentials of the sample and 30 mM KCl solution containing 0.3 mM tartaric acid. Each measuring time was set 30 s, and rinsing of electrodes after each measurement was repeated 45 times for about 180 s each time. Samples of each of the 11 drugs (1 mM solutions) were measured three times each according to a rotation procedure.

Figure 2 shows the electric potential response patterns for the 11 drugs. The data from the eight channel inputs has been reduced to a two-dimensional plane. The numbers 1—8 represent the channel numbers, and the scale on the x-axis represents the response electric potential (mV). The sensor output exhibits different patterns for each drug, although some drugs, such as quinine hydrochloride, trimebutine maleate, and dibucaine hydrochloride, show similar profiles. Figure 3 shows the relationship between the relative response electric potential (mV) of channel 1, with a negatively

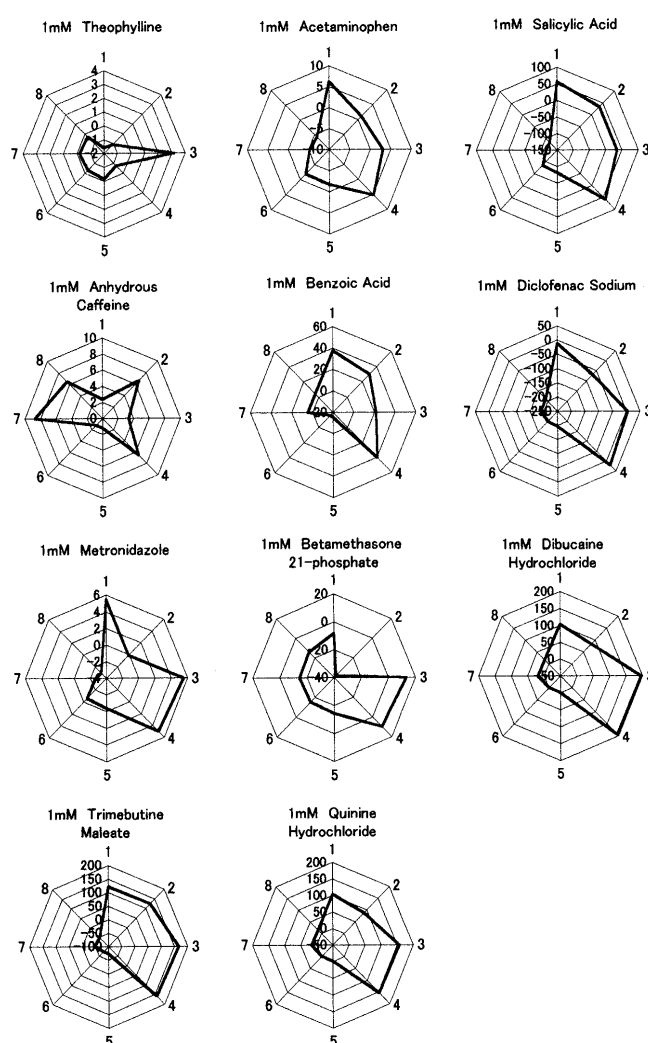


Fig. 2. Response Electric Potential Patterns for the 11 Drugs Tested

For explanation, see text.

charged membrane, and the bitterness score determined by human gustatory sensation tests as explained in Table 2. In this case, data for channel 1 was adopted as a representative data of channels 1—4 since simultaneous values for relative response electric potential (mV) were obtained in related to channels 2—4.

Although there are some exceptions, basic drugs (represented by closed symbols in Fig. 3) such as quinine, trimebutine, dibucaine, metronidazole, and betamethasone, tend to be rated as being more bitter and to have a greater relative response electric potential (mV) than acidic drugs (represented by open symbols) such as diclofenac, benzoic acid, salicylic acid, acetaminophen, theophylline, and caffeine. In particular, drugs with quaternary amino groups in the molecule, such as quinine, trimebutine and dibucaine, showed a comparatively strong electric response in the sensor. The surfaces of the membranes in channels 1—4 were charged negatively, due to dissociation of protons. An electric double layer is formed near the surface of the membrane in aqueous solution; cations such as amino groups accumulate near the surface of the negatively charged membrane. The electric potential then changes gradually from a negative value to zero. Therefore, basic drugs with amino groups are likely to show an increased relative response electric potential (mV).

Quinine was the most bitter drug tested, being scored 10/10 on the gustatory sensation test. The suppression of the bitterness of quinine by sucrose and aspartame could be quantified using the sensor, as shown in Fig. 4. The electric potential changes corresponding to channel 1 correlated well with the gustatory sensation score, thus confirming the usefulness of the sensor. Further, the ability of aspartame to suppress bitterness was found to be 100-fold greater than that of sucrose, as seen not only in the relative response electric potential but also in the gustatory sensation scores. (The effect of aspartame as an artificial sweetening agent has been reported to be 200-fold larger than the effect of sucrose.⁹⁾)

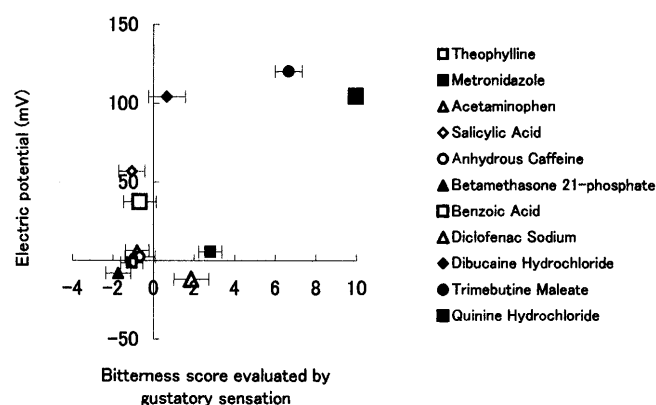


Fig. 3. The Correlation between the Relative Response Electric Potential (mV) of Channel 1 for Each of the Sample Drugs and the Bitterness Score Determined in Human Gustatory Sensation Tests

Table 2. Bitterness Score and the Corresponding Standard Quinine Hydrochloride Concentration of Bitterness

Bitterness Score ^{a)}	-10	-8	-6	-4	-2	0	2	4	6	8	10
Quinine concentration (mM)	0.0100	0.0158	0.0251	0.0398	0.0631	0.1000	0.1585	0.2512	0.3981	0.6310	1.0000

a) The value was evaluated by gustatory sensation tests in human volunteers. Before testing, the volunteer subjects kept the above standard samples in the mouth, and were told the concentration of the standard quinine solution samples and their bitterness score. Then, after tasting 1 mM sample of test drug solution, they were asked to give the sample a bitterness score. All samples were kept in the mouth for 15 s. After tasting the sample, subjects gargled well and waited for at least 20 min before tasting the next sample.

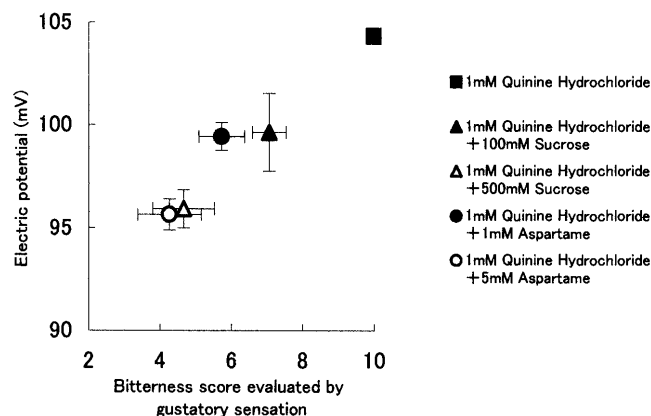


Fig. 4. The Correlation between the Relative Response Electric Potential (mV) of Channel 1 for Quinine and the Bitterness Score Determined in Human Gustatory Sensation Tests in the Presence or Absence of Sucrose or Aspartame

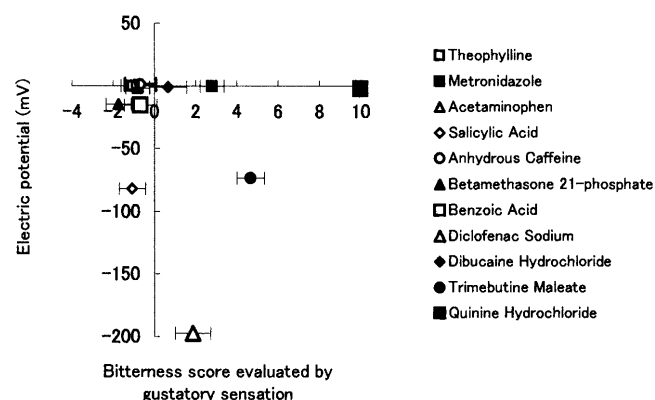


Fig. 5. The Correlation between the Relative Response Electric Potential (mV) of Channel 5 for Each of the Sample Drugs and the Bitterness Score Determined in Human Gustatory Sensation Tests

The membranes in channels 5—8 were charged positively. As a typical example, the response electric potential patterns from channel 5 for the 11 drugs was compared with the bitterness score derived from the gustatory sensation test (Fig. 5). Positively charged membranes have been used to evaluate sourness²⁾ and the correlation between the response electric potentials and bitterness scores from the gustatory sensation tests was not so good. Diclofenac sodium and salicylic acid both showed a large negative response, due to the large negative charge of the drug itself. If sourness had been adopted as a gustatory sensation criteria, a better correlation might have

been obtained. Caffeine, theophylline, and acetoaminophene contain both anionic and cationic charges in the molecule, even though they are acidic in aqueous solution. Evaluation of this type of drug using the taste sensor seems to require a more sensitive membrane than those used in the present study.

In conclusion, basic drugs with amino groups in the molecule such as quinine, show a comparatively good correlation between the relative response electric potential (mV) of channels 1 or 2 of the taste sensor, which contain negatively charged membranes, and bitterness as determined by human gustatory sensation tests. The suppression of the bitterness of quinine by sucrose and aspartame could also be quantified by this electric sensor. Secondly, for anionic drugs, such as diclofenac sodium or salicylic acid, the positively charged membrane in channel 5 or 6 seemed to be useful even though they are being sour rather than bitter (detail gustatory test in related to sourness was not shown). For drugs with both an amino (cationic) group and a carboxylic acid (anionic) group in the molecule, such as theophylline, caffeine, and metronidazole, the value of the relative response electric potential (mV) of channel 1 or 2 did not increase, even though bitterness was observed in human gustatory sensation tests. Therefore, different types of membrane component will be needed for a complete evaluation of the bitterness of medicines. For example, control of charge density and hydrophobicity of lipid membrane will dissolve the problem.

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References

- 1) Toko K., *Biosens Bioelectron*, **13**, 701—709 (1998).
- 2) Toko K., Matsuno T., Yamafuji K., Hayashi K., Ikezaki H., Sato K., Tohkubo R., Kawai S., *Biosens Bioelectron*, **9**, 359—64 (1994).
- 3) Hebbardt P., Bagla R., Doty R. L., *Behav. Res. Methods Instrum. Comput.*, **31**, 464—469 (1999).
- 4) Toko K., *IEEE IEDM Technical Digest*, 143—148 (1995).
- 5) Fukunaga T., Toko K., Mori S., Nakabayashi Y., Kanda M., *Sensors Materials*, **8**, 47—56 (1996).
- 6) American Dry Milk Institute, "Standards for Grades of Dry Milk Including Methods of Analysis," Am. Dry Milk Inst., Illinois, U.S.A., 1971, p. 4.
- 7) Iiyama S., Suzuki Y., Ezaki S., *Materials Science Engineering*, **4**, 45—49 (1996).
- 8) Takagi S., Toko K., Wada K., Yamada H., Toyoshima K., *J. Pharm. Sci.*, **87**, 552—555 (1998).
- 9) Kojima T., Osawa T., *Syokuhinn Kogyo* (in Japanese), **8**, 28—32 (1979).