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Synthesis and Thermal Cyclization Reactions of Methyl Isocrotonate Derivatives

NORIO KAWAHARA,*[,a] TAKAKO SHIMAMORI,[a] TSUNEO ITOH,[b] HIROAKI TAKAYANAGI,[b] and HARUO OGURA[b]

Hokkaido Institute of Pharmaceutical Sciences,[a] 7-1, Katsuraoka-cho, Otaru-shi, Hokkaido 047-02, Japan and School of Pharmaceutical Sciences, Kitasato University,[b] Shirokane, Minato-ku, Tokyo 108, Japan

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Methyl isocrotonate derivatives prepared from various 1,2-ethylenediamines, *o*-phenylenediamines, 2-aminoethanols or *o*-aminophenols with dimethyl acetylenedicarboxylate were easily converted to pyrido[1,2-*a*]pyrazines, pyrido[1,2-*a*]quinoxalines, pyrido[2,1-*c*]-1,4-oxazines, pyrido-[2,1-*c*]-1,4-benzoxazines, respectively, and other cyclization compounds by means of thermal reactions.

Keywords——cyclization; thermal reaction; methyl isocrotonate derivative; X-ray analysis; pyrido[1,2-*a*]pyrazine; pyrido[1,2-*a*]quinoxaline; pyrido[2,1-*c*]-1,4-oxazine; pyrido[2,1-*c*]-1,4-benzoxazine; dimethyl acetylenedicarboxylate

Acetylenic esters are widely used for the preparation of heterocyclic compounds.[1] As a part of our studies on the synthesis of heterocyclic compounds, we recently reported the results of the reactions of benzoxazoles[2] or nucleic acid bases[3] with dimethyl acetylenedicarboxylate (DMAD). Furthermore, we described a novel synthesis of pyrido[1,2-*a*]quinoxalines, pyrido[1,2-*a*]pyrazines, pyrido[2,1-*c*]-1,4-oxazine, pyrido[2,1-*c*]-1,4-benzoxazines and others.[4] Herein, we present additional information and complete experimental details of our work.

The reaction of *o*-phenylenediamine (**1**) with DMAD in MeOH at room temperature is known to produce 3-methoxycarbonylmethylene-2-oxo-1,2,3,4-tetrahydroquinoxaline (**2a**).[5] The further reaction of **2a** with DMAD under reflux in dioxane overnight afforded methyl 3-oxo-1,2,3,4-tetrahydroquinoxaline-β,γ-bismethoxycarbonyl-$\Delta^{2,\gamma}$-isocrotonate (**3a**).[4a] This compound (**3a**) could also be obtained directly by the reaction of *o*-phenylenediamine (**1a**) with DMAD in refluxing dioxane overnight[4a] as shown in Chart 1. The structure of **3a** was supported by the satisfactory elemental analysis and by comparing its spectral data with those of a similar compound, methyl 2,3-dihydro-2-oxo-4*H*-1,4-benzoxazine-β,γ-bismethoxycarbonyl-$\Delta^{2,\gamma}$-isocrotonate (**3b**, $R_1=R_2=H$).[2] Other compounds (**3b**, **6a** and **6b**) were also prepared in a similar manner. We have subsequently investigated the thermal reaction of these methyl isocrotonate derivatives.

A solution of **3a** ($R_1=R_2=H$) in dry dimethyl sulfoxide (DMSO) was refluxed for 1 h

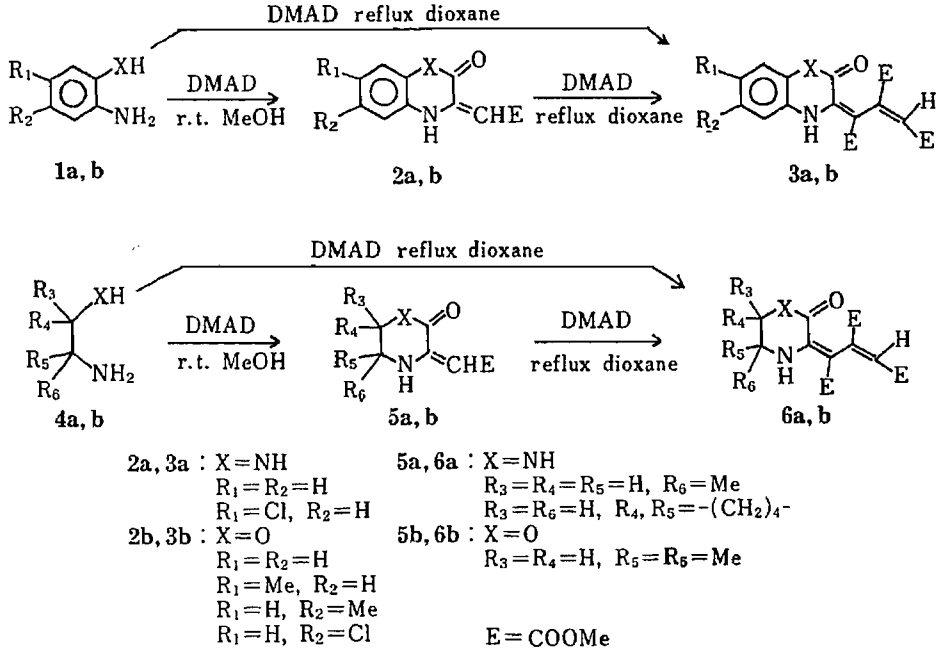


Chart 1

under a nitrogen atmosphere to give a brownish crystalline solid (63.9%), which was recrystallized from CH_2Cl_2–MeOH to afford yellow needles (**7**, $R_1=R_2=H$, mp 282—284 °C). In comparison with the starting material (**3a**), proton nuclear magnetic resonance (^{1}H-NMR) spectrum of this compound (**7**) showed a loss of one methyl ester and a downfield shift of a vinyl proton (δ 7.27). Moreover, one aromatic proton signal was observed at a very low magnetic field (δ 9.11). An empirical formula $C_{16}H_{12}N_2O_6$ was derived from elemental analysis, mass spectral analysis and the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum. From these data, the structure of this compound (**7**) was assigned as dimethyl 5,6-dihydro-6,10-dioxo-10*H*-pyrido[1,2-*a*]quinoxaline-7,8-dicarboxylate, which contains a newly formed 6-membered lactam ring. This compound was also prepared by photolysis of **3a** in MeOH in 40.5% yield. This pyrolysis resulted in the elimination of MeOH from **3a** ($C_{17}H_{16}N_2O_7$) so that intramolecular cyclization occurred. The lactone structures (A or A′) shown in Chart 2 were ruled out because of a downfield shift of one aromatic proton in the ^{1}H-NMR spectrum. It is assumed that this intramolecular cyclization proceeded *via* the conversion of the conjugated diene from the *s-trans* form to the *s-cis* form.

Similar thermal reactions of **6a** afforded similarly cyclized compounds, dimethyl 1,6-dioxo-1,2,3,4-tetrahydro-6*H*-pyrido[1,2-*a*]pyrazine-8,9-dicarboxylate derivatives (**8a**), in good yields.

Further, a solution of **6b** ($R_3=R_4=H$, $R_5=R_6=Me$) was pyrolyzed in the same manner to give **8b** in 69.6% yield. The elemental analysis and the mass spectrum (MS) of this compound (**8b**) indicated that this pyrolysis resulted in the elimination of MeOH from **6b**. The ^{1}H- and ^{13}C-NMR signals of **8b** were very similar to those of the previously prepared **8a**, suggesting that this compound is a similar cyclization product. Thus, the structure of **8b** was assigned to be dimethyl 3,4-dihydro-4,4-dimethylpyrido[2,1-*c*]-1,4-oxazine-1,6-dioxo-8,9-dicarboxylate.

On the other hand, **3b** ($R_1=H$, $R_2=Cl$) was pyrolyzed in the same manner and two crystalline compounds were isolated from the reaction mixture by silica gel column chromatography.

The major product (**9**, mp 239—241 °C, 38.8% yield) has an empirical formula

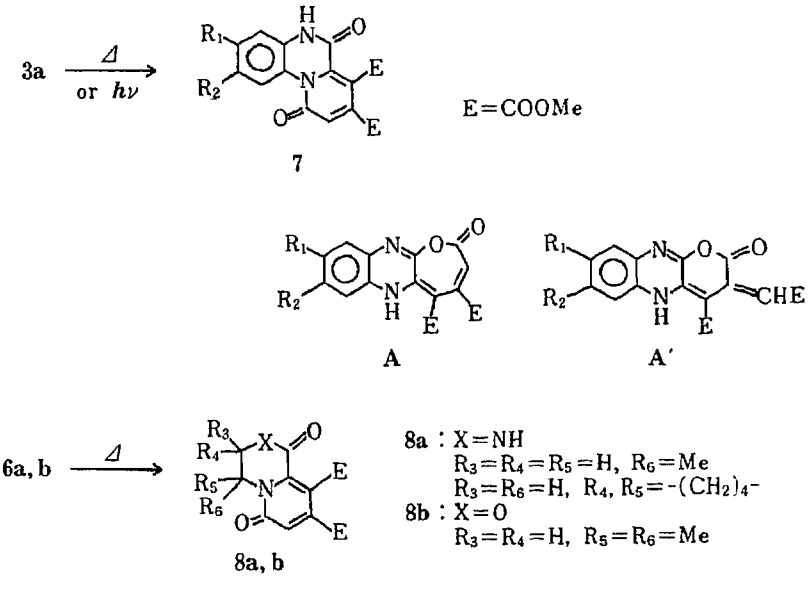


Chart 2

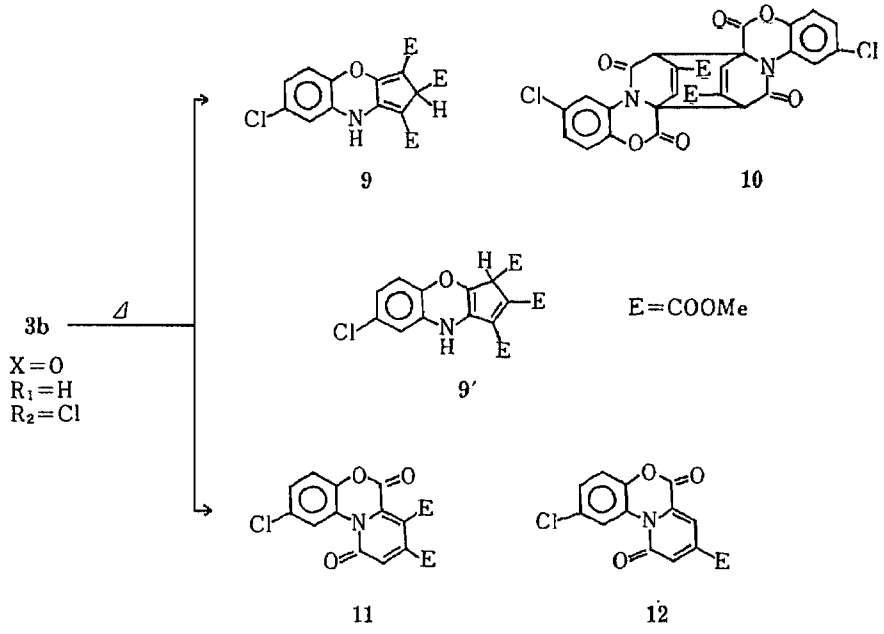
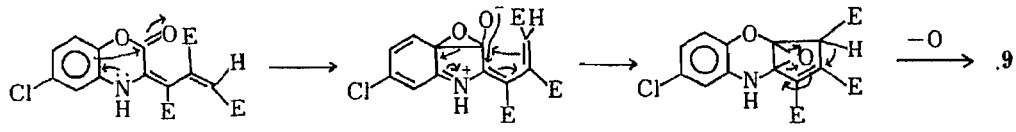


Chart 3

$C_{17}H_{14}ClNO_7$ which showed that this product is a deoxygenated compound of the starting material (**3b**, $C_{17}H_{14}ClNO_8$). The ^{1}H-NMR spectrum of this compound (**9**) showed the presence of three methyl esters at δ 3.60, 3.68 and 3.70, three aromatic protons at δ 6.89—7.51 and an amino proton at δ 10.09. A vinyl proton signal at δ 6.95 (1H, s) observed in **3b** (R_1 = H, R_2 = Cl) disappeared and instead, a signal at δ 4.47 (1H, s) appeared. The ^{13}C-NMR spectrum showed a signal at δ 49.60 which became a doublet in the single frequency off-resonance mode. These new signals (δ 4.47 and 49.60) suggested the presence of a methine carbon having a methoxycarbonyl group. Moreover, the infrared (IR) spectrum of **9** lacked the absorption at 1769 cm^{-1} observed in the case of **3b** (R_1 = H, R_2 = Cl), indicating the absence of a lactone structure in the molecule. From the data described above, this compound was considered to have a novel tricyclic structure, trimethyl 2,3-cyclopenta-1′,3′-dieno-6-chloro-1,4-

benzoxazine-3′,4′,5′-tricarboxylate (**9′**).[4b] In order to confirm the structure of this compound, we carried out an X-ray crystallographic analysis and the structure was revised as shown in Chart 3. In Fig. 1, the correct structure of **9** is shown: trimethyl 2,3-cyclopenta-1′(4′),2′-dieno-6-chloro-1,4-benzoxazine-3′,4′,5′-tricarboxylate (**9**). The atomic parameters of **9** obtained are listed in Table I, and the bond lengths and bond angles are shown in Table II. The formation of **9** can be formulated as the result of an electrocyclic reaction accompanying a deoxygenation process as shown in Chart 4.



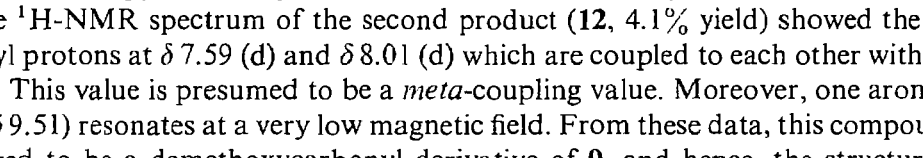
3b E=COOMe

Chart 4

A small amount (3.4% yield) of a second product was also obtained from the pyrolysis mixture as colorless fine prisms [**10**, mp 178 °C, MS *m/z* 305 and 307 ($M^+/2$)]. The novel structure of this compound was unequivocally determined by a crystallographic study. In Fig. 2, the molecular structure of **10** and the atomic numbering system are shown. The atomic parameters, bond lengths and bond angles are given in Tables III and IV. The mechanism of the formation of this dimerization product (**10**) was not clear but a compound assumed to be an intermediate in the formation of **10** was formed in the next pyrolysis.

Another pyrolysis of **3b** ($R_1=H$, $R_2=Cl$) was carried out in the same manner but for a shorter time than in the case of the previous pyrolysis. In addition to the starting material (**3b**) and **9** (17.3% yield), two new crystalline compounds were obtained. The first product (**11**, 11.9% yield) has the empirical formula $C_{16}H_{10}ClNO_7$, which indicated that this compound was prepared by elimination of MeOH from **3b**. The ^{1}H-NMR spectrum of **11** showed the presence of two methyl esters at δ 3.95 and 4.00, two aromatic protons at δ 7.19—7.46, a vinyl proton at δ 7.61 (s) and a downfield-shifted aromatic proton at δ 9.28 (d, $J=2.2$ Hz). In contrast to the result of the pyrolysis of **3a**, the structure of **11** was assigned to be dimethyl 2-chloro-6,10-dioxopyrido[2,1-*c*]-1,4-benzoxazine-7,8-dicarboxylate.

The ^{1}H-NMR spectrum of the second product (**12**, 4.1% yield) showed the presence of two vinyl protons at δ 7.59 (d) and δ 8.01 (d) which are coupled to each other with a J value of 1.95 Hz. This value is presumed to be a *meta*-coupling value. Moreover, one aromatic proton signal (δ 9.51) resonates at a very low magnetic field. From these data, this compound (**12**) was considered to be a demethoxycarbonyl derivative of **9**, and hence, the structure of **12** was elucidated to be methyl 2-chloro-6,10-dioxopyrido[2,1-*c*]-1,4-benzoxazine-8-carboxylate. This compound (**12**) may be an intermediate in the formation of the dimerized compound (**10**).



3b

R_1, R_2=H, Me

13 : $R_1=R_2=H$
15 : $R_1=H$, $R_2=Me$
17 : $R_1=Me$, $R_2=H$

14 : $R_1=R_2=H$
16 : $R_1=H$, $R_2=Me$
18 : $R_1=Me$, $R_2=H$

E=COOMe

Chart 5

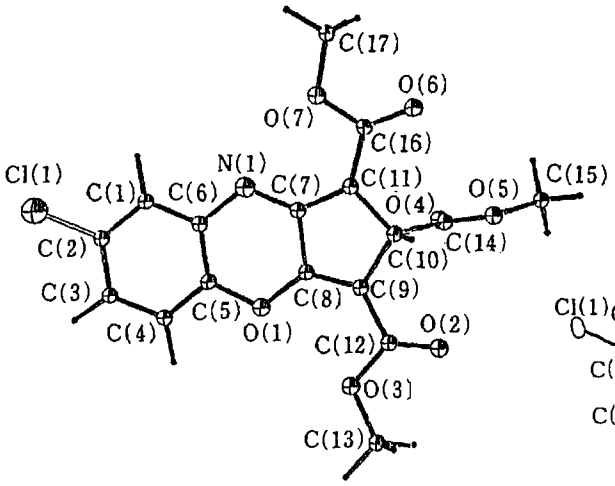


Fig. 1. The Molecular Structure of **9**

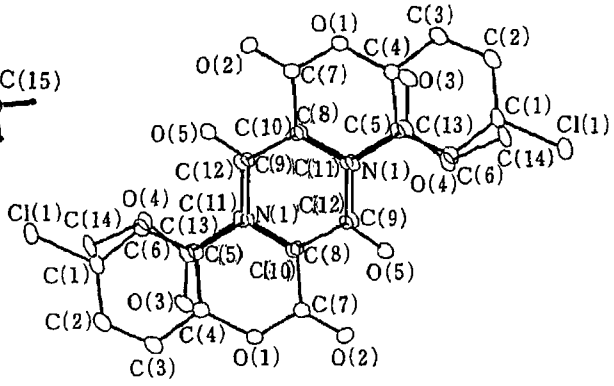


Fig. 2. The Molecular Structure of **10**

TABLE I. Positional ($\times 10^4$) and Thermal Parameters of **9** for Nonhydrogen Atoms with Their Standard Deviations in Parentheses

Atom	x	y	z	B_{eq} (Å^2)
Cl(1)	3863 (2)	75 (1)	4882 (4)	6.9
O(1)	3483 (2)	2417 (2)	5555 (6)	2.0
O(2)	3531 (3)	4158 (2)	5319 (9)	3.9
O(3)	2788 (3)	3447 (2)	6046 (8)	3.1
O(4)	5519 (3)	3701 (2)	6235 (6)	2.5
O(5)	5399 (3)	4294 (2)	4182 (7)	2.9
O(6)	6228 (3)	3360 (2)	2061 (7)	3.2
O(7)	6051 (3)	2457 (2)	2004 (7)	2.5
N(1)	4790 (3)	1999 (2)	3738 (7)	4.8
C(1)	4327 (5)	1097 (3)	4314 (10)	4.0
C(2)	3777 (4)	763 (3)	5066 (10)	3.8
C(3)	3158 (5)	961 (3)	6023 (10)	4.2
C(4)	3070 (4)	1527 (3)	6158 (10)	3.8
C(5)	3613 (4)	1864 (2)	5347 (10)	3.0
C(6)	4239 (4)	1653 (3)	4501 (10)	3.1
C(7)	4690 (4)	2541 (3)	3893 (8)	2.6
C(8)	4019 (3)	2751 (2)	4857 (8)	2.4
C(9)	4007 (3)	3287 (2)	4852 (8)	2.4
C(10)	4710 (4)	3482 (3)	3851 (9)	3.1
C(11)	5106 (4)	2966 (3)	3326 (8)	2.8
C(12)	3437 (4)	3689 (3)	5426 (11)	4.0
C(13)	2177 (5)	3833 (4)	6550 (14)	6.4
C(14)	5257 (3)	3837 (2)	4927 (9)	2.7
C(15)	5939 (5)	4653 (3)	5057 (14)	5.6
C(16)	5840 (4)	2969 (3)	2395 (10)	3.5
C(17)	6790 (4)	2411 (4)	1088 (11)	4.7

Compound **3b** ($R_1 = R_2 = H$) was pyrolyzed in the same manner as in the case of **3b** ($R_1 =$ H, $R_2 =$ Cl), and two crystalline compounds were isolated from the reaction mixture. One (69.9% yield) was the deoxygenated cyclopentadieno-1,4-benzoxazine derivative (**13**) and the other was assigned to be the pyrido[2,1-*c*]-1,4-benzoxazine derivative (**14**). When **3b** ($R_1 = H$, $R_2 =$ Me) was pyrolyzed in the same manner, **15** and **16** were obtained in 62.4% and 7.4%

TABLE II. Bond Lengths (Å) and Bond Angles (°) of **9** for Nonhydrogen Atoms with Their Standard Deviations in Parentheses

Cl(1)–C(2)	1.723 (7)	O(1)–C(5)	1.402 (8)
O(2)–C(12)	1.180 (9)	O(3)–C(12)	1.355 (9)
O(4)–C(14)	1.190 (9)	O(5)–C(14)	1.307 (8)
O(6)–C(16)	1.207 (9)	N(1)–C(7)	1.363 (8)
N(1)–C(6)	1.414 (9)	C(2)–C(3)	1.396 (11)
C(1)–C(6)	1.397 (10)	C(5)–C(6)	1.371 (9)
C(1)–C(2)	1.392 (11)	C(8)–C(9)	1.332 (8)
C(3)–C(4)	1.418 (11)	C(9)–C(12)	1.468 (10)
C(4)–C(5)	1.409 (10)	O(5)–C(15)	1.462 (10)
C(7)–C(8)	1.478 (9)	C(11)–C(16)	1.459 (9)
C(9)–C(10)	1.524 (9)	O(1)–C(8)	1.356 (7)
C(10)–C(11)	1.509 (9)	O(7)–C(17)	1.465 (9)
C(7)–C(11)	1.352 (9)	O(3)–C(13)	1.473 (11)
C(10)–C(14)	1.548 (9)	O(7)–C(16)	1.359 (9)
C(5)–O(1)–C(8)	116.2 (5)	C(12)–O(3)–C(13)	113.2 (6)
C(14)–O(5)–C(15)	115.3 (6)	C(16)–O(7)–C(17)	114.6 (6)
C(6)–N(1)–C(7)	118.5 (5)	C(2)–C(1)–C(6)	118.0 (7)
Cl(1)–C(2)–C(1)	119.8 (6)	Cl(1)–C(2)–C(3)	117.5 (6)
C(1)–C(2)–C(3)	122.7 (6)	C(2)–C(3)–C(4)	118.2 (7)
C(3)–C(4)–C(5)	118.9 (7)	O(1)–C(5)–C(4)	115.0 (6)
O(1)–C(5)–C(6)	123.9 (6)	C(4)–C(5)–C(6)	121.1 (6)
N(1)–C(6)–C(1)	118.8 (6)	N(1)–C(6)–C(5)	120.2 (6)
C(1)–C(6)–C(5)	121.0 (6)	N(1)–C(7)–C(8)	119.6 (6)
N(1)–C(7)–C(11)	132.4 (6)	C(8)–C(7)–C(11)	108.0 (6)
O(1)–C(8)–C(7)	121.7 (5)	O(1)–C(8)–C(9)	126.9 (6)
C(7)–C(8)–C(9)	111.3 (5)	C(8)–C(9)–C(10)	107.8 (5)
C(8)–C(9)–C(12)	133.6 (6)	C(10)–C(9)–C(12)	118.2 (5)
C(9)–C(10)–C(11)	103.4 (5)	C(9)–C(10)–C(14)	111.2 (6)
C(7)–C(11)–C(10)	109.4 (6)	C(11)–C(10)–C(14)	111.7 (5)
C(10)–C(11)–C(16)	121.6 (6)	C(7)–C(11)–C(16)	129.0 (6)
O(2)–C(12)–C(9)	124.0 (7)	O(2)–C(12)–O(3)	125.1 (7)
O(4)–C(14)–O(5)	125.5 (6)	O(3)–C(12)–C(9)	110.9 (6)
O(5)–C(14)–C(10)	110.6 (6)	O(4)–C(14)–C(10)	123.8 (6)
O(6)–C(16)–C(11)	126.2 (7)	O(6)–C(16)–O(7)	123.8 (6)
O(7)–C(16)–C(11)	109.9 (6)		

yields, respectively. Similarly, **3b** (R_1 = Me, R_2 = H) gave **17** and **18** in 6.9% and 43.8% yields, respectively.

In conclusion, pyrido[1,2-*a*]quinoxalines and pyrido[1,2-*a*]pyrazines were easily synthesized by the pyrolysis of the methyl isocrotonate derivatives (**3a** and **6a**), respectively. It was found that the pyrolysis of **6b** afforded the expected pyrido-[2,1-*c*]-1,4-oxazine (**8b**) in a good yield, but the pyrolysis of **3b** containing an aromatic ring afforded somewhat different results. When the substituent was H or Me, the deoxygenated cyclopentadieno-1,4-benzoxazine derivatives (**13**, **15** or **17**) and pyrido[2,1-*c*]-1,4-benzoxazine derivatives (**14**, **16** or **18**) were formed, but when chlorine was substituted on the aromatic ring (**3b**, R_1 = H, R_2 = Cl), a complex mixture containing the dimerized product was obtained.

Experimental

All melting points were determined on a micro hot-stage apparatus (Mitamura, Tokyo) and are uncorrected. IR spectra (ν_{max}) in KBr disks were recorded on a Hitachi 215 infrared spectrophotometer and data are expressed in cm^{-1}. ^1H- and ^{13}C-NMR spectra were measured on a JNM-FX 100 spectrometer (JEOL, Tokyo) at 100 MHz and

TABLE III. Positional ($\times 10^4$) and Thermal Parameters of **10** for Nonhydrogen Atoms with Their Standard Deviations in Parentheses

Atom	x	y	z	B_{eq} (Å^2)
Cl(1)	−2142 (2)	−1006 (1)	801 (2)	2.7
C(1)	−2110 (5)	−158 (4)	2794 (6)	2.1
C(2)	−3015 (5)	−715 (4)	4129 (6)	1.8
C(3)	−2922 (5)	−56 (4)	5718 (6)	2.3
C(4)	−1912 (5)	1122 (4)	5935 (5)	2.2
C(5)	−1001 (5)	1693 (4)	4602 (5)	1.6
C(6)	−1112 (5)	1048 (4)	2985 (5)	2.0
C(7)	−1169 (5)	2949 (4)	7934 (5)	2.2
C(8)	−424 (5)	3812 (4)	6431 (5)	2.0
C(9)	1053 (5)	3504 (4)	3747 (5)	1.6
C(10)	1697 (5)	5011 (4)	4142 (5)	1.5
C(11)	2105 (5)	5088 (4)	6045 (5)	1.8
C(12)	1076 (5)	4526 (4)	7175 (5)	1.9
C(13)	3563 (5)	5924 (4)	6577 (6)	1.9
C(14)	4932 (8)	6885 (7)	9030 (8)	2.2
O(1)	−1878 (4)	1674 (3)	7603 (4)	2.2
O(2)	−1201 (4)	3396 (3)	9362 (4)	2.8
O(3)	−4379 (4)	6576 (3)	5556 (4)	1.8
O(4)	3768 (4)	5878 (4)	8285 (4)	1.8
O(5)	1449 (4)	2891 (3)	2480 (4)	1.9
N(1)	−15 (4)	2924 (3)	4960 (4)	1.3

chemical shifts are expressed relative to 1% tetramethylsilane (TMS) as an internal standard; s = singlet, d = doublet, t = triplet, br = broad and m = multiplet. Low- and high-resolution MS were obtained with a GCMS-9000 spectrometer (Shimadzu, Tokyo) and JMS-DX 300 instrument (JEOL, Tokyo). Elemental analyses were done by the staff of the Analytical Center of the School of Pharmaceutical Sciences, Kitasato University (Tokyo), to whom our thanks are due. Thin-layer chromatography (TLC) was performed on Merck precoated Silica gel 60 F_{254} plates. Preparative TLC was done with the same commercial product, 20 × 20 cm, with a thickness of 0.25, 0.5 or 2.0 mm. For column chromatography, silica gel (Wakogel C-200, Wako Pure Chemical Industries, Osaka) was used.

Photolysis was carried out in a flask with a 300 W high-pressure mercury lamp covered with a Pyrex filter under nitrogen atmosphere and cooled internally with running water. All the chemicals used were of reagent grade, and were used without further purification.

Compounds **2b**, **3b**, **5b** and **6b** have already been reported.[2]

According to the literature,[4] **2a** and **5a** were easily prepared by the reaction of **1a** or **4a** with DMAD in MeOH at room temperature, respectively.

7-Chloro-3-methoxycarbonylmethylene-2-oxo-1,2,3,4-tetrahydroquinoxaline (2a, R_1 = Cl, R_2 = H)——Yellow needles, mp 246—248 °C (CH_2Cl_2–MeOH). Yield 83.2%. *Anal.* Calcd for $C_{11}H_9N_2O_3$: C, 52.29; H, 3.59; N, 11.09. Found: C, 52.23; H, 3.58; N, 11.03.

3-Methoxycarbonylmethylene-5-methyl-2-oxo-piperazine (5a, R_3 = R_4 = R_5 = H, R_6 = Me)——Pale yellow needles, mp 218—219.5 °C (CH_2Cl_2–MeOH). Yield 58.7%. *Anal.* Calcd for $C_8H_{12}N_2O_3$: C, 52.16; H, 6.57; N, 15.21. Found: C, 52.12; H, 6.60; N, 15.25. ^{13}C-NMR ($CDCl_3$) δ: 18.66 (q), 45.61 (d), 46.68 (t), 50.73 (q), 86.45 (d), 148.28 (s), 161.05 (s), 170.89 (s).

3-Methoxycarbonylmethylene-2-oxo-decahydroquinoxaline (5a, R_3 = R_6 = H, R_4, R_5 = −$(CH_2)_4$−)——White prisms, mp 198—200 °C (CH_2Cl_2–MeOH). Yield 67.1%. ^{13}C-NMR ($CDCl_3$) δ: 21.10 (t), 21.59 (t), 29.38 (t), 49.02 (d), 50.68 (d), 50.68 (q), 85.96 (d), 148.38 (s), 161.34 (s), 170.99 (s). *Anal.* Calcd for $C_{11}H_{16}N_2O_3$: C, 58.91; H, 7.19; N, 12.49. Found: C, 58.98, H, 7.28; N, 12.53.

Methyl 3-Oxo-1,2,3,4-tetrahydroquinoxaline-β,γ-bismethoxycarbonyl-$\Delta^{2,7}$-isocrotonate (3a, R_1 = R_2 = H)——Method A (**3a** from **2a**): DMAD (5 ml) was added to a dry dioxane (30 ml) solution of **2a** (R_1 = R_2 = H, 3.0 g) and the mixture was refluxed overnight under a nitrogen atmosphere. The solvent was evaporated off *in vacuo* to afford a crystalline solid, which was washed with EtOH and recrystallized from CH_2Cl_2–EtOH to give 1.54 g of **3a** (mp 220—221 °C, yellow powder). The filtrate and washings were combined and the solvent was evaporated off *in vacuo*. The residue was chromatographed on silica gel with benzene–ether (9:1) as an eluent to give 1.25 g of **3a** (total 2.79 g, 56.4%).

Method B (**3a** from **1a**): DMAD (5 ml) was added to a dry dioxane (30 ml) solution of *o*-phenylenediamine (**1a**,

TABLE IV. Bond Lengths (Å) and Bond Angles (°) of **10** for Nonhydrogen Atoms with Their Standard Deviations in Parentheses

C(1)-Cl(1)	1.738 (4)	C(9)-C(10)	1.530 (5)
C(1)-C(2)	1.375 (6)	C(9)-N(1)	1.401 (5)
C(1)-C(6)	1.396 (6)	C(9)-O(5)	1.201 (5)
C(2)-C(3)	1.378 (6)	C(10)-C(12)	1.500 (5)
C(3)-C(4)	1.384 (6)	C(11)-C(12)	1.330 (6)
C(4)-C(5)	1.381 (5)	C(11)-C(13)	1.500 (6)
C(4)-O(1)	1.391 (5)	C(13)-O(3)	1.200 (5)
C(5)-C(6)	1.392 (5)	C(13)-O(4)	1.318 (5)
C(5)-N(1)	1.429 (5)	C(14)-O(4)	1.460 (7)
C(7)-C(8)	1.526 (5)	C(7)-O(1)	1.344 (5)
C(7)-O(2)	1.183 (5)	C(8)-C(12)	1.529 (6)
C(8)-N(1)	1.473 (5)	C(8)-C(10)[a]	1.630 (6)
Cl(1)-C(1)-C(2)	119.5 (3)	N(1)-C(8)-C(10)	112.0 (3)
Cl(1)-C(1)-C(6)	117.9 (3)	C(10)-C(9)-N(1)	114.8 (3)
C(2)-C(1)-C(6)	122.6 (4)	C(10)-C(9)-O(5)	121.3 (3)
C(1)-C(2)-C(3)	118.4 (4)	N(1)-C(9)-O(5)	123.8 (3)
C(2)-C(3)-C(4)	119.6 (4)	C(9)-C(10)-C(11)	109.0 (3)
C(3)-C(4)-C(5)	122.5 (4)	C(9)-C(10)-C(8)	110.4 (3)
C(3)-C(4)-O(1)	114.7 (4)	C(11)-C(10)-C(8)	111.2 (3)
C(5)-C(4)-O(1)	122.8 (3)	C(10)-C(11)-C(12)	117.9 (4)
C(4)-C(5)-O(6)	118.1 (3)	C(10)-C(11)-C(13)	116.3 (3)
C(4)-C(5)-N(1)	118.0 (3)	C(12)-C(11)-C(13)	123.5 (4)
C(6)-C(5)-N(1)	123.9 (3)	C(8)-C(12)-C(11)	117.5 (3)
C(1)-C(6)-C(5)	118.7 (4)	C(4)-O(1)-C(7)	121.3 (3)
C(8)-C(7)-O(1)	118.9 (3)	C(5)-N(1)-C(8)	117.8 (3)
C(8)-C(7)-O(2)	121.9 (4)	C(5)-N(1)-C(9)	122.2 (3)
O(1)-C(7)-O(2)	119.1 (4)	C(8)-N(1)-C(9)	118.0 (3)
C(7)-C(8)-C(12)	106.3 (3)	C(11)-C(13)-O(3)	123.0 (4)
C(7)-C(8)-N(1)	113.1 (3)	C(11)-C(13)-O(4)	111.0 (3)
C(7)-C(8)-C(11)	105.3 (3)	O(3)-C(13)-O(4)	126.0 (4)
C(12)-C(8)-N(1)	108.8 (3)	C(13)-O(4)-C(14)	117.2 (4)
C(12)-C(8)-C(10)	111.3 (3)		

a) The bond length of C(8)-C(10) is longer than other C-C bond lengths, suggesting that this bond links the two components of the dimer.

1.08 g) and the mixture was refluxed overnight. After the same treatment as described above of the reaction mixture, 1.68 g (46.7%) of **3a** was obtained. ^{13}C-NMR (DMSO-d_6) δ: 51.51 (q), 52.34 (q), 94.00 (s), 115.00 (d), 116.00 (d), 123.60 (s), 123.78 (d), 124.65 (d), 125.28 (d), 140.68 (s), 142.58 (s), 156.27 (s), 165.34 (s), 166.12 (s), 168.65 (s). *Anal.* Calcd for $C_{17}H_{16}N_2O_7$: C, 56.67; H, 4.47; N, 7.78. Found: C, 56.52; H, 4.37; N, 7.63.

Methyl 6-Chloro-3-oxo-1,2,3,4-tetrahydroquinoxaline-β,γ-bismethoxycarbonyl-$\Delta^{2,7}$-isocrotonate (3a, R_1=Cl, R_2=H)——47.4% yield (Method A). Yellow powder, mp 195—197 °C (CH_2Cl_2-MeOH). ^{13}C-NMR ($CDCl_3$) δ: 51.75 (q), 51.95 (q), 52.78 (q), 96.29 (s), 115.15 (d), 115.88 (d), 124.26 (d), 124.65 (s), 125.53 (d), 125.53 (s), 127.87 (s), 139.17 (s), 142.49 (s), 158.42 (s), 166.17 (s), 167.14 (s), 169.48 (s). *Anal.* Calcd for $C_{17}H_{15}ClN_2O_7$: C, 51.72; H, 3.83; N, 7.10. Found: C, 51.73; H, 3.82; N, 6.97.

Methyl 6-Methyl-3-oxo-piperazine-β,γ-bismethoxycarbonyl-$\Delta^{2,7}$-isocrotonate (6a, R_3=R_4=R_5=H, R_6=Me)——53.1% yield (Method A). Yellow rods, mp 181—182.5 °C (EtOH). ^{13}C-NMR ($CDCl_3$) δ: 17.98 (q), 45.81 (d), 46.73 (t), 51.26 (q), 51.41 (q), 52.29 (q), 124.75 (d), 144.00 (s), 144.80 (s), 145.70 (s), 161.29 (s), 166.02 (s), 167.24 (s), 169.29 (s). *Anal.* Calcd for $C_{14}H_{18}N_2O_7$: C, 51.53; H, 5.56; N, 8.59. Found: C, 51.39; H, 5.64; N, 8.55.

Methyl 3-Oxo-decahydroquinoxaline-β,γ-bismethoxycarbonyl-$\Delta^{2,7}$-isocrotonate (6a, R_3=R_6=H, R_4, R_5=-$(CH_2)_4$-)——47.7% yield (Method A). Yellow needles, mp 147—149 °C (ether). ^{13}C-NMR ($CDCl_3$) δ: 21.34 (t), 28.56 (t), 29.29 (t), 49.22 (d), 49.95 (d), 51.17 (q), 51.41 (q), 52.24 (q), 94.00 (s), 124.26 (d), 143.21 (s), 146.19 (s), 161.93 (s), 166.12 (s), 167.24 (s), 169.43 (s). *Anal.* Calcd for $C_{17}H_{22}N_2O_7$: C, 55.73; H, 6.05; N, 7.65. Found: C, 55.90; H, 6.03; N, 7.58.

General Procedure for the Thermal Reaction of 3 and 6——A solution of **3** or **6** (0.082 mol) in dry DMSO (70 ml) was refluxed for 30 min or 1 h under a nitrogen atmosphere. After removal of the solvent *in vacuo* on a hot bath,

TABLE V. Spectral Data for Compounds **2**, **3** and **5**—**18**

Compd. No.	MS m/z (M^+)	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR ($CDCl_3$)[a] δ
2a ($R_1=R_2=H$)	218	1690, 1620	[a] 3.73 (3H, s), 5.57 (1H, s), 7.02—7.60 (4H, m), 9.05 (1H, br), 11.13 (1H, br)
2a ($R_1=Cl$, $R_2=H$)	252, 254	1680, 1620	[a] 3.82 (3H, s), 5.52 (1H, s), 7.03—7.53 (3H, m), 11.00 (1H, br)
3a ($R_1=R_2=H$)	360	1720, 1650, 1595	3.68, 3.71, 3.76 (each 3H, s), 6.90 (1H, s), 7.00—7.60 (4H, m), 10.57 (1H, br), 12.23 (1H, br)
3a ($R_1=Cl$, $R_2=H$)	394, 396	1690, 1620	3.72 (6H, s), 3.84 (3H, s), 6.92 (1H, s), 6.90—7.08 (3H, m), 11.22 (1H, br), 12.15 (1H, br)
5a ($R_6=Me$)	184	1680, 1620	1.28 (3H, d, $J=6.2$ Hz), 3.00—3.60 (2H, m), 3.69 (3H, s), 3.69—4.00 (1H, m), 5.61 (1H, s), 7.23 (1H, br), 8.27 (1H, br)
5a ($R_4,R_5=-(CH_2)_4-$)	222	1680, 1645, 1610	1.00—1.95 (8H, br), 3.40—3.75 (2H, br), 3.69 (3H, s), 5.60 (1H, s), 7.50 (1H, br), 8.20 (1H, br)
6a ($R_6=Me$)	326	1710, 1640 1610	1.24 (3H, d, $J=6.2$ Hz), 3.00—4.00 (3H, br), 3.61, 3.67, 3.74 (each 3H, s), 6.74 (1H, s), 7.24 (1H, br), 9.30 (1H, br)
6a ($R_4,R_5=-(CH_2)_4-$)	366	1710, 1640, 1575	1.00—1.95 (8H, br), 3.60—3.70 (2H, br), 3.60, 3.66, 3.70 (each 3H, s), 6.70 (1H, s), 8.22 (1H, br), 9.29 (1H, br)
7 ($R_1=R_2=H$)	328	1740, 1710, 1650, 1580	[a] 3.76, 3.85 (each 3H, s), 7.00—7.30 (3H, m), 7.27 (1H, s), 9.11 (1H, d, $J=9.0$ Hz), 12.02 (1H, br)
7 ($R_1=Cl$, $R_2=H$)	362, 364	1725, 1660, 1590	[a] 3.76, 3.84 (each 3H, s), 7.20—7.30 (3H, m), 7.28 (1H, s), 9.16 (1H, d, $J=9.0$ Hz), 12.09 (1H, br)
8a ($R_6=Me$)	294	1740, 1660	1.40 (3H, d, $J=6.2$ Hz), 3.33—3.90 (2H, m), 3.88, 3.90 (each 3H, s), 4.69 (1H, m), 7.35 (1H, s), 8.14 (1H, br)
8a ($R_4,R_5=-(CH_2)_4-$)	334	1740, 1700, 1660	1.30—2.30 (8H, br), 3.87, 3.90 (each 3H, s), 3.90 (1H, br), 4.90 (1H, br), 7.32 (1H, s), 8.55 (1H, br)
8b ($R_5=R_6=Me$)	309	1730, 1665, 1610	1.72 (6H, s), 3.91 (6H, s), 4.27 (2H, s), 7.27 (1H, s)
9	379, 381	1710, 1690, 1660, 1620	[a] 3.60, 3.68, 3.70 (each 3H, s), 4.47 (1H, s), 7.50 (1H, d, $J=2.4$ Hz), 7.11 (1H, d, $J=8.6$ Hz), 6.95 (1H, dd, $J=8.6$, 2.4 Hz)
11	363, 365	1760, 1740, 1685	3.95, 4.00 (each 3H, s), 7.24 (1H, d, $J=8.8$ Hz), 7.41 (1H, dd, $J=8.8$, 2.2 Hz), 9.29 (1H, d, $J=2.2$ Hz)
12	305, 307	1750, 1675, 1620	4.00 (3H, s), 7.25 (1H, d, $J=8.1$ Hz), 7.40 (1H, dd, $J=8.1$, 2.4 Hz), 7.59 (1H, d, $J=2.0$ Hz), 8.01 (1H, d, $J=2.0$ Hz), 9.51 (1H, d, $J=2.4$ Hz)
13	345	1720, 1700, 1640	3.75, 3.79, 3.83 (each 3H, s), 4.60 (1H, s), 6.80—7.20 (4H, m), 8.45 (1H, br)
14	329	1760, 1730, 1675, 1590	3.95, 3.99 (each 3H, s), 7.27—7.38 (3H, m), 7.58 (1H, s), 9.15 (1H, br)
15	359	1700, 1660, 1620	2.28 (3H, s), 3.75, 3.77, 3.81 (each 3H, s), 4.57 (1H, s), 6.64 (1H, br), 6.82 (1H, d, $J=8.1$ Hz), 7.09 (1H, d, $J=8.1$ Hz), 8.35 (1H, br)
16	343	1730, 1680	2.42 (3H, s), 3.95, 4.00 (each 3H, s), 7.19—7.26 (2H, br), 7.60 (1H, s), 8.99 (1H, br)
17	359	1700, 1655, 1615	2.29 (3H, s), 3.74, 3.78, 3.82 (each 3H, s), 4.62 (1H, s), 6.73 (1H, d, $J=10.0$ Hz), 6.88 (1H, d, $J=10.0$ Hz), 7.03 (1H, br), 8.35 (1H, br)
18	343	1755, 1730, 1670	2.42 (3H, s), 3.95, 4.00 (each 3H, s), 7.00—7.26 (2H, br), 7.60 (1H, s), 9.08 (1H, d, $J=9.3$ Hz)

a) DMSO-d_6.

extraction of the reaction product with ethyl acetate or methylene chloride followed by evaporation of the solvent *in vacuo* afforded a brownish crystalline solid or oil which was purified by column chromatography and/or preparative TLC, and finally by recrystallization from an appropriate solvent.

Dimethyl 5,6-Dihydro-6,10-dioxo-10*H*-pyrido[1,2-*a*]quinoxaline-7,8-dicarboxylate (7, $R_1=R_2=H$)——According to the general procedure, the crude product was obtained from **3a** ($R_1=R_2=H$, 3.0 g) and recrystallized from CH_2Cl_2–MeOH to afford yellow needles, mp 282—284 °C. Yield 1.75 g (63.9%). ^{13}C-NMR (DMSO-d_6) δ: 52.14 (q), 53.12 (q), 95.46 (s), 116.03 (d), 120.90 (d), 122.26 (d), 122.85 (s), 125.77 (d), 128.11 (d), 128.31 (s), 133.42 (s), 136.78 (s), 154.43 (s), 160.51 (s), 163.34 (s), 165.49 (s). *Anal.* Calcd for $C_{16}H_{12}N_2O_6$: C, 58.54; H, 3.68; N, 8.53. Found: C, 58.52; H, 3.51; N, 8.29.

Photolysis of 3a——A solution of **3a** ($R_1=R_2=H$, 0.3 g) in dry MeOH (400 ml) was irradiated for 6 h. The crystalline solid obtained upon removal of the solvent was washed with MeOH to give 0.086 g of **7**. The filtrate and washings were combined and the solvent was evaporated off *in vacuo* and purified by preparative TLC to give a further 0.025 g of 7 (total 0.111 g, 40.5%).

Dimethyl 3-Chloro-5,6-dihydro-6,10-dioxo-10*H*-pyrido[1,2-*a*]quinoxaline-7,8-dicarboxylate (7, $R_1=Cl$, $R_2=H$)——According to the general procedure, the crude product was obtained from **3a** ($R_1=Cl$, $R_2=H$, 1.0 g) and recrystallized from CH_2Cl_2–MeOH to afford a brownish powder, mp 295—296 °C. Yield 0.511 g (51.7%). *Anal.* Calcd for $C_{16}H_{11}ClN_2O_6$: C, 52.97; H, 3.06; N, 7.72. Found: C, 52.76; H, 2.89; N, 7.60.

Dimethyl 4-Methyl-1,6-dioxo-1,2,3,4-tetrahydro-6*H*-pyrido[1,2-*a*]pyrazine-8,9-dicarboxylate (8a, $R_3=R_4=R_5=H$, $R_6=Me$)——According to the general procedure, the crude product was obtained from **6a** ($R_3=R_4=R_5=H$, $R_6=Me$, 0.24 g) and recrystallized from CH_2Cl_2–ether to afford pale yellow cubes, mp 202.5—203.5 °C. Yield 0.173 g (79.7%). ^{13}C-NMR ($CDCl_3$) δ: 18.03 (q), 44.78 (d), 46.34 (t), 52.92 (q), 53.31 (q), 115.73 (s), 126.31 (d), 134.64 (s), 138.29 (s), 158.86 (s), 159.73 (s), 163.39 (s), 166.27 (s). *Anal.* Calcd for $C_{13}H_{14}N_2O_6$: C, 53.06; H, 4.80; N, 9.52. Found: C, 52.92; H, 4.81; N, 9.31.

Dimethyl 6,10-Dioxo-10*H*-pyrido[1,2-*a*]decahydroquinoxaline-7,8-dicarboxylate (8a, $R_3=R_6=H$, R_4, $R_5=-(CH_2)_4-$)——According to the general procedure, the crude product was obtained from **6a** ($R_3=R_6=H$, R_4, $R_5=-(CH_2)_4-$, 1.0 g) and recrystallized from MeOH to afford pale yellow prisms, mp 243—245 °C. Yield 0.597 g (65.4%). ^{13}C-NMR ($CDCl_3$) δ: 18.57 (t), 23.73 (t), 25.14 (t), 28.65 (t), 47.61 (d), 51.70 (d), 52.68 (q), 53.26 (q), 115.88 (s), 126.36 (d), 133.96 (s), 138.05 (s), 159.20 (s), 159.78 (s), 163.44 (s), 166.32 (s). *Anal.* Calcd for $C_{16}H_{18}N_2O_6$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.35; H, 5.35; N, 8.30.

Dimethyl 3,4-Dihydro-4,4-dimethylpyrido[2,1-*c*]-1,4-oxazine-1,6-dioxo-8,9-dicarboxylate (8b, $R_3=R_4=H$, $R_5=R_6=Me$)——According to the general procedure, the crude product was obtained from **6b** ($R_3=R_4=H$, $R_5=R_6=Me$, 0.5 g) and recrystallized from MeOH–ether to afford white rods, mp 203—205 °C. Yield 0.317 g (69.9%). *Anal.* Calcd for $C_{14}H_{15}NO_7$: C, 54.37; H, 4.89; N, 4.53. Found: C, 54.36; H, 4.86; N, 4.54.

Thermal Reaction of 3b ($R_1=H$, $R_2=Cl$)——1) A solution of **3b** ($R_1=H$, $R_2=Cl$, 3.0 g) in DMSO (30 ml) was refluxed for 1 h under a nitrogen atmosphere. According to the general procedure, the residue was subjected to silica gel column chromatography (30 g) to give **9** (eluted with 3% ether in benzene), which was recrystallized from CH_2Cl_2–MeOH to afford needles, mp 239—241 °C. Yield 1.17 g (38.8%). ^{13}C-NMR (DMSO-d_6) δ: 49.61 (d), 51.02 (q), 51.51 (q), 52.14 (q), 98.53 (s), 107.64 (s), 116.22 (d), 117.93 (d), 122.19 (d), 126.45 (s), 128.45 (s), 138.88 (s), 141.65 (s), 152.87 (s), 161.59 (s), 163.34 (s), 169.58 (s). *Anal.* Calcd for $C_{17}H_{14}ClNO_7$: C, 53.75; H, 3.69; N, 3.69. Found: C, 53.49; H, 3.71; N, 3.73. **10** (0.159 g, 3.4%) was isolated by preparative TLC (benzene : ethyl acetate = 9 : 1) of the second fraction (eluted with 5% ether in benzene) of the above column chromatography. This compound (**10**) was repeatedly recrystallized from CH_2Cl_2–ether to give pure colorless prisms (mp 178 °C).

2) A solution of **3b** ($R_1=H$, $R_2=Cl$, 3.0 g) in dry DMSO (30 ml) was refluxed for 30 min under a nitrogen atmosphere. According to the general procedure, the residue was subjected to silica gel column chromatography (30 g) to give **12** (eluted with 1% ether in benzene), which was recrystallized from CH_2Cl_2–ether to afford yellow needles, mp 176—178 °C. Yield 0.095 g (4.1%). ^{13}C-NMR ($CDCl_3$) δ: 53.41 (q), 111.98 (d), 118.56 (d), 121.39 (d), 123.09 (s), 128.79 (d), 128.98 (s), 130.26 (d), 130.69 (s), 138.29 (s), 139.95 (s), 154.13 (s), 161.01 (s), 163.20 (s). *Anal.* Calcd for $C_{14}H_8ClNO_5$: C, 54.96; H, 2.64; N, 4.58. Found: C, 54.54; H, 2.59; N, 4.50. The high-resolution MS showed *m/z* 305.00895 (Calcd 305.00903). **11**, eluted with 1.5% ether in benzene, was recrystallized from CH_2Cl_2–ether to afford pale yellow needles, mp 203—204 °C. Yield 0.429 g (11.9%). ^{13}C-NMR ($CDCl_3$) δ: 53.46 (q), 53.65 (q), 118.42 (d), 120.07 (s), 121.24 (d), 122.51 (s), 126.84 (s), 129.13 (d), 130.64 (s), 130.79 (d), 136.93 (s), 139.81 (s), 153.01 (s), 159.64 (s), 162.32 (s), 164.90 (s). *Anal.* Calcd for $C_{16}H_{10}ClNO_7$: C, 52.83; H, 2.78; N, 3.85. Found: C, 52.87; H, 2.70; N, 4.20. The high-resolution MS showed *m/z* 365.00971 (Calcd 365.01154).

X-Ray Analysis——1) The crystals of **9** are orthorhombic [$a=17.082$ (3) Å, $b=24.832$ (9) Å, $c=8.028$ (2) Å, $V=3405$ Å^3, $Z=8$, $D_{calc.}=1.482$ g cm^{-1}, space group P_{bca}]. The reflection data were collected on a Rigaku four-circle diffractometer using graphite-monochromated Cu-$K\alpha$ radiation. Those reflections having an intensity exceeding 3 times the corresponding standard deviations were collected, and 2053 reflections out of 2375 reflections were used as data. The structure was solved by the heavy-atom (chlorine) method and refined by the block-diagonal least-squares method to a final R value of 0.099.

2) The crystals of **10** are monoclinic [$a=8.5490$ Å, $b=9.3740$ Å, $c=7.6670$ Å, $V=613.61$ Å^3, $Z=1$, $D_{calc.}=$

1.654 g cm^{-1}, space group $P\bar{1}$]. The reflection data were collected on a Rigaku four-circle diffractometer using graphite-monochromated Cu-$K\alpha$ radiation. Those reflections having an intensity exceeding 3 times the corresponding standard deviations were collected and 1724 reflections out of 1984 reflections were used as data. The structure was solved by the heavy-atom (chlorine) method and refined by the block-diagonal least-squares method to a final R value of 0.074.

Thermal Reaction of 3b ($R_1=R_2=H$)——A solution of **3b** ($R_1=R_2=H$, 3.0 g) in dry DMSO (30 ml) was refluxed for 30 min under a nitrogen atmosphere. According to the general procedure, the residue was purified by silica gel column chromatography (70 g) with benzene–ether as an eluent to give **14** (2% ether–benzene), which was recrystallized from CH_2Cl_2–ether to afford pale yellow needles, mp 205—206 °C. Yield 0.166 g (6.1%). ^{13}C-NMR ($CDCl_3$) δ: 53.41 (q), 53.55 (q), 117.44 (d), 121.19 (d), 121.97 (s), 125.33 (d), 129.13 (d), 129.13 (s), 130.69 (d), 136.69 (s), 141.17 (s), 153.54 (s), 159.78 (s), 162.46 (s), 165.19 (s). *Anal.* Calcd for $C_{14}H_{11}NO_7$: C, 58.36; H, 3.37; N, 4.25. Found: C, 58.13; H, 3.29; N, 4.27. Compound **13**, eluted with 3% ether–benzene, was recrystallized from CH_2Cl_2–MeOH to afford pale yellow needles, mp 235—238 °C. Yield 1.955 g (68.2%). ^{13}C-NMR ($CDCl_3$) δ: 49.90 (d), 51.36 (q), 51.70 (q), 52.58 (q), 97.85 (s), 108.18 (s), 115.49 (d), 117.54 (d), 123.24 (d), 124.21 (s), 125.04 (d), 140.93 (s), 144.09 (s), 153.50 (s), 162.51 (s), 165.24 (s), 170.31 (s). *Anal.* Calcd for $C_{17}H_{15}NO_7$: C, 59.13; H, 4.38; N, 4.06. Found: C, 59.06; H, 4.38; N, 3.98.

Thermal Reaction of 3b ($R_1=H$, $R_2=Me$)——A solution of **3b** ($R_1=H$, $R_2=Me$, 4.0 g) in dry DMSO (40 ml) was refluxed for 1 h under a nitrogen atmosphere. According to the general procedure, the residue was purified by silica gel column chromatography (70 g) with benzene–ether as an eluent to give **16** (1% ether–benzene), which was recrystallized from ethyl acetate to afford yellow needles, mp 201–202 °C. Yield 0.272 g (7.4%). ^{13}C-NMR ($CDCl_3$) δ: 21.44 (q), 53.41 (q), 53.55 (q), 117.05 (d), 119.68 (s), 121.14 (d), 121.63 (s), 127.43 (s), 129.76 (d), 130.54 (d), 135.42 (s), 136.64 (s), 139.12 (s), 153.69 (s), 159.84 (s), 162.57 (s), 165.24 (s). *Anal.* Calcd for $C_{17}H_{13}NO_7$: C, 59.48; H, 3.82; N, 4.08. Found: C, 59.02; H, 3.76; N, 3.96. The high-resolution MS showed m/z 343.06813 (Calcd 343.06910). Compound **15**, eluted with 1.5% ether–benzene was recrystallized from CH_2Cl_2–MeOH to afford yellow plates, mp 247—249 °C. Yield 2.39 g (62.4%). *Anal.* Calcd for $C_{18}H_{17}NO_7$: C, 60.16; H, 4.79; N, 3.90. Found: C, 60.03; H, 4.73; N, 3.90.

Thermal Reaction of 3b ($R_1=Me$, $R_2=H$)——A solution of **3b** ($R_1=Me$, $R_2=H$, 1.79 g) in dry DMSO (60 ml) was refluxed for 30 min under a nitrogen atmosphere. According to the general procedure, the residue was purified by silica gel column chromatography (40 g) with benzene–ether as an eluent to give **18** (1% ether–benzene), which was recrystallized from CH_2Cl_2–ether to afford pale yellow plates, mp 251—253 °C. Yield 0.717 g (43.8%). *Anal.* Calcd for $C_{17}H_{13}NO_7$: C, 59.48; H, 3.82; N, 4.08. Found: C, 59.42; H, 3.74; N, 4.00. Compound **17**, eluted with 1.5% ether–benzene, was recrystallized from CH_2Cl_2–ether to afford pale yellow needles, mp 269—270 °C. Yield 0.118 g (6.9%). *Anal.* Calcd for $C_{18}H_{17}NO_7$: C, 60.16; H, 4.77; N, 3.90. Found: C, 60.26; H, 4.59; N, 3.30. The high-resolution MS showed m/z 359.10079 (Calcd 359.10038).

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Amino Acids and Peptides. XIV.[1,2] Synthesis and Biological Activity of Three S-Peptide Analogues of Bovine Pancreatic Ribonuclease A (RNase A)

NAOKI TENO,[a] SATOSHI TSUBOI,[a] TOMOKO SHIMAMURA,[a] YOSHIO OKADA*,[a]
YOSHIMI YANAGIDA,[b] MAKIKO YOSHINAGA,[b] KAZUKO OHGI[b]
and MASACHIKA IRIE[b]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[a] Ikawadani, Nishi-ku, Kobe 673, Japan and Department of Microbiology, Hoshi College of Pharmacy,[b] Ebara, Shinagawa-ku, Tokyo 142, Japan

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Three S-peptide analogues of bovine pancreatic ribonuclease A (RNase A), [Nle^1][Lys^7] S-peptide (I), [Lys^1][Nle^7] S-peptide (II) and [Nle^1][Nle^7] S-peptide (III), were synthesized by the fragment condensation method and their ability to reactivate S-protein was examined. It was found that Lys^1 and Lys^7 both have roles in the reactivation of S-protein.

Keywords——bovine pancreatic ribonuclease A; S-peptide; [Lys^1][Lys^7] S-peptide analogue; chemical synthesis; reactivation ability; structure–activity relationship; succinimide formation; active ester coupling

Bovine pancreatic ribonuclease A (RNase A) catalyzes the hydrolysis of ribonucleic acid or nucleotide ester through a two-step reaction, involving transesterification and hydrolysis.[3-5] The role of Asp-121 and the interaction of the free carboxyl group of Asp-121 with the imidazole group of His-119 were established by examining the reactivation of des(121—124)RNase A by synthetic C-terminal decapeptide analogues.[6] In 1958, a systematic study of the role of the N-terminal portion of RNase A in the enzymatic mechanism indicated that subtilisin cleaved the alanyl–seryl bond between residues 20 and 21. It was shown that the 20-residue N-terminal sequence of RNase A (S-peptide) could be mixed with the 104-residue C-terminal sequence (S-protein) to fully manifest RNase S activity.[7] This fact opened the way for a systematic study of the exact roles of amino acids in the N-terminal portion of RNase A. Richards and Wyckoff[5] reported that Lys-7 was located near Lys-41, His-12 and His-119 within the active site and Lys-1 was located outside the active site cleft. Hirs[8] suggested that Lys-7 might be required for generating the RNase A activity and Pares *et al.*[9] concluded, by using modified RNase A, that Lys-1 belonged to the B_3 site.

In this publication, we present the synthesis of three N-terminal eicosapeptide analogues in which the Lys-1 and/or Lys-7 residues are replaced by Nle, and we describe their ability to restore the enzymatic activity of S-protein toward ribonucleic acid (RNA) and several low-molecular-weight substrates. By the replacement of Lys with Nle, which lacks the ε-amino group, it can be determined whether or not the ε-amino groups of Lys-1 and Lys-7 are essential for the manifestation of enzymatic activity.

The synthesis was conducted by a strategy based on the use of the thioanisole-mediated TFMSA deprotection procedure[10] as shown in Fig. 1, with reference to the synthetic route to RNase A reported previously by Yajima and Fujii.[11-13] In combination with the TFA-labile Boc group for N-protection, amino acid derivatives Lys(Z), Arg(Mts), Asp(OBzl) and Ser(Bzl) bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA containing

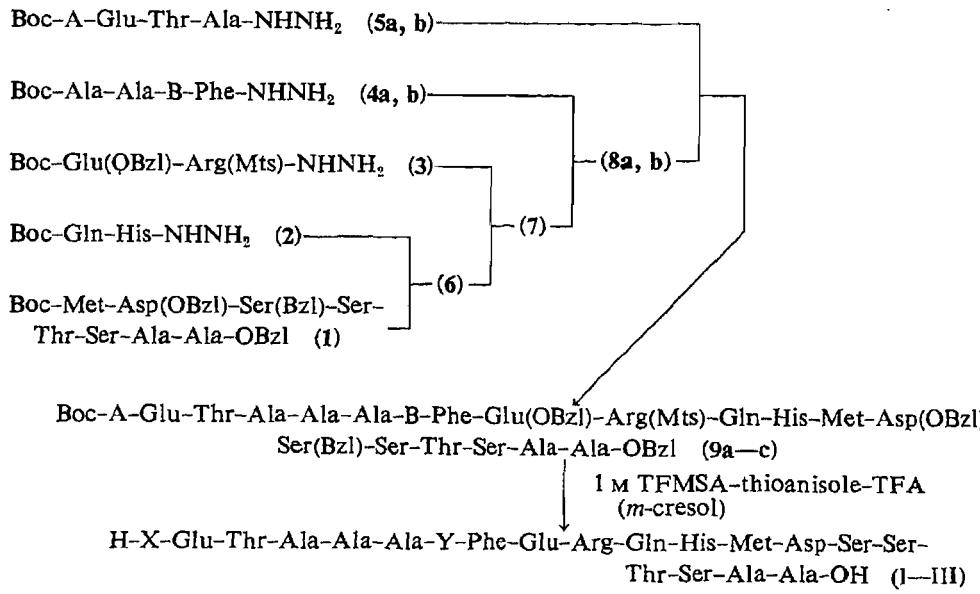

Fig. 1. Synthetic Scheme for S-Peptide Derivatives

4a, B = Nle; **4b**, B = Lys(Z); **5a**, A = Nle; **5b**, A = Lys(Boc); **8a**, B = Nle; **8b**, B = Lys(Z); **9a**, A = Nle, B = Lys(Z); **9b**, A = Lys(Boc), B = Nle; **9c**, A = Nle, B = Nle; I, X = Nle, Y = Lys; II, X = Lys, Y = Nle; III, X = Nle, Y = Nle.

m-cresol were employed. S-Peptide (13—20) possesses the Asp–Ser sequence (positions 14—15). This sequence is known to be particularly susceptible to rearrangement through the formation of succinimide,[14,15] when Asp(OBzl) is employed for the synthesis. In the trial experiment, we observed succinimide formation during the reaction between Boc–Asp(OBzl)–ONp and H–Ser–Ser–Thr–Ser–OMe. The peptide, Boc–Asc–Ser–Ser–Thr–Ser–OMe, was treated with hydrazine hydrate to give a crystalline product. From the nuclear magnetic resonance (NMR) data of this product, it was apparent that the methyl ester moiety remained and the imide had been converted to the another structure. This result indicated that the imide portion of Asc was much more susceptible to hydrazine hydrate than the methyl ester moiety. Examination of the stability of the imide structure to weak bases showed that $NaHCO_3$ (2 eq) and Et_3N (2 eq) degraded the imide moiety in Boc–Asc–Ser–Ser–Thr–Ser–OMe with half-times of 45 and 105 min, respectively. These results also support our previously finding that the conversion of Boc–Ala–Gly–Asp–Ser–OMe to Boc–Ala–Gly–Asp(–$NHNH_2$)–Ser–$NHNH_2$ by hydrazine hydrate occurs through the succinimide as an intermediate.[1] In order to avoid this base-catalyzed side reaction, Ser(Bzl) was employed in position 15; succinimide formation is suppressed due to the steric hindrance.

The common C-terminal S-peptide analogue (13—20) was prepared as shown in Fig. 2. Z–Ser–N_2H_3[16] and H–Thr–Ser–OMe[17] were coupled by the azide method to give Z–Ser–Thr–Ser–OMe, which, after catalytic hydrogenation, was condensed with Boc–Ser(Bzl)–OH[18] by the DCC method.[19] Boc–Ser(Bzl)–Ser–Thr–Ser–OMe thus obtained was exposed to hydrazine hydrate in MeOH to give the corresponding hydrazide. The Boc group of Boc–Ala–Ala–OBzl[20] was removed by TFA–anisole treatment, yielding the corresponding amine, which was coupled with Boc–Ser(Bzl)–Ser–Thr–Ser–N_3 to afford Boc–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl. This heptapeptide was treated with TFA–anisole to yield the corresponding amine, which was coupled with Boc–Asp(OBzl)–ONp[15] followed by Boc–Met–ONp to give Boc–(S-peptide 13—20)–OBzl (**1**).

The S-peptide (9—12) derivative was prepared by two methods; with Boc–Glu–Arg(Mts)–Gln–His–N_2H_3, and with Boc–Glu(OBzl)–Arg(Mts)–N_2H_2–Troc and Boc–Gln–

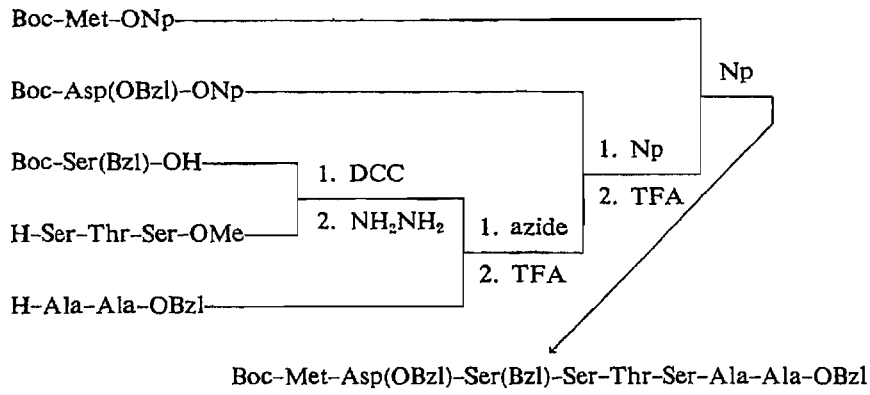

Fig. 2. Synthetic Scheme for S-Peptide (13—20) Derivative (**1**)

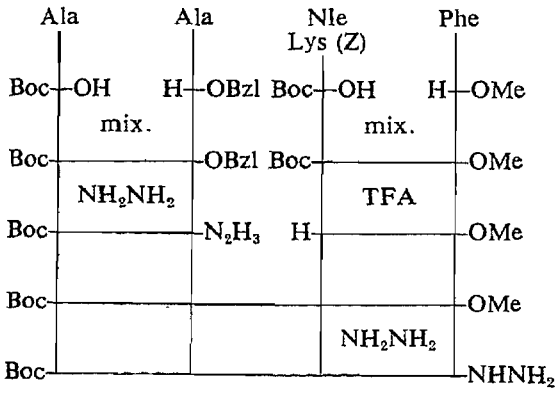

Fig. 3. Synthetic Scheme for [Nle^7] and [$Lys(Z)^7$] S-Peptide (5—8) Derivatives (**4a, b**)

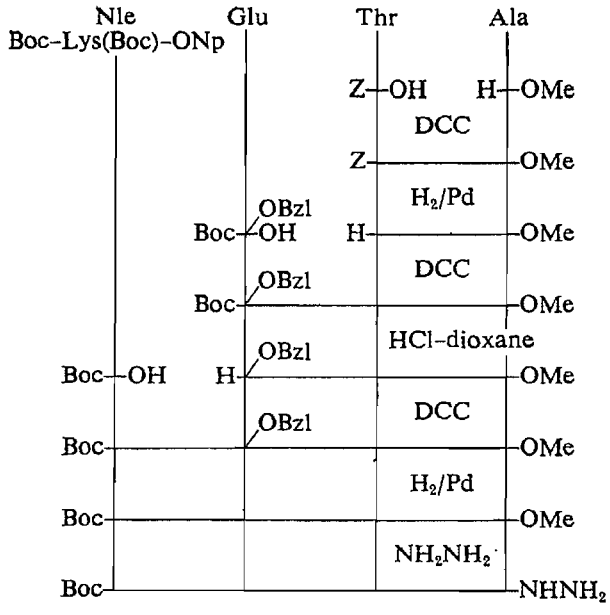

Fig. 4. Synthetic Scheme for [Nle^1] and [$Lys(Boc)^1$] S-Peptide (1—4) Derivatives (**5a, b**)

His–N_2H_3 in two fragments. The latter method was more convenient than the former, in which it was difficult to obtain the final product in a pure form due to its high solubility in water. Boc–Gln–His–OMe[21] was exposed to hydrazine in EtOH to give the corresponding hydrazide (**2**). The protected dipeptide, Boc–Glu(OBzl)–Arg(Mts)–N_2H_2–Troc, was prepared by coupling Boc–Glu(OBzl)–ONp[22] with H–Arg(Mts)–N_2H_2–Troc.[23] Boc–Glu(OBzl)–Arg(Mts)–N_2H_3 (**3**) was obtained by deprotection of the Troc group by Zn–AcOH treatment.[11]

According to the scheme shown in Fig. 3, one important intermediate in this synthesis, Boc–Ala–Ala–Nle–Phe–N_2H_3 (**4a**) or Boc–Ala–Ala–Lys(Z)–Phe–N_2H_3 (**4b**), was synthesized by the azide condensation of Boc–Ala–Ala–N_2H_3 and H–Nle–Phe–OMe or H–Lys(Z)–Phe–OMe, respectively, followed by the usual hydrazine hydrate treatment. The latter dipeptide was prepared by the mixed anhydride method from Boc–Nle–OH[24] or Boc–Lys(Z)–OH[25] and H–Phe–OMe, respectively.

The other important intermediate in this work, Boc–Nle–Glu–Thr–Ala–N_2H_3 (**5a**) or Boc–Lys(Boc)–Glu–Thr–Ala–N_2H_3 (**5b**), was prepared according to Fig. 4. Boc–Glu(OBzl)–

TABLE I. Relative Activity for Reactivation of S-Protein by Admixture with S-Peptide Analogues, with RNA, UpU, UpU>p and UpUpU>p as Substrates at pH 5.5 and 25 °C[a]

Substrate	Enzyme			
	S-Peptide + S-Protein	I + S-Protein	II + S-Protein	III + S-Protein
RNA[b]	100	60	50	32
UpU[c]	100	72	95	95
UpU>p[c]	100	64	44	26
UpUpU>p[c]	100	38	34	18

a) To ensure the binding of S-peptide analogue and S-protein, the enzyme preparation used in this experiment was a mixture of S-peptide and S-protein in a ratio of three. *b*) At pH 7.5 and 37 °C. *c*) At pH 5.5 and 25 °C.

Thr–Ala–OMe was prepared by stepwise condensation using DCC. Boc–Nle–OH or Boc–Lys(Boc)–ONp[26] was coupled with H–Glu(OBzl)–Thr–Ala–OMe, by the DCC or active ester method to afford Boc–Nle–Glu(OBzl)–Thr–Ala–OMe or Boc–Lys(Boc)–Glu(OBzl)–Thr–Ala–OMe, respectively. The desired hydrazides (**5a**) and (**5b**) were synthesized by catalytic hydrogenation, followed by the usual hydrazine hydrate treatment.

The fragments obtained above were assembled by the azide procedure starting with the C-terminal fragment, H–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl, as shown in Fig. 1. The three protected S-peptide analogues (**9a**—**c**) obtained by azide coupling were purified by washing with AcOEt.

In the final step, deprotection and subsequent purification were carried out according to the procedure described below. The protected S-peptide analogues were treated with 1 M TFMSA in the presence of thioanisole and *m*-cresol in an ice-bath for 90 min. After precipitation of the deprotected peptide with ether, the deprotected analogues were converted to the corresponding acetate form by treatment with Amberlite IRA 45 (acetate form) and then treated with 1 M ammonia at pH 8.0 in order to reverse the N→O shift in the Ser and Thr residues.[27] The final products were purified by gel-filtration on Sephadex G-25 using 3% AcOH as an eluant.

The three S-peptide analogues (I—III) were each homogeneous on thin-layer chromatography (TLC) with several solvent systems and the results of amino acid analysis and elemental analysis were in good agreement with the theoretically expected values.

The ability of the peptides to regenerate catalytic activity of S-protein toward native substrate, RNA and several low-molecular-weight substrates, UpU, UpU>p and UpUpU>p was examined and the results are summarized in Table I. From these results, it can be deduced that the ε-amino group at position 7 belongs to the P_2 site[28] and position 1 is involved in the P_3 or B_3 site.[8,29] It is concluded that the free amino group in both Lys residues plays a role in the reactivation of S-protein.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (110 °C, 18 h, 6 N HCl) were determined with an amino acid analyzer, K-101 AS (Kyowa Seimitsu Co., Ltd.). For column chromatography, a Toyo SF-160K fraction collector was used. On TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4, Rf^5 and Rf^6 values refer to the systems of $CHCl_3$–MeOH–AcOH (90:8:2), $CHCl_3$–MeOH–H_2O (89:10:1), $CHCl_3$–MeOH–H_2O (8:3:1, lower phase), *n*-BuOH–AcOH–H_2O (4:1:5, upper phase), *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2) and *n*-BuOH–AcOH–pyridine–H_2O (1:1:1:1), respectively.

Z–Ser–Thr–Ser–OMe——Z–Ser–N_3 (prepared from 3.4 g of Z–Ser–$NHNH_2$ and 1.9 ml of isopentyl nitrite as

usual) in DMF (50 ml) was added to a solution of H-Thr-Ser-OMe·HCl (prepared from 4.0g of Z-Thr-Ser-OMe by catalytic hydrogenation) in DMF (11 ml) containing Et_3N (1.6 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH, yield 3.0 g (60%), mp 165—169 °C, $[\alpha]_D^{20}$ −8.6° ($c=1.0$, MeOH), Rf^3 0.28, Rf^4 0.78. *Anal.* Calcd for $C_{19}H_{27}N_3O_9$: C, 55.6; H, 6.12; N, 9.5. Found: C, 55.5; H, 6.15; N, 9.5.

Boc-Ser(Bzl)-Ser-Thr-Ser-OMe——Boc-Ser(Bzl)-OH (prepared from 3.1 g of Boc-Ser(Bzl)-OH·CHA and 2.6 ml of 1 N HCl) and H-Ser-Thr-Ser-OMe (prepared from 3.1 g of Z-Ser-Thr-Ser-OMe by catalytic hydrogenation) were dissolved in DMF (60 ml) and cooled with ice-salt. DCC (1.8 g) was added to the cold solution, and the reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt, yield 3.0 g (72%), mp 116—120 °C, $[\alpha]_D^{20}$ −12.0° ($c=1.0$, DMF), Rf^2 0.54, Rf^3 0.66, Rf^4 0.67. *Anal.* Calcd for $C_{26}H_{40}N_4O_{11}\cdot 1/2H_2O$: C, 52.6; H, 6.91; N, 9.4. Found: C, 52.7; H, 7.17; N, 9.3.

Boc-Ser(Bzl)-Ser-Thr-Ser-NHNH$_2$——Hydrazine hydrate (90%, 0.5 ml) was added to a solution of Boc-Ser(Bzl)-Ser-Thr-Ser-OMe (2.5 g) in MeOH (10 ml). The mixture was stored at room temperature overnight. The crystals that formed were collected by filtration and recrystallized from EtOH, yield 2.2 g (84%), mp 198—200 °C, $[\alpha]_D^{20}$ +12.1° ($c=1.2$, DMF), Rf^3 0.24, Rf^4 0.70. *Anal.* Calcd for $C_{26}H_{40}N_4O_{11}$: C, 51.4; H, 6.85; N, 14.3. Found: C, 51.5; H, 6.93; N, 14.3.

Boc-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl——Boc-Ser(Bzl)-Ser-Thr-Ser-N_3 (prepared from 2.0 g of Boc-Ser(Bzl)-Ser-Thr-Ser-NHNH$_2$ and 0.48 ml of isopentyl nitrite as usual) in DMF (30 ml) was added to a solution of H-Ala-Ala-OBzl·TFA (prepared from 1.45 g of Boc-Ala-Ala-OBzl and 3.0 ml of TFA containing 0.9 ml of anisole) in DMF (11 ml) containing Et_3N (1.6 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH, yield 1.9 g (69%), mp 119—126 °C, $[\alpha]_D^{20}$ −0.15° ($c=1.3$, DMF), Rf^3 0.60, Rf^4 0.73. Rf^5 0.80. *Anal.* Calcd for $C_{38}H_{54}N_6O_{13}\cdot H_2O$: C, 55.6; H, 6.83; N, 10.2. Found: C, 55.8; H, 6.91; N, 10.2.

Boc-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl——Boc-Asp(OBzl)-ONp (0.76 g) and H-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl·TFA (prepared from 1.1 g of Boc-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl and 1.1 ml of TFA containing 0.3 ml of anisole) were dissolved in DMF (6 ml) containing Et_3N (0.3 ml). The reaction mixture was stirred at 4 °C overnight, then water was added to give crystals, which were collected by filtration and washed with ether, yield 1.1 g (78%), mp 199—202 °C, $[\alpha]_D^{20}$ −5.5° ($c=1.2$, DMF), Rf^2 0.42, Rf^3 0.65. *Anal.* Calcd for $C_{49}H_{65}N_7O_{16}\cdot 2H_2O$: C, 56.4; H, 6.61; N, 9.3. Found: C, 56.2; H, 6.25; N, 9.6. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.89, Ser 2.56, Thr 0.92, Ala 2.00 (average recovery 89.1%).

Boc-Met-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl (1)——Boc-Met-ONp (0.41 g) and H-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl·TFA (prepared from 0.9 g of Boc-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl and 0.7 ml of TFA containing 0.2 ml of anisole) were dissolved in DMF (10 ml) containing Et_3N (0.2 ml). The reaction mixture was stirred at 4 °C overnight. Water was added to the reaction mixture to give crystals, which were collected by filtration and washed with ether, yield 0.8 g (76%), mp 202—205 °C, $[\alpha]_D^{20}$ −9.7° ($c=1.3$, DMF), Rf^2 0.38, Rf^3 0.82. *Anal.* Calcd for $C_{53}H_{74}N_8O_{17}S\cdot 2H_2O$: C, 54.8; H, 6.71; N, 9.3. Found: C, 54.9; H, 6.22; N, 9.0. Amino acid ratios in a 6 N HCl hydrolysate: Met 0.72, Asp 0.85, Ser 2.91, Thr 0.98, Ala 2.00 (average recovery 85.8%).

Boc-Gln-His-NHNH$_2$ (2)——Hydrazine hydrate (90%, 0.4 ml) was added to a solution of Boc-Gln-His-OMe (1.0 g) in EtOH (8 ml). The mixture was stored at room temperature overnight. The crystals that formed were collected by filtration and recrystallized from EtOH, yield 0.6 g (60%), mp 196—198 °C, $[\alpha]_D^{20}$ −22.7° ($c=0.2$, MeOH), Rf^4 0.10, Rf^5 0.42. *Anal.* Calcd for $C_{16}H_{27}N_7O_5\cdot 1/2H_2O$: C, 47.3; H, 6.89; N, 24.1. Found: C, 47.6; H, 6.87; N, 24.4.

Boc-Glu(OBzl)-Arg(Mts)-N_2H_2-Troc——Boc-Glu(OBzl)-ONp (1.5 g) in H-Arg(Mts)-N_2H_2-Troc·HCl (prepared from 1.9 g of Z-Arg(Mts)-N_2H_2-Troc by catalytic hydrogenation) were dissolved in DMF (50 ml) containing Et_3N (0.4 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt and the AcOEt solution was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a precipitate, which was collected by filtration. The crude product in $CHCl_3$ (2 ml) was applied to a column of silica gel (3 × 16.5 cm), which was eluted with following solvents: $CHCl_3$ (500 ml) and 1% MeOH in $CHCl_3$ (300 ml). The solvent of the latter effluent was removed by evaporation. The product in EtOH (3 ml) was applied to a column of Sephadex LH-20 (3.4 × 155 cm). Individual fractions (5 g each) were collected and the solvent of the effluent in tube Nos. 118—132 was removed by evaporation. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 0.5 g (21%), mp 102—105 °C, $[\alpha]_D^{20}$ −14.5° ($c=0.6$, MeOH), Rf^1 0.47. *Anal.* Calcd for $C_{35}H_{48}Cl_3N_7O_{10}S\cdot 4H_2O$: C, 50.7; H, 5.98; N, 10.5. Found: C, 50.6; H, 5.92; N, 10.8.

Boc-Glu(OBzl)-Arg(Mts)-NHNH$_2$ (3)——Boc-Glu(OBzl)-Arg(Mts)-N_2H_2-Troc (0.8 g) in DMF (1.3 ml) and AcOH (1.3 ml) was treated with Zn powder (0.075 g, 10 eq) at room temperature for 4 h. The solvents were removed by evaporation and the residue was treated with saturated EDTA solution and washed with 5% $NaHCO_3$. The

resulting crystals were collected by filtration, yield 0.63 g (91%), mp 95—97 °C, $[\alpha]_D^{20}$ −11.2° (c=1.3, MeOH), Rf^2 0.26, Rf^3 0.78. *Anal.* Calcd for $C_{32}H_{47}N_7O_8S \cdot H_2O$: C, 53.5; H, 6.80; N, 12.1. Found: C, 53.2; H, 6.51; N, 12.1.

Boc-Ala-Ala-NHNH$_2$——Hydrazine hydrate (90%, 2.3 ml) was added to a solution of Boc-Ala-Ala-OBzl (4.9 g) in MeOH (8 ml). The mixture was kept at room temperature overnight. The crystals that formed were collected by filtration and recrystallized from EtOH, yield 2.8 g (73%), mp 193—196 °C, $[\alpha]_D^{37}$ −53.0° (c=1.0, MeOH), Rf^3 0.63. *Anal.* Calcd for $C_{11}H_{22}N_4O_4$: C, 48.2; H, 8.03; N, 20.4. Found: C, 48.4; H, 8.31; N, 20.6.

Boc-Nle-Phe-OMe——A mixed anhydride (prepared from 2.9 g of Boc-Nle-OH and 1.2 ml of ethyl chloroformate as usual) in THF (60 ml) was added to an ice-cold solution of H-Phe-OMe (prepared from 2.2 g of H-Phe-OMe·HCl and 1.7 ml of Et_3N) in DMF (60 ml). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from *n*-hexane, yield 3.9 g (73%), mp 67—71 °C, $[\alpha]_D^{20}$ −22.2° (c=0.9, MeOH), Rf^1 0.73, Rf^2 0.83. *Anal.* Calcd for $C_{21}H_{32}N_2O_5$: C, 64.3; H, 8.61; N, 7.1. Found: C, 64.4; H, 8.25; N, 7.2.

Boc-Ala-Ala-Nle-Phe-OMe——Boc-Ala-Ala-N_3 (prepared from 1.4 g of Boc-Ala-Ala-NHNH$_2$ and 0.72 ml of isopentyl nitrite as usual) in DMF (10 ml) was added to a solution of H-Nle-Phe-OMe·TFA (prepared from 1.7 g of Boc-Nle-Phe-OMe and 2.2 ml of TFA containing 0.5 ml of anisole) in DMF (30 ml) containing Et_3N (0.9 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt, yield 1.6 g (73%), mp 183—185 °C, $[\alpha]_D^{37}$ −44.6° (c=1.0, MeOH), Rf^2 0.50, Rf^3 0.59, Rf^4 0.89. *Anal.* Calcd for $C_{27}H_{42}N_4O_7 \cdot H_2O$: C, 58.7; H, 7.97; N, 10.1. Found: C, 59.0; H, 8.27; N, 10.4.

Boc-Ala-Ala-Nle-Phe-NHNH$_2$ (4a)——Hydrazine hydrate (90%, 0.5 ml) was added to a solution of Boc-Ala-Ala-Nle-Phe-OMe (1.7 g) in MeOH (8 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 0.72 g (64%), mp 255—257.5 °C, $[\alpha]_D^{37}$ −44.8° (c=1.0, MeOH), Rf^2 0.28, Rf^3 0.64. *Anal.* Calcd for $C_{26}H_{42}N_6O_6$: C, 58.5; H, 7.86; N, 15.7. Found: C, 58.1; H, 7.71; N, 15.8.

Boc-Ala-Ala-Lys(Z)-Phe-OMe——Boc-Ala-Ala-N_3 (prepared from 1.3 g of Boc-Ala-Ala-NHNH$_2$ and 0.65 ml of isopentyl nitrite as usual) in DMF (10 ml) was added to a solution of H-Lys(Z)-Phe-OMe·TFA (prepared from 2.0 g of Boc-Lys(Z)-Phe-OMe and 2.8 ml of TFA containing 0.8 ml of anisole) in DMF (20 ml) containing Et_3N (0.8 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt, yield 1.8 g (70%), mp 178—183 °C, $[\alpha]_D^{30}$ −36.2° (c=0.9, MeOH), Rf^4 0.75, Rf^5 0.82. *Anal.* Calcd for $C_{35}H_{49}N_5O_9 \cdot H_2O$: C, 59.9; H, 7.27; N, 10.0. Found: C, 59.9; H, 7.36; N, 10.1.

Boc-Ala-Ala-Lys(Z)-Phe-NHNH$_2$ (4b)——Hydrazine hydrate (90%, 0.6 ml) was added to a solution of Boc-Ala-Ala-Lys(Z)-Phe-OMe (1.6 g) in MeOH (10 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from EtOH, yield 1.0 g (49%), mp 238—241 °C, $[\alpha]_D^{20}$ −8.9° (c=0.9, MeOH), Rf^3 0.31, Rf^4 0.78. *Anal.* Calcd for $C_{34}H_{49}N_7O_8$: C, 59.7; H, 7.24; N, 14.3. Found: C, 59.5; H, 7.12; N, 14.2.

Boc-Glu(OBzl)-Thr-Ala-OMe——Boc-Glu(OBzl)-OH (7.3 g) and H-Thr-Ala-OMe (prepared from 6.0 g of Z-Thr-Ala-OMe[30] by catalytic hydrogenation) were dissolved in DMF (60 ml) and cooled with ice-salt. DCC (4.6 g) was added to the cold solution, and the reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, the residue was taken up in AcOEt and the AcOEt solution was washed with 5% Na_2CO_3, 10% citric acid and water, then dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (8 ml) was applied to a column of silica gel (4 × 36.5 cm), which was eluted with the following solvents: $CHCl_3$ (2000 ml) and 1% MeOH in $CHCl_3$ (1200 ml). The solvent of the latter effluent was removed by evaporation. Petroleum ether was added to the residue to afford crystals, yield 3.0 g (32%), mp 105—109 °C, $[\alpha]_D^{37}$ −35.9° (c=1.1, MeOH), Rf^1 0.77, Rf^3 0.75. *Anal.* Calcd for $C_{25}H_{37}N_3O_9$: C, 57.4; H, 7.07; N, 8.0. Found: C, 57.3; H, 7.25; N, 8.1.

Boc-Nle-Glu(OBzl)-Thr-Ala-OMe——Boc-Nle-OH (0.8 g) and H-Glu(OBzl)-Thr-Ala-OMe·HCl (prepared from 1.1 g of Boc-Glu(OBzl)-Thr-Ala-OMe and 3.8 ml of 5.6 N HCl/dioxane) were dissolved in DMF (18 ml) cooled with ice-salt. DCC (0.7 g) was added to the cold solution, and the reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 1.2 g (88%), mp 109—115 °C, $[\alpha]_D^{37}$ −28.4° (c=1.0, MeOH), Rf^1 0.56, Rf^3 0.90. *Anal.* Calcd for $C_{32}H_{50}N_4O_{10} \cdot H_2O$: C, 57.5; H, 7.78; N, 8.4. Found: C, 58.0; H, 7.70; N, 8.8.

Boc-Nle-Glu-Thr-Ala-OMe——Boc-Nle-Glu(OBzl)-Thr-Ala-OMe (2.5 g) in MeOH (50 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 1.2 g (58%), mp 190—194 °C, $[\alpha]_D^{37}$ −54.5° (c=1.1, MeOH), Rf^1 0.31, Rf^3 0.39. *Anal.* Calcd for $C_{25}H_{44}N_4O_{10} \cdot 1/2H_2O$: C, 52.8; H, 7.91; N, 9.8. Found: C, 52.7; H, 7.72; N, 10.1.

Boc-Nle-Glu-Thr-Ala-NHNH$_2$ (5a)——Hydrazine hydrate (90%, 0.37 ml) was added to a solution of Boc-

Nle–Glu–Thr–Ala–OMe (1.2 g) in MeOH (3 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, petroleum ether was added to the residue to afford crystals, which were collected by filtration and washed with 3% AcOH, yield 0.3 g (25%), mp 190—196 °C, $[\alpha]_D^{20}$ −3.3° (c=0.9, DMF), Rf^4 0.57, Rf^5 0.56. *Anal.* Calcd for $C_{24}H_{44}N_6O_9 \cdot H_2O$: C, 49.0; H, 7.80; N, 14.9. Found: C, 48.9; H, 7.63; N, 14.5. Amino acid ratios in a 6 N HCl hydrolysate: Nle 1.06, Glu 1.06, Thr 0.97, Ala 1.00 (average recovery 86.9%).

Boc–Lys(Boc)–Glu(OBzl)–Thr–Ala–OMe——Boc–Lys(Boc)–ONp (1.8 g) and H–Glu(OBzl)–Thr–Ala–OMe·TFA (prepared from 2.1 g of Boc–Glu(OBzl)–Thr–Ala–OMe and 2.8 ml of TFA containing 0.8 ml of anisole) were dissolved in DMF (20 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt and the AcOEt solution was washed with 5% Na_2CO_3, 10% citric acid and water, then dried over Na_2SO_4 and evaporated down. Petroleum ether and ether were added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 2.1 g (72%), mp 129—135 °C, $[\alpha]_D^{3.7}$ −38.9° (c=1.0, MeOH), Rf^1 0.50, Rf^2 0.56, Rf^3 0.77. *Anal.* Calcd for $C_{36}H_{57}N_5O_{12}$: C, 57.6; H, 7.59; N, 9.3. Found: C, 57.5; H, 7.74; N, 9.5.

Boc–Lys(Boc)–Glu–Thr–Ala–OMe——Boc–Lys(Boc)–Glu(OBzl)–Thr–Ala–OMe (2.2 g) in MeOH (50 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, petroleum ether was added to the residue to give crystals, which were collected by filtration, yield 1.9 g (99%), mp 95—100 °C, $[\alpha]_D^{18}$ −43.0° (c=1.0, MeOH), Rf^4 0.80, Rf^5 0.84. *Anal.* Calcd for $C_{29}H_{51}N_5O_{12} \cdot H_2O$: C, 51.3; H, 7.80; N, 10.3. Found: C, 51.3; H, 7.75; N, 10.3.

Boc–Lys(Boc)–Glu–Thr–Ala–NHNH$_2$ (5b)——Hydrazine hydrate (90%, 0.8 ml) was added to a solution of Boc–Lys(Boc)–Glu–Thr–Ala–OMe (1.92 g) in MeOH (5 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, petroleum ether was added to the residue to afford crystals, which were collected by filtration and washed with 3% AcOH, yield 0.9 g (44%), mp 184—186 °C, $[\alpha]_D^{18}$ −37.9° (c=1.0, MeOH), Rf^4 0.53, Rf^5 0.68. *Anal.* Calcd for $C_{28}H_{51}N_7O_{11} \cdot 1/2H_2O$: C, 50.2; H, 7.76; N, 14.6. Found: C, 49.8; H, 7.83; N, 14.7. Amino acid ratios in a 6 N HCl hydrolysate: Nle 1.08, Glu 1.09, Thr 0.92. Ala 1.00 (average recovery 106.4%).

Boc–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl (6)——Boc–Gln–His–N_3 (prepared from 0.08 g of **2** and 0.03 ml of isopentyl nitrite as usual) in DMF (6 ml) was added to a solution of H–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl·TFA (prepared from 0.11 g of **1** and 0.5 ml of TFA containing 0.07 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol)[31] in DMF (6 ml) containing Et_3N (0.014 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, water was added to the residue to give crystals, which were collected by filtration, yield 0.08 g (59%), mp 212—216 °C, $[\alpha]_D^{20}$ −11.9° (c=0.8, DMF), Rf^4 0.56, Rf^5 0.74, Rf^6 0.90. *Anal.* Calcd for $C_{65}H_{89}N_{13}O_{20}S \cdot 5H_2O$: C, 52.3; H, 6.63; N, 12.2. Found: C, 52.2; H, 6.63; N, 12.0.

Boc–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl (7)——Boc–Glu(OBzl)–Arg(Mts)–N_3 (prepared from 0.42 g of **3** and 0.02 ml of isopentyl nitrite as usual) in DMF (4 ml) was added to a solution of H–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl·TFA (prepared from 0.21 g of **6** and 0.6 ml of TFA containing 0.1 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (4 ml) containing Et_3N (0.012 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and washed with AcOEt, yield 0.19 g (64%), mp 187—189 °C, $[\alpha]_D^{20}$ −9.23° (c=0.3, DMF), Rf^4 0.67, Rf^5 0.73. *Anal.* Calcd for $C_{92}H_{124}N_{18}O_{26}S_2 \cdot 8H_2O$: C, 52.5; H, 6.27; N, 11.1. Found: C, 52.3; H, 5.88; N, 11.1.

Boc–Ala–Ala–Nle–Phe–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl (8a)——Boc–Ala–Ala–Nle–Phe–N_3 (prepared from 0.12 g of **4a** and 0.03 ml of isopentyl nitrite as usual) in DMF (12 ml) was added to a solution of H–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl·TFA (prepared from 0.11 g of **7** and 0.23 ml of TFA containing 0.04 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (6 ml) containing Et_3N (0.015 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and washed with EtOH, yield 0.11 g (85%), mp 168—171 °C, $[\alpha]_D^{20}$ −8.0° (c=0.1, DMF), Rf^4 0.62, Rf^5 0.73. *Anal.* Calcd for $C_{114}H_{156}N_{22}O_{30}S_2 \cdot 8H_2O$: C, 50.7; H, 7.11; N, 9.9. Found: C, 50.7; H, 7.52; N, 10.2.

Boc–Ala–Ala–Lys(Z)–Phe–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl (8b)——Boc–Ala–Ala–Lys(Z)–Phe–N_3 (prepared from 0.1 g of **4b** and 0.03 ml of isopentyl nitrite as usual) in DMF (10 ml) was added to a solution of H–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl·TFA (prepared from 0.1 g of **7** and 0.2 m of TFA containing 0.03 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (10 ml) containing Et_3N (0.014 ml) in the same way as described above, yield 0.04 g (30%), mp 144—150 °C, $[\alpha]_D^{20}$ −11.0° (c=0.1, DMF), Rf^4 0.65, Rf^5 0.71. *Anal.* Calcd for $C_{121}H_{161}N_{23}O_{32}S_2 \cdot 4H_2O$: C, 56.2; H, 6.54; N, 12.5. Found: C, 56.2; H, 6.45; N, 12.1.

Boc–Nle–Glu–Thr–Ala–Ala–Ala–Lys(Z)–Phe–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl (9a)——Boc–Nle–Glu–Thr–Ala–N_3 (prepared from 27.9 mg of **5a** and 0.007 ml of isopentyl nitrite as usual) in DMF (5 ml) was added to a solution of H–Ala–Ala–Lys(Z)–Phe–Glu(OBzl)–Arg(Mts)–Gln–His–Met–Asp(OBzl)–Ser(Bzl)–Ser–Thr–Ser–Ala–Ala–OBzl·TFA (prepared from 32.2 mg of **8b** and 0.05 ml of TFA

containing 0.01 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (5 ml) containing Et_3N (0.04 ml) in the same way as described above.

Boc-Lys(Boc)-Glu-Thr-Ala-Ala-Ala-Nle-Phe-Glu(OBzl)-Arg(Mts)-Gln-His-Met-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl (9b)——Boc-Lys(Boc)-Glu-Thr-Ala-N_3 (prepared from 56.4 mg of **5b** and 0.01 ml of isopentyl nitrite as usual) in DMF (6 ml) was added to a solution of H-Ala-Ala-Nle-Phe-Glu(OBzl)-Arg(Mts)-Gln-His-Met-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl·TFA (prepared from 47.6 mg of **8a** and 0.1 ml of TFA containing 0.02 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (5 ml) containing Et_3N (0.06 ml) in the same way as described above.

Boc-Nle-Glu-Thr-Ala-Ala-Ala-Nle-Phe-Glu(OBzl)-Arg(Mts)-Gln-His-Met-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl (9c)——Boc-Nle-Glu-Thr-Ala-N_3 (prepared from 50.7 mg of **5a** and 0.01 ml of isopentyl nitrite as usual) in DMF (5 ml) was added to a solution of H-Ala-Ala-Nle-Phe-Glu(OBzl)-Arg(Mts)-Gln-His-Met-Asp(OBzl)-Ser(Bzl)-Ser-Thr-Ser-Ala-Ala-OBzl·TFA (prepared from 53.6 mg of **8a** and 0.1 ml of TFA containing 0.02 ml of 3,5-dimethylanisole and 0.01 ml of 1,2-ethanedithiol) in DMF (5 ml) containing Et_3N (0.06 ml) under cooling with ice. The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and washed with EtOH.

H-Nle-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH, [Nle^1]-[Lys^7] S-Peptide, (I)——The above protected S-peptide analogue (**9a**, 26.2 mg) was treated with 1 M TFMSA-thioanisole in TFA (0.2 ml) in the presence of *m*-cresol (60 μl, 60 eq) in an ice-bath for 90 min, then dry ether was added. The resulting powder was collected by centrifugation, and dried over KOH pellets *in vacuo* for 60 min. The peptide thus obtained was dissolved in H_2O (5 ml) containing dithiothreitol (3 mg), treated with Amberlite IRA 45 (acetate form) for 30 min and then filtered. The pH of the filtrate was adjusted to 8.0 with 1 N NH_4OH, and after 30 min at 0 °C, readjusted to 6.5 with 1 N AcOH. The solution was lyophilized to give a hygroscopic powder, which was dissolved in H_2O (3 ml), and stirred with dithiothreitol (3 mg, 50 eq) at room temperature overnight. Then the solution was applied to a column of Sephadex G-25 (2.8 × 132 cm), which was eluted with 3% AcOH. Individual fractions (3 g each) were collected and the solvent of the effluent in tube Nos. 38—45 was removed by lyophilization.

H-Lys-Glu-Thr-Ala-Ala-Ala-Nle-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH, [Lys^1]-[Nle^7] S-Peptide, (II)——The above protected S-peptide analogue (**9b**, 16.6 mg) was treated with 1 M TFMSA-thioanisole in TFA (0.2 ml) in the presence of *m*-cresol (60 μl, 60 eq) in an ice-bath for 90 min in the same way as described above.

H-Nle-Glu-Thr-Ala-Ala-Ala-Nle-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala-OH, [Nle^1]-[Nle^7] S-Peptide, (III)——The above protected S-peptide analogue (**9c**, 28.8 mg) was treated with 1 M TFMSA-thioanisole in TFA (0.2 ml) in the presence of *m*-cresol (60 μl, 60 eq) in an ice-bath for 90 min in the same way as described above.

Trial Experiment Z-Ser-Ser-Thr-Ser-OMe——Z-Ser-N_3 (prepared from 4.1 g of Z-Ser-$NHNH_2$ and 2.2 ml of isopentyl nitrite as usual) in DMF (20 ml) was added to a solution of H-Ser-Thr-Ser-OMe·HCl (prepared from 5.8 g of Z-Ser-Thr-Ser-OMe by catalytic hydrogenation) in DMF (20 ml) containing Et_3N (4.5 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from MeOH, yield 4.5 g (65%), mp 233—235 °C, $[\alpha]_D^{37}$ −11.6° (c = 1.0, DMF), Rf^4 0.67, Rf^5 0.75. *Anal.* Calcd for $C_{22}H_{32}N_4O_{11}$: C, 50.0; H, 6.06; N, 10.6. Found: C, 50.2; H, 6.12; N, 10.5.

Boc-Asc-Ser-Ser-Thr-Ser-OMe——Boc-Asp(OBzl)-ONp (4.2 g) and H-Ser-Ser-Thr-Ser-OMe·HCl (prepared from 4.1 g of Z-Ser-Ser-Thr-Ser-OMe by catalytic hydrogenation) were dissolved in DMF (60 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from AcOEt, yield 3.5 g (64%), mp 194—201 °C, $[\alpha]_D^{20}$ −8.6° (c = 1.1, DMF), Rf^3 0.45, Rf^4 0.56. *Anal.* Calcd for $C_{23}H_{37}N_5O_{13}$: C, 46.7; H, 6.29; N, 11.8. Found: C, 46.9; H, 6.32; N, 11.6. NMR [$(CD_3)_2SO$]: 4.7—4.18 (7H, m, CH_2OH × 3 and succinimide CH), 3.62 (3H, s, $COOCH_3$), 3.19—2.41 (2H, m, succinimide CH_2), 1.39 (9H, s, *tert*-Bu), 1.15—0.95 (3H, m, $CH(OH)CH_3$), 8.05—7.3 (3H, m, CONH × 3). These data confirm that this compound is the succinimide derivative.

Treatment of Boc-Asc-Ser-Ser-Thr-Ser-OMe with Hydrazine Hydrate——Hydrazine hydrate (90%, 0.02 ml) was added to a solution of Boc-Asc-Ser-Ser-Thr-Ser-OMe (50 mg) in MeOH (2 ml). The mixture was stored at room temperature for 2 d. The precipitate formed was collected by filtration and recrystallized from EtOH, mp 225—231 °C, Rf^3 0.13, Rf^4 0.34, Rf^5 0.58. NMR [$(CD_3)_2SO$]: 3.62 (3H, s, $COOCH_3$), 1.39 (9H, s, *tert*-Bu), 8.05—7.3 (4H, m, CONH × 4). This compound is a hydrazide derivative at the Asp residue.

Effect of Other Bases on Boc-Asc-Ser-Ser-Thr-Ser-OMe——Boc-Asc-Ser-Ser-Thr-Ser-OMe (5 mmol) was treated with 5% aqueous $NaHCO_3$ (10 mmol) in MeOH (1 ml) or Et_3N (10 mmol) in MeOH (1 ml) at room temperature. Aliquots of the solution were examined periodically by TLC using the solvent system of Rf^5. After coloration with ninhydrin and HBr/AcOH, the ratios of two spots (degraded product and starting material) were colorimetrically determined with a Shimadzu dual-wavelength TLC scanner.

Z-Arg(Mts)-Gln-His-OMe——A mixed anhydride (prepared from 8.2 g of Z-Arg(Mts)-OH·CHA and 1.3 ml

TABLE II. Yield, Melting Point, $[\alpha]_D$, Elemental Analysis and *Rf* Data for **9a**—**c**, I, II and III

Compound	Yield (%)	mp (°C)	$[\alpha]_D^{20}$	Formula	Elemental analysis Calcd (Found) C	H	N	Rf^4	Rf^5	Rf^6
9a	74	142—146	−8.0 (*c*=0.1, DMF)	$C_{140}H_{193}N_{27}O_{39}S_2 \cdot 8H_2O$	54.5 (54.5	6.78 6.75	12.3 12.2)	0.72	0.78	
9b	48	179—183	−15.0 (*c*=0.1, DMF)	$C_{137}H_{195}N_{27}O_{39}S_2 \cdot 10H_2O$	53.5 (53.5	6.99 6.55	11.2 11.1)	0.60	0.78	
9c	51	182—186	−14.0 (*c*=0.1, DMF)	$C_{133}H_{188}N_{26}O_{37}S_2 \cdot 10H_2O$	53.5 (53.3	6.97 6.69	12.2 12.1)	0.57	0.77	
I	32	Amorphous	−62.0 (*c*=0.1, H_2O)	$C_{90}H_{145}N_{27}O_{33}S \cdot 4AcOH \cdot 20H_2O$	42.6 (42.3	6.27 5.98	13.5 13.3)		0.10	0.81
II	63	Amorphous	−55.3 (*c*=0.2, H_2O)	$C_{90}H_{145}N_{27}O_{33}S \cdot 4AcOH \cdot 20H_2O$	42.6 (42.2	6.27 6.00	13.5 13.7)		0.10	0.80
III	63	Amorphous	−66.9 (*c*=0.1, H_2O)	$C_{91}H_{146}N_{26}O_{33}S \cdot 3AcOH \cdot 23H_2O$	42.3 (42.1	6.14 5.82	12.2 12.0)		0.10	0.80

TABLE III. Amino Acid Ratios in Acid Hydrolysates of **9a**—**c**, I, II and III

Compound	Amino acid ratios in acid hydrolysates Nle	Glu	Thr	Lys	Ala	Phe	Arg	His	Met	Asp	Ser	Average recovery (%)
9a	1.06	3.20	1.84	1.12	5.00	1.02	1.16	1.14	0.72	1.26	2.03	85.3
9b	1.02	3.13	2.05	1.18	5.00	0.94	0.92	1.08	0.76	1.13	1.95	80.5
9c	2.19	3.14	2.11	—	5.00	0.88	1.02	1.14	0.73	1.10	2.26	75.1
I	1.05	2.90	1.98	1.02	5.00	0.90	1.01	1.03	0.73	1.09	2.44	81.9
II	0.88	3.28	1.93	1.09	5.00	0.96	0.91	1.09	0.78	1.09	2.47	60.6
III	1.91	2.88	2.11	—	5.00	0.88	1.02	1.14	0.73	1.01	2.26	80.5

of ethyl chloroformate as usual) in THF (120 ml) was added to an ice-cold solution of H–Gln–His–OMe (prepared from 5.1 g of Z–Gln–His–OMe by catalytic hydrogenation) in DMF (60 ml). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3 and water, then dried over Na_2SO_4 and evaporated down. The crude product in EtOH (14 ml) was applied to a column of Sephadex LH-20 (3.4 × 155 cm). Individual fractions (5 g each) were collected and the solvent of the effluent in tube Nos. 151—190 was removed by evaporation. Ether was added to the residue to afford a precipitate, which was collected by filtration, yield 2.1 g (23%), mp 105—115 °C, $[\alpha]_D^{38}$ −1.8° (*c*=0.7, MeOH), Rf^4 0.19, Rf^5 0.52. *Anal.* Calcd for $C_{35}H_{45}N_9O_{11}S$: C, 52.9; H, 6.29; N, 15.4. Found: C, 52.6; H, 6.11; N, 15.2.

Boc–Glu(OBzl)–Arg(Mts)–Gln–His–OMe——Boc-Glu(OBzl)–ONp (1.8 g) and H–Arg(Mts)–Gln–His–OMe·2HCl (prepared from 2.6 g of Z–Arg(Mts)–Gln–His–OMe by catalytic hydrogenation) were dissolved in DMF (30 ml) containing Et_3N (1.0 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt and the AcOEt solution was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford a precipitate, which was collected by filtration and reprecipitated from AcOEt and ether, yield 2.2 g (71%), mp 146—150 °C, $[\alpha]_D^{38}$ −13.2° (*c*=1.0, MeOH), Rf^3 0.67, Rf^4 0.29. *Anal.* Calcd for $C_{44}H_{62}N_{10}O_{12}S \cdot 1/2H_2O$: C, 54.9; H, 6.54; N, 14.5. Found: C, 54.8; H, 6.52; N, 14.4.

Boc–Glu–Arg(Mts)–Gln–His–OMe——Boc–Glu(OBzl)–Arg(Mts)–Gln–His–OMe (2.1 g) in MeOH (40 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether was added to the residue to give crystals, which were collected by filtration, yield 1.9 g (98%), mp 150—155 °C, $[\alpha]_D^{24}$ −20.3° (*c*=0.7, MeOH), Rf^3 0.11, Rf^4 0.18, Rf^5 0.57, Rf^6 0.89. *Anal.* Calcd for $C_{37}H_{56}N_{10}O_{12}S \cdot 3/2H_2O$: C, 49.9; H, 6.62; N, 16.0. Found: C, 49.6; H, 6.60; N, 15.8.

Boc–Glu–Arg(Mts)–Gln–His–NHNH$_2$——Hydrazine hydrate (90%, 0.32 ml) was added to a solution of Boc–Glu–Arg(Mts)–Gln–His–OMe (1.7 g) in MeOH (10 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, the oily product in 3% AcOH was applied to a column

of Sephadex G-25 (2.3 × 130 cm). Individual fractions (3 g each) were collected and the solvent of the effluent in tube Nos. 215—230 was removed by lyophilization, yield 0.2 g (8.4%), amorphous, $[\alpha]_D^{38}$ −21.6° (c=0.8, MeOH), Rf^5 0.35, Rf^6 0.82. *Anal.* Calcd for $C_{36}H_{56}N_{12}O_{11}\cdot 5/2H_2O$: C, 47.8; H, 6.71; N, 17.3. Found: C, 47.0; H, 6.89; N, 17.0. Amino acid ratios in a 6 N HCl hydrolysate: Glu 2.36, Arg 1.29, His 1.00 (average recovery 83.9%).

Enzyme Assay——(i) Enzymatic activity toward RNA. Tris-HCl buffer (2 ml, 50 mM, pH 7.5) containing RNA (5 mg) was treated with 5—15 μl of enzyme solution at 37 °C for 1 min. The reaction was stopped by addition of 1 ml of MacFadyen reagents.[32] The reaction mixture was centrifuged for 5 min at 3000 rpm. The supernatant (0.3 ml) was diluted with water (2 ml) and the absorbancy of the solution was measured at 260 nm. (ii) Oligouridylic acids as substrates. Aliquots of 1.1 ml of substrate solution (13—80 μM) in 0.1 M acetate buffer (pH 5.5) were treated with 5—15 μl of enzyme solution at 25 °C. The absorbancy change at 280 nm was measured. The final molar extinction coefficients for the transesterification of UpU, UpU>p and UpUpU>p were 750,[33] 700[34] and 700,[34] respectively.

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35(2) 479—500 (1987)]

Synthesis of *Erythrina* and Related Alkaloids. XVI.[1] Diels–Alder Approach: Total Synthesis of *dl*-Erysotrine, *dl*-Erythraline, *dl*-Erysotramidine, *dl*-8-Oxoerythraline and Their 3-Epimers

TAKEHIRO SANO,*,[a] JUN TODA,[a] NORIAKI KASHIWABA,[a]
TAKESHI OHSHIMA,[b] and YOSHISUKE TSUDA[b]

Showa College of Pharmaceutical Sciences,[a] Tsurumaki, Setagaya-ku, Tokyo 154, Japan and Faculty of Pharmaceutical Sciences, Kanazawa University,[b] Takara-machi, Kanazawa 920, Japan

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Total synthesis of erythrinan alkaloids was achieved by a strategy based on the Diels–Alder reaction of activated butadienes to a dioxopyrroline. The reaction of isoquinolinopyrrolinedione (**15**) with 1,3-bis-*O*-substituted butadienes proceeded in a regiospecific and stereoselective manner to give erythrinan derivatives (**20**) and (**21**). Lithium borohydride reduction of the adduct (**20**) or (**21**), followed by acid hydrolysis afforded the enone (**33**). Mesylation of **33** and subsequent demethoxycarbonylation of **42** under neutral conditions gave the dienone (**43**). Meerwein–Ponndorf reduction of **43** and subsequent methylation afforded erysotramidine (**2a**) and 8-oxoerythraline (**2b**). Aluminum hydride reduction of the 8-oxo derivatives (**2**) furnished *dl*-erysotrine (**1a**) and *dl*-erythraline (**1b**).

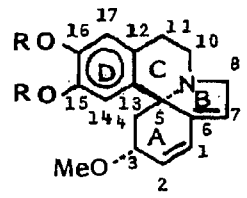
Keywords——total synthesis; erythrinan alkaloid; 1*H*-pyrrole-2,3-dione; dioxopyrroline; Diels–Alder reaction; hydroindole; erysotrine; erythraline; erysotramidine; 8-oxoerythraline

Introduction

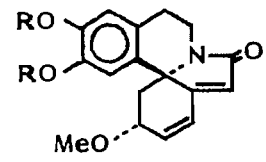
The alkaloids of the *Erythrina* species continue to attract interest because of their physiological activities, which include paralysis of smooth muscle.[2] They are classified into two groups: aromatic and non-aromatic alkaloids. Erysotrine (**1a**) and erythraline (**1b**) are typical aromatic erythrinan alkaloids widely distributed in many *Erythrina* plants.[3] Their 8-oxo derivatives, erysotramidine (**2a**) and 8-oxoerythraline (**2b**), occur in *Erythrina arborescens* ROXB.[4] and *Erythrina crysta-galli* L.[5] as minor constituents, respectively. The basic skeleton characteristically possesses a spiro carbon and a nitrogen shared by hydroindole (rings A and B) and tetrahydroisoquinoline (rings C and D) moieties. A further structural feature of these alkaloids is that they commonly possess an aromatic ring and a dienol system. Several syntheses of these alkaloids[6,7] have been reported, including ours.[8] Here, we report in detail our total synthesis of all of the alkaloids described above.

For synthesis of this unique erythrinan ring system, we have designed three different approaches: i) Diels–Alder approach,[9] ii) photochemical approach,[10] and iii) intramolecular cyclization approach.[11] All of them successfully lead to the erythrinan skeleton. In this paper we present details of the Diels–Alder approach.

This approach is based on the strategy of using an intermolecular Diels–Alder reaction of 1*H*-pyrrole-2,3-dione (dioxopyrroline) as a method of perhydroindole synthesis. The dienophilic activity of dioxopyrroline was initially demonstrated by the reaction of 3-aryldioxopyrroline (**3**) with butadiene, yielding a perhydroindole (**4**),[12] which constitutes the key step in the total synthesis of Amaryllidaceae alkaloids by Tsuda and Isobe.[13]



1

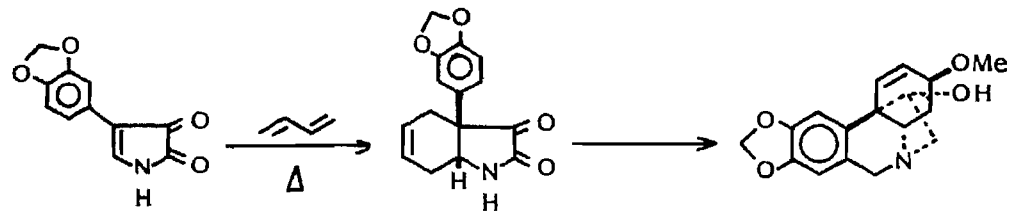


2

1a: erysotrine
1b: erythraline
2a: erysotramidine
2b: 8-oxoerythraline

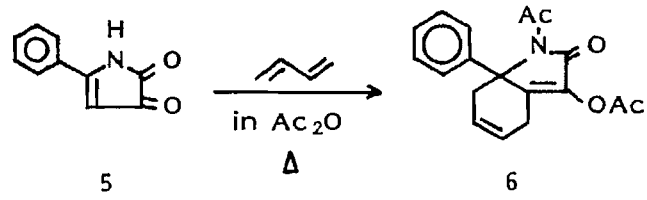
a: R=Me, b: R-R=$-CH_2-$

Chart 1



3 4 Amaryllidaceae alkaloids

Chart 2



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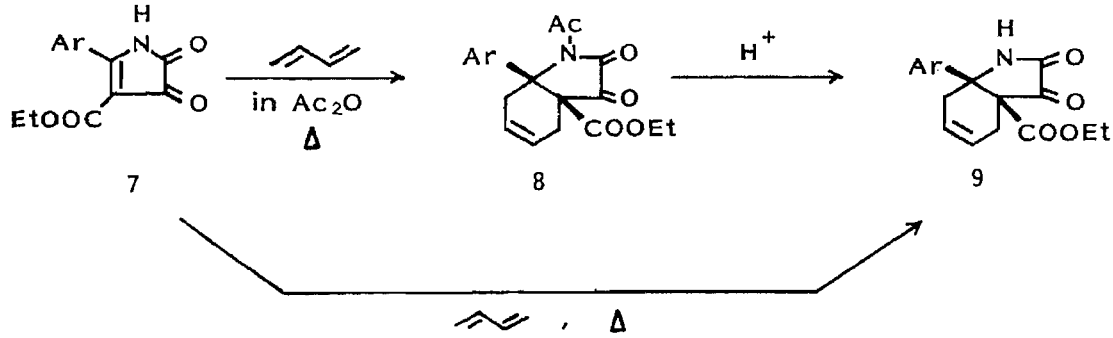


7 8 9

a: Ar = Ph , g: Ar =

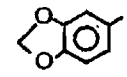


Chart 3

The same methodology is expected to be applicable to the construction of the erythrinan skeleton. The Diels–Alder reaction of 2-aryldioxopyrroline (**5**) would produce a perhydroindole which has the rings A, B, and D of an erythrinan derivative. However, in a preliminary experiment, the reactivity of 2-phenyldioxopyrroline (**5**) to butadiene was found to be quite poor, in contrast to 3-phenyldioxopyrroline.[12] Heating of **5** with butadiene in $CHCl_3$ did not give any cycloadduct. In acetic anhydride at 180 °C, the yield of the adduct (**6**) was improved to 5%. Introduction of a COOEt group on the dioxopyrroline ring enhanced

the dienophilic activity. Heating of 3-ethoxycarbonyl-2-phenyldioxopyrroline (**7a**) with butadiene in CH_2Cl_2 at 170 °C afforded the adduct (**9a**) in 10% yield.[14] The reaction of **7a** with butadiene in acetic anhydride afforded the adduct (**8a**) in 47% yield.[14] Unfortunately, the 3′,4′-methylenedioxyphenyl derivative (**7g**), which was expected to be useful as a synthon for erythraline, gave the adduct (**8g**) in only 10% yield.[9]

On heating of **7a** and its *N*-alkyl derivatives (**7b**—**f**) with an activated butadiene, 1-methoxy-3-trimethylsilyloxybutadiene, at 130 °C in toluene, the thermal cycloaddition reaction smoothly occurred to yield the ene-adduct (**10**), the product of C=C attack of the diene, and the one-adduct (**11**), the product of C=O attack of the diene.[15] The results are summarized in Table I. The ratios of ene- and one-adducts were calculated from the isolation yields of **10** and of the desilylated compounds **12**, **13**, and **14**. Apparently, the normal

Table I. Yield (%) of the Diels–Alder Adducts

7	R	ene-Adduct (10)	one-Adduct (11)
a	H	32	10
b	Me	62	25
c	Et	—	96
d	iso-Pr	—	81
e	CH_2COOEt	—	71
f	CH_2–CH=CH_2	—	57

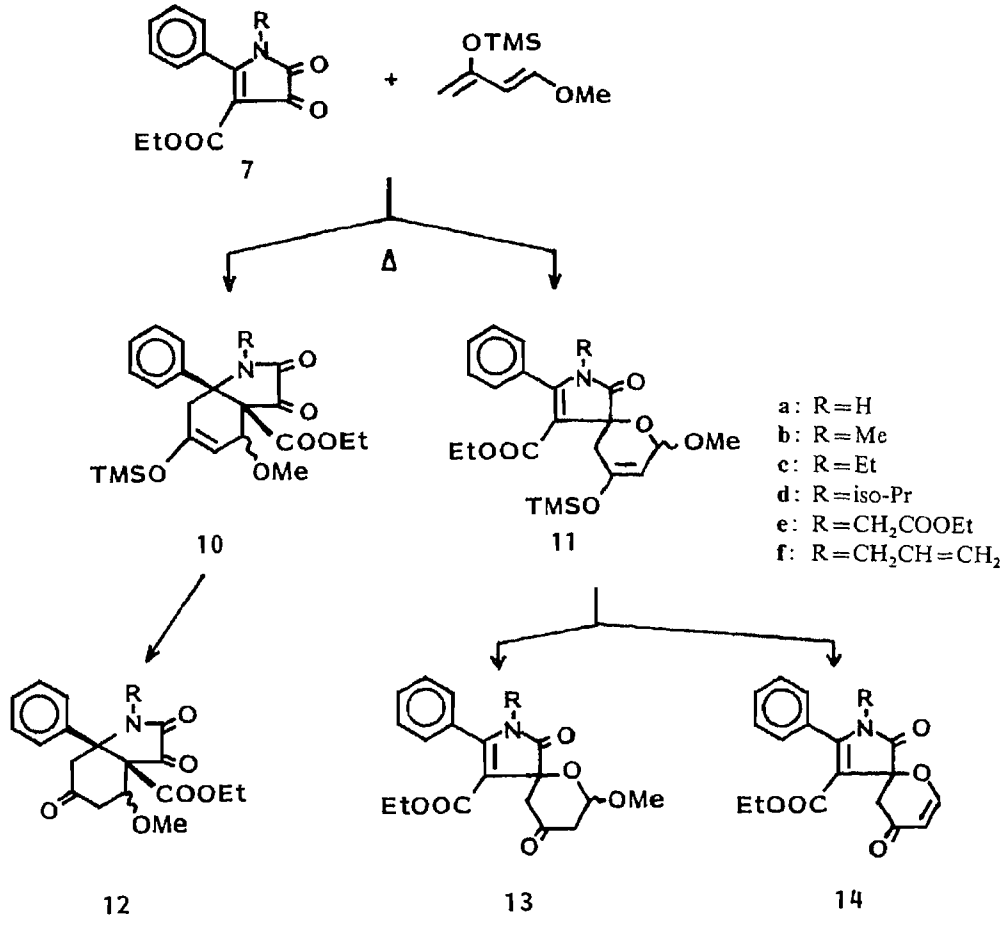


Chart 4

adducts (**10**) predominate when the *N*-substituents are small, while the unusual adducts (**11**) increase and predominate with the compounds possessing bulkier *N*-substituents. This inhibition of the approach of the diene to the C=C bond can be rationalized in terms of the steric hindrance originating from the non-coplanarity of the phenyl group to the dioxopyrroline ring in the *N*-substituted derivatives, where free rotation of the phenyl group is restricted by the *N*-substituents. This explanation is supported by the ultraviolet (UV) spectra of **7**, where inhibition of resonance of the phenyl group with the C=C of the dioxopyrroline was observed in the *N*-substituted derivatives.[16]

We therefore considered the isoquinolinopyrrolinedione (**15**). The dienophile is expected to be more reactive than the corresponding 2-aryldioxopyrrolines, since the presence of ring C restricts the free rotation of the aryl ring and forces the aromatic ring to be coplanar with the dioxopyrroline ring, thus decreasing the steric hindrance due to the aryl group. Moreover, this dienophile is attractive since the erythrinan skeleton can be constructed in one step by means of the Diels–Alder reaction. The product of this reaction with substituted 1,3-butadienes would provide ring A-functionalized erythrinans which are easily convertible to the natural alkaloids by further manipulations (decarbalkoxylation, reduction, *etc.*) of the functional groups.

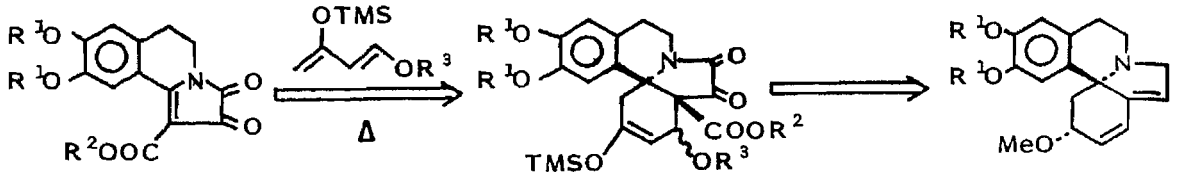
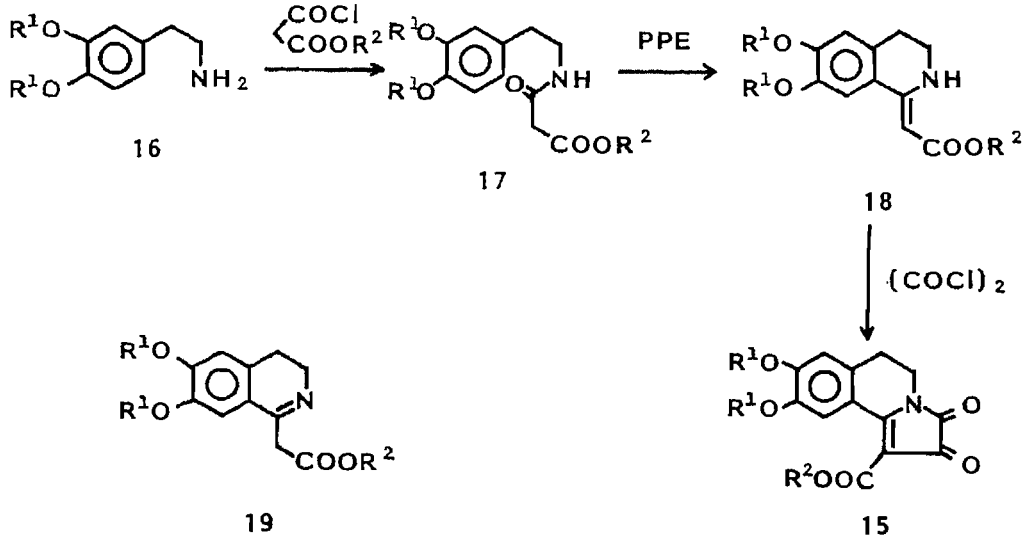


Chart 5

Synthesis of Isoquinolinopyrrolinediones

The isoquinolinopyrrolinedione (**15**) was readily prepared from arylethylamine in three steps by conventional methods. Condensation of the amine (**16**) with ethyl or methyl



a: $R^1=R^2=Me$ **b**: R^1–$R^1=-CH_2-$, $R^2=Me$ **c**: $R^1=Me$, $R^2=Et$
d: R^1–$R^1=-CH_2-$, $R^2=Et$

Chart 6

chloroformylacetate by the Schotten–Baumann method gave the amide (**17**). Bischler–Napieralski cyclization of **17** by using polyphosphate ester (PPE)[17] in $CHCl_3$ gave the tetrahydroisoquinoline (**18**) in good yield. Compound **18c** was identical with that reported by Battersby *et al.*[18] who obtained it by $POCl_3$ cyclization of **17c** in 75% yield, and suggested the imino structure (**19c**). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **18c** showed an olefinic proton signal as a singlet at δ 5.05. The infrared (IR) spectrum exhibited an ester carbonyl band in the low frequency (1660 cm^{-1}) region, indicating the pesence of intramolecular hydrogen bonding to the NH group. These data are consistent with the enamino structure (**18**) with *Z*-configuration of the double bond. In acidic media, **18** changed to the imino form **19** (protonated) as demonstrated by the UV spectral change and disappearance of the olefinic proton in the ^{1}H-NMR spectrum. Condensation of **18** with oxalyl chloride afforded the desired dioxopyrroline (**15**) as red to reddish orange crystals in good yield. Thus, four isoquinolinopyrrolinediones (**15a—d**) were prepared from homoveratrylamine and 2(3,4-methylenedioxyphenyl)-ethylamine in satisfactory yields.

Diels–Alder Reactions of Isoquinolinopyrrolinedione with 1,3-Butadienes

In order to establish the dienophilic activity of the dioxopyrrolines (**15**), we carried out Diels–Alder reaction of **15** with several 1,3-butadienes.

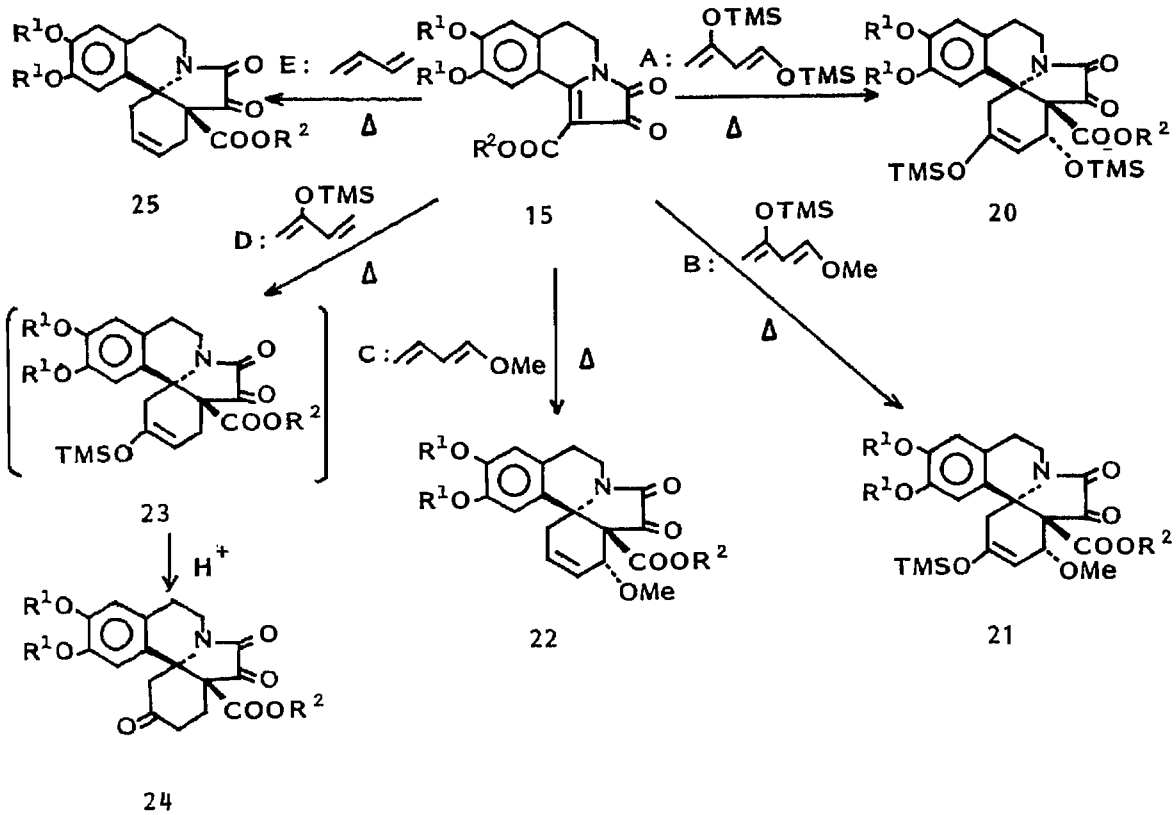
Heating of **15a** and 1,3-bis(trimethylsilyloxy)-butadiene[19] in dioxane at 130 °C for 30 min gave the adduct (**20a**) as a single product in 65% yield. 1-Methoxy-3-trimethylsilyloxybutadiene[20] also smoothly underwent cycloaddition to **15a**, giving the adduct (**21a**) in 82% yield. Similarly, **15b—d** on heating with these activated dienes gave the adducts (**20b—d**) and (**21b—d**) as sole products in comparable yields. Thus, the Diels–Alder reaction readily occurred in a highly regio- and stereospecific manner.

The Diels–Alder reaction of **15** with 1-methoxybutadiene, although more forcing conditions were required, also proceeded in a highly specific manner to give the adduct (**22**) in moderate yields. Similarly, 2-trimethylsilyloxybutadiene afforded regiospecifically the adduct (**24**), although in a lower yield. However, with butadiene they showed poor dienophilic

TABLE II. The Diels–Alder Reaction of **15** with Butadienes

Dienophile	Diene	Solvent	Temp. (°C)	Time (h)	1,4-Adduct (mp)	Yield (%)
15a	A	Dioxane	130	0.5	**20a** 141—143 °C	65
	B	Dioxane	130	1	**21a** 142—144 °C	82
15b	A	Neat	130	1	**20b** 173—176 °C	71
	B	Dioxane	120	1	**21b** 174—177 °C (dec.)	51
15c	A	Neat	140	1	**20c** 142—145 °C	58
	B	Neat	130	3	**21c** 124—126 °C	85
	C	Toluene	140	9	**22c** Gum	86[b]
	D	Toluene	150	17	**24c** 223—225 °C[a]	33
	E	Toluene	170	30	**25c** 175—176 °C	6
15d	A	Neat	130	2	**20d** 202—205 °C	76
	B	Neat	130	3	**21d** Gum	90[b]
	C	Toluene	140	5	**22d** 191—192 °C	36
	D	Toluene	170	13	**24d** 272—275 °C[a]	23
	E	Toluene	170	20	**25d** —	c)

A, 1,3-bis(trimethylsilyloxy)-1,3-butadiene; B, 1-methoxy-3-trimethylsilyloxy-1,3-butadiene; C, 1-methoxybutadiene; D, 2-trimethylsilyloxybutadiene; E, butadiene. *a*) After hydrolysis, isolated as the corresponding keto compound. *b*) Yields as crude gum. *c*) Not isolable.



a: $R^1=R^2=Me$ b: $R^1-R^1=-CH_2-$, $R^2=Me$ c: $R^1=Me$, $R^2=Et$
d: $R^1-R^1=-CH_2-$, $R^2=Et$

Chart 7

activities. For example, **15c** gave the adduct (**25c**) in only 6% yield. The above results are assembled in Table II.

Regio- and Stereochemistry of the Diels–Alder Adducts

Hydrolysis of **20d** with hydrochloric acid in aqueous tetrahydrofuran (THF) solution afforded the keto-aldehyde (**26**), an A-seco compound, the expected conjugated enone (**27**) not being produced. Similarly, acidic treatment of **21d** gave **26**. The structure of **26** was supported by the presence of an aldehydic proton signal at δ 8.07 in the ^{1}H-NMR spectrum of **26**. A similar ring cleavage reaction readily occurred in protic solvents. Trituration of **21d** in methanol afforded the enol-ether (**28**). The presence of two proton signals at δ 5.44 and 7.49 as a doublet ($J=12$ Hz) and of a methyl signal at δ 3.67 for **28** supported the assigned structure. Thus, the adducts readily underwent ring opening under acidic conditions.

On the other hand, treatment of **20d** with potassium fluoride in THF resulted in hydrolysis of the silyl enolate moiety to give the corresponding ketone (**29**) as a major product. Similar treatment of **21d** afforded the methoxy ketone (**30**). Treatment of **29** and **30** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene caused ring opening to yield **26** and **28**, respectively. Therefore, this reaction can be rationalized in terms of the reverse Michael reaction of the 1,5-diketo derivative, thus confirming the regiochemistry of the Diels–Alder adducts.

To elucidate the stereochemistry of the Diels–Alder adducts, **30** was reduced with sodium borohydride and acetylated, and the resulting diacetate (**32**) was subjected to X-ray analysis.[9] The stereochemistries of the C_1-OMe and C_3, C_7-OAc groups were determined as α and β,

respectively. The conformation of ring A is also clarified as a boat form. In solution, this ring A should also adopt a boat form since the C_1-methine proton in the ^{1}H-NMR spectrum of **32** appeared at δ 4.56 as a clean doublet with coupling constants of 11 and 6 Hz, which are consistent with the equatorial orientation of the C_1-OMe group. The structure of **22**, including the stereochemistry of the C_1-OMe group was deduced by analogy with the results described above.

Reduction of **20a** with $LiBH_4$ in THF at -70 °C, followed by dehydration of the resulting product with hydrochloric acid, afforded the hydroxy enone (**33a**) in 81% yield. Similar reduction of **21a** and acid treatment of the resulting alcohol also gave **33a** in 81% yield. The adducts (**20b, d**) on similar treatment gave **33b, d** in good yields, respectively. Compound **33** on acetylation formed the monoacetate (**34**). The structure of the enone moiety was confirmed by the ^{1}H-NMR spectrum of **33a**, which exhibited a pair of clean doublets (J = 10 Hz) at δ 6.42 and 7.53.

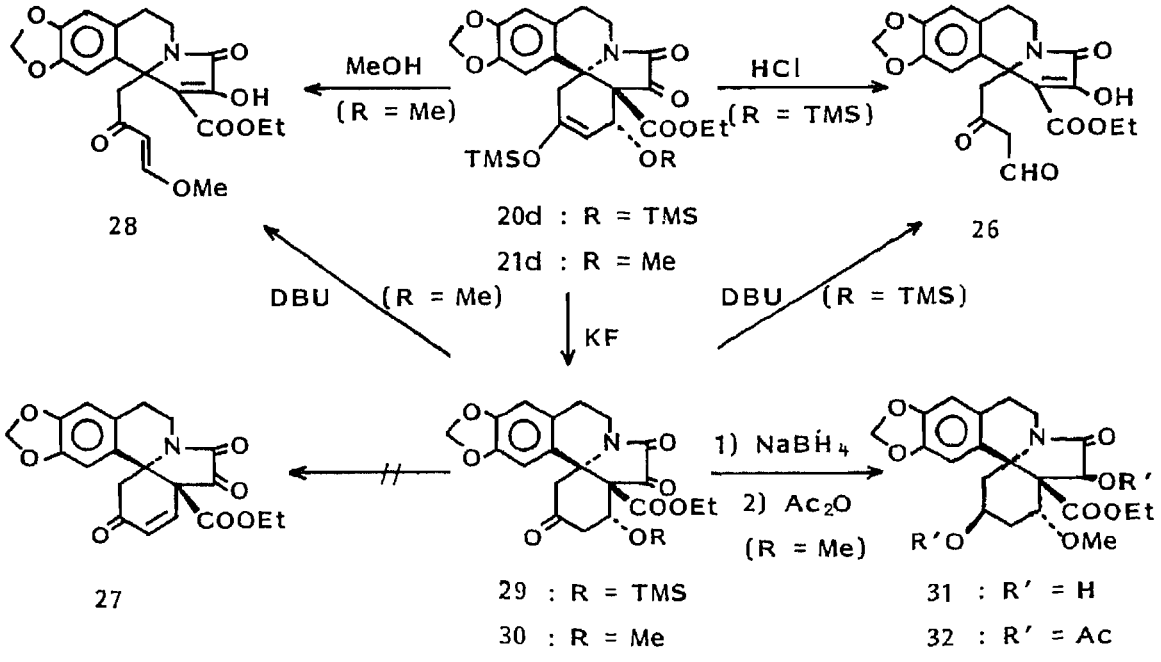


Chart 8

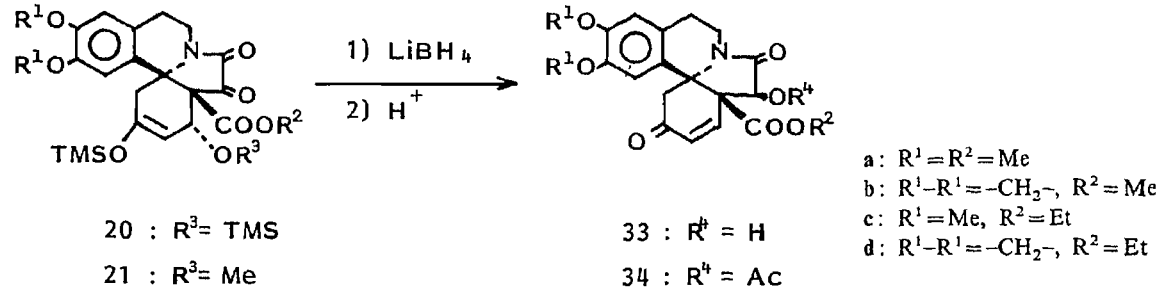


Chart 9

Stereochemical Pathways of the Diels–Alder Reaction

The stereochemistry of the C_1-substituent of the adducts indicates that the addition of the diene takes place from the *endo* face of the dioxopyrroline ring. However, when **15a** was subjected to the reaction with 1,3-bis(trimethylsilyloxy)-butadiene in CH_2Cl_2 at 180 °C for 15 min, a new cycloadduct (**35**, 70%) isomeric to **20a** was obtained. Although **35** and **20a**

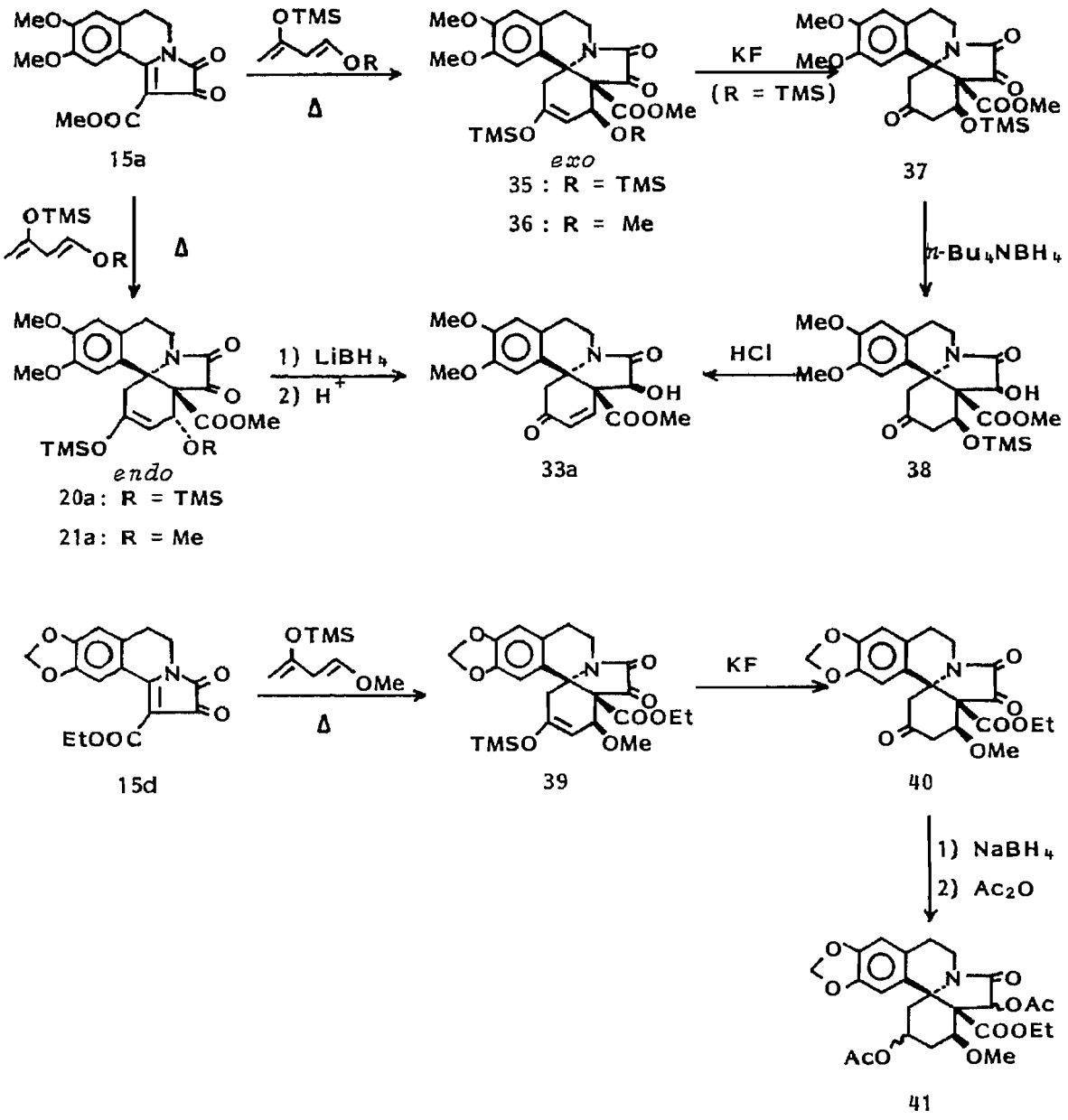
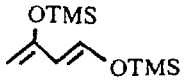
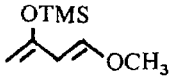


Chart 10

showed very similar ^{1}H-NMR spectra, they are clearly distinguishable by the aromatic proton signals. The C_{14}-proton of **35** (δ 7.2) appeared at lower field than that of **20a** (δ 6.5). The reaction of **15a** with 1-methoxy-3-trimethylsilyloxybutadiene under similar conditions also afforded a new adduct (**36**) as a major product. Thus, the reaction of **15a** with the activated diene was carried out in several solvents and at various temperatures, and it was concluded that the reaction yields both adducts (**20a**, **21a**, and **35**, **36**), as summarized in Table III. The ratios were roughly determined from the relative intensities of C_{14}-proton signals of the reaction mixture.

That the two cycloadducts differ only in the stereochemistry at the C_1-substituent was proved by the chemical conversion of **35** into the above described enone (**33a**). The adduct (**35**) was resistant to selective reduction at the C_7-ketone when the conditions for reduction of **20** ($LiBH_4$, -70 °C in THF) were applied. However, when the trimethylsilyloxy diketone.

TABLE III. Cycloaddition of **15a** and Dioxygenated Butadienes

Diene	Solvent	Temp., °C (min)	Adduct[a]	
	Dioxane	130 (50)	**20a** ≫ **35**	(65%)[b]
	Dioxane	180 (15)	**20a** < **35**	
	Benzene	180 (15)	**20a** > **35**	
	Toluene	180 (30)	**20a** > **35**	
	Hexane	180 (45)	**20a** < **35**	
	(Neat)	180 (15)	**20a** < **35**	
	CH_2Cl_2	130 (50)	**20a** ≪ **35**	
	CH_2Cl_2	180 (15)	**20a** ≪ **35**	(70%)[b]
	Dioxane	130 (50)	**21a**	(82%)[b]
	Dioxane	180 (50)	**21a** : **36** = 1 : 3	
	$CHCl_3$	180 (60)	**36**	(58%)[b,c]

a) *endo*/*exo* Proportion was roughly estimated by ^{1}H-NMR spectral analysis. *b*) Isolated yield of major product. *c*) 42% recovery of **15a**.

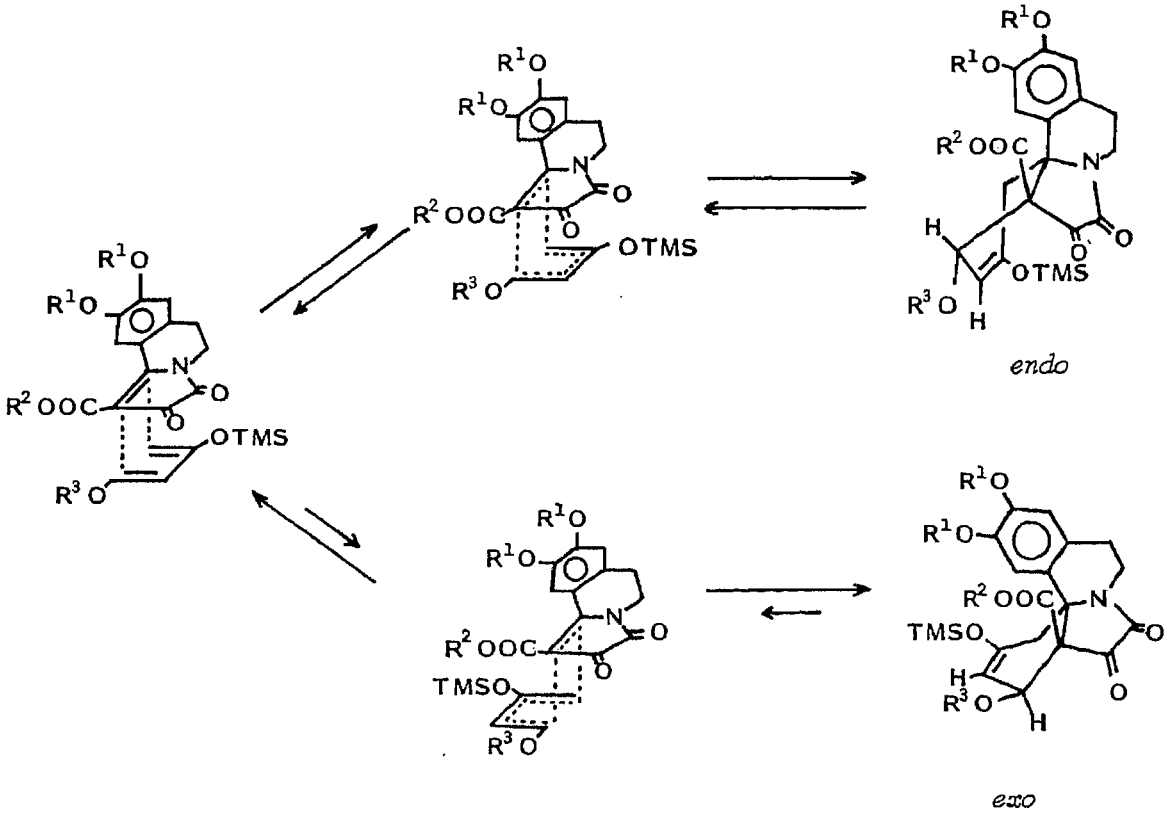


Chart 11

(**37**), which was produced by treatment of the adduct (**35**) with KF in THF, was treated with *n*-Bu_4NBH_4 in CH_2Cl_2, only the C_7-ketone was reduced to give the ketol (**38**). This, on acidic treatment, afforded the enone (**33a**), which was identical with the compound obtained from **20a**.

The adduct (**39**) obtained from **15d** was transformed to the methoxy diacetate (**41**) by desilylation with KF. This diacetate was obtained as a mixture as regards the stereochemistries of the C_3- and C_7-OAc groups. None of them was identical with **32**, confirming that the

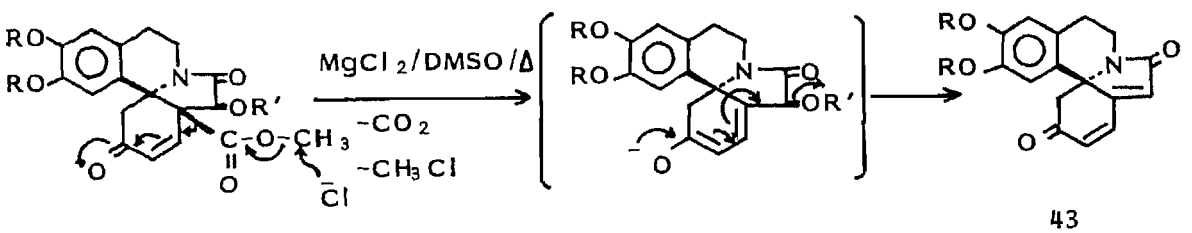
adduct (**39**) and **21d** differ in C_1-stereochemistry. Thus, it was proved that **35**, **36**, and **39** are the *cis-exo* adducts.

The results in Table III clearly demonstrate that in our Diels–Alder reaction the proportion of *exo*-isomer increases with increasing temperature. This phenomenon can be explained by cycloreversion of the kinetic product (*cis-endo*) to the original components and recombination of them to give the thermodynamic product (*cis-exo*) as shown in Chart 11. In fact, thermolysis of **21a** at 180 °C in $CHCl_3$ yielded the dioxopyrroline (**15a**, 42%) and the *cis-exo* isomer (**36**, 58%). When this thermolysis was carried out in the presence of excess diene, the isomerization of **21a** into **36** occurred quantitatively.

In the above reaction the proportion of *exo*-product also increases as the solvent polarity increases. For example, in CH_2Cl_2 either at 180 or 130 °C the *exo*-isomer (**36**) was a major product. Berson *et al.* suggested that *exo*-selectivity increases with the decrease of solvent polarity when the reaction is kinetically controlled.[21] Their suggestion appears to be in conflict with our results. Probably the result in our case may be explained by the assumption that a polar solvent such as CH_2Cl_2 or $CHCl_3$ facilitates the dissociation of the kinetically produced *endo*-isomer.

Synthesis of Erysotrine and Erythraline

The enone (**33**) was next transformed to the natural alkaloids. For this purpose, the C_6-alkoxycarbonyl group is required to be removed and a double bond must be introduced at C_{6-7}. The enone (**33**) is a compound suitable for this transformation, since it is a vinylogous β-ketoester. The conventional method, hydrolysis-decarboxylation, failed since the enone (**33**) was extensively decomposed under alkaline hydrolysis conditions. Direct dealkoxycarbonylation was achieved by applying S_N2 reaction under neutral conditions. Heating of **33a** in dimethyl sulfoxide (DMSO) at 140 °C in the presence of sodium chloride[22] slowly caused demethoxycarbonylation with concomitant dehydration and gave the dienone (**43a**) in 19% yield. Application of the modified method using magnesium chloride instead of sodium chloride, which was recently exploited by Tsuda and Sakai,[23] facilitated this demethoxycarbonylation, although the yield was still unsatisfactory (40%). The similar demethoxycarbonylation of the acetate (**34a**) gave the dienone (**43a**) in a comparable yield. This step was markedly improved by substitution of C_7-OH by a good leaving group. Mesylation of **33a** and subsequent demethoxycarbonylation of the mesylate (**42a**) in the presence of $MgCl_2$–DMSO gave **43a** in 77% yield. The dienone structure of **43a** was confirmed by the observation of three olefinic proton signals, one singlet at δ 6.36 and a pair of doublets (J = 10 Hz) at δ 6.40 and 7.73.



33 : R′ = H

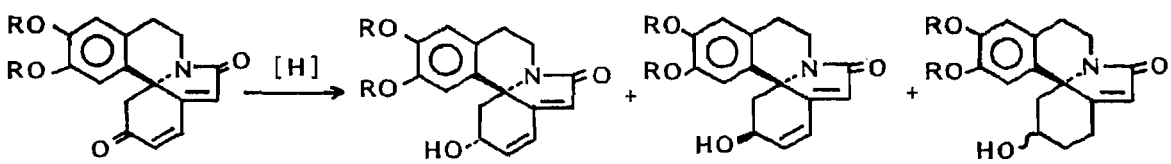
34 : R′ = Ac

42 : R′ = Ms

a: R=Me b: R-R=$-CH_2-$

Chart 12

Next we investigated the reduction of the C_3-ketone to an alcohol. Reduction of **43a** with sodium borohydride in ethanol afforded the β-alcohol (**45a**, 42%) of unnatural stereochemistry and a 1,4-reduction product (**46a**, 35%) as major products, and the α-alcohol (**44a**, 15%) of natural stereochemistry as a minor one. Reduction of **43a** with tetrabutylammonium borohydride in MeOH afforded **44a** (10%), **45a** (52%), and **46a** (13%). This reduction predominantly formed **45a** having unnatural 3β-stereochemistry. In contrast, zinc borohydride reduction of **43a** in ether afforded the 3α-isomer (**44a**, 52%) as a major product and the 3β-isomer (**45a**, 30%) as a minor one. The reduction of **43a** with the combined reagent of sodium borohydride–cerous chloride[24] in methanol also occurred stereoselectively, giving rise to an equatorial α-alcohol (**44a**) predominantly (the yields of **44a** and **45a** were 60% and 30%, respectively). No over-reduction product was observed in this case. Since the 3α-isomer is a more stable equatorial isomer, thermodynamically controlled conditions would produce the 3α-isomer predominantly. In accordance with this expectation, Meerwein–Ponndorf reduction of **43a** proceeded stereoselectively to give **44a** and **45a** in 70% and 25% yields, respectively. The above results are summarized in Table IV.



43 44 45 46

a: R=Me b: R-R=$-CH_2-$

Chart 13

TABLE IV. Reduction of 43

Substrate	Reagent	Solvent	Temp.	Time	44	45	46
43a	$NaBH_4$	EtOH	0 °C	1 h	15 (%)	42 (%)	35 (%)
	n-Bu_4NBH_4	MeOH	0 °C	5 min	10	52	13
	$Zn(BH_4)_2$	THF–Et_2O	r.t.	24 h	52	30	—
	$NaBH_4$–$CeCl_3$	MeOH	0 °C	10 min	60	30	—
	Al(iso-PrO)$_3$	iso-PrOH	Reflux	24 h	70	25	—
43b	$NaBH_4$	EtOH	0 °C	1 h	23	40	15
	Al(iso-PrO)$_3$	iso-PrOH	Reflux	24 h	58	20	—

The stereochemical assignment of the C_3-OH group was readily accomplished by the following spectral comparisons. The ^{1}H-NMR signal pattern of **44a** was very similar to that of natural erysotramidine (**2a**) except that the latter exhibited an OMe signal. Methylation of **44a** with methyl iodide in the presence of a phase transfer catalyst (KOH–Et_4NBr) furnished the *O*-methyl ether (**2a**) in 84% yield; this product was identical with *dl*-erysotramidine. Similar methylation of **45a** gave *dl*-3-epierysotramidine (**47a**, 89%).

The final step of this synthesis is removal of the C_8-lactam carbonyl group. We thought that it would be difficult to keep the double bond of the conjugated dienoid system intact in lithium aluminum hydride reduction. Mondon reported that, on treatment of the conjugated lactam (**49**) with lithium aluminum hydride, both the lactam carbonyl group and the double bond were reduced to yield the amine (**50**).[25] In fact, reduction of **2a** with lithium aluminum hydride gave an intractable material. After several fruitless attempts, we found that aluminum

hydride[26] generated from $AlCl_3$–$LiAlH_4$ was an excellent reagent for this purpose. Reduction of **2a** with this reagent in THF at room temperature gave *dl*-erysotrine (**1a**) in 80% yield. Compound **1a** was well characterized as the picrate, mp 194—196 °C (lit. mp 197—198 °C).[6b] Similar reduction of **47a** with aluminum hydride furnished *dl*-3-epierysotrine (**48a**) in 78% yield; it also formed a picrate, mp 174—175 °C (lit. mp 174—175 °C).[6b] The identities of all the products were confirmed by direct spectral comparisons with the natural alkaloids.

By the same sequences as described above we succeeded in the total synthesis of *dl*-erythraline (**1b**) from the enone (**33b**). The mesylation of the enone (**33b**) and the subsequent treatment of **42b** with $MgCl_2$–DMSO gave the dienone (**43b**) in 78% yield. Meerwein–Ponndorf reduction of **43b** yielded the α-alcohol (**44b**, 58%) and the β-alcohol (**45b**, 20%). Methylation of **44b** and **45b** with methyl iodide in the presence of Et_4NBr and KOH afforded *dl*-8-oxoerythraline (**2b**) and the 3-epimer (**47b**) in 86% and 89% yields, respectively. Reduction of **2b** and **47b** with aluminum hydride in THF afforded *dl*-erythraline (**1b**, 86%) and *dl*-3-epierythraline (**48b**, 81%). Identification of the synthetic compounds was achieved by spectral comparisons with the natural alkaloids, thus accomplishing the first total synthesis of *dl*-erythraline and *dl*-8-oxoerythraline.

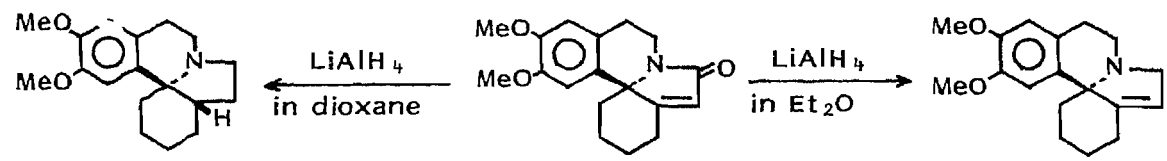
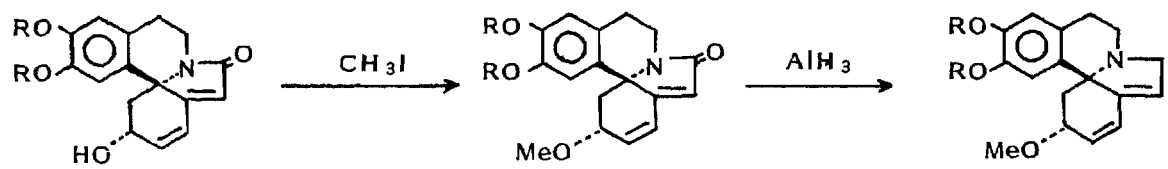
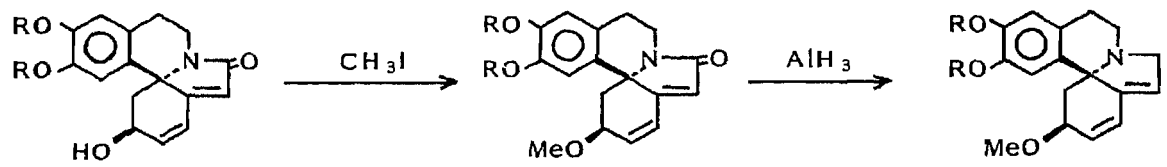


Chart 14





a: R=Me b: R–R=$-CH_2-$

Chart 15

In summary, the Diels–Alder strategy was demonstrated to be effective for the synthesis of erythrinan alkaloids. Erysotrine and erythraline were prepared in 10 steps from the commercially available arylethylamines in 10% and 13% overall yields, respectively. Thus, this synthetic method of *Erythrina* alkaloids involves the least steps and gives the highest total yield among the hitherto known methods.

Experimental

All melting points are uncorrected. IR spectra were measured with a Hitachi 215 or a Hitachi 260-10

spectrophotometer and are given as cm^{-1}. NMR spectra were taken on a Varian T-60 (60 MHz), a Hitachi R-600 (60 MHz), or a JEOL JNM-FX 100 (^1H; 100 MHz, ^{13}C; 25 MHz) NMR spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard, unless otherwise noted. The chemical shifts are given as δ values from TMS. The following abbreviations are used; s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. High-resolution mass spectra (HRMS) were determined with a JEOL JMS-D300 spectrometer at 30 eV by the direct inlet system. UV spectra were measured with a Hitachi 200-10 spectrophotometer in EtOH and given as λ_{max} nm (ε). Preparative thin layer chromatography (TLC) was performed on 20×20 cm glass plates coated with a 0.5 mm layer of Silica gel $60F_{254}$ (Merck). Column chromatography was carried out with silica gel (Wakogel C-200), alumina (Aluminiumoxid 90, Aktivitatsstufe II—III, Merck), or Florisil (60—100 mesh, Wako). Medium-pressure liquid chromatography (MPLC) was performed on a Kusano CIG prepacked silica gel column.

Diels–Alder Reaction of 5——A solution of dioxopyrroline (**5**) (1 g) and butadiene (15 ml) in Ac_2O (10 ml) was heated in a sealed tube at 180 °C for 8 h. The reaction mixture was concentrated *in vacuo* to give an oil, which was extracted with methanol. After evaporation of methanol, the residue was chromatographed over alumina and eluted with benzene to give 3-acetoxy-1-acetyl-7a-phenyl-1,2,7,7a-tetrahydro-4*H*-indole (**6**, 90 mg, 5%) as colorless prisms from methanol, mp 170—172 °C. IR (Nujol): 1775, 1740, 1700. ^1H-NMR (60 MHz): 2.32 (3H, s, N–$COCH_3$), 2.47 (3H, s, –$OCOCH_3$), 2.9—4.2 (4H, m, –CH_2–CH_2–), 5.4—5.8 (2H, m, olefinic H), 7.20 (5H, s, Ar-H). HRMS *m/z*: Calcd for $C_{18}H_{17}NO_4$ (M^+): 311.1158. Found: 311.1159.

Diels–Alder Reaction of 7a with Butadiene——1) In Methylene Chloride: A solution of dioxopyrroline (**7a**, 200 mg) and butadiene (10 ml) in CH_2Cl_2 (10 ml) was heated at 170 °C for 8 h in a sealed tube. The reaction mixture was concentrated *in vacuo* to give a brown oil, which was extracted with boiling methanol. The methanol solution was evaporated *in vacuo* to give an oily residue, which was chromatographed in benzene over alumina. Elution with CH_2Cl_2 gave a crystalline material (51 mg). Recrystallization from *n*-hexane–acetone gave (3a*S**,7a*R**)-3a-ethoxycarbonyl-2,3-dioxo-7a-phenyl-2,3,3a,4,7,7a-hexahydroindole (**9a**, 25 mg, 10%) as colorless prisms, mp 228—231 °C. IR (Nujol): 1780, 1755, 1730. ^1H-NMR (60 MHz): 0.72 (3H, t, J=7 Hz, –OCH_2CH_3), 2.95 (4H, m, –CH_2–CH_2–), 3.50 (2H, m, –OCH_2CH_3), 6.05 (2H, m, olefinic H), 7.42 (5H, m, Ar-H). HRMS *m/z*: Calcd for $C_{17}H_{17}NO_4$ (M^+): 299.1158. Found: 299.1178.

2) In Ac_2O: A solution of **7a** (2.015 g) and butadiene (10 ml) in Ac_2O (15 ml, freshly distilled) was heated at 160 °C for 10 h in a sealed tube. The reaction mixture was concentrated *in vacuo* to give a gummy material, which was extracted with methanol. Evaporation of methanol and trituration of the residue from ether gave (3a*S**,7a*R**)-1-acetyl-3a-ethoxycarbonyl-2,3-dioxo-7a-phenyl-2,3,3a,4,7,7a-hexahydroindole (**8a**, 1.305 g, 47%), colorless prisms from methanol, mp 164—166 °C. IR (Nujol): 1780, 1740, 1700. ^1H-NMR (60 MHz): 0.92 (3H, t, J=7 Hz, –OCH_2CH_3), 2.70 (3H, s, >N–$COCH_3$), 2.90, 3.25 (each 2H, m, –CH_2–), 3.55 (2H, m, –OCH_2CH_3), 5.95 (2H, t, J=2 Hz, olefinic H), 7.23 (5H, m, Ar-H). *Anal.* Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. HRMS *m/z*: 341.1263. Found: C, 66.86; H, 5.55; N, 4.00. HRMS *m/z*: 341.1295.

A solution of **8a** (23 mg) in 2% HCl–EtOH (15 ml) was heated under reflux for 30 min. After evaporation of the solvent, the residue was recrystallized from *n*-hexane–acetone to give **9a** (19 mg, 94%), mp 228—230 °C.

Diels–Alder Reaction of 7g with Butadiene——A solution of **7g** (500 mg) and butadiene (5 ml) in acetic anhydride (15 ml) was heated at 160 °C for 6 h in a sealed tube. The reaction mixture was concentrated *in vacuo* and extracted with boiling methanol. The methanol extract was concentrated *in vacuo* to give a brown oil, which was chromatographed over SiO_2. Elution with benzene gave (3a*S**,7a*R**)-1-acetyl-3a-ethoxycarbonyl-7a-(3,4-methylenedioxyphenyl)-2,3-dioxo-2,3,3a,4,7,7a-hexahydroindole (**8g**, 80 mg, 12%) as a yellow oil. IR (Nujol): 1780, 1750, 1725. ^1H-NMR (60 MHz): 1.00 (3H, t, J=7 Hz, –OCH_2CH_3), 2.67 (3H, s, >N–$COCH_3$), 1.9—3.2 (4H, m, –CH_2–CH_2–), 3.75 (2H, q, J=7 Hz, –OCH_2CH_3), 5.5—6.0 (2H, m, olefinic H), 5.93 (2H, s, –OCH_2O–), 6.6—6.8 (3H, m, Ar-H).

A solution of **8g** (80 mg) in 5% HCl–EtOH (10 ml) was heated under reflux for 30 min. The reaction mixture was diluted with water and extracted with CH_2Cl_2. The extract was washed with water, dried over Na_2SO_4 and evaporated. The residue was chromatographed over SiO_2 with benzene to give (3a*S**,7a*R**)-3a-ethoxycarbonyl-7a-(3,4-methylenedioxyphenyl)-2,3-dioxo-2,3,3a,4,7,7a-hexahydroindole (**9g**, 36 mg, 50%), colorless prisms from MeOH–ether, mp 222—224 °C. IR (Nujol): 1775, 1735, 1720. ^1H-NMR (60 MHz): 0.83 (3H, t, J=7 Hz, –OCH_2CH_3), 2.6—3.0 (4H, m, –CH_2–CH_2–), 3.73 (2H, m, –OCH_2CH_3), 5.97 (2H, m, olefinic H), 6.00 (2H, s, –OCH_2O–), 6.6—7.1 (3H, m, Ar-H). HRMS *m/z*: Calcd for $C_{18}H_{17}NO_6$ (M^+): 343.1056. Found: 343.1058.

Diels–Alder Reaction of 7 with 1-Methoxy-3-trimethylsilyloxy-1,3-butadiene——A solution of dioxopyrroline (**7**, 100 mg) and 1-methoxy-3-trimethylsilyloxy-1,3-butadiene (5 molar eq) in absolute toluene (5 ml) was heated at 130 °C for 10—20 min in a sealed tube with stirring. The reaction mixture was concentrated to dryness *in vacuo*, and purified by the following procedure. 1) In the cases of **7a** and **7b**, crystallization of the residue from *n*-hexane gave the ene-adduct (**10**). The mother liquor from the ene-adduct was treated with KF (5 molar eq) in THF at room temeprature for 15 h. The reaction mixture was extracted with CH_2Cl_2, and the organic layer was washed with H_2O, dried over Na_2SO_4, and evaporated. The residue was purified by MPLC (AcOEt : *n*-hexane = 1 : 1) to give the one-adduct (**13**) and/or (**14**).

2) In the cases of **7c**, **7d**, **7e**, and **7f**, the whole product was treated with KF in THF, and then the product was

purified by MPLC.

Products from 7a——(3a*R**,4*R**,7a*R**)-3a-Ethoxycarbonyl-4-methoxy-2,3-dioxo-7a-phenyl-6-trimethylsilyloxy-2,3,3a,4,7,7a-hexahydroindole (**10a**): mp 137—139 °C, prisms from AcOEt–*n*-hexane, 27%. IR (Nujol): 3170, 3050, 1770, 1720, 1645. ^{1}H-NMR (100 MHz): 0.27 (9H, s, $-SiMe_3$), 0.70 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.91 (2H, s, C_7-H_2), 3.30 (3H, s, C_4-OCH_3), 4.64 (1H, d, J=5 Hz, C_4-H), 5.25 (1H, d, J=5 Hz, olefinic H), 7.31 (5H, s, Ar-H), 9.61 (1H, brs, –NH–). HRMS m/z: Calcd for $C_{21}H_{27}NO_6Si$ (M^+): 417.1608. Found: 417.1636.

(3a*R**,4*R**,7a*R**)-3a-Ethoxycarbonyl-4-methoxy-2,3,6-trioxo-7a-phenyl-1-trimethylsilyloxy-2,3,3a,4,5,6,7,7a-octahydroindole (**12a**): mp 168—169 °C, prisms from AcOEt–ether, 5%. IR (Nujol): 1750, 1740, 1725. ^{1}H-NMR (100 MHz): 0.19 (9H, s, $-SiMe_3$), 1.15 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.76 (3H, s, C_4-OCH_3), 4.62 (1H, dd, J=2, 3 Hz, C_4-H), 7.4—8.0 (5H, m, Ar-H), HRMS m/z: Calcd for $C_{21}H_{27}NO_6Si$ (M^+): 417.1606. Found: 417.1595.

4-Ethoxycarbonyl-7-methoxy-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]dec-3-ene (**13a**): mp 175—177 °C, prisms from MeOH, 10%. IR (Nujol): 3170, 3100, 1730, 1690, 1630. UV: 242 (10600). ^{1}H-NMR (100 MHz): 1.06 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.59 (1H, dd, J=4, 18 Hz), 2.66, 3.20 (each 1H, d, J=16 Hz), 3.05 (1H, dd, J=4, 18 Hz), 3.50 (3H, s, $-OCH_3$), 4.06 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.20 (1H, t, J=4 Hz, >CH–OMe), 7.4—7.6 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{18}H_{19}NO_6$ (M^+): 345.1213. Found: 345.1218.

Products from 7b——(3a*R**,4*R**,7a*R**)-3a-Ethoxycarbonyl-4-methoxy-1-methyl-2,3-dioxo-7a-phenyl-6-trimethylsilyloxy-2,3,3a,4,7,7a-hexahydroindole (**10b**): mp 99—101 °C, prisms from AcOEt–*n*-hexane, 60%. IR (Nujol): 1765, 1720, 1650, ^{1}H-NMR (100 MHz): 0.30 (9H, s, $-SiMe_3$), 0.78 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.00 (3H, s, $>NCH_3$), 3.25 (3H, s, C_4-OCH_3), 4.58 (1H, d, J=5 Hz, C_4-H), 5.19 (1H, d, J=5 Hz, olefinic H), 7.0—7.4 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{22}H_{29}NO_6Si$ (M^+): 431.1762. Found: 431.1755.

(3a*R**,4*R**,7a*R**)-3a-Ethoxycarbonyl-4-methoxy-1-methyl-2,3,6-trioxo-7a-phenyl-2,3,3a,4,5,6,7,7a-octahydroindole (**12b**): mp 172—174 °C, plates from AcOEt–*n*-hexane, 1.5%. IR (Nujol): 1770, 1720, 1710. ^{1}H-NMR (100 MHz): 0.87 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.98 (3H, s, $>NCH_3$), 3.28 (3H, s, C_4-OCH_3), 4.43 (1H, dd, J=3, 4 Hz, C_4-H), 6.9—7.4 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{19}H_{21}NO_6$ (M^+): 359.1367. Found: 359.1351.

(3a*R**,4*S**,7a*R**)-3a-Ethoxycarbonyl-4-methoxy-1-methyl-2,3,6-trioxo-7a-phenyl-2,3,3a,4,5,6,7,7a-octahydroindole (**4**-epi **12b**): mp 154—155 °C, prisms from ether, 1.5%. IR (Nujol): 1775, 1705. ^{1}H-NMR (100 MHz): 0.82 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.05 (3H, s, $>NCH_3$), 3.56 (3H, s, C_4-OCH_3), 4.21 (1H, t, J=4 Hz, C_4-H), 7.1—7.5 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{19}H_{21}NO_6$ (M^+): 359.1367. Found: 359.1349.

4-Ethoxycarbonyl-7-methoxy-2-methyl-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]dec-3-ene (**13b**): mp 118—121 °C, prisms from AcOEt–*n*-hexane, 22%. IR (Nujol): 1730, 1685, 1625. UV: 238 (6700, sh), 309 (6900). ^{1}H-NMR (100 MHz): 0.87 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.61, 3.25 (each 1H, d, J=17 Hz), 2.62, 3.10 (each 1H, dd, J=4, 18 Hz), 2.84 (3H, s, $>NCH_3$), 3.57 (3H, s, $-OCH_3$), 3.93 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.22 (1H, t, J=4 Hz, >CH–OMe), 7.2—7.5 (5H, m, Ar-H). ^{13}C-NMR: 13.6 (q), 27.9 (q), 42.6 (t), 44.9 (t), 56.4 (q), 59.8 (t), 78.2 (s), 100.4 (d), 111.4 (s), 128.3 (d × 4), 129.5 (s), 129.9 (d), 157.2 (s), 162.7 (s), 176.0 (s), 202.0 (s). HRMS m/z: Calcd for $C_{19}H_{21}NO_6$ (M^+): 359.1367. Found: 359.1347.

4-Ethoxycarbonyl-2-methyl-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]deca-3,7-diene (**14b**): mp 117—118 °C, prisms from ether–*n*-hexane, 3%. IR (Nujol): 1740, 1685, 1625, 1600, 1595. UV: 247 (9600), 303 (6500). ^{1}H-NMR (100 MHz): 0.98 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.74, 3.54 (each 1H, d, J=17 Hz), 2.83 (3H, s, $>NCH_3$), 4.01 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.53 (1H, d, J=6 Hz, olefinic H), 7.2—7.6 (6H, m, Ar-H × 5, olefinic H). ^{13}C-NMR: 13.7 (q), 27.8 (q), 39.6 (t), 60.1 (t), 82.1 (s), 106.2 (d), 108.6 (s), 128.3 (d × 2), 128.5 (d × 2), 128.8 (s), 130.4 (d), 158.8 (s), 160.0 (d), 161.8 (s), 174.6 (s), 189.2 (s). HRMS m/z: Calcd for $C_{18}H_{17}NO_5$ (M^+): 327.1106. Found: 327.1096.

Products from 7c——4-Ethoxycarbonyl-2-ethyl-7-methoxy-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]dec-3-ene (**13c**): mp 83—85 °C, prisms from ether–*n*-hexane, 68%. IR (Nujol): 1730, 1690, 1625, 1595. UV: 308 (6900). ^{1}H-NMR (100 MHz): 0.85, 0.99 (each 3H, t, J=7 Hz, $-OCH_2CH_3$), 3.57 (3H, s, $-OCH_3$), 5.23 (1H, t, J=4 Hz, >CH–OMe), 7.2—7.6 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{20}H_{23}NO_6$ (M^+): 373.1525. Found: 373.1538.

4-Ethoxycarbonyl-2-ethyl-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]-deca-3,7-diene (**14c**): mp 94—95 °C, pale yellow prisms from ether–*n*-hexane, 28%. IR (Nujol): 1735, 1685, 1620, 1605, 1590. UV: 250 (10000), 300 (6900). ^{1}H-NMR (100 MHz): 0.96, 0.97 (each 3H, t, J=7 Hz, $-OCH_2CH_3$, $>NCH_2CH_3$), 2.72, 3.53 (each 1H, d, J=17 Hz), 3.34 (2H, q, J=7 Hz, $>NCH_2CH_3$), 3.99 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.52 (1H, d, J=6 Hz, olefinic H), 7.2—7.6 (6H, m, Ar-H × 5, olefinic H). HRMS m/z: Calcd for $C_{19}H_{19}NO_5$ (M^+): 341.1263. Found: 341.1258.

Product of 7d——4-Ethoxycarbonyl-2-isopropyl-7-methoxy-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]dec-3-ene (**13d**): Oil, 82%. IR ($CHCl_3$): 1730, 1685, 1630, 1595. UV: 309 (6700). ^{1}H-NMR (100 MHz): 0.83 (3H, t, J=7 Hz, $-OCH_2CH_3$), 1.31, 1.35 (each 3H, d, J=7 Hz, $-CHMe_2$), 2.59, 3.20 (each 1H, d, J=16 Hz), 2.60 (1H, dd, J=4, 18 Hz), 3.08 (1H, dd, J=4, 18 Hz), 3.57 (3H, s, $-OCH_3$), 3.88 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.22 (1H, t, J=4 Hz, >CH–OMe), 7.0—7.5 (5H, m, Ar-H). HRMS m/z: Calcd for $C_{21}H_{25}NO_6$ (M^+): 387.1682. Found: 387.1689.

Product of 7e——4-Ethoxycarbonyl-2-ethoxycarbonylmethyl-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]deca-3,7-diene (**14e**): mp 103—104 °C, prisms from ether–*n*-hexane, 57%. IR (Nujol): 1735, 1695, 1680, 1630, 1605, 1595. UV: 254 (10600), 300 (7200). ^{1}H-NMR (100 MHz): 0.96 (3H, t, J=7 Hz, $-OCH_2CH_3$), 2.74, 3.56 (each 1H, d, J=17 Hz), 3.8—3.9 (2H, m, $>NCH_2$–), 4.00 (2H, q, J=7 Hz, $-OCH_2CH_3$), 4.8—5.8 (3H, m, vinyl H), 5.52 (1H, d, J=6 Hz, olefinic H), 7.2—7.5 (6H, m, Ar-H × 5, olefinic H). HRMS m/z: Calcd for $C_{20}H_{19}NO_5$ (M^+): 353.1263. Found:

353.1278.

Product of 7f——2-Allyl-4-ethoxycarbonyl-7-methoxy-1,9-dioxo-3-phenyl-6-oxa-2-azaspiro[4.5]dec-3-ene (**13f**): Oil, 71%. IR ($CHCl_3$): 1740, 1690, 1630, 1595. UV: 302 (8400). ^{1}H-NMR (100 MHz): 0.86, 1.18 (each 3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.62 (1H, dd, J=3, 18 Hz), 2.71, 3.29 (each 1H, d, J=16 Hz, 3.11 (1H, dd, J=4, 18 Hz), 3.56 (3H, s, $-O\underline{CH_3}$), 3.87, 4.16 (each 1H, d, J=18 Hz, $>NC\underline{H_2}COOEt$), 5.24 (1H, dd, J=3, 4 Hz, $>C\underline{H}-OMe$), 7.1—7.6 (5H, m, Ar-H). ^{13}C-NMR: 13.4 (q), 13.9 (q), 42.2 (t), 42.6 (t), 44.8 (t), 56.3 (q), 59.8 (t), 61.7 (t), 78.4 (s), 100.4 (d), 112.2 (s), 128.3 (d×4), 128.9 (s), 130.0 (d), 155.8 (s), 162.5 (s), 167.1 (s), 175.9 (s), 201.6 (s). HRMS m/z: Calcd for $C_{22}H_{25}NO_8$ (M^+): 431.1580. Found: 431.1575.

Schotten–Baumann Reaction of 2-Arylethylamine (16) with Chloroformylacetate (General Procedure)——A solution of methyl or ethyl chloroformylacetate (1.2 molar equivalents) in ether (for **16c** and **16d**) or chloroform (for **16a** and **16b**) (100 ml) was added dropwise to a stirred solution of 2-arylethylamine (**16**, 20 g) in ether (or $CHCl_3$) (120 ml) and 10% K_2CO_3 (1.2 molar equivalents) at 0 °C. Stirring was continued for a further 0.5—1 h, the organic layer was separated, and the aqueous layer was extracted with $CHCl_3$. The combined organic layer was dried over Na_2SO_4 and evaporated to give a crude amide (**17**), which was purified by recrystallization or by chromatography.

N-[2(3,4-Dimethoxyphenyl)-ethyl]methoxycarbonylacetamide (**17a**): mp 75—76 °C, plates from AcOEt, 99%. IR (Nujol): 3250, 3075, 1740, 1645. ^{1}H-NMR (60 MHz): 2.78 (2H, br t, J=7 Hz, benzylic H), 3.28 (2H, s, $-COC\underline{H_2}COOMe$), 3.53 (2H, br q, J=7 Hz, $-C\underline{H_2}NH-$), 3.72 (3H, s, $-COOC\underline{H_3}$), 3.87, 3.88 (each 3H, s, $-O\underline{CH_3}$), 6.78 (3H, br s, Ar-H). HRMS m/z: Calcd for $C_{14}H_{19}NO_5$ (M^+): 281.1263. Found: 281.1269.

N-[2-(3,4-Methylenedioxyphenyl)-ethyl]methoxycarbonylacetamide (**17b**): mp 98—99 °C, needles from ether, 95%. IR (Nujol): 3350, 3270, 3100, 1750, 1650. ^{1}H-NMR (60 MHz): 2.78 (2H, br t, J=7 Hz, benzylic H), 3.28 (2H, s, $-COC\underline{H_2}COOMe$), 3.50 (2H, br q, J=7 Hz, $-C\underline{H_2}NH-$), 3.75 (3H, s, $-COOCH_3$), 5.93 (2H, s, $-O\underline{CH_2}O-$), 6.72 (3H, s, Ar-H). HRMS m/z: Calcd for $C_{13}H_{15}NO_5$ (M^+): 265.0948. Found: 265.0941.

N-[2-(3,4-Dimethoxyphenyl)-ethyl]ethoxycarbonylacetamide (**17c**): mp 57—58 °C (lit. mp 63—64 °C),[18] needles from ether–*n*-hexane, 98%. IR (Nujol): 3290, 1740, 1635. ^{1}H-NMR (60 MHz): 1.26 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.78 (2H, br t, J=7 Hz, benzylic H), 3.27 (2H, s, $-COC\underline{H_2}COOEt$), 3.54 (2H, br q, J=7 Hz, $-C\underline{H_2}NH-$), 3.88 (6H, s, $C\underline{H_3}O-\times 2$), 4.17 (2H, q, J=7 Hz, $-O\underline{CH_2}CH_3$), 6.78 (3H, br s, Ar-H). HRMS m/z: Calcd for $C_{15}H_{21}NO_5$ (M^+): 295.1419. Found: 295.1434.

N-[2(3,4-Methylenedioxyphenyl)-ethyl]ethoxycarbonylacetamide (**17d**): mp 98—100 °C, needles from ether, 86%. IR (Nujol): 3280, 1740, 1640. ^{1}H-NMR (100 MHz): 1.26 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.77 (2H, br t, J=7 Hz, benzylic H), 3.27 (2H, s, $-COC\underline{H_2}COOEt$), 3.52 (2H, br q, J=7 Hz, $-C\underline{H_2}NH-$), 4.18 (2H, q, J=7 Hz, $-O\underline{CH_2}CH_3$), 5.83 (2H, s, $-O\underline{CH_2}O-$), 6.60 (3H, s, Ar-H). HRMS m/z: Calcd for $C_{14}H_{17}NO_5$ (M^+): 279.1105. Found: 279.1105.

Bischler–Napieralski Cyclization of the Amide (17) with Polyphosphate Ester in $CHCl_3$ (General Procedure)——A solution of **17** (5 g) and PPE (50 g) in anhydrous $CHCl_3$ (50 ml) was heated under reflux for 1—5 h. After the reaction mixture had cooled to room temperature, ice was added carefully to decompose excess PPE. The mixture was basified with 10% K_2CO_3, and then extracted with $CHCl_3$. The extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was chromatographed on Florisil using benzene as an eluent to give a pure isoquinoline derivative (**18**).

6,7-Dimethoxy-1-(*Z*)-(methoxycarbonyl)-methylidene-1,2,3,4-tetrahydroisoquinoline (**18a**): Oil, 90%. ^{1}H-NMR (60 MHz): 2.80 (2H, br t, J=6 Hz, benzylic H), 3.2—3.6 (2H, m, $-C\underline{H_2}NH-$), 3.70 (3H, s, $-COOC\underline{H_3}$), 3.90 (6H, s, $C\underline{H_3}O-\times 2$), 5.05 (1H, s, olefinic H), 6.67, 7.12 (each 1H, s, Ar-H).

6,7-Methylenedioxy-1-(*Z*)-(methoxycarbonyl)-methylidene-1,2,3,4-tetrahydroisoquinoline (**18b**): mp 138—141 °C, prisms from CH_2Cl_2–ether, 94%. IR (Nujol): 3320, 1640, 1620. ^{1}H-NMR (60 MHz): 2.80 (2H, br t, J=6 Hz, benzylic H), 3.2—3.6 (2H, m, $-C\underline{H_2}NH-$), 3.70 (3H, s, $-COOC\underline{H_3}$), 5.02 (1H, s, olefinic H), 6.00 (2H, s, $-O\underline{CH_2}O-$), 6.67, 7.13 (each 1H, s, Ar-H). HRMS m/z: Calcd for $C_{13}H_{13}NO_4$ (M^+): 247.0845. Found: 247.0845.

1-(*Z*)-(Ethoxycarbonyl)-methylidene-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (**18c**): mp 82—85 °C (lit. mp 85.5—86.5 °C),[18] prisms from ether–*n*-hexane, 90%. IR (Nujol): 3320, 1660, 1610. UV: 212 (17900, sh), 227 (24700), 269 (9100), 327 (20100); UV (1% HCl–EtOH): 252 (18200), 312 (6400). ^{1}H-NMR (100 MHz): 1.31 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.83 (2H, br t, J=6 Hz, benzylic H), 3.2—3.5 (2H, m, $-C\underline{H_2}NH-$), 3.89, 3.91 (each 3H, s, $-O\underline{CH_3}$), 4.17 (2H, q, J=7 Hz, $-O\underline{CH_2}CH_3$), 5.05 (1H, s, olefinic H), 6.66, 7.13 (each 1H, s, Ar-H), 9.04 (1H, br s, $>N\underline{H}$). ^{13}C-NMR: 14.7 (q), 28.6 (t), 39.0 (t), 56.0 (q), 56.1 (q), 58.6 (t), 77.1 (d), 108.2 (d), 110.7 (d), 121.6 (s), 130.0 (s), 147.9 (s), 151.1 (s), 156.3 (s), 171.1 (s). HRMS m/z: Calcd for $C_{15}H_{19}NO_4$ (M^+): 277.1314. Found: 277.1320.

1-(*Z*)-(Ethoxycarbonyl)-methylidene-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline (**18d**): mp 148—150 °C, prisms from benzene, 80%. IR (Nujol): 3300, 1640, 1620. ^{1}H-NMR (60 MHz): 1.28 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.78 (2H, br t, J=6 Hz, benzylic H), 3.2—3.6 (2H, m, $-C\underline{H_2}NH-$), 4.14 (2H, q, J=7 Hz, $-O\underline{CH_2}CH_3$), 4.97 (1H, s, olefinic H), 5.95 (2H, s, $-O\underline{CH_2}O-$), 6.60, 7.08 (each 1H, s, Ar-H). HRMS m/z: Calcd for $C_{14}H_{15}NO_4$ (M^+): 261.1001. Found: 261.1017.

Condensation of 18 with Oxalyl Chloride (General Procedure)——A 10% oxalyl chloride ethereal solution (1.1 mol eq) was added dropwise to a stirred solution of **18** (3.0 g) in anhydrous ether (or ether–$CHCl_3$) (50 ml) at 0 °C, and the mixture was stirred for a further 1 h. The precipitated crystals were collected by filtration, dissolved in $CHCl_3$, and purified by SiO_2 chromatography to give a dioxopyrroline (**15**) as red or reddish orange crystals.

Methyl 8,9-Dimethoxy-2,3-dioxo-2,3,5,6-tetrahydropyrrolo[2,1-*a*]isoquinoline-1-carboxylate (**15a**): mp 244—246 °C, red prisms from benzene–MeOH, 60%. IR (Nujol): 1745, 1705, 1680. UV (dioxane): 212 (16100), 228 (10900, sh), 252 (11600), 322 (9600), 390 (11000), 421 (10000, sh). ^{1}H-NMR (60 MHz): 3.09 (2H, br t, J=6 Hz, benzylic H), 3.93, 3.99, 4.06 (each 3H, s, –OC$\underline{H_3}$), 6.84, 8.33 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{16}H_{15}NO_6$: C, 60.56; H, 4.77; N, 4.41. HRMS *m/z*: 317.0896. Found: C, 60.76; H, 4.68; N, 4.30. HRMS *m/z*: 317.0879.

Methyl 8,9-Methylenedioxy-2,3-dioxo-2,3,5,6-tetrahydropyrrolo[2,1-*a*]isoquinoline-1-carboxylate (**15b**): mp 221—224 °C (dec.), red needles from benzene–MeOH, 89%. IR (Nujol): 1745, 1710, 1690. UV (dioxane): 232 (14700), 253 (14600), 312 (8800), 382 (13000), 420 (9500, sh). ^{1}H-NMR (60 MHz, $CDCl_3$–DMSO-d_6): 3.05 (2H, br t, J=6 Hz, benzylic H), 3.79 (2H, br t, J=6 Hz, –C$\underline{H_2}$N<), 3.85 (3H, s, –COOC$\underline{H_3}$), 6.16 (2H, s, –OC$\underline{H_2}$O–), 6.90, 7.84 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{15}H_{11}NO_6$: C, 59.80; H, 3.68; N, 4.65. HRMS *m/z*: 301.0587. Found: C, 59.96; H, 3.75; N, 4.47. HRMS *m/z*: 301.0624.

Ethyl 8,9-Dimethoxy-2,3-dioxo-2,3,5,6-tetrahydropyrrolo[2,1-*a*]isoquinoline-1-carboxylate (**15c**): mp 209—212 °C, red prisms from benzene–CH_2Cl_2, 89%. IR (Nujol): 1760, 1710, 1690. UV (dioxane): 228 (11400), 252 (12000), 321 (9800), 388 (11500), 420 (10100, sh). ^{1}H-NMR (60 MHz): 1.37 (3H, t, J=7 Hz, –OCH$_2$C$\underline{H_3}$), 3.07 (2H, br t, J=6 Hz, benzylic H), 3.83 (2H, br t, J=6 Hz, –C$\underline{H_2}$N<), 3.93, 4.02 (each 3H, s, –OC$\underline{H_3}$), 4.34 (2H, q, J=7 Hz, –OC$\underline{H_2}$CH$_3$), 6.83, 8.20 (each 1H, s, Ar-H). ^{13}C-NMR: 13.8 (q), 55.4 (q), 55.8 (q), 59.7 (t), 98.3 (s), 111.3 (d), 114.7 (d), 114.9 (s), 135.5 (s), 147.0 (s), 154.9 (s), 156.8 (s), 162.3 (s), 165.3 (s). *Anal.* Calcd for $C_{17}H_{17}NO_6$: C, 61.63; H, 5.17; N, 4.23. HRMS *m/z*: 331.1056. Found: C, 61.84; H, 5.10; N, 4.13. HRMS *m/z*: 331.1089.

Ethyl 8,9-Methylenedioxy-2,3-dioxo-2,3,5,6-tetrahydropyrrolo[2,1-*a*]isoquinoline-1-carboxylate (**15d**): mp 219—223 °C, reddish orange needles from AcOEt, 94%. IR (Nujol): 1755, 1715, 1695. UV (dioxane): 232 (12500), 253 (12700), 312 (7100), 381 (12000), 422 (8400, sh). ^{1}H-NMR (60 MHz, $CDCl_3$–DMSO-d_6): 1.37 (3H, t, J=7 Hz, –OCH$_2$C$\underline{H_3}$), 3.02 (2H, br t, J=6 Hz, benzylic H), 3.78 (2H, br t, J=6 Hz, –C$\underline{H_2}$N<), 4.34 (2H, q, J=7 Hz, –OC$\underline{H_2}$CH$_3$), 6.10 (2H, s, –OC$\underline{H_2}$O–), 6.78, 7.78 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{16}H_{13}NO_6$: C, 60.95; H, 4.16; N, 4.44. HRMS *m/z*: 315.0759. Found: C, 61.16; H, 4.13; N, 4.43. HRMS *m/z*: 315.0743.

Diels–Alder Reaction of 15 with Various Butadienes (Formation of *cis-endo* Adduct) (Gneral Procedure)——A solution of **15** (1.0 g) and a butadiene [A: 1,3-bis(trimethylsilyloxy)-1,3-butadiene, B: 1-methoxy-3-trimethylsilyloxy-1,3-butadiene, C: 1-methoxybutadiene, D: 2-trimethylsilyloxybutadiene, E: butadiene] was heated with stirring in a sealed tube. After cooling of the mixture to room temperature, the crystalline precipitate was filtered off. The filtrate was concentrated to dryness *in vacuo* and crystallized from *n*-hexane. The combined crystals were recrystallized from an appropriate solvent to give a pure adduct (**20**—**25**).

(1*R**,5*R**,6*R**)-15,16-Dimethoxy-6-methoxycarbonyl-7,8-dioxo-1,3-bis(trimethylsilyloxy)-erythrin-2-ene (**20a**): The reaction of **15a** (1.0 g) and the diene A (4 g) was carried out in dioxane (10 ml) at 130 °C for 30 min. Yield, 1.12 g (65%). mp 141—143 °C, prisms from AcOEt–*n*-hexane. IR (Nujol): 1760, 1740, 1715, 1640. ^{1}H-NMR (100 MHz): 0.12, 0.22 (each 9H, s, –SiM$\underline{e_3}$), 3.06 (3H, s, –COOC$\underline{H_3}$), 3.77, 3.85 (each 3H, s, $C_{15,16}$–OC$\underline{H_3}$), 5.23 (1H, d, J=6 Hz, C_1-H), 5.39 (1H, br d, J=6 Hz, C_2-H), 6.56, 6.72 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{26}H_{37}NO_8Si_2$ (M^+): 547.2055. Found: 547.2048.

(1*R**,5*R**,6*R**)-6-Methoxycarbonyl-15,16-methylenedioxy-7,8-dioxo-1,3-bis(trimethylsilyloxy)-erythrin-2-ene (**20b**): The reaction of **15b** (1.0 g) and the diene A (3.8 g) was carried out without solvent at 130 °C for 1 h. Yield, 1.26 g (71%). mp 173—176 °C, prisms from AcOEt–*n*-hexane. IR(Nujol): 1765, 1740, 1720, 1645. ^{1}H-NMR (60 MHz): 0.12, 0.23 (each 9H, s, –SiM$\underline{e_3}$), 3.13 (3H, s, –COOC$\underline{H_3}$), 5.18 (1H, d, J=5 Hz, C_1-H), 6.52, 6.62 (each 1H, s; Ar-H). HRMS *m/z*: Calcd for $C_{25}H_{33}NO_8Si_2$ (M^+): 531.1744. Found: 531.1750.

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-15,16-dimethoxy-7,8-dioxo-1,3-bis(trimethylsilyloxy)-erythrin-2-ene (**20c**): The reaction of **15c** (1.0 g) and the diene A (2.1 g) was carried out without solvent at 140 °C for 1 h. Yield, 981 mg (58%). mp 142—145 °C. IR (Nujol): 1765, 1740, 1715, 1645. ^{1}H-NMR (60 MHz): 0.13, 0.22 (each 9H, s, –SiM$\underline{e_3}$), 0.60 (3H, t, J=7 Hz, –OCH$_2$C$\underline{H_3}$), 3.73, 3.82 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 5.18 (1H, d, J=6 Hz, C_1-H), 5.35 (1H, d, J=6 Hz, C_2-H), 6.50, 6.68 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{27}H_{39}NO_8Si_2$ (M^+): 561.2213. Found: 561.2248.

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-15,16-methylenedioxy-7,8-dioxo-1,3-bis(trimethylsilyloxy)-erythrin-2-ene (**20d**): The reaction of **15d** (1.0 g) and the diene A (3.5 g) was carried out without solvent at 130 °C for 2 h. Yield, 1.32 g (76%). mp 202—205 °C, prisms from AcOEt. IR (Nujol): 1760, 1730, 1710, 1645. ^{1}H-NMR (60 MHz): 0.12, 0.23 (each 9H, s, –SiM$\underline{e_3}$), 0.68 (3H, t, J=7 Hz, –OCH$_2$C$\underline{H_3}$), 5.22 (1H, d, J=6 Hz, C_1-H), 5.35 (1H, d, J=6 Hz, C_2-H), 5.92 (2H, br s, –OC$\underline{H_2}$O–), 6.57, 6.70 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{26}H_{35}NO_8Si_2$ (M^+): 545.1898. Found: 545.1891.

(1*R**,5*R**,6*R**)-1,15,16-Trimethoxy-6-methoxycarbonyl-7,8-dioxo-3-trimethylsilyloxyerythrin-2-ene (**21a**): The reaction of **15a** (0.5 g) and the diene B (810 mg) was carried out in dioxane (5 ml) at 130 °C for 1 h. Yield, 692 mg (82%). mp 142—144 °C, prisms from AcOEt. IR (Nujol): 1780, 1745, 1720, 1645. ^{1}H-NMR (60 MHz): 0.23 (9H, s, –SiM$\underline{e_3}$), 3.08 (3H, s, –COOC$\underline{H_3}$), 3.32 (3H, s, C_1-OC$\underline{H_3}$), 3.78, 3.85 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 4.76 (1H, d, J=5 Hz, C_1-H), 5.38 (1H, d, J=5 Hz, C_2-H), 6.57, 6.72 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{24}H_{31}NO_8Si$ (M^+): 489.1817. Found: 489.1787.

(1*R**,5*R**,6*R**)-1-Methoxy-6-methoxycarbonyl-15,16-methylenedioxy-3-trimethylsilyloxyerythrin-2-ene (**21b**): The reaction of **15b** (0.5 g) and the diene B (1.44 g) was carried out in dioxane (5 ml) at 120 °C for 1 h. Yield, 400 mg

(51%). mp 174—177 °C (dec.), prisms from benzene–*n*-hexane. IR (Nujol): 1760, 1720, 1710, 1645. ^{1}H-NMR (60 MHz): 0.23 (9H, s, $-SiMe_3$), 3.17 (3H, s, $-COOCH_3$), 3.32 (3H, s, C_1-OCH_3), 4.73 (1H, d, J=5 Hz, C_1-H), 5.31 (1H, d, J=5 Hz, C_2-H), 5.88 (2H, s, $-OCH_2O-$), 6.53, 6.62 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{23}H_{27}NO_8Si$ (M^+): 473.1506. Found: 473.1516.

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-1,15,16-trimethoxy-7,8-dioxo-3-trimethylsilyloxyerythrin-2-ene (**21c**): The reaction of **15c** (0.5 g) and the diene B (1.3 g) was carried out without solvent at 130 °C for 3 h. Yield, 444 mg (85%). mp 124—126 °C, prisms from benzene–*n*-hexane. IR (Nujol): 1770, 1725, 1710, 1620. ^{1}H-NMR (60 MHz): 0.22 (9H, s, $-SiMe_3$), 0.60 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.28 (3H, s, C_1-OCH_3), 3.75, 3.82 (each 3H, s, $C_{15,16}$-OCH_3), 4.72 (1H, d, J=5 Hz, C_1-H), 5.34 (1H, d, J=5 Hz, C_2-H), 6.52, 6.68 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{25}H_{33}NO_8Si$ (M^+): 503.1975. Found: 503.1995.

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-7,8-dioxo-3-trimethylsilyloxyerythrin-2-ene (**21d**): The reaction of **15d** (1.0 g) and the diene B (4.0 g) was carried out without solvent at 130 °C for 3 h. Yield, 1.4 g (90%). Oil. IR ($CHCl_3$): 1775, 1740, 1720, 1645. ^{1}H-NMR (60 MHz): 0.25 (9H, s, $-SiMe_3$), 0.72 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.35 (3H, s, C_1-OCH_3), 4.76 (1H, d, J=5 Hz, C_1-H), 5.33 (1H, d, J=5 Hz, C_2-H), 5.95 (2H, s, $-OCH_2O-$), 6.60, 6.72 (each 1H, s, Ar-H).

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-1,15,16-trimethoxy-7,8-dioxoerythrin-2-ene (**22c**): The reaction of **15c** (1.0 g) and the diene C (1.25 g, 5 mol eq) was carried out in toluene (5 ml) at 140 °C for 9 h. The product was purified by chromatography over silica gel. Yield, 1.0 g (86%). Colorless oil. IR ($CHCl_3$): 1770, 1740, 1710. ^{1}H-NMR (60 MHz): 0.65 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.62 (3H, s, C_1-OCH_3), 3.85 (6H, s, $CH_3O-\times 2$), 5.6—6.4 (2H, m, olefinic H), 6.60, 6.72 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{22}H_{25}NO_7$ (M^+): 415.1631. Found: 415.1636.

(1*R**,5*R**,6*R**)-6-Ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-7,8-dioxoerythrin-2-ene (**22d**): The reaction of **15d** (0.1 g) and the diene C (500 mg) was carried out in toluene (5 ml) at 140 °C for 5 h. The product was purified as above. Yield, 46 mg (36%). Prisms from MeOH, mp 191—192 °C. IR (Nujol): 1775, 1740, 1710. ^{1}H-NMR (60 MHz): 0.75 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.53 (3H, s, C_1-OCH_3), 3.69 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.5—6.5 (2H, m, olefinic H), 5.95 (2H, s, $-OCH_2O-$), 6.57, 6.72 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{21}H_{21}NO_7$: C, 63.15; H, 5.30; N, 3.51. HRMS *m/z*: 399.1317. Found: C, 63.04; H, 5.27; N, 3.43. HRMS *m/z*: 399.1297.

(5*R**,6*S**)-6-Ethoxycarbonyl-15,16-dimethoxy-3,7,8-trioxoerythrinane (**24c**): The reaction of **15c** (0.5 g) and the diene D (1.8 g, 5 mol eq) was carried out in toluene (5 ml) at 150 °C for 17 h. After evaporation of the solvent, the residue was treated with 5% HCl–THF (1 : 1, 12 ml) at room temperature for 30 min. The reaction mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and evaporated. The gummy residue was chromatographed over SiO_2 with CH_2Cl_2 to afford the adduct (**24c**). Yield, 200 mg (33%). mp 223—225 °C as needles from MeOH. IR (Nujol): 1780, 1745, 1725, 1710. ^{1}H-NMR (60 MHz): 0.67 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.85 (6H, s, $CH_3O-\times 2$), 6.60 (2H, s, Ar-H). *Anal.* Calcd for $C_{21}H_{23}NO_7$: C, 62.84; H, 5.78; N, 3.49. HRMS *m/z*: 401.1472. Found: C, 62.78; H, 5.74; N, 3.28. HRMS *m/z*: 401.1457.

(5*R**,6*S**)-6-Ethoxycarbonyl-15,16-methylenedioxy-3,7,8-trioxoerythrinane (**24d**): The reaction of **15d** (0.5 g) and the diene D (1.9 g) was carried out in toluene (4 ml) at 170 °C for 13 h. Work up as above gave **24d**. Yield, 138 mg (23%). mp 272—275 °C as prisms from MeOH. IR (Nujol): 1780, 1745, 1725, 1710. ^{1}H-NMR (60 MHz, $CDCl_3$–DMSO-d_6): 0.72 (3H, t, J=7 Hz, $-OCH_2CH_3$), 5.95 (2H, s, $-OCH_2O-$), 6.60, 6.73 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{20}H_{19}NO_7$: C, 62.33; H, 4.79; N, 3.64. HRMS *m/z*: 385.1160. Found: C, 62.25; H, 5.03; N, 3.65. HRMS *m/z*: 385.1159.

(5*R**,6*S**)-6-Ethoxycarbonyl-15,16-dimethoxy-7,8-dioxoerythrin-2-ene (**25c**): The reaction of **15c** (2 g) and the diene E (30 ml) was carried out in toluene (40 ml) at 170 °C for 30 h. The product was purified by chromatography in benzene over alumina to give **25c** (148 mg, 6%) as prisms from MeOH, mp 175—176 °C. IR (Nujol): 1770, 1730, 1715, 1615. ^{1}H-NMR (60 MHz): 0.68 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.61 (2H, q, J=7 Hz, $-OCH_2CH_3$), 3.85 (6H, s, $CH_3O-\times 2$), 5.6—6.3 (2H, m, olefinic H), 6.58, 6.70 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{21}H_{23}NO_5$ (M^+): 385.1523. Found: 385.1515.

Methanolysis of the Diels–Alder Adduct (21d)——The adduct (**22d**) [from **15d** (1.0 g)] was dissolved in MeOH and allowed to stand for 1 h to give a crystalline precipitate. Filtration of the precipitate gave 1-ethoxycarbonyl-2-hydroxy-10b(3-methoxyacryloylmethyl)-8,9-methylenedioxy-3-oxo-1,4,5,6-tetrahydropyrrolo[2,1-*a*]isoquinoline (**28**, 658 mg, 50% from **15d**) as prisms, mp 183—186 °C. IR (Nujol): 3100, 1710, 1690. ^{1}H-NMR (100 MHz): 1.48 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.67 (3H, s, $-OCH_3$), 4.52 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.44, 7.49 (each 1H, d, J=12 Hz, olefinic H), 5.92 (2H, d, J=2 Hz, $-OCH_2O-$), 6.56, 7.50 (each 1H, s, Ar-H), 9.50 (1H, brs, $-OH$). *Anal.* Calcd for $C_{21}H_{21}NO_8$: C, 60.72; H, 5.10; N, 3.37. HRMS *m/z*: 415.1266. Found: C, 60.66; H, 5.06; N, 3.17. HRMS *m/z*: 415.1296.

Treatment of the Adduct (20d) with HCl——A mixture of **20d** (60 mg) and 5% HCl–THF (1 : 1, 10 ml) was stirred for 1 h at room temperature. The reaction mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated to give 1-ethoxycarbonyl-10b[(3-formyl-2-oxo)-propyl]-2-hydroxy-8,9-methylenedioxy-3-oxo-4,5,6,10b-tetrahydropyrrolo[2,1-*a*]isoquinoline (**26**) as an amorphous powder. IR ($CHCl_3$): 3520, 1725, 1640. ^{1}H-NMR (60 MHz): 0.90 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.89 (2H, q, J=7 Hz, $-OCH_2CH_3$), 5.94 (2H, s, $-OCH_2O-$), 6.58, 6.68 (each 1H, s, Ar-H), 8.07 (1H, brs, $-CHO$).

Treatment of the Adduct (21d) with HCl——A mixture of **21d** (100 mg) and 5% HCl–THF (1 : 1, 10 ml) was stirred for 1 h at room temperature. The reaction mixture was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4, and concentrated. Recrystallization of the residue from ether–acetone gave the aldehyde (**26**, 45 mg, 61%) as an amorphous powder. It was identical with that obtained from the same reaction of **20d**.

Treatment of the Adduct (20d) with KF——A mixture of **20d** (300 mg) and KF (300 mg) in anhydrous THF (20 ml) was stirred for 18 h at room temperature. The reaction mixture was extracted with CH_2Cl_2. Crystallization of the residue from MeOH gave (1*R**,5*R**,6*R**)-6-ethoxycarbonyl-15,16-methylenedioxy-3,7,8-trioxo-1-trimethylsilyloxyerythrinane (**29**, 451 mg, 52%) as prisms, mp 210—213 °C. IR (Nujol): 1770, 1745, 1720, 1705. ^{1}H-NMR (100 MHz): 0.12 (9H, s, $-SiMe_3$), 0.69 (3H, t, J=7 Hz, $-OCH_2CH_3$), 5.18 (1H, t, J=3 Hz, C_1-H), 5.89 (2H, br s, $-OCH_2O-$), 6.09, 6.58 (each 1H, s, Ar-H).

Treatment of the Adduct (21d) with KF——The adduct (**21d**) obtained from the reaction of **15d** (1.0 g) with 1-methoxy-3-trimethylsilyloxy-1,3-butadiene (4.0 g) was treated with KF (500 mg) in anhydrous THF (20 ml) for 18 h at room temperature. The reaction mixture was extracted with CH_2Cl_2. The extract was washed with water, dried over Na_2SO_4, and concentrated. The residue was chromatographed over SiO_2 with CH_2Cl_2 to give (1*R**,5*R**,6*R**)-6-ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-3,7,8-trioxoerythrinane (**30**, 456 mg, 54%) as needles from MeOH, mp 208—211 °C (dec.). IR (Nujol): 1775, 1750, 1725, 1710. ^{1}H-NMR (60 MHz): 0.68 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.33 (3H, s, C_1-OCH_3), 5.93 (2H, s, $-OCH_2O-$), 6.12, 6.62 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{21}H_{21}NO_8$ (M^+): 415.1264. Found: 415.1256.

Treatment of 29 with 1,8-Diazabicyclo[5.4.0]undec-7-ene DBU——A solution of **29** (50 mg) and DBU (50 mg) in anhydrous benzene (10 ml) was stirred for 30 min at room temperature. The reaction mixture was diluted with CH_2Cl_2, washed with 5% HCl and water, dried over Na_2SO_4, and concentrated. The residue was chromatographed over SiO_2 with CH_2Cl_2 to afford **26** (23 mg, 54%) as prisms from MeOH, mp 183—186 °C. It was identical with the product obtained from the reaction of **20d** with HCl.

Treatment of 30 with DBU——A solution of **30** (50 mg) and DBU (50 mg) in anhydrous benzene (10 ml) was stirred for 30 min at room temperature. The reaction mixture was worked up as described above to give **28** (31 mg, 62%), mp 184—186 °C, prisms from MeOH. It was identical with **28** obtained from the reaction of **21d** with MeOH.

Reduction of the Diels–Alder Adduct (20 or 21) with $LiBH_4$ (General Procedure)——$LiBH_4$ (0.25—1 mol eq) was added to a solution of **20** or **21** in anhydrous THF with stirring at −60——−70 °C. The reaction mixture was stirred for 20—30 min at the same temperature, then extracted with ether. The extract was washed with brine, dried over Na_2SO_4, and concentrated. The residue in 5% HCl–THF (1 : 1, 10 ml) was heated under reflux for 1 h. The reaction mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4, then concentrated. The residue was recrystallized from MeOH to give the pure enone (**33**).

(5*R**,6*S**,7*S**)-7-Hydroxy-15,16-dimethoxy-6-methoxycarbonyl-3,8-dioxoerythrin-1-ene (**33a**): 1) The adduct (**20a**, 500 mg) was reduced with $LiBH_4$ (5 mg) and then dehydrated with HCl as described above to yield **33a** (286 mg, 81%). mp 216—218 °C, as prisms. IR (Nujol): 3530, 3400, 1730, 1690, 1670. UV: 230 (12700, sh), 293 (2400). ^{1}H-NMR (60 MHz, $CDCl_3$–DMSO-d_6): 3.28 (3H, s, $-COOCH_3$), 3.67, 3.82 (each 3H, s, $C_{15,16}$-OCH_3), 6.42, 7.53 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.47, 6.53 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{20}H_{21}NO_7$ (M^+): 387.1316. Found: 387.1311.

2) The same sequence of reactions as used for **21a** (100 mg) gave **33a** (64 mg, 81%). This was identical with **33a** obtained from **20a**.

(5*R**,6*S**,7*S**)-7-Hydroxy-6-methoxycarbonyl-15,16-methylenedioxy-3,8-dioxoerythrin-1-ene (**33b**): The adduct (**20b**, 500 mg) was reduced with $LiBH_4$ (20 mg) and then dehydrated to give the enone (**33b**, 288 mg, 82%), mp 229—231 °C, prisms. IR (Nujol): 3300 (br), 1740, 1730, 1700 (sh), 1690 (sh), 1675. UV: 230 (9400, sh), 298 (3300). ^{1}H-NMR (100 MHz, $CDCl_3$–DMSO-d_6–D_2O): 3.28 (3H, s, $-COOCH_3$), 5.85 (2H, s, $-OCH_2O-$), 6.30, 7.46 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.33, 6.53 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{19}H_{17}NO_7$ (M^+): C, 61.45; H, 4.61; N, 3.77. HRMS *m/z*: 371.1004. Found: C, 61.17; H, 4.58; N, 3.66. HRMS *m/z*: 371.0986.

(5*R**,6*S**,7*S**)-6-Ethoxycarbonyl-7-hydroxy-15,16-methylenedioxy-3,8-dioxoerythrin-1-ene (**33d**): The adduct (**20d**, 500 mg) was reduced with $LiBH_4$ (20 mg) and then dehydrated to give the enone (**33d**, 291 mg, 82%), mp 234—237 °C, prisms from MeOH. IR (Nujol): 3300 (br), 1735, 1695, 1670. ^{1}H-NMR (60 MHz): 0.88 (3H, t, J=7 Hz, $-OCH_2CH_3$), 3.85 (2H, q, J=7 Hz, $-OCH_2CH_3$), 4.73 (1H, s, C_7-H), 5.88 (2H, s, $-OCH_2O-$), 6.34, 7.55 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.45, 6.55 (each 1H, s, Ar-H).

Acetylation of 33a——The enone (**33a**, 47 mg) was acetylated with pyridine (2 ml) and Ac_2O (1 ml) without stirring for 18 h at room temperature to give (5*R**,6*S**,7*S**)-7-acetoxy-15,16-dimethoxy-6-methoxycarbonyl-3,8-dioxoerythrin-1-ene (**34a**, 44 mg, 86%), mp 197—200 °C, needles from acetone-*n*-hexane. IR (Nujol): 1765, 1710, 1690 (sh). ^{1}H-NMR (60 MHz): 2.20 (3H, s, $-OCOCH_3$), 3.32 (3H, s, $-COOCH_3$), 3.67, 3.83 (each 3H, s, $C_{15,16}$-OCH_3), 5.92 (1H, s, C_7-H), 6.45, 7.37 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.47, 6.60 (each 1H, s, Ar-H).

Reduction of 30 with $NaBH_4$——$NaBH_4$ (190 mg) was added to a solution of **30** (500 mg) in anhydrous EtOH (30 ml) at 0 °C, and the mixture was stirred for 30 min. After decomposition of excess hydride with water, the reaction mixture was extracted with CH_2Cl_2. The extract was dried over Na_2SO_4, and concentrated. The residue was purified by recrystallization from AcOEt to give (1*R**,3*S**,5*R**,6*R**,7*S**)-6-ethoxycarbonyl-3,7-dihydroxy-1-methoxy-15,16-

methylenedioxy-8-oxoerythrinane (**31**, 379 mg, 75%), mp 268—270 °C, as prisms. IR (Nujol): 3460, 3280, 1730, 1690. HRMS *m/z*: Calcd for $C_{21}H_{25}NO_8$ (M^+): 419.1581. Found: 419.1601.

The resulting diol (**31**, 100 mg) was acetylated with pyridine (2 ml) and Ac_2O (1 ml) for 18 h at room temperature. Chromatography of the product in CH_2Cl_2 over SiO_2 afforded (1*R**,3*S**,5*R**,6*R**,7*S**)-3,7-diacetoxy-6-ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-8-oxoerythrinane (**32**, 103 mg, 86%), mp 242—244 °C, as prisms from MeOH. IR (Nujol): 1760, 1740, 1710. ^{1}H-NMR (100 MHz): 0.91 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 2.01, 2.06 (each 3H, s, $-COC\underline{H_3}$), 3.42 (3H, s, C_1-$OC\underline{H_3}$), 4.58 (1H, dd, J=6, 11 Hz, C_1-H), 5.18 (1H, br t, J=4 Hz, C_3-H), 5.91 (2H, s, $-OC\underline{H_2}O-$), 6.13 (1H, s, C_7-H), 6.51, 6.89 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{25}H_{29}NO_{10}$: C, 59.63; H, 5.81; N, 2.78. HRMS *m/z*: 503.1791. Found: C, 59.64; H, 5.86; N, 2.74. HRMS *m/z*: 503.1819.

Diels–Alder Reaction of 15a with 1,3-Bis(trimethylsilyloxy)-1,3-butadiene at Higher Temperature (Formation of the *cis-exo* Adduct)——A solution of **15a** (170 mg) and 1,3-bis(trimethylsilyloxy)-1,3-butadiene (320 mg) in anhydrous CH_2Cl_2 (3 ml) was heated at 180 °C for 15 min in a sealed tube with stirring. Recrystallization of the product from CH_2Cl_2–*n*-hexane gave (1*S**,5*R**,6*R**)-15,16-dimethoxy-6-methoxycarbonyl-7,8-dioxo-1,3-bis(trimethylsilyloxy)-erythrin-2-ene (**35**, 206 mg, 70%), as prisms, mp 136—139 °C. IR (Nujol): 1760, 1740, 1710, 1640. ^{1}H-NMR (100 MHz): 0.16, 0.24 (each 9H, s, $-Si\underline{Me_3}$), 3.07 (3H, s, $-COOC\underline{H_3}$), 3.82, 3.86 (each 3H, s, $C_{15,16}$-$OC\underline{H_3}$), 5.11 (2H, m, $C_{1,2}$-H), 6.57, 7.14 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{26}H_{37}NO_8Si_2$ (M^+): 547.2055. Found: 547.2063.

Diels–Alder Reaction of 15d with 1-Methoxy-3-trimethylsilyloxy-1,3-butadiene at Higher Temperature (Formation of the *cis-exo* Adduct)——A solution of **15d** (100 mg) and 1-methoxy-3-trimethylsilyloxy-1,3-butadiene (310 mg) in anhydrous $CHCl_3$ (5 ml) was heated at 180 °C for 1 h in a sealed tube with stirring. Recrystallization of the product from benzene–*n*-hexane gave (1*S**,5*R**,6*R**)-6-ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-7,8-dioxo-3-trimethylsilyloxyerythrin-2-ene (**39**, 123 mg, 76%) as prisms, mp 176—179 °C. IR (Nujol): 1770, 1745, 1720, 1645. ^{1}H-NMR (100 MHz): 0.61 (9H, s, $-Si\underline{Me_3}$), 0.72 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 3.50 (3H, s, C_1-$OC\underline{H_3}$), 3.68 (2H, q, J=7 Hz, $-OC\underline{H_2}CH_3$), 4.63 (1H, d, J=7 Hz, C_1-H), 5.24 (1H, dt, J=2, 2, 7 Hz, C_2-H), 5.90 (2H, dd, J=1, 2 Hz, $-OC\underline{H_2}O-$), 6.54, 7.35 (each 1H, s, Ar-H).

Thermal Isomerization of the *cis-endo* Adduct (21a) to the *cis-exo* Adduct (36)——1) Without Diene: A solution of **21a** (100 mg) in anhydrous $CHCl_3$ (5 ml) was heated at 180 °C for 1 h with stirring in a sealed tube. The reaction mixture was concentrated to dryness to give a residue, which was recrystallized from *n*-hexane to give **15a** (27 mg, 42%), mp 243—246 °C. Evaporation of the mother liquor from **15a** gave (1*S**,5*R**,6*R**)-1,15,16-trimethoxy-6-methoxycarbonyl-7,8-dioxo-3-trimethylsilyloxyerythrin-2-ene (**36**) as a colorless gum (58 mg, 58%). ^{1}H-NMR (60 MHz): 3.05 (3H, s, $-COOC\underline{H_3}$), 3.49 (3H, s, C_1-$OC\underline{H_3}$), 3.78, 3.82 (each 3H, s, $C_{15,16}$-$OC\underline{H_3}$), 4.59 (1H, d, J=7 Hz, C_1-H), 5.23 (1H, br d, J=7 Hz, C_2-H), 6.53, 7.30 (each 1H, s, Ar-H).

2) In the Presence of 1-Methoxy-3-trimethylsilyloxy-1,3-butadiene: A solution of **21a** (100 mg) and 1-methoxy-3-trimethylsilyloxy-1,3-butadiene (175 mg, 5 mol eq), in anhydrous $CHCl_3$ (5 ml) was heated at 180 °C for 1 h with stirring in a sealed tube. The reaction mixture was concentrated to dryness to give a gummy residue (98 mg), which was proved to be identical with **36** obtained by the procedure described above.

Treatment of the *exo*-Adduct (35) with KF——A mixture of **35** (200 mg) and KF (210 mg) in THF (10 ml) was stirred for 41 h at room temperature. The reaction mixture was extracted with CH_2Cl_2. The extract was washed with water, dried over Na_2SO_4, and concentrated. Recrystallization of the residue from acetone–*n*-hexane gave (1*S**,5*R**,6*R**)-15,16-dimethoxy-6-methoxycarbonyl-3,7,8-trioxo-1-trimethylsilyloxyerythrinane (**37**, 165 mg, 90%) as needles, mp 220—223 °C. IR (Nujol): 1775, 1750, 1720. ^{1}H-NMR (60 MHz): 0.30 (9H, s, $-Si\underline{Me_3}$), 3.02 (3H, s, $-COOC\underline{H_3}$), 3.75, 3.77 (each 3H, s, $C_{15,16}$-$OC\underline{H_3}$), 4.99 (1H, t, J=3 Hz, C_1-H), 6.44, 6.83 (each 1H, s, Ar-H).

***n*-Bu_4NBH_4 Reduction of 37 to 38**——A solution of **37** (80 mg) and *n*-Bu_4NBH_4 (130 mg) in anhydrous CH_2Cl_2 (10 ml) was stirred for 1 h at 0 °C. The reaction mixture was extracted with ether, washed with brine, dried over Na_2SO_4, and concentrated. Recrystallization of the product from *n*-hexane–acetone gave (1*S**,5*R**,6*R**,7*S**)-7-hydroxy-15,16-dimethoxy-6-methoxycarbonyl-3,8-dioxo-1-trimethylsilyloxyerythrinane (**38**, 40 mg, 50%) as needles, mp 113—115 °C. IR (Nujol): 3350, 1755, 1715, 1700. ^{1}H-NMR (60 MHz): 0.32 (9H, s, $-Si\underline{Me_3}$), 3.10 (3H, s, $-COOC\underline{H_3}$), 3.75 (6H, s, $C\underline{H_3}O-\times 2$), 4.37 (1H, s, C_5-H), 4.97 (1H, m, C_1-H), 6.38, 7.25 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{23}H_{31}NO_8Si$ (M^+): 477.1816. Found: 477.1816.

Transofmation of 38 into 33a with HCl——A solution of **38** (37 mg) in 5% HCl–THF (1 : 1, 14 ml) was heated under reflux for 30 min. The reaction mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and concentrated. The residue was chromatographed over SiO_2 with CH_2Cl_2 to afford **33a** (26 mg, 87%), mp 213—216 °C, which was identical with that obtained from **20a**.

Treatment of 39 with KF——A mixture of **39** (93 mg), and KF (50 mg) in anhydrous THF (10 ml) was stirred for 1.5 d at room temperature. The reaction mixture was extracted with $CHCl_3$. The extract was washed with water, dried over Na_2SO_4, and concentrated. The residue was recrystallized from AcOEt–ether to afford (1*S**,5*R**,6*R**)-6-ethoxycarbonyl-1-methoxy-15,16-methylenedioxy-3,7,8-trioxoerythrinane (**40**, 69 mg, 91%) as prisms, mp 202—203 °C. IR (Nujol): 1765, 1740, 1725, 1705. ^{1}H-NMR (100 MHz): 0.74 (3H, t, J=7 Hz, $-OCH_2\underline{CH_3}$), 3.70 (3H, s, C_1-$OC\underline{H_3}$), 5.93 (2H, s, $-OC\underline{H_2}O-$), 6.56, 7.22 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{21}H_{21}NO_8$ (M^+): 415.1268. Found: 415.1278.

Reduction of 40 with $NaBH_4$——$NaBH_4$ (27 mg) was added to a solution of **40** (60 mg) in absolute EtOH (10 ml)

at 0 °C. The mixture was stirred for 25 min at the same temperature. After addition of ice-water, the mixture was extracted with CH_2Cl_2. The extract was dried over Na_2SO_4, and concentrated. The residue was acetylated with Ac_2O (1 ml) and pyridine (2 ml) to give the diacetate (**41**) as a colorless gum. This was proved to be a mixture of stereoisomers by the ^{1}H-NMR spectrum, although attempts at separation by either preparative TLC or MPLC failed. Neither product was identical with **32** on TLC.

Demethoxycarbonylation of 33a——1) A mixture of **33a** (45 mg), NaCl (35 mg), and DMSO (5 ml) was heated at 140 °C for 10 h in a sealed tube with stirring. The reaction mixture was extracted with $CHCl_3$, washed with water, dried over Na_2SO_4, and concentrated. The residue was chromatographed over SiO_2 in CH_2Cl_2 to give 15,16-dimethoxy-3,8-dioxoerythrina-1,6-diene (**43a**, 7 mg, 19%), mp 193—195 °C, as pale yellow needles from acetone–*n*-hexane. IR (Nujol): 1690. UV: 230 (11300, sh), 276 (14200). ^{1}H-NMR (100 MHz): 2.78, 3.27 (each 1H, d, J=15 Hz, C_4-H), 3.72, 3.84 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 6.37 (1H, s, C_7-H), 6.41, 7.75 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.66, 6.85 (each 1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{18}H_{17}NO_4$ (M^+): 311.1158. Found: 311.1165.

2) Twelve sealed tubes each containing **33a** (50 mg), anhydrous $MgCl_2$ (50 mg), and DMSO (2 ml) were heated at 130 °C for 2 h with stirring. All of the reaction mixtures were combined and extracted with $CHCl_3$. The extract was washed with water, dried over Na_2SO_4, and evaporated. The residue was chromatographed over SiO_2 in CH_2Cl_2 to afford the dienone (**43a**, 195 mg, 40%), which was identical with the specimen obtained above.

Demethoxycarbonylation of the Acetate (34)——A mixture of **34a** (206 mg), anhydrous $MgCl_2$ (230 mg), and DMSO (6 ml) was heated at 140 °C for 2 h in a sealed tube with stirring. The mixture was worked up as described above to afford **43a** (25 mg, 17%).

Mesylation of 33——A mixture of **33** (200—300 mg) and methane sulfonylchloride (600—950 mg) in pyridine (5 ml) was stirred for 3 h at room temperature. The reaction mixture was basified with 5% K_2CO_3 and extracted with $CHCl_3$. The extract was washed with water, dried over Na_2SO_4, and concentrated. Chromatography of the product over SiO_2 gave the mesylate (**42**).

(5*R**,6*S**,7*S**)-7-Methanesulfonyloxy-15,16-dimethoxy-6-methoxycarbonyl-3,8-dioxoerythrin-1-ene (**42a**): Chromatography with AcOEt–*n*-hexane (1 : 1) as an eluent gave **42a** (209 mg, 86%) from **33a** (200 mg), as prisms from AcOEt, mp 243—244 °C. IR (Nujol): 1750, 1730, 1715, 1690, 1520. ^{1}H-NMR (100 MHz): 2.81, 3.23 (each 1H, d, J=16 Hz, C_4-H), 3.32, 3.38 (each 3H, s, –COOC$\underline{H_3}$, –$SO_2C\underline{H_3}$), 3.67, 3.83 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 5.50 (1H, s, C_7-H), 6.44, 6.56 (each 1H, s, Ar-H), 6.51, 7.45 (each 1H, d, J=11 Hz, $C_{2,1}$-H). HRMS *m/z*: Calcd for $C_{21}H_{23}NO_9S$ (M^+): 465.1091. Found: 465.1085.

(5*R**,6*S**,7*S**)-7-Methanesulfonyloxy-6-methoxycarbonyl-15,16-methylenedioxy-3,8-dioxoerythrin-1-ene (**44b**): MPLC with AcOEt–*n*-hexane (3 : 1) as an eluent gave **42b** (354 mg, 95%) from **33b** (308 mg), as prisms from MeOH, mp 217—219 °C. IR (Nujol): 3475 (br), 3375 (br), 1740, 1730, 1690. ^{1}H-NMR (100 MHz): 2.80, 3.22 (each 1H, d, J=16 Hz, C_4-H), 3.37, 3.39 (each 3H, s, –COOC$\underline{H_3}$, –$SO_2C\underline{H_3}$), 5.48 (1H, s, C_7-H), 5.90 (2H, br s, –OC$\underline{H_2}$O–), 6.44, 6.56 (each 1H, s, Ar-H), 6.50, 7.44 (each 1H, d, J=11 Hz, $C_{2,1}$-H). HRMS *m/z*: Calcd for $C_{20}H_{19}NO_9S$ (M^+): 449.0779. Found: 449.0768.

Demethoxycarbonylation of the Mesylate (42a)——A mixture of **42a** (52 mg) and anhydrous $MgCl_2$ (65 mg) in DMSO (5 ml) was heated at 140 °C for 2.5 h in a sealed tube with stirring. The reaction mixture was worked up as described above to give the dienone (**43a**, 30 mg, 89%).

Demethoxycarbonylation of the Mesylate (42b)——The reaction was carried out with **42b** (52 mg) and anhydrous $MgCl_2$ (55 mg) as described above to yield 15,16-methylenedioxy-3,8-dioxoerythrina-1,6-diene (**43b**, 28 mg, 82%), mp 193—196 °C, as pale yellow prisms from acetone–*n*-hexane. IR (Nujol): 1670. UV: 277 (14600). ^{1}H-NMR (100 MHz): 2.76, 3.24 (each 1H, d, J=15 Hz, C_4-H), 5.88 (2H, s, –OC$\underline{H_2}$O–), 6.35, 7.72 (each 1H, d, J=10 Hz, $C_{2,1}$-H), 6.36 (1H, s, C_7-H), 6.65, 6.77 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. HRMS *m/z*: 295.0844. Found: C, 69.28; H, 4.30; N, 4.60. HRMS *m/z*: 295.0874.

$NaBH_4$ Reduction of 43a——$NaBH_4$ (14 mg) was added to a solution of **43a** (59 mg) in anhydrous EtOH (10 ml) at 0 °C. The mixture was stirred for 50 min at the same temperature. After decomposition of excess hydride by ice, the reaction mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4, and concentrated. The products was separated by preparative TLC developing with $CHCl_3$–MeOH to give the β-alcohol (**45a**, 25 mg, 42%, upper zone), the tetrahydro derivative (**46a**, 21 mg, 35%, middle zone), and the α-alcohol (**44a**, 9 mg, 15%, lower zone).

(3*R**,5*R**)-3-Hydroxy-15,16-dimethoxy-8-oxoerythrina-1,6-diene (**44a**): Needles from acetone, mp 232—235 °C. IR ($CHCl_3$): 3300 (br), 1670. ^{1}H-NMR (100 MHz): 1.69 (1H, dd, J=10.5, 11 Hz, C_4-H), 3.75, 3.85 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 4.1—4.4 (1H, m, C_3-H), 6.00 (1H, s, C_7-H), 6.30 (1H, br d, J=10 Hz, C_1-H), 6.71, 6.79 (each 1H, s, Ar-H), 6.91 (1H, dd, J=3, 10 Hz, C_2-H). HRMS *m/z*: Calcd for $C_{18}H_{19}NO_4$ (M^+): 313.1313. Found: 313.1268.

(3*S**,5*R**)-3-Hydroxy-15,16-dimethoxy-8-oxoerythrina-1,6-diene (**45a**): Prisms from ether–acetone, mp 181—183 °C. IR (Nujol): 3300, 1660. ^{1}H-NMR (100 MHz): 2.10 (1H, dd, J=6, 14 Hz, C_1-H), 2.68 (1H, br d, J=14 Hz, C_1-H), 3.79, 3.86 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 4.50 (1H, m, C_3-H), 5.98 (1H, s, C_7-H), 6.31 (1H, dd, J=5, 10 Hz, C_2-H), 6.76, 6.99 (each 1H, s, Ar-H), 6.92 (1H, d, J=10 Hz, C_1-H). HRMS *m/z*: Calcd for $C_{18}H_{19}NO_4$ (M^+): 313.1313. Found: 313.1310.

3-Hydroxy-15,16-dimethoxy-8-oxoerythrin-6-ene (**46a**): Prisms from acetone, mp 193—194 °C. IR (Nujol): 3400, 1660. ^{1}H-NMR (100 MHz): 3.84, 3.86 (each 3H, s, $C_{15,16}$-OC$\underline{H_3}$), 5.91 (1H, d, J=2 Hz, C_7-H), 6.65, 7.50 (each

1H, s, Ar-H). HRMS *m/z*: Calcd for $C_{18}H_{21}NO_4$ (M^+): 315.1469. Found: 315.1458.

$NaBH_4$ Reduction of 43b——Compound **43b** (47 mg) was similarly reduced by $NaBH_4$ and worked up as above to give the β-alcohol (**45b**, 19 mg, 40%), the tetrahydro derivative (**46b**, 7 mg), and the α-alcohol (**44b**, 11 mg, 23%).

(3*R**,5*R**)-3-Hydroxy-15,16-methylenedioxy-8-oxoerythrina-1,6-diene (**44b**): Prisms from MeOH, mp 122—125 °C. IR (Nujol): 3350, 3120, 1640. ^{1}H-NMR (100 MHz, $CDCl_3$–DMSO-d_6): 5.90 (2H, br s, $-OCH_2O-$), 5.96 (1H, s, C_7-H), 6.29 (1H, br d, J=10 Hz, C_1-H), 6.71, 6.74 (each 1H, s, Ar-H), 6.80 (1H, dd, J=2, 10 Hz, C_2-H). HRMS *m/z*: Calcd for $C_{17}H_{15}NO_4$ (M^+): 297.1001. Found: 297.1003.

(3*S**,5*R**)-3-Hydroxy-15,16-methylenedioxy-8-oxoerythrina-1,6-diene (**45b**): Prisms from acetone, mp 205—207 °C. IR (Nujol): 3350, 1650. ^{1}H-NMR (100 MHz): 2.07 (1H, dd, J=5, 14 Hz, C_4-H), 2.69 (1H, br d, J=14 Hz, C_4-H), 4.48 (1H, br s, C_3-H), 5.91 (2H, s, $-OCH_2O-$), 5.98 (1H, s, C_7-H), 6.26 (1H, dd, J=5, 10 Hz, C_2-H), 6.73, 6.87 (each 1H, s, Ar-H), 6.88 (1H, d, J=10 Hz, C_1-H).

3-Hydroxy-15,16-methylenedioxy-8-oxoerythrin-6-ene (**46b**): Prisms from acetone, mp 245—247 °C. IR (Nujol): 3350, 1650, 1620. ^{1}H-NMR (100 MHz): 5.91 (2H, dd, J=1, 2 Hz, $-OCH_2O-$), 5.96 (1H, d, J=2 Hz, C_7-H), 6.66, 7.21 (each 1H, s, Ar-H). *Anal.* Calcd for $C_{17}H_{17}NO_4$: C, 68.22; H, 5.72; N, 4.68. HRMS *m/z*: 299.1158. Found: C, 68.29; H, 5.79; N, 4.68. HRMS *m/z*: 299.1198.

***n*-Bu_4NBH_4 Reduction of 43a**——*n*-Bu_4NBH_4 (220 mg) was added to a solution of **43a** (131 mg) in MeOH (10 ml) at 0 °C. The mixture was stirred for 5 min at the same temperature and worked up as described above to afford **45a** (69 mg, 52%), **46a** (17 mg, 13%), and **44a** (13 mg, 10%).

$Zn(BH_4)_2$ Reduction of 43a——An ethereal solution of $Zn(BH_4)_2$ (large excess) was added to a solution of **43a** (50 mg) in anhydrous THF (3 ml). The mixture was stirred for 2 d at room temperature. After decomposition of excess hydride with water, the mixture was extracted with ether. The ethereal layer was washed with water, dried over Na_2SO_4, and concentrated. Separation of the product by preparative TLC as described above gave the starting material (**43a**, 4 mg), **45a** (15 mg, net yield 30%), and **44a** (26 mg, net yield 52%).

$NaBH_4$–$CeCl_3$ Reduction of 43a——$CeCl_3 \cdot 7H_2O$ (98 mg) and $NaBH_4$ (10 mg) was added to a solution of **43a** (39 mg) in MeOH (10 ml) at 0 °C. The mixture was stirred for 5 min at room temperature. After decomposition of excess hydride with ice, the mixture was extracted with CH_2Cl_2. The extract was worked up as described above to afford **45a** (9 mg, 30%), and **44a** (18 mg, 60%).

Meerwein–Ponndorf Reduction of 43a——A mixture of **43a** (20 mg) and Al (iso-PrO)$_3$ (260 mg) in anhydrous iso-PrOH (20 ml) was heated under reflux for 24 h with stirring. The reaction mixture was concentrated and extracted with $CHCl_3$. The extract was washed with 5% HCl and water, dried over Na_2SO_4, and concentrated. The residue was worked up as described above to give **45a** (5 mg, 25%) and **44a** (14 mg, 70%).

Meerwein–Ponndorf Reduction of 43b——The reaction was carried out with **43b** (50 mg) and Al (iso-PrO)$_3$ (345 mg) in iso-PrOH (30 ml), and work-up as above gave **45b** (10 mg, 20%) and **44b** (29 mg, 58%).

Methylation of 44 and 45 with CH_3I Catalyzed by Tetraethylammonium Bromide (General Procedure)——A mixture of **44** or **45** (40—100 mg), KOH, Et_4NBr, and CH_3I in THF was stirred for 18 h at room temperature. The mixture was extracted with $CHCl_3$. The extract was washed with water, dried over Na_2SO_4, and concentrated. The residue was chromatographed over SiO_2 with CH_2Cl_2 to give the corresponding methyl ether.

(±)-8-Oxoerysotrine [(±)-Erysotramidine] (**2a**): The reaction was carried out with **44a** (100 mg), KOH (210 mg), Et_4NBr (210 mg), and CH_3I (454 mg). Yield, 88 mg (84%). A colorless oil. IR ($CHCl_3$): 1670, 1610. ^{1}H-NMR (100 MHz): 3.34 (3H, s, C_3-OCH_3), 3.76, 3.86 (each 3H, s, $C_{15,16}$-OCH_3), 6.02 (1H, s, C_7-H), 6.32 (1H, br d, J=10 Hz, C_1-H), 6.72, 6.80 (each 1H, s, Ar-H), 6.90 (1H, dd, J=3, 10 Hz, C_2-H). HRMS *m/z*: Calcd for $C_{19}H_{21}NO_4$ (M^+): 327.1468. Found: 327.1466.

This was identical with natural erysotramidine in terms of the IR ($CHCl_3$) and ^{1}H-NMR spectra.

(±)-3-Epi-8-oxoerysotrine (**47a**): The reaction was carried out with **45a** (68 mg), KOH (143 mg), Et_4NBr (210 mg), and CH_3I (310 mg). Yield, 63 mg (89%). mp 168—170 °C as prisms from acetone. IR (Nujol): 1680, 1610. ^{1}H-NMR (100 MHz): 1.96 (1H, dd, J=6, 14 Hz, C_4-H), 2.75 (1H, br d, J=14 Hz, C_4-H), 3.18 (3H, s, C_3-OCH_3), 3.78, 3.84 (each 3H, s, $C_{15,16}$-OCH_3), 5.94 (1H, s, C_7-H), 6.35 (1H, dd, J=5, 10 Hz, C_2-H), 6.61, 7.20 (each 1H, s, Ar-H), 6.92 (1H, br d, J=10 Hz, C_1-H). HRMS *m/z*: Calcd for $C_{19}H_{21}NO_4$ (M^+): 327.1468. Found: 327.1448.

(±)-8-Oxoerythraline (**2b**): The reaction was carried out with **44b** (67 mg), KOH (150 mg), Et_4NBr (210 mg), and CH_3I (398 mg). Yield, 60 mg (80%). A colorless oil. IR ($CHCl_3$): 1670. ^{1}H-NMR (100 MHz): 3.33 (3H, s, C_3-OCH_3), 5.89 (2H, dd, J=1.5, 4 Hz, $-OCH_2O-$), 5.99 (1H, s, C_7-H), 6.27 (1H, br d, J=10 Hz, C_1-H), 6.68, 6.71 (each 1H, s, Ar-H), 6.83 (1H, dd, J=2, 10 Hz, C_2-H). HRMS *m/z*: Calcd for $C_{18}H_{17}NO_4$ (M^+): 311.1158. Found: 311.1193.

(±)-3-Epi-8-oxoerythraline (**47b**): The reaction was carried out with **45b** (43 mg), KOH (50 mg), Et_4NBr (24 mg), and CH_3I (200 mg). Yield, 26 mg (60%). mp 161—165 °C as prisms from ether–acetone. IR (Nujol): 1675, 1610. ^{1}H-NMR (100 MHz): 1.90 (1H, dd, J=5, 14 Hz, C_4-H), 2.77 (1H, br d, J=14 Hz, C_4-H), 3.12 (3H, s, C_3-OCH_3), 4.06 (1H, t, J=5 Hz, C_3-H), 5.88 (2H, dd, J=1.5, 4 Hz, $-OCH_2O-$), 5.96 (1H, s, C_7-H), 6.30 (1H, br dd, J=5, 10 Hz, C_2-H), 6.62, 6.93 (each 1H, s, Ar-H), 6.89 (1H, d, J=10 Hz, C_1-H). HRMS *m/z*: Calcd for $C_{18}H_{17}NO_4$ (M^+): 311.1158. Found: 311.1183.

Reduction of the 8-Oxo Derivatives with AlH_3 (General Procedure)——A ethereal solution of AlH_3 (prepared from $LiAlH_4$: $AlCl_3$ = 3 : 1, excess) was added to a solution of the 8-oxo derivative in anhydrous THF, and the

mixture was stirred at room temperature. The reaction mixture was extracted with $CHCl_3$. The extract was washed with 5% NH_4OH, dried over Na_2SO_4, and concentrated to give the corresponding base.

(±)-Erysotrine (**1a**): The reaction was carried out with **2a** (50 mg) in THF (5 ml) under stirring for 45 min. Yield, 38 mg (80%). A colorless oil. IR ($CHCl_3$): 1610, 1510. ^{1}H-NMR (100 MHz): 1.85 (1H, dd, J=11, 12 Hz, C_4-H), 2.54 (1H, dd, J=5, 12 Hz, C_4-H), 3.33 (3H, s, C_3-OCH_3), 3.76, 3.86 (each 3H, s, $C_{15,16}$-OCH_3), 3.9—4.2 (1H, m, C_3-H), 5.73 (1H, br s, C_7-H), 6.00 (1H, br d, J=10 Hz, C_1-H), 6.58 (1H, dd, J=2, 10 Hz, C_2-H), 6.62, 6.83 (each 1H, s, Ar-H). The picrate: yellowish brown prisms from EtOH, mp 194.5—196 °C [lit. mp 197—198 °C].[6b] IR (Nujol): 1630, 1610, 1570, 1560, 1510.

(±)-3-Epierysotrine (**48a**): The reaction was carried out with **47a** (58 mg) in THF (10 ml) under stirring for 30 min at room temperature. Yield: 43 mg, 78%. A colorless oil. ^{1}H-NMR (100 MHz): 3.21 (3H, s, C_3-OCH_3), 3.75, 3.78 (each 3H, s, $C_{15,16}$-OCH_3), 3.99 (1H, br t, J=5, 10 Hz, C_3-H), 5.80 (1H, br s, C_7-H), 6.15 (1H, dd, J=5, 10 Hz, C_2-H), 6.50, 7.14 (each 1H, s, Ar-H), 6.70 (1H, d, J=10 Hz, C_1-H). The picrate: brown prisms from EtOH, mp 174—175 °C [lit. mp 174—175 °C].[6b] IR (Nujol): 1625, 1605, 1535, 1510.

(±)-Erythraline (**1b**): The reaction was carried out with **2b** (45 mg) in THF (10 ml) under stirring for 2 h at room temperature. Yield, 35 mg (81%). A colorless oil. IR ($CHCl_3$): 1500, 1480. ^{1}H-NMR (100 MHz): 1.83 (1H, t, J=11 Hz, C_4-H), 2.94 (1H, dd, J=6, 11 Hz, C_4-H), 3.32 (3H, s, C_3-OCH_3), 3.7—4.1 (1H, m, C_3-H), 5.71 (1H, br s, C_7-H), 5.87 (2H, dd, J=1.5, 3 Hz, -OCH_2O-), 5.95 (1H, br d, J=10 Hz, C_1-H), 6.53 (1H, dd, J=2, 10 Hz, C_2-H), 6.61, 6.76 (each 1H, s, Ar-H). The picrate: brown prisms from EtOH, mp 196—199 °C (dec.). IR (Nujol): 1625, 1605, 1560.

(±)-3-Epierythraline (**48b**): The reaction was carried out with **47b** (26 mg) in THF (5 ml) under stirring for 30 min. Yield, 20 mg (81%). A colorless oil. IR (Nujol): 1505, 1480. ^{1}H-NMR (100 MHz): 2.05 (1H, dd, J=6, 14 Hz, C_4-H), 2.63 (1H, br d, J=14 Hz, C_4-H), 3.16 (3H, s, C_3-OCH_3), 3.92 (1H, br t, J=6 Hz, C_3-H), 5.8—5.9 (3H, m, -OCH_2O-, C_7-H), 6.11 (1H, dd, J=5, 10 Hz, C_2-H), 6.52, 6.95 (each 1H, s, Ar-H), 6.67 (1H, d, J=10 Hz, C_1-H).

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Gas-Liquid Chromatographic Separation of Aldose Enantiomers as Trimethylsilyl Ethers of Methyl 2-(Polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates[1)]

SHUUJI HARA, HIKARU OKABE and KUNIHIDE MIHASHI*

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma 8–19–1, Jonan-ku, Fukuoka 814–01, Japan

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Pairs of enantiomers of nine aldoses were separated by gas-liquid chromatography on an OV-17 capillary column as the trimethylsilyl ethers of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates, which were obtained by the reaction of aldoses with L-cysteine methyl ester. This method was applied to the determination of the absolute configurations of the component monosaccharides of a *Thladiantha* saponin.

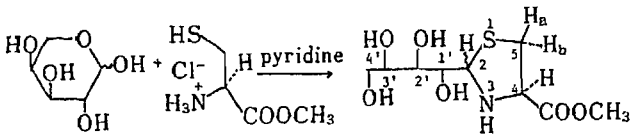
Keywords——sugar enantiomer separation; GLC; OV-17 capillary column; L-cysteine methyl ester; thiazolidine derivative; methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylate

In the field of saccharide and glycoside chemistry, it is necessary to determine the absolute configurations (D or L) of the component monosaccharides. This has been a serious problem in the determination of glycoside structures, especially in cases where the amount of the sugar available is small, and the sugar moiety is composed of several kinds of monosaccharides, because the ordinary determination by measurement of the optical rotation requires considerable amounts of pure sugar samples.

Several investigators have achieved the separation of sugar enantiomers at the microgram level by employing gas-liquid chromatography (GLC) or high-performance liquid chromatography (HPLC). Some have separated enantiomers by GLC on chiral stationary phases.[2)] However, application of these methods is still not widespread because of poor availability and high cost of materials. Other workers have tried separation by GLC or HPLC after conversion of sugars to chiral derivatives. The chiral derivatives so far reported for separation of enantiomers include glycosides of (−)-2-butanol and (+)-2-octanol,[3)] bis[(+)-1-phenylethyl]dithioacetals[4)] and 1-deoxy-1-α-methylaminoalditols[5)] (obtained by the reaction of aldoses with chiral α-methylbenzylamine in the presence of sodium cyanoborohydride). Among these chiral derivatives, the last one seemed to be preferable for sugar analysis of glycosides having several kinds of component sugars because of the commercial availability and stability of the chiral reagent, the ease of derivation and the simplicity of the chromatogram. Recently, this method has been effectively employed by Kasai *et al.*[6)] for determination of the absolute configurations (D/L) of the component sugars of several glycosides.

We have also tried to use this method, but found that it is still unsatisfactory in some cases for separation of enantiomers and differentiation of sugar species. This finding has led us to search for other chiral derivatives that might allow clear separation of sugars and determination of the absolute configuration of each sugar.

Bognár *et al.*[7)] have reported that aldoses react quantitatively with L-cysteine or its methyl ester to give thiazolidine derivatives. They suggested the applicability of these derivatives for separation of sugars and their enantiomers.



Chart

This report deals with the GLC separation of enantiomers of nine aldoses on an OV-17 capillary column after converting the sugars to the trimethylsilyl (TMS) ethers of the corresponding methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates.

Experimental

Materials——The following sugar samples were commercially obtained; D-xylose and L-arabinose (Wako Pure Chemicals, Co., Ld., Tokyo), L-xylose and L-rhamnose (Aldrich Chemical Co., Inc., Wisconsin), D-arabinose (Katayama Chemical Co., Ltd., Osaka), D-lyxose and D-ribose (Tokyo Kasei Kogyo Co., Ltd., Tokyo), D-fucose, L-lyxose, L-fucose,.L-glucose, L-ribose and L-galactose (Sigma Chemical Co., Ltd., St. Louis), D-glucose and D-mannose (Yoneyama Yakuhin Kogyo Co., Ltd., Osaka), D-galactose (Ishizu Pharmaceutical Co., Ltd., Osaka). D-Rhamnose was synthesized by catalytic hydrogenation of methyl 6-deoxy-6-iodo-α-D-mannopyranoside followed by hydrolysis.[8] L-Cysteine methyl ester hydrochloride and TMS-HT kit (hexamethyldisilazane–trimethylchlorosilane (HMDS–TMCS)) were obtained from Wako Pure Chemicals Co., Ltd., and Tokyo Kasei Kogyo Co., Ltd., respectively.

Apparatus——Gas chromatographic (GC) analysis was performed with a Shimadzu GC-8 gas chromatograph equipped with an H_2 flame ionization detector. The column was G-SCOT Silicone OV-17 on Silanox (0.3 mm i.d. × 50 m). Analytical conditions were as described in the caption of Fig. 3.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken in pyridine-d_5 solution with a JEOL JNM GX-400 spectrometer using tetramethylsilane as an internal standard. Fast-atom bombardment mass (FAB-MS) spectra were recorded with a JEOL JMS DX-300 spectrometer using a glycerol matrix containing NaI.

Derivation and Analytical Procedure——Pyridine solutions (100 μl each) of the sugar (0.04 mol/l) and L-cysteine methyl ester hydrochloride (0.06 mol/l) were mixed, and warmed at 60 °C for 1 h. The trimethylsilylation reagent HMDS–TMCS (150 μl) was added, and the warming at 60 °C was continued for another 30 min. The precipitate was centrifuged off, and the supernatant (1 μl) was subjected to GLC analysis. When the sample was analyzed as an acetate, the HMDS–TMCS was replaced by acetic anhydride (150 μl) and the mixture was warmed at 90 °C for 1 h. After evaporation of pyridine and acetic anhydride by air-blowing, the residue was dissolved in acetone (350 μl) and the solution (1 μl) was subjected to GLC.

Preparation of Methyl 2-(D- and L-*arabino*-Tetrahydroxybutyl)-thiazolidine-4(*R*)-carboxylates——The standard samples of thiazolidine derivatives of D- and L-arabinoses were prepared by the method reported by Bognár *et al.*[9] Thus, 0.58 g of L-cysteine methyl ester hydrochloride and 0.5 g of L-arabinose were dissolved in water (1 ml) and 0.3 ml of pyridine was added. The mixture was left to stand at room temperature overnight, then ethanol (2 ml) was added to complete the precipitation of the reaction product, and the precipitate was filtered off, washed with ethanol and dried *in vacuo*. The yield was 75%; amorphous powder, mp 155—156 °C, FAB-MS *m/z*: 290.068 ($[M+Na]^+$) (Calcd for $C_9H_{17}NNaO_6S$: *m/z* 290.067). *Anal.* Calcd for $C_9H_{17}NO_6S$: C, 40.44; H, 6.41; N, 5.24. Found: C, 39.96; H, 6.37; N, 5.23.

D-Arabinose was treated in the same manner to give the corresponding derivative: crystalline powder, mp 156—159 °C, FAB-MS *m/z*: 290.067 ($[M+Na]^+$). *Anal.* Found: C, 40.56; H, 6.44; N, 5.28.

Acetylation of Methyl 2-(L-*arabino*-Tetrahydroxybutyl)-thiazolidine-4(*R*)-carboxylate——The sample (0.1 g) was dissolved in pyridine (0.5 ml) and acetic anhydride (0.5 ml) was added. The reaction mixture was stirred at room temperature for 12 h. Water (3 ml) was added and the precipitate was filtered off and recrystallized from ethanol to give colorless needles, mp 126—127 °C, $[\alpha]_D^{17}$ −49.1° (c = 1.12, $CHCl_3$). The melting point and optical rotation were in good agreement with those reported (127—128 °C, $[\alpha]_D$ −50°) for methyl 3-acetyl-2(*R*)-(L-*arabino*-1′,2′,3′,4′-tetraacetoxybutyl)-thiazolidine-4(*R*)-carboxylate.[9]

Determination of Absolute Configurations of Component Monosaccharides of a *Thladiantha* Saponin——The *Thladiantha* saponin (4 mg) was heated in 1 N HCl (0.5 ml) at 90 °C for 2 h. The precipitate that deposited on cooling was removed by centrifugation. The supernatant was neutralized with Ag_2CO_3. After centrifugation of the precipitate, the supernatant was bubbled through with H_2S and concentrated *in vacuo* to give a sugar fraction (*ca.* 1 mg). The derivation to the thiazolidine derivative and GC analysis were carried out according to the standard procedure. The result is shown in Fig. 3B.

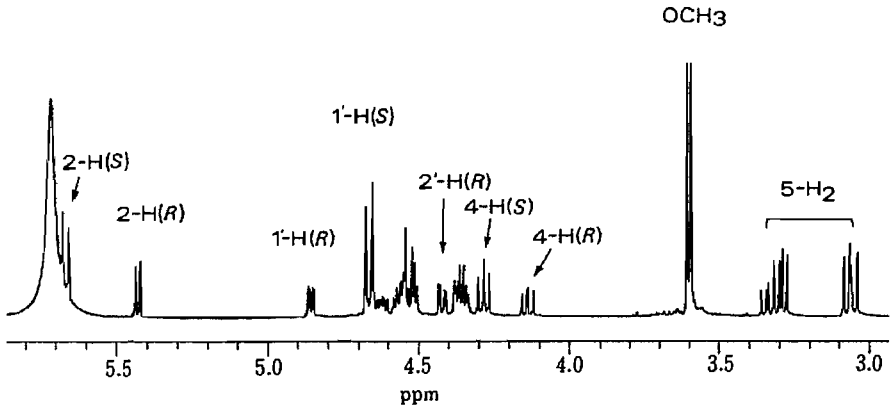


Fig. 2. ^{1}H-NMR Spectrum of Methyl 2-(L-*arabino*-Tetrahydroxybutyl)-thiazolidine-4(*R*)-carboxylate

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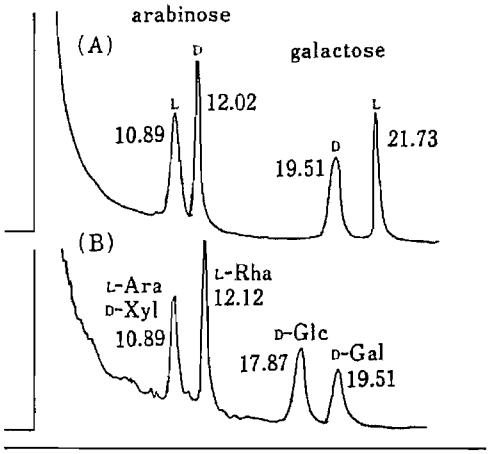

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GLC conditions: column, G-SCOT OV-17 on Silanox (0.3 mm i.d. × 50 m); column bath temperature, 200 °C; injection temperature, 270 °C; carrier gas, He (0.8 ml/min); split ratio, 1/75; make-up gas, He (50 ml/min).

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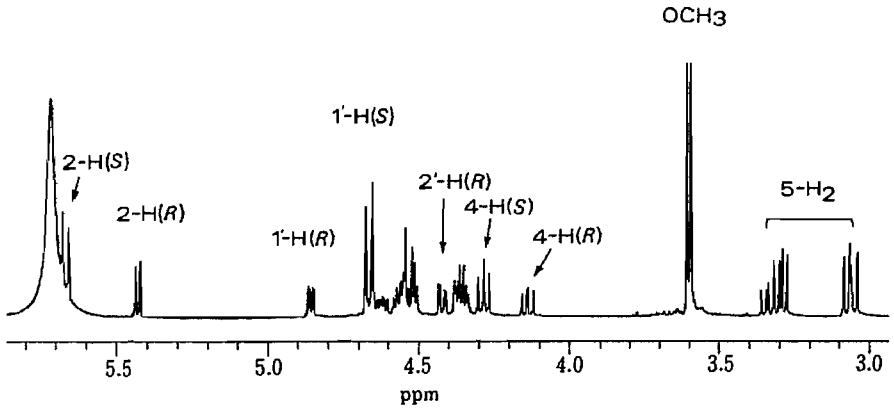

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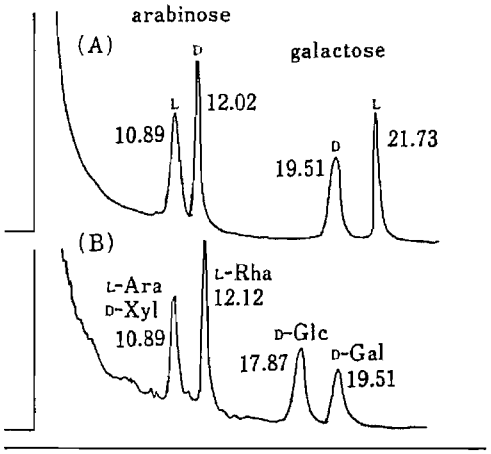

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The separation of enantiomers of all sugar species was quite clear, though the derivatives

TABLE I. Retention Times, Separation Factors and Resolutions of TMS Ethers of Methyl 2-(Polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylates Derived from Aldoses

Sugars	t_R		γ	*Rs*
	D-	L-		
Xylose	11.05 (*R*)	12.01 (*S*)	1.087	1.66
Arabinose	12.02 (*S*)	10.89 (*R*)	1.104	2.05
Lyxose	11.71 (*S*)	11.32 (*R*)	1.034	0.98
Ribose	11.25 (*R*)	12.45 (*S*)	1.107	3.16
Fucose	13.06 (*R*)	14.48 (*S*)	1.109	3.16
Rhamnose	12.60 (*S*)	12.14 (*R*)	1.038	1.10
Glucose	17.87 (*R*)	19.19 (*S*)	1.074	1.65
Galactose	19.51 (*R*)	21.73 (*S*)	1.114	3.31
Mannose	18.03 (*S*)	17.51 (*R*)	1.030	1.04

t_R, retention time (min); γ, separation factor (t_{R_2}/t_{R_1}); *Rs*, resolution $[2(t_{R_2}-t_{R_1})/(W_1+W_2)]$ (*R* or *S* in parentheses is the configuration of C-1′).

of some different sugar species (*e.g.* D-xylose and L-arabinose; L-xylose and D-arabinose; L-lyxose and D-ribose) gave similar t_R values and were difficult to separate.

It seemed that the absolute configuration (D or L) of the original sugar species had no correlation to the t_R value: the derivatives of D-enantiomers have smaller t_R values than those of L-enantiomers in some cases, while in other cases, the L-enantiomers were eluted first. However, when the t_R values and the structures of the products were examined in detail, it became apparent that the absolute configuration at C-1′ had a significant influence on the elution order. A sugar derivative which has the *R*-configuration at C-1′ has a smaller t_R value than the counterpart, without exception. The influence of the configuration of other carbons could not be separated because of the limited number of sugars investigated, but the small separation factors (γ) for lyxose, rhamnose and mannose, which have the same absolute configurations at C-1′ and C-2′, and the large separation factor for ribose, which has the same absolute configurations at C-1′, -2′ and -3′, imply that the absolute configurations of carbons of the polyol moiety or the steric relationship of the trimethylsilylated hydroxyl groups may have a significant influence on the separation of a pair of enantiomers.

As an application, this method was utilized to determine the absolute configurations of component sugars of a quillaic acid glycoside isolated from the tuber of *Thladiantha dubia*.[12] The sugar moiety was previously determined to be composed of 1 mol each of arabinose, xylose, rhamnose, glucose and galactose by GLC. The hydrolysate of the glycoside was treated as described for the standard sugar samples and checked by GLC. The result is shown in Fig. 3B. By comparison of the data with the t_R values of the standard samples, the absolute configurations were determined to be D for xylose, glucose and galactose, and L for arabinose and rhamnose.

This method is superior to the methods hitherto reported for separation of enantiomers as chiral sugar derivatives in that (a) the derivation procedure is simpler and it is easier to get a quantitative yield of the derivative, and (b) a clear separation of enantiomers is obtained for almost all sugars under ordinary GLC conditions, even though it is still not possible to achieve the identification of all sugar species and determination of the absolute configuration of each sugar by a single GLC run.

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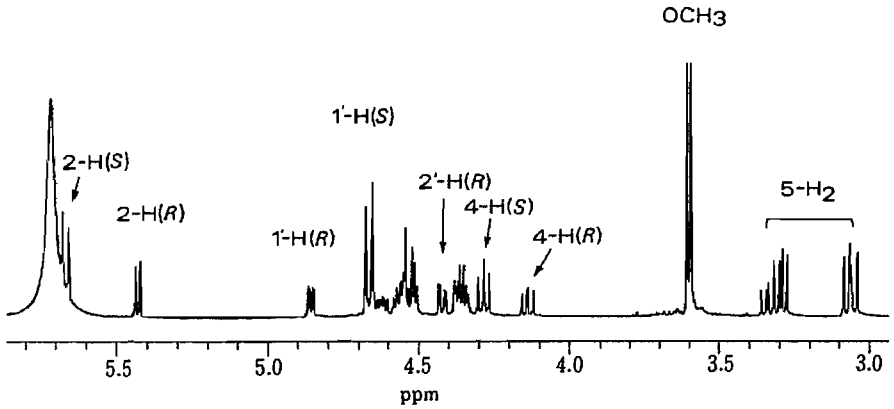

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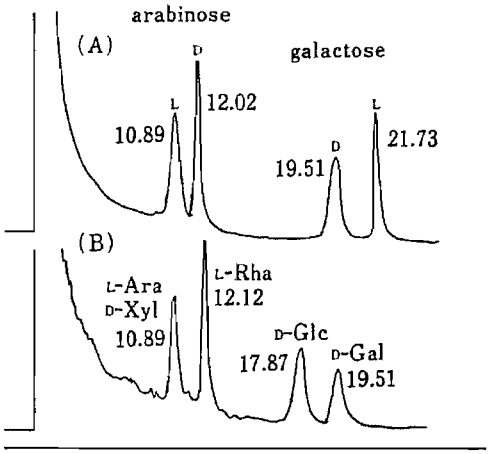

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Photochemistry of Conjugated Nitrogen–Carbonyl Systems. III.[1] Photosensitized Oxygenation of 3- and 6-Substituted 2-Pyridones[2]

EISUKE SATO, YOSHIYA IKEDA and YUICHI KANAOKA*

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

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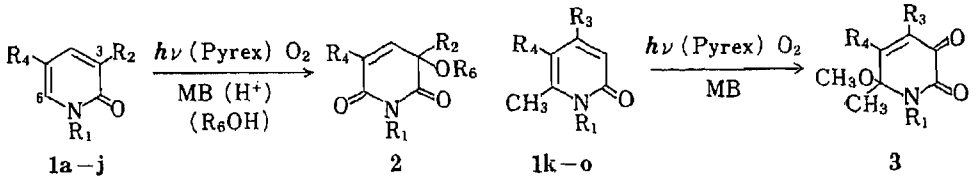
Irradiation of solutions of 3- and 6-substituted 2-pyridones (**1**) in the presence of methylene blue as a sensitizer under an oxygen atmosphere gave 3-substituted pyridine-2,6-(1*H*,3*H*)-diones (**2**) and 6-substituted pyridine-2,3-(1*H*,6*H*)-diones (**3**), respectively, which are probably derived from the intermediate endoperoxides (**6**).

Keywords——photosensitized oxygenation; 2-pyridone; pyridine-2,6-(1*H*,3*H*)-dione; pyridine-2,3-(1*H*,6*H*)-dione; endoperoxide; singlet oxygen

In the course of our systematic studies on the photochemistry of nitrogen–carbonyl systems such as amides and imides,[2] we have been exploring photoreactions of conjugated nitrogen–carbonyl systems.[1] As a continuation of this work, we are interested in the photochemistry of 2-pyridones, the most fundamental member in the family of the conjugated nitrogen–carbonyl systems.

Although photooxygenation reactions of 5- and 7-membered nitrogen–heterocycles such as pyrroles, indoles[3] and diazepines[4] are well known, little is known about the photochemistry of 6-membered nitrogen–heterocycles, except for 2-pyrazinone,[5] and 1,2-dihydropyridine derivatives.[6] Photochemical reactions of 2-pyridones such as [4+4]dimerization,[7]



1a–j **2** **1k–o** **3**

	R_1	R_2	R_4	R_6
a	H	CH_3	H	H
b	CH_3	CH_3	H	H
c	Et	CH_3	H	H
d	Bzl	CH_3	H	H
e	CH_3	CH_3	CH_3	H
f	Bzl	CH_3	CH_3	H
g	H	OCH_3	H	CH_3
h	H	OCH_3	H	Et
i	H	OCH_3	H	iso-Pr
j	H	OCH_3	H	allyl

	R_1	R_3	R_4
k	H	H	H
l	H	CH_3	H
m	CH_3	H	H
n	CH_3	CH_3	H
o	H	H	CH_3

Chart 1

TABLE I. Photooxygenation Products from 2-Pyridones (**1**, **4**)

Product	Solvent	Acid catalyst (H^+)	Yield (%)
2a	CH_2Cl_2	+	14
2b	CH_2Cl_2	+	50
2c	CH_2Cl_2	+	27
2d	CH_2Cl_2	+	31
2e	CH_2Cl_2	+	21
2f	CH_2Cl_2	+	41
2g	CH_3OH	−	65
2h	EtOH	−	42
2i	iso-PrOH	−	42
2j	Allyl-OH	−	19
3k	CH_3OH	−	12
3l	CH_3OH	−	23
3m	CH_3OH	−	11
3n	CH_3OH	−	20
3o	CH_3OH	−	26
5p	CH_2Cl_2	+	16
5q	CH_2Cl_2	+	28

valence isomerization,[8] and [2+2]cycloaddition to olefins[9] have been extensively studied. However, photooxygenation of 2-pyridones, which seems interesting from the view point of synthetic utility, has attracted no attention.[3] In this paper we wish to report the photosensitized oxygenation reactions of 3- and 6-substituted 2-pyridones (**1**).

Irradiation of 3-methyl-2-pyridone derivatives (**1a**—**f**) with a 500 W halogen lamp through a Pyrex filter, in methylene chloride with methylene blue (MB) as sensitizer in the presence of a small amount of *p*-toluenesulfonic acid under an oxygen atmosphere, afforded 3-hydroxypyridine-2,6-diones (**2a**—**f**) as shown in Chart 1 and Table I.

In contrast, when **1b** and **1e** were irradiated under similar conditions but in the absence of the acid catalyst, the yields of **2b** and **2e** were very low and most of the starting materials were recovered. The mechanism of these reactions may be explained in terms of involvement of endoperoxides (**6**) as common intermediates. In the presence of an acid catalyst, protonation may promote the O–O bond fission, leading to the products **2** through path A.

Presumably the first step of oxygen addition to form the endoperoxide (**6**) is reversible (Chart 2), and the back process may be predominant in the absence of an acid catalyst. Alternatively, the formation of **2** could also be explained by attack of a water molecule at the 3-position of **6** followed by dehydration. If this mechanism is correct, one might expect a 3-methoxylated product in place of the 3-hydroxy product in the reaction of **1** in methanol. However, irradiation of **1b** in methanol in the absence of an acid catalyst gave intractable mixtures of products including a trace of **2b**, detected by thin layer chromatography (TLC). In the presence of an acid catalyst either in methylene chloride or in methanol, the reaction proceeded smoothly, giving **2b**, not the 3-methoxy product.[10] These results support the mechanism in which the acid catalyst promotes the O–O bond fission along path A as shown in Chart 2.

Since singlet oxygen possesses an electrophilic character,[11] 2-pyridone derivatives with an electron-donating substituent may be expected to undergo efficient oxygenation. 3-Methoxy-2-pyridone (**1g**) bearing an electron-donating methoxy group at the 3-position was then subjected to the photooxygenation in an alcoholic medium. Irradiation of **1g** in methanol gave 3,3-dimethoxypyridine-2,6-(1*H*,3*H*)-dione (**2g**), though the reaction in methylene chloride, either in the presence or absence of acid catalyst, gave a complex mixture on TLC.[10]

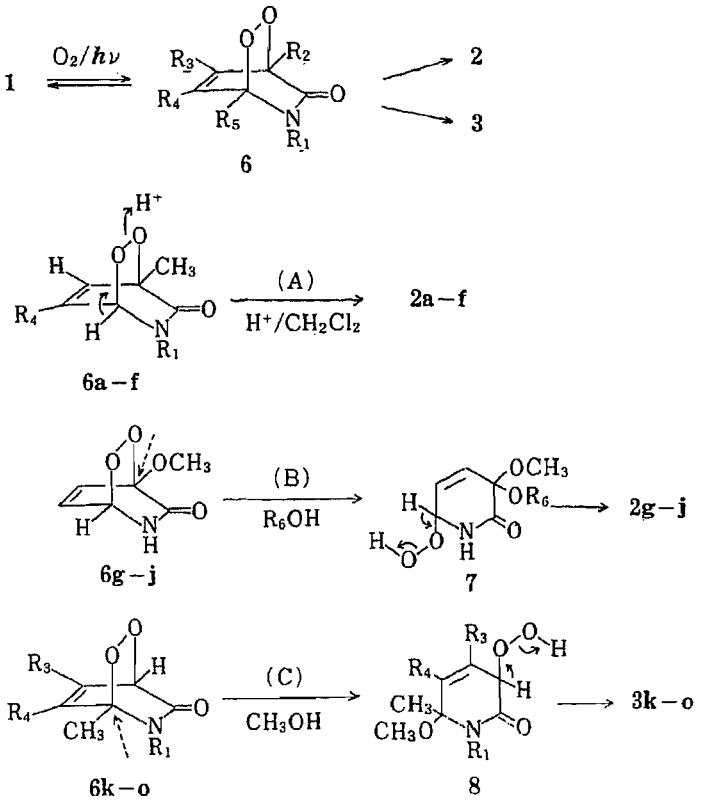
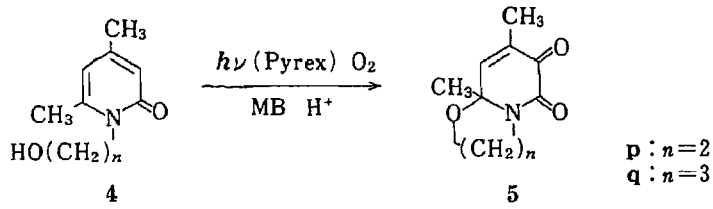


Chart 2

Similar products **2h**—**j** were also obtained by irradiation of **1h**—**j** in other alcohols such as ethanol, isopropanol and allyl alcohol, as shown in Table I. Apparently, when R_2 is a methoxy group, nucleophilic attack of an alcohol molecule occurs at the 3-position of the endoperoxide (**6**) (path B) to form a hydroperoxide (**7**) which is then dehydrated to give **2a**—**f**. The site of alcohol attack can be explained by the stabilizing effect of the methoxy substituent on the intermediary carbonium ion, assisted by the adjacent double bond, if S_N1-character is assumed for the alcoholysis.

To examine the relation between the substituents and the reactivities of the postulated endoperoxides **6**, the photo reactions of **1k**—**o**, which have a methyl group at the 6-position, and are therefore structural isomers of **1a**, **b** with a methyl at the 3-position, were examined. These substrates **1k**—**o** would lead to the endoperoxides **6k**—**o** having a methyl group at the bridgehead 6-position. Upon photooxygenation in methanol, 6-methyl-2-pyridones (**1k**—**o**) gave 6-methoxypyridine-2,3-dione derivatives (**3k**—**o**). The formation of 2,3-diones (**3**) can be explained on the basis of dehydration of the hydroperoxide (**8**) formed by nucleophilic attack of methanol on the endoperoxide (**6**) (path C). The fact that the reaction site is the tertiary carbon suggests that the reaction mode is again of S_N1 type, in contrast to that in the reactions of the isomeric pyridones **1a**—**f**, where the O–O bond fission seems to be an initial step. Even in the presence of an acid catalyst, **6k**—**o** mainly undergo the S_N1-like substitution, giving similar results.[10]

Since the introduction of an oxygen function at the 3- or 6-position of 2-pyridones by incorporation of solvent alcohol is now possible, intramolecular alcoholysis was examined for the synthetic application of this reaction. Thus, *N*-hydroxylalkyl-2-pyridones **4p** and **4q** in methylene chloride were treated under the photooxygenation conditions. As expected, the bicyclic products **5p** and **5q** were obtained, respectively, as shown in Table I and Chart 3. In



4 5

Chart 3

the absence of an acid catalyst, only traces of **5p** and **5q** were detected.

Dilling *et al.* reported that oxygen had no effect on the photoreactions of 2-pyridones such as [4+4]dimerization and valence isomerization.[8] Therefore, it is interesting that the 3- and 6-substituted 2-pyridones underwent photosensitized oxygenation to produce oxygenated products in moderate yields. The formation of all the products can be explained by assuming the endoperoxides (**6**) as common intermediates which have not yet been isolated. Further synthetic application of this reaction is under investigation.

Experimental

All melting points are uncorrected. Vacuum distillation was carried out by using a Büchi Kugelrohr apparatus and boiling points are the uncorrected bath temperatures, nuclear magnetic resonance (NMR) spectra were taken on a JEOL JNM-FX 100 FT-NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were obtained with a JEOL JMS-D 300 mass spectrometer. Infrared (IR) spectra were recorded with JASCO IRA-1 infrared spectrometer.

General Procedure for the Photolysis——A solution of **1** or **4** (10 mmol) in 400 ml of solvent (Table I) was irradiated with a 500 W halogen lamp through a Pyrex filter for 3 h under an oxygen atmosphere using methylene blue trihydrate (20 mg) as a sensitizer in the presence of *p*-toluenesulfonic acid (100 mg) as a catalyst for **1a—f**, **5p—q**, and in the absence of the acid for **1g—o**. After removal of the solvent under reduced pressure, the residue was subjected to column chromatography on silica gel (80 g), followed by recrystallization from AcOEt–*n*-hexane or distillation under reduced pressure. Spectral data are given in Table II.

3-Hydroxy-3-methylpyridine-2,6-(1*H*,3*H*)-dione (2a)——From 1.09 g of **1a**. Chromatography: eluted with *n*-hexane–acetone (3 : 1). Recrystallization gave colorless needles, 192 mg (14%), mp 132—134 °C. *Anal.* Calcd for $C_6H_7NO_3$: C, 51.06; H, 5.00; N, 9.93. Found: C, 50.93; N, 4.92; N, 9.85.

3-Hydroxy-1,3-dimethylpyridine-2,6-(1*H*,3*H*)-dione (2b)——From 1.23 g of **1b**. Chromatography: eluted with CH_2Cl_2–AcOEt (5 : 1). Distilled *in vacuo*, bp 100 °C/0.7 mmHg to give a colorless solid, mp 82—86 °C, 772 mg (50%). *Anal.* Calcd for $C_7H_9NO_3$: C, 54.19; H, 5.85; N, 9.03. Found: C, 54.15; H, 5.79; N, 9.03.

1-Ethyl-3-hydroxy-3-methylpyridine-2,6-(1*H*,3*H*)-dione (2c)——From 1.37 g of **1c**. Chromatography: eluted with CH_2Cl_2–AcOEt (6 : 1). Distilled under reduced pressure, bp 150 °C/0.45 mmHg to give a colorless solid, mp 64—67 °C, 464 mg (27%). *Anal.* Calcd for $C_8H_{13}NO_3$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.48; H, 6.43; N, 8.16.

1-Benzyl-3-hydroxy-3-methylpyridine-2,6-(1*H*,3*H*)-dione (2d)——From 1.99 g of **1d**. Chromatography: eluted with CH_2Cl_2–AcOEt (20 : 1). Distilled *in vacuo*, bp 220 °C/0.9 mmHg, to give a colorless oil, 711 mg (31%). High-resolution MS: Calcd for $C_{13}H_{13}NO_3$: 231.08950. Found: 231.08949 (M^+).

3-Hydroxy-1,3,5-trimethylpyridine-2,6-(1*H*,3*H*)-dione (2e)——From 1.37 g of **1e**. Chromatography: eluted with CH_2Cl_2–AcOEt (20 : 1). Distilled *in vacuo*, bp 125 °C/0.8 mmHg to give a colorless solid, mp 62—65 °C, 358 mg (21%). *Anal.* Calcd for $C_8H_{13}NO_3$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.57; H, 6.53; N, 8.14.

1-Benzyl-3-hydroxy-3,5-dimethylpyridine-2,6-(1*H*,3*H*)-dione (2f)——From 2.13 g of **11f**. Chromatography: eluted with CH_2Cl_2–AcOEt (30 : 1). Distilled *in vacuo*, bp 220 °C/0.6 mmHg, to give a colorless oil, 960 mg (41%). High-resolution MS: Calcd for $C_{14}H_{15}NO_3$: 245.10525. Found: 245.10345 (M^+).

3,3-Dimethoxypyridine-2,6-(1*H*,3*H*)-dione (2g)——From 1.25 g of **1g** in 400 ml of MeOH. Chromatography: eluted with CH_2Cl_2–AcOEt (8 : 1). Recrystallization gave colorless prisms, mp 86—87 °C. 1.11 g (65%). *Anal.* Calcd for $C_7H_9NO_4$: C, 49.12; H, 5.30; N, 8.12. Found: C, 49.07; H, 5.35; N, 8.24.

3-Ethoxy-3-methoxypyridine-2,6-(1*H*,3*H*)-dione (2h)——From 1.25 g of **1g** in 400 ml of EtOH. Chromatography: eluted with CH_2Cl_2–AcOEt (10 : 1). Distilled *in vacuo*, bp 165 °C/0.8 mmHg, to give a colorless oil, 780 mg (42%). *Anal.* Calcd for $C_8H_{11}NO_4$: C, 51.88; H, 5.99; N, 8.28. Found: C, 51.70; H, 5.95; N, 8.13.

3-Isopropyloxy-3-methoxypyridine-2,6-(1*H*,3*H*)-dione (2i)——From 1.25 g of **1g** in 400 ml of iso-PrOH. Chromatography: eluted with CH_2Cl_2–AcOEt (10 : 1). Distilled *in vacuo*, bp 175 °C/0.9 mmHg, to give a colorless oil, 828 mg (42%). *Anal.* Calcd for $C_9H_{13}NO_4$: C, 54.26; H, 6.58; N, 7.03. Found: C, 54.02; H, 6.58; N, 7.08.

TABLE II. Spectral Data for the Products (2, 3 and 5)

Product	IR (Nujol) ν_{max} (cm^{-1})	MS, *m/z*	^{1}H-NMR (solvent) δ (ppm), *J* (Hz)
2a	3390 (NH) 3160 (NH) 3070 (NH) 1705 (C=O) 1690 (C=O) 1625 (C=C)	141 (M^+) 126 98 (base)	(DMSO-d_6): 1.37 (3H, s), 5.98 (1H, d, J=10), 6.13 (1H, s), 6.80 (1H, d, J=10), 11.0—12.0 (1H, s)
2b	3340 (OH) 1710 (C=O) 1670 (C=O) 1630 (C=C)	155 (M^+) 140 98 (base)	($CDCl_3$): 1.56 (3H, s), 3.24 (3H, s), 3.30 (1H, s), 6.15 (1H, d, J=10), 6.83 (1H, d, J=10)
2c	3350 (OH) 1710 (C=O) 1670 (C=O) 1630 (C=C)	169 (M^+) 154 98 (base)	($CDCl_3$): 1.17 (3H, t, J=7), 1.55 (3H, s), 3.34 (1H, s), 3.89 (2H, q, J=7), 6.13 (1H, d, J=10), 6.82 (1H, d, J=10)
2d	3400 (OH) 1720 (C=O) 1690 (C=O) 1680 (C=O) 1640 (C=C) (Neat)	231 (M^+) 98 (base)	($CDCl_3$): 1.52 (3H, s), 3.34 (1H, s), 5.01 (2H, s), 6.04 (1H, d, J=10), 6.83 (1H, d, J=10), 7.23—7.41 (5H, m)
2e	3420 (OH) 1710 (C=O) 1680 (C=O) 1640 (C=C)	170 (M^++1) 154	($CDCl_3$): 1.53 (3H, s), 1.99 (3H, d, J=1.5), 3.24 (3H, s), 3.27 (1H, s), 6.57 (1H, q, J=1.5)
2f	3440 (OH) 1720 (C=O) 1690 (C=O) 1665 (C=O) 1650 (C=C) (Neat)	245 (M^+) 112 (base)	($CDCl_3$): 1.50 (3H, s), 1.97 (3H, d, J=1.5), 3.26 (1H, s), 5.01 (2H, s), 6.56 (1H, q, J=1.5), 7.23—7.41 (5H, m)
2g	3180 (NH) 3100 (NH) 1730 (C=C) 1715 (C=O) 1640 (C=C)	140 (M^+−31) 128	($CDCl_3$): 3.48 (6H, s), 6.26 (1H, dd, J=10, 2), 6.81 (1H, d, J=10), 6.98—7.36 (1H, s)
2h	3220 (NH) 3100 (NH) 1720 (C=O) 1710 (C=O) 1640 (C=C) (Neat)	186 (M^++1) 154 142 (base) 140	($CDCl_3$): 1.25 (3H, t, J=7), 3.48 (3H, s), 3.76 (2H, q, J=7), 6.26 (1H, dd, J=10.5, 2), 6.82 (1H, d, J=10.5), 8.2—8.5 (1H, s)
2i	3220 (NH) 3110 (NH) 1720 (C=O) 1705 (C=O) 1640 (C=C) (Neat)	156 (M^+−43) 140	($CDCl_3$): 1.20 (3H, d, J=6), 1.22 (3H, d, J=6), 3.46 (3H, s), 4.40 (1H, dq, J=6.6), 6.24 (1H, dd, J=10, 2), 6.79 (1H, d, J=10), 7.9—8.3 (1H, s)
2j	3210 (NH) 3100 (NH) 1720 (C=O) 1705 (C=O) 1640 (C=C) (Neat)	154 (M^+−43) 140	($CDCl_3$): 3.48 (3H, s), 4.29 (2H, dt, J=7, 1), 5.15—5.39 (2H, m), 5.73—6.06 (1H, m), 6.26 (1H, dd, J=10, 2), 6.83 (1H, d, J=10), 8.0—8.4 (1H, s)

TABLE II. (continued)

Product	IR (Nujol) ν_{max} (cm^{-1})	MS, *m/z*	^{1}H-NMR (solvent) δ (ppm), *J* (Hz)
3k	3260 (NH) 1685 (C=O) 1635 (C=O)	155 (M^+) 140 124	($CDCl_3$): 1.72 (3H, s), 3.23 (3H, s), 6.58 (1H, d, *J*=10), 6.89 (1H, dd, *J*=10, 2), 6.76—6.90 (1H, s)
3l	3240 (NH) 1700 (C=O) 1690 (C=O) 1660 (C=C)	169 (M^+) 154 139 138 126	($CDCl_3$): 1.68 (3H, s), 2.01 (3H, d, *J*=1), 3.20 (3H, s), 6.60 (1H, q, *J*=1), 7.1—7.5 (1H, s)
3m	1700 (C=O) 1670 (C=O) 1640 (C=C)	169 (M^+) 154 138 126	($CDCl_3$): 1.64 (3H, s), 3.05 (3H, s), 3.12 (3H, s), 6.58 (1H, d, *J*=10.5), 6.90 (1H, d, *J*=10.5)
3n	1690 (C=O) 1660 (C=O) 1610 (C=C)	183 (M^+) 168 152 126	($CDCl_3$): 1.61 (3H, s), 2.01 (3H, d, *J*=1.5), 3.04 (3H, s), 3.08 (3H, s), 6.65 (1H, q, *J*=1.5)
3o	3210 (NH) 3110 (NH) 1720 (C=O) 1715 (C=O) 1650 (C=C)	169 (M^+) 138 137 126 (base)	($CDCl_3$): 1.68 (3H, s), 2.08 (3H, d, *J*=1), 3.13 (3H, s), 6.46 (1H, q, *J*=1)
5p	1690 (C=O) 1670 (C=O) 1595 (C=C)	181 (M^+) 166 (base) 138	($CDCl_3$): 1.57 (3H, s), 1.97 (3H, d, *J*=1.5), 3.64—3.73 (1H, m), 4.10—4.36 (3H, m), 6.97 (1H, q, *J*=1.5)
5q	1700 (C=O) 1665 (C=O) 1600 (C=C)	195 (M^+) 180 (base) 152	($CDCl_3$): 1.75 (3H, s), 1.65—1.77 (1H, m), 1.97 (3H, d, *J*=1), 1.84—2.04 (1H, m), 3.19 (1H, ddd, *J*=12, 12, 5), 3.78—4.00 (1H, m), 4.08 (1H, ddd, *J*=12, 12, 5), 4.64—4.90 (1H, m), 6.75 (1H, q, *J*=1)

3-Allyloxy-3-methoxypyridine-2,6-(1*H*,3*H*)-dione (2j)——From 1.25 g of **1g** and 11.6 g (20 mmol) of allyl alcohol in 400 ml of CH_2Cl_2. Chromatography: eluted with CH_2Cl_2–AcOEt (10:1). Distilled *in vacuo*, bp 175 °C/0.6 mmHg, to give a colorless oil, 384 mg (19%). *Anal.* Calcd for $C_9H_{11}NO_4$: C, 54.82; H, 5.62; N, 7.10. Found: C, 54.75; H, 5.66; N, 7.14.

6-Methoxy-6-methylpyridine-2,3-(1*H*,6*H*)-dione (3k)——From 1.09 g of **1k**. Chromatography: eluted with CH_2Cl_2–AcOEt (3:1). Recrystallization gave colorless needles, mp 162.5—163 °C (dec.), 186 mg (12%). *Anal.* Calcd for $C_6H_7NO_3$: C, 51.06; H, 5.00; N, 9.93. Found: C, 51.16; H, 4.93; N, 9.81.

6-Methoxy-4,6-dimethylpyridine-2,3-(1*H*,6*H*)-dione (3l)——From 1.23 g of **1l**. Chromatography: eluted with CH_2Cl_2–AcOEt (3:1). Recrystallization gave colorless needles, mp 140—141 °C (dec.), 385 mg (23%). *Anal.* Calcd for $C_8H_{11}NO_3$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.80; H, 6.56; N, 8.32.

6-Methoxy-1,6-dimethylpyridine-2,3-(1*H*,6*H*)-dione (3m)——From 1.23 g of **1m**. Chromatography: eluted with CH_2Cl_2–AcOEt (3:1). Recrystallization gave colorless needles, mp 116—118 °C, 181 mg (11%). *Anal.* Calcd for $C_8H_{11}NO_3$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.65; H, 6.53; N, 8.24.

6-Methoxy-1,4,6-trimethylpyridine-2,3-(1*H*,6*H*)-dione (3n)——From 1.37 g of **1n**. Chromatography: eluted with CH_2Cl_2–AcOEt (4:1). Recrystallization gave fine colorless needles, mp 116—118 °C (dec.), 370 mg (20%). *Anal.* Calcd for $C_9H_{13}NO_3$: C, 59.00; H, 7.15; N, 7.65. Found: C, 58.93; H, 7.01; N, 7.59.

6-Methoxy-5,6-dimethylpyridine-2,3-(1*H*,6*H*)-dione (3o)——From 1.23 g of **1o**. Chromatography: eluted with CH_2Cl_2–AcOEt (3:1). Recrystallization gave colorless needles, mp 131—133 °C, 436 mg (26%). *Anal.* Calcd for $C_8H_{11}NO_3$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.73; H, 6.52; N, 8.29.

7,8a-Dimethyl-5,6-dioxo-2,3,5,6-tetrahydro-8a*H*-oxazolo[3,2-*a*]pyridine (5p)——From 1.67 g of **4p**. Chromatography: eluted with CH_2Cl_2–AcOEt (6:1). Recrystallization gave fine colorless needles, mp 111—112 °C, 286 mg (16%). *Anal.* Calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.54; H, 6.05; N, 7.71.

8,9a-Dimethyl-6,7-dioxo-3,4,6,7-tetrahydro-2*H*,9a*H*-pyrido[2,1-*b*][1,3]oxazine (5q)——From 1.93 g of **4q**. Chromatography: eluted with CH_2Cl_2–AcOEt (6:1). Recrystallization gave fine colorless needles, mp 152—154 °C,

581 mg (28%). *Anal.* Calcd for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.55; H, 6.73; N, 7.23.

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Condensed Pyridazines. V.[1] Reactions of 7-(Methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine with Methoxide Ion, Grignard Reagents, Hydrazines, and Amines

ETSUO ŌISHI,* AKIYA YAMADA, EISAKU HAYASHI, and TAKEO HIGASHINO

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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Nucleophiles were found to react with 7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]-pyridazine (**1**) in three ways, depending on the nature of the reagent. One is substitution of the methylsulfonyl group in **1** by the reagent. The second one is addition of the reagent to the 4,5-double bond in **1**. The third one is the addition of the reagent, followed by ring fission of the pyridazine moiety.

Thus, the reaction of **1** with sodium methoxide resulted in substitution of the methylsulfonyl group, giving 7-methoxy-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**2**). The addition was found to proceed in the reaction with alkylmagnesium halides and hydrazines, giving the corresponding 4-alkyl- (**3a—d**) and 4-hydrazino-4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]-pyridazines (**4a**, **b**), respectively. The reaction with primary and cyclic amines resulted in ring fission, giving the corresponding 4-(alkyliminomethyl)- (**5b**, **c**) and 4-[di(cyclic amino)methyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazoles (**6d—f**), respectively.

On the other hand, the reaction of 7-chloro-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**7**) with sodium methoxide, hydrazine, and amines resulted in the substitution of the chlorine atom, giving **2**, 7-hydrazino- (**11**), and 7-alkylamino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines (**12a—g**), respectively, but neither addition nor ring fission was observed.

Keywords——(methylsulfonyl)triazolopyridazine; nucleophile; addition; ring fission; substitution; triazole

It was reported by Ōishi, one of the authors of this paper, that the treatment of 1-(methylsulfonyl)phthalazine (A) with nucleophiles results in two types of reactions depending on the reagent used.[2] For example, butylamine reacted with A by replacing the methylsulfonyl substituent, giving 1-(butylamino)phthalazine (B),[2a] while methylmagnesium iodide added to the 4,5-double bond in A, giving 1,2-dihydro-1-methyl-4-(methylsulfonyl)-2-(1-

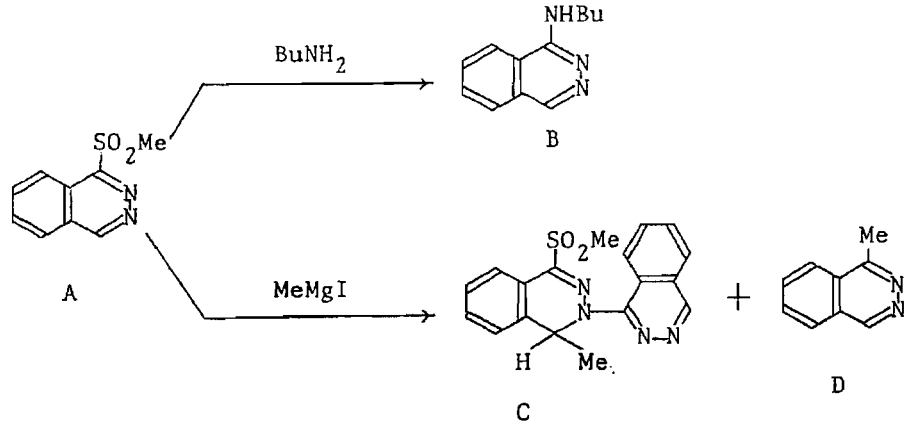


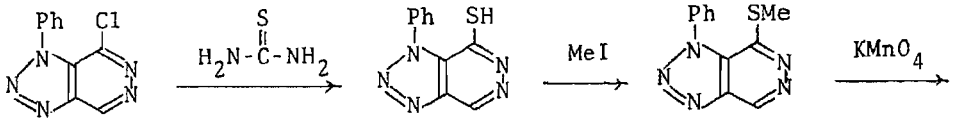
Chart 1

phthalazinyl)phthalazine (C) as a main product together with 1-methylphthalazine (D) as a by-product.[2b]

In the expectation that similar substitution and addition reactions would take place, we examined the reaction of 7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**1**) with nucleophiles, and found that the reaction proceeded in three ways depending on the reagent used. The first one is the expected substitution reaction, leading to 7-substituted 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**2**). The second one is the addition reaction, giving 4-substituted 4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines (**3** and **4**). The third one is the nucleophilic addition, followed by ring fission, giving triazoles (**5** and **6**).

In the present paper, we describe the above reaction in detail as well as the reaction of 7-chloro-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**7**)[3] with nucleophiles.

Compound **1** was prepared from **7** by way of **8** and **9** as shown in Chart 2, in an overall yield of 55%.



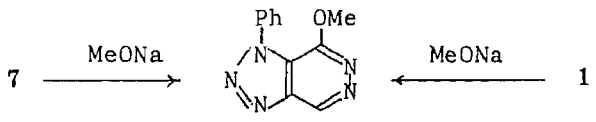
7 8 9

1

Chart 2

The Reaction with Sodium Methoxide

When **7** reacted with sodium methoxide in methanol, 7-methoxy-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**2**) was obtained in 78.5% yield. A similar substitution was found to occur in the reaction between **1** and sodium methoxide, giving **2** in 73.9% yield.



2

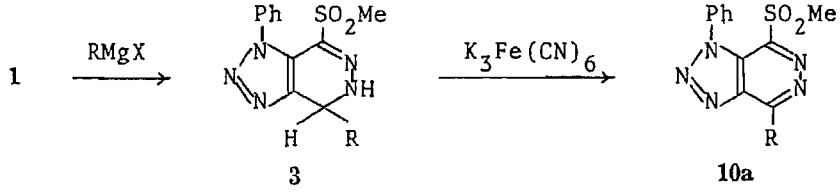
Chart 3

The Reaction with Grignard Reagents

When **1** was stirred with Grignard reagents in benzene, the reagent added to the 4,5-double bond in **1**, resulting in the formation of 4-alkyl-4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines (**3**). Thus, the reactions with phenylmagnesium bromide, methylmagnesium iodide, ethylmagnesium bromide, and benzylmagnesium chloride gave the corresponding 4-phenyl- (**3a**, 53.0%), 4-methyl- (**3b**, 47.7%), 4-ethyl- (**3c**, 1.2%), and 4-benzyl-4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**3d**, 15.4%), respectively. The structures of the dihydrocompounds (**3a—d**) were suggested by their elemental analyses and mass spectra (MS), and confirmed by their infrared (IR) absorption and proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR),

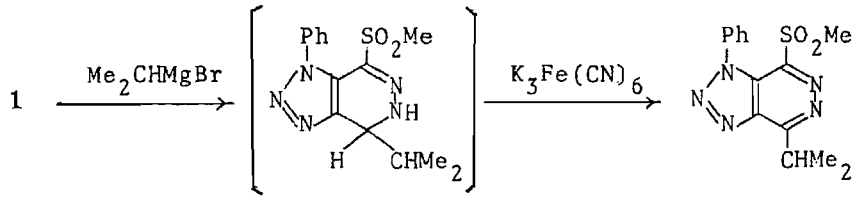
spectral data, as described in the experimental section. Moreover, the dihydro compound (**3a**) was converted into 1,4-diphenyl-7-(methylsulfonyl)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**10a**) in 87.2% yield by oxidation with potassium ferricyanide.

However, in the case of isopropylmagnesium bromide, the corresponding dihydro compound (**3e**) could not be isolated, and a tarry product was obtained. It is assumed that the first product in the reaction is **3e**, which could not be isolated due to its high susceptibility to oxidation, because when the reaction mixture was subjected to potassium ferricyanide oxidation, **10e** was obtained, although in low yield (1.3%).



3 10a

a : R=Ph, X=Br **b** : R=Me, X=I **c** : R=Et, X=Br **d** : R=$PhCH_2$, X=Cl

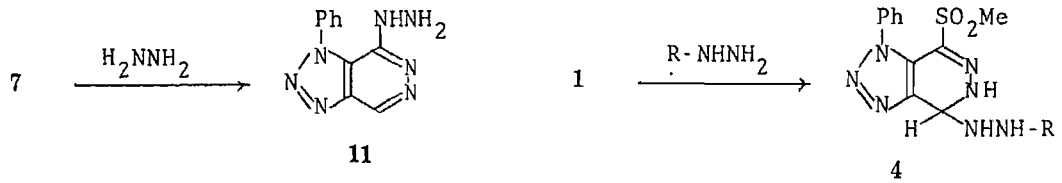


3e 10e

Chart 4

The Reaction with Hydrazines

When **7** was refluxed with hydrazine in methanol, the reaction proceeded to give 7-hydrazino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**11**). On the other hand, in the reaction of **1** with hydrazine and phenylhydrazine a nucleophilic addition proceeded, resulting in the formation of 4-hydrazino- (**4a**) and 4-(2-phenylhydrazino)-4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**4b**), respectively.



11 4

a : R=H **b** : R=Ph

Chart 5

The Reaction with Amines

When **7** was heated with primary and secondary cyclic amines, the reaction proceeded in the same way as with hydrazines, and resulted in the formation of 7-(substituted amino)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines (**12**). Thus, aniline, butylamine, cyclohexylamine, morpholine, piperidine, 4-methylpiperidine, and pyrrolidine gave 7-anilino- (**12a**, 45.8%), 7-(butylamino)- (**12b**, 62.2%), 7-(cyclohexylamino)- (**12c**, 62.2%), 7-morpholino- (**12d**, 69.7%),

7-piperidino- (**12e**, 44.6%), 7-(4-methylpiperidino)- (**12f**, 53.5%), and 7-pyrrolidino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12g**, 58.3%), respectively.

On the other hand, reactions with **1** were found to proceed in a different way from those with **7**, resulting in the fission of the pyridazine ring with elimination of nitrogen. Thus, butylamine and cyclohexylamine gave 4-(butyliminomethyl)- (**5b**, 57.6%) and 4-(cyclohexyliminomethyl)-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**5c**, 32.6%), respectively. In the case of cyclic amines, morpholine, piperidine, and 4-methylpiperidine, 4-(dimorpholinomethyl)- (**6d**, 95.4%), 4-(dipiperidinomethyl)- (**6e**, 88.3%), and 4-[bis(4-methylpiperidino)methyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**6f**, 77.8%) were afforded, respectively.

The structures of **5**, **6**, and **12** were well supported by their elemental analyses, and ^{1}H- and ^{13}C-NMR spectral data, as shown in Tables I to III.

The formation of **5** and **6** is considered to occur through the mechanism shown in Chart 7. The first step is undoubtedly an addition of amines to the 4,5-double bond in **1**, giving an adduct (E) corresponding to **4**. The subsequent ring fission of E with elimination of nitrogen leads to an intermediate (G) by way of an intermediate (F). Then, G is readily tautomerized to the more stable compound **5**.

In the case of cyclic amines, G does not have a shifting hydrogen at the nitrogen in the cyclic amine moiety, favoring an addition of a second molecule of cyclic amine. This second addition leads to **6**.

In methanol solution, **6d** was more stable than **6e** and **6f**. For example, **6d** could be recrystallized from methanol. However, when **6e** and **6f** were dissolved in methanol at ordinary temperature, methanol reacted with **6e** and **6f** by replacing one of the cyclic amino substituents, giving 4-[α-(piperidino)methoxymethyl]- (**13e**) and 4-[α-(4-methylpiperidino)-

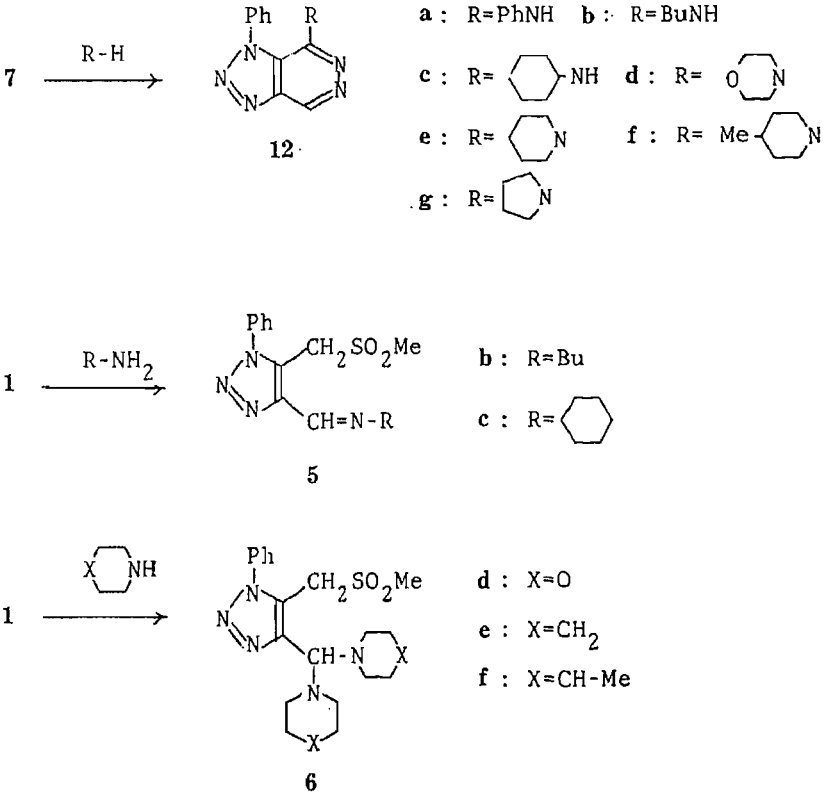


Chart 6

TABLE I. MS and Elemental Analysis Data for **5**, **6**, and **12**

Compd.	MS *m/z* (M^+)	Formula	Analysis (%) Calcd (Found)		
			C	H	N
5b	320	$C_{15}H_{20}N_4O_2S$	56.23	6.29	17.49
			(56.10	6.27	17.19)
5c	346	$C_{17}H_{22}N_4O_2S$	58.94	6.40	16.17
			(58.74	6.42	16.14)
6d		$C_{19}H_{27}N_5O_4S$	54.14	6.46	16.61
			(53.85	6.42	16.53)
6e		$C_{21}H_{31}N_5O_2S$	60.40	7.48	16.77
			(60.38	7.46	16.78)
6f		$C_{23}H_{35}N_5O_2S$	61.99	7.92	15.72
			(62.29	7.94	15.74)
12a	288	$C_{16}H_{12}N_6$	66.66	4.20	29.15
			(66.22	4.15	29.06)
12b	268	$C_{14}H_{16}N_6$	62.67	6.01	31.32
			(62.32	5.98	31.00)
12c	294	$C_{16}H_{18}N_6$	65.29	6.16	28.55
			(65.13	6.20	28.68)
12d	282	$C_{14}H_{14}N_6O$	59.56	5.00	29.77
			(59.42	4.99	29.68)
12e	280	$C_{15}H_{16}N_6$	64.27	5.75	29.98
			(64.52	5.75	29.69)
12f	294	$C_{16}H_{18}N_6$	65.29	6.16	28.55
			(65.27	6.18	28.72)
12g	266	$C_{14}H_{14}N_6$	63.14	5.30	31.56
			(63.12	5.28	31.66)

methoxymethyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**13f**), respectively.

On reaction with silica gel (Wakogel C-200) in the presence of water, **6d**—**f** were hydrolyzed to 5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole-4-carboxaldehyde (**14**). The aldehyde **14** reacted with butylamine and cyclohexylamine in the presence of *p*-toluenesulfonic acid (TsOH) to form **5b** and **5c**, respectively. Similarly, the reaction with morpholine gave **6d**.

The experimental results may be summarized as follows. i) On reaction with various nucleophiles, **7** undergoes nucleophilic substitution of the chlorine atom rather than addition of nucleophiles to the 4,5-double bond, resulting in the formation of 7-substituted 1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines. ii) In the reaction with alkylmagnesium halide and hydrazines, **1** favors nucleophilic addition rather than nucleophilic substitution of the methylsulfonyl substituent, resulting in the formation of the corresponding 4-substituted 4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazines. iii) In the reaction with amines, fission of the pyridazine ring of **1** occurs with elimination of nitrogen, giving 1-phenyl-1*H*-1,2,3-triazoles. This ring fission of **1** with amines is the first such finding in the condensed pyridazine ring system.

Experimental

All melting points are uncorrected. IR spectra were recorded on a Jasco A-102 diffraction grating IR spectrometer. ^{1}H-NMR spectra were measured at 60 MHz on a Hitachi R-24B high-resolution NMR spectrometer, and ^{13}C-NMR spectra were taken at 90 MHz on a JEOL JNM-FX90Q FTNMR spectrometer. Chemical shifts are quoted in parts per million (ppm) with tetramethylsilane as an internal standard, and coupling constants (*J*) are given

TABLE II. IR and ^{1}H-NMR Data for **5**, **6**, and **12**

Compd.	IR ν_{max}^{KBr} cm^{-1}	^{1}H-NMR ($CDCl_3$)
5b	1305, 1145 (SO_2)	8.62 (1H, s, CH=N), 7.56 (5H, s, Ph-H), 4.86 (2H, s, CH_2SO_2), 3.91—3.50 (2H, m, NCH_2), 2.89 (3H, s, SO_2CH_3), 1.97—0.78 (7H, m, $CH_2CH_2CH_3$)
5c	1315, 1140 (SO_2)	8.66 (1H, s, CH=N), 7.56 (5H, s, Ph-H), 4.88 (2H, s, CH_2SO_2), 3.49—3.05 (1H, m, NCH), 2.90 (3H, s, SO_2CH_3), 2.15—0.98 (10H, m, $(CH_2)_5$)
6d	1320, 1145 (SO_2)	7.58 (5H, s, Ph-H), 4.56 (2H, s, CH_2SO_2), 4.32 (1H, s, N-CH-N), 3.98—3.26 (8H, m, $(CH_2$-O-$CH_2)_2$), 3.02—2.15 (11H, m, $(CH_2$-N-$CH_2)_2$, SO_2CH_3)
6e	1310, 1145 (SO_2)	7.54 (5H, s, Ph-H), 4.70 (2H, s, CH_2SO_2), 4.25 (1H, s, N-CH-N), 3.15—2.10 (11H, m, $(CH_2$-N-$CH_2)_2$, SO_2CH_3), 2.10—1.11 (12H, m, $(CH_2CH_2CH_2)_2$)
6f	1310, 1125 (SO_2)	7.53 (5H, s, Ph-H), 4.70 (2H, s, CH_2SO_2), 4.28 (1H, s, N-CH-N), 3.40—2.82 (8H, m, $(CH_2$-N-$CH_2)_2$), 2.62 (3H, s, SO_2CH_3), 2.50—0.74 (16H, m, $(CH_2CH_2)_2$, $(CH)_2$, $(CH_3)_2$)
12a[a]	3400 (NH)	9.56 (1H, s, C^4-H), 8.01—6.99 (10H, m, Ph-H)
12b	3380—3120 (NH)	9.23 (1H, s, C^4-H), 7.60 (5H, s, Ph-H), 4.60—4.28[b] (1H, br, NH), 3.78—3.35 (2H, m, NCH_2), 1.83—0.70 (7H, m, $CH_2CH_2CH_3$)
12c	3410 (NH)	9.28 (1H, s, C^4-H), 8.03—7.46 (5H, m, Ph-H), 4.65—3.94[b] (1H, br, NH), 3.02—2.48 (1H, m, NCH), 2.86—0.74 (10H, m, $(CH_2)_5$)
12d		9.57 (1H, s, C^4-H), 7.63 (5H, s, Ph-H), 4.13—3.89 (2H, m, OCH_2), 3.75—3.40 (2H, m, OCH_2), 3.40—3.09 (4H, m, $(NCH_2)_2$)
12e		9.48 (1H, s, C^4-H), 7.61 (5H, s, Ph-H), 3.43 (4H, m, $(NCH_2)_2$), 1.82—1.07 (6H, m, $CH_2CH_2CH_2$)
12f		9.47 (1H, s, C^4-H), 7.57 (5H, s, Ph-H), 3.80—2.54 (4H, m, $(NCH_2)_2$), 1.70—0.71 (8H, m, $(CH_2)_2$, CH, CH_3)
12g		9.29 (1H, s, C^4-H), 7.53 (5H, s, Ph-H), 3.45—3.08 (4H, m, $(NCH_2)_2$), 2.04—1.61 (4H, m, $(CH_2)_2$)

a) ^{1}H-NMR in $CDCl_3$-CF_3COOD. *b*) Exchangeable with CD_3OD.

TABLE III. ^{13}C-NMR Data for **5** and **6**

Compd.	^{13}C-NMR ($CDCl_3$)
5b	153.93 (d, CH=N), 143.47 (s, Ph), 135.29 (s, C^4), 130.52 (d, Ph), 129.65 (d, Ph), 126.46 (d, Ph), 126.29 (s, C^5), 61.60 (t, N-CH_2),[a] 49.74 (t, CH_2SO_2),[a] 41.72 (q, SO_2CH_3), 33.05, 20.48 (t, CH_2CH_2), 13.76 (q, CH_3)
5c	151.86 (d, CH=N), 143.58 (s, Ph), 135.34 (s, C^4), 130.52 (d, Ph), 129.65 (d, Ph), 126.51 (d, Ph), 126.24 (s, C^5), 70.16 (d, NCH), 49.79 (t, CH_2SO_2), 41.77 (q, SO_2CH_3), 34.62 (t, CH_2), 25.57 (t, CH_2), 24.60 (t, CH_2)
6d	142.93 (s, Ph), 135.83 (s, C^4), 130.63 (d, Ph), 129.93 (d, Ph), 126.40 (d, Ph), 124.72 (s, C^5), 80.73 (d, N-CH-N), 67.18 (t, O-CH_2), 49.68 (t, N-CH_2, CH_2SO_2), 42.21 (q, SO_2CH_3)
6e	144.49 (s, Ph), 136.21 (s, C^4), 130.30 (d, Ph), 129.76 (d, Ph), 126.40 (d, Ph), 124.40 (s, C^5), 81.97 (d, N-CH-N),[a] 50.55 (t, NCH_2), 49.90 (t, CH_2SO_2), 42.26 (q, SO_2CH_3), 26.55, 25.14 (t, CH_2)
6f	144.44 (s, Ph), 136.10 (s, C^4), 130.20 (d, Ph), 129.65 (d, Ph), 126.29 (d, Ph), 124.29 (s, C^5), 81.27 (d, N-CH-N),[a] 49.73 (t, CH_2SO_2),[a] 48.93 (t, N-CH_2), 42.21 (q, SO_2CH_3), 34.84 (t, CH_2), 31.32 (d, CH), 22.00 (q, CH_3)

a) Assignment based on selective decoupling.

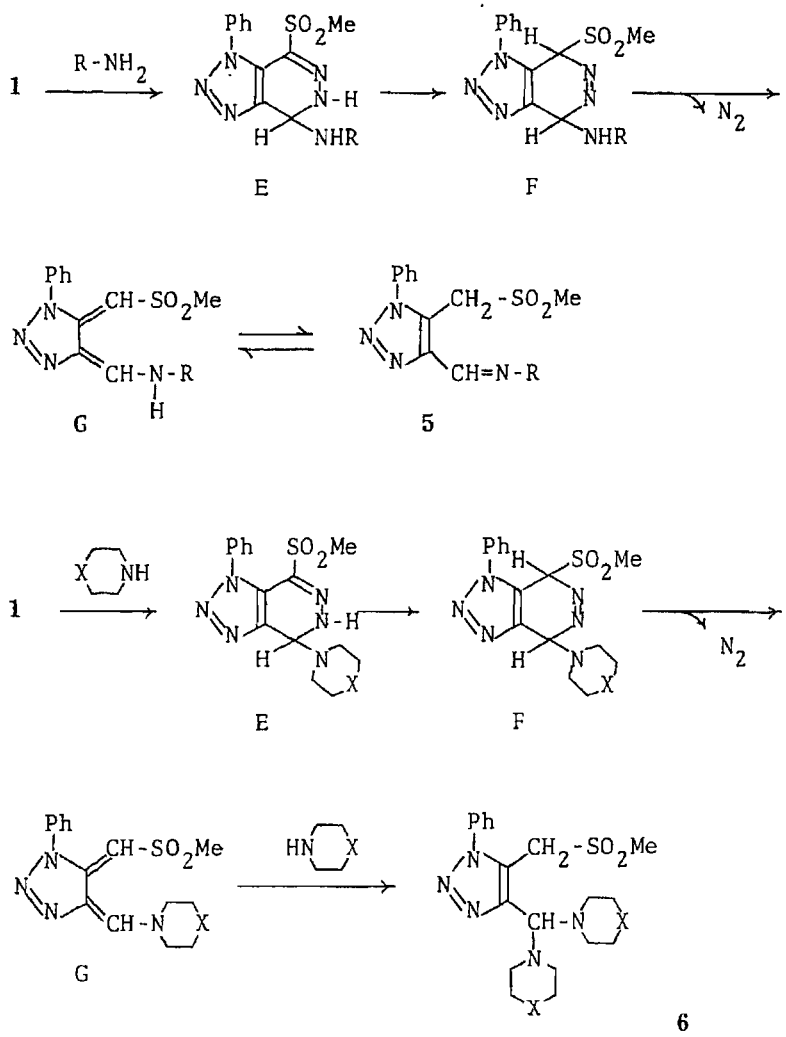


Chart 7

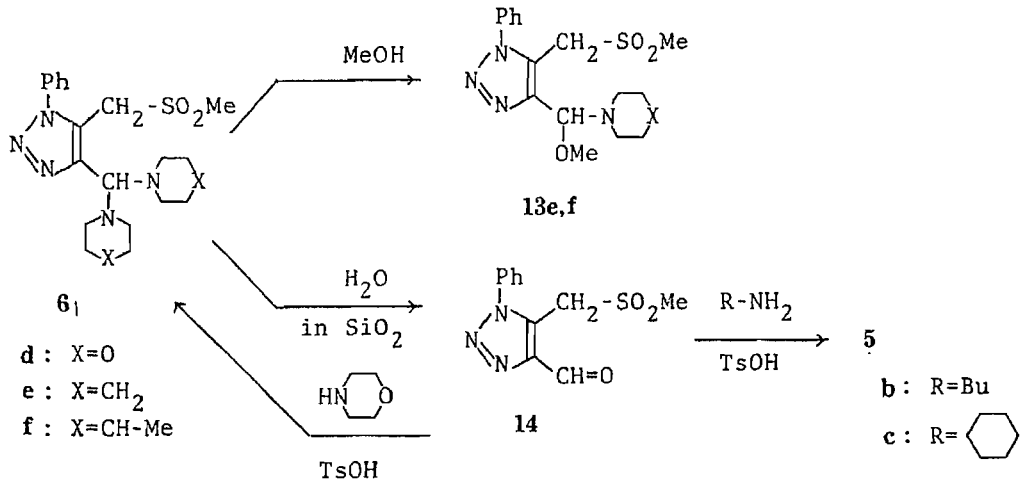


Chart 8

in Hz. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, sept=septet, m=multiplet, and br s=broad singlet. MS were recorded on a JEOL JMS D-100 mass spectrometer. Exact mass measurement was carried out on a JEOL JMS-01SG-2 mass spectrometer combined with a JEC-6 spectrum computer. Samples were vaporized in a direct inlet system. Column chromatography was carried out on SiO_2,

Wakogel C-200 (200 mesh).

7-Mercapto-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (8)——Thiourea (1.2 g, 16 mmol) was added to a solution of **7** (3.2 g, 14 mmol) in MeOH (100 ml), and the mixture was refluxed for 20 min. On cooling, crystals separated. They were collected by suction and recrystallized from MeOH to give a yellow powder, mp 259—261 °C, in 82.4% yield (2.6 g). *Anal.* Calcd for $C_{10}H_7N_5S$: C, 52.39; H, 3.08; N, 30.55. Found: C, 52.28; H, 3.04; N, 30.57. IR ν_{max}^{KBr} cm^{-1}: 3200—3000 (NH). ^{1}H-NMR ($(CD_3)_2SO$): 14.50 (1H, br s, SH), 9.14 (1H, s, C^4-H), 7.59 (5H, s, Ph-H).

7-(Methylthio)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (9)——Methyl iodide (2.3 g, 16 mmol) was added to a solution of **8** (1.8 g, 8 mmol) in 2 N KOH (15 ml), and the mixture was shaken for 10 min at room temperature. The reaction mixture was extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was recrystallized from benzene–petr. ether to give colorless needles, mp 163—164 °C, in 94.2% yield (1.8 g). *Anal.* Calcd for $C_{11}H_9N_5S$: C, 54.31; H, 3.73; N, 28.79. Found: C, 54.20; H, 3.72; N, 28.36. ^{1}H-NMR ($CDCl_3$): 9.68 (1H, s, C^4-H), 7.80—7.25 (5H, m, Ph-H), 2.75 (3H, s, SCH_3). ^{13}C-NMR ($CDCl_3$): 149.59 (s, C^7), 142.01 (d, C^4), 140.71 (s, Ph), 135.29 (s, C^{3a}), 131.12 (d, Ph), 129.28 (d, Ph), 127.11 (d, Ph), 124.18 (s, C^{7a}), 13.11 (q, CH_3).

7-(Methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (1)——A solution of 3% $KMnO_4$ was added drop by drop to a stirred solution of **9** (1000 mg, 4 mmol) in AcOH (1 ml) until the purple color of the permanganate persisted. The amount of $KMnO_4$ solution required was about 40 ml. The mixture was stirred for an additional 1 h, decolorized with Na_2SO_3, and extracted with $CHCl_3$. The extract was well washed with H_2O, dried over Na_2SO_4, and passed through a column of SiO_2 to remove impurities. Recrystallization from benzene–petr. ether gave colorless plates, mp 225—228 °C (dec.), in 70.7% yield (800 mg). *Anal.* Calcd for $C_{11}H_9N_5O_2S$: C, 47.99; H, 3.30; N, 25.44. Found: C, 48.03; H, 3.24; N, 25.44. IR ν_{max}^{KBr} cm^{-1}: 1315, 1145 (SO_2). ^{1}H-NMR ($CDCl_3$): 10.00 (1H, s, C^4-H), 7.56 (5H, s, Ph-H), 3.49 (3H, s, SO_2CH_3). ^{13}C-NMR ($(CD_3)_2SO$): 149.21 (s, C^7), 148.18 (d, C^4), 144.23 (s, Ph), 136.59 (s, C^{3a}), 130.68 (d, Ph), 128.79 (d, Ph), 127.16 (d, Ph), 126.68 (s, C^{7a}), 41.18 (q, CH_3).

7-Methoxy-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (2)——i) Compound **7** (100 mg, 0.43 mmol) was added to a solution of MeONa [prepared from Na (40 mg, 1.72 mmol) and MeOH (5 ml)], and the mixture was refluxed for 4 h. After removal of MeOH under reduced pressure, the residue was poured into H_2O (5 ml), neutralized with AcOH, and extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was recrystallized from benzene–petr. ether to give **2**, mp 155—156 °C,[3] in 78.5% yield (77 mg).

ii) Compound **2** was obtained in 73.9% yield (61 mg) from the reaction of **1** (100 mg, 0.36 mmol) with MeONa [prepared from Na (30 mg, 1.3 mmol) and MeOH (3 ml)] in essentially the same manner as described for the reaction of **7** with MeONa (i).

Reaction of 1 with RMgX——The procedure for the reaction of **1** with PhMgBr is described in detail as a typical example.

4,5-Dihydro-1,4-diphenyl-7-(methylsulfonyl)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**3a**): A solution of PhMgBr [prepared from PhBr (350 mg, 2.23 mmol) and Mg (55 mg, 2.26 mmol) in ether (3 ml)] was added to a stirred solution of **1** (200 mg, 0.73 mmol) in benzene (10 ml), and the mixture was stirred for 1 h at room temperature. Aqueous NH_4Cl–NH_3 (a solution of NH_4Cl (3 g) and 28% NH_3 (0.5 ml) in H_2O (10 ml)) was added to the reaction mixture. The aqueous solution was extracted with $CHCl_3$. The $CHCl_3$ solution was combined with the benzene–ether solution, and the combined solution was dried over Na_2SO_4, then concentrated to dryness. The residue was chromatographed on a column of SiO_2 eluting with $CHCl_3$. The first fraction gave biphenyl, and the second fraction gave recovered **1** (79 mg). The third fraction gave **3a** in 53.0% yield (137 mg). Compound **3a** was recrystallized from $CHCl_3$–MeOH to give colorless needles, mp 220—223 °C (dec.). *Anal.* Calcd for $C_{17}H_{15}N_5O_2S$: C, 57.78; H, 4.28; N, 19.82. Found: C, 57.52; H, 4.20; N, 19.85. MS *m/z*: 353 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3310 (NH), 1320, 1130 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.54 (5H, s, Ph-H), 7.41 (5H, s, Ph-H), 7.15 (1H, br s, exchangeable with CD_3OD, NH), 6.27 (1H, s, C^4-H), 2.94 (3H, s, SO_2CH_3).

From MeMgI, 4,5-dihydro-4-methyl-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**3b**) was obtained as colorless needles from MeOH, mp 169—170 °C (dec.), in 47.7% yield (101 mg). *Anal.* Calcd for $C_{12}H_{13}N_5O_2S$: C, 49.47; H, 4.50; N, 24.04. Found: C, 49.60; H, 4.46; N, 24.12. MS *m/z*: 291 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3310 (NH), 1315, 1135 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.47 (5H, s, Ph-H), 6.85 (1H, br s, exchangeable with CD_3OD, NH), 5.27 (1H, q, J=8 Hz, C^4-H), 2.85 (3H, s, SO_2CH_3), 1.65 (3H, d, J=8 Hz, C^4-CH_3).

From EtMgBr, 4,5-dihydro-4-ethyl-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**3c**) was obtained as a white powder from MeOH, mp 173—175 °C (dec.), in 1.2% yield (2.7 mg). MS *m/z* Calcd for $C_{13}H_{15}N_5O_2S$: 305.0947 (M^+). Observed: 305.0964. IR ν_{max}^{KBr} cm^{-1}: 3310 (NH), 1310, 1135 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.65—6.75 (6H, m, Ph-H, NH), 5.14 (1H, t, J=8 Hz, C^4-H), 2.28 (3H, s, SO_2CH_3), 2.00 (2H, m, C^4-CH_2Me), 1.09 (3H, t, J=8 Hz, $CH_2\underline{CH_3}$).

From $PhCH_2MgBr$, 4-benzyl-4,5-dihydro-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**3d**) was obtained as colorless plates from MeOH, mp 161—165 °C (dec.), in 15.4% yield (41 mg). *Anal.* Calcd for $C_{18}H_{17}N_5O_2S$: C, 58.84; H, 4.66; N, 19.06. Found: C, 58.93; H, 4.64; N, 19.22. MS *m/z*: 276 ($M^+ - 91$). IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 1320, 1135 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.63—7.00 (10H, m, Ph-H), 6.97—6.86 (1H, br, exchangeable with CD_3OD, NH), 5.52—5.29 (1H, m, C^4-H), 3.33—3.10 (2H, m, $\underline{CH_2}$Ph), 2.76 (3H, s, SO_2CH_3).

1,4-Diphenyl-7-(methylsulfonyl)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (10a)——A solution of $K_3Fe(CN)_6$ (500 mg) in 1 N KOH (2 ml) was added to a suspension of **3a** (30 mg, 0.08 mmol) in benzene (2 ml), and the mixture was shaken for 20 min at room temperature. The reaction mixture was neutralized with AcOH and extracted with $CHCl_3$. The extract was washed with H_2O, dried over Na_2SO_4, and concentrated to dryness. The residue was recrystallized from benzene to give colorless needles, mp 226.5—228 °C, in 87.2% yield (26 mg). *Anal.* Calcd for $C_{17}H_{13}N_5O_2S$: C, 58.11; H, 3.73; N, 19.93. Found: C, 57.87; H, 3.65; N, 19.81. MS *m/z*: 351 (M^+). IR ν_{max}^{KBr} cm^{-1}: 1320, 1140 (SO_2). ^{1}H-NMR ($CDCl_3$): 9.05—8.71, 7.80—7.44 (10H, m, Ph-H), 3.49 (3H, s, SO_2CH_3).

Reaction of 1 with $Me_2CHMgBr$——A solution of $Me_2CHMgBr$ prepared from Me_2CHBr (274 mg, 2.23 mmol) and Mg (55 mg, 2.26 mmol) in ether (3 ml) was added to a stirred solution of **1** (200 mg, 0.73 mmol) in benzene (10 ml), and the mixture was stirred for 1 h at room temperature. The residue, obtained by work-up of the reaction mixture as described for **3a**, was subjected to $K_3Fe(CN)_6$ oxidation in essentially the same manner as described for the oxidation of **3a**, to give 4-isopropyl-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**10e**) as slightly yellow needles from $CHCl_3$–MeOH, mp 145—149 °C, in 1.3% yield (3.0 mg). *Anal.* Calcd for $C_{14}H_{15}N_5O_2S$: C, 52.98; H, 4.76; N, 22.07. Found: C, 52.68; H, 4.58; N, 21.80. MS *m/z*: 317 (M^+). IR ν_{mx}^{KBr} cm^{-1}: 1320, 1150 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.52 (5H, s, Ph-H), 4.15 (1H, sept, J=7 Hz, C<u>H</u>Me_2), 3.45 (3H, s, SO_2CH_3), 1.69 (6H, d, J=7 Hz, CH(C<u>H</u>$_3$)$_2$).

7-Hydrazino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (11)——Hydrazine hydrate (90 mg, 1.80 mmol) was added to a solution of **7** (100 mg, 0.43 mmol) in MeOH (6 ml), and the mixture was refluxed for 3 h. The reaction mixture was concentrated to a volume of about 2 ml and allowed to cool. The separated crystals were recrystallized from MeOH to give slightly yellow needles, mp 197—200 °C,[3] in 65.2% yield (64 mg).

4,5-Dihydro-4-hydrazino-7-(methylsulfonyl)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (4a)——Hydrazine hydrate (73 mg, 1.46 mmol) was added to a solution of **1** (100 mg, 0.36 mmol) in MeOH (4 ml), and the mixture was refluxed for 10 min. On cooling, crystals separated. They were collected by suction, washed with MeOH, and recrystallized from MeOH to give pale yellow needles, mp 165—167 °C (dec.), in 49.0% yield (55 mg). *Anal.* Calcd for $C_{11}H_{13}N_7O_2S$: C, 42.99; H, 4.26; N, 31.90. Found: C, 43.02; H, 4.22; N, 32.07. IR ν_{max}^{KBr} cm^{-1}: 3400—3200 (NH), 1315, 1130 (SO_2). ^{1}H-NMR ($(CD_3)_2SO$): 8.64 (2H, br s, exchangeable with CD_3OD, NH), 7.84 (1H, s, C^4-H), 7.51 (5H, br s, Ph-H), 6.95 (2H, br s, exchangeable with CD_3OD, NH), 2.87 (3H, s, SO_2CH_3). ^{13}C-NMR ($(CD_3)_2SO$): 145.04 (s, Ph), 135.99 (s, C^{3a}), 129.55 (d, Ph), 129.17 (d, Ph), 126.62 (s, C^7), 123.69 (d, Ph), 120.12 (s, C^{7a}), 78.83[4] (d, C^4), 42.53 (q, CH_3).

4,5-Dihydro-7-(methylsulfonyl)-1-phenyl-4-(2-phenylhydrazino)-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (4b)——Phenylhydrazine (158 mg, 1.46 mmol) was added to a solution of **1** (100 mg, 0.36 mmol) in EtOH (4 ml), and the mixture was refluxed for 8 h. After removal of the solvent (2 ml), the separated crystals were collected by suction and recrystallized from EtOH to give slightly yellow needles, mp 202—203 °C (dec.), in 44.5% yield (62 mg). *Anal.* Calcd for $C_{17}H_{17}N_7O_2S$: C, 53.25; H, 4.47; N, 25.57. Found: C, 53.24; H, 4.47; N, 25.60. MS *m/z*: 383 (M^+). ^{1}H-NMR ($(CD_3)_2SO$): 10.45 (1H, br s, exchangeable with CD_3OD, NH), 8.89 (2H, br s, exchangeable with CD_3OD, NH), 8.01 (1H, s, C^4-H), 7.51 (5H, s, Ph-H), 7.40—6.64 (5H, m, Ph-H), 2.69 (3H, s, SO_2CH_3). IR ν_{max}^{KBr} cm^{-1}: 3400—3170 (NH), 1315, 1140 (SO_2). ^{13}C-NMR ($(CD_3)_2SO$): 144.99 (s, Ph), 144.56 (s, Ph), 135.89 (s, C^{3a}), 129.44 (d, Ph), 129.11 (d, Ph), 128.79 (d, Ph), 126.73[4] (d, C^4), 126.35 (s, C^7), 123.53 (d, Ph), 120.01 (s, C^{7a}), 119.03 (d, Ph), 111.99 (d, Ph), 42.53 (q, CH_3).

Reaction of 7 with Amines——Standard Method: A mixture of **7** (100 mg, 0.43 mmol) and an amine (1 mmol) was heated for 1 h on a water bath (90 °C). After the mixture had cooled, ether was added, and the separated crystals were recrystallized from MeOH to give **12**.

From the reaction with aniline, 7-anilino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12a**) was obtained as yellow needles, mp 212—213 °C, in 45.8% yield (57 mg).

From the reaction with cyclohexylamine, 7-(cyclohexylamino)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12c**) was obtained as colorless prisms, mp 187—188 °C, in 62.2% yield (79 mg).

From the reaction with butylamine, 7-(butylamino)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12b**) was obtained as colorless prisms, mp 117—118 °C, in 62.2% yield (72 mg).

From the reaction with morpholine, 7-morpholino-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12d**) was obtained as slightly yellow plates, mp 207—208.5 °C, in 69.7% yield (85 mg).

From the reaction with piperidine, 1-phenyl-7-piperidino-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12e**) was obtained as yellow plates, mp 170—171 °C, in 44.6% yield (54 mg).

From the reaction with 4-methylpiperidine, 7-(4-methylpiperidino)-1-phenyl-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12f**) was obtained as colorless needles, mp 137—138 °C, in 53.5% yield (68 mg).

From the reaction with pyrrolidine, 1-phenyl-7-pyrrolidino-1*H*-1,2,3-triazolo[4,5-*d*]pyridazine (**12g**) was obtained as colorless prisms, mp 190—191 °C, in 58.3% yield (67 mg).

Elemental analysis, IR, and ^{1}H-NMR spectral data for **12a—g** are summarized in Tabels I and II.

Reaction of 1 with Primary Amines——Standard Method: A mixture of **1** (100 mg, 0.36 mmol) and a primary amine (0.9 mmol) was heated for 10 min on a water bath (90 °C). After rhe mixture had cooled, ether (5 ml) was added, and the separated **5** was recrystallized from ether.

The reaction with butylamine gave 4-(butyliminomethyl)-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**5b**) as colorless needles, mp 124—125 °C, in 57.6% yield (58 mg).

The reaction with cyclohexylamine gave 4-(cyclohexyliminomethyl)-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**5c**) as colorless needles, mp 155—156 °C, in 32.6% yield (41 mg).

The elemental analyses, IR, ^{1}H-NMR, and ^{13}C-NMR spectral data for **5b, c** are summarized in Tables I, II, and III.

Reaction of 1 with Cyclic Amines——Standard Method: The procedure for the reaction of **1** (100 mg, 0.36 mmol) with a cyclic amine (0.9 mmol) was essentially the same as that described for the reaction with primary amines, and the following 4-[di(cyclic amino)methyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazoles (**6d—f**) were obtained.

4-(Dimorpholinomethyl)-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**6d**) from morpholine: mp 174—176 °C (dec.), slightly yellow plates, yield 95.4% (146 mg).

4-(Dipiperidinomethyl)-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**6e**) from piperidine: mp 139—140 °C (dec.), colorless needles, yield 88.3% (134 mg).

4-[Bis(4-methylpiperidino)methyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**6f**) from 4-methylpiperidine: mp 146—148 °C, colorless needles, yield 77.8% (126 mg).

The elemental analysis, IR, ^{1}H-NMR, and ^{13}C-NMR spectral data are summarized in Tables I, II, and III.

Reaction of 6e with MeOH——A solution of **6e** (100 mg, 0.24 mmol) in MeOH (1 ml) was allowed to stand at room temperature overnight, and the separated crystals were recrystallized from MeOH to give 4-[α-(piperidino)methoxymethyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**13e**) as colorless needles, mp 116—118 °C, in 51.6% yield (45 mg). *Anal.* Calcd for $C_{17}H_{24}N_4O_3S$: C, 56.02; H, 6.64; N, 15.37. Found: C, 56.06; H, 6.62; N, 15.40. IR ν_{max}^{KBr} cm^{-1}: 1315, 1140 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.53 (5H, s, Ph-H), 5.14 (1H, s, O–CH–N), 4.64 (2H, s, Ar-CH_2SO_2), 3.52 (3H, s, OCH_3), 2.88 (3H, s, SO_2CH_3), 3.06—2.25 (4H, m, CH_2NCH_2), 1.82—1.10 (6H, m, $(CH_2)_3$). ^{13}C-NMR ($CDCl_3$): 145.53 (s, Ph), 135.56 (s, C^4), 130.03 (d, Ph), 129.44 (d, Ph), 126.19 (d, Ph), 124.34 (s, C^5), 92.76[4] (d, O–CH–N), 56.40 (q, OCH_3), 49.36 (t, CH_2SO_2), 48.55 (t, N–CH_2), 41.34 (q, SO_2CH_3), 26.01 (t, CH_2), 24.49 (t, CH_2).

Reaction of 6f with MeOH——4-[α-(4-Methylpiperidino)methoxymethyl]-5-(methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole (**13f**) was obtained as colorless needles, mp 137—138.5 °C, in 94.2% yield (80 mg) from the reaction of **6f** (100 mg, 0.22 mmol) with MeOH (1 ml) in essentially the same manner as described for the reaction of **6e** with MeOH. *Anal.* Calcd for $C_{18}H_{26}N_4O_3S$: C, 57.12; H, 6.92; N, 14.80. Found: C, 57.10; H, 6.90; N, 14.75. IR ν_{max}^{KBr} cm^{-1}: 1315, 1140 (SO_2). ^{1}H-NMR ($CDCl_3$): 7.55 (5H, s, Ph-H), 5.15 (1H, s, O–CH–N), 4.64 (2H, s, CH_2SO_2), 3.53 (3H, s, OCH_3), 3.29—2.00 (7H, m, $N(CH_2)_2$, SO_2CH_3), 1.80—0.76 (8H, m, CH, $2CH_2$, CH_3). ^{13}C-NMR ($CDCl_3$): 145.74 (s, Ph), 135.83 (s, C^4), 130.30 (d, Ph), 129.65 (d, Ph), 126.46 (d, Ph), 124.51 (s, C^5), 92.92 (d, O–CH–N), 56.51[4] (q, OCH_3), 49.68[4] (t, CH_2SO_2), 48.33 (t, NCH_2), 48.00 (t, NCH_2), 41.50 (q, SO_2CH_3), 34.62 (t, CH_2), 31.10 (d, CH), 21.89 (q, CH_3).

5-(Methylsulfonylmethyl)-1-phenyl-1*H*-1,2,3-triazole-4-carboxaldehyde (14)——i) A solution of **6d** (100 mg, 0.24 mmol) in $CHCl_3$ was passed through a column of SiO_2. The $CHCl_3$ eluate gave **14** as colorless prisms from MeOH, mp 98.5—99 °C, in 82.8% yield (52 mg). *Anal.* Calcd for $C_{11}H_{11}N_3O_3S$: C, 49.80; H, 4.18; N, 15.84. Found: C, 49.67; H, 4 29; N, 15.71. MS *m/z*: 265 (M^+). IR ν_{max}^{KBr} cm^{-1}: 1695 (CO), 1310, 1140 (SO_2). ^{1}H-NMR ($CDCl_3$): 10.24 (1H, s, CHO), 7.58 (5H, s, Ph-H), 4.73 (2H, s, CH_2SO_2), 2.96 (3H, s, SO_2CH_3). ^{13}C-NMR ($CDCl_3$): 186.60 (d, CHO), 144.61 (s, Ph), 134.42 (s, C^4), 131.17 (d, Ph), 129.98 (d, Ph), 126.51 (d, Ph), 126.24 (s, C^5), 49.47 (t, CH_2SO_2), 42.26 (q, SO_2CH_3).

ii) By the same procedure as described in i), **6e** (100 mg, 0.24 mmol) gave **14** in 68.2% yield (43 mg).

iii) By the same procedure as described in i), **6f** (100 mg, 0.22 mmol) gave **14** in 58.8% yield (35 mg).

Reaction of 14 with Amines——Standard Method: A solution of **14** (50 mg, 0.19 mmol), an amine (0.42 mmol), and a catalytic amount of TsOH in EtOH (1 ml) was refluxed for 30 min. After removal of the solvent, ether (2 ml) was added to the residue, and the separated crystals were recrystallized.

From the reaction with butylamine, **5b** was obtained in 39.7% yield (24 mg).

From the reaction with cyclohexylamine, **5c** was obtained in 67.4% yield (44 mg).

From the reaction with morpholine, **6d** was obtained in 84.3% yield (67 mg).

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New Triterpene Saponins from *Ilex pubescens*

KAZUYUKI HIDAKA,[a] MIYOKO ITO,[a] YOKO MATSUDA,[a] HIROSHI KOHDA,*[,a]
KAZUO YAMASAKI,[a] JOHJI YAMAHARA,[b] TAKESHI CHISAKA,[b]
YOSHIYUKI KAWAKAMI,[c] TADASHI SATO[c]
and KENGO KAGEI[c]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[a] 1–2–3, Kasumi, Minami-ku, Hiroshima 734, Japan, Department of Pharmacognosy, Kyoto Pharmaceutical University,[b] Misasagi, Yamashina-ku, Kyoto 607, Japan, and Tsukuba Research Laboratory, Eisai Co., Ltd.,[c] 5–1–3, Tokodai, Toyosato-cho, Tsukuba-gun, Ibaragi 300–26, Japan

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Novel triterpenoid saponins, ilexsaponins B_1, B_2 and B_3, have been isolated from the roots of *Ilex pubescens*. Degradative and spectroscopic studies have established their structures as shown in formulae **5**, **6**, and **7**. Ilexsaponin B_3 was found to possess an activity against experimental hypercholesteremia in mice.

Keywords——*Ilex pubescens*; Aquifoliaceae; triterpene; saponin; antihypercholesteremia; ^{13}C-NMR

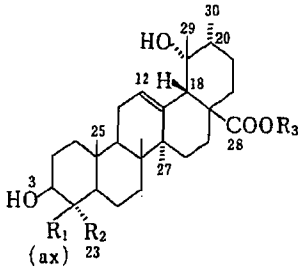
In China, "mao-dong-qing" (毛冬青), the root of *Ilex pubescens* HOOK. *et* ARN. (Aquifoliaceae), is widely used for the treatment of cardiovascular diseases, hypercholesteremia, *etc.*[1] In a preliminary screening test for the antihypercholesteremic action of several crude drugs, this plant showed significant activity. For the purpose of identifying the active principle, the chemical constituents of mao-dong-qing were investigated. This plant is known to contain several simple phenolics, *i.e.* 3,4-dihydroxyacetophenone, hydroquinone, scopoletin, esculetin, homovanillic acid and glaberide I, a natural degradation product of syringaresinol.[2] From a closely related species, *I. cornuta*, two glycosides of pomolic acid (**3**),[3] and from *I. rotunda*, oleanolic acid and rotundic acid (**4**)[4] have been isolated.

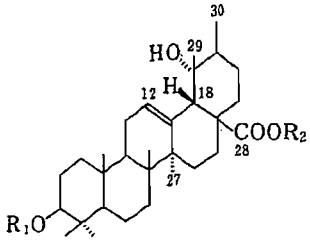
During the course of our investigations on the chemical constituents of *Ilex pubescens*, we have isolated and elucidated the structure of a triterpene, ilexgenin A (**1**), and its glucoside, ilexsaponin A_1 (**2**) from the EtOAc-soluble fraction of the MeOH extract of the roots.[5] In the present study we report the isolation and the structural elucidation of three new triterpene glycosides having a new aglycone, from the BuOH-soluble fraction of the MeOH extract of the roots. One of the compounds showed antihypercholesteremic activity.

Mao-dong-qing, the dried roots of *Ilex pubescens* were first extracted with benzene, followed by MeOH. The MeOH extract was suspended in water, then extracted with benzene, EtOAc, 1-BuOH–H_2O successively, and the antihypercholesteremic activity of each fraction was assayed. The BuOH-soluble fraction showed significant activity.

From the EtOAc fraction, compounds **1** and **2** were isolated by reversed-phase and normal-phase column chromatography.[5] Neither of them showed antihypercholesteremic activity.

From the active BuOH-soluble fraction, in addition to compound **1**, three new compounds, ilexsaponins B_1 (**5**), B_2 (**6**), and B_3 (**7**), were isolated by chromatography on Diaion HP-20, followed by silica gel chromatography. By the carbon-13 nuclear magnetic





	R_1	R_2	R_3
1	COOH	CH_3	H
2	COOH	CH_3	Glc
3	CH_3	CH_3	H
3a	CH_3	CH_3	CH_3
4	CH_3	CH_2OH	H
9	CH_2OH	CH_2OH	H

	R_1	R_2
5	-Xyl^2-Glc	H
6	-Xyl^2-Glc^2-Rha	H
7	-Xyl^2-Glc	Glc
8	H	H
8a	H	CH_3
10	-Xyl	H

Xyl: β-D-xylopyranosyl
Glc: β-D-glucopyranosyl
Rha: α-L-rhamnopyranosyl

TABLE I. ^{13}C-NMR Spectral Data for Genin Moieties of Ilexsaponins B_1 (**5**), B_2 (**6**) and B_3 (**7**) and Related Compounds

	3a[a]	**8a**[a]	**8**	**5**	**6**	**7**	**10**
C-1	38.7	38.4	39.0	38.8	38.8	38.8	38.7
C-2	27.4	27.1	28.1	27.0	27.0	26.8	27.0
C-3	79.0	79.0	78.2	88.9	89.7	88.8	88.7
C-4	38.5	38.7	39.4	39.5	39.7	39.6	39.5
C-5	55.2	55.1	55.9	55.9	56.0	55.9	55.9
C-6	18.4	18.4	19.0	18.6	18.6	18.7	18.6
C-7	32.8	32.7	33.6	33.5	33.5	33.5	33.5
C-8	39.9	39.8	40.3	40.2	40.2	40.3	40.2
C-9	47.2	47.3	47.8	47.7	47.7	47.7	47.7
C-10	36.9	37.0	37.4	36.9	37.0	36.9	37.0
C-11	23.7	23.9	24.9	24.8	24.9	24.6	24.8
C-12	129.1	128.5	127.3	127.2	127.3	127.5	127.2
C-13	138.0	137.7	139.5	139.5	139.5	138.8	139.4
C-14	41.1	41.1	42.2	42.0	42.1	42.1	42.1
C-15	28.1	28.1	29.3	29.2	29.2	29.1	29.2
C-16	25.5	26.1	27.1	26.6	26.6	26.7	26.7
C-17	47.9	47.4	48.0	47.8	47.9	48.3	47.9
C-18	53.2	46.6	47.4	47.3	47.4	47.1	47.4
C-19	73.1	74.0	73.4	73.4	73.4	73.4	73.4
C-20	41.1	41.3	43.1	42.8	43.0	42.6	42.9
C-21	26.0	23.6	24.0	23.9	23.9	23.8	24.0
C-22	37.4	31.2	32.4	32.3	32.5	31.7	32.4
C-23	28.1	28.1	28.8	28.0	28.4	28.1	28.2
C-24	15.2	15.2	15.6	15.5	15.4	15.6	15.5
C-25	15.5	15.6	16.2	16.1	16.1	16.0	16.1
C-26	16.6	16.7	17.3	17.2	17.3	17.4	17.3
C-27	24.5	24.5	24.4	24.3	24.4	24.3	24.4
C-28	178.3	178.4	180.7	180.7	180.7	177.1	180.9
C-29	27.2	30.1	29.9	29.8	29.8	29.7	29.8
C-30	16.1	15.8	16.6	16.6	16.7	16.7	16.9
$COO\underline{CH_3}$	51.5	51.6					

δ relative to TMS in C_6D_5N (except **3a** and **8a**). *a*) Measured in $CDCl_3$; data for **3a** are cited from ref. 7.

TABLE II. ^{13}C-NMR Spectral Data for Sugar Moieties of Ilexsaponins B_1 (5), B_2 (6) and B_3 (7) and the Prosapogenin (10)

	10	5	6	7
3-*O*-Sugar				
Xyl-1	107.6	105.7	105.9	105.8
Xyl-2	75.4	82.8	79.0	83.0
Xyl-3	78.5	77.8	77.8	77.5
Xyl-4	71.2	71.5	71.3	71.5
Xyl-5	67.0	66.5	66.6	66.7
Glc-1		105.8	102.3	105.8
Glc-2		76.8	79.4	76.9
Glc-3		78.1	79.0	78.3
Glc-4		70.8	72.3	70.9
Glc-5		77.8	78.5	77.9
Glc-6		62.6	63.3	62.6
Rha-1			102.0	
Rha-2			72.6	
Rha-3			72.6	
Rha-4			74.3	
Rha-5			69.5	
Rha-6			18.9	
28-*O*-Sugar				
Glc-1				95.7
Glc-2				74.0
Glc-3				79.1
Glc-4				70.9
Glc-5				78.8
Glc-6				62.1

δ relative to TMS in C_6D_5N.

resonance (^{13}C-NMR) analysis, **5**, **6**, and **7** were proved to have the same aglycone (Tables I and II).

Structure of the Aglycone (8)

Enzymic hydrolysis of **5** afforded the aglycone (**8**), $C_{30}H_{48}O_4$. The ^{13}C-NMR spectrum of **8** showed 30 carbon signals, *i.e.* $CH_3-\times 7$, $-CH_2-\times 9$, $>CH-\times 4$, $>C<\times 5$, $>CH-O\times 1$, $>C-O\times 1$, $>C=CH-\times 1$, $CO\times 1$. Its infrared (IR) spectrum indicated the presence of –OH (3400 cm^{-1}), and –COO (1690 cm^{-1}) groups. The electron impact mass spectra (EIMS) of **8** revealed the fragment peak at *m/z* 264, which is characteristic of retro-Diels–Alder cleavage of olean-12-ene or urs-12-en-28-oic acid having a hydroxy group on the D or E ring. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum in pyridine-d_5 showed a 1H singlet signal at δ 3.30 (1H, s), assignable to 18-H of 19-hydroxylated ursane. The spectroscopic data for the 8-methyl ester (**8a**) were compared with those of methyl pomolate (**3a**).[6] The EIMS of **8a** and **3a** were nearly identical, as were those of **8** and **3** (Table III).

The ^{13}C-NMR spectrum of **8a** was very similar to that of **3a**,[7] and A, B and C ring carbon signals of both compounds were essentially the same, though some carbon signals in rings D and E were significantly different (Table I). Among them, the carbon signals assignable to C-18 and C-22 of **8a** were shifted to high field by 6.6 and 6.2 ppm, respectively, from the corresponding carbon signals of **3a**. This can be explained in terms of the γ-effect of the 30-β-(axial) methyl group in place of α-(equatorial) methyl in **3a**. To confirm this, *J* and nuclear Overhauser effect (NOE) correlated 2-dimensional ^{1}H-NMR spectra of **8a** and acid (**4**) were measured at 400 MHz. Assignments of these spectra were mainly based on the reference

TABLE III. MS Data for 3, 3a, 8 and 8a

3[a]	8	3a[a]	8a
472 (M^+, 9%)	472 (M^+, 6%)	486 (M^+, 8%)	486 (M^+, 10%)
454 (M^+-H_2O, 12%)	454 (M^+-H_2O, 9%)	468 (M^+-H_2O, 9%)	468 (M^+-H_2O, 7%)
439 ($M^+-H_2O-CH_3$, 8%)	439 ($M^+-H_2O-CH_3$, 3%)	453 ($M^+-H_2O-CH_3$, 5%)	453 ($M^+-H_2O-CH_3$, 3%)
264 (16%)	264 (6%)	426 ($M^+-HCOOCH_3$, 21%)	426 ($M^+-HCOOCH_3$, 20%)
207 (40%)	207 (33%)	278 (8%)	278 (5%)
146 (100%)	146 (100%)	207 (31%)	207 (29%)
		179 (100%)	179 (100%)

a) Data are cited from ref. 6.

data for clethric acid (**9**).[7] However, the original assignments of the C-27 and C-29 methyl groups should be reversed, since, in the NMR experiments on the stereochemically similar compound **4**, on irradiation of 18-H (δ2.60), NOE was observed at δ1.21 (29-Me), not at δ1.27 (27-Me). With this change, the stereochemistry of **4** was reasonably explained. In the ^{1}H-NMR spectrum of **8a**, NOE was observed between 12-H (δ5.32) and 18-H (δ2.82), and irradiation of 18-H gave NOE at 29-CH_3 (δ1.16) and 30-CH_3 (δ0.99). On irradiation of 29-CH_3, NOE was observed at 12-H, 18-H and 30-CH_3. Therefore, it was concluded that all of these atoms or functional groups have the same configuration, β. It follows that **8** can be formulated as (30*S*)-3β,19α-dihydroxyurs-12-en-28-oic acid, and we named it ilexgenin B.

Structures of Ilexsaponins B_1 (5), B_2 (6) and B_3 (7)

In the ^{13}C-NMR spectrum of **5**, two sets of monosaccharide signals were observed, and the aglycone carbon signals of the B, C, D and E rings were nearly identical to those of ilexgenin B (**8**). A significantly different chemical shift was observed at C-3, which was shifted downfield by 10.7 ppm on going from **8** to **5** (Tables II and III). Acid hydrolysis of **5** afforded xylose and glucose, identified by gas liquid chromatography (GLC) analysis. By ^{13}C- and ^{1}H-NMR analyses, these sugars were identified as β-xylopyranose (anomeric H, δ4.82, d, J=6.3 Hz) and β-glucopyranose (anomeric H, δ5.36, d, J=7.6 Hz). The absolute configurations of these sugars were assumed to be both D, by analogy with the glycosylation shift of holothurins, 2-quinovosylxylosides.[8,9] Gas chromatography-mass spectrometry (GC-MS) analysis of the acetates of the acid hydrolysis products of permethylated **5** revealed 2-linked xylopyranose and terminal glucopyranose. The EIMS of the acetate gave a peak at *m/z* 331 [glucose(Ac)$_4$]$^+$ and 547 [glucose(Ac)$_4$ − xylose(Ac)$_2$]$^+$. It follows that **5** can be formulated as the 3-*O*-β-D-glucopyranosyl(1→2)-β-D-xylopyranoside of **8**.

On enzymic hydrolysis, ilexsaponin B_2 (**6**) afforded the aglycone (**8**), the prosapogenin (**10**), and a saponin (**5**). In the ^{13}C-NMR spectrum of **6**, three sets of monosaccharide signals were observed, and the aglycone signals are identical with those of **5**. The structure of the oligosaccharide at the C-3-*O*-position was determined as above. Xylose, glucose, and rhamnose were detected by GLC, and the form of the sugars was deduced from the ^{13}C-NMR (Table II) and ^{1}H-NMR spectra. In the ^{1}H-NMR spectrum of **6**, anomeric signals of the sugars appear at δ4.91 (1H, d, J=5.4 Hz), δ5.83 (1H, d, J=7.1 Hz), δ6.41 (1H, brs) for xylose, glucose and rhamnose, respectively, confirming them to be β-xylopyranose, β-glucopyranose and α-rhamnopyranose. The GC-MS analysis revealed 2-linked xylopyranose, 2-linked glucopyranose, and terminal rhamnopyranose. In addition, the EIMS of the acetate of **6** showed peaks at *m/z* 273 [rhamnose(Ac)$_3$]$^+$ and 561 [rhamnose(Ac)$_3$−glucose(Ac)$_3$]$^+$. It follows that **6** can be formulated as the 3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-xylopyranoside of **8**.

The ^{13}C-NMR spectrum of **7** is very similar to that of **5**. The difference is that in the spectrum of **7**, the C-28 signal was shifted upfield by 3.6 ppm and a set of esterified β-glucopyranosyl signals was observed. In the ^{1}H-NMR spectrum, three anomeric signals were observed at δ 4.82 (1H, distorted d by virtual coupling), δ 5.39 (1H, d, J=6.3 Hz) and δ 6.37 (1H, d, J=6.8 Hz). Selective ester glycoside cleavage reaction[10] of **7** afforded ilexsaponin B_1 (**5**) and methyl glucopyranoside. It follows that **7** can be formulated as the 28-*O*-β-D-glucopyranoside of **5**.

Among the ilexsaponins isolated from *Ilex pubescens*, only ilexsaponin B_3 (**7**) showed the antihypercholesteremic activity. The value of inhibition by **7** was 68% of the treated control at the oral dose of 300 mg/kg.

Experimental

General Procedure——NMR spectra were taken at 25 °C using tetramethylsilane (TMS) as an internal standard; ^{13}C-NMR at 25.15 MHz and ^{1}H-NMR at 100 MHz unless otherwise stated. EIMS were taken at 75 eV. Melting points were taken on a micro hot stage and are uncorrected. Acid hydrolysis of each glycoside was carried out in the usual way, and the resulting monosaccharides were identified by GC as their trimethylsilyl ethers.

Plant Materials——A sample of "Mao-dong-qing," the root of *Ilex pubescens* HOOK. *et* ARN., was purchased from Mikuni Co., Ltd. (Osaka), in 1983.

Bioassay for Anti-hypercholesteremic Activity——The activity was examined in male dd-k mice (weighing *ca.* 23 g) which were fed on "fat emulsion" (olive oil, 32.6%; cholesterol, 2.5%; sucrose, 42.0%; sodium cholate, 1.8%, water, 21.1%). Just after, and 7 h after the administration of fat emulsion, samples to be tested were administered orally in the form of a suspension in Gumi Arabicum. At 17 h after the last feeding, the animals were anesthetized with ether, and the blood samples for cholesterol determination were collected from the abdominal aorta. The blood samples were centrifuged at 3000 *g*, and the total cholesterol content of the blood serum was measured by the cholesterol oxidase–*p*-chlorophenol method, using the Cholesterol C II-Test Wako (Wako Pure Chemical Co., Ltd.). As positive control drugs, Niceritol and Nicomol were used.

Extraction of Triterpene and Glycosides——Dried roots (4.7 kg) were crushed and extracted with benzene (20 l ×2), the residue was extracted with MeOH (20 l × 4) and the MeOH extract was evaporated to dryness (414 g). A suspension of the resulting extract in H_2O was washed with benzene, then extracted with EtOAc and BuOH/H_2O successively to give the extracts, 103 and 256 g, respectively.

A portion of the BuOH extract (32 g) was chromatographed on a column of Diaion HP-20 (Mitsubishi Chemical Ind. Co.) and eluted with 15% MeOH, 30% MeOH, MeOH and $CHCl_3$–MeOH (1 : 1), successively. The MeOH eluate was divided into two fractions before and after the appearance of a yellow band. This Diaion-chromatography was repeated 8 times to separate all the BuOH extract, affording 171 g of crude glycoside fraction as the second MeOH eluate. The glycoside fraction (156 g) was chromatographed on silica gel using the $CHCl_3$–MeOH–H_2O system, and divided into 31 fractions. From fractions 4 and 5 ($CHCl_3$–MeOH–H_2O, 15 : 3 : 0.2 eluate), compound **1** (3.1 g) was obtained. From fractions 10 and 11 ($CHCl_3$–MeOH–H_2O, 15 : 4 : 0.4 eluate), **5** (6.7 g) was obtained as colorless crystals. Fraction 15 ($CHCl_3$–MeOH–H_2O, 15 : 6 : 1 eluate) was chromatographed on ODS-Gel using MeOH–H_2O gradient to give **7** (610 mg, from 70% MeOH) and **6** (480 mg, from 60% EtOH).

Ilexsaponin B_1 (5)——mp 246—248 °C (MeOH), $[\alpha]_D^{20}$ −9.6° (c=1.01, pyridine). *Anal.* Calcd for $C_{41}H_{66}O_{13}\cdot 2H_2O$: C, 61.32; H, 8.79. Found: C, 61.32; H, 8.79. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1690. EIMS of acetate[11] *m/z*: 331 $[Glc(Ac)_4]^+$, 547 $[Glc(Ac)_4-Xyl(Ac)_2]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.90, 1.09, 1.10, 1.26, 1.44, 1.75 (3H × 6, s, 23, 24, 25, 26, 27 and 29-CH_3). 1.13 (3H, d, J=7.3 Hz, 30-CH_3), 3.29 (1H, s, 18-H), 4.82 (1H, d, J=6.3 Hz, Xyl-1-H), 5.05 (1H, s, 19α-OH), 5.36 (1H, d, J=7.6 Hz, Glc-1-H), 5.56 (1H, br t, 12-H). ^{13}C-NMR see Tables I and II.

Enzymic Hydrolysis of Ilexsaponin B_1 (5)——A few drops of toluene were added to a solution of **5** (370 mg) and crude hesperidinase (300 mg, Tanabe Pharm. Ind. Co., Ltd., Osaka) in McIlvaine buffer (pH 4.0, 200 ml). The mixture was incubated at 38 °C for 90 h and then extracted with $CHCl_3$ and BuOH saturated with water, successively. Each organic layer was washed with water and concentrated to dryness, affording 85 and 171 mg of residue, respectively. Both fractions were chromatographed on silica gel, affording the aglycone **8** (58 and 37 mg, respectively), and prosapogenin **10** (6 and 57 mg, respectively).

Ilexgenin B (8)——mp 276—279 °C (MeOH), $[\alpha]_D^{20}$ +28.1° (c=0.48, pyridine). *Anal.* Calcd for $C_{30}H_{48}O_4\cdot 1/2H_2O$: C, 74.80; H, 10.25. Found: C, 74.98; H, 10.47. High-resolution MS *m/z*: 472.3529, Calcd for $C_{30}H_{48}O_4$, 472.3553. EIMS see Table I. IR ν_{max}^{Nujol} cm^{-1}: 3450, 1690. ^{1}H-NMR (in pyridine-d_5) δ: 0.93, 1.04, 1.11, 1.24, 1.47, 1.76 (3H, s × 6), 1.14 (3H, d, J=6.4 Hz, 30-CH_3), 3.30 (1H, s, 18-H), 5.14 (1H, br s, 19-OH), 5.58 (1H, br s, 12-H). ^{13}C-NMR see Table I.

Methylation of 8——**8** (27 mg) was methylated with diazomethane to afford **8a** (9 mg). mp 119—123 °C from

MeOH, $[\alpha]_D^{20}$ +28.1° (c=0.48, $CHCl_3$). High-resolution MS m/z: 486.3709, Calcd for $C_{31}H_{50}O_4$, 486.3709. ^{1}H-NMR (in $CDCl_3$, 400 MHz) δ: 0.67, 0.78, 0.90, 0.98, 1.16, 1.23 (each 3H, s, 26-CH_3, 25-CH_3, 23-CH_3, 24-CH_3, 29-CH_3, 27-CH_3, respectively), 0.99 (3H, d, J=6 Hz, 30-CH_3), 2.82 (1H, s, 18-H), 3.22 (1H, dd, J=4, 10 Hz, 3αH), 3.62 (3H, s, -COOMe), 5.32 (1H, t, J=3 Hz, 12-H). ^{13}C-NMR see Table 1.

Prosapogenin (10)——^{13}C-NMR see Tables I and II.

Ilexsaponin B_2 (6)——mp 240—243 °C (EtOH), $[\alpha]_D^{20}$ −15.3° (c=1.20, pyridine). *Anal.* Calcd for $C_{47}H_{76}O_{17}\cdot 5/2H_2O$: C, 58.91; H, 8.35. Found: C, 58.94; H, 8.35. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1685. EIMS of acetate[11] m/z: 273 $[Rha(Ac)_3]^+$, 561 $[Rha(Ac)_3-Glc(Ac)_3]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.86, 1.08, 1.35, 1.43, 1.76 (3H×6, s), 1.12 (3H, d, J=9.0 Hz, 30-CH_3), 1.79 (3H, d, J=6.1 Hz, Rha-6-H), 3.30 (1H, s, 18-H), 4.91 (1H, d, J=5.4 Hz, Xyl-1H), 5.08 (1H, s, 19α-OH), 5.56 (1H, br t, 12-H), 5.83 (1H, d, J=7.1 Hz, Glc-1-H), 6.41 (1H, br s, Rha-1-H). ^{13}C-NMR see Tables I and II.

Enzymic Hydrolysis of Ilexaponin B_2 (6)——A few drops of toluene were added to a solution of **6** (100 mg) and crude hesperidinase (98 mg, Tanabe Pharm. Ind. Co., Ltd., Osaka) in McIlvaine buffer (pH 4.0, 100 ml). The mixture was incubated at 37 °C for 48 h and then extracted with $CHCl_3$ and BuOH saturated with water, successively. Each organic layer was washed with water and concentrated to dryness, affording 3 and 68 mg of residue, respectively. The latter was chromatographed on silica gel, affording the aglycone **8** (19 mg), the prosapogenin **10** (4 mg) and ilexsaponin B_1 (**5**) (11 mg).

Ilexsaponin B_3 (7)——A white powder, $[\alpha]_D^{20}$ +10.0° (c=0.40, MeOH). *Anal.* Calcd for $C_{47}H_{76}O_{18}\cdot 5/2H_2O$: C, 57.95; H, 8.38. Found C, 57.98; H, 8.31. IR ν_{max}^{Nujol} cm^{-1}: 3300, 1720. EIMS of peracetate[11] m/z: 331 $[Glc(Ac)_4]^+$, 547 $[Glc(Ac)_4-Xyl(Ac)_2]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.95, 1.13, 1.21, 1.26, 1.41, 1.72 (3H×6, s), 0.99 (3H, d, J=8.8 Hz, C-30-Me), 3.20 (1H, s, 18-H), 4.82 (1H, distorted d by virtual coupling, Xyl-1-H), 5.19 (1H, s, 19α-OH), 5.39 (1H, d, J=6.3 Hz, 3-*O*-Glc-1-H), 5.56 (1H, br t, 12-H), 6.37 (1H, d, J=6.8 Hz, 28-*O*-Glc-1-H). ^{13}C-NMR see Tables I and II.

Selective Hydrolysis of Ester Sugar of Ilexsaponin B_3 (7)——A solution of **7** (51 mg) and anhydrous LiI (40 mg) in 2,6-lutidine (3 ml) and anhydrous MeOH (2 ml) was refluxed under N_2 gas for 13 h. After addition of H_2O (1 ml), the reaction mixture was deionized by passing it through a column of Amberlite MB-3 and then chromatographed on silica gel to give ilexsaponin B_1 (**5**, 20 mg) and methyl glucopyranoside (9 mg) by elution with chloroform–methanol–H_2O (150:25:1).

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11) A saponin (*ca.* 1 mg) was heated with 5 drops of pyridine and 3 drops of Ac_2O in a sealed tube at 100 °C for 1 h. The reaction mixture was concentrated to dryness by blowing N_2 gas over it at room temperature. The residue was directly used for MS measurement.

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Studies on 1-Alkyl-2(1*H*)-pyridone Derivatives. XXX.[1] Reactions of 1-Alkyl-2(1*H*)-pyridones with Fumaric Acid Monomethyl Ester and Acrylic Acid

HIROSHI TOMISAWA,* HIROSHI HONGO, TOSHIMITSU HATANO, HIROTO NAKANO, and REIKO FUJITA

Tohoku College of Pharmacy, 4–4–1, Komatsushima, Sendai 983, Japan

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Studies aimed at the synthesis of 6-azabicyclo[3.2.1]octane derivatives from 1-alkyl-2(1*H*)-pyridones (Ia—d) were carried out. Reactions of Ia—d with fumaric acid monomethyl ester and acrylic acid gave 6-alkyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acids (VIIa—d) and 6-alkyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acids (Xa—d), respectively.

Keywords——1-alkyl-2(1*H*)-pyridone; fumaric acid monomethyl ester; acrylic acid; 6-azabicyclo[3.2.1]octane; 6-alkyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acid; 6-alkyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acid

Diels–Alder adducts of 1-substituted-2-pyridones are interesting as possible synthetic intermediates[2] of iboga alkaloids. Our previous work[3] in this series has shown that the reactions of 1-methyl-2(1*H*)-pyridone (Ia) with dimethyl fumarate (II) and fumaric acid (III) gave dimethyl 2-methyl-3-oxo-2-azabicyclo[2.2.2]oct-7-ene-5-*endo*-6-*exo*-dicarboxylate (IV) and 6-methyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2,8-*endo*-dicarboxylic acid (Va) (which would be formed by transformation from the Diels–Alder adduct), respectively, as shown in Chart 1. These results prompted us to examine the reactions of 1-substituted-2-pyridone with dienophiles having a carboxyl group in the expectation that they might give 6-azabicyclo[3.2.1]octene derivatives. This expectation was realized, and the results are reported in the present paper (Chart 2).

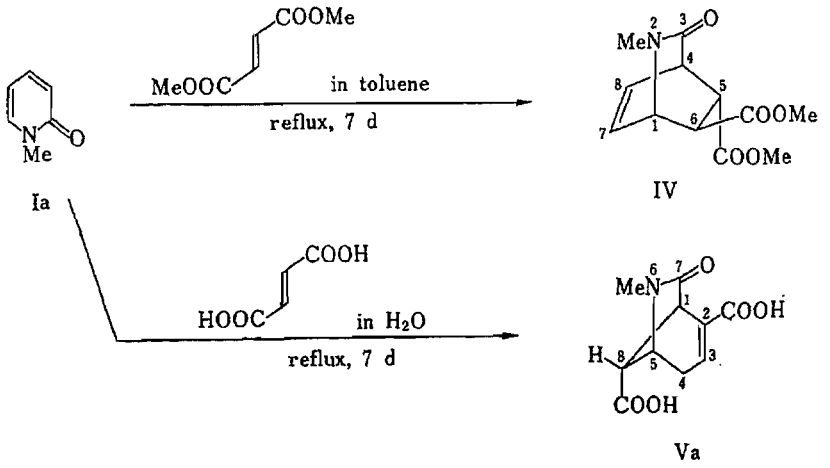
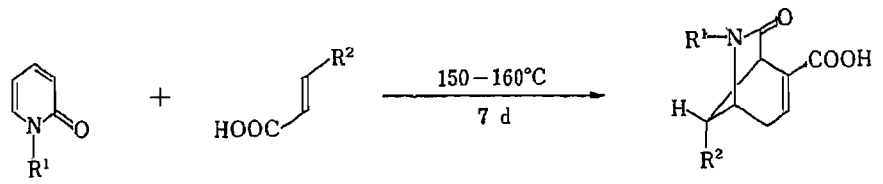


Chart 1



Ia : R^1=Me
Ib : R^1=$C_6H_5CH_2$
Ic : R^1=Et
Id : R^1=$C_6H_5CH_2CH_2$

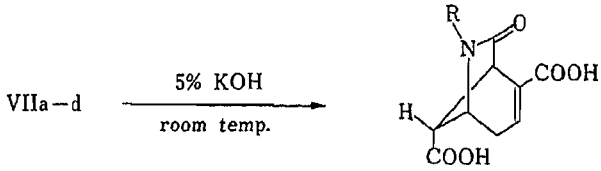
VI : R^2=COOMe
IX : R^2=H

VIIa—d and Xa—d

Chart 2

Reactions of 1-Benzyl-2(1*H*)-pyridone with Fumaric Acid Monomethyl Ester and Acrylic Acid

The reaction of 1-benzyl-2(1*H*)-pyridone (Ib) with fumaric acid monomethyl ester (VI) was carried out at 150—160 °C for 7 d to produce 6-benzyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acid (VIIb), as shown in Table I. The structure of VIIb was confirmed in the following way. The elemental analysis and the spectral examinations indicated that VIIb was a 6-azabicyclo[3.2.1]octane derivative formed from the adduct of Ib and VI. Hydrolysis of VIIb gave 6-benzyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2,8-*endo*-dicarboxylic acid (Vb)[4] (Chart 3). Next, the location of the methoxycarbonyl group was determined by inspection of the infrared (IR) spectra of VIIb and the diester (VIII). Esterification of VIIb with MeOH and $SOCl_2$ afforded dimethyl 6-benzyl-7-oxo-6-azabicyclo [3.2.1]oct-2-ene-2,8-*endo*-dicarboxylate (VIII), the IR spectrum of which showed absorptions due to ester carbonyl (8-position) at 1724 cm^{-1} and ester conjugated with a double bond (2-position) at 1716 cm^{-1}. Based on a comparison of the IR spectrum of VIIb with that of VIII, the carbonyl absorptions at 1725 and 1710 cm^{-1} in the IR spectrum of VIIb could be assigned to an ester and a carboxyl group conjugated with a double bond, respectively. Consequently, it was proved that the methoxycarbonyl group was at the 8-position in VIIb.



Va—d

Va[3] : R=Me
Vb[4] : R=$C_6H_5CH_2$
Vc[4] : R=Et
Vd[4] : R=$C_6H_5CH_2CH_2$

Chart 3

Heating of Ib and acrylic acid (IX) at 150—160 °C for 7 d afforded 6-benzyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic acid (Xb), as shown in Table I. Confirmation of its structure was provided by the elemental analysis and spectral examinations. The IR spectrum of Xb exhibited absorptions due to lactam carbonyl at 1645 cm^{-1} and a carboxyl group conjugated with a double bond at 1705 cm^{-1}. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of Xb in pyridine-d_5 showed the signals due to one proton at δ 7.00 and two protons at δ 1.95—2.35, which could be assigned to the C_3-proton at the double bond conjugated with the carboxyl group and methylene at the 4-position, respectively. Moreover, the ^{1}H-NMR spectral characteristics of Xb were quite similar to those of Vb and VIIb.

The mechanism of the formation of Xb was investigated as follows. 2-Benzyl-2-azabicyclo[2.2.2]oct-7-ene-5-*exo*-carboxylic acid (XI)[2] was suggested as a possible intermediate in a speculative mechanism for the synthesis of the 6-azabicyclo[3.2.1]octene system

reported in our previous paper,[3] as shown in Chart 4. Heating of XI at 150—160 °C for 48 h gave Xb in 66.7% yield, whereas methyl 2-benzyl-2-azabicyclo[2.2.2]oct-7-ene-5-*exo*-carboxylate[2] was recovered in 60% yield under the same reaction conditions. The above results offer a chemical basis for the mechanism and provide the location of the methoxycarbonyl group in VIIb, as shown in Chart 4.

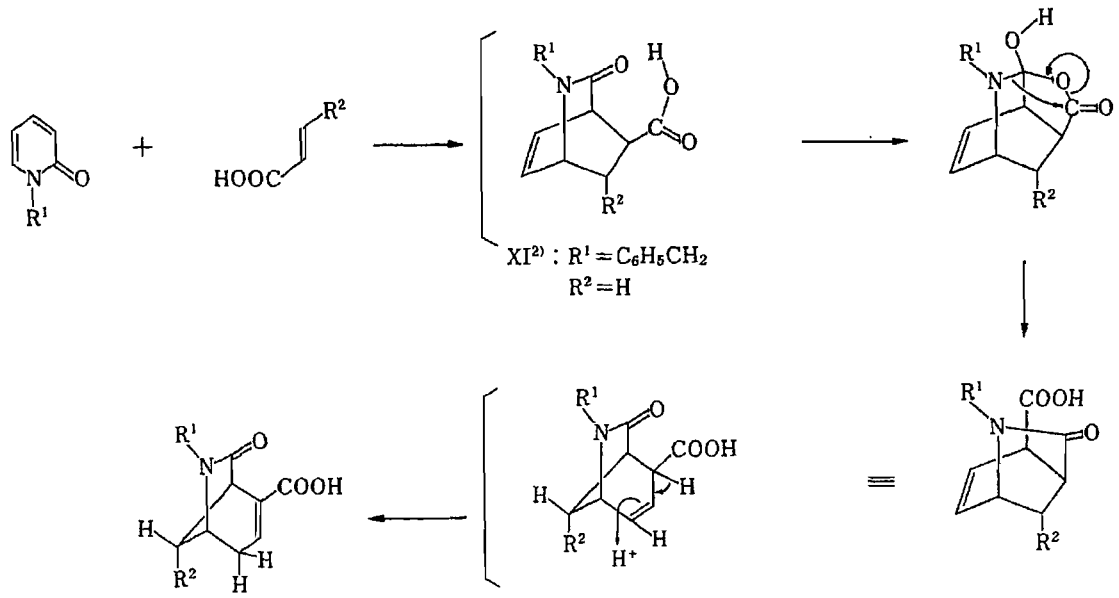


Chart 4

Reactions of 1-Alkyl-2(1*H*)-pyridones with VI and IX

The reactions of 1-alkyl-2(1*H*)-pyridones (Ia, c, d) with VI and IX gave VIIa, c, d and Xa, c, d, respectively, as shown in Table I. These structures were confirmed by the elemental analyses and spectral examinations. Furthermore, VIIa, c, d were hydrolyzed to give the corresponding dicarboxylic acids (Va, c, d) (Chart 3), which were identical with the corresponding authentic samples.[3,4]

Table I. Reactions of 1-Alkyl-2(1*H*)-pyridones (Ia—d) with VI and IX

Starting material I	R^1	Dienophile (VI or IX) R^2	Product (VII or X) Yield (%)	Recovery of I (%)
a	Me	COOMe	20.8	40.4
a	Me	H	15.2	48.3
b	$C_6H_5CH_2$	COOMe	22	56.9
b	$C_6H_5CH_2$	H	21.8	56.8
c	Et	COOMe	9.4	58.5
c	Et	H	9.9	56.7
d	$C_6H_5CH_2CH_2$	COOMe	14.3	64.7
d	$C_6H_5CH_2CH_2$	H	30.1	52

Experimental

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu IR-430 spectrophotometer. Mass spectra (MS) were taken on a Hitachi RMU-6MG spectrometer. ^{1}H-NMR spectra were taken at 60 MHz with tetramethylsilane (TMS) as an internal standard on

a JEOL JNM-PMX 60 spectrometer, in C_5D_5N unless otherwise noted. The chemical shifts are expressed as ppm downfield from TMS. The following abbreviations are used: s=singlet; d=doublet; t=triplet; q=quartet; m=multiplet; br=broad and Ar=aromatic. The unit (Hz) of coupling constants (*J* Hz) is omitted.

General Procedure for the Preparation of 6-Alkyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acids (VIIa—d)——A mixture of a 1-alkyl-2(1*H*)-pyridone (Ia—d, 30 mmol) and fumaric acid monomethyl ester (VI, 15 mmol) was heated in a sealed tube at 150—160 °C (an oil bath) for 7 d. The reaction mixture was washed with hot isopropyl ether. The resulting residue was chromatographed on a column of silica gel. The fraction eluted with $CHCl_3$–MeOH (50 : 1) was evaporated to give VIIa—d (Table I).

8-*endo*-Methoxycarbonyl-6-methyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (VIIa): Colorless prisms, mp 239—240 °C (EtOH). *Anal.* Calcd for $C_{11}H_{13}NO_5$: C, 55.23; H, 5.48; N, 5.86. Found: C, 55.06; H, 5.46; N, 5.75. MS *m/z*: 239 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1742 (COOMe), 1708 (COOH), 1660 (NC=O). ^{1}H-NMR δ: 2.33 (1H, br d, *J*=18, C_4-H_{endo}), 2.67 (1H, br d, *J*=18, C_4-H_{exo}), 2.77 (3H, s, N-Me), 3.40—3.63 (1H, m, C_8-H), 3.50 (3H, s, COOMe), 3.87 (1H, m, C_5-H), 4.33 (1H, d, *J*=5, C_1-H), 7.00 (1H, m, C_3-H).

6-Benzyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (VIIb): Colorless prisms, mp 194—196 °C (EtOH). *Anal.* Calcd for $C_{17}H_{17}NO_5$: C, 64.75; H, 5.45; N, 4.44. Found: C, 64.79; H, 5.48; N, 4.35. MS *m/z*: 315 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1725 (COOMe), 1710 (COOH), 1655 (NC=O), 732, 702 (δCH). ^{1}H-NMR δ: 2.43 (1H, br d, *J*=20, C_4-H_{endo}), 2.63 (1H, br d, *J*=20, C_4-H_{exo}), 3.45—3.77 (1H, m, C_8-H), 3.53 (3H, s, COOMe), 4.00 (1H, m, C_5-H), 4.37 (1H, d, *J*=15, N-C<u>H</u>-C_6H_5), 4.40—4.57 (1H, m, C_1-H), 4.85 (1H, d, *J*=15, N-C<u>H</u>-C_6H_5), 7.10 (1H, br s, C_3-H), 7.37 (5H, s, Ar-H).

6-Ethyl-8-*endo*-methoxycarbonyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (VIIc): Colorless prisms, mp 189—190 °C (Me_2CO). *Anal.* Calcd for $C_{12}H_{15}NO_5$: C, 56.91; H, 5.97; N, 5.53. Found: C, 56.69; H, 5.95; N, 5.55. MS *m/z*: 253 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1740 (COOMe), 1710 (COOH), 1660 (NC=O). ^{1}H-NMR δ: 1.10 (3H, t, *J*=7, CH_2-C<u>H</u>$_3$), 2.37 (1H, br d, *J*=20, C_4-H_{endo}), 2.73 (1H, br d, *J*=20, C_4-H_{exo}), 3.13 (2H, q, *J*=7, C<u>H</u>$_2$-CH_3), 3.40—3.80 (1H, m, C_8-H), 3.60 (3H, s, COOMe), 4.13 (1H, m, C_5-H), 4.37 (1H, d, *J*=5, C_1-H), 7.13 (1H, m, C_3-H).

8-*endo*-Methoxycarbonyl-7-oxo-6-phenethyl-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (VIId): Colorless prisms, mp 212—213 °C (EtOH). *Anal.* Calcd for $C_{18}H_{19}NO_5$: C, 65.64; H, 5.82; N, 4.25. Found: C, 65.67; H, 5.81; N, 4.14. MS *m/z*: 329 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1735 (COOMe), 1705 (COOH), 1658 (NC=O), 742, 698 (δCH). ^{1}H-NMR δ: 2.40 (1H, br d, *J*=20, C_4-H_{endo}), 2.50—3.20 (3H, m, C_4-H_{exo}, N-CH-C<u>H</u>$_2$-C_6H_5), 3.30—4.15 (4H, m, C_5-H, C_8-H, N-C<u>H</u>$_2$-CH-C_6H_5), 3.53 (3H, s, COOMe), 4.33 (1H, d, *J*=5, C_1-H), 7.10 (1H, m, C_3-H), 7.30 (5H, s, Ar-H).

Preparation of Va—d by Hydrolysis of VIIa—d——A mixture of VIIa—d (1 mmol), 10% KOH (3 ml) and MeOH (3 ml) was stirred at room temperature for 24 h. The reaction mixture was acidified with 10% HCl (3 ml). The resulting solid was collected and recrystallized from EtOH to give Va—d (Chart 3) in 63.8, 73.8, 68.5 and 73.1% yields, respectively. The products (Va—d) were identified by comparison with the corresponding authentic samples.[3,4]

Preparation of VIII—Compound VIIb (0.32 g) was added to an ice-cooled mixrure of $SOCl_2$ (0.3 g) and MeOH (3 ml), and the mixture was stirred overnight at room temperature. The reaction mixture was poured into ice-water and made basic with $NaHCO_3$, and the basic mixture was extracted with benzene. The benzene extract was evaporated to give a solid, which was recrystallized from benzene to afford dimethyl 6-benzyl-7-oxo-6-azabicyclo-[3.2.1]oct-2-ene-2,8-*endo*-dicarboxylate (VIII, 0.26 g), mp 86—87 °C, in 77.8% yields as colorless prisms. *Anal.* Calcd for $C_{18}H_{19}NO_5$: C, 65.64; H, 5.82; N, 4.25. Found: C, 65.67; H, 5.89; N, 4.13. MS *m/z*: 329 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1724, 1716 (COOMe), 1672 (NC=O), 724, 700 (δCH). ^{1}H-NMR ($CDCl_3$) δ: 2.27 (1H, br d, *J*=20, C_4-H_{endo}), 2.63 (1H, br d, *J*=20, C_4-H_{exo}), 3.27 (1H, t, *J*=5, C_8-H), 3.63 (3H, s, COOMe), 3.80—4.07 (2H, m, C_1-H, C_5-H), 3.83 (3H, s, COOMe), 4.20 (1H, d, *J*=14, N-C<u>H</u>-C_6H_5), 4.77 (1H, d, *J*=14, N-C<u>H</u>-C_6H_5), 6.80 (1H, br s, C_3-H), 7.27 (5H, s, Ar-H).

General Procedure for the Preparation of 6-Alkyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acids (Xa—d)——A mixture of a 1-alkyl-2(1*H*)-pyridone (Ia—d, 30 mmol) and acrylic acid (IX, 15 mmol) was heated in a sealed tube at 150—160 °C (an oil bath) for 7 d. The reaction mixture in each case was worked up in the following way to give Xa—d (Table I), respectively.

6-Methyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (Xa): The reaction mixture was washed with benzene. The residue was treated with Et_2O. The resulting solid was collected and recrystallized from EtOH to give Xa, mp 207—208 °C, as colorless prisms. *Anal.* Calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.45; H, 6.22; N, 7.75. MS *m/z*: 181 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1700 (COOH), 1640 (NC=O). ^{1}H-NMR δ: 1.57 (1H, d, *J*=12, C_8-H_{endo}), 1.90—2.50 (3H, m, C_4-H×2, C_8-H_{exo}), 2.73 (3H, s, N-Me), 3.53 (1H, m, C_5-H), 3.83 (1H, d, *J*=5, C_1-H), 6.97 (1H, m, C_3-H).

6-Benzyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (Xb): The reaction mixture was washed with hot isopropyl ether. The residue was dissolved in $CHCl_3$ and the $CHCl_3$ solution was washed with 10% HCl. The $CHCl_3$ layer was dried over $MgSO_4$ and evaporated. The resulting solid was recrystallized from EtOH to give Xb, mp 171—172 °C, as colorless prisms. *Anal.* Calcd for $C_{15}H_{15}NO_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 69.72; H, 5.86; N, 5.31. MS *m/z*: 257 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1705 (COOH), 1645 (NC=O), 732, 700 (δCH). ^{1}H-NMR δ: 1.60 (1H, d, *J*=11, C_8-H_{endo}), 1.95—2.35 (3H, m, C_4-H×2, C_8-H_{exo}), 3.60 (1H, m, C_5-H), 3.97 (1H, d, *J*=5, C_1-H), 4.23 (1H, d, *J*=15,

N-CH-C_6H_5), 4.80 (1H, d, J=15, N-CH-C_6H_5), 7.00 (1H, m, C_3-H), 7.23 (5H, s, Ar-H).

6-Ethyl-7-oxo-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (Xc): The reaction mixture was worked up as described for Xa, to give Xc, mp 182—183 °C (EtOH), as colorless prisms. *Anal.* Calcd for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.36; H, 6.58; N, 7.12. MS *m/z*: 195 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1710 (COOH), 1640 (NC=O). ^{1}H-NMR δ: 1.03 (3H, t, J=7, CH_2-CH_3), 1.65 (1H, d, J=12, C_8-H_{endo}), 1.95—2.50 (3H, m, C_4-H×2, C_8-H_{exo}), 2.80—3.65 (2H, m, CH_2-CH_3), 3.70 (1H, m, C_5-H), 3.87 (1H, d, J=5, C_1-H), 7.03 (1H, m, C_3-H).

7-Oxo-6-phenethyl-6-azabicyclo[3.2.1]oct-2-ene-2-carboxylic Acid (Xd): The reaction mixture was worked up as described for Xb, to give Xd, mp 197—198 °C (EtOH), as colorless prisms. *Anal.* Calcd for $C_{16}H_{17}NO_3$: C, 70.83; H, 6.32; N, 5.16. Found: C, 70.57; H, 6.39; N, 5.33. MS *m/z*: 271 (M^+). IR ν_{max}^{Nujol} cm^{-1}: 1718 (COOH), 1645 (NC=O), 740, 704 (δ CH). ^{1}H-NMR δ: 1.60 (1H, d, J=11, C_8-H_{endo}), 1.93—2.45 (3H, m, C_4-H×2, C_8-H_{exo}), 2.70—3.17 (2H, m, N-CH-CH_2-C_6H_5), 3.43—4.05 (3H, m, C_5-H, N-CH_2-CH-C_6H_5), 3.85 (1H, d, J=6, C_1-H), 7.03 (1H, m, C_3-H), 7.25 (5H, s, Ar-H).

Preparation of Xb by Heating of XI——2-Benzyl-2-azabicyclo[2.2.2]oct-7-ene-5-*exo*-carboxylic acid (XI,[2] 0.03 g) was heated at 150—160 °C (an oil bath) for 2 d in a sealed tube. The resulting solid was recrystallized from EtOH to give Xb (0.02 g) in 66.7% yield.

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Studies on Peptides. CXLVII.[1,2] Synthesis of Valosin, a Novel 25-Residue Peptide from Porcine Intestine

KENICHI AKAJI,[a] MICHINORI TANAKA,[a] SHOICHIRO SUMI,[b] MASAFUMI KOGIRE,[b] KYOICHI TAKAORI,[b] RYUICHIRO DOI,[b] KAZUTOMO INOUE,[b] TAKAYOSHI TOBE,[b] MOTOYUKI MORIGA,[c] MITSURU AONO,[c] and HARUAKI YAJIMA*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] First Department of Surgery,[b] and First Department of Internal Medicine,[c] Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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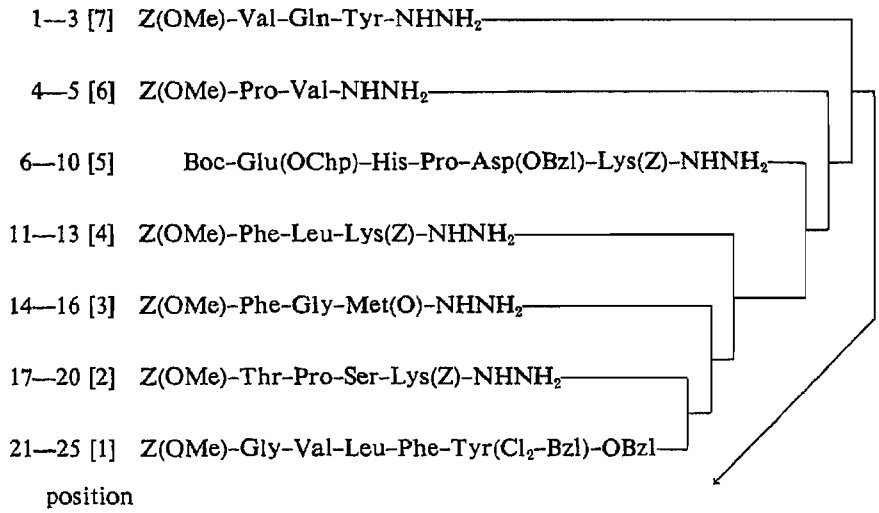
A 25-residue peptide isolated from porcine intestine, designated valosin, was synthesized by assembling seven peptide fragments of established purity, followed by deprotection with 1 M trifluoromethanesulfonic acid in trifluoroacetic acid. γ-Cycloheptylglutamate, [Glu(OChp)], was employed to suppress pyrrolidone formation during fragment condensation. Before deprotection, Met(O) was reduced with phenylthiotrimethylsilane and trimethylsilyl trifluoromethanesulfonate. The effect of the synthetic peptide on arterial pressure, blood flow in the superior mesenteric artery, pancreatic capillary blood flow and pancreatic exocrine secretion was examined in dogs, but no significant dose-dependent response was observed. In rats, synthetic valosin stimulated pancreatic secretion, but showed no anti-gastric activity.

Keywords——valosin synthesis; porcine intestinal polypeptide; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; γ-cycloheptylglutamate; Met(O) reduction; pancreatic secretion

In 1985, Schmidt *et al.*[3] isolated, using an N-terminal sequence screening strategy, a new 25-residue peptide from a side-fraction of the porcine secretin purification scheme.[4] This peptide was designated "valosin" from N-terminal valine and C-terminal tyrosine. In preliminary screening tests, this peptide seemed to exert a dose-dependent stimulatory action on exocrine pancreatic secretion in dogs, like cholecystokinin[5] (CCK) or bombesin.[6] However, the putative regulatory function of valosin in the gastrointestinal tract remains to be clarified.

We wish to report the synthesis of valosin, for which seven peptide fragments were selected as building blocks to construct the entire 25 amino acid sequence of this newly found gastrointestinal polypeptide (Fig. 1). In combination with the TFA-labile Z(OMe) or Boc group, amino acid derivatives bearing protecting groups removable by 1 M TFMSA-thioanisole/TFA[7] were employed, *i.e.*, Lys(Z), Asp(OBzl), Glu(OChp), and Tyr(Cl_2–Bzl). Of these, Glu(OChp)[8] was employed to minimize pyrrolidone formation during fragment coupling and Tyr(Cl_2–Bzl)[9] (C-terminus) to prevent *O*-acylation in each chain elongation step. The Asp(OBzl)–Lys(Z) sequence (positions 9—10) is not sensitive to base-catalyzed succinimide formation.[10] Thus, we decided not to apply any other particular protecting group for the Asp residue at this position. The Met residue located in the middle portion of valosin was protected as its sulfoxide[11] to prevent air-oxidation during the synthesis.

The C-terminal fragment, Z(OMe)–Gly–Val–Leu–Phe–Tyr(Cl_2–Bzl)–OBzl [1], was prepared according to the scheme illustrated in Fig. 2. Z(OMe)–Val–Leu–$NHNH_2$, converted from the corresponding Me ester[12] by the usual hydrazine treatment, was condensed with a



H-Val-Gln-Tyr-Pro-Val-Glu-His-Pro-Asp-Lys-Phe-Leu-Lys-Phe-Gly-Met-Thr-Pro-Ser-Lys-Gly-Val-Leu-Phe-Tyr-OH

Fig. 1. Synthetic Route to Valosin

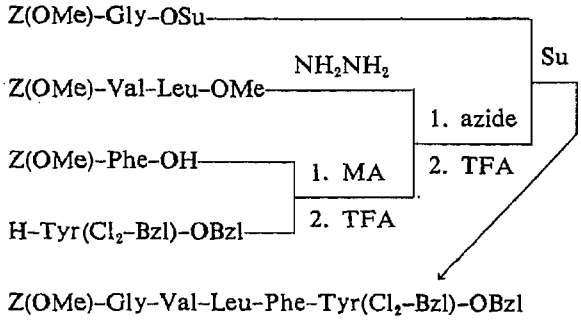


Fig. 2. Synthetic Scheme for the C-Terminal Pentapeptide Ester [1] (Positions 21—25)

TFA-treated sample of Z(OMe)–Phe–Tyr–(Cl_2–Bzl)–OBzl, prepared by the mixed anhydride (MA) procedure.[13] The resulting tetrapeptide ester was treated with TFA, then condensed with Z(OMe)–Gly–OH by the Su procedure[14] to give the protected pentapeptide ester [1]. The purity of [1] was ascertained by thin layer chromatography (TLC), elemental analysis and amino acid analysis after 6 N HCl hydrolysis, as was done with the other fragments.

Fragment [2], Z(OMe)–Thr–Pro–Ser–Lys(Z)–$NHNH_2$ (positions 17—20), was prepared in a stepwise manner starting from a TFA-treated sample of Z(OMe)–Ser–Lys(Z)–OMe,[15] onto which two residues, Pro and Thr, were introduced by the MA and the azide procedures[16] respectively. The resulting protected tetrapeptide ester was converted to [2] by the usual hydrazine treatment.

For preparation of fragment [3], Z(OMe)–Phe–Gly–Met(O)–$NHNH_2$ (positions 14—16), Z(OMe)–Gly–Met–OMe prepared by the DCC procedure[17] was oxidized with $NaIO_4$[18] and the resulting sulfoxide, Z(OMe)–Gly–Met(O)–OMe, after being treated with TFA, was condensed with Z(OMe)–Phe–OH by the Np procedure[19] to give the protected tripeptide ester, which was converted to [3] by the usual hydrazine treatment as stated above.

Fragment [4], Z(OMe)–Phe–Leu–Lys(Z)–$NHNH_2$ (positions 11—13), was prepared by the azide condensation of Z(OMe)–Phe–Leu–$NHNH_2$[20] with H–Lys(Z)–OMe, followed by the usual hydrazine treatment of the resulting tripeptide ester.

Fragment [5], Boc–Glu(OChp)–His–Pro–Asp(OBzl)–Lys(Z)–$NHNH_2$ (positions 6—10), was prepared in a stepwise manner starting from H–Lys(Z)–NHNH–Troc as shown in Fig. 3.

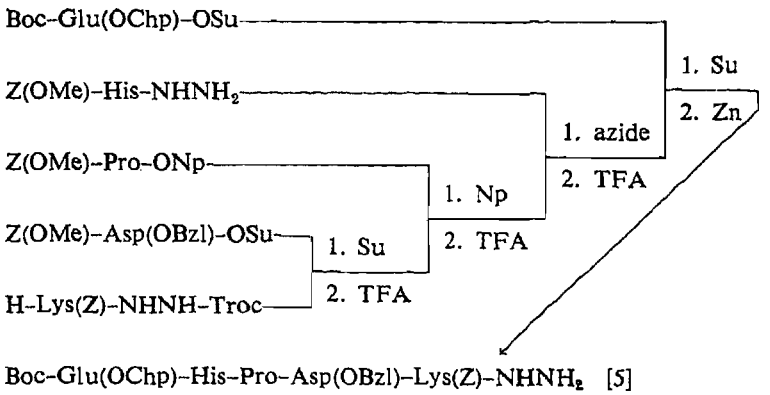


Fig. 3. Synthetic Scheme for the Protected Pentapeptide Hydrazide [5] (Positions 6—10)

The Su method was employed to introduce Asp(OBzl) and Glu(OChp), the Np method for Pro and the azide procedure for His. The Troc group[21] was removed from the resulting protected pentapeptide derivative by treatment with Zn powder.[22]

Next, we attempted to prepare Z(OMe)–Val–Gln–Tyr–Pro–Val–$NHNH_2$ (positions 1—5) by hydrazine treatment of the corresponding Me ester. However, this reaction was incomplete, even after 48 h at 40 °C. Thus we decided to subdivide this pentapeptide unit into two units. At the dipeptide stage, Z(OMe)–Pro–Val–OMe prepared by the Np method was smoothly converted to the corresponding hydrazide, Z(OMe)–Pro–Val–$NHNH_2$ [6] (positions 4—5). The N-terminal fragment, Z(OMe)–Val–Gln–Tyr–$NHNH_2$ [7], was prepared in a stepwise manner starting from H–Tyr–OMe by successive condensations of the Gln and the Val residues *via* the Np and the Su active esters, respectively, followed by the usual hydrazine treatment of the resulting protected tripeptide ester.

Seven peptide fragments thus obtained were assembled successively, according to the route shown in Fig. 1, by the azide procedure to minimize racemization. Every reaction could be performed in DMF or DMF–DMSO without particular solubility problems. The amount

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic Valosin and Its Protected Intermediates

	Protected peptides							Syn. valosin	Residue
	21—25	17—25	14—25	11—25	6—25	4—25	1—25		
Asp					1.02	0.88	1.03	1.00	(1)
Thr		0.89	0.92	1.09	1.00	0.95	1.04	0.87	(1)
Ser		0.84	0.92	1.08	0.96	0.90	0.98	0.75	(1)
Glu					0.97	0.86	2.07	1.81	(2)
Pro		1.12	0.96	1.14	2.11	2.89	3.22	2.82	(3)
Gly	0.99	0.99	1.88	2.31	2.14	2.00	2.05	1.97	(2)
Val	0.97	0.99	0.94	0.97	0.93	1.77	2.98	2.66	(3)
Met			0.64	0.91	0.85	0.82	0.89	0.71	(1)
Leu	1.00	1.00	1.00	2.00	2.00	2.00	2.00	2.00	(2)
Tyr	1.02	0.96	0.99	1.06	0.96	1.00	1.73	1.79	(2)
Phe	1.06	1.02	1.90	3.33	3.16	3.00	3.10	3.03	(3)
Lys		0.97	1.04	2.12	2.98	2.68	2.93	2.96	(3)
His					0.93	0.79	0.94	0.96	(1)
Recov.	95%	80%	83%	82%	74%	78%	78%	70%	

of the acyl component was increased from 1.1 to 4.5 as chain elongation progressed. Products were purified either by precipitation from DMF with EtOH or MeOH or by gel-filtration on Sephadex LH-60 using DMF as an eluant. Throughout the condensations, Leu was used as a diagnostic amino acid in acid hydrolysis (Table I). By comparison of the recovery of Leu with those of newly incorporated amino acids, satisfactory incorporation of each fragment was ascertained.

In the final step, protected valosin was treated with phenylthiotrimethylsilane[23] and trimethylsilyl trifluoromethanesulfonate[24] to reduce the Met(O) residue, then with 1 M TFMSA–thioanisole/TFA in the presence of *m*-cresol and ethanedithiol to remove all protecting groups employed. In a preliminary experiment, we confirmed that the Cl_2–Bzl group could be cleaved from Tyr by the above acid treatment in an ice-bath within 60 min.[7] The deprotected peptide was treated with dil. ammonia to reverse any possible N→O shift[25] at the Ser and Thr residues and purified by gel-filtration on Sephadex G-25, followed by high performance liquid chromatography (HPLC) on a TSK gel LS-410KG column with gradient elution (27—32% acetonitrile in 0.1% TFA). The purity of synthetic valosin thus obtained was ascertained by TLC, disk isoelectrofocusing, analytical HPLC and amino acid analyses after acid hydrolysis (Table I) and enzymatic digestion.

To determine the biological activity of synthetic valosin, three mongrel dogs were used for measurement of arterial pressure, blood flow in the superior mesenteric artery, and pancreatic capillary blood flow. Pancreatic exocrine secretion was also measured in two dogs. However, no significant dose-related response was observed. In rats, synthetic valosin (4 μg/kg) stimulated pancreatic secretion, but had no anti-gastric activity. Further studies are required to elucidate the physiological functions of valosin.

Experimental

General experimental procedures employed in this investigation are essentially the same as described in Part LXXXVIII[26] of this series.

Prior to each condensation reaction, the N^{α}-protecting group, Z(OMe) or Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of peptide) in the presence of anisole (2 eq or more) at ice-bath temperature for 60 min. After evaporation of the TFA *in vacuo* at 15—20 °C, the residue was treated with *n*-hexane, then with ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h, then used for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

The active ester reaction was performed at room temperature. An azide was prepared with isoamyl nitrite according to Honzl and Rudinger[16] and the reaction was conducted at 4 °C. A mixed anhydride was prepared with isobutyl chloroformate according to Vaughan[13] and the reaction was conducted in an ice-bath.

Unless otherwise stated, products were purified by either one of the following procedures.

A (Extraction Procedure): After evaporation of the solvent, the product was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O, dried over Na_2SO_4, and concentrated. The residue was recrystallized or precipitated from appropriate solvents.

B (Washing Procedure): After evaporation of the solvent, the residue was treated with 5% citric acid and ether and the resulting powder was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O and precipitated from appropriate solvents.

C (Gel-Filtration Procedure): The product obtained in B was dissolved in a small amount of DMF (*ca.* 5—8 ml) and the solution was applied to a column of Sephadex LH-60, which was eluted with the same solvent. Fractions (8 ml each) corresponding to the front main peak [monitored by measurement of the ultraviolet (UV) absorption at 275 nm] were collected and the solvent was removed by evaporation. The residue was precipitated from DMF with ether to obtain the product as a powder. For brevity, details of the experimental scale are omitted in the preparation of dipeptides.

Rf values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1, $CHCl_3$–MeOH (10:0.5); Rf_2 $CHCl_3$–MeOH–H_2O (8:3:1); Rf_3 $CHCl_3$–MeOH–AcOH (9:1:0.5); Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2); Rf_5 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1). HPLC was conducted with a Waters 204 compact model. Leucine aminopeptidase (LAP) was purchased from Sigma (Lot. No. 62F-8000).

Z(OMe)–Phe–Tyr(Cl_2–Bzl)–OBzl——This compound was prepared by the MA procedure and purified by precipitation from DMF with AcOEt; yield 55%, mp 164—167 °C, $[\alpha]_D^{29}$ −15.2° (c=1.3, DMF). Rf_1, 0.95. *Anal.* Calcd for $C_{41}H_{38}Cl_2N_2O_7$: C, 66.40; H, 5.16; N, 3.78. Found: C, 66.11; H, 5.12; N, 3.48.

Z(OMe)–Val–Leu–$NHNH_2$——Z(OMe)–Val–Leu–OMe[11] in MeOH was treated with 80% hydrazine hydrate (10 eq) for 12 h. The solid formed during the treatment was collected by filtration and precipitated from DMF with MeOH; yield 77%, mp 207—211 °C, $[\alpha]_D^{29}$ −6.5° (c=0.9, DMF). Rf_2, 0.72. *Anal.* Calcd for $C_{20}H_{32}N_4O_5$: C, 58.81; H, 7.90; N, 13.72. Found: C, 58.70; H, 8.03; N, 13.83.

Z(OMe)–Val–Leu–Phe–Tyr(Cl_2–Bzl)–OBzl——The azide [prepared from 3.20 g (7.83 mmol) of Z(OMe)–Val–Leu–$NHNH_2$] in DMF (50 ml) and Et_3N (1.20 ml, 8.54 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Phe–Tyr(Cl_2–Bzl)–OBzl (5.28 g, 7.12 mmol) in DMF (30 ml) containing Et_3N (1.0 ml, 7.12 mmol), then the mixture was stirred overnight. The solvent was removed by evaporation and the residue was purified by procedure B, followed by precipitation from DMSO with MeOH; yield 6.22 g (92%), mp 225—228 °C, $[\alpha]_D^{29}$ −15.3° (c=1.0, DMSO). Rf_1, 0.78. *Anal.* Calcd for $C_{52}H_{58}Cl_2N_4O_9$: C, 65.47; H, 6.13; N, 5.87. Found: C, 65.28; H, 6.10; N, 5.74.

Z(OMe)–Gly–Val–Leu–Phe–Tyr(Cl_2–Bzl)–OBzl [1] (Positions 21—25)——A mixture of Z(OMe)–Gly–OSu (0.54 g, 7.55 mmol), Et_3N (1.32 ml, 9.43 mmol), and a TFA-treated sample of Z(OMe)–Val–Leu–Phe–Tyr(Cl_2–Bzl)–OBzl (6.00 g, 6.29 mmol) in DMF (100 ml) was stirred overnight and concentrated. The residue was purified by precipitation from DMF with MeOH; yield 4.46 g (70%), mp 212—215 °C, $[\alpha]_D^{29}$ −11.6° (c=0.6, DMF). Rf_1, 0.56. Amino acid ratios in a 6 N HCl hydrolysate: Gly 0.99, Val 0.97, Leu 1.00, Phe 1.06, Tyr 1.02 (recovery of Leu, 95%). *Anal.* Calcd for $C_{54}H_{61}Cl_2N_5O_{10}$: C, 64.15; H, 6.08; N, 6.93. Found: C, 64.32; H, 6.05; N, 6.88.

Z(OMe)–Pro–Ser–Lys(Z)–OMe——A MA [prepared from 3.12 g (8.25 mmol) of Z(OMe)–Pro–OH, CHA] in THF (50 ml) was added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Ser–Lys(Z)–OMe[14] (3.00 g, 5.50 mmol) in DMF (50 ml) containing Et_3N (0.85 ml, 6.05 mmol) and the mixture was stirred for 3 h. The solvent was removed by evaporation and the residue was purified by procedure A, followed by recrystallization from MeOH and ether; yield 2.42 g (69%), mp 136—138 °C, $[\alpha]_D^{25}$ −27.5° (c=0.9, DMF). Rf_1, 0.28. *Anal.* Calcd for $C_{32}H_{42}N_4O_{10}$: C, 59.80; H, 6.59; N, 8.72. Found: C, 59.61; H, 6.64; N, 8.73.

Z(OMe)–Thr–Pro–Ser–Lys(Z)–OMe——The azide [prepared from 4.72 g (15.9 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (60 ml) and Et_3N (2.22 ml, 15.9 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Pro–Ser–Lys(Z)–OMe (8.50 g, 13.2 mmol) in DMF (80 ml) containing Et_3N (3.70 ml, 26.5 mmol) and the solution was stirred overnight. The solvent was removed by evaporation and the residue was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 5.41 g (55%), mp 65—71 °C, $[\alpha]_D^{20}$ −53.9° (c=1.1, MeOH). Rf_2, 0.75. *Anal.* Calcd for $C_{36}H_{49}N_5O_{12}$: C, 58.13; H, 6.64; N, 9.42. Found: C, 57.88; H, 6.79; N, 9.03.

Z(OMe)–Thr–Pro–Ser–Lys(Z)–$NHNH_2$ [2] (Positions 17—20)——The above protected tetrapeptide ester (5.00 g, 6.72 mmol) in MeOH (50 ml) was treated with 80% hydrazine hydrate (4.03 ml, 10 eq) for 36 h. The solution was concentrated and the residue was precipitated from DMF with MeOH; yield 2.32 g (46%), mp 118—120 °C, $[\alpha]_D^{20}$ −28.5° (c=1.4, DMF). Rf_2, 0.57. Amino acid ratios in a 6 N HCl hydrolysate: Thr 0.92, Pro 0.99, Ser 0.91, Lys 1.00 (recovery of Lys, 90%). *Anal.* Calcd for $C_{35}H_{49}N_7O_{11}$: C, 56.52; H, 6.64; N, 13.18. Found: C, 56.26; H, 6.72; N, 13.46.

Z(OMe)–Gly–Met–OMe——The title compound was prepared by the DCC procedure and purified by procedure A followed by column chromatography on silica using $CHCl_3$ as an eluant. The product was finally recrystallized from $CHCl_3$ and n-hexane; yield 63%, mp 49—51 °C, $[\alpha]_D^{20}$ −13.3° (c=0.8, MeOH). Rf_1 0.56. *Anal.* Calcd for $C_{17}H_{24}N_2O_6S$: C, 53.11; H, 6.29; N, 7.29. Found: C, 53.03; H, 6.41; N, 7.17.

Z(OMe)–Gly–Met(O)–OMe——A mixture of Z(OMe)–Gly–Met–OMe (4.45 g, 11.6 mmol) in AcOEt (50 ml) and $NaIO_4$ (2.97 g, 1.2 eq) in H_2O (20 ml) was stirred for 5 h and filtered. The organic layer was washed with a small amount of 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized from MeOH and AcOEt; yield 3.67 g (79%), mp 89—92 °C, $[\alpha]_D^{20}$ +3.85° (c=0.8, MeOH). Rf_1, 0.33. *Anal.* Calcd for $C_{17}H_{24}N_2O_7S$: C, 50.99; H, 6.04; N, 7.00. Found: C, 51.14; H, 6.03; N, 6.98.

Z(OMe)–Phe–Gly–Met(O)–OMe——A mixture of a TFA-treated sample of the above dipeptide ester (6.50 g, 16.2 mmol), Et_3N (4.95 ml, 35.6 mmol), Z(OMe)–Phe–ONp (8.76 g, 19.4 mmol), and HOBt (0.26 g, 1.94 mmol) in DMF (60 ml) was stirred for 14 h and concentrated. The residue was extracted with n-BuOH. The organic phase was washed with 5% Na_2CO_3, 5% citric acid and H_2O, dried over $MgSO_4$ and concentrated. The residue was further purified by column chromatography on silica (7 × 10 cm), which was eluted with $CHCl_3$–MeOH (10 : 1). The product was finally recrystallized from AcOEt and ether; yield 6.93 g (78%); mp 134—137 °C, $[\alpha]_D^{20}$ −12.5° (c=1.9, MeOH). Rf_3, 0.44. *Anal.* Calcd for $C_{26}H_{33}N_3O_8S \cdot 0.5H_2O$: C, 56.10; H, 6.16; N, 7.55. Found: C, 55.99; H, 6.04; N, 7.58.

Z(OMe)–Phe–Gly–Met(O)–$NHNH_2$ [3] (Positions 14—16)——The above protected tripeptide ester (2.0 g, 3.65 mmol) in MeOH (20 ml) was treated with 80% hydrazine hydrate (1.83 ml, 10 eq) at 37 °C overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with EtOH; yield 1.98 g (99%), mp 183—186 °C, $[\alpha]_D^{20}$ −20.3° (c=0.7, DMF). Rf_2, 0.23. Amino acid ratios in a 6 N HCl hydrolysate: Phe 1.01, Gly 1.00, Met+Met(O) 0.76 (recovery of Gly, 93%). *Anal.* Calcd for $C_{25}H_{33}N_5O_7S$: C, 54.83; H, 6.07; N, 12.79. Found: C, 54.73; H, 6.13; N, 12.50.

Z(OMe)–Phe–Leu–Lys(Z)–OMe——The azide [prepared from 10.30 g (22.6 mmol) of Z(OMe)–Phe–Leu–$NHNH_2$[20]] in DMF (100 ml) and Et_3N (3.14 ml, 22.6 mmol) were added to an ice-chilled solution of H–Lys(Z)–OMe [prepared from 8.21 g (24.8 mmol) of the HCl salt] in DMF (80 ml) and the mixture was stirred for 14 h. The solvent was removed by evaporation and the residue was purified by procedure A, followed by recrystallization from MeOH and ether; yield 11.65 g (66%), mp 130—135 °C, $[\alpha]_D^{20}$ −14.3° (c=1.5, DMF). Rf_1, 0.78. *Anal.* Calcd for $C_{39}H_{50}N_4O_9 \cdot H_2O$: C, 65.16; H, 7.01; N, 7.79. Found: C, 64.93; H, 6.95; N, 8.08.

Z(OMe)–Phe–Leu–Lys(Z)–$NHNH_2$ [4] (Positions 11—13)——The above tripeptide ester (6.00 g, 8.35 mmol) in DMF–MeOH (1 : 1, 60 ml) was treated with 80% hydrazine hydrate (4.18 ml, 83.5 mmol) for 48 h and the solvent was removed by evaporation. The residue was purified by precipitation from DMF with EtOH; yield 5.42 g (90%), mp 198—202 °C, $[\alpha]_D^{20}$ −15.3° (c=0.9, DMF). Rf_3, 0.56. Amino acid ratios in a 6 N HCl hydrolysate: Phe 1.00, Leu 1.03, Lys 0.93 (recovery of Phe, 94%). *Anal.* Calcd for $C_{38}H_{50}N_6O_8 \cdot 0.5H_2O$: C, 62.70; H, 7.06; N, 11.55. Found: C, 62.99; H, 6.84; N, 11.71.

Z(OMe)–Asp(OBzl)–Lys(Z)–NHNH–Troc——The title compound was prepared by the Su procedure and purified by procedure A, followed by recrystallization from MeOH and ether; yield 77%, mp 103—105 °C, $[\alpha]_D^{15}$ −17.6° (c=1.0, MeOH). Rf_1, 0.42. *Anal.* Calcd for $C_{37}H_{42}Cl_3N_5O_{11}$: C, 52.96; H, 5.05; N, 8.35. Found: C, 52.98; H, 5.02; N, 8.17.

Z(OMe)–Pro–Asp(OBzl)–Lys(Z)–NHNH–Troc——A mixture of a TFA-treated sample of Z(OMe)–Asp(OBzl)–Lys(Z)–NHNH–Troc (5.00 g, 5.96 mmol), Et_3N (1.81 ml, 13.1 mmol), and Z(OMe)–Pro–ONp (3.29 g, 7.14 mmol) was stirred for 40 h and concentrated. Trituration of the residue with ether afforded a powder which was recrystallized from MeOH and benzene; yield 3.30 g (59%), mp 150—152 °C, $[\alpha]_D^{17}$ −38.2° (c=1.0, DMF). Rf_1, 0.31. *Anal.* Calcd for $C_{42}H_{49}Cl_3N_6O_{12}$: C, 53.88; H, 5.28; N, 8.98. Found: C, 54.10; H, 5.26; N, 8.77.

Z(OMe)–His–Pro–Asp(OBzl)–Lys(Z)–NHNH–Troc——The azide [prepared from 1.78 g (5.34 mmol) of Z(OMe)–His–$NHNH_2$] in DMF (20 ml) and Et_3N (0.74 ml, 5.34 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tripeptide derivative (3.34 g, 3.57 mmol) in DMF (20 ml) containing Et_3N (0.50 ml, 3.57 mmol) and the mixture was stirred for 20 h. The solution was concentrated and the residue was purified by procedure A, followed by column chromatography on silica gel (5 × 20 cm), which was eluted with $CHCl_3$–MeOH–AcOEt (9 : 1 : 0.5). The product was finally recrystallized from AcOEt and ether; yield 3.00 g (78%), mp 112—115 °C, $[\alpha]_D^{18}$ −49.5° (c=1.0, MeOH), Rf_3, 0.18; Rf_2, 0.87. *Anal.* Calcd for $C_{48}H_{56}Cl_3N_9O_{13} \cdot 3H_2O$: C, 51.13; H, 5.54; N, 11.18. Found: C, 51.06; H, 5.20; N, 10.84.

Boc–Glu(OChp)–His–Pro–Asp(OBzl)–Lys(Z)–NHNH–Troc——A mixture of Boc–Glu(OChp)–OSu (1.47 g, 3.34 mmol), a TFA-treated sample of the above tetrapeptide derivative (3.00 g, 2.79 mmol), Et_3N (0.78 ml, 5.58 mmol) and HOBt (0.51 g, 3.34 mmol) in DMF (20 ml) was stirred overnight and concentrated. The residue was purified by procedure A, followed by column chromatography on silica gel (4.5 × 15 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was finally recrystallized from AcOEt and ether; yield 2.35 g (68%), mp 104—108 °C, $[\alpha]_D^{20}$ −78.0° (c=0.8, MeOH). Rf_2, 0.62. *Anal.* Calcd for $C_{56}H_{75}Cl_3N_{10}O_{15}$: C, 54.48; H, 6.12; N, 11.35. Found: C, 54.43; H, 6.17; N, 11.22.

Boc–Glu(OChp)–His–Pro–Asp(OBzl)–Lys(Z)–$NHNH_2$ [5] (Positions 6—10)——The above pentapeptide derivative (2.00 g, 1.62 mmol) in AcOH (20 ml) was treated with Zn powder (1.06 g, 10 eq) for 2 h and the solution was filtered. The solvent was removed by evaporation and the residue was extracted with *n*-BuOH. The organic phase was washed with 5% EDTA and H_2O, dried over $MgSO_4$ and concentrated. The residue was recrystallized from MeOH and ether; yield 1.68 g (98%), mp 114—119 °C, $[\alpha]_D^{20}$ −37.5° (c=1.4, DMF). Rf_2, 0.59. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.02, His 0.97, Pro 1.04, Asp 1.04, Lys 1.00 (recovery of Lys, 79%). *Anal.* Calcd for $C_{53}H_{74}N_{10}O_{13} \cdot 3H_2O$: C, 57.18; H, 7.24; N, 12.58. Found: C, 57.23; H, 6.88; N, 11.98.

Z(OMe)–Pro–Val–$NHNH_2$ [6] (Positions 4—5)——Z(OMe)–Pro–Val–OMe was prepared by the DCC procedure and purified by procedure A. The oily product thus obtained was dissolved in MeOH and treated with 80% hydrazine hydrate (10 eq) overnight. The product was precipitated from DMF with MeOH; yield 73%, mp 192—194 °C, $[\alpha]_D^{20}$ −40.3° (c=0.8, DMF). Rf_2, 0.67. Amino acid ratios in a 6 N HCl hydrolysate: Val 1.00, Pro 1.06 (recovery of Val, 92%). *Anal.* Calcd for $C_{19}H_{28}N_4O_5$: C, 58.15; H, 7.19; N, 14.28. Found: C, 58.10; H, 7.25; N, 14.58.

Z(OMe)–Gln–Tyr–OMe——The title compound was prepared by the Np method and purified by procedure B, followed by precipitation from DMF with AcOEt; yield 81%, mp 190—197 °C, $[\alpha]_D^{20}$ +11.2° (c=0.8, DMF). Rf_2, 0.76. *Anal.* Calcd for $C_{24}H_{29}N_3O_8$: C, 59.13; H, 6.00; N, 8.62. Found: C, 58.89; H, 5.93; N, 8.63.

Z(OMe)–Val–Gln–Tyr–OMe——A mixture of Z(OMe)–Val–OSu (9.31 g, 24.6 mmol), Et_3N (5.74 ml, 41.2 mmol), and a TFA-treated sample of Z(OMe)–Gln–Tyr–OMe (10.00 g, 20.5 mmol) in DMF (100 ml) was stirred overnight and concentrated. The residue was precipitated from DMF with MeOH; yield 8.75 g (73%), mp 197—205 °C, $[\alpha]_D^{20}$ +4.8° (c=1.7, DMF), *Anal.* Calcd for $C_{29}H_{38}N_4O_9 \cdot 0.5H_2O$: C, 58.47; H, 6.60; N, 9.41. Found: C, 58.42; H, 6.62; N, 9.91.

Z(OMe)–Val–Gln–Tyr–$NHNH_2$ [7] (Positions 1—3)——The above protected tripeptide ester (4.00 g, 6.82 mmol) in DMF–MeOH (2 : 1, 60 ml) was treated with 80% hydrazine hydrate (4.3 ml, 10 eq) overnight. The solid formed during the treatment was collected by filtration and precipitated from DMF with MeOH; yield 3.71 g (93%), mp 245—249 °C, $[\alpha]_D^{20}$ −1.7° (c=1.8, DMSO). Rf_2, 0.25. Amino acid ratios in a 6 N HCl hydrolysate: Val 1.00, Glu

1.00, Tyr 0.91 (recovery of Val, 66%). *Anal.* Calcd for $C_{28}H_{38}N_6O_8 \cdot 0.5H_2O$: C, 56.46; H, 6.60; N, 14.11. Found: C, 56.67; H, 6.67; N, 14.34.

Z(OMe)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr(Cl_2-Bzl)-OBzl (Positions 17—25)——The azide [prepared from 3.63 g (4.89 mmol) of fragment [2]] in DMF (40 ml) and Et_3N (0.63 ml, 4.51 mmol) were added to an ice-chilled solution of a TFA-treated sample of fragment [1] (3.80 g, 3.76 mmol) in DMF (30 ml) containing Et_3N (0.53 ml, 3.76 mmol), then the mixture was stirred overnight. H_2O (300 ml) was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.48 g (94%), mp 190—195 °C, $[\alpha]_D^{20}$ −26.6° (c=0.7, DMF). Rf_2, 0.49. *Anal.* Calcd for $C_{80}H_{98}Cl_2N_{10}O_{18}$: C, 61.65; H, 6.34; N, 8.99. Found: C, 61.52; H, 6.35; N, 8.83.

Z(OMe)-Phe-Gly-Met(O)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr(Cl_2-Bzl)-OBzl (Positions 14—25)——The azide [prepared from 1.37 g (2.50 mmol) of fragment [3]] in DMF (20 ml) and Et_3N (0.35 ml, 2.50 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected nonapeptide (3.00 g, 1.92 mmol) in DMF (30 ml) containing Et_3N (0.27 ml, 1.92 mmol) and the solution was stirred overnight. H_2O (300 ml) was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.08 g (84%), mp 188—195 °C, $[\alpha]_D^{20}$ −31.7° (c=0.9, DMF). Rf_2, 0.69. *Anal.* Calcd for $C_{96}H_{119}Cl_2N_{13}O_{22}S \cdot H_2O$: C, 59.80; H, 6.33; N, 9.44. Found: C, 59.64; H, 6.29; N, 9.33.

Z(OMe)-Phe-Leu-Lys(Z)-Phe-Gly-Met(O)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr(Cl_2-Bzl)-OBzl (Positions 11—25)——The azide [prepared from 1.58 g (2.20 mmol) of fragment [4]] in DMF (30 ml) and Et_3N (0.31 ml, 2.20 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected dodecapeptide (3.00 g, 1.57 mmol) in DMF (30 ml) containing Et_3N (0.22 ml, 1.57 mmol) and the solution was stirred overnight. H_2O (300 ml) was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.58 g (68%), mp 248—252 °C, $[\alpha]_D^{20}$ −24.1° (c=1.0, DMF). Rf_2, 0.72. *Anal.* Calcd for $C_{125}H_{157}Cl_2N_{17}O_{27} \cdot H_2O$: C, 61.26; H, 6.54; N, 9.72. Found: C, 61.08; H, 6.61; N, 9.89.

Boc-Glu(OChp)-His-Pro-Asp(OBzl)-Lys(Z)-Phe-Leu-Lys(Z)-Phe-Gly-Met(O)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr(Cl_2-Bzl)-OBzl (Positions 6—25)——The azide [prepared from 368 mg (347 μmol) of fragment [5]] in DMF (2 ml) and Et_3N (54 μl, 386 μmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected pentadecapeptide (570 mg, 232 μmol) in DMF–DMSO (1 : 1, 4 ml) containing Et_3N (32 μl, 232 μmol) and the solution was stirred at −15 °C for 48 h. Additional azide (1 eq) and Et_3N (1 eq) were added and stirring was continued overnight at 4 °C. H_2O (100 ml) was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with MeOH; yield 0.51 g (66%), mp 205—209 °C, $[\alpha]_D^{20}$ −33.1° (c=0.2, DMF). Rf_2, 0.58. *Anal.* Calcd for $C_{169}H_{219}Cl_2N_{25}O_{37}S \cdot 5H_2O$: C, 59.95; H, 6.82; N, 10.34. Found: C, 59.95; H, 6.86; N, 10.74.

Z(OMe)-Pro-Val-Glu(OChp)-His-Pro-Asp(OBzl)-Lys(Z)-Phe-Leu-Lys(Z)-Phe-Gly-Met(O)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr-(Cl_2-Bzl)-OBzl (Positions 4—25)——The azide [prepared from 198 mg (505 μmol) of fragment [6]] in DMF (2 ml) and Et_3N (78 μl, 557 μmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected eicosapeptide (480 mg, 144 μmol) in DMF–DMSO (1 : 1, 4 ml) containing Et_3N (20 μl, 144 μmol) and the solution was stirred at −15 °C for 48 h and then at 4 °C overnight. H_2O (100 ml) was added and the resulting powder was purified by procedure B, followed by precipitation from DMF with EtOH; yield 420 mg (81%), mp 211—216 °C, $[\alpha]_D^{20}$ −36.3° (c=0.3, DMF). Rf_2, 0.41. *Anal.* Calcd for $C_{183}H_{235}Cl_2N_{27}O_{40}S \cdot 3.5H_2O$: C, 60.73; H, 6.74; N, 10.45. Found: C, 60.72; H, 6.75; N, 10.55.

Z(OMe)-Val-Gln-Tyr-Pro-Val-Glu(OChp)-His-Pro-Asp(OBzl)-Lys(Z)-Phe-Leu-Lys(Z)-Phe-Gly-Met(O)-Thr-Pro-Ser-Lys(Z)-Gly-Val-Leu-Phe-Tyr(Cl_2-Bzl)-OBzl, Protected Valosin——The azide [prepared from 298 mg (508 μmol) of fragment [7]] in DMF (2 ml) and Et_3N (78 μl, 557 μmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected docosapeptide (404 mg, 113 μmol) in DMF–DMSO (1 : 1, 4 ml) containing Et_3N (16 μl, 113 μmol) and the solution was stirred at −15 °C for 48 h and then at 4 °C overnight. H_2O (100 ml) was added and the resulting powder was purified by procedure C, followed by precipitation from DMF with EtOH; yield 150 mg (34%), mp 172—177 °C, $[\alpha]_D^{20}$ −9.9° (c=0.1, DMF). Rf_2, 0.48. *Anal.* Calcd for $C_{202}H_{261}Cl_2N_{31}O_{45}S \cdot 4H_2O$: C, 60.37; H, 6.75; N, 10.81. Found: C, 60.40; H, 6.70; N, 10.73.

Synthetic Valosin——The above protected pentacosapeptide (54 mg, 13.6 μmol) in DMF (1 ml) was treated with phenylthiotrimethylsilane (248 μl, 100 eq) and trimethylsilyl trifluoromethanesulfonate (12 μl, 4 eq) at ice-bath temperature for 30 min under an Ar atmosphere, while the starting material disappeared and a new spot, Rf_2 0.51, was detected on TLC. The solution, after being neutralized with Et_3N, was concentrated and the residue was treated with ether to form a powder, yield 52 mg (97%).

The reduced peptide (50 mg, 12.7 μmol) was treated with 1 M TFMSA—thioanisole in TFA (3.8 ml) in the presence of *m*-cresol (118 μl, 90 eq) and EDT (47 μl, 45 eq) in an ice-bath for 180 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O (5 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH and after 30 min to 5.0 with 1 N AcOH. The solution was applied to a column of Sephadex G-25 (2.2 × 132 cm), which was eluted with 1 N AcOH. The fractions (6.5 ml each) corresponding to the front main peak (monitored by UV measurement at 275 nm) were combined and the solvent was removed by lyophilization to give a powder; yield, 37 mg (98%).

The crude product thus obtained (13.1 mg) was purified by HPLC on a TSK gel LS-410KG column

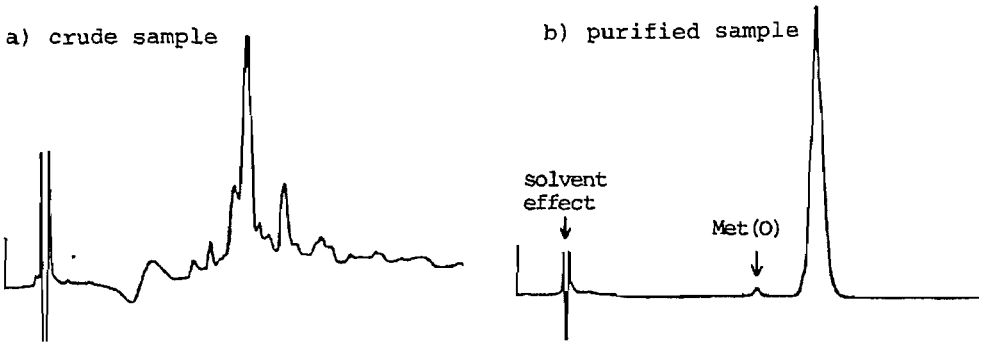


Fig. 4. HPLC of Synthetic Valosin

Fig. 5. Disk Isoelectrofocusing of Synthetic Valosin

(21.5 × 300 mm) with gradient elution (27—32% acetonitrile in 0.1% TFA over 90 min) at a flow rate of 5 ml/min (Fig. 4-a). The eluate corresponding to the main peak (t_R, 77.4 min) was collected and the solvent was removed by lyophilization. The resulting powder was dissolved in 1 N AcOH (0.5 ml) and subjected to gel-filtration on Sephadex G-25 (1.8 × 55 cm) using 1 N AcOH as an eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 3.2 mg (28%). The rest of the sample was similarly purified; yield 5.7 mg. The overall yield from the protected peptide was 24%. $[\alpha]_D^{20}$ −97.1° (c=0.2, 1 N AcOH). Rf_4, 0.31; Rf_5, 0.36. t_R 15.8 min in HPLC on an analytical Nucleosil 5C18 column (4 × 150 mm) on gradient elution with acetonitrile (27—32%, 15 min) in 0.1% TFA at a flow rate of 0.8 ml per min (Fig. 4-b); a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 6.5 cm) containing Pharmalyte (pH 3—10), mobility 5.1 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (Fig. 5). Amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 0.90 (1), Thr 0.98 (1), Ser 0.95 (1), Glu 0.76 (1), Pro 2.54 (3), Gly 1.89 (2), Val 2.30 (3), Met 0.85 (1), Leu 2.00 (2), Tyr 1.75 (2), Phe 3.00 (3), Lys 2.84 (3), His 0.90 (1), Gln was not determined; digestion of the Pro–Val bond (positions 4—5) was incomplete (recovery of Leu, 77%).

Biological Assays——Three mongrel dogs, weighing from 13.5 to 22 kg, were used for measurement of arterial pressure, blood flow in the superior mesenteric artery, and pancreatic capillary blood flow. Pancreatic exocrine secretion was also measured in two dogs. Dogs were anesthetized with calcium ethyl-methylbutyl-barbiturate (Nembutal) and placed on a mechanical ventilator. Femoral artery and vein canulation was performed. The ultrasonic flow probe of an ultrasonic transit time flow meter[27] was applied on the superior mesenteric artery. A laser Doppler capillary perfusion monitor[28] was applied to the pancreatic tissue. In two dogs, canulation of the pancreatic duct was performed with the accessory pancreatic duct ligated; continuous intravenous infusion of secretin (Eisai, Japan) at a rate of 1 U/kg·h was performed during the study. Pancreatic juice was collected every 10 min and the volume was measured. After these preparations, valosin was given intravenously as a bolus to each dog. The doses employed were 0.01 μg/kg (n=2), 0.05 μg/kg (n=1), 0.1 μg/kg (n=3), 0.5 μg/kg (n=2), 1 μg/kg (n=3), 4 μg/kg (n=2), 6 μg/kg (n=1), and 32 μg/kg (n=1). Arterial pressure, superior mesenteric arterial blood flow, and pancreatic capillary blood flow did not show any significant response to various doses between 0.01 to 6 μg/kg. At the dose of 32 μg/kg, a slight increase of arterial pressure and 20% decrease of superior mesenteric arterial blood flow were noted for about 1 min. Valosin did not cause any significant change in the volume of pancreatic juice. The studies conducted here indicate that synthetic valosin does not exert any significant biological effect on the circulation or pancreatic exocrine secretion at physiological doses.

Next, the effects of synthetic valosin were examined in rats (female Wistar albino rats weighing 200—250 g). The effect on pancreatic secretion was tested in the Love–Tachibana rat preparation.[29] Synthetic valosin (1, 4, and 16 μg/kg) was given as a bolus injection, then pancreatic juice was collected every 30 min and the volume was measured. The first increase of pancreatic flow (7% increase) occurred with a dose of 1 μg/kg of synthetic valosin. The dose of 4 μg/kg appeared to be the lowest dose causing the maximal response (42%). A further increase in synthetic valosin dose (16 μg/kg) did not result in a significantly higher pancreatic response (34%). Next, the effect on gastric secretion was tested in the Ghosh–Lai rat preparation.[30] Gastric secretion was stimulated by an intravenous infusion of pentagastrin (ICI, England, 1.5 μg/kg/h), then synthetic valosin was infused for 1 h in graded doses of 4 and 16 μg/kg/h, but no inhibitory action was observed.

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Total Synthesis of (±)-Solavetivone and Aglycone A_3. Regio- and Stereo-Selective Birch Reduction of 6,10-Dimethyl-2-hydroxyspiro[4.5]deca-6,9-dien-8-one[1)]

CHUZO IWATA,* MINORU YAMADA, TAKAFUMI FUSAKA, KAZUYUKI MIYASHITA, AYATSUGU NAKAMURA, TETSUAKI TANAKA, TAKAJI FUJIWARA, and KEN-ICHI TOMITA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565, Japan

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Total synthesis of (±)-solavetivone (**1**), a spirovetivane phytoalexin, was achieved. The metal catalyzed decomposition of the phenolic diazoketone (**8**) gave the spiro-dienone (**6**), selective reduction of which afforded the hydroxy-enone (**5**). The regio- and stereo-selective Birch reduction of **5** provided a high yield of the hydroxy-spiro-enone (**9**), whose structure was determined by X-ray crystal analysis. Compound **9** was transformed to (±)-**1** and the aglycone A_3 in several steps.

Keywords——solavetivone; total synthesis; Birch reduction; X-ray crystal analysis; (2*S**,5*S**,10*R**)-6,10-dimethyl-2-hydroxy-spiro[4.5]dec-6-en-8-one; neighboring hydroxyl group participation; phytoalexin; antibacterial activity; spirovetivane sesquiterpene; spiro-annelation

In 1974, solavetivone (**1**), a spirovetivane sesquiterpene, was isolated from the tuber tissues of white potatoes infected with a blight fungus *Phytophthora infestans*,[2)] and it was also isolated from air-cured Burley tobacco (*Nicotiana tabacum* cv. BURLEY) in 1977.[3)] Its structure was synthetically determined by Yamada *et al.* in 1977.[4,5)] In 1982, **1** was proved to be a biogenetic precursor of other antibacterial sesquiterpenes, lubimin (**2**), oxylubimin (**3**), and rishitin (**4**), by Murai *et al.*[6)] The most noteworthy characteristic is that solavetivone (**1**) is one of the phytoalexins,[7)] which are antibacterial compounds produced in plant tissues when the plant is infected with bacteria. Furthermore, the spirovetivane family includes a number of phytoalexins.

In connection with our synthetic studies on spirocyclic natural products, we set about establishing a general method for synthesizing spirovetivane sesquiterpenes. Considering the functionalities and C_2-substituents, we selected the hydroxy-spiro-dienone (**5**) as an important synthetic intermediate. In this paper, we describe the regio- and stereo-selective Birch reduction of the hydroxy-spiro-dienone (**5**), and the total synthesis of (±)-solavetivone (**1**).

We applied our spiro-annelation method[8)] to the synthesis of 6,10-dimethylspiro-[4.5]deca-6,9-diene-2,8-dione (**6**), which is the precursor of **5**. The acetoxy-diazoketone (**7**)[9)] was hydrolyzed with buffered aqueous sodium carbonate[10)] to give the phenolic diazoketone (**8**), which was subjected to thermolysis in the presence of copper(I) chloride in benzene to afford the spiro-dienone (**6**)[11)] in 56% yield. Compound **6** was selectively reduced with lithium tri-*tert*-butoxyaluminum hydride to provide 6,10-dimethyl-2-hydroxyspiro[4.5]-deca-6,9-dien-8-one (**5**) in 80% yield.

In spite of numerous examples of the Birch reduction of α,β-unsaturated carbonyl compounds,[12,13)] there are few examples of neighboring group participation in the protonation at the β-position,[14)] and the effect on the stereochemistry is still uncertain. In the Birch reduction of **5**, intramolecular protonation from the neighboring hydroxyl group is

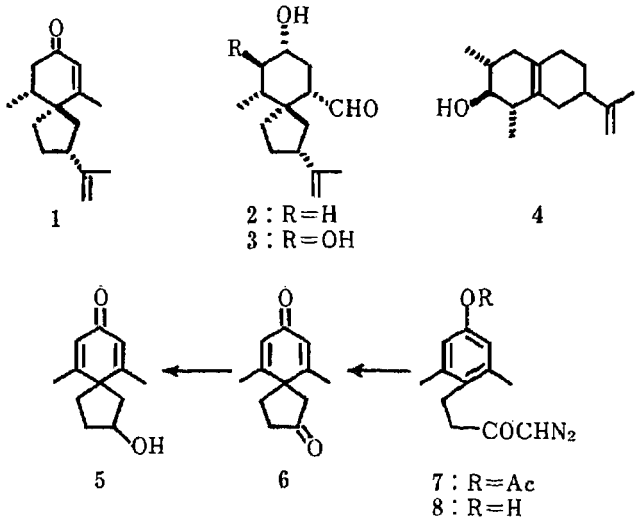
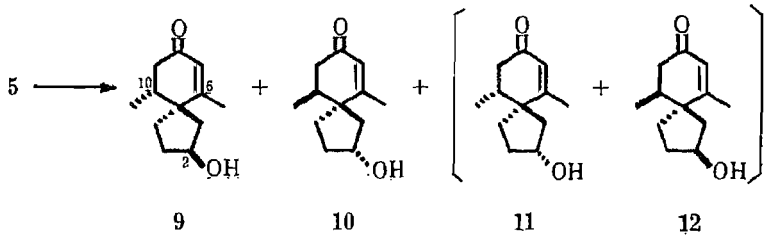
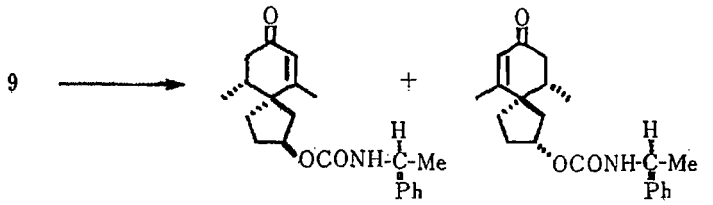
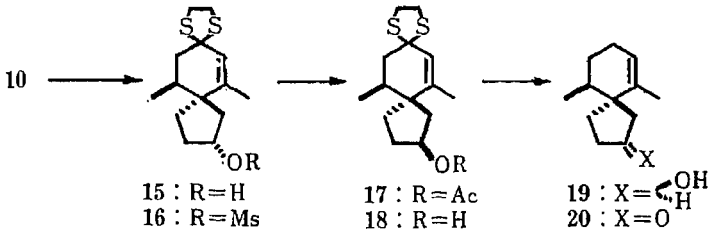


Chart 1







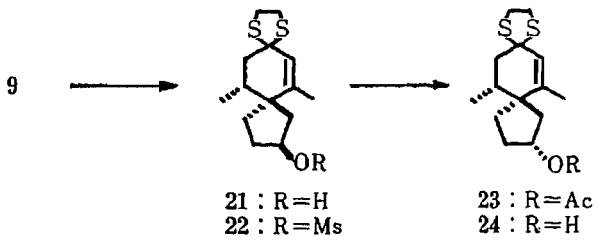


Chart 2

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(δ 1.05) appeared at lower field than that of **15** (δ 0.94) owing to the influence of the hydroxyl group.[19] The signal of the vinylic methyl group of **15** (δ 1.79) appeared at lower field than that of **18** (δ 1.75). Therefore, it was concluded that the configuration of the hydroxyl group of **15** is α.[18] Accordingly, the stereostructure of **10** was determined. Furthermore, we synthesized the thioacetal derivative (**21**) and its hydroxyl inverted isomer (**24**) and the acetate (**23**) from **9** in a manner similar to that used for **15** and **18**, and compared their ^{1}H-NMR spectra. The signal of the *sec*-methyl of **21** (δ 1.05) appeared at lower field than that of **24** (δ 0.93), and that of the vinylic methyl of **24** (δ 1.78) appeared at lower field than that of **21** (δ 1.66). These tendencies are similar to those of **15** and **18**, and support the structure of **15**, thereby reconfirming the structure of **10**.

Judging from the 400 MHz ^{1}H-NMR spectrum of the crude product of the Birch reduction, there are two minor *sec*-methyl signals other than those of **9** and **10**. They seem to have originated from **11** and **12**, although neither compound was isolated. Finally, the product ratio was estimated from the integrals of the ^{1}H-NMR (400 MHz) signals to be *ca.* 91 : 7 : 1 : 1 (**9** : **10** : **11** : **12**).

The mechanism of this regio- and stereo-selective Birch reduction might be explained as follows. Two possible epimeric dianion (or radical anion) transition states, A (chair form) and B (boat or half-chair form), can be considered in the reduction process *via* intramolecular protonation.[13] There is a strong interaction between the alcohol oxygen and the methyl group in the transition state A, but not in B. Therefore, the reduction will proceed predominantly *via* the transition state B, thus producing the isomer **9**.

As described above, we synthesized compound (**9**) as an intermediate for the synthesis of spirovetivane sesquiterpenes in good yield. Now we set about synthesizing solavetivone (**1**).[20] The mesylate (**22**) was treated with sodio diethyl malonate in dimethoxyethane (DME) to give the diester (**25**) in 67% yield, and this was hydrolyzed with potassium hydroxide in ethanol to afford the half-ester (**26**). Compound **26** was heated with diethylamine and 35% aqueous formaldehyde and then further heated with sodium acetate and glacial acetic acid[21] to form the α-methylene ester (**27**) in 92% yield. The ester (**27**) was reduced with diisobutylaluminum hydride (DIBAH) in toluene at -70 °C to give the allylic alcohol (**28**) in 87% yield, and this was treated with hexachloroacetone and triphenylphosphine in tetrahydrofuran (THF) at 20 °C[22] to provide the unstable allylic chloride (**29**) in 90% yield. Immediate treatment of **29**

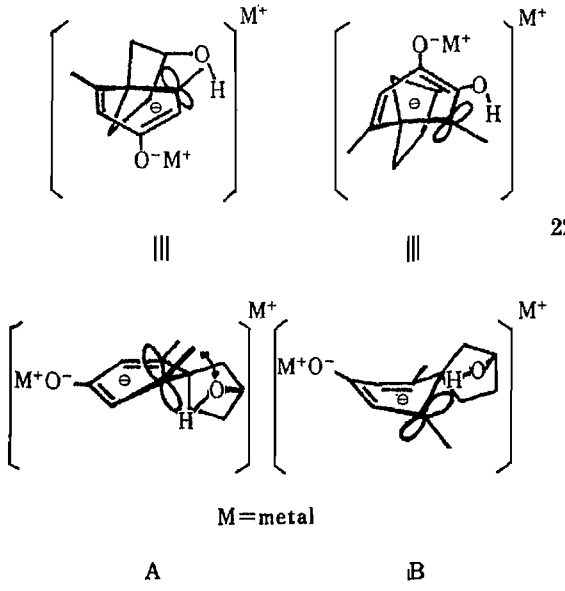

Chart 3

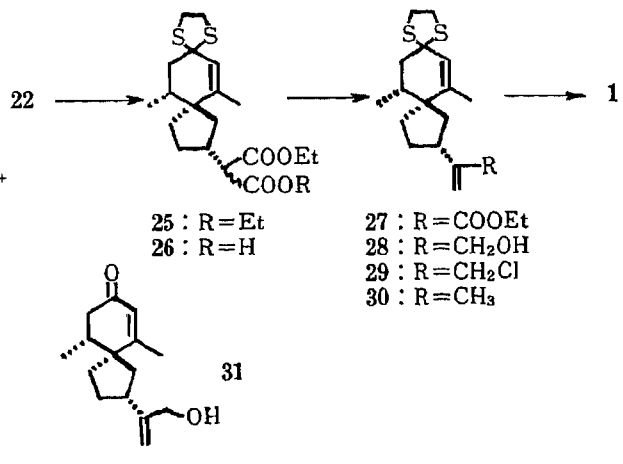

Chart 4

with zinc powder in a mixture of glacial acetic acid, benzene, and ethanol at 80 °C afforded the isopropenyl compound (**30**) in 74% yield. Finally, deprotection of **30** with methyl iodide[23] gave (±)-solavetivone (**1**) in 63% yield, the identity of which was confirmed by comparing its IR, ^{1}H-NMR, and mass spectra with those of an authentic sample.[2,3] Further, deprotection[23] of **28** afforded the spiro-enone (**31**) in 63% yield, and this product was found to be identical with the aglycone A_3[24] isolated from flue-cured Virginia tobacco.

Experimental

Melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. IR and ultraviolet (UV) spectra were recorded on Hitachi EPI-G3 and Hitachi 124 spectrophotometers, respectively. ^{1}H-NMR spectra were recorded on a Hitachi R-22 (90 MHz) instrument with tetramethylsilane as an internal standard. The following abbreviations for the signal patterns are used: s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, and br = broad. Mass spectra (MS) and high resolution mass spectra (High MS) were obtained with Hitachi RMU-6E and JEOL JMS D300 mass spectrometers. For column and preparative thin layer chromatography (PTLC), Merck Kieselgel 60 (70—230 mesh) and Merck Kieselgel PF_{254} were used, respectively.

Diazomethyl 2-(4-Hydroxy-2,6-dimethylphenyl)ethyl Ketone (8)——A solution of $NaHCO_3$ (2.1 g), Na_2CO_3 (3.5 g) in H_2O (35 ml) was added to a solution of **7**[9] (7.5 g) in MeOH (200 ml), and the whole was stirred for 2 h at room temperature. After evaporation of the MeOH, the mixture was extracted with Et_2O. The aqueous layer was acidified with oxalic acid, then extracted with Et_2O. The combined Et_2O extracts were washed with H_2O and brine, then dried, and evaporated to give **8** (5.8 g; 90% yield), mp 83—85 °C (from benzene). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 3340, 2110, 1635. ^{1}H-NMR ($CDCl_3$) δ: 2.0—3.0 (10H, m with s at 2.17), 5.21 (1H, s, CHN_2), 6.51 (2H, s, aromatic H). MS *m/z*: 218 (M^+, 28), 135 (100).

6,10-Dimethylspiro[4.5]deca-6,9-diene-2,8-dione (6)——A solution of **8** (5.8 g) in dry benzene (200 ml) was added dropwise during 30 min to refluxing dry benzene containing CuCl (2 g). After the addition, the mixture was cooled to room temperature, then filtered through filter-paper. The solids on the filter-paper were washed with $CHCl_3$. The combined filtrates were evaporated to give the crude product, which was purified by column chromatography (AcOEt) to give **6** (2.53 g; 56% yield), mp 115—116 °C (from AcOEt–benzene) (lit.[11] mp 116 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1747, 1668, 1626. UV λ_{max}^{EtOH} nm (ε): 247 (17100). ^{1}H-NMR ($CDCl_3$) δ: 1.8—2.4 (8H, m with s at 2.03), 2.4—2.8 (4H, m, CH_2COCH_2), 6.07 (2H, s, olefinic H). MS *m/z*: 190 (M^+, 59), 106 (100). *Anal.* Calcd for $C_{12}H_{14}O_2$: C, 75.76; H, 7.42. Found: C, 75.79; H, 7.45.

2-Hydroxy-6,10-dimethylspiro[4.5]deca-6,9-dien-8-one (5)——A solution of **6** (50 mg) was added to a suspension of LiAlH (*tert*-BuO)$_3$ (4 eq) in THF (1.5 ml), and the whole was stirred for 24 h at room temperature. After the addition of H_2O, the mixture was extracted with AcOEt. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC to give **5** (40 mg; 80% yield), mp 120—122 °C (from AcOEt). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3605, 3420, 1665, 1620. UV λ_{max}^{EtOH} nm (ε): 247 (15100). ^{1}H-NMR ($CDCl_3$) δ: 1.6—2.4 (12H, m with d, J=1.2 Hz, at 2.00 and d, J=1.2 Hz, at 2.17), 2.77 (1H, br s, OH), 4.63 (1H, qn, J=6 Hz, C_2-H), 5.97 and 6.04 (each 1H, d, J=1.2 Hz, olefinic H). MS *m/z*: 192 (M^+, 8), 184 (100). *Anal.* Calcd for $C_{12}H_{16}O_2$: C, 74.97; H, 8.39. Found: C, 74.79; H, 8.51.

(2*S,5*S**,10*R**)- and (2*R**,5*S**,10*S**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one (9 and 10)**——A solution of **5** (255 mg) in THF (15 ml)–toluene (15 ml) was added to a solution of Li (28 mg) in liq. NH_3 (40 ml) at −85 °C. After 2 min, the reaction was quenched by adding NH_4Cl. After NH_3 was allowed to evaporate at room temperature, H_2O was added, and the whole was extracted with AcOEt. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC (AcOEt) to give a mixture of four stereoisomers (210 mg; 78% yield), which was purified again by PTLC (Et_2O; developed 4 times) to give **9** (Rf=0.42) and **10** (Rf=0.49) as colorless prisms (both from benzene–hexane), mp 80—81 and 85—86 °C, respectively. **9**: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3608, 3440, 1662, 1613. UV $\lambda_{max}^{Et_2O}$ nm (ε): 234 (13000). ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, d, J=6.2 Hz, C_{10}-Me), 1.4—2.8 (13H, m with s at 2.04), 4.40 (1H, m C_2-H), 5.78 (1H, br s, C_7-H). MS *m/z*: 194 (M^+). *Anal.* Calcd for $C_{12}H_{18}O_2$: C, 74.18; H, 9.34. Found: C, 73.91; H, 9.44. **10**: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3608, 3450, 1665, 1618. UV $\lambda_{max}^{Et_2O}$ nm (ε): 238 (15500). ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, d, J=6.2 Hz, C_{10}-Me), 1.5—2.8 (12H, m with s at 1.89), 3.27 (1H, br s, OH), 4.42 (1H, m, C_2-H), 5.71 (1H, br s, C_7-H). MS *m/z*: 194 (M^+). *Anal.* Calcd for $C_{12}H_{18}O_2$: C, 74.18; H, 9.34. Found: C, 73.99; H, 9.41.

Resolution of the Racemic (2*S,5*S**,10*R**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one (9)**——A mixture of **9** (110 mg), (*S*)-(−)-phenethyl isocyanate (95 mg), Et_3N (3 drops), and dry benzene (10 ml) was heated at 80 °C for 24 h. After evaporation of the solvent, the residue was purified by PTLC (Aluminum Oxide 150 PF_{254}; CH_2Cl_2 : AcOEt : petr. ether = 80 : 2.5 : 17.5) to give the carbamates, **13** and **14** (Rf=0.40 and 0.32), one of which (Rf=0.32) shows the following physical data. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3425, 1715, 1660, 1624. ^{1}H-NMR ($CDCl_3$) δ: 0.96 (3H, d, J=6 Hz, C_{10}-Me), 1.47 (3H, d, J=6 Hz, NCHC$\underline{H}_3$), 1.6—2.8 (12H, m), 4.5—5.3 (3H, m), 5.70 (1H, br s,

olefinic H), 7.27 (5H, s, aromatic H). MS *m/z*: 341 (M^+, 20), 120 (100).

The carbamate (*Rf*=0.32) was dissolved in CH_2Cl_2, then Et_3N and $HSiCl_3$ were added, and the whole was stirred for 48 h at room temperature, washed with satd. NH_4Cl, H_2O, and brine, dried, and evaporated. The residue was purified by PTLC (AcOEt) to give (−)-**9** ($[\alpha]_D^{18}$ −57° (*c*=0.14, $CHCl_3$)), mp 78—80 °C (colorless plates from Et_2O–petr. ether).

(2*R,5*S**,10*S**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (15)**——A mixture of **10** (45 mg), 1,2-ethanedithiol (0.3 ml), $BF_3 \cdot Et_2O$ (2 drops), and MeOH (2 ml) was stirred for 3 h at room temperature. After the addition of 5% NaOH, the mixture was extracted with CH_2Cl_2. The extract was washed with 5% NaOH, H_2O, and brine, then dried, and evaporated to give the crude product, which was purified by column chromatography (benzene–AcOEt) to provide **15** (55 mg; 88% yield), mp 157—158 °C (from benzene–hexane). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3612, 3450, 1645. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, d, *J*=6 Hz, C_{10}-Me), 1.1—2.3 (13H, m with s at 1.72), 3.0—3.5 (4H, m, SCH_2CH_2S), 4.19 (1H, m, C_2-H), 5.49 (1H, br s, C_7-H). MS *m/z*: 270 (M^+, 98), 209 (100). *Anal.* Calcd for $C_{14}H_{22}OS_2$: C, 62.20; H, 8.21; S, 23.67. Found: C, 61.92; H, 8.26; S, 23.77.

(2*R,5*S**,10*S**)-2-Mesyloxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (16)**——Mesyl chloride (0.06 ml) was added to a solution of **15** (55 mg) in pyridine (1 ml), and the whole was stirred overnight at room temperature. After the addition of $NaHCO_3$ solution, the whole was extracted with benzene. The extract was washed with satd. $NaHCO_3$, H_2O, cold 5% HCl (3 times), H_2O, and brine, then dried, and evaporated. The residue was purified by column chromatography (benzene) to give **16** (69 mg; 90% yield) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1646, 1362. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, d, *J*=6 Hz, C_{10}-Me), 1.4—2.5 (12H, m with d, *J*=1.4 Hz, at 1.76), 2.97 (3H, s, $MeSO_3$), 3.0—3.5 (4H, m, SCH_2CH_2S), 4.95 (1H, m, C_2-H), 5.52 (1H, br s, C_7-H). MS *m/z*: 348 (M^+, 40), 191 (100). High MS *m/z*: 348.089 (Calcd for $C_{15}H_{24}O_3S_3$: 348.089).

(2*S,5*S**,10*S**)-2-Acetoxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (17)**——A mixture of **16** (20 mg), TEAA[25] (60 mg), and acetone (1.5 ml) was refluxed for 8 h. After cooling, the solvent was evaporated off. The residue was purified by PTLC (benzene) to give **17** (10 mg; 56% yield) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1734, 1642. ^{1}H-NMR ($CDCl_3$) δ: 0.99 (3H, d, *J*=6 Hz, C_{10}-Me), 1.3—2.4 (15H, m with d, *J*=1.4 Hz, at 1.67 and s at 1.98), 2.9—3.5 (4H, m, SCH_2CH_2S), 5.15 (1H, m, C_2-H), 5.45 (1H, br s, C_7-H). MS *m/z*: 312 (M^+, 65), 43 (100). High MS *m/z*: 312.122 (Calcd for $C_{16}H_{24}O_2S_2$: 312.122).

(2*S,5*S**,10*S**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (18)**——An ether solution of MeLi was added to a solution of **17** (37 mg) in Et_2O (2 ml). After 5 min, the excess MeLi was decomposed by the addition of AcOEt. After the addition of H_2O, the whole was extracted with benzene. The extract was washed with H_2O and brine, then dried, and evaporated to give **18** (26 mg; 81% yield) as colorless crystals. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3620, 3444, 1640. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, d, *J*=6 Hz, C_{10}-Me), 1.2—2.4 (13H, m with d, *J*=1.4 Hz, at 1.75), 3.1—3.6 (4H, m, SCH_2CH_2S), 4.38 (1H, m, C_2-H), 5.44 (1H, br s, C_7-H). MS *m/z*: 270 (M^+, 100). High MS *m/z*: 270.110 (Calcd for $C_{14}H_{22}OS_2$: 270.111).

(2*S,5*S**,10*S**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-ene (19)**——Sodium metal (100 mg) was added to a stirred solution of **18** (26 mg), THF (5 ml), and liq. NH_3 (20 ml) at −70 °C, and the whole was stirred for 10 min.[26] EtOH (1 ml) was added, and stirring was continued until the blue color disappeared. NH_3 was allowed to evaporate at room temperature, then H_2O was added, and the whole was extracted with Et_2O. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC (Et_2O : petr. ether. = 3 : 2) to give **19** (14 mg; 81% yield) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3627, 1657. ^{1}H-NMR (CCl_4) δ: 0.93 (3H, d, *J*=6.5 Hz, C_{10}-Me), 1.2—2.4 (14H, m with br s at 1.60), 4.23 (1H, m, C_2-H), 5.17 (1H, br s, C_7-H). MS *m/z*: 180 (M^+, 28), 162 (100). High MS *m/z*: 180.151 (Calcd for $C_{12}H_{20}O$: 180.151).

(5*S,10*S**)-6,10-Dimethylspiro[4.5]dec-6-en-2-one (20)**——PCC was added portionwise to a stirred solution of **19** (12 mg) in CH_2Cl_2 (2 ml) at 10 °C until the starting material was no longer detected on TLC. After filtration, the filtrate was evaporated. The residue was purified by PTLC (Et_2O : petr. ether = 2 : 3) to give **20** (9 mg; 76% yield) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1752, 1655. ^{1}H-NMR (CCl_4) δ: 0.90 (3H, d, *J*=6.8 Hz, C_{10}-Me), 1.3—2.6 (14H, m), 5.34 (1H, m, C_7-H). MS *m/z*: 178 (M^+, 54), 107 (100). High MS *m/z*: 178.137 (Calcd for $C_{12}H_{18}O$: 178.136).

(2*S,5*S**,10*R**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (21)**——Compound **21** was prepared from **9** in a manner similar to that described for **15** in 84% yield as colorless crystals, mp 150—151.5 °C (from MeOH). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3613, 3450, 1645. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, d, *J*=6 Hz, C_{10}-Me), 1.1—2.4 (13H, m with d, *J*=1 Hz, at 1.66), 3.0—3.6 (4H, m, SCH_2CH_2S), 4.28 (1H, m, C_2-H), 5.46 (1H, br s, C_7-H). MS *m/z*: 270 (M^+, 100). *Anal.* Calcd for $C_{14}H_{22}OS_2$: C, 62.20; H, 8.21; S, 23.67. Found: C, 61.97; H, 8.27; S, 23.87.

(2*S,5*S**,10*R**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (22)**——Compound **22** was prepared from **21** in a manner similar to that described for **16** in 98% yield as colorless plates, mp 129—131 °C (from acetone–Et_2O). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1645, 1360, 1335. ^{1}H-NMR ($CDCl_3$) δ: 1.07 (3H, d, *J*=6 Hz, C_{10}-Me), 1.3—2.4 (12H, m with d, *J*=1.5 Hz, at 1.69), 2.99 (3H, s, $MeSO_3$), 3.1—3.5 (4H, m, SCH_2CH_2S), 5.03 (1H, m, C_2-H), 5.51 (1H, br s, C_7-H). MS *m/z*: 348 (M^+, 50), 191 (100). *Anal.* Calcd for $C_{15}H_{24}O_3S_3$: C 51.69; H, 6.94; S, 27.60. Found: C, 51.51; H, 7.02; S, 27.70.

(2*R,5*S**,10*R**)-2-Acetoxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (23)**——Compound **23** was prepared from **22** in a manner similar to that described for **17** in 60% yield as colorless plates, mp 81—82 °C

(from hexane). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1745, 1643. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, d, J=6 Hz, C_{10}-Me), 1.4—2.3 (15H, m with s at 1.71 and s at 1.89), 2.9—3.5 (4H, m, SCH_2CH_2S), 5.11 (1H, m, C_2-H), 5.46 (1H, br s, C_7-H). MS m/z: 312 (M^+, 83), 43 (100). High MS m/z: 312.121 (Calcd for $C_{16}H_{24}O_2S_2$: 312.122).

(2*R,5*S**,10*R**)-2-Hydroxy-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (24)**——Compound **24** was prepared from **23** in a manner similar to that described for **18** in 97% yield, as colorless crystals. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3603, 3440, 1641. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, d, J=6 Hz, C_{10}-Me), 1.4—2.4 (13H, m with d, J=1.4 Hz, at 1.78), 3.0—3.6 (4H, m, SCH_2CH_2S), 4.38 (1H, m, C_2-H), 5.52 (1H, br s, C_7-H). MS m/z: 270 (M^+, 99), 209 (100). High MS m/z: 270.111 (Calcd for $C_{14}H_{22}OS_2$: 270.111).

(2*R,5*S**,10*R**)-2-Bis(ethoxycarbonyl)methyl-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (25)**——Diethyl malonate (0.17 ml) was gradually added to a stirred suspension of NaH (26 mg) in DME (4 ml) at 0 °C, and the whole was stirred for 30 min. Compound **22** (95 mg) in DME (1 ml) was added to the sodio malonate solution, and the whole was refluxed for 17 h. After cooling, satd. NH_4Cl solution was added, and the whole was extracted with benzene. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC to give **25** (75 mg) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1762, 1743, 1644. ^{1}H-NMR (CCl_4) δ: 1.01 (3H, d, J=6 Hz, C_{10}-Me), 1.1—2.2 (18H, m with t, J=7 Hz, at 1.23 and s at 1.68), 2.2—2.8 (1H, m, C_2-H), 2.9—3.5 (5H, m, SCH_2CH_2S and $CH(COOEt)_2$), 4.10 (4H, q, J=7 Hz, OCH_2CH_3), 5.34 (1H, br s, C_7-H). MS m/z: 412 (M^+, 100). High MS m/z: 412.171 (Calcd for $C_{24}H_{32}O_4S_2$: 412.174).

The Half Ester (26)——A mixture of **25** (92 mg), KOH (56 mg), and EtOH (4 ml) was stirred overnight at room temperature. After the addition of satd. NH_4Cl, the whole was extracted with AcOEt. Cold 5% HCl was added to the aqueous layer, then the whole was extracted with AcOEt. The combined organic layers were washed with brine, then dried, and evaporated. The residue was purified by PTLC (benzene : EtOH = 82 : 4) to give **26** (84 mg, 99% yield) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3300—2350, 1737. ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, br d, J=5 Hz, C_{10}-Me), 1.1—2.3 (15H, m with t, J=7 Hz, at 1.29 and s at 1.72), 2.3—2.9 (1H, m, C_2-H), 3.0—3.5 (5H, m, SCH_2CH_2S and $CH(COOEt)_2$), 4.27 (2H, q, J=7 Hz, OCH_2CH_3), 5.40 (1H, s, C_7-H), 10.73 (1H, br s, COOH). MS m/z: 384 (M^+, 88), 224 (100). High MS m/z: 384.143 (Calcd for $C_{19}H_{28}O_4S_2$: 384.143).

(2*R,5*S**,10*R**)-2-(1-Ethoxycarbonylvinyl)-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (27)**——A mixture of **26** (83 mg), Et_2NH (0.12 ml), and 35% HCHO solution (0.2 ml) was refluxed for 20 min. After cooling, NaOAc (21 mg) and AcOH (0.21 ml) were added, and the whole was refluxed for 15 min. After cooling, H_2O was added, then the mixture was extracted with benzene. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC (petr. ether : Et_2O = 4 : 1) to give **27** (71 mg; 93% yield) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1722, 1642, 1631. UV λ_{max}^{EtOH} nm (ε): 207 (17000). ^{1}H-NMR (CCl_4) δ: 1.05 (3H, d, J=6 Hz, C_{10}-Me), 1.3—2.3 (15H, m with t, J=7 Hz, at 1.30 and d, J=1.6 Hz, at 1.71), 2.6—3.4 (5H, m, SCH_2CH_2S and C_2-H), 4.15 (2H, q, J=7 Hz, OCH_2CH_3), 5.38 (1H, br s, C_7-H), 5.46 and 6.02 (each 1H, d, J=1.4 Hz, $=CH_2$). MS m/z: 352 (M^+, 100). High MS m/z: 352.151 (Calcd for $C_{19}H_{28}O_2S_2$: 352.153).

(2*R,5*S**,10*R**)-2-(3-Hydroxypropen-2-yl)-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (28)**——DIBAH (0.8 ml; 25 g in 100 ml hexane) was gradually added to a solution of **27** (112 mg) in toluene (4 ml) at −70 °C. After 5 min, the reaction was quenched by the addition of satd. NH_4Cl, and 5% HCl solution was added. The mixture was extracted with benzene. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC (petr. ether : Et_2O = 1 : 1) to give **28** (86 mg; 82% yield) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3625, 1655, 1647. ^{1}H-NMR (CCl_4) δ: 1.01 (3H, d, J=6 Hz, C_{10}-Me), 1.2—2.2 (14H, m with d, J=1.5 Hz, at 1.71), 2.51 (1H, m, C_2-H), 2.9—3.4 (4H, m, SCH_2CH_2S), 4.00 (2H, s, CH_2OH), 4.82 and 4.93 (each 1H, br s, $=CH_2$), 5.39 (1H, br s, C_7-H). MS m/z: 310 (M^+, 100). High MS m/z: 310.142 (Calcd for $C_{17}H_{26}OS_2$: 310.142).

(2*R,5*S**,10*R**)-2-(3-Chloropropen-2-yl)-6,10-dimethylspiro[4.5]dec-6-en-8-one Ethylene Dithioacetal (29)**——Triphenylphosphine (50 mg) was added portionwise to a solution of **28** (25 mg) and hexachloroacetone (132 mg) in THF (1 ml), and the whole was stirred for 10 min. The solvent was evaporated off, and the residue was purified by PTLC (hexane : benzene = 10 : 1) to give **29** (24 mg; 90% yield) as an unstable oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1642. ^{1}H-NMR (CCl_4) δ: 0.97 (3H, d, J=6 Hz, C_{10}-Me), 1.3—2.3 (13H, m with s at 1.65), 2.4—3.0 (1H, m, C_2-H), 3.0—3.5 (4H, m, SCH_2CH_2S), 4.00 (2H, s, CH_2Cl), 4.96 and 5.07 (each 1H, br s, $=CH_2$), 5.43 (1H, br s, C_7-H). MS m/z: 328 (M^+, 76), 267 (100). High MS m/z: 328.109 (Calcd for $C_{17}H_{25}S_2Cl$: 328.109).

(2*R,5*S**,10*R**)-6,10-Dimethyl-2-(2-propenyl)spiro[4.5]dec-6-en-8-one Ethylene Diethioacetal (30)**——Zinc powder (60 mg) was added to a solution of **29** (15 mg) in benzene–EtOH–AcOH (10 : 10 : 1) (1 ml), and the whole was refluxed for 30 min under stirring. After cooling, the mixture was filtered. The filtrate was concentrated, and purified by PTLC (benzene : hexane = 1 : 5) to give **30** (10 mg; 74% yield) as a colorless oil. IR $\nu_{max}^{CCl_4}$ cm^{-1}: 1648. ^{1}H-NMR (CCl_4) δ: 1.01 (3H, d, J=6 Hz, C_{10}-Me), 1.3—2.2 (15H, m), 2.44 (1H, m, C_2-H), 2.9—3.4 (4H, m, SCH_2CH_2S), 4.65 (2H, br s, $=CH_2$), 5.38 (1H, br s, C_7-H). MS m/z: 294 (M^+, 100). High MS m/z: 294.147 (Calcd for $C_{17}H_{26}S_2$: 294.147).

(±)-Solavetivone (1)——Compound **30** (15 mg) was dissolved in MeCN–H_2O (5 : 1, 1 ml), then MeI (0.5 ml) and anhydrous $CaCO_3$ (26 mg) were added, and the whole was refluxed for 8 h during which time MeI (0.2 ml) was added twice. After cooling, H_2O was added, and the mixture was extracted with Et_2O. The extract was washed with H_2O and brine, then dried, and evaporated. The residue was purified by PTLC (petr. ether : Et_2O = 3 : 1) to give (±)-

solavetivone (1) (7 mg; 63% yield) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1660, 1613. ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, d, J= 6 Hz, C_{10}-Me), 1.3—2.9 (16H, m), 4.75 (2H, s, $=CH_2$), 5.75 (1H, s, C_7-H). MS *m/z*: 218 (M^+, 62), 108 (100). High MS *m/z*: 218.166 (Calcd for $C_{15}H_{22}O$: 218.167).

Aglycone A_3 (31)——Compound **31** was obtained from **28** in a manner similar to that described for **1**. The crude product was purified by PTLC (Et_2O) to give **31** (62% yield). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3625, 3450, 1680, 1619. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, d, J=7 Hz, C_{10}-Me), 1.4—2.9 (14H, m), 4.13 (2H, s, CH_2OH), 4.94 and 5.07 (each 1H, s, $=CH_2$), 5.75 (1H, s, C_7-H). MS *m/z*: 234 (M^+, 8), 41 (100). High MS *m/z*: 234.162 (Calcd for $C_{15}H_{22}O_2$: 234.162).

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Studies on the Constituents of *Actinostemma lobatum* MAXIM. I.[1] Structures of Actinostemmosides A, B, C and D, Dammarane Triterpene Glycosides Isolated from the Herb

MASAYO IWAMOTO, TOSHIHIRO FUJIOKA, HIKARU OKABE,* KUNIHIDE MIHASHI and TATSUO YAMAUCHI

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma 8–19–1, Jonan-ku, Fukuoka 814–01, Japan

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From the dried herb of *Actinostemma lobatum* MAXIM. (Cucurbitaceae), four dammarane-type triterpene glycosides named actinostemmosides A, B, C and D were isolated and their structures were elucidated on the basis of chemical and spectral evidence. Actinostemmosides A, B and C were identified as 20-*O*-*β*-D-glucopyranosides of 3*β*,6*α*,20,27-tetrahydroxy-(20*S*)-dammar-24-ene, 3*β*,7*β*,20,27-tetrahydroxy-(20*S*)-dammar-24-ene and 3*β*,7*β*,18,20,27-pentahydroxy-(20*S*)-dammar-24-ene, respectively, and actinostemmoside D, as the *α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside of 3*β*,6*α*,20,27-tetrahydroxy-(20*R*)-dammar-24-ene.

Keywords——*Actinostemma lobatum*; Cucurbitaceae; dammarane; triterpene glycoside; 3*β*,6*α*,20,27-tetrahydroxy-(20*S*)-dammar-24-ene; 3*β*,7*β*,20,27-tetrahydroxy-(20*S*)-dammar-24-ene; 3*β*,7*β*,18,20,27-pentahydroxy-(20*S*)-dammar-24-ene; 3*β*,6*α*,20,27-tetrahydroxy-(20*R*)-dammar-24-ene; ^{13}C-NMR spectrometry; FAB-MS

Actinostemma lobatum MAXIM. (Cucurbitaceae) is a vine which grows in the area from China to Japan. The herb has been traditionally used in China, though not in Japan, as a diuretic for the treatment of nephrotic edema, and as an antidote (applied externally) for poisonous snake bite.[2] In the course of screening of Cucurbitaceous plants for saponin constituents, it was found that the seeds and herb of the title plant contain a considerable amount of saponins. The preliminary check by thin-layer chromatography (TLC) showed that the MeOH extract of the herb contains a large amount of polar saponins (tentatively named lobatosides) which stain dark blue when the plate is sprayed with sulfuric acid followed by heating, and a small amount of less polar saponins (named actinostemmosides) which strain violet. This paper deals with isolation and characterization of four actinostemmosides.

The extraction and fractionation are summarized in Chart 1 and described in the experimental section.

Actinostemmoside A (I), $C_{36}H_{62}O_9$, was obtained as colorless needles in the yield of 0.00085%. The fast atom bombardment mass spectrum (FAB-MS) of I showed the $[M+Na]^+$ ion at *m/z* 661. The enzymatic hydrolysis of I with cellulase gave the aglycone (II) and D-glucose. The FAB-MS of II showed the $[M+Na]^+$ ion at *m/z* 499. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited signals due to seven methyl groups (δ 0.97, 0.97, 1.08, 1.40, 1.48, 2.01 and 2.04, all singlets), one hydroxymethylene group (δ 4.54, s-like) linked to an olefinic carbon, two hydroxymethine groups (δ 3.56, dd, J=11, 6 Hz; δ 4.40, ddd, J=10, 10, 4 Hz) and a proton (δ 5.50, t, J=7 Hz) on a trisubstituted double bond next to a methylene group (δ 2.55, m). The ^{13}C-NMR spectrum showed the signals of four C–C bonded quaternary carbons (δ 39.5, 40.4, 41.8 and 50.6), one oxygenated quaternary carbon (δ 74.0) and the functional groups assigned on the basis of the ^{1}H-NMR sepctrum. These spectral data

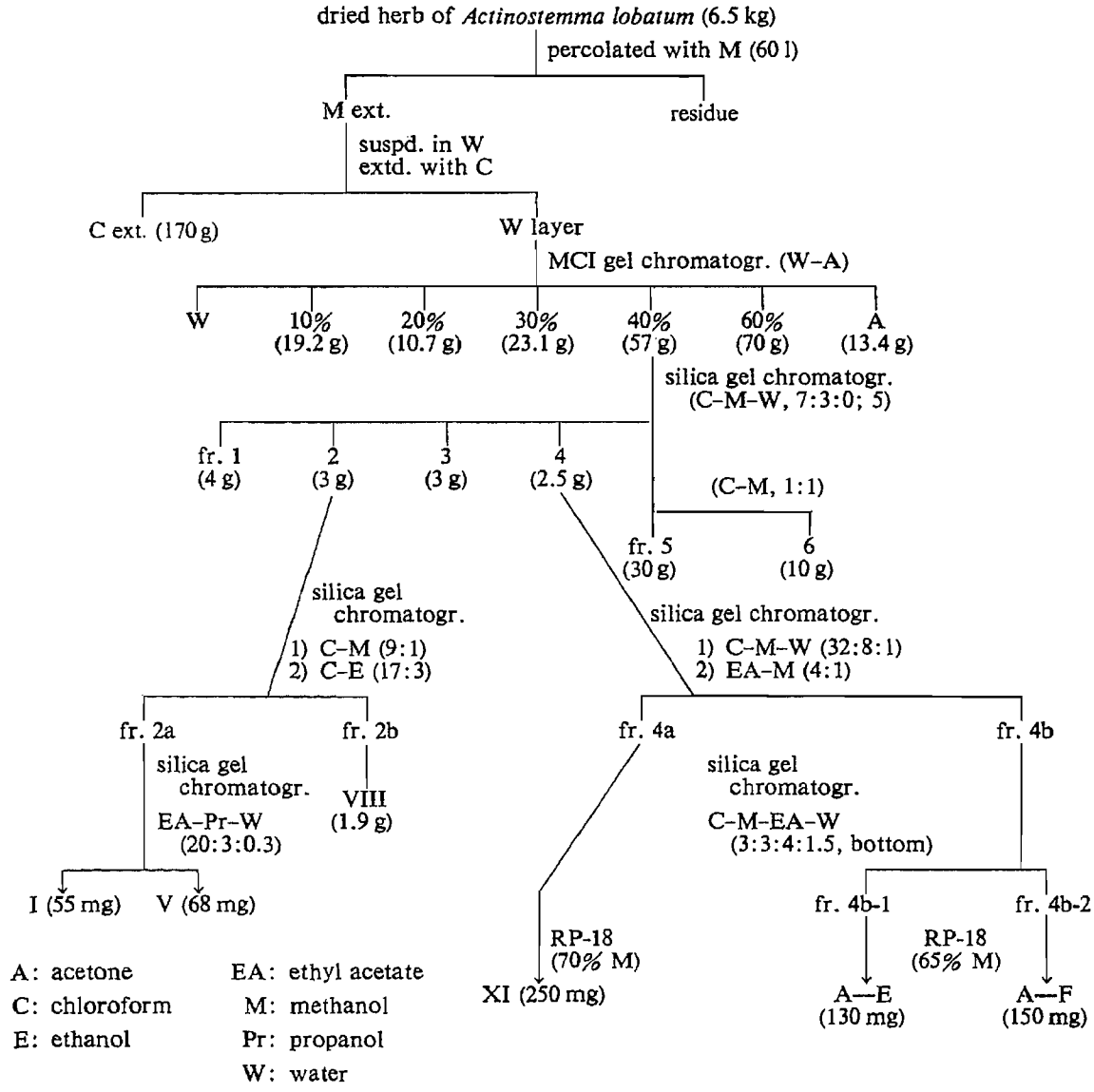

Chart 1. Fraction and Isolation of Glycosides

strongly suggest that II is a tetrahydroxy-(20*S*)-dammarene having two secondary hydroxyl groups in the saturated tetracyclic moiety, and a tertiary hydroxyl group, a primary hydroxyl group and a trisubstituted double bond in the side chain.

The ^{13}C-NMR spectrum of II was quite similar to that of 3β,6α,20,26-tetrahydroxy-(20*S*)-dammar-24-ene (III), the aglycone of Kizuta saponin K_9 (IV), which was isolated from *Hedera rhombea* BEAN (Araliaceae) by Tomimori *et al.*[3] The ^{13}C-NMR spectral differences between II and III are that the signals of C_{27} (δ 14.0) and C_{26} (δ 68.2) of III were not observed, and instead, the signals of a methyl group (δ 21.8) and a hydroxymethylene group (δ 60.9) were observed in the spectrum of II. The two-dimensional ^{1}H-NMR spectrum (^{1}H–^{1}H shift-correlated spectrum) measured in the NOESY mode showed the cross-peak arising from the nuclear Overhauser effect (NOE) between the olefinic proton and methyl protons on an olefinic carbon, indicating that the olefinic proton and the methyl group are in the *cis* configuration. These spectral data unequivocally show that II is 3β,6α,20,27-tetrahydroxy-(20*S*)-dammar-24-ene.

The chemical shift of the anomeric carbon (δ 98.6), the glycosylation shift (Δ 8.2 ppm) of

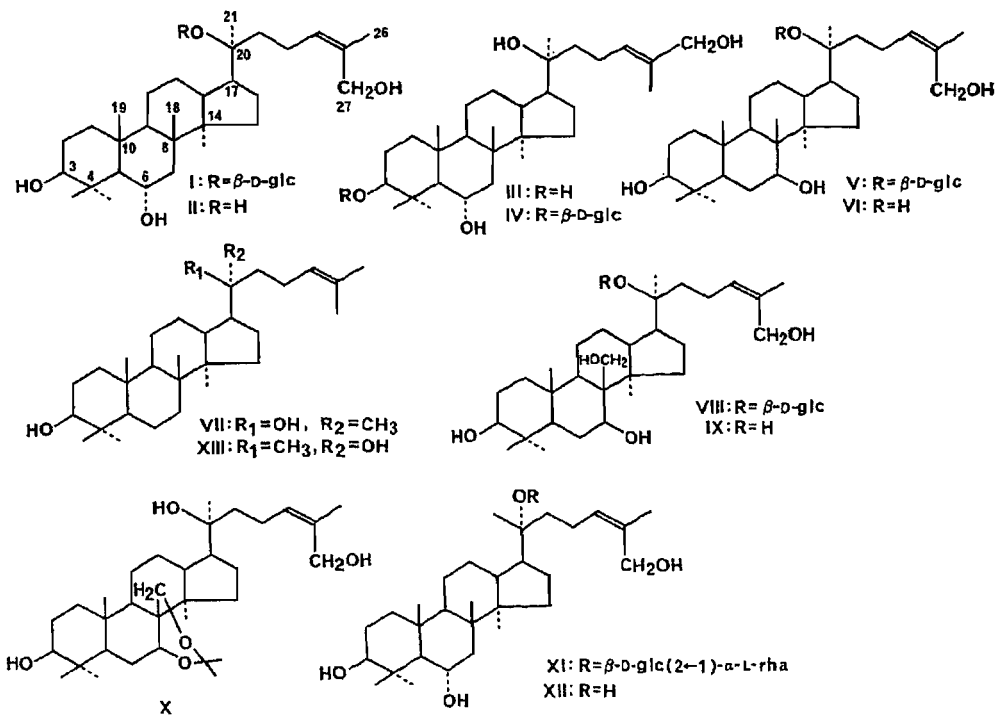

Chart 2

the oxygenated quaternary carbon and the anomeric proton signal (δ 5.03, d, J=8 Hz) indicate that D-glucose is linked to the C_{20}-hydroxyl group in the β-pyranoside form. Thus, actinostemmoside A (I) is the 20-*O*-β-D-glucopyranoside of 3β,6α,20,27-tetrahydroxy-(20*S*)-dammar-24-ene.

Actinostemmoside B (V), $C_{36}H_{62}O_9 \cdot 1.5H_2O$, was obtained as colorless needles (yield: 0.001%). The FAB-MS of V showed the $[M+Na]^+$ ion at m/z 661. Enzymatic hydrolysis of V gave D-glucose and the aglycone (VI), FAB-MS: m/z 499 ($[M+Na]^+$). The ^{13}C- and ^{1}H-NMR data of VI indicated the presence of the same functional groups as those of II. Comparison of the ^{13}C-NMR spectra of II and VI showed that II and VI have the same side-chain carbon signals, indicating that they have the same side-chain structure. The other carbon signals were considerably different from those of II. The ^{1}H-NMR signals of two hydroxymethine protons appeared at δ 3.48 (t, J=8 Hz) and δ 4.08 (dd, J=11, 5 Hz). The former is the signal of C_3-H, and the latter should be that of the proton adjacent to the unlocated hydroxyl group. The splitting pattern indicates that the hydroxymethine group is between a methylene group and a quaternary carbon atom. The positions which satisfy this requirement are C_1, C_7 and C_{15}, among which the C_1 is ruled out because the proton signal (δ 1.9—2.0) of the methylene group next to the hydroxymethine group in question was not influenced by irradiation at the frequency of C_3-H.

The ^{13}C-NMR spectra of VI and dammarenediol II (VII)[4] were compared. Compound VI exhibited quaternary carbon signals at δ 37.4, 39.4, 46.6 and 50.4, and the first, second and fourth signals correspond to those of C_{10} (δ 37.4), C_4 (δ 39.5) and C_{14} (δ 50.6) of VII, but the third one, which should be that of C_8, is shifted downfield by 5.9 ppm compared to that of VII (δ 40.7). If the hydroxyl group is at C_{15}, the downfield shift of the C_8 signal and unchanged chemical shift of C_{14} cannot be explained. If the hydroxyl group is located at C_7, the downfield shifts of the C_6 signal (Δ 10.6 ppm) and the C_8 signal (Δ 5.9 ppm) compared to those of VII, and upfield shifts of signals due to C_5 (Δ 2.5 ppm) and one of the methyl carbons on quaternary carbon (Δ 5.3—6.0 ppm) are well rationalized. The anomalous downfield shift of

TABLE I. ^{13}C-NMR Chemical Shifts of Actinostemmosides and Their Aglycones[a]

	I	II	III[3]	V	VI	VII[4]	XI	XII	X	VIII	IX	XIII[4]
C-1	39.6	39.5	39.5	39.6	39.5	39.5	39.5	39.5	39.1	39.8	39.8	39.5
C-2	27.9	28.0	28.1	28.4	28.4	28.3	27.5	28.0	28.2	28.5	28.5	28.1
C-3	78.5	78.5	78.6	78.1	78.0	78.0	78.5	78.5	78.0	78.0	78.0	78.0
C-4	40.4	40.4	40.4	39.4	39.4	39.5	40.4	40.3	39.4	39.5	39.5	39.5
C-5	61.9	61.9	62.0	54.1	54.0	56.5	61.8	61.9	53.1	54.6	54.7	56.5
C-6	67.7	67.7	67.9	29.4	29.4	18.8	67.8	67.7	29.2	30.3	30.4	18.8
C-7	48.1	48.1	48.1	74.8	74.8	35.7	48.1	48.1	74.0	77.8	78.2	35.7
C-8	41.8	41.8	41.9	46.6	46.6	40.7	41.9	41.8	45.2	49.2	49.3	40.8
C-9	50.8	50.7	50.9	51.0	51.0	51.1	50.9	50.7	51.5	52.2	52.2	51.2
C-10	39.5	39.5	39.5	37.0	37.4	37.4	39.5	39.5	37.0	37.8	37.9	37.4
C-11	22.0	21.9	22.0	22.2	22.1	21.9	21.8	21.8	21.1	22.9	22.9	21.9
C-12	25.5	25.3	25.4	26.1	25.8	25.8	25.3	25.7	25.7	25.8	25.7	25.8
C-13	42.2	42.1	42.2	43.7	43.6	42.6	42.4	42.2	43.9	44.4	44.6	42.6
C-14	50.7	50.6	50.7	50.4	50.4	50.6	50.2	50.4	50.5	50.4	50.3	50.4
C-15	31.5	31.6	31.7	35.4	35.5	31.7	31.2	31.5	36.3	36.5	36.5	31.5
C-16	28.2	28.2	28.2	28.1	28.2	28.1	28.2	28.2	28.2	28.2	28.2	28.1
C-17	48.4	50.4	50.5	47.6	49.8	50.3	48.1	50.0	49.7	47.4	49.5	49.9
C-18	17.7[b]	17.6[b]	17.7[b]	10.5	10.5	16.5[b]	17.6[b]	17.6[b]	59.6	62.0	61.7	16.5[b]
C-19	17.4[b]	17.3[b]	17.4[b]	16.7[b]	16.6[b]	16.3[b]	17.3[b]	17.6[b]	14.4[b]	16.9[b]	16.9[b]	16.3[b]
C-20	82.2	74.0	74.2	82.5	74.2	74.0	82.6	74.3	74.4	82.4	74.4	74.4
C-21	21.4	26.1	26.1	21.8	26.2	25.3	22.7	24.4	26.3	21.9	26.4	24.5
C-22	40.7	42.1	41.8	40.5	42.0	41.9	38.9	43.2	42.1	40.9	41.8	42.9
C-23	22.7	22.8	22.9	22.7	22.9	23.3	22.2	22.6	22.9	22.9	22.9	23.0
C-24	127.7	127.7	125.7	127.9	127.7	126.0	127.8	127.6	127.6	127.8	127.8	126.0
C-25	136.0	136.1	136.3	135.9	136.1	130.6	136.1	136.1	136.2	136.0	136.1	130.6
C-26	21.8	21.8	68.2	21.4	21.8	26.1	21.9	21.8	21.8	21.8	21.9	25.8
C-27	61.0	60.9	14.0	61.1	60.9	17.7	61.1	60.9	60.9	61.0	60.9	17.7
C-28	32.0	32.0	32.0	28.7	28.6	28.7	32.0	32.0	28.5	28.7	28.8	28.7
C-29	16.5[c]	16.5[c]	16.5	16.5[b]	16.5[b]	15.8[b]	16.5[c]	16.5[c]	16.0[b]	16.6[b]	16.7[b]	15.8[b]
C-30	16.8[c]	16.8[c]	16.8	16.8[b]	16.7[b]	16.9	16.9[c]	16.7[c]	17.3[b]	17.1[b]	16.9[b]	16.8
–O–C(Me)–Me									26.7			
									31.2			

a) Assignments are essentially based on the reports by Tanaka *et al.*[4] and Tomimori *et al.*[3] The chemical shifts of the sugar moieties of the glycosides are shown in the experimental section. b, c) Values in each column may be interchangeable.

the C_{15} signal (Δ 3.8 ppm) can be explained in terms of the δ_1-shift which was reported by Eggert *et al.*[5] in the case of several hydroxylated steroids. The ^{1}H–^{1}H shift-correlated spectrum measured in the NOESY mode revealed the presence of NOE between the hydroxymethine proton and a methyl group on C_{14} indicating that the hydroxyl group is linked to C_7 in the β-configuration. The splitting pattern (dd, J=11, 5 Hz) of the hydroxymethine proton supports this configuration.

The glucose linkage was judged to be at the C_{20}-hydroxyl group in the β-configuration for the same reasons as in the case of I. Thus, actinostemmoside B (V) was determined to be the 20-*O*-β-D-glucopyranoside of 3β,7β,20,27-tetrahydroxy-(20*S*)-dammar-24-ene.

Actinostemmoside C (VIII), $C_{36}H_{62}O_{10}\cdot 1/2H_2O$, was obtained as colorless needles (yield: 0.027%). The FAB-MS showed the $[M+Na]^+$ ion at *m/z* 677. Enzymatic hydrolysis gave D-glucose and the aglycone (IX). The FAB-MS of IX showed the $[M+Na]^+$ ion at *m/z* 515, 16 mass units more than that of VI. When the ^{13}C- and ^{1}H-NMR spectra of IX were compared with those of VI, it was found that IX has one more hydroxymethylene group and one less methyl group on a quaternary carbon atom than VI. The ^{13}C-NMR spectrum indicated that IX has the same side chain, and therefore the new hydroxymethylene group

TABLE II. ^{1}H-NMR Chemical Shifts of Actinostemmosides

	I	V	VIII	XI	
H-3	3.53 dd (6, 11)	3.46 t (8)	3.45 t (8)	3.50 dd (7, 10)	
H-5	1.21 d (11)			1.17 d (11)	
H-6	4.38 ddd (11, 11, 4)			4.35 ddd (11, 11, 3)	
H-7		4.05 dd (11, 4)	4.11 dd (11, 3)		
H-13			2.33 ddd (11, 11, 2)		
H-17	2.15 ddd (11, 11, 6)	2.17 m		2.22 ddd (11, 11, 6)	
H-18	0.96 s[a)]	1.26 s	4.49 d, 4.68 d (12) (12)	0.85 s[a)]	
H-19	1.10 s[a)]	0.88 s[a)]	1.05 s[a)]	1.08 s[a)]	
H-21	1.47 s	1.50 s	1.52 s	1.49 s	
H-23	2.47 m, 2.53 m	2.52 m, 2.62 m		2.45 m, 2.70 m	
H-24	5.50 t (7)	5.50 t (7)	5.45 t (7)	5.50 t (7)	
H-26	2.02 s	2.02 s	1.99 s	2.00 s	
H-27	4.49 d, 4.57 d (12) (12)	4.50 d, 4.57 d (12) (12)	4.47 d, 4.52 d (12) (12)	4.54 d, 4.64 d (12) (12)	
H-28	1.97 s	1.20 s	1.20 s	1.92 s	
H-29	1.44 s[b)]	1.05 s[a)]	1.02 s[a)]	1.42 s[b)]	
H-30	0.94 s[b)]	1.13 s[a)]	1.16 s[a)]	1.03 s[b)]	
	Glc	Glc	Glc	Glc	Rha
Sug-1	5.03 d (8)	5.03 d (8)	5.02 d (8)	4.98 d (8)	6.40 d (s-like)
Sug-2	3.97 dd (8, 9)	3.97 dd (8, 9)	3.92 dd (8, 9)	4.13 dd (8, 10)	4.77 t (3)
Sug-3	4.26 t (9)	4.26 t (9)	4.20 t (9)	4.25 t (10)	4.57 dd (3, 9)
Sug-4	4.17 t (9)	4.17 t (9)	4.11 t (9)	3.99 t (10)	4.28 t (9)
Sug-5	3.88 ddd (9, 5, 3)	3.88 ddd (9, 5, 3)	3.84 ddd (9, 5, 3)	3.87 ddd (10, 7, 2)	4.65 dd (9, 6)
Sug-6	4.29 dd (5, 12) 4.46 dd (3, 12)	4.29 dd (5, 12) 4.46 dd (3, 12)	4.25 dd (5, 12) 4.41 dd (2, 12)	4.18 dd (7, 12) 4.45 dd (2, 12)	1.76 d (6)

The spectra were measured in pyridine-d_5+D_2O, and the numbers in parentheses are coupling constants in Hz. *a, b)* Values in each column may be interchangeable, although those given here are preferred.

should be at the tetracyclic moiety. Among the signals of the quaternary carbons (δ 37.9, 39.5, 49.3 and 50.3), the first, second and fourth signals have almost the same chemical shifts as those of C_{10}, C_4 and C_{14} of VI, whereas the signal of the third quaternary carbon is shifted downfield by 2.7 ppm from the C_8 signal (δ 46.6) of VI. These spectral data suggested that IX is 18-hydroxy-VI, namely 3β,7β,18,20,27-pentahydroxy-(20*S*)-dammar-24-ene. When IX was treated with acetone in the presence of anhydrous $CuSO_4$, it gave, as expected, an acetonide (X). The glucose linkage was determined to be at the C_{20}-hydroxyl group, in the β-configuration, on the same bases as in the case of V. Thus, actinostemmoside C (VIII) was

identified as the 20-*O*-β-D-glucopyranoside of 3β,7β,18,20,27-pentahydroxy-(20*S*)-dammar-24-ene.

Actinostemmoside D (XI), $C_{42}H_{72}O_{13}\cdot H_2O$, was obtained as colorless needles in the yield of 0.0038%. The FAB-MS of XI showed the $[M+Na]^+$ ion at *m/z* 807. The NMR spectra exhibited two anomeric carbon signals at δ 97.4 and 101.5 ppm and two anomeric proton signals at δ 4.98 (d, $J=8$ Hz) and δ 6.40 (s-like), and the C–H shift correlated spectrum revealed that the former proton is on the anomeric carbon which appeared at δ 97.4 and the latter proton is on the carbon having the chemical shift δ 101.5.

On heating in 10% acetic acid, XI gave the aglycone (XII) and a biose; the latter furnished methyl glycosides of α-L-rhamnopyranose and α-D-glucopyranose on methanolysis. The ^{13}C-NMR chemical shifts of the oxygenated quaternary carbon (δ 82.6 ppm) shifted upfield (δ 74.3 ppm) on going from XI to XII. These data show that XI is a glycoside having a rhamnosylglucose moiety linked to the tertiary hydroxyl group of the aglycone.

The FAB-MS of XII exhibited the $[M+Na]^+$ ion at *m/z* 499. The ^{13}C- and ^{1}H-NMR spectra of XII are quite similar to those of II indicating that XII is a compound closely related to II. The differences in the ^{13}C-NMR chemical shifts are that the C_{23}, C_{21}, C_{17} and C_{14} signals are shifted upfield by 0.2, 1.7, 0.4 and 0.2 ppm, respectively, and C_{12}, C_{22} and C_{20} signals are shifted downfield by 0.4, 1.1 and 0.3 ppm, respectively. The same differences in the chemical shifts were observed between VII and its (20*R*)-epimer, dammarenediol I (XIII).[4] These data indicate that XII is 3β,6α,20,27-tetrahydroxy-(20*R*)-dammar-24-ene.

Compound XI was enzymatically hydrolyzed with crude hesperidinase in a neutral medium and the ^{13}C-NMR spectrum of the aglycone was measured without crystallization. The ^{13}C-NMR spectrum was superimposable on that of XII obtained by acetic acid hydrolysis, and no sign of the presence of the (20*S*)-epimer was observed.

Compound XI was fully methylated by the modified Hakomori's method and the product was methanolyzed to give methyl glycosides of 2,3,4-tri-*O*-methyl-α-L-rhamnopyranose and 3,4,6-tri-*O*-methyl-α-D-glucopyranose. From the ^{13}C-NMR chemical shifts of the anomeric carbons and ^{1}H-NMR coupling constants of the component sugars, coupled with the glycosylation shift of the oxygenated quaternary carbon, the position of the biose linkage was determined to be at the C_{20}-hydroxyl group and the configurations of the component sugars were identified as α for rhamnose and β for glucose. Thus, the structure of actinostemmoside D was determined to be the 20-*O*-α-L-rhamnopyranosyl-(1$\rightarrow$2)-β-D-glucopyranoside of 3β,6α,20,27-tetrahydroxy-(20*R*)-dammar-24-ene.

It is well-known that a 20-*O*-glycosylated dammarane triterpene is readily hydrolyzed even under mild acidic conditions to give a C_{20}-epimeric mixture of the corresponding sapogenin. (20*R*)-Dammarane saponins have been reported as constituents of red ginseng,[6] but are generally considered to be artifacts formed during the processing procedure.

Actinostemmoside D gave only a (20*R*)-epimer on both acid and enzymatic hydrolyses. This shows that XII is the genuine aglycone and thus, actinostemmoside D is the first naturally occurring (20*R*)-dammarane glycoside.

Actinostemmosides E and F seem to have similar partial structures to above-mentioned actinostemmosides, but they are different in that they have no tertiary hydroxyl group and that the C_3-hydroxyl group seems to be glycosylated. Characterization of their structures is in progress.

Experimental[7]

Extraction and Isolation of Actinostemmosides A—F——The air-dried herb (6.5 kg) of *Actinostemma lobatum* MAXIM. collected in the suburbs of Fukuoka city in September 1984 was packed in a glass tube and percolated with MeOH (60 l). Water (6 l) was added to the MeOH solution and the MeOH was evaporated off. The aqueous solution

was set aside, and the water-insoluble dark resinous material was dissolved in $CHCl_3$ (1 l). This solution was washed with water (1 l). The $CHCl_3$ layer was evaporated *in vacuo* to give a dark resin (170 g). The aqueous solution and the washing were combined and 1/7 of it was passed through an MCI gel (polystyrene gel) column (300 ml). The column was washed with water (1 l) and then eluted with 1 l each of aqueous acetone solutions containing increasing proportions of acetone. The rest of the aqueous solution was treated in the same manner. The yield of each fraction is shown in Chart 1.

The 40% acetone eluate contained actinostemmosides and lobatosides, and this fraction was roughly fractionated by silica gel (10 times the weight of the material) column chromatography, first eluted with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) (frs. 1—4) and then with $CHCl_3$–MeOH (1 : 1) (frs. 5—6). Actinostemmosides were contained in frs. 2 (3 g) and 4 (2.5 g). Fraction 2 was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH (9 : 1) and $CHCl_3$–EtOH (17 : 3) to separate two fractions (frs. 2a and 2b). Separation of the glycosides was monitored by TLC ($CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5). Fraction 2a contained actinostemmosides A (I) and B (V). Fraction 2b was a thin-layer chromatographically homogeneous sample of actinostemmoside C (VIII, 1.9 g). Fraction 2a was chromatographed on silica gel (Lobar column, 31 × 2.5 cm i.d.; three columns were connected) using EtOAc–PrOH–H_2O (20 : 3 : 0.3) to give I (55 mg) and V (68 mg).

Fraction 4 was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (32 : 8 : 1) and EtOAc–MeOH (4 : 1) and separated into two fractions (frs. 4a and 4b) on the basis of TLC monitoring. Fraction 4a was chromatographed on silica gel ($CHCl_3$–MeOH–EtOAc–H_2O, 3 : 3 : 4 : 1.5, bottom layer) and then on an RP-18 column (70% MeOH) to give thin-layer chromatographically homogeneous XI (250 mg). Fraction 4b was chromatographed on silica gel ($CHCl_3$–MeOH–EtOAc–H_2O, 3 : 3 : 4 : 1.5, bottom layer) to give two fractions (frs. 4b-1 and 4b-2). Both fractions were passed through the RP-18 column using 65% MeOH as the eluant to give actinostemmoside E (130 mg) from fr. 4b-1, and F (150 mg) from fr. 4b-2.

Actinostemmoside A (I): Colorless needles from dil. EtOH, mp 125—130 °C, $[\alpha]_D^{16}$ +32.3° (c=0.3, MeOH). FAB-MS m/z: 661.428 ($[M+Na]^+$). $C_{36}H_{62}NaO_9$ requires m/z 661.429. ^{1}H-NMR: shown in Table II. ^{13}C-NMR: sugar moiety; 98.6 (1), 75.6 (2), 79.0 (3), 71.9 (4), 77.9 (5), 63.0 (6).

Actinostemmoside B (V): Colorless needles from dil. EtOH, mp 142—145 °C, $[\alpha]_D^{19}$ +15.4° (c=0.5, MeOH). *Anal.* Calcd for $C_{36}H_{62}O_9 \cdot 1.5H_2O$: C, 64.93; H, 9.84. Found: C, 65.01; H, 9.80. FAB-MS m/z: 661 ($[M+Na]^+$). ^{1}H-NMR: shown in Table II. ^{13}C-NMR: sugar moiety; 98.6 (1), 75.6 (2), 79.0 (3), 71.9 (4), 77.9 (5), 63.0 (6).

Actinostemmoside C (VIII): Colorless needles from dil. MeOH, mp 194—197 °C, $[\alpha]_D^{17}$ +3.3° (c=1.0, MeOH). *Anal.* Calcd for $C_{36}H_{62}O_{10} \cdot 1/2H_2O$: C, 65.13; H, 9.57. Found: C, 64.93; H, 9.99. FAB-MS m/z: 661 ($[M+Na]^+$). ^{1}H-NMR: shown in Table II. ^{13}C-NMR: sugar moiety; 98.7 (1), 75.6 (2), 78.9 (3), 71.9 (4), 77.9 (5), 62.9 (6).

Actinostemmoside D (XI): Colorless needles from dil. EtOH, mp 168—171 °C, $[\alpha]_D^{17}$ −2.2° (c=1.0, MeOH). *Anal.* Calcd for $C_{42}H_{72}O_{13} \cdot H_2O$: C, 62.82; H, 9.29. Found: C, 62.40; H, 9.53. FAB-MS m/z: 807 ($[M+Na]^+$). ^{1}H-NMR: shown in Table II. ^{13}C-NMR: sugar moiety; 97.4 (G-1), 77.9 (G-2), 80.2 (G-3), 72.7 (G-4), 77.3 (G-5), 63.2 (G-6), 101.5 (R-1), 72.3 (R-2), 72.4 (R-3), 74.2 (R-4), 69.6 (R-5), 19.4 (R-6).

Enzymatic Hydrolysis of I, V and VIII——Compound I (40 mg) was suspended in 20% MeOH (50 ml). After addition of cellulase (100 mg), the mixture was stirred at 38 °C for 40 h. MeOH was evaporated off, and the aqueous solution was extracted with EtOAc. The EtOAc extract was purified by silica gel column chromatography ($CHCl_3$–MeOH–H_2O, 32 : 8 : 1; benzene–acetone, 3 : 1) followed by crystallization of the product from ether to give II (20 mg): Colorless needles, mp 148—150 °C, $[\alpha]_D^{20}$ +52.3° (c=0.13, MeOH). FAB-MS m/z: 499 ($[M+Na]^+$). ^{1}H-NMR (pyridine-d_5 + D_2O) δ: 3.56 (H, dd, J=11, 6 Hz, C_3-H), 4.40 (H, ddd, J=10, 10, 4 Hz, C_6-H), 2.55 (2H, m, C_{23}-H), 5.50 (H, t, J=7 Hz, C_{24}-H), 4.54 (2H, s-like, C_{27}-H). Methyl signals: 0.97, 0.97, 1.08, 1.40, 1.48, 2.01, 2.04. ^{13}C-NMR: shown in Table I. The aqueous layer was evaporated to dryness and then extracted with MeOH. The MeOH extract was dissolved in 1 N HCl–MeOH and the solution was refluxed for 1 h. The HCl was neutralized by adding Ag_2CO_3, the precipitates were removed by filtration, and the MeOH was evaporated off. The residue was acetylated in the usual manner and purified by silica gel chromatography (hexane–AcOEt, 2 : 1) to give a syrup (10 mg); $[\alpha]_D^{21}$ +104.8° (c=0.5, $CHCl_3$). The ^{1}H-NMR spectrum was superimposable on that of methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside.

Compound V (40 mg) was treated in the same manner to give VI (13 mg) and methyl glycoside acetate (7 mg). VI: Colorless needles from ether, mp 180—183 °C, $[\alpha]_D^{21}$ +24.5° (c=0.1, MeOH). FAB-MS m/z: 499 ($[M+Na]^+$). ^{1}H-NMR (pyridine-d_5 + D_2O): 0.90, 1.05, 1.14, 1.22, 1.27, 1.43, 2.04 (CH_3), 2.55 (2H, m, C_{23}-H), 3.48 (H, t, J=8 Hz, C_3-H), 4.08 (H, dd, J=11, 5 Hz, C_7-H), 4.54 (2H, s-like, C_{27}-H), 5.50 (H, t, J=8 Hz, C_{24}-H). ^{13}C-NMR: shown in Table I.

Methyl glycoside acetate: $[\alpha]_D^{21}$ +110.0° (c=0.3, $CHCl_3$). The ^{1}H-NMR spectrum was the same as that of methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside.

Compound VIII (300 mg) and cellulase (150 mg) were dissolved in 20% MeOH (20 ml) and the mixture was stirred at 38 °C for 4 d. After evaporation of the solvent, the residue was dissolved in dilute MeOH and filtered. The filtrate was evaporated and chromatographed on silica gel (20 g). Elution with $CHCl_3$–MeOH–H_2O (32 : 8 : 1) gave the aglycone fraction (95 mg) and VIII (130 mg). Further elution with $CHCl_3$–MeOH–H_2O (25 : 17 : 3) gave the sugar fraction (31 mg). Rechromatography of the aglycone fraction on silica gel (10 g) using benzene–acetone (2 : 1) gave

thin-layer chromatographically homogeneous IX (86 mg): colorless needles from $CHCl_3$, mp 119—121 °C, $[\alpha]_D^{17}$ +7.6° (c=0.87, MeOH). FAB-MS m/z: 515 ($[M+Na]^+$). ^{1}H-NMR (pyridine-d_5 + D_2O) δ: 1.05, 1.05, 1.20, 1.22, 1.45, 2.02 (CH_3), 2.55 (2H, m, C_{23}-H), 3.49 (H, t, J=8 Hz, C_3-H), 4.18 (H, dd, J=11, 4 Hz, C_7-H), 4.54 (2H, s-like, C_{27}-H), 4.71, 4.48 (H each, d, J=12 Hz, C_{18}-H), 5.50 (H, t, J=8 Hz, C_{24}-H). ^{13}C-NMR: shown in Table I.

The sugar fraction was treated with 1 N HCl–MeOH and then acetylated. The product ($[\alpha]_D^{17}$ +111.1° (c=1.3, MeOH)) was identified as methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside from the ^{1}H-NMR spectrum and specific rotation.

Treatment of IX with $CuSO_4$ in Acetone; Preparation of the Acetonide (X) from IX——Compound IX (50 mg) and anhydrous $CuSO_4$ (250 mg) were stirred in acetone (5 ml) at room temperature for 2 d. After filtration of $CuSO_4$, the filtrate was concentrated to dryness and then the residue was chromatographed on silica gel (benzene–acetone, 4:1) to give the acetonide (X, 33 mg). Crystallization from dilute acetone gave colorless needles, mp 132—134 °C. FAB-MS m/z: 555 ($[M+Na]^+$). ^{1}H-NMR (pyridine-d_5) δ: 1.00, 1.11, 1.12, 1.25, 1.41, 1.46, 1.66, 2.04 (CH_3), 3.98, 4.03 (H, each, d, J=12 Hz, C_{18}-H), 4.07 (H, dd, J=12, 6 Hz, C_7-H), 3.49 (H, m, C_3-H). ^{13}C-NMR: shown in Table I.

Acid Hydrolysis of XI——Compound XI (95 mg) was suspended in 10% acetic acid (4 ml) and heated at 75 °C for 1 h. The solvent was evaporated off and the residue was chromatographed on silica gel. Elution with $CHCl_3$–MeOH (3:1) gave an aglycone fraction (50 mg). Further elution with $CHCl_3$–MeOH (1:1) gave a sugar fraction (40 mg). Rechromatography of the aglycone fraction on silica gel (benzene–acetone, 3:1) gave a thin-layer chromatographically homogeneous compound (48 mg). Crystallization from dilute MeOH gave colorless needles, mp 183—185 °C, $[\alpha]_D^{17}$ +49.0° (c=0.1, MeOH). FAB-MS m/z: 499 ($[M+Na]^+$). ^{1}H-NMR (pyridine-d_5 + D_2O) δ: 0.96, 0.98, 1.10, 1.37, 1.47, 2.00, 2.04 (CH_3), 2.55 (2H, m, C_{23}-H), 3.60 (H, dd, J=11, 6 Hz, C_3-H), 4.40 (H, ddd, J=10, 10, 3 Hz, C_6-H), 4.53 (2H, s-like, C_{27}-H), 5.50 (H, t, J=7 Hz, C_{24}-H) ^{13}C-NMR: shown in Table I.

Treatment of the sugar fraction with 0.1 N HCl–MeOH at room temperature for 40 h and chromatography of the product on silica gel ($CHCl_3$–MeOH–H_2O, 32:8:1) gave methyl glycoside-I (8 mg) and -II (8 mg). The acetates were identified as methyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside and methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside by comparison of the ^{1}H-NMR spectra and specific rotations with those of authentic samples. Acetate of methyl glycoside-I: $[\alpha]_D^{21}$ −54.4° (c=0.57, $CHCl_3$). Acetate of methyl glycoside-II: $[\alpha]_D^{21}$ +107.0° (c=0.33, $CHCl_3$).

Methylation of XI and Identification of Component Methylated Sugar——Compound XI (10 mg) and NaH (in oil, 50%) (30 mg) were stirred in freshly distilled tetrahydrofuran (2 ml) at room temperature for 10 min. CH_3I (2 ml) was added and the mixture was stirred at room temperature for 50 h. The reaction mixture was poured into water (5 ml) and extracted with $CHCl_3$ (5 ml). The $CHCl_3$ layer was chromatographed on silica gel (benzene–acetone, 9:1) to give a methylation product (5 mg). The product (2 mg) was dissolved in 1 N HCl–MeOH (1 ml) and this solution was refluxed for 3 h. The acid was neutralized by adding Ag_2CO_3, the precipitate was filtered off, and the filtrate was concentrated to dryness. TLC (benzene–acetone, 2:1) showed two spots. The Rf values of the spots were same as those of authentic samples of methyl 2,3,4-tri-*O*-methyl-α-L-rhamnopyranoside and methyl 3,4,6-tri-*O*-methyl-α-D-glucopyranoside. The methanolysate was acetylated in a usual manner and the acetylation product was analyzed by gas chromatography-chemical ionization-MS (GC-CI-MS). The reconstructed GC-CI-MS chromatogram showed two peaks and the CI-MS of the corresponding peaks were identical with those of methyl 2,3,4-tri-*O*-methyl-α-L-rhamnopyranoside and methyl 2-*O*-acetyl-3,4,6-tri-*O*-methyl-α-D-glucopyranoside.

Enzymatic Hydrolysis of XI——Compound XI (70 mg) and crude hesperidinase (70 mg) were dissolved in water (10 ml) and stirred at 37 °C for 10 d. The reaction solution was passed through an Amberlite XAD-2 (5 ml) column. After being washed with water (20 ml), the column was eluted with MeOH (30 ml). The MeOH eluate was evaporated and the residue was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (32:8:1) and 15 mg of a thin-layer chromatographically homogeneous compound was obtained. The ^{1}H- and ^{13}C-NMR spectra were superimposable on those of XII obtained by acid hydrolysis of XI.

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References and Notes

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7) The instruments and materials used in this work were as follows: Yanaco micro melting point apparatus

(melting point, uncorrected), JASCO DIP-4 digital polarimeter (specific rotations), JEOL JNM GX-400 spectrometer (100 MHz for ^{13}C-NMR spectra and 400 MHz for ^{1}H-NMR spectra), JEOL JMS DX-300 mass spectrometer (mass spectra), Auto GCMS-6020 with GC-MSPAC 500 FDG data analyzer (GC-CI-MS), Kieselgel 60 (70—230 mesh, E. Merck), LiChroprep RP-18 (25—40 μm, E. Merck), MCI Gel CHP 20P (150—300 μ, Mitsubishi Chemical Industries Ltd.), precoated Kieselgel 60 F_{254} plate (E. Merck). The cellulase was Type II from *Aspergillus niger* (Sigma Chemical Co., Ltd.). The crude hesperidinase was a gift from Prof. T. Nohara of Kumamoto University. ^{1}H- and ^{13}C-NMR spectra were measured in pyridine-d_5 or pyridine-d_5 containing D_2O and chemical shifts were expressed on the δ scale using TMS as an internal standard. The FAB-MS were obtained in a glycerol matrix containing NaI.

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Chemistry of *O*-Silylated Ketene Acetals.[1] Novel Chemical Transformation of Vinyl Sulfoxides and Related Sulfoxides

YASUYUKI KITA,* OSAMU TAMURA, FUMIO ITOH, HITOSHI YASUDA, TAKASHI MIKI, and YASUMITSU TAMURA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565, Japan

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It is shown that vinyl sulfoxides undergo two novel modes of reactions induced selectively by the *O*-silylated ketene acetal used. Vinyl sulfoxides, on treatment with bulky *tert*-butyldimethylsilyl ketene acetals, undergo a Michael–Pummerer-type reaction to give γ-siloxy-γ-(phenylthio)esters, while with less bulky trimethylsilyl ketene acetals they undergo a double carbon–carbon bond-forming reaction to give 3-(phenylthio)adipates. The reaction of related sulfoxides with *O*-silylated ketene acetals was also examined.

Keywords——*O*-methyl-*O*-*tert*-butyldimethylsilyl ketene acetal; vinyl sulfoxide; Michael–Pummerer-type reaction; methyl 4-(phenylthio)-4-siloxybutanoate; *O*-methyl-*O*-trimethylsilyl ketene acetal; double carbon–carbon bond forming reaction; dimethyl 3-(phenylthio)adipate

Recently, we reported[2] that the treatment of alkyl phenyl sulfoxides (**1**) with *O*-methyl-*O*-*tert*-butyldimethylsilyl ketene acetals (**2a**) in dry acetonitrile in the presence of a catalytic amount of zinc iodide caused a Pummerer-type rearrangement to give α-siloxysulfides (**3**), while the *O*-methyl-*O*-trimethylsilyl ketene acetals (**2c, d**) under similar conditions gave the carbon–carbon bond-formed products, β-(phenylthio)ester derivatives (**4**). We have now examined the reaction of vinyl sulfoxides (**5a—f**) with the *O*-silylated ketene acetals (**2a—d**) and report here two novel modes of reaction of **5** with **2**: i) Michael–Pummerer-type reaction leading to methyl 4-(phenylthio)-4-siloxybutanoates (**6a—e**) (route a) and ii) the double carbon–carbon bond-forming reaction leading to dimethyl 3-(phenylthio)adipates (**8a, b**) (route b).

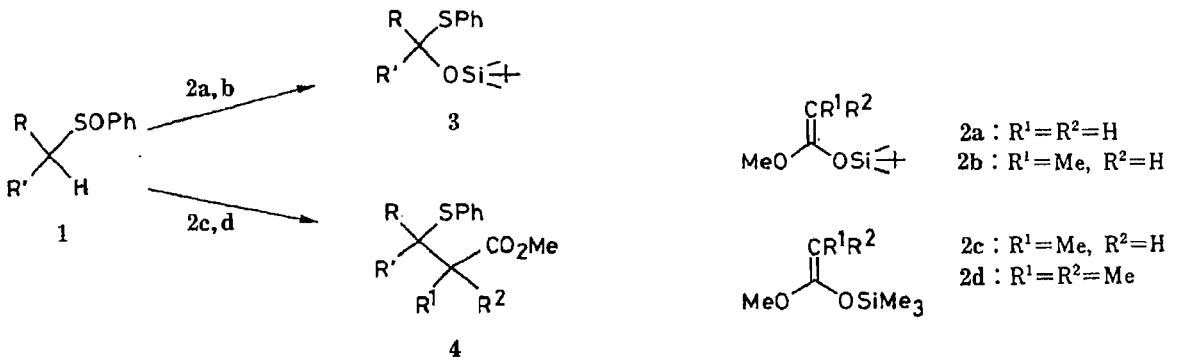


Chart 1

Results and Discussion

While much attention has been focussed on the additive Pummerer-type rearrangement

of vinyl sulfoxides,[3] there is no report on the initial activation of the sulfinyl group of vinyl sulfoxides with *O*-silylated ketene acetal. We have found that *tert*-butyldimethylsilyl ketene acetals (**2a, b**) are quite effective for the initial activation of the sulfinyl group of vinyl sulfoxides (**5a—d**), followed by a Michael–Pummerer-type reaction to give methyl 4-(phenylthio)-4-siloxybutanoates (**6a—e**) in moderate yields.

A typical experimental procedure is as follows for the reaction of *O*-methyl-*O*-*tert*-butyldimethylsilyl ketene acetal (**2a**) with phenyl vinyl sulfoxide (**5a**). A solution of **2a**, **5a**, and a catalytic amount of zinc iodide in dry acetonitrile was stirred at room temperature for 5 h, followed by usual work-up after addition of one drop of pyridine, to give methyl 4-*tert*-butyldimethylsiloxy-4-(phenylthio)butanoate (**6a**). Similarly, vinyl sulfoxides (**5a—d**) reacted with **2a** or **2b** to give the corresponding methyl 4-(phenylthio)-4-siloxybutanoates (**6b—e**). All these products (**6a—e**) were purified by distillation and characterized by proton nuclear magnetic resonance (^{1}H-NMR), infrared (IR), and analytical measurements.

The reaction of the sulfoxides (**5a—d**) with **2a, b** presumably proceeds *via* the siloxysulfonium ylide intermediates (B) shown in Chart 2: initial silicon transfer from the *O*-silylated ketene acetals (**2a, b**) to the sulfoxides (**5a—d**) and subsequent Michael addition by the generated ester enolate anions (A) would give B (↔B′), which then rearranges by the usual Pummerer pathway to give the methyl 4-(phenylthio)-4-siloxybutanoates (**6a—e**). Since the siloxysulfide moiety can be readily converted to a keto group by treatment with fluoride anion, the present route (route a) provides a useful method for the preparation of the γ-ketoester derivatives. Treatment of **6c** with tetra-*n*-butylammonium fluoride in tetrahydrofuran (THF) at room temperature for 5 min gave methyl 3-formyl-3-phenylpropanoate (**7**) in 61% yield.

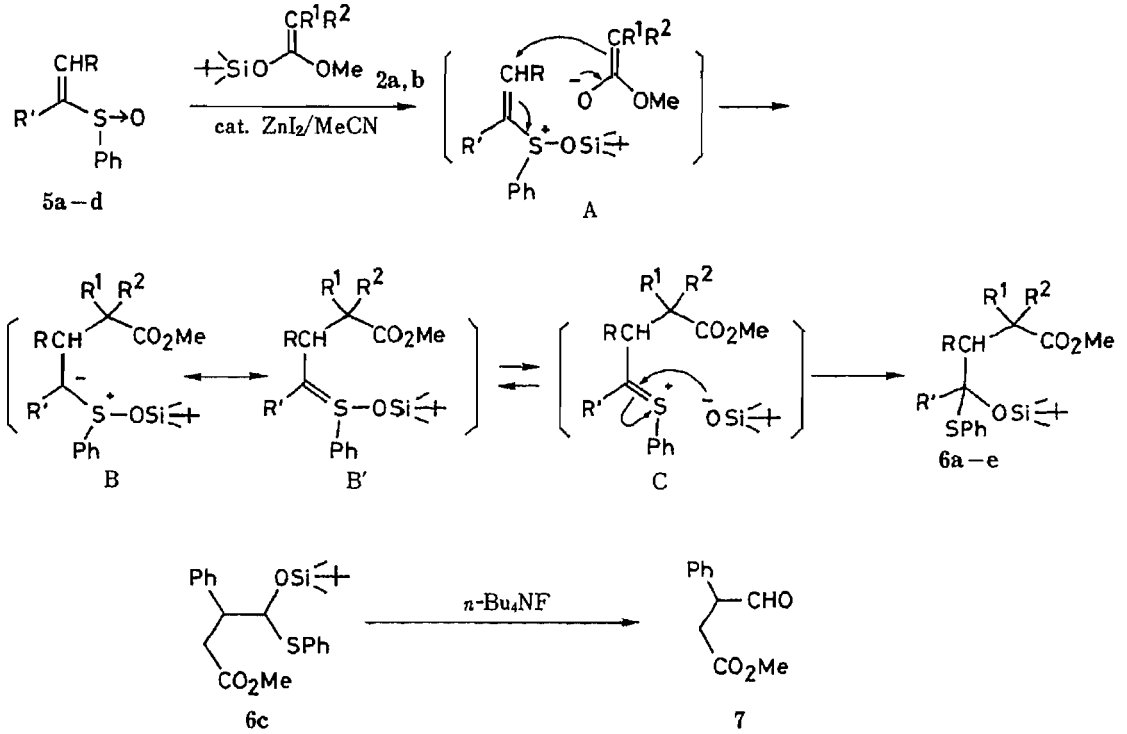


Chart 2

In the reaction of vinyl sulfoxides (**5**) with *O*-silylated ketene acetals (**2**), the bulkiness of the substituents on the silicon atom of **2** has an important effect on the reaction course. Thus, the reaction of **5** with bulky *tert*-butyldimethylsilyl ketene acetals (**2a, b**) gives the Michael–

Pummerer-type reaction products (**6**), while with the less bulky trimethylsilyl ketene acetals (**2c, d**) it gives the double carbon–carbon bond-formed products. For example, phenyl vinyl sulfoxide (**5a**) was treated with *O*-methyl-*O*-trimethylsilyl ketene acetals (**2c, d**) under conditions similar to those described for the reaction of **5** with **2a, b** to give dimethyl 3-(phenylthio)adipates (**8a, b**) in moderate yields (Chart 3).

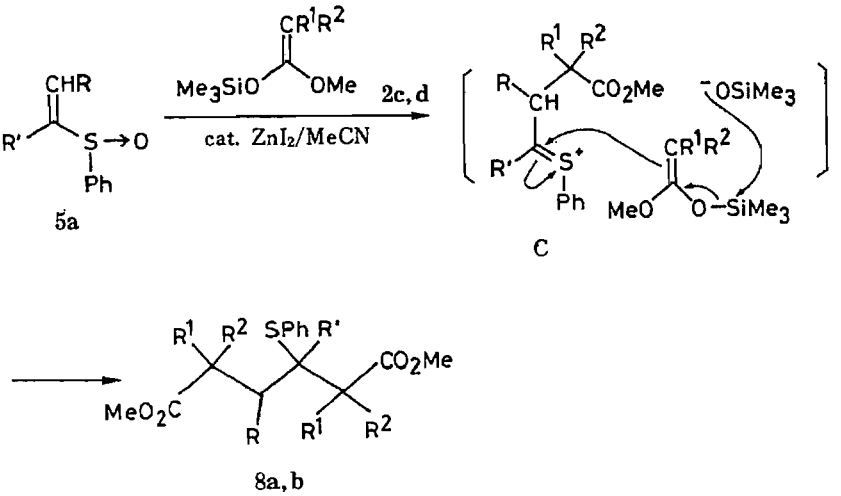


Chart 3

The marked contrast with the result that the reaction of vinyl sulfoxides (**5a—d**) with **2a, b** gives the Michael–Pummerer-type products (**6a—e**) can be explained by consideration of the common initial intermediate (C). The carbon–carbon bond-forming reaction of **2c, d** leading to **8** is greatly facilitated by the strong silicon–oxygen affinity as compared with oxygen–carbon affinity. On the other hand, in the case of **2a, b** having a bulky *tert*-butyl group on silicon, strong steric hindrance causes the siloxy anion to attack the carbon atom of the thionium intermediate (C), leading to **6**, rather than the silicon atom of the starting reagents (**2a, b**), as shown in Chart 4.

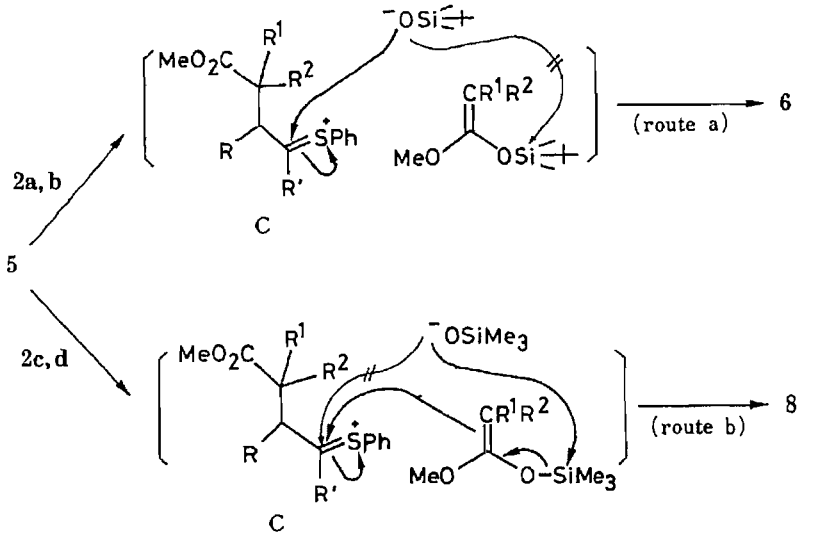
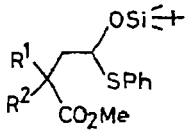
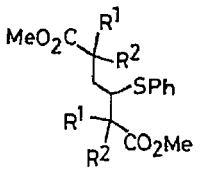
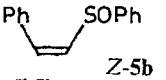
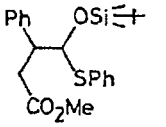
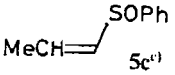
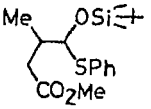
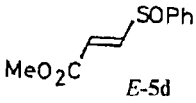
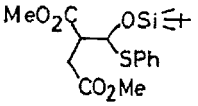
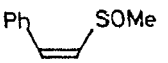
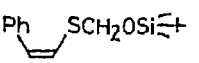
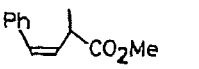
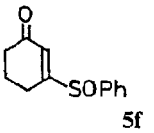
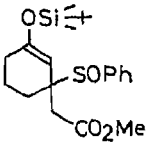


Chart 4

Next, we examined the reaction of the sulfoxide (*Z*-**5e**) having both vinyl and alkyl groups adjacent to the sulfur atom with **2a** and found that the Pummerer-type rearrange-

TABLE I. Reaction of Vinyl Sulfoxides (**5a**—**f**) with *O*-Silylated Ketene Acetals (**2a**—**d**)

Runs	Sulfoxides	Conditions	Products		Yield (%)
1	 **5a**	1.2 eq **2a** r.t. 5 h		**6a** ($R^1=R^2=H$)	65
2	**5a**	1.5 eq **2b** r.t. 12 h		**6b** ($R^1=Me$, $R^2=H$) (1 : 1)[a]	66[b]
3	**5a**	3.0 eq **2c** r.t. 5 min		**8a** ($R^1=Me$, $R^2=H$)[a]	58
4	**5a**	3.0 eq **2d** 80 °C 5 h		**8b** ($R^1=R^2=Me$)	45
5	 *Z*-**5b**	1.2 eq **2a** 70 °C 5 h		**6c** (1 : 1.7)[a]	32
6	*E*-**5b**	1.2 eq **2a** r.t. 15 h		**6c** (1 : 1.6)[a]	41[b]
7	 **5c**[c]	1.2 eq **2a** r.t. 15 h		**6d** (1 : 1)[a]	40
8	 *E*-**5d**	1.2 eq **2a** r.t. 36 h		**6e** (1 : 1)[a]	53[b]
9	 *Z*-**5e**	1.2 eq **2a** r.t. 11 h		**9**	43
10	*Z*-**5e**	4.5 eq **2c** r.t. 3 h[d]		**10**	40
11	 **5f**	1.2 eq **2a** r.t. 12 h		**11**	76

a) The stereochemistry of the diastereomers has not been determined. *b*) Yield is based on the reacted vinyl sulfoxide. *c*) A mixture of *Z*- and *E*-isomers (*Z*/*E* = *ca.* 1/1) was used. *d*) The reaction was carried out in the presence of a catalytic amount of tetra-*n*-butylammonium fluoride. r.t.: room temperature.

ment[2] of the alkyl sulfoxide part predominates over the Michael–Pummerer-type reaction of the vinyl sulfoxide part. Thus, treatment of *Z*-**5e** with **2a** in the presence of a catalytic amount of zinc iodide in anhydrous acetonitrile at room temperature for 11 h gave *Z*-*tert*-butyldimethylsiloxymethyl styryl sulfide (**9**) and none of the Michael–Pummerer-type reaction product was obtained. The product directly substituted by the ester enolate anion, methyl *Z*-2-methyl-4-phenyl-3-butenoate (**10**), was obtained in moderate yield on treatment of **5e** with **2c** in the presence of a catalytic amount of tetra-*n*-butylammonium fluoride in THF, although the mechanism is unclear.

To establish the course of the reaction of the *O*-silylated ketene acetal with γ-ketovinylsulfoxide, which has two possible orientations of Michael addition based on the α,β-unsaturated ketone[4] and vinyl sulfoxide moieties, the sulfoxide (**5f**) was reacted with **2a** in the presence of a catalytic amount of zinc iodide in acetonitrile at room temperature for 12 h to give the silyl enol ether (**11**) by the conjugate addition of **2a** to the α,β-unsaturated ketone moiety, accompanied by a silyl group transfer. These results are summarized in Table I.

Finally, we examined the reaction of *O*-silylated ketene acetal with some related

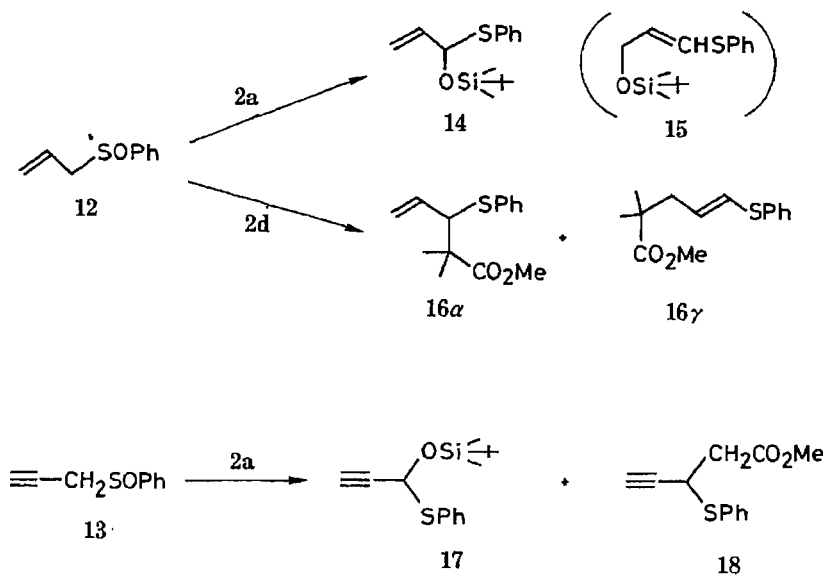


Chart 5

sulfoxides, such as allyl phenyl sulfoxide (**12**) and propargyl phenyl sulfoxide (**13**). Treatment of **12** with **2a** under conditions similar to those described for the reaction of **5** and **2** furnished the Pummerer-type rearrangement product (**14**) in a moderate yield and none of the vinylogous Pummerer-type rearrangement product (**15**) could be obtained.[5] On the other hand, treatment of **12** with **2d** gave an inseparable mixture of carbon–carbon bond-formed products (**16α** and **16γ**) at both the α and γ positions. The γ-substituted isomer (**16γ**) predominated, the **16α**:**16γ** ratio being *ca.* 2:3, based on the relative intensities of the well separated ^{1}H-NMR signals due to the ester–methyl protons of the constituent isomers.

Treatment of propargyl phenyl sulfoxide (**13**) with **2a** gave a mixture of α-substituted products, from which the major constituents (**17** and **18**) were separated in 36 and 24% yields, respectively, by careful column chromatography (Chart 5).

As is clear from the examples cited above, the reaction of vinyl sulfoxides or the related sulfoxides with *O*-silylated ketene acetals, together with the previous results on the Pummerer-type rearrangement of sulfoxides,[2] could be of considerable utility for the preparation of a wide variety of α-siloxy phenyl sulfide derivatives.

Experimental

All melting and boiling points are uncorrected. IR absorption spectra were recorded on a JASCO HPIR-102 spectrometer, and ^{1}H-NMR spectra on a Hitachi R-22 (90 MHz), or a JEOL JNM-FX 90Q FT-NMR (90 MHz) spectrometer (with tetramethylsilane as an internal standard). Low- and high-resolution mass spectra (MS) were obtained with a JEOL JMS D-300 instrument, with a direct inlet system. For column chromatography, E. Merck Silica-gel (70—230 mesh ASTM) was used.

Vinyl Sulfoxides (5a—f)——The sulfoxide **5a** is commercially available. Other sulfoxides (*Z*-**5b**,[6] *E*-**5b**,[6b,7] **5c**,[8] **5d**[9] *Z*-**5e**[10] and **5f**[11]) were prepared by the reported methods.

General Procedure for the Preparation of Methyl 4-Phenylthio-4-siloxybutanoates (6a—e) and Dimethyl 3-(Phenylthio)adipates (8a—e)——The following procedure is typical. A solution of the ketene silyl acetal (**2**, 6 mmol) was added dropwise to a stirred solution of the vinyl sulfoxide (**5**, 5 mmol) and zinc iodide (38 mg, 0.12 mmol) in dry acetonitrile (7 ml) at room temperature under nitrogen. The mixture was stirred at the temperature and for the period indicated in Table I, then one drop of pyridine was added to the solution. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in *n*-hexane (30 ml) with vigorous shaking and undissolved material was filtered off. The filtrate was concentrated under reduced pressure to give a residue, which was subjected to column chromatography on silica gel with benzene and purified by distillation under reduced pressure to give the pure product (**6** or **8**).

Methyl 4-*tert*-Butyldimethylsiloxy-4-(phenylthio)butanoate (6a)——This (1.10 g) was prepared from **2a** (1.13 g, 6 mmol), ZnI_2 (38 mg, 0.12 mmol) and phenyl vinyl sulfoxide (**5a**, 760 mg, 5 mmol) in dry CH_3CN (7 ml). Distillation under reduced pressure gave pure **6a** as a colorless liquid, bp 240—245 °C/10.2 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1070, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.05 (s, 6H, Me_2Si), 0.89 (s, 9H, *tert*-BuSi), 1.8—2.7 (m, 4H, $CH_2 \times 2$), 3.63 (s, 3H, OMe), 5.11 (t, 1H, J=6 Hz, C<u>H</u>SPh), 7.1—7.6 (m, 5H, SPh). *Anal.* Calcd for $C_{17}H_{28}O_3SSi$: C, 59.96; H, 8.27; S, 9.41. Found: C, 59.86; H, 8.44; S, 9.58.

Methyl 4-*tert*-Butyldimethylsiloxy-2-methyl-4-(phenylthio)butanoate (6b)——This (156 mg) was prepared from **2b** (312 mg, 1.5 mmol) ZnI_2 (20 mg, 0.063 mmol) and **5a** (152 mg, 1 mmol) in dry CH_3CN (3 ml). There was a 34% (52 mg) recovery of **5a**. Distillation under reduced pressure gave a 1 : 1 diastereo-mixture of **6b** as a colorless liquid, bp 135—140 °C/0.20 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 835. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.05 (s, 3H, MeSi), 0.09 (s, 3H, MeSi), 0.89 (s, 9H, *tert*-BuSi), 1.15 (d, 3H, J=7.2 Hz, Me), 1.7—2.9 (m, 3H, C<u>H</u>$_2$C<u>H</u>CO$_2$Me), 3.64 (s, 3/2H, OMe × 1/2), 3.66 (s, 3/2H, OMe × 1/2), 4.95—5.3 (m, 1H, CHSPh), 7.1—7.7 (m, 5H, SPh). MS *m/z*: 339 ($M^+ - Me$), 323 ($M^+ - OMe$), 297 ($M^+ -$ *tert*-Bu). *Anal.* Calcd for $C_{18}H_{30}O_3SSi$; C, 60.97; H, 8.53; S, 9.04. Found: C, 61.22; H, 8.80; S, 9.03.

Methyl 4-*tert*-Butyldimethylsiloxy-3-phenyl-4-(phenylthio)butanoate (6c)——i) From *Z*-Phenyl Styryl Sulfoxide (*Z*-**5b**): This (530 mg) was prepared from **2a** (789 mg, 4.2 mmol) ZnI_2 (27 mg, 0.08 mmol) and *Z*-phenyl styryl sulfoxide (*Z*-**5b**, 798 mg, 3.5 mmol) in dry CH_3CN (5 ml). Distillation under reduced pressure gave a 5 : 3 diastereo-mixture of **6c** as a colorless liquid, bp 180—185 °C/0.18 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1085, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: −0.14 (s, 3H, MeSi), −0.05 (s, 9/8H, MeSi × 3/8), 0.02 (s, 15/8H, MeSi × 5/8), 0.81 (s, 27/8H, *tert*-BuSi × 3/8), 0.89 (s, 45/8H, *tert*-BuSi × 5/8), 2.7—3.2 (m, 2H, CH_2), 3.3—3.6 (m, 1H, CHPh), 3.53 (s, 3H, OMe), 5.16 (d, 5/8H, J=4.4 Hz, CHSPh × 5/8), 5.29 (d, 3/8H, J=5.8 Hz, CHSPh × 3/8), 6.9—7.5 (m, 10H, Ph × 2). MS *m/z*: 401 ($M^+ - Me$). 359 ($M^+ -$ *tert*-Bu). Exact mass Calcd for $C_{23}H_{32}O_3SSi$–*tert*-Bu: 359.1135. Found: 359.1112.

ii) From *E*-**5b**: This (186 mg) was prepared from **2a** (452 mg, 2.4 mmol), ZnI_2 (36 mg, 0.11 mmol) and *E*-**5b** (456 mg, 2 mmol), in dry CH_3CN (5 ml). There was a 45% (204 mg) recovery of **5b**. Distillation under reduced pressure gave an 8 : 5 diastereo-mixture of **6c** as a colorless liquid, bp 180—185 °C/0.18 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1085, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: −0.14 (s, 3H, MeSi), −0.05 (s, 15/13H, MeSi × 5/13), 0.02 (s, 24/13H, MeSi × 8/13), 0.81 (s, 45/13H, *tert*-BuSi × 5/13), 0.89 (s, 72/13H, *tert*-BuSi × 8/13), 2.7—3.2 (m, 2H, CH_2), 3.3—3.6 (m, 1H, CHPh), 3.53 (s, 3H, OMe), 5.16 (d, 8/13H, J=4.4 Hz, CHSPh × 8/13), 5.29 (d, 5/13H, J=5.8 Hz, CHSPh × 5/13), 6.9—7.5 (m, 10H, Ph × 2). MS *m/z*: 401 ($M^+ - Me$), 359 ($M^+ -$ *tert*-Bu). Exact mass Calcd for $C_{23}H_{32}O_3SSi$–*tert*-Bu: 359.1138. Found: 359.1143.

Methyl 4-*tert*-Butyldimethylsiloxy-3-methyl-4-(phenylthio)butanoate (6d)——This (710 mg) was prepared from **2a** (1.128 g, 6 mmol), ZnI_2 (38 mg, 0.12 mmol) and a 1 : 1 mixture of *Z*- and *E*-phenyl 1-propenyl sulfoxide (**5c**, 831 mg, 5 mmol) in dry CH_3CN (6 ml). Distillation under reduced pressure gave a 1 : 1 diastereo-mixture of **6d** as a colorless liquid, bp 145—150 °C/0.18 mmHg (bath temp). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725, 1070, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.01 (s, 3H, MeSi), 0.03 (s, 3/2H, MeSi × 1/2), 0.07 (s, 3/2H, MeSi × 1/2), 0.92 (s, 9H, *tert*-BuSi), 1.08 (d, 3/2H, J= 5.0 Hz, C-Me × 1/2), 1.14 (d, 3/2H, J=5.0 Hz, C-Me × 1/2), 2.1—2.9 (m, 3H, C<u>H</u>C<u>H</u>$_2$CO$_2$Me), 3.65 (s, 3/2H, OMe × 1/2), 3.67 (s, 3/2H, OMe × 1/2), 5.06 (d, 1/2H, J=7.0 Hz, CHSPh × 1/2), 5.13 (d, 1/2H, J=7.0 Hz, CHSPh × 1/2), 7.0—7.6 (m, 5H, SPh). MS *m/z*: 297 ($M^+ -$ *tert*-Bu). *Anal.* Calcd for $C_{18}H_{30}O_3SSi$: C, 60.97; H, 8.53; S, 9.04. Found: C, 61.11; H, 8.63; S, 8.97.

Dimethyl 2-[*tert*-Butyldimethylsiloxy(phenylthio)methyl]succinate (6e)——This (224 mg) was prepared from **2a** (452 mg, 2.4 mmol), ZnI_2 (38 mg, 0.12 mmol) and *E*-methyl 3-(phenylsulfinyl)acrylate (*E*-**5d**, 421 mg, 2 mmol) in dry CH_3CN (5 ml). There was a 47% (198 mg) recovery of *E*-**5d**. Distillation under reduced pressure gave a 1 : 1 diastereo-mixture of **6e** as a colorless liquid, bp 190—195 °C/0.17 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1735, 1730, 1080, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.00 (s, 3H, MeSi), 0.03 (s, 3/2H, MeSi × 1/2), 0.09 (s, 3/2H, MeSi × 1/2), 0.85 (s, 9H, *tert*-BuSi), 2.7—3.7 (m, 3H, C<u>H</u>C<u>H</u>$_2$CO$_2$Me), 3.64 (s, 3H, OMe), 3.68 (s, 3H, OMe), 5.38 (d, 1/2H, J=6.5 Hz, CHSPh × 1/2), 5.52 (d, 1/2H, J=3.5 Hz, CHSPh × 1/2), 7.0—7.6 (m, 5H, SPh). MS *m/z*: 341 ($M^+ -$ *tert*-Bu). *Anal.* Calcd for $C_{19}H_{30}O_5SSi$: C, 57.25; H, 7.59; S, 8.04. Found: C, 57.45; H, 7.65; S, 8.20.

Methyl 3-Formyl-3-phenylpropanoate (7)——A solution of tetra-*n*-butylammonium fluoride (86 mg, 0.33 mmol) was added to a solution of **6c** (114 mg, 0.27 mmol) in dry THF (2 ml) at room temperature under nitrogen. The mixture was stirred for 5 min under the same conditions and concentrated *in vacuo*. The residue was subjected to column chromatography on silica gel with *n*-hexane : ethyl acetate = 10 : 1 to give a 61% yield (32 mg) of **7** as a colorless liquid, bp 105 °C/0.3 mmHg (bath temp.) (lit.[12] bp 103 °C/0.2 mmHg). All spectral data were identical with those of an authentic sample.

Dimethyl 2,5-Dimethyl-3-(phenylthio)adipate (8a)——This (358 mg) was prepared from **2c** (960 mg, 6 mmol), ZnI_2 (36 mg, 0.11 mmol) and **5a** (304 mg, 2 mmol) in dry CH_3CN (5 ml). Distillation under reduced pressure gave **8a** as a colorless liquid, bp 155—160 °C/0.18 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.0—1.35 (m, 6H, -CMe × 2), 1.5—2.2 (m, 2H, CH_2), 2.5—3.1 (m, 2H, MeC<u>H</u> × 2), 3.2—3.85 (m, 1H, C<u>H</u>SPh), 3.52 (s, 9/5H, OMe × 3/5), 3.61 (s, 6/5H, OMe × 2/5), 3.65 (s, 3H, OMe), 7.0—7.5 (m, 5H, SPh). MS *m/z*: 310 (M^+). *Anal.* Calcd for $C_{16}H_{22}O_4S$: C, 61.91; H, 7.14; S, 10.33. Found: C, 61.76; H, 7.18; S, 10.26.

Dimethyl 2,2,5,5-Tetramethyl-3-(phenylthio)adipate (8b)——This (304 mg) was prepared from **2d** (1.04 g, 6 mmol), ZnI_2 (36 mg, 0.11 mmol) and **5a** (304 mg, 2 mmol) in dry CH_3CN (5 ml). Distillation under reduced pressure gave **8b** as a colorless liquid, bp 185—190 °C/0.18 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.15 (s, 3H, C–Me), 1.19 (s, 3H, C–Me), 1.26 (s, 6H, C–Me × 2), 1.60—2.12 (m, 2H, CH_2), 3.40 (s, 3H, OMe), 3.60 (s, 3H, OMe), 3.3—3.8 (m, 1H, CHSPh), 7.0—7.5 (m, 5H, SPh). Exact mass Calcd for $C_{18}H_{26}O_4S$: 338.1550. Found: 338.1545.

***Z-tert*-Butyldimethylsiloxymethyl Styryl Sulfide (9)**——This (800 mg) was prepared from **2a** (1.579 g, 8.4 mmol), ZnI_2 (54 mg, 0.17 mmol) and *Z*-methyl styryl sulfoxide (*Z*-**5e**, 742 mg, 7 mmol) in dry CH_3CN (7 ml). Distillation under reduced pressure gave pure **9** as a colorless liquid, bp 225—230 °C/0.2 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1600, 1260, 1085, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.14 (s, 6H, Me_2Si), 0.90 (s, 9H, *tert*-BuSi), 4.94 (s, 2H, OCH_2S), 6.46 (s, 2H, CH=CH), 7.1—7.6 (m, 5H, SPh). Exact mass Calcd for $C_{15}H_{24}OSSi$: 280.1318. Found: 280.1319.

Methyl *Z*-2-Methyl-4-phenyl-3-butenoate (10)——A 1 M solution of tetra-*n*-butylammonium fluoride in THF (36 μl, 0.036 mmol) was added dropwise to a solution of *Z*-**5e** (318 mg, 3 mmol) in dry THF (4 ml) at room temperature under nitrogen. After addition of **2c** (598 mg, 13.6 mmol), the reaction mixture was stirred for 3 h under the same conditions and worked up according to the general procedure to give **10**. Distillation under reduced pressure gave pure **10** as a colorless liquid, bp 140—150 °C/12 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 1.29 (d, 3H, J=7.0 Hz, –CMe), 3.3—4.1 (m, 1H, CHCO$_2$Me), 3.69 (s, 3H, OMe), 5.67 (dd, 1H, J=10 and 11.5 Hz, =CH), 6.54 (d, 1H, J=11.5 Hz, PhCH=), 7.26 (s, 5H, Ph). MS *m/z*: 190 (M^+). Exact mass Calcd for $C_{12}H_{14}O_2$: 190.0980. Found: 190.0968.

1-*tert*-Butyldimethylsiloxy-3-methoxycarbonylmethyl-3-(phenylsulfinyl)cyclohexene (11)——Compound **2a** (452 mg, 2.4 mmol) was added to a stirred solution of 3-(phenylsulfinyl)-2-cyclohexenone (**5f**, 440 mg, 2 mmol) and ZnI_2 (36 mg, 0.11 mmol) in dry CH_3CN (5 ml) at room temperature under nitrogen. The mixture was stirred for 12 h under the same conditions, diluted with ether (20 ml) and washed with aqueous $NaHCO_3$. The organic layer was washed with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated *in vacuo* to give a residue, which was purified by column chromatography on silica gel with *n*-hexane : benzene = 2 : 3 to give a 76% yield (621 mg) of **11** as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1730, 1640, 1095, 1040, 835. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.07 (s, 3H, MeSi), 0.16 (s, 3H, MeSi), 0.86 (s, 9H, *tert*-BuSi), 1.4—2.4 (m, 6H, CH_2 × 3), 2.63 (s, 2H, CH_2CO_2Me), 3.66 (s, 3H, OMe), 6.61—6.71 (brs, 1H, CH=), 7.3—7.8 (m, 5H, Ph). MS *m/z*: 351 (M^+ – *tert*-Bu). Exact mass Calcd for $C_{21}H_{32}O_4SSi$–*tert*-C_4H_9: 351.1069: Found: 351.1084.

1-*tert*-Butyldimethylsiloxyallyl Phenyl Sulfide (14)——This (163 mg, 54% yield) was prepared from **2a** (290 mg, 1.54 mmol), ZnI_2 (13 mg, 0.04 mmol) and allyl phenyl sulfoxide[13] (**12**, 178 mg, 1.07 mmol) in dry CH_3CN (1 ml) at room temperature for 15 min. Distillation under reduced pressure gave pure **14** as a colorless liquid, bp 100—110 °C/0.17 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1645, 1030, 840. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.00 (s, 6H, Me_2Si), 0.88 (s, 9H, *tert*-BuSi), 4.98 (dt, 1H, J=11 and 1.5 Hz, CH=CH$_2$), 5.16 (dt, 1H, J=17 and 1.5 Hz, CH=CH$_2$), 5.50 (dt, 1H, J=5 and 1.5 Hz, CHSPh), 5.90 (ddd, 1H, J=17, 11 and 5 Hz, CH=CH$_2$), 7.1—7.6 (m, 5H, SPh). *Anal.* Calcd for $C_{15}H_{24}OSSi$: C, 64.23; H, 8.62; S, 11.43. Found: C, 64.25; H, 8.85; S, 11.50.

Methyl 2,2-Dimethyl-3-(phenylthio)-4-pentenoate (16α) and Methyl *E*-2,2-Dimethyl-5-(phenylthio)-4-pentenoate (16γ)——An inseparable mixture (47%, 178 mg) of **16α** and *E*-**16γ** was obtained from **2d** (800 mg, 4.59 mmol), ZnI_2 (27 mg, 0.085 mmol) and **12** (254 mg, 1.53 mmol) in dry CH_3CN (2 ml). Distillation under reduced pressure gave a 2 : 3 mixture of **16α** and *E*-**16γ** as a colorless liquid, bp 150 °C/0.18 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725. ^{1}H-NMR (10% solution in $CDCl_3$) δ: signals due to **16α**; 1.24 (s, 6/5H, Me × 2/5), 1.34 (s, 6/5H, Me × 2/5), 3.75 (s, 6/5H, OMe × 2/5), 3.85 (d, 2/5H, J=9.5 Hz, CHSPh × 2/5), 4.89 (dd, 2/5H, J=17.3 and 3 Hz, CH$_2$=CH × 2/5), 5.05 (dd, 2/5H, J=10 and 3 Hz, CH$_2$=CH × 2/5), 5.89 (ddd, 2/5H, J=17.3, 10, and 9.5 Hz, CH$_2$=CH × 2/5), 7.0—7.6 (m, 2H, SPh × 2/5); signals due to *E*-**16γ**; 1.21 (s, 18/5H, CMe_2 × 3/5), 2.40 (d, 6/5H, J=7.2 Hz, CH$_2$CH= × 3/5), 3.73 (s, 9/5H, OMe × 3/5), 5.92 (dt, 3/5H, J=15.5 and 7.2 Hz, CH= × 3/5), 6.29 (d, 3/5H, J=15.5 Hz, =CHSPh × 3/5), 7.0—7.6 (m, 3H, SPh × 3/5). ^{1}H-NMR (10% solution in C_6D_6) δ: signals due to **16α**; 1.24 (s, 6/5H, Me × 2/5), 1.37 (s, 6/5H, Me × 2/5), 3.49 (s, 6/5H, OMe × 2/5), 3.95 (d, 2/5H, J=9.5 Hz, CHSPh × 2/5), 4.78 (dd, 2/5H, J=17 and 3.5 Hz, CH$_2$=CH × 2/5), 4.87 (dd, 2/5H, J=11.2 and 3.5 Hz, CH$_2$=CH × 2/5), 5.86 (ddd, 2/5H, J=17, 11.2, and 9.5 Hz, CH$_2$=CH × 2/5), 6.9—7.6 (m, 2H, SPh × 2/5); signals due to *E*-**16γ**; 1.10 (s, 18/5H, CMe_2 × 3/5), 2.25 (d, 6/5H, J=6.8 Hz, CH$_2$CH= × 3/5), 3.41 (s, 9/5H, OMe × 3/5), 5.95 (dt, 3/5H, J=15.3 and 6.8 Hz, CH= × 3/5), 6.22 (d, 3/5H, J=15.3 Hz, =CHSPh × 3/5), 6.9—7.6 (m, 3H, SPh × 3/5). These assignments are in good accord with those of related sulfides.[14] *Anal.* Calcd for $C_{14}H_{18}O_2S$: C, 67.17; H, 7.25; S, 12.81. Found: C, 67.16; H, 7.39; S, 12.68.

1-*tert*-Butyldimethylsiloxy-2-propynyl Phenyl Sulfide (17) and Methyl 3-(Phenylthio)-4-pentynoate (18)——These compounds were obtained from phenyl propargyl sulfoxide[15] (**13**, 246 mg, 1.5 mmol) and **2a** (338 mg, 1.79 mmol) in anhydrous CH_3CN (3 ml) at room temperature for 1 h. Work-up as usual gave a residue, which was subjected to column chromatography on silica gel with *n*-hexane : ether = 5 : 1 to give a 36% yield (152 mg) of **17** and a 24% yield (80 mg) of **18**. **17**: colorless syrup, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3300, 2120. ^{1}H-NMR (10% solution in $CDCl_3$) δ: 0.05 (s, 6H, Me_2Si), 0.87 (s, 9H, *tert*-BuSi), 2.67 (d, 1H, J=2.8 Hz, C≡CH), 5.70 (d, 1H, J=2.8 Hz, OCHSPh), 7.2—7.8 (m, 5H, SPh). Exact mass Calcd for $C_{15}H_{22}OSSi$, 278.1158. Found: 278.1153. **18**: colorless syrup, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3300, 1730.

^{1}H-NMR (10% solution in $CDCl_3$) δ: 2.37 (d, 1H, J=2.4 Hz, C≡CH), 2.78 (d, 2H, J=7.5 Hz, CH_2CO_2Me), 3.72 (s, 3H, CO_2Me), 4.20 (dt, 1H, J=7.5 and 2.4 Hz, CHSPh), 7.2—7.8 (m, 5H, SPh). Exact mass Calcd for $C_{12}H_{12}O_2S$: 220.0555. Found: 220.0543.

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Hypervalent Iodine Oxidation of α,β-Unsaturated Carbonyl Compounds

YASUMITSU TAMURA,* TAKAYUKI YAKURA, HIROAKI TERASHI, JUN-ICHI HARUTA, and YASUYUKI KITA

Faculty of Pharmaceutical Sciences, Osaka University, 1-6, Yamada-oka, Suita, Osaka 565, Japan

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Oxidation of non-enolizable α,β-unsaturated carbonyl compounds and enolizable β-monosubstituted α,β-unsaturated carbonyl compounds with phenyl iodine(III) diacetate (PIDA) in methanolic potassium hydroxide gave α-hydroxydimethylacetal β-methoxy products, and oxidation of enolizable β,β-disubstituted α,β-unsaturated carbonyl compounds under the same conditions gave α′-hydroxy products.

Keywords——phenyl iodine(III) diacetate; oxidation of α,β-unsaturated carbonyl compound; α-hydroxydimethylacetal β-methoxy product; α′-hydroxy-α,β-unsaturated ketone; methanolic potassium hydroxide solution

In spite of considerable interest in the hypervalent iodine oxidation of ketones leading to α-hydroxydimethylacetals (**1**),[1] only a few reports on the oxidation of α,β-unsaturated ketones have appeared. The reaction of $PhI(OAc)_2$ [phenyl iodine(III) diacetate, PIDA] in methanolic potassium hydroxide with non-enolizable α,β-unsaturated ketones which cannot form anions by α-hydrogen abstraction, such as chromones, flavone, and chalcone, was shown to give α-hydroxydimethylacetal β-methoxy products.[2] Further, an enolizable α,β-unsaturated ketone, 3,5,5-trimethyl-2-cyclohexenone (isophorone), was shown to give the α-hydroxy product, 2-hydroxy-3,5,5-trimethyl-2-cyclohexenone.[3] As a part of our study of the chemistry of hypervalent iodine reagents,[4] we have examined the reaction of PIDA with various types of α,β-unsaturated carbonyl systems, some of which possess α′ and γ CH_2-units. It was found that both non-enolizable α,β-unsaturated carbonyl compounds (**2a, b**) and enolizable β-monosubstituted α,β-unsaturated carbonyl compounds (**2c—f**) react with PIDA in KOH–MeOH to give α-hydroxydimethylacetal β-methoxy products (**3a, b** and **3c—f**), and enolizable β,β-disubstituted α,β-unsaturated carbonyl compounds (**7a—c**) react with PIDA/KOH–MeOH to give α′-hydroxy products (**8a—c**, **9a, b** and **10**).

Results and Discussion

To establish the generality of the oxidation of non-enolizable α,β-unsaturated carbonyl compounds with PIDA/KOH–MeOH, which was shown to give α-hydroxydimethylacetal β-methoxy products,[2] we first examined the reaction of acyclic systems (**2a, b**) with PIDA/KOH–MeOH. The oxidation of *trans*-cinnamaldehyde (**2a**) takes place at the α-position at room temperature for 30 min to give the α-hydroxydimethylacetal β-methoxy product (**3a**) in 62% yield. The oxidation of **2b** with PIDA was accomplished under similar conditions to give a 78% yield of a 1:2 mixture of α-hydroxydimethylacetal β-methoxy product (**3b**) and its deacetalization product (**3b′**), from which only the latter compound was isolated in a pure state because of the ready deacetalization of **3b** (Chart 1).

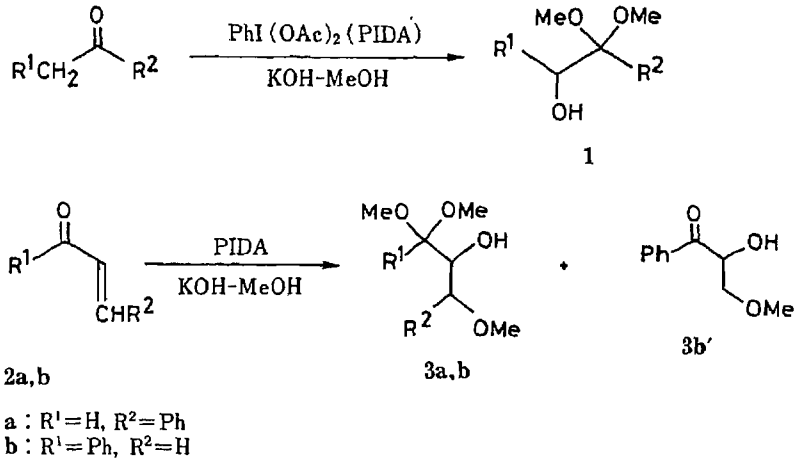
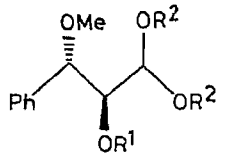
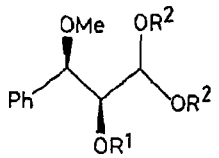


Chart 1

The present oxidation of **2a** gave a mixture of diastereomers with no stereoselectivity. Thus, stereoisomers [*threo*-**3a** (**3a**-*syn*) and *erythro*-**3a** (**3a**-*anti*)] were separated by column chromatography on silica gel in a ratio of 53 : 47. A tentative assignment of the stereostructures was made on the basis of nuclear magnetic resonance (NMR) studies of the derivatives (**4**—**6**) from each stereoisomer, since Heathcock and co-workers have pointed out[5] that the C-3 proton in the *anti*-aldol adducts generally resonates at higher field than the corresponding proton in the *syn*-diastereomers, and the vicinal coupling constant ($J_{2,3}$) in the *anti*-isomers has a larger value than that of the *syn*-isomers. Data for all these derivatives (**4, 5**) are in good accord with the expected values. The stereochemistry of **3a** was finally confirmed by direct comparison of the methylated **3a**-*syn* (**6**-*syn*) with an authentic specimen prepared from *trans*-cinnamaldehyde dimethylacetal[6] by stereospecific *cis*-hydroxylation with osmium tetroxide followed by dimethylation of the resultant *vic*-glycol, as shown in Chart 2.



3a,4,5-*anti*



3a, 4,5-*syn*

	anti $\delta(J_{2,3})$	*syn* $\delta(J_{2,3})$
3a : R1=H, R2=Me	4.25 (5.0)	4.31 (4.5)
4 : R1=Ac, R2=Me	4.38 (6.3)	4.49 (4.0)
5 : R1=Ac, R2=-(CH2)2-	4.37 (7.0)	4.45 (4.0)

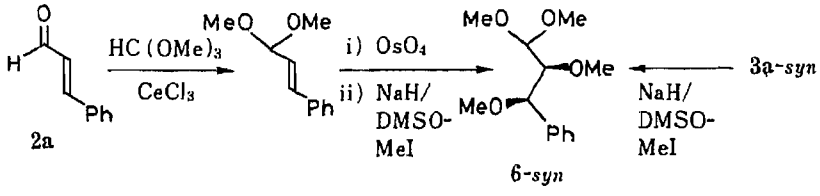


Chart 2

Next, we examined the oxidation of enolizable β-monosubstituted α,β-unsaturated carbonyl compounds (**2c—f**) with PIDA/KOH–MeOH. The oxidation takes place at the α-position under similar conditions to give the corresponding α-hydroxydimethylacetal β-methoxy products (**3c—f**) in moderate yields. The *cis*-relationship of the C(2)-hydroxy and C(3)-methoxy groups of **3c—e** was deduced from a comparison of the coupling constants with those of related compounds.[2a] The formation of **3c—e** from **2c—e** is best explained by the same mechanistic pathway as proposed for the reaction of non-enolizable α,β-unsaturated carbonyl compounds with PIDA/KOH–MeOH by Moriarty *et al.*,[2] as shown in Chart 3.

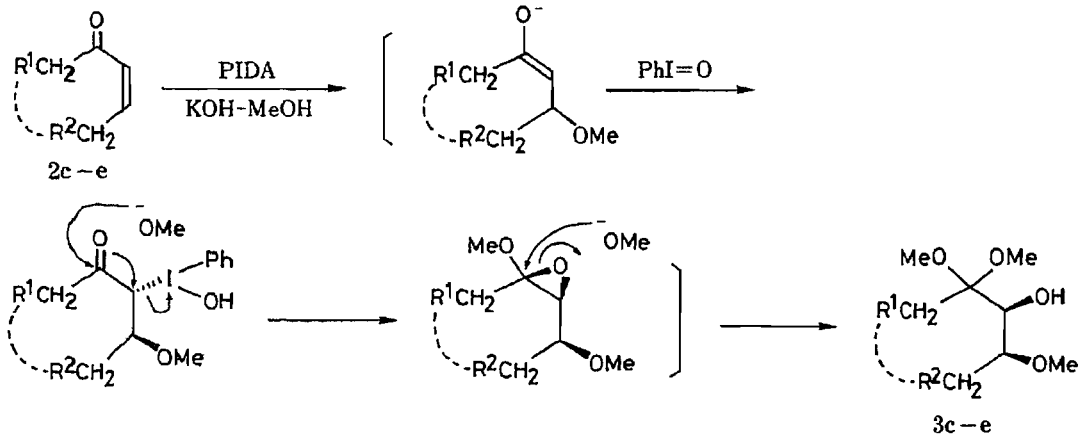
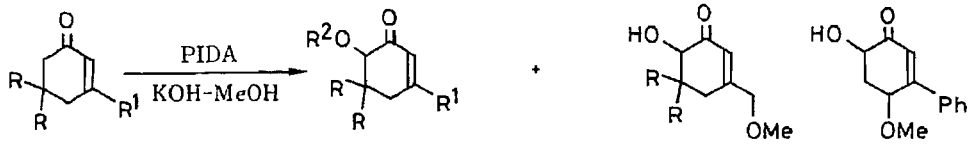


Chart 3

On the other hand, the oxidation of β,β-disubstituted α,β-unsaturated ketones **7a—c** with PIDA takes place at the α′-position to give α′-hydroxy products (**8a—c**) together with α′-hydroxy-γ-methoxy products (**9a, b** and **10**) (Chart 4).[7] The structures of **8a, b** were confirmed by direct comparison of their *O*-acetates (**11a, b**) with authentic specimens prepared by an alternative method[8] and those of other novel compounds (**3a—f, 4—6, 8c, 9a, b** and **10**) were fully supported by satisfactory spectral data[9] (given in the experimental section). Although the mechanism of formation of these α′-hydroxy products (**8—10**) is not rigorously defined, it is suggested that Michael addition of the methoxy anion to the enones is restricted by steric hindrance, and α′- and γ-enolizations occur to give the products.



7a: R=H, R^1=CH_3
7b: R=R^1=CH_3
7c: R=H, R^1=C_6H_5

8a: R=R^2=H, R^1=CH_3
8b: R=R^1=CH_3, R^2=H
8c: R=R^2=H, R^1=C_6H_5
11a: R=H, R^1=CH_3, R^2=$COCH_3$
11b: R=R^1=CH_3, R^2=$COCH_3$

9a: R=H
9b: R=CH_3

10

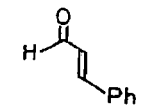
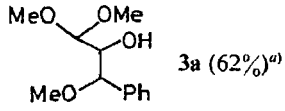
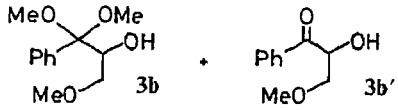
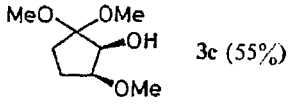
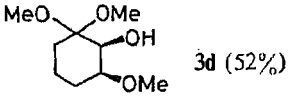
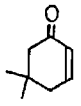
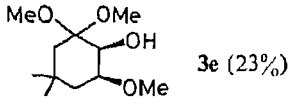
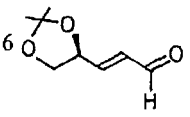
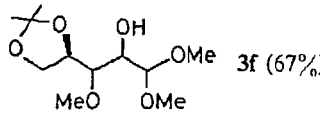
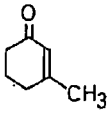
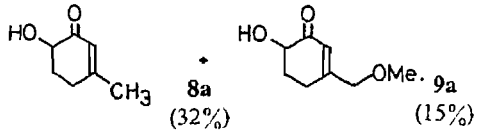
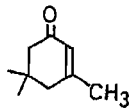
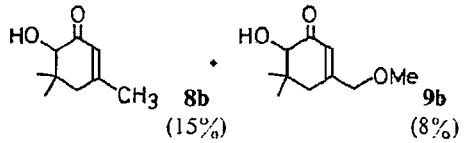
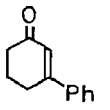
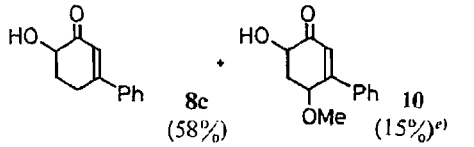
Chart 4

The reaction conditions, products, and yields are summarized in Table I.

Experimental

All melting and boiling points are uncorrected. Infrared (IR) absorption spectra were recorded on a JASCO

TABLE I. Oxidation of α,β-Unsaturated Carbonyl Compounds with PIDA/KOH–MeOH

Run	α,β-Unsaturated carbonyl compounds	Reaction conditions	Products (Yields)
1	 **2a**	2 eq PIDA r.t. 30 min	 **3a** (62%)[a]
2	 **2b**	1.1 eq PIDA r.t. 20 min	 **3b** + **3b'** 3b/3b' = 1/2 (76%),[b] 2/1 (69%)[c]
3	 **2c**	1.1 eq PIDA 0 °C, 40 min	 **3c** (55%)
4	 **2d**	1.1 eq PIDA 0 °C, 1 h	 **3d** (52%)
5	 **2e**	1.1 eq PIDA r.t. 30 min	 **3e** (23%)
6	 **2f**	2 eq PIDA r.t. 40 min	 **3f** (67%)[d]
7	 **7a**	3 eq PIDA r.t. 1 h	 **8a** (32%) + **9a** (15%)
8	 **7b**	3 eq PIDA r.t. 30 min	 **8b** (15%) + **9b** (8%)
9	 **7c**	2 eq PIDA r.t. 30 min	 **8c** (58%) + **10** (15%)[e]

a) A diastereo-mixture of **3a**-*syn* and **3a**-*anti* (**3a**-*syn* : **3a**-*anti* = 53 : 47) was obtained. *b*) The reaction mixture was worked-up by the general procedure. *c*) The reaction mixture was concentrated and subjected directly to column chromatography without washing with saturated aqueous NaCl. *d*) A 15 : 5 : 5 : 3 diastereo-mixture was obtained. *e*) A 3 : 2 diastereo-mixture was obtained.

HPIR-102 spectrometer, and ^{1}H-NMR spectra (10% solution in $CDCl_3$) on a Hitachi R-22 (90 MHz) spectrometer (with tetramethylsilane as an internal standard). Low- and high-resolution mass spectra (MS) were obtained with a JEOL JMS D-300 instrument, with a direct inlet system at 70 eV. Column chromatography was carried out on Merck Silica gel 60.

General Procedure for the Oxidation of α,β-Unsaturated Carbonyl Compounds (2a—f and 7a—c) with PIDA/KOH–MeOH——A solution of α,β-unsaturated carbonyl compound (2 mmol) in anhydrous MeOH (3 ml) and a solution of KOH (12—18 mmol) in anhydrous MeOH (5 ml) were added dropwise to a stirred solution of PIDA (4 mmol) in anhydrous MeOH (12 ml) at room temperature under nitrogen. The reaction mixture was stirred for 30 min under the same conditions, concentrated under reduced pressure, and partitioned between water (20 ml) and

methylene chloride (20 ml). The aqueous layer was extracted with methylene chloride (10 ml × 2). The combined extract was washed with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude oxidized product, which contains iodobenzene. A pure sample was isolated by column chromatography on silica gel with *n*-hexane–ethyl acetate (2 : 1—10 : 1).

2-Hydroxy-3-methoxy-3-phenylpropanal Dimethylacetal (3a), 2-Acetoxy-3-methoxy-3-phenylpropanal Dimethylacetal (4) and 2-Acetoxy-3-methoxy-3-phenylpropanal-1′,3′-dioxolane (5)——Compound **3a** (280 mg) was obtained from **2a** (264 mg, 2 mmol), PIDA (1.288 g, 4 mmol) and KOH (672 mg, 12 mmol) as a diastereo-mixture of **3a**-*anti* and **3a**-*syn* in a ratio of 53 : 47. The mixture was chromatographed on silica gel to give pure **3a**-*anti* (148.5 mg) and **3a**-*syn* (131.6 mg). **3a**-*anti*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3500, 1595, 1450. ^{1}H-NMR δ: 2.32 (brs, 1H, OH, disappeared on addition of D_2O), 3.22 (s, 3H, OMe), 3.34 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.9—4.1 (m, 1H, C_2-H), 4.13 (d, 1H, J=4.5 Hz, C_1-H), 4.25 (d, 1H, J=5 Hz, C_3-H), 7.30 (s, 5H, ArH). Exact mass Calcd for $C_{12}H_{18}O_4$–H_2O: 208.1097. Found: 208.1081. **3a**-*syn*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3500, 1600, 1450. ^{1}H-NMR δ: 2.75 (brs, 1H, OH, disappeared on addition of D_2O), 3.26 (s, 3H, OMe), 3.38 (s, 6H, OMe × 2), 3.6—3.8 (m, 1H, C_2-H), 4.24 (d, 1H, J=5 Hz, C_1-H), 4.31 (d, 1H, J=4.5 Hz, C_3-H), 7.31 (s, 5H, ArH). Exact mass Calcd for $C_{12}H_{18}O_4$–H_2O: 208.1099. Found: 208.1102. The acetates (**4**-*anti* and **4**-*syn*) were obtained from **3a**-*anti* and **3a**-*syn* by treatment with Ac_2O–pyridine. **4**-*anti*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1745. ^{1}H-NMR δ: 1.94 (s, 3H, OCOMe), 3.25 (s, 3H, OMe), 3.37 (s, 3H, OMe), 3.41 (s, 3H, OMe), 4.29 (d, 1H, J=5 Hz, C_1-H), 4.38 (d, 1H, J=6.3 Hz, C_3-H), 5.33 (dd, 1H, J=5 and 6.3 Hz, C_2-H), 7.32 (s, 5H, ArH). Exact mass Calcd for $C_{14}H_{20}O_5$–CH_3CO_2H: 208.1097. Found: 208.1081. **4**-*syn*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740. ^{1}H-NMR δ: 1.96 (s, 3H, OCOMe), 3.26 (s, 3H, OMe), 3.30 (s, 3H, OMe), 3.42 (s, 3H, OMe), 4.43 (d, 1H, J=7.5 Hz, C_1-H), 4.49 (d, 1H, J=4 Hz, C_3-H), 5.12 (dd, 1H, J=4 and 7.5 Hz, C_2-H), 7.28 (s, 5H, ArH). Exact mass Calcd for $C_{14}H_{20}O_5$–CH_3CO_2H: 208.1100. Found: 208.1122. The 1,3-dioxolanes (**5**-*anti* and **5**-*syn*) were obtained from **3a**-*anti* and **3a**-*syn*, respectively, by treatment with ethylene glycol in the presence of a catalytic amount of *p*-TsOH in methylene chloride at room temperature overnight, followed by acetylation with Ac_2O–pyridine. **5**-*anti*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740. ^{1}H-NMR δ: 1.84 (s, 3H, OCOMe), 3.22 (s, 3H, OMe), 3.8—4.0 (m, 4H, CH_2 × 2), 4.37 (d, 1H, J=7 Hz, C_3-H), 5.1—5.3 (m, 2H, C_1-H and C_2-H), 7.28 (s, 5H, ArH). Exact mass Calcd for $C_{12}H_{18}O_5$–CH_3CO_2H: 206.0943. Found: 206.0946. **5**-*syn*: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740. ^{1}H-NMR δ: 2.01 (s, 3H, OCOMe), 3.24 (s, 3H, OMe), 3.8—4.1 (m, 4H, CH_2 × 2), 4.45 (d, 1H, J=4 Hz, C_3-H), 4.9—5.1 (m, 2H, C_1-H and C_2-H), 7.27 (s, 5H, ArH). Exact mass Calcd for $C_{12}H_{18}O_5$–CH_3CO_2H: 206.0944. Found: 206.0945.

***threo*-2,3-Dimethoxy-3-phenylpropanal Dimethylacetal (6-*syn*)**——i) From *threo*-2-Hydroxy-3-methoxy-3-phenylpropanal Dimethylacetal (**3a**-*syn*): The acetal (**3a**-*syn*) was methylated by the Hakomori method.[10] Anhydrous dimethyl sulfoxide (2 ml) was added dropwise to oil-free NaH (100 mg) with stirring under nitrogen at 60—70 °C until hydrogen was no longer evolved (about 1 h). Then **3a**-*syn* (118 mg, 0.5 mmol) in dimethyl sulfoxide (2 ml) was added dropwise to a stirred solution of sodium methylsulfinylmethide at room temperature under nitrogen. The mixture was stirred for 1 h under the same conditions and methyl iodide (0.5 ml) was added. After being stirred for 30 min, the reaction mixture was poured into ice-water (20 ml) and extracted with methylene chloride. The extract was washed with aqueous 10%-$Na_2S_2O_3$ and saturated aqueous NaCl, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to column chromatography on silica gel to give a 98% yield (122 mg) of **6**-*syn* as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1600, 1450, 1355, 1185, 1100. ^{1}H-NMR δ: 3.17 (s, 3H, OMe), 3.24 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.0—3.2 (m, 1H, C_2-H), 4.33 (d, 1H, J=4.8 Hz, C_1-H), 4.38 (d, 1H, J=1.8 Hz, C_3-H), 7.29 (s, 5H, ArH). Exact mass Calcd for $C_{13}H_{20}O_4$–CH_3OH: 208.1100. Found: 208.1123.

ii) From *trans*-Cinnamaldehyde Dimethylacetal: *trans*-Cinnamaldehyde dimethylacetal, obtained by the reaction of *trans*-cinnamaldehyde with $CeCl_3 \cdot 7H_2O$ and trimethyl orthoformate,[6] was converted into **6**-*syn* by osmium tetroxide-catalyzed *cis*-hydroxylation[11] followed by dimethylation of the resultant *vic*-glycol by the Hakomori method.[10] Then 2 mol-equivalents of $Me_3N \rightarrow O \cdot 2H_2O$ (222 mg) and 0.4 M osmium tetroxide in *tert*-butanol (25 ml) were added to a stirred solution of *trans*-cinnamaldehyde dimethylacetal (178 mg, 1 mmol) in THF/H_2O (9 : 1, 5 ml) cooled to 0 °C. The mixture was allowed to warm to room temperature, stirred for 5 h, quenched by addition of solid $NaHSO_3$ with vigorous stirring for 30 min, and concentrated under reduced pressure. Methylene chloride was added to the residue, and the mixture was filtered through a short celite column. The filtrate was concentrated under reduced pressure to give a residue, which was purified by column chromatography on silica gel to give *threo*-2,3-dihydroxy-3-phenylpropanal dimethylacetal in 71% yield (150 mg). Recrystallization from *n*-hexane gave a pure sample as colorless crystals, mp 79—81 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3500, 1450, 1205. ^{1}H-NMR δ: 2.5—2.7 (brs, 1H, OH, disappeared on addition of D_2O), 2.9—3.1 (brs, 1H, OH, disappeared on addition of D_2O), 3.44 (s, 6H, OMe × 2), 3.6—3.8 (m, 1H, C_2-H), 4.32 (d, 1H, J=4.8 Hz, C_1-H), 4.8—4.9 (m, 1H, C_3-H), 7.31 (brs, 5H, ArH). *Anal.* Calcd for $C_{11}H_{16}O_4$: C, 62.25; H, 7.60. Found: C, 62.10; H, 7.63. The *threo*-glycol (53 mg, 0.25 mmol) was dimethylated by the method described for i) to give an 85% yield (51 mg) of **6**-*syn* as a colorless oil. This was identical with the sample obtained above.

2-Hydroxy-3-methoxy-1-phenyl-1-propanone Dimethylacetal (3b) and 2-Hydroxy-3-methoxy-1-phenyl-1-propanone (3b′)——A solution of **2a** (132 mg, 1 mmol) in anhydrous MeOH (1 ml) and a solution of KOH (185 mg, 3.3 mmol) in anhydrous MeOH (1.5 ml) were added dropwise to a solution of PIDA (354 mg, 1.1 mmol) in anhydrous MeOH (2.5 ml) at room temperature under nitrogen. The reaction mixture was worked up according to the general

procedure to give a residue, which was subjected to column chromatography on silica gel with methylene chloride to give a 78% yield (153 mg) of an inseparable mixture of **3b** and **3b'** in a ratio of 1 : 2 (calculated from the relative intensities of the methoxy signals in the ^{1}H-NMR spectra). When the reaction mixture was concentrated under reduced pressure without treatment with water, only crude **3b** was obtained. The crude **3b** was subjected to column chromatography on silica gel with *n*-hexane : ethyl acetate = 5 : 1 to give a 2 : 1 ratio of **3b** and **3b'** in 69% yield. Crude **3b**: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3550, 1595, 1450, 1225, 1115. ^{1}H-NMR δ: 3.29 (s, 3H, OMe), 3.31 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.5—3.7 (m, 1H, C_3-H), 3.7—3.85 (m, 1H, C_3-H), 3.85—4.05 (br s, 1H, OH, disappeared on addition of D_2O), 4.28 (dd, 1H, J=8.5 and 2 Hz, C_2-H), 7.3—7.6 (m, 5H, ArH). Exact mass Calcd for $C_{12}H_{18}O_4$–CH_3O: 195.1020. Found: 195.1010. The 1 : 2 mixture of **3b** and **3b'** was converted into **3b'** in a quantitative yield by stirring in methylene chloride for 1 week or by passage through a long silica gel column in methylene chloride : *n*-hexane = 2 : 1. **3b'**: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3450, 1685, 1600, 1450, 1265, 1130. ^{1}H-NMR δ: 3.36 (s, 3H, OMe), 3.78 (dd, 2H, J=4 and 3 Hz, CH_2OMe), 3.9—4.1 (br s, 1H, OH, disappeared on addition of D_2O), 5.1—5.4 (m, 1H, CHOH), 7.5—7.8 (m, 3H, ArH), 8.0—8.15 (m, 2H, ArH). Exact mass Calcd for $C_{10}H_{12}O_3$: 180.0784. Found: 180.0761.

2-Hydroxy-3-methoxycyclopentanone Dimethylacetal (3c)——This (98 mg) was obtained from **2c** (84 mg, 1 mmol), PIDA (354 mg, 1.1 mmol) and KOH (185 mg, 3.3 mmol). Distillation under reduced pressure gave pure **3c** as a colorless oil, bp 135—145 °C/10 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3500, 1460, 1090, 910. ^{1}H-NMR δ: 1.7—2.1 (m, 4H, CH_2 × 2), 2.6—2.8 (br s, 1H, OH, disappeared on addition of D_2O), 3.30 (s, 3H, OMe), 3.38 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.8—4.1 (m, 2H, C_2-H and C_3-H). MS *m/z*: 176 (M^+). *Anal.* Calcd for $C_8H_{16}O_4$: C, 54.53; H, 9.15. Found: C, 54.68; H, 9.34. The acetate (**3c**·acetate) was obtained from **3c** by treatment with Ac_2O–pyridine. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1735. ^{1}H-NMR δ: 1.8—2.1 (m, 4H, CH_2 × 2), 2.18 (s, 3H, OCOMe), 3.24 (s, 3H, OMe), 3.26 (s, 3H, OMe), 3.36 (s, 3H, OMe), 3.8—4.1 (m, 1H, C_3-H), 5.31 (d, 1H, J=4 Hz, C_2-H).

2-Hydroxy-3-methoxycyclohexanone Dimethylacetal (3d)——This (303 mg) was obtained from **2d** (297 mg, 3.1 mmol), PIDA (1.09 g, 3.4 mmol) and KOH (571 mg, 10 mmol). Distillation under reduced pressure gave pure **3d** as a colorless oil, bp 140—150 °C/15 mmHg (bath temp.). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3500, 1460, 1140, 1085. ^{1}H-NMR δ: 1.3—1.7 (m, 6H, CH_2 × 3), 2.16 (br s, 1H, OH, disappeared on addition of D_2O), 3.14 (s, 3H, OMe), 3.24 (s, 3H, OMe), 3.35 (s, 3H, OMe), 3.3—3.4 (m, 1H, –CHOMe), 4.02 (d, 1H, J=3 Hz, –CHOH). MS *m/z*: 190 (M^+). *Anal.* Calcd for $C_9H_{18}O_4$: C, 56.82; H, 9.54. Found: C, 56.80; H, 9.69. The acetate (**3d**·acetate) was obtained from **3d** by treatment with Ac_2O–pyridine. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725. ^{1}H-NMR δ: 1.2—1.8 (m, 6H, CH_2 × 3), 2.10 (s, 3H, OCOMe), 3.16 (s, 6H, OMe × 2), 3.33 (s, 3H, OMe), 3.3—3.4 (m, 1H, –CHOMe), 5.43 (d, 1H, J=3 Hz, –CHOAc). Exact mass Calcd for $C_{11}H_{20}O_5$: 232.1308. Found: 232.1280.

2-Hydroxy-3-methoxy-5,5-dimethyl-2-cyclohexanone Dimethylacetal (3e)——This (76.5 mg) was obtained from **2e** (191 mg, 1.5 mmol), PIDA (731 mg, 1.7 mmol) and KOH (285 mg, 5.1 mmol) as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3550, 1460, 1145, 1085. ^{1}H-NMR δ: 0.95 (s, 3H, Me), 1.01 (s, 3H, Me), 1.4—1.8 (m, 4H, CH_2 × 2), 2.22 (br s, 1H, OH, disappeared on addition of D_2O), 3.16 (s, 3H, OMe), 3.25 (s, 3H, OMe), 3.38 (s, 3H, OMe), 3.4—3.7 (m, 1H, –CHOMe), 4.00 (d, 1H, J=3 Hz, –CHOH). The acetate (**3e**·acetate) was obtained from **3e** by treatment with Ac_2O–pyridine. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1725. ^{1}H-NMR δ: 0.96 (s, 3H, Me), 1.03 (s, 3H, Me), 1.4—1.7 (m, 4H, CH_2 × 2), 2.08 (s, 3H, OCOMe), 3.11 (s, 3H, OMe), 3.15 (s, 3H, OMe), 3.33 (s, 3H, OMe), 3.4—3.7 (m, 1H, –CHOMe), 5.38 (br s, 1H, –CHOAc). Exact mass Calcd for $C_{13}H_{24}O_5$: 260.1621. Found: 260.1615.

4,5-*O*-Isopropylidene-2,4,5-trihydroxy-3-methoxypentanal Dimethylacetal (3f)——This (84 mg) was obtained from **2f** (78 mg, 0.5 mmol), PIDA (322 mg, 1.0 mmol) and KOH (168 mg, 3.0 mmol) as a mixture of four inseparable diastereomers (checked by ^{1}H-NMR). A small amount of one major diastereomer was isolated in a pure state by chromatography on a long column of silica gel with *n*-hexane : ethyl acetate = 3 : 1, colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3400, 1450, 1380, 1375, 1235. ^{1}H-NMR δ: 1.34 (s, 3H, Me), 1.43 (s, 3H, Me), 2.35—2.45 (m, 1H, OH), 3.43 (s, 3H, OMe), 3.48 (s, 6H, OMe × 2), 3.7—4.4 (m, 5H, C_2-H, C_3-H, C_4-H, C_5-2H), 4.47 (d, 1H, J=6 Hz, C_1-H). Exact mass Calcd for $C_{11}H_{22}O_6$–CH_3: 235.1179. Found: 235.1169. The acetate (**3f**·acetate) was obtained from **3f** by treatment with Ac_2O–pyridine. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1450, 1370. ^{1}H-NMR δ: 1.34 (s, 3H, Me), 1.40 (s, 3H, Me), 2.09 (s, 3H, OCOMe), 3.33 (s, 3H, OMe), 3.37 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.4—3.6 (m, 1H, C_5-H), 3.7—4.4 (m, 3H, C_3-H, C_4-H, C_5-H), 4.57 (d, 1H, J=7 Hz, C_1-H), 5.26 (dd, 1H, J=3 and 7 Hz, C_2-H). Exact mass Calcd for $C_{13}H_{24}O_7$–CH_3: 277.1284. Found: 277.1281.

6-Hydroxy-3-methyl-2-cyclohexenone (8a), 6-Hydroxy-3-methoxymethyl-2-cyclohexenone (9a) and 6-Acetoxy-3-methyl-2-cyclohexenone (11a)——Compounds **8a** (73 mg) and **9a** (45 mg) were obtained from **7a** (223 mg, 2.0 mmol), PIDA (1.921 g, 6.0 mmol) and KOH (1.00 g, 18 mmol), **8a**; colorless oil, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3400, 1670. ^{1}H-NMR δ: 1.99 (s, 3H, Me), 2.2—2.6 (m, 5H, CH_2 × 2 and OH), 4.11 (dd, 1H, J=6 and 12 Hz, –CHOH), 5.94 (m, 1H, –CH=). The acetate (**11a**) was obtained from **8a** by treatment with Ac_2O–pyridine. Recrystallization from pet. ether gave pure **11a** as colorless crystals, mp 67.5—68.5 °C (lit.[8a] 60—62 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1690, 1240. ^{1}H-NMR δ: 1.98 (s, 3H, Me), 2.2—2.6 (m, 4H, CH_2 × 2), 2.18 (s, 3H, OCOMe), 5.18 (dd, 1H, J=6 and 12 Hz, –CHOAc), 5.90 (m, 1H, CH=). MS *m/z*: 168 (M^+). **9a**: Colorless oil, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3450, 1680. ^{1}H-NMR δ: 2.3—2.6 (m, 4H, CH_2 × 2), 3.38 (s, 3H, OMe), 3.73 (br s, 1H, OH, disappeared on addition of D_2O), 4.01 (br s, 2H, CH_2OMe), 4.16 (dd, 1H, J=5 and 13 Hz, –CHOH), 6.13 (m, 1H, –CH=). The acetate (**9a**·acetate) was obtained from **9a** by

treatment with Ac_2O–pyridine as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1690, 1230. ^{1}H-NMR δ: 2.16 (s, 3H, OCOMe), 2.2—2.6 (m, 4H, $CH_2 \times 2$), 3.36 (s, 3H, OMe), 4.00 (br s, 2H, CH_2OMe), 5.31 (dd, 1H, J=6 and 13 Hz, –CHOAc), 6.10 (m, 1H, –CH=). Exact mass Calcd for $C_{10}H_{14}O_4$: 198.0889. Found: 198.0871.

6-Hydroxy-3,5,5-trimethyl-2-cyclohexenone (8b), 6-Hydroxy-3-methoxymethyl-5,5-dimethyl-2-cyclohexenone (9b) and 6-Acetoxy-3,5,5-trimethyl-2-cyclohexenone (11b)——Compounds **8b** (46 mg) and **9b** (32 mg) were obtained from **7b** (283 mg, 2.1 mmol), PIDA (1.980 g, 6.2 mmol) and KOH (1.03 g, 18 mmol). **8b**: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3400, 1670. ^{1}H-NMR δ: 0.82 (s, 3H, Me), 1.19 (s, 3H, Me), 1.95 (s, 3H, Me), 2.2—2.4 (m, 2H, CH_2), 3.67 (br s, 1H, OH, disappeared on addition of D_2O), 3.93 (br s, 1H, –CHOH), 5.9—6.0 (m, 1H, –CH=). The acetate (**11b**) was obtained from **8b** by treatment with Ac_2O–pyridine. Recrystallization from pet. ether gave pure **11b** as colorless crystals, mp 77—78 °C (lit.[8b] 77—77.5 °C, lit.[8c] 76—78 °C). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1680, 1635, 1235. ^{1}H-NMR δ: 0.98 (s, 3H, Me), 1.09 (s, 3H, Me), 1.96 (s, 3H, Me), 2.20 (s, 3H, OCOMe), 2.2—2.7 (m, 2H, CH_2), 5.19 (s, 1H, –CHOAc), 5.8—5.9 (m, 1H, –CH=). MS m/z: 196 (M^+). **9b**: Pale yellow oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600—3400, 1680. ^{1}H-NMR δ: 0.82 (s, 3H, Me), 1.21 (s, 3H, Me), 2.2—2.4 (m, 2H, CH_2), 3.37 (s, 3H, OMe), 3.67 (br s, 1H, OH, disappeared on addition of D_2O), 3.98 (m, 3H, $-CH_2OMe$ and –CHOH), 6.1—6.2 (m, 1H, –CH=). The acetate (**9b**·acetate) was obtained from **9b** by treatment with Ac_2O–pyridine as a colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1690, 1640, 1230. ^{1}H-NMR δ: 0.98 (s, 3H, Me), 1.11 (s, 3H, Me), 2.21 (s, 3H, OCOMe), 2.3—2.5 (m, 2H, CH_2), 3.39 (s, 3H, OMe), 4.20 (s, 2H, CH_2OMe), 5.23 (s, 1H, –CHOAc), 6.1—6.2 (m, 1H, –CH=). Exact mass Calcd for $C_{12}H_{18}O_4$: 226.1205. Found: 226.1205.

6-Hydroxy-3-phenyl-2-cyclohexenone (8c) and 6-Hydroxy-4-methoxy-3-phenyl-2-cyclohexenone (10)——Compound **8c** (110 mg) and a 3:2 diastereo-mixture of **10** (32 mg) were obtained from **7c** (172 mg, 1 mmol), PIDA (644 mg, 2 mmol) and KOH (336 mg, 6 mmol). Recrystallization of **8c** from benzene–*n*-hexane gave the pure sample as colorless crystals, mp 71—74 °C (lit.[12] no physical or spectral data). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3450, 1670, 1605, 1105, 910. ^{1}H-NMR δ: 1.9—2.3 (m, 1H, C_5-H), 2.5—2.8 (m, 1H, C_5-H), 2.9—3.1 (m, 2H, C_4-H), 3.8—4.0 (br s, 1H, OH, disappeared on addition of D_2O), 4.32 (dd, 1H, J=6 and 14 Hz, C_6-H), 6.57 (s, 1H, C_2-H), 7.4—7.7 (m, 5H, ArH). Exact mass Calcd for $C_{12}H_{12}O_2$: 188.0835. Found: 188.0823. A 3:2 diastereo-mixture of **10**: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3550—3450, 1680, 1605, 1425, 1265, 1080. ^{1}H-NMR δ: 1.5—1.8 (br s, 1H, OH), 1.9—2.3 (m, 1H, C_5-H), 2.8—3.2 (m, 1H, C_5-H), 3.37 (s, 9/5H, OMe × 3/5), 3.59 (s, 6/5H, OMe × 2/5), 4.2—5.0 (m, 2H, C_4-H and C_6-H), 6.42 (d, 3/5H, –CH= × 3/5), 6.52 (s, 2/5H, –CH= × 2/5), 7.3—7.8 (m, 5H, ArH). Exact mass Calcd for $C_{13}H_{14}O_3$: 218.0941. Found: 218.0913. The acetate (**10**·acetate) was obtained from **10** by treatment with Ac_2O–pyridine. Careful separation of the 3:2 diastereo-mixture of **10**·acetate by column chromatography on silica gel with *n*-hexane:ethyl acetate=5:1 gave two isomers (**10**·acetate-A and **10**·acetate-B) in a 3:2 ratio. **10**·acetate-A: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1690, 1600, 1425, 1375, 1230. ^{1}H-NMR δ: 2.25 (s, 3H, OCOMe), 2.3—2.55 (m, 1H, C_5-H), 2.8—3.1 (m, 1H, C_5-H), 3.38 (s, 3H, OMe), 4.8—5.1 (m, 1H, C_4-H), 5.53 (dd, 1H, J=14 and 5 Hz, C_6-H), 6.38 (d, 1H, J=2 Hz, C_2-H), 7.52 (s, 5H, ArH). Exact mass Calcd for $C_{15}H_{16}O_4$: 260.1049. Found: 260.1071. **10**·acetate-B: Colorless oil. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1740, 1685, 1610, 1420, 1375, 1230. ^{1}H-NMR δ: 2.25 (s, 3H, OCOMe), 2.7—2.9 (m, 1H, C_5-H), 3.2—3.5 (m, 1H, C_5-H), 3.59 (s, 3H, OMe), 4.5—4.7 (m, 1H, C_4-H), 5.88 (dd, 1H, J=13 and 5 Hz, C_6-H), 6.49 (s, 1H, C_2-H), 7.4—7.8 (m, 5H, ArH). Exact mass Calcd for $C_{15}H_{16}O_4$: 260.1048. Found: 260.1063.

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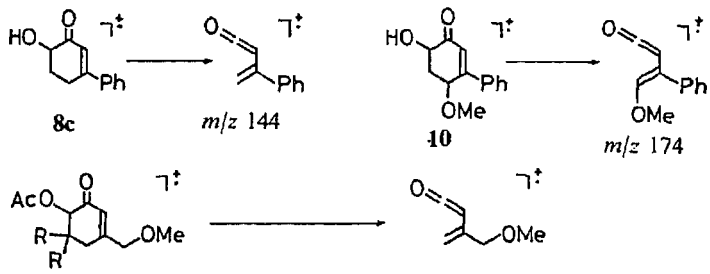

11a: R=H
11b: R=CH_3

m/z 112

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Chaetochromins B, C and D, Bis(naphtho-γ-pyrone) Derivatives from *Chaetomium gracile*

KIYOTAKA KOYAMA and SHINSAKU NATORI*

Meiji College of Pharmacy, Yato-cho, Tanashi-shi, Tokyo 188, Japan

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Besides chaetochromin A (**1**), a known mycotoxin and antitumor agent, three related bis(naphtho-γ-pyrone) derivatives named chaetochromins B (**2**), C (**3**), and D (**4**) were isolated from *Chaetomium gracile*. The structures were elucidated as the diastereomer, the 3-demethyl derivative, and the 2,3-dehydro derivative of **1**, respectively, chiefly on the basis of nuclear magnetic resonance data.

Keywords——*Chaetomium gracile*; chaetochromin; bis(naphtho-γ-pyrone); mycotoxin; antitumor agent; ^{1}H-NMR; ^{13}C-NMR

Chaetochromin (**1**) is a mycotoxin, first isolated from *Chaetomium thielavioideum* CHEN NHL2829, and was identified as 2,2′,3,3′-tetrahydro-5,5′,6,6′,8,8′-hexahydroxy-2,2′,3,3′-tetramethyl-[9,9′-bi-4*H*-naphtho[2,3-*b*]pyran-4,4′-dione].[1] The synonomy of the species, *Chaetomium cellulolyticum* CHAHAL and *Achaetomiella virescens* v. ARX with *C. virescens* (v. ARX) UDAGAWA was later proved[2] and the species was characterized by the production of the set of mycotoxins, chaetochromin (**1**), chaetocin, and sterigmatocystin.[3] Chaetochromin (**1**) was also isolated from *Chaetomium caprinum* BAINIER, *C. gracile* UDAGAWA, and *C. tetrasporum* HUGHES.[3] Chaetochromin was demonstrated to induce delayed liver injuries, bone marrow aplasia, and atrophy of lymphatic tissue in mice,[4] and to exhibit teratogenicity (*e.g.*, inducing exencephalus) on administration to pregnant mice.[5] The compound was effective in the treatment of MX-1 breast xenograft, M5076 ovarian carcinoma, and P388 lymphocytic leukemia in mice.[6]

To perform detailed biological testing, large amounts of the material were required and the strain (73-S-T-Y-3)[3] of *C. gracile* was selected among the available strains as the best producer of chaetochromin (**1**).

This paper presents the structures of three congeners of chaetochromin isolated in the course of the study and also the relative stereochemistry of chaetochromin (**1**), which remained unsettled at the time of the structure elucidation.[1] Preparation of some derivatives of **1** for the examination of structure–activity relationship is also presented.

Dichloromethane extract of the strain of *C. gracile* cultured on wheat grains afforded, besides the major metabolite chaetochromin (this compound will hereafter be referred to as chaetochromin A (**1**)), three congeners named chaetochromins B (**2**), C (**3**), and D (**4**) by silica gel chromatography and high-performance liquid chromatography (HPLC) on Nucleosil 50-5. Chaetochromins B (**2**), C (**3**), and D (**4**) showed nearly the same ultraviolet (UV) and infrared (IR) absorptions as chaetochromin A (**1**), indicating the presence of the same naphtho-γ-pyrone chromophores in the molecules. Molecular formulae determined by high-resolution mass spectroscopy (MS) indicated $C_{30}H_{26}O_{16}$, $C_{29}H_{24}O_{10}$, and $C_{30}H_{24}O_{10}$ for **2**, **3**, and **4**, respectively. The values correspond to an isomer and demethyl and dehydro derivatives of **1**, respectively.

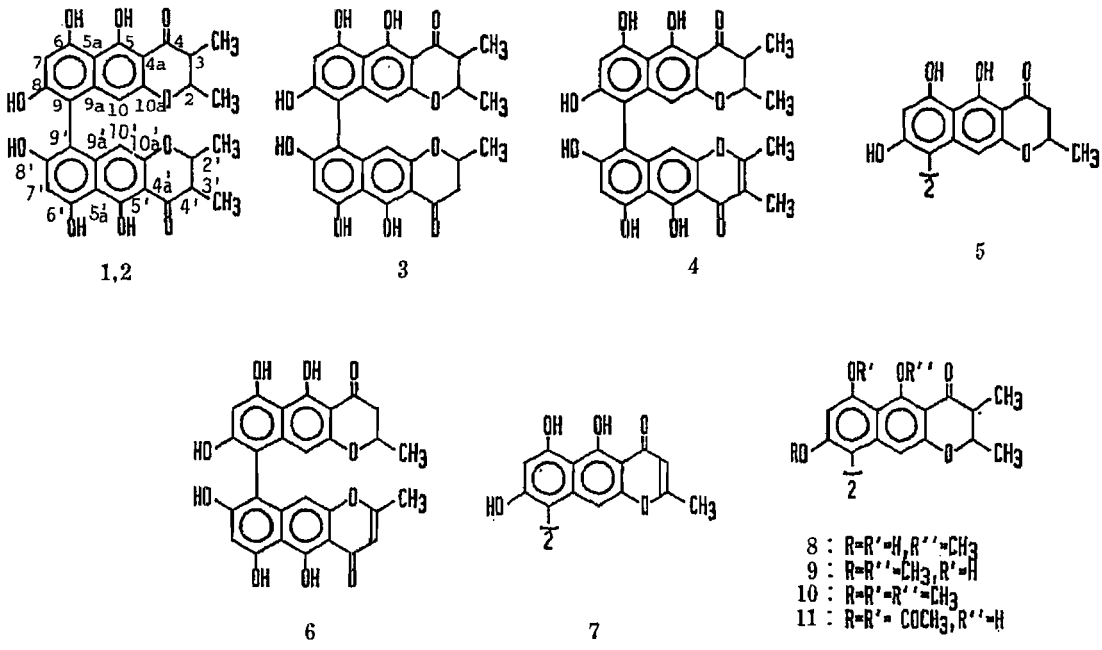

Chart 1

TABLE I. ^{1}H-NMR Data for Chaetochromins (*in* $CDCl_3$)[a]

Proton at	Chaetochromins			
	A (1)	B (2)	C (3)	D (4)
2, 2′	4.17 (dq, J=11.0, 6.1)	4.18 (dq, J=11.0, 6.1) 4.53 (dq, J=3.1, 6.7)	4.17 (dq, J=11.0, 6.1) 4.49 (ddq, J=9.8, 5.5, 6.1)	4.16 (dq, J=11.3, 6.1)
3, 3′	2.62 (dq, J=11.0, 6.7)	2.64 (dq, J=11.0, 6.7) 2.62 (dq, J=3.1, 7.3)	2.62 (dq, J=11.0, 6.7) 2.681 (d, J=9.8) 2.676 (d, J=5.5)	2.61 (dq, J=11.3, 6.7)
7, 7′	6.48 (s)	6.50 (s) 6.49 (s)	6.50 (s) 6.47 (s)	6.55 (s) 6.47 (s)
10, 10′	5.93 (s)	5.95 (s) 5.93 (s)	5.93 (s) 5.93 (s)	5.94 (s) 6.33 (s)
2-CH_3, 2′-CH_3	1.42 (d, J=6.1)	1.43 (d, J=6.1) 1.31 (d, J=6.7)	1.42 (d, J=6.1) 1.38 (d, J=6.1)	1.40 (d, J=6.1) 2.29 (s)
3-CH_3, 3′-CH_3	1.24 (d, J=6.7)	1.25 (d, J=6.7) 1.18 (d, J=7.3)	1.24 (d, J=6.7)	1.24 (d, J=6.7) 1.99 (s)
5-OH, 5′-OH	15.27 (s)	15.28 (s) 15.18 (s)	15.25 (s) 15.06 (s)	16.26 (s) 15.26 (s)
6-OH, 6′-OH	9.65 (s)	9.68 (s) 9.65 (s)	9.63 (s) 9.63 (s)	9.96 (s) 9.62 (s)
8-OH, 8′-OH	5.70 (br)	5.76 (br) 5.76 (br)	5.84 (br) 5.84 (br)	5.80 (br) 5.80 (br)

a) Chemical shifts are given on the δ (ppm) scale with tetramethylsilane as an internal standard and coupling constants are given in Hz (s, singlet; d, doublet; t, triplet; q, quartet; br, broad).

The proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra of these compounds (Tables I and II) clearly indicated the structural differences of these compounds. Chaetochromin A (1) is a symmetric dimer and all the NMR signals represent the summation of two identical halves in the molecule. The NMR spectra of the three congeners

Table II. ^{13}C-NMR Data for Chaetochromins (in $CDCl_3$)

Carbon atom at	Chaetochromins			
	A (1)	B (2)	C (3)	D (4)
2, 2′	78.4	78.4	78.4	162.5 (s)
		75.5	73.4	78.4 (d)
3, 3′	46.2	46.2	46.2 (d)	113.1 (s)
		44.4	43.3 (t)	46.2 (d)
4, 4′	200.8	202.7	200.8	200.8
		200.8	198.3	183.3
4a, 4a′	101.8	101.9	101.9	102.6
		101.8	101.6	100.9
5, 5′	164.4	165.1	164.7	165.0
		164.5	164.5	164.4
5a, 5a′	105.6	105.6	105.6	106.9
		105.6	105.6	105.5
6, 6′	159.8	160.0	160.0	159.9
		159.9	159.8	158.9
7, 7′	99.7	99.8	99.8[a]	99.9
		99.8	99.5[a]	99.7
8, 8′	160.8	160.9	161.1	160.7
		160.8	161.0	160.2
9, 9′	102.0	102.0	102.6	102.0
		101.4	102.0	102.0
9a, 9a′	141.9	142.0	142.0	141.9
		141.9	141.9	139.7
10, 10′	99.3	99.3	99.7[a]	99.3
		99.3	99.3[a]	98.3
10a, 10a′	156.3	156.2	156.4	156.3
		156.0	156.3	152.9
2-CH_3, 2′-CH_3	19.6	19.6	20.9	19.6
		16.6	19.6	18.7
3-CH_3, 3′-CH_3	9.9	9.9	10.1	9.8
		9.5		9.0

a) Assignments may be interchanged.

(2—4) showed that the two halves in these molecules are not the same. In the ^{13}C-NMR spectrum of chaetochromin A (1) fifteen signals appear, while in that of chaetochromin B (2) thirty signals, two corresponding to each signal in 1, were observed. The ^{1}H-NMR spectrum of 2 showed that the relative stereochemistry of the two secondary methyl groups at the 2- and 3-positions in one half of the molecule is the same as that of the two methyl groups in 1 (J_{2H-3H}, 11.0 Hz both in 1 and 2), but the other 2,3-dimethyl group in 2 showed a different coupling constant (J_{2H-3H}, 3.1 Hz). In the previous paper[1] the stereochemistry of chaetochromin A (1) remained unsettled. Comparison of these coupling constants clearly indicated that both of the vicinal methyl groups in 1 and one of the two in 2 are *trans*, while the other in 2 is *cis*. Reexamination of the ^{13}C-NMR data, such as $^3J_{CH}$ of the C-3 proton and C-2 methyl carbon (1.8 Hz) in 1[7] did not show any discrepancy in the assignments. Thus, the structure of chaetochromin B (2) was established as the stereoisomer of chaetochromin A (1), where one of the two *trans*-2,3-dimethyl groups was replaced by a *cis*-2,3-dimethyl group.

In chaetochromin C (3), the next congener, one of the four secondary methyl groups is lacking (^{1}H- and ^{13}C-NMR), and the ^{1}H-NMR spectrum indicated the presence of one methylene group at the 3-position (δ 2.681 and 2.676, each 1H). The spectral data indicated that the other part of the molecule is the same as in 1. Thus, chaetochromin C (3) is composed

of half of **1** having a 2,3-dimethyl group and half having a 2-methyl group but lacking the 3-methyl group. Cephalochromin (**5**), a metabolite of *Cephalosporium* sp.,[8,9] *Verticillium* sp.,[10] *Nectria viridescens*,[11] and *N. flavoviridis*,[11] is a known dimer of the latter half of chaetochromin C (**3**).

The third compound, chaetochromin D (**4**), showed the presence of one γ-pyrone moiety instead of one dihydro-γ-pyrone unit in the molecule of chaetochromin A (**1**): one of the two pairs of two sp^3 carbons at the 2- and 3-positions is replaced by one pair of two sp^2 carbons (^{13}C-NMR of **4**, δ: 162.4 and 113.1 ppm) and one of the two pairs of vicinal secondary methyl groups is replaced by a dimethyl group on a double bond (^{1}H-NMR of **4**, δ: 1.99 (3H, s) and 2.29 (3H, s)). These facts indicated that chaetochromin D (**4**) corrresponds to the 2,3-dehydro compound of chaetochromin A (**1**). In the case of cephalochromin (**5**), the bisdemethyl compound of chaetochromin A (**1**), coproduction of the bisdehydro compound (**6**) and the tetrakis-dehydro compound (isoustilaginoidin A) (**7**) by *Verticillium* sp. was reported.[10]

The circular dichroism (CD) curves of these compounds (**1**—**4**) showed positive Cotton effects around 295 nm and negative effects around 265 nm, as shown in Fig. 1, indicating the same stereostructure around the 9-9′ positions, which is antipodal to that in ustilaginoidins,

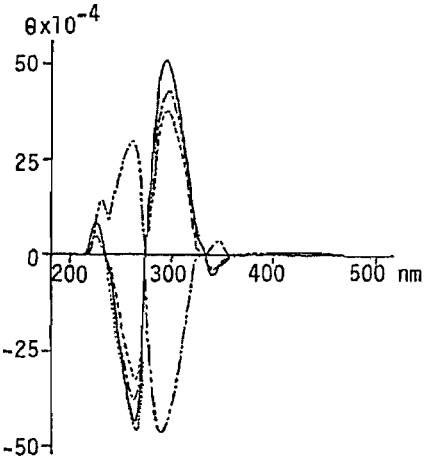

Fig. 1. CD Spectra of Chaetochromins (in Dioxane)

——, chaetochromin A (**1**); – – –, chaetochromin B (**2**); – – –, chaetochromin C (**3**); – – – –, chaetochromin D (**4**); – – – – –, ustilaginoidin A (**7**).

TABLE III. ^{1}H-NMR Data for Chaetochromin A Derivatives (in $CDCl_3$)

Proton at	Chaetochromin A			
	5,5′-Dimethyl ether (**8**)	5,5′,8,8′-Tetramethyl ether (**9**)	5,5′,6,6′,8,8′-Hexamethyl ether (**10**)	6,6′,8,8′-Tetra-acetate (**11**)
2, 2′	4.18 (dq, J=11.0, 6.1)	4.13 (dq, J=11.0, 6.1)	4.11 (dq, J=11.0, 6.1)	4.14 (dq, J=11.0, 6.1)
3, 3′	2.50 (dq, J=11.0, 6.6)	2.50 (dq, J=11.0, 7.0)	2.51 (dq, J=11.0, 7.0)	2.63 (dq, J=11.0, 7.2)
7, 7′	6.54 (s)	6.69 (s)	6.63 (s)	6.92 (s)
10, 10′	6.35 (s)	6.32 (s)	6.32 (s)	6.08 (s)
2, 2′-CH_3	1.42 (d, J=6.1)	1.39 (d, J=6.1)	1.37 (d, J=6.1)	1.41 (d, J=6.1)
3, 3′-CH_3	1.20 (d, J=6.6)	1.19 (d, J=7.0)	1.17 (d, J=7.0)	1.21 (d, J=7.2)
5, 5′-OH				14.19 (s)
6, 6′-OH	10.14 (s)	10.17 (s)		
8, 8′-OH	5.69 (br)			
5, 5′-OCH_3	4.09 (s)	4.14 (s)	4.07 (s)	
6, 6′-OCH_3			4.00 (s)	
8, 8′-OCH_3		3.76 (s)	3.79 (s)	
6, 6′-$OCOCH_3$				1.93 (s)
8, 8′-$OCOCH_3$				2.41 (s)

TABLE IV. ^{13}C-NMR Data for Chaetochromin A Derivatives (in $CDCl_3$)

Carbon atom at	Chaetochromin A			
	5,5′-Dimethyl ether (**8**)	5,5′,8,8′-Tetra-methyl ether (**9**)	5,5′,6,6′,8,8′-Hexamethyl ether (**10**)	6,6′,8,8′-Tetra-acetate (**11**)
2, 2′	77.9	77.7	77.7	78.1
3, 3′	48.4	48.5	48.5	46.6
4, 4′	192.8	193.0	193.1	201.3
4a, 4′a	109.7[b]	109.4[b]	112.6[b]	104.6
5, 5′	160.3[a]	160.4[a]	161.3[a]	162.8
5a, 5′a	109.7[b]	109.2[b]	112.3[b]	119.5[b]
6, 6′	159.3[a]	159.9[a]	160.3[a]	150.3[a]
7, 7′	100.4	97.2	93.8	113.7
8, 8′	158.3[a]	158.5[a]	158.3[a]	150.0[a]
9, 9′	100.7	108.4[b]	110.0[b]	112.2[b]
9a, 9′a	140.3	139.9	140.7	140.4
10, 10′	105.5	105.8	105.2	101.1
10a, 10′a	158.3[a]	157.0[a]	157.3[a]	156.3
2, 2′-CH_3	19.7	19.7	19.8	21.1[c]
3, 3′-CH_3	10.5	10.7	10.6	9.8
5, 5′-OCH_3	64.5	64.4	63.0	
6, 6′-OCH_3			56.4[c]	
8, 8′-OCH_3		56.2	56.3[c]	
6,6′,8,8′-$OCOCH_3$				169.7
				168.3
6,6′,8,8′-$OCOCH_3$				20.5[c]
				19.5[c]

a—*c*) Assignments may be interchanged in each column.

metabolites of *Claviceps virens* (anamorph state: *Ustilaginoidea virens*).[12] The absolute configurations of these compounds will be the subject of a forthcoming paper.

To examine the structure–activity relationship of the chaetochromin and related compounds, partial methylation and acetylation of chaetochromin A (**1**) were attempted. The 5,5′-di- (**8**), 5,5′,8,8′-tetra- (**9**), and 5,5′,6,6′,8,8′-hexa-methyl ethers (**10**) and the 6,6′,8,8′-tetra-acetate (**11**) were prepared and the structures were confirmed by ^{1}H- and ^{13}C-NMR as shown in Tables III and IV. The results of biological tests will also be reported in a forth coming paper.

Experimental

All melting points were determined on a Yanagimoto MP micromelting point apparatus and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GX-400 (^{1}H 400 MHz and ^{13}C 100 MHz) spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. Chemical shifts are recorded in ppm (δ). MS were taken on a JEOL JMS-D300. UV and IR spectra were meausred with a Shimadzu UV-240 spectrophotometer and a JASCO A-102 infrared spectrophotometer. The $[\alpha]_D$ values were measured with a JASCO DIP-140 digital polarimeter. CD spectra were recorded on a JASCO J-20 spectropolarimeter.

Kiesel gel $60F_{254}$ (Merck) precoated plates were used for thin-layer chromatography (TLC) and the spots were detected by UV illumination. Column chromatography was carried out on 70—230 mesh silica gel (Merck). HPLC were carried out by using a Waters M45J pump with a Oyo-Bunko Uvilog-5IIIA UV detector.

Isolation of Metabolites from *Chaetomium gracile* ——The strain (73-S-T-Y-3) was incubated in stationary culture on sterilized wheat (150 g) at 26 °C for 5 d. The moldy wheat was extracted twice with CH_2Cl_2 (250 ml) for 24 h at room temperature. The extract was chromatographed over silica gel (treated with 3% oxalic acid) using CH_2Cl_2 as the developing solvent to afford fractions 1 and 2. Purification by HPLC (Nucleosil 50-5, treated with 3%

oxalic acid) using EtOAc–hexane (1 : 5) as the developing solvent gave chaetochromin A (**1**) (125 mg) from fraction 1 and chaetochromins A (**1**) (103 mg), B (**2**) (1 mg), C (**3**) (15 mg), and D (**4**) (4 mg) from fraction 2. Chaetochromin A (**1**) was recrystallized from $CHCl_3$–hexane as a yellow powder, mp 207—210 °C, $[\alpha]_D^{20}$ +600° (c=0.06, dioxane). MS m/z: 546.1515 (M^+, Calcd for $C_{30}H_{26}O_{10}$: 546.1526). UV $\lambda_{max}^{dioxane}$ nm (ε): 235 (43680), 270 (48230), 292 (59150), 325 (15470), 335 (10740), 412 (10470). IR ν_{max}^{KBr} cm^{-1}: 3400, 1640, 1630, 1587, 1445, 1383, 1360, 1348, 1257, 1150, 1133, 1091, 1025, 915, 840. CD (dioxane) $[\theta]^{20}$ (nm): +91400 (226), 0 (236), −63700 (243), −436800 (266), 0 (275), +196600 (281), +509600 (294), +50100 (326), 0 (333), −49900 (339), 0 (354), 9100 (415). Its identity with an authentic sample of chaetochromin A[1] was confirmed by IR and TLC.

Chaetochromin B (2)——Recrystallized from $CHCl_3$–hexane as a yellow powder, mp 204—206 °C, $[\alpha]_D^{20}$ +524° (c=0.05, dioxane). MS m/z: 546.1496 (M^+, Calcd for $C_{30}H_{26}O_{10}$: 546.1526). UV $\lambda_{max}^{dioxane}$ nm (ε): 230 (37200), 270 (52100), 292 (62900), 325 (10300), 340 (6500), 412 (9500). IR ν_{max}^{KBr} cm^{-1}: 3400, 1638, 1628, 1585, 1443, 1380, 1357, 1340, 1149, 1140, 1085, 1025, 1018, 908, 839. CD (dioxane) $[\theta]^{20}$ (nm): +86500 (226), 0 (235), −64900 (240), −459500 (266), 0 (275), +216200 (283), +502800 (294), +46500 (327), 0 (334), −47000 (340), 0 (356), +7600 (415).

Chaetochromin C (3)——Recrystallized from $CHCl_3$–hexane as a yellow powder, mp 214—217 °C, $[\alpha]_D^{20}$ +454° (c=0.08, dioxane). MS m/z: 532.1392 (M^+, Calcd for $C_{29}H_{24}O_{10}$: 532.1369). UV $\lambda_{max}^{dioxane}$ nm (ε): 233 (33700), 270 (37700), 293 (45200), 325 (12000), 338 (8100), 414 (7900). IR ν_{max}^{KBr} cm^{-1}: 3380, 1638, 1630, 1585, 1443, 1381, 1347, 1145, 1128, 1085, 1020, 867, 840. CD (dioxane) $[\theta]^{20}$ (nm): +83450 (226), 0 (236), −41700 (243), −326900 (266), 0 (275), +149500 (282), +375500 (295), +37600 (326), 0 (333), −38900 (340), 0 (354), +8400 (412).

Chaetochromin D (4)——Recrystallized from EtOAc–hexane as an orange powder, mp >300 °C, $[\alpha]_D^{20}$ +411° (c=0.06, dioxane). MS m/z: 544.1323 (M^+, Calcd for $C_{30}H_{24}O_{10}$: 544.1369). UV $\lambda_{max}^{dioxane}$ nm (ε): 234 (36300), 270 (38800), 293 (47500), 325 (13000), 338 (8820), 416 (8700). IR ν_{max}^{KBr} cm^{-1}: 3400, 1639, 1633, 1590, 1362, 1261, 1145, 1138, 1090, 920, 840, 802. CD (dioxane) $[\theta]^{20}$ (nm): +51400 (226), 0 (234), −68500 (242), −376900 (265), 0 (275), +428300 (294), +24800 (324), 0 (333), −30400 (341), 0 (358), +3400 (393).

Chaetochromin 5,5′-Dimethyl Ether (8)——A CH_2Cl_2 solution of **1** (57.6 mg) was methylated with ethereal CH_2N_2 in an ice bath for 1 h and the reaction product was purified by HPLC (Nucleosil 50-5) with benzene–AcOEt (6 : 1) to give **8** (32.1 mg), yellow powder (from EtOAc–hexane), mp 222—224 °C, $[\alpha]_D^{20}$ +615° (c=0.06, dioxane). MS m/z: 574.1895 (M^+, Calcd for $C_{32}H_{30}O_{10}$: 574.1839). UV $\lambda_{max}^{dioxane}$ nm (ε): 205 (14900), 230 (24300), 260 (29500), 290 (45100), 325 (6400), 390 (4600). IR ν_{max}^{KBr} cm^{-1}: 3400, 1635, 1620, 1565, 1455, 1380, 1340, 1135, 1085, 1070, 880. CD (dioxane) $[\theta]^{20}$ (nm): −302400 (255), 0 (267), +10300 (270), +548400 (290), 0 (326), −39500 (342), 0 (356), +5600 (393).

Chaetochromin 5,5′,8,8′-Tetramethyl Ether (9)——A CH_2Cl_2 solution of **1** (52.3 mg) was methylated with ethereal CH_2N_2 in a water bath for 4 h and the reaction mixture was purified by HPLC (Nucleosil 50-5) with hexane–EtOAc (2 : 1) to give **9** (36.8 mg), yellow needles (from $CHCl_3$–hexane), mp 171—176 °C, $[\alpha]_D^{20}$ +485° (c=0.1, dioxane). MS m/z: 602.2183 (M^+, Calcd for $C_{34}H_{34}O_{10}$: 602.2152). UV $\lambda_{max}^{dioxane}$ nm (ε): 232 (41200), 264 (41200), 292 (73500), 332 (8900), 348 (6300), 400 (7600). IR ν_{max}^{KBr} cm^{-1}: 3440, 1745, 1695, 1615, 1590, 1490, 1470, 1460, 1435, 1400, 1345, 1330, 1270, 1260, 1175, 1130, 1115, 1100, 1070, 1010, 750. CD (dioxane) $[\theta]^{20}$ (nm): −373200 (255), −48200 (275), 0 (280), +812700 (292), 0 (328), −57800 (346), 0 (360), +9600 (408).

Chaetochromin 5,5′,6,6′,8,8′-Hexamethyl Ether (10)——A CH_2Cl_2 solution of **9** (54.6 mg) was methylated with ethereal CH_2N_2 overnight at room temperature and the reaction product was purified by HPLC (Nucleosil 50-5) with hexane–EtOAc (5 : 6) to give **10** (40 mg), yellow fine prisms (from hexane–EtOAc), mp 276—279 °C, $[\alpha]_D^{20}$ +277° (c=0.12, dioxane). MS m/z: 630.2478 (M^+, Calcd for $C_{36}H_{38}O_{10}$: 630.2465). UV $\lambda_{max}^{dioxane}$ nm (ε): 235 (50200), 260 (53500), 285 (83400), 325 (12500), 340 (9700), 383 (10300). IR ν_{max}^{KBr} cm^{-1}: 1685, 1610, 1565, 1560, 1370, 1350, 1330, 1275, 1215, 1125. CD (dioxane) $[\theta]^{20}$ (nm): +52900 (219), 0 (228), −582400 (252), −63500 (270), 0 (274), +1090600 (287), 0 (318), −84700 (339), 0 (363), +6350 (398).

Chaetochromin 6,6′,8,8′-Tetraacetate (11)——**1** (56.6 mg) was dissolved in pyridine (1.5 ml) and acetic anhydride (1.5 ml), and the solution was left at room temperature for 10 min. The reaction product was chromatographed on a silica gel column with hexane–EtOAc (6 : 5) to give the acetate (56 mg), which was recrystallized from hexane–EtOAc to provide yellow crystals, mp 267—278 °C, $[\alpha]_D^{20}$ +354° (c=0.19, dioxane). MS m/z: 714.1920 (M^+, Calcd for $C_{38}H_{34}O_{14}$: 714.1948). UV $\lambda_{max}^{dioxane}$ nm (ε): 206 (22400), 230 (69400), 270 (74300), 280 (73600), 305 (19500), 400 (7100) IR ν_{max}^{KBr} cm^{-1}: 3450, 1770, 1630, 1580, 1430, 1370, 1340, 1190, 1160, 1125, 1025. CD (dioxane) $[\theta]^{20}$ (nm): +12470((224), 0 (234), −861300 (267), 0 (275), +1076700 (283), 0 (316), −34000 (321), 0 (336), +9100 (395).

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Total Synthesis of 12α- and 12β-Carboxylated Estrogens *via* the Thermal Elimination of β-Ketosulfoxide[1)]

TAKAO KUROSAWA, UTAKO NIITSU and MASAHIKO TOHMA*

Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Ishikari-Tobetsu, Hokkaido 061-02, Japan

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The thermal elimination of β-ketosulfoxide (**2**) afforded the α,β-unsaturated γ-ketoester (**3**) as a Michael acceptor (analogous to the key intermediate in Smith's estrone synthesis), which was condensed with 2-methylcyclopentane-1,3-dione to give the adduct (**4**) having all carbon units of the aromatic steroidal skeleton. The Michael adduct was then cyclized in the presence of methanesulfonic acid to yield the novel estrapentaene (**6**) with a methoxycarbonyl group at the C-12 position. The estrapentaene (**6**) was converted to 12β- and 12α-methoxycarbonylestrones (**25** and **26**) and their estradiol derivatives (**27** and **28**) by selective reductions followed by demethylation.

Keywords——β-ketosulfoxide; thermal elimination; total synthesis; Michael reaction; carbonyl intramolecular reduction; 12α-methoxycarbonylestrone; 12β-methoxycarbonylestrone; 12α-methoxycarbonylestradiol diacetate; 12β-methoxycarbonylestradiol diacetate

Modified estrogens having a substituent group in ring C are not only expected to be useful as haptens for the preparation of specific antisera in radioimmunoassay of estrogenic hormones,[2)] but also are of interest in connection with studies of the interaction between estrogens and their receptors.[3)] However, it is difficult to introduce a substituent group into ring C of natural estrogens. Total synthetic approaches, therefore, have been attempted by the use of a biomimetic polyene cyclization[4)] and the Torgov method.[5)] The Smith method[6)] (an efficient synthesis of estrone) has not been yet applied to substituted estrone derivatives because of difficulty in the preparation of an effective intermediate. As a part of our studies on the synthetic application of β-ketosulfoxides,[7)] we wish to report here the total synthesis of 12α- and 12β-carboxyestrone and estradiol derivatives by means of the thermal elimination of a substituted β-ketosulfoxide.

The β-ketosulfoxide (**1**), prepared from methyl 4-(3-methoxyphenyl) butyrate,[7)] was

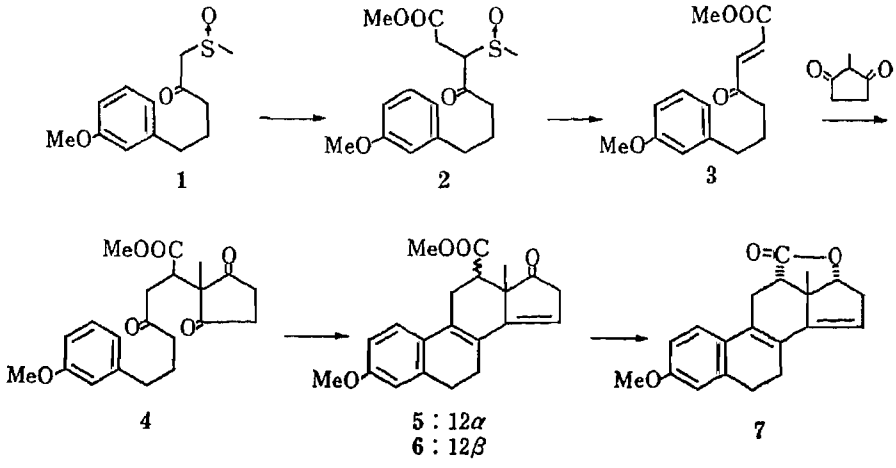

Chart 1

treated with methyl bromoacetate in the presence of potassium hydride to give the α-substituted β-ketosulfoxide (**2**) as an enone precursor in high yield. The nuclear magnetic resonance (NMR) spectrum showed that **2** was a diastereomeric mixture (attributable to the two asymmetric centers, methine and S). The thermal elimination of **2** by heating in dioxane gave the α,β-unsaturated γ-ketoester (**3**) as an acceptor for Michael condensation. Its NMR spectrum showed two doublet signals at 6.60 and 7.05 ppm ($J=14$ Hz) due to the *trans*-oriented olefinic protons. The Michael reaction of **3** with 2-methylcyclopentane-1,3-dione was readily achieved in the presence of catalytic amounts of triethylamine to afford the triketone (**4**). The acid-catalyzed cyclization of the triketone (**4**) was carried out with methanesulfonic acid at 0 °C to give a mixture of 12α- and 12β-methoxycarbonylestrapentaene (**5** and **6**) in the ratio of 1 : 20. In the NMR spectrum of **5**, the signal at 3.55 ppm of the methyl ester group indicated that the α-oriented (axial) ester group is strongly shielded by the double bonds, whereas the other compound (**6**) showed the signal of the β-oriented (equatorial) group at 3.80 ppm. The result of the above NMR analysis was further confirmed by the following chemical reaction. Although the 12β-ester (**6**) gave the 17β-alcohol (**8**) on treatment with sodium borohydride owing to the steric hindrance of the C-18 methyl group, the 12α-epimer (**5**) afforded the lactone (**7**). The formation of the lactone (**7**) could be explained as follows: the C-17 carbonyl group of **5** is initially reduced to a 17α-hydroxy group owing to the steric hindrance of the 12α-ester group to hydride attack from the β-side of the molecule, and the 17α-alcohol is subsequently lactonized with the 12α-substituent. This finding of the α-oriented ester group in **5** is consistent with the reported result of configurational analysis of the 12α-hydroxy group in ethiocholanoic acid.[8] The 12β-carboxylated pentaene (**6**) was hydrogenated with a catalytic amount of palladium on charcoal to give the 14α-estratetraene (**9**) accompanied with a small amount of the 14β-isomer (**10**). The ultraviolet (UV) spectrum of **9** showed an absorption maximum at 280 nm, suggesting the desired C/D-*trans* juncture because of the small bathochromic shift of the absorption maximum compared with that of the *cis* isomer (**10**) at 275 nm.[6,9] The structures of these compounds (**9** and **10**) were also

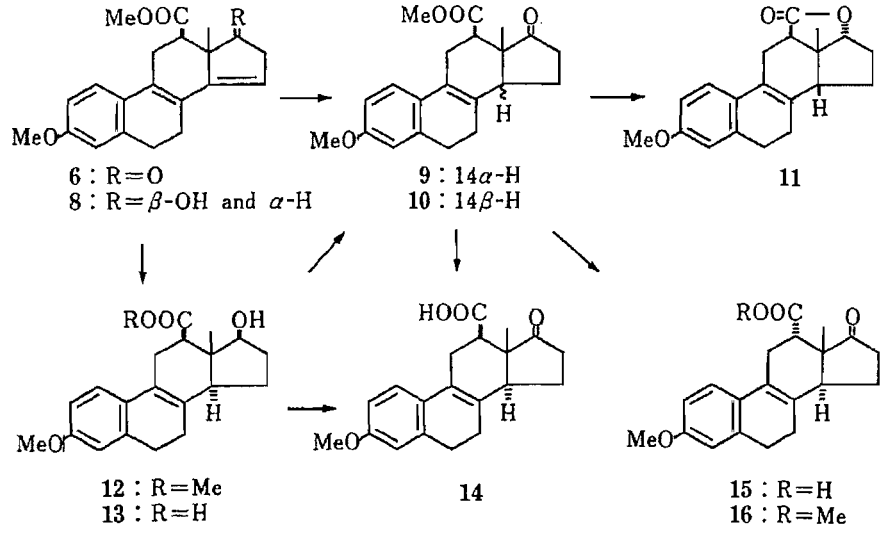

Chart 2

supported by the reported observations and following chemical reaction. Ruffer *et al.*[10] reported that the 14β-isomer (C/D-*cis*) of estrone derivatives gave a 17α-hydroxy compound on reduction with sodium borohydride owing to steric hindrance from the deflection of ring D to the α-side. Actually, the 14β-isomer (**10**) was reduced to give the lactone (**11**), indicating the formation of a 17α-alcohol. In order to avoid the formation of the undesired 14β-isomer,[11]

the 17β-hydroxyestrapentaene (**8**) was hydrogenated to give the 14α-estratetraene (**12**) in high yield (99%). The structure of **12** was defined by its transformation into the 14α-estratetraen-17-one (**9**). The two estratetraene esters (**9** and **12**) were hydrolyzed in order to prevent an unfavorable reaction prior to the following double bond reduction. The 17-hydroxy compound (**12**) was treated with potassium hydroxide in methanol to give the 12β-carboxyestratetraen-17-ol (**13**), which was converted into the 12β-carboxyestratetraen-17-one (**14**) by chromic oxidation. The hydrolysis of the 17-oxo compound (**9**) under the same conditions gave, interestingly, the epimeric 12α-carboxyestratetraen-17-one (**15**) accompanied with a small amount of the 12β-isomer (**14**). The orientation of the 12α-carboxy group in **15** was defined by NMR spectral analysis after conversion to the methyl ester (**16**). The ester (**16**) showed a signal at 3.63 ppm which suggested an α-oriented substituent because of the shielding effect of the unsaturated rings A and B compared with that of 12β-isomer (**9**) at 3.80 ppm. The 17β-hydroxy-tetraene (**13**) was then reduced with sodium in liquid ammonia to give the 12β-carboxyestratrien-17-ol (**17**) and the 17-oxo carboxylic acids (**14** and **15**) also gave the corresponding estratrienes (**18** and **19**). The 12β-carboxy methyl ester (**18**) was converted into the 12β-carboxyestratriene (**20**) by acid-catalyzed hydrolysis since basic hydrolysis gave an epimeric 12α-carboxyestratriene (**21**) as a major product,[12] which was also derived from **19** by using methanolic potassium hydroxide. The 12β-carboxyestratriene (**20**) was additionally prepared by the oxidation of the 17-hydroxy compound (**17**) with chromic

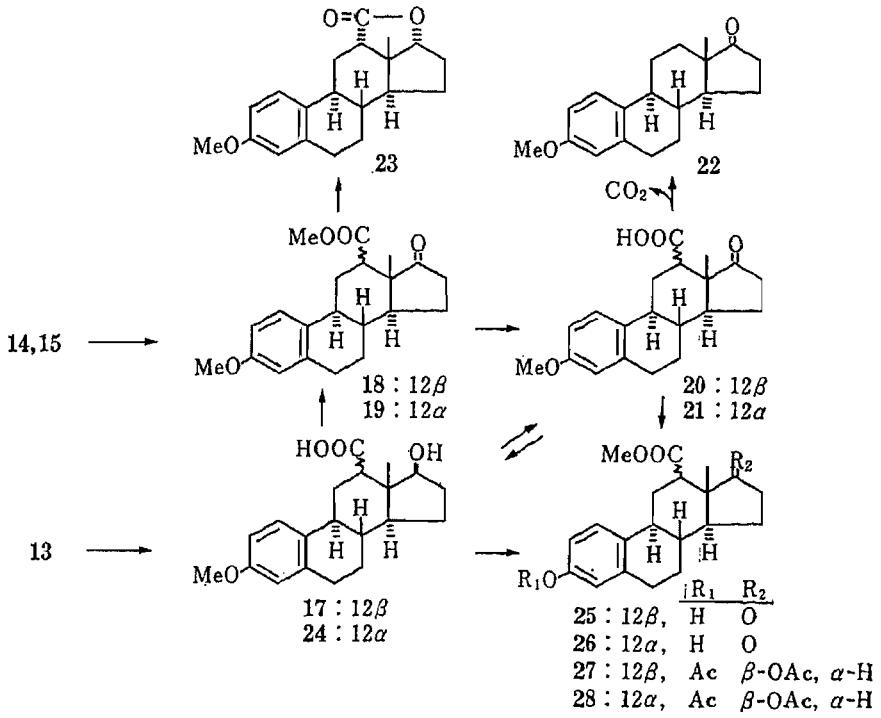

Chart 3

acid. The structures of these trienes (**20** and **21**) were determined by conversion into estrone methyl ether (**22**) using the decarboxylation method.[13] The reduction of the 12α-ester (**19**) with sodium borohydride gave the lactone (**23**) after initial formation of the 17α-ol because of the presence of a 12α-ester group as described above. On the other hand, the 12α-carboxylic acid (**21**) gave the 17β-alcohol (**24**) on reduction with sodium borohydride in dioxane. The β-orientation of the 17-hydroxy group in **24** was deduced from the NMR spectrum, which showed the triplet-like signal of the 17α-proton at 4.60 ppm (J=9 Hz) in pyridine.[14] The

difference between the ester (**19**) and the carboxylic acid (**21**) in the reduction with sodium borohydride can be explained as follows. Brown and Subba Rao[15] reported that sodium borohydride forms a complex with carboxylic acids, and the complex has been used as a reducing agent.[16] The 12α-carboxylic acid (**21**), presumably formed such a complex with sodium borohydride on the α-side of the molecule through the α-oriented carboxyl group and then the intramolecular reduction of the 17-carbonyl group occurred to give the 17β-alcohol (**24**). Finally, 12-carboxyestrone methyl ethers (**20** and **21**) were demethylated with chlorotrimethylsilane and sodium iodide[17] to give 12β-methoxycarbonylestrone (**25**) and 12α-methoxycarbonylestrone (**26**) after esterification. The 17-hydroxy compounds (**17** and **24**) were also converted into 12-methoxycarbonylestradiol derivatives (**27** and **28**), which were isolated after esterifications.

These compounds were utilized as new haptens conjugated at the C-12 position with bovine serum albumin for immunoassay of estradiol; details of the investigation on these haptens were reported in this journal.[18] These 12-carboxylated compounds are also expected to be useful as new estrogenic hormones for studies of the interaction between estrogens and their receptors.

Experimental

All melting points were taken on a micro hot stage apparatus and are uncorrected. UV spectra were measured on a Shimadzu UV-200 spectrometer. Infrared (IR) spectra were obtained on a JASCO IR A-102 spectrometer. NMR spectra were recorded on a Hitachi R-40 spectrometer and a JEOL 90-Q spectrometer at 90 MHz using tetramethylsilane as an internal standard. (s=singlet, br s=broad singlet, d=doublet, t=triplet, m=multiplet). Mass spectral (MS) measurements were run on a Shimadzu LKB 9000 spectrometer with the ionizing voltage at 20 eV. For column chromatography, silica gel (70—230 mesh) was used.

Methyl 7-(3-Methoxyphenyl)-3-methylsulfinyl-4-oxoheptanoate (2)——5-(3-Methoxyphenyl)-1-methylsulfinylpentan-2-one[7] (**1**, 7.62 g) in tetrahydrofuran (THF) (20 ml) was added to a stirred solution of KH (1.2 g) in THF (30 ml) at 0 °C under an Ar atmosphere. The stirring was continued for 30 min and $BrCH_2COOMe$ (4.8 g) was added dropwise. After 20 min, the reaction mixture was poured into ice-water and extracted three times with $CHCl_3$. The combined extracts were washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography (AcOEt : *n*-hexane = 2 : 1) to yield **2** as a colorless oil (9.2 g, 94%).[19] IR ν_{max}^{neat} cm^{-1}: 1740 (COOMe), 1710 (CO), 1050 (SO). NMR ($CDCl_3$) δ: 2.0 (2H, s), 2.42 and 2.45 (1.4 and 1.6H, respectively, s, CH_3), 3.68 (3H, s, $COOCH_3$), 3.79 (3H, s, OCH_3), 4.02 and 4.24 (0.47 and 0.53H, respectively, t, J = 10 Hz, methine).

Methyl 7-(3-Methoxyphenyl)-4-oxo-2-heptenoate (3)——A solution of **2** (3.62 g) in dioxane (10 ml) was refluxed for 15 min. The reaction mixture was evaporated and the residue was purified by column chromatography (*n*-hexane : AcOEt = 9 : 1) to yield 2.31 g (88%) of **3** as a colorless oil. IR ν_{max}^{neat} cm^{-1}: 1730 (COOMe), 1700 (CO), 1660 (C=C). NMR ($CDCl_3$) δ: 2.0 (2H, m), 2.65 (4H, t, J = 6 Hz), 3.80 (6H, s, $COOCH_3$ and OCH_3), 6.60 (1H, d, J = 14 Hz olefinic), 7.05 (1H, d, J = 14 Hz, olefinic). MS *m/z* (%): 262 (M^+, 4), 230 (6), 134 (100), 121 (33).

2-[6-(3-Methoxyphenyl)-1-methoxycarbonyl-3-oxohexyl]-2-methylcyclopentane-1,3-dione (4)——A solution of **3** (2.62 g) and 2-methylcyclopentane-1,3-dione (1.46 g) in 5% triethylamine–AcOEt (10 ml) was refluxed for 4.5 h. The reaction mixture was evaporated and the residue was recrystallized from MeOH to yield **4** as colorless needles (3.3 g, 88%). mp 123—123.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1760 (cyclic ketone), 1740 (COOMe), 1720 (CO), 1600, 1580. NMR ($CDCl_3$) δ: 0.98 (3H, s, CH_3), 1.7—2.1 (2H), 2.48 (4H, br s, cyclic methylene), 3.55 (3H, s, $COOCH_3$), 3.77 (3H, s, OCH_3), 3.83 (1H, t, J = 6 Hz, methine). *Anal.* Calcd for $C_{21}H_{26}O_6$: C, 67.36; H, 7.00. Found: C, 67.40; H, 7.00.

(±)-12α-Methoxycarbonyl-3-methoxyestra-1,3,5(10),8,14-pentaen-17-one (5) and (±)-12β-Methoxycarbonyl-3-methoxyestra-1,3,5(10),8,14-pentaen-17-one (6)——Methanesulfonic acid (2 ml) was added to a stirred solution of **4** (3.74 g) in CH_2Cl_2 (15 ml) at 0 °C. The mixture was stirred for 20 min and then poured into ice-water. After extraction with CH_2Cl_2, the extract was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was recrystallized from MeOH to give colorless plates of **6** (3.05 g, 90%). mp 139—140.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1745 (CO), 1720 (COOMe), 1560. UV λ_{max}^{EtOH} nm (ε): 314 (41000). NMR ($CDCl_3$) δ: 1.30 (3H, s, CH_3), 3.80 (3H, s, $COOCH_3$), 3.82 (3H, s, OCH_3), 5.93 (1H, t, J = 3 Hz, olefinic). MS *m/z* (%): 338 (M^+, 80), 317 (82), 310 (36), 261 (31), 251 (100). *Anal.* Calcd for $C_{21}H_{22}O_4$: C, 74.53; H, 6.55. Found: C, 74.51; H, 6.51. The mother liquor was subjected to column chromatography (*n*-hexane : AcOEt = 8 : 1) to give **5** (148 mg, 4%) as colorless prisms from MeOH. mp 175.5—177 °C. IR ν_{max}^{Nujol} cm^{-1}: 1740, 1730, 1560. UV λ_{max}^{EtOH} nm (ε): 314 (32000). NMR ($CDCl_3$) δ: 1.15 (3H, s, CH_3), 3.55 (3H, s, $COOCH_3$), 3.82 (3H, s, OCH_3), 6.02 (1H, t, J = 3 Hz, olefinic). MS *m/z* (%): 338 (M^+, 100), 320 (28), 299 (70), 263 (40), 251 (90). *Anal.* Calcd for $C_{21}H_{22}O_4$: C, 74.53; H, 6.55. Found: C, 74.53; H, 6.57.

(±)-3-Methoxyestra-1,3,5(10),8,14-pentaene-12α,17α-carbolactone (7)——A mixture of **5** (70 mg) and $NaBH_4$ (25 mg) in EtOH (30 ml) was stirred for 4 h at room temperature. The reaction mixture was evaporated and the residue was heated in 2 N HCl (5ml) for 30 min. The mixture was then cooled to room temperature and the precipitate was recrystallized from MeOH to give **7** (44 mg) as colorless prisms. mp 146—148.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1770 (lactone), 1560. NMR ($CDCl_3$) δ: 1.21 (3H, s, CH_3), 3.78 (3H, s, OCH_3), 4.61 (1H, d, J=3 Hz, 17β-H), 5.52 (1H, t, J=3 Hz, olefinic). MS *m/z* (%): 308 (M^+, 100), 263 (7), 248 (24). *Anal.* Calcd for $C_{20}H_{20}O_3$: C, 77.90; H, 6.54. Found: C, 77.63; H, 6.50.

(±)-12β-Methoxycarbonyl-3-methoxyestra-1,3,5(10),8,14-pentaen-17β-ol (8)——A solution of **6** (520 mg) and $NaBH_4$ (18 mg) in benzene (5 ml) and MeOH (20 ml) was stirred for 15 min at 0 °C. The reaction mixture was concentrated and the residue was extracted with CH_2Cl_2. The extract was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was recrystallized from MeOH to give **8** (497 mg, 95%) as colorless prisms. mp 138—142 °C. IR ν_{max}^{Nujol} cm^{-1}: 3380 (OH), 1720 (COOMe), 1610, 1570. NMR ($CDCl_3$)·δ: 0.96 (3H, s, CH_3), 3.75 (6H, s, $COOCH_3$ and OCH_3), 3.85 (1H, br s, 12α-H), 4.30 (1H, m, 17α-H), 5.55 (1H, t, J=3 Hz, olefinic). *Anal.* Calcd for $C_{21}H_{24}O_4$: C, 74.54; H, 6.55. Found: C, 74.52; H, 6.49.

(±)-12β-Methoxycarbonyl-3-methoxy-14α-estra-1,3,5(10),8-tetraen-17-one (9) and (±)-12β-Methoxycarbonyl-3-methoxy-14β-estra-1,3,5(10),8-tetraen-17-one (10)——(a) A mixture of **6** (170 mg) and 10% Pd/C (20 ml) in benzene (10 ml) was hydrogenated under H_2. After removal of the catalyst by filtration, the solvent was removed *in vacuo*. The residue was recrystallized from MeOH to give **9** (136 mg, 80%) as colorless crystals. mp 122.5—123 °C. IR ν_{max}^{Nujol} cm^{-1}: 1740 (CO and COOMe), 1600, 1570. UV λ_{max}^{EtOH} nm (ε): 280 (19600). NMR ($CDCl_3$) δ: 1.10 (3H, s, CH_3), 3.80 (6H, s, $COOCH_3$ and OCH_3). MS *m/z* (%): 340 (M^+, 100), 325 (5), 280 (15), 224 (38), 223 (35). *Anal.* Calcd for $C_{21}H_{24}O_4$: C, 74.09; H, 7.11. Found: C, 74.10; H, 7.11. The mother liquor was subjected to column chromatography (*n*-hexane : AcOEt = 6 : 1) to yield **10** (4 mg) as colorless needles from MeOH. mp 186.5—190 °C. IR ν_{max}^{Nujol} cm^{-1}: 1725, 1600, 1570. UV λ_{max}^{EtOH} nm (ε): 275 (17000). NMR ($CDCl_3$) δ: 1.10 (3H, s, CH_3), 3.65 (3H, s, $COOCH_3$), 3.80 (3H, s, OCH_3). *Anal.* Calcd for $C_{21}H_{24}O_4$: C, 74.09; H, 7.11. Found: C, 74.18; H, 7.08.

(b) A solution of **12** (230 mg) and CrO_3 (380 mg) in pyridine (8 ml) was stirred for 3 h at 50 °C and diluted with AcOEt (25 ml). The resulting suspension was filtered with Al_2O_3 on a sintered glass filter funnel and the filtrate was evaporated. The residue was recrystallized from MeOH to give colorless crystals of **9** (205 mg, 89%). mp 123—124.5 °C.

(±)-3-Methoxy-14β-estra-1,3,5(10),8-tetraen-12β,17α-carbolactone (11)——A solution of **10** (70 mg) and $NaBH_4$ (40 mg) in EtOH was stirred for 2 h at room temperature and evaporated. The oily residue was heated with 2 N HCl (5 ml) for 20 min and cooled to give a precipitate. The precipitate was recrystallized from MeOH to give **11** (42 mg) as pale yellow needles. mp 147—148 °C. IR ν_{max}^{Nujol} cm^{-1}: 1765 (lactone), 1600. NMR ($CDCl_3$) δ: 1.27 (3H, s, CH_3), 3.75 (3H, s, OCH_3), 4.55 (1H, br s, 17β-H). MS *m/z* (%): 310 (M^+, 100), 308 (6), 265 (7), 223 (5). *Anal.* Calcd for $C_{20}H_{22}O_3$: C, 77.39; H, 7.14. Found: C, 77.30; H, 7.22.

(±)-12β-Methoxycarbonyl-3-methoxyestra-1,3,5(10),8-tetraen-17β-ol (12)——A mixture of **8** (340 mg) and 10% Pd/C (50 mg) in benzene (20 ml) was hydrogenated under H_2. Filtration and evaporation gave a crude product which was recrystallized from MeOH to yield **12** (338 mg, 99%) as colorless prisms. mp 121—121.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 3480 (OH), 1710 (COOMe), 1570. NMR ($CDCl_3$) δ: 0.78 (3H, s, CH_3), 3.75 (6H, s, $COOCH_3$ and OCH_3), 3.90 (1H, br s, 12α-H), 4.10 (1H, m, 17α-H). MS *m/z* (%): 342 (M^+, 100), 309 (16), 265 (33), 223 (32). *Anal.* Calcd for $C_{21}H_{26}O_4$: C, 73.66; H, 7.66. Found: C, 73.51; H, 7.61.

(±)-12β-Carboxy-3-methoxyestra-1,3,5(10),8-tetraen-17β-ol (13)——A solution of **12** (342 mg) in 2 N KOH–MeOH (30 ml) was refluxed for 2 h and then concentrated *in vacuo*. The residue was dissolved in H_2O and acidified with 2 N HCl to give a precipitate, which was recrystallized from MeOH to give **13** (325 mg, 99%) as colorless needles. mp 227—229 °C. IR ν_{max}^{Nujol} cm^{-1}: 3350 (OH), 2700 (COOH), 1705 (COOH). NMR (pyridine-d_5) δ: 1.07 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 4.36 (1H, dd, J=7 and 10 Hz, 17α-H). *Anal.* Calcd for $C_{20}H_{24}O_4$: C, 73.14; H, 7.37. Found: C, 73.11; H, 7.38.

(±)-12β-Carboxy-3-methoxyestra-1,3,5(10),8-tetraen-17-one (14) and (±)-12α-Carboxy-3-methoxyestra-1,3,5-(10),8-tetraen-17-one (15)——(a) A solution of **9** (340 mg) in 2 N KOH–MeOH (20 ml) was refluxed for 2 h. The reaction mixture was concentrated and acidified with 2 N HCl. Then the mixture was extracted with CH_2Cl_2 twice and the combined extracts were washed with saturated NaCl, dried ($MgSO_4$) and evaporated. The residue was subjected to column chromatography ($CHCl_3$: iso-PrOH = 40 : 1) to afford two fractions. The first fraction gave **14** (7 mg, 2%) as colorless plates from MeOH. mp 205—207 °C. IR ν_{max}^{Nujol} cm^{-1}: 2700 (COOH), 1740 (CO), 1700 (COOH). NMR ($CDCl_3$) δ: 1.00 (3H, s, CH_3), 3.80 (3H, s, OCH_3). MS *m/z* (%): 326 (M^+, 100), 311 (28), 280 (13), 263 (10), 237 (9), 224 (20). *Anal.* Calcd for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.52; H, 6.82. The second fraction gave **15** (293 mg, 90%) as colorless prisms from MeOH. mp 187—189 °C. IR ν_{max}^{Nujol} cm^{-1}: 2700 (COOH), 1750 (CO), 1695 (COOH). NMR ($CDCl_3$) δ: 0.90 (3H, s, CH_3), 3.80 (3H, s, OCH_3). MS *m/z* (%): 326 (M^+, 30), 311 (28), 280 (20), 263 (55), 237 (27), 233 (100). *Anal.* Calcd for $C_{20}H_{22}O_4$: C, 73.60; H, 6.79. Found: C, 73.59; H, 6.80.

(b) A solution of **9** (170 mg) in EtOH (2 ml) and 10% H_2SO_4 (2 ml) was heated under reflux for 4 h. The reaction mixture was extracted with CH_2Cl_2. The extract was washed with H_2O, dried ($MgSO_4$) and evaporated. The residue was recrystallized from MeOH to give **14** (152 mg, 93%) as colorless plates. mp 206—207 °C.

(±)-12α-Methoxycarbonyl-3-methoxyestra-1,3,5(10),8-tetraen-17-one (16)——A solution of **15** (98 mg) in MeOH (5 ml) was treated with diazomethane in ether and then evaporated. The residue was recrystallized from MeOH to give **16** (89 mg) as colorless prisms. mp 167—170 °C. IR ν_{max}^{Nujol} cm^{-1}: 1750 (CO), 1700 (COOMe), 1600, 1570. NMR ($CDCl_3$) δ: 0.87 (3H, s, CH_3), 3.63 (3H, s, $COOCH_3$), 3.76 (3H, s, OCH_3). *Anal.* Calcd for $C_{21}H_{24}O_4$: C, 74.09; H, 7.11. Found: C, 74.00; H, 7.22.

(±)-12β-Carboxyestradiol 3-Methyl Ether (17)——Sodium (100 mg) was added to stirred liquid NH_3 (70 ml) at −50 °C. After 5 min, **13** (328 mg) in THF (25 ml) and aniline (2 ml) were added to the above solution and stirring was continued for 20 min. After the addition of solid NH_4Cl (5 g), the NH_3 was evaporated off. The residue was dissolved in H_2O, and acidified with 2 N HCl to give a white precipitate, which was recrystallized from MeOH to give **17** (298 mg, 90%) as colorless prisms. mp 212—213 °C. IR ν_{max}^{Nujol} cm^{-1}: 3350, (OH), 1690 (COOH). NMR (pyridine-d_5) δ: 1.02 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 4.25 (1H, t, J=9 Hz, 17α-H), 6.6—6.9 (2H, m, 2,4-H), 7.25 (1H, d, J=9 Hz, 1-H). *Anal.* Calcd for $C_{20}H_{26}O_4$: C, 66.28; H, 7.23. Found: C, 66.25; H, 7.22.

(±)-12β-Methoxycarbonylestrone Methyl Ether (18)——Sodium (100 mg) was added to stirred liquid NH_3 (60 ml) at −40 °C. After 5 min, a mixture of **14** (300 mg) and aniline (2 ml) in THF (25 ml) was added to the above solution. After 20 min, further sodium (100 mg) was added and the reaction mixture was stirred for an additional 20 min. Solid NH_4Cl (5 g) was added to the mixture and the NH_3 was evaporated off. The residue was dissolved in 27% HCl-MeOH (10 ml) and allowed to stand for 1 h at room temperature. The mixture was concentrated and the residue was extracted with CH_2Cl_2. The extract was washed with H_2O, dried (Na_2SO_4) and evaporated to give an oily residue, which was oxidized with 8 N H_2CrO_4 in acetone at 0 °C. After usual work-up, the crude product was subjected to column chromatography (*n*-hexane: AcOEt = 3 : 1) to give **18** (201 mg, 67%) as colorless prisms. mp 144.5—145.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1740 (CO), 1730 (COOMe), 1600. NMR ($CDCl_3$) δ: 1.13 (3H, s, CH_3), 3.75 (6H, s, $COOCH_3$ and OCH_3), 6.6—6.8 (2H, m, 2, 4-H), 7.15 (1H, d, J=9 Hz, 1-H). MS *m/z* (%): 342 (M^+, 100), 282 (40), 267 (21), 225 (27). *Anal.* Calcd for $C_{21}H_{26}O_4$: C, 73.66; H, 7.66. Found: C, 73.52; H, 7.68.

(±)-12α-Methoxycarbonylestrone Methyl Ether (19)——Sodium (200 mg) was added to stirred liquid NH_3 (60 ml) at −40 °C. After 5 min, a mixture of **15** (330 mg) and aniline (2 ml) in THF (10 ml) was added to the above solution. Stirring was continued for 40 min and then NH_4Cl (5 g) was added. After evaporation of the NH_3, the same work-up as used in the preparation of **18** yielded **19** (261 mg, 79%) as colorless needles from MeOH. mp 167.5—170 °C. IR ν_{max}^{Nujol} cm^{-1}: 1745 (CO), 1730 (COOMe). NMR ($CDCl_3$) δ: 1.05 (3H, s, CH_3), 3.71 (3H, s, $COOCH_3$), 3.74 (3H, s, OCH_3), 6.6—6.75 (2H, m, 2, 4-H), 7.00 (1H, d, J=9 Hz, 1-H). MS *m/z* (%): 342 (M^+, 20), 282 (100), 225 (41), 211 (24). *Anal.* Calcd for $C_{21}H_{26}O_4$: C, 73.66; H, 7.66. Found: C, 73.48; H, 7.67.

(±)-12β-Carboxyestrone Methyl Ether (20)——(a) A solution of **18** (170 mg) in EtOH (4 ml) and 10% H_2SO_4 (4 ml) was refluxed for 4 h. The reaction mixture was poured into ice-water and the precipitate was recrystallized from MeOH to give **20** (141 mg, 86%) as colorless prisms. mp 182—184 °C. IR ν_{max}^{Nujol} cm^{-1}: 1730 (CO), 1695 (COOH), 1600. NMR (pyridine-d_5) δ: 1.30 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 6.7—6.8 (2H, m, 2, 4-H), 7.20 (1H, d, J=9 Hz, 1-H). *Anal.* Calcd for $C_{20}H_{24}O_4$: C, 73.14; H, 7.37. Found: C, 73.14; H, 7.39.

(b) An 8 N H_2CrO_4 solution (1 ml) was added to a stirred solution of **17** (80 mg) in acetone (3 ml) and CH_2Cl_2 (1 ml) at 0 °C. The reaction mixture was stirred for 25 min and extracted with CH_2Cl_2. The extract was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was recrystallized from MeOH to give **20** (74 mg, 93%) as colorless prisms. mp 180—183 °C.

(±)-12α-Carboxyestrone Methyl Ether (21)——A solution of **19** (171 mg) in 2 N KOH-MeOH (10 ml) was refluxed for 30 min and the reaction mixture was concentrated. The residue was dissolved in H_2O and acidified with 2 N HCl to give a precipitate, which was recrystallized from MeOH to afford **21** (147 mg, 90%) as colorless prisms. mp 227—231 °C. IR ν_{max}^{Nujol} cm^{-1}: 1740 (CO), 1700 (COOH), 1605. NMR (pyridine-d_5) δ: 1.04 (3H, s, CH_3), 3.71 (3H, s, OCH_3), 6.7—6.9 (2H, m, 2, 4-H), 7.10 (1H, d, J=9 Hz, 1-H). *Anal.* Calcd for $C_{20}H_{24}O_4$: C, 73.14; H, 7.37. Found: C, 73.08; H, 7.33.

Decarboxylation of 20 and 21——A solution of the carboxylic acid (**20** or **21**, 40 mg) and $SOCl_2$ (200 mg) in $CHCl_3$ (5 ml) was refluxed for 1 h and the reaction mixture was concentrated *in vacuo*. The residue was dissolved in *p*-cymene (5 ml) and pyridine (5 ml). A solution of *tert*-BuOOH (190 mg) in *p*-cymene (2 ml) was added dropwise to the above acid chloride solution with stirring at 0 °C, and stirring was continued for 1 h. The reaction mixture was poured into ice-water and the separated organic layer was dried ($MgSO_4$). The organic layer was then heated to 140 °C for 1 h and evaporated. The oily residue was subjected to column chromatography (*n*-hexane: AcOEt = 10 : 1) to give estrone methyl ether (**22**). The 12β-carboxylic acid (**20**) gave **22** (14 mg, 140—142 °C from MeOH) and the 12α-isomer (**21**) gave **22** (18 mg, mp 141—142.5 °C from MeOH, lit.[6] mp 142—143 °C). The spectral data of both were identical with that of authentic *dl*-estrone methyl ether.

(±)-3-Methoxyestra-1,3,5(10)-triene-12α,17α-carbolactone (23)——A solution of **19** (34 mg) and $NaBH_4$ (15 mg) in EtOH (10 ml) was heated at 60 °C for 1 h. The reaction mixture was acidified with 4 N HCl (5 ml), heated for 10 min, diluted with H_2O and extracted with ether. The extract was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was subjected to column chromatography (*n*-hexane: AcOEt = 6 : 1) to give **23** (21 mg, 68%) as colorless prisms from MeOH. mp 178—179.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 1760 (lactone), 1600. *Anal.* Calcd for $C_{20}H_{24}O_3$: C, 76.89; H, 7.74. Found: C, 76.83; H, 7.72.

(±)-12α-Carboxyestradiol 3-Methyl Ether (24)——A solution of **21** (328 mg) and $NaBH_4$ (40 mg) in dioxane (20 ml) was stirred for 10 min at 4 °C. Stirring was continued for 30 min at room temperature and then the mixture was refluxed for 30 min. The mixture was concentrated and acidified with 2N HCl, and the precipitate was recrystallized from MeOH to yield **24** (301 mg, 91%) as colorless prisms. mp 188—190.5 °C. IR ν_{max}^{Nujol} cm^{-1}: 3450 (OH), 2700 (COOH), 1710 (COOH). NMR (pyridine-d_5) δ: 1.20 (3H, s, CH_3), 3.70 (3H, s, OCH_3), 4.60 (1H, t, J= 10 Hz, 17α-H), 6.8—7.0 (2H, m, 2, 4-H), 7.15 (1H, d, J=9 Hz, 1-H). *Anal.* Calcd for $C_{20}H_{26}O_4$: C, 66.28; H, 7.23. Found: C, 66.21; H, 7.22.

(±)-12β-Methoxycarbonylestrone (25)——A solution of **20** (110 mg), chlorotrimethylsilane (280 mg) and NaI (350 mg) in CH_3CN (6 ml) was heated at 60 °C for 4 h with stirring under N_2. The reaction mixture was diluted with H_2O and extracted with AcOEt. The extract was washed with H_2O, 5% $Na_2S_2O_3$ dried (Na_2SO_4) and evaporated. The residue was dissolved in 10% HCl–MeOH (5 ml) and then this solution was heated at 60 °C for 10 min. The mixture was evaporated and the residue was recrystallized from $CHCl_3$–isopropyl ether to yield **25** (96 mg, 87%) as colorless needles. mp 231—235 °C. IR ν_{max}^{Nujol} cm^{-1}: 3400 (OH), 1740 (CO), 1720 (COOMe), 1605. NMR (DMSO-d_6) δ: 0.72 (3H, s, CH_3), 3.70 (3H, s, $COOCH_3$), 6.4—6.6 (2H, m, 2, 4-H), 7.00 (1H, d, J=8 Hz, 1-H). *Anal.* Calcd for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 72.11; H, 7.62.

(±)-12α-Methoxycarbonylestrone (26)——A solution of **21** (164 mg), chlorotrimethylsilane (540 mg) and NaI (1 g) in CH_3CN (8 ml) was heated at 60 °C with stirring under N_2 for 4 h. After the same work-up as described above, **26** (134 mg) was obtained as colorless prisms, mp 254—257 °C. IR ν_{max}^{Nujol} cm^{-1}: 3450 (OH), 1730 (CO), 1725 (COOMe), 1605. NMR (DMSO-d_6) δ: 1.01 (3H, s, CH_3), 3.66 (3H, s, $COOCH_3$), 6.5—6.7 (2H, m, 2, 4-H), 6.80 (1H, d, J=9 Hz, 1-H). *Anal.* Calcd for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 72.10; H, 7.70.

(±)-12β-Methoxycarbonylestradiol Diacetate (27)——A solution of **17** (100 mg), chlorotrimethylsilane (190 mg) and NaI (220 mg) in CH_3CN (15 ml) was heated at 40 °C for 6 h with stirring under N_2. After the same work-up as described above, the oily residue was dissolved in Ac_2O (1 ml) and pyridine (2 ml). The mixture was kept at room temperature for 24 h, poured into ice-water and extracted with ether. The extract was washed with 2N HCl and 5% $NaHCO_3$, then dried (Na_2SO_4) and evaporated. The crude product was purified by column chromatography (*n*-hexane : AcOEt = 2 : 1) to yield **27** (94 mg, 75%) as colorless prisms from MeOH. mp 162.5—163.5 °C. NMR ($CDCl_3$) δ: 0.90 (3H, s, CH_3), 2.00 and 2.23 (each 3H, s, OAc), 3.63 (3H, s, $COOCH_3$), 4.81 (1H, dd, J=8 and 9 Hz, 17α-H), 6.7—6.8 (2H, m, 2, 4-H), 7.2 (1H, d, J=9 Hz, 1-H). MS *m/z* (%): 414 (M^+, 48), 372 (100), 312 (7), 269 (5), 253 (10). *Anal.* Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.51; H, 7.30.

(±)-12α-Methoxycarbonylestradiol Diacetate (28)——A solution of **24** (170 mg), chlorotrimethylsilane (280 mg) and NaI (300 mg) in CH_3CN (8 ml) was heated at 40 °C for 6 h with stirring under N_2. After the same work-up as described above, **28** (94 mg, 44%) was obtained as colorless crystals. mp 194—195.5 °C. NMR ($CDCl_3$) δ: 1.02 (3H, s, CH_3), 2.01 and 2.23 (each 3H, s, OAc), 3.65 (3H, s, $COOCH_3$), 4.81 (1H, t, J=9 Hz, 17α-H), 6.7—6.8 (2H, m, 2, 4-H), 7.07 (1H, d, J=9 Hz, 1-H). MS *m/z* (%): 414 (M^+, 4), 354 (61), 312 (100), 294 (22), 252 (57). *Anal.* Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.70; H, 7.44.

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19) The work-up procedure was carried out at below 10 °C, and the crude product was sufficiently pure for next reaction step. Purification by column chromatography at room temperature gave a lower yield (80%).

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One-Pot Synthesis of *N*-Alkylpyrrolidines by 1,3-Dipolar Cycloaddition

NOBUYUKI IMAI and KAZUO ACHIWA*

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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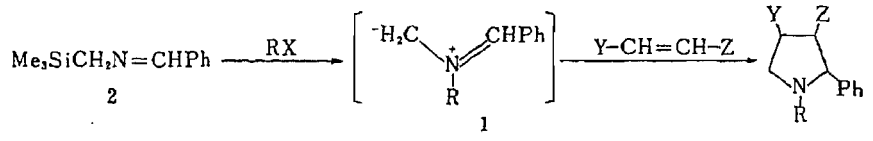
N-Alkylpyrrolidines were obtained by one-pot synthesis through 1,3-dipolar cycloaddition of the *N*-alkylazomethine ylide intermediates generated from *N*-(benzylidene)trimethylsilylmethylamine and alkyl halide or tosylate.

Keywords——1,3-dipolar cycloaddition; *N*-alkylpyrrolidine; *N*-unsubstituted pyrrolidine; *N*-alkylazomethine ylide; *N*-trimethylsilylazomethine ylide; *N*-(benzylidene)trimethylsilylmethylamine; Schiff base

Recently, we and other groups have developed a number of 1,3-dipolar cycloadditions[1-3] involving intermediary azomethine ylides derived from organosilicon compounds by heterolytic cleavage of the silicon–carbon bond, and have applied them to the synthesis of alkaloids bearing a pyrrolidine nucleus.[2] In a short communication, we have also reported a new one-pot synthesis of *N*-alkylpyrrolidine derivatives by 1,3-dipolar cycloaddition of the intermediary *N*-alkylazomethine ylides (**1**) formed from *N*-(benzylidene)trimethylsilylmethylamine (**2**) and alkyl halide or tosylate.[3] This new methodology includes direct and one-pot formation of *N*-alkylpyrrolidines from three components: the organosilicon compound **2**, alkyl halide, and conjugated olefin. The details of this reaction are the subject of this paper.

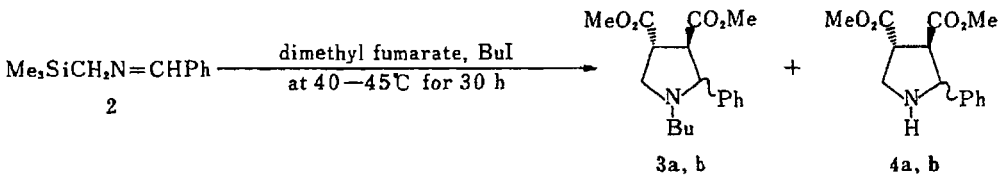


RX: alkyl halide or tosylate

Preliminary experiments included examination of the solvent effect using **2** as a substrate, dimethyl fumarate as a dipolarophile, butyl iodide as an alkyl halide and hexamethylphosphoramide (HMPA), tetramethylurea (TMU), *N*,*N*-dimethylformamide (DMF), acetonitrile or tetrahydrofuran (THF) as a solvent. These results are summarized in Table I. The reaction of **2** with butyl iodide and dimethyl fumarate proceeded smoothly at 40—45 °C for 30 h in HMPA, TMU or DMF to give the corresponding *N*-alkylpyrrolidine derivatives (**3a**, **b**) along with the *N*-unsubstituted ones (**4a**, **b**) as minor products. HMPA was selected as the most suitable solvent for this cycloaddition because it gave a high yield with little by-product. Next, examination of the relative reactivities of **2** with dimethyl fumarate and butyl halide or tosylate in HMPA was carried out; the data obtained are listed in Table II. Interestingly, marked differences among butyl halides and tosylate were observed. The reaction with butyl iodide proceeded smoothly to give **3a**, **b** exclusively, whereas that with butyl chloride gave **4a**, **b**.

The 1,3-dipolar cycloaddition of the Schiff base **2** with other olefinic dipolarophiles in the

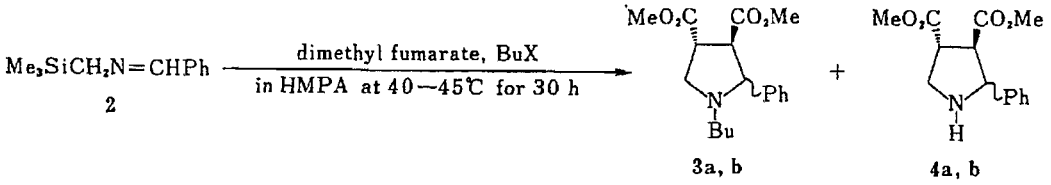
TABLE I. Solvent Effect[a]



Solvent	Total yield (%)	Molar proportion 3a, b	4a, b
HMPA	100	99	1
TMU	90	70	30
DMF	70	65	35
Acetonitrile	20	70	30
THF	0	—	—

a) The yields and molar proportions were determined by vapar phase chromatography (VPC) analysis.

TABLE II. Relative Reactivities of Butyl Halides and Tosylate[a]



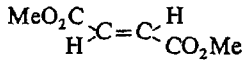
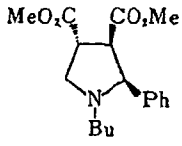
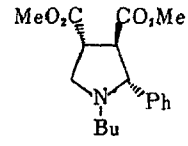
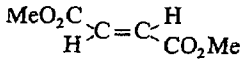
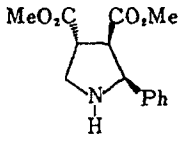
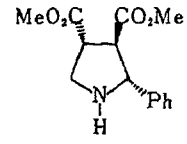
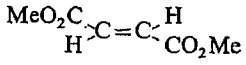
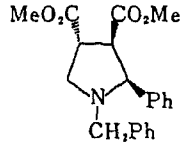
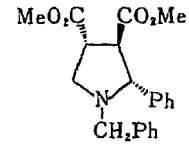
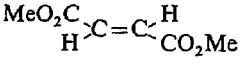
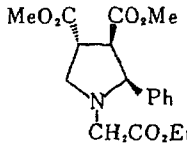
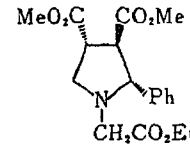
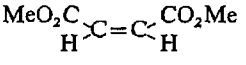
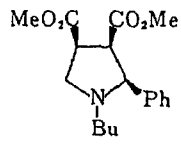
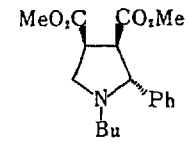
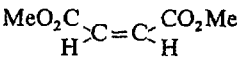
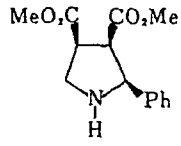
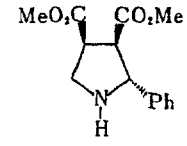
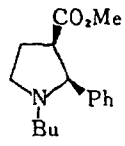
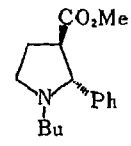
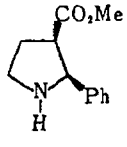
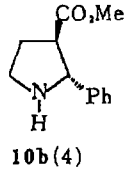
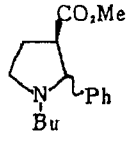
BuX	Total yield (%)	Molar proportion 3a, b	4a, b
BuI	100	99	1
BuBr	100	63	37
BuCl	66	—	100
BuOTs	79	50	50

a) The yields and molar proportions were determined by VPC analysis.

presence of several alkyl halides in HMPA at 80—85 °C for 2 h also proceeded smoothly to give the corresponding pyrrolidine derivatives. The results of extensive experiments are summarized in Table III. In all cases, the products were obtained as mixtures of two stereoisomers which could not be separated by silica gel column chromatography (SGCC), except for the products **3a** and **3b** (entry 1), **5a** and **5b** (entry 3), **10a** and **10b** (entry 8), which were separated by SGCC using the eluent indicated in Experimental. The structures of these products were determined on the basis of proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data (see Table IV), and other spectral and physical data (see Table V). The ratios of the stereoisomers were determined from the signal ratios of the separated chemical shifts of the methyl protons of the esters, because the signals of the methyl protons oriented *cis* to the phenyl groups in the five-membered ring appear at higher magnetic field owing to the shielding effect of the phenyl groups.[4]

Mechanistically, the Schiff base **2** is presumed to react first with alkyl halide or tosylate to give the *N*-alkyliminium salt (**11**), which is then easily converted into the intermediary *N*-alkylazomethine ylide **1** (1,3-dipole), and the subsequent cycloaddition of the 1,3-dipole **1** with a dipolarophile gives the corresponding *N*-alkylpyrrolidine, whereas the formation of *N*-

TABLE III. Synthesis of Pyrrolidine Derivatives[a]

Entry	Olefin	RX	Products[b]	Total yield (%)
1		BuI	 **3a** (3)  **3b** (2)	82
2		BuCl	 **4a** (1)  **4b** (1.2)	76
3		$PhCH_2Br$	 **5a** (1.2)  **5b** (1)	79
4		EtO_2CCH_2Br	 **6a** (1.1)  **6b** (1)	74
5		BuI	 **7a** (1)  **7b** (2)	61
6		BuCl	 **8a** (3)  **8b** (7)	76
7	$H_2C=CHCO_2Me$	BuI	 **9a** (3)  **9b** (2)	45
8	$H_2C=CHCO_2Me$	BuCl	 **10a** (5.4)  **10b** (4)  **9a, b** (1)	65

a) Reaction conditions: molar ratio, 2: RX: olefin = 1 : 1 : 1.2; solvent, HMPA; temp, 80—85 °C; time, 2 h. *b*) Ratio of the products is given in parentheses.

TABLE IV. ^{1}H-NMR and ^{13}C-NMR Spectral Data (3—10)

Compound No	^{1}H-NMR δ ($CDCl_3$, J=Hz)	^{13}C-NMR δ ($CDCl_3$)
3a	0.60—1.04 (3H, m, $NCH_2CH_2CH_2C\underline{H}_3$), 1.04—1.70 (4H, m, $NCH_2C\underline{H}_2C\underline{H}_2CH_3$), 1.79—2.81 (4H, m, $N(CH_2)_2$), 3.03 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group), 3.29—4.01 (3H, m, 2-CH, 3-CH, 4-CH), 3.65 (3H, s, 4-CO_2CH_3), 7.20 (5H, s, C_6H_5).	13.9 (q, $NCH_2CH_2CH_2\underline{C}H_3$), 20.4 (t, $NCH_2CH_2\underline{C}H_2CH_3$), 30.5 (t, $NCH_2\underline{C}H_2CH_2CH_3$), 44.4 (d, 4-C), 51.1 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 51.9 (q, OCH_3), 52.3 (q, $O\underline{C}H_3$), 53.4 (d, 3-C), 55.4 (t, 5-C), 71.6 (d, 2-C), 127.5, 127.9, 128.5, 139.4 (d, d, d, s, C_6H_5), 171.9 (s, CO), 173.4 (s, CO).
3b	0.56—1.03 (3H, m, $NCH_2CH_2CH_2C\underline{H}_3$), 1.03—1.60 (4H, m, $NCH_2C\underline{H}_2C\underline{H}_2CH_3$) 1.60—2.80 (4H, m, $N(CH_2)_2$), 3.14—4.10 (3H, m, 2-CH, 3-CH, 4-CH), 3.55 (3H, s, 3-CO_2CH_3 oriented *trans* to 2-phenyl group), 3.67 (3H, s, 4-CO_2CH_3), 7.21 (5H, s, C_6H_5).	13.9 (q, $NCH_2CH_2CH_2\underline{C}H_3$), 20.3 (t, $NCH_2CH_2\underline{C}H_2CH_3$), 30.3 (t, $NCH_2\underline{C}H_2CH_2CH_3$), 45.1 (d, 4-C), 51.8 (q, OCH_3), 52.2 (q, OCH_3), 52.8 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 55.0 (d, 3-C), 55.3 (t, 5-C), 73.5 (d, 2-C), 127.7, 128.0, 128.4, 141.1 (d, d, d, s, C_6H_5), 173.6 (s, CO), 173.9 (s, CO).
4a[a]	2.48 (2H, s, NH × 2), 3.13 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group of **4a**), 3.23—3.44 (6H, m, 4-CH × 2, 5-CH_2 × 2), 3.60 (3H, s, 3-CO_2CH_3 oriented *trans*	49.3 (d, 4-C), 51.0 (t, 5-C), 51.3 (q, OCH_3), 52.0 (q, OCH_3), 55.4 (d, 3-C), 67.8 (d, 2-C), 126.9, 127.5, 127.7, 139.0 (d, d, d, s, C_6H_5), 173.6 (s, CO), 174.1 (s, CO).
4b[a]	to 2-phenyl group of **4b**), 3.66 (6H, s, 4-CO_2CH_3 × 2), 4.27 (2H, br d, J=8.4, 3-CH × 2), 4.50 (2H, d, J=8.4, 2-CH × 2), 7.12—7.50 (10H, m, C_6H_5 × 2).	47.7 (d, 4-C), 50.8 (t, 5-C), 52.0 (q, OCH_3), 52.1 (q, OCH_3), 53.3 (d, 3-C), 66.4 (d, 2-C), 126.9, 128.1, 128.6, 141.2 (d, d, d, s, C_6H_5), 172.7 (s, CO), 173.6 (s, CO).
5a	2.88—4.13 (7H, 2-CH, 3-CH, 4-CH, 5-$C\underline{H}_2$, $NC\underline{H}_2Ph$), 3.07 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group), 3.63 (3H, s, 4-CO_2CH_3), 7.08—7.52 (5H, m, $NCH_2C_6\underline{H}_5$), 7.22 (5H, s, C_6H_5).	44.2 (d, 4-C), 51.3 (d, 3-C), 51.9 (q, OCH_3), 52.3 (q, OCH_3), 55.1 (t, 5-C), 57.3 (t, $N\underline{C}H_2Ph$), 70.7 (d, 2-C), 127.1, 127.8, 128.1, 128.2, 128.5, 128.6, 138.3, 138.8 (d, d, d, d, d, d, s, s, C_6H_5 × 2), 171.8 (s, CO), 173.3 (s, CO).
5b	2.84—4.07 (7H, 2-CH, 3-CH, 4-CH, 5-CH_2, $NC\underline{H}_2Ph$), 3.56 (3H, s, 3-CO_2CH_3 oriented *trans* to 2-phenyl group), 3.63 (3H, s, 4-CO_2CH_3), 6.97—7.57 (5H, m, $NCH_2C_6\underline{H}_5$), 7.15 (5H, s, C_6H_5).	45.0 (d, 4-C), 51.9 (q, OCH_3), 52.1 (q, OCH_3), 54.9 (d, 3-C), 55.1 (t, 5-C), 56.8 (t, $N\underline{C}H_2Ph$), 72.5 (d, 2-C), 126.9, 127.9, 128.1, 128.2, 128.4, 128.6, 138.6, 140.7 (d, d, d, d, d, d, s, s, C_6H_5 × 2), 173.4 (s, CO), 173.7 (s, CO).
6a[a]	1.20 (6H, t, J=7.9, $CO_2CH_2C\underline{H}_3$ × 2), 2.86—4.38 (14H, 2-CH × 2, 3-CH × 2, 4-CH × 2, 5-CH_2 × 2, NCH_2CO_2 × 2), 3.07 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group of **6a**), 3.59 (3H, s, 3-CO_2CH_3 oriented *trans* to 2-phenyl group of **6b**), 3.67 (3H, s, 4-CO_2CH_3 of **6a**),	14.2 (q, $OCH_2\underline{C}H_3$), 44.4 (d, 4-C), 51.3 (d, 3-C), 52.0 (q, OCH_3), 52.1 (t, $O\underline{C}H_2CH_3$), 52.2 (q, OCH_3), 54.6 (t, 5-C), 60.3 (t, $N\underline{C}H_2CO_2$), 68.6 (d, 2-C), 127.9, 128.1, 128.4, 138.5, (d, d, d, s, C_6H_5), 170.2 (s, CO), 171.6 (s, CO), 172.9 (s, CO).
6b[a]	3.73 (3H, s, 4-CO_2CH_3 of **6b**), 4.09 (4H, q, J=7.9, $CO_2C\underline{H}_2CH_3$ × 2), 7.27 (5H, s, C_6H_5 of **6a**), 7.35 (5H, s, C_6H_5 of **6b**).	14.2 (q, $OCH_2C\underline{H}_3$), 45.1 (d, 4-C), 52.0 (q, OCH_3), 52.1 (t, $O\underline{C}H_2CH_3$), 52.2 (q, OCH_3), 52.3 (d, 3-C), 54.6 (t, 5-C), 60.3 (t, $N\underline{C}H_2CO_2$), 70.8 (d, 2-C), 127.9, 128.1, 128.6, 139.7 (d, d, d, s, C_6H_5), 170.2 (s, CO), 172.9 (s, CO), 173.4 (s, CO).

TABLE IV. (continued)

Compound No.	^{1}H-NMR δ ($CDCl_3$, J=Hz)	^{13}C-NMR δ ($CDCl_3$)
7a[a]	0.67—0.99 (6H, m, $NCH_2CH_2CH_2C\underline{H}_3\times2$), 0.99—1.60 (8H, m, $NCH_2C\underline{H}_2C\underline{H}_2CH_3\times2$), 1.91—2.96 (8H, m, $N(CH_2)_2\times2$), 2.96—4.06 (6H, m, 2-CH×2, 3-CH×2, 4-CH×2), 3.17 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group of **7a**), 3.60 (3H, s, 3-CO_2CH_3 oriented *trans*	14.0 (q, $NCH_2CH_2CH_2\underline{C}H_3$), 20.4 (t, $NCH_2CH_2\underline{C}H_2CH_3$), 31.0 (t, $NCH_2\underline{C}H_2CH_2CH_3$), 44.2 (d, 4-C), 50.9 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 52.0 (q, OCH_3), 52.9 (q, OCH_3), 53.6 (d, 3-C), 54.1 (t, 5-C), 72.4 (d, 2-C), 127.7, 128.1, 128.5, 139.1 (d, d, d, s, C_6H_5), 171.1 (s, CO), 172.2 (s, CO).
7b[a]	to 2-phenyl group of **7b**), 3.63 (3H, s, 4-CO_2CH_3 of **7a**), 3.66 (3H, s, 4-CO_2CH_3 of **7b**), 7.17—7.55 (10H, m, $C_6H_5\times2$).	13.9 (q, $NCH_2CH_2CH_2\underline{C}H_3$), 20.4 (t, $NCH_2CH_2\underline{C}H_2CH_3$), 30.6 (t, $NCH_2\underline{C}H_2CH_2CH_3$), 44.8 (d, 4-C), 51.7 (q, $OCH_3\times2$), 53.3 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 54.8 (d, 3-C), 55.1 (t, 5-C), 72.0 (d, 2-C), 127.7, 128.4, 141.8 (d, d, s, C_6H_5), 172.6 (s, CO×2).
8a[a]	2.31 (2H, s, NH×2), 3.14 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group of **8a**), 3.19—3.54 (6H, m, 4-CH×2, 5-$CH_2\times2$), 3.63 (6H, s, 4-CO_2CH_3 of **8a**, 3-CO_2CH_3	47.7 (d, 4-C), 51.0 (t, 5-C), 51.9 (q, OCH_3), 52.1 (q, OCH_3), 55.4 (d, 3-C), 67.8 (d, 2-C), 126.5, 126.9, 128.5, 141.0 (d, d, d, s, C_6H_5), 172.8 (s, CO), 174.1 (s, CO).
8b[a]	oriented *trans* to 2-phenyl group of **8b**), 3.67 (3H, s, 4-CO_2CH_3 of **8b**), 4.16—4.68 (4H, m, 2-CH×2, 3-CH×2), 7.15 (5H, s, C_6H_5 of **8b**), 7.24 (5H, s, C_6H_5 of **8a**).	47.8 (d, 4-C), 49.9 (t, 5-C), 51.9 (q, OCH_3), 52.0 (q, OCH_3), 55.2 (d, 3-C), 64.9 (d, 2-C), 126.5, 126.9, 128.5, 142.8 (d, d, d, s, C_6H_5), 172.6 (s, CO), 173.6 (s, CO).
9a, b[a]	0.74—0.98 (6H, m, $NCH_2CH_2CH_2C\underline{H}_3\times2$), 0.98—1.65 (8H, m, $NCH_2C\underline{H}_2C\underline{H}_2CH_3\times2$), 1.76—2.67 (8H, m, $N(CH_2)_2\times2$), 3.06 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group of **9a**), 3.13—3.55 (8H, m, 2-CH×2, 3-CH×2, 4-$CH_2\times2$), 3.60 (3H, s, 3-CO_2CH_3 oriented *trans* to 2-phenyl group of **9b**), 7.27 (5H, s, C_6H_5 of **9a**), 7.31 (5H, s, C_6H_5 of **9b**).	13.9 (q, $NCH_2CH_2CH_2\underline{C}H_3\times2$), 20.5 (t, $NCH_2CH_2\underline{C}H_2CH_3\times2$), 26.5, 27.5 (t, t, 4-C×2), 30.7, 30.8 (t, t, $NCH_2\underline{C}H_2CH_2CH_3\times2$), 49.8 (d, 3-C), 50.8 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 51.5 (d, 3-C), 52.5 (q, OCH_3), 52.6 (q, OCH_3), 52.8 (t, $N\underline{C}H_2CH_2CH_2CH_3$), 53.6, 54.0 (t, t, 5-C×2), 71.8, 73.1 (d, d, 2-C×2), 127.2, 127.8, 128.4, 140.4, 142.3 (d, d, d, s, s, $C_6H_5\times2$), 173.3 (s, CO×2).
10a	1.90—2.30 (2H, m, 4-CH_2), 2.30 (1H, s, NH), 2.73—3.66 (3H, m, 3-CH, 5-CH_2), 3.14 (3H, s, 3-CO_2CH_3 oriented *cis* to 2-phenyl group), 4.30 (1H, d, J=7.2, 2-CH), 7.17 (5H, s, C_6H_5).	29.6 (t, 4-C), 46.7 (t, 5-C), 49.7 (d, 3-C), 50.9 (q, OCH_3), 66.3 (d, 2-C), 126.8, 127.1, 127.9, 139.9 (d, d, d, s, C_6H_5), 174.1 (s, CO).
10b	1.72—2.52 (2H, m, 4-CH_2), 2.16 (1H, s, NH), 2.66—3.36 (3H, m, 3-CH, 5-CH_2), 3.58 (3H, s, 3-CO_2CH_3 oriented *trans* to 2-phenyl group), 4.31 (1H, d, 7.2, 2-CH), 7.23 (5H, s, C_6H_5).	31.0 (t, 4-C), 46.8 (t, 5-C), 51.7 (q, OCH_3), 52.1 (d, 3-C), 66.2 (d, 2-C), 126.7, 127.3, 128.5, 143.0 (d, d, d, s, C_6H_5), 175.2 (s, CO).

a) The NMR spectra of these compounds were obtained with the mixture of two stereoisomers in each case.

TABLE V. Physical and Spectral Data for Pyrrolidine Derivatives (3—10)

Compound No.	IR (liq. or KBr) cm^{-1}	bp/°C (mmHg) mp/°C (Recry. solv.)	MS M^+ (m/z)	Formula	Analysis (%) Calcd (Found)		
					C	H	N
3a, b[a]	1740 (CO)	110—111 (0.2)	319	$C_{18}H_{25}NO_4$	67.69	7.89	4.39
					(67.43	7.84	4.30)
4a, b[a]	3335 (NH) 1732 (CO)	116—117 (0.15)	263	$C_{14}H_{17}NO_4$	63.86	6.51	5.32
					(63.60	6.53	5.26)
5a	1732 1743 (CO)	82—83 (Hexane)	353	$C_{21}H_{23}NO_4$	71.37	6.56	3.96
					(71.52	6.63	3.90)
5b	1735 1745 (CO)	60—62 (Hexane)	353	$C_{21}H_{23}NO_4$	71.37	6.56	3.96
					(71.39	6.57	3.86)
6a, b[a]	1739 (CO)	142—143 (0.1)	349	$C_{18}H_{23}NO_6$	61.88	6.64	4.01
					(61.66	6.59	3.94)
7a, b[a]	1738 (CO)	104—105 (0.25)	319	$C_{18}H_{25}NO_4$	67.69	7.89	4.39
					(67.60	7.94	4.35)
8a, b[a]	3345 (NH) 1738 (CO)	141—142 (0.15)	263	$C_{14}H_{17}NO_4$	63.86	6.51	5.32
					(63.72	6.40	5.15)
9a, b[a]	1738 (CO)	103—104 (0.25)	261	$C_{16}H_{23}NO_2$	73.53	8.87	5.36
					(73.51	8.87	5.28)
10a, b[a]	3335 (NH) 1728 (CO)	85—86 (0.2)	205	$C_{12}H_{15}NO_2$	70.22	7.37	6.82
					(70.02	7.34	6.81)

a) The physical and spectral data were obtained for the mixture of two stereoisomers in each case.

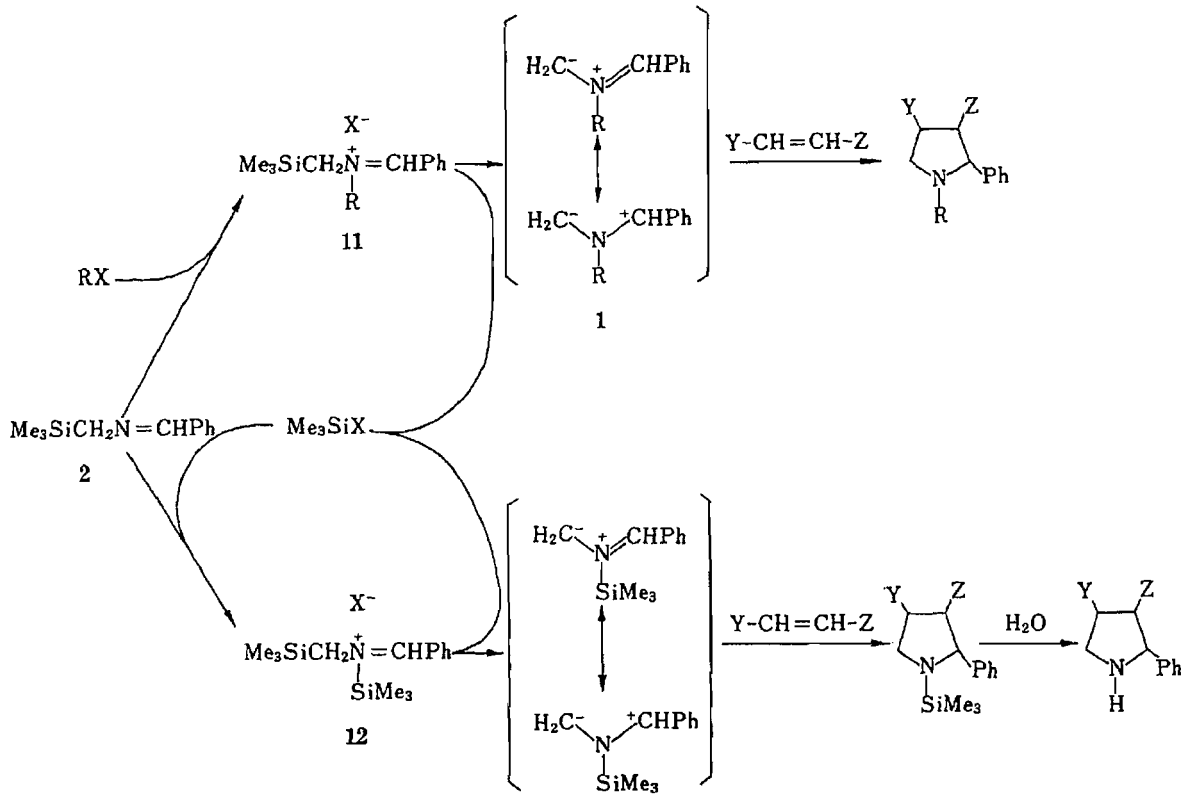


Chart I

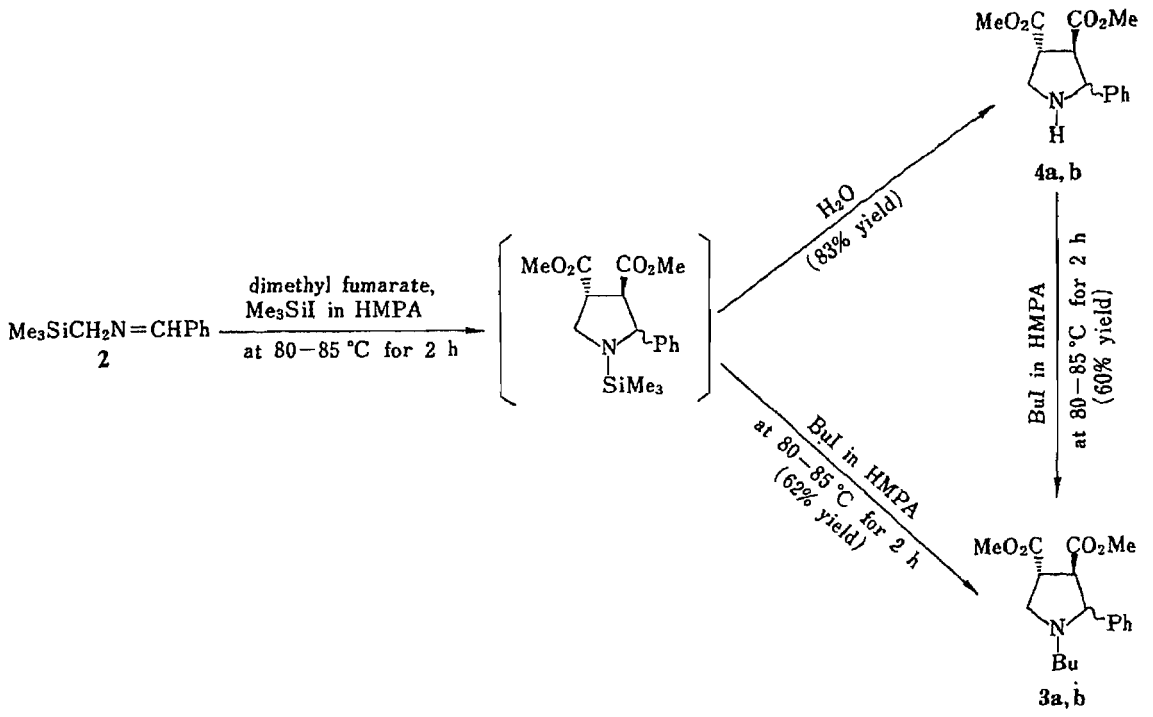


Chart 2

unsubstituted pyrrolidine would proceed *via* the *N*-trimethylsilyliminium salt (**12**) formed from **2** in the presence of trimethylsilyl halide or tosylate, which is also formed from the reaction of **11** (see Chart 1). The findings that a mixture of **4a, b** was obtained by the reaction of **2** with dimethyl fumarate in the presence of a catalytic amount of trimethylsilyl iodide under the same reaction conditions, and that addition of butyl iodide to the above reaction mixture gave *N*-butyl-substituted pyrrolidines (**3a, b**) in a moderate yield (62% combined yield from **2**) indicate the possibility of another reaction pathway in which the replacement of the *N*-trimethylsilyl group by the *N*-butyl group is involved, at least in part, in the course of the formation of **3a, b**, as shown in Chart 2.

The present 1,3-dipolar cycloaddition involving heterolysis of the silicon–carbon bond of the intermediary *N*-alkyltrimethylsilylmethyleneiminium salts (**11**) provides a new method for simple synthesis of *N*-alkylpyrrolidine derivatives (**3**—**10**).

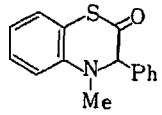
Experimental

All melting and boiling points are uncorrected. Infrared (IR) spectra were measured with a Hitachi EPI-G2 infrared spectrometer. NMR spectra were taken on a JEOL JNM90Q spectrometer (90 MHz) and all chemical shifts are given downfield from tetramethylsilane (TMS). Mass spectral (MS) data were recorded on a Hitachi RMS-4 mass spectrometer.

Solvent Effect on 1,3-Dipolar Cycloaddition of 2 with Dimethyl Fumarate in the Presence of Butyl Iodide——General Procedure: A solution of *N*-(benzylidene)trimethylsilylmethylamine (**2**, 0.96 g, 5 mmol), dimethyl fumarate (0.86 g, 6 mmol), and butyl iodide (1.10 g, 6 mmol) in 25 ml of the solvent indicated in Table I was stirred at 40—45 °C for 30 h under a nitrogen atmosphere. Work-up of the reaction mixture was carried out as follows. In the case of HMPA, DMF or TMU, benzene (100 ml) was added to the reaction mixture and washed with a mixture of sat. aq. NaCl (50 ml) and 10% aq. $KHCO_3$ (25 ml), and then sat. aq. NaCl (75 ml). On the other hand, in the case of acetonitrile or THF, the solvent was evaporated off and the residue was treated with benzene (100 ml) and 10% aq. $KHCO_3$ (50 ml).

The water layer was extracted with benzene (25 ml). The benzene extracts were combined, dried over $MgSO_4$ and filtered. The filtrate was transferred into a 250 ml volumetric flask and 3-phenyl-4-methyl-2,3-dihydro-1,4-

benzothiazine-2-one (**13**)[5] weighed exactly as a standard material in *ca.* 125 ml of benzene was then added to the flask and made up to 250 ml. The resulting benzene solution was submitted to gas liquid chromatographic (GLC) analysis (10% SE-30 on Chromosorb-W, 1 m). The solvent efficiency in this reaction was assessed in terms of the conversion percentage, which was calculated in comparison with the standard material (**13**), and the product yields were obtained by GLC analysis. These data are listed in Table I



13

Relative Reactivities of Butyl Halides and Tosylate on 1,3-Dipolar Cycloaddition of 2 with Dimethyl Fumarate——General Procedure: A solution of the Schiff base **2** (0.96 g, 5 mmol), dimethyl fumarate (0.86 g, 6 mmol), and butyl halide or tosylate (6 mmol) in 25 ml of HMPA was stirred at 40—45 °C for 30 h under a nitrogen atmosphere. The treatment of the reaction mixture and the calculation of the product yields were carried out in the same manner as above. The experimental data are listed in Table II.

The Pyrrolidines (3—10)——General Procedure: A solution of **2** (0.96 g, 5 mmol), a dipolarophile (6 mmol), and an alkyl halide (5 mmol) in 20 ml of HMPA was stirred at 80—85 °C for 2 h under a nitrogen atmosphere. The reaction mixture was diluted with benzene (100 ml) and washed with a mixture of sat. aq. NaCl (50 ml) and 10% aq. $KHCO_3$ (25 ml), and then with sat. aq. NaCl (50 ml), and dried over $MgSO_4$. After removal of the benzene, the residual oil was subjected to SGCC with the following eluent. In entries 1 through 8, the eluents employed were benzene, benzene–ethyl acetate (5 : 1), benzene–iso-Pr_2O (9 : 1), benzene–iso-Pr_2O (9 : 1), benzene, benzene–ethyl acetate (4 : 1), benzene, and benzene–ethyl acetate (1 : 2), respectively. The samples for elemental analysis were obtained by distillation except in the cases of **5a** and **5b** (entry 3). These compounds, **5a** and **5b**, were subjected to elemental analysis after recrystallization from hexane.

The ratios of the isomers, shown in Table III, were calculated on the basis of GLC or ^{1}H-NMR spectra of the crude products. The spectral and physical data are collected in Tables IV and V.

***N*-Butylation of the *N*-Unsubstituted Pyrrolidines (4a, b)**——A solution of the above mixture of **4a** and **4b** (0.39 g, 1.48 mmol) and butyl iodide (0.33 g, 1.78 mmol) in 10 ml of HMPA was stirred at 80—85 °C for 2 h. The reaction mixture was diluted with benzene (50 ml) and washed with a mixture of sat. aq. NaCl (25 ml) and 10% aq. $KHCO_3$ (12 ml), and then sat. aq. NaCl (25 ml), and dried over $MgSO_4$. After removal of the benzene, the residue was submitted to SGCC using benzene–iso-Pr_2O (4 : 1) as an eluent to give a mixture of **3a** and **3b**, in 60% combined yield.

Reaction of 2 with Dimethyl Fumarate in the Presence of Trimethylsilyl Iodide——A 1 mol/l solution of trimethylsilyl iodide in dichloromethane (0.25 mmol) was added at room temperature to a solution of **2** (0.96 g, 5 mmol) and dimethyl fumarate (0.86 g, 6 mmol) in HMPA (20 ml). The whole was stirred at 80—85 °C for 2 h. The reaction mixture was treated as described for the preparation of the pyrrolidines (**3—10**). The residual oil was submitted to SGCC using benzene–ethyl acetate (1 : 1) as an eluent to give a mixture of **4a** and **4b** in 83% combined yield.

Reaction of the Intermediary *N*-Trimethylsilylpyrrolidines with Butyl Iodide——A 1 mol/l solution of trimethylsilyl iodide in dichloromethane (0.25 mmol) was added at room temperature to a solution of **2** (0.96 g, 5 mmol) and dimethyl fumarate (0.86 g, 6 mmol) in HMPA (20 ml). The whole was stirred at 80—85 °C for 2 h, then butyl iodide (0.92 g, 5 mmol) was added at room temperature and the reaction mixture was heated at 80—85 °C with stirring for 2 h. The reaction mixture was treated as described for the preparation of the pyrrolidines (**3—10**). The residual oil was submitted to SGCC using benzene–iso-Pr_2O (4 : 1) as an eluent to give a mixture of **3a** and **3b** in 62% combined yield.

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Tannins and Related Compounds. LV.[1] Isolation and Characterization of Acutissimins A and B, Novel Tannins from *Quercus* and *Castanea* Species

KANJI ISHIMARU, GEN-ICHIRO NONAKA, and ITSUO NISHIOKA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

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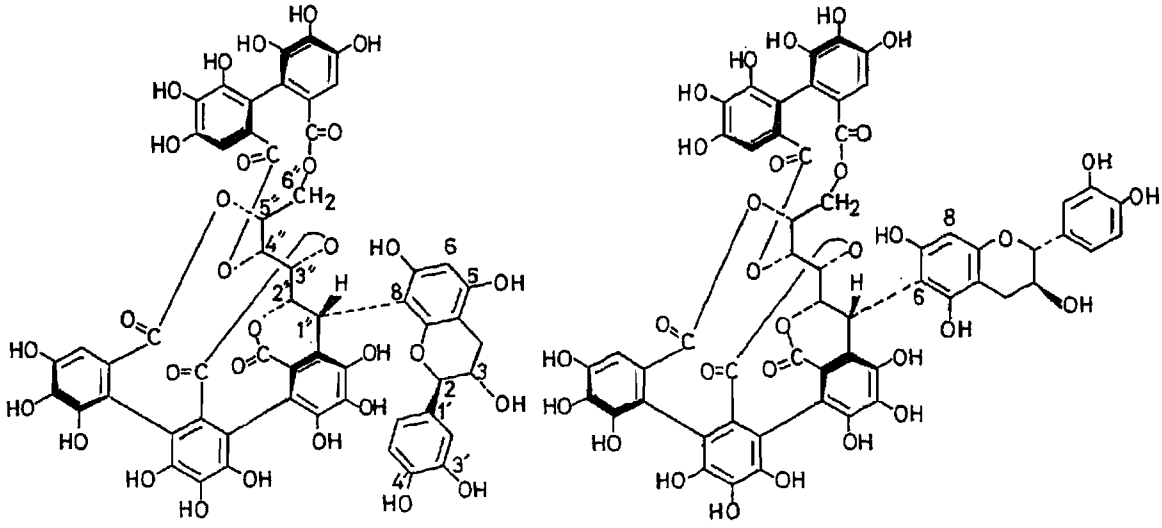
Two novel and closely related tannins, designated as acutissimins A (**1**) and B (**2**), have been isolated from various Fagaceous plants: *Quercus acutissima*, *Q. miyagii*, *Q. stenophylla*, *Q. mongolica* var. *grosseserrata* and *Castanea crenata*. Degradative and synthetic studies combined with ^{1}H- and ^{13}C-NMR spectrometry and various mass spectral measurements have permitted the assignments of the structures, in which (+)-catechin (**4**) and the *C*-glycosylated ellagitannin, castalagin (**3**), are connected through a carbon–carbon linkage.

Keywords——*Quercus* sp.; *Castanea crenata*; Fagaceae; acutissimin A; acutissimin B; tannin; flavan-3-ol; *C*-glycosylated ellagitannin

In previous papers, we demonstrated that the bark of *Quercus stenophylla* MAKINO (Fagaceae) produces a homologous series of unusual tannins (stenophyllanins A, B and C[2] and stenophynins A and B[3]) in which a flavan-3-ol (catechin) unit, one of the component units of condensed tannins, is connected through a carbon–carbon linkage to a hydrolyzable tannin. Taking the structural features into account, these tannins can not be classified into either condensed or hydrolyzable tannins, and we therefore believe that the recognition of these tannins as a new class is an interesting extension of the phytochemistry of tannins. In a continuing systematic chemical examination of the polyphenolic constituents in various Fagaceous plants, we have now isolated two new tannins, acutissimins A (**1**) and B (**2**), which are structurally related to the above compounds, from *Quercus acutissima* CARRUTH. (Japanese name: Kunugi), *Q. miyagii* KOIDZ. (Okinawa-urajirogashi), *Q. stenophylla* MAKINO (Urajirogashi), *Q. mongolica* FISCHER var. *grosseserrata* (BL.) REHD. *et* WILS. (Mizunara) and *Castanea crenata* SIEB. *et* ZUCC. (Kuri), and in this paper we present a detailed account of the structural determination of these compounds.

Typical procedures for the isolation of acutissimins A (**1**) and B (**2**) from each plant material are as follows. Initially, the aqueous acetone extract was subjected to Sephadex LH-20 chromatography. Stepwise elution with water containing increasing amounts of methanol effected fairly good fractionation. Earlier fractions contained simple phenolic glycosides and lower-molecular-weight tannins, while acutissimins were less mobile with this solvent system and were almost invariably eluted in the final fractions. The acutissimin-containing fractions were subsequently chromatographed with a mixture of methanol–water on reversed-phase gels such as MCI-gel CHP-20P, Bondapak C_{18} Porasil B and/or Fuji-gel ODS-G3 to yield pure samples.

Acutissimin A (**1**), obtained as an off-white amorphous powder, $[\alpha]_D$ −74.0° (acetone), gave a reddish pink coloration on treatment with the anisaldehyde–sulfuric acid reagent,[4] suggesting the presence of a flavan-3-ol framework in the molecule, while a brown coloration



1 2

Chart 1

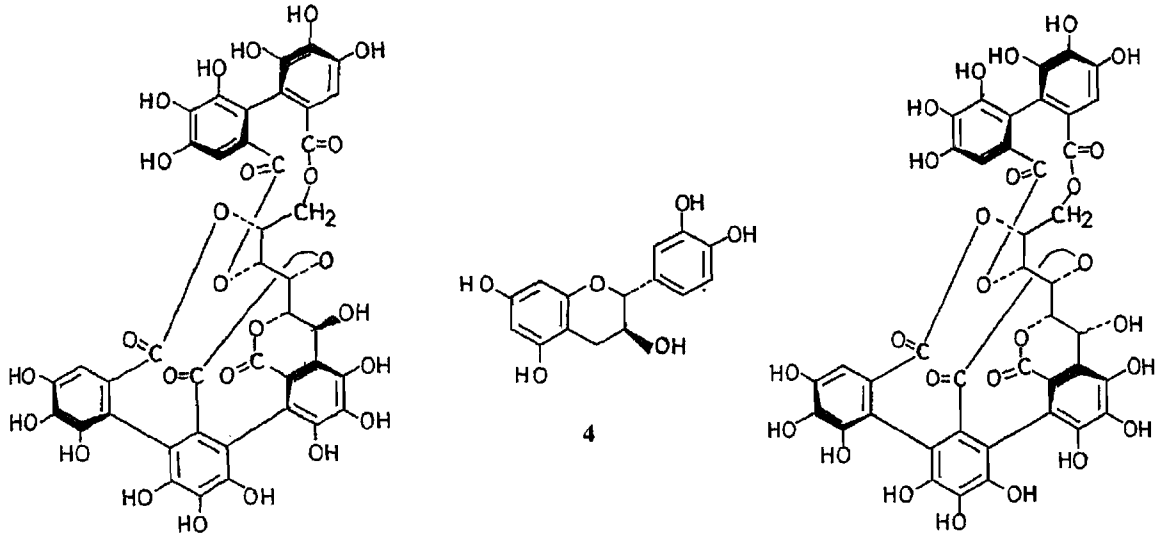
TABLE I. ^{1}H-NMR Data for Acutissimins A (1) and B (2) and the Hydrolysate (10)[a]

	1	2	10
Catechin moiety			
2-H	5.48 (br s)	4.58 (d, J=8)	4.82 (d, J=8)
3-H	4.56 (m)	—	—[b]
4-H	2.40 (br d, J=16)	2.3—3.0 (m)	2.50—2.84 (m)
	2.90 (br d, J=16)		
6-H	6.32 (s)	—	6.03 (s)
8-H	—	6.10 (s)	—
2′-H	6.90 (br s)	6.96 (br s)	6.82 (br s)
5′-H	6.76 (d, J=8)	6.76 (d, J=8)	6.78 (d, J=8)
6′-H	6.94 (br d, J=8)	6.88 (br d, J=8)	6.70 (br d, J=8)
Polyalcohol moiety			
1″-H	4.84 (s)	4.72 (s)	4.60 (s)
2″-H	5.20 (s)	5.12 (s)	5.33 (s)
3″-H	4.76 (d, J=8)	4.87 (d, J=8)	4.64 (d, J=8)
4″-H	5.28 (t, J=8)	5.28 (t, J=8)	—
5″-H	5.60 (d, J=8)	5.68 (d, J=8)	5.16 (m)
6″-H	4.12 (d, J=12)	3.98 (d, J=12)	—[b]
	4.60 (d, J=12)	—[b]	
Biphenoyl H	6.56 (s)	6.64 (s)	—
	6.76 (s)	6.80 (s)	
Triphenoyl H	7.08 (s)	7.10 (s)	6.70 (s)

a) Measured at 100 MHz in acetone-d_6+D_2O; J-values are expressed in Hz. *b*) Overlapped with an HOD or H_2O signal which lies in the range of δ 4.0—4.5.

in the nitrous acid test[5] was consistent with an ellagitannin. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed three one-proton aromatic singlets at δ 6.56, 6.76 and 7.08, two of which were attributable to the protons of a hexahydroxydiphenoyl ester group. Other aromatic resonances appeared as an ABX-type signal at δ 6.76 (d, J=8 Hz), 6.90 (br s) and 6.94 (br d, J=8 Hz), and as a high-field singlet at δ 6.32, the chemical shifts suggesting the presence of catechol and phloroglucinol rings, respectively. In the aliphatic proton region, the

appearance of a pair of broad doublets at δ 2.40 (J = 16 Hz) and 2.90 (J = 16 Hz) assignable to the C-4 benzylic methylene was characteristic of a flavan-3-ol derivative. In addition, the pattern of the low-field signals due to a polyalcohol methylene and methines bearing acyl groups was closely analogous to that found in a *C*-glycosylated ellagitannin, castalagin (**3**).[6]



3 4 5

Chart 2

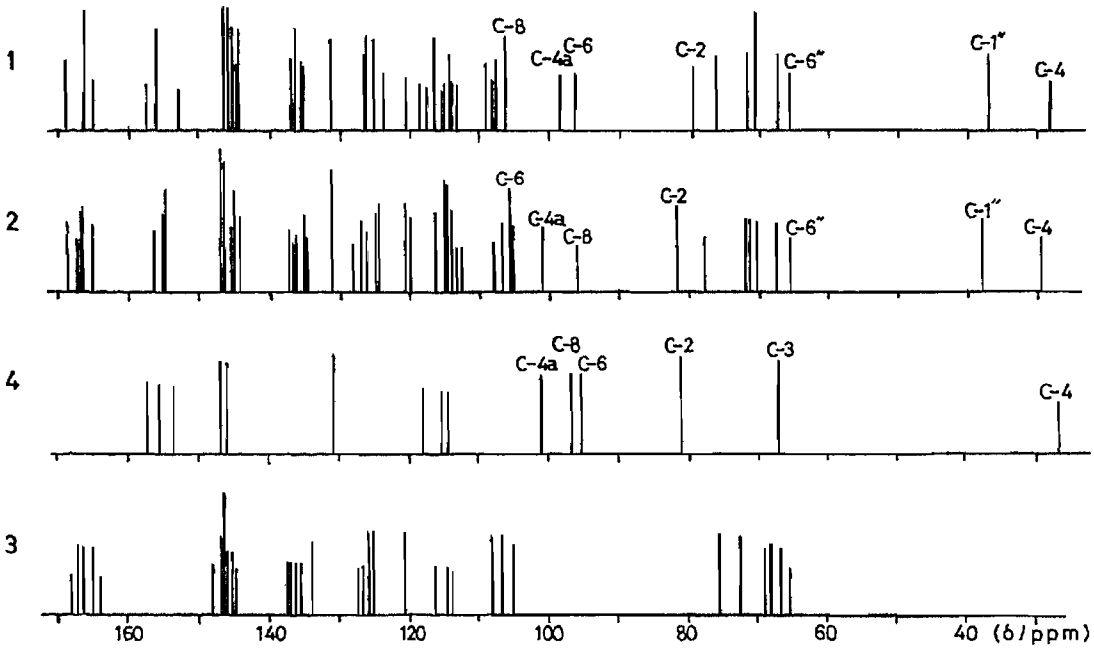


Fig. 1. ^{13}C-NMR Spectra of Acutissimins A (**1**) and B (**2**), (+)-Catechin (**4**) and Castalagin (**3**) (in Acetone-d_6 + D_2O)

These ^{1}H-NMR observations suggested that a 5,7,3′,4′-tetrahydroxy-flavan-3-ol is attached to a *C*-glycosylated ellagitannin at the C-6 or C-8 position. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) data were in good agreement with these observations, but were more informative. Almost the same chemical shifts for flavan C-ring carbons as those in (+)-

catechin (**4**) (Fig. 1) implied the 2,3-*trans* configuration of the flavan unit,[7] while the difference in the chemical shifts of the A-ring carbons, especially in the low-field shift (δ 106.9) of the C-6 or C-8 carbon, clearly indicated the location of a substituent at the A-ring. Other signals, except those arising from polyalcohol carbons, were almost in line with those of **3** plus **4** (Fig. 1). These observations coupled with the significant upfield shift (δ 37.9) of the polyalcohol C-1 atom as compared with that in **3** suggested that acutissimin A (**1**) is a condensation product of **3** and **4**. This was supported by the appearance of the $[M+H]^+$ peak at *m/z* 1207 in the fast atom bombardment mass spectrum (FAB-MS) of **1**.

On treatment with mineral acids, **1** yielded a complex mixture of degradation products, and no information about the structure was obtained. However, oxidative degradation of **1** with 10% ferric chloride afforded glucose and arabinose, thus confirming that the polyalcohol carbons, except for the C-1 atom, possess the same configuration as those of glucose. On the other hand, refluxing of **1** in ethanol containing acetic acid (20%) for a long period,[2] followed by repeated chromatography over Sephadex LH-20, yielded, among many uncharacterized products, a crystalline compound, mp 143 °C, $[\alpha]_D$ +16.7° (acetone), which was shown to be identical with (+)-catechin (**4**).

The ^{1}H-NMR spectrum of **1** showed a broad singlet at δ 4.84 due to the polyalcohol C-1 proton, the coupling pattern suggesting that the dihedral angle between the C-1 and C-2 protons is close to 90°. Inspection of a Dreiding model revealed that when the C-1 proton occupied the β-position, the dihedral angle was about 85° (Fig. 2), which is consistent with the ^{1}H-NMR data. Furthermore, in a comparison of the ^{1}H-NMR coupling constant of the C-1 proton in **1** with those in **3** and its C-1 epimer, vescalagin (**5**),[6] the *J*-value of **1** was similar to that (J = 1 Hz) of **5** rather than that (J = 5 Hz) of **3**, thus indicating that the C-1 atom has the same configuration as that of **5**.

The differentiation of C-6 and C-8 substituted catechin derivatives by ^{13}C-NMR spectroscopy of their methyl ethers has been described repeatedly.[2,8] To determine the point of the linkage between the catechin and *C*-glycosylated ellagitannin moieties, this method was applied. Methylation of **1** with dimethyl sulfate and anhydrous potassium carbonate in dry acetone furnished the nonadecamethyl ether (**1a**), the field-desorption mass spectrum (FD-MS) of which exhibited a prominent M^+ peak at *m/z* 1472, consistent with the proposed structure. The ^{13}C-NMR spectrum of **1a** aided by an off-resonance technique showed an unsubstituted A-ring carbon signal at δ 89.5 (d), the chemical shift being in good agreement with those of C-8 substituted catechin derivatives[8] [*e.g.*, gambiriin A_1 nonamethyl ether (**6**)[9]: δ 88.6] rather than those of the C-6 substituted alternatives [*e.g.*, gambiriin A_3 nonamethyl ether (**7**)[9]: δ 96.1] (Fig. 3). Based on these observations, the ellagitannin was concluded to be connected through a carbon–carbon linkage to the C-8 position of the catechin nucleus.

The chiralities of the hexahydroxydiphenoyl and nonahydroxytriphenoyl ester groups were determined as follows. Alkaline methanolysis of the methyl ether (**1a**) with sodium methoxide in methanol yielded optically active dimethyl hexamethoxydiphenoate (**8**) ($[\alpha]_D$ −31.6° ($CHCl_3$)), together with the methanolysate (**9**) [FD-MS *m/z* 1118 $(M)^+$]. The sign of

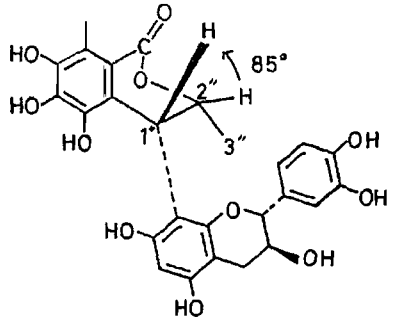
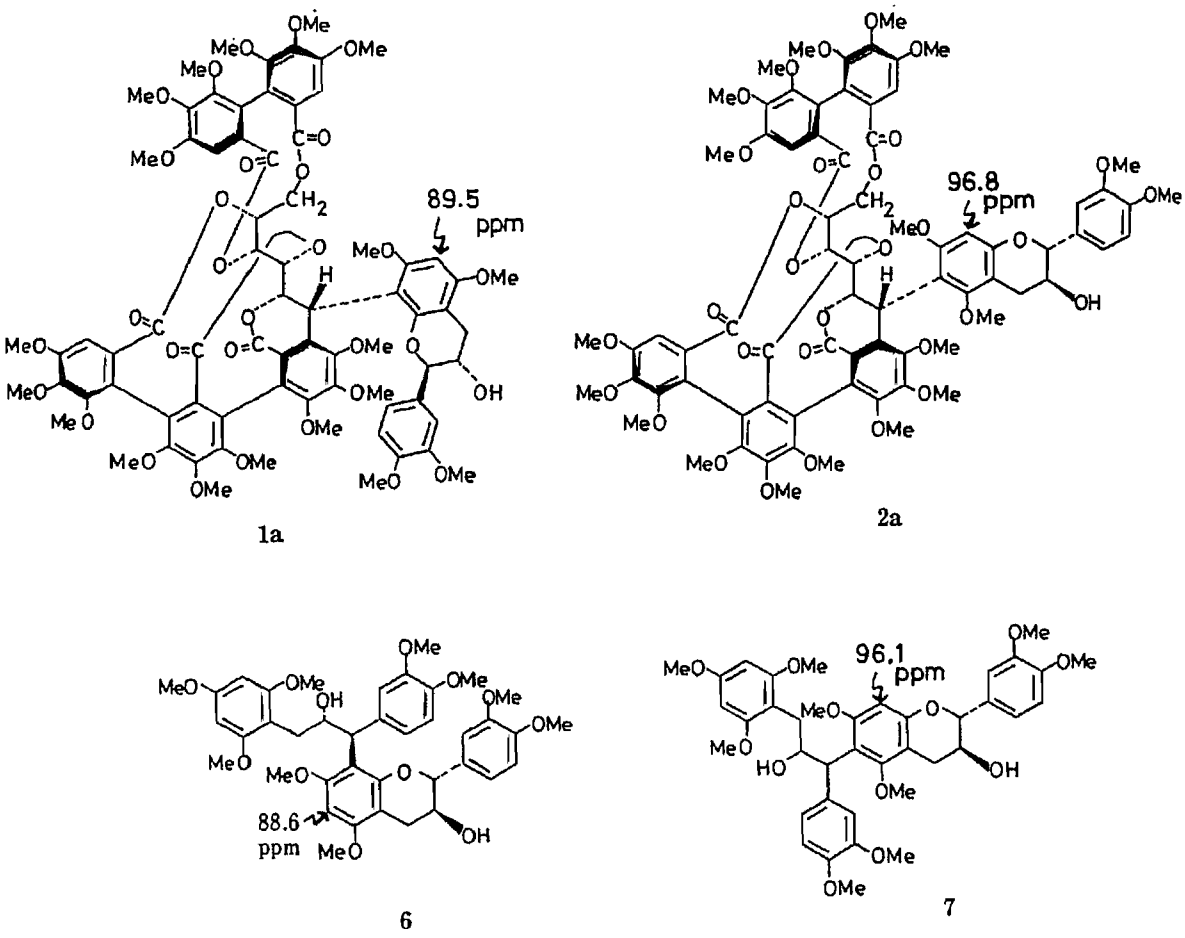
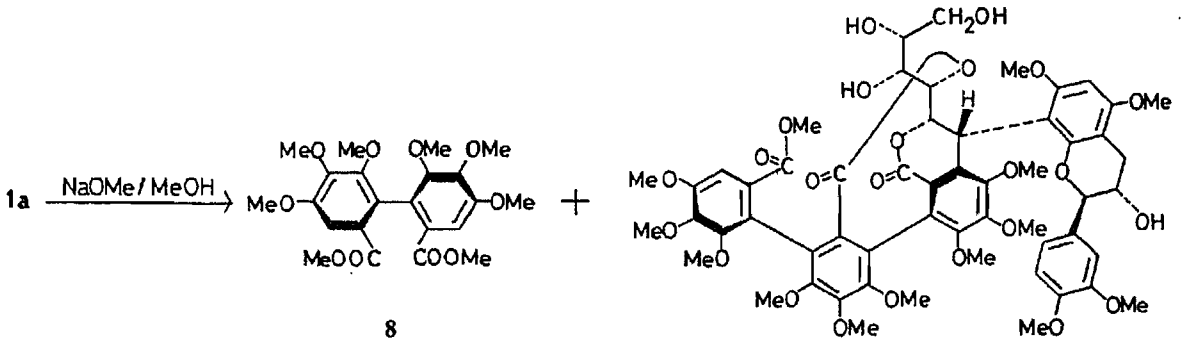


Fig. 2. Configuration at C-1″ in Acutissimin A (**1**)



1a 2a 6 7

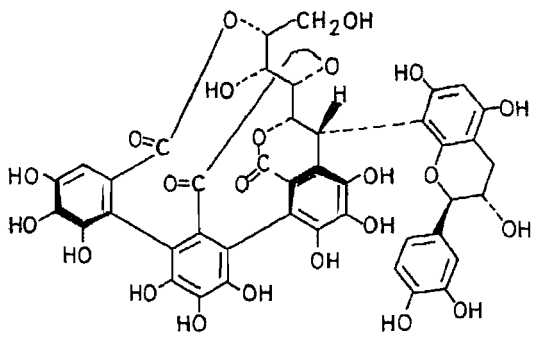
Fig. 3



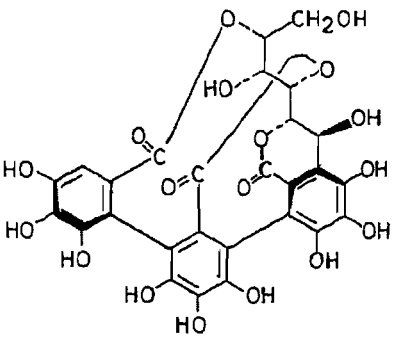
8 9

Chart 3

the specific optical rotation of **8** thus established unequivocally the atropisomerism to be in the *S*-series.[10] On the other hand, upon enzymatic hydrolysis with tannase, **1** liberated ellagic acid and an amorphous compound (**10**), whose molecular mass [*m/z* 903 $(M-H)^-$ in the negative FAB-MS] confirmed its deshexahydroxydiphenoyl structure. The circular dichroism (CD) spectrum (Fig. 4) of **10** showed an intense positive Cotton effect at 237 nm and a negative one at 263 nm, both corresponding well to those found in castalin (**11**), whose triphenoyl ester moiety had been established to possess the *S*,*S*-configuration.[6b] Thus, the



10



11

Chart 4

atropisomerism in the triphenoyl group was concluded to be in the *S,S*-series.

Unequivocal structural assignment of **1** was successfully achieved by condensation of **3** and **4**. Refluxing of the mixture in dry acetone containing *p*-toluenesulfonic acid, followed by repeated chromatography over Sephadex LH-20 with ethanol and 60% aqueous methanol, afforded, together with a large amount of unreacted **3**, a condensation product, which was found to be identical with **1**.

Acutissimin B (**2**), obtained as an off-white amorphous powder, $[\alpha]_D$ $-5.5°$ (acetone), showed chromatographic properties and color reactions similar to those of **1**. The FAB-MS with the $[M+H]^+$ peak at *m/z* 1207 indicated the same molecular mass as that of **1**. The ^{1}H- and ^{13}C-NMR spectra were also closely correlated with those of **1** (Table I and Fig. 1): the almost identical signal patterns in the aromatic fields showed that similar functional groups exist in the molecule, while the chemical shifts for the polyalcohol carbons confirmed the presence of the same substitution system in the polyalcohol moiety. The ^{1}H-NMR coupling patterns of the flavan C-2 and C-4 protons clearly indicated the presence of a catechin moiety. In addition, the appearance of a singlet at δ 4.72 due to the polyalcohol C-1 proton confirmed the configuration of the C-1 atom to be the same as that of **1**.

On methylation in the same way as described for **1**, **2** formed the nonadecamethyl ether (**2a**). Subsequent alkaline methanolysis of **2a** with sodium methoxide in methanol yielded a methanolysate (**12**) and (−)-dimethyl hexamethoxydiphenoate (**8**) ($[\alpha]_D$ $-31.0°$ ($CHCl_3$)), thus establishing unambiguously the atropisomerism of the hexahydroxydiphenoyl ester group to be in the *S*-series. The spectral data of **12** including FD-MS [*m/z* 1118 $(M)^+$] were

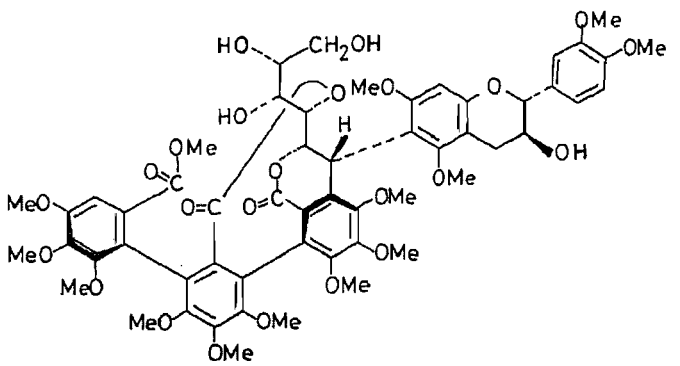


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Chart 5

consistent with its deshexahydroxydiphenoyl structure (similar to that of **9**).

The ^{13}C-NMR spectrum of **2a** aided by an off-resonance experiment showed an

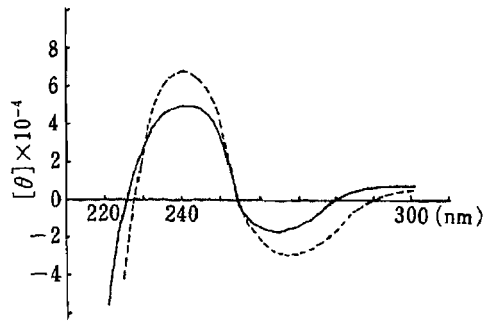

Fig. 4. CD Spectra of **10** and **11** (in MeOH)
——, **10**; -----, **11**.

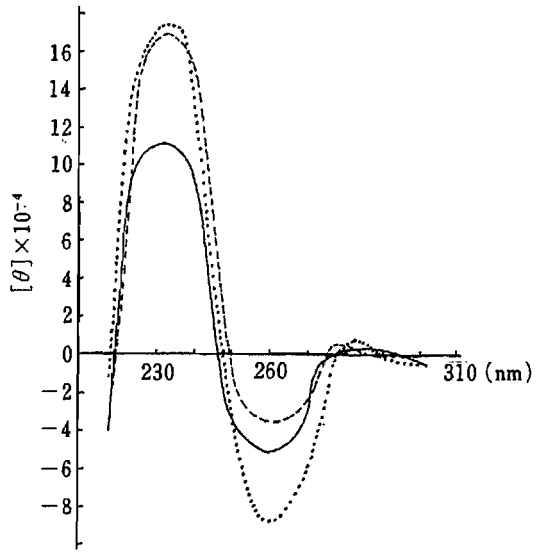

Fig. 5. CD Spectra of **1**, **2** and **3** (in MeOH)
——, **1**; - - - -, **2**; -----, **3**.

unsubstituted flavan A-ring singlet at δ 96.8, the chemical shift being in good agreement with that (δ 96.1) of the C-6 substituted catechin derivative, gambiriin A_3 nonamethyl ether (**7**).[9] Furthermore, the ^{1}H-NMR chemical shift for the flavan C-2 proton supported the C-6 substitution in the catechin moiety[11]: the chemical shift (δ 4.58) for the C-2 proton was almost identical with that (δ 4.54) found in (+)-catechin (**4**), whereas in the case of **1**, the C-2 proton signal appeared considerably downfield (δ 5.48), indicating that in **2** there is no magnetic through-space interaction between the C-2 proton and the substituent, and that the substituent is therefore located at the remote C-6 position.

The atropisomerism of the triphenoyl ester group was determined to be in the *S*,*S*-series from the close similarities of the CD spectra of **2** and **1** (Fig. 5). The stronger intensities of the Cotton effects at 230—235 and 260 nm in **2** and **1** than those observed in **10** and **11** also supported the view that all the biphenyl and triphenyl chiralities are in the *S*-series.

From the chemical and spectral data described above, the whole structure was concluded to be represented by the formula **2**.

Several Fagaceous plants contain both hydrolyzable and condensed tannins, though their contents and compositions differ remarkably among the species and also even in different parts of the plants. Considering that acutissimins A (**1**) and B (**2**) occur almost invariably in association with (+)-catechin (**4**) and castalagin (**3**), they are likely to be biosynthesized by condensation of these compounds.

Experimental

The following instruments were used to obtain physical and spectral data. A Yanagimoto micro-melting point apparatus (melting points), a JASCO DIP-4 digital polarimeter (optical rotations), a Hitachi 100-50 type spectrophotometer, JEOL D-300 and DX-300 spectrometers (FD- and FAB-MS), JEOL PS-100 and FX-100 spectrometers [^{1}H (100 MHz)- and ^{13}C (25.05 MHz)-NMR spectra], and a JASCO J-20 apparatus (CD spectra). Column chromatography was performed using Sephadex LH-20 (25—100 μm, Pharmacia Fine Chemicals), MCI-gel CHP-20P (75—150 μm, Mitsubishi Chemical Industries Ltd.), Bondapak C_{18} Porasil B (Waters Associates), Fuji-gel ODS-G3 (43—65 μm, Fuji-gel Hanbai Co., Ltd.), and Kieselgel 60 (70—230 mesh, Merck). Thin-layer chromatography (TLC) was conducted on precoated Kieselgel 60 F_{254} plates (0.20 mm, Merck) and precoated cellulose F_{254} plates (0.10 mm, Merck), and spots were detected by their blue fluorescence under ultraviolet (UV) light and with

ferric chloride, anisaldehyde–sulfuric acid or 10% sulfuric acid reagent spray. Analytical gas-liquid chromatography (GLC) for sugars was carried out over 1.5% SE-30 (2 m × 4 mm) with nitrogen as the carrier gas.

Isolation of Acutissimins A (1) and B (2)——Due to significant differences in the polyphenolic constituents from plant to plant, general procedures for the isolation of acutissimins were not available, and typical isolation procedures (from *Quercus acutissima* CARRUTH.) are described herein.

The fresh bark (6.1 kg) of *Q. acutissima* was chopped into small pieces and extracted at room temperature with acetone–water (4 : 1). Concentration of the extract under reduced pressure afforded an aqueous solution, which deposited a resinous precipitate. After filtration, the filtrate was subjected to Sephadex LH-20 chromatography. Elution with water containing increasing proportions of methanol and finally with water–acetone (1 : 1) furnished three fractions. Fraction (fr.) I contained relatively lower-molecular-weight polyphenols. Fraction II was chromatographed over Sephadex LH-20 with a solvent system of ethanol–water–acetone[12] to yield five further fractions; frs. II-1 (5.5 g), II-2 (5.1 g), II-3 (0.3 g), II-4 (7.3 g) and II-5 (32 g). The final fraction II-5 was repeatedly chromatographed over reversed-phase gels; MCI-gel CHP-20P, Bondapak C_{18} Porasil B and Fuji-gel, with water containing increasing amounts of methanol, to yield acutissimin A (1) (2.0 g). Similarly, fraction III was subjected to rechromatography over Sephadex LH-20 in water containing increasing amounts of methanol to afford four fractions; frs. III-1 (5 g), III-2 (8 g), III-3 (12 g) and III-4 (2.4 g). Repeated chromatography of fraction III-1 over the above reversed gels gave acutissimin B (2) (0.48 g).

Acutissimin A (1)——An off-white amorphous powder, $[\alpha]_D^{32}$ −74.0° (c=1.2, acetone). *Anal.* Calcd for $C_{56}H_{38}O_{31}\cdot 5H_2O$: C, 51.85; H, 3.73. Found: C, 51.84; H, 4.13. FAB-MS m/z: 1207 $[M]^+$. UV λ_{max}^{MeOH} nm (log ε): 275 sh (4.93), 225 sh (4.46). ^{13}C-NMR (acetone-d_6) ppm: 23.8 (C-4), 37.9 (C-1″), 65.7 (C-6″), 67.6 (C-3), 71.0 (×2), 72.2, 77.6 (C-2″, C-3″, C-4″ and C-5″), 80.0 (C-2), 96.8 (C-6), 98.3 (C-4a), 106.9 (C-8), 107.1, 107.4, 108.9 (unsubstituted biphenoyl and triphenoyl C), 131.5 (C-1′), 152.9, 155.9, 157.1 (C-5, C-7 and C-8a), 165.9, 167.3 (×3), 169.4 (–COO–). CD (c=1.6×10^{-5}, MeOH) [θ] (nm): −5.29×10^4 (260), 0 (247), +1.13×10^5 (233).

Oxidative Degradation of 1——A solution of 1 (14 mg) in 10% ferric chloride (2 ml) was heated under reflux for 5 d. The dark blue reaction mixture was neutralized with Amberlite MB-3 resins and the solvent was evaporated off under reduced pressure. The residue was passed through a Sep-pak (ODS) column, and the eluent was concentrated to dryness to yield a colorless syrup, which was treated with *N*-trimethylsilylimidazole. The trimethylsilyl derivatives were analyzed by GLC (flow rate: 40 ml/min), and peaks corresponding to glucose [t_R: 14.7 and 23.6 min (column temp.: 150 °C)] and arabinose [t_R: 7.6 and 8.8 min (column temp.: 130 °C)] were confirmed.

Acid-Catalyzed Degradation of 1——A solution of 1 (0.11 g) in ethanol (6 ml) containing acetic acid (1.5 ml) was heated under reflux for 5 d. The solvent was evaporated off under reduced pressure, and the residue was repeatedly chromatographed over Sephadex LH-20 with ethanol to yield (+)-catechin (**4**) as colorless needles (1.2 mg), mp 143 °C, $[\alpha]_D^{29}$ +16.7° (c=0.12, acetone).

Methylation of 1——A mixture of 1 (0.3 g), dimethyl sulfate (2 ml) and anhydrous potassium carbonate (2.5 g) in dry acetone was heated under reflux for 3 h. After removal of the inorganic salts by filtration, the filtrate was concentrated under reduced pressure, and subjected to silica gel chromatography. Stepwise elution with benzene containing increasing proportions of acetone furnished the nonadecamethyl ether (**1a**) as a white amorphous powder (0.19 g), $[\alpha]_D^{29}$ −140.0° (c=0.54, $CHCl_3$). *Anal.* Calcd for $C_{75}H_{76}O_{31}\cdot 1/2H_2O$: C, 60.76; H, 5.24. Found: C, 60.69; H, 5.35. FD-MS m/z: 1472 $(M)^+$. ^{1}H-NMR[13] ($CDCl_3$) ppm: 5.70 (1H, d, J=8 Hz, 5″-H), 6.26 (1H, s, 6-H), 6.60—6.92 (6H, m, aromatic H). ^{13}C-NMR[13] ($CDCl_3$) ppm: 27.7 (C-4), 38.0 (C-1″), 65.6 (C-6″), 68.0 (C-3), 69.4, 70.1, 71.4, 76.4 (C-2″, C-3″, C-4″ and C-5″), 82.4 (C-2), 89.5 (C-6).

Alkaline Methanolysis of 1a——A solution of **1a** (0.13 g) in methanol (4 ml) was treated with sodium methoxide (0.25 g) in methanol (12 ml) at room temperature for 3 d. After neutralization with Amberlite IR-120B (H^+ form) resins, the reaction products were separated by silica gel chromatography with benzene–acetone (4 : 1—1 : 1) to give *S*-dimethyl hexamethoxydiphenoate (**8**) as a colorless syrup, $[\alpha]_D^{29}$ −31.6° (c=0.55, $CHCl_3$), and the methanolysate (**9**). **9**: A white amorphous powder, $[\alpha]_D^{29}$ −32.7° (c=0.55, $CHCl_3$). *Anal.* Calcd for $C_{56}H_{62}O_{24}\cdot 1/2H_2O$: C, 59.62; H, 5.63. Found: C, 59.62; H, 5.70. FD-MS m/z: 1118 $(M)^+$. ^{1}H-NMR ($CDCl_3$) ppm: 2.20—3.04 (2H, m, 4-H), 4.48 (1H, brs, 1″-H), 4.86 (1H, d, J=8 Hz, 2-H), 6.04 (1H, s, 6-H), 6.68—7.16 (4H, m, aromatic H). ^{13}C-NMR ($CDCl_3$) ppm: 26.8 (C-4), 37.5 (C-1″), 63.6 (C-6″), 67.3 (C-3), 70.6, 74.4, 77.0, 79.3 (C-2″, C-3″, C-4″ and C-5″), 81.6 (C-2), 89.0 (C-6), 102.1 (C-4a), 106.2 (C-8), 165.2, 170.4, 170.5 (–COO–).

Tannase Hydrolysis of 1——A solution of **1** (0.13 g) in water was incubated overnight with tannase at 37 °C. The solvent was evaporated off under reduced pressure, and the residue was treated with ethanol. The ethanol-soluble portion was subjected to chromatography over Sephadex LH-20 with water containing increasing amounts of methanol and then over Fuji-gel with water–methanol (7 : 3) to yield ellagic acid, the hydrolysate (**10**) (8 mg) and the starting material (**1**) (55 mg). **10**: An off-white amorphous powder, $[\alpha]_D^{29}$ −7.3° (c=0.9, MeOH). *Anal.* Calcd for $C_{42}H_{32}O_{23}\cdot 11/2H_2O$: C, 50.25; H, 4.31. Found: C, 50.10; H, 4.06. Negative FAB-MS m/z: 903 $(M-H)^-$ UV λ_{max}^{MeOH} nm (log ε): 275 sh (4.89), 227 sh (4.44).

Preparation of 1——A mixture of (+)-catechin (**4**) (2.9 g) and castalagin (**3**) (2.9 g) in dry dioxane (150 ml) containing *p*-toluenesulfonic acid (0.13 g) was heated under reflux for 24 h. The solvent was evaporated off under reduced pressure, and the residue was repeatedly chromatographed over Sephadex LH-20 with 60% aqueous

methanol and ethanol to give a condensation product (0.14 g), which was shown to be identical with acutissimin A (**1**) by $[\alpha]_D$ and ^{1}H- and ^{13}C-NMR spectral comparisons.

Acutissimin B (2)——An off-white amorphous powder, $[\alpha]_D^{32}$ −5.5° (c=0.84, acetone). *Anal.* Calcd for $C_{56}H_{38}O_{31}\cdot 7/2H_2O$: C, 52.96; H, 3.57. Found: C, 53.23; H, 4.01. FAB-MS *m/z*: 1207 $(M+H)^+$. UV λ_{max}^{MeOH} nm (log ε): 275 sh (4.89), 225 sh (4.40). ^{13}C-NMR (acetone-d_6 + D_2O) ppm: 29.4 (C-4), 38.0 (C-1″), 65.8 (C-6″), 70.2, 71.3, 71.8, 78.2 (C-2″, C-3″, C-4″ and C-5″), 82.2 (C-2), 96.2 (C-8), 101.2 (C-4a), 107.5 (C-6), 107.2, 108.8, 110.0 (unsubstituted biphenoyl and triphenoyl C), 115.5, 115.9 (C-2′ and C-5′), 120.3 (C-6′), 131.4 (C-1′), 154.9 (×2), 156.2 (C-5, C-7 and C-8a), 165.9, 167.1, 167.4, 167.7, 169.4 (–COO–). CD ($c=1.5\times10^{-5}$, MeOH) [θ] (nm): -5.58×10^4 (260), 0 (251), $+1.74\times10^5$ (235).

Methylation of 2——A mixture of **2** (0.15 g), dimethyl sulfate (1.5 ml) and anhydrous potassium carbonate (1.5 g) in dry acetone (10 ml) was refluxed for 3 h with stirring. After cooling, the inorganic precipitate was filtered off, and the filtrate was concentrated to dryness under reduced pressure to give a residue, which was purified by silica gel chromatography. Elution with benzene–acetone (2 : 1) yielded the nonadecamethyl ether (**2a**) as a white amorphous powder, $[\alpha]_D^{29}$ −25.4° (c=0.55, $CHCl_3$). *Anal.* Calcd for $C_{75}H_{76}O_{31}\cdot H_2O$: C, 60.39; H, 5.27. Found: C, 60.46; H, 5.39. FD-MS *m/z*: 1472 $(M)^+$. ^{1}H-NMR ($CDCl_3$) ppm: 4.68 (1H, br s, 1″-H), 4.80 (1H, d, J=8 Hz, 2-H), 4.88 (1H, d, J=8 Hz, 3″-H), 5.06 (1H, br s, 2″-H), 5.32 (1H, t, J=8 Hz, 4″-H), 5.72 (1H, d, J=8 Hz, 5″-H), 6.28 (1H, s, 8-H), 6.74, 6.88, 7.16 (each 1H, s, aromatic H), 6.92—7.04 (3H, m, 2′-H, 5′-H and 6′-H). ^{13}C-NMR ($CDCl_3$) ppm: 28.8 (C-4), 37.9 (C-1″), 65.3 (C-6″), 68.2 (C-3), 70.1, 70.3, 71.1, 77.0 (C-2″, C-3″, C-4″ and C-5″), 82.3 (C-2), 96.8 (C-8), 106.1 (C-4a), 104.8, 107.4 (unsubstituted biphenoyl C), 110.0, 110.7, 111.3 (C-2′, C-5′ and unsubstituted triphenoyl C), 113.7 (C-6), 120.3 (C-6′), 155.3, 156.7, 159.0 (C-5, C-7 and C-8a), 163.9, 164.0, 165.0, 166.1, 167.7 (–COO–).

Alkaline Methanolysis of 2a——A solution of **2a** (40 mg) in methanol (8 ml) containing sodium methoxide (0.05 g) was stirred at room temperature for 7 d. The reaction mixture was neutralized with Amberlite IR-120B (H^+ form), and the solvent was evaporated off under reduced pressure. The residue was chromatographed over silica gel with benzene–acetone (4 : 1—1 : 1) to yield *S*-dimethyl hexamethoxydiphenoate (**8**) as a colorless syrup (11 mg), $[\alpha]_D^{24}$ −31.0° (c=0.1, $CHCl_3$), and the methanolysate (**12**) (22 mg). **12**: A white amorphous powder, $[\alpha]_D^{24}$ +52.4° (c=0.41, $CHCl_3$). *Anal.* Calcd for $C_{56}H_{62}O_{24}\cdot 1/2H_2O$: C, 59.62; H, 5.63. Found: C, 59.63; H, 5.94. FD-MS *m/z*: 1118 $(M)^+$. ^{1}H-NMR ($CDCl_3$) ppm: 6.22 (1H, s, 8-H), 6.84—7.04 (3H, m, 2′-H, 5′-H and 6′-H), 7.16 (1H, s, aromatic H).

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Tannins and Related Compounds. LVI.[1] Isolation of Four New Acylated Flavan-3-ols from Oolong Tea. (1)

FUMIO HASHIMOTO, GEN-ICHIRO NONAKA, and ITSUO NISHIOKA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

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A chemical examination of the aqueous acetone extract of commercial oolong tea has led to the isolation of four new acylated flavan-3-ols **1**—**4**, together with the known compounds **5**—**13**. On the basis of chemical and spectroscopic evidence, compounds **1**—**4** have been characterized as (−)-epiafzelechin 3-*O*-gallate (**1**), (−)-epicatechin 3-*O*-(4-*O*-methyl)-gallate (**2**), (−)-epicatechin 3-*O*-*p*-hydroxybenzoate (**3**) and (−)-epigallocatechin 3-*O*-cinnamate (**4**).

Keywords——oolong tea; *Camellia sinensis* var. *assamica*; Theaceae; acylated flavan-3-ol; tea catechin; tannin

In the course of our systematic chemical examination on polyphenolic constituents in various beverage teas, we previously demonstrated the occurrence of hydrolyzable tannins in green tea,[2] and of benzotropolone-type red pigments (theaflagallins) in black tea,[3] together with several proanthocyanidin gallates and a new class of dimeric flavan-3-ol gallates (theasinensins A and B) in fresh green tea leaf.[4] We now wish to report on the isolation and characterization of four new acylated flavan-3-ol derivatives from oolong tea, which is semi-fermented before being dried (the fermentation stage is considered to be intermediate between those in green tea and black tea).

Preliminary examination of several varieties of commercial oolong teas by thin-layer chromatography (TLC) revealed that, among others, the variety (commercial name: Shiraore, 白折) produced in Fukien (福建) province (China) contains relatively large amounts of polyphenols, though the component patterns in these varieties are closely related to each other. We have therefore chosen this commercial sample as a representative for examination of the polyphenolic constituents.

The extraction and isolation procedures are summarized in Chart 1. Among the thirteen phenolic compounds **1**—**13** isolated here, compounds **5**—**13** were found to be identical with (+)-catechin (**5**),[4] (−)-epicatechin (**6**),[4] (−)-epigallocatechin (**7**),[4] (−)-epicatechin 3-*O*-gallate (**8**),[4] (−)-epigallocatechin 3-*O*-gallate (**9**),[4] (−)-epicatechin 3-*O*-(3-*O*-methyl)-gallate (**10**),[4] (−)-epigallocatechin 3-*O*-*p*-coumaroate (**11**),[4] (−)-epigallocatechin 3,3′-di-*O*-gallate (**12**)[4] and (−)-epigallocatechin 3,4′-di-*O*-gallate (**13**).[4]

Compound **1**, an off-white amorphous powder, $[\alpha]_D$ −181.7° (acetone), $C_{22}H_{18}O_9 \cdot H_2O$, was positive to the ferric chloride and anisaldehyde–sulfuric acid reagents (blue and orange colorations, respectively), suggesting the presence of a pyrogallol moiety and a flavan-3-ol nucleus. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum (Table I) showed the presence of a flavan skeleton with a 3,5,7,4′-tetrahydroxy substitution pattern. The appearance of a two-proton singlet at δ 7.03 suggested the presence of a galloyl group. These observations were confirmed by enzymatic hydrolysis of **1** with tannase, which gave (−)-epiafzelechin[5] and gallic acid. The lowfield shift of flavan 3-H in the ^{1}H-NMR spectrum of **1**, analogous to that found in **8** or **9**, indicated the location of the galloyl group at this position,

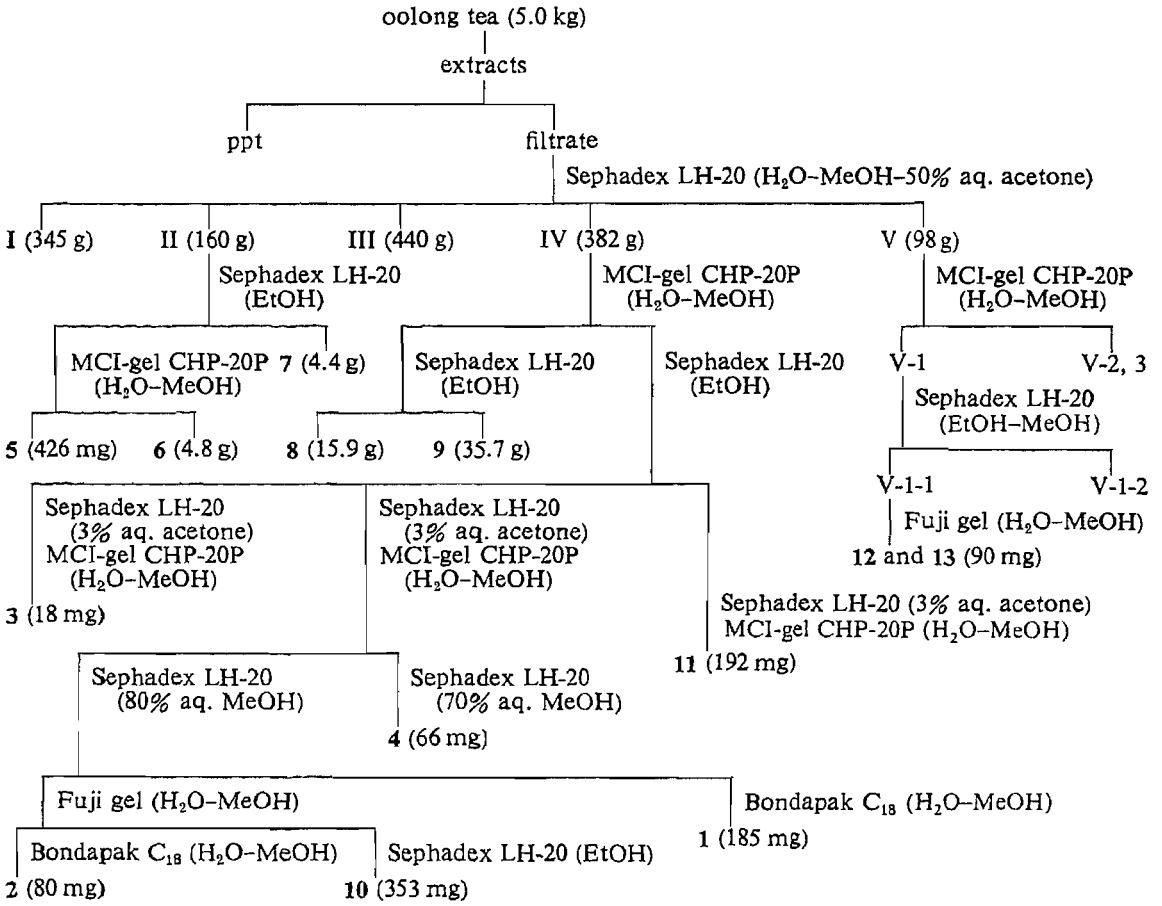
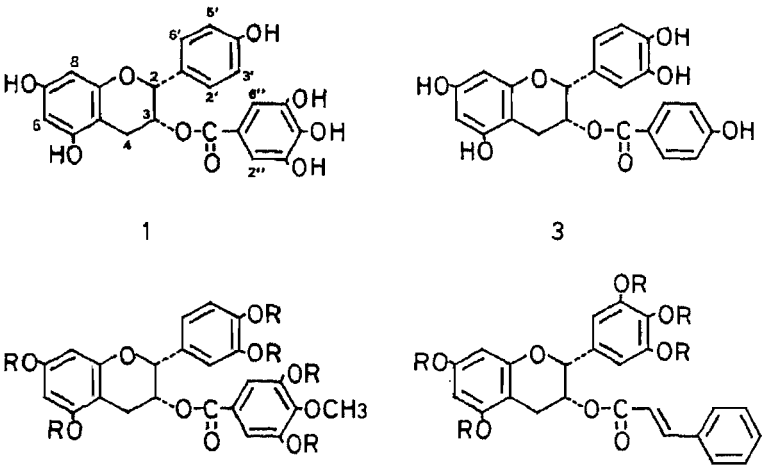


Chart 1



1 3

2: R=H
2a: R=Et

4: R=H
4a: R=Me

thus establishing the structure of **1** as (−)-epiafzelechin 3-*O*-gallate.

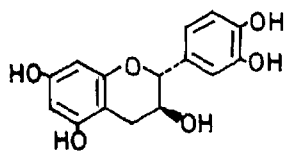
Compound **2**, an off-white amorphous powder, $[\alpha]_D$ −160.1° (acetone), gave a light green coloration on TLC with the ferric chloride reagent, suggestive of the occurrence of a catechol ring in the molecule. The molecular formula, $C_{23}H_{20}O_{10}$, confirmed by elemental analysis and fast atom bombardment mass spectrometry (FAB-MS) [$(M+H)^+$ *m/z*: 457],

differs from that of **8** in having an extra CH_2. The ^{1}H-NMR spectrum (Table I) of **2** was almost identical with that of **8** except for the presence of a methoxy signal at δ 3.83. Ethylation of **2** with diethyl sulfate and potassium carbonate in dry acetone furnished a hexaethyl ether (**2a**), $[\alpha]_D$ $-138.5°$ ($CHCl_3$), whose electron-impact mass spectrum (EI-MS) showed prominent fragment peaks at *m/z* 384 and 223, arising from tetraethoxyflaven and 4-*O*-methyl-3,5-di-*O*-ethyl galloyl cations, respectively. On the other hand, enzymatic hydrolysis of **2** with tannase afforded **6** and 4-*O*-methyl gallic acid. The location of the 4-*O*-methyl galloyl group in the epicatechin nucleus was deduced from the lowfield shift of 3-H in the ^{1}H-NMR spectrum of **2**. Based on these observations, compound **2** was characterized as (−)-epicatechin 3-*O*-(4-*O*-methyl)-gallate.

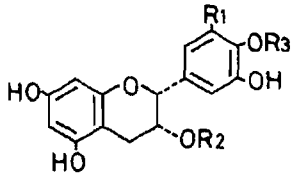
TABLE I. ^{1}H-NMR Spectral Data for Compounds 1—4[a]

	1	2	3	4
C-Ring				
C_2-H	5.19 (s)	5.16 (s)	5.13 (s)	5.03 (s)
C_3-H	5.54 (m)	5.59 (m)	5.55 (m)	5.50 (m)
C_4-H	2.79—3.24 (m)	2.89—3.24 (m)	2.79—3.22 (m)	2.75—3.17 (m)
A-Ring				
C_6-H	6.05 (d, J=2 Hz)	6.07 (s)	6.04 (d, J=2 Hz)	6.02 (d, J=2 Hz)
C_8-H	6.09 (d, J=2 Hz)	6.07 (s)	6.08 (d, J=2 Hz)	6.09 (d, J=2 Hz)
B-Ring				
$C_{2'}$-H	7.38 (d, J=8 Hz)	7.09 (d, J=2 Hz)	7.07 (d, J=2 Hz)	6.63 (s)
$C_{3'}$-H	6.78 (d, J=8 Hz)	—	—	—
$C_{5'}$-H	6.78 (d, J=8 Hz)	6.87 (d, J=8 Hz)	6.76 (d, J=8 Hz)	—
$C_{6'}$-H	7.38 (d, J=8 Hz)	6.93 (dd, J=8, 2 Hz)	6.89 (dd, J=8, 2 Hz)	6.63 (s)
Galloyl				
$C_{2'',6''}$-H	7.03 (s)	—	—	—
4-*O*-Methylgalloyl				
$C_{2'',6''}$-H	—	7.00 (s)	—	—
OCH_3	—	3.83 (s)	—	—
p-Hydroxybenzoyl				
$C_{2'',6''}$-H	—	—	7.76 (d, J=8 Hz)	—
$C_{3'',5''}$-H	—	—	6.84 (d, J=8 Hz)	—
Cinnamoyl				
$C_{2'',6''}$-H	—	—	—	7.32—7.80 (m)
-CH=CH-	—	—	—	6.21, 7.52 (each d, J=16 Hz)

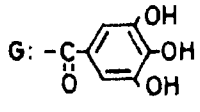
a) Spectra were measured in acetone-d_6+D_2O at 100 MHz.



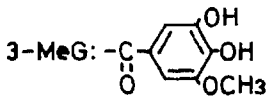
5



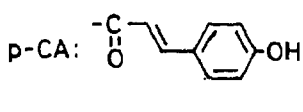
6: R1=R2=R3=H
7: R1=OH, R2=R3=H
8: R1=R3=H, R2=G
9: R1=OH, R2=G, R3=H
10: R1=R3=H, R2=3-MeG
11: R1=OH, R2=p-CA, R3=H
12: R1=OG, R2=G, R3=H
13: R1=OH, R2=R3=G



G:



3-MeG:



p-CA:

TABLE II. ^{13}C-NMR Spectral Data for Compounds 1—4[a]

	1	2	3	4
C-Ring				
C_2	78.0	77.8	77.9	77.8
C_3	69.7	70.1	69.7	69.7
C_4	26.4	26.4	26.5	26.5
A-Ring				
C_{4a}	98.6	98.6	98.6	98.6
$C_{6,8}$	95.5	95.5	95.5	95.6
	96.5	96.5	96.4	96.5
$C_{5,7,8a}$	157.7 (3C)	156.7	156.7	156.5
		157.3	157.3 (2C)	157.3
		157.4		157.5
B-Ring				
$C_{1'}$	130.2	130.9	130.9	130.3
$C_{2'}$	128.8	114.2[b]	114.7[b]	106.6
$C_{3'}$	115.6	145.3	145.3[c]	146.0[b]
$C_{4'}$	157.7	145.3	145.4[c]	133.0
$C_{5'}$	115.6	115.7[b]	115.7[b]	146.1[b]
$C_{6'}$	128.8	118.6	118.7	106.6
Acyl group				
-COO-	166.6	166.3	166.4	167.0
$C_{1''}$	121.2	126.1	121.7	134.9
$C_{2'',6''}$	109.9	109.8	132.4	128.9
$C_{3'',5''}$	145.9	151.1	116.0	129.7
$C_{4''}$	139.1	140.6	162.8	131.3
OCH_3	—	60.6	—	—
-CH=CH-	—	—	—	118.6
				146.1

a) Spectra were measured in acetone-d_6 + D_2O at 25.05 MHz. *b*—*c*) Assignments may be interchanged in each column.

Compound **3**, an off-white amorphous powder, $[\alpha]_D$ −144.4° (acetone), $C_{22}H_{18}O_8$ $4/3H_2O$, gave the same coloration as **2** with the ferric chloride reagent. The ^{1}H-NMR spectrum was also correlated closely with that of **8**, but A_2B_2-type aromatic signals were found at δ 6.84 and 7.76 (each 2H, d, J=8 Hz) instead of a two-proton galloyl singlet. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (Table II) showed the presence of a carboxyl group and a *para*-hydroxyphenyl ring, in addition to fifteen carbon resonances assignable to an epicatechin moiety. From these observations, **3** was considered to be (−)-epicatechin 3-*O*-*p*-hydroxybenzoate. Further support for the structure was obtained by field-desorption mass spectrum (FD-MS), which showed the $(M+H)^+$ ion peak at *m/z* 411, accompanied by prominent peaks at *m/z* 272 and 121 arising from flaven and *p*-hydroxybenzoyl cations, respectively. Thus, **3** was concluded to be (−)-epicatechin 3-*O*-*p*-hydroxybenzoate.

Compound **4**, an off-white amorphous powder, $[\alpha]_D$ −144.1° (acetone), $C_{24}H_{20}O_8 \cdot 4/3H_2O$, showed, with the ferric chloride reagent, a blue coloration characteristic of a pyrogallol derivative. In the ^{1}H-NMR spectrum (Table I), the observation of five aromatic multiplets at δ 7.32—7.80 and two *trans*-olefinic doublets at δ 6.21 and 7.52 (J= 16 Hz) suggested the occurrence of a cinnamoyl group in **4**. In addition, the resonances at δ 2.57—3.17 (2H, m), 5.03 (1H, s), 5.50 (1H, m), 6.02, 6.09 (each 1H, d, J=2 Hz) and 6.63 (2H, s) suggested the presence of an epigallocatechin moiety to which the acyl group is attached at the C-3 position. The ^{13}C-NMR data (Table II) were consistent with these ^{1}H-

NMR observations, showing the presence of (−)-epigallocatechin and cinnamoyl moieties. Ordinary phenolic methylation of **4** yielded the pentamethyl ether (**4a**), $[\alpha]_D$ −146.9° ($CHCl_3$), the EI-MS of which exhibited the molecular ion peak at *m/z* 506, together with significant peaks at *m/z* 358 and 131 derived from pentamethoxyflaven and cinnamoyl cations, respectively. Based on these results, the structure of **4** was assigned as (−)-epigallocatechin 3-*O*-cinnamate.

In conclusion, although oolong tea is regarded as a partially fermented product in terms of tea manufacture, significant differences from black tea were found in its phenolic pattern, particularly in the contents and compositions of monomeric flavan-3-ol derivatives. Further chemical examination of other polyphenolic constituents in oolong tea is under way.

Experimental

Details of the instruments and chromatographic conditions used in this study were essentially the same as described in the previous paper,[6] except for the ratio (5 : 4 : 1 or 3 : 6 : 1) of the solvent system, benzene–ethyl formate–formic acid, used in TLC of free phenolics.

Extraction and Isolation——Commercial oolong tea (commercial name; Shiraore) (5.0 kg) was extracted three times with 80% aqueous acetone at room temperature. The acetone was removed by evaporation under reduced pressure (*ca.* 40 °C), and the resulting aqueous solution afforded dark green precipitates consisting mainly of chlorophylls, which were removed by filtration. The filtrate was applied to a column of Sephadex LH-20, preswollen with H_2O. Elution with H_2O containing increasing amounts of MeOH and then with H_2O–acetone (1 : 1) afforded five fractions (frs.), frs. I (345 g), II (160 g), III (440 g), IV (382 g) and V (98 g). Fraction I consisted largely of caffeine contaminated with simple phenolics such as theogallin. Rechromatography of fraction II on columns of Sephadex LH-20 and MCI-gel CHP-20P gave compounds **5** (426 mg), **6** (4.8 g) and **7** (4.4 g). Repeated column chromatography of fraction IV over Sephadex LH-20, MCI-gel CHP-20P, Bondapak C_{18} and Fuji gel with various solvent systems furnished compounds **1** (185 mg), **2** (80 mg), **3** (18 mg), **4** (66 mg), **8** (15.9 g), **9** (35.7 g), **10** (353 mg) and **11** (192 mg). Rechromatography of fraction V on columns of MCI-gel CHP-20P, Sephadex LH-20 and Fuji gel afforded an inseparable mixture of compounds **12** and **13** (90 mg).

(−)-Epiafzelechin 3-*O*-Gallate (1)——An off-white amorphous powder, $[\alpha]_D^{21}$ −181.7° (*c* = 0.9, acetone). *Anal.* Calcd for $C_{22}H_{18}O_9 \cdot H_2O$: C, 59.46; H, 4.54. Found: C, 59.96; H, 4.27. FD-MS *m/z*: 427 $[(M+H)^+, 4\%]$. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Enzymatic Hydrolysis of 1 with Tannase——A solution of **1** (20 mg) in H_2O (4 ml) was shaken for 10 min with tannase (2 mg) at room temperature. The reaction mixture was concentrated by evaporation under reduced pressure and treated with EtOH, and the precipitate formed was filtered off. The filtrate was concentrated, and the residue was applied to a Sephadex LH-20 column. Elution with EtOH yielded gallic acid (4 mg) and a hydrolysate, which was shown to be identical with (−)-epiafzelechin (7 mg).

(−)-Epicatechin 3-*O*-(4-*O*-Methyl)-gallate (2)——An off-white amorphous powder, $[\alpha]_D^{22}$ −160.1° (*c* = 1.1, acetone). *Anal.* Calcd for $C_{23}H_{20}O_{10} \cdot 2/3H_2O$: C, 58.97; H, 4.59. Found: C, 58.71; H, 4.73. FAB-MS *m/z*: 457 $[(M+H)^+, 0.7\%]$, 184, 167. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Enzymatic Hydrolysis of 2 with Tannase——A solution of **2** (42 mg) in H_2O (5 ml) was shaken for 10 min with tannase (2 mg) at room temperature. The reaction mixture was worked up as described above to give 4-*O*-methyl gallic acid (5 mg) and **6** (12 mg).

Ethylation of 2——A mixture of **2** (27 mg), diethyl sulfate (0.4 ml) and anhydrous potassium carbonate (0.5 g) in dry acetone (5 ml) was refluxed for 2 h with stirring. After removal of inorganic salts by filtration, the filtrate was concentrated to a syrup, which was chromatographed over silica gel. Elution with benzene–acetone (50 : 1, v/v) gave crude **2a**, which was purified by chromatography over a silica gel column with benzene–ethyl acetate (40 : 1, v/v) to yield the ethyl ether (**2a**) (31 mg) as an off-white amorphous powder, $[\alpha]_D^{18}$ −138.5° (*c* = 0.6, $CHCl_3$). *Anal.* Calcd for $C_{35}H_{44}O_{10} \cdot 3H_2O$: C, 61.93; H, 7.43. Found: C, 62.25; H, 7.49. EI-MS *m/z*: 624 (M^+, 0.2%), 384, 223. ^{1}H-NMR ($CDCl_3$): 1.23—1.51 (18H, m, $6 \times OCH_2\underline{CH_3}$), 3.03 (2H, d, *J* = 4 Hz, C_4-H), 3.84 (3H, s, OCH_3), 3.84—4.20 (12H, m, $6 \times O\underline{CH_2}CH_3$), 5.09 (1H, s, C_2-H), 5.59 (1H, m, C_3-H), 6.08, 6.21 (each 1H, d, *J* = 2 Hz, $C_{6,8}$-H), 6.79 (1H, d, *J* = 8 Hz, $C_{5'}$-H), 6.96 (1H, dd, *J* = 8, 2 Hz, $C_{6'}$-H), 7.01 (1H, br s, $C_{2'}$-H), 7.11 (2H, s, $C_{2'',6''}$-H).

(−)-Epicatechin 3-*O*-*p*-Hydroxybenzoate (3)——An off-white amorphous powder, $[\alpha]_D^{21}$ −144.4° (*c* = 1.0, acetone). *Anal.* Calcd for $C_{18}H_{18}O_7 \cdot H_2O$: C, 59.33; H, 5.53. Found: C, 59.77; H, 5.70. FAB-MS *m/z*: 347 $[(M+H)^+, 0.7\%]$, 272, 121. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

(−)-Epigallocatechin 3-*O*-Cinnamate (4)——An off-white amorphous powder, $[\alpha]_D^{23}$ −144.1° (*c* = 1.0, acetone). *Anal.* Calcd for $C_{24}H_{20}O_8 \cdot 4/3H_2O$: C, 62.60; H, 4.96. Found: C, 62.86; H, 4.87. FAB-MS *m/z*: 437 $[(M+H)^+, 0.7\%]$. ^{1}H-NMR: Table I. ^{13}C-NMR: Table II.

Methylation of 4——A mixture of **4** (37 mg), dimethyl sulfate (0.2 ml) and anhydrous potassium carbonate (0.3 g) in dry acetone (5 ml) was refluxed for 1 h with stirring. The reaction mixture was worked up in the same way as described for **2** to give the methyl ether (**4a**) (22 mg) as an off-white amorphous powder, $[\alpha]_D^{18}$ −176.9° (c=0.9, $CHCl_3$). *Anal.* Calcd for $C_{29}H_{30}O_8 \cdot 1/3H_2O$: C, 67.95; H, 6.03. Found: C, 68.16; H, 5.95. EI-MS m/z: 506 (M^+, 0.3%), 358, 131. ^{1}H-NMR ($CDCl_3$): 3.20 (2H, d, J=4 Hz, C_4-H), 3.78—3.86 (15H, m, 5×OCH_3), 5.05 (1H, s, C_2-H), 5.63 (1H, m, C_3-H), 6.14, 6.27 (each 1H, d, J=2 Hz, $C_{6,8}$-H), 6.37, 7.58 (each 1H, d, J=16 Hz, olefinic-H), 6.73 (2H, s, $C_{2',6'}$-H), 7.26—7.48 (5H, m, arom-H).

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Antiproliferating Polyquinanes. V.[1] Di- and Triquinanes Involving α-Methylene or α-Alkylidene Cyclopentanone, Cyclopentenone, and γ-Lactone Systems

KIYOMI KAKIUCHI,*,[a] MASAKI UE,[a] MITSUNORI TAKEDA,[a] TOSHIHIRO TADAKI,[a] YUJI KATO,[a] TERUHISA NAGASHIMA,[a] YOSHITO TOBE,[a] HIROSHI KOIKE,[b] NOBUTAKA IDA,[b] and YOSHINOBU ODAIRA[a]

Department of Applied Fine Chemistry, Faculty of Engineering, Osaka University,[a] Suita, Osaka 565, Japan and Basic Research Laboratories, Toray Industries, Inc.,[b] Kamakura, Kanagawa 248, Japan

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New analogues related to quadrone (**3**), 3-methylenetricyclo[4.3.2.0^{1,5}]undecan-4-one (**14**), 4-methylenetricyclo[4.3.2.0^{1,5}]undecan-3-one (**15**), and 2-methylenetricyclo[4.3.2.0^{1,5}]undec-4-en-3-one (**16**), and novel propellane- and angular-type triquinanes, 3-methylenetricyclo[3.3.3.0]undecan-2-one (**17**), (*E*)-3-propylidene- and (*E*)-3-(5-carbomethoxy)pentylidenetricyclo[3.3.3.0]undecan-2-ones (**18** and **19**), (*E*)-5-(2-oxotricyclo[3.3.3.0]undecan-3-ylidene)pentyl (*E*)-cinnamate (**20**), 2-methylenetricyclo[3.3.3.0]undecan-3-one (**21**), 4-methylene-2-oxatricyclo[3.3.3.0]undecan-3-one (**22**), and 7-methylenetricyclo[6.3.0.0^{1,5}]undecan-6-one (**23**), were readily synthesized through skeletal transformations, and showed antiproliferating activity. Biomimetic reactions of a model compound of **3**, 2-methylenetricyclo[4.3.2.0^{1,5}]undecan-3-one (**8**), with propanethiol, *etc.* were undertaken. Correlations of the activity of some polyquinanes with second-order rate constants of addition of L-cysteine and with the carbon-13 nuclear magnetic resonance chemical shifts of *exo*-methylene β-carbons are discussed.

Keywords——diquinane; triquinane; α-methylene cyclopentanone; antiproliferating activity; quadrone; cytotoxic functional group; structure–activity relationship; biomimetic reaction; second-order rate constant; ^{13}C-NMR chemical shift

Introduction

The potent cytotoxic action of many terpenoid plant products and their ability to inactivate certain enzymes *in vitro* have been attributed to the presence of the α-methylene γ-butyrolactone moiety.[2] The biological activities of these lactones are apparently derived from the significant chemical affinity of this moiety for thiols and other biological nucleophiles.[3] In particular, interest in α-methylene γ-lactones as medicinal agents has been stimulated by the possibility that some of them might show enough selective toxicity against neoplastic cells to be of therapeutic value as anticancer agents.[3,4] It should be mentioned, however, that the biological activity of α-methylene γ-lactones is not confined to the complex polyfunctional sesquiterpene lactones only. For example, it has been shown that artificial α-methylene γ-lactone derivatives containing no other reactive functional groups can in some instances have growth-inhibitory activity comparable to that of multifunctional natural products.[5]

The structurally related five-membered ring systems, α-methylene cyclopentanone and α-methylene cyclopentenone groups, have also been demonstrated to show intriguing biological activities.[2] Simple representatives of these types (monoquinane: one cyclopentane ring) are sarkomycin (**1**)[6] and methylenomycin B (**2**),[7] possessing antitumor and antimicrobial activities, respectively.

Quadrone (**3**), isolated from the fermentation broth of *Aspergillus terreus* in 1978, was found to display significant activities against KB human epidermoid carcinoma of the nasopharynx *in vitro* and P388 lymphocytic leukemia *in vivo*.[8] The diquinane-type lactone **3** can be classified into the above category, in spite of the absence of functional groups commonly associated with antitumor agents (as described above) in the tetracyclic structure, because the retrolactonization product, terrecyclic acid A (**4**), isolated from the same fungus together with **3**, has been regarded as the carrier of the activities of **3** due to the α-methylene cyclopentanone moiety in **4**.[9] Moreover, as their synthetic analogue, descarboxyquadrone (**5**), having the active center and C-11 geminal methyls on a novel tricyclo[4.3.2.$0^{1,5}$]undecane skeleton, has been synthesized by several groups[10] including ours[11] and found to possess cytotoxicity against HeLa cell at almost the same level as **3**.[10a]

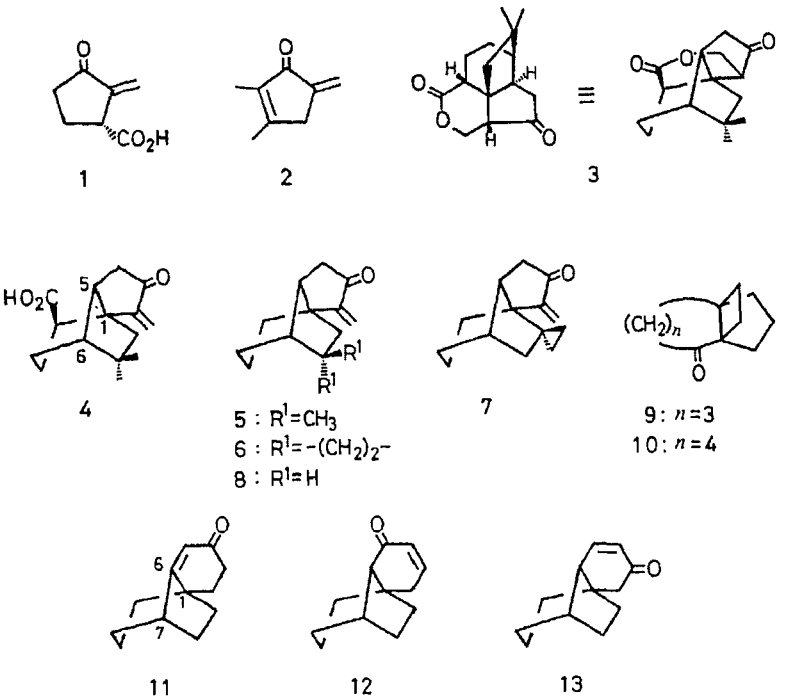


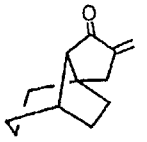
Chart 1

Recently we have reported the synthesis of not only **5**[11] but also some related compounds,[12] 2-methylene-11- and 10-spirocyclopropanotricyclo[4.3.2.$0^{1,5}$]undecan-3-ones (**6** and **7**),[12] and a model compound **8**[11a] by using the unique skeletal transformation of [4.3.2]propellan-2-one (**9**) to this novel framework.[13] As an application of the rearrangement to the higher homologue, [5.3.2]propellan-2-one (**10**), we have also synthesized the tricyclo[5.3.2.$0^{1,6}$]dodecenones **11**, **12**, and **13** containing a cyclohexenone moiety at various positions.[14] In view of the antiproliferating activity of these compounds against P388 and L1210 lymphocytic leukemic cells of mice (also listed in Table I), the results supported the tentative conclusion that, in cell culture as opposed to whole animal assays, the α-methylene cyclopentanone moiety is sufficient to confer cytotoxic properties upon the diquinane structure even in the absence of other functional groups, like the α-methylene γ-lactone system.[5]

On the basis of the above conclusion, in order to elucidate the relationship between cytotoxic activity and the polycyclic structure surrounding the α-methylene cyclopentanone system, and, furthermore, to search for potent cytotoxic functional groups, we describe here the synthesis of the following ten polyquinanes **14**—**23** having an α-methylene or α-alkylidene

carbonyl group and their antiproliferating activities. The diquinanes **14** and **15** with a tricyclo[4.3.2.$0^{1,5}$]undecane framework, the common skeleton of quadrone (**3**) and the related compounds **4**—**8**, possess an α-methylene carbonyl group at different positions from that of **4**—**8**. On the other hand, **16** has an α-methylene cyclopentenone group in place of cyclopentanone in **4**—**8**, and its methylene and carbonyl group are located at the same positions as those of **4**—**8**. These diquinanes **14**—**16** were prepared easily using the skeletal transformations of [4.3.2]propellanone (**9**) as in the cases of **5**—**8**.[11,12] Structurally different triquinane-type α-methylene cyclopentanones **17**, **21**, and **23** were also prepared on the basis of acid-catalyzed rearrangement of [4.3.2]propellanones. Namely, propellane-type triquinanes **17** and **21** were derived from [3.3.3]propellan-2-one (**24**), which was obtained selectively by the rearrangement of **9**.[13a] The oxapropellane **22** having an α-methylene γ-lactone group was also prepared for the purpose of comparison of its activity with that of **21**. The angular-type triquinane **23** was prepared by utilizing the two-step rearrangement method, such as the transformation of **9** to tricyclo[6.3.0.$0^{1,5}$]undecan-5-ol (**25**).[13b,c] In view of our previous results on the biological activities of some α-alkylidene γ-lactones,[15] the propellane-type triquinanes **18**—**20** having an α-alkylidene cyclopentanone group were prepared. The antiproliferating activities of these new polyquinanes would be informative concerning not only the effect of the polycyclic structure and position of the active moiety on the biological activity but also potent cytotoxically active functions.

Next, in order to elucidate the reason for the appearance of the antiproliferating activity of the α-methylene cyclopentanone derivatives, biomimetic reactions of **8** with model compounds of biological nucleophiles are also described herein. Moreover, in an attempt to estimate the magnitude of the cytotoxicity of α-methylene cyclopentanones by chemical methods, comparisons of the activity of some polyquinanes with the reaction rates with L-cysteine and with the carbon-13 nuclear magnetic resonance (^{13}C-NMR) chemical shifts of *exo*-methylene β-carbons are discussed.



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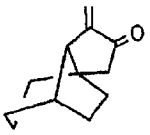

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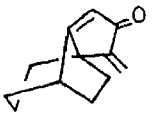

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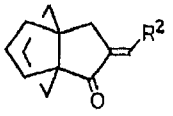
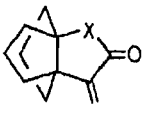
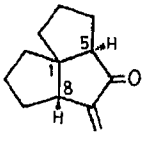


17: R^2=H
18: R^2=C_2H_5
19: R^2=-$(CH_2)_4CO_2Me$
20: R^2=-$(CH_2)_4OCOCH$=CHC_6H_5



21: X=CH_2
22: X=O



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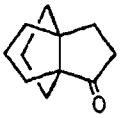

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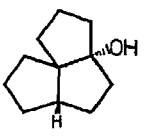

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Chart 2

Preparation of Polyquinanes

The diquinanes **14**—**16** were prepared from the synthetic intermediate, tricyclo-[4.3.2.$0^{1,5}$]undec-4-ene (**26**),[11a] used in the synthesis of **8** (Chart 3). The diquinane **14** was obtained in 42% overall yield by hydroboration-oxidation of **26** followed by oxidation and subsequent α-methylenation of the ketone **27**[13b] in the following manner[16]: i) treatment of the enolate of **27** with chlorotrimethylsilane, ii) carbon–carbon bond formation of the silyl enol ether **28** and chloromethyl phenyl sulfide with titanium(IV) chloride ($TiCl_4$), iii) oxidation of the sulfide **29** with *m*-chloroperbenzoic acid (MCPBA), and iv) thermolysis of the sulfoxide **30**. Similarly, α-methylenation of the saturated ketone **32**, which was derived by allylic oxidation of **26** and then hydrogenation of the enone **31**,[11a] gave the diquinane **15** in 21% overall yield along with **8** in 20% overall yield. Dehydration of the alcohol[11a] prepared by hydroxymethylation of **31** *via* the mesylate afforded the diquinane **16** in 70% overall yield.

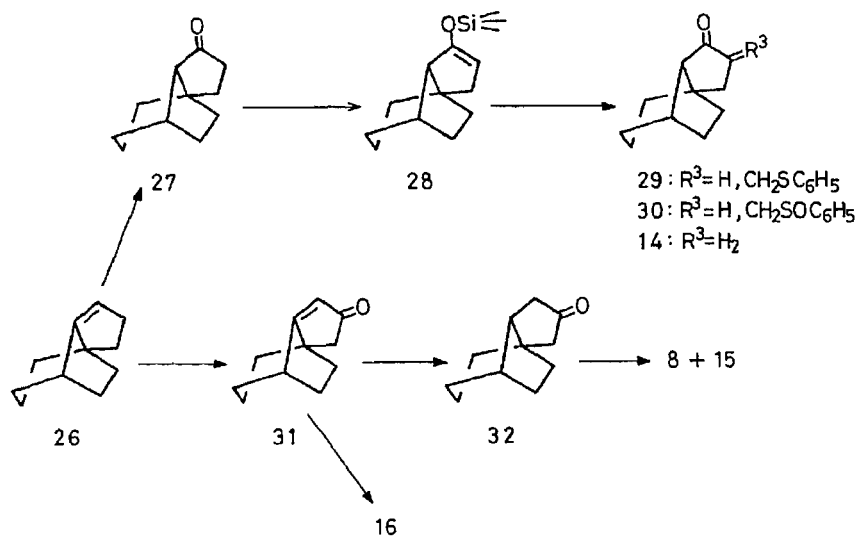


Chart 3

The propellane-type triquinane **17** was produced in 42% overall yield together with a small amount of the dimer (*vide infra*), by α-methylenation of **24** as described for the preparation of **14**. α-Alkylidene cyclopentanones **18**—**20** were synthesized by reaction of the enolate of **24** with the corresponding aldehydes (propionaldehyde, methyl 6-oxohexanoate,[17] and 5-oxopentyl cinnamate) in 71—85% yields. The *E*-geometry of the double bond was deduced from a comparison of the proton nuclear magnetic resonance (^{1}H-NMR) chemical shifts of their vinyl protons (δ, 6.30) with that of (*E*)-2-propylidenecyclopentanone (δ, 6.37).[18] Moreover, under these conditions, products with *E*-geometry are generally obtained.[19] The synthesis of **21** was carried out in two ways starting from **24** (Chart 4). Reaction with methyllithium (MeLi)[20] followed by dehydration of the tertiary alcohol **33** gave the olefin **34** in 86% overall yield. Epoxidation of **34** with MCPBA and the subsequent base-induced isomerization[21] of the epoxide **35** followed by oxidation of the allylic alcohol **36** furnished **21** in 44% overall yield. Alternatively, Wittig olefination of **24** followed by allylic oxidation[22] of the olefin **37** gave **21** in 49% overall yield and **36** in 47% overall yield.

The oxa-analogue of **22** was synthesized by ring contraction[23] of **24** and subsequent transformation to the corresponding γ-lactone **46**[24] followed by α-methylenation (Chart 5). Namely, α-formylation[25] of **24** followed by treatment of the crude **38** with tosyl azide[26] and subsequent UV-irradiation of the diazo ketone **39** in methanol gave methyl [3.3.2]propellane-9-carboxylate (**40**) in 61% overall yield. Saponification of **40** and then reaction of the

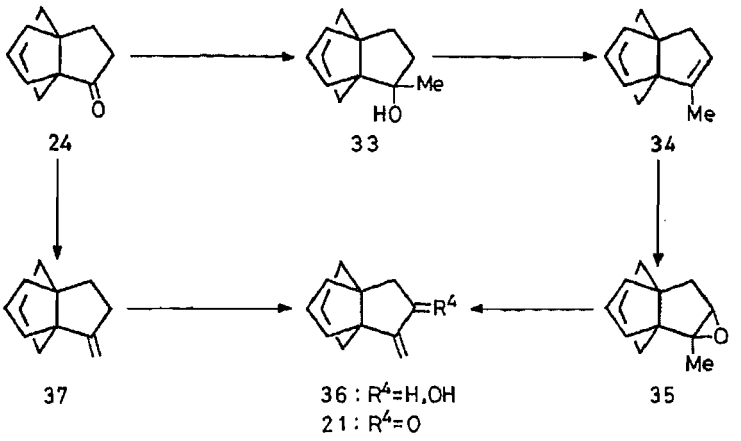


Chart 4

carboxylic acid **41** with MeLi afforded the methyl ketone **42** in 63% overall yield. Baeyer–Villiger oxidation of **42** followed by reduction of the acetate **43** with lithium aluminum hydride ($LiAlH_4$) and then oxidation of the secondary alcohol **44** furnished the cyclobutanone **45**[24] in 78% overall yield. Baeyer–Villiger oxidation[27] of **45** followed by α-methylenation of **46** as described for the preparation of **16** gave the α-methylene γ-lactone **22** in 47% overall yield.

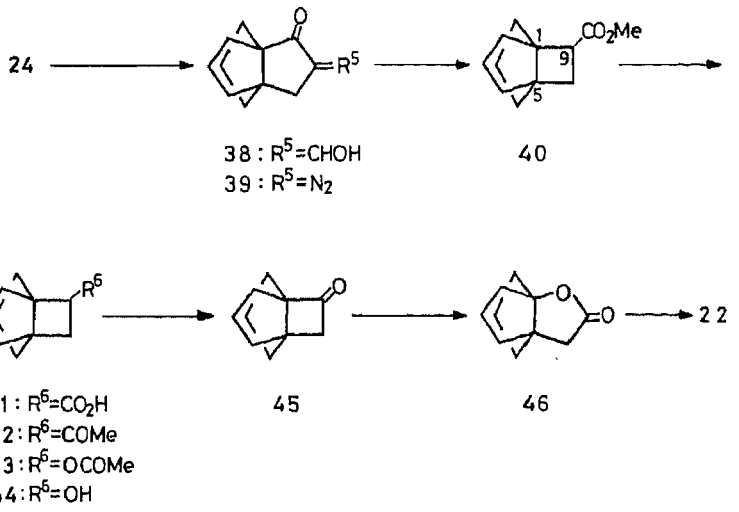


Chart 5

For the synthesis of the angular-type triquinane **23**, the straightforward route from **25** was unsuccessful, because dehydration[13c] of **25** followed by hydroboration-oxidation and subsequent oxidation gave an inseparable mixture of the desired ketone **54** and its isomer, tricyclo[6.3.0.$0^{1,5}$]undecan-4-one,[28] in an approximately 1 : 1 ratio. Therefore, **54** was prepared in a selective manner from [4.3.2]propell-10-en-2-one (**47**)[13b] according to our rearrangement protocol as follows (Chart 6): i) acid-catalyzed rearrangement of **47**, ii) reduction of the chlorohydrin **48** with tributyltin hydride (*n*-Bu_3SnH), iii) acid-catalyzed rearrangement of the tertiary alcohol **49**, which was confirmed by hydrogenation to give the known saturated alcohol,[13b] iv) chlorination of the allylic alcohol **50**, v) reduction of the

chloride **51** to give the olefin **52**,[29] vi) hydroboration-oxidation of **52** to afford the two epimeric alcohols **53** (3.5 : 1),[29] and vii) oxidation of **53**. Alternatively, we also obtained **54** from **52** and **53** produced by solvolytic rearrangement of *endo*-tricyclo[6.3.0.$0^{1,6}$]undecan-5-yl tosylate (**55**).[29] Finally, α-methylenation of **54** was undertaken by the same procedure as used for **14** to give **23** in 40% overall yield.

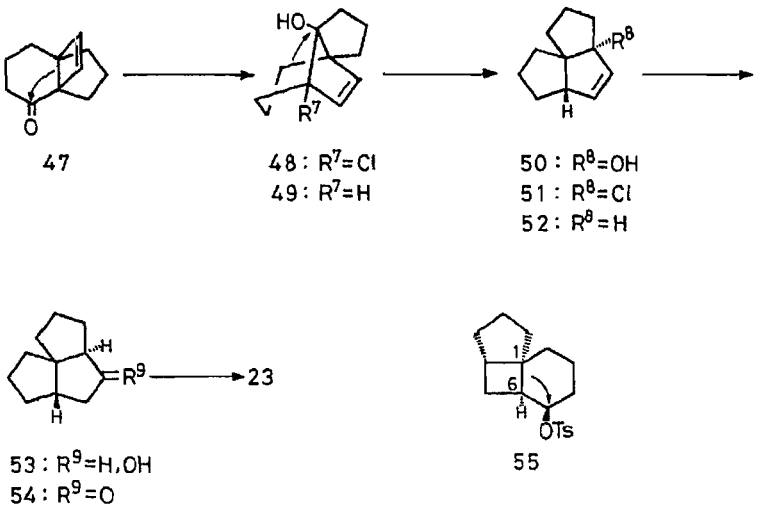


Chart 6

Biological Results and Discussion

The antiproliferating activities of the synthetic compounds **14**—**16** and **18**—**23** against P388, L1210, 3LL, and LY cells in culture are summarized in Table I. The assay procedure is described in the experimental section. The results on quadrone (**3**) and terrecyclic acid A (**4**), which were kindly provided for comparison by Prof. H. Sakai, are also listed in Table I along with those for the other diquinanes **5**—**8** and the cyclohexenones **11**—**13** prepared previously. The propellane-type triquinane **17** was not assayed because of its insolubility in the test media. This may be due to the fact that **17** readily formed the crystalline dimer **56**, when it was allowed to stand at room temperature. The structure of **56** was deduced from the similar dimerization of α-methylene cyclopentanone[30] and was established by spectroscopic and elemental analyses.

In the diquinane series related to quadrone (**3**), **14** showed almost the same level of activity as **3** but was slightly less active than **8**, while **15** was more active than **3** and even slightly more active than **8**, against the leukemic cells. On the other hand, the α-methylene cyclopetenone derivative **16** displayed a lower activity by at least one order of magnitude against P388 cell than the dihydro derivative **8**. These results suggest that the position of the α-methylene carbonyl group in the tricyclic structure has an important effect on the activity, and the α-methylene cyclopentanone group is more effective for inhibition of the cell proliferation than α-methylene cyclopentenone.

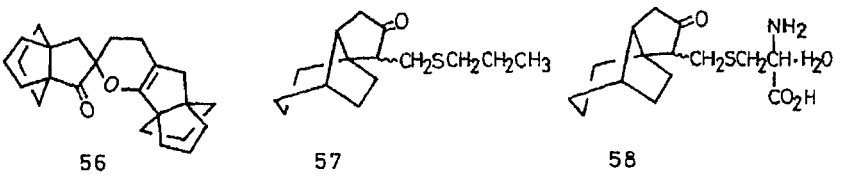
It is of great interest that the variation of tricyclic structure from the diquinane framework to the triquinanes **21** and **23** resulted in enhancement of the activity against almost all cells in the α-methylene cyclopentanone series. In particular, the propellane-type triquinane **21** is the most active of all polyquinanes tested. In contrast, the activity of the α-methylene γ-lactone derivative **22**, an oxa-analogue of **21**, was remarkably depressed. Furthermore, the α-alkylidene cyclopentanone derivatives **18** and **19** had no effect on cell proliferation, like the cyclohexenone derivatives **11**—**13**, whereas **20** having the cinnamyl

TABLE I. Antiproliferating Activity of Di- and Triquinane-Type Compounds[a]

Compound	IC_{50} (μg/ml)			
	P388	L1210	3LL	LY
3	0.19	0.65	0.39	>1.0
4[b]	<1.0	<1.0	<1.0	<1.0
5	0.14	0.06	NT	NT
6	0.17	0.11	0.08	0.26
7	0.42	0.49	0.36	>1.0
8	0.09	0.19	0.07	0.58
11	>1.0	>1.0	>1.0	>1.0
12	>1.0	>1.0	>1.0	>1.0
13	>1.0	>1.0	>1.0	>1.0
14	0.26	0.26	NT	NT
15	0.06	0.05	NT	NT
16	1.05	0.33	NT	NT
18	>1.0	>1.0	NT	NT
19	>1.0	>1.0	NT	NT
20	0.90	>1.0	NT	NT
21	0.02	0.02	0.01	0.08
22	4.00	1.45	NT	NT
23	0.05	0.04	0.08	0.23

a) >1.0 shows no effect on cell proliferation. NT means not tested. *b*) IC_{50} was not determined. The values as % of control at 1 μg/ml for **4** are comparable with those of **8** and were as follows: for **4**, 7.5%, 6.2%, 4.6%, and 10.4%, and for **8**, 6.4%, 3.0%, 1.4%, and 6.6%, against P388, L1210, 3LL, and LY cells, respectively.

moiety, which is inferred to be an active site for antitumor activity,[31] showed very weak activity against P388 cells. These findings indicate that the tricyclic skeleton surrounding the α-methylene carbonyl group plays a significant role in the appearance of the antiproliferating activity, and also demonstrate that the most potent cytotoxic functional group is the α-methylene cyclopentanone group.



56 57 58

Chart 7

In order to confirm that our polyquinane-type compounds serve as Michael acceptors of biological nucleophiles during the appearance of the growth-inhibitory activity, like α-methylene γ-lactones,[5] biomimetic reactions were carried out according to the literature.[32] The reaction conditions and the results are summarized in Table II.

Adenosine as the nucleic acid model compound, and propanethiol, L-cysteine, and L-lysine, and L-serine as the enzyme model compounds were allowed to react with the diquinane-type α-methylene cyclopentanone **8**. The diquinane **8** did not react with adenosine under the conditions employed and was recovered unchanged, as were L-lysine and L-serine. Reactions of **8** with the two compounds containing an SH group, on the other hand, took place smoothly to give adducts **57** and **58** in high yields (82—95%). The structures were established by spectroscopic and elemental analyses. Our biomimetic reactions suggest that the appearance of the activity may be due to irreversible S-alkylation of either L-cysteine,

TABLE II. Biomimetic Reactions of the Diquinane-Type Compound **8** with a Nucleic Acid Model Compound and with Enzyme Model Compounds

Model compound	Solvent system	Product and yield (%)
Adenosine	pH 7.4 potassium phosphate buffer solution–EtOH (1 : 1)	Recovery of **8**
Propanethiol	pH 9.2 borate buffer–THF (2 : 3)	**57**, 95%[a]
L-Cysteine	pH 7.4 potassium phosphate buffer solution–EtOH (1 : 2)	**58**, 82%[b]
L-Lysine	pH 7.4 potassium phosphate buffer solution–EtOH (1 : 1)	Recovery of **8**
L-Serine	pH 7.4 potassium phosphate buffer solution–EtOH (1 : 1)	Recovery of **8**

a) Isolated yield based on **8**. *b*) Isolated yield based on L-cysteine.

TABLE III. Second-Order Rate Constants at 1 °C of Di- and Triquinane-Type Compounds with L-Cysteine at pH 7.4[a]

	5	**8**	**16**	**18**	**19**	**21**
k_2 (l/mol·s)	0.71	0.49	0.24[b]	0.09[b]	0.08[b]	0.83

a) The second-order kinetics for addition of cysteine to polyquinanes were nonlinear. The rate constants were calculated from initial rates. *b*) Extrapolated from Arrhenius plots.

TABLE IV. ^{13}C-NMR Chemical Shifts of *exo*-Methylene β-Carbons of Di- and Triquinane-Type Compounds

Compound	Chemical shift (ppm)
4	116.1[a]
5	114.2[b]
6	112.3[c]
7	112.8[c]
8	112.7[d]
14	117.9
15	115.9
16	113.5
17	117.7
21	117.7
23	117.7

a) Ref. 9. *b*) Ref. 11*b*. *c*) Ref. 12. *d*) Ref. 11*a*.

which is an absolute nutritional requirement for leukemic cells grown in culture,[33] or SH enzymes, as in the case of α-methylene γ-lactones.[3,34]

Based on the above results, it is reasonable to consider that the rate of thiol addition would influence the cytotoxicity. Consequently, the second-order rate constants (Table III) for some synthetic di- and triquinanes with L-cysteine were determined by using the methods of Kupchan *et al.*[3a,34] and Grassetti and Murray.[35] An interesting tendency is apparent from Tables I and III, although only a limited number of our polyquinanes was examined. It seems clear that, in the cases of the simple synthetic compounds, the more active compounds showed faster reaction rates with L-cysteine, though there is no correlation of the rate of cysteine addition with the cytotoxicity of various multifunctional antitumor agents.[3a]

It is a well-accepted view that intramolecular neighboring group participation, such as hydrogen bonding to the carbonyl group of an α,β-unsaturated system, catalyzes the Michael

addition[36] or is a factor enhancing the activity of many antitumor agents.[3] The enhancement is ascribed to the increase in electrophilicity at the *exo*-methylene β-carbon.[3] Therefore, the ^{13}C-NMR chemical shifts of β-carbon which reflect the electrophilicity, *i.e.*, δ^+ nature,[37] were examined for some of our α-methylene cyclopentanone series (Table IV).

Interestingly, a rather wide range ($\Delta\delta = 5.6$) of chemical shifts was found in spite of the absence of functional groups showing neighboring group participation. When these results are compared with the antiproliferating activity (Table I), no rigorous relationship between chemical shift and activity is apparent. It seems interesting, however, that the chemical shifts of **21** and **23**, possessing higher activity, appeared at lower field than those of the diquinanes except for **14**.

Experimental

All melting and boiling points are uncorrected. Infrared (IR) spectra were recorded on a Hitachi 260-10 spectrometer as liquid films unless otherwise stated. ^{1}H-NMR spectra were obtained on a JEOL JNM-PS-100 spectrometer in CCl_4, and ^{13}C-NMR spectra were taken on a JEOL JNM-FX-60S spectrometer in $CDCl_3$. Chemical shifts are reported as δ-values in parts per million relative to Me_4Si (δ, 0.0) as the internal standard. Mass spectra (MS) were measured with a Hitachi RMU-6E spectrometer and are given in terms of *m/z* (relative intensity) compared with the base peak. Ultraviolet (UV) spectra were recorded on a Hitachi 356 dual-wavelength double-beam spectrometer for kinetic measurement. Analytical gas liquid chromatography (GLC) was carried out on a Hitachi 163 gas chromatograph, and preparative GLC was conducted on a Varian Aerograph 920 gas chromatograph with a 10% FFAP column or a 30% SE-30 column. Column chromatography was performed with Wako C-200 silica gel. Flash chromatography[38] was carried out with Merck Silica gel 60. Yields were calculated based on the consumed starting materials.

Materials——[3.3.3]Propellanone (**24**),[13a] tricyclo[4.3.2.0^{1,5}]undecan-4-one (**27**),[13b] tricyclo[4.3.2.0^{1,5}]undec-4-en-3-one (**31**) and its 2-hydroxymethyl derivatives,[11a] and tricyclo[4.3.2.0]undec-10-en-2-one (**47**),[13b] were prepared as described previously. Adenosine, L-serine, L-lysine·HCl·H_2O, and pH 9.2 borae buffer solution were purchased from Wako Pure Chemical Industries. L-Cysteine was obtained from Sigma Chemical Company. 2,2′-Dipyridyl disulfide and 2-thiopyridone were purchased from Aldrich Chemical Company, Inc. Potassium phosphate buffer solution (pH 7.4) was obtained from Tokyo Kasei Kogyo Co., Ltd.

Diquinane-Type Compound 14——A 1.5 M solution of butyllithium (*n*-BuLi, 7.80 ml, 12.2 mmol) in hexane was added to a stirred solution of diisopropylamine (1.69 ml, 12.2 mmol) in dry tetrahydrofuran (THF, 5 ml) *via* a syringe at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred for 15 min and cooled to −78 °C. A solution of **27**[13b] (1.00 g, 6.10 mmol) in dry THF (10 ml) was added dropwise during 25 min. The solution was maintained at −78 °C for 30 min and chlorotrimethylsilane (2.41 ml, 18.3 mmol) was added *via* a syringe. The reaction mixture was allowed to warm to room temperature and then stirred for 30 min. The mixture was filtered and the residue was washed with petroleum ether. The combined filtrates were concentrated *in vacuo* to give the residue, which was diluted with petroleum ether. The mixture was filtered again. The filtration was repeated until the residue became a clear solution, to give **28**. IR: 1620 (C=C), 1250 (C–O) cm^{-1}.

A solution of $TiCl_4$ (0.80 ml, 7.32 mmol) in dichloromethane (CH_2Cl_2, 5 ml) was added to a stirred solution of the crude ether **28** and chloromethylphenyl sulfide (1.23 ml, 9.15 mmol) in CH_2Cl_2 (6 ml) *via* a syringe at −23 °C under a nitrogen atmosphere. The reaction mixture was stirred for 1.5 h and then poured into saturated sodium bicarbonate ($NaHCO_3$) solution (30 ml). The resulting mixture was extracted with ether. The combined extract were washed with brine and dried over magnesium sulfate ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give recovered **27** (0.16 g, ether : petroleum ether = 5 : 95) and **29** (1.28 g, 88% yield from **27**, ether : petroleum ether = 3 : 97). IR: 1730 (C=O), 1580 (C_6H_5) cm^{-1}.

A solution of MCPBA (0.91 g, 70%, 4.49 mmol) in chloroform ($CHCl_3$, 15 ml) was added to a stirred solution of the above sulfide **29** in $CHCl_3$ (15 ml) at −15 °C. The reaction mixture was stirred for 2 h and allowed to stand at room temperature overnight. Water was added and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2. The combined extracts were washed with saturated sodium bisulfite ($NaHSO_3$) solution, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **30**. IR: 1730 (C=O), 1040 (S=O) cm^{-1}.

A solution of the crude sulfoxide **30** in toluene (70 ml) was heated at reflux with stirring for 2 h and then concentrated *in vacuo*. The residue was chromatographed to give **14** (0.52 g, 66% yield from **29**, ether : petroleum ether = 4 : 96). *Anal.* Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.25. Found: C, 81.40; H, 9.27. IR: 1725 (C=O), 1635 (C=C) cm^{-1}. ^{1}H-NMR: 1.1—1.9 (11H, m), 2.49 (2H, m, 2-CH_2), 2.66 (1H, m, 5-CH), 5.22 (1H, m, C=CH_2), 5.94 (1H, m, C=CH_2). ^{13}C-NMR: 19.3 (t), 28.6 (t), 32.3 (t), 32.8 (t), 37.1 (d, 6-C), 37.6 (t), 38.5 (t), 45.3 (s, 1-C), 65.0 (d, 5-

C), 117.9 (t, C=$\underline{C}H_2$), 144.7 (s, 3-C), 206.3 (s, 4-C). MS *m/z*: 176 (M^+, 100), 107 (59), 79 (88).

Tricyclo[4.3.2.0^{1,5}]undecan-3-one (32)——A mixture of **31**[11a] (5.15 g, 31.8 mmol) and 10% palladized charcoal (1.00 g) in methanol (MeOH, 80 ml) was stirred at room temperature for 76 h under atmospheric pressure of hydrogen. After filtration, the filtrate was concentrated *in vacuo* and the residue was chromatographed to give **32** (4.84 g, 91% yield, ether : petroleum ether = 4 : 96), which was purified by preparative GLC, mp 40—41 °C. Semicarbazone, recrystallized from MeOH, mp 216 °C (dec.). *Anal.* Calcd for $C_{12}H_{19}N_3O$: C, 65.12; H, 8.65; N, 18.99. Found: C, 64.75; H, 8.66; N, 19.12. IR: 1740 (C=O) cm^{-1}. ^{1}H-NMR: 1.1—2.3 (m). ^{13}C-NMR: 19.2 (t), 28.0 (t), 32.6 (t), 32.8 (t), 37.3 (t), 38.5 (d, 6-C), 40.6 (t), 47.9 (s, 1-C), 49.8 (t), 53.1 (d, 5-C), 217.9 (s, 3-C). MS *m/z*: 164 (M^+, 97), 121 (100), 108 (68), 79 (64).

Diquinane-Type Compound 15——α-Methylenation of **32** (1.01 g, 6.10 mmol) as described for the preparation of **14** gave recovered **32** (0.15 g), **8** (0.23 g, 22% yield, ether : petroleum ether = 4 : 96), and **15** (0.25 g, 23% yield, ether : petroleum ether = 3 : 97) after chromatography. *Anal.* Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.54; H, 9.41. IR: 1725 (C=O), 1640 (C=C) cm^{-1}. ^{1}H-NMR: 1.1—2.0 (10H, m), 2.14 (2H, ABq, J = 17 Hz, 2-CH_2), 2.28 (1H, m, 6-CH), 2.46 (1H, m, 5-CH), 5.06 (1H, m, C=CH_2), 5.88 (1H, m, C=CH_2). ^{13}C-NMR: 19.5 (t), 28.3 (t), 32.4 (t), 33.6 (t), 36.8 (t), 38.6 (d, 6-C), 46.1 (s, 1-C), 48.6 (t, 2-C), 58.4 (d, 5-C), 115.8 (t, C=$\underline{C}H_2$), 147.3 (s, 4-C), 206.7 (s, 3-C). MS *m/z*: 176 (M^+, 100), 148 (90), 119 (86), 91 (100), 79 (81).

Diquinane-Type Compound 16——Methanesulfonyl chloride (5.81 ml, 74.8 mmol) was added dropwise to a stirred solution of the primary alcohol[11a] (4.79 g, 24.9 mmol), derived from **31** by hydroxymethylation, in pyridine (50 ml) *via* a syringe at 0 °C. The reaction mixture was stirred at room temperature for 14 h. Ice-water was added and the mixture was extracted with ether. The combined extracts were washed with two portions of 5% hydrochloric acid (HCl), saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give the mesylate. IR: 1700 (C=O), 1640 (C=C), 1355, 1175 (SO_2) cm^{-1}.

1,8-Diazabicyclo[5.4.0]undec-7-ene (11.2 ml, 74.8 mmol) was added to a stirred solution of the crude mesylate in benzene (50 ml) at room temperature. The reaction mixture was stirred for 21 h and water was added. The mixture was extracted with ether. The combined extracts were washed with two portions of 5% HCl, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **16** (3.01 g, 70% yield from the starting alcohol, ether : petroleum ether = 3 : 97). *Anal.* Calcd for $C_{12}H_{14}O$: C, 82.72; H, 8.10. Found: C, 82.65; H, 8.30. IR: 1700 (C=O), 1630 (C=C) cm^{-1}. ^{1}H-NMR: 1.2—2.2 (10H, m), 3.02 (1H, m, 6-CH), 5.11 (1H, s, C=CH_2), 5.75 (1H, s, 4-CH), 5.81 (1H, s, C=CH_2). ^{13}C-NMR: 18.0 (t), 29.6 (t), 29.7 (t), 31.8 (t), 31.4 (d, 6-C), 39.3 (t), 51.4 (s, 1-C), 113.5 (t, C=$\underline{C}H_2$), 118.0 (d, 4-C), 150.9 (s, 2-C), 188.9 (s, 5-C), 197.6 (s, 3-C). MS *m/z*: 174 (M^+, 48), 146 (100).

Propellane-Type Triquinane Compound 17——α-Methylenation of **24**[13a] (1.50 g, 9.14 mmol) as described for the preparation of **14** gave the dimer **56** (0.10 g, 4% yield), **17** (0.56 g, 42% yield), and recovered **24** (0.26 g) after chromatography (ether : petroleum ether = 3 : 97). *Anal.* Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.54; H, 9.33. IR: 1715 (C=O), 1625 (C=C) cm^{-1}. ^{1}H-NMR: 1.3—2.1 (12H, m), 2.49 (2H, t, J = 2.5 Hz, 4-CH_2), 5.16 (1H, m, C=CH_2), 5.80 (1H, m, C=CH_2). ^{13}C-NMR: 26.5 (2C, t), 38.3 (2C, t), 40.9 (t, 4-C), 41.4 (2C, t), 55.9 (s, 5-C), 68.0 (s, 1-C), 117.7 (t, C=$\underline{C}H_2$), 147.0 (s, 3-C), 212.8 (s, 2-C). MS *m/z*: 176 (M^+, 59), 107 (100), 65 (53).

Dimer **56**, mp 72.5—74.0 °C, recrystallized from CH_2Cl_2. *Anal.* Calcd for $C_{24}H_{32}O_2$: C, 81.77; H, 9.15. Found: C, 81.59; H, 9.15. IR (KBr): 1730 (C=O), 1695 (C=C–O) cm^{-1}. ^{1}H-NMR: 1.0—2.4 (m). ^{13}C-NMR: 19.3 (t), 26.0 (t), 26.2 (2C, t), 26.5 (2C, t), 27.6 (t), 36.9 (t), 37.0 (t), 38.2 (t), 38.5 (t), 41.6 (2C, t), 41.9 (t), 44.8 (t), 47.1 (t), 55.6 (s), 57.5 (s), 65.5 (s), 66.8 (s), 84.7 (s, spiro carbon), 104.4 (s, $\underline{C}$=C–O) 150.4 (s, C=$\underline{C}$–O), 218.8 (s, C=O). MS *m/z*: 352 (M^+, 100), 177 (87), 176 (64).

(*E*)-Propylidene Triquinane Compound 18——A solution of **24** (2.76 g, 16.8 mmol) in dry THF (40 ml) was added dropwise to a stirred solution of lithium diisopropylamide (LDA, 26.9 mmol) in dry THF (60 ml) (prepared as described above) at −78 °C during 1 h under a nitrogen atmosphere. The reaction mixture was stirred for 1 h and propionaldehyde (1.23 ml, 16.8 mmol) was added *via* a syringe. After 1 h, the mixture was allowed to warm to room temperature and then stirred for 1 h. Saturated ammonium chloride solution was added and the resulting mixture was extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **18** (2.05 g, 69% yield, ether : petroleum ether = 3 : 97), recovered **24** (0.37 g), and aldols (0.49 g, ether : petroleum ether = 3 : 7). Dehydration of the aldols as described for the preparation of **16** gave **18** (0.13 g, 4% yield from **24**). *Anal.* Calcd for $C_{14}H_{20}O$: C, 82.30; H, 9.87. Found: C, 82.27; H, 10.12. IR: 1710 (C=O), 1640 (C=C) cm^{-1}: ^{1}H-NMR: 1.08 (3H, t, J = 8 Hz, CH_3), 1.3—2.0 (12H, m), 2.14 (2H, qd, J = 8, 2 Hz, $C\underline{H}_2CH_3$), 2.42 (2H, m, 4-CH_2), 6.30 (1H, m, C=CH). ^{13}C-NMR: 12.3 (q, CH_3), 24.4 (t, $\underline{C}H_2CH_3$), 26.1 (2C, t), 37.9 (2C, t), 38.3 (t, 4-C), 41.4 (2C, t), 55.4 (s, 5-C), 67.9 (s, 1-C), 137.7 (d, C=$\underline{C}$H), 138.4 (s, 3-C), 211.6 (s, 2-C). MS *m/z*: 204 (M^+, 100), 175 (50), 96 (57).

(*E*)-Carbomethoxypentylidene Triquinane Compound 19——Methyl 6-oxohexanoate was prepared by using the method of Ballini and Petrini,[17] bp 66—68 °C/3 mmHg (lit.[39] 59—61 °C/1 mmHg). Reaction of **24** (0.50 g, 3.01 mmol) and the aldehyde (0.48 g, 3.35 mmol) as described above gave recovered **24** (0.06 g) and **19** (0.64 g, 85% yield, ether : petroleum ether = 2 : 8) after chromatography. *Anal.* Calcd for $C_{18}H_{26}O_3$: C, 74.44; H, 9.03. Found: C, 74.11; H, 9.15. IR: 1740 (C=O), 1710 (C=O), 1640 (C=C), 1165 (O=C–O) cm^{-1}. ^{1}H-NMR: 1.2—1.9 (16H, m),

2.0—2.4 (4H, m), 2.46 (2H, m, 4-CH_2), 3.60 (3H, s, OCH_3), 6.30 (1H, m, C=CH). ^{13}C-NMR: 24.2 (t), 26.1 (2C, t), 27.3 (t), 28.9 (t), 33.2 (t), 38.0 (2C, t), 38.5 (t, 4-C), 41.4 (2C, t), 50.8 (q, OCH_3), 55.5 (s, 5-C), 68.0 (s, 1-C), 135.9 (d, C=CH), 139.4 (s, 3-C), 173.1 (s, O=C-O), 211.7 (s, 2-C). MS *m/z*: 290 (M^+, 100), 203 (60), 79 (51).

5-Oxopentyl (*E*)-Cinnamate——A solution of (*E*)-cinnamoyl chloride (8.01 g, 48.1 mmol) in dry ether (50 ml) was added dropwise to a stirred solution of 1,5-pentanediol (5.00 g, 48.1 mmol) and pyridine (3.89 ml, 48.1 mmol) in dry ether (200 ml) at room temperature during 6 h under a nitrogen atmosphere. The reaction mixture was heated at reflux with stirring for 2 h and water was added to the cooled mixture. The organic layer was separated and the aqueous layer was extracted with ether. The combined extracts were washed with 10% sulfuric acid (H_2SO_4) and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give 5-hydroxypentyl (*E*)-cinnamate (5.61 g, 50% yield, ether: petroleum ether = 7 : 3). IR: 3450 (OH), 1710 (C=O), 1640 (C=C), 1580 (C_6H_5) cm^{-1}.

A solution of pyridine (23.3 ml, 288 mmol) in CH_2Cl_2 (200 ml) was stirred with a mechanical stirrer at 0 °C and anhydrous chromium(VI) oxide (14.4 g, 144 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and then a solution of the above hydroxy ester in CH_2Cl_2 (50 ml) was added. The reaction mixture was stirred for 1 h and decanted from the residue, which was washed with ether. The combined organic solutions were washed with two portions of 10% sodium hydroxide (NaOH) solution, two portions of 5% HCl, saturated $NaHCO_3$ solution, and brine successively, then dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give 5-oxopentyl (*E*)-cinnamate (3.32 g, 60% yield, ether : petroleum ether = 15 : 85). IR: 2820, 2720 (CHO), 1725, 1710 (C=O), 1640 (C=C), 1580 (C_6H_5) cm^{-1}. ^{1}H-NMR: 1.4—1.9 (4H, m, CH_2CH_2), 2.43 (2H, m, CH_2CHO), 4.12 (2H, m, OCH_2), 6.33 (1H, d, J = 16 Hz, C=CHC=O), 7.2—7.5 (5H, m, C_6H_5), 7.57 (1H, d, J = 16 Hz, C=CHC$_6$H$_5$), 9.72 (1H, m, CHO). MS *m/z*: 232 (M^+, trace), 132 (100), 103 (54).

(*E*)-[(*E*)-Cinnamyloxy]pentylidene Triquinane Compound 20——Reaction of **24** (0.48 g, 2.93 mmol) and the above aldehyde (0.68 g, 2.93 mmol) as described in the literature[19] gave recovered **24** (0.13 g) and **20** (0.57 g, 71% yield, ether: petroleum ether = 1 : 9) after chromatography, mp 37—38 °C, recrystallized from pentane. *Anal.* Calcd for $C_{25}H_{30}O_3$: C, 79.33; H, 7.99. Found: C, 79.03; H, 8.03. IR (KBr): 1710 (C=O), 1655, 1640 (C=C), 1580 (C_6H_5) cm^{-1}. ^{1}H-NMR: 1.3—2.3 (18H, m), 2.40 (2H, m, 4-CH_2), 4.10 (2H, t, J = 6 Hz, OCH_2), 6.30 (1H, m, C=CHCH$_2$), 6.32 (1H, d, J = 16 Hz, C=CHC=O), 7.2—7.5 (5H, m, C_6H_5), 7.57 (1H, d, J = 16 Hz, C=CHC$_6$H$_5$). ^{13}C-NMR: 24.7 (t), 26.4 (2C, t), 28.3 (t), 29.0 (t), 38.3 (2C, t), 38.8 (t, 4-C), 41.7 (2C, t), 55.8 (s, 5-C), 63.9 (t, OCH_2), 68.4 (s, 1-C), 118.0 (d, C=CHC=O), 127.9, 128.8, 130.1, 134.3 (d, d, d, s, C_6H_5), 136.1 (d, C=CHCH$_2$), 139.8 (s, 3-C), 144.5 (d, C=CHC$_6$H$_5$), 166.7 (s, O=C-O), 212.3 (s, 2-C). MS *m/z*: 378 (M^+, 10), 131 (100).

2-Methyltricyclo[3.3.3.0]undecan-2-ol (33)——A 1.2 N solution of MeLi (4.34 ml, 5.20 mmol) in ether was added to a stirred solution of **24** (0.57 g, 3.47 mmol) in dry ether (20 ml) *via* a syringe at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred for 15 min and then MeOH (0.21 ml, 5.20 mmol) was added *via* a syringe. After 1 min, MeLi (4.34 mmol) was added and the reaction mixture was stirred for 15 min. This procedure was repeated a total of eight times.[20] Water was added and the organic layer was separated. The aqueous layer was extracted with ether. The combined extracts were washed with brine and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give recovered **24** (0.10 g) and **33** (0.46 g, 89% yield, ether: petroleum ether = 5 : 95), which was purified by preparative GLC, mp 96.5—98.5 °C. *Anal.* Calcd for $C_{12}H_{20}O$: C, 79.94; H, 11.18. Found: C, 79.68; H, 11.23. IR (KBr): 3450 (OH) cm^{-1}. ^{1}H-NMR: 0.8—2.0 (m, containing s at 1.18). MS *m/z*: 180 (M^+, 11), 109 (59), 108 (100).

2-Methyltricyclo[3.3.3.0]undec-2-ene (34)——Thionyl chloride (0.62 ml, 8.50 mmol) was added to a stirred solution of **33** (0.90 g, 5.00 mmol) and pyridine (2.80 ml, 35.0 mmol) in CH_2Cl_2 (10 ml) *via* a syringe at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 4 h. Ice-water was carefully added and the resulting mixture was extracted with CH_2Cl_2. The combined extracts were washed with 5% HCl, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was carefully removed *in vacuo* and the residue was chromatographed to give **34** (0.78 g, 96% yield, petroleum ether). *Anal.* Calcd for $C_{12}H_{18}$: C, 88.82; H, 11.18. Found: C, 88.67; H, 11.44. IR: 3020, 790 (C=C) cm^{-1}. ^{1}H-NMR: 1.2—1.8 (15H, m), 2.14 (2H, m, 4-CH_2), 4.98 (1H, m, 3-CH). MS *m/z*: 162 (M^+, 51), 133 (100).

2-Methyl-3-oxatetracyclo[4.3.3.0.0^{2,4}]dodecane (35)——A solution of MCPBA (1.09 g, 7.68 mmol) in $CHCl_3$ (30 ml) was added to a stirred mixture of **34** (0.78 g, 4.81 mmol) and disodium hydrogen phosphate (1.09 g, 7.68 mmol) in $CHCl_3$ (15 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 4 h and then water was added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined extracts were washed with saturated $NaHSO_3$ solution, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in cacuo* and the subsequent flash chromatography (ether : peptroleum ether = 1 : 9) gave **35** (0.56 g, 65% yield). *Anal.* Calcd for $C_{12}H_{18}O$: C, 80.85; H, 10.18. Found: C, 80.70; H, 10.40. IR: 3000, 835 (epoxide) cm^{-1}. ^{1}H-NMR: 1.2—2.1 (17H, m, containing s at 1.29), 3.18 (1H, d, J = 2 Hz, 4-CH). MS *m/z*: 178 (M^+, 63), 108 (68), 107 (100).

Propellane-Type Triquinane Compound 21——A 1.5 M solution of *n*-BuLi (15.1 ml, 22.7 mmol) in hexane was added to a stirred solution of diethylamine (2.53 ml, 22.7 mmol) in dry ether (25 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and then at room temperature. A solution of **35** (2.02 g, 11.3 mmol) in dry ether (10 ml) was

added to the above lithium diethylamide solution.[21] The mixture was heated at reflux with stirring for 6 h and then water was added to the cooled mixture. The organic layer was separated and the aqueous layer was extracted with ether. The combined extracts were washed with 5% HCl, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **36**. IR: 3300 (OH), 1660 (C=C) cm^{-1}.

The crude alcohol **36** was oxidized with Collins reagent as described above to give recovered **35** (0.34 g) and **21** (1.12 g, 68% yield from **35**) after flash chromatography (ether : petroleum ether = 15 : 85). *Anal.* Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.58; H, 9.38. IR: 1715 (C=O), 1625 (C=C) cm^{-1}. ^{1}H-NMR: 1.2—2.0 (12H, m), 2.31 (2H, s, 4-CH_2), 5.18 (1H, s, C=CH_2), 5.82 (1H, s, C=CH_2). ^{13}C-NMR: 26.0 (2C, t), 42.1 (2C, t), 42.9 (2C, t), 52.4 (t, 4-C), 54.3 (s, 5-C), 62.3 (s, 1-C), 117.7 (t, C=CH_2), 156.3 (s, 2-C), 209.5 (s, 3-C). MS *m/z*: 176 (M^+, 12), 134 (100).

2-Methylenetricyclo[3.3.3.0]undecane (37)——A 2 N solution of sodium *tert*-amylate (30.5 ml, 61.0 mmol) in benzene was added to a stirred mixture of triphenylmethylphosphonium bromide (16.3 g, 45.7 mmol) in dry benzene (50 ml) at room temperature under a nitrogen atmosphere, then a solution of **24** (2.50 g, 15.2 mmol) in dry benzene (20 ml) was further added. The reaction mixture was heated at reflux with stirring for 3 h and then water was added to the cooled mixture. The organic layer was separated and the aqueous layer was extracted with ether. The combined extracts were washed with brine and dried ($MgSO_4$). The solvent was carefully removed *in vacuo* and the subsequent flash chromatography (petroleum ether) gave **37** (2.42 g, 98% yield). *Anal.* Calcd for $C_{12}H_{18}$: C, 88.82; H, 11.18. Found: C, 88.84; H, 11.31. IR (CCl_4): 3050, 1645 (C=C) cm^{-1}. ^{1}H-NMR: 1.3—1.8 (14H, m), 2.26 (2H, td, J=7, 2 Hz, 3-CH_2), 4.67 (2H, t, J=2 Hz, C=CH_2). MS *m/z*: 162 (M^+, 38), 134 (100).

An Alternative Synthesis of 21——A mixture of selenium(IV) oxide (0.77 g, 7.00 mmol) and 80% *tert*-butylhydroperoxide (3.50 ml, 28 mmol) in CH_2Cl_2 (11 ml) was stirred at 25 °C for 30 min.[22] The mixture was cooled to 0 °C, and **37** (2.26 g, 14.0 mmol) was added. This mixture was stirred at 25 °C for 2 h, then saturated $NaHSO_3$ solution and water were added. The resulting mixture was extracted with ether. The combined extracts were washed with brine and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **21** (1.20 g, 49% yield, ether : petroleum ether = 1 : 9) and **36** (1.18 g, 47% yield, ether : petroleum ether = 2 : 8).

Methyl Tricyclo[3.3.2.0]decan-9-carboxylate (40)——A mixture of **24** (5.00 g, 30.5 mmol) and ethyl formate (3.70 ml, 45.7 mmol) was added dropwise to a stirred ice-cooled suspension of sodium hydride (1.47 g, 50%, 30.5 mmol) and ethanol (EtOH, 1.5 ml) in dry ether (90 ml). The mixture was stirred at room temperature for 3 h and then allowed to stand overnight. Water was added and the organic layer was separated and washed with three portions of 10% NaOH solution. The combined aqueous layer was acidified with conc. HCl. The solid that separated was taken up in ether. The combined extracts were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo* to give **38** as a white solid. IR (KBr): 1680, 1600, 1520, 1180 (O=C–C=C–OH) cm^{-1}.

p-Toluenesulfonyl azide (7.40 g, 37.5 mmol) was added dropwise to a stirred solution of the crude hydroxymethylene ketone **38** in triethylamine (9.50 ml, 68.2 mmol) and CH_2Cl_2 (27 ml) cooled in an ice-salt bath. The mixture was stirred at that temperature for 2 h. A solution of potassium hydroxide (KOH, 3.00 g) in water (27 ml) was then added and the whole was stirred at room temperature for 30 min. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined extracts were washed with KOH solution and two portions of water, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **39**. IR: 2070 (N_2), 1655 (C=O) cm^{-1}.

A solution of the crude diazoketone **39** in MeOH (300 ml) was irradiated in a Pyrex vessel with a 500 W high-pressure mercury lamp for 36 h. The solvent was removed *in vacuo* and the residue was chromatographed to give **40** (3.62 g, 61% yield from **24**, ether : petroleum ether = 5 : 95). *Anal.* Calcd for $C_{12}H_{18}O_2$: C, 74.19; H, 9.34. Found: C, 74.58; H, 9.43. IR: 1730 (C=O), 1165 (O=C–O) cm^{-1}. ^{1}H-NMR: 1.1—2.3 (14H, m), 2.63 (1H, dd, J=10, 7 Hz, 9-CH), 3.61 (3H, s, OCH_3). ^{13}C-NMR: 29.5 (t), 30.1 (t), 30.6 (t), 35.0 (t), 38.5 (t), 39.2 (t), 39.8 (t), 41.3 (d, 9-C), 51.2 (q, OCH_3), 53.3 (s, 5-C), 60.1 (s, 1-C), 174.6 (s, C=O). MS *m/z*: 194 (M^+, 2), 108 (100), 80 (56).

9-Acetyltricyclo[3.3.2.0]decane (42)——A mixture of **40** (3.42 g, 17.6 mmol) and KOH (2.50 g) in MeOH (35 ml) was heated at reflux with stirring for 3 h. The solvent was removed *in vacuo* and water was added to the residue. The mixture was extracted with petroleum ether. The combined extracts were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo* to give recovered **40** (0.21 g). The aqueous layer was acidified with 6 N HCl and extracted with ether. The combined extracts were washed with brine, dried ($MgSO_4$), and concentrated *in vacuo* to give **41**, as a white solid. IR (KBr): 3500—2500 (CO_2H), 1685 (C=O) cm^{-1}.

A 1.2 N solution of MeLi (35.8 ml, 43.0 mmol) in ether was added dropwise to a stirred solution of the crude carboxylic acid **41** in dry ether (70 ml) at 0 °C during 1 h under a nitrogen atmosphere. The reaction mixture was stirred at room temperature overnight and then water was carefully added at 0 °C. The organic layer was separated and the aqueous layer was extracted with ether. The combined extracts were washed with brine and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **42** (1.86 g, 63% yield form **40**, ether : petroleum ether = 5 : 95). IR: 1700 (C=O) cm^{-1}. ^{1}H-NMR: 1.1—2.3 (17H, m, containing s at 1.91), 2.68 (1H, dd, J=9, 8 Hz, 9-CH). MS *m/z*: 178 (M^+, 5), 108 (100), 107 (70), 80 (67).

2-Oxatricyclo[3.3.3.0]undecan-3-one (46)[24]——A solution of **42** (1.78 g, 10.0 mmol) and MCPBA (1.96 g, 12.0 mmol) in $CHCl_3$ (50 ml) was stirred at 40 °C for 40 h. The mixture was washed with saturated $NaHSO_3$ solution, saturated $NaHCO_3$ solution, and brine successively, and dried ($MgSO_4$). The solvent was removed *in vacuo* to give **43**. IR: 1735 (C=O), 1240, 1040 (O=C–O) cm^{-1}.

A solution of the crude acetate **43** in dry ether (15 ml) was added to a stirred solution of $LiAlH_4$ (0.95 g, 25.0 mmol) in dry ether (15 ml) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. Water was carefully added to the cooled mixture at 0 °C and then 5% HCl was added. The organic layer was separated and the aqueous layer was extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **44** (1.42 g, 87% yield from **42**, ether : petroleum ether = 15 : 85), which was identical with a sample prepared by an alternative method.[24]

Oxidation of **44** (0.80 g, 5.25 mmol) with Collins reagent as described above gave **45**[24] (0.70 g, 89% yield, ether : petroleum ether = 5 : 95) after chromatography. Baeyer-Villiger oxidation[27] of **45** (4.88 g, 29.3 mmol) with 30% hydrogen peroxide and 9.3 N NaOH in MeOH afforded **46** (4.53 g, 86% yield, ether : petroleum ether = 2 : 8) after chromatography.

Propellane-Type Oxatriquinane Compound 22——α-Hydroxymethylation of **46** (2.00 g, 12.1 mmol) as described previously[11,15] gave recovered **46** (1.23 g) and the primary alcohol (0.65 g, 70% yield, ether : petroleum ether = 8 : 2) after chromatography. IR: 3450 (OH), 1745 (C=O) cm^{-1}. ^{1}H-NMR: 1.3—2.3 (12H, m) 2.53 (1H, t, J = 7 Hz, 4-CH), 2.82 (1H, br s, OH), 3.80 (2H, d, J = 7 Hz, CH_2OH). Dehydration of the alcohol was carried out as described for the preparation of **16** to give **22** (0.44 g, 78% yield from the alcohol, ether : petroleum ether = 2 : 8) after chromatography. *Anal.* Calcd for $C_{11}H_{14}O_2$: C, 74.13; H, 7.92. Found: C, 73.95; H, 8.10. IR: 1745 (C=O), 1650 (C=C) cm^{-1}. ^{1}H-NMR: 1.5—2.2 (12H, m), 5.51 (1H, s, C=CH_2), 6.09 (1H, s, C=CH_2). ^{13}C-NMR: 25.8 (2C, t), 38.3 (2C, t), 41.0 (2C, t), 59.8 (s, 5-C), 102.1 (s, 1-C), 122.2 (t, C=$\underline{C}H_2$), 146.2 (s, 4-C), 171.3 (s, 3-C). MS *m/z*: 178 (M^+, 80), 150 (100), 122 (89), 79 (84).

5-Hydroxytricyclo[4.3.2.0^{1,5}]undec-10-ene (49)——A solution of **47**[13b] (7.18 g, 44.3 mmol) and conc. HCl (15 ml) in ether (120 ml) was heated at reflux with stirring for 10 h. Water was added and the organic layer was separated. The aqueous layer was extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **48** (6.97 g, 79% yield, ether : petroleum ether = 5 : 95). IR: 3470 (OH), 725 (C=C) cm^{-1}

A solution of *n*-Bu_3SnH (15.3 g, 52.6 mmol) in cyclohexane (100 ml) was added to a stirred solution of **48** (6.96 g, 35.1 mmol) and 2,2′-azobisisobutyronitrile (1.0 g) in cyclohexane (50 ml) at room temperature under a nitrogen atmosphere. The reaction mixture was heated at reflux with stirring for 9 h. The solvent was removed *in vacuo* and the residue was chromatographed to give **49** (4.71 g, 82% yield, ether : petroleum ether = 2 : 8), which was purified by preparative GLC, mp 42—43 °C. IR (KBr): 3380 (OH), 3030 (C=C), 1120 (C–OH), 725 (C=C) cm^{-1}: 1.0—2.2 (13H, m), 2.50 (1H, m, 6-CH), 5.42 (1H, d, J = 6 Hz, 10-CH), 5.68 (1H, dd, J = 6, 2.5 Hz, 11-CH). ^{13}C-NMR: 17.2 (t), 20.0 (t), 22.8 (t), 24.1 (t), 32.6 (t), 37.3 (t), 47.6 (d, 6-C), 55.9 (s, 1-C), 87.8 (s, 5-C), 131.1, 137.6 (d, d, C-10 and C-11). MS *m/z*: 164 (M^+, 93), 108 (96), 97 (100). Hydrogenation of **49** (0.25 g, 1.55 mmol) as described above gave a saturated alcohol (0.23 g, 92% yield) which was identical (mp, IR, and ^{13}C-NMR spectra) with the known alcohol.[13b]

Angular-Type Triquinane Compound 23——A solution of **49** (1.00 g, 6.10 mmol) and 50% H_2SO_4 (8 ml) in THF (20 ml) was stirred at *ca.* 60 °C for 24 h. Water was added and the resulting mixture was extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give recovered **49** (0.06 g) and **50** (0.21 g, 22% yield, ether : petroleum ether = 1 : 9). IR: 3450 (OH), 3030, 740 (C=C) cm^{-1}.

Thionyl chloride (0.42 ml, 5.79 mmol) was added to a stirred solution of **50** (0.63 g, 3.86 mmol) in dry ether (7 ml) *via* a syringe at 0 °C. The reaction mixture was stirred at 0 °C for 4 h and then ice-water was carefully added. The resulting mixture was extracted with ether. The combined extracts were washed with saturated $NaHCO_3$ solution and brine, and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was chromatographed to give **51** (0.37 g, 53% yield, petroleum ether). IR: 3030, 760 (C=C) cm^{-1}.

Reduction of **51** (0.37 g, 2.03 mmol) with *n*-Bu_3SnH as described above gave **52** (0.24 g, 80% yield, elution with petroleum ether) which was identical (IR and ^{1}H-NMR spectra) with the sample obtained from **55**.[29] The ketone **54** was prepared as reported[29] by hydroboration-oxidation of **52** followed by Collins oxidation of **53** (3.5 : 1, the epimer mixture).

α-Methylenation of **54** (3.11 g, 18.9 mmol) as described for the preparation of **14** gave recovered **54** (0.85 g) and **23** (0.95 g, 40% yield from **54**) after chromatography (ether : petroleum ether = 3 : 97). *Anal.* Calcd for $C_{12}H_{16}O$: C, 81.77; H, 9.15. Found: C, 81.66; H, 9.44. IR: 1715 (C=O), 1625 (C=C) cm^{-1}. ^{1}H-NMR: 1.2—2.1 (12H, m), 2.13 (1H, dd, J = 12, 6 Hz, 5-CH), 2.70 (1H, m, 8-CH), 5.14 (1H, m, C=CH_2), 5.80 (1H, m, C=CH_2). ^{13}C-NMR: 26.2 (t), 26.6 (t), 31.6 (t), 36.1 (t), 40.9 (t), 41.7 (t), 51.3 (d, 8-C), 55.6 (s, 1-C), 59.7 (d, 5-C), 117.7 (t, C=$\underline{C}H_2$), 152.0 (s, 7-C), 217.7 (s, 6-C). MS *m/z*: 176 (M^+, 100), 148 (49), 119 (45).

Antiproliferation Assay *in Vitro*——The assay was performed according to our previous method[40] with a slight modification. Test murine cells maintained in our laboratory (Toray) were cultured in RPMI 1640 medium containing 2-mercaptoethanol (2×10^{-5} M) for P388 (lymphocytic leukemia) and L1210 (lymphocytic leukemia), RPMI 1640 medium for 3LL (Lewis lung tumor), or Eagle's MEM for LY (subcutaneous tissue). Each medium was supplemented with 10% precolostrum newborn calf serum (Mitsubishi Chemical Industries Ltd., Tokyo). Polyquinanes were dissolved in acetone at a concentration of 10 mg/ml followed by dilution with dimethylsulfoxide

(DMSO) at 1 mg/ml. Alternatively, five test compounds (**3**, **4**, **8**, **12**, and **13**) were directly dissolved in DMSO at 1 mg/ml. These solutions were further diluted with the cell culture medium to appropriate concentrations. Test cells (2×10^4/0.9 ml/well) were seeded into culture plates (24 flat-bottomed wells, Flow Laboratories, Inc., U.S.A.) and sample solutions (0.1 ml/well) were added simultaneously. In the control group, corresponding amounts of the organic solvents were added. After 4 d of culture at 37 °C in a 5% CO_2 incubator (NAPCO, U.S.A.), the cell number was determined with a Coulter counter (model TA II, Coulter Electronics, Inc., U.S.A.). The IC_{50} values (μg/ml) required to produce 50% reduction of cell number *versus* control culture were determined and are summarized in Table I.

Biomimetic Reactions[32]——(1) Treatment of **8** with Adenosine: A solution of **8** (49 mg, 0.28 mmol) and adenosine (91 mg, 0.34 mmol) in a mixture of pH 7.4 potassium phosphate buffer solution (2.5 ml) and EtOH (2.5 ml) was stirred at room temperature for 7 d. Ethanol was removed *in vacuo* and the residue was extracted with ethyl acetate. The combined extracts were washed with brine and dried ($MgSO_4$). The solvent was removed *in vacuo* to give recovered **8** (41 mg).

(2) Treatment of **8** with Propanethiol: Propanethiol (0.77 ml, 8.52 mmol) was added *via* a syringe to a stirred solution of **8** (50 mg, 0.28 mmol) in a mixture of pH 9.2 borate buffer solution (1.6 ml) and THF (2.4 ml) at room temperature. The reaction mixture was stirred for 13 h and then water was added. The mixture was extracted with ether. The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo*. Flash chromatography (ether : petroleum ether = 8 : 92) of the residue gave **57** (68 mg, 95% yield), mp 22—23 °C. *Anal.* Calcd for $C_{15}H_{24}OS$: C, 71.38; H, 9.58. Found: C, 71.33; H, 9.63. IR: 1730 (C=O) cm^{-1}. ^{1}H-NMR: 0.99 (3H, t, J=4 Hz, CH_3), 1.2—2.3 (18H, m), 2.44 (2H, t, J=7 Hz, SCH_2CH_2), 2.88 (1H, t, J=4 Hz, 2-CH). MS *m/z*: 252 (M^+, 34), 177 (100).

(3) Treatment of **8** with L-Cysteine: A solution of L-cysteine (35 mg, 0.29 mmol) in pH 7.4 potassium phosphate buffer solution (0.7 ml) was added to a stirred solution of **8** (53 mg, 0.30 mmol) in EtOH (1.3 ml) at room temperature, with bubbling of nitrogen through the solution. A white precipitate appeared immediately. The mixture was further stirred for 15 min and subjected to filtration to give a white solid. The solid was washed with water, EtOH, and ether successively, to give **58** (74 mg, 82% yield based on L-cysteine), which seemed to be a mixture of diastereomers. We could not determine the ratio or separate them due to their insolubility in organic solvents and only slight solubility in water. Recrystallization from water gave an analytical sample as fine needles, which seemed to be one of the diastereomers, mp 113—115 °C (dec.). *Anal.* Calcd for $C_{15}H_{24}NO_4S$: C, 57.12; H, 7.99; N, 4.44. Found: C, 56.72; H, 7.70; N, 4.34. IR (KBr): 3650—3200 (CO_2H), 1730 (C=O), 1620 (H_2O) cm^{-1}. MS *m/z*: 279 ($M^+ - 2H_2O$, 6), 44 (75), 18 (100).

(4) Treatment of **8** with L-Lysine: Reaction of **8** (50 mg, 0.28 mmol) and L-lysine·HCl·H_2O (57 mg, 0.31 mmol) as described above gave recovered **8** (46 mg).

(5) Treatment of **8** with L-Serine: Reaction of **8** (51 mg, 0.29 mmol) and L-serine (33 mg, 0.31 mmol) as described above gave recovered **8** (47 mg).

Kinetic Measurement——The second-order kinetics for addition of L-cysteine to polyquinanes were measured according to the methods in the literature.[3a,34,35] To determine the quantity of remaining L-cysteine, the λ_{max} (343 nm) and the molar extinction (6.96×10^3, lit.[35] 7.06×10^3) of 2-thiopyridone, which was produced by reaction of L-cysteine and 2,2'-dipyridyl disulfide, were used. Since the second-order kinetics were nonlinear, the rate constants were calculated from initial rates (25—40% reaction),[34] and were the averages of at least three runs at the same sample concentration. In the cases of **16**, **18**, and **19**, the reactions proceeded too slowly at 1 °C to give accurate rate constants. Therefore, these were extrapolated from Arrhenius plots obtained by measurement at different temperatures. The rate constants are as follows: **16**, k_2=0.59 (l/mol·s) at 10 °C, 0.36 at 5 °C; **18**, k_2=0.32 at 10 °C, 0.16 at 5 °C; **19**, k_2=0.51 at 15 °C, 0.27 at 10 °C.

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Synthesis and Hypotensive Activity of Benzopyran Derivatives[1)]

MASAMI SHIRATSUCHI,*,[a] KIYOSHI KAWAMURA,[a] TOSHIHIRO AKASHI,[a]
MIKIO FUJII,[a] HIROSHI ISHIHAMA,[a]
and YASUMI UCHIDA[b]

Tokyo Research Laboratories, Kowa Company, Ltd.,[a] Noguchi-cho, Higashimurayama, Tokyo 189, Japan and Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo,[b] Bunkyo-ku, Tokyo 113, Japan

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A series of dihydrobenzopyranyloxypropanolamines and dihydrobenzopyranylethanolamines containing a nitroxy moiety was synthesized. The cardiovascular effects of these compounds were investigated in anesthetized dogs. Some of the compounds exhibited hypotensive activity in combination with β-adrenergic blocking and vasodilating action. The structure–activity relationships are discussed.

Keywords——β-adrenergic blocking activity; vasodilating activity; antihypertensive drug; dihydrobenzopyran; nitrate; nipradilol

β-Adrenergic receptor antagonists (β-blockers) have been applied to the treatment of hypertension, but the onset of their actions is often slow and they are effective in only about 50% of hypertensive patients. Vasodilators that were similarly applied to the treatment of hypertension cause tachycardia, and so β-blockers are often prescribed in combination with vasodilators for the therapy of hypertension. In an attempt to obtain both β-blocking and vasodilative actions in a single molecule, we synthesized novel dihydrobenzopyran derivatives having the side chain characteristic of β-blockers and a nitroxyalkyl moiety that was expected to have vasodilating activity, and tested them for hypotensive activity in dogs.

Chemistry

The phenolic precursors (**25**—**28**) were prepared from methoxybenzopyrans[2)] (**21**—**24**) by catalytic hydrogenation and subsequent demethylation (Chart 1).

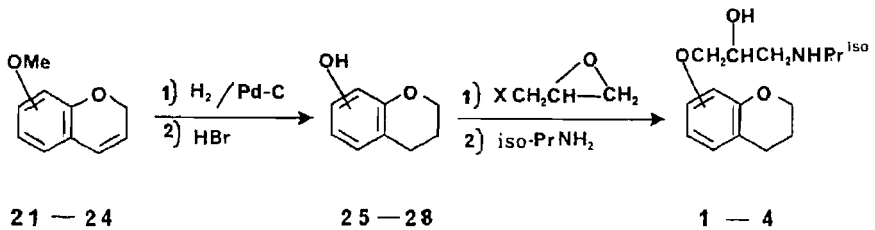
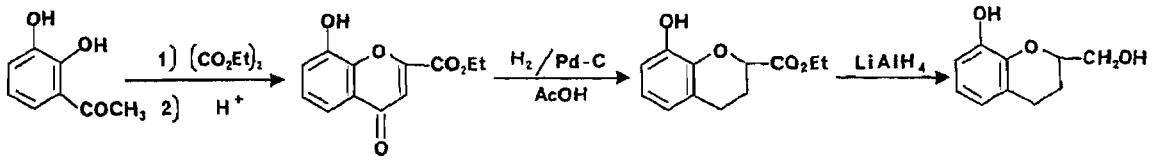


Chart 1

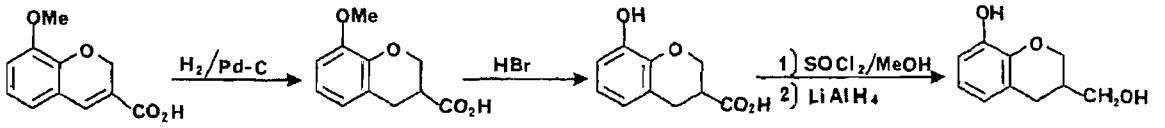
3,4-Dihydro-8-hydroxy-2-hydroxymethyl-2*H*-1-benzopyran (**54**) was prepared from 2′,3′-dihydroxyacetophenone (**29**) (Chart 2). Thus, condensation of **29** with diethyl oxalate and subsequent ring closure with conc. HCl gave chromone (**30**), which was hydrogenated on 10% Pd–C and subsequently reduced with $LiAlH_4$ to give **54**. 3,4-Dihydro-8-hydroxy-3-hydroxymethyl-2*H*-1-benzopyran (**55**) was prepared from 8-methoxy-2*H*-1-benzopyran-3-carboxylic acid[3)] (**32**) (Chart 3). Thus, catalytic hydrogenation of **32** on 10% Pd–C gave 3,4-

dihydro-8-methoxy-2*H*-1-benzopyran-3-carboxylic acid (**33**), which was demethylated with 47% HBr, followed by esterification and reduction with $LiAlH_4$ to give **55**. Similarly, ethyl 3,4-dihydro-8-methoxy-2*H*-1-benzopyran-4-carboxylate[4] (**35**) was led to 3,4-dihydro-8-hydroxy-4-hydroxymethyl-2*H*-1-benzopyran (**56**) (Chart 4).



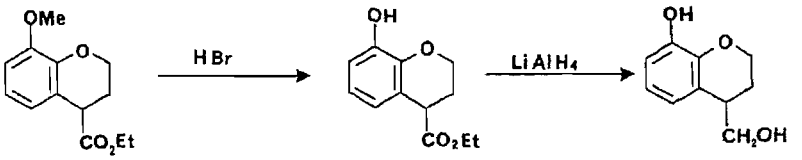
29 **30** **31** **54**

Chart 2



32 **33** **34** **55**

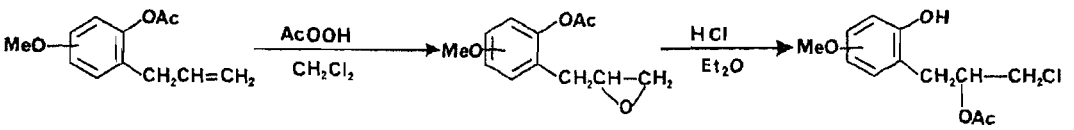
Chart 3



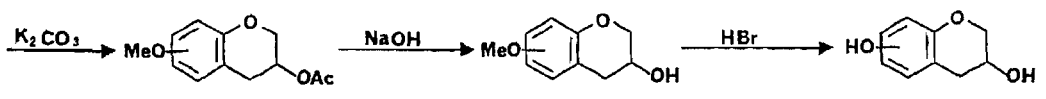
35 **36** **56**

Chart 4

Dihydroxybenzopyrans (**57**—**59**) were prepared from methoxy-2-allylphenylacetates[5] (**37**—**39**) (Chart 5). Thus, treatment[6] of **37**—**39** with 40% (w/v) peracetic acid in methylene chloride afforded epoxides (**40**—**42**), which were led to chlorohydrins (**43**—**45**), with opening of the epoxide ring and rearrangement of the acetyl moiety, by treatment with HCl. Ring closure of **43**—**45** with K_2CO_3 gave acetoxymethoxybenzopyrans (**46**—**48**), followed by hydrolysis and demethylation to give **57**—**59**.



37—**39** **40**—**42** **43**—**45**



46—**48** **49**—**51** **57**—**59**

Chart 5

6-Acetyl-3,4-dihydro-3,8-dihydroxy-2*H*-1-benzopyran (**60**) was obtained by acetylation of the diacetoxy compound (**52**) with acetyl chloride and $AlCl_3$ and subsequent hydrolysis with NaOH (Chart 6).

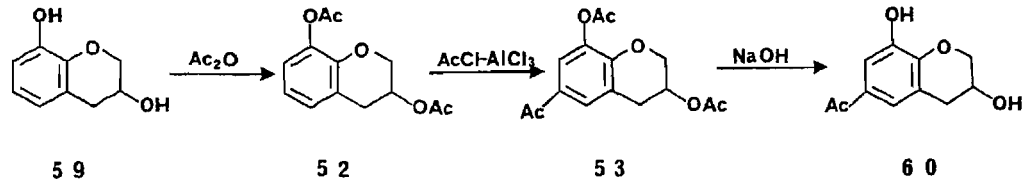


Chart 6

The nitrates (**61**—**67**) were prepared by acylation of hydroxyalkyl-3,4-dihydro-2*H*-1-benzopyrans (**54**—**60**) with acyl chloride and subsequent esterification with fuming nitric acid in acetic anhydride at low temperature and subsequent hydrolysis with NaOH. Glycidylation of compounds **25**—**28**, and **61**—**67** with epihalohydrin gave the epoxides, which were then aminated with the appropriate amines to produce the desired compounds (**1**—**17**) (Charts 1 and 7).

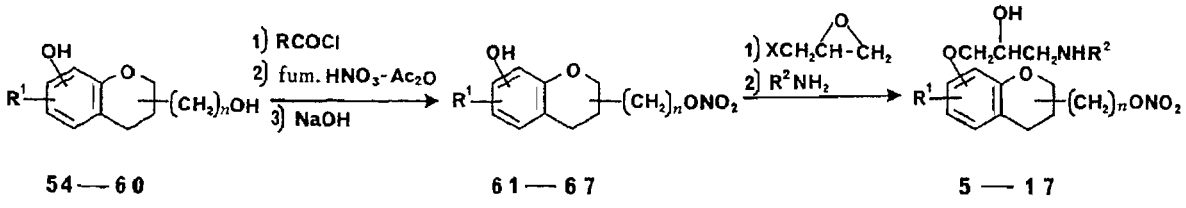
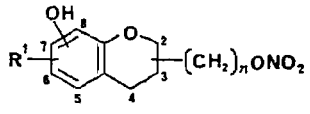


Chart 7

TABLE I. Hydroxynitroxyalkyl-3,4-dihydro-2*H*-1-benzopyrans

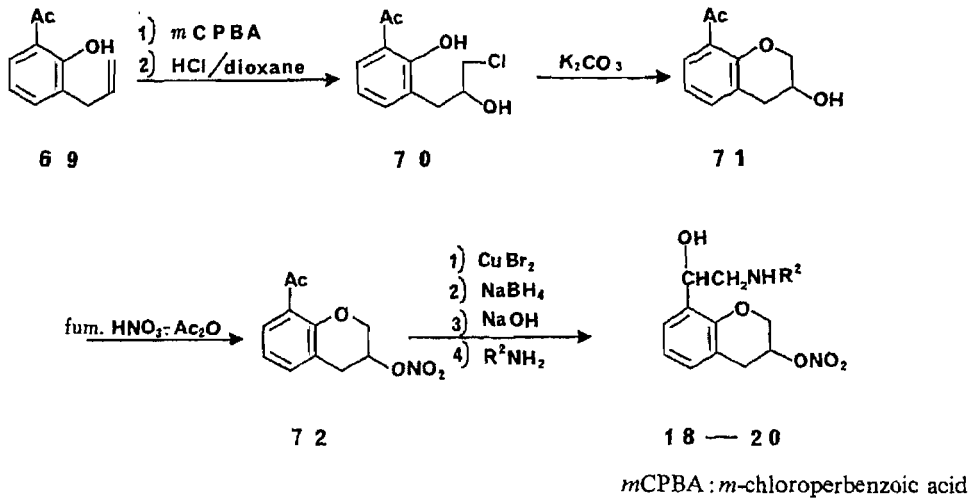


Compd. No.	OH	R^1	$(CH_2)_nONO_2$	Yield[a] (%)	mp (°C)	Formula[b]
61	8-OH	H	2-CH_2ONO_2	67	72—74	$C_{10}H_{11}NO_5$
62	8-OH	H	3-CH_2ONO_2	71	58—61	$C_{10}H_{11}NO_5$
63	8-OH	H	4-CH_2ONO_2	49	Oil	$C_{10}H_{11}NO_5$
64	5-OH	H	3-ONO_2	66	129	$C_9H_9NO_5$
65	6-OH	H	3-ONO_2	42	115—117	$C_9H_9NO_5$
66	8-OH	H	3-ONO_2	65	101—103	$C_9H_9NO_5$
67	8-OH	6-$COCH_3$	3-ONO_2	66	116—118	$C_{11}H_{11}NO_5$
68	8-OH	5,6-Br_2 (or 5,7-, or 6,7-)	3-ONO_2	73[c]	148—150	$C_9H_7Br_2NO_5$

a) Total yields of acylation, esterification and hydrolysis. *b*) All compounds were analyzed for C, H and N; the analytical results were within ±0.4% of the calculated values. *c*) Yield of bromination of **66** with *N*-bromosuccinimide.

The benzopyranylethanolamines (**18**—**20**) in Table IV were prepared by the route shown in Chart 8. Thus, epoxidation of 2-hydroxy-3-(2-propenyl)acetophenone[7] (**69**) with *m*-chloroperbenzoic acid, followed by treatment with HCl and cyclization with K_2CO_3 yielded 8-acetyl-3,4-dihydro-3-hydroxy-2*H*-1-benzopyran (**71**). The nitrate (**72**) was obtained from **71**

by treatment with fuming nitric acid in acetic anhydride. Compounds **18**—**20** were synthesized from **72** according to the literature.[8]



*m*CPBA : *m*-chloroperbenzoic acid

Chart 8

Pharmacological Test Methods

Mongrel dogs (3 to 7 dogs per group), whose systolic blood pressure was 104±3 mmHg and heart rate was 133±3 bpm, were anesthetized with intravenous sodium pentobarbital. A cuffed endotracheal tube was inserted into the trachea, and respiration was maintained by means of a Harvard respirator. Catheters were placed in the aortic arch *via* the femoral artery for measurement of systemic blood pressure, and in the left femoral vein for drug administration. Systemic blood pressure was measured by a pressure transducer (Statham, P23ID). Heart rate was measured with a cardiotachometer triggered by the *R* wave of the electrocardiogram (the second limb lead). Pressure and heart rate were recorded on a linear recorder (Nihon Koden, WT 683G). A compound was administered by bolus intravenous injection in two or three dogs at a dose of 100 μg/kg, and the results are shown in Tables II—IV. Each compound was dissolved in saline or equimolar diluted hydrochloric acid, and the solution was diluted with saline to a final volume of 0.1 ml/kg.

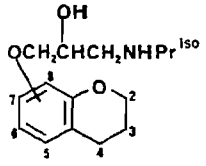
Results and Discussion

The effects of compounds **1**—**20** on blood pressure and heart rate are summarized in Tables II—IV. The introduction of an isopropylaminohydroxypropoxy side chain at the 5- or 8- position of the benzopyran ring resulted in a strong suppressive action on heart rate (compound **1** or **4**).

The nitrates are known to be typical vasodilators, and so we synthesized compounds **5**—**7**, introducing a nitroxyalkyl moiety into compound **4**. The hypotensive activity of these compounds was slightly stronger than that of compound **4**. However, the hypotensive activity was transient. It is likely that the nitrates of primary alcohols are easily reduced enzymatically. Therefore we synthesized the nitrates of secondary alcohols **8**—**10**. Compound **9** showed the strongest hypotensive activity among the tested compounds and the duration of action was long. However, it caused an increase in heart rate due to sympathetic reflex. Compound **10** showed strong hypotensive and negative chronotropic actions. In addition, the duration of action was very long (systemic blood pressure (SBP) reduction, 16±3 mmHg; heart rate (HR) decrease, 16±4 bpm).

Compounds **11**—**12**, derivatives of compound **10**, and compounds **13**—**17**, in which the

TABLE II. Dihydrobenzopyranyloxypropanolamines



Compd. No.	Position	Yield[a] (%)	mp (°C)	Formula[b]	SBP[c] Change	SBP[c] Duration (min)	HR[d] Change	HR[d] Duration (min)
Propranolol[e]					−1	0	−3	30
Nitroglycerin[f]					−4	10	+3	3
1	5	13	65—68	$C_{15}H_{23}NO_3$	−1	30	−3	>30
2	6	59	78—81	$C_{15}H_{23}NO_3$	±	—	−1	30
3	7	48	97—99	$C_{15}H_{23}NO_3$	±	—	−1	30
4	8	15	78—79	$C_{15}H_{23}NO_3$	−1	30	−3	>30

a) Total yields of glycidylation and amination. *b*) See footnote *b* in Table I. *c*) Systemic blood pressure level is indicated as follows: (−1), 1—5 mmHg reduction; (−2), 6—10 mmHg reduction; (−3), 11—20 mmHg reduction; (−4), >20 mmHg reduction. *d*) Heart rates are indicated showed as follows: (+1), 1—5 beats per minute (bpm) increase; (+2), 6—10 bpm increase; (+3), 11—20 bpm increase; (−1), 1—5 bpm decrease; (−2), 6—10 bpm decrease; (−3), 11—20 bpm decreases; (−4), >20 bpm decrease. *e*) Dose: 100 μg/kg. *f*) Dose: 20 μg/kg.

isopropylamino moiety was replaced by other alkylamino groups, showed a weaker hypotensive activity than compound **10**. The order of potency of hypotensive action for amino substituents was iso-Pr > *tert*-Bu > Et > H.

Compounds **18**—**20**, aminoethanol derivatives, showed strong hypotensive and positive chronotropic actions.

These results suggest that the nitroglycerin-like action of a compound whose β-blocking action is weak may be strong. This study showed that compound **10** had the most desirable actions among the tested compounds. Therefore we selected compound **10** as a clinical candidate, and named it nipradilol. Furthermore, Uchida and coworkers[9] found that nipradilol had β-blocking and vasodilating actions in SHR not only on arterial but also on venous vessels, resulting in regulatory effects on pre- and after-load. Therefore, nipradilol may be a new type of antihypertensive drug.

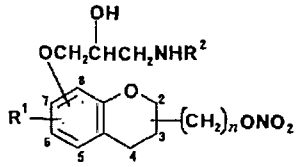
Experimental

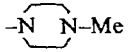
All melting points are uncorrected. Infrared (IR) spectra were measured with JASCO IRA-1 and Shimadzu IR-435 spectrometers. Proton magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz with a Varian EM-360 spectrometer and at 100 MHz with a JEOL JNM-MH-100 spectrometer. Chemical shifts are expressed in δ (ppm) values. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, and br = broad. Mass spectra (MS) were measured with JEOL JMS-D-300 and JMS-D-100 mass spectrometers.

Hydroxy-3,4-dihydro-2*H*-1-benzopyrans (25—28) (Table V)——General Procedure: A solution of **21**—**24** (2.0 g) in AcOH (20 ml) was hydrogenated over 10% Pd–C (1.0 g) at room temperature under atmospheric pressure overnight. The catalyst was removed by filtration and washed with EtOH (20 ml). The filtrate and washings were combined and concentrated *in vacuo*. AcOH (5 ml) and 47% (w/v) HBr (20 ml) were added to the oily residue. The mixture was stirred at 100—110 °C for 1—2 h. The reaction mixture was concentrated to one-third of its original volume, then AcOEt (20 ml) and satd. brine were added. The AcOEt layer was washed with satd. brine and dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was then chromatographed on a silica gel column using benzene as an eluent to give pure **25**—**28**.

Ethyl 8-Hydroxy-4-oxo-4*H*-1-benzopyran-2-carboxylate (30)——A solution of 2′,3′-dihydroxyacetophenone

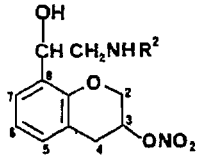
TABLE III. Nitroxyalkyldihydrobenzopyranyloxypropanolamines



Compd. No.	R^1	$-OCH_2\underset{OH}{C}HCH_2NHR^2$ Position	R^2	$(CH_2)_nONO_2$	Yield[a] (%)	mp (°C)	Formula[b]	SBP[c] Change	SBP[c] Duration (min)	HR[d] Change	HR[d] Duration (min)
5	H	8	iso-Pr	2-CH_2ONO_2	49	85—86	$C_{16}H_{24}N_2O_6$	−1	5	−3	30
6	H	8	iso-Pr	3-CH_2ONO_2	64	59—61	$C_{16}H_{24}N_2O_6$	−2	5	−3	30
7	H	8	iso-Pr	4-CH_2ONO_2	39	122—123	$C_{16}H_{24}N_2O_6 \cdot H_2O$	−2	15	±	0
8	H	5	iso-Pr	3-ONO_2	52	82—85	$C_{15}H_{22}N_2O_6$	−1	5	−3	>30
9	H	6	iso-Pr	3-ONO_2	50	113—118	$C_{15}H_{22}N_2O_6$	−4	>30	+2	>30
10[e]	H	8	iso-Pr	3-ONO_2	61	110—122	$C_{15}H_{22}N_2O_6$	−3	>30	−3	>30
11	6-$COCH_3$	8	iso-Pr	3-ONO_2	61	107—110	$C_{17}H_{24}N_2O_7$	−1	5	±	0
12	5,6-Br_2 (or 6,7- or 7,8-)	8	iso-Pr	3-ONO_2	46	130—136	$C_{15}H_{20}Br_2N_2O_6$	−2	3	+3	3
13	H	8	*tert*-Bu	3-ONO_2	46	135—143	$C_{16}H_{24}N_2O_6(COOH)_2$	−2	20	−3	>30
14	H	8	Et	3-ONO_2	30	117—124	$C_{14}H_{20}N_2O_6$	−1	15	−4	>30
15	H	8	H	3-ONO_2	5	140—144	$C_{12}H_{16}N_2O_6 \cdot HCl \cdot H_2O$	−1	5	−3	>30
16	H	8	CH_2Ph	3-ONO_2	43	138—144	$C_{19}H_{22}N_2O_6$	−1	1	+1	3
17	H	8		3-ONO_2	58	115—125	$C_{17}H_{25}N_3O_6 \cdot 2HCl \cdot H_2O$	±	0	±	0

a) Total yields of glycidylation and amination. *b*) See footnote *b* in Table I. *c*, *d*) See footnotes *c*, *d* in Table II. *e*) Nipradilol.

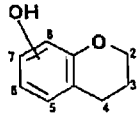
TABLE IV. Nitroxydihydrobenzopyranylethanolamines



Compd. No.	R^2	Yield[a] (%)	mp (°C)	Formula[b]	SBP[c] Change	SBP[c] Duration (min)	HR[d] Change	HR[d] Duration (min)
18	iso-Pr	31	125—134	$C_{14}H_{20}N_2O_5$	−4	>30	+3	>30
19	CH_2Ph	26	90—101	$C_{18}H_{20}N_2O_5$	−3	5	+3	3
20	$CH_2CH_2CH_2Ph$	15	93—101	$C_{20}H_{24}N_2O_5$	−3	5	+2	3

a) Total yields of bromination, reduction, hydrolysis and amination. *b*) See footnote *b* in Table I. *c*, *d*) Footnotes, *c*, *d* in Table II.

TABLE V. Hydroxy-3,4-dihydro-2*H*-1-benzopyrans



Compd. No.	R	Yield (%)	mp (°C)	^{1}H-NMR ($CDCl_3$)
25	5-OH	49	68—70	1.8—2.1 (2H, m), 2.5—2.8 (2H, m), 4.0—4.3 (2H, m), 5.7—6.1 (1H, br s), 6.2—6.5 (2H, m), 6.8—7.0 (1H, m)
26	6-OH	88	Oil	1.8—2.1 (2H, m), 2.5—2.8 (2H, m), 3.9—4.3 (2H, m), 6.4—6.8 (4H, m)
27	7-OH	23	Oil	1.8—2.1 (2H, m), 2.5—2.8 (2H, m), 4.0—4.2 (2H, m), 6.2—6.4 (2H, m), 6.8—6.9 (1H, m)
28	8-OH	60	Oil	1.8—2.1 (2H, m), 2.6—2.8 (2H, m), 4.1—4.3 (2H, m), 5.6 (1H, s), 6.4—6.8 (3H, m)

(**29**) (1.53 g) and diethyl oxalate (5.5 g) in EtOH (10 ml) was added to NaOEt (1.5 g) in EtOH (30 ml) at room temperature during 5 min, and the mixture was heated under reflux for 3 h. Conc. HCl (15 ml) was added to the reaction mixture, and the whole was heated under reflux for 3 h. A precipitate was removed by filtration, the filtrate was concentrated *in vacuo*, and AcOEt (150 ml) was added to the oily residue. The AcOEt extract was washed with H_2O, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was then purified on a silica gel column using $CHCl_3$–MeOH (10 : 1) as an eluent. Recrystallization from MeOH and AcOEt gave **30** (1.70 g, 72.6%) as colorless prisms, mp 196—200 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.47 (3H, t, J=6 Hz, CH_3), 4.45 (2H, q, J=6 Hz, CH_3), 6.98 (1H, s, C_3-H), 7.18—7.55 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1720 (COOEt), 1630 (CO). MS *m/z*: 234 (M^+).

3,4-Dihydro-8-hydroxy-2-hydroxymethyl-2*H*-1-benzopyran (54)——Compound **30** (11.4 g) was dissolved in AcOH (150 ml), and hydrogenated over 10% Pd–C (13.7 g) at 70 °C under atmospheric pressure for 4 h. The catalyst was filtered off, the filtrate was concentrated *in vacuo*, and anhydrous ether (140 ml) was added to the oily residue (**31**). The ether solution was added dropwise to $LiAlH_4$ (4.65 g) in anhydrous ether (70 ml) with stirring at 5 °C, and the whole was stirred at 10 °C for 2.5 h. The reaction mixture was added to an ice-cold mixture of H_2O (200 ml), AcOEt (200 ml) and 1 N HCl (50 ml). The organic layer was washed with H_2O, dried, and concentrated *in vacuo* to leave a brown solid. Recrystallization from AcOEt and hexane provided **54** (1.84 g, 21.0%) as colorless prisms, mp 146—148 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 1.50—2.05 (2H, m, C_3-H), 3.60—3.96 (2H, m, CH_2O), 4.00—4.30 (1H, m, C_2-H), 6.44—6.73 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3340 (OH). MS *m/z*: 180 (M^+).

3,4-Dihydro-8-methoxy-2*H*-1-benzopyran-3-carboxylic Acid (33)——Compound **32** (21.0 g) in AcOH (300 ml) was hydrogenated over 10% Pd–C (10.0 g) at room temperature under atmospheric pressure for 12 h. The catalyst was filtered off, and the filtrate was concentrated *in vacuo*. Recrystallization of the crystalline residue from MeOH

provided **33** (7.5 g, 35.4%) as colorlss needles, mp 180—182 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 2.80—3.10 (3H, m, C_3-H and C_4-H), 3.80 (3H, s, OCH_3), 3.90—4.70 (2H, m, C_2-H), 6.60—6.80 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1698 (COOH). MS *m/z*: 208 (M^+).

3,4-Dihydro-8-hydroxy-2*H*-1-benzopyran-3-carboxylic Acid (34)——Compound **33** (10.4 g) was heated in 47% HBr (105 ml) at 120 °C for 1 h. The reaction mixture was cooled to 5 °C, and the resulting precipitate was filtered. Recrystallization from AcOEt provided **34** (6.75 g, 74.1%) as colorless prisms, mp 177—179 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 2.90—3.20 (3H, m, C_3-H and C_4-H), 3.98—4.70 (2H, m, C_2-H), 6.50—6.80 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1700 (COOH). MS *m/z*: 194 (M^+).

3,4-Dihydro-8-hydroxy-3-hydroxymethyl-2*H*-1-benzopyran (55)——Thionyl chloride (3.24 g) was added to **34** (3.53 g) in MeOH (35 ml) at 2 °C, and the reaction mixture was heated under reflux for 1 h, then evaporated *in vacuo*. The residue was dissolved in Et_2O (40 ml), and the solution was added to $LiAlH_4$ (2.08 g) in Et_2O (60 ml) at 1—3 °C. The mixture was stirred at 10 °C for 5 h and extracted with AcOEt (80 ml) after addition of aqueous HCl (50 ml). The AcOEt extract was washed with H_2O, dried over anhydrous sodium sulfate and concentrated *in vacuo* to give **55** (2.92 g, 89.5%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 2.00—2.90 (3H, m, C_3-H and C_4-H), 3.40—3.70 (2H, m, CH_2OH), 3.80—4.35 (2H, m, C_2-H), 6.40—6.80 (3H, m, Ar-H). IR ν_{max}^{film} cm^{-1}: 3350 (OH). MS *m/z*: 180 (M^+).

3,4-Dihydro-8-hydroxy-4-hydroxymethyl-2*H*-1-benzopyran (56)——Compound **35** (4.10 g) was dissolved in AcOH (10 ml), the solution was added to 47% HBr (80 ml), and the mixture was stirred at 110 °C for 4 h, then concentrated *in vacuo*. The residue was dissolved in $CHCl_3$ (100 ml). The $CHCl_3$ solution was washed with satd. brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo* to give ethyl 3,4-dihydro-8-hydroxy-2*H*-1-benzopyran-4-carboxylate (**36**) (2.81 g) as a brown viscous oil. $LiAlH_4$ (2.21 g) was added to **36** (2.81 g) in anhydrous Et_2O (50 ml) at 1—3 °C, and the mixture was stirred at room temperature for 2 h. The reaction mixture was added to ice-cold water (100 ml), and the whole was extracted with AcOEt (100 ml). The organic layer was washed with satd. brine, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to leave a brown viscous oil. The oil was chromatographed on a silica gel column using $CHCl_3$–MeOH (100 : 1, v/v) to give **56** (2.12 g, 67.9%) as a colorless viscous oil. ^{1}H-NMR ($CDCl_3$) δ: 1.96—2.20 (2H, m, C_3-H), 2.80—3.10 (1H, m, C_4-H), 3.70—3.96 (2H, m, CH_2O), 4.15—4.36 (2H, m, C_2-H), 6.60—6.85 (3H, m, Ar-H). IR ν_{max}^{film} cm^{-1}: 3350 (OH). MS *m/z*: 180 (M^+).

2-(2,3-Epoxy)propyl-6-methoxyphenyl Acetate (42)——A 40% (w/v) peracetic acid solution (3.0 ml) and AcOK (0.18 g) were added to 2-allyl-6-methoxyphenyl acetate (1.82 g) in CH_2Cl_2 (20 ml), and the mixture was stirred at room temperature for 48 h. The reaction mixture was added to $CHCl_3$ (100 ml) and 5% aqueous Na_2SO_3 (50 ml), and the $CHCl_3$–CH_2Cl_2 layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to give **42** (1.74 g, 88.7%) as a pale yellow viscous oil. ^{1}H-NMR ($CDCl_3$) δ: 2.36 (3H, s, $-OCOCH_3$), 2.47—2.96 (4H, m, $-CH_2-CH-CH_2$), 3.00—3.35 (1H, m, $-CH_2-CH-CH_2$), 3.88 (3H, s, $-OCH_3$), 6.90—7.40 (3H, m, Ar-H).

2-(2-Acetoxy-3-chloro)propyl-6-methoxyphenol (45)——A 20% (w/v) ethereal HCl solution (5.0 ml) was added to **42** (1.74 g) in Et_2O (10 ml) at 0 °C, and the mixture was stirred at room temperature for 12 h. Et_2O (100 ml) was added to the reaction mixture, which was neutralized with saturated aqueous $NaHCO_3$. The Et_2O layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to give **45** (1.83 g, 90.4%) as a pale yellow viscous oil. NMR ($CDCl_3$) δ: 2.09 (3H, s, $-OCOCH_3$), 2.90—3.34 (2H, m, $-CH_2-CH-CH_2Cl$), 3.67—3.87 (2H, m, $-CH-CH_2Cl$), 3.94 (3H, s, $-OCH_3$), 5.25—5.66 (1H, m, $-HC<$), 6.08 (1H, m, OH), 6.83—7.17 (3H, m, Ar-H).

3-Acetoxy-3,4-dihydro-8-methoxy-2*H*-1-benzopyran (48)——K_2CO_3 (1.26 g) was added to **45** (1.82 g) in *N*,*N*-dimethylformamide (DMF) (10 ml) with stirring at room temperature for 2 h. H_2O (50 ml) and benzene (100 ml) were then added to the reaction mixture, and the benzene layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to give **48** (1.53 g) as a pale yellow viscous oil. ^{1}H-NMR ($CDCl_3$) δ: 2.04 (3H, s, $-OCOCH_3$), 2.70—3.32 (2H, m, C_4-H), 3.88 (3H, s, $-OCH_3$), 4.08—4.48 (2H, m, C_2-H), 5.20—5.40 (1H, m, C_3-H), 6.60—6.98 (3H, m, Ar-H). IR ν_{max}^{film} cm^{-1}: 1731 ($OCOCH_3$). MS *m/z*: 222 (M^+).

3,4-Dihydro-3-hydroxy-8-methoxy-2*H*-1-benzopyran (51)——Compound **48** (1.53 g) was dissolved in MeOH (30 ml), and then 1 N NaOH (10 ml) was added to the MeOH solution. The mixture was stirred at room temperature for 1 h, added to 1 N HCl (12 ml), and concentrated *in vacuo*. The residue was extracted with benzene (30 ml). The benzene layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to leave a brown viscous oil. Crystallization of the oil from benzene–hexane provided **51** (0.66 g, 52.1%) as colorless prisms, mp 79—82 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.40—3.26 (3H, m, C_4-H and OH), 3.85 (1H, s, OCH_3), 3.87—4.20 (3H, m, C_2-H and C_3-H), 6.35—6.87 (3H, m, Ar-H). MS *m/z*: 180 (M^+).

3,4-Dihydro-3,8-dihydroxy-2*H*-1-benzopyran (59)——Compound **51** (1.50 g) was added to 47% HBr (8.60 g), and the mixture was stirred at 90 °C for 9 h. The reaction mixture was cooled to room temperature, made weakly acidic with aqueous NaOH, and extracted with AcOEt (30 ml). The AcOEt layer was washed with satd. brine, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to leave a brown solid, which was crystallized from acetone. Recrystallization from acetone and hexane gave **59** (1.23 g, 88.9%) as colorless needles, mp 126—129 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.56—3.20 (2H, m, C_4-H), 3.90—4.30 (4H, m, C_2-H, C_3-H and OH), 6.44—6.80 (3H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3360 (OH). MS *m/z*: 166 (M^+).

3,4-Dihydro-3,5-dihydroxy-2*H*-1-benzopyran (57)——Compound **57** was synthesized from 2-allyl-3-methoxyphenyl acetate (**37**) *via* a route similar to that described above. Pale yellow viscous oil. ^{1}H-NMR (CD_3OD) δ:

2.40—3.10 (2H, m, C_4-H), 3.60—4.30 (3H, m, C_2-H and C_3-H), 6.24—6.44 (2H, m, Ar-H), 6.76—6.96 (1H, m, Ar-H). MS *m/z*: 166 (M^+).

3,4-Dihydro-3,6-dihydroxy-2*H*-1-benzopyran (58)——Compound **58** was synthesized from 2-allyl-4-methoxyphenyl acetate (**38**) *via* a route similar to that described above. Colorless needles (from acetone), mp 110—111 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 2.50—3.20 (2H, m, C_4-H), 3.70—4.50 (4H, m, C_2-H, C_3-H and OH), 6.45—6.80 (3H, m, Ar-H). MS *m/z*: 166 (M^+).

3,8-Diacetoxy-3,4-dihydro-2*H*-1-benzopyran (52)——Triethylamine (4.05 g) and Ac_2O (40.9 g) were successively added to a solution of **59** (16.7 g) in $CHCl_3$ (100 ml), and the mixture was stirred at room temperature for 12 h. Then 10% aqueous K_2CO_3 was added to the reaction mixture, and the $CHCl_3$ layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to leave pale yellow crystals. Recrystallization of the crystals from benzene and hexane gave **52** (21.0 g, 83.5%) as colorless prisms, mp 101—103 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.00 (3H, s, C_3-$OCOCH_3$), 2.28 (3H, s, C_8-$OCOCH_3$), 2.65—3.30 (2H, m, C_4-H), 4.10—4.35 (2H, m, C_2-H), 5.12—5.30 (1H, m, C_3-H), 6.80—7.00 (3H, m, Ar-H).

6-Acetyl-3,8-diacetoxy-3,4-dihydro-2*H*-1-benzopyran (53)——$AlCl_3$ (13.5 g) was added to **52** (12.6 g) in nitrobenzene (100 ml) at 1 °C during 0.5 h, and the mixture was stirred for 10 h at room temperature. Petroleum ether (800 ml) was added to the reaction mixture, the solvent was decanted off, and the residual brown viscous oil was partitioned between benzene (500 ml) and aqueous HCl (800 ml). The benzene layer was washed with H_2O, dried over anhydrous sodium sulfate, and evaporated *in vacuo*. The brown residue was chromatographed on a silica gel column using $CHCl_3$–benzene (1 : 1, v/v) as an eluent. Recrystallization of the crude crystals from benzene gave **53** (4.68 g, 31.8%) as colorless prisms, mp 155—158 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.03 (3H, s, C_3-$OCOCH_3$), 2.30 (3H, s, C_8-$OCOCH_3$), 2.50 (3H, s, C_6-$COCH_3$), 2.80—3.38 (2H, m, C_4-H), 4.10—4.46 (2H, m, C_2-H), 5.20—5.38 (1H, m, C_3-H), 7.49 (1H, d, J=2 Hz, Ar-H), 7.56 (1H, d, J=2 Hz, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 1735 (CH_3COO), 1670. MS *m/z*: 292 (M^+).

6-Acetyl-3,4-dihydro-3,8-dihydroxy-2*H*-1-benzopyran (60)——A 2 N NaOH solution (15 ml) was added to **53** (3.03 g) in MeOH (25 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was adjusted to pH 4 with aqueous HCl, and extracted with AcOEt (350 ml). The AcOEt layer was washed with saturated brine, dried over anhydrous sodium sulfate, and then evaporated *in vacuo*. Recrystallization of the solid from AcOEt provided **60** as colorless needles, mp 185—187 °C. ^{1}H-NMR (CD_3OD–$CDCl_3$) δ: 2.50 (3H, s, $COCH_3$), 2.62—3.24 (2H, m, C_4-H), 7.20—7.30 (2H, m, Ar-H). IR ν_{max}^{KBr} cm^{-1}: 3390 (OH), 3160 (OH), 1655 ($COCH_3$). MS *m/z*: 208 (M^+).

Nitroxyalkyl-3,4-dihydro-2*H*-1-benzopyrans (61—67) (Table I)——General Procedure: A solution of 23 mmol of ethyl chloroformate (or trichloroacetyl chloride) in tetrahydrofuran (THF) (35 ml) was added to a solution of the appropriate hydroxyalkyl-3,4-dihydro-2*H*-1-benzopyran (**54—60**) (20 mmol) and triethylamine (23 mmol) in THF (20 ml) at 0—5 °C and the mixture was stirred at the same temperature for 0.5 h, then filtered. The filtrate was concentrated under reduced pressure. The residue (4.85 g) was dissolved in acetonitrile (80 ml), the solution was cooled to −8—−10 °C, and acetyl nitrate [prepared from Ac_2O (2.22 g) and fum. HNO_3 (1.36 g)] was added to the solution at −8 °C. The mixture was stirred at the same temperature for 0.5 h, and once again acetyl nitrate [prepared from Ac_2O (1.36 g) and fum. HNO_3 (0.85 g)] was added at −8 °C. The mixture was stirred at the same temperature for 0.5 h, quenched with aqueous $NaHCO_3$ and extracted with AcOEt (180 ml). The organic layer was washed with aqueous $NaHCO_3$ and satd. brine, dried, and evaporated to dryness. The residual brown oil was dissolved in a mixture of MeOH (10 ml) and a solution of NaOH (0.93 g) in H_2O (5 ml), and the mixture was stirred at room temperature for 0.5 h. The reaction mixture was acidified with conc. HCl, freed of MeOH *in vacuo*, and extracted with AcOEt (60 ml). The extract was washed with satd. brine, dried, and evaporated to dryness. The residual dark brown oil was purified by silica gel column chromatography using benzene as an eluent. The solid eluate was crystallized from benzene and hexane to give the corresponding **61—67** as colorless crystals. The yields were generally 60—75%. 3,4-Dihydro-8-hydroxy-3-nitroxy-2*H*-1-benzopyran (**66**) afforded the following spectral data as an example of this class of compounds. IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1620 and 1280 (ONO_2). ^{1}H-NMR ($CDCl_3$) δ: 2.80—3.40 (2H, m, C_4-H), 4.08—4.49 (2H, m, C_2-H), 5.28—5.48 (1H, m, C_3-H), 5.49 (1H, s, OH), 6.48—6.89 (3H, m, Ar-H).

3,4-Dihydro-2*H*-1-benzopyranyloxypropanolamines (1—4) (Table II) and Nitroxyalkyl-3,4-dihydro-2*H*-1-benzopyranyloxypropanolamines (5—17) (Table III)——General Procedure: A solution of 4 mmol of the appropriate hydroxybenzopyran (**25—29**, **61—67**) in 1 N NaOH (15 ml) was treated with epichlorohydrin (16 mmol) and the whole was heated at 50 °C for 2 h. AcOEt (30 ml) was added to the reaction mixture and the organic layer was washed with 1 N NaOH (10 ml) and satd. brine (10 ml), dried, and evaporated to dryness. The residual oil was used without further purification. Thin layer chromatography (TLC) usually indicated a single product. 3,4-Dihydro-8-(2,3-epoxy)propoxy-3-nitroxy-2*H*-1-benzopyran afforded the following spectral data as an example of this class of compounds. ^{1}H-NMR ($CDCl_3$) δ: 2.64—3.26 (4H, m, C_4-H and $-\underset{\underset{}{}}{CH}-C\underline{H}_2O$), 3.26—3.50 (1H, m, $-\underset{\underset{}{}}{CH}-CH_2$), 3.86—4.56 (4H, m, C_2-H and $-OCH_2-$), 5.32—5.52 (1H, m, C_3-H), 6.60—6.90 (3H, m, Ar-H). A solution of 4 mmol of the epoxide in MeOH (30 ml) was treated with alkylamine (20 mmol). The reaction mixture was heated at 70 °C for 1 h and evaporated to dryness. The residual brown oil was purified by Al_2O_3 column chromatography ($CHCl_3$). The solid eluate was crystallized from AcOEt and hexane. The total yield from the corresponding hydroxybenzopyran was generally 65—75%. The following spectral data were obtained for compound **10** (nipradilol) as an example of this class of compounds. IR ν_{max}^{KBr} cm^{-1}: 3270 (NH), 3100 (OH), 1620 and 1280 (ONO_2). ^{1}H-NMR ($CDCl_3$) δ: 1.06 (6H, d,

J=6 Hz, $-CH(CH_3)_2$), 2.40—3.42 (7H, m, C_4-H, $-CH_2NHCH$ and OH), 5.30—5.52 (1H, m, C_3-H), 6.54—6.86 (3H, m, Ar-H).

3-(3-Chloro-2-hydroxy)propyl-2-hydroxyacetophenone (70)——*m*CPBA (6.6 g) in CH_2Cl_2 (30 ml) was added to a solution of **69** (1.8 g) in CH_2Cl_2 (30 ml) at 3—8 °C, and the mixture was stirred at room temperature for 10 h. The reaction mixture was diluted with $CHCl_3$ (30 ml), treated with 10% aqueous K_2CO_3 (30 ml) and 10% aqueous Na_2SO_3 (50 ml), washed with H_2O, and then dried over anhydrous sodium sulfate. Removal of the solvent from the filtrate under reduced pressure furnished the epoxide (1.83 g, 93.3%). A solution of the epoxide (1.83 g) in THF (10 ml) was treated with 18.5% HCl–dioxane (5 ml) at 5 °C for 1.5 h. The reaction mixture was poured into ice-cold H_2O (20 ml) and extracted with AcOEt (30 ml). Work-up of the AcOEt extract in the usual manner gave a product (2.36 g), which was purified by column chromatography (SiO_2 50 g, hexane : AcOEt = 15 : 1) to furnish **70** (1.21 g, 55.8%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 2.60 (3H, s, CH_3), 2.96 (2H, d, J=6 Hz, CH_2), 3.50—3.65 (2H, m, CH_2Cl), 3.88—4.45 (1H, m, C<u>H</u>(OH)), 6.80—7.85 (3H, m, Ar-H). MS *m/z*: 228 (M^+).

8-Acetyl-3,4-dihydro-3-hydroxy-2*H*-1-benzopyran (71)——K_2CO_3 (0.56 g) was added to a solution of **70** (0.46 g) in DMF (3 ml) at room temperature and the mixture was stirred at room temperature for 2.5 h. The reaction mixture was diluted with H_2O (10 ml), and extracted with benzene (15 ml), and the extract was washed with H_2O (5 ml), then dried over sodium sulfate. Removal of the solvent from the filtrate under reduced pressure gave a product, which was purified by column chromatography (SiO_2 10 g, $CHCl_3$: MeOH = 100 : 1) to furnish **71** (0.25 g, 65.1%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 2.60 (3H, s, CH_3), 2.85—3.10 (2H, m, C_4-H), 3.95—4.55 (3H, m, C_2-H and C_3-H), 6.90—7.85 (3H, m, Ar-H). MS *m/z*: 192 (M^+).

8-Acetyl-3,4-dihydro-3-nitroxy-2*H*-1-benzopyran (72)——Compound **71** (0.98 g) in acetonitrile (10 ml) was cooled to −35 °C and acetyl nitrate [prepared from Ac_2O (0.27 g) and fum. HNO_3 (0.13 ml)] was added to the solution at −30 °C. The mixture was stirred at the same temperature for 0.5 h, and once again the same quantity of acetylnitrate was added at −30 °C. The mixture was stirred at the same temperature for 0.5 h, quenched with aqueous K_2CO_3 and extracted with $CHCl_3$ (45 ml). The organic layer was washed with aqueous K_2CO_3 and satd. brine, dried and evaporated to dryness. The residual brown oil was purified by column chromatography (SiO_2 30 g, benzene : AcOEt = 50 : 1) to furnish **72** (0.95 g, 78.8%), which was crystallized from AcOEt–hexane to give colorless prisms, mp 80—83 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.60 (3H, s, CH_3), 2.90—3.55 (2H, m, C_4-H), 4.30—4.80 (2H, m, C_2-H), 5.45—5.65 (1H, m, C_3-H), 6.92—7.80 (3H, m, Ar-H). MS *m/z*: 237 (M^+).

8-(2-Alkylamino-1-hydroxy)ethyl-3,4-dihydro-3-nitroxy-2*H*-1-benzopyran (18—20) (Table IV)——Compounds **18—20** were prepared by the literature procedure. IR ν_{max}^{KBr} cm^{-1}: 1620—1622 and 1275—1278 (ONO_2). ^{1}H-NMR ($CDCl_3$) δ: 4.10—4.40 (2H, m, C_2-H), 4.90—5.20 (1H, m, C<u>H</u>(OH)), 5.30—5.60 (1H, m, C_3-H).

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References and Notes

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Studies on Prodrugs. VI. Preparation and Characterization of (5-Substituted 2-Oxo-1,3-dioxol-4-yl)methyl Esters of Mecillinam

FUMIO SAKAMOTO,* SHOJI IKEDA, RYOICHI HIRAYAMA, MASARU MORIYAMA, MIKIO SOTOMURA and GORO TSUKAMOTO

Pharmaceuticals Research Center, Kanebo Co., Ltd., 1-5-90, Tomobuchi-cho, Miyakojima-ku, Osaka 534, Japan

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As a new type of mecillinam prodrug, mecillinam (5-substituted 2-oxo-1,3-dioxol-4-yl)methyl esters were prepared. These esters were found to produce at least 4-fold higher mecillinam levels in blood than mecillinam itself after oral administration to mice. Mecillinam (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester hydrochloride was hygroscopic, but its L-tartrate was not.

Keywords——(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester; mecillinam prodrug; oral absorbability; pro-moiety; mecillinam ester

Mecillinam is a unique penicillin derivative which exhibits strong antimicrobial activities against *Escherichia coli* and other gram-negative bacilli.[1] As it is not absorbed orally,[2] its sodium salt is used parenterally and two of its esters, namely pivmecillinam[3] and bacmecillinam[4] are clinically used as oral prodrugs mainly for urinary tract infections.

We have been developing the (5-substituted 2-oxo-1,3-dioxol-4-yl)methyl group as a new, useful pro-moiety, and the effectiveness of the prodrugs of ampicillin[5] and norfloxacin[6] has been reported recently. As an extension of this prodrug study we prepared some new mecillinam (5-substituted 2-oxo-1,3-dioxol-4-yl)methyl esters and examined their oral absorbabilities. The present paper describes the preparation and characterization of these mecillinam esters.

Chemistry

6-Aminopenicillanic acid triethylammonium salt (**1**) was reacted with activated *N*-formylhexamethyleneimine (**2**)[7] to give crude mecillinam (**3**). This product was esterified with the halides (**4**) without further purification to give mecillinam ester hydrochlorides (**5**) as shown in Chart 1. The method for preparing the halides, (5-substituted 4-halomethyl-2-oxo-1,3-dioxoles (**4**), was reported previously.[5,8] We have already reported a convenient and practical method for preparing (5-substituted 2-oxo-1,3-dioxol-4-yl)methyl 6-aminopenicillanate,[9] and the mecillinam esters can also be synthesized by the reaction of these esters and activated *N*-formylhexamethyleneimine (**2**).

Crystals of ester hydrochlorides (**5a**·HCl and **5b**·HCl) were found to have equimolar alcohol of crystallization (isopropanol or ethanol), and **5a**·HCl was hygroscopic. In an attempt to obtain a non-hygroscopic derivative, the hydrochloride was converted to other acid salts. Compound **5a** was found to form crystalline salts with L-tartaric acid, and L- and D-dibenzoyltartaric acid, but not with D-tartaric acid, malonic acid, maleic acid, *p*-toluenesulfonic acid or sulfuric acid. Among the crystalline salts, the L-tartrate did not have solvent of crystallization and was not hygroscopic.

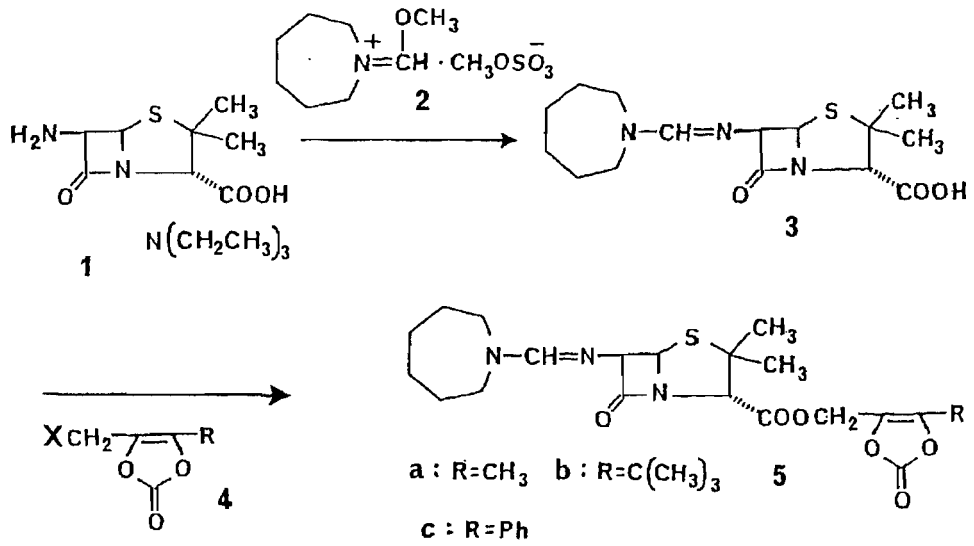

Chart 1. Synthesis of Mecillinam Esters

TABLE I. Serum Concentrations of Mecillinam (μg/ml)

Compd.	15	30	60	90	(min)
5a·HCl·EtOH	24.0	19.0	7.3	3.3	
5a·HCl·iso-PrOH	23.5	17.8	9.6	4.5	
5a·L-tartrate	25.5	22.8	11.9	3.2	
5b·HCl·iso-PrOH	10.1	7.0	3.2	0.8	
5c·HCl	11.2	5.8	2.1	0.6	
Mecillinam	2.3	2.4	2.0	0.7	

Oral Absorption in Mice

In order to estimate the oral absorbability of the mecillinam esters (**5**), serum concentrations of mecillinam were measured in mice after oral administration of these esters, and compared with serum levels after administration of the parent drug (mecillinam). The results are summarized in Table I.

The mecillinam (5-substituted 2-oxo-1,3-dioxol-4-yl)methyl esters were found to be effective as mecillinam prodrugs, showing at least 4-fold higher serum concentrations of mecillinam as compared with mecillinam itself. In particular, (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester (**5a**) showed the highest C_{max} and a normal absorption pattern. There was no significant difference between the oral absorbabilities of the hydrochloride and L-tartrate of **5a**.

Stability Test

To estimate the stability of the mecillinam esters in the digestive tract, hydrolysis tests in artificial gastric juice (pH 1.2) and intestinal juice (pH 6.8)[10] were performed by a bioautography method, as described in the experimental section. The predetermined *Rf* values of mecillinam and its esters (**5**) separately obtained on bioautograms were 0.5 and about 0.9, respectively. All of the test solutions sampled after 10, 30 and 50 min showed the inhibitory zone only at *Rf* about 0.9, and not at *Rf* 0.5. Therefore, it is concluded that these mecillinam esters are stable for a period of more than 50 min in the artificial digestive juices.

Recovery of Mecillinam

In order to confirm the effective hydrolysis of the mecillinam esters to the parent drug *in vivo*, the hydrolysis test in 40% mouse blood was carried out in the same way as the above-mentioned stability tests. Each of the mecillinam esters was dissolved in 40% mouse blood which had been heparinized and diluted with 1/15 M phosphate buffer (pH 7.4). Each solution was shaken at 37 °C and bioautographed after 5 and 10 min.

In the cases of **5a** and **5c**, the inhibitory zones were observed only at *Rf* 0.5 and not at *Rf* about 0.9. On the other hand, the inhibitory zones from **5b** were observed at both *Rf* 0.5 and *Rf* about 0.9, though the latter zone was small at 10 min. From these results, **5a** and **5c** were concluded to be hydrolyzed readily within 5 min at 37 °C in mouse blood *in vitro*, while **5b** was more stable.

Discussion

(5-Substituted 2-oxo-1,3-dioxol-4-yl)methyl groups, which have been developed as pro-moieties of ampicillin prodrugs were applied to mecillinam. The mecillinam esters obtained were well absorbed orally and showed at least 4-fold higher serum concentrations of mecillinam as compared with mecillinam itself.

The 5-methyl derivative (**5a**) was the best compound among these esters and showed a 10-fold higher C_{max} than that of mecillinam, and a normal absorption pattern. Some of the mecillinam ester hydrochlorides were found to contain alcohol of crystallization and were hygroscopic. These properties are different from those of the ampicillin ester hydrochlorides with the same pro-moiety, as well as those of other conventional mecillinam ester hydrochlorides, such as pivmecillinam hydrochloride and bacmecillinam hydrochloride. Formulation of this prodrug was expected to be difficult because of the hygroscopicity. Among the other acid salts of **5a** prepared in an attempt to eliminate this hygroscopicity, the L-tartrate was found to have no solvent of crystallization and was not hygroscopic. Further, it showed good oral absorbability. L-Tartaric acid is widely used as a acidulant for soft drinks and confectionary products,[11] and is considered to be safe.

The 5-*tert*-butyl derivative (**5b**) exhibited poorer oral absorbability than the 5-methyl derivative (**5a**), analogously with the ampicillin ester.[5] This may be partially attributable to the lack of lability in mouse blood, as noted before. On the other hand, the 5-phenyl derivative (**5c**) was labile in mouse blood and stable in artificial digestive juices, but it was not absorbed as well as **5a**. The reason for this poor absorbability is not clear.

The degradation products of mecillinam esters *in vivo* have not yet been examined, but it can be predicted that the mecillinam ester **5a** would be hydrolyzed in the same way as the corresponding ampicillin ester, lenampicillin.[12] Thus, the metabolites of **5a** should be mecillinam, acetoin and 2,3-butanediol.

Experimental

Melting points were determined on a Yamato capillary melting point apparatus, model MR-21, and all melting points are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a Nihon Denshi PS-100 NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Infrared (IR) spectra were recorded with a Shimadzu IR-440 machine. The esters were analyzed for C, H, N, and the values were within 0.4% of the calculated theoretical ones. No attempts were made to maximize the yields.

Mecillinam (5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl Ester Hydrochloride Isopropanoate (5a·HCl·iso-PrOH)—A mixture of 6-aminopenicillanic acid (6.5 g) and triethylamine (12.6 ml) in 60 ml of dichloromethane was stirred at 35 °C for 30 min, then cooled to 0—5 °C. *N*-Formylhexamethylene dimethylsulfate (9.1 g) was added, and the whole was stirred for 1 h at the same temperature. The solvent was evaporated off to give a pale yellow syrup, which was dissolved in a mixture of ethyl acetate (70 ml) and *N*,*N*-dimethylformamide (30 ml). Then, sodium iodide (0.5 g) and 4-chloromethyl-5-methyl-2-oxo-1,3-dioxole (**4a**, X = Cl, 6 g) were added and the mixture was stirred for 20 h at room

temperature. Ethyl acetate (70 ml) and ice water (100 ml) were added, and after vigorous stirring, the organic layer was separated, washed with ice water, mixed with ice water (150 ml) and adjusted to pH 2.5 in the aqueous layer with 2 N hydrochloric acid. The aqueous layer was separated, and sodium chloride was added to saturation. The resulting oily product was extracted with dichloromethane (100 and 50 ml), and the combined dichloromethane extracts were washed with saturated aqueous sodium chloride, dried with anhydrous magnesium sulfate, and concentrated under reduced pressure. The resulting syrup was dissolved in isopropanol (50 ml) and the solution was allowed to stand overnight at 0—5 °C. The solid was filtered off and washed with cold isopropanol to give 8 g of the title compound as colorless crystals (50%). mp 70—71 °C. IR ν_{max}^{KBr} cm^{-1}: 1820, 1790, 1755, 1690. ^{1}H-NMR (DMSO-d_6) δ: 1.4—1.9 (14H, m, 2-CH_3 and $(CH_2)_4$), 2.20 (3H, s, C=C-CH_3), 3.5—4.6 (4H, m, $(CH_2)_2N$), 4.6 (1H, s, 3-H), 5.14 (2H, s, C=C-CH_2), 5.5—5.65 (2H, m, 5- and 6-H), 8.22 (1H, s, CH=N). *Anal.* Calcd for $C_{20}H_{27}N_3O_6S \cdot HCl \cdot C_3H_7OH$: C, 51.72; H, 6.79; N, 7.86. Found: C, 51.46; H, 6.75; N, 7.91.

Mecillinam (5-*tert*-Butyl-2-oxo-1,3-dioxol-4-yl)methyl Ester Hydrochloride Isopropanoate (5b·HCl·iso-PrOH)——According to the above procedure, crude mecillinam was treated with 4-bromomethyl-5-*tert*-butyl-2-oxo-1,3-dioxole (**4b**, X=Br), and the obtained syrup was crystallized from a mixture of isopropanol and ethyl ether to give the title compound as colorless crystals (35%). mp 75—80 °C. IR ν_{max}^{KBr} cm^{-1}: 1835, 1775, 1755, 1690. ^{1}H-NMR (DMSO-d_6) δ: 1.28 (9H, s, $C(CH_3)_3$), 1.4—1.9 (14H, m, 2-CH_3 and $(CH_2)_4$), 3.5—3.8 (4H, m, $(CH_2)_2N$), 4.62 (1H, s, 3-H), 5.17 (2H, s, C=C-CH_2), 5.58 (2H, s, 5- and 6-H), 8.22 (1H, s, CH=N). *Anal.* Calcd for $C_{23}H_{33}N_3O_6S \cdot HCl \cdot C_3H_7OH$: C, 54.20; H, 7.34; N, 7.29. Found: C, 54.01; H, 7.13; N, 7.33.

Mecillinam (2-Oxo-5-phenyl-1,3-dioxol-4-yl)methyl Ester Hydrochloride (5c·HCl)——According to the above procedure, crude mecillinam was treated with 4-bromomethyl-2-oxo-5-phenyl-1,3-dioxole (**4c**, X=Br), and the obtained residue was crystallized from a mixture of acetone and ethyl ether to give the title compound as colorless crystals (30%). mp 85—90 °C. IR ν_{max}^{KBr} cm^{-1}: 1835, 1795, 1760, 1690. ^{1}H-NMR (DMSO-d_6) δ: 1.5—2.1 (14H, m, 2-CH_3 and $(CH_2)_4$), 3.6—4.1 (4H, m, $(CH_2)_2N$), 4.53 (1H, s, 3-H), 5.20 (2H, s, C=C-CH_2), 5.52—5.68 (2H, m, 5- and 6-H), 7.4—7.6 (5H, m, arom. H), 7.74 (1H, s, CH=N). *Anal.* Calcd for $C_{25}H_{29}N_3O_6S \cdot HCl \cdot H_2O$: C, 54.20; H, 5.82; N, 7.58. Found: C, 54.10; H, 6.00; N, 7.32.

Mecillinam (5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl Ester Hydrochloride Ethanolate (5a·HCl·EtOH)——A solution of 10 g of **5a**·HCl·iso-PrOH in ice water (50 ml) was treated with active carbon under ice cooling and filtered. The aqueous solution was saturated with sodium chloride, and the separated oily product was extracted with dichloromethane (60 and 20 ml). The combined dichloromethane extracts were washed with saturated aqueous sodium chloride, dried and concentrated under reduced pressure to give a colorless syrup. The syrup was dissolved in ethanol (10 ml) and mixed with diisopropyl ether (25 ml), and the mixture was allowed to stand overnight at 0—5 °C. The resulting solid was filtered off and washed with a cold mixed solvent (diisopropyl ether : ethanol, 2 : 1) to give 8 g of the title compound as colorless crystals. mp 89—91 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 1820, 1780, 1750, 1680. ^{1}H-NMR (DMSO-d_6) δ: 1.4—1.9 (14H, m, 2-CH_3 and $(CH_2)_4$), 2.22 (3H, s, C=C-CH_3), 3.3—4.2 (4H, m, $(CH_2)_2N$), 4.64 (1H, s, 3-H), 5.18 (2H, s, C=C-CH_2), 5.63 (2H, brs, 5- and 6-H), 8.35 (1H, s, CH=N). *Anal.* Calcd for $C_{20}H_{27}N_3O_6S \cdot HCl \cdot C_2H_5OH$: C, 50.75; H, 6.29; N, 8.45. Found: C, 50.54; H, 6.07; N, 8.49.

Mecillinam (5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl Ester L-Tartrate (5a·L-Tartrate)——A solution of 10 g of **5a**·HCl·iso-PrOH in ice water (50 ml) was treated with active carbon under ice cooling and filtered. Ethyl acetate (60 ml) was added to the filtrate with stirring under ice cooling, and the pH of the aqueous layer was adjusted to 7.3 with 5% aqueous sodium bicarbonate. The organic layer was separated, washed with cold saturated aqueous sodium chloride, and dried with anhydrous magnesium sulfate. A methanol solution (15 ml) of L-tartaric acid (2.8 g) was added to the organic layer, and the mixture was allowed to stand overnight at −10 °C. The separated solid was filtered off, washed with cold ethyl acetate, suspended in diisopropyl ether (100 ml) and refluxed for 5 min. After cooling, the solid was filtered off and washed with diisopropyl ether to give 8 g of the title compound as colorless crystals. mp 75—79 °C. IR ν_{max}^{KBr} cm^{-1}: 1820, 1780, 1740, 1680. ^{1}H-NMR (DMSO-d_6) δ: 1.3—1.9 (14H, m, 2-CH_3 and $(CH_2)_4$), 2.21 (3H, s, C=C-CH_3), 3.3—3.7 (4H, m, $(CH_2)_2N$), 4.23 (2H, s, HOCH-CHOH), 4.4 (1H, s, 3-H), 5.15 (2H, s, C=C-CH_2), 5.22 (1H, d, J=4.0 Hz, 5-H), 5.52 (1H, d, J=4.0 Hz, 6-H), 7.6 (4H, br, OH and COOH), 7.80 (1H, s, CH=N). *Anal.* Calcd for $C_{20}H_{27}N_3O_6S \cdot C_4H_6O_6$: C, 49.05; H, 5.66; N, 7.15. Found: C, 49.18; H, 5.72; N, 7.01.

Mecillinam (5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl Ester Dibenzoyl-L-tartrate (5a·Dibenzoyl-L-tartrate)——By the same procedure as described above, **5a**·HCl·iso-PrOH was treated with dibenzoyl-L-tartaric acid and the title compound was solidified with ethyl acetate as colorless crystals (35%). mp 163—165 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 1825, 1790, 1765, 1740, 1725, 1690. ^{1}H-NMR (DMSO-d_6) δ: 1.3—1.9 (14H, m, 2-CH_3 and $(CH_2)_4$), 2.19 (3H, s, C=C-CH_3), 3.3—3.7 (4H, m, $(CH_2)_2N$), 4.40 (1H, s, 3-H), 5.10 (2H, s, C=C-CH_2), 5.30 (1H, d, J=4.0 Hz, 5-H), 5.50 (1H, d, J=4.0 Hz, 6-H), 5.70 (2H, s, PhCOOCH-CHOCOPh), 7.3—8.1 (11H, m, arom. H and CH=N), 8.5—10.5 (2H, br, COOH). *Anal.* Calcd for $C_{20}H_{27}N_3O_6S \cdot C_{18}H_{14}O_8$: C, 57.35; H, 5.19; N, 5.28. Found: C, 57.41; H, 5.04; N, 5.06.

Oral Absorption Test——An aqueous solution of a mecillinam ester (**5**) or mecillinam was given to groups of five fasted male ddY mice (about 21 g body weight) at a dose of 50 mg equivalent of mecillinam per kg body weight. Blood was taken from the cut axilla region at 15, 30, 60 and 90 min after dosing, and allowed to stand for 30 min at 0 °C. The serum was obtained by centrifugation. Serum specimens obtained as the same time were combined and assayed on the

day of sampling. Concentrations of mecillinam were measured by bioassay using *E. coli* NIHJ as a test organism.[13]

Stability Test——Each of the mecillinam esters was dissolved in artificial digestive juices and the solutions were shaken at 37 °C, and bioautographed after 10, 30 and 50 min, in the following way. The test solution was spotted on a cellulose plate (Art 5718 supplied by Merck & Co.) for thin layer chromatography, and the plate was developed with the upper layer of a mixture of *n*-butanol, ethanol and water in a ratio of 4 : 1 : 5. The plate was dried in air, sprayed with a 30% aqueous mouse serum solution and left to stand at 37 °C for 30 min to convert the unchanged ester to mecillinam, which was detectable on the bioautogram. The thin layer plate was kept in intimate contact with a nutrient agar plate inoculated with *E. coli* NIHJ for 30 min, then the agar plate was incubated for 18 h at 37 °C. The *Rf* value of the inhibitory zone on the plate against *E. coli* NIHJ was determined for mecillinam and mecillinam esters.

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Synthesis and Biological Activity of Fluorine-Modified Platelet Activating Factors[1)]

KAGARI FUJITA,[a] SUSUMU KOBAYASHI,[a] ICHIRO KUDO,[a] KEIZO INOUE,[a] SHOSHICHI NOJIMA,[a] MASAJI OHNO,* YOSHIRO KOBAYASHI,*[,b] MAKOTO ODAGIRI,[b] and TAKEO TAGUCHI[b]

Faculty of Pharmaceutical Sciences, University of Tokyo,[a] Hongo, Bunkyo-ku, Tokyo 113, Japan and Tokyo College of Pharmacy,[b] Horinouchi, Hachioji, Tokyo 192-03, Japan

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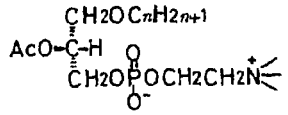
Fluorine-modified acetyl glyceryl ether phosphorylcholines (platelet activating factors; PAFs) were efficiently synthesized in an enantioselective manner by the coupling of a D-threitol derivative with fluorinated long-chain alkoxy methanesulfonates. The introduction of a trifluoromethyl group at the terminal of the alkyl ether chain decreased the hypotensive activity and platelet activation considerably. As the number of fluorine atoms in the alkyl ether chain was increased, both activities were gradually restored, but no selective agonist was obtained from among the fluorinated PAFs.

Keywords——platelet activating factor; fluorine-modified platelet activating factor; D-threitol derivative; alkylation

Since the characterization of platelet activating factors (PAFs) (**1a**, **1b**) by Hanahan *et al.*,[2)] and Benveniste *et al.*,[3)] in 1979, this new class of biologically active lipids has been the subject of a great deal of synthetic study in the field of medicinal chemistry. They act as powerful mediators in physiological or pathological processes such as anaphylaxis and inflammation.[4)] We reported in a previous communication[5)] an efficient methodology for the preparation of both enantiomers of C_{16}- and C_{18}-PAFs, starting from D- and/or L-tartaric acids as chiral synthons, and found that the unnatural PAFs possess far lower biological activities than the natural ones. Some analogues with modifications of the hydrophilic quaternary ammonium moiety were also synthesized and their biological activities were investigated. The modification of the cationic moiety of PAF caused a large diminution or enhancement of both biological activities (platelet activation and hypotension), in the same direction.



platelet activating factor
1a: $n=16$
1b: $n=18$

We were next interested in modification of the lipophilic alkyl moiety, especially the introduction of fluorine atoms into the chain. In general, introduction of fluorines into biologically active compounds is expected to change the properties of the parent hydrogen-substituted compounds in the direction of enhanced lipophilicity and metabolic stability

without appreciable change of steric bulkiness.[6,7]

Thus, to examine the substituent effect of fluorine on the biological activities of PAF, some analogues having fluorine atoms in the 1-*O*-alkyl chain were synthesized and their biological activities were investigated.

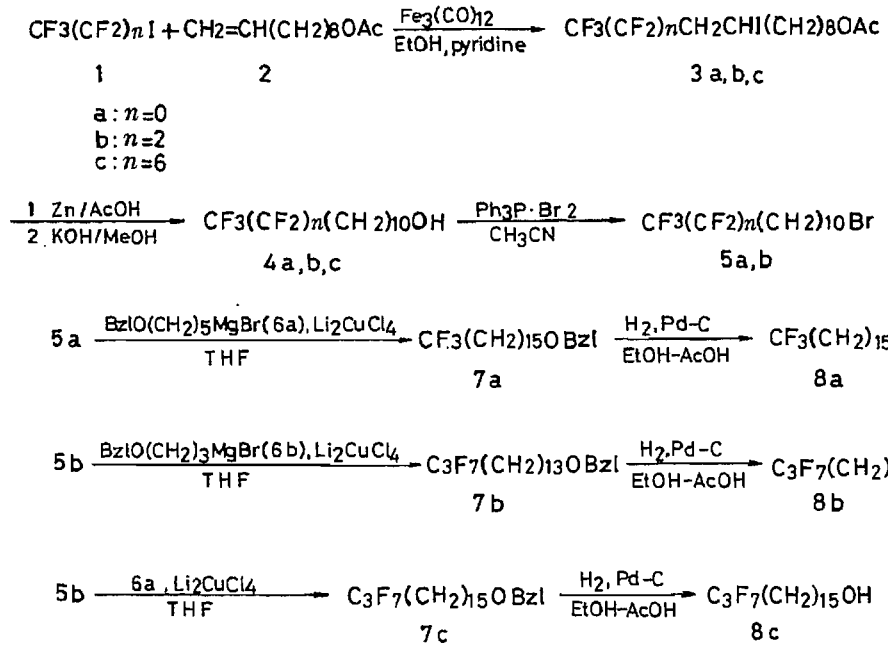


Chart 1

Synthesis of Fluorinated C_{16}–C_{18} Alcohols (Chart 1)

The C_{16}–C_{18} alcohols (**4c** and **8a**—**c**) were prepared as follows. The radical addition reaction of perfluoroalkyl iodide (**1a**—**c**) to the terminal olefinic compound (**2**) catalyzed by

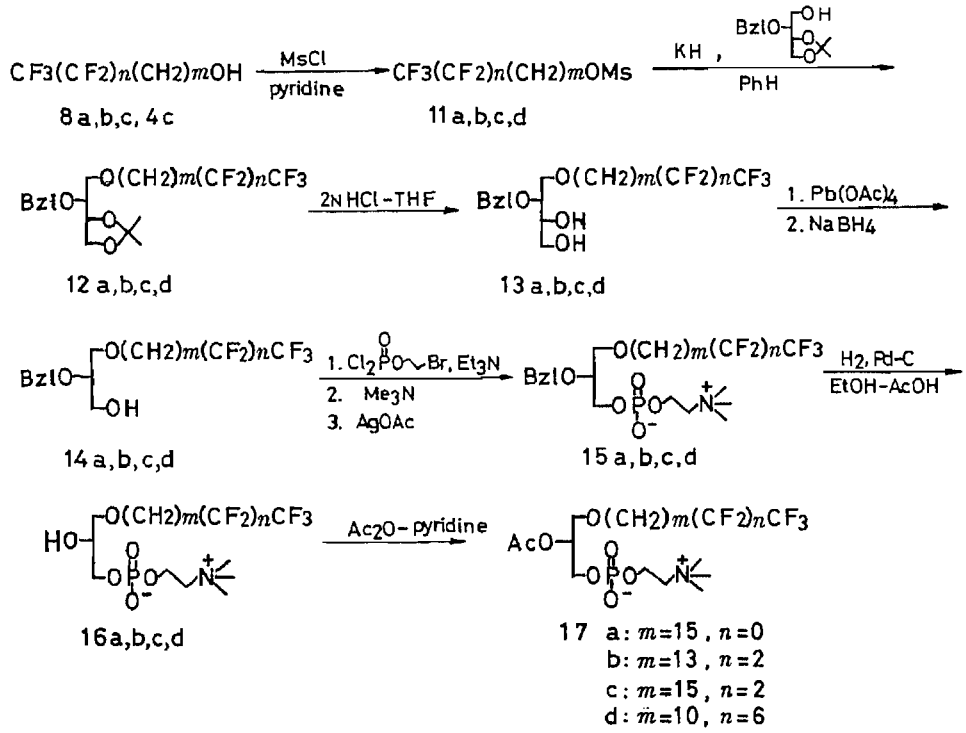


Chart 2

$Fe_3(CO)_{12}$[8] afforded the adducts (**3a—c**), which were in turn reduced with Zn dust in acetic acid followed by saponification to give the fluorinated alcohols (**4a—c**). The Grignard coupling reaction of ω-benzyloxyalkyl magnesium bromide (**6a, b**) with the bromides (**5a, b**) derived from **4a** and **4b** proceeded in the presence of a catalytic amount of Li_2CuCl_4[9] to give the benzyl ethers (**7a—c**), which were converted to the C_{16}–C_{18} alcohols (**8a—c**) by debenzylation (H_2/Pd–C).

Synthesis of 1-*O*-Fluoroalkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholines (Fluorine-Modified PAFs) (Chart 2)

The C_{16}–C_{18} alcohols were converted to the methanesulfonyl esters and coupled with 2-*O*-benzyl-3,4-*O*-isopropylidene-D-threitol, and then converted to 1-*O*-fluoroalkyl-2-*O*-benzyl-*sn*-glycerol following the methods described in the previous communication.[5]

Then phosphorylcholine groups were introduced into the *sn*-3-position of the glycerols, and the benzyl groups were removed and acetylated to afford fluorine-modified PAFs.

Biological Activities of Fluorine-Modified PAFs (Table I)

The biological activities (platelet activation and hypotensive) of fluorine-modified PAFs were investigated. The results are summarized in Table I, showing the relative biological activities of the analogues. Their ability to induce irreversible platelet aggregation was determined by measuring aggregation and the release of ^{14}C-serotonin from rabbit platelets[10] and their hypotensive activities were examined by using male Wistar strain rats.[11] The activities of the analogues are expressed relative to that of C_{16}-PAF (reciprocals of the concentrations required).

The results showed that no selective analogue with high hypotensive activity but with low platelet activation was present among the modified PAFs with fluorinated alkyl ether chains. However, it should be noted that the introduction of a trifluoromethyl group at the terminal of the alkyl ether chain (**17a**) decreased both activities considerably, and as the number of fluorine atoms in the alkyl ether chain was increased, both activities were gradually restored (**17b**, **17c**, **17d**). Although the results obtained here are difficult to explain in terms of lipophilicity and metabolic stability, the structure–activity relationships of PAF should be explored further, not only from the viewpoint of constitutional modification, but also by conformational analysis.[12]

TABLE I. Biological Activities of Fluorine-Modified PAFs

	Relative activity	
	Platelet activation	Hypotension
C_{16}-PAF	1	1
17a	0.10	0.10
17b	0.33	0.33
17c	0.58	1
17d	0.63	0.30

Experimental

All the reactions were performed under an argon atmosphere unless otherwise specified, using a standard syringe technique for the transfer of materials. The solvents were generally redistilled before use. Tetrahydrofuran (THF) and ether were dried over sodium and distilled before use. Thin-layer chromatography (TLC) was performed on Merck 60 F_{254} (0.25 mm) sheets, and the spots were visualized with molybdophosphoric acids in sulfuric acid. Wako-gel C-200 was employed for the column chromatography. Melting points were taken on a hot-stage microscope (Yanagimoto) and are uncorrected. Infrared (IR) spectra were recorded using a Jasco IRA-1 spectrophotometer.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian EM 390L instrument and the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane. ^{19}F-NMR spectra were recorded on a Varian EM 360L spectrometer and the chemical shifts are reported in parts per million relative to benzotrifluoride as an internal standard. The optical rotations were measured with a JASCO DIP 140 polarimeter. The mass spectra (MS) were recorded with a JEOL JMS-DX 300 apparatus. Perfluoroalkyl iodides were purchased from PCR Inc., Gainesville, Florida.

11,11,11-Trifluoroundecan-1-ol (4a)——A 30 ml test-tube containing 9-decenyl acetate (6.0 g, 30.3 mmol), ethanol (3 ml), pyridine (0.9 ml) and $Fe_3(CO)_{12}$ (150 mg) was placed in a stainless-steel autoclave, and trifluoromethyl iodide (7.42 ml, 89 mmol) was introduced. The autoclave was heated at 80 °C for 2 h, then at 110 °C for 16 h. The reaction mixture was diluted with ether, washed successively with 1 N HCl and brine, then dried over $MgSO_4$. After removal of the solvent *in vacuo*, the residue was treated with Zn powder (6.53 g) in a mixture of acetic acid (60 ml) and ether (60 ml) at room temperature for 15 h. The precipitate was filtered off through Celite and washed with ether. The filtrate was washed with 1 N HCl, $NaHCO_3$ solution and brine, then dried over $MgSO_4$. The solvent was removed *in vacuo* to leave crude 11,11,11-trifluoroundecanyl acetate (5.95 g), which was saponified by treatment with 4.3% methanolic KOH (50 ml) at room temperature for 2 h. The reaction mixture was evaporated to remove the solvent and the residue was diluted with water. This was extracted with ether, then the extract was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was submitted to column chromatography (SiO_2, 10% ethereal hexane as the eluent) to give 11,11,11-trifluoroundecan-1-ol (**4a**, 2.556 g, 37%) as a colorless oil. **4a**: ^{1}H-NMR ($CDCl_3$) δ: 3.63 (2H, t, J=6 Hz, $-CH_2OH$). ^{19}F-NMR ($CDCl_3$) δ: −3.67 (t, J=9.4 Hz). IR (neat): 3300, 2920, 2850, 1270, 1140 cm^{-1}. MS *m/z*: 208 ($M^+ - H_2O$), 180, 166, 152, 138, 97, 83, 69. High-resolution MS Calcd for $C_{11}H_{19}F_3$: 208.14438. Found: 208.14448.

1-Bromo-11,11,11-trifluoroundecane (5a)——A dimethylformamide (DMF) solution (10 ml) of **4a** (2.056 g, 8.7 mmol) was added to a suspension of triphenylphosphine dibromide [prepared from triphenylphosphine (3.10 g, 11.8 mmol) and bromine (1.746 g, 10.9 mmol)] in acetonitrile (10 ml) at 0 °C. The reaction mixture was stirred for 12 h at room temperature, diluted with water and extracted with ether. The organic layer was washed with water, dried over $MgSO_4$, and then chromatographed (SiO_2, 10% ethereal hexane) to give the bromide (**5a**, 2.465 g, 94%) as a colorless oil. **5a**: ^{1}H-NMR ($CDCl_3$) δ: 3.10 (2H, t, J=7 Hz, $-CH_2Br$). MS *m/z*: 290 $M^+(^{81}Br)$, 288 $M^+(^{79}Br)$, 209, 135, 133. High-resolution MS Calcd for $C_{11}H_{20}BrF_3$: 289.0699. Found: 289.0692.

1-Benzyloxy-16,16,16-trifluorohexadecane (7a)——A 0.1 M THF solution of Li_2CuCl_4 (0.5 ml) was added to a solution of the Grignard reagent [prepared from 5-benzyloxypentyl bromide (780 mg) and Mg (100 mg)] in THF (10 ml), followed by the addition of the bromide **5a** (300 mg, 1.04 mmol). The reaction mixture was stirred for 8 h at room temperature. After the usual extractive work-up (ether for extraction), the extract was purified by column chromatography (SiO_2, 0.5% AcOEt in hexane) to give the coupling product **7a** (350 mg, 88%) as a colorless oil. **7a**: ^{1}H-NMR ($CDCl_3$) δ: 3.50 (2H, m, $-CH_2OBn$), 4.53 (2H, s, $-OCH_2Ph$), 7.37 (5H, s, Ph). ^{19}F-NMR ($CDCl_3$) δ: −3.3 (t, J=11.3 Hz). MS *m/z*: 386 (M^+), 295, 276. High-resolution MS Calcd for $C_{23}H_{37}F_3O$: 386.2793. Found: 386.2781. IR (neat): 3030, 2935, 2860, 1100 cm^{-1}.

16,16,16-Trifluorohexadecan-1-ol (8a)——Hydrogenolysis of the benzyl ether **7a** (291 mg, 0.75 mmol) on 5% Pd–C in EtOH (5 ml) under a pressure of 3 atm at room temperature followed by recrystallization from *n*-hexane gave the alcohol **8a** (156 mg, 70%) as colorless crystals. **8a**: mp 56—56.5 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.17—1.85 (26H, m), 1.85 (1H, s, –OH), 1.80—2.40 (2H, m, CF_3CH_2-), 3.65 (2H, t, J=5 Hz, $-CH_2O-$). ^{19}F-NMR ($CDCl_3$) δ: −3.8 (t, J=10.3 Hz). IR (KBr): 3360, 2918, 2842, 1155 cm^{-1}. MS *m/z*: 278 ($M^+ - 18$), 250. High-resolution MS Calcd for $C_{16}H_{29}F_3$: 278.2219. Found: 278.2202.

11,11,12,12,13,13,13-Heptafluorotridecan-1-ol (4b)——A mixture of heptafluoro-1-iodopropane (6.694 g, 22.6 mmol), 9-decenyl acetate (3.775 g, 19 mmol) and $Fe_3(CO)_{12}$ (100 mg) in a Pyrex glass sealed tube was heated at 80 °C for 5.5 h. The reaction mixture was dissolved in a mixture of acetic acid (33 ml) and ether (33 ml), and treated with Zn powder (1.95 g) at room temperature for 1 h. Extractive work-up (ether for extraction) followed by chromatographic purification (SiO_2, 20% ethereal pentane) gave crude heptafluorotridecanyl acetate (5.268 g, 76%), which was saponified by treatment with 5% methanolic KOH (35 ml) at room temperature for 2 h to give **4b** (4.569 g, 71% overall yield) as a colorless oil. **4b**; bp 135—142 °C/4 mm (Kugelröhr). ^{1}H-NMR ($CDCl_3$) δ: 3.69 (2H, t, J=6 Hz, $-CH_2OH$). ^{19}F-NMR ($CDCl_3$) δ: −18.1 (3F, t, J=9.4 Hz), −52.5 (2F, m), −65.1 (2F, m). IR (neat): 3300 cm^{-1}. MS *m/z*: 308 ($M^+ - 18$), 280.

1-Bromo-11,11,12,12,13,13,13-heptafluorotridecane (5b)——By the same method as used for the preparation of **5a**, **5b** was obtained in 85% yield. **5b**: Colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 3.43 (2H, t, J=7 Hz, $-CH_2Br$). ^{19}F-NMR ($CDCl_3$) δ: −17.3 (3F, t, J=9.4 Hz), −52.3 (2F, m), −65.0 (2F, m). IR (neat): 2920, 2850, 1225, 1170 cm^{-1}. MS *m/z*: 390 $M^+(^{81}Br)$, 388 $M^+(^{79}Br)$.

1-Benzyloxy-14,14,15,15,16,16,16-heptafluorohexadecane (7b)——In the same manner as described for the preparation of **7a**, the reaction of the Grignard reagent [prepared from 3-benzyloxypropyl bromide (3.054 g, 13.3 mmol) and Mg (470 mg, 20 mmol)] with **5b** (1.577 g, 4.1 mmol) in the presence of Li_2CuCl_4 (0.05 mmol) in THF at room temperature for 5 h gave the benzyl ether **7b** (742 mg, 40%) as a colorless oil. **7b**: ^{1}H-NMR ($CDCl_3$) δ: 3.50 (2H, t, J=7 Hz, $-CH_2OBn$), 4.53 (2H, s, $-OCH_2Ph$), 7.40 (5H, s, Ph). ^{19}F-NMR ($CDCl_3$) δ: −17.7 (3F, t, J=9.4 Hz),

−51.8 (2F, m), −64.5 (2F, m). MS *m/z*: 458 (M^+), 367, 348, 321, 91. High-resolution MS Calcd for $C_{23}H_{33}F_7O$: 458.2416. Found: 458.2390.

14,14,15,15,16,16,16-Heptafluorohexadecan-1-ol (8b)——Hydrogenolysis of the benzyl ether **7b** (433 mg, 0.95 mmol) on 5% Pd–C in EtOH (2 ml) and acetic acid (0.5 ml) under atmospheric pressure, followed by recrystallization from *n*-hexane gave **8b** (281 mg, 80%). **8b**: mp 49.5—51 °C. ^{1}H-NMR ($CDCl_3$)·δ: 1.20—1.85 (23H, m), 1.92—2.30 (2H, m, $C_3F_7CH_2$–), 3.67 (2H, t, J=7 Hz, –CH_2OH). ^{19}F-NMR ($CDCl_3$) δ: −17.8 (3F, t, J=9.4 Hz), −51.8 (2F, m), −64.5 (2F, m). MS *m/z*: 350 (M^+ − 18), 322, 294, 280. *Anal.* Calcd for $C_{16}H_{27}F_7O$: C, 52.17; H, 7.39; F, 36.10. Found: C, 52.23; H, 7.16; F, 36.15.

1-Benzyloxy-16,16,17,17,18,18,18-heptafluorooctadecane (7c)——85% yield. ^{1}H-NMR ($CDCl_3$) δ: 3.50 (2H, t, J=6 Hz, –CH_2OBn), 4.53 (2H, s, –OCH_2Ph), 7.43 (5H, s, Ph). ^{19}F-NMR ($CDCl_3$) δ: −17.9 (3F, t, J=9.4 Hz), −52.3 (2F, m), −65.0 (2F, m). MS *m/z*: 486 (M^+), 395, 376, 91.

16,16,17,17,18,18,18-Heptafluorooctadecan-1-ol (8c)——87% yield; mp 55—57 °C (*n*-hexane). ^{1}H-NMR ($CDCl_3$) δ: 1.20—1.85 (27H, m), 1.92—2.30 (2H, m, $C_3F_7CH_2$–), 3.67 (2H, t, J=7 Hz, –CH_2OH). ^{19}F-NMR ($CDCl_3$) δ: −17.6 (3F, t, J=9.4 Hz), −52.3 (2F, m), −65.0 (2F, m). MS *m/z*: 378 (M^+ − 18), 350, 322. *Anal.* Calcd for $C_{18}H_{31}F_7O$: C, 54.54; H, 7.88; F, 33.55. Found: C, 54.33; H, 7.90; F, 33.72.

Pentadecafluoroheptadecan-1-ol (4c)——A mixture of perfluoroheptyl iodide (7.18 g, 14.5 mmol), 9-decenyl acetate (2.41 g, 12 mmol) and $Fe_3(CO)_{12}$ (50 mg) was heated at 60 °C for 1 h, and then at 80 °C for 1 h. The reaction mixture was dissolved in a mixture of acetic acid (30 ml) and ether (30 ml), and treated with Zn powder at room temperature for 3 h to give the crude acetate (4.82 g), which was saponified with 2.5% methanolic KOH (35 ml) at room temperature for 5 h. After extractive work-up (ether for extraction) and evaporation of the solvent, the residue was purified by sublimation (57—59 °C/3 mmHg) to give **4c** (2.751 g, 43% overall yield). **4c**: mp 61—63 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.23—1.90 (16H, m), 1.42 (1H, s, –OH), 1.92—2.50 (2H, m, $C_7F_{15}CH_2$–), 3.68 (2H, t, J=6 Hz, –CH_2OH). ^{19}F-NMR ($CDCl_3$) δ: −17.3 (3F, t, J=9.4 Hz), −50.2 (2F, m), −58.0 (8F, m), −62.0 (2F, m). *Anal.* Calcd for $C_{17}H_{21}F_{15}O$: C, 38.80; H, 4.02; F, 54.14. Found: C, 38.76; H, 4.00; F, 53.93.

Methanesulfonylated Fluoroalcohols (11a—d)——The alcohols **8a**, **8b**, **8c** and **4c** (0.5 mmol) were each dissolved in dry pyridine (5 ml) and methanesulfonyl chloride (MsCl) (63 mg, 0.55 mmol) was added at 0 °C. Each mixture was stirred at room temperature for 1 h. The reaction mixture was poured into ice-water, and extracted with ether. The ethereal solution was washed with dil. HCl and brine, then dried on $MgSO_4$, and concentrated to afford **11a**, **11b**, **11c** or **11d**, respectively as white crystals.

11a: (m=15, n=0) yield (Y.) 83%, mp 45—46 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.2—1.48 (m, 24H), 1.90—2.24 (m, 2H), 1.84—2.36 (m, 2H), 3.00 (s, 3H), 4.20 (t, J=7 Hz, 2H). IR (KBr): 3070, 2950, 2880, 1480, 1355, 1192, 1140 cm^{-1}. MS *m/z*: 374 (M^+).

11b: (m=13, n=2) Y. 87%, mp 39—42 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.10—1.90 (m, 22H), 1.90—2.24 (m, 2H), 2.98 (s, 3H), 4.20 (t, J=7 Hz, 2H). IR (KBr): 2940, 2860, 1473, 1356, 1172, 1126, 1115, 1050 cm^{-1}. MS *m/z*: 446 (M^+).

11c: (m=15, n=2) Y. 100%, mp 49—51 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.08—1.27 (m, 24H), 1.27—1.87 (m, 2H), 1.87—2.20 (m, 2H), 2.97 (s, 3H), 4.20 (t, J=7 Hz, 2H). IR (KBr): 2940, 2860, 1473, 1356, 1172, 1126, 1115, 1050 cm^{-1}. MS *m/z*: 474 (M^+).

11d: (m=10, n=6) Y. 87%, mp 59.5—62.5 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.13—1.44 (m, 14H), 1.44—1.88 (m, 2H), 1.89—2.25 (m, 2H), 2.97 (s, 3H), 4.20 (t, J=7 Hz, 2H). IR (KBr): 2940, 2860, 1472, 1357, 1170, 1126, 1116, 1052, 1006 cm^{-1}. MS *m/z*: 604 (M^+).

1-*O*-Fluoroalkyl-2-*O*-benzyl-3,4-*O*-isopropylidene-D-threitols (12a—d)——A 22% potassium hydride oil dispersion (0.27 ml, 1.5 mmol) was slowly added to a solution of 2-*O*-benzyl-3,4-*O*-isopropylidene-D-threitol[5] (175 mg, 0.7 mmol) in benzene (10 ml) at room temperature. The reaction mixture was stirred for an additional 30 min, and then **11** (0.46 mmol) in benzene (10 ml) was added. The reaction mixture was stirred for 10 h at room temperature, cooled to 0 °C, diluted with hexane (10 ml), and quenched by successive addition of EtOH (1 ml) and water (5 ml). The organic layer was separated, and the aqueous layer was washed with AcOEt. The combined organic layer was dried on $MgSO_4$, concentrated and purified by silica gel column chromatography (benzene) to afford **12** as a colorless oil.

12a: (m=15, n=0) Y. 66%. ^{1}H-NMR ($CDCl_3$) δ: 1.17—1.34 (m, 24H), 1.39 (s, 3H), 1.43 (s, 3H), 1.57—1.87 (m, 2H), 1.87—2.31 (m, 2H), 3.41 (t, J=7 Hz, 2H), 3.51—4.40 (m, 6H), 4.78 (s, 2H), 7.27—7.47 (m, 5H). IR (neat): 3450, 2920, 2860, 1455, 1370, 1220, 1160 cm^{-1}. MS *m/z*: 530 (M^+ − 15).

12b: (m=13, n=2) Y. 75%. ^{1}H-NMR ($CDCl_3$) δ: 1.16—1.72 (m, 22H), 1.36 (s, 3H), 1.42 (s, 3H), 1.90—2.40 (m, 2H), 3.38 (t, J=12 Hz, 2H), 3.47—4.40 (m, 6H), 4.71 (s, 2H), 7.16—7.48 (m, 5H). IR (neat): 3450, 2920, 2860, 1445, 1370, 1150 cm^{-1}. MS *m/z*: 602 (M^+), 587 (M^+ − 15).

12c: (m=15, n=2) Y. 89%. ^{1}H-NMR ($CDCl_3$) δ: 1:16—1.72 (m, 26H), 1.36 (s, 3H), 1.40 (s, 3H), 1.89—2.28 (m, 2H), 3.41 (t, J=7 Hz, 2H), 3.51—4.40 (m, 6H), 4.72 (s, 2H), 7.28 (m, 5H). IR (neat): 3450, 2920, 2860, 1455, 1370, 1160 cm^{-1}. MS *m/z*: 630 (M^+), 615 (M^+ − 15).

12d: (m=10, n=6) Y. 66%. ^{1}H-NMR ($CDCl_3$) δ: 1.16—1.48 (m, 14H), 1.34 (s, 3H), 1.43 (s, 3H), 1.49—1.88 (m, 2H), 1.89—2.25 (m, 2H), 3.40 (t, J=7 Hz, 2H), 3.44—4.10 (m, 5H), 4.10—4.30 (m, 1H), 4.75 (s, 2H), 7.22—7.48 (s, 5H). IR (neat): 3450, 2920, 2840, 1455, 1370, 1160 cm^{-1}. MS *m/z*: 760 (M^+), 745 (M^+ − 15).

1-*O*-Fluoroalkyl-2-*O*-benzyl-D-threitols (13a—d)——The *O*-alkylated derivative **12** (0.4 mmol) was stirred in

THF (15 ml) and 2 N HCl (5 ml) at room temperature for 2 h, then the solution was concentrated to a small volume (*ca.* 5 ml) under reduced pressure. After water (10 ml) had been added, the mixture was extracted with ethyl acetate (20 ml × 3). Evaporation of the solvent gave a residue, which was chromatographed on silica gel to give **13** as white crystals.

13a: Y. 73%, mp 36—38 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.17—1.48 (m, 24H), 1.48—1.76 (m, 2H), 1.87—2.31 (m, 4H), 3.41 (t, J=7 Hz, 2H), 3.60—3.88 (m, 6H), 4.38 (d, J=12 Hz, 1H), 4.78 (d, J=12 Hz, 1H), 7.27—7.37 (m, 5H). IR (KBr): 3300, 2920, 2860, 1460, 1210, 1150 cm^{-1}.

13b: Y. 77%, mp 32—34 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.15—1.85 (m, 22H), 1.92—2.20 (m, 2H), 2.85 (br s, 2H), 3.42 (t, J=7 Hz, 2H), 3.55—3.90 (m, 6H), 4.53 (d, J=12 Hz, 1H), 4.73 (d, J=12 Hz, 1H), 7.17—7.52 (m, 5H). IR (KBr): 3350, 2940, 2880, 1240, 1210, 1160 cm^{-1}.

13c: Y. 76%, mp 41—44 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.15—1.72 (m, 26H), 1.91—2.20 (m, 4H), 3.42 (t, J=7 Hz, 2H), 3.53—3.87 (m, 6H), 4.55 (d, J=12 Hz, 1H), 4.75 (d, J=12 Hz, 1H), 7.29 (s, 5H). IR (KBr): 3450, 2920, 2840, 1470, 1355, 1210, 1150 cm^{-1}. CI-MS *m/z*: 591 (M^++1).

13d: Y. 88%, mp 51—54 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.16—1.48 (m, 14H), 1.49—1.88 (m, 2H), 1.89—2.25 (m, 2H), 2.79 (br s, 2H), 3.44 (t, J=7 Hz, 2H), 3.55—3.88 (m, 6H), 4.56 (d, J=12 Hz, 1H), 4.76 (d, J=12 Hz, 1H), 7.32 (s, 5H). IR (KBr): 3350, 2940, 2880, 1240, 1210, 1150 cm^{-1}.

1-*O*-Fluoroalkyl-2-*O*-benzyl-*sn*-glycerols (14a—d)——Under vigorous stirring **13** (0.3 mmol) in benzene (10 ml) was added to a solution of $Pb(OAc)_4$ (200 mg, 0.45 mmol) in benzene (20 ml) over a 2 h period at room temperature. After being stirred for an additional hour, the reaction mixture was cooled to 0 °C and saturated aqueous sodium bicarbonate was added. The resulting insoluble material was filtered through a celite layer and washed with benzene. The organic layer was separated, dried over Na_2SO_4, and concentrated to afford the corresponding crude aldehyde in quantitative yield as a colorless oil. This was used without further purification for the next step. $NaBH_4$ (113 mg, 3 mmol) was slowly added to a solution of the crude aldehyde (0.3 mmol) in methanol (10 ml) at room temperature. The reaction mixture was allowed to stand at room temperature overnight, cooled to 0 °C, acidified with dil. hydrochloric acid, and concentrated to dryness under reduced pressure. The product was extracted with ethyl acetate (20 ml × 3), and the organic layer was dried over Na_2SO_4. After the removal of the solvent, the residue was chromatographed on silica gel to afford **14** as a colorless oil (**14a**, **b**, **d**) or white crystals (**14c**).

14a: Y. 81%, oil. ^{1}H-NMR ($CDCl_3$) δ: 1.17—1.48 (m, 24H), 1.49—1.76 (m, 2H), 1.87—2.31 (m, 3H), 3.41 (t, J=7 Hz, 2H), 3.44—3.80 (m, 5H), 4.62 (d, J=12 Hz, 1H), 4.64 (d, J=12 Hz, 1H), 7.28 (s, 5H). IR (neat): 3400, 2910, 2840, 1460, 1450, 1230, 1135 cm^{-1}. $[\alpha]_D^{20}$ −5.98° (c=0.57, $CHCl_3$). MS *m/z*: 460 (M^+).

14b: Y. 72%, oil. ^{1}H-NMR ($CDCl_3$) δ: 1.15—1.85 (m, 22H), 1.86—2.37 (m, 3H), 3.43 (t, J=7 Hz, 2H), 3.53—3.97 (m, 5H), 4.67 (s, 2H), 7.21—7.51 (m, 5H). IR (neat): 3400, 2920, 2840, 1460, 1450, 1230, 1140 cm^{-1}. $[\alpha]_D^{20}$ −12.4° (c=0.54, $CHCl_3$). MS *m/z*: 532 (M^+).

14c: Y. 72%, white crystals, mp 36—38 °C. ^{1}H-NMR ($CDCl_3$) δ: 1.16—1.72 (m, 26H), 1.91—2.20 (m, 2H), 3.41 (t, J=7 Hz, 2H), 3.51—3.95 (m, 5H), 4.64 (s, 2H), 7.21—7.47 (m, 5H). IR (KBr): 3450, 2920, 2840, 1470, 1355, 1205, 1140 cm^{-1}. $[\alpha]_D^{20}$ −9.28° (c=1.12, $CHCl_3$). MS *m/z*: 560 (M^+).

14d: Y. 73%, oil. ^{1}H-NMR ($CDCl_3$) δ: 1.16—1.48 (m, 14H), 1.49—1.88 (m, 2H), 1.89—2.25 (m, 2H), 3.40 (t, J=7 Hz, 2H), 3.44—3.80 (m, 5H), 4.62 (d, J=12 Hz, 1H), 4.64 (d, J=12 Hz, 1H), 7.28 (s, 5H). IR (neat): 3350, 2920, 2840, 1465, 1452, 1320, 1240, 1210, 1140 cm^{-1}. $[\alpha]_D^{20}$ −6.20° (c=1.37, $CHCl_3$). MS *m/z*: 704 (M^+).

1-*O*-Fluoroalkyl-2-*O*-benzyl-*sn*-glycero-3-phosphocholines (15a—d)——A mixture of **15** (0.19 mmol), 2-bromoethyl phosphoryl dichloride (69 mg, 0.29 mmol) and triethylamine (39 mg, 0.38 mmol) in dry ether (10 ml) was stirred under reflux for 12 h. Then water (0.2 ml) and triethylamine (0.1 ml) were added, and the reaction mixture was heated under reflux for 1 h. After cooling, 1 N HCl (5 ml) was added and the mixture was extracted with chloroform (20 ml × 3). The organic layer was dried on $MgSO_4$ and concentrated to afford crude 2-bromoethyl 2-*O*-benzyl-L-1-*O*-fluoroalkyl-glyceryl hydrogen phosphate. The product was used without further purification in the next step. The phosphate was heated with trimethylamine (1 ml) and methanol (2 ml) to 55 °C in a sealed tube for 12 h. After removal of the solvent, AgOAc (80 mg, 0.48 mmol) in 90% aqueous methanol (20 ml) was added, and the reaction mixture was stirred for 2 h. The resulting precipitate was removed by filtration, and the filtrate was concentrated to give crude **15**. The product was purified by silica gel column chromatography to afford **15** as a white powder.

15a: Y. 52%, mp *ca.* 190 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.17—1.48 (m, 24H), 1.49—1.76 (m, 2H), 1.87—2.31 (m, 2H), 3.09 (s, 9H), 3.28—3.64 (m, 9H), 3.88—4.10 (m, 2H), 4.69 (s, 2H), 7.18—7.40 (m, 5H). IR (KBr): 3390, 3070, 3040, 2930, 2860, 1660, 1460, 1320, 1220, 1150 cm^{-1}. $[\alpha]_D^{20}$ +1.20° (c=0.40, $CHCl_3$–MeOH, 1:1).

15b: Y. 86%, mp 135—142 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.10—1.85 (m, 22H), 1.90—2.33 (m, 2H), 3.26 (s, 9H), 3.41 (t, J=7 Hz, 2H), 3.30—3.70 (m, 4H), 3.70—4.37 (m, 5H), 4.70 (s, 2H), 7.18—7.50 (m, 5H). IR (KBr): 3420, 2940, 2850, 1465, 1230, 1085 cm^{-1}. $[\alpha]_D^{20}$ +3.81° (c=0.42, $CHCl_3$–MeOH, 1:1).

15c: Y. 68%, mp 229—233 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.10—1.73 (m, 26H), 1.91—2.33 (m, 2H), 3.11 (s, 9H), 3.41 (t, J=7 Hz, 2H), 3.30—3.70 (m, 4H), 3.70—4.37 (m, 5H), 4.70 (s, 2H), 7.18—7.50 (m, 5H). IR (KBr): 3420, 2940, 2850, 1465, 1230, 1085 cm^{-1}. $[\alpha]_D^{20}$ +2.61° (c=0.94, $CHCl_3$–MeOH, 1:1).

15d: Y. 60%, mp 223—226 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.16—1.83 (m, 16H), 1.90—2.37 (m, 2H), 3.09 (s, 9H), 3.28—3.64 (m, 9H), 3.88—4.10 (m, 2H), 4.69 (s, 2H), 7.23 (s, 5H). IR (KBr): 3400, 2940, 2860, 1460,

1230, 1085 cm^{-1}. $[\alpha]_D^{20}$ +2.11° (c=1.11, $CHCl_3$–MeOH, 1:1).

1-*O*-Fluoroalkyl-*sn*-glycero-3-phosphocholines (16a—d)——A mixture of **15** (0.13 mmol) and palladium black (10 mg) in ethanol was vigorously stirred under a hydrogen atmosphere (1 atm) for 6 h at 50 °C. The catalyst was filtered off, and the filtrate was concentrated. The residual powder was purified on silica gel column chromatography to afford **16** as a white powder.

16a: Y. 100%, mp 230 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.17—1.48 (m, 24H), 1.49—1.76 (m, 2H), 1.87—2.31 (m, 2H), 3.21 (s, 9H), 3.30—3.76 (m, 9H), 3.88—4.10 (m, 2H). IR (KBr): 3420, 2940, 2850, 1465, 1210, 1150 cm^{-1}. $[\alpha]_D^{20}$ −2.40° (c=0.35, $CHCl_3$–MeOH, 1:1).

16b: Y. 85%, mp 196 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.10—1.85 (m, 22H), 1.90—2.35 (m, 2H), 3.26 (s, 9H), 3.38—3.76 (m, 7H), 3.77—4.09 (m, 2H), 4.10—4.46 (m, 2H). IR (KBr): 3400, 2940, 2860, 1472, 1360, 1220, 1145 cm^{-1}. $[\alpha]_D^{20}$ −2.00° (c=1.17, $CHCl_3$–MeOH, 1:1).

16c: Y. 78%, mp 226—234 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.10—1.73 (m, 26H), 1.91—2.33 (m, 2H), 3.25 (s, 9H), 3.33—3.76 (m, 7H), 3.77—3.93 (m, 2H), 3.94—4.40 (m, 2H). IR (KBr): 3400, 2940, 2860, 1472, 1360, 1220, 1140 cm^{-1}. $[\alpha]_D^{20}$ −3.61° (c=0.64, $CHCl_3$–MeOH, 1:1).

16d: Y. 90%, mp 222—224 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.16—1.83 (m, 16H), 1.90—2.37 (m, 2H), 3.21 (s, 9H), 3.30—3.94 (m, 2H). IR (KBr): 3420, 2940, 2850, 1465, 1210, 1140 cm^{-1}. $[\alpha]_D^{20}$ −2.55° (c=0.55, $CHCl_3$–MeOH, 1:1).

1-*O*-Fluoroalkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholines (17a—d)——A solution of **16** (0.1 mmol) in acetic anhydride was mixed with pyridine, and the reaction mixture was heated to 70 °C for 2 h, then cooled to 50 °C. Pyridine and acetic anhydride were removed under reduced pressure, and the resulting residue was chromatographed on silica gel to afford **17** as a white powder.

17a: Y. 55%, mp 272—276 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.17—1.48 (m, 24H), 1.49—1.76 (m, 2H), 1.87—2.31 (m, 2H), 2.12 (s, 3H), 3.21 (s, 9H), 3.45 (t, J=7 Hz, 2H), 3.59 (d, J=5 Hz, 2H), 3.64 (m, 2H), 4.00 (m, 2H), 5.13 (quintet, J=5 Hz, 1H). IR (KBr): 3420, 2920, 2850, 1732, 1627, 1465, 1374, 1235, 1140 cm^{-1}. $[\alpha]_D^{20}$ −0.46° (c=0.11, $CHCl_3$–MeOH, 1:1). FAB-MS (glycerine) 578 (M^++1).

17b: Y. 38%, mp 272—276 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.12—1.54 (m, 22H), 1.55—1.78 (m, 2H), 2.20 (s, 3H), 3.34 (s, 9H), 3.52—4.00 (m, 8H), 4.20—4.40 (m, 2H), 5.10—5.28 (m, 1H). IR ($CHCl_3$): 2930, 2850, 1735, 1220, 1080 cm^{-1}. $[\alpha]_D^{20}$ −4.86° (c=0.10, $CHCl_3$–CD_3OD, 1:1). FAB-MS (glycerine) 650 (M^++1).

17c: Y. 75%, mp 208—212 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.10—1.73 (m, 26H), 1.91—2.35 (m, 2H), 2.07 (s, 3H), 3.18 (s, 9H), 3.28—3.70 (m, 9H), 3.88—4.10 (m, 2H), 4.10—4.40 (m, 2H), 5.10—5.25 (m, 1H). ^{19}F-NMR ($CDCl_3$): −17.9 (t, J=9.5 Hz, 3F), −52.6 (m, 2F), −65.1 (m, 2F). IR (KBr): 3350, 2940, 2860, 1735, 1465, 1230, 1150 cm^{-1}. $[\alpha]_D^{20}$ −1.59° (c=0.63, $CHCl_3$–MeOH, 1:1). FAB-MS (glycerine) 678 (M^++1).

17d: Y. 57%, mp 196—199 °C (dec.). ^{1}H-NMR ($CDCl_3$–CD_3OD, 3:1) δ: 1.16—1.83 (m, 16H), 1.90—2.37 (m, 2H), 2.90 (s, 3H), 3.20 (s, 9H), 3.45 (t, J=7 Hz, 2H), 3.53—3.70 (m, 2H), 3.84—4.06 (m, 2H), 4.06—4.40 (m, 2H), 5.12 (quintet, J=5 Hz, 1H). ^{19}F-NMR ($CDCl_3$): −18.0 (t, J=9.7 Hz, 3F), −51.2 (m, 2F), −57.7——60.0 (m, 8F), −63.0 (m, 2F). IR (KBr): 3340, 2940, 2860, 1735, 1465, 1230, 1150 cm^{-1}. $[\alpha]_D^{20}$ −4.75° (c=0.33, $CHCl_3$–MeOH, 1:1). FAB-MS (glycerine) 808 (M^++1).

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Hypotensive Compounds Isolated from Alcohol Extract of the Unossified Horn of *Cervus elaphus* L. var. *xanthopygus* MILNE-EDWARG (Rokujo). I.[1] Isolation of Lysophosphatidyl Choline as a Hypotensive Principle and Structure–Activity Study of Related Compounds

HIROSHI TSUJIBO,[a] YOSHIAKI MIYAKE,[a] KOSHI MARUYAMA[b] and YOSHIHIKO INAMORI*,[a]

Osaka University of Pharmaceutical Sciences,[a] Kawai, Matsubara-shi, Osaka 580, Japan and Shinwa Bussan & Co., Ltd.,[b] 2-chome, Doshomachi, Higashi-ku, Osaka 541, Japan

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By the use of spontaneously hypertensive rats (SH rats) as a screening system, two hypotensive compounds were isolated from alcohol extract of the unossified horn of *Cervus elaphus* L. var. *xanthopygus* (Rokujo). One of the two compounds was identified as lysophosphatidyl choline (LPC). It was shown by gas chromatography that palmitic acid ($C_{16:0}$) accounted for about half of the fatty acid content. Next, the effects of various LPCs on SH rats were examined. Eleven LPCs with $C_{10:0}$ to $C_{20:0}$ fatty acids were examined, and all except for those with $C_{10:0}$ and $C_{20:0}$ fatty acids showed the hypotensive effect (3 mg/kg, i.v.). In particular, $C_{14:0}$ and $C_{16:0}$-LPC showed rather potent activity. On the other hand, of the six LPCs with unsaturated fatty acids, only $C_{16:1}$-LPC showed the hypotensive effect (3 mg/kg). It was concluded that at least a part of the hypotensive action of alcohol extract of Rokujo is due to LPC.

Keywords——Rokujo; *Cervus elaphus* L. var. *xanthopygus*; lysophosphatidyl choline; spontaneously hypertensive rat; hypotensive compound; hypotensive action; Pantocrin; fatty acid composition; pharmacological action

Cervus elaphus L. var. *xanthopygus* and related animals which belong to Cervidae in Artiodactyla are widely distributed in Siberia and China. The unossified horn of these animals, so-called "Rokujo" has been used as a valuable drug in Chinese medicine from ancient times. The pharmacological actions of Pantocrin, alcohol extract of Rokujo, on the neuro-muscular and endocrine systems were examined by Pavlenko *et al.*[2] In Japan, there have been studies[3,4] on the effect of Pantocrin on experimental whiplash injury and on unidentified clinical syndrome. Pantocrin is clinically used as an injection. Takigawa *et al.*[4] also reported that Pantocrin had a hypotensive effect as one of its pharmacological actions. However, no detailed work has yet been done on the active principles of alcohol extract of Rokujo.

In the present study, two hypotensive compounds were isolated from alcohol extract of the unossified horn of *Cervus elaphus* L. var. *xanthopygus*. One of the two compounds was identified as lysophosphatidyl choline (LPC), and its fatty acid composition was clarified. Furthermore, the hypotensive effects of LPC and related compounds were examined in spontaneously hypertensive rats (SH rats).

Materials and Methods

Chemicals——L-α-Monocapryl lysophosphatidyl choline ($C_{10:0}$-LPC), L-α-monolauroyl lysophosphatidyl cho-

line ($C_{12:0}$-LPC), L-α-monomyristoyl lysophosphatidyl choline ($C_{14:0}$-LPC), L-α-monopalmitoyl lysophosphatidyl choline ($C_{16:0}$-LPC), L-α-monostearoyl lysophosphatidyl choline ($C_{18:0}$-LPC) and L-α-monooleoyl lysophosphatidyl choline ($C_{18:1}$-LPC) were obtained from Avanti Polar Lipid, Inc. L-α-Diundecanoyl phosphatidyl choline ($C_{11:0}$-PC), L-α-ditridecanoyl phosphatidyl choline ($C_{13:0}$-PC), L-α-dipentadecanoyl phosphatidyl choline ($C_{15:0}$-PC), L-α-diheptadecanoyl phosphatidyl choline ($C_{17:0}$-PC), L-α-dinonadecanoyl phosphatidyl choline ($C_{19:0}$-PC), L-α-diarachidoyl phosphatidyl choline ($C_{20:0}$-PC), L-α-dimyristoleoyl phosphatidyl choline ($C_{14:0}$-PC), L-α-dilinoleoyl phosphatidyl choline ($C_{18:2}$-PC), L-α-dilinolenoyl phosphatidyl choline ($C_{18:3}$-PC) and L-α-dieicosenoyl phosphatidyl choline ($C_{20:1}$-PC) were also obtained from Avanti Polar Lipid, Inc. $C_{11:0}$-LPC, $C_{13:0}$-LPC, $C_{15:0}$-LPC, $C_{17:0}$-LPC, $C_{19:0}$-LPC, $C_{20:0}$-LPC, $C_{14:1}$-LPC, $C_{18:2}$-LPC, $C_{18:3}$-LPC and $C_{20:1}$-LPC were obtained by the hydrolysis of the respective phosphatidyl choline with phospholipase A_2 according to the method of Wells and Hanahan.[6] L-α-Palmitelaidoyl lysophosphatidyl choline ($C_{16:1}$-LPC) was kindly supplied by Nippon Shoji Kaisha, Ltd.

Animals——Male SH rats weighing about 250—300 g (12—14 weeks old) from the colony of the Department of Pharmacology, Jichi Medical School, were used.

Isolation of the Hypotensive Principles from Alcohol Extract of *Cervus elaphus* L. var. *xanthopygus* MILNE-EDWARG——The unossified horn of *C. elaphus* L. var. *xanthopygus* (1000 g) was finely cut and extracted three times with 50% EtOH (10 l) at room temperature for 18 h. The extracted solution was filtered and the filtrate (7 l) was evaporated to dryness under reduced pressure. The residue (50 g) was extracted with H_2O (500 ml) and lyophilized. The lyophilized powder was extracted with various organic solvents. Among them, $CHCl_3$ extract showed the hypotensive effect (−30 mmHg, 5 mg/kg, i.v.) on SH rats. The $CHCl_3$ extract (5 g) was applied to a column of silica gel (200 g). Stepwise elusion of the column was carried out with $CHCl_3$ (a), $CHCl_3$–MeOH (8:2, b), $CHCl_3$–MeOH (6:4, c), $CHCl_3$–MeOH (4:6, d), $CHCl_3$–MeOH (2:8, e) and MeOH (f) to give Fr-a (150 mg), Fr-b (1500 mg), Fr-c (300 mg), Fr-d (400 mg), Fr-e (300 mg) and Fr-f (500 mg). The Fr-f, eluted with MeOH, showed the hypotensive effect. The active fraction (f, 500 mg) was subjected to gel filtration on a Sephadex LH-20 column (2.0 × 135 cm) using MeOH as an eluent to give Fr-1 (300 mg), Fr-2 (100 mg) and Fr-3 (100 mg). According to the method of Rouser *et al.*,[7] the active fraction (Fr-1, 300 mg) was chromatographed on a diethylaminoethyl (DEAE)-cellulose column (2.5 × 30 cm) with $CHCl_3$–MeOH (9:1, 700 ml, A), $CHCl_3$–MeOH (9:1, 300 ml, B), $CHCl_3$–MeOH (7:3, 1000 ml, C), MeOH (500 ml, D), $CHCl_3$–AcOH (3:1, 500 ml, E), AcOH (1000 ml, F) and MeOH (300 ml, G) as eluents to give Fr-1-A (150 mg), Fr-1-B (100 mg), Fr-1-C (10 mg), Fr-1-D (10 mg), Fr-1-E (10 mg), Fr-1-F (10 mg) and Fr-1-G (10 mg). Two active fractions (Fr-1-A and Fr-1-B) were further purified by preparative high-performance liquid chromatography (HPLC). Apparatus, high-performance liquid chromatograph (Toyo Soda HLC 803 Series A); column, YMC-Pack A-312 ODS (6 × 150 mm); detector, ultraviolet (UV) 215 nm; mobile phase, 95% MeOH; flow rate, 1 ml/min; temperature, ambient. Two active principles with hypotensive activity were designated as Fr-1-A-III (100 mg) and Fr-1-B-IV (5 mg). The *Rf* value of Fr-1-A-III on thin layer chromatogram was similar to that of lysophosphatidyl choline, and infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectral data of Fr-1-A-III were identical with those of an authentic sample of lysophosphatidyl choline (Serdary Research Laboratories) isolated from bovine brain.

Hypotensive Activity in SH Rats——SH rats were anesthetized with sodium pentobarbital 40 mg/kg, i.p. with supplemental doses as necessary. The trachea was isolated and cannulated with a short piece of polyethylene tubing. The systemic arterial blood pressure was measured *via* a carotid catheter connected to a pressure transducer (Nihon Kohden P 23 ID, with WI-621 G chart recorder). Samples were dissolved in 0.9% saline and administered through a cannula in the femoral vein. SH rats, which showed the blood pressure above 160 mmHg, were used for the assay of the hypotensive activity.

Analysis of Fatty Acid Composition——Fatty acid methyl esters were prepared by methanolysis,[8] and determined on a Shimadzu GC-7A gas chromatograph. The inlet system was equipped with a glass column (3.0 mm × 5.0 m) packed with 5% Advance DS on 60—80 mesh Chromosorb W. Column temperature, 200 °C; injection temperature, 250 °C; nitrogen flow rate, 28 ml/min.

Results

Isolation of Hypotensive Principles from Alcohol Extract of *Cervus elaphus* L. var. *xanthopygus* MILNE-EDWARG (Rokujo)

Two hypotensive principles were purified by extraction with 50% ethanol, water and chloroform in that order, followed by the combination of column chromatography and preparative HPLC as shown in Chart 1. The effect of each fraction on the blood pressure in SH rats is summarized in Table I.

Identification of Fr-1-A-III

The physico-chemical properties of Fr-1-A-III were as follows: Dittmer–Lester and

TABLE I. Effect of Each Fraction on the Blood Pressure in Anesthetized SH Rats

Sample	Dose (mg/kg, i.v.)	Mean arterial blood pressure (mmHg)
$CHCl_3$ extract	5	−30
MeOH fraction (Fr-f)	5	−35
Fr-1	5	−50
Fr-1-A	5	−60
Fr-1-B	5	−30
Fr-1-A-III (LPC)	5	−90
Fr-1-B-IV	5	−80

Body weight: 250—300 g. Anesthetic: pentobarbital-Na (40 mg/kg, i.p.). Each value represents the mean of three rats.

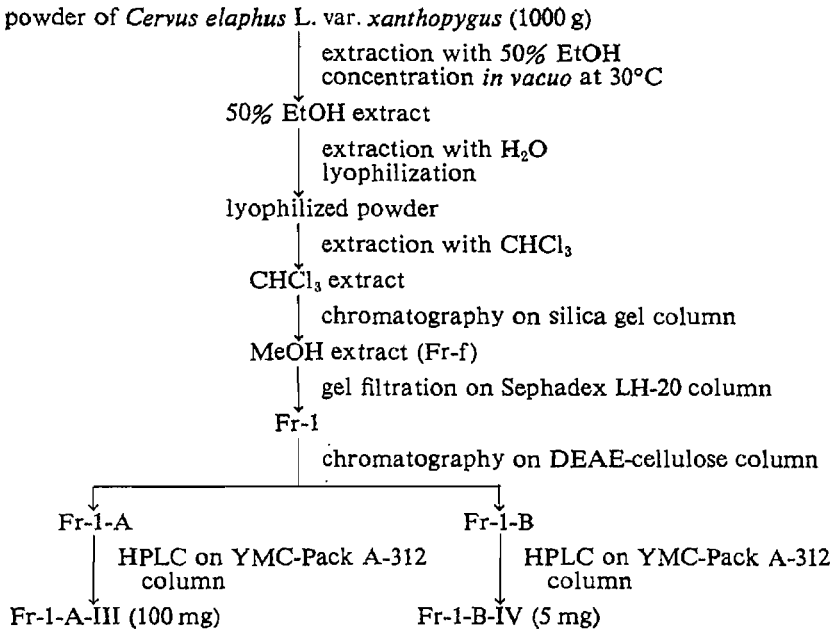

Chart 1

Dragendorff reagents gave bluish and yellow colors on the thin layer chromatogram (TLC), respectively. These findings indicate that Fr-1-A-III may be a phospholipid containing choline. Furthermore, the *Rf* values of Fr-1-A-III (0.15 in solvent system A, $CHCl_3$: MeOH : AcOH : H_2O = 50 : 30 : 8 : 4; 0.13 in solvent system B, $CHCl_3$: MeOH: 28% NH_4OH = 65 : 35 : 5; 0.08 in solvent system C, $CHCl_3$: MeOH : H_2O = 65 : 25 : 4) on TLC were similar to those of LPC. Thus, the ^{1}H-NMR and IR spectra of Fr-1-A-III were measured. The ^{1}H-NMR and IR spectra of Fr-1-A-III were identical with those of an authentic sample of LPC. Thus, Fr-1-A-III was identified as LPC.

Fatty Acid Composition of the Hypotensive Compound, Fr-1-A-III

As shown in Table II, the methyl esters of fatty acids were separated into 8 components by gas chromatography. The fatty acid composition consisted of myristic, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic and linoleic acids. Among them, the predominant fatty acid was palmitic acid and its content was 46.1%. The contents of the unsaturated fatty acids, palmitoleic, oleic and linoleic acids, were 6.8, 31.5 and 5.6%, respectively. The ratio of saturated and unsaturated fatty acids was approximately 1 : 1.

TABLE II. Fatty Acid Composition of the Hypotensive Compound, Fr-1-A-III

Fatty acid	Content (%)
$C_{14:0}$	1.20
$C_{15:0}$	2.00
$C_{16:0}$	46.10
$C_{16:1}$	6.79
$C_{17:0}$	0.24
$C_{18:0}$	0.58
$C_{18:1}$	31.50
$C_{18:2}$	5.60

TABLE III. Effect of LPC Purified from Rokujo on the Blood Pressure in Anesthetized SH Rats

Sample	Dose (mg/kg, i.v.)	Mean arterial blood pressure (mmHg)
LPC	0.5	0
	1.0	−31.7±7.2
	3.0	−53.3±8.3
	5.0	−90.0±2.9
	10.0	−95.0±5.0

Body weight: 250—300 g. Anesthetic: pentobarbital-Na (40 mg/kg, i.p.). Each value represents the mean±S.E. (n=3).

TABLE IV. Effect of Various LPCs on the Blood Pressure in Anesthetized SH Rats

Sample	Dose (mg/kg, i.v.)	Mean arterial blood pressure (mmHg)
10:0-LPC	3	0
11:0-LPC	3	−15.0±5.0
12:0-LPC	3	−38.3±8.3
13:0-LPC	3	−58.3±7.3
14:0-LPC	3	−81.7±1.6
15:0-LPC	3	−38.3±14.5
16:0-LPC	3	−76.7±7.3
17:0-LPC	3	−26.7±7.3
18:0-LPC	3	−40.0±7.6
19:0-LPC	3	−12.5±7.5
20:0-LPC	3	0

Body weight: 250—300 g. Anesthetic: pentobarbital-Na (40 mg/kg, i.p.). Each value represents the mean±S.E. (n=3).

TABLE V. Effect of Various LPCs on the Blood Pressure in Anesthetized SH Rats

Sample	Dose (mg/kg, i.v.)	Mean arterial blood pressure (mmHg)
14:1-LPC	3	0
16:1-LPC	3	−37.5±2.5
18:1-LPC	3	0
18:2-LPC	3	0
18:3-LPC	3	0
20:1-LPC	3	0

Body weight: 250—300 g. Anesthetic: pentobarbital-Na (40 mg/kg, i.p.). Each value represents the mean±S.E. (n=3).

Effects of Various Lysophosphatidyl Cholines (LPCs) on the Blood Pressure in Anesthetized SH Rats

Table III shows the correlation between dose and hypotensive activity of LPC in SH rats. Hypotensive activity was seen at dose levels higher than 1.0 mg/kg, and this activity increased strongly with increasing dose of LPC. LPC showed a transient hypotensive effect (−53 mmHg, 3 mg/kg, i.v.) and the blood pressure recovered to the original level within one minute. LPC also caused pronounced hypotension in normotensive rats (data not shown).

Next, the relationship between fatty acid composition and hypotensive activity was examined in SH rats. The results are summarized in Tables IV and V. Of eleven LPCs with $C_{10:0}$ to $C_{20:0}$ fatty acids, all the LPCs except for $C_{10:0}$ and $C_{20:0}$ fatty acids showed the hypotensive effect, as shown in Table IV. Among them, $C_{14:0}$-LPC showed the strongest hypotensive activity, followed by $C_{16:0}$-LPC and then by $C_{13:0}$-LPC.

On the other hand, of the six LPCs with unsaturated fatty acids, only $C_{16:1}$-LPC showed the hypotensive effect, as shown in Table V. Its activity tended to be weaker than those of LPCs with saturated fatty acids.

Discussion

Two hypotensive compounds were isolated from alcohol extract of Rokujo by the use of

SH rats as a screening system (Table I). One of the two compounds was identified as LPC. D-1,[9] which occurred in acetone extract of bovine brain and might be LPC or closely related compound, was already reported to have hypotensive activity. In addition to the hypotensive effect,[10,11] LPC has antitumor activity,[12] causes activation of various enzymes[13] and has hemolytic activity.[14] However, the isolation of LPC as a hypotensive factor from alcohol extract of Rokujo has been reported for the first time in this paper. It was found that at least a part of the hypotensive action of alcohol extract of Rokujo is due to LPC. Next, the fatty acid composition of LPC isolated from alcohol extract of Rokujo was examined (Table II). The predominant fatty acid was the $C_{16:0}$ acid and its content was 46.1%. The contents of unsaturated fatty acids such as $C_{18:1}$, $C_{16:1}$ and $C_{18:2}$ were 31.5, 6.8 and 5.6%, respectively. The effects of various LPCs on blood pressure in anesthetized SH rats were next examined (Tables IV and V). Among the sample tested, $C_{14:0}$-LPC showed the strongest hypotensive activity (-81.7 ± 1.6 mmHg, 3 mg/kg, i.v.), followed by $C_{16:0}$-LPC (-76.7 ± 7.3 mmHg, 3 mg/kg, i.v.). Among LPCs with unsaturated fatty acids, only $C_{16:1}$-LPC showed the hypotensive effect (-37.5 ± 2.5 mmHg, 3 mg/kg, i.v.). LPC isolated from alcohol extract of Rokujo is rich in $C_{16:0}$ and $C_{16:1}$ acids, which showed rather strong hypotensive effect. The relationship between the fatty acid composition in LPC isolated from alcohol extract of Rokujo and the hypotensive activity is of considerable interest, since it may cast light on the mechanism of the hypotensive action of LPC. As mentioned above, it is well known that LPC has hemolytic activity. Thus, the relationship between the hemolytic and the hypotensive activities of LPC was investigated. First, the hemolytic activity of various LPCs was examined with the red blood cells of rats. $C_{18:0}$-LPC, $C_{18:1}$-LPC and $C_{16:0}$-LPC showed the strongest hemolytic activity among LPCs tested. On the other hand, $C_{14:0}$-LPC, which showed the highest hypotensive activity, had moderate hemolytic activity, and $C_{18:1}$-LPC completely lacked hypotensive activity (data not shown). Further, the hypotensive activity of LPC was decreased by the addition of bovine serum albumin or rat plasma, which completely inhibited the hemolytic action of LPC. However, LPC still retained weak hypotensive activity (data not shown). Therefore, it is unlikely that the hypotensive effect of LPC depends only on the hemolytic activity. The mechanism of the hypotensive activity of LPC and the chemical structure of Fr-1-B-IV will be reported in a subsequent paper.

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Flavonoids Syntheses. V.[1] Synthesis of Flavonoids with Three Hydroxy and Four Methoxy Groups and Their Spectral Properties

MUNEKAZU IINUMA,* TOSHIYUKI TANAKA, KAZUHIRO ITO and MIZUO MIZUNO

Gifu Pharmaceutical University, 6–1 Mitahora-higashi 5-chome, Gifu 502, Japan

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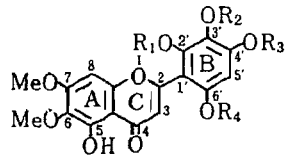
Twelve flavonoids with three hydroxy and four methoxy groups located at C-2′,3′,4′,5,6,6′,7 (**1**—**4**), C-2′,3′,4′,5,6′,7,8 (**5**), C-2′,3,4′,5,5′,6,7 (**6**—**11**) and C-2′,3,4′,5,5′,7, 8 (**12**) were synthesized to confirm the structure of NAS-3 and to investigate the spectral properties of the hepta-oxygenated flavonoids. The methods used to prepare the tetra-oxygenated benzaldehydes (**14**—**19**) for the B ring moieties are also described.

Keywords——flavone synthesis; trihydroxy-tetramethoxyflavone; 2′,3′,4′,5,6,6′,7-heptaoxygenated flavone; 2′,3′,4′,5,6′,7,8-heptaoxygenated flavone; 2′,3,4′,5,5′,6,7-heptaoxygenated flavone; 2′,3,4′,5,5′,7,8-heptaoxygenated flavone

The syntheses of flavonoids hepta-oxygenated at C-2′,3′,4′,5,6,6′,7 and C-2′,3,4′,5,5′, 6,7 with two hydroxy and five methoxy groups were discussed in connection with the structure of brickellin (2′,5-dihydroxy-3,4′,5′,6,7-pentamethoxyflavone) in our previous papers[2,3] The flavonoids with three hydroxy and four methoxy groups located at C-2′,3′,4′,5,6,6′,7, C-2′,3′,4′,5,6′,7,8, C-2′,3,4′,5,5′,6,7 and C-2′,3,4′,5,5′,7,8 were synthesized to confirm the structure of NAS-3.[4] The results of a comparison with the synthetic products (4′,5,6′-trihydroxy-2′,3′,6,7-tetramethoxy- (1), 2′,4′,5-trihydroxy-3′,6,6′,7-tetramethoxy- (**3**) and 3′,5,6′-trihydroxy-2′,4′,6,7-tetramethoxyflavone (**4**)) and identification of NAS-3 with synthetic 2′,4′,5-trihydroxy-3,5′,7,8-tetramethoxyflavone (**13**) were reported in our previous publications.[5,6] In this paper, the synthesis of the flavonoids (**1**—**12**), the preparation of their starting materials and the characteristic spectral features are described.



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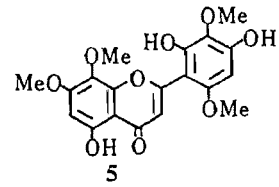

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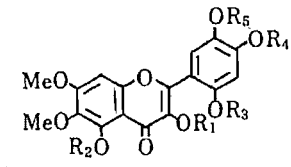
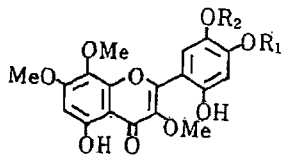
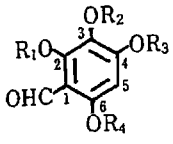


6: $R_1=R_2=R_3=H$, $R_4=R_5=Me$
7: $R_2=R_3=R_4=H$, $R_1=R_5=Me$
8: $R_1=R_3=R_4=H$, $R_2=R_5=Me$
9: $R_1=R_2=R_4=H$, $R_3=R_5=Me$
10: $R_2=R_3=R_5=H$, $R_1=R_4=Me$
11: $R_1=R_3=R_5=H$, $R_2=R_4=Me$



12: $R_1=Me$, $R_2=H$
13: $R_1=H$, $R_2=Me$

Chart 1



14: $R_3=R_4=Bz$, $R_1=R_2=Me$
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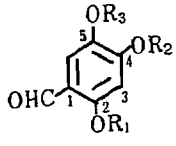
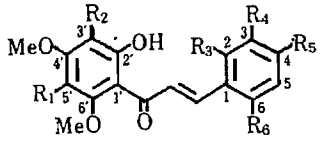
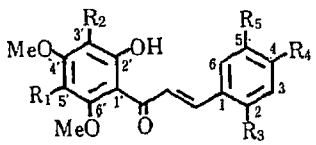
16: $R_3=R_4=\prec$, $R_1=R_2=Me$
17: $R_1=R_4=\prec$, $R_2=R_3=Me$
18: $R_1=R_3=Bz$, $R_2=R_4=Me$
19: $R_2=R_4=Bz$, $R_1=R_3=Me$



20: $R_1=\prec$, $R_2=R_3=Me$
21: $R_1=R_2=\prec$, $R_3=Me$
22: $R_1=R_2=Bz$, $R_3=Me$
23: $R_2=\prec$, $R_1=R_3=Me$
24: $R_1=R_3=\prec$, $R_2=Me$
25: $R_1=R_3=Bz$, $R_2=Me$



34: $R_2=H$, $R_1=R_3=R_4=OMe$, $R_5=R_6=OBz$
35: $R_2=H$, $R_1=R_4=R_5=OMe$, $R_3=R_6=OBz$
36: $R_2=H$, $R_1=R_3=R_4=OMe$, $R_5=R_6=O\prec$
37: $R_2=H$, $R_1=R_4=R_5=OMe$, $R_3=R_6=O\prec$
38: $R_2=H$, $R_1=R_4=R_6=OMe$, $R_3=R_5=OBz$
39: $R_2=H$, $R_1=R_3=R_5=OMe$, $R_4=R_6=OBz$
40: $R_1=H$, $R_2=R_4=R_6=OMe$, $R_3=R_5=OBz$



41: $R_2=H$, $R_1=R_4=R_5=OMe$, $R_3=O\prec$
42: $R_2=H$, $R_1=R_5=OMe$, $R_3=R_4=O\prec$
43: $R_2=H$, $R_1=R_5=OMe$, $R_3=R_4=OBz$
44: $R_2=H$, $R_1=R_3=R_5=OMe$, $R_4=O\prec$
45: $R_2=H$, $R_1=R_4=OMe$, $R_3=R_5=O\prec$
46: $R_2=H$, $R_1=R_4=OMe$, $R_3=R_5=OBz$
47: $R_1=H$, $R_2=R_4=OMe$, $R_3=R_5=O\prec$

$Bz=CH_2C_6H_5$

Chart 2

Preparation of Benzaldehydes (14—19)

The requisite aldehyde for synthesis of **1**, 4,6-dibenzyloxy-2,3-dimethoxybenzaldehyde (**14**) was prepared as follows; bromination of 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin), followed by methylation and the Baeyer–Villiger reaction gave 1-bromo-3-hydroxy-4,5-dimethoxybenzene (**26**), which was converted to 3-hydroxy-4,5-dimethoxybenzaldehyde (**27**).[7] The aldehyde **27** was further subjected to the Baeyer–Villiger reaction to give the diphenol, which was benzylated to obtain 1,5-dibenzyloxy-2,3-dimethoxybenzene (**28**). The Vilsmeier reaction of **28** gave a mixture of **14** and its isomer, 2,6-dibenzyloxy-3,4-dimethoxybenzaldehyde (**15**) in a ratio of *ca.* 1 : 1. Each structure was determined by nuclear Overhauser effect (NOE) techniques in the proton nuclear magnetic resonance (^{1}H-NMR) spectra. In the case of **15**, 23% NOE at the aromatic proton was observed on irradiation of the methoxy groups. On the other hand, no NOE was observed in **14** on similar irradiation. The

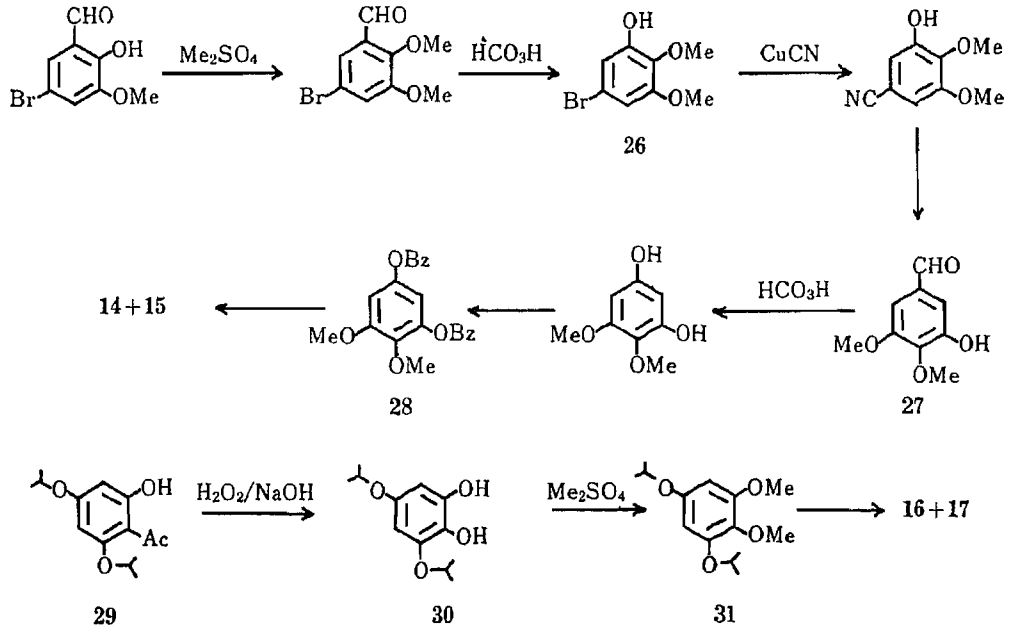


Chart 3

total yield of **14** and **15** was 12% from *o*-vanillin. This method of preparation for **1** was improved by using the isopropyl group instead of the benzyl group. That is, 2-hydroxy-4,6-diisopropyloxyacetophenone (**29**)[8] was subjected to the Dakin reaction to give 1,2-dihydroxy-3,5-diisopropyloxybenzene (**30**), which was easily oxidized to the *o*-quinone in air. The *o*-quinone was immediately methylated after reduction with sodium hydrosulfite to 3,5-diisopropyloxy-1,2-dimethoxybenzene (**31**). The Vilsmeier reaction of **31** gave a mixture of 4,6-diisopropyloxy-2,3-dimethoxy- (**16**) and 2,6-diisopropyloxy-3,4-dimethoxybenzaldehyde (**17**) in a ratio of *ca.* 1 : 1. Each structure was confirmed by the same technique as mentioned above (**16**, 0%; **17**, 23% NOE). The total yield of a mixture of **16** and **17** was 63% from **30**. 2,4-Dibenzyloxy-3,6-dimethoxy- (**18**) and 3,6-dibenzyloxy-2,4-dimethoxybenzaldehyde (**19**) were then prepared by oxidation with nitric acid, reduction with sodium hydrosulfite, methylation (or benzylation) and finally by the Vilsmeier reaction of 1,2,3-tribenzyloxy- or 1,2,3-trimethoxybenzene (see Experimental).

Syntheses of Flavones (1—5) and Flavonols (6—12)

The aldehydes (**14**—**19**) were condensed with 2-hydroxy-4,5,6-trimethoxy- (**32**) or 2-hydroxy-3,4,6-trimethoxyacetophenone (**33**) in the presence of piperidine in pyridine to give the corresponding chalcones, 4,6-dibenzyloxy-2′-hydroxy-2,3,4′,5′,6-pentamethoxy- (**34**), 2,6-dibenzyloxy-2′-hydroxy-3,4,4′,5′,6′-pentamethoxy- (**35**), 2′-hydroxy-4,6-diisopropyloxy-2,3,4′,5′,6′-pentamethoxy- (**36**), 2′-hydroxy-2,6-diisopropyloxy-3,4,4′,5′,6′-pentamethoxy- (**37**), 2,4-dibenzyloxy-2′-hydroxy-3,4′,5′,6,6′-pentamethoxy- (**38**), 3,6-dibenzyloxy-2′-hydroxy-2,4,4′,5′,6′-pentamethoxy- (**39**), and 2,4-dibenzyloxy-2′-hydroxy-3,3′,4′,6,6′-pentamethoxychalcone (**40**). The chalcone **34** was directly oxidized with 2,3-dichloro-5,6-di-

TABLE I. Elemental Analysis and Mass Spectral Data for Synthetic Flavones

Compound	Found[a]		MS (*m/z*) (rel. int.)
	C	H	
1	58.18	4.69	390 (100), 375 (76), 360 (12), 354 (10), 197 (5), 195 (12), 181 (19), 180 (7), 153 (17)
2	58.30	4.71	390 (100), 375 (83), 361 (15), 354 (12), 197 (4), 195 (22), 181 (22), 180 (9), 153 (19)
3	58.42	4.67	390 (100), 375 (60), 361 (13), 354 (12), 197 (7), 195 (12), 181 (15), 180 (13), 153 (20)
4	58.19	4.72	390 (100), 375 (65), 361 (13), 360 (18), 354 (15), 197 (7), 195 (17), 181 (24), 180 (5), 153 (25)
5	58.28	4.61	390 (63), 375 (100), 361 (17), 360 (18), 355 (15), 197 (7), 195 (10), 181 (12), 180 (17), 153 (15)
6	58.22	4.65	390 (100), 375 (52), 361 (10), 359 (33), 357 (17), 347 (13), 341 (11), 329 (10), 195 (13), 194 (14), 181 (17), 179 (25)
7	58.40	4.70	390 (100), 375 (62), 373 (25), 359 (33), 357 (17), 347 (13), 341 (11), 329 (10), 195 (13), 194 (14), 181 (17), 179 (25)
8	58.80	4.71	390 (100), 375 (61), 373 (14), 357 (22), 355 (16), 347 (12), 329 (8), 211 (31), 195 (12), 180 (8), 167 (12), 153 (10)
9	58.50	4.65	390 (100), 375 (20), 359 (22), 197 (32), 192 (16), 181 (12), 179 (22)
10	58.52	4.72	390 (100), 375 (34), 373 (27), 360 (9), 359 (41), 341 (12), 195 (10), 194 (15), 181 (12), 180 (7), 179 (25), 167 (10), 153 (12)
11	58.20	4.73	390 (100), 375 (19), 355 (41), 329 (16), 211 (41), 195 (16), 181 (11), 167 (22), 153 (16)
12	58.22	4.66	390 (100), 375 (8), 359 (20), 355 (10), 347 (6), 195 (55), 194 (73), 181 (33), 179 (29), 167 (10), 153 (18)

a) $C_{19}H_{18}O_9$ requires C; 58.46; H, 4.56%.

TABLE II. Melting Points and ^{1}H-NMR Spectral Data[a] for Synthetic Flavones

Compound	mp (°C)	^{1}H-NMR (ppm)						
		OMe[b]	H-3	H-6	H-8	H-3′	H-5′	H-6′
1	281	3.74, 3.79, 3.85, 3.95	6.28		6.75		6.33	
2	239 (dec.)	3.75 (2), 3.80, 3.92	6.19		6.73		6.28	
3	241	3.69 (2), 3.75, 3.91	6.13		6.74		6.21	
4	232—233	3.75 (2), 3.80, 3.90	6.28		6.75		6.21	
5	220 (dec.)	3.68, 3.73, 3.90 (2)	6.11	6.53			6.20	
6	191—193	3.90 (3), 3.96			6.66	6.54		7.16
7	157—158	3.86, 3.90, 3.93 (2)			6.68	6.49		7.08
8	208—210	3.73, 3.78, 3.86, 3.92			6.95	6.45		6.96
9	203—205	3.81 (3), 3.94			6.62	6.55		7.05
10	220—222	3.73 (2), 3.80, 3.89			6.69	6.52		6.85
11	199—201	3.78 (2), 3.86, 3.90			6.88	6.50		6.91
12	203—205	3.73 (2), 3.80, 3.91		6.55		6.55		6.80

a) All spectra were measured in DMSO-d_6. *b*) The numbers in parentheses are the numbers of methoxy groups.

TABLE III. UV Spectral Data for Synthetic Flavones

Compound	λ_{max}^{MeOH} (nm)	+$AlCl_3$	+$AlCl_3$/HCl	+NaOMe
1	263, 329	275, 298 sh, 362	275, 297 sh, 362	263, 295 sh, 390
2	263, 327	277, 297 sh, 360	276, 297 sh, 360	264, 345
3	263, 325	273, 299, 364	273, 298 sh, 364	263, 385
4	263, 330	276, 386	276, 383	263, 390 sh (dec.)
5	263, 290, 335	275, 305, 390	275, 305 sh, 388	263, 295, 386
6	262, 374	272, 332 sh, 427	272, 329 sh, 424	267, 406
7	262, 206 sh, 350	276, 325, 400	275, 320, 392	268, 300 sh, 408
8	255 sh, 334	290, 325, 428	290, 325, 426	266 sh, 335, 430
9	260, 302 sh, 358	272, 326, 436	268, 324, 410	255 sh, 266, 416
10	261, 355	276, 338, 425	274, 334, 425	269, 380 (dec.)
11	260, 325	270, 333, 430	265, 327, 425	262, 305, 375 (dec.)
12	263, 363	278, 305 sh, 420	277, 305 sh, 416	267, 374 (dec.)

cyano-1,4-benzoquinone (DDQ)[9] to 4′,6′-dibenzyloxy-2′,3′,5,6,7-pentamethoxyflavone (**34a**). Debenzylation, followed by partial demethylation of **34a** gave the desired flavone **1**. Analogous treatment of the chalcones **35**—**40** gave the corresponding flavones **35a**—**40a** which were further led to 2′,5,6′-trihydroxy-3′,4′,6,7-tetramethoxy- (**2**), 2′,4′,5-trihydroxy-3′,6,6′,7-tetramethoxy- (**3**), 3′,5,6′-trihydroxy-2′,4′,6,7-tetramethoxy- (**4**) and 2′,4′,5-trihydroxy-3′,6′,7,8-tetramethoxyflavone (**5**), respectively.

On the other hand, the aldehydes (**20**—**25**), synthesis of which was described in a previous paper,[10] were also condensed with **32** or **33** in the presence of potassium hydroxide to give 2′-hydroxy-2-isopropyloxy-4,4′,5,5′,6′-pentamethoxy- (**41**), 2′-hydroxy-2,4-diisopropyloxy-4′,5,5′,6′-tetramethoxy- (**42**), 2,4-dibenzyloxy-2′-hydroxy-4′,5,5′,6′-tetramethoxy- (**43**), 2′-hydroxy-4-isopropyloxy-2,4′,5,5′,6′-pentamethoxy- (**44**), 2′-hydroxy-2,5-diisopropyloxy-4,4′,5′,6′-tetramethoxy- (**45**), 2,5-dibenzyloxy-2′-hydroxy-4,4′,5′,6′-tetramethoxy- (**46**) and 2′-hydroxy-2,5-diisopropyloxy-3′,4,4′,6′-tetramethoxychalcone (**47**), respectively. The chalcone **41** was subjected to the Algar–Flynn–Oyamada (AFO) flavonol synthesis[2] to afford 3-hydroxy-2′-isopropyloxy-4′,5,5′,6,7-pentamethoxyflavone (**41a**), which

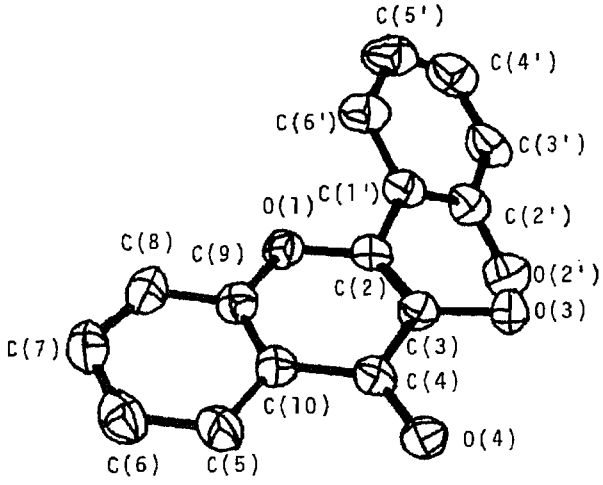


Fig. 1. The X-Ray Model of 2'-Hydroxyflavonol

was treated with boron trichloride to afford 2',3,5-trihydroxy-4',5',6,7-tetramethoxyflavone (**6**). By similar procedures, the chalcones (**42**—**47**) were also led to the respective flavonols (**42a**—**47a**). After methylation, debenzylation, deisopropylation and/or partial demethylation, the above flavonols were converted to 2',4',5-trihydroxy-3,5',6,7-tetramethoxy- (**7**), 2',3,4'-trihydroxy-5,5',6,7-tetramethoxy- (**8**), 3,4',5-trihydroxy-2',5',6,7-tetramethoxy- (**9**), 2',5,5'-trihydroxy-3,4',6,7-tetramethoxy- (**10**), 2',3,5'-trihydroxy-4',5,6,7-tetramethoxy- (**11**) and 2',5,5'-trihydroxy-3,4',7,8-tetramethoxyflavone (**12**), respectively. Tables I, II and III show the physical and the spectroscopic properties of the flavones (**1**—**5**) and the flavonols (**6**—**12**) thus obtained.

In the ^{1}H-NMR spectra of the flavones tetraoxygenated at C-2',3',4',6' in the B ring, the C-3 proton signal is observed at rather high field (6.11—6.28 ppm), and sometimes overlaps the signal of the C-5' proton (6.21—6.33 ppm). In the case of the flavonols trioxygenated at C-2',4',5', the C-3' proton signals is observed at *ca.* 6.55 ppm, the region in which the C-3 proton signal usually appears in flavones lacking substituents at C-2' and/or C-6'. In the ultraviolet (UV) spectra, the absorptions based on band I are characteristically observed at 330 nm in the flavones. In the case of the flavonols, these bands are at 350 nm. The conspicuous UV absorption of the flavonoids oxygenated at C-2' and/or C-6' can be explained as follows: when C-3 and C-2' and/or C-6' are oxygenated, the mesomeric effects between the B ring and C ring diminish because of steric hindrance. This is supported by the results of X-ray analysis of a model compound, 2',3-dihydroxyflavone (2'-hydroxyflavonol): the torsion angles are O1, C2, C1', C2' = −140.54°, O1, C2, C1', C6' = 37.63°, C3, C2, C1', C2' = 43.69° and C3, C2, C1', C6' = −138.14° (Fig. 1).

Further detailed structural characterization of the flavones oxygenated at C-3 and C-2' and/or C-6' is in progress.

Experimental

Melting points were determined on a Büchi melting point apparatus and are uncorrected. ^{1}H-NMR spectra were determined with a Hitachi-Perkin-Elmer R-20B 60 MHz instrument, using tetramethylsilane as an internal standard. UV spectra were recorded on a Hitachi 323 spectrometer. Mass spectra (MS) were taken on a JEOL JMS-D300 machine at 70 eV.

4,6-Dibenzyloxy-2,3-dimethoxy- (14) and 2,6-Dibenzyloxy-3,4-dimethoxybenzaldehyde (15)——5-Bromo-2-hydroxy-3-methoxybenzaldehyde (30 g) was refluxed with Me_2SO_4 (16.8 g) and K_2CO_3 (50 g) in acetone (300 ml) for 3 h to give 5-bromo-2,3-dimethoxybenzaldehyde (29 g) as colorless needles, mp 130 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ: 3.88, 3.95 (3H, each s, OMe), 7.21, 7.49 (1H, each d, J = 2.3 Hz, H-4 and 6), 10.25 (1H, s, CHO). A formic acid solution (30 ml) containing the aldehyde (5 g) was added to HCO_3H prepared from 85% HCO_2H (13 ml) and 30%

H_2O_2 (4.6 g) at 5 °C, and the solution was left in a refrigerator overnight. After addition of Na_2SO_3 (2 g) and water, the reaction mixture was extracted with Et_2O. The ethereal solution was added to 5% KOH (200 ml) and the solution was stirred for 2 h. The water layer was acidified with 5% HCl and extracted with AcOEt. The organic phase was evaporated *in vacuo*, and the residue was chromatographed on silica gel (eluent, $CHCl_3$: MeOH = 10 : 1) to give 1-bromo-3-hydroxy-4,5-dimethoxybenzene (**26**, 3.1 g) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 3.76, 3.78 (3H, each s, OMe), 5.78 (1H, br s, OH), 6.54, 6.96 (1H, each d, J = 2.3 Hz, H-4 and 6). **26** (2.33 g) was refluxed with CuCN (900 mg) in dimethyl formamide (DMF) (50 ml) for 4 h, and allowed to cool then $FeCl_3$ (2 g) in water (10 ml) was added. The reaction mixture was extracted with AcOEt under reflux 90 min. The organic phase was concentrated *in vacuo* to give a brown residue (1.3 g). Without purification, the residue was refluxed with Raney-Ni in 70% HCO_2H for 2 h. The reaction mixture was filtered after cooling, and the filtrate was extracted with AcOEt. The organic phase was evaporated and purified by chromatography to give 3,4-dimethoxy-5-hydroxybenzaldehyde (**27**, 0.9 g) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 3.88, 3.94 (3H, each s, OMe), 6.98 (1H, br s, OH), 7.03, 7.10 (1H, each d, J = 2.3 Hz, H-2 and 6), 9.75 (1H, s, CHO). **27** (2 g) was subjected to the Baeyer–Villiger reaction in the manner described above to give 2,3-dimethoxy-1,6-dihydroxybenzene (1.2 g) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 3.71, 3.76 (3H, each s, OMe), 5.93 (2H, br s, 2 × OH), 5.95, 6.08 (1H, each d, J = 3 Hz, H-4 and 6). The diphenol (1 g), benzyl chloride (1.5 g) and K_2CO_3 (4.5 g) were refluxed in DMF (30 ml) at 120 °C for 1 h. By usual work-up, 1,2-dimethoxy-3,5-dibenzyloxybenzene (**28**) was obtained as a pale brown oil (1.8 g). ^{1}H-NMR ($CDCl_3$) δ: 3.71, 3.76 (3H, each s, OMe), 4.88, 4.99 (2H, each s, $-CH_2-$), 6.20 (2H, br s, H-4 and 6), 7.30 (10H, m, 2 × C_6H_5). **28** (1.2 g) was allowed to react with Vilsmeier reagent, prepared from $POCl_3$ (1 g) and DMF (3 ml), at room temperature overnight. The reaction mixture was poured into 1 N NaOH (40 ml) and extracted with AcOEt. After evaporation of the organic phase *in vacuo*, the oily residue was chromatographed with $CHCl_3$ to give 4,6-dibenzyloxy-2,3-dimethoxybenzaldehyde (**14**, 400 mg) and 2,6-dibenzyloxy-3,4-dimethoxybenzaldehyde (**15**, 420 mg). **14**: A pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 3.88, 3.93 (3H, each s, OMe), 5.04, 5.10 (2H, each s, $-CH_2-$), 6.33 (1H, s, H-5), 7.35 (10H, br s, 2 × C_6H_5), 10.30 (1H, s, CHO). **15**: A pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 3.78 (6H, s, 2 × OMe), 5.04, 5.10 (2H, each s, $-CH_2-$), 6.33 (1H, s, H-5), 7.35 (10H, br s, 2 × C_6H_5), 10.30 (1H, s, CHO).

4,6-Diisopropyloxy-2,3-dimethoxy- (16) and 2,6-Diisopropyloxy-3,4-dimethoxybenzaldehyde (17)——A mixture of 2-hydroxy-4,6-diisopropyloxyacetophenone (**29**, 6.5 g), 6% NaOH (20 ml), pyridine (20 ml) and 3% H_2O_2 (27 ml) was stirred overnight at room temperature. The reaction mixture was extracted with AcOEt after acidification, and the organic phase was washed with 5% sodium hydrosulfite and evaporated to give **30** as a yellow oil (3.8 g). The oily residue was dissolved in acetone (100 ml) and refluxed with Me_2SO_4 (2.52 g) and K_2CO_3 (10 g) to give 2,3-dimethoxy-1,5-diisopropyloxybenzene (**31**, 3.5 g) as a pale brown oil. ^{1}H-NMR ($CDCl_3$) δ: 1.30, 1.35 (6H, each d, J = 6 Hz, $CH(CH_3)_2$), 4.00, 4.09 (3H, each s, OMe), 4.72 (2H, m, 2 × CH<), 6.15 (2H, br s, H-4 and 6). **31** (1 g) was allowed to react with the Vilsmeier reagent, prepared in manner described above, to give 2,3-dimethoxy-4,6-diisopropyloxybenzaldehyde (**16**, 410 mg) and 3,4-dimethoxy-2,6-diisopropyloxybenzaldehyde (**17**, 420 mg). **16**: A pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.38, 1.43 (6H, each d, J = 6 Hz, $CH(CH_3)_2$), 3.82, 3.93 (3H, each s, OMe), 4.55 (2H, m, 2 × CH<), 6.29 (1H, s, H-5), 10.30 (1H, s, CHO). **17**: A pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.38, 1.43 (6H, each d, J = 6 Hz, $CH(CH_3)_2$), 3.82, 3.93 (3H, each s, OMe), 4.65 (2H, hept, 2 × CH<), 6.30 (1H, s, H-5), 10.33 (1H, s, CHO).

2,4-Dibenzyloxy-3,6-dimethoxy- (18) and 3,6-Dibenzyloxy-2,4-dimethoxybenzaldehyde (19)——2,6-Dibenzyloxy-1,4-dimethoxybenzene[12] (4 g) was allowed to react with the Vilsmeier reagent (DMF 10 ml + $POCl_3$ 2.5 g) to give 2,4-dibenzyloxy-3,6-dimethoxybenzaldehyde (**18**, 3.7 g) as colorless needles, mp 93—94 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.78, 3.83 (3H, each s, OMe), 5.13, 5.20 (2H, each s, $-CH_2-$), 6.30 (1H, s, H-5), 7.36, 7.40 (5H, each br s, C_6H_5), 10.23 (1H, s, CHO). 1,2,3-Trimethoxybenzene (25.2 g) was dissolved in warm AcOH (15 ml). With stirring, 30% HNO_3 (60 ml) was added dropwise over 1 h. After cooling, the reaction mixture was filtered, and the collected precipitate was washed with a small amount of MeOH to give 2,6-dimethoxy-1,4-benzoquinone (16.1 g) as yellow needles, mp 260 °C (C_6H_6). The phenol (19.5 g) was benzylated with BzCl (32 g) and K_2CO_3 (52.4 g) in DMF (150 ml) to give 1,4-dibenzyloxy-2,6-dimethoxybenzene (33 g). The benzylated compound (12.1 g) was allowed to react with the Vilmseier reagent prepared in manner described above to give 3,6-dibenzyloxy-2,4-dimethoxybenzaldehyde (**19**, 7.2 g) as colorless needles, mp 64—66 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.80, 3.93 (3H, each s, OMe), 4.93, 5.14 (2H, each s, $-CH_2-$), 7.40 (10H, br s, 2 × C_6H_5), 10.36 (1H, s, CHO).

4′,5,6′-Trihydroxy-2′,3′,6,7-tetramethoxyflavone (1)——The aldehyde (**14**, 900 mg) and an acetophenone (**32**, 1.5 g) were added to pyridine (10 ml) containing piperidine (10 drops), and the solution was stirred overnight. The reaction mixture was diluted with water, acidified with HCl, and extracted with AcOEt. The organic phase was evaporated and the residue was recrystallized to give 4,6-dibenzyloxy-2′-hydroxy-2,3,4′,5′,6′-pentamethoxychalcone (**34**, 1.2 g) as orange-red rectangles, mp 136 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.73, 3.74, 3.83, 3.85, 3.94 (3H, each s, OMe), 5.04, 5.06 (2H, each s, $-CH_2-$), 6.25, 6.36 (1H, each s, H-3′ and 5), 7.33 (10H, br s, 2 × C_6H_5), 8.25 (2H, s, H-α and β), 13.85 (1H, s, OH). **34** (800 mg) was refluxed with (DDQ, 1.2 g) in dry dioxane (20 ml) for 10 h. Usual work-up and purification by silica gel column chromatography (eluent, C_6H_6 : acetone = 5 : 1) gave 4′,6′-dibenzyloxy-2′,3′,5,6,7-pentamethoxyflavone (**34a**) as a pale brown oil (500 mg). ^{1}H-NMR ($CDCl_3$) δ: 3.86, 3.90, 3.93 (3H, each s, OMe), 4.03 (6H, s, 2 × OMe), 5.03, 5.13 (2H, each s, $-CH_2-$), 6.31, 6.43, 6.66 (1H, each s, H-3, 5′ and 8), 7.28, 7.38 (5H, each br s, C_6H_5). **34a** (500 mg) was debenzylated in AcOEt (50 ml) with 10% Pd–C in an H_2 atmosphere to afford

4′,6′-dihydroxy-2′,3′,5,6,7-pentamethoxyflavone (220 mg) as a colorless powder, mp 241 °C (MeOH). The dihydroxyflavone (200 mg) was suspended in CH_2Cl_2 (20 ml) and BCl_3 was added at −60 °C. Purification of the reactant by column chromatography (eluent, C_6H_6 : acetone = 3 : 1) gave **1** (110 mg). An acetophenone (**32**, 1.5 g) and **16** (700 mg) were added to pyridine (10 ml) containing 10 drops of piperidine, and the solution was stirred overnight. After usual work-up, a chalcone (**36**, 1.2 g) was obtained as a red oil. **36** (600 mg) was oxidized with DDQ (1.1 g) in dry dioxane (30 ml) to give 4′,6′-diisopropyloxy-2′,3′,5,6,7-pentamethoxyflavone (**36a**, 340 mg) as a brown oil. ^{1}H-NMR ($CDCl_3$) δ: 1.25, 1.43 (6H, each d, J = 6 Hz, $CH(CH_3)_2$), 3.85, 3.91 (3H, each s, OMe), 3.93 (6H, s, 2 × OMe), 4.50 (2H, m, 2 × CH), 6.28, 6.70 (1H, each s, H-3, 5′ and 8). **36a** (100 mg) was deisopropylated and partially demethylated by using BCl_3 (1 ml) in CH_2Cl_2 at −60 °C to give **1** as a pale yellow powder (45 mg) (AcOEt–C_6H_{14}).

2′,5,6′-Trihydroxy-3′,4′,6,7-tetramethoxyflavone (2)——Condensation of **15** (900 mg) with **32** (1.5 g) gave 2,6-dibenzyloxy-2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone (**35**, 1.5 g) as orange rectangles, mp 136—137 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.60, 3.68 (3H, each s, OMe), 3.85 (6H, s, 2 × OMe), 3.80 (3H, s, OMe), 5.04, 5.15 (2H, each s, $-CH_2-$), 6.23, 6.33 (1H, each s, H-5 and 5′), 7.35 (10H, br s, 2 × C_6H_5), 8.08, 8.13 (1H, each d, J = 16 Hz, H-α and β), 13.85 (1H, s, OH). **35** (500 mg) was oxidized with DDQ (700 mg) to give 2′,6′-dibenzyloxy-3′,4′,5,6,7-pentamethoxyflavone (**35a**, 290 mg) as a pale brown oil. ^{1}H-NMR ($CDCl_3$) δ: 3.86, 3.88, 3.90, 3.95, 4.03 (3H, each s, OMe), 5.11, 5.12 (2H, each s, $-CH_2-$), 6.28, 6.43, 6.53 (1H, each s, H-3, 5′ and 8). **35a** (200 mg) was debenzylated in the manner described above to give 2′,6′-dihydroxy-3′,4′,5,6,7-pentamethoxyflavone (95 mg) as a colorless powder, mp 233 °C (MeOH). The flavone (50 mg) was partially demethylated with BCl_3 (1 ml) to give **2** as a pale yellow powder (AcOEt–C_6H_{14}).

2′,4′,5-Trihydroxy-3′,6,6′,7-tetramethoxyflavone (3)——Condensation of **32** (0.9 g) with **18** (1.5 g) gave 2,4-dibenzyloxy-2′-hydroxy-3,4′,5′,6,6′-pentamethoxychalcone (**38**, 1.8 g) as orange needles, mp 126—127 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.76, 3.80, 3.85, 3.88, 3.98 (3H, each s, OMe), 5.09, 5.12 (2H, each s, $-CH_2-$), 6.25, 6.36 (1H, each s, H-5′ and 5), 7.40 (10H, br s, 2 × C_6H_5), 8.21 (2H, s, H-α and β), 13.98 (1H, s, OH). **38** (1 g) was oxidized with DDQ (1.2 g) to give 2′,4′-dibenzyloxy-3′,5,6,6′,7-pentamethoxyflavone (**38a**, 450 mg) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 3.69, 3.81, 3.86, 3.87, 3.89 (3H, each s, OMe), 5.10, 5.30 (2H, each s, $-CH_2-$), 6.16, 6.38, 6.50 (1H, s, H-3, 5′ and 8), 7.18, 7.45 (5H, br s, C_6H_5). **38a** (450 mg) was debenzylated to give 2′,4′-dihydroxy-3′,5,6,6′,7-pentamethoxyflavone (190 mg) as pale yellow needles, mp 210—211 °C (MeOH). ^{1}H-NMR (DMSO-d_6) δ: 3.69 (6H, s, 2 × OMe), 3.79, 3.84, 3.93 (3H, each s, OMe), 5.99, 6.11, 6.94 (1H, each s, H-3,5′ and 8). The above flavone (450 mg) was partially demethylated to give **3** (350 mg) as a pale yellow powder (AcOEt–C_6H_{14}).

3′,5,6′-Trihydroxy-2′,4′,6,7-tetramethoxyflavone (4)——Condensation of **32** (0.9 g) with **19** (1.5 g) gave 3,6-dibenzyloxy-2′-hydroxy-2,4,4′,5′,6′-pentamethoxychalcone (**39**, 2 g) as orange rectangles, mp 127—128 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.76, 3.78, 3.88 (3H, each s, OMe), 3.96 (6H, s, 2 × OMe), 4.95, 5.20 (2H, each s, $-CH_2-$), 6.25, 6.31 (1H, each s, H-5 and 5′), 7.40 (10H, br s, 2 × C_6H_5), 8.27 (2H, s, H-α and β). A DDQ oxidation of **39** (1 g) gave 3′,6′-dibenzyloxy-2′,4′,5,6,7-pentamethoxyflavone (**39a**, 550 mg) as colorless needles, mp 141—143 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.81, 3.86, 3.89 (3H, each s, OMe), 3.99 (6H, s, 2 × OMe), 4.96, 5.06 (2H, each s, $-CH_2-$), 6.30, 6.38, 6.63 (1H, each s, H-3, 5′ and 8), 7.40 (10H, br s, 2 × C_6H_5). By usual debenzylation, **39a** (200 mg) was derived to 3′,6′-dihydroxy-2′,4′,5,6,7-pentamethoxyflavone (95 mg) as pale yellow prisms, mp 245 °C (dec.) (C_6H_6). ^{1}H-NMR (DMSO-d_6) δ: 3.73, 3.75 (3H, each s, OMe), 3.80 (6H, s, 2 × OMe), 3.95 (3H, s, OMe), 6.03, 6.35, 6.95 (1H, each s, H-3, 5′ and 8). The above flavone (100 mg) was partially demethylated to give **4** (65 mg) as a pale yellow powder (AcOEt–C_6H_{14}).

2′,4′,5-Trihydroxy-3′,6′,7,8-tetramethoxyflavone (5)——Condensation of **33** (0.9 g) with **18** (1.5 g) gave 2,4-dibenzyloxy-2′-hydroxy-3,3′,4′,6,6′-pentamethoxychalcone (**40**, 2 g) as orange needles, mp 127—128 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.63, 3.91, 3.83, 3.86, 3.91 (3H, each s, OMe), 5.06, 5.18 (2H, each s, $-CH_2-$), 5.98, 6.35 (1H, each s, H-5 and 5′), 7.40 (10H, br s, 2 × C_6H_5), 8.22 (2H, s, H-α and β). By DDQ oxidation, **40** (1 g) was converted to 2′,4′-dibenzyloxy-3′,5,6′,7,8-pentamethoxyflavone (**40a**, 520 mg) as colorless needles, mp 152 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.70, 3.73, 3.88 (3H, each s, OMe), 3.98 (6H, s, 2 × OMe), 5.10, 5.20 (2H, each s, $-CH_2-$), 6.18, 6.38, 6.43 (1H, each s, H-3, 5′ and 6), 7.18, 7.43 (5H, each br s, C_6H_5). **40a** (500 mg) was debenzylated to give 2′,4′-dihydroxy-3′,5,6′,7,8-pentamethoxyflavone (230 mg) as a pale brown oil. ^{1}H-NMR (DMSO-d_6) δ: 3.70, 3.85, 3.93 (3H, each s, OMe), 3.65 (6H, each s, 2 × OMe), 5.90, 6.08, 6.60 (1H, each s, H-3, 5′ and 6). The above flavone (200 mg) was partially demethylated to give **5** (105 mg) as a pale yellow powder (AcOEt–C_6H_{14}).

2′,3,5-Trihydroxy-4′,5′,6,7-tetramethoxyflavone (6)——A solution of 2′-hydroxy-2-isopropyloxy-4,4′,5,5′,6′-pentamethoxychalcone[10] (**41**, 1.5 g) dissolved in MeOH (60 ml) was added to 35% H_2O_2 (9 ml) and 30% NaOH (20 ml) to give 3-hydroxy-2′-isopropyloxy-4′,5,5′,6,7-pentamethoxyflavone (**41a**, 900 mg) after purification by silica gel chromatography (eluent, C_6H_6 : acetone = 3 : 1) as yellow prisms, mp 155—156 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.37 (6H, d, J = 6 Hz, $CH(CH_3)_2$), 3.89, 3.90 (3H, each s, OMe), 3.92 (6H, s, 2 × OMe), 4.05 (3H, s, OMe), 4.27 (1H, hept, J = 6 Hz, CH), 6.67, 6.76, 7.14 (1H, each s, H-3′, 6′ and 8). The above flavone (100 mg) was deisopropylated by treatment with BCl_3 to give **6** (45 mg) as pale yellow needles (MeOH).

2′,4′,5-Trihydroxy-3,5′,6,7-tetramethoxyflavone (7)——2′-Hydroxy-2,4-diisopropyloxy-4′,5,5′,6′-tetramethoxychalcone[10] (**42**, 2.5 g) was worked up in the manner described above to give 3-hydroxy-2′,4′-diisopropyloxy-5,5′,6,7-tetramethoxyflavone (**42a**, 1.2 g) as yellow prisms, mp 180—182 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.25, 1.39 (6H,

each d, J=6 Hz, $CH(CH_3)_2$), 3.89 (3H, s, OMe), 3.91 (6H, s, 2×OMe), 4.01 (3H, s, OMe), 4.20—4.70 (2H, m, 2×CH<), 6.69, 6.74, 7.03 (1H, each s, H-3′, 6′ and 8). **42a** (500 mg) was methylated to give 2′,4′-diisopropyloxy-3,5,5′,6,7-pentamethoxyflavone (420 mg) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.27, 1.42 (6H, each d, J=6 Hz, $CH(CH_3)_2$), 3.81, 3.83 (3H, each s, OMe), 3.91 (6H, s, 2×OMe), 4.02 (3H, s, OMe), 4.25—4.85 (2H, m, 2×CH<), 6.69, 6.74, 7.03 (1H, each s, H-3′, 6′ and 8). The flavone (100 mg) was deisopropylated and partially demethylated with BCl_3 to give **7** (30 mg) as yellow needles (MeOH).

2′,3,4′-Trihydroxy-5,5′,6,7-tetramethoxyflavone (8)——2,4-Dibenzyloxy-2-′-hydroxy-4′,5,5′,6′-tetramethoxychalcone[10] (**43**, 3.4 g) was derived to 2′,4′-dibenzyloxy-3-hydroxy-5,5′,6,7-tetramethoxyflavone (**43a**, 1.7 g) as yellow prisms, mp 159—160 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 3.86, 3.88, 3.90, 4.02 (3H, each s, OMe), 5.01, 5.19 (2H, each s, $-CH_2-$), 6.62, 6.70, 7.20 (1H, each s, H-3′, 6′ and 8), 7.35, 7.40 (5H, each brs, C_6H_5). **43a** (150 mg) was debenzylated to give **8** (55 mg) as yellow needles (MeOH).

3,4′,5-Trihydroxy-2′,5′,6,7-tetramethoxyflavone (9)——3-Hydroxy-4′-isopropyloxy-2′,5,5′,6,7-pentamethoxyflavone[2] (**44a**, 350 mg) was deisopropylated and partially demethylated to give **9** (170 mg) as a pale yellow powder (MeOH).

2′,5,5′-Trihydroxy-3,4′,6,7-tetramethoxyflavone (10)——2′,5′-Dihydroxy-3,4′,5,6,7-pentamethoxyflavone (apulein)[2] (100 mg) was partially demethylated to give **10** (65 mg) as yellow needles (MeOH).

2′,3,5′-Trihydroxy-4′,5,6,7-tetramethoxyflavone (11)——2′,5′-Dibenzyloxy-3-hydroxy-4′,5,6,7-tetramethoxyflavone[2] (**45a**, 1.5 g) was debenzylated to give **11** (410 mg) as a yellow powder (AcOEt–C_6H_{14}).

2′,5,5′-Trihydroxy-3,4′,7,8-tetramethoxyflavone (12)——Condensation of **33** (1 g) with **24** (1.2 g) gave 2,5-diisopropyloxy-2′-hydroxy-3′,4,5′,6′-tetramethoxychalcone (**47**, 1.4 g) as an orange powder, mp 141 °C (MeOH). ^{1}H-NMR ($CDCl_3$) δ: 1.38, 1.40 (6H, each d, J=6 Hz, $CH(CH_3)_2$), 3.83, 3.88, 3.90, 3.93 (3H, each s, OMe), 4.40—4.70 (2H, m, 2×CH<), 5.99, 6.48, 7.13 (1H, each s, H-3, 5′ and 6), 7.63, 8.13 (1H, each d, J=16 Hz, H-α and β), 14.01 (1H, s, OH). **47** (600 mg) was derived to 2′,5′-diisopropyloxy-3-hydroxy-4′,5,7,8-tetramethoxyflavone (**47a**, 390 mg) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.15, 1.33 (6H, each d, J=6 Hz, $CH(CH_3)_2$), 3.85 (6H, s, 2×OMe), 3.94 (6H, s, 2×OMe), 4.45 (2H, hept, J=6 Hz, 2×CH<), 6.40, 6.63, 7.19 (1H, each s, H-3′, 6 and 6′). **47a** (300 mg) was methylated to give 2′,5′-diisopropyloxy-3,4′,5,7,8-pentamethoxyflavone (250 mg) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 1.20, 1.35 (6H, each d, J=6 Hz, $CH(CH_3)_2$), 3.78, 3.84, 3.88, 3.94, 3.98 (3H, each s, OMe), 4.50—4.75 (2H, m, CH<), 6.45, 6.64, 7.03 (1H, each s, H-3′, 6 and 6′). The above flavone (200 mg) was deisopropylated and partially demethylated to give **12** (90 mg) as a yellow powder (AcOEt–C_6H_{14}).

Crystallographic Measurement——A single crystal of 2′-hydroxyflavonol was grown in EtOH solution. It was colorless. The crystal data of the compound are: a=10.864, b=10.035, c=8.787 Å, α=97.302, β=104.914, γ=111.32°. Space group $C2/C$.

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New Acyclic Bis-phenylpropanoids from the Aril of *Myristica fragrans*[1)]

MASAO HATTORI, SUMITRA HADA, YUE-ZHONG SHU, NOBUKO KAKIUCHI and TSUNEO NAMBA*

Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama, Japan

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From a methanolic extract of the aril of *Myristica fragrans* HOUTT. (mace), the following new *threo* and *erythro* acyclic bis-phenylpropanoids were isolated: *threo*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**1a**), *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropane (**2b**), *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-ol (**3b**), 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propane (**4**), *erythro*-2-(4-allyl-2-methoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**5b**), *threo*-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-[2-methoxy-4-(1(*E*)-propenyl)phenoxy]propan-1-ol (**6a**) and *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3-hydroxy-4,5-dimethoxyphenyl)propan-1-ol (**7b**). Contrary to a previous finding that *Myristica* species contain only an *erythro* type of acyclic bis-phenylpropanoids, we isolated both *threo* and *erythro* derivatives.

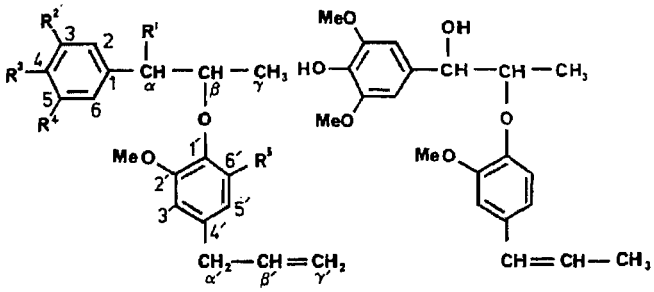
Keywords——acyclic bis-phenylpropanoid; mace; Myristicaceae; *Myristica fragrans*; neolignan

The aril of *Myristica fragrans* HOUTT. (Myristicaceae), mace, has been widely used as a spice and a valuable remedy in Ayurvedic medicine for treatment in the low stage of fever, in consumptive complaints, humoral asthma, and when mixed with aromatics, in wasting and long-term bowel complaints.[2)] There have been numerous reports on the constituents of mace as well as nutmeg (the seed kernels of *Myristica fragrans*) in the literature. These include essential oils,[3)] fats, glycerides,[4)] cyclic and acyclic bis-phenylpropanoids[5)] and a pigment.[6)] Pharmacological studies have also been conducted by many workers as to hallucinogenic effect,[7)] and inhibitory effects on the growth of silkworm larvae, *Bombyx mori*,[5a,b)] and on prostaglandin biosynthesis.[8)] Recently, we have reported that dehydrodiisoeugenol and 5′-methoxydehydrodiisoeugenol from mace have antibacterial action against a primary cariogenic bacterium, *Streptococcus mutans*.[9)] In a continuation of this investigation, we report the isolation of new *threo* and *erythro* diastereomers of acyclic bis-phenylpropanoids (**1a**, **2b**, **3b**, **5b**, **6a** and **7b**; but with **a**=*threo* and **b**=*erythro*), as well as **4**.

Results and Discussion

A methanolic extract of the aril of *Myristica fragrans* was subjected to solvent fractionation and silica gel column chromatography. This procedure led to the isolation of new compounds (**1a**, **2b**, **3b**, **4**, **5b**, **6a** and **7b**) along with the known 4-propenylphenols, dehydrodiisoeugenol derivatives, guaiacin,[9)] and acyclic bis-phenylpropanoids.[9)] The structures of the new compounds were determined as follows:

Compound **1a** was isolated as an oily substance with the molecular formula $C_{21}H_{26}O_6$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum (in CD_3COOD) showed signals



6a

Fig. 1. Structures of Acyclic Bis-phenylpropanoids

	R^1	R^2	R^3	R^4	R^5
1a, b	OH	OMe	OH	H	OMe
2a, b	OMe	OMe	OH	H	OMe
3b	OH	OMe	OH	OMe	OMe
4	H	OMe	OH	H	OMe
5b	OH	OMe	OH	H	H
7b	OH	OH	OMe	OMe	OMe

a, *threo* derivative; b, *erythro derivative.*

TABLE I. ^{13}C-NMR Spectral Data for Acyclic Bis-phenylpropanoids

Carbon No.	1a	2b	3b	4	5b	6a	7b
1	135.3	135.3	133.7	134.6	133.6	131.2	133.1
2	109.6	109.5	103.0	112.2	109.0	104.3	105.9
3	146.3	146.3	147.0	146.1	146.4	147.0	148.8
4	145.2	144.7	140.0	143.8	144.8	137.5	136.1[a]
5	114.0	113.7	147.0	113.9	113.9	147.0	152.2
6	120.6	120.4	103.0	122.1	119.1	104.3	101.9
α	86.4	85.2	82.3	42.9	82.3	84.1	82.0
β	79.0	82.4	73.1	79.9	73.6	78.4	72.7
γ	17.4	13.9	12.7	19.5	13.4	17.1	12.7
1′	135.8	135.3	136.1	135.3	144.8	146.8	136.3[a]
2′	152.7	153.4	153.5	153.6	145.6	149.8	153.4
3′	105.6	105.7	105.7	105.8	112.6	109.5	105.6
4′	132.7	131.5	131.2	131.0	131.9	133.6	130.6
5′	105.6	105.7	105.7	105.8	120.0	119.1	105.6
6′	152.7	153.4	153.5	153.6	119.0	118.8	153.4
α′	40.4	40.4	40.5	40.4	39.9	130.5	40.4
β′	137.0	137.2	137.0	137.2	137.2	124.8	137.0
γ′	116.0	115.7	116.1	115.7	115.8	18.2	116.0
–OMe	55.9	55.9	56.1	56.0	55.9	55.8	55.9
		57.3	56.3	55.8	55.8	56.4	56.1
							60.8

a) Assignments may be interchanged. a, *threo* derivative; b, *erythro* derivative.

of one *sec*-methyl (δ 1.16, J=6.3 Hz), three methoxyls (δ 3.83 and 3.86; 3H and 6H, respectively), one allyl group (δ 3.35, 2H, d, J=6.7 Hz; 5.06—5.15, 2H, m; 5.80—6.03, 1H, m), one methine substituted by oxygen (β-methine; δ 4.02, br dq, J=6.3 and 8.4 Hz), one benzylic methine substituted by oxygen (α-methine; δ 4.66, d, J=8.4 Hz), and five aromatic protons (δ 6.50, 2H, s; 6.82—6.93, 3H). On irradiation at δ 4.02, the two doublets at δ 4.66 (α-H) and 1.16 (γ-H) each became singlets. On irradiation at δ 4.66, the double quartet at δ 4.02 (β-H) became a sharp quartet (J=*ca.* 6 Hz). Further, the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum (Table I) showed the presence of 4-allyl-2,6-dimethoxyphenoxyl and 4-hydroxy-3-methoxyphenyl groups in the molecule. These findings indicate that **1a** is an acyclic bis-phenylpropanoid. The molecular ion peak and mass fragmentation pattern (Table II) were identical with those of a known constituent of mace, *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol (**1b**).[5] The coupling constant between the α- and β-methine protons in **1a** ($J_{\alpha,\beta}$=8.4 Hz in CD_3COOD) was, however,

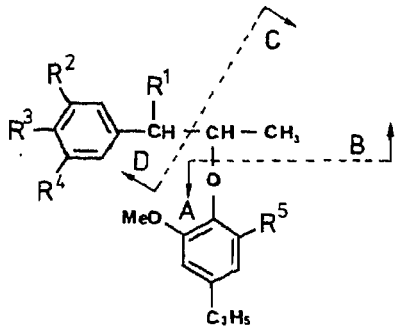


Fig. 2. Mass Fragmentation Pattern of Acyclic Bis-phenylpropanoids

Table II. Mass Spectral Data for Acyclic Bis-phenylpropanoids

Compound	$[M]^+$	$[M-HR^1]^+$	$[A+H]^+$	$[C]^+$	Others
1a	374 (8%)	356 (10%)	194 (100%)	221 (36%)	
2b	388 (38%)	356 (38%)	194 (100%)	221 (4%)	167 (30%), 195 (35%)
3b	404 (6%)	386 (13%)	194 (100%)	221 (30%)	167 (70%)
4	358 (21%)		194 (100%)	221 (8%)	137 (40%), 165 (100%)
5b	344 (2%)	326 (16%)	164 (100%)	191 (4%)	
6a	374 (30%)		164 (100%)	191 (40%)	181 (100%)
7b	404 (18%)		194 (100%)	221 (46%)	181 (58%)

R^1=OH for **1a**, **3b** and **5b**; R^1=OMe for **2b**.

larger than that in **1b** ($J_{\alpha,\beta}$=2.9 Hz).[10] Consequently, **1a** was concluded to be *threo*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol. This is the second example of the isolation of *threo* acyclic bis-phenylpropanoids from *Myristica fragrans*.[11]

Compound **2b** was isolated as an oily substance with the molecular formula $C_{22}H_{28}O_6$. The ^{1}H-NMR spectrum (in $CDCl_3$) showed signals due to one *sec*-methyl (δ 1.25, d, J=6.6 Hz), one aliphatic methoxyl (δ 3.37),[5h,9] three aromatic methoxyls (δ 3.79 and 3.87; 6H and 3H, respectively), two methines substituted by oxygen (δ 4.16, dq, J=3.4 and 6.6 Hz; δ 4.40, d, J=3.4 Hz), one allyl group, and five aromatic protons (δ 6.38 and 6.72—6.93, 2H and 3H, respectively). The spectral features were similar to those of **1b** except for the aliphatic methoxyl signal, suggesting that **2b** is an alpha-(*O*)-methyl ether of **1b**. The molecular ion peak at *m/z* 388 and the mass fragmentation pattern of **2b** (Table II) were identical with those of a known constituent of mace, *threo*-2-(4-allyl-2,6-dimethoxy)-1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropane (**2a**), which we have previously isolated from mace.[9] The ^{1}H-NMR spectrum of **2b** was, however, not identical with that of **2a**, but in both chemical shifts and coupling constants was similar to that of a synthetic *erythro*-isomer as reported by Nishiyama *et al.*[12]; the $J_{\alpha,\beta}$ value was smaller in **2b** ($J_{\alpha,\beta}$=3.4 Hz) than in **2a** ($J_{\alpha,\beta}$=6.6 Hz in CD_3COOD), indicating that **2b** is *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropane.

Compound **3b** was isolated as an oily substance with the molecular formula $C_{22}H_{28}O_7$. The mass spectrum (MS) showed a characteristic pattern ascribed to an acyclic bis-phenylpropanoid (Table II). The molecular ion peak was 30 mass units higher than that of **1a** and **1b**, suggesting a monomethoxylated derivative of either **1a** or **1b**. The ^{13}C-NMR spectrum showed the presence of 4-allyl-2,6-dimethoxyphenoxyl and 4-hydroxy-3,5-dimethoxyphenyl groups (Table I), which were also confirmed by the observation of two distinct singlet signals (δ 6.46 and 6.55, 2H each) and four methoxyl signals (δ 3.870 and 3.874, 6H each) in the ^{1}H-NMR spectrum. Based on the above evidence together with the small $J_{\alpha,\beta}$ value (2.8 Hz),

3b was concluded to be *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-ol. This compound was first suggested to occur in mace by Davis *et al.*[13] and Harvey[14] on the basis of mass spectrometry-mass spectrometry and gas chromatography-mass spectrometry, respectively, but has not previously been isolated.

Compound **4** was isolated as an oily substance with the molecular formula $C_{21}H_{26}O_5$. The ^{1}H-NMR spectrum showed characteristic peaks due to a 4-allyl-2,6-dimethoxyphenoxyl group, but no peak ascribable to an α-methine proton in an acyclic bis-phenylpropanoid. Instead, two double-doublet signals (each one proton) were observed at δ 2.72 (α-H_a; J = 13.4 and 8.3 Hz) and 3.12 (α-H_b; J = 13.4 and 5.1 Hz). On irradiation at δ 4.33 (β-methine), the two double-doublets at δ 2.72 and 3.12 became two doublets (J = *ca.* 13 Hz each) and the doublet at δ 1.20 (J = 6.1 Hz, γ-H) became a singlet, suggesting a partial structure of Ph–CH_2–CH(R)–CH_3 (but with R = 4-allyl-2,6-dimethoxyphenoxyl). The ^{13}C-NMR spectrum showed the presence of a 4-hydroxy-3-methoxyphenyl group (Table I). Based on these findings as well as the mass fragmentation pattern (Table II), the structure of **4** was determined to be 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propane.

Compound **5b** was isolated as an oily substance. The MS showed a molecular ion peak at *m/z* 344 in agreement with a molecular formula of $C_{20}H_{24}O_5$. The ^{1}H-NMR spectrum showed the presence of *sec*-methyl (δ 1.17, d, J = 6.3 Hz), α- and β-methines (δ 4.82, br s; δ 4.32, m; respectively), an allyl group and six aromatic protons. The ^{13}C-NMR spectrum showed the presence of both 4-hydroxy-3-methoxyphenyl and 4-allyl-2-methoxyphenoxyl groups (Table I), the latter of which was also confirmed by the observation of an intense mass fragment peak at *m/z* 164 (Table II). On acetylation, **5b** yielded a diacetate (^{1}H-NMR, aliphatic and aromatic acetoxyls at δ 2.06 and 2.23, respectively). The small $J_{\alpha,\beta}$ values of **5b** and its acetate (*ca.* 2—3 Hz and 4.4 Hz, respectively) were indicative of *erythro* derivatives. Based on these findings, **5b** was concluded to be *erythro*-2-(4-allyl-3-methoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol.

Compound **6a** was isolated as an oily substance, $C_{21}H_{26}O_6$. In contrast to the spectra of the series of acyclic bis-phenylpropanoids mentioned above, the ^{1}H-NMR spectrum showed no signals due to an allyl group but showed characteristic ABX_3-type signals at δ 1.88, *ca.* 6.15 and 6.36 (J_{AB} = 15.8 Hz, J_{AX} = 1.5 Hz and J_{BX} = 6.6 Hz) due to a 1(*E*)-propenyl group. Further, the spectrum showed signals of one *sec*-methyl (δ 1.17), three methoxyls (δ 3.89 and 3.92; 6H and 3H, respectively), two α- and β-methine protons (δ 4.59, d, J = 8.3 Hz; δ *ca.* 4.09, dq, J = 8.3 and 6.2 Hz) and five aromatic protons. The aromatic proton signal at high field, δ 6.61 (2H, s) and the methoxyl signals at δ 3.89 (6H, s), as well as the ^{13}C-NMR signals (Table I) indicated the presence of a 4-hydroxy-3,5-dimethoxyphenyl group in the molecule. In addition, the ^{13}C-NMR spectrum indicated the presence of a 2-methoxy-4-(1(*E*)-propenyl)-phenoxyl group. These findings and the large $J_{\alpha,\beta}$ value (8.3 Hz) led to the structure of *threo*-1-(4-hydroxy-3,5-dimethoxyphenyl)-2-[2-methoxy-4-(1(*E*)-propenyl)phenoxy]propan-1-ol for **6a**.

Compound **7b** had the molecular formula $C_{22}H_{28}O_7$. The ^{1}H-NMR spectrum showed characteristic signals indicative of a bis-phenylpropanoid derivative; one *sec*-methyl (δ 1.12, d, J = 6.4 Hz), four methoxyls (δ 3.89), α- and β-methines (δ 4.75, br s; δ 4.35, dq, J = 2.8 and 6.4 Hz), an allyl group and four aromatic protons. A 4-allyl-2,6-dimethoxyphenoxy group was apparent from the ^{1}H-NMR, ^{13}C-NMR (Table I) and mass (Table II) spectra, but common substituents such as 4-hydroxy-3,5-dimethoxyphenyl and 4-hydroxy-3-methoxyphenyl groups were absent in the molecule. The coupling constants (J = *ca.* 1.4 Hz) of the signals at δ 6.42 and 6.58 suggested that these aromatic protons were located in the meta position with respect to each other. Further, the two methoxy signals at δ 55.9 and lower field, 60.8, in the ^{13}C-NMR spectrum suggested the presence of a 3-hydroxy-4,5-dimethoxyphenyl group.[15] The ^{13}C-NMR signals assignable to this group were in good agreement with those reported.[15]

These findings and the small $J_{\alpha,\beta}$ value (2.7 Hz) led to the structure of *erythro*-2-(4-allyl-2,6-dimethoxyphenoxy)-1-(3-hydroxy-4,5-dimethoxyphenyl)propan-1-ol for **7b**.

A ^{1}H-NMR technique provided a convenient way to assign *threo* and *erythro* diastereomers of acyclic bis-phenylpropanoids, $J_{\alpha,\beta}$ values being in the range of 8.0—8.6 Hz[15] for the *threo* derivatives and 2.7—4.4 Hz for the *erythro* derivatives.[5a,b,e,h,9,15] However, the diagnostic α-methine proton did not show a simple doublet for such compounds as **2a**, **5b** and **7b**, due to overlapping with a β-proton signal or spin–spin coupling with an α-hydroxyl proton. The difficulty in determining $J_{\alpha,\beta}$ values could be overcome in general by using CD_3COOD instead of $CDCl_3$ as a solvent. On the other hand, the diastereomers were quite simply assignable on the basis of a diagnostic methyl signal in the ^{13}C-NMR spectrum, this signal appearing at δ 16.5—17.4 for the *threo* derivatives and at 12.6—13.9 for the *erythro* derivatives.[9,15]

The acyclic bis-phenylpropanoids, Ph–CH(OH)–CH(O–Ph)–CH_3, have been isolated exclusively from plants in the family of Myristicaceae.[16] Among them, all of the compounds isolated so far from *Virola surinamensis* (ROL.) WARD. belong to the *threo* series carrying a 2-methoxy-4-(1(*E*)-propenyl)phenoxyl group, while those from *Myristica fragrans* belong to the *erythro* series carrying a 4-allyl-2,6-dimethoxyphenoxyl group.[15] However, we have isolated *threo* and *erythro* acyclic derivatives with a 4-allyl-2,6-dimethoxyphenoxyl (**1a** and **1b**; **2a** and **2b**; **3b**; **7b**), a 4-allyl-2-methoxyphenoxyl (**5b**) or a 2-methoxy-4-(1(*E*)-propenyl)phenoxyl group (**6a**) from the aril of *Myristica fragrans*. In addition, we have isolated a new derivative (**7b**) possessing a 3-hydroxy-4,5-dimethoxyphenyl group as well as α-methoxy (**2a** and **2b**) and α-dihydro (**4**) derivatives.

In conclusion, seven acyclic bis-phenylpropanoids have been isolated as new natural products from the aril of *Myristica fragrans*. This is the first report of the co-existence of both *threo* and *erythro* diastereomers of acyclic bis-phenylpropanoids in the same plant.

Experimental

Apparatus——All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were measured with JEOL GX-270 (^{1}H, 270 MHz) and JEOL FX-90Q (^{13}C, 22.5 MHz) spectrometers with tetramethylsilane as an internal standard. MS were measured with a JMS-DX 300 mass spectrometer (JEOL) at an ionization voltage of 70 eV. Ultraviolet (UV) spectra were measured with a Shimadzu UV-210 digital double beam spectrophotometer. Infrared (IR) spectra were taken on a Hitachi 260-10 infrared spectrometer.

Plant Material——The arils of *Myristica fragrans* (mace) were purchased from W. Wilbert and Co. (Colombo, Sri Lanka) in 1983, and were ground before extraction.

Chromatography——Silica gel, Wako gel C-200, was used for column chromatography. Merck Kieselgel 60 F_{254} plates were used for thin layer chromatography (TLC) and Merck PSC-60 F_{254} plates for preparative TLC. Solvent systems used were as follows: A, $CHCl_3$–MeOH (9 : 1, v/v); B, benzene–EtOAc (9 : 1, v/v); C, benzene–acetone (9 : 1, v/v). Spots on the plate were detected under ultraviolet light.

Isolation of Components of Mace——Crude powder of mace (936 g) was extracted with MeOH at room temperature as described in a previous paper.[9] The extract (290 g) was partitioned between 95% MeOH and *n*-hexane. A portion of the 95% MeOH soluble (84.5 g) was separated into acidic, phenolic and neutral fractions. The phenolic fraction (31.5 g) was chromatographed on silica gel with benzene containing increasing amounts of ethyl acetate, and the new compounds **1a**, **2b**, **3b**, **4**, **5b**, **6a** and **7b** were obtained in yields of 110, 120, 240, 50, 30, 110 and 30 mg, respectively, along with the known constituents.[9]

***threo*-2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol (1a)**——High-resolution MS, Calcd for $C_{21}H_{26}O_6$, *m/z*: 374.1730 (M^+). Found, *m/z*: 374.1746. IR (KBr) cm^{-1}: 3475 (OH), 1615, 1540, 1525 (aromatic). ^{1}H-NMR ($CDCl_3$) δ: 1.18 (3H, d, J=6.3 Hz, γ-H×3), 3.35 (2H, d, J=6.6 Hz, α′-H×2), 3.87 (9H, s, -OMe×3), *ca.* 3.85—3.95 (β-H, overlapped with the peaks of methoxy groups), 4.60 (1H, d, J=8.5 Hz, α-H), 5.08—5.16 (2H, m, α′-H×2), 5.85—6.01 (1H, m, β′-H), 6.44 (2H, s, 3′-H and 5′-H), 6.84—6.87 (3H, 2-H, 5-H and 6-H). ^{1}H-NMR (CD_3COOD) δ: 1.16 (3H, d, J=6.3 Hz, γ-H×3), 3.35 (2H, d, J=6.7 Hz, α′-H×2), 3.83 (3H, s, 3-OMe), 3.86 (6H, s, 2′-OMe and 5′-OMe), 4.02 (1H, br dq, J=6.3, 8.4 Hz, β-H), 4.66 (1H, d, J=8.4 Hz, α-H), 5.06—5.15 (2H, m, γ′-H×2), 5.80—6.03 (1H, m, β′-H), 6.50 (2H, s, 3′-H and 5′-H), 6.82—6.93 (3H, aromatic H).

***erythro*-2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropane (2b)**——High-resolution MS, Calcd for $C_{22}H_{28}O_6$, *m/z*: 388.1886 (M^+). Found, *m/z*: 388.1896. ^{1}H-NMR ($CDCl_3$) δ 1.25 (3H, d, J=6.6 Hz, γ-H × 3), 3.33 (2H, d, J=6.8 Hz, α'-H × 2), 3.37 (3H, s, α-OMe), 3.79 (6H, s, 2'-OMe, 6'-OMe), 3.87 (3H, s, 3-OMe), 4.16 (1H, dq, J=3.4, 6.6 Hz, β-H), 4.40 (1H, d, J=3.4 Hz, α-H), 5.06—5.14 (2H, m, γ'-H × 2), 5.55 (1H, s, -OH), 5.89—6.07 (1H, m, β'-H), 6.38 (2H, s, 3'-H and 5'-H), 6.72—6.93 (3H, m, 2-H, 5-H and 6-H).

***erythro*-2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-ol (3b)**——High-resolution MS, Calcd for $C_{22}H_{28}O_7$, *m/z*: 404.1835 (M^+). Found, *m/z*: 404.1800. ^{1}H-NMR ($CDCl_3$) δ: 1.11 (3H, d, J=6.4, γ-H × 3), 3.37 (2H, d, J=6.8 Hz, α'-H × 2), 3.870 (6H, s, -OMe × 2), 3.874 (6H, s, -OMe × 2), 4.31 (1H, dq, J=2.8, 6.4 Hz, β-H), 4.78 (1H, br d, J=*ca.* 2.8 Hz, α-H), 5.10—5.17 (2H, m, γ'-H × 2), 5.44 (1H, s, -OH), 5.9—6.1 (1H, m, β'-H), 6.46 (2H, s, 3'-H and 5'-H), 6.55 (2H, s, 2-H and 6-H).

2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propane (4)——High-resolution MS, Calcd for $C_{21}H_{26}O_5$, *m/z*: 358.1779 (M^+). Found, *m/z*: 358.1779. ^{1}H-NMR ($CDCl_3$) δ: 1.20 (3H, d, J=6.1 Hz, γ-H × 3), 2.72 (1H, dd, J=13.4, 8.3 Hz, α-H_a), 3.12 (1H, dd, J=13.4, 5.1 Hz, α-H_b), 3.34 (2H, d, J=6.8 Hz, α'-H × 2), 3.80 (6H, s, 3'-OMe and 5'-OMe), 3.82 (3H, s, 3-OMe), 4.33 (1H, m, β-H), 5.07—5.15 (2H, m, γ'-H × 2), 5.48 (1H, s, -OH), 5.92—6.02 (1H, m, β'-H), 6.40 (2H, s, 2'-H and 6'-H), 6.70 (1H, dd, J=8.1, 1.7 Hz, 6-H), 6.77 (1H, d, J=1.7 Hz, 2-H), 6.82 (1H, d, J=8.1 Hz, 5-H).

***erythro*-2-(4-Allyl-2-methoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)propan-1-ol (5b)**——MS *m/z*: 344 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, d, J=6.3 Hz, γ-H × 3), 3.36 (2H, d, J=6.8, α'-H × 2), 3.89 (6H, s, -OMe × 2), 4.32 (1H, m, β-H), 4.82 (1H, br s, α-H), 5.13—5.07 (2H, m, γ'-H × 2), 5.57 (-OH), 6.2—5.9 (1H, m, β'-H), 6.7—7.0 (6H, m, aromatic H). On acetylation, **5b** yielded a diacetate, *erythro*-1-acetoxy-1-(4-acetoxy-3-methoxyphenyl)-2-(4-allyl-2-methoxyphenoxy)propane. High-resolution MS, Calcd for $C_{24}H_{28}O_7$, *m/z*: 428.1834 (M^+). Found, *m/z*: 428.1789. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, d, J=6.3 Hz, γ-H × 3), 2.06 (3H, s, α-OAc), 2.23 (3H, s, 4-OAc), 3.25 (2H, d, J=6.6 Hz, α'-H × 2), 3.71 (3H, s, -OMe), 3.76 (3H, s, -OMe), 4.45 (1H, m, β-H), 4.97—5.03 (2H, m, γ'-H × 2), 5.86 (1H, d, J=4.4 Hz, α-H), 5.8—6.0 (1H, m, β'-H), 6.6—7.0 (6H, aromatic H). Assignments of α-, β- and γ-H were confirmed by double resonance experiments.

***threo*-1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-[2-methoxy-4-(1(*E*)-propenyl)phenoxy]propan-1-ol (6a)**——High-resolution MS, Calcd for $C_{21}H_{26}O_6$, *m/z*: 374.1730 (M^+). Found, *m/z*: 374.1765. ^{1}H-NMR ($CDCl_3$) δ: 1.17 (3H, d, J=6.2 Hz, γ-H × 3), 1.88 (3H, dd, J=6.6, 1.5 Hz, γ'-H × 3), 3.89 (6H, s, 3-OMe and 5-OMe), 3.92 (3H, s, 2'-OMe), 4.09 (1H, dq, J=8.3, 6.2 Hz, β-H), 4.59 (1H, d, J=8.3 Hz, α-H), 5.50 (1H, s, -OH), 6.15 (1H, dq, J_{AB}=15.8 Hz, J_{BX}=6.6 Hz, β'-H), 6.36 (1H, dd, J_{AB}=15.8 Hz, J_{AX}=1.5 Hz, α'-H), 6.61 (2H, s, 2-H and 6-H), 6.84—6.95 (3H, m, 3'-H, 5'-H and 6'-H).

***erythro*-2-(4-Allyl-2,6-dimethoxyphenoxy)-1-(3-hydroxy-4,5-dimethoxyphenyl)propan-1-ol (7b)**——An oily substance. High-resolution MS, Calcd for $C_{22}H_{28}O_7$, *m/z*: 404.1835 (M^+). Found, *m/z*: 404.1800. ^{1}H-NMR ($CDCl_3$) δ: 1.12 (3H, d, J=6.4 Hz, γ-H × 3), 3.37 (2H, d, J=6.6 Hz, α'-H × 2), 3.89 (12H, s, 4-OMe, 5-OMe, 2'-OMe and 6'-OMe), 4.08 (1H, br d, -OH), 4.35 (1H, dq, J=2.8, 6.4 Hz, β-H), 4.75 (1H, br s, α-H), 5.09—5.16 (2H, m, γ'-H × 2), 5.71 (1H, s, -OH), 5.93—6.03 (1H, m, β'-H), 6.42 (1H, d, J=*ca.* 1.4 Hz, ArH), 6.45 (2H, s, 3'-H and 5'-H), 6.58 (1H, d, J=*ca.* 1.4 Hz, ArH).

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10) ^{1}H-NMR spectral data for **1b** in CD_3COOD were as follows: δ: 1.11 (3H, d, J=6.4 Hz, γ-H×3), 3.36 (2H, d, J=6.6 Hz, α'-H×2), 3.85 (3H, s, 3-OMe), 3.86 (6H, s, 2′-OMe and 6′-OMe), 4.32 (1H, m, β-H), 4.84 (1H, d, J=2.9 Hz, α-H), 5.06—5.15 (2H, m, γ'-H), 5.98—6.05 (1H, m, β'-H), 6.52 (2H, s, 3′-H and 5′-H), 6.68—6.98 (3H, m, 2-H, 5-H and 6-H).

11) In a preceding paper, we reported the first isolation of *threo*-2-(4-allyl-2,5-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropane from mace (see ref. 9).

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Inhibitors of Adenosine 3′,5′-Cyclic Monophosphate Phosphodiesterase in *Daphne genkwa* SIEB. *et* ZUCC.[1)]

TAMOTSU NIKAIDO,[a] TAICHI OHMOTO,*[,a] and USHIO SANKAWA[b]

School of Pharmaceutical Sciences, Toho University,[a] Funabashi, Chiba 274, Japan and Faculty of Pharmaceutical Sciences, University of Tokyo,[b] Hongo, Bunkyo-ku, Tokyo 113, Japan

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Luteolin, luteolin-7-methylether and kaempferol 3-*O*-β-D-(6″-*p*-coumaroyl)glucopyranoside-(tiliroside) were identified as adenosine 3′,5′-cyclic monophosphate phosphodiesterase inhibitors contained in the flowers and buds of *Daphne genkwa* SIEB. *et* ZUCC. The assignments of the proton and carbon-13 nuclear magnetic resonance signals of tiliroside were confirmed by two-dimensional ^{1}H–^{13}C chemical shift correlation spectroscopy.

Keywords——*Daphne genkwa*; luteolin; luteolin-7-methylether; kaempferol 3-*O*-β-D-(6″-*p*-coumaroyl)glucopyranoside; tiliroside; cAMP phosphodiesterase; inhibitor

Adenosine 3′,5′-cyclic monophosphate (cAMP) phosphodiesterase inhibition testing provides a useful means for the screening of biologically active compounds contained in medicinal plants. We have identified cAMP phosphodiesterase inhibitors contained in various medicinal plants.[2a–k)] The present paper deals with the identification of cAMP phosphodiesterase inhibitors contained in the flowers and buds of *Daphne genkwa* SIEB. *et* ZUCC. (Japanese name "Genka," Thymelaeaceae), which have been used as purgative, diuretic and expectorant agents in traditional medicine. As constituents, flavones[3)] and a diterpene[4)] have beeen isolated. Apigenin and luteolin have been reported to be strong xanthine oxidase inhibitors.[5)]

Results and Discussion

Hot methanol extract of commercial Genkwa Flos was fractionated as shown in Chart 1.

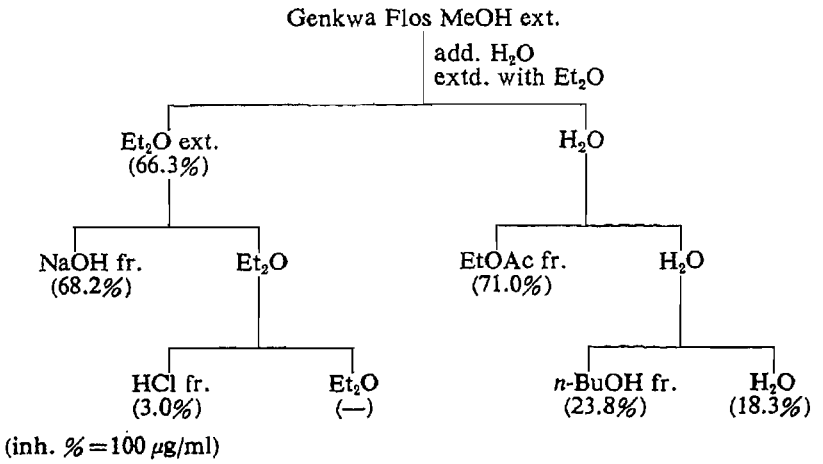


Chart 1

The alkali-soluble fraction, which showed high inhibitory activity, was fractionated repeatedly by column chromatography, centrifugal chromatography ("Chromatotron," Harrison Research Inc.) and preparative thin-layer chromatography (TLC, silica gel) with monitoring for inhibitory activity against cAMP phosphodiesterase.

Two active constituents in this fraction were identified as luteolin (**1**)[6,7] and luteolin-7-methyl ether (**2**)[5,8] on the basis of melting points and spectral comparison with authentic samples. These flavonoids have been isolated from Genkwa Flos.[5]

The active ethyl acetate soluble fraction was further fractionated by Diaion HP-20 column chromatography and medium pressure liquid chromatography with monitoring for inhibitory activity against cAMP phosphodiesterase. The most active constituent (**3**) was isolated as pale yellow needles, mp 263 °C (dec.). Compound **3** was positive for $FeCl_3$, Mg+HCl and Zn+HCl reactions. The ultraviolet (UV) spectrum of **3** showed absorption maxima at 266 and 310 nm which gave bathochromic shifts on addition of base or aluminum chloride. The fast atom bombardment mass spectrum (FAB-MS) of **3** showed the molecular ion peak ($M^+ + 1$) at *m/z* 595. The proton nuclear magnetic resonance (^{1}H-NMR) (Table I) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) (Table II) spectra suggested that **3** might be a flavone acylglycoside derivative. Acid hydrolysis of **3** in ethanol gave kaempferol.

TABLE I. ^{1}H-NMR Chemical Shifts of **3** in DMSO-d_6

Position	Chemical shifts (ppm)
Kaempferol moiety	
6	6.41 (1H, d, J=1.8)
8	6.18 (1H, d, J=1.8)
2′,6′	6.90 (2H, d, J=8.8)
3′,5′	8.02 (2H, d, J=8.8)
5-OH	12.60 (1H, br s)
7-OH	10.15 (1H, br s)
Glucose moiety	
1″	5.48 (1H, d, J=8)
2″	3.23—3.47 (4H, m)
3″	
4″	
5″	
6″	4.08 (1H, dd, J=2, 12)
	4.35 (1H, dd, J=6, 12)
p-Coumaric acid moiety	
2‴,6‴	6.83 (2H, d, J=8.8)
3‴,5‴	7.39 (2H, d, J=8.8)
7‴	7.38 (1H, d, J=16.1)
8‴	6.14 (1H, d, J=16.1)

TABLE II. ^{13}C-NMR Chemical Shifts of Kaempferol, *p*-Coumaric Acid and **3** in DMSO-d_6

Position	Chemical shifts (ppm)		
	Kaempferol	*p*-Coumaric acid	**3**
Kaempferol moiety			
2	146.1		155.7 (s)
3	135.0		132.5 (s)
4	175.0		176.5 (s)
5	155.4		155.7 (s)
6	93.0		93.2 (d)
7	163.2		163.6 (s)
8	97.7		98.3 (d)
9	159.9		160.4 (s)
10	102.5		103.3 (s)
1′	121.1		120.2 (s)
2′,6′	128.9		130.1 (d)
3′,5′	114.8		114.5 (d)
4′	158.4		159.2 (s)
Glucose moiety			
1″			100.6 (d)
2″			73.9 (d)
3″			76.0 (d)
4″			69.7 (d)
5″			73.9 (d)
6″			62.7 (t)
p-Coumaric acid moiety			
1‴		124.7	124.3 (s)
2‴,6‴		129.4	129.4 (d)
3‴,5‴		115.2	115.2 (d)
4‴		158.8	159.0 (s)
7‴		143.4	143.8 (d)
8‴		114.8	113.1 (d)
9‴		167.1	165.3 (s)

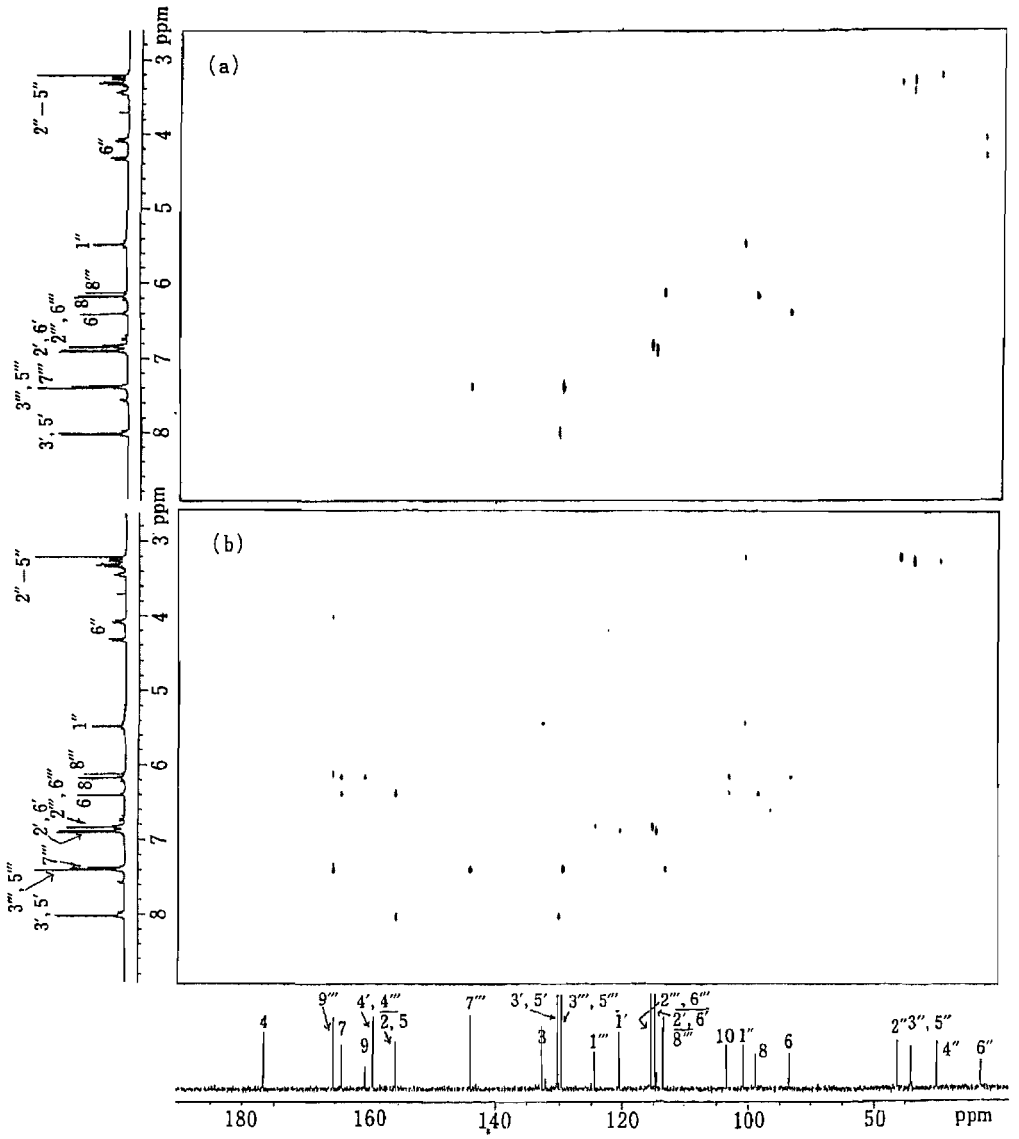


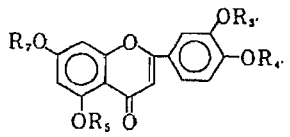
Fig. 1. (a) Contour Map of the ^{1}H–^{13}C Shift-Correlated Spectrum of Tiliroside (**3**).
(b) Contour Map of the Long-Range ^{1}H–^{13}C Shift-Correlated Spectrum of Tiliroside (**3**)

Alkaline hydrolysis of **3** gave *p*-coumaric acid, and kaempferol and glucose were identified in the aqueous layer after hydrolysis with sulfuric acid. Enzymic hydrolysis of **3** with *β*-glucosidase was carried out, and kaempferol was identified.

We next wish to describe the structural determination of **3** by analysis of the ^{1}H- and ^{13}C-NMR spectra. All protons and carbons of **3** were assigned on the basis of proton–proton and proton–carbon shift correlation spectroscopy (Tables I and II). The chemical shifts of the kaempferol moiety were compared with those of authentic kaempferol, and clear glucosylation shifts[7] at C-2 and C-3 were observed. On the basis of the signals of hydroxyl groups at C-5 (12.60 ppm) and C-7 (10.15 ppm) in the ^{1}H-NMR spectrum and the bathochromic shifts in the UV spectrum, it was suggested that the glucose is attached at C-3 of kaempferol. This was confirmed by the long-range carbon–proton shift correlation between the C-3 carbon (132.5 ppm) of kaempferol and the anomeric proton (5.48 ppm) of glucose. Moreover, a clear long-range carbon–proton shift correlation between the carbonyl carbon (165.3 ppm) of *p*-

coumaric acid and the C-6 methylene protons (4.08 ppm) of glucose was observed. (Fig. 1), and the C-6 carbon signal of glucose in the ^{13}C-NMR spectrum was shifted downfield. Thus, it was concluded that *p*-coumaric acid was esterified with glucose at the C-6 position. Therefore **3** was identified as kaempferol 3-*O*-*β*-D-(6''-*p*-coumaroyl)glucopyranoside (tiliroside), which has been isolated from *Rosa canina*[9] and *Tilia argentea*[10] and from several Tiliaceae and other plants. Tiliroside was isolated from *Daphne genkwa* for the first time in this study, and the assignment of proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra were confirmed by ^{1}H–^{13}C shift-correlated two dimensional (2D) NMR experiments. There are some differences from the assignments which have been reported by Natori *et al.*[11] and

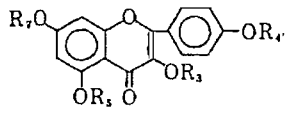
TABLE III. Inhibitory Activity of Luteolin, and Methyl Ethers and Glucosides of Luteolin on cAMP Phosphodiesterase



Compound No.	R_5	R_7	$R_{3'}$	$R_{4'}$	IC_{50} ($\times 10^{-5}$ M)	Source
1	H	H	H	H	20.8	D.g.
2	H	Me	H	H	34.3	D.g.
4	H	H	Me	H	35.3	2c)
5	Me	H	Me	Me	50.3	2c)
6	glu	H	H	H	62.5	Sw.
7	H	glu	H	H	32.3	G.j.
8	H	H	glu	H	17.3	R.l.
9	H	H	H	glu	7.9	T.a.
10	glu-Ac	Ac	Ac	Ac	189.9	Sw.
Papaverine					3.0	Com.

glu, glucose; D.g., *Daphne genkwa*; Sw., *Swertia* sp.[13]; G.j., *Gleditsia japonica*[14]; R.l., *Reseda luteola*[15]; T.a., *Trachelospermum asiaticum* var. *intermedium*[16]; Com., commercial reagent (Tokyo Kasei).

TABLE IV. Inhibitory Activity of Kaempferol, and Methyl Ethers and Glycosides of Kaempferol on cAMP Phosphodiesterase



Compound No.	R_3	R_5	R_7	$R_{4'}$	IC_{50} ($\times 10^{-5}$ M)	Source
11	H	H	H	H	22.5	Com.
12	H	H	Me	H	>500	A.c.
13	Me	H	H	Me	33.4	2c)
14	Me	H	Me	Me	56.4	Syn.
15	Me	Me	Me	Me	26.2	Syn.
16	H	H	rham	H	18.9	H.m.
17	gal-glu	H	H	H	>500	P.g.
3	glu-pca	H	H	H	4.9	D.g.
18	glu-pcaAc	Ac	Ac	Ac	>500	D.g.
Papaverine					3.0	Com.

rham, rhamnose; gal, galactose; glu, glucose; pca, *p*-coumaric acid; Com., commercial reagent (Tokyo Kasei); A.c., *Artemisia capillaris*; Syn., synthesized from kaempferol by treatment with CH_2N_2; H.m., *Hibiscus mutabilis*[17]; P.g., *Panax ginseng*[18]; D.g., *Daphne genkwa*.

Wagner *et al.*[12] The assignments of C-6 and C-8 of the kaempferol moiety were different from ours in both papers, and those of C-5 and C-9 were also different in the paper by Wagner *et al.*

In order to investigate the effect of substituent groups, derivatives of luteolin and kaempferol were examined for ability to inhibit cAMP phosphodiesterase. The results are shown in Tables III and IV. The flavones (**2**, **4**, **5**, **12**—**15**) having a methoxyl group were less active than luteolin (**1**) or kaempferol (**11**). The luteolin congeners (**6**, **7**), with glucose in the A-ring were less active than luteolin, but those (**8**, **9**) with glucose in the B-ring were more active than luteolin, and the 4′-glucoside (**9**) showed especially high activity. This is the most active flavone glycoside (in terms of cAMP phosphodiesterase inhibition) that we have so far found. Thus, tiliroside (**3**) and luteolin 4′-glucoside (**9**) were found to be strong inhibitors of cAMP phosphodiesterase. We intend to examine more flavone glycosides and to investigate the correlation between inhibitory activity and glycosylation of flavones.

Experimental

All melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. The UV spectra were recorded with a Hitachi 340 spectrophotometer. The FAB-MS was recorded on a JEOL JMS-DX 303 mass spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded with JEOL JNM-4H-100 and JEOL GX-400 spectrometers; chemical shifts are given on the δ scale (ppm) with tetramethylsilane (TMS) as an internal standard, and coupling constants are given in Hz. The ^{1}H–^{13}C shift-correlated spectra were measured under the following conditions. The spectral width in the f_2 domain was 14836.8 Hz and that in the f_1 domain was 17234.8 Hz. The amount of sample used was 10 mg in dimethyl sulfoxide (DMSO)-d_6 at 50 °C. Total data collection required 5 h for the 2048 × 256 data point matrix. The normal ^{1}H–^{13}C shift-correlated spectrum was assumed to be an optimal setting which gave values for the delays of 3.6 and 1.8 ms, respectively. The long-range ^{1}H–^{13}C shift-correlated spectrum was assumed to be an optimal setting which gave values for the delays of 50.0 and 25.0 ms, respectively. The following abbreviations are used: s = singlet, br s = broad singlet, d = doublet, dd = double doublet, t = triplet and m = multiplet. Column chromatography was carried out on silica gel (Fuji-Davison, BW-820MH) and ion exchange resin (DIAION HP-20, Mitsubishi Chemical Industry Ltd.). TLC and preparative TLC were performed on precoated Silica gel 60 F plates (Merck) and Wako gel B-5 (Wako Pure Chemical Industry Ltd.), respectively, and detection was achieved by illumination with a UV lamp or by spraying 10% H_2SO_4 followed by heating. Centrifugal chromatography ("Chromatotron," Harrison Research Inc.) was carried out on silica gel (Wako)-coated plates (2 m/m) developed with gradient solvents (MeOH–H_2O).

Assay Method for cAMP Phosphodiesterase——The liquid scintillation counter used was an Aloka LSC-903. Samples were tested for cAMP phosphodiesterase activity in duplicate by the method described in the previous paper.[2a] All the inhibitors were added as solutions in DMSO. The presence of DMSO in the assay medium at up to 2% concentration is known to have no effect on the enzyme activity. The IC_{50} value is the concentration of a compound required for 50% inhibition of cAMP phosphodiesterase activity.

Enzymes and Chemicals——Beef heart phosphodiesterase was purchased from Boehringer. Snake venom nucleotidase and cAMP were obtained from Sigma, and [^{3}H]cAMP from the Radiochemical Centre. Papaverine, a reference inhibitor, was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo).

Extraction and Separation——The dried flowers and buds of *Daphne genkwa* (4.5 kg, purchased from Uchida Pharmacy for Oriental Medicine, Tokyo) were extracted with hot MeOH (36 l) for 48 h. The extract was evaporated to dryness and the residue (750 g) was partitioned between water and ether. The ether solution was extracted with 5% NaOH solution. The NaOH extract was acidified with diluted HCl solution and extracted with ether. The ether solution was dried over Na_2SO_4 and concentrated to give the NaOH-soluble fraction (129.4 g). The ether solution which had been extracted with NaOH, was then extracted with 5% HCl solution. The HCl extract was made alkaline with diluted NaOH solution and extracted with ether. The ether solution was dried over Na_2SO_4 and concentrated to give the HCl-soluble fraction (1.6 g). The ether solution which had been extracted with NaOH and HCl was washed with water, and the neutral ether solution was dried over Na_2SO_4 and concentrated to give a neutral fraction (30.0 g). The aqueous layer was extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4 and evaporated to give the AcOEt-soluble fraction (24.0 g). The residual aqueous layer was extracted with *n*-BuOH. The *n*-BuOH extract was dried over Na_2SO_4 and evaporated to give the *n*-BuOH-soluble fraction (210.0 g). A part of the residual aqueous layer was frozen and dried to give a powder (H_2O fraction). These fractions were tested for inhibitory effect on cAMP phosphodiesterase.

The NaOH-soluble fraction showed inhibitory activity, and the two active compounds of Rf = 0.41 and 0.62 were separated by preparative TLC with $CHCl_3$–MeOH as an eluent, yielding **1** and **2**.

The AcOEt-soluble fraction was also active and was further fractionated on Diaion using H_2O–MeOH as an

eluent. The most active fraction (eluted with MeOH) was further chromatographed on silica gel using MeOH as an eluent. The active compound was isolated as compound **3**.

Luteolin (1)——Yellow needles from MeOH, mp >300 °C. This was identical with an authentic sample of luteolin[6] on the basis of spectral comparisons. TLC, Rf=0.41 ($CHCl_3$: MeOH = 10 : 1).

Luteolin-7-methylether (2)——Yellow needles from MeOH, mp 283 °C. This was identical with an authentic sample of luteolin-7-methyl-ether[7] on the basis of spectral comparisons and melting point. TLC, Rf=0.62 ($CHCl_3$: MeOH = 10 : 1).

Kaempferol 3-*O*-β-D-(6″-*p*-Coumaroyl)glucopyranoside (Tiliroside, 3)——Pale yellow needles from aq. MeOH, mp 263 °C (dec.). $FeCl_3$, dark green; Mg+HCl, red; Zn+HCl, red. UV λ_{max}^{EtOH} nm (log ε): 266 (3.32), 310 (3.34). λ^{+NaOH}: 270, 364. λ^{+AlCl_3}: 272, 302, 392. FAB-MS *m/z*: 595 (M^++1), 493, 287, 93. ^{1}H- and ^{13}C-NMR: see Tables I and II.

Acetate of 3——**3** was acetylated with Ac_2O–pyridine in the usual way. The crude acetate was purified by prep. TLC to give the acetate as white needles (recrystallized from MeOH). mp 174—177 °C. ^{1}H-NMR (DMSO-d_6) (ppm): 1.97, 1.98, 2.07, 2.13, 2.30, 2.32, 2.37 (each 3H, s, OAc × 7).

Acid Hydrolysis of 3——**3** (7 mg) in 3% H_2SO_4–EtOH solution was refluxed in a water bath for 3 h. The reaction mixture was extracted with AcOEt. The AcOEt layer was washed and evaporated to dryness. Kaempferol in the residue was identified by comparison with an authentic sample (Tokyo Kasei) by TLC.

Enzyme Hydrolysis of 3——**3** (7 mg) was incubated with β-glucosidase (50 units, from Almonds, Nakarai Chemicals Ltd.) in acetate buffer (15 ml, pH 4.8) solution at 30 °C for 48 h. The reaction mixture was extracted with AcOEt. The AcOEt layer was washed and evaporated to dryness. Kaempferol in the residue was identified by comparison with an authentic sample (Tokyo Kasei) by TLC.

Alkaline Treatment of 3 Followed by Acid Hydrolysis——**3** (10 mg) in 2% NaOH solution was kept overnight with stirring at room temperature. The reaction mixture was acidified with dilute HCl solution and extracted with Et_2O. The Et_2O solution was washed and evaporated to dryness. The residue was recrystallized from aq. EtOH to give white needles, mp 217—219 °C; this product was identified as *p*-coumaric acid by mixed melting point determination and comparison of the TLC and NMR spectra with those of an authentic sample (Tokyo Kasei).

The aqueous layer was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated to dryness. The residue in 2% H_2SO_4 solution was heated on a water bath for 1 h, then cooled, and extracted with AcOEt. The AcOEt was evaporated off and kaempferol in the residue was identified by comparison with an authentic sample using TLC.

The aqueous layer was neutralized with Amberlite IRC-50 (Organo) and evaporated to dryness. The residue was identified as D-glucose by TLC and gas chromatography [the TMS ether was prepared with TMS-HT (Tokyo Kasei)].

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Direct Liquid Chromatographic Resolution of Racemic Compounds. Use of Ovomucoid as a Column Ligand

TOSHINOBU MIWA,*[,a] MASAKI ICHIKAWA,[a] MASANORI TSUNO,[b] TEIICHI HATTORI,[b] TAKESHI MIYAKAWA,[a] MASANORI KAYANO,[a] and YASUO MIYAKE[b]

Eisai Co., Ltd., Pharmaceutical Research Laboratory,[a] 2–1 Takehaya-machi, Kawashima-cho, Hashima-gun, Gifu 483, Japan and Eisai Tsukuba Laboratory,[b] 5–1–3 Tokodai, Toyosato-cho, Tsukuba-gun, Ibaragi 300–06, Japan

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A new chiral recognition column, with conjugated ovomucoid as the ligand, was developed. This column may be employed for the chiral resolution of acids as well as amines without derivatization. The retention time, capacity factor and resolution factor were dependent on pH, buffer strength and 2-propanol concentration of the mobile phase. For chlorpheniramine, a resolution factor of 1.5 was obtained.

Keywords——chiral resolution; affinity column; HPLC; enantiomer; ovomucoid

Introduction

Diastereomeric derivatization has been the preferred method for resolution of optically active compounds by high performance liquid chromatography (HPLC), but recently, the direct resolution of racemic compounds on certain stationary phases has been achieved by many researchers. For example, a 3,5-dinitrobenzoyl amino acid-conjugated silica gel,[1] a poly(triphenylmethyl methacrylate)-conjugated stationary phase,[2] a chiral charge-transfer complex,[3] and a crown ether-conjugated silica gel[4] have been used in normal-phase HPLC. In addition, columns that may be used in reversed-phase HPLC have been developed: a cyclodextrin-conjugated stationary phase,[5] a ligand exchange column[6] and a protein-conjugated silica gel have been reported. The application of the chiral recognition ability of a protein to chromatographic resolution of racemic compounds started at the beginning of this century. The usefulness of protein-conjugated columns in HPLC has been demonstrated by Allenmark and Bomgren,[7] who employed a bovine serum albumin (BSA)-conjugated column, and by Hermansson,[8] who used an α_1-acid glycoprotein-conjugated column. The α_1-acid glycoprotein column is preferred for the resolution of racemic amines because this protein has a low pI value, but by using the ion pairing method, this column can also be used for resolution of racemic acids.[9] However, the availability of some of these proteins is very limited. For example, α_1-acid glycoprotein is present in Cohn V fraction at a level of only approximately 0.3 mg/ml.

In this report we describe a new chiral resolution column whose chiral recognition ligand is conjugated ovomucoid. The nature of this ovomucoid has been thoroughly researched[10] and its property of trypsin inhibition[11] is well known. Methods for its purification have been reported by many researchers.[12] Ovomucoid is readily available, and is relatively stable to variation of pH, to heat and to organic solvents. For these reasons, we chose chicken ovomucoid as the chiral recognition ligand and developed a method for conjugation which is

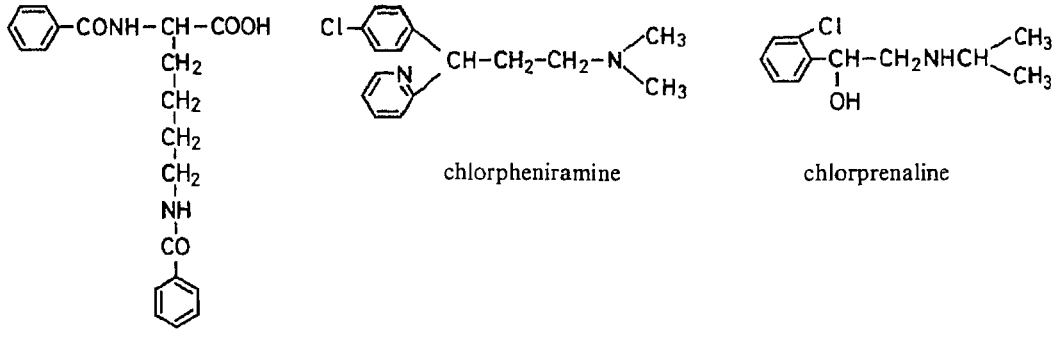
relatively easy to perform and uses succinimide derivatives.

The resolution of some racemic compounds and the characteristics of the column are also described.

Materials and Methods

Apparatus——A Hitachi L-5000 system (a 655A-11 liquid chromatograph with a 655A variable-wavelength UV monitor) equipped with a 655A-40 automatic sample injector and a 655A-52 column oven was used. Stainless-steel columns of 4.6 (i.d.) × 150 mm were packed with ovomucoid conjugated silica gel. The pH was measured with a Toa electrode pH meter. A JASCO DIP-4 digital polarimeter was used.

Chemicals——α,ε-Dibenzoyllysine was purchased from Tokyo Kasei Co. Chlorpheniramine maleate, chlorprenaline hydrochloride and phenylpropanolamine were of pharmaceutical grade. *d*-Chlorpheniramine maleate and *l*-chlorprenaline hydrochloride were purified by different crystallization methods. The structures of these compounds are presented in Fig. 1.



α,ε-dibenzoyllysine chlorpheniramine chlorprenaline

Fig. 1. Structures of Compounds Resolved by HPLC on an Ovomucoid-Conjugated Column

Purification of Ovomucoid——Ovomucoid was purified from chicken egg white by the ethanol-precipitation method described by Fredericq and Deutsch.[12]

Synthesis of Ovomucoid-Conjugated Silica Gel——Aminopropyl silica gel was activated with *N*,*N*-disuccinimidyl carbonate. The procedure was as follows: Unisil Q NH_2 (2 g) and *N*,*N*-disuccinimidyl carbonate (3 g) were reacted overnight in a coupling buffer (0.1 M $NaHCO_3$, pH 6.8) at room temperature using a rotary evaporator. After the activated silica gel had been washed with water and then with the coupling buffer, 30 ml of ovomucoid solution (2 g in 30 ml of coupling buffer) was added dropwise. The ovomucoid-conjugated silica gel was packed into the column.

Resolution Conditions——A water–organic solvent mixture was used as the eluent. Five to 50 mM potassium phosphate buffer (pH 3.0 to 6.7) containing 2-propanol as the organic solvent was used, and the change of retention time (k') and the resolution were calculated. Column temperature was maintained at 25 °C.

Results and Discussion

Ovomucoid is one of the most stable proteins present in egg white. Accordingly, the ovomucoid-conjugated silica gel column prepared in this study was stable over a wide range of pH values and was stable to organic solvents. This column was also stable at room temperature for more than 3 months.

Resolution of the acidic compound dibenzoyllysine was achieved with a 15 cm column eluted with 20 mM potassium phosphate buffer (pH 6.0), as shown in Fig. 2. Amines (chlorpheniramine and chlorprenaline) were resolved on the same column and with the same buffer at different pH values and different 2-propanol concentrations. (Figs. 3 and 4).

The pH of the mobile phase greatly affected the retention. Table I shows that higher pH values caused stronger retention of chlorprenaline and phenylpropanolamine. α,ε-Dibenzoyllysine was strongly retained by the ovomucoid column at lower pH values, as

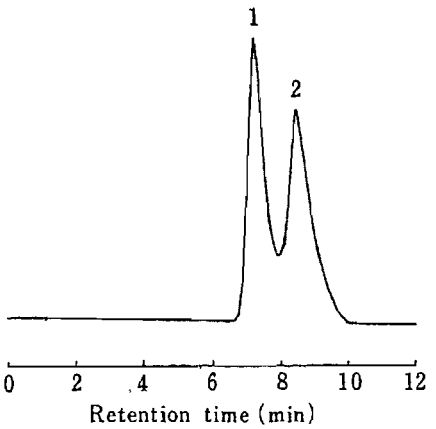


Fig. 2. Separation of the Enantiomers of α,ε-Dibenzoyllysine

Mobile phase, 20 mM potassium phosphate buffer (pH 6.0); detection, 220 nm; flow rate, 1.0 ml/min. The earlier eluted peak is that of *d*-α,ε-dibenzoyllysine.

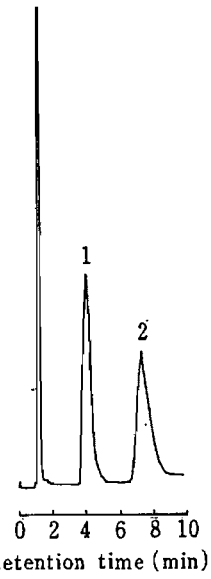


Fig. 3. Separation of *d,l*-Chlorpheniramine on an Ovomucoid-Conjugated Column

Mobile phase, 20 mM potassium phosphate buffer (pH 5.5) containing 6% 2-propanol; detection, 220 nm; flow rate, 1.2 ml/min; sample amount, 2.5 μg as the mixture.

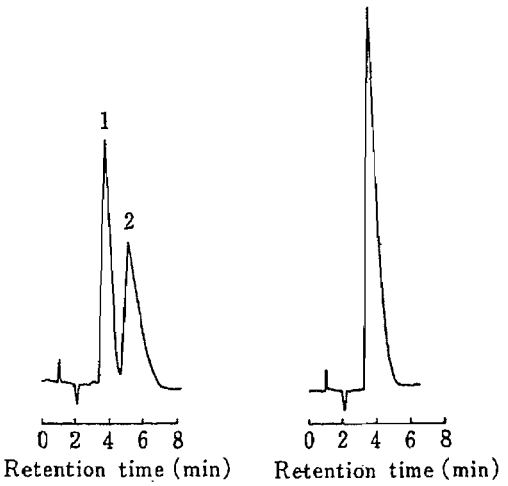


Fig. 4. Separation of *d,l*-Chlorprenaline and Chromatogram of *l*-Chlorprenaline on an Ovomucoid-Conjugated Column

Mobile phase, 20 mM potassium phosphate buffer (pH 6.1); detection, 210 nm; flow rate, 1.2 ml/min; sample amount, 1 μg as the mixture.

shown in Table II.

These results show that the ovomucoid column exhibits a strong hydrophobic interaction with solutes. Allenmark and Bomgren[7] stated, in their report on a BSA-conjugated silica gel HPLC column, that the k' values of aroyl-amino acids are influenced by hydrophobic interaction, coulombic interaction and hydrogen bonding. To elucidate the coulombic interaction of the ovomucoid-conjugated column, the effect of the ionic strength of the mobile phase on the retention of solutes was examined (Table III). Tropic acid is strongly retained on this column at lower ionic strength. The retention of mandelic acid methyl ester, a non-ionic substance, was not influenced by mobile phase concentration. Higher k' values for chlorpheniramine were obtained at high buffer strengths. Though we have not examined these interactions in detail, it can be considered that they may be controllable by adjusting the protein and buffer conditions.

Hermansson[8] stated that, in an orosomucoid-conjugated column, oxazoline derivatives

TABLE I. Separation of Racemic Chlorprenaline and Phenylpropanolamine by HPLC on an Ovomucoid-Conjugated Silica Gel Column

	pH	k_1'	k_2'	α
Chlorprenaline	6.1	2.63	3.97	1.51
	6.4	4.65	7.42	1.59
Phenylpropanolamine	6.1	0.186	0.186	1
	6.4	0.346	0.415	1.2
	6.7	0.47	0.47	1

Mobile phase: 20 mM potassium phosphate. Column: 4.6 × 150 mm.

TABLE II. Separation of Racemic α,ε-Dibenzoyllysine by HPLC on an Ovomucoid-Conjugated Silica Gel Column

pH	k_1'	k_2'	α	Rs
5	10.9	13.2	1.21	0.93
5.5	9.97	11.6	1.16	0.62
6	5.42	6.48	1.20	0.68
6.5	2.60	3.10	1.10	0.64

Mobile phase: 20 mM potassium phosphate. Column: 4.6 × 150 mm.

TABLE III. Influence of Buffer Strength on the Retention of an Acid, a Non-ionic Substance, and an Amine

	pH	5 mM	20 mM	50 mM
Tropic acid	4.5	3.37	1.31	0.75
	6.0	1.04	0.32	0.50
CH_3-mandelate	3.0	0.39	0.32	0.36
	5.0	0.45	0.20	0.50
Chlorpheniramine	4.5	0.35	0.93	1.55
	6.0	3.75	3.60	6.50
		6.65	6.82	11.36

Mobile phase: potassium phosphate. Column: 4.6 × 150 mm.

TABLE IV. Influence of 2-Propanol Concentration on the Resolution of Chlorprenaline and Chlorpheniramine

	2-Propanol (%)	k_1'	k_2'	α	Rs
Chlorprenaline	0	2.55	3.75	1.47	
	1	1.33	1.79	1.35	
	2	1.04	1.21	1.16	
	4	0.80	0.80	1	
Chlorpheniramine	0	5.12	10.29	2.01	0.87
	1	2.42	4.82	1.99	1.17
	2	2.93	5.75	1.96	1.24
	4	1.30	2.05	1.58	0.95
	6	3.29	6.64	2.02	1.52

Mobile phase: For chlorprenaline, 20 mM potassium phosphate (pH 6.1); for chlorpheniramine, 20 mM potassium phosphate (pH 6.5). Column: 4.6 × 150 mm.

of racemic β-blockers (*e.g.* propranolol), acetylated primary amines, and the ethyl ester of mandelic acid can be resolved with excellent separation factors. More recently, Hermansson and Eriksson,[9] and Schill *et al.*[13] also found that some amines and acidic compounds without derivatization could be separated on an orosomucoid-conjugated column. An ovomucoid-conjugated column could resolve chlorpheniramine and chlorprenaline without derivatization. Though the two amines were resolved by this method, the k' values were strongly influenced by the pH of the mobile phase, and at high pH values (7 and above) the amines were retained on the column. Phenylpropanolamine was resolved slightly when the pH of the mobile phase was 6.4, but not at 6.1 or 6.7. The reason for this phenomenon is not yet clear.

The influence of a mobile phase additive (2-propanol) on the resolution of chlorprenaline and chlorpheniramine is shown in Table IV. Addition of 2-propanol caused a decrease in the retention times of both compounds. The separation factor of chlorprenaline enantiomers decresed when the retention time was shorter. Although the resolution factor of chlorpheniramine tended to become lower as the concentration of 2-propanol was increased to 4%, a 2-propanol concentration of 6% resulted in an increase of k' value to a level higher than that when no 2-propanol was present, and gave excellent resolution. Though we have not tested higher concentration of 2-propanol, an optimum concentration may exist.

Conclusion

Ovomucoid, a stable protein present in egg white, is useful for chiral recognition. It is readily available, and from this viewpoint, an ovomucoid column is preferable to an orosomucoid column. Though ovomucoid is stable at various pH values, a high pH will cause decomposition of the silica gel, so the use of a porous polymers as the stationary phase may allow the more extensive use of ovomucoid as a chiral stationary-phase ligand.

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1,2-Diamino-4,5-methylenedioxybenzene as a Highly Sensitive Fluorogenic Reagent for α-Keto Acids

MASARU NAKAMURA,*[,a] SHUUJI HARA,[a] MASATOSHI YAMAGUCHI,[a] YASUYO TAKEMORI[a] and YOSUKE OHKURA[b]

Faculty of Pharmaceutical Sciences, Fukuoka University,[a] Nanakuma, Jonan-ku, Fukuoka 814-01, Japan and Faculty of Pharmaceutical Sciences, Kyushu University,[b] 62, Maidashi, Higashi-ku, Fukuoka 812, Japan

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The reactivity of pyruvic acid with 1,2-diaminobenzenes was investigated as part of a survey of fluorogenic reagents for α-keto acids of biological importance. Of eight 1,2-diaminobenzenes tested, 1,2-diamino-4,5-methylenedioxybenzene was found to be the best reagent in terms of sensitivity and reactivity. The reagent reacts with α-keto acids in an acidic solution to produce the corresponding fluorescent derivatives, which fluorescent most intensely in a neutral medium. The derivatives of ten α-keto acids can be separated within 18 min by reversed-phase high-performance liquid chromatography with isocratic elution. The detection limits for the acids are at femtomole levels for an injection volume of 10 μl. The fluorescent product from pyruvic acid was characterized as 3-methyl-6,7-methylenedioxy-2(1*H*)-quinoxalinone. The fluorescence properties of the product are also described.

Keywords——1,2-diaminobenzene derivative; 1,2-diamino-4,5-methylenedioxybenzene; fluorogenic reagent; α-keto acid; HPLC; precolumn derivatization

o-Phenylenediamine (OPD) was first used as a fluorogenic reagent for the determination of α-keto acids,[1] and was applied as a reagent in high-performance liquid chromatography (HPLC) with fluorescence detection.[2,3] Recently, we found that 1,2-diamino-4,5-dimethoxybenzene (DDB) reacts with α-keto acids to give much more strongly fluorescent compounds as compared with OPD.[4,5] Thus, DDB has been applied to the sensitive determination of α-keto acids in human urine and serum by fluorimetric HPLC.[6]

We examined the reactions of eight 1,2-diaminobenzenes (DBs) including the above-mentioned compounds with pyruvic acid as part of a survey of effective fluorogenic reagents for α-keto acids of biological importance. We found that, of the DBs, 1,2-diamino-4,5-methylenedioxybenzene (DMB) is the best reagent for sensitive and rapid determination of α-keto acids.

Experimental

Reagents and Solutions——All chemicals and solvents used were of reagent grade, unless otherwise specified. Deionized and distilled water was used. Sodium salts of α-keto acids listed in Table II were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). OPD·2HCl, 1,2-diamino-4,5-dimethylbenzene, 1,2-diamino-4-methoxybenzene·HCl and 1,2-diaminobenzene-4-carboxylic acid were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 1,2-Diamino-3-methoxybenzene·HCl was synthesized by the method of Lane and Williams.[7] DDB·HCl was prepared as described previously[8]; it is now available from Dojindo Labs. (Kumamoto, Japan). The 0.7 mM solutions of DMB and other DBs were prepared in 0.7 M HCl containing 1.0 M β-mercaptoethanol and 28 mM $Na_2S_2O_4$.

Apparatus and HPLC Conditions——Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter in 10 × 10 mm quartz cells; spectral bandwidths of 10 nm were used in both the

excitation and emission monochromators. Infrared (IR) spectra were recorded with a Shimadzu 430 IR spectrophotometer in KBr pellets. ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-90 spectrometer at 90 MHz in the indicated solvents with sodium trimethylsilylpropionate or tetramethylsilane as an internal standard. Splitting patterns were designated as follows: s, singlet; br, broad. Electron impact mass spectra (EI-MS) were taken with a JEOL DX-300 spectrometer. Uncorrected melting points were measured with a Yazawa melting point apparatus.

A Hitachi 635A high-performance liquid chromatograph equipped with a sample injector (10-μl loop) was used. A Shimadzu RF-530 fluorescence spectromonitor equipped with a 12-μl flow cell was operated at an excitation wavelength of 367 nm and an emission wavelength of 446 nm. The column was a Radial Pak cartridge C_{18} (100 × 8 mm i.d.; particle size, 5 μm; Waters Assoc., Milford, Mass., U.S.A.). The mobile phase was a mixture of CH_3OH, CH_3CN and 0.04 M potassium phosphate buffer (pH 7.0) (13 : 12 : 25, v/v). The flow rate was 1.5 ml/min. The column temperature was ambient (*ca.* 25 °C).

Synthesis of DMB·2HCl——1,2-Dinitro-4,5-methylenedioxybenzene[9] (5 g) was dissolved in 200 ml of benzene. To this solution, iron powder (100 g; 80 mesh; Kishida Kagaku, Osaka, Japan) mixed with 20 ml of concentrated HCl was added in small portions over 1 h under reflux. The mixture was then refluxed for 4 h, 10 ml of H_2O was added, and the whole was further refluxed for 2 h. After cooling, the mixture was made alkaline with 2.6 M NaOH and extracted 3 times with 200 ml portions of benzene. The extracts were combined and the solvent was removed. The residue was mixed with *ca.* 10 ml of concentrated HCl and the resulting salt was recrystallized from C_2H_5OH to give DMB·2HCl (*ca.* 3 g; yield *ca.* 57%) as colorless needles, mp 176—179 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3410 and 3360 (NH_2). ^{1}H-NMR (D_2O) δ: 6.03 (2H, s, OCH_2O), 6.86 (2H, s, aromatic protons). MS *m/z*: 153 (MH^+), 152 (M^+, base peak). *Anal.* Calcd for $C_7H_{10}Cl_2N_2O_2$: C, 37.36; H, 4.38; N, 12.45. Found: C, 37.24; H, 4.62; N, 12.24.

Synthesis of 1,2-Diamino-4,5-ethylenedioxybenzene (DEB)·2HCl——1,2-Dinitro-4,5-ethylenedioxybenzene[10] (5 g) was treated in the same way as described for the synthesis of DMB to give DEB·2HCl (*ca.* 3 g; yield *ca.* 57%) as colorless needles, mp 215—218 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3400 and 3310 (NH_2). ^{1}H-NMR (D_2O) δ: 4.33 (4H, s, $O(CH_2)_2O$), 6.95 (2H, s, aromatic protons). MS *m/z*: 167 (MH^+), 166 (M^+). *Anal.* Calcd for $C_8H_{12}Cl_2N_2O_2$: C, 40.19; H, 5.06; N, 11.72. Found: C, 40.45; H, 5.49; N, 11.51.

Both DMB·2HCl and DEB·2HCl were stable in the crystalline state at room temperature for at least a year in a desiccator containing phosphorus pentoxide.

Isolation of the Reaction Product of Pyruvic Acid with DMB——DMB·2HCl (560 mg) and pyruvic acid (250 mg) were dissolved in 30 ml of 0.5 M HCl, and the mixture was heated for 50 min in a boiling water bath. The precipitate that separated on cooling the mixture in ice-water was filtered off, washed with water, dried *in vacuo* and then recrystallized from CH_3OH to give compound I (*ca.* 300 mg; yield *ca.* 65%) as pale yellow needles, mp 273—274 °C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3450 (lactam NH), 1660 (lactam C=O), 1630 (aromatic C=N and/or C=C). ^{1}H-NMR (Me_2SO-d_6) δ: 6.10 (2H, s, OCH_2O), 6.75 and 7.16 (1H each, s each, aromatic protons), 2.34 (3H, s, CH_3), 12.17 (1H, brs, NH). MS *m/z*: 204 (M^+, based peak). *Anal.* Calcd for $C_{10}H_8N_2O_3$: C, 58.82; H, 3.95; N, 13.72. Found: C, 58.97; H, 3.84; N, 13.83.

Isolation of the Reaction Product of Pyruvic Acid with DEB——DEB·2HCl (600 mg) and pyruvic acid (250 mg) were treated as described for the isolation of compound I to give compound II (*ca.* 310 mg, yield *ca.* 63%) as pale yellow needles, mp 264—265 °C. IR ν_{max}^{KBr} cm^{-1}: 3420 (lactam NH), 1655 (lactam C=O), 1630 (aromatic C=N and/or C=C). ^{1}H-NMR (Me_2SO-d_6) δ: 4.28 (4H, s, $O(CH_2)_2O$), 6.70 and 7.14 (1H each, s each, aromatic proton), 2.33 (3H, s, CH_3), 11.98 (1H, brs, NH). MS *m/z*: 218 (M^+, base peak). *Anal.* Calcd for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.62; N, 12.84. Found: C, 60.60; H, 4.65; N, 12.72.

Procedure for Screening of DBs (Manual Assay Procedure)——A screw-capped 10-ml vial containing 1.0 ml of a DB solution and 1.0 ml of an aqueous test solution, was tightly closed and heated at 100 °C for 45 min. The reaction mixture was cooled in ice-water to stop the reaction, and then adjusted to pH 6.6—7.4 by adding 1.0 ml of 0.4 M potassium phosphate buffer (pH 7.0) and 0.5 ml of 1.4 M NaOH. The reagent blank was prepared in the same way except that the sample solution (1.0 ml) was replaced with 1.0 ml of H_2O. The fluorescence intensities of the test and blank were measured at the emission maximum wavelength with irradiation at the excitation maximum (see Tables I and II).

Procedure for the Separation of the DMB Derivatives of α-Keto Acids by HPLC (HPLC Procedure)——A mixture (1.0 ml) of α-keto acids was treated as in the manual assay procedure except that the neutralization with the phosphate buffer and NaOH solution was omitted. The reaction mixture (10 μl) was subjected to HPLC.

Results and Discussion

Screening of DBs

The screening of DBs as fluorogenic reagents was carried out by using pyruvic acid as a model compound of biogenic α-keto acids. The fluorescence excitation and emission maxima and intensities of the reaction mixtures of pyruvic acid and DBs are shown in Table I. This

Table I. Excitation (Ex) and Emission (Em) Maxima and Relative Intensities (RFI) of the Fluorescence Produced by the Reactions of Pyruvic Acid with DBs

DB	Maximum[a] (nm)		RFI[b]	
	Ex	Em	Test	Blank
DMB	367	445	100	1.8
DEB	361	457	10.8	0.3
DDB	361	448	60.2	1.2
OPD	337	417	1.4	0.1
1,2-Diamino-4,5-dimethylbenzene	346	433	2.6	0.7
1,2-Diamino-3-methoxybenzene	342	410	0.2	0.1
1,2-Diamino-4-methoxybenzene	323	406	0.7	0.9
1,2-Diaminobenzene-4-carboxylic acid	331	406	1.2	300

a) Portions (1.0 ml) of 10 μM pyruvic acid solution (or water for the blank) were treated according to the manual assay procedure. *b*) The intensity obtained by the reaction with DMB was taken as 100.

table indicates that the DBs having methyleneoxy groups at both the 4- and 5-positions (DMB, DEB and DDB) give stronger fluorescence with longer excitation and emission wavelengths as compared with the other DBs. DMB gave the highest intensity with an equimolar amount of pyruvic acid; the intensity was *ca.* 70 and 1.6 times those with OPD and DDB, respectively. DBs other than DMB, DEB and DDB were not favorable reagents because of high values of their reagent blanks or low yields of fluorescence from pyruvic acid. Therefore, DMB was selected for further investigation to establish suitable reaction conditions for an analytical procedure for α-keto acids.

Conditions for the Fluorescence Reaction of Pyruvic Acid with DMB

The excitation and emission spectra of the fluorescence from pyruvic acid and the reagent

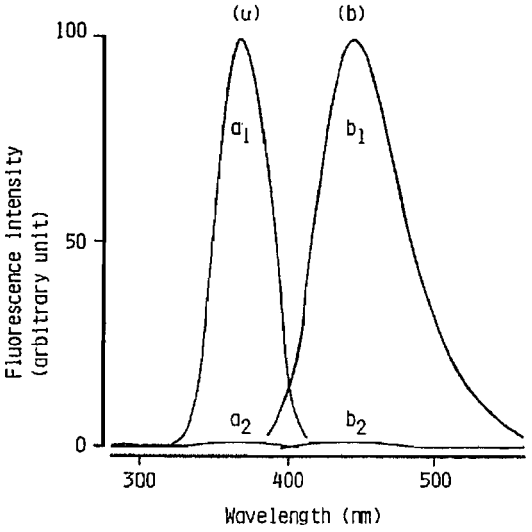


Fig. 1. Fluorescence Excitation and Emission Spectra of the Final Reaction Mixture of Pyruvic Acid with DMB

a, excitation spectra; b, emission spectra.
a_1 and b_1: a portion (1.0 ml) of 10 μM pyruvic acid solution was treated according to the manual assay procedure.
a_2 and b_2: reagent blanks corresponding to a_1 and b_1.

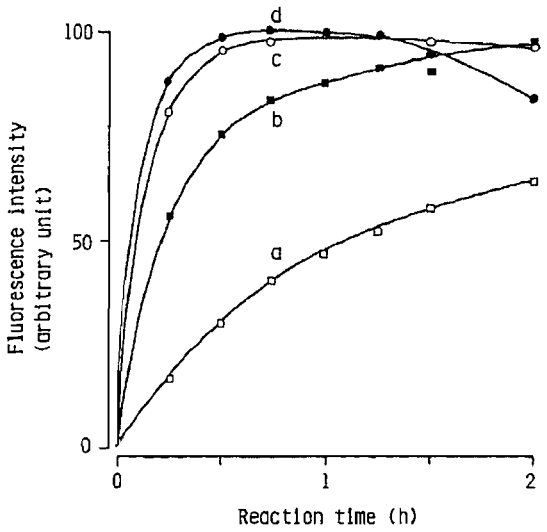


Fig. 2. Effect of Reaction Temperature and Time on the Fluorescence Reaction of Pyruvic Acid with DMB

Portions (1.0 ml) of 10 μM pyruvic acid solution were treated as in the manual assay procedure with DMB at various temperatures for various periods. Temperature: a, 37 °C; b, 60 °C; c, 80 °C; d, 100 °C.

blank are shown in Fig. 1. Pyruvic acid reacted with DMB in dilute hydrochloric acid solution, but not in neutral or alkaline solution. Although sulfuric acid also allowed the same fluorescence to develop, DMB was only slightly soluble in sulfuric acid. Thus, DMB was dissolved in dilute hydrochloric acid. Hydrochloric acid at 0.6—1.0 M gave almost maximum fluorescence intensity; 0.7 M was selected as the optimum. DMB gave the most intense fluorescence at concentrations greater than *ca.* 5.0 mM in the reagent solution, whereas the blank fluorescence increased slightly in proportion to DMB concentration; 7.0 mM was used as a sufficient concentration. β-Mercaptoethanol and sodium hydrosulfite most effectively stabilized DMB during the reaction at the concentrations of 1.0 M and 28 mM, respectively. The DMB solution was usable for at least 2 weeks even when stored at room temperature in daylight. If either of these antioxidants is absent, the reaction mixture becomes reddish, which may interfere with the assay of pyruvic acid.

The fluorescence reaction occurred at temperatures above *ca.* 37 °C; higher temperatures allowed the fluorescence to develop more rapidly (Fig. 2). At 100 °C, which was selected for convenience, the maximum and constant intensity of the fluorescence from pyruvic acid was achieved by heating for 45—75 min (Fig. 2), while the reaction with DDB required 2 h or more to attain constant and maximum intensity.[4] When the acidic reaction mixture was neutralized to pH 6.6—7.4 with 0.4 M potassium phosphate buffer (pH 7.0) and 1.4 M sodium hydroxide, the fluorescence from pyruvic acid became at least 30 times more intense than that in the acidic reaction mixture. The fluorescence in the final mixtures was stable for at least 5 h in daylight at room temperature.

Fluorescence from Other α-Keto Acids in the Reaction with DMB

Many α-keto acids fluoresced under the recommended conditions (manual assay procedure). The excitation and emission maxima, and the relative fluorescence intensities are shown in Table II. The excitation and emission spectra of the fluorescences from these α-keto acids were very similar in shapes and maxima to those of the fluorescence from pyruvic acid and were not characteristic of individual α-keto acids. The relative fluorescence intensities were nearly independent of the individual α-keto acids, except for *p*-hydroxyphenylpyruvic acid and phenylpyruvic acid. The intensity from *p*-hydroxyphenylpyruvic acid was much

TABLE II. Excitation and Emission Maxima and Relative Intensities of the Fluorescence from the Reactions of α-Keto Acids with DMB, and Their Detection Limits in the HPLC

Compound	Maximum[a] (nm)		RFI[a,b]	Detection limit[c] (fmol/10 μl)
	Ex	Em		
α-Ketoglutaric acid	368	446	131	4
Pyruvic acid	367	445	100	10
p-Hydroxyphenylpyruvic acid	370	448	1	440
α-Ketobutyric acid	372	445	85	13
α-Ketovaleric acid	369	445	106	11
α-Ketoisovaleric acid	368	444	71	38
α-Ketoisocaproic acid	370	445	120	14
Phenylpyruvic acid	375	449	26	33
α-Ketocaproic acid	370	445	97	21
α-Keto-β-methylvaleric acid	372	446	93	39

a) Portions (1.0 ml) of 10 μM α-keto acid solutions were treated as in the manual assay procedure with DMB. *b*) The intensity obtained from pyruvic acid was taken as 100. *c*) Defined as the concentration giving a peak detectable at a signal-to-noise ratio of 2.

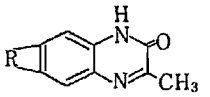
smaller than those from the other acids. The same phenomenon has been observed for OPD[4] and DDB.[4,5]

Reaction of Other Substances with DMB

Alloxan, diacetyl and ascorbic acid gave weak responses with the reagent at a concentration of 5 nmol/ml; the intensities were about double that of the reagent blank. None of the other biologically important substances examined fluoresced under the recommended conditions at a concentration of 10 nmol/ml. The compounds tested were benzaldehyde, 3-tolualdehyde, 2-, 3- and 4-hydroxybenzaldehydes, 2,4- and 3,4-dihydroxybenzaldehydes, 2-, 3- and 4-methoxybenzaldehydes, 2-chlorobenzaldehyde, glycine, 17 different L-α-amino acids, glutathione, creatine, creatinine, histamine, tyramine, octopamine, dopamine, thiamine, citrulline, allantoin, uric acid, urea, *N*,*N*-dimethylurea, acetone, cyclohexane, 4-methylcyclohexane, acetylacetone, acetophenone, benzil, lactic acid, 3-hydroxybutyric acid, acetoacetic acid, homogentisic acid, inositol, D-xylose, D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, epiandrosterone, dehydroepiandrosterone, cortisone and cholesterol. This suggests that the manual assay procedure with DMB is fairly selective for α-keto acids.

Reaction Products of Pyruvic Acid with DMB and DEB (Compounds I and II, Respectively), and Their Fluorescence Properties

The fluorescent product in the reaction of pyruvic acid with DDB has been characterized as 3-methyl-6,7-dimethoxy-2(1*H*)-quinoxalinone.[5] Accordingly, compounds I and II should be 3-methyl-6,7-methylenedioxy-2(1*H*)-quinoxalinone and 3-methyl-6,7-ethylenedioxy-2(1*H*)-quinoxalinone, respectively (Chart 1); these identifications were confirmed by the analytical and spectral data described in the experimental section.



I: R=O-CH_2-O
II: R=O-$(CH_2)_2$-O

Chart 1

TABLE III. Excitation and Emission Maxima and Relative Intensities[a] of the Fluorescence of Compounds I and II in Various Solvents

Compound	Water Maximum (nm)	Methanol Maximum (nm)	Acetonitrile Maximum (nm)	Acetone Maximum (nm)	Chloroform Maximum (nm)	*n*-Hexane Maximum (nm)
	Ex Em (RFI)	Ex Em (RFI)	Ex Em (RFI)	Ex Em (RFI)	Ex Em (RFI)	Ex Em (RFI)
I	365 445 (97)	363 439 (100)	360 430 (71)	361 428 (61)	363 428 (76)	359 423 (48)
II	360 457 (10)	361 449 (8)	358 437 (5)	359 443 (4)	360 436 (5)	358 428 (4)

a) The intensity of compound I in methanol was taken as 100.

The fluorescence spectra and intensities of compounds I and II were measured in several solvents that have been used as mobile phases in HPLC (Table III). A red shift of the emission wavelength with increasing solvent polarity was observed for both compounds. The fluorescence intensities also varied with solvent polarity, the maximum values being found in water and methanol. The intensity of compound II was almost one-tenth of that of compound I in each solvent.

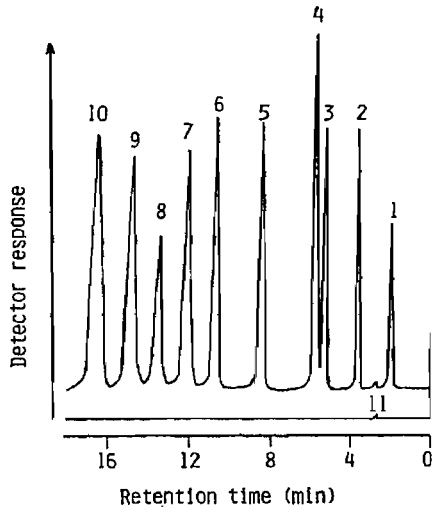


Fig. 3. Chromatograms of the DMB Derivatives of α-Keto Acids and the Reagent Blank

a, test; b, blank.
A portion (1.0 ml) of a mixture of ten α-keto acids or water was treated according to the HPLC procedure. Peaks (pmol per injection volume): 1, α-ketoglutaric acid (25); 2, pyruvic acid (50); 3, *p*-hydroxyphenylpyruvic acid (4000); 4, α-ketobutyric acid (100); 5, α-ketovaleric acid (100); 6, α-ketoisovaleric acid (200); 7, α-ketoisocaproic acid (100); 8, phenylpyruvic acid (300); 9, α-ketocaproic acid (150); 10, α-keto-β-methylvaleric acid (200); 11, blank.

HPLC Separation of the DMB Derivatives of α-Keto Acids

DMB was applied to the fluorescence derivatization of a mixture of ten α-keto acids of biological importance. The DMB derivatives could be separated by reversed-phase HPLC on a Radial Pak cartridge C_{18} with isocratic elution using a mixture of methanol, acetonitrile and potassium phosphate buffer (pH 7.0) as a mobile phase. The optimum HPLC conditions were as described in the experimental section.

Figure 3 shows typical chromatograms obtained with a standard mixture of α-keto acids of biological importance, and with water as the reagent blank. The individual α-keto acids each gave a single peak in the chromatogram. The peaks for the DMB derivatives of the acids and the small peak due to the reagent blank could be completely separated within 18 min. This suggests that DMB may be used for the determination all the α-keto acids, tested here.

Linear relationships were observed between the peak heights and the amounts of α-keto acids up to at least 5 nmol per injection volume (10 μl). The precision was established by repeated determination ($n=10$) using a mixture of ten α-keto acids (10 nmol/ml). The coefficient of variation did not exceed 3% for all the acids. The detection limits are listed in Table II. The sensitivity was at least 1.6 and 150 times better than those of the HPLC methods with DDB and OPD, respectively.

DMB has excellent properties as regards reactivity, selectivity and sensitivity for the derivatization of α-keto acids in comparison with the reagents reported so far. Thus, the reagent may be applicable to the determination of α-keto acids in small amounts of biological samples.

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Urea Sensor Based on an Ion-Sensitive Field Effect Transistor. IV. Determination of Urea in Human Blood

JUN-ICHI ANZAI,[a] SACHIKO TEZUKA,[a] TETSUO OSA,*[,a] HIDEKI NAKAJIMA,[b] and TADAYUKI MATSUO[b]

Pharmaceutical Institute, Tohoku University,[a] Aobayama, Sendai 980, Department of Electronic Engineering, Tohoku University,[b] Aoba, Aramaki, Sendai 980, Japan

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A potentiometric method for the determination of urea in human blood with an ion-sensitive field effect transistor coated with a urease–albumin membrane is described. The urea sensor exhibits a potentiometric response to urea concentrations in the range of 0.1—10 mM with a response time of *ca.* 3 min. Urea concentrations of diluted serum and plasma can be determined accurately with the sensor. Comparison with an official method (*i.e.* the urease–indophenol method) gave satisfactory results.

Keywords——urea sensor; ion-sensitive field effect transistor; urease; enzyme sensor; human blood

Much effort has been directed to the selective determination of physiologically important substrates in biological fluids by means of spectroscopic, chromatographic, and electrochemical methods.[1,2] Recently, ion-selective electrodes equipped with an enzyme membrane have been studied extensively because of their high selectivity originating from the highly specific enzymatic reaction.[3] To date, many kinds of electrodes such as the pH-sensitive glass electrode, ammonia electrode, and oxygen electrode, have been used to construct such enzyme electrodes.

The ion sensitive field effect transistor (ISFET)[4-6] can be endowed with sensitivity to biological compounds by covering the gate surface with an appropriate enzyme, and an ISFET coated with ionophore-containing polymer membrane was successfully applied to the determination of K^+ and Ca^{2+} ions.[7] Caras and Janata, from this point of view, developed a penicillin-sensitive FET by immobilizing penicillinase on the gate of a pH-sensitive FET.[8] The penicillin sensor exhibited a linear response to penicillin solutions over the range of 0.2—25 mM. They have recently reported a quantitative analysis of the performance parameters of ISFET-based enzyme sensors.[9-11] We[12-15] and Miyahara *et al.*[16] have independently developed urea sensors by covering the ISFET gate with a urease membrane. The urea sensors are based on the urease-catalyzed reaction (1), in which urea is decomposed to ammonia and carbon dioxide with consumption of H^+. The urease-bearing ISFET can detect the amount of H^+ consumed through the reaction (1) as a pH change around the gate surface. The previous papers described the effects of some operating variables such as capacity and pH of the working buffer and the membrane thickness upon the performance characteristics of the sensor.[12-15]

This paper is concerned with the construction of an ISFET-based urea sensor and its application to the determination of urea in human blood.

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4 + HCO_3 \qquad (1)$$

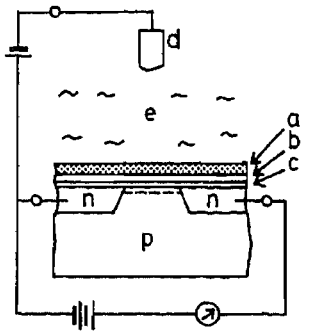

Fig. 1. Schematic Representation of the Urease Membrane-Coated ISFET

a) urease membrane; b) Si_3N_4 layer; c) SiO_2 layer; d) SCE; e) sample solution.

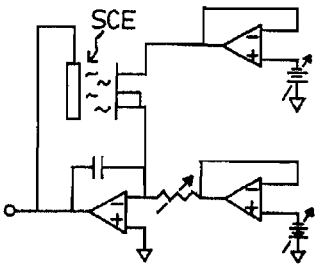

Fig. 2. Circuit for Operating the Sensor

Experimental

Fabrication of ISFET——The pH-sensitive FET was fabricated on a p^--silicon wafer, which was 0.5 mm wide, 6.5 mm long and 0.2 mm thick. The Si_3N_4 gate (1000 Å) was grown on an SiO_2 layer by the chemical vapor deposition method using SiH_4 and NH_3 as materials. The ISFET thus prepared exhibited a near-Nernstian response over the pH range of 1—13. The procedure for making the ISFET and the properties were reported previously.[17]

Chemicals——Urease (E.C. 3.4.1.5, from jack beans) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. as lyophilized powder. All chemicals used were of reagent grade. Distilled water was used throughout.

Preparation of Urease Membrane on the ISFET Gate——The gate surface of the ISFET was washed with water immediately before immobilizing the enzyme on it. About 20% urease and 10% BSA solutions were prepared in Tris–HCl buffer. Aqueous glutaraldehyde (GA) solution (8%) was prepared. An appropriate amount of the mixture was applied to the ISFET gate before gelation began. The probe was air-dried at ambient temperature for about 30 min, and was bathed in the working buffer for at least 30 min before use. The thickness of the enzyme membrane thus prepared was typically about 3 μm in the dry state. A schematic representation of the urease-bearing ISFET is illustrated in Fig. 1.

Measurement——Calculated amounts of urea were dissolved in 10 mM Tris–HCl buffer (pH 7.35), which contained 160 mM NaCl to compensate for the difference in ionic strength from that of blood samples, to prepare the standard urea solutions. From potentiometric measurements on these solutions, a calibration graph was obtained.

The serum and plasma samples were prepared in the usual manner from whole blood obtained from members of this laboratory. The samples were diluted with the working buffer, and pH adjustment, if necessary, was done by adding a small amount of HCl. Solutions were not stirred during the measurements and the probe was rinsed with the working buffer after each measurement. All measurements were conducted at 23 °C. A circuit for operating the sensor in the constant drain current mode with a saturated calomel electrode is depicted in Fig. 2.

The value of gate voltage recorded for serum or plasma without urea, after pretreatment with urease to decompose urea, was employed as a base-line value of potential. Urea determination of the samples by the urease–indophenol method was performed as described elsewhere.[18]

Results and Discussion

We have already reported in the previous papers that a pH-sensitive FET coated with a urease–albumin membrane serves as a urea sensor if operated in a low capacity buffer.[12] In 1 mM Tris–HCl buffer, for example, the urea sensor exhibited near-Nernstian response for urea solutions over the range of 0.1—10 mM. One of the most difficult problems encountered when we employed the pH-sensitive device as a transducer for an enzyme electrode is that the performance characteristics of the sensor depend markedly upon the buffer capacity of the medium. This situation is inevitable since such pH-sensitive devices detect the pH change originating from the enzymatic reaction at the interior of the enzyme membrane in spite of the buffered medium. This is the case for the ISFET-based urea sensor. In order to obtain a higher response of the sensor to urea, it is preferable to operate the sensor in a low concentration of buffer (*e.g.* 1 mM buffer) in the neutral or slightly acidic pH region.[13] These

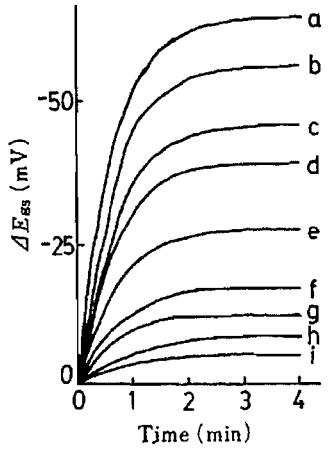

Fig. 3. Typical Response Curves of the Sensor for Urea Solutions

Tris-HCl buffer (pH 7.35, 10 mM) was used.

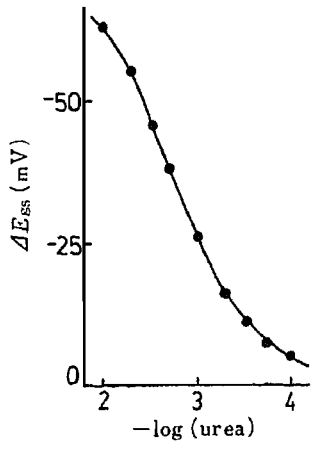

Fig. 4. A Calibration Graph for Urea Determination with the Sensor

Tris-HCl buffer (pH 7.35, 10 mM) was used.

conditions, however, are inapplicable to the determination of urea in human serum and plasma, because, with such a low capacity buffer, the pH values of blood samples can not be maintained constant at that of the standard urea solutions used for constructing the calibration graph. The deviation of pH values from sample to sample will make it impossible to determine the blood level of urea accurately due to the pH-dependence of urease activity. Accordingly, we used here 10 mM Tris-HCl buffer to keep the pH values constant at the cost of poorer potentiometric response of the sensor.

Potentiometric Response of the Sensor

In Fig. 3 the response curves of the sensor for 0.1—10 mM urea standard solutions are illustrated. Before measurements, the dried probe was immersed in the working buffer to ensure swelling of the enzyme membrane. After swelling of the enzyme layer, the probe was dipped into urea solutions of known concentration. The output voltages (E_{gs}) changed rapidly at the initial stage of the measurements and declined to reach steady-state potentials after about 3 min. The potential shifts of the sensor were induced by the pH changes around the gate surface, which in turn resulted from the enzymatic reaction (1) in the membrane layer. Reproducibility of the potentiometric response of the sesnor for urea solutions was fairly good, the deviation of E_{gs} values being within 0.5 mV.

A calibration graph was constructed in Fig. 4 by plotting the ΔE_{gs} (ΔE_{gs} is the difference between the E_{gs} value for sample solution and that for base line) values read from Fig. 3 against the logarithm of urea concentration. The graph shows that the sensor exhibits a potentiometric response in the 0.1—10 mM urea concentration range, the slope of the linear part of the graph being about −40 mV/decade. This calibration graph can be used for determining urea in human blood in view of the fact that the urea concentration in blood is normally at millimolar level.

Determination of Urea in Human Blood

We applied the sensor for the determination of urea in blood serum after appropriate dilution. The potentiometric response of the sensor with diluted sera is illustrated in Fig. 5; the response characteristics of the sensor were almost the same as those observed for the urea standard solutions. After each measurement of a blood sample, the base line value of E_{gs} was recovered by dipping the probe into the working buffer. Reproducibility of the response to the blood samples was good. These results imply that the enzymatic activity of the membrane-

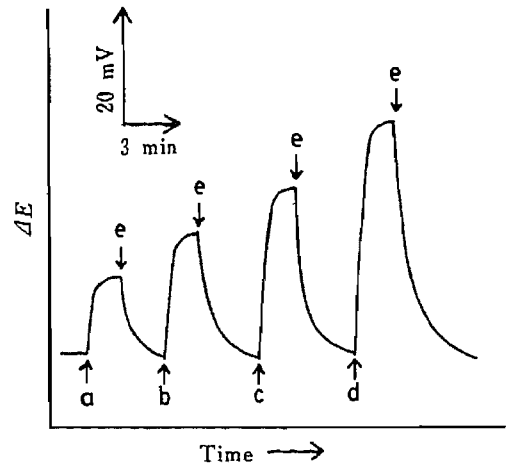

Fig. 5. Potentiometric Response of the Sensor with Diluted Sera

The probe was dipped into 10-fold (a), 6-fold (b), 4-fold (c), and 2-fold (d) diluted sera, and rinsed with the working buffer (e).

TABLE I. Determination of Urea in Blood Serum and Plasma[a]

Sample	Urea found (mM)		Difference (mM)
	Presented method	Urease–indophenol method	
10-fold dilution			
A[b]	4.1	4.2	−0.1
B[b]	3.7	3.6	0.1
C[b]	3.7	3.9	−0.2
D[c]	4.0	3.9	0.1
E[d]	3.8	3.9	−0.1
F[e]	4.3	4.1	0.2
20-fold dilution			
A[b]	4.0	4.2	−0.2
B[b]	3.8	3.6	0.2
C[b]	4.0	3.9	0.1
D[c]	3.8	3.9	−0.1
E[d]	4.0	3.9	0.1
F[e]	4.4	4.1	0.3

a) Tris–HCl buffer (pH 7.33, 10mM) was used. *b*) Blood serum without lipid. *c*) Blood serum. *d*) Hemolyzed serum. *e*) Blood plasma.

bound urease remained unchanged even after exposing the membrane repeatedly to blood samples containing possible interfering substrates such as proteins and lipids.

Table I lists typical results of determination of urea in human blood. The values obtained by the urease–indophenol method are also listed for reference. Samples were diluted 10-fold or 20-fold with the working buffer prior to the potentiometric analysis due to the necessity of pH adjustment. The agreement between the values of urea concentration, as measured by the conventional urease–indophenol method and by the potentiometric method with the urea sensor, is acceptable for all samples. Both serum and plasma could be analyzed with the urea sensor. Further, hemolysis did not cause any disturbance in the potentiometric determination of urea with the sensor. We also analyzed sera diluted 2-fold, 4-fold, 6-fold, and 8-fold with the buffer. For 2-fold and 4-fold diluted samples, typically about 10% deviation of the values from those obtained with the urease–indophenol method was observed. This might be due to undesirable effects of proteins on the potentiometric response. These results suggest that it is preferable to analyze the blood serum and plasma after diluting them 10-fold or more with the

TABLE II. Recovery Test with Diluted Serum and Plasma[a)]

Amount of urea (mM)		Recovery (%)
Added	Found	
10-fold diluted serum		
0	0.40	—
0.50	0.89	98
1.00	1.38	98
2.50	2.75	94
3.75	4.26	103
20-fold diluted plasma		
0	0.43	—
0.50	0.93	100
1.00	1.41	98
2.50	2.85	98
3.75	4.37	105

a) Tris–HCl buffer (pH 7.33, 10 mM) was used.

working buffer in order to determine urea accurately.

Recovery

Recovery tests were carried out on 10-fold diluted serum and plasma in order to check the accuracy of the present method. Table II shows the results. The recoveries varied between 94% and 105%, which is satisfactory for analytical purposes. There was no significant difference between the results for serum and plasma.

Thus, we have shown that the urea concentration in blood samples can be determined potentiometrically by the use of the ISFET-based urea sensor. To obtain the maximum performance of the sensor, it is recommended to dilute the sample 10-fold or more with the buffer. Studies to further improve in the performance of the sensor are now in progress in this laboratory.

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Suppressive Effect of Zinc on Some Functions of Neutrophils: Studies with Carrageenan-Induced Inflammation in Rats

JUN YATSUYANAGI,* KAZUHIKO IWAI, and TAKETO OGISO

Hokkaido Institute of Pharmaceutical Sciences, Katsuraoka-cho 7–1, Otaru 047–02, Japan

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The effect of zinc administration to rats on the acute inflammatory response to carrageenan in the pleural cavity was studied. The number of neutrophils mobilized into the pleural cavity was markedly reduced in a dose-dependent manner, whereas the volume of exudate formed significantly increased during the development of pleurisy. In the neutrophils isolated from zinc-treated rats, the mobilization capacity, phagocytic activity and concomitant O_2^- production were strongly inhibited accompanied with a slight increase in zinc content in the cells. These inhibitory effects of zinc on neutrophil functions and the increase in zinc content of cells were also observed in an *in vitro* system.

The viability of neutrophils obtained from zinc-treated rats slightly decreased, as was evidenced from leakage of cytoplasmic lactate dehydrogenase during the course of phagocytosis, suggesting that the neutrophils from zinc-treated rats may be more susceptible to cell damage during phagocytosis.

Keywords——carrageenan; pleurisy; neutrophil; zinc; chemotaxis; phagocytosis; O_2^- production; lactate dehydrogenase leakage

Zinc has been shown to influence many functions of various cells associated with the inflammatory process.[1] Various effects of zinc have been demonstrated *in vitro*, including stabilization of the lysosomal membrane,[2] inhibition of macrophage and neutrophil migration and phagocytic activity,[3] inhibition of histamine release from mast cells[4] and inhibition of prostaglandin synthesis.[5] On the other hand, zinc administration to rats has been shown to inhibit the mobilization and phagocytosis of leukocytes in the inflammatory response to intraperitoneal injection of mineral oil.[6] The effect of zinc on these inflammatory cells has been postulated as being the result of interaction of zinc with some functional groups of intrinsic components in the plasma membrane,[1] but the exact mechanism still remains to be elucidated.

The experiments to be described here involve studies of the effect of zine administration to rats on functional parameters of neutrophils, using carrageenan pleurisy, a model of acute inflammation which allows precise quantitative evaluations of both neutrophil migration and exudate volume. The results indicate the ability of zinc to interfere with chemotactic function, phagocytic activity and superoxide anion (O_2^-) production of neutrophils.

Experimental

Materials——Cytochrome c (horse heart type III), superoxide dismutase, catalase (horse heart), zymosan A, N-f-Met-Leu-Phe (FMLP) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., lambda-carrageenan from Minsei Rikagaku Co. and nicotinamide adenine dinucleotide (NAD^+) from Oriental Yeast Co., Japan. Hematoxylin and Giemsa solutions were purchased from Merck Co. Other reagents used were of analytical grade.

Rat Pleurisy——Pleurisy was induced by the method described by Vineger *et al.*[7] Male Wistar rats, weighing 200 ± 20 g, were given a single, intraperitoneal injection of various doses of zinc (as $ZnCl_2$) dissolved in isotonic saline.

Control rats were received the same volume of saline vehicle. At 60 min thereafter, both groups of rats were anesthetized with chloroform, and 0.25 ml of 0.2% carrageenan (500 μg) in sterilized saline was injected into the pleural cavity. At the specified time, rats were exsanguinated under chloroform anesthesia, then the pleural exudate was collected, and its volume was measured. Neutrophils were obtained from the pleural exudate by centrifugation at $170 \times g$ for 10 min. Residual erythrocytes were eliminated by hypotonic lysis. The cell fractions obtained from both control and zinc-treated rats contained about 95% neutrophils with more than 95% viability as determined by exclusion of trypan blue dye. Leukocyte numbers and types were determined from hemocytometer counts and from May-Grünwald-Giemsa-stained smears, respectively.

Chemotaxis——The *in vitro* chemotaxis assay was done by a modification of the Boyden technique.[8] Cell chambers (Bio-Rad. Lab., Richmond, Calif.) and Millipore filters with 3.0 μm pore size (Millipore Corp., Bedford, Mass.) were used in these experiments. Neutrophils obtained from pleural exudate were suspended at 1.5×10^6 cells/ml in modified Hank's solution, devoided of Ca^{2+}, containing 0.1% BSA and placed above the filter in the chambers. A similar cell-free solution containing a chemotactic agent, 10^{-8} M FMLP, was placed in the lower compartment of the chambers. The chambers were incubated for 3 h at 37 °C in 5% CO_2. The filters were fixed and stained with hematoxylin, and chemotactic activity was assessed by counting the numbers of neutrophils that had migrated to the lower surface of the filter under high-power magnification.

Opsonization of Zymosan——Serum-treated zymosan (STZ) was prepared by the method described by Cheson *et al.*[9] with a minor modification as follows: a suspension of 25 mg of zymosan in 1.0 ml of fresh rat serum was incubated for 30 min at 37 °C, and then washed and resuspended in modified Hank's solution (10 mg/ml).

Phagocytosis——STZ was added to a neutrophil suspension (5×10^6 cells/ml) to make 0.5 mg/ml final concentration and the mixture was incubated for 30 min at 37 °C. The uptake of zymosan particles was quantitated microscopically after staining with May-Grünwald-Giemsa solution. Cells with two or more zymosan particles were counted as phagocytic.[10]

O_2^- Production——The O_2^- released from neutrophils was assayed by measuring the SOD-inhibitable reduction of ferricytochrome c at 550—540 nm on a dual-wavelength spectrophotometer (Hitachi, 556) with a constant-temperature cuvette holder kept at 37 °C.[11] The assay mixture contained 25 μM cytochrome c, 5 μg/ml catalase and 5×10^6 neutrophils in 1.0 ml of modified Hank's solution. After the addition of STZ to the reaction mixture (0.5 mg/ml), the time course of cytochrome c reduction was followed on the recorder. The rate of O_2^- production by neutrophils was calculated from the linear portion of the chart, based on a molar extinction coefficient of $19.1 \times 10^3 \cdot M^{-1} \cdot cm^{-1}$.

Enzyme Assay——Lactate dehydrogenase (LDH) was assayed by measuring the conversion of NAD^+ to reduced nicotinamide adenine dinucleotide (NADH) during the reaction of lactate to pyruvate.[13] The enzyme activities were expressed as percent of total enzyme activity in the cells, which was determined after disruption of the cells with 0.2% Triton X-100.

Zinc Determination——The concentrations of zinc in neutrophils and serum were measured with an atomic absorption spectrophotometer (Hitachi, 180-80).

Results

Effect of Zinc Administration on Pleurisy

The time courses of number of neutrophils mobilized and volume of exudate formed in the pleural cavity after intrapleural injection of carrageenan (500 μg) are shown in Fig. 1. In control rats, the number of neutrophils increased rapidly between 3 and 4 h after the injection of carrageenan and then increased more slowly until 6 h. Similarly, the volume of exudate continued to increase until 6 h. In comparison with the control group, the pretreatment with zinc (8.2 mg/kg) 1 h before the carrageenan injection markedly reduced the number of neutrophils, whereas the volume of exudate formed significantly increased during the development of pleurisy.

As can be seen in Table I, pretreatment with various doses of zinc (8.2—33.0 mg/kg) produced a dose-dependent increase in zinc content in the serum and a decrease in the number of neutrophils mobilized at 5 h after the injection of carrageenan. At a dose of zinc of 16.4 mg/kg, the inhibition of neutrophil mobilization to the pleural cavity was about 50%, while the volume of exudate increased to the extent of about 42%. This magnitude of increase in the exudate was slightly reduced at the zinc dose of 33.0 mg/kg. Although the data are not shown, the administration of zinc (33.0 mg/kg) did not have any significant effect on the number of peripheral neutrophils during the development of pleurisy. These results indicate

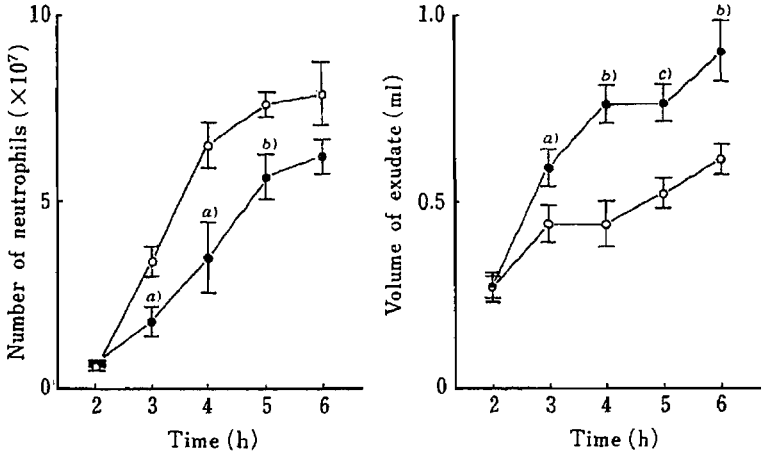

Fig. 1. Time Course of Neutrophil Mobilization and Exudate Formation in Carrageenan Pleurisy of Zinc-Treated Rats

Rats were injected intrapleurally with carrageenan (500 μg) 1 h after the i.p. injection of zinc (8.2 mg/kg) or the vehicle saline (control). The number of neutrophils and the volume of exudate in the pleural cavity were estimated at the indicated time after the injection of carrageenan. Each point represents the mean value of 5 to 23 estimations. The vertical bars represent the standard error of the mean.

○, control. ●, zinc-treated; *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

TABLE I. Effect of Various Doses of Zinc on Neutrophil Mobilization and Exudate Formation in Pleurisy

Zinc dose (mg/kg)	Serum zinc (μg/ml)	Number of neutrophils ($\times 10^7$)	(%)[a]	Volume of exudate (ml)	(%)[b]
0	1.74±0.13 (18)	7.61±0.32 (23)	—	0.52±0.04 (23)	—
8.2	10.02±0.97 (11)	5.65±0.58 (18)	−26	0.76±0.05 (17)	+46
16.4	13.49±0.86 (11)	3.89±0.44 (10)	−49	0.74±0.06 (11)	+42
33.0	22.00±1.22 (6)	2.32±0.28 (6)	−70	0.64±0.09 (6)	+23

a) Change in number of neutrophils. *b*) Change in volume of exudate. Rats were injected intrapleurally with carrageenan (500 μg) 1 h after the i.p. injection of various doses of zinc or the vehicle saline. After 5 h, the number of neutrophils and the volume of exudate in the pleural cavity were estimated. Each value represents the mean ± S.E. of the number of animals indicated in parenthesis.

that zinc administration to rats suppresses one of the features of the inflammatory response, *i.e.*, the mobilization of neutrophils to the inflammatory site.

Effect of Zinc Administration on Functions of Pleural Neutrophils

We next evaluated the functions of pleural neutrophils isolated from zinc-treated rats (16.4 mg/kg) at 5 h after the intrapleural injection of carrageenan (500 μg); the results are summarized in Table II. Zinc contents in the neutrophils isolated from the normal and zinc-treated rats were about 0.67 and 0.86 μg/10^8 cells, respectively. Chemotactic response to an attractant, FMLP, of neutrophils from zinc-treated rats markedly decreased by about 47% as compared to the control. LDH release by cells from zinc-treated rats was minimal (<3% of total activity in the cell) and was unaltered during the period of chemotactic assay, suggesting that the inhibition of cell mobilization was not due to direct toxic action of zinc on the cells. The zinc treatment also caused a significant inhibition of phagocytic activity and production of O_2^- stimulated with STZ by about 21 and 50%, respectively. These results indicated that the mobilization capacity, phagocytic activity and concomitant O_2^- production are all strongly inhibited in the neutrophils isolated from zinc-treated rats.

TABLE II. Some Functions of Pleural Neutrophils Obtained from Zinc-Treated Rats

	Zn content ($\mu g/10^8$ cells)	Chemotaxis (neutrophils/field)		Phagocytosis (%)		O_2^- production (nmol/min/5×10^6)	
Control	0.67 ± 0.01	53.3 ± 5.2		28.9 ± 1.1		1.29 ± 0.050	
Zinc-treated	0.86 ± 0.01	28.5 ± 2.3	46.5[a]	22.9 ± 1.1	20.8[a]	0.65 ± 0.090	49.6[a]

a) Inhibition %. Neutrophils were obtained from the rats injected i.p. with zinc (16.4 mg/kg) or vehicle saline as described in the legend to Table I. Chemotaxis, phagocytosis and O_2^- production of neutrophils were measured by the methods described in the text. Each value represents the mean $\pm$ S.E. of 6 to 15 experiments.

TABLE III. Effect of Zinc on Some Functions of Neutrophils *in Vitro*

Zn^{2+} (μM) in medium	Chemotaxis (neutrophils/field)	Phagocytosis (%)	O_2^- production (nmol/min/5×10^6)
0	53.3 ± 5.2 —	28.9 ± 1.1 —	1.29 ± 0.050 —
100	33.4 ± 7.6 (37.7)	17.3 ± 4.2 (40.1)	0.32 ± 0.009 (75.2)
200	13.7 ± 3.4 (74.3)	11.3 ± 2.4 (60.9)	0.10 ± 0.009 (92.2)
300	7.0 ± 1.4 (86.9)	7.6 ± 0.8 (73.7)	0.07 ± 0.006 (94.6)

Neutrophils were obtained from rats as described in the legend to Table I. Chemotaxis, phagocytosis and O_2^- production of neutrophils were measured by the methods described in the text, in the presence of various amounts of zinc. Values in parenthesis represent the inhibition %. Each value represents the mean $\pm$ S.E. of 5 to 6 experiments.

Effect of Zinc on Functions of Neutrophils *in Vitro*

Experiments were performed to test the effect of zinc addition on the functions of pleural neutrophils harvested from normal rats injected intrapleurally with carrageenan (500 μg). As shown in Table III, the addition of zinc (as $ZnCl_2$) to the medium at concentrations of 100 to 300 μM resulted in a concentration-dependent depression of chemotaxis toward FMLP. There was no significant release of cytoplasmic LDH (about 3% of total cell activity) during the period of incubation. Similarly, the phagocytic activity and O_2^- production induced by STZ were strongly inhibited by the addtion of zinc in a concentration-dependent fashon. Incubation of cells for 30 min in medium with 200 μM $ZnCl_2$ resulted in an increase of zinc content in cells to the same level (0.86 $\mu g/10^8$ cells) as that of the cells from zinc-treated rats (16.4 mg/kg). The inhibitory actions of zinc on cell functions as described above were also demonstrated on peritoneal neutrophils harvested from normal rats injected intraperitoneally with 6% sterilized casein (data not shown). These results indicate that the depletion of neutrophil functions by zinc also occurred in the *in vitro* system.

Release of LDH from Neutrophils

In the course of these experiments, we observed that the viability of neutrophils obtained from zinc-treated rats slightly decreased during the course of phagocytosis stimulated with STZ. Therefore, the leakage of the cytoplasmic LDH from cells stimulated with STZ, as a measure of cell damage, was assessed and the results are shown in Fig. 2. In neutrophils from zinc-treated rats, the release of LDH progressively increased with time of incubation and reached the level of about 12% of the total cell activity at 30 min, while the release of LDH was about 3% in cells from normal rats. As described above, the administration of zinc (16.4 mg/kg) caused the inhibition of phagocytic activity and O_2^- production stimulated with STZ by about 21 and 51%, respectively (Table III). These results indicate that the cell membrane tends to be disrupted in spite of the inhibition in the phagocytic response to STZ,

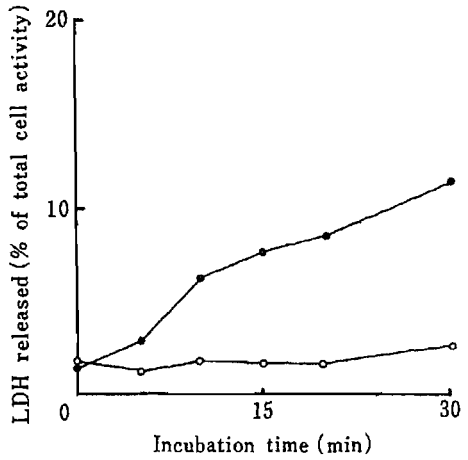

Fig. 2. Time Course of LDH Release from Neutrophils Stimulated with STZ

Pleural neutrophils obtained from zinc-treated and normal rats as described in Experimental were incubated for various times in the presence of STZ (0.5 mg) and the LDH activities in the supernatants were measured. ●, neutrophils from zinc-treated rats (16.4 mg/kg); ○, normal neutrophils. Each point represents the mean of duplicated experiments.

suggesting that the neutrophils from zinc-treated rats may be more susceptible to cell damage during phagocytosis.

Discussion

The injection of carrageenan into the pleural cavity is followed by an increase in the vascular permeability, the accumulation of exudate and infiltration of neutrophils.[7] The results described in this paper demonstrate that zinc administration to rats modified the acute inflammatory response to carrageenan in the pleural cavity: neutrophil mobilization was inhibited and exudate formation was augmented. The magnitude of inhibition of neutrophil mobilization into the pleural cavity was related to the dose of zinc (Table I). These effects of zinc on cell mobilization are consistent with the report that the intraperitoneal injection of mineral oil into rats treated with zinc resulted in the inhibition of cell mobilization into the peritoneal cavity accompanied with an increase of serum zinc level. The observed inhibition of cell mobilization did not seem to be mediated by an antimitotic action of zinc, since a high dose of zinc (33.0 mg/kg) failed to affect the peripheral cell count. In addition, marked suppression of chemotactic response to a chemoattractant was demonstrated in neutrophils isolated from zinc-treated rats or incubated in medium containing zinc ion by Millipore filter assay (Tables II and III), suggesting that zinc can inhibit some processes of neutrophil activation, resulting in the suppression of neutrophil chemotaxis and mobilization into the inflammed area. Previous workers[3] have shown that in dog peripheral granulocytes, zinc inhibits the *in vitro* phagocytosis of yeast particles and concomitant respiratory burst, with an increase in cellular zinc content, suggesting that the actual quantity of zinc bound to cell structures is critical for the activity of cells. In agrement with their studies, the present results indicate that zinc treatment in *in vivo* as well as *in vitro* situations inhibited both phagocytosis and O_2^- production of neutrophils stimulated with STZ. Futhermore, our results showed that the suppression of neutrophil functions coincided with an increase in the zinc content in cells. The mechanism by which neutrophil functions could be inhibited by zinc is unknown, though several possibilities can be considered, such as blocking of membrane receptors, changing the fluidity of the membrane components, interfering with cytoskeletal elements and antagonizing Ca^{2+}, as suggested by the previous authors.[3]

Chvapil *et al.* suggested that the increase in viability of macrophages pretreated *in vivo* with zinc was related to the decrease of phagocytic activity.[14] These findings differ from the present results, which indicate that the viability of neutrophils from zinc-treated rats decreased, despite the inhibition of phagocytic activity for STZ (Fig. 2). This apparent

discrepancy may be attributable to the difference in the particles being phagocytosed, in cell types or in the viability index employed. Neutrophils are known to be susceptible to damage by self-produced oxygen metabolites[15] and the viability of phagocytosing neutrophils could be protected by defense mechanisms, namely, superoxide dismutase, catalase and glutathione peroxidase.[16] Moreover, a similar protective role of intracellular glutathione against oxidative damage has been proposed.[17] Our finding that cell damage was potentiated in neutrophils from zinc-treated rats may reflect impairment of any of these defence systems. Further experiments are required to clarify the mechanism of cell damage in neutrophils from zinc-treated rats.

The present experiments demonstrated that zinc-treatment augmented the volume of exudate in spite of the dose-related decrease in mobilization of neutrophils (Table I). It has been suggested that although the mobilized neutrophils are required to initiate the process of exudate formation, the cells of the pleura produce reactive prostaglandin intermediates that increase vascular permeability.[18] Further study demonstrated that prostaglandin E_2 potentiated the activity of bradykinin to initiate the accumulation of pleural exudate.[19] On the other hand, zinc has been shown to inhibit *in vitro* prostaglandin synthesis in rabbit peritoneal neutrophils.[5] Therefore, we can not yet explain the augmenting effect of zinc on the formation of exudate.

Several workers have observed the beneficial effect of zinc therapy in patients with rheumatoid arthritis,[20] sickle cell anemia[21] and venous leg ulcer.[22] The results presented here demonstrate a depressive effect of zinc upon neutrophil functions, so the possibility must be considered that under certain conditions, zinc treatment may render the organism more susceptible to bacterial infection. In fact, parenteral administration of $ZnCl_2$ has been reported to potentiate lethality in rats following challenge with *Salmonella typhimurium*.[23]

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Glycyrrhizin β-D-Glucuronidase of *Eubacterium* sp. from Human Intestinal Flora

TAIKO AKAO, TERUAKI AKAO and KYOICHI KOBASHI*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan

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A bacterial strain capable of hydrolyzing glycyrrhizin (GL) to glycyrrhetic acid (GA) was isolated from human feces. This bacterium was identified as *Eubacterium* sp. The GL-hydrolyzing activity increased in parallel with the growth of this bacterium, which also produced β-D-glucuronidase (EC 3.2.1.31) acting on β-D-glucuronides of phenolic compounds such as phenolphthalein mono-β-D-glucuronide. GL-hydrolyzing activity was recovered in the supernatant fraction after disruption of this bacterium with a French press and was partially purified by means of ammonium sulfate fractionation, and Sephadex G-200 and octyl-Sepharose column chromatographies.

GL-hydrolyzing enzyme was separated from the β-D-glucuronidase which hydrolyzes β-D-glucuronides of phenolic compounds by octyl-Sepharose column chromatography, indicating that the GL-hydrolyzing enzyme is a novel type of β-D-glucuronidase.

Keywords——glycyrrhizin β-D-glucuronidase; *Eubacterium* sp.; human intestinal bacteria; glycyrrhizin; β-D-glucuronidase

Introduction

Glycyrrhizin (GL), an active principle of liquorice, *Glycyrrhiza Glabra* L. (Leguminosae), is hydrolyzed to 18β-glycyrrhetic acid (GA), the aglycone, and then transformed to 3-*epi*-18β-glycyrrhetic acid *via* 3-dehydro-18β-glycyrrhetic acid by human intestinal flora.[1]

β-D-Glucuronidase activities (EC 3.2.1.31) occur widely in nature from humans to bacteria, and some of these enzymes have been purified.[2~12] However, it is not yet known whether or not these enzymes hydrolyzing monoglucuronide conjugates such as phenolphthalein mono-β-D-glucuronide also show activitity towards GL, the diglucuronide of GA, though it has recently been reported by Muro *et al.*[13] that *Aspergillus niger* produces an enzyme which hydrolyzes GL, but not phenolphthalein mono-β-D-glucuronide.

In the present paper, we report the isolation of a human intestinal bacterium capable of hydrolyzing GL to GA, and the partial purification of GL β-D-glucuronidase.

Materials and Methods

Chemicals——GL monoammonium and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo. Glycyrrhetic acid mono-β-D-glucuronide was a gift from Dr. M. Kanaoka of the Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical and Pharmaceutical University. Phenolphthalein mono-β-D-glucuronide (PPG), 4-methylumbelliferyl mono-β-D-glucuronide (MUG), pregnenolone mono-β-D-glucuronide (PNG) and D-saccharic acid-1,4-lactone were purchased from Sigma Chemical Co., U.S.A. *p*-Nitrophenol mono-β-D-glucuronide (*p*NPG) was purchased from Nakarai Chemicals, Ltd., Kyoto. General anaerobic medium (GAM) was a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the best commercial quality available.

Isolation of an Intestinal Bacterium Hydrolyzing GL and Culture of the Bacterium——A suspension of human feces was diluted with GAM medium containing 2 mM GL to prepare a series of 10-fold dilutions. An aliquot of the medium producing GA from GL after cultivation was inoculated on GAM agar plates and then incubated at 37 °C for 5 d in an anaerobic box. Well-separated colonies were taken and individually inoculated into about two hundred

tubes of GAM medium containing 2 mM GL. A strain capable of hydrolyzing GL was isolated by repeating these procedures. The bacterium isolated was maintained in GAM semisolid agar.

Thin-Layer Chromatography (TLC)——TLC for GA and GA mono-β-D-glucuronide was performed on silica gel plates (Merck, Silica gel 60 F-254, layer thickness 0.25 mm) with the solvent system of chloroform–petroleum ether–acetic acid (5 : 5 : 1, v/v). GA and GA mono-β-D-glucuronide were detected on TLC plates under ultraviolet (UV) light. The quantity was analyzed with a TLC scanner ($\lambda_s = 250$ nm, $\lambda_r = 400$ nm) by using calibration lines obtained with authentic samples.

Enzyme Assay——The enzyme activity for hydrolysis of GL was measured as follows. The assay mixture for hydrolysis contained 50 nmol of GL monoammonium salt, 0.1 M acetate buffer (pH 5.6), and 5—50 μl of enzyme solution in a final volume of 0.2 ml. The mixture was incubated at 37 °C for 10—20 min and the reaction was stopped by adding 0.1 ml of 1 N HCl. Then, the mixture was extracted twice with 2 ml of ethyl acetate. The ethyl acetate solution was concentrated to a small volume and the amount of GA was determined by TLC as described above.

The enzyme activities for hydrolysis of PPG and *p*NPG were measured by the following method: The assay mixture contained 0.5 μmol of substrate, 50 mM potassium phosphate buffer (pH 6.3), and 5—50 μl of enzyme solution in a final volume of 1.0 ml. The mixture was incubated at 37 °C for 10—30 min and the reaction was stopped by adding 0.25 ml of 5% Na_2CO_3. The enzyme activities were measured at 565 and 405 nm for hydrolysis of PPG and *p*NPG, respectively. The reaction rates were calculated by using calibration lines for phenolphthalein and *p*-nitrophenol, respectively.

Determination of Acetic Acid——*Eubacterium* sp. precultured at 37 °C for 48 h was inoculated into 10 ml of peptone yeast extract Fildes broth containing glucose and cultured at 37 °C for 4 d. An aliquot of the culture broth was acidified with 1/10 volume of 10 N HCl and then extracted with a half volume of ether. An aliquot (3 μl) of the extract treated with Na_2SO_4 was injected into a GC column (4 mm i.d. × 2 m) of Diasolid L-1 (Nippon Chromat. Co., 60—80 mesh) coated with 10% SP-1200 and 1% H_3PO_4. GC was carried out with a linear increase of column temperature from 100 to 160 °C (8 °C/min).

Identification of β-D-Glucuronic Acid by High Performance Liquid Chromatography (HPLC)——Enzyme solution from the octyl-Sepharose column was mixed with 60 μg of glycyrrhizin in 80 μl of 10 mM Tris–HCl buffer (pH 7.4) and then incubated at 37 °C for 1 h. A 50 μl aliquot of the supernatant fraction obtained by centrifugation at 6000 rpm for 20 min was applied to a Synchropack AX-300 column (250 × 4.1 i.d.) on a Gilson HPLC system. The column was eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.5) at a flow rate of 1 ml/min and monitored at 225 nm.

Determination of Molecular Weight of GL β-D-Glucuronidase——The molecular weight of the enzyme was estimated by Sephadex G-200 column chromatography according to the method of Andrews[14] using phenol red, ovalbumin, bovine serum alubmin, rabbit muscle fructose-1,6-bisphosphate aldolase, and blue dextran as molecular weight markers.

Results

Isolation and Characterization of a Human Intestinal Bacterium Capable of Hydrolyzing GL into GA

From several hundred colonies, which were formed on GAM agar inoculated with a diluted suspension of feces, no GL-hydrolyzing bacteria were isolated. However, a bacterium hydrolyzing GL, strain A-1, was isolated by repeated colonization of a bacterial suspension capable of hydrolyzing GL. This bacterium in GAM medium converted GL completely to GA. The strain was identified as the genus *Eubacterium*; it was strictly anaerobic, gram-

Table I. Fermentation Reactions of a GL-Metabolizing Strain Isolated from Human Feces

Glycerol	−	Rhamnose	+	Raffinose	−
Adonitol	−	Glucose	+	Starch pH	−
Erythritol	−	Mannose	−	Esculin pH	−
Inositol	−	Fructose	+	Salicin	w
Mannitol	−	Galactose	+	Amygdalin	−
Arabinose	+	Sucrose	−	Starch hyd.	−
Xylose	+	Maltose	+	Esculin hyd.	+

Symbols: −, negative; w, weakly positive; +, positive reaction.

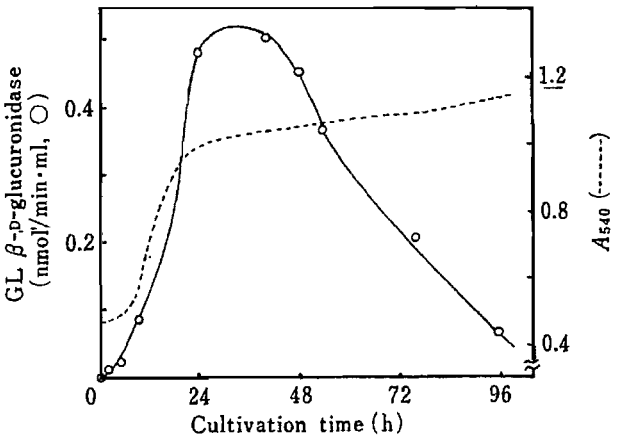

Fig. 1. GL-Hydrolyzing Activity of *Eubacterium* sp. Cultured in GAM Medium

Eubacterium sp. was inoculated into GAM medium and precultured for 48 h at 37 °C in an anaerobic box. The bacterial suspension was added to 9 vol. of GAM medium, and cell growth was monitored by measuring the absorbance at 540 nm. An aliquot of the medium (10 ml) taken from the culture bottle at each sampling time was centrifuged at 6000×*g* for 10 min and the precipitate was suspended in 1 ml of 20 mM potassium phosphate buffer (pH 7.2) and then sonicated. GL-hydrolyzing activities were measured as described in Materials and Methods.

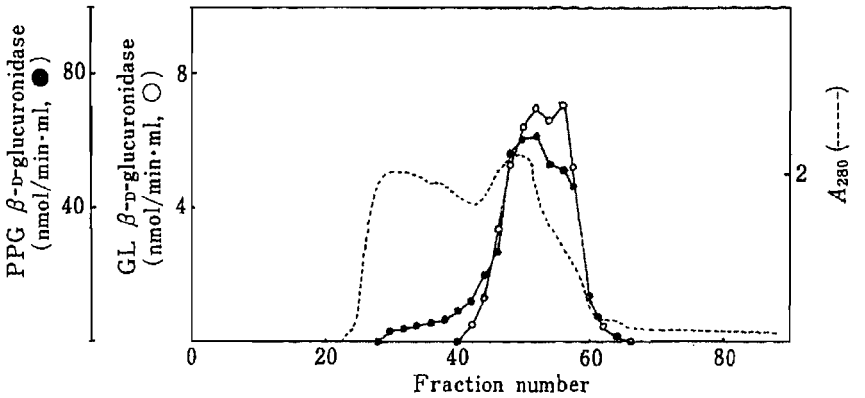

Fig. 2. Sephadex G-200 Gel Filtration of GL β-D-Glucuronidase and PPG β-D-Glucuronidase

Fractions of 3 ml were collected. GL- and PPG-hydrolyzing activities were measured as described in Materials and Methods.

positive and rod-shaped, showed sugar fermentation activities as listed in Table I and produced acetic acid from glucose. The strain showed similar characteristics to *Eubacterium contorium*, but the species was not identified.

Culture and GL-Hydrolyzing Activity of the Isolated Bacterium

The bacterial culture reached the stationary phase at 24 h after inoculation as shown in Fig. 1, indicating that this bacterium grow very slowly. GL-hydrolyzing activity increased with cell growth, continued to increase even after the stationary phase, reached the maximal activity 36 h after inoculation, and then decreased gradually.

Purification of GL β-D-Glucuronidase

Eubacterium grown for 37 h in 5 l of GAM medium under anaerobic conditions was used as a starting material for enzyme purification. All fractionation steps were carried out at 0—4 °C. Bacteria collected were disrupted with a French press (Ohtake Factory Co.) and then centrifuged at 12000×*g* for 20 min. Most of the GL-hydrolyzing activity was recovered in the supernatant fraction. It was fractionated with ammonium sulfate of 35—60% saturation. The precipitate obtained was dissolved in 20 mM potassium phosphate buffer (pH 7.2) and passed through a column of Sephadex G-200 (2.2×80 cm, Pharmacia Fine Chemicals) equilibrated with 10 mM glycine–NaOH buffer (pH 8.5). β-D-Glucuronidase activities toward GL and PPG were eluted in the same fractions as two peaks. The latter peak showed a molecular weight of about 65 kilodaltons (Fig. 2). When the diluted enzyme solution

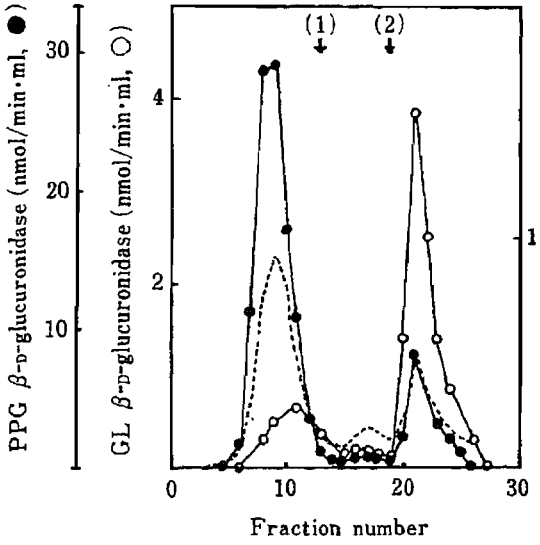

Fig. 3. Octyl-Sepharose Column Chromatography of GL β-D-Glucuronidase and PPG β-D-Glucuronidase

An enzyme solution from Sephadex G-200 column chromatography was applied as described in Results. After being washed with 10 ml of 10 mM glycine–NaOH buffer (pH 8.5) containing 50 mM NaCl, the column was eluted with 6 ml of 10 mM glycine–NaOH buffer (pH 8.5) containing 10 mM NaCl (arrow 1) and then 20 ml of 10 mM glycine–NaOH buffer (pH 8.5) (arrow 2). Fractions of 2 ml each were collected. Hydrolyzing activities toward PPG, *p*NPG, MUG and PNG showed almost the same elution profile. One-half of the GA mono-β-D-glucuronide-hydrolyzing activity was eluted in the same fractions and one-half in the fractions containing GL β-D-glucuronidase.

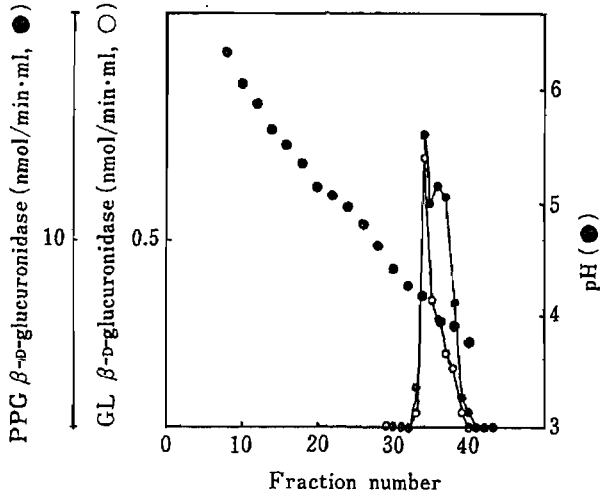

Fig. 4. Isoelectric Focusing Column Chromatography of GL β-D-Glucuronidase and PPG β-D-Glucuronidase

The supernatant fraction obtained from disrupted bacteria was applied to a column containing Ampholine (LKB, pH 4—6). The column was electrophoresed at 700 V for 2 d. After electrophoresis, 2 ml fractions were collected.

was applied to the column, the former peak of these hydrolyzing activities decreased or disappeared, suggesting the presence of monomer and dimer of the enzyme(s).

Fractions containing these glucuronidase activities were collected and applied to a column of octyl-Sepharose (1.5 × 7.5 cm, Pharmacia Fine Chemicals) equilibrated with 10 mM glycine–NaOH buffer (pH 8.5) containing 50 mM NaCl. A large amount of PPG-hydrolyzing activity passed through the column with the same buffer containing 50 mM NaCl without absorption on the gel, though a little GL-hydrolyzing activity remained. By rechromatography of this void fraction, PPG-hydrolyzing activity was obtained virtually without contamination by GL-hydrolyzing activity. Most of the GL-hydrolyzing activity was eluted with the same buffer, together with a small amount of PPG-hydrolyzing activity (Fig. 3). The PPG-hydrolyzing activity and GL β-D-glucuronidase were eluted as separate but partly mutually contaminated peaks in other preparations. *p*NPG-hydrolyzing activity showed almost the same elution profile as PPG-hydrolyzing activity. The result showed that GL- and PPG-hydrolyzing activities are due to different enzymes, and *Eubacterium* produced two kinds of β-D-glucuronidases having a similar molecular weight, GL β-D-glucuronidase and β-D-glucuronidase (EC 3.2.1.31). In these steps of purification, GL β-D-glucuronidase was purified 6-fold with a yield of 16% (specific activity of the enzyme, 12 nmol/min·mg of protein).

Properties of GL β-D-Glucuronidase

As shown in Fig. 4, GL- and PPG-β-D-glucuronidases had the same pI value of 4.1, though another shoulder of PPG-hydrolyzing activity was observed at a lower pI region.

Accordingly, these two enzymes were not separable by diethylaminoethyl (DEAE)-cellulose column chromatography.

The pH optimum of GL β-D-glucuronidase was determined to be 5.6 and K_m for GL was 0.11 mM under the standard assay conditions. After hydrolysis of GL with the enzyme, GA was detected on a thin layer plate, but GA mono-β-D-glucuronide was not detected. D-Glucuronic acid was detected by HPLC using an anion-exchange column, but diglucuronic acid was not detected. Therefore, it was not clear whether GL was converted to GA and D-glucuronic acid *via* GA and diglucuronic acid or *via* GA monoglucuronide and glucuronic acid.

The enzyme was able to hydrolyze GA mono-β-D-glucuronide twice as fast as GL. It could not be concluded that GL β-D-glucuronidase does not hydrolyze PPG and *p*NPG, because the enzyme preparation still contained β-D-glucuronidase activity (EC 3.2.1.31).

Effects of Various Reagents on GL β-D-Glucuronidase

The enzyme was inactivated by trypsin digestion (1 mg/ml, a 25 °C for 30 min) and by heating (at 100 °C for 1 min). Powerful inhibition was obtained with D-saccharic acid 1,4-lactone (2.5×10^{-3} M). Sulfhydryl reagents such as *p*-chloromercuribenzoic acid (1.0×10^{-4} M) and 5,5′-dithio-bis(2-nitrobenzoic acid) (1.0×10^{-3} M) were found to inhibit completely the enzyme activity. On the other hand, diisopropylfluorophosphate had no effect on the enzyme activity. These results suggest that GL β-D-glucuronidase has sulfhydryl group(s) but not reactive hydroxyl group(s) in its active site.

Discussion

Eubacterium sp. isolated from human feces contained two kinds of β-D-glucuronidases as shown in the elution profile of octyl-Sepharose column chromatography (Fig. 3). One was β-D-glucuronidase (EC 3.2.1.31) showing a substrate specificity similar to that reported hitherto. Namely, the enzyme hydrolyzed PPG, *p*NPG, MUG and PNG, but not GL. The other was a new type of β-D-glucuronidase, GL β-D-glucuronidase. This enzyme could hydrolyzed GL and GA mono-β-D-glucuronide to GA. However, it was not clear whether or not the enzyme was able to hydrolyze PPG, *p*NPG, MUG and PNG. These two glucuronidases showed similar physical properties, such as isoelectric point (4.1) and molecular weight (65000), and had the same susceptibility to various reagents, except for the affinity to octyl-Sepharose gel. However, the molecular weight of these enzymes was different from those of β-D-glucuronidases from rat liver lysosomes (280000, 310000),[7,10] rat liver microsomes (310000, 290000),[10,11] female rat preputial gland (320000),[9] bovine liver (290000),[8] human placenta (310000)[12] and GL hydrolase from *Aspergillus niger* (150000).[13] Further, the isoelectric point of the present enzymes was more acidic than those of β-D-glucuronidases from rat liver lysosomes (5.8—6.6),[10] rat liver microsomes (6.9—7.6, 6.7),[10,11] female rat preputial gland (6.15, 6.7 and 6.8),[6,9] bovine liver (5.1)[8] and GL hydrolase from *Aspergillus niger* (about 6).[13]

Human intestinal flora metabolized GL to GA and 3-*epi*-GA.[1] Twelve strains isolated from human intestine did not hydrolyze GL except for a weak hydrolysis of GL by *Peptostreptococcus intermedius*.[15] We found that the newly isolated *Eubacterium* sp. could convert GL to GA. *Ruminococcus* sp. isolated from human feces was also capable to hydrolyzing GL to GA.[15] However, there seem to be very few strains capable of hydrolyzing GL in human intestinal flora in comparison with the numerous strains capable of hydrolyzing monoglucuronides of phenolic compounds. Other bacterial strains hydrolyzing GL were not isolated from human feces although we surveyed more than five hundred colonies. Accordingly, GL glucuronidase may be rare, whereas β-D-glucuronidase (EC 3.2.1.31) seems to be abundant in intestinal bacteria. In fact, β-D-glucuronidases of *Escherichia coli* purchased

from Sigma Chemical Co. and of *Bacteroides clostridiiformis* isolated from rat feces in our laboratory could hydrolyze PPG and *p*NPG but not GL (data not shown). *Ruminococcus* sp. capable of hydrolyzing GL also had GL β-D-glucuronidase and β-D-glucuronidase (EC 3.2.1.31) activities, like *Eubacterium* sp. (unpublished data).

Although β-D-glucuronidase of bovine liver purchased from Sigma Chemical Co. could hydrolyzed GL to GA at less than one-hundredth of the rate of hydrolysis of *p*NPG (data not shown), it was not clear whether the enzyme preparation contained GL β-D-glucuronidase in addition to β-D-glucuronidase (EC 3.2.1.31). GL hydrolase purified from *Aspergillus niger*[13] could hydrolyze GL but not PPG or *p*NPG. That enzyme is different from our enzyme, judging from the molecular weight and the isoelectric point.

The absence of GL-hydrolyzing activity with rat liver, and the high activity in rat intestinal flora (unpublished data), means that intestinal bacteria play an important role in GL metabolism, suggesting that intestinal flora may contribute greatly to GL metabolism in humans, too.

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Purification and Characterization of Sarcosine Oxidase of *Bacillus* Origin

YASUCHIKA MATSUDA,*[,a] HIDEMI HOSHIKA,[a] YOSHIO INOUYE,[a] SHIGERU IKUTA,[b] KAZUO MATSUURA[b] and SHOSHIRO NAKAMURA[a]

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[a] 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan and Research Laboratory, Toyo Jozo Co., Ltd.,[b] 632-1 Mifuku, Ohito, Shizuoka 410-23, Japan

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Sarcosine oxidase (EC 1.5.3.1) produced by *Bacillus* sp. B-0618 was purified by ion exchange chromatography on diethyl aminoethyl-cellulose and gel filtration on Sephadex G-100 and G-150. The molecular weight of the enzyme was estimated to be 42000 by gel filtration on Sephadex G-150 and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme exhibited an absorption spectrum characteristic of flavoprotein. The enzyme showed the maximum activity at pH 8.5—9 and was stable at pH 7—10. The pI value was 4.7 as determined by isoelectric focusing. Although sarcosine is the preferred substrate, the enzyme also oxidized *N*-methyl-DL-alanine, *N*-methyl-L-leucine and *N*-methyl-DL-valine to lesser extents. The apparent K_m values of sarcosine, *N*-methyl-DL-alanine, *N*-methyl-L-leucine and *N*-methyl-DL-valine were 12.2, 6.8, 106 and 173 mM, respectively. The enzyme was inactivated by *N*-bromosuccinimide, Zn^{2+}, Fe^{3+} and Hg^{2+}, but not by ethylenediaminetetraacetate, *p*-chloromercuribenzoate, monoiodoacetate or *p*-toluenesulfonyl-chloride.

Keywords——sarcosine oxidase (EC 1.5.3.1); sarcosine; *Bacillus*; clinical diagnosis; flavoprotein

The Folin method[1] is most commonly used in the clinical diagnostic analysis of creatinine in serum and urine. However, this method has the disadvantages of poor specificity and requirement for deproteinization. This is the reason why enzymatic measurement systems[2-5] are gradually replacing the Folin method. Some of the enzymatic systems consist of creatinine amidohydrolase (creatininase, EC 3.5.2.10), creatine amidinohydrolase (creatinase, EC 3.5.3.3) and sarcosine oxidase (EC 1.5.3.1). The first one catalyzes interconversion between creatinine and creatine, the second hydrolyzes creatine to sarcosine and urea and the last further hydrolyzes sarcosine to glycine and formaldehyde, generating hydrogen peroxide. Formaldehyde can be determined by using formaldehyde dehydrogenase, on one hand, and peroxidase can be conveniently used for the determination of hydrogen peroxide, on the other hand.

We have already reported the purification and characterization of creatininase and creatinase of *Alkaligenes* origin.[6,7] Recently, we observed the high yield production of sarcosine oxidase by strain B-0618. As mentioned above, the enzyme might complement two other creatinine-catabolic enzymes, creatininase and creatinase, in the determination of creatinine and/or creatine in clinical samples. The details of the purification and characterization of sarcosine oxidase produced by strain B-0618 and the taxonomy of the producing organism are presented in this paper.

Materials and Methods

Materials——Horseradish peroxidase was obtained from Wako Chemical Ind., Ltd. Formaldehyde dehy-

drogenase was a product of Oriental Yeast Co., Ltd. Sephadex G-100 and G-150 were purchased from Pharmacia Fine Chemicals. *N*-Methyl-L-leucine, *N*-methyl-DL-valine and *N*-methyl-DL-alanine were obtained from Sigma Chemical Co., Ltd. All other materials were commercial products of analytical grade.

Cultivation of Strain B-0618——Strain B-0618 was cultured in 500 ml Erlenmeyer flasks containing 100 ml of a medium composed of 0.5% creatine, 0.5% yeast extract, 0.5% fish meat extract, 0.3% KCl, 0.2% $(NH_4)_2HPO_4$ and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7.0) at 30 °C for 20 h.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Assay of Sarcosine Oxidase——Assay Method I: A mixture of an enzyme solution (0.1 ml) and 0.1 M Tris–HCl buffer (pH 8.5, 0.9 ml) containing 0.27 mmol of sarcosine was incubated at 37 °C for 10 min. The reaction was terminated by adding 0.5 N acetic acid (0.5 ml). Formaldehyde formed was measured by the method of Nash.[9] Briefly, the reaction mixture (1.5 ml) was further incubated with 10% ammonium acetate (3 ml) containing 0.02% acetylacetone at 37 °C for 40 min and the absorbance at 410 nm was read against the blank.

Assay Method II: Hydrogen peroxide formed by the enzyme reaction was measured by following the formation of quinone imine dye in the presence of 4-aminoantipyrine, phenol and peroxidase. An enzyme solution (0.05 ml) was incubated at 37 °C for 10 min with a mixture of 0.2 M Tris–HCl (pH 8.5, 0.3 ml), 0.9 M sarcosine (0.3 ml), 30 mM 4-aminoantipyrine (0.25 ml), 1.5% phenol (0.05 ml), and 150 units/ml horseradish peroxidase (0.05 ml). The reaction was terminated by adding ethanol (1 ml) and the absorbance at 480 nm was read against the blank.

In both methods, one unit was defined as the amount of enzyme which catalyzed the oxidation of 1 μmol of substrate per min under the conditions described above. Although assay method II took less time than assay method I, the former could not be used when the direct effect on the sarcosine oxidase activity was measrued because the system consisted of two enzymes.

Electrophoresis——Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed in 10% polyacrylamide according to the method of Weber and Osborn.[10] Bovine serum albumin (MW 68000), α-chymotrypsinogen (MW 25700) and lysozyme (MW 14300) were used as references to determine the molecular weight of the enzyme.

Polyacrylamide disc gel electrophoresis was performed according to the method of Williams and Reisfeld.[11] Specific staining of sarcosine oxidase activity in the gel was carried out by diformazane formation as follows. After electrophoresis, the gel was soaked in 0.1 M Tris–HCl buffer (pH 8.5, 10 ml) containing 7 mg of nicotinamide adenine dinucleotide, 3.5 mg of nitrotetrazolium blue, 0.2 mg of phenazine methosulfate, 15 units of formaldehyde dehydrogenase and 2 mmol of substrate at 37 °C for 1 h in the dark.

Isoelectric focusing was done with carrier ampholite giving the pH gradient of 3 to 10 according to the method of Vesterberg.[12]

Results

Taxonomic Studies of Strain B-0618

Strain B-0618 was isolated from a soil sample collected in Fukuchiyama, Kyoto. Morphological and physiological characteristics of strain B-0618 are summarized in Tables I and II, and the results for the cleavage of carbohydrates are shown in Table III.

By consulting "Bergey's Manual of Determinative Bacteriology,"[13] this strain was classified in genus *Bacillus*. Further, *Bacillus badius, B. freudenreichii* and *B. macroides* were selected as the species most resembling strain B-0618. On the basis of items such as urease production, esculin hydrolysis, production of hydrogen sulfide and acid formation from fructose, glycerin and mannitol, *B. badius* was excluded as a candidate. In addition, the properties of *B. macroides* were not compatible with those of strain B-0168 in gelatin hydrolysis, oxidase and urease production, esculin hydrolysis, production of hydrogen sulfide and acid formation from fructose, glycerin and mannitol. *B. freudenreichii* was the most similar species, though it was differentiated from strain B-0618 in the following items: esculin hydrolysis, production of hydrogen sulfide and acid formation from fructose, glycerin and mannitol.

Purification of Sarcosine Oxidase

All procedures were carried out at 4 °C unless otherwise specified. The mycelial cake (12 g) was washed with 10 mM phosphate buffer (pH 7.0), suspended in the same buffer (100 ml) containing 2 mM ethylenediamine tetraacetate (EDTA) and then digested with 4200

TABLE I. Morphological Characteristics of Strain B-0618

	Characteristics
Vegetative cells	
Shape	Rods with round ends
Size	1.0—1.5 × 2.0—5.0 μm
Motility	Positive
Flagella	Peritrichous
Spores	
Shape	Column or ellipitical
Size	0.8—1.0 × 1.2—1.6 μm
Position	Terminal or central
Gram-strain	Positive
Acid-fast	Negative

TABLE II. Physiological Characteristics of Strain B-0618

	Characteristics
Reduction of nitrate	Negative
Denitrification	Negative
MR test	Negative
Voges-Proskauer reaction	Negative
Indole formation	Negative
Hydrogen sulfide formation	Positive
Hydrolysis of starch	Negative
Hydrolysis of gelatin	Positive
Hydrolysis of casein	Negative
Hydrolysis of esculin	Negative
Hydrolysis of cellulose	Negative
Utilization of citrate	
Simons medium	Negative
Christensen medium	Positive
Utilization of inorganic nitrogen source	
Nitrate nitrogen	Positive
Ammonium nitrogen	Negative
Catalase reaction	Positive
Oxidase reaction	Positive
Urease reaction	Positive
Temperature range for growth	10—42 °C
pH range for growth	pH 6.4—9.6

TABLE III. Cleavage of Carbohydrate by Strain B-0618

Carbon source	Acid formation	Gas formation
Fructose	Positive	Negative
Galactose	Negative	Negative
Glucose	Negative	Negative
Glycerol	Positive	Negative
Inositol	Negative	Negative
Lactose	Negative	Negative
Maltose	Negative	Negative
Mannitol	Positive	Negative
Mannose	Negative	Negative
Sorbitol	Negative	Negative
Starch	Negative	Negative
Sucrose	Negative	Negative
Xylose	Negative	Negative

units/ml lysozyme at 37 °C for 30 min. The supernatant (97 ml) was obtained by centrifugation at 3000 × *g* for 15 min. Two percent aqueous protamine sulfate (2.5 ml) was added to this supernatant with stirring. After centrifugation at 3000 × *g* for 15 min, the supernatant (96 ml) was obtained. The precipitate with ammonium sulfate at 50—70% saturation was collected by centrifugation at 3000 × *g* for 15 min, and dissolved in 10 mM Tris-HCl buffer (pH 8.0, 20 ml). After desalting by Sephadex G-25 column chromatography, the crude enzyme solution was charged on a column of diethyl aminoethyl (DEAE)-cellulose (2.0 × 18 cm) equilibrated with 10 mM Tris-HCl buffer. The column was thoroughly washed with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl, and the enzyme was eluted with a linear gradient of KCl from 0.1

to 0.5 M in the same buffer. The partially purified enzyme from DEAE-cellulose column chromatography was concentrated to 5 ml by ultrafiltration and passed through columns of Sephadex G-100 (2.7 × 50 cm) with 10 mM Tris–HCl buffer (pH 8.0) and Sephadex G-150 (1.6 × 85 cm) with the same buffer containing 0.1 M NaCl, in that order. The purified enzyme was dialyzed against 0.01 M Tris–HCl buffer (pH 8.0) for 15 h and stored in a frozen state for further characterization. The purification of the enzyme is summarized in Table IV.

The enzyme was purified 25.6-fold in terms of specific activity, recovering 20.7% of the original activity. The purified enzyme showed a single major band and a trace amount of two minor bands on SDS–polyacrylamide gel electrophoresis (Fig. 1). The position of the major band in gel showed fluorescence under ultraviolet illumination (360 nm).

Analysis of Sarcosine Metabolites

The metabolites of sarcosine formed by this enzyme were analyzed. Sarcosine (90 mmol, 0.9 ml) was incubated with the enzyme solution (0.4 unit, 0.1 ml) at 37 °C for 10 min and the reaction mixture was subjected to thin layer chromatography on Kieselgel 60 F_{254} (Merck) developed with a solvent system of phenol–ethanol–water (14 : 4 : 1). The chromatogram showed two ninhydrin-positive spots with *Rf* values identical with those of sarcosine and glycine.

The consumption of oxygen was measured at 37 °C with a Clark type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) and the formation of formaldehyde and hydrogen peroxide, which could be converted stoichiometrically to formaldehyde in the presence of catalase and methanol,[14] was determined by the method of Nash.[9] Table V shows that the molarity of oxygen consumed is equivalent to those of formaldehyde and hydrogen peroxide produced. From these results, it was concluded that this enzyme converts sarcosine to glycine and formaldehyde, generating hydrogen peroxide from molecular oxygen in air.

TABLE IV. Purification of Sarcosine Oxidase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Lysozyme-digested cell sup.	980	1400	1.4	100
Protamine sulfate sup.	610	1190	2.0	85
Ammonium sulfate ppt.	240	970	4.0	69
DEAE-cellulose	43	540	13	39
Sephadex G-100	32	485	15	35
Sephadex G-150	8.1	290	36	21

Fig. 1. SDS–Polyacrylamide Gel Electrophorogram of Purified Sarcosine Oxidase

The enzyme (75 μg as protein) was subjected to electrophoresis at a constant current of 10 mA per gel for 4 h, and then the gel was stained with 0.25% Coomassie Brilliant Blue R-250.

TABLE V. Stoichiometry of Sarcosine Oxidase Reaction

Oxygen consumed	0.440 μmol
Formaldehyde formed	0.448 μmol
Hydrogen peroxide formed	0.420 μmol

The reaction mixture (2.925 ml), containing 810 μmol of sarcosine in 0.1 M Tris–HCl buffer (pH 8.5) (further 2800 units of catalase and 0.18 ml of methanol in the case of determination of hydrogen peroxide), was incubated at 37 °C for 10 min with 0.075 ml of sarcosine oxidase (0.6 unit/ml). Formaldehyde formed was measured by the method of Nash.[9] For the determination of hydrogen peroxide, it can be converted stoichiometrically to formaldehyde in the presence of catalase and methanol, and the molarity of hydrogen peroxide was calculated from the molarities of formaldehyde estimated in the presence and absence of catalase and methanol.

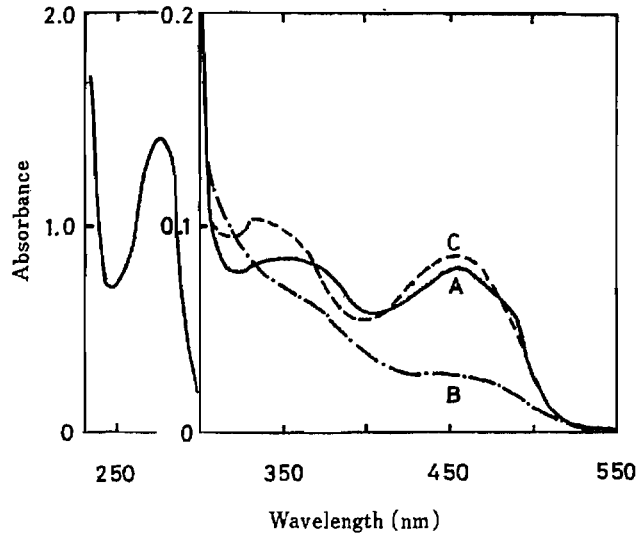

Fig. 2. Absorption Spectra of Native, Reduced and Pronase-Digested Sarcosine Oxidase

Curve A: The native enzyme (1.1 mg) in 0.05 M phosphate buffer (pH 8.0, 2 ml). Curve B: The enzyme (1.1 mg) treated with 0.2 mmol of sarcosine in the same buffer (2 ml) under anaerobic conditions. Curve C: The enzyme (1.1 mg) digested with 4 mg/ml pronase in the same buffer (2 ml) at 37 °C for 16 h.

Prosthetic Groups of Sarcosine Oxidase

The enzyme showed a characteristic absorption spectrum of flavoprotein with absorption maxima at 276, 360 and 453 nm (Fig. 2A). When sarcosine was added to the enzyme solution under anaerobic conditions, the peaks at 360 and 453 nm disappeared (Fig. 2B). The enzyme solution was kept in a boiling water bath for 5 min in the presence of 10% trichloroacetic acid. The supernatant obtained by centrifugation did not contain any colored material. However, the enzyme digested by pronase released a yellowish material in the supernatant. The absorption spectrum shows peaks at 333 and 450 nm (Fig. 2C), suggesting the existence of covalently bound flavin.

Estimation of Molecular Weight

By both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-150, the molecular weight of the enzyme was estimated to be 42000.

Isoelectric Point of Sarcosine Oxidase

The pI value was determined to be 4.7 by isoelectric focusing.

Effects of pH on Sarcosine Oxidase Activity and Stability

The effects of pH on the enzyme activity and stability were tested by assay method I. The optimum activity was observed at pH 8.5—9. Although the enzyme was stable at pH 7—10, it seemed especially labile at pH below 5.

Effects of Various Chemicals and Metal Ions

The sarcosine oxidase activity was measured by assay method I. The enzyme was inactivated by *N*-bromosuccinimide, Zn^{2+}, Fe^{3+}, and Hg^{2+}, but not by *p*-chloromercuribenzoate (PCMB), EDTA or *p*-toluenesulfonylchloride (Tables VI and VII).

Substrate Specificity and Kinetics

The relative rates of the oxidation of various substrates by the enzyme were measured by assay method II. The enzyme was active toward sarcosine, *N*-methyl-DL-alanine, *N*-methyl-DL-valine and *N*-methyl-L-leucine, but completely inactive toward amino acids, *β*-alanine, *N*-methylethanolamine, *N*,*N*-dimethylethanolamine, choline, betaine, *N*-methylhydantoin, 2-methylalanine, 1,3-dimethylurea, 1-methylguanidine, methoxyacetate, creatine and creatinine. The apparent K_m and relative V_{max} values of *N*-methylamino acids were estimated from Lineweaver-Burk plots[15] and the results are shown in Table VIII.

TABLE VI. Effects of Various Chemicals on Sarcosine Oxidase Activity

Chemical	Residual activity (%) 1 mM	10 mM
None	100	100
Ethylenediaminetetraacetate	100	99
8-Hydroxyquinoline	94	98
o-Phenanthroline hydrochloride	95	93
α,α'-Dipyridyl	100	99
p-Chloromercuribenzoate	93	NT
Monoiodeacetate	100	95
Glutathione (oxidized)	104	107
5,5'-Dithio-bis(2-nitrobenzoate)	97	94
N-Ethylmaleimide	91	94
p-Toluenesulfonylchloride	103	99
Diisopropyl fluorophosphate	100	95
Phenylmethanesulfonyl fluoride	102	103
N-Bromosuccinimide	0	NT
Glutathione (reduced)	108	93
Dithiothreitol	102	68
2-Mercaptoethanol	101	85
Hydroxylamine hydrochloride	84	0
Sodium dodecyl sulfate	72	0

A mixture of 0.2 ml of enzyme solution (0.7 unit/ml) in 0.1 M Tris–HCl buffer (pH 8.5) and 0.2 ml of chemical (2 or 20 mM) solution in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I. NT: not tested.

TABLE VII. Effects of Various Metal Ions on Sarcosine Oxidase Activity

Metal salt (1 mM)	Residual activity (%)
None	100
$ZnSO_4$	12
$MnCl_2$	101
$CoCl_2$	91
$NiCl_2$	94
$FeCl_3$	39
$FeSO_4$	69
$CuSO_4$	98
$MgSO_4$	98
$CaCl_2$	95
$HgCl_2$	23

A mixture of 0.2 ml of enzyme solution (0.7 unit/ml) in 0.1 M Tris–HCl buffer (pH 8.5) and 0.2 ml of metal salt (2 mM) solution in the same buffer was incubated at 37 °C for 30 min and the residual activity was measured by assay method I.

TABLE VIII. Kinetic Parameters of Sarcosine Oxidase

Substrate	K_m (mM) (*a*)	Relative V_{max} (%) (*b*)	*b*/*a*
Sarcosine	12.2	100	8.2
N-Methyl-DL-alanine	6.8	14	2.0
N-Methyl-L-leucine	106	31	0.29
N-Methyl-DL-valine	173	40	0.23

The K_m and relative V_{max} values were calculated by assay method I, using a reaction mixture composed of 0.9 ml substrate solution (10—300 mM) in 0.1 M Tris–HCl buffer (pH 8.5) and 0.1 ml of aqueous enzyme solution (0.5, 2.0, 1.5 or 1.5 unit/ml).

On polyacrylamide gel electrophoresis, the enzymatic staining gave a single band with the same mobility for each *N*-methylamino acid substrate. Furthermore, the mobilities of the diformazan bands were the same as that of the major protein band. Among the above-mentioned chemicals, only β-alanine inhibited sarcosine oxidase, and the type of inhibition was characterized as partially non-competitive.

Discussion

To date, sarcosine oxidase from *Corynebacterium* sp. U-96[16] and *Cylindrocarpon didymum* M-1[17] have been reported. The former was found to have a molecular weight of 174000 and be composed of four non-identical subunits, and the latter was reported to be a monomer with a molecular weight of 45000. Both enzymes were inactivated by PCMB and monoiodoacetate. However, sarcosine oxidase from strain B-0618 is a monomer with a molecular weight of 42000 and is not inactivated by PCMB, monoiodoacetate or *N*-ethylmaleimide. Further, the K_m values of the enzymes from Corynebacterium, Cylindrocarpon and strain B-0618 for sarcosine were 3.4, 1.8 and 12.2 mM, respectively, and the corresponding specific activities were 8.93, 27.6 and 35.8 units/mg protein.

Sarcosine oxidase from strain B-0618 was active toward *N*-methyl-DL-alanine, *N*-methyl-L-leucine and *N*-methyl-DL-valine as well as sarcosine. On polyacrylamide gel electrophoresis, the mobilities of the diformazan band which showed the position of enzyme activity were identical for all above-mentioned substrates. Further, the diformazan band had the same mobility as the major protein band. This implies that the oxidation of sarcosine, *N*-methyl-DL-alanine, *N*-methyl-L-leucine, *N*-methyl-DL-valine is catalyzed by the same component, although the enzyme preparation still contained a trace amount of minor components.

It was reported that *N*-methyl-L-amino acid oxidase (EC 1.5.3.2) of rabbit kidney catalyzed the oxidation of *N*-methyl-L-amino acid to formaldehyde and L-amino acid, generating hydrogen peroxide with consumption of oxygen, but is not active toward sarcosine.[18] In this respect, sarcosine oxidase of strain B-0618 is distinguished from *N*-methyl-L-amino acid oxidase of rabbit kidney.

The sarcosine oxidase of *Bacillus* origin should be useful in the enzymatic determination of creatinine and/or creatine in clinical samples in combination with creatininase and/or creatinase, and a study aimed at the clinical application of the enzymes is in progress in our laboratory.

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A Physiologically Based Pharmacokinetic Model for Biperiden in Animals and Its Extrapolation to Humans

EMI NAKASHIMA,[a] KOICHI YOKOGAWA,[a] FUJIO ICHIMURA,*[,a] KOUICHI KURATA,[b] HIDEKI KIDO,[b] NARIYOSHI YAMAGUCHI,[b] and TSUKINAKA YAMANA[a]

Hospital Pharmacy,[a] and Department of Neuropsychiatry,[b] School of Medicine, Kanazawa University, Takara-machi, Kanazawa 920, Japan

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The disposition characteristics of biperiden were investigated in rats, rabbits, beagles, and humans, and a physiologically based pharmacokinetic model was established by using the hepatic intrinsic clearance of unbound drug concentration and the tissue-to-plasma unbound concentration ratios. Protein-binding parameters and blood-to-plasma concentration ratios were determined, and linear parameters were obtained in beagles and humans over a wide concentration range. The hepatic intrinsic clearance of humans was predicted from the animal data. The coincidence of each tissue-to-plasma unbound concentration ratio between rats and rabbits was confirmed in the steady state, and the mean tissue-to-plasma unbound concentration ratios were used for the prediction of the plasma concentration–time courses of beagles and humans. The predicted lines fitted the observed plasma concentrations of beagles and a patient well after a single intravenous injection and repeated intramuscular administrations, respectively.

Keywords——biperiden; physiological pharmacokinetics; animal scale up; rat; rabbit; beagle; human; tissue-to-plasma unbound concentration ratio; hepatic intrinsic clearance

Introduction

Biperiden (BP) is an anticholinergic drug applied in the treatment of parkinsonian syndrome.[1] In contrast to the extensive clinical use of BP, knowledge of its pharmacokinetic behavior, metabolism and body distribution in man is still limited. Only two reports of BP serum concentration in man after single oral administration have been made to date,[2,3] while no data are available on serum concentration after repeated parenteral administration of BP in man.

The characteristics of BP pharmacokinetics were thought to be large distribution volume and high plasma clearance in rabbits.[4,5] In order to predict the plasma concentration in humans from limited data, it is important to use animal data to establish a suitable pharmacokinetic model of BP.

Physiologically based pharmacokinetics have the advantage of interspecies scalability. For the prediction of the intrinsic clearance of an unbound drug in humans, the function of body size of animals was reported to be useful.[6] Moreover, it has been claimed that the tissue-to-plasma unbound concentration ratios in animals are important to predict the concentrations of several drugs in human plasma.[7,8]

The purpose of this study was to establish the physiologically based pharmacokinetics of BP by using the hepatic intrinsic clearance of unbound drug and the tissue-to-plasma unbound concentrations. To predict the human plasma concentrations after repeated administrations of BP, the animal data were examined, and the possibilities of animal scale-up are discussed.

Materials and Methods

Materials——Biperiden was used as supplied by Dainippon Pharmaceutical Co., Osaka, Japan. Diazepam, which was chosen as the internal reference standard, was kindly supplied by Takeda Pharmaceutical Co., Osaka, Japan. All other chemicals were of reagent grade and were used without further purification.

Determination of Serum Protein Binding——Venous blood (60 ml) was taken from three normal subjects into disposable polyethylene syringes. Each sample was allowed to stand for 30 min at room temperature and then centrifuged at 1300*g* for 10 min; the serum was stored at 4 °C, and the experiment was finished within 12 h after blood collection. The extent of binding of BP to serum proteins in beagles and humans was measured by the equilibrium dialysis technique using two compartment plates with a 0.8 ml sample volume as described previously.[5] The equilibrium concentration of BP in both the serum and buffer compartments was measured after more than 8 h at 37 °C. The free BP fraction was obtained by calculating the ratio between the concentrations of the drug in the buffer and serum compartments. In this study, since the volume shift between the two compartments was within 6%, it was assumed to be negligible.

Animals and Patient——Male Wistar rats (290±4 g; mean±S.D.), adult male albino rabbits (2.1±0.2 kg; mean±S.D.) and beagles (9.0—13.0 kg) were utilized. A patient (42.3 kg), who was being concomitantly treated with BP and haloperidol, participated in the study.

The pharmacokinetic studies were undertaken as follows. BP was infused over precisely 2 min with an infusion pump *via* the antecubital vein of beagles. Blood samples were withdrawn through a cannula *via* the opposite antecubital vein at specified time intervals after the infusion, and collected in heparinized tubes, and plasma samples were separated. In the case of the patient, 3.88 mg of BP was administered intramuscularly every 8 h. Blood was drawn from the antecubital vein of the patient, allowed to clot for several h and spun to obtain the plasma. The sample was stored in a frozen state at −30 °C until assay.

Tissue Distribution——To determine the tissue-to-plasma partition coefficients at the steady state (K_p), infusion was performed at the rate of 0.765 ml/h (5.32 mg/ml BP saline solution) after intravenous bolus injection of the priming dose (3.2 mg/kg BP saline solution) to rats. At 16 h after the commencement of the infusion studies, the rats were sacrified for tissue sampling. The tissues were quickly excised, rinsed well with ice-cold saline, blotted, and weighed. The procedure for obtaining tissue homogenate was essentially the same as that described previously.[9]

Determination of Blood-to-Plasma Concentration Ratio (*RBP*)——A conventional *in vitro* method was performed as follows. The venous blood was collected from the beagles and humans *via* the antecubital vein into heparinized syringes. Aliquots (0.1 ml) of isotonic buffer solution containing various amounts of BP were added to 5 ml of whole blood. The samples were incubated with slow shaking for 30 min at 37 °C. The concentrations of BP in the plasma after separation by centrifugation and in whole blood were then assayed.

Analytical Procedures——Drug concentrations of BP in plasma were determined by gas liquid chromatography (GLC) assay as described in the preceding paper.[4] In order to detect the low concentration of BP in plasma, 3-fold more plasma was used in the GLC assay. The detection limit of the method was 0.3 ng/ml, and the coefficient of variation was less than 10%. To determine the concentrations in tissues, the same method was applied to the tissue homogenates. Calibration curves were obtained by the same method for each biological sample. The detection limit of the method was 20 ng/ml for the homogenized samples.

Data Analysis——The BP data in beagles were analyzed according to the model-independent moment analysis procedure, as described previously.[5] The data for the patient after repeated administrations were fitted to a three-compartment pharmacokinetic model by using the MULTI computer program.[10] The model prediction by using differential equations was performed using a previously described program[7,9,11] with an appropriate modification according to the equations in "Appendix." A FACOM M360AP digital computer at the Data Processing Center, Kanazawa University, was used.

The measure of the fit between the observed (C_{obs}) and the predicted (C_{pred}) concentrations of BP was based on the coefficient of determination, r^2, calculated from the equation: $r^2 = 1 - \Sigma dev^2/Sy^2$, where $Sy^2 = \Sigma Y_{obs}^2 - (\Sigma Y_{obs})^2/N$, $dev^2 = (Y_{obs} - Y_{pred})^2$, and N represents the number of determinations. In this calculation, the logarithmic values of C_{obs} and C_{pred} were employed as Y_{obs} and Y_{pred}.[12]

Results

Table I shows the volume of distribution in the steady state per body weight (Vd_{ss}/BW) and total body plasma clearance per body weight (CL_{tot}/BW) in animals. The values of Vd_{ss}/BW in animals were in the range of 9.5 to 19.3 l/kg, which indicate that extensive tissue distribution of BP is a common characteristic among the tested animals. The value of Vd_{ss}/BW of beagles was about half that of rabbits. The value of CL_{tot}/BW in the patient was considerably lower than those of rats, rabbits, and beagles. The rank order of CL_{tot}/BW was:

TABLE I. Pharmacokinetic Parameters of Biperiden

Parameters	Rats[a]	Rabbits[b]	Beagles[c]	Patient[d]
Vd_{ss}/BW (l/kg)	14.0±1.4	19.3±3.0	9.5±1.4	6.2
CL_{tot}/BW (ml/min/kg)	67.7±3.0	78.7±3.8	26.7±1.2	15.3

a) Ref. 13. *b*) Ref. 5. *c*) Determined by model-independent moment analysis. Data are presented as the mean±S.D. Calculated from a set of mean plasma concentrations and S.D. at each time after drug administration. *d*) Determined by three-compartment model analysis of the plasma concentration time course after repeated administration of BP.

TABLE II. Physiological and Pharmacokinetic Parameters of Biperiden

Parameters	Rats	Rabbits	Beagles	Humans
f_p[a]	0.110±0.041[d] (16)	0.393±0.04[f] (11)	0.096±0.005 (16)	0.097±0.032 (17)
RBP[b]	1.15±0.06[d] (12)	1.17±0.05[f] (6)	0.97±0.05 (9)	0.95±0.03 (6)
ER[c]	0.910±0.037[d] (4)	0.940±0.038[f] (6)	—	—
$CL_{uint,H}/BW$ (ml/min/kg)	6220[e]	3170[e]	943[g]	176[h]

a) f_p represents the unbound fraction of BP in plasma. *b*) *RBP* represents the blood-to-plasma concentration ratio of BP. *c*) *ER* represents the hepatic extraction ratio. Each value represents the mean±S.D. The number of experiments is given in parentheses. *d*) Ref. 13. *e*) The value of the hepatic intrinsic clearance of unbound drug ($CL_{uint,H}$) was calculated by using Eq. 1. *f*) Ref. 5. *g*) The value of the hepatic intrinsic clearance of unbound drug ($CL_{uint,H}$) was calculated by using Eq. 2. *h*) The value of the hepatic intrinsic clearance of unbound drug ($CL_{uint,H}$) of humans was predicted by using those in animals. See the text for details.

rabbits > rats > beagles > man. Although the values of CL_{tot}/BW in rats and rabbits could be well explained in terms of hepatic blood flow rate-limited elimination, those of beagles and human were about half of the respective blood flow rate.

Constant serum protein binding and *RBP* were observed for BP in beagles and humans over a wide BP concentration range (25—10000 ng/ml). All parameters are given in Table II, together with the reported values in rats[13] and rabbits.[5] Similar *RBP* values were obtained in animals and in humans. The values of the plasma unbound fraction (f_p) in beagles and humans were nearly equal to that of rats, rather than that of rabbits.

The hepatic intrinsic clearance ($CL_{uint,H}$) of BP was calculated by

$$CL_{uint,H} = RBP \cdot Q_1 \cdot ER/(f_p \cdot (1-ER)) \quad (1)$$

where Q_1 and ER are hepatic blood flow rate and hepatic extraction ratio, respectively. The calculated values for rats and rabbits are listed in Table II.

By assuming that the elimination of BP was occurring only in the liver, the hepatic clearance of BP in beagles was calculated by means of the following equation without the determination of *ER*.

$$CL_{uint,H} = RBP \cdot Q_1 \cdot CL_{tot}/(f_p \cdot (RBP \cdot Q_1 - CL_{tot})) \quad (2)$$

The calculated value of $CL_{uint,H}$ in beagles is listed in Table II. In our previous[5] and present experiments on the oral administration of BP in animals, BP was detected in the plasma only in the case of beagles. This may be due to the fact that $CL_{uint,H}$ of beagles was the smallest among the tested animals.

The tissue-to-plasma concentration ratios (K_p) of BP in rabbits were reported pre-

viously.[5] In order to determine the tissue-to-plasma concentration ratio of BP in rats, infusion studies were performed. The values thus obtained for rabbits and rats are listed in Table III. The tissue-to-plasma unbound concentration ratios (K_{pu}) were obtained by dividing the tissue-to-plasma concentration ratio by the value of the serum unbound fraction. The relationship between the K_{pu} values of BP in rats and rabbits is shown in Fig. 1. Although the f_p value of BP in rats was about one-fourth of that of rabbits, similar K_{pu} values for each tissue were obtained between rats and rabbits. High K_{pu} values were obtained in lung and adipose tissue. The K_{pu} values of some viscera, such as the brain, heart, kidney, and gut, were in the range from 50 to 100.

Figure 2 shows the physiological pharmacokinetic model utilized here for calculation. This model was built on the basis of the following assumptions: (1) each tissue acts as a well-stirred compartment; (2) BP distribution is blood-flow limited; (3) elimination of BP occurs only from the liver in a linear manner; and (4) the K_{pu} of BP is independent of the drug concentration. The mass balance equations developed according to the flow diagram in Fig. 2 are given in Appendix II. The mean values of K_{pu} between rats and rabbits were used to

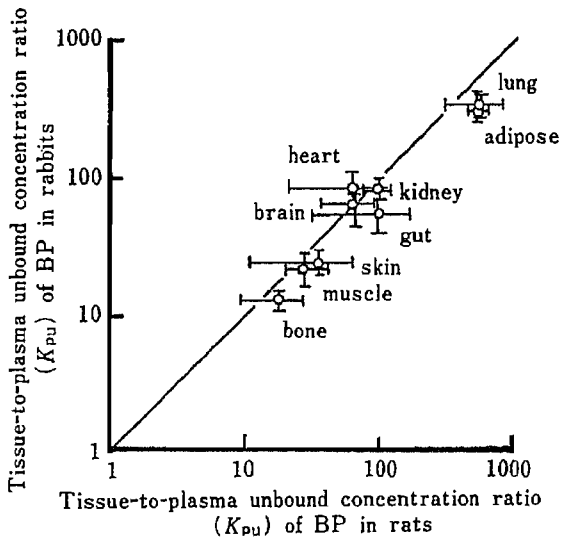

Fig. 1. The Relationship between the Tissue-to-Plasma Unbound Concentration Ratios (K_{pu}) of Biperiden in Rabbits and Rats

The line shows a positive correlation. $r=0.933$.

TABLE III. Tissue-to-Plasma Concentration Partition Coefficients (K_p) of Biperiden for Various Tissues of Animals

Tissue	K_p[a]		K_{pu}[b]
	Rabbit[c]	Rat	Mean
Blood	1.17±0.05	1.16±0.02[d]	6.8
Lung	131.0±6.0	60.9±25.1	445.0
Brain	25.7±8.8	7.0±3.0	64.8
Heart	34.3±8.7	7.0±4.7	75.8
Kidney	31.3±4.6	11.0±2.9	90.2
Gut	22.5±8.4	11.0±7.7	78.9
Muscle	8.5±2.5	3.05±1.0[d]	24.7
Adipose	120.0±16.0	58.0±6.1[d]	418.0
Skin	9.9±2.0	4.0±2.5	30.9
Bone	5.2±0.6	2.0±0.99	15.8

a) The value of K_p is the tissue-to-plasma concentration ratio at the steady state. Data are presented as the mean±S.D. *b*) The value of K_{pu} is the tissue-to-plasma unbound concentration ratio at the steady state. *c*) Ref. 5. *d*) Ref. 13.

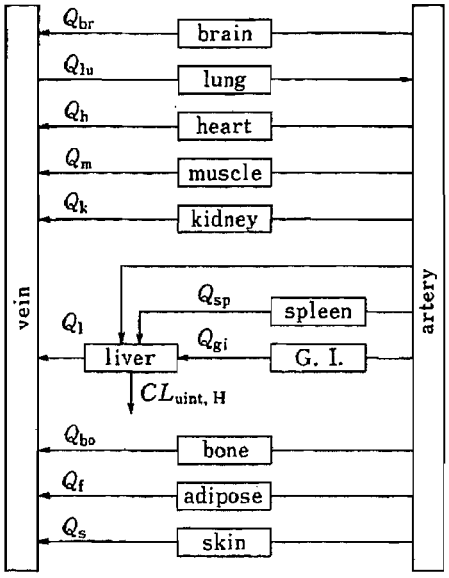

Fig. 2. Physiologically Based Pharmacokinetic Model Fully Diagramming the Blood Circulation through Various Tissues Studied for Biperiden

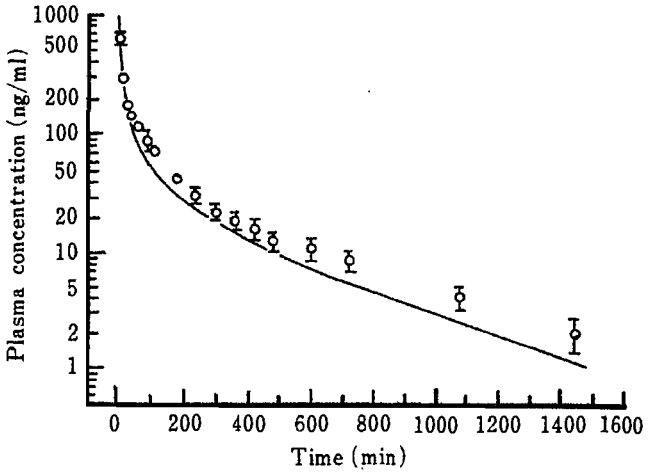

Fig. 3. Model-Predicted (Lines) *versus* Observed (Points) Plasma Biperiden Concentrations after a 6.7 mg Intravenous 2 min Infusion into 2.1 kg Rabbits

Each point represents the mean of three rabbits, and bars represent the standard errors of the mean. $r=0.940$. $p<0.001$. See text for details.

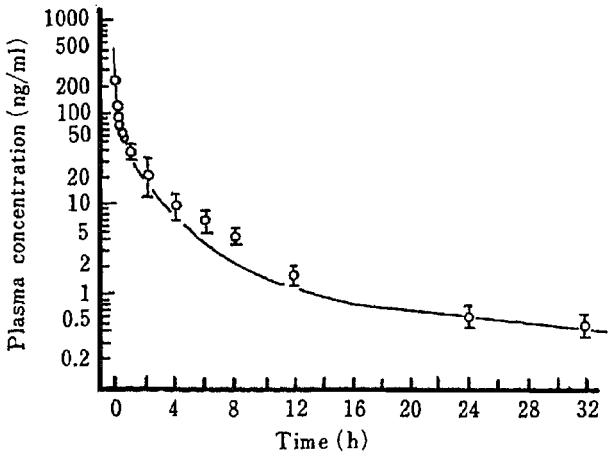

Fig. 4. Model-Predicted (Lines) *versus* Observed (Points) Plasma Biperiden Concentrations after a 3.2 mg/kg Intravenous 2 min Infusion into Beagles

Each point represents the mean of three data and bars represent the standard errors of the mean. $r=0.985$. $p<0.001$. See the text for details.

simulate beagles and humans. As a substitute value of K_{pu} in the liver and spleen, the mean value of viscera was used for the calculation.

Figure 3 shows the calculated variations of BP concentration in the plasma of 2.1-kg rabbits as a function of time. The initial dosage was 6.7 mg. The observed plasma concentration of BP agreed well with the simulated levels ($r=0.940$, $p<0.001$).

The results for plasma concentrations in beagles after intravenous administration of 3.2 mg/kg of BP are shown in Fig. 4. The observed plasma concentrations of BP coincided well with the simulated levels ($r=0.985$, $p<0.001$).

In order to predict the $CL_{uint, H}$ in humans, a regression analysis was adopted using the function of body size of animals as reported.[6] In Fig. 5, we show a log-log plot of $CL_{uint} \times MLP$ *vs.* body weight in rats, rabbits, and beagles. *MLP* is the maximum life-span potential in years. From the regression of the BP data in Fig. 5, the following equation is obtained:

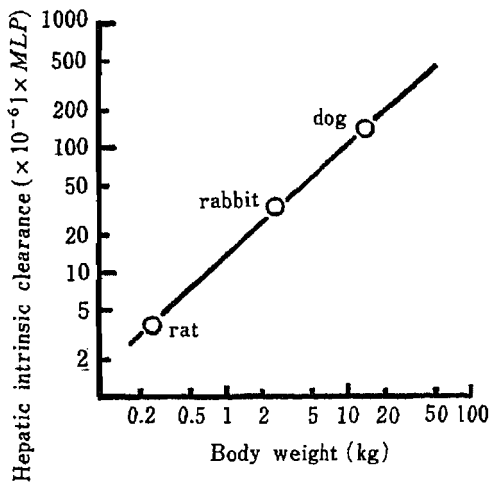

Fig. 5. Allometric Relationship between Unbound Biperiden Intrinsic Clearance per Maximum Lifespan Potential and Body Weight

$r=0.999$. $p<0.05$. See text for details.

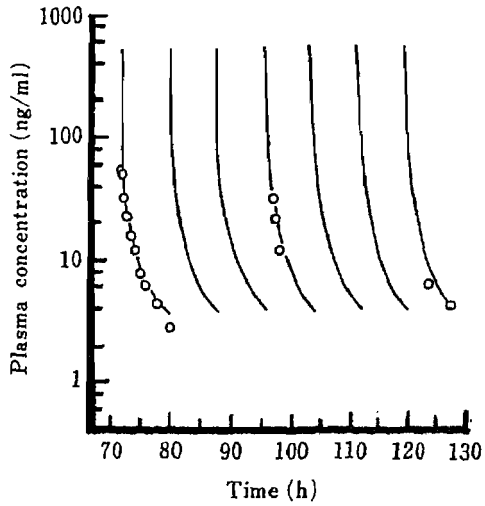

Fig. 6. Model-Predicted (Lines) *versus* Observed (Points) Plasma Biperiden Concentrations after Repeated Administration of 3.88 mg of Biperiden Intramuscularly to the Patient Weighing 42.3 kg

The model predictions were performed by using the physiological parameters, which were corrected by multiplying by the ratio of actual body weight to standard body weight. $r=0.980$. $p<0.001$.

$$CL_{uint} \times MLP = (1.36 \times 10^7) \times BW^{0.892} \quad (3)$$

where BW is the standard body weight of animals in kg, and CL_{uint} is the intrinsic clearance of unbound drug in one year. In this analyzing procedure, the values of MLP as reported by Boxenbaum were used.[6] The correlation coefficient was high and statistically significant ($r=0.999$, $p>0.05$). According to the regression parameters, $CL_{uint,H}$ in humans was calculated as listed in Table II. The values of $CL_{uint,H}$ of rats and rabbits were more than 10 times greater than that of humans.

Figure 6 shows the predicted and observed plasma concentration profile after intramuscular administrations of BP to the patient. The administration was repeated every 8 h. A previous study carried out at this laboratory[5] showed that the absorption from the injection site in the muscle into the systemic circulation was rapid and complete. Moreover, the pharmacokinetics of BP after intravenous injection and intramuscular administration were almost equal in rabbits.[5] Thus, the input function in humans was assumed to be equivalent to rapid infusion into the venous plasma. The predicted line agreed well with the observed plasma concentration in the patient. The correlation coefficient was high and statistically significant ($r=0.980$, $p<0.001$).

Discussion

This study demonstrates that a physiologically based pharmacokinetic model based on the hepatic intrinsic clearance of unbound drug and the tissue-to-plasma unbound concentration ratios was applicable to BP.

In our previous studies using the rabbit,[5] we have already shown that the high plasma clearance of BP could be well explained in terms of hepatic blood flow rate-limited elimination. Although the same explanation could be applied to rats, the value of CL_{tot}/BW in beagles and humans seemed to be smaller than the hepatic blood flow rate. By using the

estimated value of $CL_{uint,H}$ in humans in Table II, the value of CL_{tot}/BW was calculated as 10 ml/min/kg. This value is similar to the observed CL_{tot}/BW (=15.3 ml/min/kg) in humans (Table III). It was reported that no unchanged BP is excreted *via* the kidney in man.[2] In our experiments, the urinary recovery of BP in beagles was almost negligible.(=0.017% of the administered dose) within 48 h. Thus, it is reasonable to conclude that the route of elimination of intact BP was mainly hepatic elimination, not only in rats and rabbits, but also in beagles and humans. As shown in Table II, humans have a lower capacity for hepatic metabolism of BP. Man's lesser quantitative ability to metabolize appears to apply to many drugs.[6] Several regression parameters of the allometric exponentials have been reported, *i.e.*, 1.09, 1.23, and 0.922 for antipyrine, phenytoin, and clonazepam, respectively.[6] The parameter for BP coincides well with these values.

In order to construct a physiologically based pharmacokinetic model using K_{pu}, the coincidence of the K_{pu} value of each tissue in several animals must be confirmed. We attempted to establish this most important postulate by using rats and rabbits. This is because the f_p value of rabbits was the largest among the animals tested, and rats were suitable and convenient animals for determining the K_{pu} values. The values of each tissue were in good agreement between rats and rabbits. Recently, Sawada *et al.*[8] reported that, in general, there is little difference between the unbound volume of distribution of various basic drugs in tissues of animals and humans. From the good coincidence in tissue distribution characteristic between rats and rabbits, as shown in Fig. 1, this generality was confirmed to be applicable to the BP tissue distribution in rats and rabbits. However, the value of $Vd_{ss}/BW/f_p$ in rats was somewhat larger than that of rabbits. We examined the correlation between Vd_{ss} and f_p in animals and humans according to the reported prediction rule of basic drugs.[14] The correlation was high, but not significant ($r=0.867$). An explanation for this is not yet available.

It is reasonable to assume that the equilibrium conditions in each tissue phase are approached in a simple, linear manner, with characteristic terms determined by tissue volumes and blood flow rates. The assumption of perfusion-limited transport is applicable to lipid-soluble drugs, for which diffusion and movement across lipoid membranes should be relatively rapid. The physiological parameters for a 70-kg human were used as described bofore.[11] On this basis, the calculated result is in fair agreement with the observation in the patient given repeated intramuscular administrations.

In conclusion, the physiologically based pharmacokinetic model derived by using the plasma unbound concentration was useful to predict the human plasma concentration. Moreover, the hepatic intrinsic clearance of humans was well explained by the animal data. In the future, in order to clarify the pharmacodynamic action of BP, we can take into account the simulated target tissue concentrations in various disease states by using the present model.

Appendix

I: Nomenclature

General

C = concentration in plasma or tissue, ng/ml
C_u = unbound concentration in plasma or tissue, ng/ml
$CL_{uint,H}$ = hepatic intrinsic clearance of unbound drug, ml/min
f_p = unbound fraction in plasma
K_{pu} = tissue-to-plasma unbound concentration ratio
RBP = blood-to-plasma partition coefficient
Q = blood flow rate through tissue, ml/min
V = volume of plasma or tissue, ml

Subscripts

b=blood; a=arterial plasma; l=liver; k=kidney; gi=gastrointestinal tract; lu=lung; h=heart; m=muscle; br=brain; f=fat; bo=bone; v=venous; s=skin; t=tissue.

II. Model Equations for BP

Noneliminating organ or tissue

$$V_i \cdot dC_i/dt = RBP \cdot Q_i \, (C_a - C_i/(f_p \cdot K_{pu,i}))$$

Venous blood compartment

$$V_b \cdot dC_v/dt = Q_i \cdot C_i/(f_p \cdot K_{pu,i}) - Q_b \cdot C_v + IJ/RBP$$

$$Q_b = Q_{br} + Q_l + Q_k + Q_h + Q_m + Q_f + Q_{bo} + Q_s$$

Arterial blood

$$V_a \cdot dC_a/dt = Q_{lu}(C_{lu}/(f_p \cdot K_{pu,lu}) - C_a)$$

Lung

$$V_{lu} \cdot dC_{lu}/dt = RBP \cdot Q_{lu}(C_v - C_{lu}/(f_p \cdot K_{pu,lu}))$$

Liver

$$V_l \cdot dC_l/dt = RBP((Q_l - Q_{gi} - Q_{sp})C_a + Q_{gi} \cdot C_{gi}/(f_p \cdot K_{pu,gi}) + Q_{sp} \cdot C_{sp}/(f_p \cdot K_{pu,sp}) - Q_l \cdot C_l/(f_p \cdot K_{pu,l})) - CL_{uint,H} \cdot C_l/K_{pu,l}$$

IJ is the injection functions give by

$$IJ = D/2$$

(D is the dose; this function was used only within 2 min)

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Effect of Azone on the Percutaneous Absorption of 5-Fluorouracil from Gels in Hairless Rats[1)]

KEN-ICHI HOSOYA,[2a)] NORIMASA SHUDO,[2b)] KENJI SUGIBAYASHI and YASUNORI MORIMOTO*

Faculty of Pharmaceutical Sciences, Josai University, 1–1, Keyakidai, Sakado, Saitama 350–02, Japan

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Poly(acrylic acid) gels containing 5-fluorouracil (5-FU) and Azone were prepared and the effects of Azone on 5-FU release from the gels and on 5-FU permeation across the skin were studied by *in vitro* and *in vivo* methods. 5-FU was released rapidly from the gels. The release rate of 5-FU from the gels was higher than that from a commercial ointment. Azone did not affect the *in vitro* drug release from the gels. Experiments on *in vitro* permeation of 5-FU across the hairless rat skin with vertical diffusion cells showed that addition of Azone to the gels markedly enhanced the 5-FU permeability, although a lag time of approximately 6 h was observed. Increasing the Azone concentration in the gels to 15% proportionally increased the permeability of 5-FU. More than 10 h was required to reach a steady-state blood level of 5-FU after administration of 5-FU–Azone gel topically. Pretreatment with Azone, however, shortened the lag time so that a steady-state level was reached sooner. The area under the blood concentration–time curve (*AUC*) after topical administration was comparable to that after oral administration.

These results suggest not only that Azone would be very useful for increasing the skin permeability and blood level of 5-FU, but also that Azone might be useful for developing transdermal therapeutic systems for the delivery of practically unabsorbable drugs.

Keywords——5-fluorouracil; Azone; poly(acrylic acid) gel; drug release; percutaneous absorption; hairless rat

Development of a new drug requires much research, a long development time and also coordinated team efforts of a large group of researchers in various fields. Instead of searching for new drugs using a random, hit-or-miss approach, the development of superior drug delivery systems (DDS), which enhance the therapeutic efficacy of conventional drugs by controlling the release rate and/or targeting to the disease sites, may be an effective approach to improve the efficacy of chemotherapeutic agents.

In the present study, the usefulness of the transdermal route for absorption of antitumor agents was investigated, since the application of transdermal controlled-release medication is one of the most potentially valuable DDS development programs. The effects of vehicles and an absorption enhancer, Azone (laurocapram, 1-dodecylazacycloheptan-2-one, or 1-dodecylhexahydro-2*H*-azepin-2-one), on the permeability of a model antitumor agent, 5-fluorouracil (5-FU), across the hairless rat skin were measured *in vitro* and *in vivo*. Poly(acrylic acid) (Carbopol 934) gels were chosen as a vehicle and compared with a commercial ointment.

Experimental

Materials——Azone was supplied by Nelson Research and Development (Irvine, CA, U.S.A.). 5-FU powder and 5-FU ointment were kindly supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Poly(acrylic acid) (Carbopol 934) (CP) was purchased from B. F. Goodrich Chemical Co. (Cleveland, OH, U.S.A.). Propylene glycol (PG) and polyethylene glycol (PEG) 400 were purchased from Wako Pure Chemical Industries (Osaka, Japan). All

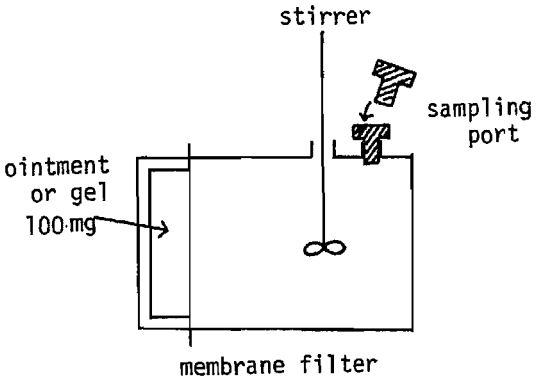

Fig. 1. Apparatus for Drug Release Experiments with a Half Cell

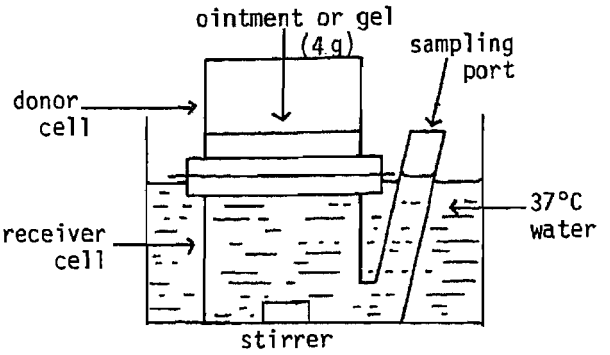

Fig. 2. Apparatus for Drug Permeation Experiments with a Vertical Diffusion Cell

other chemicals were of reagent grade quality and were obtained commercially.

Animals——Male hairless rats (WBN/kob strain) weighing between 150 and 250 g, supplied by Saitama Laboratory Animals (Sugito, Saitama, Japan), were used in all animal experiments.

Preparation of Poly(Acrylic Acid) Gels——CP was dispersed either in water (CP aq gel), PG (CP pg gel), or 50% PEG 400 in water (CP peg gel). In addition, 10% NaOH or diisopropanolamine was added to thicken CP aq gel and CP peg gel or CP pg gel, respectively. The concentration of CP was 0.8% (w/v), and the pH was adjusted to 7.0. 5-FU and Azone were added when required as a solution (or emulsion) in water, PG, or 50% PEG 400 in water. Final concentrations of 5-FU and Azone in gels were 1% and 3—15%, respectively.

Determination of Viscosity of Gels——The viscosities of CP gels and commercial 5-FU ointment were measured with a rotational viscometer (Rotovisko RV 100/M 150, Haake, West Germany) at a shear rate of 0—4650 s^{-1} and 37 °C.

***In Vitro* 5-FU Release from Gels**——The *in vitro* release of 5-FU from CP gels and 5-FU ointment was measured at 37 °C with half of a 2-chamber diffusion cell, as shown in Fig. 1.[3] A membrane filter, (SCWP 02400, pore size 8.0 μm, Millipore Corp., Bedford, MA, U.S.A.) was used to prevent loss of the gel into the sink. The weight of gel was 100 mg and the volume of water in the sink was 2.0 ml. Stirring of the sink solution was carried out with a Teflon propeller driven at 150 rpm with an electric motor. At appropriate intervals, a 100 μl sample was withdrawn from the sink solution for analysis and the same volume of water was added to keep the sink volume constant. Adsorption of 5-FU on the membrane filter was ignored. 5-FU concentration was measured spectrophotometrically at 266 nm.[4]

Procedure for *in Vitro* 5-FU Permeation across Skin——The hair of the abdominal region of hairless rats was carefully shaved with an electric razor, and a donor cap from the vertical diffusion cell set (diameter available for drug permeation: 25 mm) was fixed to the skin with surgical tissue cement (Aron Alpha, Toa Gosei Chemical Co., Ltd., Tokyo). The skin was excised with the cell cap from the rat and clamped to a receiver. The receiver compartment was filled with 20 ml of normal saline and the donor cell was filled with 4 g of ointment. The experiments were done at 37 °C in a water bath. A schematic diagram of the cell set during the experiment is shown in Fig. 2. At appropriate times, 100 μl of solution was withdrawn from the receiver compartment for analysis. After sampling, 100 μl of saline was added to the receiver to keep the volume constant.

Procedure for *in Vivo* 5-FU Percutaneous Absorption——CP aq gel or ointment (2 g) containing 20 mg of 5-FU (1% of total weight) and 60 mg of Azone (3%) with a backing of Cateripad (Nichiban Co., Tokyo) was applied to 22 cm^2 (4.0 × 5.5 cm) of the abdominal skin in rats weighing about 250 g. The concentration of 3% was chosen for Azone in gels because the same concentration had been used in emulsion formulations in the previous work.[5] To ensure adequate fixation, an elastic bandage (Elastopore No. 50, Nichiban Co.) was wrapped around the body. A Bollman cage (KN-326, Natsume Seisakusho, Tokyo) was also used for the long term experiments. Azone gel (2 g) without 5-FU was applied to the abdominal skin over 24 h in the pretreatment experiments just before the 5-FU–Azone gel was applied. 5-FU–Azone gel was applied to the same site of the abdominal skin after the Azone gel without 5-FU had been wiped off. For comparison purposes, 5-FU was also administered intravenously *via* the tail vein or perorally by gastric intubation in rats. Food was withheld for 24 h before peroral administration. Each dose in the various dosage forms was 80 mg/kg body weight. A 0.5 ml aliquot of blood was withdrawn with a heparinized syringe from the jugular vein at appropriate times for analysis.

Analysis of 5-FU——Concentrations of 5-FU in the receiver compartment and in serum were determined by high performance liquid chromatography (HPLC).

A 100 μl of acetonitrile was added to 100 μl of the receiver solution for deproteinization and mixed. The solution was centrifuged at 16000 rpm at 4 °C for 5 min. Then 25 μl of the resulting supernatant was injected into the HPLC apparatus.

Blood samples were centrifuged at 16000 rpm for 15 min at 4 °C to obtain serum. Then 0.1 ml of 0.5 M NaH_2PO_4, 0.75 ml of water and 4 ml of ethyl acetate were mixed with 0.25 ml of the serum and shaken for 10 min. The same extraction was repeated twice and the supernatant obtained by centrifugation was dried under nitrogen gas. A 100 μl of 1 mM KH_2PO_4 : methanol (9 : 1) was added to the vial and 25 μl of the supernatant obtained by centrifugation was chromatographed through a stainless steel precolumn (30 mm × 4.6 mm, i.d.) and a main column (250 mm × 4.6 mm, i.d.) of microporous silica with octyl and octadecyl chains (RP-8, Braunlee Lab., Inc., Santa Clara, CA, U.S.A. and Nucleosil $10C_{18}$, Nergel, West Germany, respectively). The eluting solvent was 1 mM KH_2PO_4–methanol (9 : 1) and the flow rate was 1.0 ml/min. Detection was done by ultraviolet (UV) absorption measurement at 266 nm. A pump control unit (Type 635-s, Hitachi Co.), a UV detector (Type Ubilog-5III, Oyobunko Kiki Co., Ltd., Tokyo) and an integrator (Type 3390A, Hewlett Packard Co., Avondale, PA, U.S.A) were used.

Results

Effect of Azone on the Rheological Properties of Gels and on *in Vitro* 5-FU Release from Ointment and Gels

The rheological properties of CP gels were found to depend on the kind and amount of neutralizing agents and the concentration of CP. In the present study, we adjusted the concentration of CP and pH to 0.8% and pH 7. Figure 3 shows the viscosity *versus* shear rate

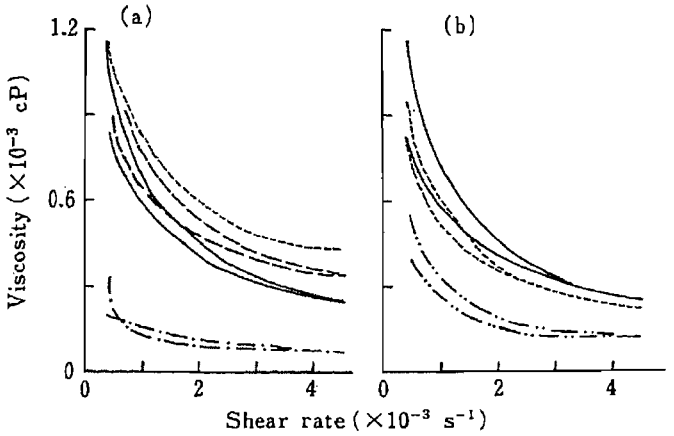

Fig. 3. Rheograms of CP Gels and Ointment

(a) CP aq gel (——), CP pg gel (------), CP peg gel (– – – –) and commercial ointment (— · —).
(b) CP aq gel containing 0% (——), 3% (------) and 10% (– – – –) Azone.

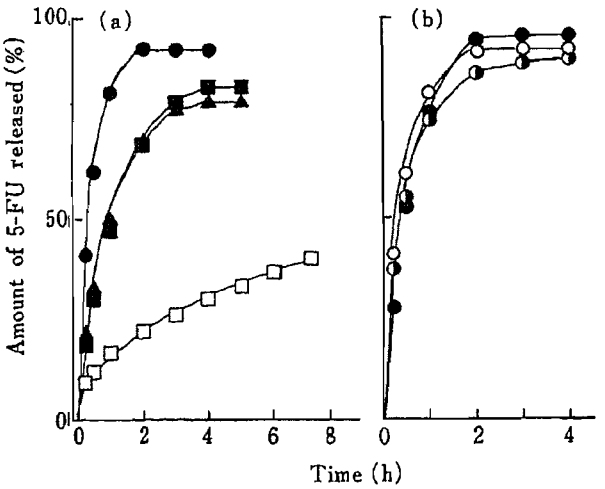

Fig. 4. Effect of Bases on the 5-FU Release from CP Gels and Ointment

(a) CP aq gel (●), CP pg gel (▲), CP peg gel (■) and commercial ointment (□).
(b) CP aq gel containing 0% (○), 3% (◑) and 10% (●) Azone.
Each point represents the mean of 3 experiments.

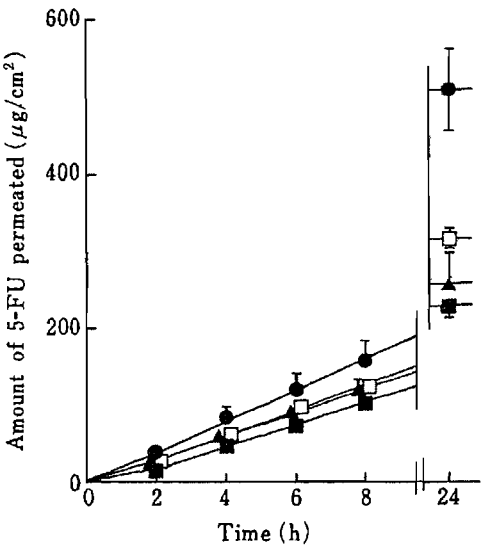

Fig. 5. Effect of Bases on the 5-FU Permeation across Skin

CP aq gel (●), CP pg gel (▲), CP peg gel (■) and commercial ointment (□).
Each point represents the mean ± S.E. of 5 experiments.

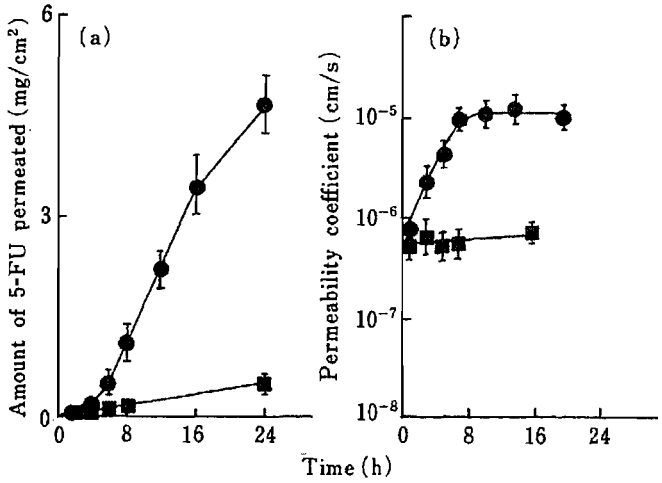

Fig. 6. Effect of Azone on the 5-FU Permeation across Skin

(a) Time course of cumulative amount of drug permeated with (●) and without (■) Azone.
(b) Time course of P; symbols as in (a).
Each point represents the mean ± S.E. of 5 experiments.

curves of a commercial ointment and CP gels. Every gel and ointment showed non-Newtonian flow. Thixotropy was observed, except for CP pg gel. The viscosity of CP pg gel was the highest, whereas that of the ointment was the lowest (Fig. 3a). Addition of Azone to CP aq gel (3% and 10%) decreased the viscosity (Fig. 3b).

In vitro release rates of 5-FU from gels were very fast and most of the drug was released from the gels within 2 h, as shown in Fig. 4. The release rate from the commercial ointment was slower than those from CP gels (Fig. 4a). Addition of Azone to CP gels had no effect on the release properties of 5-FU (Fig. 4b).

5-FU Permeation across Skin from Ointment and Gels

Figure 5 shows the effect of bases on the *in vitro* 5-FU permeation acorss skin. Among 3 kinds of gels, the permeation from CP aq gel was the fastest. The amounts of 5-FU that permeated during the initial 24 h were 504, 258 and 229 $\mu g/cm^2$, and the average permeability coefficients[5] were calculated to be 6.11, 3.09 and 2.72×10^{-7} cm/s for CP aq gel, pg gel and peg gel, respectively. In spite of the higher drug content (5%) in the commercial ointment compared to the gels (1%), the cumulative amount of drug that permeated from the ointment (312 $\mu g/cm^2$) over 24 h was smaller than that from CP aq gel (504 $\mu g/cm^2$). These results suggest the usefulness of CP gels, especially CP aq gel, for 5-FU topical therapy. Only CP aq gel was used for the following experiments.

Effect of Azone on 5-FU Permeation across Skin

Figure 6a shows the time course of 5-FU that permeated across the skin from CP aq gel containing Azone at a concentration of 10%. A marked enhancing effect of Azone on 5-FU permeation was observed. Cumulative amounts of 5-FU that permeated over 24 h were 4.68 mg/cm^2 with Azone and 0.50 mg/cm^2 without Azone. A lag time of approximately 6 h was seen with Azone treatment, which is almost the same as that observed in the case of Azone emulsion system.[5] The permeation rate was decreased after 16 h compared to that during 6—16 h, and this can be explained in terms of the decrease of 5-FU concentration in the gels due to the permeation of more than 50% of the drug into the receiver cell through the skin.

Figure 6b shows the time course of the permeability coefficients.[5] The steady-state permeabilities were 1.3×10^{-5} and 6.1×10^{-7} cm/s with and without Azone, respectively, a difference of about 20 times.

Figure 7 shows the effect of Azone concentration on the *in vitro* permeation of 5-FU across the skin. Increasing Azone concentration in the gels increased 5-FU permeation. After reaching a steady-state level,[5] the permeability *versus* Azone concentration curve was almost

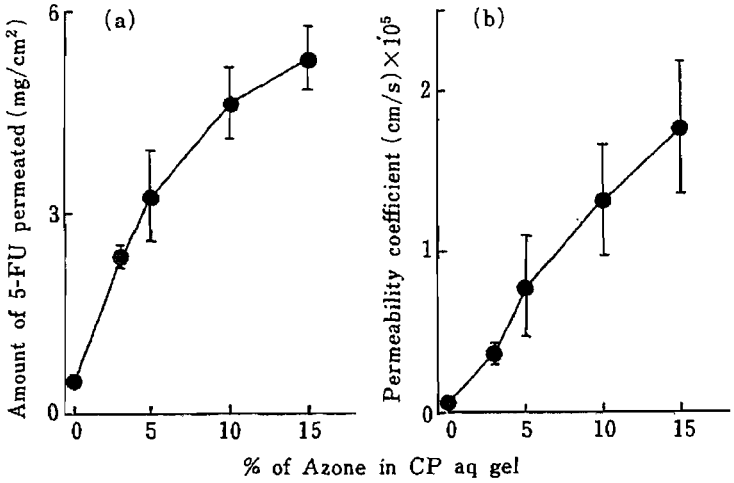

Fig. 7. Effect of Concentration of Azone on the 5-FU Permeation across Skin

(a) Cumulative amount of drug permeated over 24 h; (b) P. Each point represents the mean ± S.E. of 5 experiments.

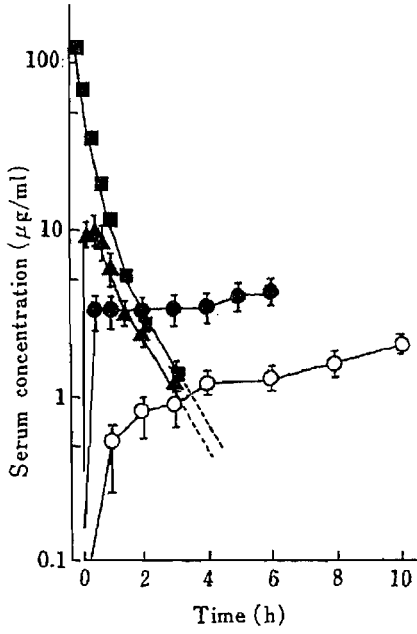

Fig. 8. Time Course of 5-FU Serum Level after Administration of Several Dosage Forms

Dose; 80 mg/kg.
i.v. (■), *p.o.* (▲), topical application with (●) and without (○) pretreatment.
Each point represents the mean ± S.E. of 5 rats.

liner, whereas the curve of the cumulative amount of 5-FU permeated over 24 h against 5-FU concentration in gels was not. The reason is that higher Azone concentrations cause a faster decrease of 5-FU concentration in gels. If a drug-suspending gel was used, straight lines should be obtained for both the cumulative drug transport and permeability data.

Comparison of 5-FU Serum Levels after Topical Administration with Those after i.v. and *p.o.* Administrations

5-FU gel containing Azone at a concentration of 3% was applied to the abdominal skin of rats and the 5-FU serum level after topical application was compared with those after intravenous (i.v.) and *peroral* (*p.o.*) administrations.

After application of 5-FU–Azone gel without Azone gel pretreatment, the serum concentration of 5-FU increased up to 10 h, as shown in Fig. 8. In contrast, a steady-state blood level of about 3—4 μg/ml was found immediately after application of 5-FU–Azone gel onto abdominal skin which had been pretreated with Azone gel without 5-FU for 24 h before

TABLE I. Comparison of AUCs

Route of administration	AUC_{0-6h} ($\mu g\,ml^{-1}\,h$)
i.v. injection	62.41
p.o. administration	13.64
Topical applications	
Without pretreatment	5.22
With pretreatment	20.12

Each value was calculated from the average serum concentrations of 5 rats as shown in Fig. 8.

the drug permeation experiment. 5-FU was not detectable in blood after topical application of CP aq gel without Azone, or commercial ointment. On the other hand, the same dose (80 mg/kg) given i.v. and *p.o.* resulted in a much higher peak concentration, but drug elimination was also much faster than after topical administration of 5-FU–Azone gel.

Table I compares the area under the serum concentration *vs.* time curves (AUCs) for 0—6 h after i.v., *p.o.* and topical administrations. AUC_{0-6h} after topical application without pretreatment was 5.22 $\mu g\,ml^{-1}\,h$. AUC_{0-6h} after topical application with pretreatment was 20.12 $\mu g\,ml^{-1}\,h$. AUC_{0-6h} values after i.v. and *p.o.* treatments were 62.41 and 13.64 $\mu g\,ml^{-1}\,h$, respectively. AUC_{3-6h} values for both treatments were calculated by dividing the serum concentration at 3 h by the terminal elimination constant. AUCs for other periods for which serum concentration data were available were calculated by using the trapezoidal rule.

Addition of Azone to CP gel increased the bioavailability of 5-FU and the AUC after topical administration was comparable to that after oral administration, especially in the case of topical administration after pretreatment with Azone gel.

Discussion

In vitro release of 5-FU from CP aq gel was very fast and no significant effect of the viscosity of gels on the release was found. Since Azone did not promote drug release from the gels, the Azone effect on 5-FU transport across the skin should be caused in the skin. We suggested in the previous papers[5] that Azone affects the skin, especially the stratum corneum. However, the mechanism of the skin penetration enhancing effect of Azone is still unknown.

Stoughton[6a] examined the effect of Azone concentration on the skin permeation of erythromycin and sodium fusidate. The penetration of erythromycin was enhanced by Azone in a monotonic fashion, rising to an apparent plateau at concentrations of Azone above 30—40%. In the case of sodium fusidate, a low concentration of Azone produced a 2- to 3-fold enhancement, but higher concentrations showed less enhancement. Stoughton and McClure[6b] also examined the effect of Azone on 5-FU permeation from water and propylene glycol formulations. A maximum effect was observed at 1.8% Azone; higher concentrations (9.0 and 45.0%) were less effective. From these results and the present data, it is clear that the concentration of Azone which is required to produce optimum enhancement varies from one drug and/or formulation to another. Each drug and formulation should be studied over a range of concentrations of Azone in order to optimize skin permeation.

The blood concentration of 5-FU after administration of 5-FU with Azone gel was very high and comparable to that achieved by systemic therapies (i.v. and *p.o.*) at the same dosage level. In general, the steady-state blood level, C_{ss}, after topical application can be estimated by using the following equation;

$$C_{ss} = J/CL \quad (1)$$

where J is percutaneous absorption flux and CL is total clearance. Flux, J, is expressed as follows;

$$J = P\,Cs\,A \quad (2)$$

where P, Cs and A are the permeability coefficient of the drug across the skin, the solubility of the drug in the formulation and its application area.[7] From both equations, P can be written as follows.

$$P = \frac{C_{ss}CL}{CsA} \quad (3)$$

After topical application of Azone gel with pretreatment, C_{ss} was 3.3 μg/ml, as shown in Fig. 8. Total clearance in hairless rats can be calculated as 0.0890 ml/s from the $AUC_{i.v.}$ (62.41 $\mu g\,ml^{-1}\,h$) as shown in Table I and the dose (20 mg per head) by using the following equation.

$$CL = \frac{\text{dose}}{AUC_{i.v.}} \quad (4)$$

A concentration of 1% (10 mg/ml) was used as the initial concentration of 5-FU in formulations in the present experiments. Since the drug concentration in formulations is almost constant throughout the short-term experiments, this initial concentration can be used instead of Cs. A is 22 cm^2. Substituting these values into Eq. 3 yields 1.34×10^{-6} cm/s as the P value in hairless rat skin.

Phillips *et al.*[8] reported that the CL value of 5-FU in humans was 21.6 ml/s. If we suppose that a saturated aqueous gel is administered (Cs is 17 mg/ml at 37 °C)[9] on 100 cm^2 of human skin and that the permeability across the human skin is the same as that across the hairless rat skin, C_{ss} in humans can be calculated as 105 ng/ml according to Eqs. 1 and 2. This blood level is thought to be a clinically effective concentration.[10] The flux, J, in humans, however, was reported to be smaller than that in rats for many drugs.[11] Thus, J in humans may be estimated as 0.1 to 1 times that in rats. In the present *in vivo* experiments, 3% Azone gel was used. From the *in vitro* permeation experiments (Fig. 7), it is suggested that higher Azone concentrations in the gel may produce higher blood concentrations. As a next step, clinical experiments using dermal formulations containing Azone should be carried out.

In conclusion, Azone appears to be effective for enhancing transdermal transport of hydrophilic compounds such as 5-FU. Transdermal systemic treatment with many drugs may be feasible by using Azone in the near future.

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References and Notes

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2) Present address: *a) Omiya Research Laboratory, Nikken Chemical Co., Ltd., Kitabukuro, Omiya, Saitama, 330, Japan*; *b) Biological & Chemical Research Laboratory, Nissan Chemical Industry Co., Ltd., Shiraoka, Minamisaitama-gun, Saitama 349-02, Japan.*
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Effects of Drug Bindings on the Esterase-like Activity of Human Serum Albumin. VII.[1] Subdivision of R-Type Drugs Inhibiting the Activity towards *p*-Nitrophenyl Acetate

YUKIHISA KURONO,* YOSHIRO OZEKI, HIDETO YAMADA, TOSHIMASA TAKEUCHI, and KEN IKEDA

Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467, Japan

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The subdivision of R-type drugs, which inhibit the reaction of *p*-nitrophenyl acetate with an active site (R site) located near the tyrosine-411 residue of human serum albumin (HSA), is proposed based on the results of kinetic and fluorometric studies. We found two kinds of R-type drugs with regard to the influence on the reaction rate of 3,5-dinitroaspirin (DA) at a site (U site) near the lysine-199 and tryptophan-214 (Trp-214) residues of HSA. One of them (R_2-type drug, *e.g.*, diazepam or medazepam) greatly accelerates the reaction of DA with HSA, and the other (R_1-type drug, *e.g.*, clofibric acid or octanoic acid) does not affect the reaction. The R_2-type drug quenches the fluorescence originating from the Trp-214 residue in the U site of HSA, and the R_1-type drug hardly influences this fluorescence. The acceleration of the reaction was considered to be due to the conformational change of the U site caused by binding of the R_2-type drug to a part of the R site on HSA. Five benzodiazepines, 3 sulfonylureas, and 3 other drugs were classified into R_1-type and R_2-type drugs.

Keywords——human serum albumin; esterase-like activity; drug binding site; classification; benzodiazepine; sulfonylurea; *p*-nitrophenyl acetate; 3,5-dinitroaspirin; fluorescence quenching; conformational change

In the previous papers[2-4] it was reported that human serum albumin (HSA) has esterase-like activity towards *p*-nitrophenyl acetate (NPA)[2,3] and 2,4-dinitrophenyl diethyl phosphate (DDP),[4] and the active site was found to be located close to the tyrosine-411 residue (named the R site[3]) of the HSA amino acid sequence.[5] Drugs which inhibit the reaction of NPA with HSA were referred to as R-type drugs.[3] Since the R-type drugs were found to correspond to Sudlow's Site II drugs,[6,7] the R site is considered to be identical with Site II.

HSA has another reactive site for substituted aspirins (*e.g.*, 3,5-dinitroaspirin, DA), and this site was found to be located near the lysine-199 residue (named the U site) of the sequence.[8,9] The single tryptophan-214 (Trp-214) residue is also located in the U site and the binding of a drug to the U site decreased the fluorescence intensity of this residue.[3,8] Drugs, which inhibit the reaction of DA with HSA and quench the fluorescence due to the Trp-214 residue, were referred to as U-type drugs. Because the U-type drugs correspond to Sudlow's Site I drugs, the U site appears to be identical with Site I.

Sjöholm *et al.*[10] and Fehske *et al.*[11] reported that two drugs, diazepam and warfarin, function as specific markers for two specific drug-binding sites, *i.e.*, the diazepam site and the warfarin site. Diazepam inhibited the reaction of NPA with HSA[12,13] and it was, thus, considered to be an R-type drug.[12] However, diazepam was, unlike other R-type drugs, found to cause acceleration of the reaction with DA and quenching of the fluorescence due to the Trp-214 residue of HSA. In this paper, we describe the basis for the subdivision of the R-type

drugs, and report the dissociation constants of the complex between the R site and various drugs including 5 benzodiazepines, 3 sulfonylureas, and *n*-octanoic acid. The data for clofibric acid and ibuprofen reported previously[3,8] are also presented for comparison.

Experimental

Materials and Apparatus——HSA (Sigma Chem. Co., Fraction V, lots 47C-04423, 100F-02061, and 64F-9309) was used after purification by Chen's method.[14] The concentration of HSA was determined from the molar absorptivity ($\varepsilon = 3.66 \times 10^4\ \text{M}^{-1}\,\text{cm}^{-1}$) at 278 nm, assuming a molecular weight of 69000.[15,16] DA[8,9] and DDP[4] were synthesized according to the methods of Zaugg *et al.*[17] and Gulick and Geske,[18] respectively. Diazepam (Takeda Chemical Industries, Ltd.), medazepam (Shionogi and Co., Ltd.), chlordiazepoxide (Takeda Chemical Industries, Ltd.), flunitrazepam (Nippon Roche Co., Ltd.), bromazepam (Nippon Roche Co., Ltd.), acetohexamide (Shionogi and Co., Ltd.), tolbutamide (Yamanouchi Pharmaceutical Co., Ltd.), and chlorpropamide (Ono Pharmaceutical Co., Ltd.) were gifts from the respective manufacturers. All other chemicals were commercial reagent-grade products.

A Hitachi UV-124 spectrophotometer and a Union-Giken RA-401 stopped-flow spectrophotometer were used for the measurements of the reaction rates. A Shimadzu RF-520 spectrofluorophotometer was employed for the measurement of the fluorescence spectra.

Kinetic Runs——The buffer used was pH 7.4, 0.067 M phosphate and the ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C. The reactions of substrate (1.00×10^{-5} M) with HSA (5.00×10^{-5} M) in the presence and absence of a drug were followed spectrophotometrically by monitoring the appearance of product at an appropriate wavelength. The wavelengths used were 380, 360, and 360 nm for *p*-nitrophenol,[3] 2,4-dinitrophenol,[4] and 3,5-dinitrosalicylic acid,[8] respectively. The drug concentration was varied as required. The pseudo first-order rate constant (k_{obs}) was determined from a plot of $\log(A_\infty - A)$ against time, where A_∞ and A are the absorbances at completion of the reaction and at time t, respectively.

For experimental convenience, stock solutions of the substrates were prepared in acetonitrile (for NPA and DA) or dioxane (for DDP) so that the reaction solution for the rate measurement always contained 0.5% (v/v) of the organic solvent. Because of the low solubilities of benzodiazepines, 1% (v/v) ethanol was added to the reaction solution. As described previously,[19] the effect of the solvent on the reaction rate with NPA could be interpreted in terms of competitive inhibition of the reactive site on HSA by the solvent. As will be shown later (Figs. 1 and 3), the inhibition (or acceleration) pattern of the reaction caused by the drug was not much affected by the presence of the solvent (1% ethanol). When the concentration of the solvent was fixed for a series of reactions, therefore, the effects of other factors (*e.g.*, the addition of the drug) on the reaction could reasonably be compared, *i.e.*, the solvent effect could be ignored in the comparison.

Fluorescence Measurements——Fluorescence spectra of HSA were measured in the presence and absence of the drug. An excitation wavelength of 300 nm was used for the measurements, since the single Trp-214 residue of HSA was excited at this wavelength.[20] The inner filter effect of the drug on the fluorescence intensity was corrected as required.[21,22]

Results and Discussion

Effects of Drugs on the Reaction Rates of NPA and DDP with HSA

Figure 1 shows the effects of four drugs on the reaction rate of NPA with HSA. In this figure, k'_{obs} on the ordinate is the rate constant in the presence of drug and the concentrations with subscript 0 on the abscissa show the initial concentrations of drug and HSA. All these drugs inhibit the reaction of NPA with HSA. Figure 1 also illustrates the theoretical inhibition curves assuming different values of the dissociation constant ($K_{I,R}$) of the complex between the drug and the R site of HSA. The curves were obtained by analog computer simulation of the reaction of NPA with HSA in the presence and absence of the drug. The details of the production of the inhibition curve are described elsewhere.[23] From the inhibition curve giving the best fit to the experimental results, the dissociation constant ($K_{I,R}$) was estimated, and the values are listed in Table I.

Some of the reciprocal values ($1/K_{I,R}$, M^{-1}) of $K_{I,R}$ (M) in Table I are similar to the binding constants found in the literature, and the others are significantly different.[13,24] The discrepancies of the parameters presumably arise from the differences of experimental

TABLE I. Dissociation Constants of the Complex between Drug and R site of HSA[a]

Drug	$K_{I,R}$ (M)
Medazepam[b]	1×10^{-6}
Diazepam[b]	1×10^{-5}
Chlordiazepoxide[b]	2×10^{-5}
Flunitrazepam[b]	2×10^{-4}
Bromazepam[b]	8×10^{-5}
Acetohexamide	3×10^{-5}
Tolbutamide	6×10^{-5}
Chlorpropamide	8×10^{-5}
n-Octanoic acid	1×10^{-5}
Clofibric acid[c]	5×10^{-6}
Ibuprofen[c]	5×10^{-6}

a) pH 7.4, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) containing 0.5% (v/v) acetonitrile at 25 °C. *b*) The organic solvent content was 0.5% (v/v) acetonitrile and 1% (v/v) ethanol. *c*) The data were reported previously.[23]

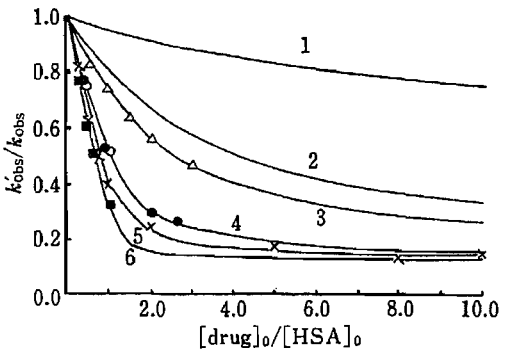

Fig. 1. Effects of Drugs on the Reaction Rate of NPA with HSA, and R-Type Inhibition Curves at Different Assumed Dissociation Constants ($K_{I,R}$)

△, tolbutamide; ×, clofibric acid; ■, medazepam; ●, diazepam; ○, diazepam without ethanol; curve 1, $K_{I,R} = 1 \times 10^{-3}$ M; 2, 1×10^{-4} M; 3, 6×10^{-5} M; 4, 1×10^{-5} M; 5, 5×10^{-6} M; 6, 1×10^{-6} M; $[NPA]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; pH 7.4, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) containing 0.5% (v/v) acetonitrile and 25 °C; $k_{obs} = 6.20 \times 10^{-3}\,s^{-1}$; k_{obs} for the case containing 1% ethanol $= 4.95 \times 10^{-3}\,s^{-1}$; data for clofibric acid were reported previously.[23]

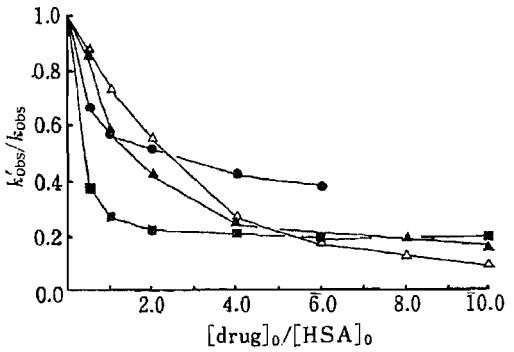

Fig. 2. Effects of Drugs on the Reaction Rate of DDP with HSA

△, tolbutamide; ▲, octanoic acid; ■, medazepam; ●, diazepam; $[DDP]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; pH 7.4, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) containing 0.5% (v/v) dioxane and 25 °C; $k_{obs} = 9.25 \times 10^{-3}\,s^{-1}$; k_{obs} for the case containing 1% ethanol $= 7.36 \times 10^{-3}\,s^{-1}$.

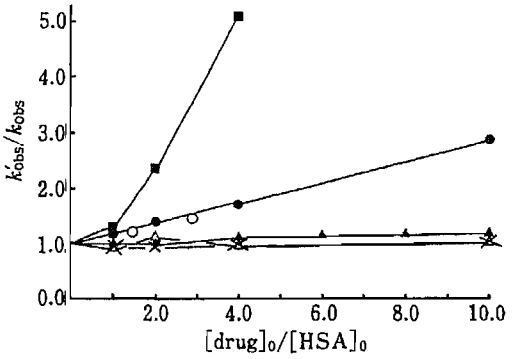

Fig. 3. Effects of Drugs on the Reaction Rate of DA with HSA

△, tolbutamide; ▲, octanoic acid; ×, clofibric acid; ■, medazepam; ●, diazepam; ○, diazepam without ethanol; $[DA]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; pH 7.4, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) containing 0.5% (v/v) acetonitrile and 25 °C; $k_{obs} = 2.30 \times 10^{-1}\,s^{-1}$; k_{obs} for the case containing 1% ethanol $= 2.17 \times 10^{-1}\,s^{-1}$; data for clofibric acid were reported previously.[23]

procedures, such as equilibrium dialysis, ultrafiltration, and spectroscopy, and also from slight differences of the experimental conditions such as buffer components, ionic strength, and source of HSA.

Figure 2 shows the effects of the drugs on the reaction rate of DDP with HSA. All these drugs inhibit the reaction, and the primary binding site for these drugs seems to be the R site.

Influence of Drug Binding on the U Site of HSA

The effects of the drugs on the reaction rate of DA with HSA are represented in Fig. 3. There were two kinds of drugs in terms of the effects on the above reaction, one of which (named R_2-type drugs, *e.g.*, medazepam and diazepam) markedly accelerates the reaction and

the other (named R_1-type drugs, *e.g.*, octanoic acid and tolbutamide) hardly affects the reaction. In the absence of HSA, medazepam or diazepam does not accelerate the hydrolysis of DA itself. Thus, the acceleration mechanism may be explained by the reaction scheme shown in Chart 1.

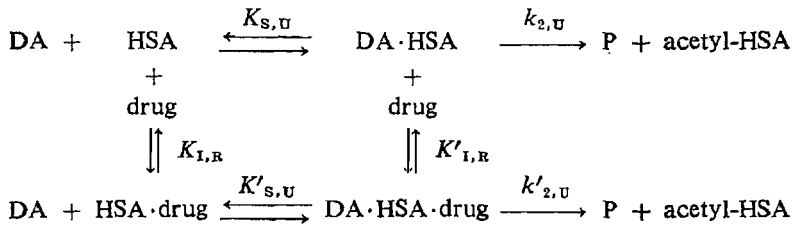

Chart 1

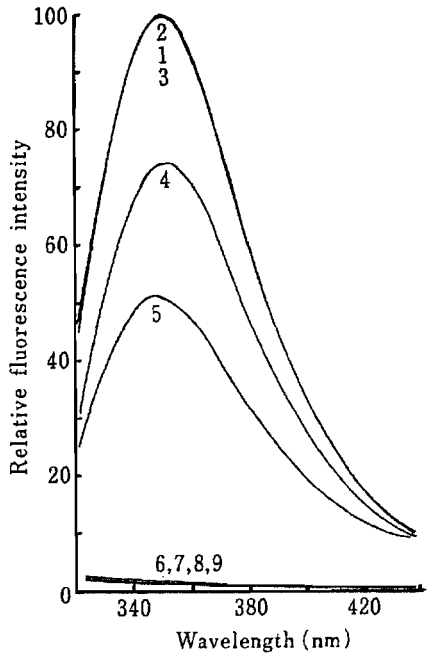

Fig. 4. Fluorescence Emission Spectra Excited at 300 nm

$[HSA]_0 = 1.00 \times 10^{-5}$ M; $[drug]_0 = 1.00 \times 10^{-5}$ M; 1, HSA alone; 2, HSA + octanoic acid; 3, HSA + clofibric acid; 4, HSA + diazepam; 5, HSA + medazepam; 6, 7, 8 and 9, each drug alone; pH 7.4, 0.067 M phosphate buffer (μ=0.2 with NaCl) and 25 °C.

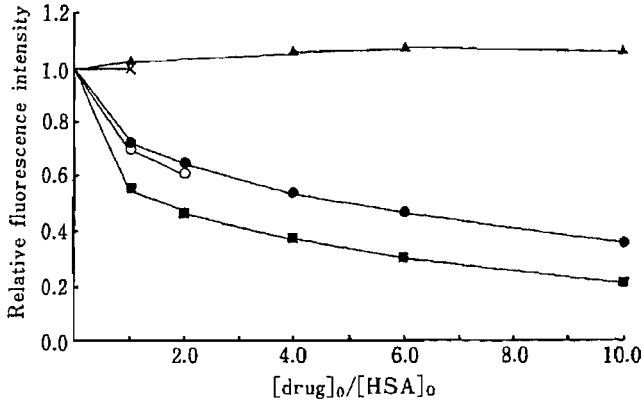

Fig. 5. Effect of Drug Concentration on the Fluorescence Intensity

▲, octanoic acid; ×, clofibric acid; ■, medazepam; ●, diazepam; ○, diazepam without ethanol; $[HSA]_0 = 1.00 \times 10^{-5}$ M; pH 7.4, 0.067 M phosphate buffer (μ=0.2 with NaCl) and 25 °C.

In Chart 1, DA·HSA and HSA·drug are the complexes between DA (substrate, S) and the U site of HSA and between the drug (inhibitor, I, and/or accelerator) and the R site of HSA, respectively. $K_{S,U}$ and $K_{I,R}$ are the dissociation constants of DA·HSA and HSA·drug, respectively. DA·HSA·drug is the ternary complex. $K'_{S,U}$ and $K'_{I,R}$ are the respective dissociation constants of the ternary complex. The rate constants of the complexes, DA·HSA and DA·HSA·drug, are represented by $k_{2,U}$ and $k'_{2,U}$, respectively. P and acetyl-HSA are 3,5-dinitrosalicylic acid and acetylated HSA, respectively.[8] For the acceleration ($(k'_{obs}/k_{obs}) > 1$ in Fig. 3) of the reaction of DA with HSA, the following conditions are necessary: $k'_{2,U} > k_{2,U}$ and/or $K_{S,U} > K'_{S,U}$. Since, as reported previously,[8] $K_{S,U}$ seems to be much smaller than $[HSA]_0$ (initial concentration of HSA employed, 5×10^{-5} M), $K_{S,U}$ does not contribute to

TABLE II. Subdivision of R-Type Drugs

R_1	R_2
Clofibric acid	Medazepam
Ibuprofen	Diazepam
n-Octanoic acid	Chlordiazepoxide
Tolbutamide	Flunitrazepam
Bromazepam	Acetohexamide
Chlorpropamide	

k_{obs}, that is, $k_{obs}=k_{2,U}\cdot[HSA]_0/(K_{S,U}+[HSA]_0)\fallingdotseq k_{2,U}$. Even if $K'_{S,U}<K_{S,U}$, therefore, it may not be observable. Consequently, the acceleration is considered to result from $k'_{2,U}>k_{2,U}$ in our present case. The conformational change of the U site induced by the binding of the R_2-type drug to a part of the R site of HSA probably increases the reactivity of the lysine-199 residue in the U site.

The conformational change caused by the R_2-type drug may be supported by the quenching of the fluorescence due to the Trp-214 residue located in the U site of HSA. Figure 4 shows the fluorescence spectral change in the presence of the drug. Figure 5 illustrates the effect of the drug concentration on the fluorescence intensity (corrected for the inner filter effect of the drug).[21,22] The strong quenching of the fluorescence by diazepam or medazepam indicates conformational change of the U site. On the other hand, octanoic acid or clofibric acid does not affect the fluorescence intensity of the residue located in the U site of HSA. On the basis of these fluorescence (Fig. 5) and kinetic (Fig. 3) results, we propose the subdivision of the R-type drugs, that is, one group (R_2-type drugs) affects the U site and the other (R_1-type drugs) does not influence the U site.

Concluding Remarks on the Subdivision of R-Type Drugs

Table II summarizes the subdivision of the R-type drugs so far examined. Bruderlein and Bernstein[25] suggested that Site I (U site) and Site II (R site) are overlapping. This is consistent with the results on diazepam or medazepam in this study. However, octanoic acid and clofibric acid seem to bind to the R site independently of the U site (see Figs. 1 and 3). On the basis of the finding that there are considerable differences in the displacing effect of bilirubin (another binding site marker) between diazepam and Site II drugs, Maruyama *et al.*[26] recently reported that the diazepam site (considered as the R site here) can be regraded as the diazepam site–Site II overlapping area, and they also suggested that the binding of benzodiazepines to the diazepam site may cause limited conformational changes around the bilirubin site. Our results in Table II are similar to those of Maruyama *et al.*[26] Sudlow *et al.*[7] also reported that Site II drugs might be divided into two groups. Since the three dimensional structure of HSA is not known, we can say at present only that there are two kinds of R-type drugs, depending on whether the drug influences the U site or not.

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Effect of Ricin or Ricin A-Chain Encapsulated in Anti-carcinoembryonic Antigen (CEA) Antibody-Bearing Liposomes on CEA-Producing Tumor Cells

YOSHIFUMI WATANABE and TOSHIAKI OSAWA*

Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

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Small and large liposomes containing ricin or ricin A-chain (a toxic subunit of ricin) and having monoclonal anti-carcinoembryonic antigen (CEA) antibodies on the surface were prepared, and their effects on CEA-producing tumor cells, were examined in order to evaluate the efficacy of these liposomes as drug delivery systems. It was found that the large unilamellar liposomes (average diameter: 3000 Å) could not be internalized by the tumor cells, but were ruptured after specific binding to tumor cells through the monoclonal antibodies on the surface. On the other hand, the small unilamellar liposomes (average diameter: 800 Å) were found to be easily internalized by tumor cells.

Keywords——ricin; ricin A-chain; carcinoembryonic antigen; anti-carcinoembryonic antigen antibody; liposome; tumoricidal activity

In the field of tumor chemotherapy, "drug targeting" has recently been attempted to specifically kill tumor cells by conjugating a cytotoxic agent to a tumor-specific antibody in order to ensure the arrival of the agent at the tumor site.[1-11] Such conjugates can be classified into the following three types: (i) a drug directly bond to an antibody; (ii) a drug bound to an antibody through a spacer; and (iii) a drug encapsulated in an antibody-bearing liposome. In the previous paper,[11] we reported the effects of a monoclonal anti-carcinoembryonic antigen (CEA) antibody-ricin A-chain conjugate on human CEA-producing tumor cells *in vitro* and *in vivo*. We found that this conjugate did not show significant antitumor activity against solid tumors *in vivo*, mainly due to the rapid rupture of the linkage between ricin A-chain and the monoclonal antibody and the inactivation of the resulting free ricin A-chain *in vivo*. These results indicate the need for some measures such as encapsulation in liposomes to protect the biologically active substances to *in vivo*.

On the other hand, in the field of cancer therapy, various attempts are being made to enhance the response of hosts against tumors by the use of so-called biological response modifiers (BRM) including lymphokines.[9,12-14] Recent progress in biotechnology enabling these lymphokines to be obtained in relatively large amounts has facilitated research on the clinical application of these substances. However, if these substances are simply injected into tumor-bearing animals, they are generally not so effective because of dilution in the blood, degradation by various enzymes and so on. Therefore, it is important to develop a delivery system to ensure the arrival of sufficient amounts of these substances at the sites of tumors, and this system should also incorporate some means of protecting the factors from inactivation *in vivo*.

In this study, we prepared ricin-containing small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) bearing anti-CEA antibody on their surface and compared their effects on target cells as model experiments for the development of a delivery system for

biologically active proteins, *e.g.* lymphokines, because the effect is easily discernable when these strongly toxic ricin-containing liposomes are tested against CEA-producing tumor cells.

Experimental

Chemicals——Cholesterol and egg phosphatidylcholine (PC) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Egg phosphatidylethanolamine (PE) was purified from egg yolk by the standard method.[15] *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was purchased from Pharmacia (Uppsala, Sweden). *N*-[3-(2-Pyridyldithio)propionyl]phosphatidylethanolamine (PDP-PE) was synthesized from PE and SPDP by the method of Martin *et al.*[16] Glutathione (reduced) was kindly provided by Mochida Pharmaceutical Co. (Tokyo). Ricin and ricin A-chain were purified as described previously.[11]

Cell Lines——Four different types of cells were used. CEA-producing cells employed in this study were from a human colonic carcinoma cell line, C-1,[17] a human pancreatic exocrine adenocarcinoma cell line, T3M-4,[18] and a human lung carcinoma cell line, HLC-2.[19] The non-CEA-producing cells used were from a human melanoma cell line, A375,[20] kindly provided by Dr. Fidler, University of Texas System Cancer Center, Houston, Texas, U.S.A.

Protein Analysis——Protein was determined by the method of Lowry *et al.*[21] or with Biorad Protein Assay kits (Bio-Rad Co., Ltd.).

The Antibody and Its Modification——A cell clone, 5F10-2, which secretes monoclonal anti-CEA immunoglobulin G (IgG) antibody, was established by Tsunoda *et al.*[22] from somatic cell hybrids between NS-1 myeloma cells and spleen cells from BALB/c mice immunized with CEA. The antibody was purified from mouse ascites fluid by gel filtration on a column of Sephacryl S-300, $(NH_4)_2SO_4$ fractionation and ion exchange chromatography on a diethylaminoethyl (DEAE)-52 column. The purified anti-CEA antibody belongs to the IgG1 subclass and show virtually no cross reaction with non-specific cross-reacting antigens and very weak binding to human granulocytes in assays carried out by the method of Hatada *et al.*[23]

A 5 mM ethanolic solution (150 μl) of SPDP was added dropwise to 5 ml of anti-CEA IgG solution (5 mg/ml) in 10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), and then the mixture was incubated at 23 °C for 30 min. After extensive dialysis against 0.1 M acetate buffered saline (pH 4.5), about 40 mg of dithiothreitol (DTT) was added to the resulting solution of IgG (final conc., about 50 mM), and the mixture was incubated at 23 °C for 20 min. The resulting solution was dialyzed against 10 mM sodium phosphate buffered saline (pH 8.0), and stored as the SH-IgG solution. The number of SH-groups introduced into IgG was determined by measuring the absorbance at 343 nm after adding DTT.

Modification of Ricin A-Chain——The sulfhydryl group of the ricin A-chain must be blocked so that it does not react with PDP-PE. We modified the ricin A-chain with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and glutathione to block it. Briefly, 1 ml of ricin A-chain (3.4 mg/ml) in PBS was added to 300 μl of 10 mM DTNB in 1 M triethanolamine adjusted to pH 8.5 with 0.1 M NaOH. After 30 min, the mixture was dialyzed against PBS to remove unreacted materials. Then it was reacted with excess glutathione. After removal of free glutathione by dialysis against PBS, the product was stored as the modified ricin A-chain. We confirmed that it retained the protein synthesis inhibitory activity in a cell-free system when assayed by the method described previously.[24]

Preparation of Anti-CEA Antibody Bearing Ricin or Ricin A-Chain Containing Liposomes——LUV were prepared by the reverse phase evaporation method of Szoka and Papahadjopoulos.[25] Briefly, 10 μmol of cholesterol, 9 μmol of PC and 1 μmol of PDP-PE were dissolved in 1 ml of diethyl ether. Then ricin (3—5 mg/ml) or modified ricin A-chain (13 mg/ml) in PBS (300 μl) was added, and the two phases were emulisified by sonication for 1 min at 20 °C in a bath-type sonicator. Ether was removed under reduced pressure at 23 °C. The resulting vesicle suspension was supplemented with 4.7 ml of PBS and then centrifuged at 50000 × *g* for 1 h to remove unentrapped protein. The pellets were resuspended in 2 ml of the SH-IgG solution (5 mg/ml) and the suspension was incubated at 4 °C for 24 h. Then, it was recentrifuged at 50000 × *g* for 1 h to remove unreacted SH-IgG. The precipitated pellet was suspended in 2 ml of saline. After passage through a 0.45 μm Millipore membrane, the suspension was used as the solution of antibody-bearing toxin-containing LUV (AbT-LUV).

SUV were prepared by bath sonication for 2 to 3 h of toxin-containing multilamellar vesicles (MLV). For the preparation of toxin-containing MLV, a lipid mixture identical in composition to that used for the preparation of LUV was dried under vacuum onto the walls of a glass tube. Then 2 ml of a modified ricin A-chain solution (13 mg/ml) in PBS was added, the mixture was dispersed by vortex agitation, and the MLV preparation was centrifuged at 3000 × *g* for 20 min to remove free toxins. In some experiments, the lipid mixture used contained a trace amount of [^{3}H]PC. Large vesicles were removed from the sonicated mixture by centrifugation at 100000 × *g* for 1 h. The SUV obtained were conjugated as above with thiolated antibody or reduced glutathione at 4 °C overnight. The conjugated vesicles were then separated from unbound substances by chromatography on a column of Sepharose CL-6B which had been equilibrated with PBS. The fraction containing the conjugated SUV was passed through a 0.2 μm Millipore membrane. For determination of the amount of entrapped ricin, vesicles were prepared in the presence of ^{125}I-ricin.

Binding Assay——The specific binding activity of the anti-CEA antibody-bearing toxin-liposomes was determined by the direct fluorescence test using liposomes in which fluorescein-labeled ricin was entrapped (Ab-FITC-lipo). Glutaraldehyde-fixed CEA-producing cells were incubated at 4 °C for 1 h with Ab-FITC-lipo in PBS in the presence of lactose to prevent the binding of FITC-ricin that had leaked from the liposomes. After being washed with PBS to remove unbound Ab-FITC-lipo, the stained cells were observed under a fluorescence microscope.

Cytotoxic Assay for AbT-LUV——Various cells (5×10^4 cells/well) were incubated with various concentrations of AbT-LUV (50 μl/well) at 37 °C for 1 h in RPMI 1640 medium containing 10% fetal calf serum (FCS) (RPMI-FCS). After being washed with the same medium, the cell suspension (200 μl/well) was further incubated at 37 °C for 48 h. The cells were then pulsed with L-[^{3}H]leucine for an additional 6 h and harvested with a multiple cell harvester, and the radioactivity incorporated into the cells was measured.

Reversal of the Cytotoxicity of AbT-LUV by Lactose——HLC-2 cells (5×10^4 cells/well) were incubated with a doubly diluted solution of AbT-LUV (50 μl/well) at 37 °C for 1 h. The cells were washed with RPMI-FCS, and suspended in RPMI-FCS containing lactose (0—50 mM). [^{3}H]Leucine incorporation was assayed as described above and compared with that of a positive control (without lactose).

Cytotoxicity Assay of AbT-SUV——CEA-producing cells (T3M-4) or non-CEA-producing cells (A375) (5×10^4 cells/well) were incubated with various concentrations of two kinds of toxin-SUVs (anti-CEA-bearing ricin A-chain-containing SUV or glutathione-bearing ricin A-chain-containing SUV) at 37 °C for 48 h. Then the cells were pulsed with [^{3}H]leucine and harvested as above for the assay.

Vesicle Size Estimation——A liposome mixture of AbT-LUV and AbT-SUV was separated by gel filtration on a column (2×90 cm) of Sepharose CL-2B, and their sizes were determined by observation under an electron-microscope after negative staining.

Cytotoxic Assay *in Vivo*——Human CEA-producing cells, T3M-4 (5×10^6 cells/mouse) were implanted into the subcutaneous space in the inguinal region of nude mice (BALB/c nu/nu). The effect of antibody-bearing liposomes on tumor growth was evaluated in terms of the area (mm^2) of a tumor, calculated from its largest and smallest diameters.

Results

Coupling of SH-Antibodies to PDP-PE Liposomes

The series of reactions for the preparation of AbT-liposomes (AbTL) is schematically depicted in Fig. 1. LUV (PC : cholesterol : PDP-PE = 9 : 10 : 1) were prepared by the reverse

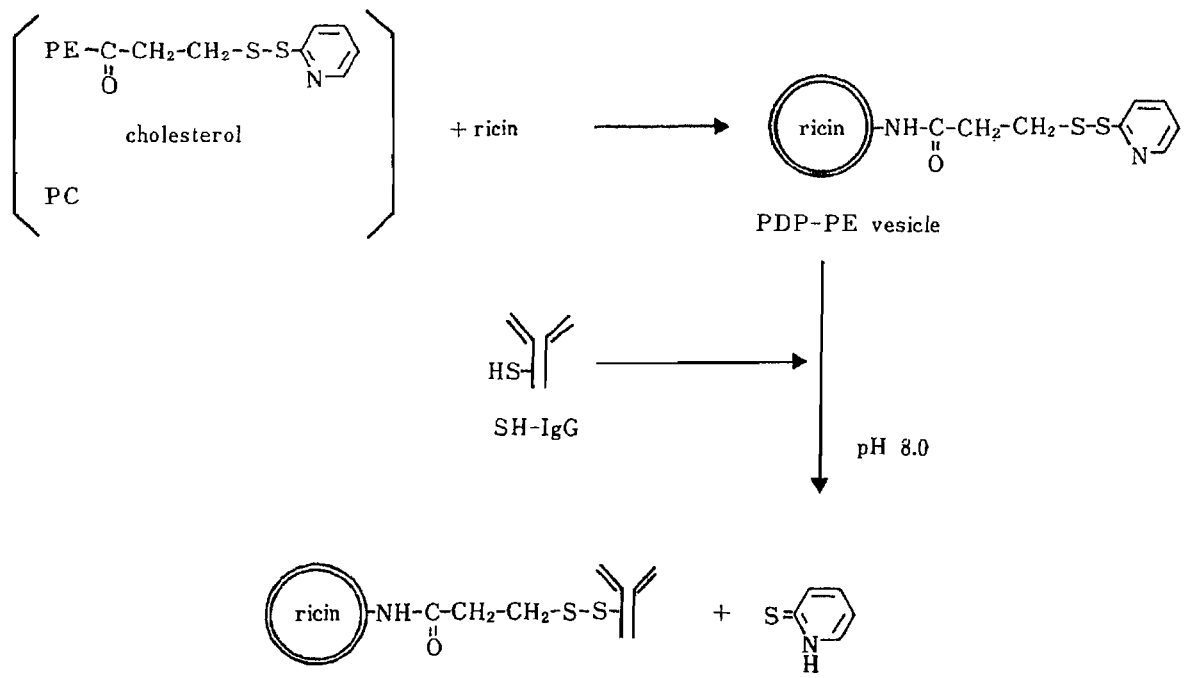

Fig. 1. Scheme for the Preparation of Anti-CEA Antibody-Bearing Ricin-Containing Liposomes

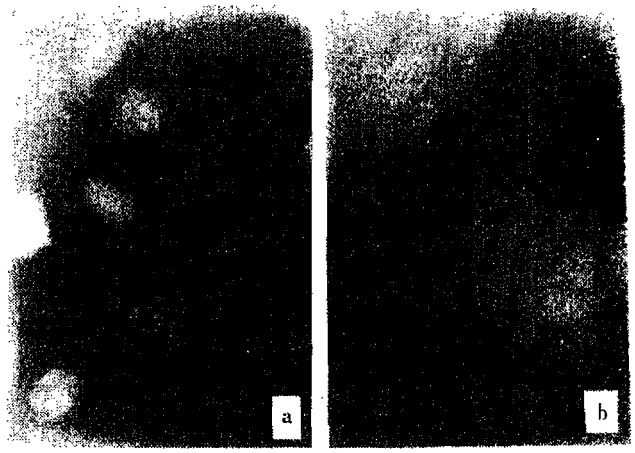

Fig. 2. Assay of Binding of Ab-FITC-Liposomes to CEA-Producing Cells (C-1 or T3M-4) (×400)

The fixed cells were incubated at 4 °C for 1 h with Ab-FITC-liposomes in the presence of 0.1 M lactose. The other experimental details are given in the text. a) C-1 cells; b) T3M-4 cells.

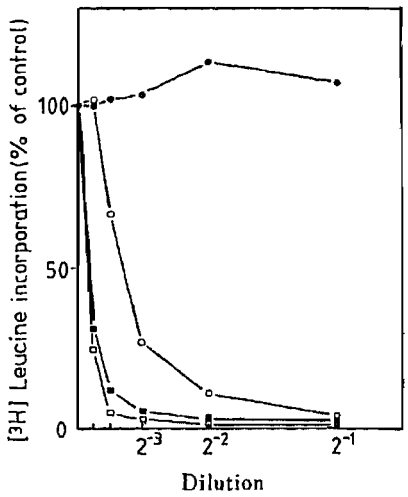

Fig. 3. Antitumor Activity of AbT-LUV on Various Cell Lines

A series of AbT-LUV solutions was prepared by successive two-fold dilutions of the original liposome solution (40 μg protein/5 μmol lipid/ml). The other experimental details are given in the text. ●—●, A375; ○—○, HLC-2; ■—■, C-1; □—□, T3M-4.

phase evaporation method with entrapment of about 14% of the original ricin solution, as judged from the radioactivity of ^{125}I-ricin entrapped in the liposomes. On the other hand, SUV could entrap less than 1% of the original ricin solution. Thiolated anti-CEA antibodies prepared as described in the experimental section contained an average of 1.8 SH groups/molecule. The PDP-PE liposomes were reacted with freshly prepared SH-IgG (5 mg/ml) at 4 °C for 24 h. Then, the LUV suspension was centrifuged at 50000×*g* for 1 h to remove unreacted SH-IgG. By measuring the absorbance of the supernatant at 343 nm, we calculated that the amount of SH-IgG coupled to the modified liposomes was about 30% of the total SH-IgG employed in the coupling reaction. In the case of SUV after separation by Sepharose CL-2B column chromatography, the coupling efficiency for SH-IgG was about 40—50%.

Assay of Binding of AbTL to CEA-Producing Cells

Glutaraldehyde fixed CEA-producing cells (T3M-4 or C-1) or non-CEA-producing cells (A375) were incubated with Ab-FITC-lipo in 0.1 M lactose–PBS to prevent the binding of free FITC-ricin that had leaked from liposomes to the cells. The cells were washed and observed under a fluorescence microscope (×400). Only CEA-producing cells were stained with the Ab-FITC-liposomes (Fig. 2). Therefore, AbT-liposomes bind to CEA-producing cells through the specific antibodies on the surface, and not through nonspecific interaction of the liposomes with the cells.

***In Vitro* Cytotoxicity of Abt-LUV toward Various Cell Lines**

CEA-producing cells (HLC-2, C-1 or T3M-4) or non-CEA-producing cells (A375) were incubated with AbT-LUV (60 μl/well) at 37 °C for 1 h. The tumor cells were washed and further cultured for 48 h, then pulsed with [^{3}H]leucine. The AbT-LUV showed cytotoxicity only toward CEA-producing cells (Fig. 3).

Effect of Various Toxin-LUV on CEA-Producing Cells (HLC-2)

CEA-producing cells (HLC-2) were incubated with ricin-LUV, anti-CEA-bearing ricin-containing LUV or anti-CEA-bearing ricin A-chain-containing LUV at 37 °C for 1 h, then

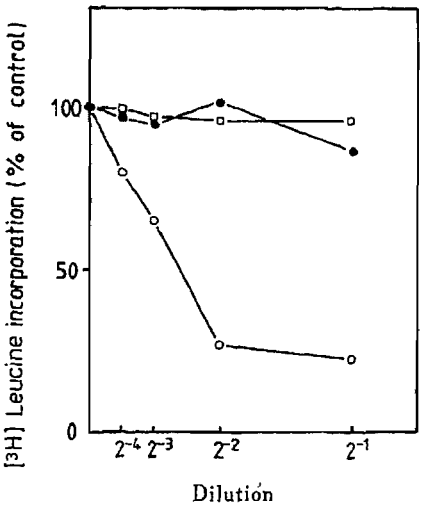

Fig. 4. Effects of Various Toxin-LUV on CEA-Producing Cells (HLC-2)

A series of liposome solutions was prepared by successive two-fold dilutions of the original liposome solution (40 μg protein/5 μmol lipid/ml). The other experimental details are given in the text. □—□, ricin-containing LUV (without antibody); ●—●, anti-CEA-bearing ricin A-chain-containing LUV; ○—○, anti-CEA-bearing ricin-containing LUV.

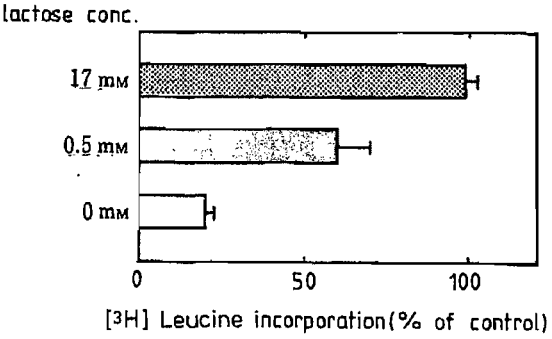

Fig. 5. Effect of Lactose on the Cytotoxicity of Anti-CEA-Bearing Ricin-Containing LUV toward HLC-2 Cells

The experimental details are given in the text.

washed. The inhibition of [^{3}H]leucine incorporation was assayed as described in the experimental section. Only anti-CEA-bearing ricin-containing LUV showed cytotoxicity toward the tumor cells, the other liposomes tested being not toxic at all (Fig. 4). Ricin A-chain is a toxic subunit of ricin and shows toxic activity only in a cell-free system or when it is internalized by cells. From these results, it can be concluded that AbT-LUV are neither internalized by tumor cells nor fused with tumor cells.

Reversal of AbT-LUV Cytotoxicity by Lactose

Lactose is known to inhibit the cytotoxicity of ricin through inhibition of the binding of the ricin B-chain (a binding subunit) to the cell surface.[26] In order to confirm that the cytotoxicity of AbT-LUV is due to the ricin released from them, HLC-2 cells were incubated with AbT-LUV at 37 °C for 1 h, washed with the medium, and then suspended in the same medium containing lactose at various concentrations (0—50 mM), and the cell suspension was further incubated at 37 °C for 48 h. As shown in Fig. 5, lactose (17 mM) completely inhibited the cytotoxicity of AbT-LUV, suggesting that the cytotoxicity of AbT-LUV was actually due to ricin which was released into the microenvironment from AbT-LUV after specific binding of the AbT-LUV to the tumor cell surface.

Effects of Various Toxin-SUV on CEA-Producing Cells (T3M-4)

CEA-producing cells (T3M-4) or non-CEA-producing cells (A375) were incubated at 37 °C for 48 h with two kinds of toxin-SUVs, anti-CEA-bearing ricin A-chain-containing SUV or glutathione bearing ricin A-chain-containing SUV, and then pulsed with [^{3}H]leucine. As shown in Fig. 6, tumor cytotoxicity was only seen when anti-CEA-bearing ricin A-chain-containing SUVs were incubated with CEA-producing cells. Anti-CEA-bearing ricin A-chain-containing SUV incubated with non-CEA-producing tumor cells, and glutathione-bearing ricin A-chain-containing SUV incubated with CEA-producing tumor cells had no effect on the tumor cells.

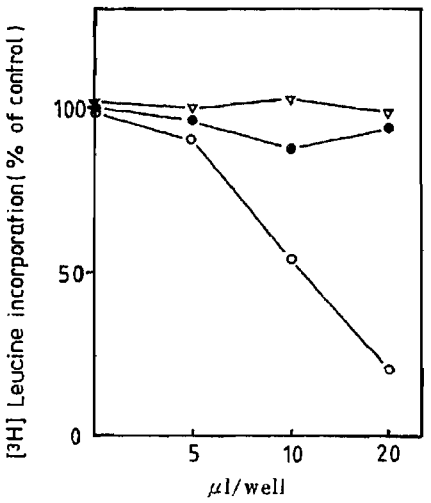

Fig. 6. Cytotoxic Activity of Two Kinds of Ricin A-Chain Containing SUV

The experimental details are given in the text. ▽—▽, glutathione-bearing ricin A-chain-containing SUV on A375 cells; ○—○, anti-CEA antibody-bearing ricin A-chain-containing SUV on T3M-4 cells.

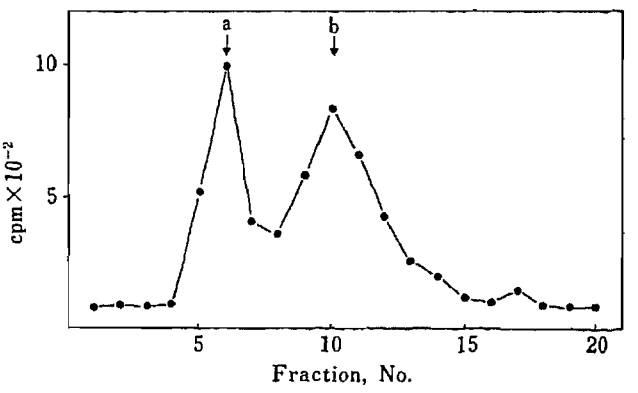

Fig. 7. Chromatography of Antibody-Bearing Liposomes on a Sepharose CL-2B Column

The experimental details are given in the text.

Vesicle Size Estimation

^{3}H-Labeled AbT-LUV and AbT-SUV were mixed and subjected to chromatography on a column of Sepharose CL-2B. The radioactivity of the vesicles was spearated into two peaks (Fig. 7). The vesicles in peaks a and b were observed under an electionmicroscope after negative staining. It was found that the average diameter of vesicles in peak a was about 3000 Å (range: 2000—4500 Å) and that of vesicles in peak b was about 800 Å (range: 600—1200 Å).

Discussion

We reported in the previous paper[11] the preparation of a conjugate between monoclonal anti-CEA antibody and ricin A-chain, and its antitumor activity. However, the results *in vivo* were not so satisfactory. One of the reasons was confirmed to be the instability of the ricin A-chain moiety of the conjugate.

On the other hand, many attempts have recently been made to attack tumors through the activation of the host's immunesystem with BRM, including lymphokines.[9,12-14] However, direct administration of BRM to the host is generally not effective, partly due to dilution in the serum and partly due to destinction by various enzymes *in vivo*.

Our major aim in the preparation of monoclonal anti-CEA antibody-bearing ricin-containing liposomes was to analyze the interaction of those liposomes target cells, particularly with regard to the size of the liposomes, and to establish a model for BRM-targeting that would allow accumulation of a BRM at a sufficient concentration around tumor cells by using a monoclonal antibody as a carrier, with protection of the BRM from enzymic attack through its encapsulation in liposomes.

Since most BRM (including lymphokines) are proteins and a protein for such a model should be one whose effect is easily detectable, we encapsulated a strong cytotoxin, ricin, in LUV which were large enough to hold a considerable amount of the protein,[25] and then bound anti-CEA antibody molecules to the surface of the liposomes. After these modifi-

cations, the activities of both the antibody on the liposome surface and ricin in the liposomes were ascertained to be retained, because AbT-liposomes bound to CEA-producing cells specifically and killed them effectively *in vitro* (Figs. 2 and 3). We also prepared anti-CEA-bearing LUV with entrapped ricin A-chain, a toxic subunit of ricin, but they did not show cytotoxicity toward CEA-producing cells (Fig. 4). Moreover, the cytotoxicity of the AbT-LUV with entrapped ricin was found to be inhibited by lactose, which is an inhibitor of ricin (Fig. 5).

On the basis of these results, the mechanism of the cytotoxicity of AbT-liposomes may be explained as follows: i) AbT-LUV bind to CEA moieties on the target cell surface; ii) the AbT-LUV burst on the tumor cell surface, releasing ricin; iii) the released ricin binds to the nearest tumor cell; and iv) the bound ricin is internalized by the tumor cell and kills it. In conclusion, AbT-LUV exert their cytotoxicity through increasing the toxin concentration around the tumor cells, *i.e.*, not through fusion or internalization.

On the other hand, AbT-SUV containing ricin A-chain can kill CEA-producing cells (Fig. 6), indicating that the ineffectiveness of AbT-LUV containing ricin A-chain is not due to the absence of B-chain. This also suggests that AbT-SUV are internalized by tumor cells, unlike AbT-LUV. This difference between LUV and SUV may be due to the difference in their diameters. The diameter of AbT-LUV was found to be about four times greater than that of AbT-SUV. There may be a threshold as to the size of liposomes for internalization into cells. The internalization of AbT-SUV was most probably effected by endocytosis, not by fusion, because AbT-LUV with the same lipid composition as AbT-SUV could not be internalized. Matthay *et al.*[27] also confirmed that small liposomes were pinocytosed more effectively, while larger liposomes could associated with the cell surface but were not subsequently internalized. Other investigators came to the same conclusion.[8,28]

Therefore, if we want to deliver drugs into cells directly, we have to use SUV, but if it is sufficient to produce an enhanced concentration of drugs around cells, we can use LUV. Various low-molecular-weight antitumor metabolic inhibitors should be suitable for delivery in SUV, while various BRM (including lymphokines) may be effectively delivered by means of LUV.

Considering that a large amount of liposomes is trapped in the reticuloendothelial system (kidneys, liver, *etc.*), BRM which activate immunocytes are more suitable than chemotherapeutic agents or cytotoxins as substances for entrapment in liposomes. The antitumor effects of antibody-bearing liposomes with various entrapped lymphokines *in vivo* are now under study in our laboratory.

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Lyophilized Liposomes Prepared by a Modified Reversed-Phase Evaporation Method

TETSUROU HANDA,* HIROFUMI TAKEUCHI, YUICHI OHOKUBO, and YOSHIAKI KAWASHIMA

Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan

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A modification of the reversed-phase evaporation method (modified REV method) was developed for the preparation of lyophilized unilamellar liposomes. The encapsulation efficiencies of the liposomes after a dehydration (lyophilization)–rehydration procedure were satisfactorily high, and the liposome sizes were maintained nearly constant throughout the procedure. These results are different from those obtained with liposome samples prepared by the reversed-phase evaporation method. In the latter case, marked enlargement of liposome size and extensive leakage from liposomes were observed. The small amount of residual ether in the modified REV liposomes keeps the lipid membranes fluid even at freezing temperature. The fluidity is considered to play an important role in the protection of liposomes against aggregation, fusion and leakage.

Keywords——lyophilized liposome; modified REV liposome; encapsulation in liposome; liposome size; membrane fluidity; membrane charge

Introduction

Liposomes have been considered to have great potential as local and systemic drug delivery vehicles.[1-3] From the pharmaceutical stand-point, the chemical and physical stabilities of "liposome particles" are critically important parameters affecting the performance of drug loaded liposomes *in vivo*. Freezing[4-6] and lyophilization[5,7,8] of liposomes have been considered as possible ways to improve the stability. As regards freezing of liposomes, the rupture of liposomes and leakage from liposomes are considerably reduced in glucose or dimethyl sulfoxide (DMSO) solution.[9] Frozen storage, however, is inconvenient, inappropriate in many situations, and lowers the usefulness of the liposomes. Lyophilization of liposomes has been investigated by Kirby and Gregoriadis,[7] Shulkin *et al.*[5] and Ohsawa *et al.*[10] The problems recognized in these studies[4,5,10] were remarkable enlargement of liposome size during lyophilization and the consequent destabilization of liposomes as colloidal particles. The lyophilized liposomes of large size were difficult to resuspend, and could be harmful if injected intravenously.

The reversed-phase evaporation method (REV method) yields unilamellar liposomes with a size distribution around 500 nm and with very high efficiency of encapsulation.[11,12] In this study, a modified REV method was developed for the preparation of lyophilized liposomes with high encapsulation efficiency. The liposomes manufactured by this method showed resistance to aggregation, fusion and leakage during the freeze-drying procedure. Encapsulation of reagents before and after a dehydration–rehydration cycle was examined after the separation of liposomes from the aqueous medium containing free reagent. The liposome size and the size distribution before and after the lyophilization–rehydration procedure were monitored by measurements of dynamic light scattering and freeze-fracture electron micrography.

Experimental

Materials——Soybean phosphatidylcholine (PC) was supplied by Nikko Chemicals Co., Ltd. (Epikuron 200, PC content: more than 97%). The sample was analyzed by high-pressure liquid chromatography, and showed a small peak due to lysophosphatidylcholine (less than 3%) besides the main peak of PC. Dicetylphosphate (DCP) was purchased from Sigma Chemicals Co. Stearylamine (SA) and cholesterol (Cho) were obtained from Nakarai Chemicals Ltd. Pyranine and methylene blue were purchased from Eastman Kodak Co. All solvents were obtained from Nakarai Chemicals Co., Ltd. Sepharose 4B was from Pharmacia Fine Chemicals AB.

Preparation and Lyophilization of Liposomes——The REV liposomes were manufactured according to the method of Szoka and Papahadjopoulos.[11] The modified REV liposomes were prepared as follows: PC, or lipid mixtures of PC and DCP or SA (66 μmol of total lipid) in chloroform solution were deposited on the side of a round-bottomed flask by removal of the solvent by rotary evaporation with water aspiration. The lipid was then redissolved in 3 ml of diethylether. An aqueous solution (1 ml) of pyranine or methylene blue in 5 mM Tris–HCl buffer (pH = 7.0) was added. The mixture was sonicated to give a W/O emulsion with a probe-type sonicator (UR-200P from Tomy Seiko Co., Ltd.) under N_2 at 0 °C for 5 min. A part of the ether was gently evaporated to give a viscous gel. The phase change from W/O emulsion to liposomes was induced by the dropwise addition of an excess amount of buffer (5 mM Tris–HCl, pH = 7.0, 45 ml) on a vibration-mixer. Therefore, in the liposomes prepared by the above method, a small amount of ether remained. Although the exact amount of residual ether was not determined, the sudden gelation could be taken as a clear end point of solvent removal (the reduction of the ether phase to a similar volume to the aqueous phase results in the gelation of W/O emulsion). In the REV method, on the other hand, the phase change was triggered by the more complete removal of ether, and an excess amount of buffer was added after the phase change. A brief comparison of the modified REV and REV methods is given in Fig. 1.

Liposomes were lyophilized after addition of mannitol as an adjunct for drying (5% of liposome suspension). Prefrozen liposome suspensions (at −70 °C) were dried under vacuum for 4 h. The temperature of the solvent condenser of the apparatus (Neocool from Yamato Co.) was kept at −110 °C. The rehydration of lyophilized liposomes was carried out by gentle combination with distilled water to give a volume of liposome suspension equal to that of the original suspension.

Determination of Percent Encapsulation——The encapsulation efficiencies of pyranine and methylene blue were

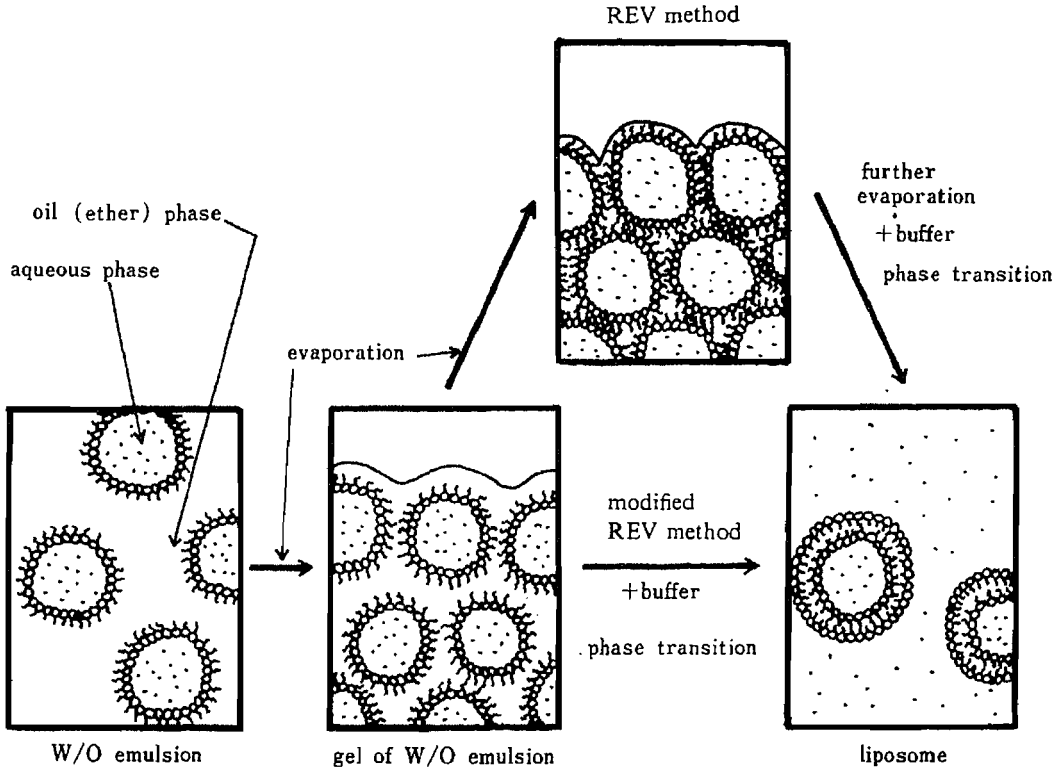

Fig. 1. Schematic Representations of Modified REV and REV Methods (See the Text)

The phase change (phase transition) from W/O emulsion to liposomes is induced in different ways in these methods.

estimated after separation of liposomes from the medium. Gel filtration was carried out on a Sepharose 4B column with 5 mM Tris–HCl. A part of the liposomes (with larger size) was not eluted through the column (*e.g.* 65% of liposomes composed of PC remained on the column). The separation was complete on centrifugation of the liposome suspension at 105000*g* for 30 min. Only a few percent of lipid remained in the supernatant fraction. The liposome fractions were solubilized in a surfactant solution (hexaethyleneglycolmonododecylether from Nikko Chemicals Co.) to eliminate turbidity. The fluorescence intensity of pyranine (excitation 400 nm, emission 510 nm) was measured with a Hitachi 650-60 spectrofluorometer, and the absorbance of methylene blue (662 nm) was recorded on a Hitachi 100-60 spectrophotometer.

Phosphate Assay——The amount of PC in the eluate from the Sepharose 4B column was determined by phosphate assay. The eluted PC sample was ashed in 5 N H_2SO_4–2 N HNO_3 and the concentration of inorganic phosphate was assayed by the Fiske–Subbarow method.[13] KH_2PO_4 solutions were employed as standards.

Measurement of Liposome Size——Freeze-fracture electron micrographs of liposomes before and after the lyophilization–rehydration procedure were taken with a Hitachi H-700 electron microscope. The detailed procedures have been presented elsewhere.[14] The dynamic (quasi elastic) light scattering was also measured and the data were analyzed by a cumulant method.[15]

Results

Percent Encapsulation

Liposomes encapsulating pyranine or methylene blue were separated from aqueous medium, which contained free reagents, by centrifugation at 10500*g* for 30 min. The percent encapsulation was defined as (moles of encapsulated reagent in liposomes)/(total moles of reagent in liposome suspension), and the results are shown in Table I. The anionic reagent, pyranine, was entrapped efficiently in both REV and modified REV liposomes composed of PC and 5 mol% of cationic lipid, SA. During the dehydration–rehydration procedure, however, remarkable reductions in the percent encapsulation were noted in the REV liposomes containing SA. The modified REV liposomes composed of PC + 5 mol% of anionic lipid, DCP, showed the reduction of encapsulation through the freeze-drying process. The electrical repulsion between pyranine, which has three negative charges, and the negatively charged liposomes prevents the effective association of the reagent with the membrane. Leakage may take place through possible small defects of the modified REV liposomes in the frozen state.

In the case of cationic methylene blue, although the REV liposomes composed of PC or PC and SA showed high entrapment, leakage took place during the dehydration–rehydration procedure. When 5 mol% of anionic lipid, DCP, was mixed with PC, both REV and modified REV liposomes gave good encapsulations. The modified REV liposomes showed more stable encapsulation than the REV liposomes through the lyophilization–rehydration procedure.

TABLE I. Percent Encapsulation Obtained by the REV and Modified REV Methods (Centrifugation Method)

Lipid	PC + 5 mol% SA		PC		PC + 5 mol% DCP	
	Before D–R[a]	After D–R[a]	Before D–R[a]	After D–R[a]	Before D–R[a]	After D–R[a]
Pyranine						
REV method	76.6	23.4	38.9	8.6	35.9	5.5
Modified REV method	72.0	79.4	13.1	8.8	21.2	0
Methylene blue						
REV method	24.6	14.2	50.0	17.2	29.2	20.3
Modified REV method	12.3	13.1	27.9	25.0	33.3	31.6

a) D–R = dehydration (lyophilization)–rehydration.

TABLE II. Effects of Lipid Amount in W/O Emulsion[a] on Percent Encapsulation (Centrifugation Method)

Lipid amount[b] (μmol)	Pyranine Percent encapsulation		Methylene blue Percent encapsulation	
	Before D–R[c]	After D–R[c]	Before D–R[c]	After D–R[c]
66	72.0	79.4	33.3	31.6
125	84.9	87.4	33.8	30.9
251	83.5	88.9	34.4	32.6
502	92.0	88.1	40.0	37.5

a) Oil phase, 3 ml of diethylether; water phase, 1 ml of 5 mM Tris–HCl. *b*) PC+5 mol% SA for pyranine and PC+5 mol% DCP for methylene blue. *c*) D–R=dehydration (lyophilization)–rehydration.

TABLE III. Effects of Cholesterol Addition on Percent Encapsulation (Centrifugation Method)

Lipid	Pyranine Percent encapsulation after D–R[a]	Lipid	Methylene blue Percent encapsulation after D–R[a]
PC+5 mol% SA	79.4	PC+5 mol% DCP	31.0
PC+5 mol% SA +33 mol% Cho[b]	57.0	PC+5 mol% DCP +33 mol% Cho[b]	35.5

a) D–R=dehydration (lyophilization)–rehydration. *b*) Cho=cholesterol.

These results on pyranine and methylene blue indicate that when an ionic lipid with electrical charge of opposite sign to that of the encapsulated reagent is mixed with PC, the modified REV method yields lyophilized liposomes of high percent encapsulation. Liposomes prepared by other methods (*e.g.* REV, surfactant dialysis and freeze-thawing methods) exhibited large reductions in the encapsulation amount.[16,17]

The effects of lipid amount on the encapsulation were investigated and the results are summarized in Table II. Slight increases of pyranine and methylene blue encapsulations with increase in lipid amount were seen. Here, the volumes of ether and water in the W/O emulsion were kept constant at 3 and 1 ml, respectively. The addition of cholesterol to lipid mixtures can affect the clearance of liposomes from blood.[18] The addition of cholesterol (33 mol% of total lipid) to the modified REV liposomes caused a reduction in the encapsulation efficiency of pyranine (Table III).

As described above, liposomes manufactured by the modified REV method were incompletely eluted through the Sepharose 4B column. The lipid amounts in the eluted fractions were 37, 36, and 90 mol% of the total lipid in liposomes composed of PC+5 mol% SA, PC, and PC+5 mol% DCP, respectively. Small unilamellar liposomes (prepared by sonication) were completely recovered in the solution eluted through the column. These results are similar to those reported by Sharma *et al.*[19] Electrical charges on the lipid membrane surface were found to affect the recovery of liposomes. Therefore, besides the size distribution of liposomes, the interaction between membrane charges and the Sepharose 4B gel bed seems to be important in relation to the recovery of liposomes. The maximum size of eluted liposomes depended on the sign of the surface charge. Further, when methylene blue was encapsulated in the PC–DCP liposomes, the lipid eluted was reduced to an amount similar to those in the cases of cationic and neutral liposomes.

TABLE IV. Comparison of Percent Encapsulations Obtained by the Centrifugation and Gel Chromatography Methods

Lipid	Centrifugation method		Gel chromatography method[a]	
	Before D–R[b]	After D–R[b]	Before D–R[b]	After D–R[b]
Pyranine				
PC+5mol% SA	72.0	79.4	15.0	22.0
PC+5mol% DCP	21.2	0	15.0	6.0
Methylene blue				
PC+5mol% SA	12.3	13.1	2.0	2.0
PC	27.9	25.0	3.0	5.0
PC+5mol% DCP	33.3	31.6	13.0	19.0

a) Calculated on the basis of Sepharose 4B column chromatography; the errors in the values are ±5%. *b*) D–R = dehydration (lyophilization)–rehydration.

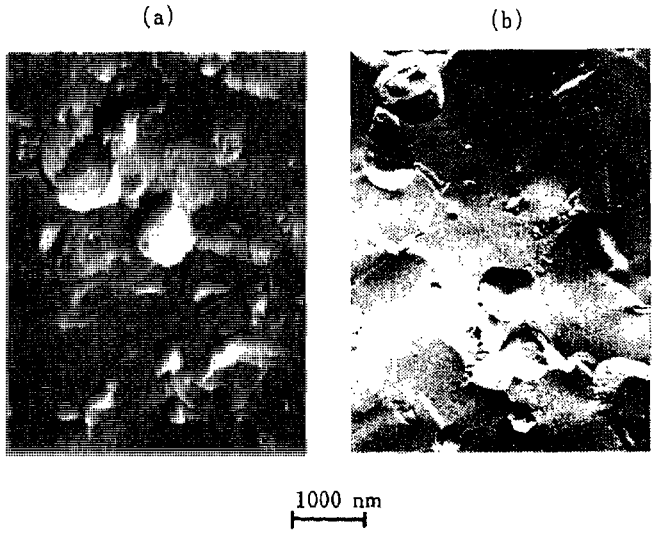

Fig. 2. Freeze-Fracture Electron Micrographs of Modified REV Liposomes before (a) and after (b) the Dehydration (Lyophilization)–Rehydration Procedure

The sizes and shapes of the liposomes were not changed significantly.

The percent encapsulations estimated after the separation of liposomes from the medium by centrifugation and by gel chromatography are compared in Table IV. Here, the percent encapsulations based on the gel chromatography method were calculated as (moles of reagent eluted with liposomes/fraction of lipid eluted)/(total moles of reagent in liposome suspension). It was found that the percent encapsulations calculated on the basis of the gel column chromatography were considerably lower than the values obtained by the centrifugation method. These results suggested that the smaller liposomes (eluted through the sepharose 4B column) had lower encapsulation efficiencies (*i.e.* lower encapsulation per unit weight of lipid). Larger liposomes remained on the column could have the larger internal aqueous volume and could have higher efficiencies of entrapment.

Freeze-Fracture Electron Microscopy

The sizes and shapes of the modified REV liposomes before and after the lyophilization–rehydration procedure were investigated on freeze-fracture electron micrographs. The results for modified REV liposomes containing PC and SA are presented in Fig. 2. It was found that the liposome sizes were not changed during the dehydration–rehydration procedure and were in the range of 300—800 nm (not homogeneous). These results are distinct from those obtained by the REV, surfactant dialysis[17] and freeze-thawing[6,10] methods. In the latter cases, the

increases in liposome size are very significant.

Dynamic Light Scattering

Dynamic (quasi elastic) light scattering of modified REV liposomes was measured. The cumulant analysis gave average diameters of 350—400 nm and polydispersity indexes of 0.5—0.6. These values were not appreciably changed through the freeze-drying process. The rather larger values of the indexes suggested broad size distributions of liposomes, probably with multiple peaks. When particles have a broad size distribution with multiple peaks, cumulant analysis does not give the real diameter; measurement by electron microscopy is more useful. The electron micrographs in Fig. 2 showed that the modified REV liposomes were composed of smaller (diameter = 300 nm) and larger (diameter = 800 nm) liposome particles.

Discussion

Liposomes in the Frozen State

In the frozen state, ice structure formation in the liposome suspension causes the separation of liposomes from the ice domains and leads to very high concentrations of liposomes in confined regions. This leads to aggregation and fusion of the liposomes. In the freeze-drying process, the sublimation of ice through the rigidly frozen membranes is also responsible for ruptures of the membrane. The creation of defects in the rigid liposome membrane during the freeze-drying process is considered to be a primary factor in liposome–liposome interactions.[20,21] Small unilamellar liposomes (20—50 nm) were frozen and thawed, and the sizes of the liposomes were monitored on electron micrographs; remarkable aggregations, fusions and enlargements of liposomes (1000 nm—) were found. It has been reported that cryoprotective substances (*e.g.* glucose) dissolved in the medium of liposome suspension help to maintain liposomes intact during a freezing.[9] Glucose and trehalose dissolved in an aqueous solution result in an amorphous glassy state at freezing temperature (0——70 °C) and, may decrease the stress of ice structure on the liposomes. These carbohydrates are also considered to prevent the crystallization and solidification of membrane lipids at low temperature, and to maintain the membrane structure. Trehalose and glucose are effective for the prevention of aggregation and fusion of liposomes at very low temperature.[7,22,23] The sublimation of ice from the glassy state of carbohydrate solution, however, was delayed markedly. Bubbling in the glassy state of liposome suspension was observed during the freeze-drying procedure and led to the destruction of liposomes and the leakage of encapsulated compounds from the liposomes. In conclusion, glucose and trehalose seems to be useful only for the storage of frozen liposomes.

In the modified REV liposomes, a small amount of ether remains and keeps the lipid membranes fluid even at low temperature where the aqueous medium is frozen. The fluidity plays an important role in the resistance of modified REV liposomes to rupture, aggregation and fusion during lyophilization. The residual ether in the modified REV liposomes can be completely removed by prolonged drying under vacuum.

Electrical Interaction between Liposome and Reagent

The results in Table I suggest that the electrical attractions between liposome membrane and reagent (SA^+-pyranine^{3-}, DCP^--methyleneblue$^+$) are also important for effective encapsulation in lyophilized liposomes. The electrical attractions enhanced the initial encapsulation both in the modified REV and in the REV liposomes. The binding and partitioning of the reagent to the oppositely charged lipid membrane may account for this. In the frozen state, the lipid membranes of the REV liposomes are solidified and, thereby, the binding and partitioning of the reagent are reduced, and leakage through ruptures or defects

of the membranes occurs. The decreased binding and partitioning coefficients of various compounds to rigid lipid membrane are well known.[24,25] On the other hand, in the modified REV liposomes, the fluidity of the lipid membranes and the electrical interactions between reagent and membranes act cooperatively to keep the reagent inside the liposomes. Cholesterol reduces the fluidity of liposomes and lowers the partitioning of hydrophilic reagent, pyranine, to the membrane, causing a decrease in encapsulation. The more hydrophobic reagent, methylene blue, was not affected by the addition of cholesterol.

Size and Homogeneity of Liposome Particles

As can be seen in Fig. 2, the size of liposomes manufactured by the modified REV method remained nearly constant throughout the lyophilization–rehydration process. The fragility of liposomes in the frozen state was greatly reduced by the enhanced fluidity due to the residual ether. Both the high efficiency of encapsulation and the resistance to aggregation and fusion during dehydration–rehydration of the modified REV liposomes are closely correlated with the fluidity of the liposomes at freezing temperature.

The results in Table IV show that the encapsulation efficiencies based on the centrifugation and gel chromatography methods are different. In the latter method, the values are calculated from the encapsulated amounts in the eluted smaller liposomes. These results indicate the dependence of encapsulation on liposome size. Liposomes of different size are known to show different behavior *in vivo*.[26,27] Therefore, the preparation of stable lyophilized liposomes of homogeneous size is critically important for the controlled delivery of drugs *in vivo*. Further investigations on the size control of liposomes are in progress in this laboratory.

Conclusion

1. A modification of the REV method was developed for the preparation of lyophilized unilamellar liposomes.
2. The method gave lyophilized liposomes of high encapsulation efficiency.
3. During a lyophilization–rehydration procedure, the size of the modified REV liposomes remained nearly constant and in the range of 300—800 nm.

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Interaction of Aminoglycosides with Heparan Sulfate from Rat Kidney

YUKIHIKO ARAMAKI,*[,a] ASAICHI INABA,[a] JUN-ICHI HOSODA[b] and SEISHI TSUCHIYA[a]

Tokyo College of Pharmacy,[a] 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan and Tokyo Medical College,[b] 7-1 Nishishinjuku, Shinjuku, Tokyo 160, Japan

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Heparan sulfate was purified from rat kidney cortex by a combination of affinity chromatography and ion exchange chromatography, and identified by cellulose acetate electrophoresis. As determined by the equilibrium dialysis method, heparan sulfate has two classes of binding sites for dibekacin and gentamicin, and has one class of binding site for amikacin. From the results of equilibrium dialysis using dextran sulfate and hyaluronic acid, the high affinity sites of heparan sulfate for gentamicin and dibekacin were considered to be the sulfate groups, and the low affinity sites to be the carboxyl groups.

Keywords——aminoglycoside; gentamicin; dibekacin; amikacin; rat kidney; heparan sulfate; acidic mucopolysaccharide; binding

Introduction

It is well known that aminoglycoside antibiotics are specifically accumulated within the kidney, and cause severe nephrotoxicity.[1,2] On account of the amino groups of aminoglycosides, they easily interact ionically with negatively charged substances, such as phosphatidylinositol,[3,4] chondroitin sulfate A[5] and adenosine triphosphate (ATP).[6] While these interactions are related to the accumulation and nephrotoxicity of aminoglycosides, there is no specificity of their localization in the kidney, and the compounds are distributed in various organs. Thus, it is still unclear why aminoglycosides have a high affinity for the kidney and are specifically accumulated there. Seno *et al.*[7] and Parthasarathy and Spiro[8] reported that one of the acidic mucopolysaccharides (AMPs), heparan sulfate, was present in large amount in rat kidney, especially in the renal glomerular basement membrane.

In this report, AMPs were prepared from rat kidney cortex, and purified by affinity chromatography. The purified AMP was identified as heparan sulfate by cellulose acetate electrophoresis. The binding of aminoglycosides to heparan sulfate was examained by the equilibrium dialysis method.

Materials and Methods

Material——Dibekacin (DKB), gentamicin (GM) and amikacin (AMK) were kindly given by Meiji Seika Ltd. (Tokyo), Shionogi Pharmaceutical Co., Ltd. (Osaka) and Banyu Pharmaceutical Co., Ltd. (Tokyo), respectively. Heparan sulfate (calf kidney) and standard AMP were obtained from Seikagaku Kogyo Co., Ltd. (Tokyo). Sephadex G-50, G-100, Sepharose CL-4B, and diethylaminoethyl (DEAE)-Sephadex A-50 were purchased from Pharmacia (Sweden). Male Wistar rats weighing 200—250 g were purchased from Tokyo Jikken Dobutsu (Tokyo).

Preparation of Acid Mucopolysaccharides——The preparation of AMPs was carried out by the method reported by Seno *et al.*[7] In brief, the kidney cortexes (21.6 g) isolated from 20 rats were homogenized with a Waring blender (15000 × rpm, 15 min) in cold acetone–ethanol (1 : 1) and stirred at 4 °C for 24 h. The homogenate was washed 3 times with ether and dried (yield 4.02 g). The defatted dried powder was suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ and treated with Pronase-P (Kaken Pharmaceutical Co., Ltd.) at 37 °C for 24 h. The digested

protein was deproteinized with trichloroacetic acid and dialyzed against water. The lyophilizate was applied to a Sephadex G-50 column equilibrated with distilled water to obtain crude AMPs (carbazol positive fraction in Fig. 1).

Preparation of DKB-Conjugated Sepharose——The preparation of DKB-conjugated Sepharose CL-4B was carried out by the method described in our previous report[9]; 3.9 nmol of DKB was bound to 1.0 mg of Sepharose CL-4B gel.

Electrophoresis——Cellulose acetate membrane (Sepharax, Jookoo Co., Ltd., Tokyo) electrophoresis was carried out with 0.25 M calcium acetate buffer for 4 h at the constant current of 1 mA/cm or with 1 M pyridine-acetic acid, pH 3.5 for 25 min at the current of 0.5 mA/cm.[10] The AMP was stained with alcian blue aqueous solution (0.5%).

Binding Study——The equilibrium dialysis method was employed. An aqueous solution (1 ml) of 1.0 mg of heparan sulfate containing various concentrations of aminoglycosides was dialyzed against 2.0 ml of 10 mM Tris-HCl buffer (pH 7.0) at 25 °C for 24 h. The amount of aminoglycosides bound to heparan sulfate was determined from the concentration of aminoglycosides in the outside compartment. The non-specific binding of aminoglycosides to the dialysis membrane or vessels was negligible.

Analytical Method——The concentration of aminoglycosides was determined by the high performance liquid chromatography (HPLC) method.[11] Hexuronic acids and protein were determined by means of the carbazole reaction[12] and the method of Lowry *et al.*,[13] respectively.

Results

Purification of Acidic Mucopolysaccharide——The carbazole-positive fractions (fr. 10—fr. 25) of the Sephadex G-50 eluates of AMP were collected and lyophilized (Fig. 1). The lyophilizate (35 mg) was applied to an affinity column of DKB-conjugated Sepharose CL-4B pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Ninety percent of the applied sample was adsorbed on the DKB-conjugated affinity column, and eluted with Tris-HCl buffer containing 1.0 M NaCl (Fig. 2). The carbazole-positive fraction of Sepadex G-50 chromatography had no affinity for the Sepharose CL-4B column. The bound fraction (28 mg) in the affinity column was loaded onto a DEAE-Sephadex A-50 column (2.5 × 40 cm) equilibrated with distilled water, and was eluted stepwise with various concentrations of NaCl. An about

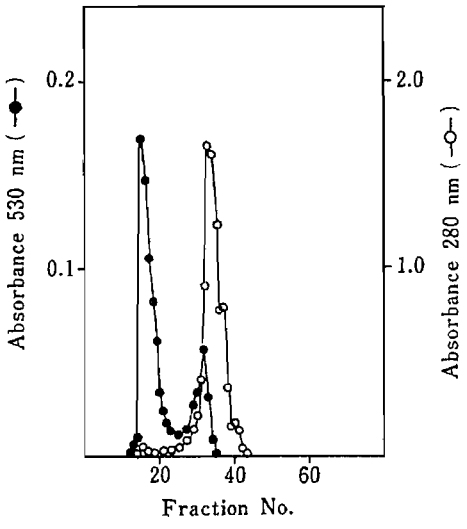

Fig. 1. Gel Filtration of AMP on Sephadex G-50

The crude AMP was extracted from rat kidney cortexes.
●, hexuronic acids; ○, proteins.

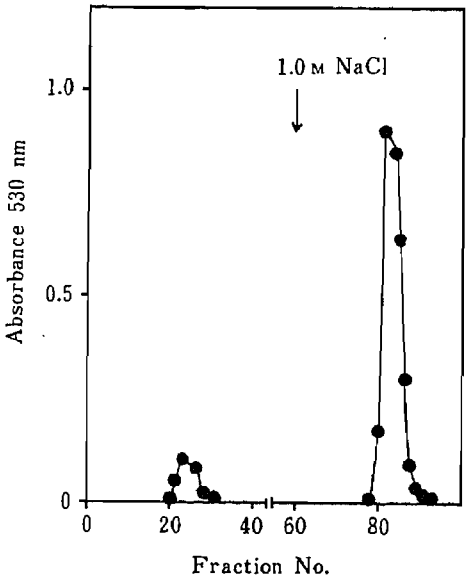

Fig. 2. DKB-Conjugated Sepharose CL-4B Column Chromatography of AMP

The arrow indicates the change of the elution buffer from 10 mM Tris-HCl buffer (pH 7.0) to the same buffer containing 1.0 M NaCl. The column size was 2.0 × 12.0 cm, and fraction of 2.0 ml each were collected.

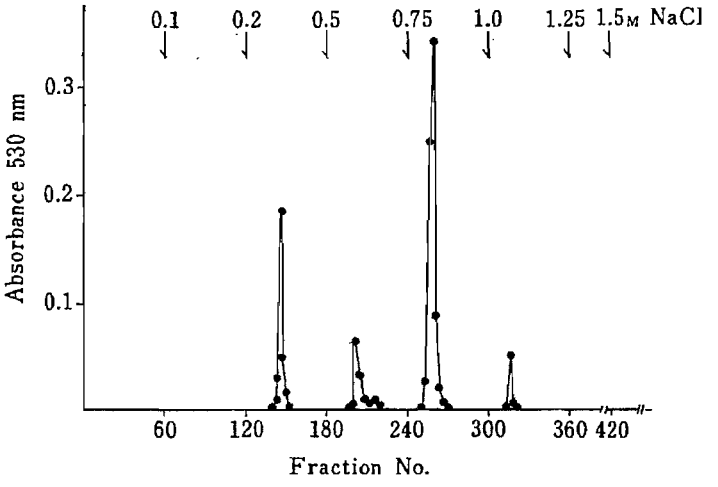

Fig. 3. DEAE-Sephadex A-50 Column Chromatography of AMP Having Affinity for DKB-Conjugated Sepharose CL-4B

A DEAE-Sephadex A-50 column was equilibrated with distilled water and was eluted stepwise with various concentrations of NaCl. The column size was 2.5×40 cm and fractions of 5.0 ml each were collected.

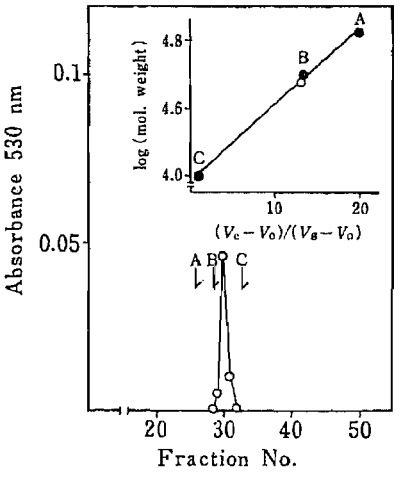

Fig. 4. Determination of the Molecular Weight of Heparan Sulfate on Sephadex G-100

Dextrans with molecular weights of 7×10^4 (A), 4×10^4 (B) and 1×10^4 (C) were used as standards, and the elution positions are indicated by the arrows. Heparan sulfate from rat kidney cortex is indicated by open circles.

70% of the applied sample was recovered in the 0.75 M NaCl-eluted fraction (Fig. 3). This fraction was further applied to a Sephadex G-100 column pre-equilibrated with 0.5 M NaCl solution and eluted at the rate of 0.4 ml/min. A single peak was obtained and its molecular weight was determined by using dextran of various molecular weights as standards (Fig. 4). From the elution volume, the molecular weight of the purified AMP was estimated to be 3.6×10^4.

On cellulose acetate electrophoresis, the purified AMP gave a single spot as detected by alcian blue staining. The migration was compared with those of six kinds of AMPs as standards, and the migration distance was the same as that of heparan sulfate from calf kidney (Fig. 5). Furthermore, the chromatographic behavior of heparan sulfate in DKB-conjugated affinity and DEAE-Sephadex A-50 chromatography was the same as that of the purified AMP from rat kidney (data not shown).

Binding of Aminoglycosides to Heparan Sulfate——The bindings of aminoglycosides to heparan sulfate purified from rat kidney and commercially available heparan sulfate from calf kidney were studied by the equilibrium dialysis method. Curved Scatchard plots were obtained for GM and DKB, and a linear regression line was obtained for AMK. These results

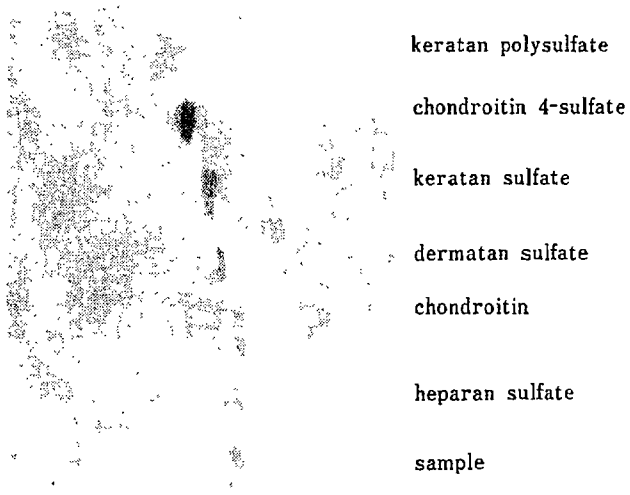

Fig. 5. Cellulose Acetate Electrophoresis of AMP

Electrophoresis was carried out using 0.25 M calcium acetate buffer.
AMPs were stained with 5% alcian blue.

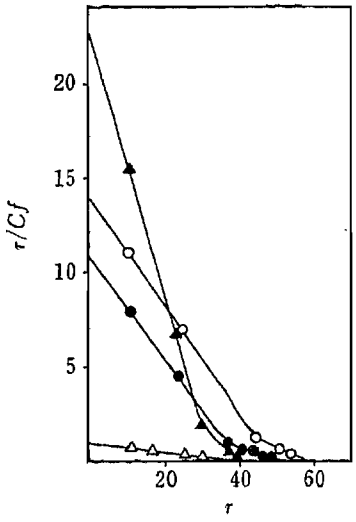

Fig. 6. Scatchard Plots of Aminoglycoside Binding to Heparan Sulfate

●, DKB-heparan sulfate (rat kidney); ○, DKB-heparan sulfate (calf kidney); △, AMK-heparan sulfate (calf kidney); ▲, GM-heparan sulfate (calf kidney).

TABLE I. Binding Parameters of Aminoglycosides and Heparan Sulfate

Antibiotic		Rat kidney HS		Calf kidney HS	
		$K^{a)}$	n	$K^{a)}$	n
Gentamicin	High	—	—	3.42	9.6
	Low	—	—	0.27	28.2
Dibekacin	High	0.46	24.0	0.59	13.0
	Low	0.13	29.6	0.14	31.7
Amikacin		—	—	0.05	36.0

a) $\times 10^6\,M^{-1}$

TABLE II. Binding Parameters of Dibekacin and Dextran Sulfate or Hyaluronic Acid

Substance	Binding parameter	
	$K^{a)}$	n
Dextran sulfate	0.80	8.5
Hyaluronic aicd	0.24	73.5

a) $\times 10^4\,M^{-1}$.

suggest that heparan sulfate has at least two classes of binding sites for GM and DKB with different affinities, and has a single class of binding site for AMK. The binding parameters are summarized in Table I, and almost the same association constants were obtained for DKB with calf heparan sulfate and with the purified AMP from rat kidney.

Binding of GM to Dextran Sulfate or Hyaluronic Acid——The binding affinities of GM for sulfate and carboxyl groups in dextran sulfate and hyaluronic acid were compared by the equilibrium dialysis method. The Scatchard plot was linear in both cases, and the binding parameters are shown in Table II. The binding constant (K) of GM to dextran sulfate was 3-times higher than that to hyaluronic acid.

Discussion

The acidic mucopolysaccharides (AMPs) were isolated from rat kidney. The purification of AMPs was carried out by affinity chromatography on DKB-conjugated Sepharose CL-4B,

because AMP has affinity for DKB. This purified acidic mucopolysaccharide was further purified by using a DEAE-Sephadex A-50 column, and about 70% of the applied AMP was recovered in the 0.75 M NaCl fraction. On cellulose acetate electrophoresis, the migration distance of this fraction was the same as that of heparan sulfate from calf kidney. Small amounts of AMP were recovered in the 0.2, 0.5 and 1.0 M NaCl fractions. Seno *et al.*[7] reported the purification of AMP obtained from rat kidney by Dowex 1 column chromatography; they recovered heparan sulfate in the 1.25 M NaCl fraction and small amounts of hyaluronic acid, dermatan sulfate, and chondroitin sulfate were obtained in the 0.25, 0.5 and 1.5 M NaCl fractions, respectively. Thus, the three minor peaks on DEAE-Sephadex A-50 chromatography may represent hyaluronic acid, dermatan sulfate and chondroitin sulfate. The molecular weight of the purified AMP was determined to be 36000 from the elution volume on the Sephadex G-100 column. Praag *et al.*[14] reported that the molecular weight of heparan sulfate obtained from rabbit kidney was 14000. This difference may be caused by the difference in the animal species or in the preparation method.

Heparan sulfate has two different negatively charged groups, carboxyl and sulfate groups, in its molecule. GM and DKB bind to heparan sulfate with two different association constants (high and low affinity constants), and the high affinity binding may represent the interaction between the amino group of the aminoglycoside and the sulfate group of heparan sulfate. Thus, the binding of GM with sulfate and carboxyl groups was examined by using dextran sulfate and hyaluronic acid. Indeed, GM has a higher affinity for dextran sulfate than hyaluronic acid, as shown in Table II, and this difference is almost the same as that of the high and low affinity sites of heparan sulfate (Table I). Thus, the high and low affinity sites represent the sulfate and the carboxyl groups, respectively. The difference of binding sites (n) probably arise from the difference in the molecular weights, because dextran sulfate (mean M.W. = 5000) and hyaluronic acid (mean M.W. = 50000) were used in this experiment. In the case of AMK, heparan sulfate has one class of binding site, and the binding constant (K) was very small compared with those of DKB and GM (Table I). These differences among GM, DKB, and AMK may be attributed to the structural differences of aminoglycoside. As regards the intensity of high affinity binding ($n \times K$), values of 33.0, 7.7, and 0.9 were obtained for GM, DKB, and AMK, respectively. The order of these values corresponds to that of nephrotoxicity (GM > DKB > AMK). Thus, further studies of the interaction between aminoglycoside and heparan sulfate should improve our understanding the mechanism of nephrotoxicity.

The above results suggest that the binding of aminoglycoside to heparan sulfate may be one of the initial steps involved in the manifestation of nephrotoxicity

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Adsorption of Drugs on Microcrystalline Cellulose Suspended in Aqueous Solutions

SATOSHI OKADA,* HIROSHI NAKAHARA, and HIROSHI ISAKA

National Institute of Hygienic Sciences, Osaka Branch, 1-1-43, Hoenzaka, Higashi-ku, Osaka 540, Japan

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The adsorption of various drugs on microcrystalline cellulose (MCC) suspended in aqueous solutions was investigated. Adsorption of diphenhydramine, chlorpheniramine, isoniazid, and *p*-aminobenzoic acid was very slight or negligible, whereas the adsorption of four phenothiazine derivatives (PTZs) was considerable, and that of acrinol was quite large. Except for chlorpromazine sulfoxide, adsorption isotherms for three PTZs and acrinol were all of the Freundlich type. Based on the Freundlich constants, k and $1/n$, the adsorbability of those drugs on MCC was in the order acrinol $\gg$ chlorpromazine > triflupromazine > promazine. The slight differences in $1/n$ for those drugs suggest differences in the adsorption mechanisms. Further it was observed that the adsorption increases with increase of pH, and decreases with increase of ionic strength. In addition, the pH values for MCC suspended solutions decrease gradually with the addition of neutral salts. Thus, in the adsorption of PTZs and acrinol on MCC, the ion-exchange mechanism appeared to play an important role, but adsorption due to non-electrostatic forces should also be appreciable. Marked adsorption of acrinol on MCC can be interpreted in terms of the presence of polar groups capable of hydrogen-bond formation, and the coplanar arrangement of the acridine ring.

Keywords——microcrystalline cellulose; phenothiazine derivative; acrinol; adsorption isotherm; Freundlich-type; adsorption mechanism

Microcrystalline cellulose (MCC) has been widely used in the field of drug formulations as a diluent, disintegrant, and binder. It is considered to be an excellent excipient in the preparation of direct-compressed tablets. Despite its extensive utilization, very few studies have been undertaken to clarify the interaction of drugs with MCC.[1] Certain steroids, phenothiazines, antihistaminics, and antibiotics were reported to be absorbed on MCC from aqueous solutions, but the adsorption mechanism is still obscure. For example, as regards the effect of pH on adsorption and desorption of phenothiazines by MCC, contradictory results were obtained by Nyqvist *et al.*[1a] and Frantz and Peck.[1b] In addition, the adsorption experiments were performed only in the relatively low concentration range (below 4×10^{-5} mol/l),[1b] and the adsorption equilibrium was apparently attained in the short time of 30—60 min. Furthermore, neither the molecular specificity of drug adsorption on MCC nor the relation between the fibrous structure of MCC and the molecular structures of drugs were taken into account in their studies.

In the present study, it was intended to clarify the characteristics of drug–MCC interactions by studying the adsorption behavior of several drugs under various conditions of pH, ionic strength, and temperature. Elucidation of general features of drug–MCC interaction should facilitate the more effective utilization of the excipient.

Experimental

Materials——MCC, PH-101 and PH-301, were provided by Asahi Chemical Industry Co., Ltd. and used

without further purification. Four kinds of phenothiazine derivatives (PTZs) were used; chlorpromazine (CPZ)·HCl and chlorpromazine sulfoxide (CPZSO)·HCl were supplied by Yoshitomi Pharmaceutical Co., Ltd., promazine (PZ)·HCl by Shiratori Pharmaceutical Co., Ltd., and triflupromazine (TFPZ)·HCl by Nippon Squibb Co., Ltd. Ethacridine lactate monohydrate(acrinol)[2] was purchased from Wako Pure Chemical Industry Co., Ltd. Two antihistaminics, diphenhydramine·HCl (DPH) and chlorpheniramine maleate (CPM), were supplied by Tokyo Kasei Kogyo Co., Ltd. and Iwaki Pharmaceutical Co., Ltd., respectively. Isoniazid (INAH) and *p*-aminobenzoic acid (PABA) were obtained from Shionogi Pharmaceutical Co., Ltd. and Wako Pure Chemical Industry Co., Ltd., respectively. Except for PABA, all drugs used in this study were of either JP or specific reagent grade, and were used without further purification. PABA was purified twice from methanol/water. All other chemicals were of specific reagent grade and were used as received.

Adsorption Studies——Analytical Procedure: Drug concentrations were determined spectrophotometrically on a Hitachi 100-60 spectrophotometer. The analytical wavelengths(nm) used in the spectrophotometric determination of the drugs were as follows; PZ (251), CPZ (254), TFPZ (254). CPZSO (239), acrinol (269), DPH (230), CPM (261), INAH (261), and PABA (264). The Ultraviolet (UV) spectra of PZ, CPZ, TFPZ, and acrinol were independent of pH in the range of pH 2—8, because of their strong basicity. Thus, pH adjustment within pH 2—8 was not necessary in spectrophotometry.

Adsorption Isotherms: The desired quantity of 5×10^{-3} or 2×10^{-2} mol/l stock solution of a drug was added to a mixture of 24 ml of phosphate buffer solution and a weighed amount of MCC (0.2 g for the acrinol–MCC system and 1.0 g for all other drug–MCC systems) in an amber 50 ml centrifugal tube with a glass stopper.

Initial drug concentration ranged from 1×10^{-5} to 3.2×10^{-4} mol/l. Except for special adsorption experiments described below, the final pH, ionic strength, and volume (ml) were adjusted to 6.92, 0.04 and 25, respectively. The tubes were placed in a thermostatted incubator set at 15, 25, 35, or 45±0.1 °C, and shaken for 12—60 h. After the attainment of adsorption equilibrium, about 3—5 ml of the supernatant was taken and filtered through a membrane filter (0.45 μm, Millipore HV013NS). Control experiments, in which no drug was added, were done in parallel, and the filtrate was used as a reference solution. With or without dilution of the filtrate to a drug concentration of 0.5×10^{-5}—2×10^{-5} mol/l, the concentration at equilibrium, $[D]_f$ was determined by the above-mentioned procedure. Hence the adsorbed amount, $[D]_b$ can be represented by Eq.1,

$$[D]_b = ([D]_i - [D]_f)V/1000m \quad (1)$$

where $[D]_i$ is the initial drug concentration (mmol/l), m the amount of the adsorbent (g), and V the volume of the drug–MCC suspension. Then $[D]_b$ is expressed in the unit of mmol/g MCC.

Effect of pH on Adsorption——The effect of pH on the adsorption of PTZs and acrinol on MCC was studied in the range of pH 1.7—7.8 at 25 °C. Values of pH were adjusted by means of HCl–NaCl or NaOH–NaCl solutions, holding the ionic strength constant at $I=0.05$. Initial drug concentrations were fixed at 2×10^{-5} mol/l for PTZs and 1.5×10^{-5} mol/l for acrinol. The magnitude of drug adsorbability to MCC was conventionally expressed as fraction bound $(\%)=([D]_b/[D]_i) \times 100$.

Effect of Ionic Strength on Adsorption——The effect of ionic strength on the adsorption of CPZ on MCC was investigated at constant pH 6.92 at 25 °C. Ionic strength was adjusted by the addition of NaCl in the range of 0.01—0.52. Initial drug concentration was 2×10^{-5} mol/l, and the expression of the adsorbability was the same as that described above.

Cation-Exchange Ability of MCC——The cation-exchange ability of MCC was examined simply by monitoring the pH of MCC suspended aqueous solutions (1 g/25 ml) upon addition of a neutral salt such as NaCl, KCl, CPZ·HCl, and acrinol (ethacridine lactate), using a precision pH meter (EA 510) equipped with a combined glass electrode (EA 125) (Metrohm Co., Ltd.).

Results and Discussion

Adsorption Isotherms

Figure 1 shows adsorption isotherms for PZ, CPZ, CPZSO, acrinol, and CPM at pH 6.92 and at 25 °C; the abscissa indicates the equilibrium drug concentration, and the ordinate shows the adsorbed amount of drug. As shown in Fig. 1, the adsorption of CPM on MCC was very slight, that of PTZs (PZ, CPZ, CPZSO) was considerable, and that of acrinol was quite large. Although the adsorption isotherms for DPH, INAH, PABA, and TFPZ on MCC are not shown in Fig. 1, significant adsorption was not found for the first three drugs, while for the last drug the adsorption was comparable with that of CPZ and the isotherm is omitted for clarity. Adsorption isotherms for three PTZs and acrinol appeared to be not of Langmuir type, but of Freundlich type. However, the isotherm for CPZSO was sigmoid and clearly

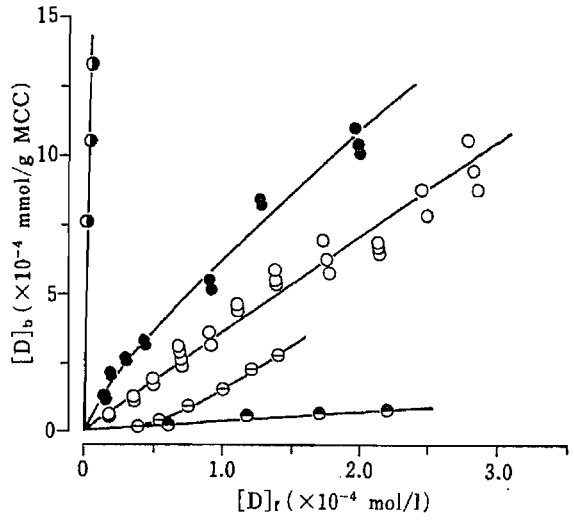

Fig. 1. Adsorption Isotherms for Several Drugs on MCC, PH-101, at pH 6.92 at 25 °C

○, PZ; ●, CPZ; ⊖, CPZSO; ⊜, CPM; ◑, acrinol.

different from those for other PTZs. Though this type of adsorption is often seen in the cooperative binding of surfactants to protein and/or polymer,[3] further analysis of the adsorption was not performed in this study.

The Freundlich type adsorption is described by Eq. 2, where x is the adsorbed amount of drug per unit weight of

$$x=k[D]_f^{1/n} \tag{2}$$

MCC (mmol/g), $[D]_f$ the equilibrium drug concentration (mol/l), and k and $1/n$ are constants. Equation 2 is an empirical one, and the physicochemical meanings of those constants are not always clear, but it is likely that the former gives a rough measure of the relative adsorbent capacity for a given adsorbate, while the latter reflects the affinity of the adsorbate for the adsorbent.[4] The logarithmic expression of Eq. 2 is:

$$\log x=\log k+(1/n)\log [D]_f \tag{3}$$

Thus, if a linear relation between $\log x$ and $\log [D]_f$ is obtained, the constants k and $1/n$ are given by the values of the intercept and the slope, respectivity. Figures 2 and 3 show Freundlich plots, according to Eq. 3, for the CPZ–MCC and acrinol–MCC systems at pH 6.92 and at various temperatures, respectively. In these plots data points are shown only for the adsorption system at 15 °C, for simplicity. Linear relations were obtained for all of the drug–MCC adsorption systems, and the intercept and the slope were calculated by linear regression analysis.

Freundlich constants thus obtained for various drug–MCC systems are summarized in Table I. As mentioned above, since the constant k may be a rough measure of the relative adsorbent capacity for a given adsorbate, it was considered that the greater the value of k, the larger the adsorption of a given drug. Thus the magnitude of the adsorption of the present drugs on MCC is in the order,

$$\text{acrinol} \gg \text{CPZ} > \text{TFPZ} > \text{PZ}$$

regardless of the kind of MCC, PH-101 or PH-301. The rather strong adsorption of acrinol by MCC will be discussed later. It is interesting that the order of adsorption on MCC among three PTZs is different from that of hydrophobicity. That is, although TFPZ has the greatest hydrophobicity, its adsorption seems to be smaller than that of CPZ. Thus, it may be considered that although the hydrophobicity of the adsorbate is important as a driving force for the penetration into narrow inter-fiber space of the cellulose, the bulkiness of the

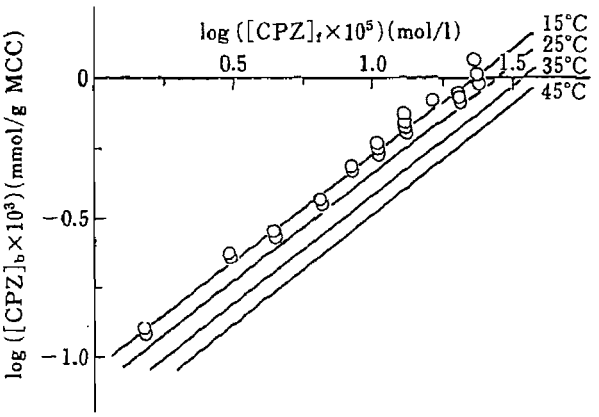

Fig. 2. Freundlich Plots for the Adsorption of CPZ on MCC, PH-101, at pH 6.92 at Various Temperatures

Data points are shown only for the adsorption system at 15 °C. Linear regression analysis gave the indicated solid lines at different temperatures (15, 25, 35, 45 °C).

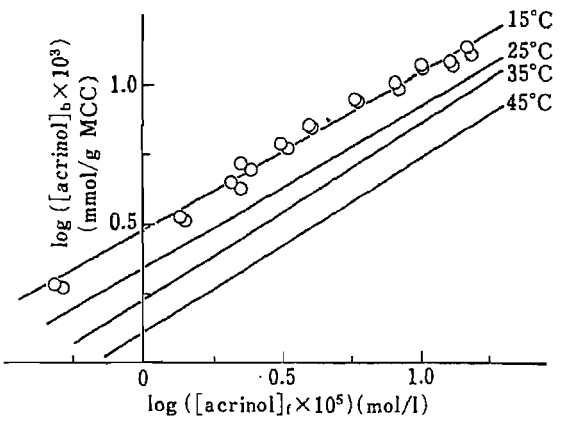

Fig. 3. Freundlich Plots for the Adsorption of Acrinol on MCC, PH-101, at pH 6.92 at Various Temperatures

Details are the same as described in Fig. 2.

TABLE I. Freundlich Constants[a] of Three PTZs and Acrinol Adsorbed on MCC, (A) PH-101 and (B) PH-301, from Aqueous Solutions at pH 6.92 and at Various Temperatures

Drug	Freundlich constant	Temperature (°C)			
		15	25	35	45
			(A) PH-101		
PZ	1/n	0.944	0.985	1.01	
	k	3.69	2.57	2.04	
CPZ	1/n	0.744	0.775	0.780	0.805
	k	9.12	7.67	6.17	5.13
TFPZ	1/n		0.647		
	k		5.68		
Acrinol	1/n	0.563	0.580	0.637	0.626
	k	30.1	21.8	16.8	12.8
			(B) PH-301		
PZ	1/n		1.04		
	k		2.76		
CPZ	1/n	0.715	0.683	0.789	
	k	10.9	10.6	11.6	
TFPZ	1/n		0.702		
	k		8.63		
Acrinol	1/n		0.599		
	k		17.4		

a) Freundlich constant k is shown as 10^5 times the value, in units of mmol/g MCC.

substituent at the 2-position (-CF_3 group for TFPZ) probably inhibits deep penetration. Furthermore the tendency of the value of k to decrease in all adsorption systems with increasing temperature might mean that the adsorption process is exothermic, as is the case for general adsorption systems.

As regards the kind of MCC, PH-101 is a standard grade, while PH-301 possesses a higher density and flow-ability. Differences between them appear mainly in the powder

characteristics, and may arise from differences in the physicochemical structure of cellulose microcrystallite and/or aggregates. That is, in the process of preparation, aggregation or clustering of cellulose microcrystallites, which are assumed to be the minimum unit forming MCC particles and to be composed of crystalline (or micellar) and amorphous regions, occurs to a significant extent. The values of k for PTZs appear to be higher for PH-301 than PH-101, while the reverse relation is found for acrinol. The small differences in adsorbent capacity of the two MCCs may reflect the difference in the cellulose microcrystallite and/or cluster structure, but further study is necessary to establish this.

The physical meaning of the constant, $1/n$, is not clear at present. If the constant is assumed to be a measure of the affinity of the adsorbent for the adsorbate,[4] PZ will have the strongest affinity with MCC. This conclusion is inconsistent with the observed low adsorption of PZ, so variations of the constant may reflect differences in the adsorption mechanisms.[5] In the present case, $1/n$ for PZ–MCC is nearly equal to unity, which indicates that the adsorption is of partition type rather than Freundlich type.

The adsorption mechanisms for CPZ and TFPZ on MCC were suggested to be similar, and slightly different from that for PZ. Further, the relatively low value of $1/n$ for acrinol–MCC indicates that the adsorption isotherm is moderately curved with increasing drug concentration, and shows some similarity to Langmuir-type adsorption. In the work of El-Samaligy *et al.* on the adsorption of antibiotics(ampicillin and amoxycillin) on MCC, the adsorption was assumed to be of Langmuir type, though the saturated adsorption region was not clearly shown in their adsorption isotherms.[1c] Thus, the adsorption can probably also be described by a Freundlich-type equation. Since the intermediate part in Langmuir-type adsorption can usually be approximated by Freundlich-type adsorption,[6] the possibility that the present adsorption isotherms represent a Langmuir-type adsorption cannot be ruled out completely. On the other hand, the temperature dependence of $1/n$ was very small, and a slight increasing tendency with increase of temperature was observed for CPZ–MCC and acrinol–MCC systems. This might reflect a slight structural change of cellulose microcrystallites.

pH Dependence of Adsorption

Adsorption of CPZ was pH-dependent, as shown in Fig. 4. It was lower in the acidic region below pH 4, and increased gradually with increase of pH, becoming nearly to a constant above pH 6.5. Similar pH dependence was observed for other PTZ–MCC and acrinol–MCC systems. The present results support the data of Frantz and Peck[1b] but not the findings of Nyqvist *et al.*[1a]

The large pH-dependence and the shape of it suggest the existence of dissociable groups with weak acidity in either the drug or MCC. Since both PTZs and acrinol are strongly basic drugs (pK_as for PTZs and acrinol are 9.2—9.4[7] and 11.6,[8] respectively), most of the PTZ and acrinol may be present in monoprotic form below pH 7.5.[9] In the case of acrinol, the existence of a diprotic form is possible due to protonation of the 6-NH_2 group in the acridine ring. However, since the UV spectrum of acrinol (210—320 nm) did not change with pH in the range of pH 2—8, most of the acrinol seemed to be in the monoprotic form, and the amount of the diprotic form may be negligible. On the other hand, it was reported that the cellulose surface has a negative charge due to the ionization of carboxyl groups formed by oxidation of the hydroxy groups on anhydro-glucose units.[9] Hence MCC should have a negative surface, a conclusion which is also supported by the negative value of the zeta-potential (−20 mV) in aqueous solutions.[10] Thus, the pH-dependence of adsorption seems to result from dissociation of carboxyl groups on the MCC surface with increasing pH of the MCC suspension.

Effect of Ionic Strength on Adsorption

Since the electrostatic force is clearly involved in the adsorption of PTZs and acrinol on

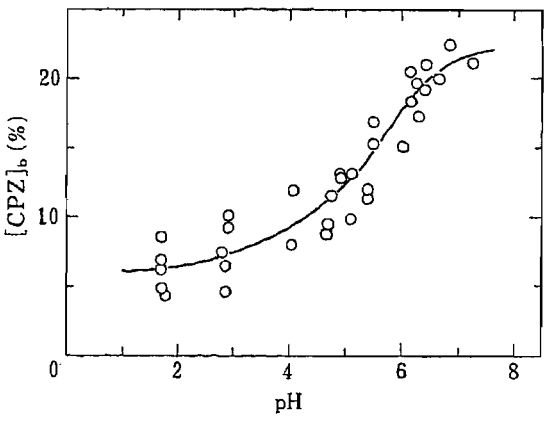

Fig. 4. pH Dependence of the Adsorption of CPZ on MCC, PH-101, at 25 °C at Ionic Strength of $I=0.05$

Initial drug concentration, $[CPZ]_i$, was 2×10^{-5} mol/l. Change of the adsorbed amount was expressed as the fraction bound (%), $[CPZ]_b$.

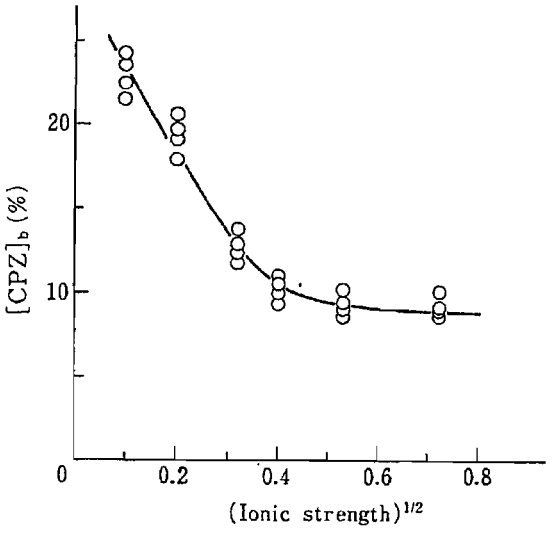

Fig. 5. Effect of Ionic Strength on the Adsorption of CPZ on MCC, PH-101, at pH 6.92 at 25 °C

Initial drug concentration, $[CPZ]_i$, was 2×10^{-5} mol/l. Ionic strength was adjusted with sodium chloride. $[CPZ]_b$ (%) is the same as in Fig. 4.

MCC, it was expected that the adsorption would be affected by ionic strength. As can be seen in Fig. 5, the adsorption rapidly decreases with increase of ionic strength, and approaches a constant value above $(I)^{1/2}=0.4$. Similar results were reported by Frantz and Peck on the adsorption of promethazine and fluphenazine on MCC.[1b] The result indicates that electrostatic binding of a cationic drug to the anionic site on the MCC surface may be inhibited by increasing ionic strength due to the restriction of the electric double layer around the cationic and/or anionic center. An alternative view of the adsorption is possible, *i.e.*, that a cationic drug is adsorbed at an anionic site on MCC by ion-exchange. Cationic drugs such as PTZ and acrinol may be preferentially adsorbed at such a site.

The fact that a certain level of adsorption was maintained even at a large excess of salt (Fig. 5), suggests that non-electrostatic binding is involved in the adsorption. This assumption is also supported by the previous result on the pH-dependence of adsorption (Fig. 4), which indicated that the adsorption is not zero even in strongly acidic regions, in which all of the carboxyl groups on MCC should be completely protonated. This non-electrostatic adsorption appears to occur also in the acrinol–MCC system, since the effects of pH and ionic strength on the adsorption are similar to those in the CPZ–MCC system, as shown in Figs. 4 and 5. The non-electrostatic forces involved in the adsorption may be hydrogen-bonding and van der Waals forces, of which the former should be major, as described below, while the latter may be minor and complementary.

Cation-Exchange Properties of MCC

Since MCC has carboxyl groups on its surface, it should act as a weakly acidic cation-exchanger above pH 4. Figure 6 shows the pH changes of MCC suspensions with the addition of neutral salt. The pH values gradually decrease with increase of salt concentration, except in the acrinol–MCC system. This indicates that H^+ is released from the MCC surface by cation-exchange adsorption of Na^+, K^+, or $CPZH^+$. Since the pH-lowering magnitude at a constant salt concentration was in the order,

$$CPZH^+ > K^+ > Na^+$$

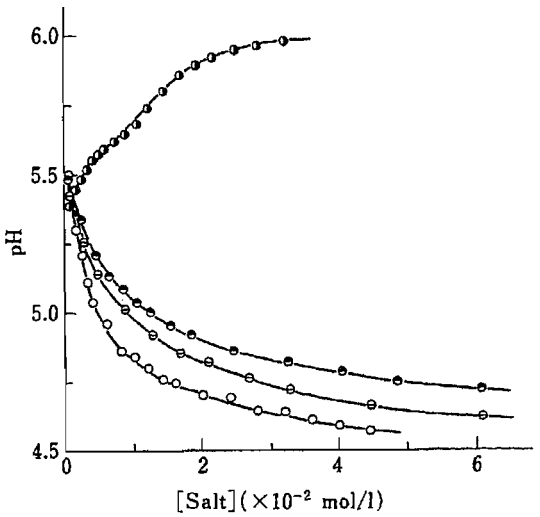

Fig. 6. pH Change of the MCC Aqueous Suspensions on Addition of NaCl (○), KCl (⊖), CPZ·HCl (◓), and Acrinol[2] (◑)

the above cations cannot be equivalent with respect to the MCC anionic site, and the order seems to reflect the site specificity.

The addition of acrinol induced an increase of pH, as shown in Fig. 6. Although the different result with acrinol as compared with the other salts cannot be clearly interpreted, a probable explanation is the buffering action of acrinol. That is, since acrinol is a salt of basic ethacridine ($pK_{a1}=11.6$)[8] and lactic acid ($pK_{a2}=3.86$), at higher salt concentrations the value of pH should approach $1/2\ (pK_{a1}+pK_{a2})=7.73$, provided that monomolecular dispersion can be assumed. Thus, the deprotonation effect of cation-exchange adsorption may be overcome by the buffering action of the drug itself.

Conclusion

If MCC were composed of clusters of pure cellulose microcrystals, adsorption of drugs or other low-molecular adsorbates would be slight. However, since appreciable amorphous regions remain in the microcrystallites, considerable amounts of drugs can be adsorbed at these regions. As for dye adsorption to cellulosic fiber, it is considered that fairly large dyes cannot penetrate between fibers arranged in a regular close-spaced array.[11] Cationic drugs are adsorbable on the surface by an ion-exchange mechanism, due to the negatively charged MCC surface. However, since not all of the cationic drugs were equally adsorbed on MCC, penetration of the drugs into the intermicellar space of microcrystallites may be important in the adsorption. That is, nonadsorbable drugs seem not to be capable of such penetration, because of chemical and structural factors. Nonelectrostatic binding of drugs to MCC also cannot be neglected, as mentioned above.

In the field of dye chemistry, there have been numerous studies on the use of direct dyes for cellulose, and the excellent dyes have some common structural features. Vickerstaff states the requirements for the molecular structure as follows; a) a linear configuration, b) a coplanar arrangement, c) groups capable of hydrogen-bonding, d) the presence of a conjugated system of double bonds.[12] Hence acrinol has the molecular structure of a typical dye for cellulose (it has been used as a disinfectant for gauze and absorbent cotton). On the other hand, the PTZs examined in this study do not contain groups capable of hydrogen-bonding, and there is no conjugated system of double bonds through the molecule. The tricyclic PTZ ring thus cannot form a planar arrangement, but takes a boat conformation with a dihedral angle of about 140°.[13] Therefore the large difference in adsorption on MCC between PTZs and acrinol may arise mainly from the differences in their molecular structure

and arrangement.

The results of the present study indicate that the bioavailability of drugs may be modified by MCC employed as an excipient. Since acrinol is commonly used externally, the effect should be unimportant. However, PTZs are mainly prescribed for internal use over a long period. Therefore, it may be necessary to reconsider the combined use of MCC with at least some of the PTZs.

Acknowledgement The present work was supported in part by the Science and Technology Agency. We are grateful to Dr. Uchiyama, Director of the Department of Drugs, National Institute of Hygienic Sciences, for his kind advice and encouragement.

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Hydrogen Fluoride Adduct Formation of Medicinal Compounds and Its Application to Particle Size Reduction[1)]

YASUYUKI TSUDA,* ETSUKO SUZUKI and KEIJI SEKIGUCHI

School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108, Japan

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It was confirmed that griseofulvin, sulfanilamide and theophylline form HF adducts with combining ratios of 1 : 1 (griseofulvin : HF), 1 : 2 (sulfanilamide : HF) and 1 : 3 and 1 : 2 (theophylline : HF). The thermal and other physico-chemical properties of these adducts and the recovered medicinals were investigated by differential scanning calorimetry, thermogravimetry, X-ray powder diffractometry, infrared spectroscopy and microscopy. Effective particle size reduction was achieved by desorbing HF from the adducts.

Keywords——griseofulvin; sulfanilamide; theophylline; hydrogen fluoride adduct; hydrogen chloride salt; hydrogen fluoride adduct thermal behavior; hydrogen fluoride adduct physico-chemical property; hydrogen fluoride sorption; hydrogen fluoride desorption; particle size reduction

In a series of studies, the authors have reported a method of particle size reduction by making use of the phase conversion from a solvate or adduct to the original medicinal compound. In the present study, adduct formation was attempted between anhydrous HF, which is known to be one of the most acidic substances, and four medicinal compounds (griseofulvin, sulfanilamide, theophylline and phenacemide). Thermal and other physico-chemical properties of the HF adducts and the recovered medicinals were investigated by differential scanning calorimetry, thermogravimetry, X-ray powder diffractometry, infrared spectroscopy and microscopy. The HCl salt of sulfanilamide was prepared and its properties were determined for reference.

Experimental

Materials——Griseofulvin: Recrystallized from acetone.

Sulfanilamide: Needle crystals (polymorphic α form) recrystallized from *n*-butyl alcohol were used.

Theophylline: The anhydrous form was used. Monohydrate crystallized from water was dried at 100 °C for several hours before use.

Phenacemide: Commercial product was used without further purification.

Anhydrous Hydrogen Fluoride: Commercially available liquid HF in a steel bomb (Daikin Kogyo Co., Ltd.) was used. The guaranteed purity was over 99.99%.

Hydrogen Chloride: Commercially available liquid HCl in a steel bomb (Tsurumi Soda Co., Ltd.) was used without further purification. The guaranteed purity was over 99.7%.

Preparation of HF Adducts——An HF-reaction apparatus type I provided by Peptide Institute, Inc. was used. The apparatus, made of polychlorotrifluoroethylene, was evacuated first, then a vessel (inert to HF) containing the sample material was cooled with an acetone-dry ice bath. When anhydrous HF gas was introduced gradually into the vessel by single step distillation, it was liquefied and dissolved the sample material. After several minutes, the acetone-dry ice bath was removed from the vessel. The apparatus was connected to an HF vacuum line equipped with an absorption cylinder (CaO) and the excess of HF in the vessel was allowed to evaporate through the vacuum line. Several hours of additional evacuation were done from the point where almost all the liquid HF had been eliminated. Thereafter, samples were stored in tight containers, if necessary in a refrigerator.

Preparation of Sulfanilamide HCl Salt——When dried HCl gas was introduced gradually into an ethyl alcohol solution of sulfanilamide in a flask at room temperature, immediate crystallization occurred. Small needle-like transparent crystals were obtained by filtration.

Differential Scanning Calorimetry (DSC) and Thermogravimetry (TG)——Simultaneous measurements of DSC and TG were done by using a Thermoflex TG-DSC (Rigaku Denki Co., Ltd.) under a nitrogen gas flow of 50 ml/min. Eliminated HF gas was trapped at the gas outlet. For the measurements under closed conditions, an SSC/580-DSC 20 differential scanning calorimeter (Seiko Instruments & Electronics) was used, and each sample was sealed into a container capable of withstanding a pressure of about 50 atm.

X-Ray Powder Diffractometry——The instrument used was a JEOL JDX-7F X-ray diffractometer (Cu-$K\alpha$ radiation, $\lambda = 1.542$ Å, Ni filter).

Infrared (IR) Spectroscopy——IR spectra (Nujol mull method) were measured with a Jasco IRA-1 grating infrared spectrophotometer.

Microscopic Observation——An FHF-Tr-IV microscope (Olympus Optical Co., Ltd.) was used. For hot-stage microscopy, an FP 52 micro-furnace (Mettler) was installed on the microscope.

Scanning Electron Microscopy (SEM)——An MSM-4 scanning electron microscope (Hitachi-Akashi) was used.

Results and Discussion

HF Adduct of Griseofulvin

Anhydrous HF solution of griseofulvin showed a reddish yellow tinge, and was evaporated to yield yellow crystals. This coloration phenomenon may be ascribed to molecular interaction of griseofulvin with HF. The crystals, however, turned white gradually during the process of HF evacuation. In the present study, we used the white crystals obtained by additional evacuation (*ca.* 1 h).

Determination of the Combining Ratio and X-Ray Diffractometry——The TG curve of the white crystals from HF solution showed a gradual weight decrease immediately after the measurement run and the weight loss (attributable to HF dissociation) continued until about 170 °C. From 6 measurements, the value of the combining ratio was within the range of 1 : 1.0—1.3 mol (Fig. 1(c)). When sample crystals were held at room temperature (24±1 °C) under open conditions, HF dissociation proceeded gradually and the composition changed to 1 : 0.8—1.0 mol after a few hours. As is shown in Fig. 2, the powder X-ray diffraction pattern of the sample material was completely different from that of griseofulvin itself, which

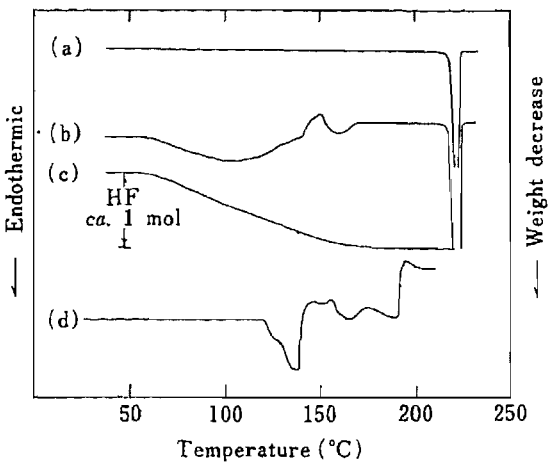

Fig. 1. DSC and TG Curves of Griseofulvin and Its 1 : 1 HF Adduct

(a), DSC curve of griseofulvin under semiclosed conditions: sample weight, 7.99 mg; heating rate, 5 °C/min. (b) and (c), DSC and TG curves of 1 : 1 HF adduct under open conditions: sample weight, 13.93 mg; heating rate, 10 °C/min. (d), DSC curve of 1 : 1 HF adduct under closed conditions: sample weight, 6.15 mg; heating rate, 2 °C/min.

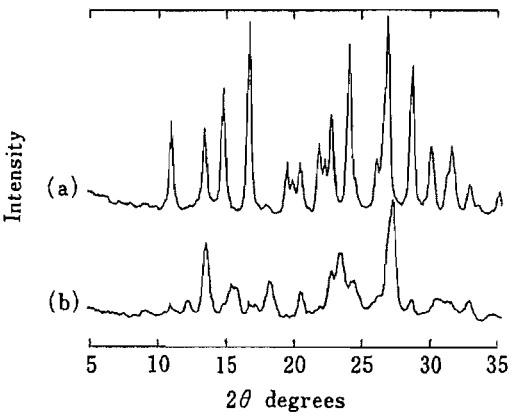

Fig. 2. X-Ray Diffraction Patterns of Griseofulvin and Its 1 : 1 HF Adduct (2θ scan speed: 4 °/min)

(a), griseofulvin; (b), 1 : 1 HF adduct.

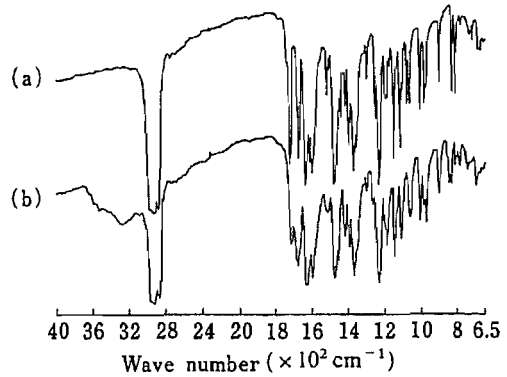

Fig. 3. Infrared Spectra of Griseofulvin and Its 1 : 1 HF Adduct
(a), griseofulvin; (b), 1 : 1 HF adduct.

indicates the formation of an HF adduct possessing an intrinsic crystal structure. No difference was found between the diffraction pattern of the HF adduct possessing a stoichiometric combining ratio of 1 : 1 and that of a sample having more HF, such as 1 : 1.3. On the basis of these results, we may conclude that the HF adduct formation of griseofulvin occurs at least with equimolar ratio (1 : 1). Combining of excessive HF may be caused by adhesion on the surface or to small cavities of the sample material.

Thermal Behavior——When DSC measurement of the HF adduct was done under open conditions, the DSC curve showed three endothermic peaks and an exothermic peak, as depicted in Fig. 1(b). Since the last endothermic peak at about 220 °C is due to the melting of griseofulvin, the first shallow endothermic peak from 60 to 140 °C and the exothermic peak at about 150 °C may be attributable to dissociation of HF and retarded crystallization of recovered griseofulvin, respectively. An endothermic peak at about 160 °C may suggest the possibility of existence of a lower adduct.

On the other hand, when DSC was done under closed conditions (Fig. 1(d)), a sharp endothermic peak with a shoulder appeared at about 120—140 °C and no heat effect occurred below this temperature. Since the sample can be treated as a condensed system under such conditions, this peak would be attributable to peritectic transition of the HF adduct. Successive broad endothermic heat effects at about 150—190 °C must reflect fusion of griseofulvin in the presence of the liquid derived from the adduct.

IR Spectra——The IR spectra of griseofulvin and its HF adduct are depicted in Fig. 3. The HF adduct showed a new absorption band in the wide range of 3680—3060 cm^{-1}. The absorption band of griseofulvin at 1715 cm^{-1}, which is attributable to the C=O group in the benzofuran ring (3-ketone stretching band), showed a slight downward frequency shift (*ca.* 5 cm^{-1}) and was less intense after HF adduct formation. At the same time, the 4'-ketone stretching band (in the cyclohexene ring) of griseofulvin at 1665 cm^{-1} was broadened and extended toward the higher frequency region by about 15 cm^{-1}. Changes in the IR spectrum in the finger-print region on HF adduct formation are very similar to those of the chloroform solvate.[2] From this spectral evidence, it is supposed that HF adduct formation can be ascribed to hydrogen bonding between griseofulvin and HF.

HF Adduct of Sulfanilamide

When crystals of sulfanilamide were dissolved in HF solution and excess HF was eliminated, crystallization did not occur immediately. After standing at room temperature for a few days, gradual solidification proceeded and a white material was obtained.

Determination of the Combining Ratio and Thermal Behavior——From four TG measurements, the combining ratio of HF was determined to be 1 : 2.00 ± 0.01 (sulfanilamide : HF), as shown in Fig. 4(c). In DSC measurements under closed conditions, only a sharp endothermic peak appeared at about 142 °C (curve (h)). On the other hand, under open conditions, three to

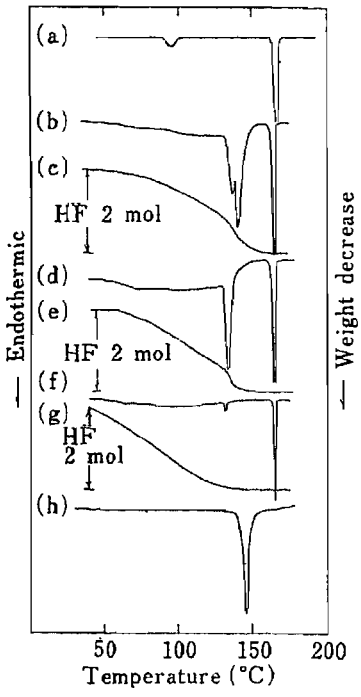

Fig. 4. DSC and TG Curves of Sulfanilamide and Its 1 : 2 HF Adduct

(a), DSC curve of sulfanilamide (polymorphic α form) under semiclosed conditions: sample weight, 8.20 mg; heating rate, 5 °C/min. (b) and (c), DSC and TG curves of 1 : 2 HF adduct under open conditions: sample weight, 9.30 mg; heating rate, 5 °C/min. (d) and (e), DSC and TG curves of 1 : 2 HF adduct under open conditions: sample weight, 6.58 mg; heating rate, 5 °C/min. (f) and (g), DSC and TG curves of 1 : 2 HF adduct under open conditions: sample weight, 7.70 mg; heating rate, 1 °C/min. (h), DSC curve of 1 : 2 HF adduct under closed conditions: sample weight, 4.23 mg; heating rate, 5 °C/min.

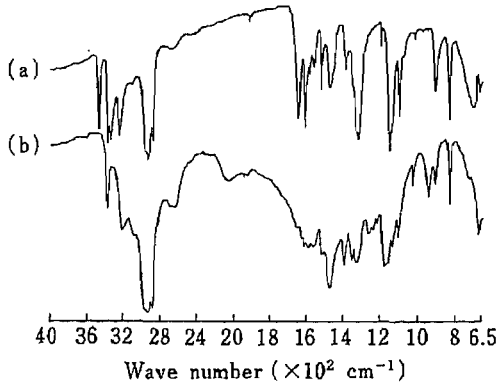

Fig. 5. Infrared Spectra of Sulfanilamide and Its 1 : 2 HF Adduct

(a), sulfanilamide (polymorphic α form). (b), 1 : 2 HF adduct.

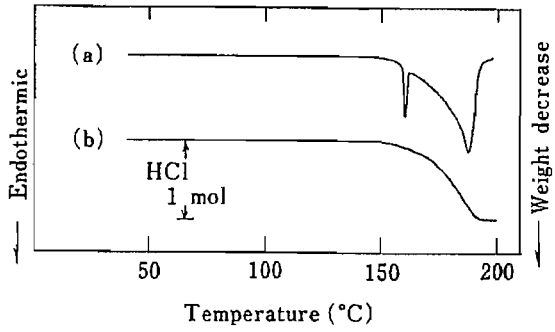

Fig. 6. DSC and TG Curves of Sulfanilamide HCl Salt

(a) and (b), DSC and TG curves under open conditions: sample weight, 3.31 mg; heating rate, 5 °C/min.

four endothermic heat effects were exhibited in the DSC curve. The first broad endotherm, accompanied with a gradual weight decrease, began at about 50—60 °C and the HF elimination proceeded till the appearance of the second endothermic peak. At the temperature of the sharp endothermic peak (132 °C), complete dissociation of HF occurred (curves (c) and (e)). Depending on the magnitude of the HF dissociation below 132 °C, the DSC patterns varied considerably as shown in Fig. 4(b), (d) and (f). In the DSC curve (b) measured at a heating rate of 5 °C/min, the HF content was about 0.8 mol at that temperature, and a successive endothermic peak appeared at 141 °C (peak maximum). The slower the heating rate, the smaller the endothermic peak, and it finally disappeared when complete elimination had occurred prior to that temperature. The highest endothermic peak at 164 °C is due to melting of recovered sulfanilamide.

On the basis of these results, the following conclusions can be drawn.

1. Sulfanilamide forms an HF adduct with a molar ratio of 1 : 2 (sulfanilamide : HF), and the HF adduct has a congruent melting point at 142 °C.

2. Sulfanilamide and the HF adduct show a eutectic temperature of 132 °C, below which temperature HF dissociation from the adduct proceeds as a solid-gas phase reaction.

IR Spectra——In the IR spectrum of the HF adduct (Fig. 5(b)), a characteristic broad absorption band appeared at about 2180—1900 cm^{-1}. Absorption spread to the region of 1900—1540 cm^{-1} on adduct formation, instead of two sharp absorption bands at 1635 and 1595 cm^{-1} (attributable to NH deformation) in the spectrum of sulfanilamide (curve (a)). Further, a new absorption band appeared between 1280 and 1230 cm^{-1}. These absorption bands caused by HF adduct formation were not recognized in the spectrum of the HCl salt of sulfanilamide, as is depicted later (Fig. 7). In the NH stretching frequency region, three absorption bands appeared between 3400 and 3040 cm^{-1} (curve (b)). Although the number and position of absorptions in this region for sulfanilamide are affected somewhat by the various physical forms, each of the polymorphic α, γ and δ forms shows four bands (the higher two are attributed to ν_{NH} and the lower two to ν_{SO_2NH}[3]), the β form shows three and the monohydrate five within 3600—3100 cm^{-1}.[4] The spectrum of the HF adduct exhibited strong downward shifts of the ν_{NH} bands or disappearance of one or two of the bands. These IR data suggest that dipole-induced dipole interaction or hydrogen bonding between sulfanilamide and HF plays a major role in the adduct formation.

HCl Salt of Sulfanilamide

Simultaneous TG-DSC curves of the HCl salt of sulfanilamide are shown in Fig. 6(a) and (b). A broad endothermic peak appeared between 150 and 200 °C which corresponds to a weight decrease of 1 mol of HCl. A small endothermic peak that appeared at about 160 °C is attributable to the melting of sulfanilamide recovered partially by dissociation of HCl on heating. It can be said that the HCl salt of sulfanilamide melts at a higher temperature than the original sulfonamide, accompanied with decomposition of the HCl salt. This conclusion was supported by visual observation by hot-stage microscopy.

In the IR spectrum of the HCl salt, the NH deformation bands near 1600 cm^{-1} were extremely weak (Fig. 7).

HF Adducts of Theophylline

Determination of the Combining Ratio and Thermal Behavior——Sample material obtained from an anhydrous HF solution of theophylline was confirmed to exist as a 1 : 3 HF adduct (theophylline : HF = 1 : 2.9 $\pm$ 0.2) by simultaneous TG-DSC measurements under open conditions (Fig. 8(c) and (d)) and direct weighing of the sample before and after measurement by using a semimicrobalance. The DSC curve of the 1 : 3 adduct showed an endothermic heat effect between 40 and 75 °C, and the TG curve indicated a first step of weight decrease corresponding to 1 mol of HF. Then HF dissociation proceeded up to about 180—200 °C. The recovery of theophylline was proved by the appearance of the highest endothermic DSC peak at 270 °C (melting of theophylline). This thermal behavior suggests the formation of a lower 1 : 2 adduct during heating. Incidentally, the DSC curve exhibited unusual heat effects, some abrupt endothermic peaks that might be attributable to the escape of dissociated HF molecules entrapped in the solid phase, between 70 and 150 °C in the process of HF dissociation from the 1 : 2 adduct. On hot-stage microscopy, however, no change of appearance was noticed in the adduct crystals during heating in the temperature range of this complicated heat effect. It was indicated that the particle size of the HF adduct influences the DSC patterns. In the case of a sample composed of finer particles (curve (e)), the first endothermic heat effect became smaller, another broad endotherm followed by an exotherm appeared at about 100 °C, and no abrupt irregular endothermic heat effect was shown. Therefore, it is supposed that the composition of the 1 : 3 adduct is easily changed merely by

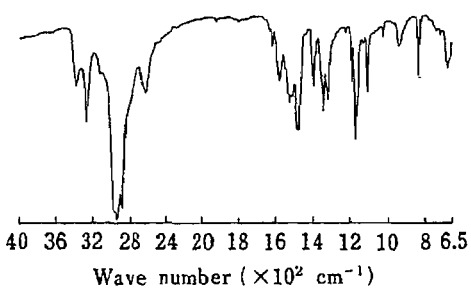

Fig. 7. Infrared Spectrum of Sulfanilamide HCl Salt

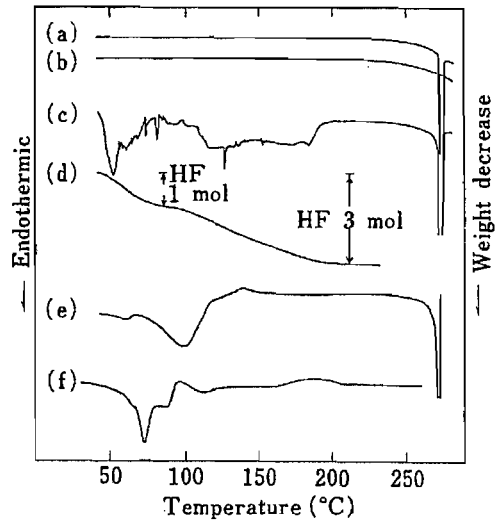

Fig. 8. DSC and TG Curves of Theophylline and Its 1 : 3 HF Adduct

(a) and (b), DSC and TG curves of anhydrous theophylline under semiclosed conditions: sample weight, 8.52 mg; heating rate, 5 °C/min. (Weight decrease above 230 °C is attributed to sublimation.) (c) and (d), DSC and TG curves of 1 : 3 HF adduct under open conditions: sample weight, 11.46 mg; heating rate, 5 °C/min. (e), DSC curve of 1 : 3 HF adduct under open conditions after trituration: sample weight, 6.10 mg; heating rate, 5 °C/min. (f), DSC curve of 1 : 3 HF adduct under closed conditions: sample weight, 14.37 mg; heating rate, 5 °C/min.

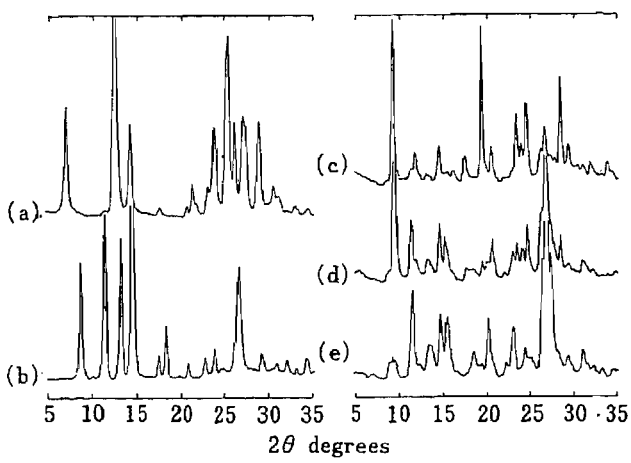

Fig. 9. X-Ray Diffraction Patterns of Anhydrous Theophylline, the Monohydrate, and 1 : 3 and 1 : 2 HF Adducts

(a), anhydrous theophylline; (b), monohydrate; (c), 1 : 3 HF adduct, immediately after sampling; (d), after 20 min; (e), after 70 min (1 : 2 HF adduct).

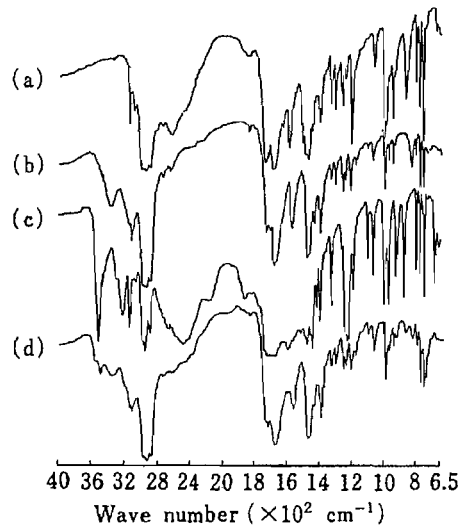

Fig. 10. Infrared Spectra of Anhydrous Theophylline, the Monohydrate, and 1 : 3 and 1 : 2 HF Adducts

(a), anhydrous theophylline; (b), monohydrate; (c), 1 : 3 HF adduct; (d), 1 : 2 HF adduct.

trituration, and the broad endothermic and successive exothermic peaks may be ascribed to dissociation of the 1 : 2 adduct to HF-free theophylline and retarded crystallization, respectively.

When DSC measurement of the 1:3 adduct was done under closed conditions, as is shown by curve (f), the first sharp endothermic peak with a shoulder appeared at about 70 °C, and an endothermic peak and a rather flat exothermic peak followed between 90 and 210 °C. In the closed heating system, evaporation of HF was restricted inside the container, unless the container was ruptured by the pressure increase; therefore, the change in the sample composition would be small. The endothermic peak at about 70 °C is probably attributable to liquefaction caused by a peritectic or eutectic phase reaction, while the shoulder peak at about 85 °C should represent melting either of HF-free theophylline or of the HF adduct. The appearance of the following endothermic peak suggests the partial formation of the 1:2 adduct under the conditions adopted.

X-Ray Diffractometry——The HF dissociation process of the 1:3 adduct was demonstrated by repeated powder X-ray diffractometry at room temperature (Fig. 9). The X-ray pattern of the sample changed gradually to a different one (curve (e)) over several tens of minutes. On TG measurement, the sample giving pattern (e) was found to correspond to the 1:2 adduct.

IR Spectra——The IR spectra of anhydrous theophylline, the monohydrate, and the 1:3 and 1:2 HF adducts are shown in Fig. 10(a)—(d). The spectrum of the 1:3 adduct exhibited three characteristic strong absorption bands at 3490, 3210 and 3115 cm^{-1}, which indicate the presence of hydrogen bonds between theophylline and HF. The two broad absorption bands between 2800—2000 cm^{-1} may indicate the presence of a positively charged nitrogen atom[5] in the 1:3 adduct molecule.

The IR spectrum of the 1:2 adduct was very similar to that of the monohydrate except for the absorption at 3485 cm^{-1}.

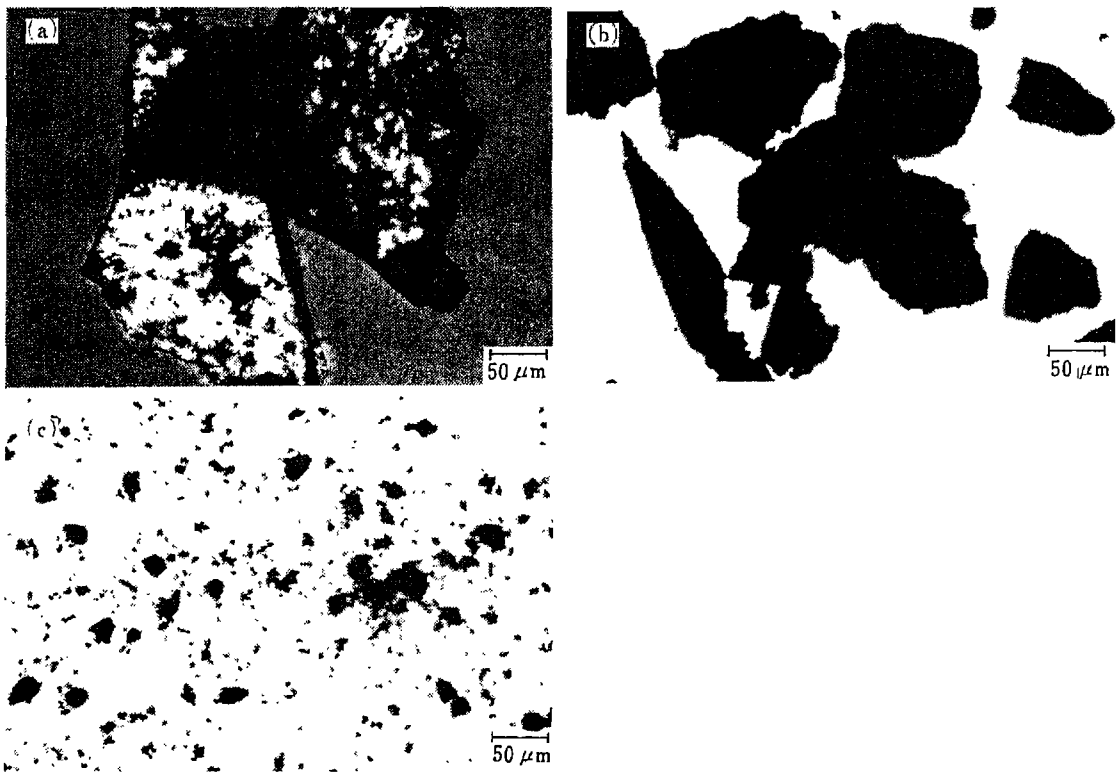

Fig. 11. Micrographs of Sulfanilamide 1:2 HF Adduct and Its Desorbed Form

(a), 1:2 HF adduct (partially desorbed), 100×. (b), after desorption (standing at room temperature under atmospheric pressure), 100×. (c), lightly pulverized state of (b), 100×.

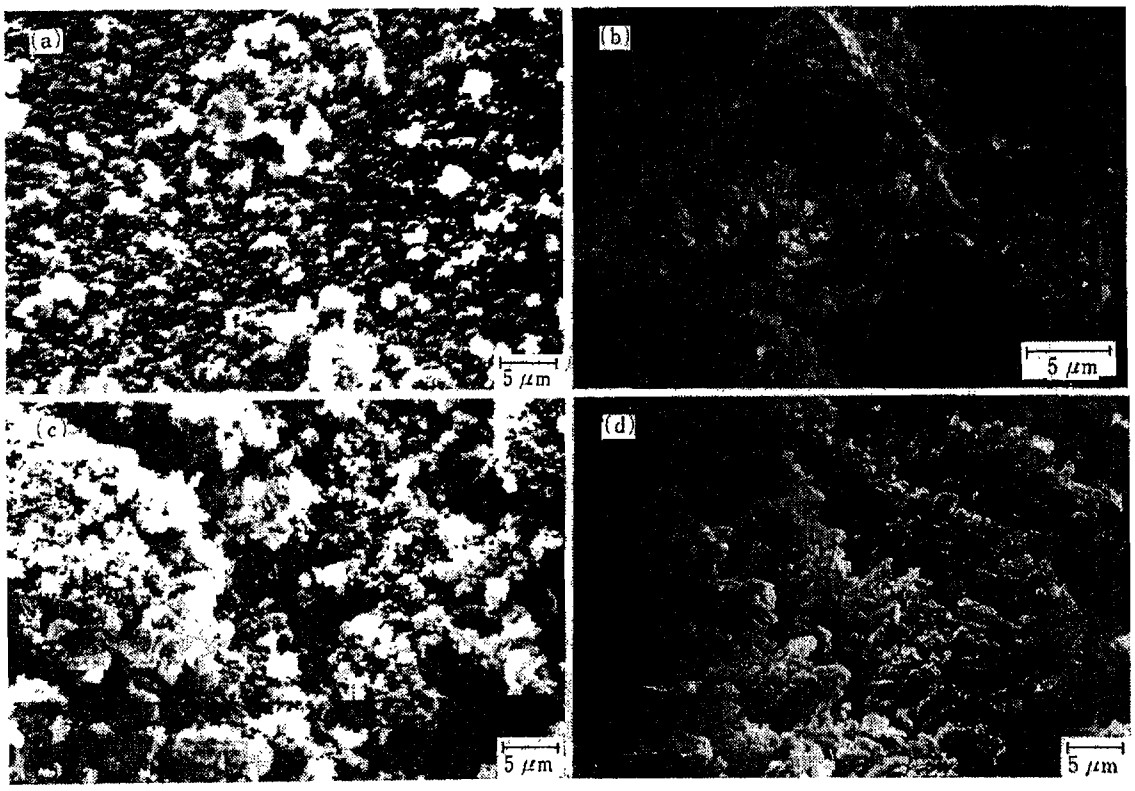

Fig. 12. Scanning Electron Micrographs of Griseofulvin, Sulfanilamide, Theophylline and Phenacemide Particles Recovered *via* Their HF Adducts

(a), griseofulvin, 2000×; (b), sulfanilamide, 3000×; (c), theophylline, 2000×; (d), phenacemide, 2000×.

((a), (b) and (d) were recovered after standing at room temperature under atmospheric pressure, and (c) after heating up to 158 °C on TG.)

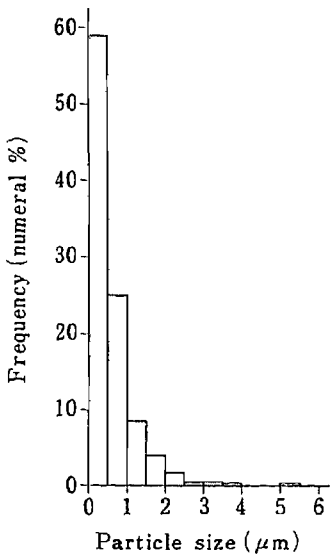

Fig. 13. Particle Size Distribution of Griseofulvin Recovered *via* Its 1:2 HF Adduct

HF desorption proceeded under atmospheric pressure at room temperature in a desiccator containing CaO. Particle size measured was the Green diameter.

$$\frac{\Sigma nd}{\Sigma n}=0.6\,\mu m \quad (n=625)$$

Particle Size Reduction by Desorption of HF from the HF Adducts

Desorption of HF from HF adducts of griseofulvin, sulfanilamide and theophylline occurred completely and each original medicinal compound was recovered. Optical and

scanning electron micrographs of samples obtained *via* the HF adducts and particle size distribution data are shown in Figs. 11, 12 and 13. From these results, it is clear that effective particle size reduction is possible.

Further, as is shown in Fig. 12(d), the external appearance of crystals of phenacemide obtained from its anhydrous HF solution revealed agglomerates that consisted of primary fine particles, which may be formed *via* the HF adduct, although adduct formation of phenacemide could not be confirmed because of low stability.

Conclusion

Griseofulvin, sulfanilamide and theophylline were confirmed to form HF adducts having combining ratios of 1:1 (griseofulvin:HF), 1:2 (sulfanilamide:HF) and 1:3 and 1:2 (theophylline:HF). The mechanisms of HF adduct formation are supposed to involve hydrogen bonding or dipole-induced dipole interaction. The particle size of each medicinal was effectively reduced by formation of the HF adduct and subsequent desorption of HF.

References and Notes

1) This paper forms Part XXIV of the series entitled "Studies on Methods of Particle Size Reduction of Medicinal Compounds." The preceding paper, Part XXIII: E. Suzuki, K. Shirotani, Y. Tsuda and K. Sekiguchi, *Chem. Pharm. Bull.*, **33**, 5028 (1985).
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Potentiation of Barbiturate-Induced Narcosis by *N,N'*- (or *N,S*-) Diallyl-Substituted Derivatives of Phenobarbital, Amobarbital and Thiopental in Mice

YUJI TATEOKA,[a] TOSHIYUKI KIMURA,[a] KAZUHITO WATANABE,[a] IKUO YAMAMOTO*,[a] and ING KANG HO[b]

Department of Hygienic Chemistry, School of Pharmacy, Hokuriku University,[a] 3, Ho Kanagawa-machi, Kanazawa 920-11, Japan and Department of Pharmacology and Toxicology, The University of Mississippi Medical Center,[b] 2500 North State Street, Jackson, Mississippi 39216, U.S.A.

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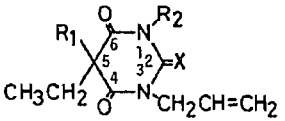
N- (or *S*-) Allyl derivatives of phenobarbital (PheB), amobarbital (AB) and thiopental (TP) were prepared, and their pharmacological activities (hypnotic activity and anticonvulsant activity against pentylenetetrazol-induced seizures) were investigated in mice. *N*-Monoallyl-substituted derivatives (MAPheB, MAAB and MATP) of PheB, AB and TP exhibited hypnotic and anticonvulsant activities. *N,S*-Diallylthiopental (*N,S*-DATP) possessed hypnotic activity, but failed to show anticonvulsant activity even at a dose of 250 mg/kg (i.p.). *N,N'*-Diallyl-substituted derivatives (DAPheB, DAAB and DATP) were devoid of not only the hypnotic activity, but also the anticonvulsant activity. The interactions of these allyl derivatives with various barbiturates were then studied in order to characterize whether the allyl compounds have antagonistic or potentiating properties. MAPheB, DAPheB, MAAB and DAAB showed dose-dependent potentiation of the sleep induced by the corresponding mother compounds. All the allyl compounds tested prolonged the pentobarbital-induced sleeping time more than 7- to 19-fold as compared with the control. MAAB markedly prolonged the PheB-induced sleeping time by 25-fold, but it did not potentiate the barbital-induced sleep. These results indicate that *N*- (or *S*-) allyl derivatives of PheB, AB and TP interact differently with the barbiturates.

Keywords——*N,N'*-diallylphenobarbital; *N,N'*-diallylamobarbital; *N,N'*-diallylthiopental; *N,S*-diallylthiopental; *N*-monoallylbarbiturate; central depressant effect; hypnotic activity; anticonvulsant activity; acute toxicity; barbiturate-induced sleep

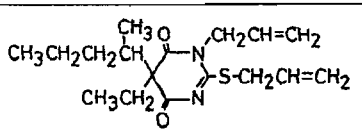
In previous studies,[1-3] we demonstrated that *N*-allyl substituted derivatives of pentobarbital (PB), barbital (B) and barbituric acid exhibited different pharmacological activities in mice. *N*-Monoallylbarbital displayed more potent hypnotic activity than B.[2] Further, these allyl derivatives possessed a potent synergistic effect on sedative hypnotic-induced responses. In contrast, *N,N'*-diallylpentobarbital apparently showed a shortening effect on the B-induced sleeping time.[1] These results indicate the complexity of action of allyl-substituted barbiturates. In connection with the above findings, the present study was designed to investigate systematically the pharmacological activities of allyl-substituted phenobarbital (PheB), amobarbital (AB) and thiopental (TP), although syntheses and/or studies of the hypnotic activity of *N*-allyl derivatives of PheB and AB have already been reported.[4-7]

In order to evaluate the antagonistic or potentiating properties of these allyl compounds and to compare the results with those obtained previously,[1-3] we further examined the pharmacological activities of allyl derivatives of PheB, AB and TP. For this purpose, three kinds of barbiturates having an ethyl group and a phenyl, 3-methylbutyl or 1-methylbutyl group at the 5-position, and having =O or =S at the 2-position of the barbiturate ring were chosen.

TABLE I. *N*- (or *S*-)Allyl-Substituted Derivatives of Barbiturates



Compd.	R_1	R_2	X	Yield (%)	mp (°C) (Lit.)	Recryst. solvent	Formula	Analysis (%) Calcd (Found) C	H	N	IR ν_{max}^{KBr} (cm^{-1})	^{1}H-NMR (in $CDCl_3$) δ
MAPheB		H	O	73	68—70 (68—69)[4]	MeOH : H_2O (1 : 1)	$C_{15}H_{16}N_2O_3$	66.16 (66.15	5.92 6.00	10.29 10.25)	3120 (NH) 1690 (C=O)	4.58 (2H, d, J=6 Hz, N-CH_2-), 5.04—5.44 (2H, m, =CH_2), 5.72—6.06 (1H, m, -CH=)
DAPheB		CH_2=$CHCH_2$-	O	80	36—40 (40)[4]	MeOH : H_2O (1 : 1)	$C_{18}H_{20}N_2O_3$	69.21 (69.18	6.45 6.55	8.97 8.95)	1680 (C=O)	4.61 (4H, d, J=6 Hz, (N-CH_2-)$_2$), 5.18—5.45 (4H, m, (=CH_2)$_2$), 5.76—6.06 (2H, m, (-CH=)$_2$)
MAAB	$(CH_3)_2C_3H_5$-	H	O	68	37—40	MeOH : H_2O (1 : 1)	$C_{14}H_{22}N_2O_3$	63.14 (63.08	8.33 8.46	10.52 10.53)	3250 (NH) 1685 (C=O)	4.57 (2H, d, J=6 Hz, N-CH_2-), 5.23—5.54 (2H, m, =CH_2), 5.74—6.07 (1H, m, -CH=)
DAAB	$(CH_3)_2C_3H_5$-	CH_2=$CHCH_2$-	O	19	Oil[a]		$C_{17}H_{26}N_2O_3$	66.64 (66.59	8.55 8.64	9.14 9.10)	1690 (C=O)	4.61 (4H, d, J=6 Hz, (N-CH_2-)$_2$), 5.25—5.54 (4H, m, (=CH_2)$_2$), 5.87—6.20 (2H, m, (-CH=)$_2$)
MATP	$C_3H_7(CH_3)CH$-	H	S	7	Oil[a]		$C_{14}H_{22}N_2O_2S$	59.55 (59.33	7.85 7.93	9.92 9.79)	3250 (NH) 1680 (C=O)	4.96 (2H, d, J=6 Hz, N-CH_2-), 5.18—5.46 (2H, m, =CH_2), 5.68—6.11 (1H, m, -CH=)
DATP	$C_3H_7(CH_3)CH$-	CH_2=$CHCH_2$-	S	2	Oil[a]		$C_{17}H_{26}N_2O_2S$	63.32 (63.41	8.12 8.15	8.68 8.41)	1690 (C=O)	5.04 (4H, d, J=6 Hz, (N-CH_2-)$_2$), 5.19—5.32 (4H, m, (=CH_2)$_2$), 5.68—6.10 (2H, m, (-CH=)$_2$)
N,*S*-DATP[b]				70	Oil[a]		$C_{17}H_{26}N_2O_2S$	63.32 (63.00	8.12 8.17	8.68 8.69)	1680 (C=O)	3.92 (2H, d, J=7 Hz, S-CH_2-), 4.54 (2H, d, J=6 Hz, N-CH_2-), 5.14—5.48 (4H, m, (=CH_2)$_2$) 5.62—6.15 (2H, m, (-CH=)$_2$)

a) Oily compounds were purified by column chromatography on silica gel. *b*) This novel compound has the following structure: 

Results

1) Pharmacological Activity

Analytical data for *N*- (or *S*-) allyl barbiturates synthesized for the studies are listed on Table I. Table II summarizes the pharmacological activities of *N*- (or *S*-) allyl-substituted drivatives. *N*-Monoallyl-substituted derivatives of PheB, AB and TP (MAPheB, MAAB and MATP) possessed some hypnotic and anticonvulsant activities. However, the activities of these *N*-monoallyl compounds were less potent than those of the parent compounds. MATP had the most potent anticonvulsant activity among these allyl compounds. *N*,*S*-Diallylthiopental (*N*,*S*-DATP) possessed some hypnotic activity, although the compound did not show any anticonvulsant activity even at 250 mg/kg, i.p. On the other hand, *N*,*N'*-diallyl-substituted derivatives of PheB, AB and TP (DAPheB, DAAB and DATP) exhibited neither hypnotic nor anticonvulsant activity. The acute toxicities of these allyl compounds were lower than those of the corresponding parent compounds.

2) Interaction

2.1) MAPheB and DAPheB——The time course (1, 15, 30 and 60 min) of the effect of pretreatment with MAPheB and DAPheB (80 mg/kg, i.p.) on the PheB (150 mg/kg, i.p.)-induced sleeping time was examined. The peak effects of MAPheB and DAPheB on the PheB-

Table II. Pharmacological Activities of *N*- (or *S*-) Allyl-Substituted Derivatives of Barbiturates

Compd.	HD_{50} (mg/kg, i.p.)	PTZ-ED_{50} (mg/kg, i.p.)	LD_{50} (mg/kg, i.p.)
PheB	96.3 (77.0—120)[a]	19.2 (13.8—26.6)	226 (213—240)
MAPheB	186 (173—200)	62.0 (51.0—75.3)	488 (456—522)
DAPheB	None (1400)[b]	>250	1270 (1207—1336)
AB	67.0 (53.4—84.1)	17.5 (15.6—19.6)	185 (171—200)
MAAB	176 (147—210)	75.5 (62.1—91.8)	650 (620—682)
DAAB	None (1200)	>250	855 (770—949)
TP	53.0 (42.7—65.7)	7.10 (4.73—10.7)	193 (183—203)
MATP	107 (84.7—135)	29.1 (24.2—35.0)	>320
DATP	None (240)	>160	>240
N,*S*-DATP	275 (215—351)	>250	575 (501—660)

a) The 95% confidence limits are shown in parentheses. *b*) The word "None" means that there was no loss of righting reflex even at the dose indicated in parentheses.

Table III. Effects of MAPheB and DAPheB on Barbiturate-Induced Sleep

Barbiturate	Sleeping time (min)		
	Control	MAPheB 80 mg/kg, i.p.	DAPheB 80 mg/kg, i.p.
B (300 mg/kg, i.p.)	32± 6 (9)	63± 6[b] (12)	51± 6[a] (11)
PheB (150 mg/kg, i.p.)	76±18 (8)	233±24[b] (10)	146±34 (8)
AB (100 mg/kg, i.p.)	82± 7 (8)	555±56[b] (8)	324±26[b] (7)
PB (40 mg/kg, i.p.)	21± 3 (8)	259±29[b] (8)	253±36[b] (8)
TP (50 mg/kg, i.p.)	9± 2 (7)	87±29[a] (8)	15± 3 (7)

MAPheB and DAPheB were administered 15 min prior to the injection of barbiturates. Data are expressed as the mean±S.E. Numbers in parentheses represent numbers of animals used. *a*) Significantly different from the control ($p<0.05$). *b*) Significantly different from the control ($p<0.01$).

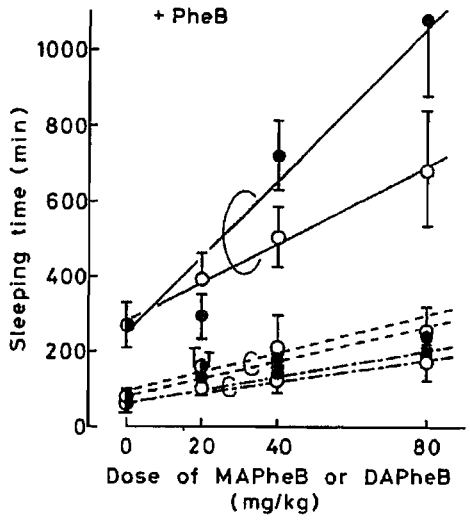


Fig. 1. Dose–Response Curves for Effect of MAPheB and DAPheB on the PheB-Induced Sleep

MAPheB and DAPheB were administered i.p. 15 min prior to the i.p. injection of PheB. The control group was pretreated with 1% Tween 80–saline (vehicle). Each point corresponds to the mean sleeping time of 8 mice. Vertical bars indicate S.E. of the mean.

●—●, MAPheB+PheB 180 mg/kg; ●---●, MAPheB+PheB 150 mg/kg; ●---●, MAPheB+PheB 120 mg/kg; ○—○, DAPheB+PheB 180 mg/kg; ○---○, DAPheB+PheB 150 mg/kg; ○---○, DAPheB+PheB 120 mg/kg.

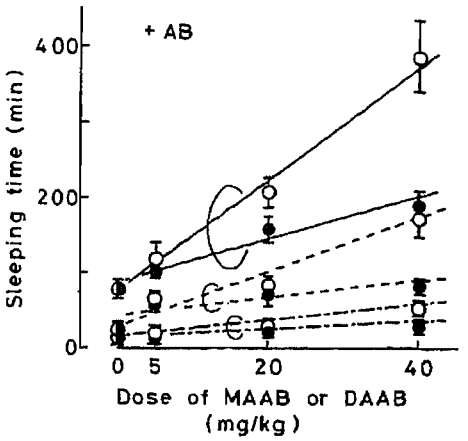


Fig. 2. Dose–Response Curves for Effect of MAAB and DAAB on the AB-Induced Sleep

MAAB and DAAB were administered i.p. 15 min prior to the i.p. injection of AB. The control group was pretreated with 1% Tween 80–saline (vehicle). Each point corresponds to the mean sleeping time of 8 mice. Vertical bars indicate S.E. of the mean.

●—●, MAAB+AB 100 mg/kg; ●---●, MAAB+AB 80 mg/kg; ●---●, MAAB+AB 60 mg/kg; ○—○, DAAB+AB 100 mg/kg; ○---○, DAAB+AB 80 mg/kg; ○---○, DAAB+AB 60 mg/kg.

TABLE IV. Effects of MAAB and DAAB on Barbiturate-Induced Sleep

Barbiturate	Sleeping time (min)		
	Control	MAAB 80 mg/kg, i.p.	DAAB 80 mg/kg, i.p.
B (300 mg/kg, i.p.)	32±6 (9)	39± 8 (8)	103±29[a] (11)
PheB (150 mg/kg, i.p.)	61±9 (6)	1573±61[b] (5)	405±95[a] (6)
AB (100 mg/kg, i.p.)	79±7 (8)	317±33[b] (8)	784±72[b] (8)
PB (40 mg/kg, i.p.)	13±2 (7)	110±21[b] (8)	97±21[b] (8)
TP (50 mg/kg, i.p.)	4±1 (5)	11± 1[b] (8)	10± 2 (8)

MAAB and DAAB were administered 15 min prior to the injection of barbiturates. Data are expressed as the mean±S.E. Numbers in parentheses represent numbers of animals used. *a*) Significantly different from the control ($p<0.05$). *b*) Significantly different from the control ($p<0.01$).

induced sleep were obtained at 15—30 min, as reported previously.[1,2] As shown in Table III, MAPheB (80 mg/kg, i.p.) markedly prolonged the barbiturate-induced sleeping time in all cases. DAPheB (80 mg/kg, i.p.) also significantly prolonged the B- ($p<0.05$), AB- ($p<0.01$) and PB- ($p<0.01$) induced sleeping times, but not that of PheB or TP. Potentiation of the PB-induced sleep by DAPheB was about the same as that of MAPheB, although the effect of DAPheB on the AB-induced sleeping time was only about half of that by MAPheB. Figure 1 shows the dose–response relationship for the effects of MAPheB and DAPheB on the parent compound (PheB)-induced sleep. MAPheB and DAPheB (20, 40 and 80 mg/kg, i.p.) prolonged the PheB (120, 150 and 180 mg/kg, i.p.)-induced sleeping time. Potentiation by

TABLE V. Effects of MATP, DATP and *N*,*S*-DATP on the PB-Induced Sleep

Compd.	Dose (mg/kg, i.p.)	Sleeping time (min)
Control		34± 9 (5)
MATP	80	423± 43[b] (6)
DATP	80	438±102[a] (6)
N,*S*-DATP	80	663± 45[b] (6)

MATP, DATP and *N*,*S*-DATP were administered 15 min prior to the 40 mg/kg, i.p. injection of PB. Data are expressed as the mean±S.E. Numbers in parentheses represent numbers of animals used. *a*) Significantly different from the control ($p<0.05$). *b*) Significantly different from the control ($p<0.01$).

both compounds of the PheB-induced sleep was dose-dependent. When 150 or 180 mg/kg, i.p. of PheB was given, MAPheB exhibited a more potent prolonging effect than DAPheB.

2.2) MAAB and DAAB——The peak effects of MAAB and DAAB on the AB-induced sleep were at 30 and 15 min, respectively. As shown in Table IV, MAAB significantly ($p<0.01$) prolonged the barbiturate-induced sleeping times, except in the case of B. In particular, MAAB potentiated the PheB-induced sleep 25-fold over the control level. DAAB also exhibited a prolonging effect on the barbiturate-induced sleep, except in the case of TP. DAAB was more potent than MAAB in potentiating B- and AB-induced sleeping times. Figure 2 shows the dose–response relationship (5, 20 and 40 mg/kg, i.p.) for the effects of MAAB and DAAB on the AB (60, 80 and 100 mg/kg, i.p.)-induced sleep. Potentiation of the AB-induced sleep by MAAB was evident at doses lower than HD_{50} of MAAB. The slope of the plot for the DAAB-treated group is steeper than that in the case of the MAAB-treated group. Potentiation of the AB-induced sleeping time by DAAB was stronger than that by MAAB.

2.3) MATP, DATP and *N*,*S*-DATP——Table V shows the interactions of MATP, DATP and *N*,*S*-DATP with PB. MATP and DATP significantly ($p<0.01$ and $p<0.05$, respectively) prolonged the PB-induced sleeping time 12-fold and 13-fold, respectively, as compared with the control. The novel compound, *N*,*S*-DATP, also markedly prolonged the PB-induced sleeping time 20-fold. Potentiation of the PB-induced sleep by DATP was as effective as that by MATP.

Discussion

MAPheB, MAAB, MATP, and *N*,*S*-DATP exhibited some hypnotic activity, although these derivatives were less active than the parent barbiturates. MAPheB, MAAB and MATP also showed some anticonvulsant activity. It has been reported that monomethoxymethyl derivatives of PheB possess potent anticonvulsant activity[8] and *N*-monoallyl-B has potent hypnotic activity.[2] However, the present study indicated that introduction of an allyl group into the barbiturate ring resulted in a decrease of the depressant effect of the parent compound. The results were the same as in the case of PB reported previously.[1] DAPheB, DAAB and DATP did not show hypnotic and anticonvulsant activities. The acute toxicities (LD_{50}'s) of allyl compounds were lower than those of the corresponding parent compound.

MAPheB, which has some hypnotic activity, prolonged the sleeping times induced by all barbiturates. Among all allyl derivatives of barbiturates tested, MAAB had the most potent prolonging effect on the PheB-induced sleep. However, the compound did not show any prolonging effect on the B-induced sleep. The reason for this difference is not clear. B and PheB have been classified as long-acting barbiturates. However, each barbiturate may have a

different mechanism of action. On the other hand, DAPheB potentiated the B-induced sleeping time, as well as the AB- and PB-induced sleep, and DAAB also showed potentiation of the B-, PheB- and PB-induced sleep. Further, MAPheB, DAPheB, MAAB and DAAB dose-dependently potentiated the parent compound (PheB or AB)-induced sleeping time. The prolonging effect of DAAB on the AB-induced sleep was stronger than that of MAAB. Therefore, it is suggested that DAPheB and DAAB possess some depressant effect on the central nervous system. DAAB did not attenuate the B-induced sleep, while *N*,*N*′-diallylpentobarbital antagonized the B-induced sleep.[1] The difference in the effect of the *N*,*N*′-diallyl derivatives on the B-induced sleep indicates that the position of the methyl group on the methylbutyl side chain of AB and PB may affect the antagonistic action against B. Allyl derivatives of TP markedly prolonged the PB-induced sleeping time. DATP and MATP showed a similar effect on the PB-induced sleep. The central depressant effect of MATP and *N*,*S*-DATP is responsible for their prolonging effect on the PB-induced sleep. It has been reported that *N*-methyl-TP has a very high lipid/water partition coefficient[9] and that the concentration of *N*-methyl-TP in fat was 40 times the plasma concentration at 6 h after administration in dogs.[10] TP can bind to serum albumin more strongly than comparable barbiturates and will partially displace the latter from serum albumin.[11] Allyl derivatives of TP, which have higher lipid solubility, may affect the binding of PB to serum albumin, so that the concentration of free PB in plasma increases, leading to a higher concentration of PB in the brain.

In conclusion, the results of the present study indicate that allyl derivatives of PheB, AB and TP have different pharmacological activities and that diallyl derivatives of these barbiturates, as well as the *N*-monoallylbarbiturates, potentiate the barbiturate-induced sleep *via* their central depressant effect.

Experimental

Materials——Animals: Male ddN strain mice weighing 22—28 g were used. Animals were housed under a normal light-dark cycle (7:00—19:00) at ambient temperature (23±1 °C). Food and water were given *ad lib*.

Chemicals: Preparations of allyl compounds were carried out according to the method described in our previous paper.[3] Sodium PheB (Linasen, Daiichi Seiyaku Co., Ltd.), AB (Isomytal, Nippon Shinyaku Co., Ltd.), sodium TP (Ravonal, Tanabe Seiyaku Co., Ltd.), sodium B (E. Merck A G.), sodium PB (Nembutal, Abbott Laboratories) and pentylenetetrazol (PTZ) (K & K Laboratories) were used. All allyl compounds and AB were suspended in 1% Tween 80–saline solution and the other drugs used were dissolved in saline. Allyl compounds and barbiturates were administered intraperitoneally (i.p.) to mice.

Animal Experiments——Measurement of Sleeping Time: Sleeping time was considered to be the interval between loss and recovery of an effective righting reflex. An effective righting reflex was considered to be recovery from a side position within 1 min.

Hypnotic Activity [50% Hypnotic Dose (HD_{50})]: Hypnotic activity was determined as the loss of righting reflex regardless of the duration of the effect. The number of mice that slept was recorded for each dose, and the dose required to induce sleep in 50% of the animals (HD_{50} with 95% confidence limits) was determined.

Anticonvulsant Activity [50% Effective Dose against PTZ-induced Seizures (PTZ-ED_{50})]: The anticonvulsant activity was evaluated in terms of the protection against PTZ-induced seizures, by using a modification of the method described by Vida *et al.*[8] Compounds were administered 20 min prior to the subcutaneous injection of PTZ, 120 mg/kg. The blocking of tonic-extensor convulsion was considered to be evidence of anticonvulsant activity.

Acute Toxicity [50% Lethal Dose (LD_{50})]: The LD_{50} values of the compounds tested were also determined. Mortality was observed for a period of 3 d.

Interaction with Barbiturates: Allyl derivatives were injected 15 min prior to the administration of barbiturates. The effects of MAPheB, DAPheB, MAAB and DAAB (80 mg/kg, i.p.) on the sleep induced by several barbiturates (B, PheB, AB, PB and TP) were studied. Control groups received the vehicle, 1% Tween 80–saline solution. Dose-response experiments on the effects of MAPheB and DAPheB (20, 40 and 80 mg/kg, i.p.) on the PheB (120, 150 and 180 mg/kg, i.p.)-induced sleeping time were carried out. In the experiments on the interaction of MAAB and DAAB with AB, the allyl compound (5, 20 and 40 mg/kg, i.p.) was administered prior to the injection of AB (60, 80 and 100 mg/kg, i.p.). The effects of MATP, DATP and *N*,*S*-DATP (80 mg/kg, i.p.) on the PB (40 mg/kg, i.p.)-induced

sleep were also evaluated.

Statistical Analysis——HD_{50}, PTZ-ED_{50}, LD_{50} and their 95% confidence limits were calculated by the method of Litchfield and Wilcoxon.[12] Statistical significance was analyzed by using Student's *t*-test [a), $p<0.05$; b) $p<0.01$].

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Metabolism of the Hair Dye Component, Nitro-*p*-phenylenediamine, in the Rat

MITSUO NAKAO,*[,a] YUKIKO GOTOH,[a] YASUHIKO MATSUKI,[a]
AKIRA HIRATSUKA,[b] and TADASHI WATABE[b]

Hatano Research Institute, Food and Drug Safety Center,[a] 729–5 Ochiai, Hadano, Kanagawa 257, Japan and Laboratory of Drug Metabolism and Toxicology, Department of Hygienic Chemistry, Tokyo College of Pharmacy,[b] Horinouchi, Hachioji, Tokyo 192–03, Japan

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The metabolism of nitro-*p*-phenylenediamine (I), a constituent of hair dyes, was studied following its administration to male rats. Two major metabolites, N^4-acetyl-2-nitro-1,4-diaminobenzene (II) and N^1,N^4-diacetyl-1,2,4-triaminobenzene (IV), were isolated from the urine and identified. However, other *N*-acetyl regioisomers of II and IV, and the fully *N*-acetylated metabolite, 1,2,4-triacetylaminobenzene, could not be detected by high-pressure liquid chromatography. Further, N^4-acetyl-1,2,4-triaminobenzene (III), a possible precursor of IV, could also not be detected in the urine.

After the administration of the possible intermediates produced through the metabolism of I, the metabolite IV was isolated and identified from the urine of the animals. Based on the results of the present investigation, the hair dye constituent I appears to be metabolized successively to II, III and IV by regioselective N^4-acetylation and subsequent nitro reduction, followed by regioselective N^1-acetylation, because rats given II or III excreted IV in the urine. Compound I might be also metabolized through an alternative pathway to IV *via* 1,2,4-triaminobenzene (VI) formed by direct nitro reduction, because IV was excreted as a urinary metabolite in rats given VI or its N^1-monoacetylated product (III-1). Thus, the metabolism of I appears to proceed through regioselective *N*-acetylation of the amino groups.

Keywords——hair dye; nitro-*p*-phenylenediamine; metabolism; rat urine; *N*-acetylation; regioselectivity; HPLC

Nitro-*p*-phenylenediamine (2-nitro-1,4-diaminobenzene: I), one of the essential ingredients in commercially available hair dyes, has been reported to be mutagenic to the TA1538 and TA98 strains of *Salmonella typhimurium*[1,2] and carcinogenic to the mouse.[3-5] The metabolism of this compound, however, has remained unclarified. Nakao and Takeda have isolated and tentatively identified two major metabolites from the urine of male Sprague-Dawley rats given I intraperitoneally; one was N^4-acetyl-2-nitro-1,4-diaminobenzene (II), and the other was N^1,N^4-diacetyl-1,2,4-triaminobenzene (IV).[6] Their study suggests that the rat *N*-acetylates I and its reduced form, N^4-acetyl-1,2,4-triaminobenzene (III), regioselectively *in vivo*.

The present study was undertaken to survey the urinary excretion of the regioisomers of II and IV and of the fully acetylated metabolite, 1,2,4-triacetylaminobenzene (V), in the rat. A survey was also made in the present study of the probable routes of metabolism of I, on the basis of the detection, identification and quantification of urinary metabolites following the administration of possible intermediates to the rat.

Experimental

Chemicals——I, 1,2,4-triaminobenzene (VI) dihydrochloride, N^1, N^4-diacetyl-2-nitro-1,4-diaminobenzene (II-2), N^4-acetylsulfanilamide and acetanilide were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. II and IV were synthesized from I and II-2, respectively, according to our published procedures.[6] N^1-Acetyl-2-nitro-1,4-

diaminobenzene (II-1) was synthesized by the method of Atkinson *et al.*[7] III, N^1-acetyl-1,2,4-triaminobenzene (III-1) and N^2-acetyl-1,2,4-triaminobenzene (III-2) were synthesized by the method described by Kehrmann and Mermod.[8] For convenience, the reaction mixture of Kehrmann and Mermod,[8] containing the *N*-acetyl-1,2,4-triaminobenzene (III, III-1, or III-2), was directly used for quantitative derivatization to the corresponding dipropionate; this was carried out by agitating the mixture with propionic anhydride (0.5 vol.) at room temperature for 30 min. The reaction mixture was then concentrated *in vacuo*. The residue was recrystallized from acetone. The melting points and mass spectral (MS) data for the *N*-acetyl-1,2,4-triaminobenzene dipropionate derivatives can be summarized as follows: III dipropionate—mp 200 °C, MS *m/z* (relative intensity, %): 277 (M^+, 73), 259 (15), 221 (52), 179 (18), 165 (100), 161 (86), 123 (59), 122 (82); III-1 dipropionate—mp 204 °C, MS *m/z* (relative intensity, %): 277 (M^+, 44), 259 (30), 221 (25), 203 (31), 179 (38), 165 (32), 161 (100), 147 (96), 123 (41), 122 (67); and III-2 dipropionate—mp 200 °C, MS *m/z* (relative intensity, %): 277 (M^+, 48), 259 (22), 221 (34), 203 (26), 179 (31), 165 (49), 161 (58), 147 (100), 123 (43), 122 (76). N^2,N^4-Diacetyl-1,2,4-triaminobenzene (IV-1) and N^1,N^2-diacetyl-1,2,4-triaminobenzene (IV-2) were synthesized from N^2,N^4-diacetyl-1-nitro-2,4-diaminobenzene[9] and N^1,N^2-diacetyl-4-nitro-1,2-diaminobenzene[9] in yields of 62.1 and 56.2%, respectively, after recrystallizations from acetone, by the same method as used for the synthesis of IV.[6] The melting points and spectral data for the *N*,*N'*-diacetyl-1,2,4-triaminobenzenes can be summarized as follows: IV-1—mp 232—233 °C, UV $\lambda_{max}^{ethanol}$ nm (ε): 213 (15900), 260 (12000), MS *m/z* (relative intensity, %): 207 (M^+, 72), 189 (29), 165 (33), 147 (76), 123 (88), 122 (100), 95 (52); and IV-2—mp 204—205 °C, UV $\lambda_{max}^{ethanol}$ nm (ε): 223 (18800), 255 (10900), MS *m/z* (relative intensity, %): 207 (M^+, 39), 165 (16), 147 (51), 123 (46), 122 (100), 95 (45). V was prepared by acetylation with acetic anhydride from the *N*,*N'*-diacetyl-1,2,4-triaminobenzenes.[9]

Dosage and Urine Collection——Male Sprague-Dawley rats, 6 weeks of age, each weighing about 200 g (Charles River Japan, Inc., Atsugi), were used throughout the study. The rats were injected intraperitoneally with various compounds dissolved in a 2% carboxymethyl cellulose sodium salt solution. I, II and IV were administered at 100 mg/5 ml/kg, and III, III-1 and VI at 30 mg/5 ml/kg because of their higher toxicity.

The animals had free access to food and water in individual metabolism cages. Following the injection of each compound into 5 rats, the urine was collected for 24 h. Prior to the collection of the urine, a concentrated solution of sodium bisulfite had been put in the urine container, so that at the end of 24 h, its final concentration would be 0.2—0.5%. The combined urine was frozen until use.

Extraction of Metabolites——Aliquots (2 ml each) of urine samples were mixed with a solution (0.1 ml) of an internal standard (N^4-acetylsulfanilamide or acetanilide) for subsequent high-pressure liquid chromatography (HPLC). The samples were adjusted to pH 10 with 5 N NaOH and extracted three times with two volumes of *n*-butanol–ethyl acetate (1 : 1, v/v; saturated with H_2O before use). The combined extracts were evaporated *in vacuo* below 50 °C. The residue was dissolved in methanol (0.5 ml) and poured onto a column (1.0 × 10 cm) of Silica gel 60 (E. Merck, Darmstadt) packed with ethyl acetate, and the column was eluted with ethyl acetate–ethanol (10 : 1, v/v; 130 ml). The eluate was evaporated to dryness *in vacuo* below 50 °C. The residue was redissolved in 50% aqueous methanol and subjected to HPLC.

For the determination of *N*-monoacetates of 1,2,4-triaminobenzene (VI), aliquots (2 ml each) of urine samples were agitated with propionic anhydride (0.5 ml) for 30 min. The propionated products were extracted with *n*-butanol–ethyl acetate (1 : 1, v/v; saturated with H_2O before use) and analyzed by HPLC, following the same treatment on the silica column as described above.

Chromatography——Separation of the metabolites from the urinary extracts was carried out by HPLC (Hitachi model 635 chromatograph). The columns used were a Shim-pack CLC-ODS (6 × 150 mm, 5 μm in particle size, Shimadzu) and a Shim-pack CLC-SIL (6 × 150 mm, 5 μm in particle size, Shimadzu). Monitoring was done at 254 nm with an ultraviolet (UV) detector.

Thin-layer chromatography (TLC) was carried out on precoated silica gel plates (0.25 mm in thickness, Silica gel 60 F_{254}, E. Merck, Darmstadt). The developing solvent used was chloroform–methanol–conc. NH_4OH (60 : 10 : 1, v/v). Thin layer chromatograms were visualized with a UV lamp (254 nm) or by spraying with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde).

Spectral Measurements——UV absorption spectra were recorded in ethanol with a Shimadzu UV-201 spectrophotometer. MS were obtained with a Shimadzu model QP-1000 mass spectrometer at an ionization voltage of 70 eV and an ion source temperature of 290 °C in the electron impact mode with direct insertion.

Results and Discussion

Identification of the Urinary Metabolites of Nitro-*p*-phenylenediamine (I)

A urinary extract, obtained at pH 10 by extraction with a polar solvent mixture of the 24 h urine of rats given I, was found by HPLC to contain two major and two minor compounds arising from I (Fig. 1). Of the two minor materials appearing in the chromatogram shown in Fig. 1A, the peak 1 material was identified as unchanged compound I by TLC,

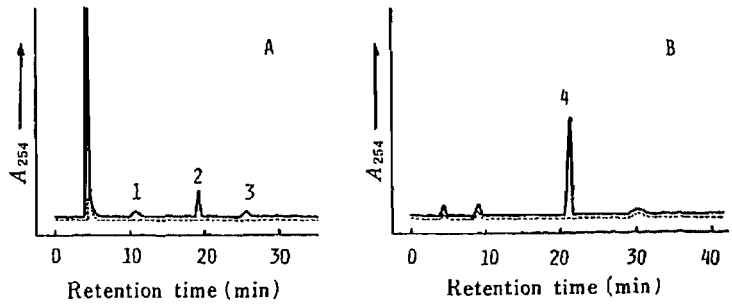

Fig. 1. Urinary Metabolites of Nitro-*p*-phenylenediamine Separated by Reverse Partition HPLC

Mobile phases used were MeOH–H_2O (3:7, v/v) and MeOH–H_2O (1:9, v/v) in chromatograms A and B, respectively. Other chromatographic conditions are described in Table I. Solid lines represent the extract from urine of rats given nitro-*p*-phenylenediamine (I), and broken lines the extract from urine of the same rats prior to the administration of I.

TABLE I. Chromatographic Data of Nitro-*p*-phenylenediamine and Related Compounds

Compound[a]				TLC[b]	HPLC[c]		
R_1	R_2	R_3		*Rf* value	Retention times (min)		
					1	2	3
NH_2	NO_2	NH_2	(I)	0.65	10.6		
NH_2	NO_2	NHAc	(II)	0.46	19.2		
NHAc	NO_2	NH_2	(II-1)	0.74	10.0		
NHAc	NO_2	NHAc	(II-2)	0.66	23.2		
NHAc	H	H[d]		0.72	32.2		
NH_2	NH_2	NHAc	(III)[e]	0.52			16.8
NHAc	NH_2	NH_2	(III-1)[e]	0.51			16.8
NH_2	NHAc	NH_2	(III-2)[e]	0.52			16.8
NHAc	NH_2	NHAc	(IV)	0.33	7.0	21.2	
NH_2	NHAc	NHAc	(IV-1)	0.27	7.0	14.0	
NHAc	NHAc	NH_2	(IV-2)	0.23	7.0	12.0	
NHAc	NHAc	NHAc	(V)	0.37	8.8	39.6	
NHAc	H	SO_2NH_2[d]		0.32		26.0	

a) R_1, R_2, and R_3 represent the substituents at the 1-, 2-, and 4-positions of the benzene nucleus, respectively. *b*) Mobile phase: $CHCl_3$–MeOH–conc. NH_4OH (60:10:1, v/v). Plates: Merck Silica gel plates 60 F_{254}. *c*) 1: Mobile phase, MeOH–H_2O (3:7, v/v); column, Shim-pack CLC-ODS; flow rate, 0.6 ml/min. 2: Mobile phase, MeOH–H_2O (1:9, v/v); column, Shim-pack CLC-ODS; flow rate, 0.6 ml/min. 3: Mobile phase, *n*-hexane–isopropanol (8:2, v/v); column, Shim-pack CLC-SIL; flow rate, 1.0 ml/min. *d*) Internal standard. *e*) *N,N'*-Dipropionate derivative.

UV absorption and MS spectra measurements. The two major urinary metabolites of I appeared as major peaks 2 and 4 in the chromatograms (Fig. 1A and B) obtained under different conditions that allowed separation of all the possible *N*-monoacetates and the N^1,N^4-diacetate of I, the *N,N'*-diacetates, of 1,2,4-triaminobenzene (VI), and the triacetate V of VI (Table I).

The peak 2 material eluted from the reverse partition HPLC column showed a single spot at *Rf* 0.46 on silica gel TLC which was visualized as a bright yellow spot by Ehrlich's reagent and also as a quenching spot under UV light. This metabolite was chromatographically identical with an authentic specimen of the N^4-monoacetate II of the parent compound I. In addition, the UV absorption and MS spectra of the metabolite and II were also superimposable on each other; $\lambda_{max}^{ethanol}$ nm (ε): 253 (23000); *m/z* (relative intensity, %): 195 (M^+, 47), 153

TABLE II. Urinary Metabolites of Nitro-*p*-phenylenediamine and Its Derivatives in the Rat

Metabolite[a] R_1	R_2	R_3		Administration[b] I	II	III	III-1	IV	VI
				% of dose[c]					
NH_2	NO_2	NHAc	(II)	4.7	4.8	—	—	—	—
NHAc	NO_2	NH_2	(II-1)	ND	ND	—	—	—	—
NHAc	NO_2	NHAc	(II-2)	ND	ND	—	—	—	—
NH_2	NH_2	NHAc	(III)	ND	ND	ND	ND	ND	ND
NHAc	NH_2	NH_2	(III-1)	ND	ND	ND	ND	ND	ND
NH_2	NHAc	NH_2	(III-2)	ND	ND	ND	ND	ND	ND
NHAc	NH_2	NHAc	(IV)	16.9	15.4	31.7	28.0	61.8	9.7
NH_2	NHAc	NHAc	(IV-1)	ND	ND	ND	ND	ND	ND
NHAc	NHAc	NH_2	(IV-2)	ND	ND	ND	ND	ND	ND
NHAc	NHAc	NHAc	(V)	ND	ND	ND	ND	ND	ND

a) R_1, R_2, and R_3 represent the substituents at the 1-, 2-, and 4-positions of the benzene nucleus, respectively. *b*) The same number is used for representing each compound as shown in Table I. *c*) ND: Not detectable ($\leq 0.1\%$ of dosed compounds).

(100), 107 (88), 80 (33).

The more polar peak 4 material showed a UV-absorbing and Ehrlich's reagent-positive (yellow) single spot at *Rf* 0.33 on silica gel TLC under the same conditions as mentioned above. This metabolite was identical with an authentic specimen of the N^1,N^4-diacetate IV of the triaminobenzene VI on co-chromatography (HPLC and TLC). In addition, their UV absorption and MS spectra were also superimposable on each other; $\lambda_{max}^{ethanol}$ nm (ε): 225.5 (29600), 255 (12000); *m/z* (relative intensity, %): 207 (M^+, 66), 189 (21), 165 (29), 147 (57), 123 (89), 122 (100), 95 (52). The peak 3 material was not identical with any of the authentic specimens used.

The present investigation not only gives solid support to our previous study carried out by using radioactive I, in which we demonstrated that only metabolites II and IV are excreted in the urine of rats given I intraperitoneally,[6] but also provides direct evidence for regioselectivity in the *N*-acetylation of I, since the N^1-monoacetate II-1 of I, a regioisomer of II, and the N^1,N^4-diacetate II-2 of I were not detected by HPLC. More direct evidence for the highly regioselective *N*-acetylation of I to II has very recently been obtained from a study *in vitro* in our laboratory. Our unpublished data indicated that I was specifically acetylated at N^4 in rat liver cytosol fortified with acetyl-CoA to afford II as the sole metabolite in the presence and in the absence of the carboxyesterase inhibitor, paraoxon (0.1 mM), and also that 2-nitroaniline did not undergo *N*-acetylation under the same conditions *in vitro*, whereas 3-nitroaniline was readily *N*-acetylated. It is of interest that, of the three possible diacetates of the triamine VI, only the single isomer IV was excreted in the urine of rats given I without any detectable amount of the N^1- and N^4-monoacetates (III and III-1), possible precursors of IV (Table II). The fully *N*-acetylated product V of the triamine VI was also not detectable in the urine (Table II), although authentic specimens of these undetectable mono-, di- and tri-acetates added to the urine of untreated rats were all recovered in ratios higher than 80% by the analytical procedures used. As to the mono-acetates, they were extracted as stable *N*,*N'*-dipropionates from the neutral urine treated directly with propionic anhydride.

Obligatory Intermediacy of Nitro-*p*-phenylenediamine N^4-Monoacetate (II) in the Metabolism of I to 1,2,4-Triaminobenzene N^1,N^4-Diacetate (IV)

The N^4-monoacetate II of I was injected into rats in order to confirm whether or not II is an obligatory intermediate in the biotransformation of I to the N^1,N^4-diacetate IV. From the urine of the animals, IV was isolated and identified as the sole *N*,*N'*-diacetylated metabolite

by the aforementioned chromatographic and spectroscopic methods. However, the triaminobenzene N^4-monoacetate III, a possible precursor of IV, could not be detected in the urine by HPLC (Table II).

Injection of III or III-1 into the animals resulted in the excretion of a significant amount of IV without concomitant excretion of any detectable amount of the *N,N'*-diacetylregioisomers IV-1 and IV-2 (Table II). No trace of the triacetate V was detected in the urine of rats given II, III, and III-1 (Table II).

1,2,4-Triaminobenzene (VI) as Another Obligatory Intermediate in the Metabolism of I to IV

In spite of many attempts, no suitable method has been developed for the detection and isolation of 1,2,4-triaminobenzene (VI), a reduction product of I and a possible intermediate to the major metabolite IV, because of the extreme instability of the triamine VI in rat urine. However, the animals given intraperitoneally a stable dihydrochloride salt of the triamine VI excreted the N^1,N^4-diacetate IV in their urine as the sole metabolite, which was isolated and identified in the same manner as mentioned above. This rat urine did not contain any detectable amount of the *N*-monoacetates (III, III-1 and III-2), other *N,N'*-diacetates (IV-1 and IV-2), or the N^1,N^2,N^4-triacetate (V) (Table II).

1,2,4-Triaminobenzene N^1,N^4-Diacetate (IV) as a Final Acetylated Metabolite in the Metabolism of I

The N^1,N^4-diacetate IV of triaminobenzene VI was administered to rats to examine whether IV is a final acetylated metabolite in the biotransformation of I. The N^1,N^4-diacetate IV, however, did not undergo further acetylation, as is apparent from the fact that the triacetate V, an expected final acetylated metabolite, could not be detected by HPLC (Table II). Thus, there was no evidence for N^2-acetylation taking place in rats injected with any of the triamine VI, the diamines III and III-1, and the monoamine IV.

The possibility, however, should not be excluded of facile disposition of the metabolites with the 2-acetylamino group by different types of metabolic reactions such as aromatic ring oxidation and *O*- or *N*-conjugations of the resulting aminophenols. Actually, only 22.8% of I given to rats was recovered as the unchanged form (1.2%), II (4.7%), and IV (16.9%) from the urine. Aminophenols in general may be too polar and unstable to be recovered substantially from urine. Nakao and Takeda[6] failed to extract more polar metabolites than II and IV from acid-treated and subsequently neutralized urine of rats given [^{14}C]I.

In the present study, carried out using a polar solvent mixture and sodium bisulfite added as an antioxidant to urine, no appreciable peaks probably due to phenols or aminophenols arising from I were detected by HPLC even after direct treatment of the urine with propionic anhydride, which would stabilize them by formation of the *N*-propionates. Actually, the unstable *N*-monoacetates (III, III-1 and III-2) of VI added to rat urine were recovered as stable *N,N'*-dipropionates in the presence of the bisulfite, while the authentic triamine VI used as a free base was not recovered from the urine by this method.

It is of interest that metabolic *N*-acetylation *in vivo* is likely to take place only at the N^4- and N^1,N^4-positions for nitro-*p*-phenylenediamine (I) and 1,2,4-triaminobenzene (VI), respectively, as judged simply from the isolated metabolites. Based on this assumption, a probable pathway in the metabolism of I to the N^1,N^4-diacetate IV in the rat may be summarized as illustrated in Chart 1. The pathway may involve the formation of two essential precursors from I; one (II), an isolated metabolite, is regioselectively formed by direct N^4-acetylation, and the other (VI), not detected, formed by direct nitro reduction of I. The precursor II administered to rats is reduced to the obligatory intermediate III and excreted in the urine after regioselective N^1-acetylation to IV. The N^4-monoacetate III is also excreted as IV when administered to rats. The other possible precursor VI may be metabolized to IV *via* two obligatory intermediates III and III-1. The undetected urinary metabolite III-1 is also

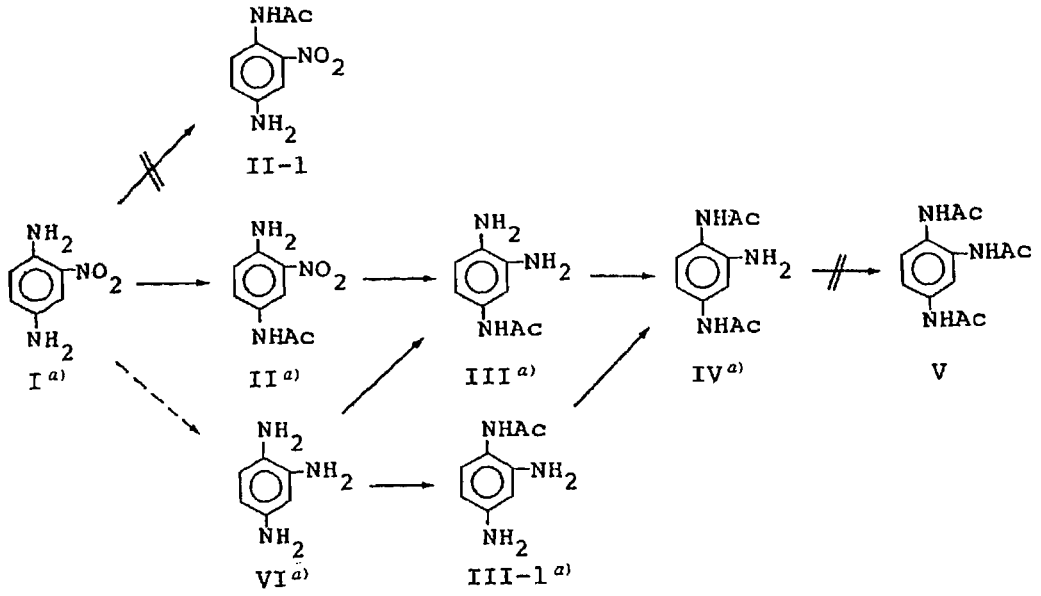

Chart 1. Proposed Pathway for the Metabolism of Nitro-*p*-phenylenediamine in the Rat

Compounds marked *a*) were administered to the animals to study their intermediacy.

excreted as IV after administration to rats.

N-Deacetylation of the acetylamino group[10,11] may also occur in these probable routes for the metabolism of I, but the present data do not provide any evidence for this. As to the urinary metabolites II and IV, no detectable amount of I, III or III-1 was excreted in the urine of the animals injected with the metabolites. This would suggest that *N*-deacetylation may not take place with II and IV *in vitro*. In connection with this, Bray *et al.*[12] have demonstrated that a rat liver extract is hardly capable of *N*-deacetylation of 3-nitroaniline *N*-acetate, while 2-nitroaniline *N*-acetate is readily *N*-deacetylated under the same conditions.

The regioselectivity in *N*-acetylation *in vivo* suggests the participation of a steric hindrance factor in enzymatic transacylation of an active acetyl group to these aromatic amines. In connection with this, Grantham *et al.* have demonstrated that rats given 2,4-diaminotoluene excrete the N^4-mono-acylated metabolites, 4-acetylamino-2-aminotoluene and 4-acetylamino-2-aminobenzoic acid,[13] and also that 2,4-diaminoanisole given to rats is regioselectively acetylated at the N^4-position and excreted mainly as 4-acetylamino-2-aminoanisole in the urine.[14] They did not detect the regioisomeric metabolite, 2-acetylamino-4-aminoanisole, in the rat urine.

An *in vitro* study of enzymatic *N*-acetylation of the aromatic amines used in the present investigation is now in progress in our laboratories.

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The Effects of Metabolic Activation on the Mutagenicity of Aminoacridines

MASAHITO FUKUNAGA,*,[a] YASUO MIZUGUCHI[b] and LERENA W. YIELDING[c]

Faculty of Pharmacy and Pharmaceutical Science, Fukuyama University,[a] Sanzo 985, Higashimuramachi, Fukuyamashi 729-02, Japan, Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Japan,[b] Iseigaoka 1-1, Yahatanishiku, Kitakyushushi 807, Japan and Department of Anatomy, College of Medicine, University of South Alabama,[c] Mobile, Alabama 36688, U.S.A.

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The mutagenic activity of a series of aminoacridines was examined by the preincubation method with and without mammalian metabolizing enzymes in *Salmonella* tester strains. Without metabolizing enzymes, three acridines having an amino substituent at position 9 showed high activity in *Salmonella typhimurium* TA1537 and TA1977, and other acridines with amino substituents at position 2 or 3 were mutagenic but were less effective than the 9-aminoanalogs. The liver microsomal enzymes generally deactivated acridine mutagenicity in TA1537, but created a broad spectrum of mutagenicity if tested by the reversion of TA1535, the strain detecting base-pair substitution mutagens, and TA1538, the strain detecting covalently bound frameshift mutagens.

Keywords——acridine; mutagenicity; chemical mutagen; Ames test; metabolic activation

Introduction

The aminoacridines are classic deoxyribonucleic acid (DNA)-intercalating compounds which induce frameshift mutagenesis.[1] They have been examined extensively in an effort to determine the underlying molecular processes responsible for their mutagenic activity.[2] The frameshift mutagenic activity, amply demonstrated by reversion of the Ames *Salmonella* tester strain TA1537, is regarded as characteristic of 9-aminoacridine.[3-6] However, when metabolically activated with a mammalian liver microsomal preparation, proflavine (3,6-diaminoacridine) exhibits another frameshift mutagenic activity characterized by the reversion of the *Salmonella* tester strain TA1538.[3]

In this study, in an effort to determine the effects of metabolism on the mutagenic potential of aminoacridines, a series of acridines was examined using the Ames strains with an without mammalian metabolic enzymes incorporated into the assay.

Materials and Methods

Compounds——Acridine (Aldrich Chemical Company, Milwaukee, WI) was recrystallized from 50% ethanol; 9-aminoacridine and 3,6-diaminoacridine (proflavine, Sigma Chemical Company, St. Louis, MO) were recrystallized from acetone and water, respectively; 10-methylacridinium chloride (Eastman Kodak Company, Rochester, NY) was used without further purification; and 3,6-diamino-10-methylacridinium chloride (acriflavine, Aldrich Chemical Company), obtained as a mixture of acriflavine and proflavine, was purified by anion exchange chromatography according to the procedure of Gupta *et al.*[7] All the other acridine compounds were synthesized in our laboratory[8] and were confirmed to be pure by thin-layer chromatography on Silica Gel "G" plates (Brinkman Instruments, Inc., Westbury, NY) using visible and short or long wavelength UV-light for detection. Structures for all compounds synthesized were confirmed by proton nuclear magnetic resonance (^{1}H-NMR) prior to testing. All chemicals were

dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt) at a concentration of 1—2 mg/ml. Dilutions were made in DMSO and 20 μl of each concentration was used. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase (G-6-PDH) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Salmonella Tester Strains——The *Salmonella typhimurium* LT-2 tester strains developed by Dr. B. N. Ames (University of California, Berkeley, CA) are histidine-requiring mutants (*his*$^-$), and the reversion assay was used to examine the mutagenic activity of the acridines. TA1535 is reverted by mutagenic agents which cause base-pair substitutions; the other strains, TA1537 and TA1538, are reverted by mutagens which cause frameshifts. Strains TA1975, TA1977 and TA1978, unlike the corresponding strains TA1535, TA1537 and TA1538, possess a normal *uvrB* repair system.[5]

Media and Culturing——The strains were handled essentially as recommended by Ames.[5,9-11] Overnight cultures were grown to densities of *ca.* 1×10^9 cells/ml in 10 ml of L-broth (1% Bactotryptone, 0.5% yeast extracts, 0.5% NaCl and 0.1% dextrose) as determined by dilution plating.

Mutagen Screening——Mutagenicity was examined by the preincubation method of Yahagi,[12] a modification of Ames' method.[10] This procedure involves a preincubation period for the bacteria (*ca.* 10^8 cells) and the acridine (20 μl in DMSO) in 0.5 ml of sodium phosphate (0.1 M, pH 7.4) buffer at 37 °C for 20 min. During this preincubation, the bacteria were not growing, but the acridine was allowed ample time to permeate into the bacterial cells. All of the bacteria were exposed to the same acridine concentration, in contrast to the spot-test method where a concentration gradient is formed by the acridine on the plate and only a fraction of the bacteria are in contact with the highest acridine concentration.

When mammalian microsomal enzymes were included in the assay, the same preincubation method was used. Microsomal enzymes (S9) were prepared from male Wistar rats (*ca.* 200 g) treated with 500 mg/kg of tetrachlorobiphenyl (Wako Pure Chemical Co., Ltd., Tokyo, Japan). About 10^8 *Salmonella* cells (0.1 ml of overnight culture in nutrient L-broth) were incubated with 20 μl of samples for 20 min at 37 °C in 0.5 ml of medium, containing 4 μmol of $MgCl_2$, 16.5 μmol of KCl, 2.5 μmol of G-6-P, 2 μmol of NADPH, 0.25 unit of G-6-PDH, 50 μmol of phosphate buffer (pH 7.4) and 0.15 ml of S9 rat liver homogenate (3 nmol as cytochrome P450). After the treatment, 2 ml of top agar (0.7% Bactoagar, 0.5% NaCl, 0.1 μmol each of histidine and biotin) was used for spreading the sample on Vogel-Bonner minimal medium[13] plates. Subsequently, the plates were incubated in the dark for 2 d at 37 °C. Four plates were used for each concentration of acridine compound in the experiments, and each experiment was performed three times on different days.

Results

The mutagenicity of a series of actidines was examined in the Ames *Salmonella* tester strains using the preincubation method of Yahagi.[12] These same acridines had been examined previously by the spot-test method.[4] The two methods produced different results, as might be expected for a variety of reasons, including the steep dose-response curve for aminoacridines and limited diffusion of chemicals in agar as required by the spot-test method.

Without S9 microsomal enzymes in the preincubation assay, only acridine, the parent compound, was not mutagenic, although 2-aminoacridine and 10-methylacridinium chloride showed minimal activity (Table I). 2,9-Diaminoacridine, 3,9-diaminoacridine, 3,6-diaminoacridine, 2-amino-10-methylacridinium chloride, 3-amino-10-methylacridinium chloride and 3,6-diamino-10-methylacridinium chloride were all potent mutagens in TA1537. In addition, 2-amino-10-methylacridinium chloride produced lower reversion levels in the excision repair-proficient strain, TA1977. 2-Amino-10-methylacridinium chloride, and to a lesser extent 3,9-diaminoacridinine, showed activity in TA1538 (203 revertants per μg and five revertants per μg, respectively). The *uvrB*-proficient strain, TA1978 was much less sensitive to reversion by these compounds.

The preincubation procedure has been recommended as the best assay procedure, because it provides for a greater exposure of the bacteria to the compound being tested. This exposure of the bacteria to the compound prior to its addition to the growth medium was

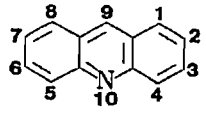

Fig. 1. Acridine Structure and Numbering System

TABLE I. The Effects of Mamalian Microsomal Activation on the Mutagenicity of Acridines in *Salmonella*

Chemicals	Concentration (μg/plate)	Strains										
		TA1535 −S9	TA1535 +S9	TA1975 +S9	TA1537 −S9	TA1977 −S9	TA1537 +S9	TA1977 +S9	TA1538 −S9	TA1978 −S9	TA1538 +S9	TA1978 +S9
Control ± S.D.[a]	0	26±5[b]	28±10	25±10	17±5	20±5	15±5	12±5	21±5	17±5	37±10	27±10
Acridine	20	30±10	29±5	—	25±5	—	41±10	—	23±5	—	44±10	—
1-Aminoacridine	20	27±5	32±5	—	319±60	255±50	440±60	80±20	18±5	—	260±50	32±10
	10	—	—	—	22±5	20±5	—	—	—	—	—	—
2-Aminoacridine	20	30±5	778±90	40±15	51±10	20±5	>1000	45±15	20±5	—	>1000	650±110
	10	24±5	305±30	—	26±5	—	*ca.*1000	31±10	24±5	—	>1000	170±40
	4	—	90±15	—	—	—	190±20	—	—	—	898±130	60±15
	2	—	39±10	—	—	—	53±15	—	—	—	320±65	30±10
	1	—	—	—	—	—	—	—	—	—	120±25	—
3-Aminoacridine	20	36±5	160±40	32±10	*ca.*1000	*ca.*1000	*ca.*1000	*ca.*1000	47±10	—	912±120	120±25
	15	—	—	—	—	—	204±30	261±45	—	—	—	—
	10	27±5	—	—	>1000	>1000	57±15	30±10	26±5	—	455±40	31±10
	4	—	—	—	*ca.*1000	*ca.*1000	18±5	15±5	—	—	146±20	—
	2	—	—	—	585±20	553±60	—	—	—	—	—	—
	1	—	—	—	153±40	38±10	—	—	—	—	—	—
9-Aminoacridine	20	38±10	32±10	—	*ca.*1000	*ca.*1000	*ca.*1000	*ca.*1000	24±5	—	44±10	—
	10	—	—	—	>1000	>1000	275±40	160±25	—	—	—	—
	4	—	—	—	>1000	>1000	35±10	33±10	—	—	—	—
	2	—	—	—	*ca.*1000	*ca.*1000	—	—	—	—	—	—
	1	—	—	—	492±95	496±85	—	—	—	—	—	—
	0.4	—	—	—	20±5	27±10	—	—	—	—	—	—
1,9 Diaminoacridine	20	—	95±15	60±10	*ca.*1000	*ca.*1000	>1000	>1000	—	—	152±20	55±15
	10	15±5	—	—	>1000	>1000	*ca.*1000	*ca.*1000	14±5	—	—	—
	4	35±10	—	—	>1000	>1000	130±25	28±10	28±5	—	—	—
	2	—	—	—	>1000	>1000	35±10	—	—	—	—	—
	1	—	—	—	*ca.*1000	*ca.*1000	—	—	—	—	—	—
	0.4	—	—	—	73±20	52±20	—	—	—	—	—	—
2,9-Diaminoacridine	20	36±10	32±10	—	>1000	>1000	245±40	255±20	27±5	—	170±30	29±5
	10	25±5	—	—	>1000	>1000	—	—	20±5	—	—	—
	4	—	—	—	*ca.*1000	775±150	—	—	—	—	—	—
	2	—	—	—	224±40	50±15	—	—	—	—	—	—
3,9-Diaminoacridine	20	38±10	25±5	—	*ca.*1000	*ca.*1000	55±15	28±10	124±20	29±5	162±20	28±5
	10	22±5	—	—	>1000	>1000	—	—	40±10	18±5	—	—
	4	—	—	—	*ca.*1000	*ca.*1000	—	—	—	—	—	—
	2	—	—	—	172±42	220±45	—	—	—	—	—	—
	1	—	—	—	23±5	28±5	—	—	—	—	—	—

3,6-Diaminoacridine	20	—	25±5	—	21±5	16±5	*ca.*1000	*ca.*1000	—	—	>1000	153±30
	10	31±10	—	—	160±40	150±40	216±35	150±25	10±5	—	*ca.*1000	60±20
	4	28±5	—	—	*ca.*1000	*ca.*1000	30±10	24±10	11±5	—	510±40	28±10
	2	30±10	—	—	*ca.*1000	*ca.*1000	—	—	10±5	—	154±30	—
	1	—	—	—	175±35	150±35	—	—	—	—	60±20	—
	0.4	—	—	—	25±5	32±10	—	—	—	—	—	—
10-Methylacridinium chloride	20	32±10	46±15	—	40±10	—	80±20	—	23±5	—	88±15	26±5
1-Amino-10-methyl-acridinium chloride	20	21±5	22±5	—	>1000	>1000	38±10	—	35±10	—	41±10	—
	10	—	—	—	*ca.*1000	*ca.*1000	—	—	26±5	—	—	—
	4	—	—	—	283±35	55±15	—	—	—	—	—	—
	2	—	—	—	40±10	26±5	—	—	—	—	—	—
2-Amino-10-methyl-acridinium chloride	20	36±10	34±10	—	>1000	224±50	290±60	85±20	>1000	295±40	>1000	>1000
	10	—	—	—	642±110	78±25	—	—	>1000	150±35	>1000	795±90
	4	—	—	—	317±55	20±5	—	—	833±130	65±20	>1000	160±40
	2	—	—	—	42±15	—	—	—	378±74	—	>1000	70±15
	1	—	—	—	—	—	—	—	217±40	—	*ca.*1000	26±5
	0.4	—	—	—	—	—	—	—	—	—	520±80	—
	0.2	—	—	—	—	—	—	—	—	—	220±40	—
	0.1	—	—	—	—	—	—	—	—	—	140±45	—
3-Amino-10-methyl-acridinium chloride	20	28±5	25±5	—	16±5	14±5	*ca.*1000	*ca.*1000	29±5	—	410±45	28±5
	15	—	—	—	—	—	237±25	198±30	—	—	—	—
	10	24±5	—	—	*ca.*1000	*ca.*1000	70±15	35±10	21±5	—	—	—
	4	—	—	—	>1000	>1000	—	—	—	—	—	—
	2	—	—	—	545±65	535±58	—	—	—	—	—	—
	1	—	—	—	84±20	22±5	—	—	—	—	—	—
9-Amino-10-methyl-acridinium chloride	20	75±15	77±25	—	*ca.*1000	*ca.*1000	>1000	>1000	25±5	—	42±10	—
	10	22±5	—	—	>1000	>1000	*ca.*1000	*ca.*1000	24±5	—	—	—
	4	—	—	—	>1000	>1000	30±10	19±5	—	—	—	—
	2	—	—	—	>1000	>1000	—	—	—	—	—	—
	1	—	—	—	1000	730±52	—	—	—	—	—	—
	0.4	—	—	—	50±20	39±15	—	—	—	—	—	—
3,6-Diamino-10-methyl-acridinium chloride	20	—	26±5	—	12±5	26±5	*ca.*1000	*ca.*1000	—	—	350±35	50±10
	10	16±5	—	—	120±25	176±35	240±50	225±45	10±5	18±5	—	—
	4	29±5	—	—	*ca.*1000	*ca.*1000	24±10	15±5	12±5	20±5	—	—
	2	25±5	—	—	*ca.*1000	*ca.*1000	—	—	16±5	—	—	—
	1	—	—	—	*ca.*1000	*ca.*1000	—	—	—	—	—	—
	0.4	—	—	—	25±5	22±5	—	—	—	—	—	—

a) Control ± S.D. indicates the mean and standard deviation for spontaneous bacterial *his*$^+$ revertants in the absence of acridine. *b*) The number of *his*$^+$ revertants generated per 1×10^8 bacteria when an acridine solution was incubated with 0.1 ml of late log culture of *Salmonella* in phosphate buffer (0.1 M, pH 7.4) with (+S9) and without (−S9) 0.15 ml of S9 rat liver homogenate (3 nmol as cytochrome P-450). Additional information is given in Materials and Methods. Experiments were done in triplicate and each experiment included 4 plates. −, not tested.

found to be critical in our experiments. For 2,9-diaminoacridine and 2-amino-10-methylacridinium chloride, the reversion frequencies without a preincubation period were 3.5 and 2.5 revertants per μg, respectively. However, a 5-min preincubation increased the mutagenic activity of these compounds to >50 revertants/μg (data not shown).

The effect of metabolism by microsomal enzymes on the mutagenic activity of these acridines was examined, and the results are given in Table I. Again, the preincubation method was used, as described for the previous experiments. Thus, the bacteria were exposed to the mammalian metabolizing enzymes and acridine in the absence of growth medium for 20 min at 37 °C prior to plating. Most of the acridines that were mutagenic in TA1537 without exposure to the mammalian enzymes showed much lower activities following exposure to these enzymes. For example, 1-amino-10-methylacridinium chloride was rendered completely non mutagenic by the enzyme. However, there were three exceptions; the low activity of 2-aminoacridine was greatly enhanced following S9 activation; the moderate activity of 1-aminoacridine was increased slightly; and the low activity of 10-methylacridinium chloride was doubled.

An interesting observation from the S9-metabolic activation studies was the enhanced activity for TA1538. The more effective compounds had amino substitution at the 2 and 3 (6) positions. A methyl substituent at position 10 further enhanced the activity of those compounds with an amino group at position 2 but suppressed the activity of those compounds with an amino group at position 3 (6). Only acridine, which never showed mutagenic activity with any of the experimental protocols, 9-aminoacridine, 1-amino-10-methylacridinium chroride, and 9-amino-10-methylacridinium chloride showed no activity for TA1538, and 10-methylacridinium chloride was only slightly mutagenic.

2-Aminoacridine demonstrated high activity in TA1535, the strain detecting base-pair substitution mutagens after incubation with S9 enzymes. 3-Aminoacridine produced modest activity, and 1,9-diaminoacridine and 9-amino-10-methylacridinium chloride were slightly active.

Discussion

When the compounds were examined by the preincubation method of Yahagi[12] without the S9 enzyme preparation, some acridine derivatives (3,9-diaminoacridine and 2-amino-10-methylacridinium chloride) showed mutagenic activity in TA1538, and almost all compounds were highly mutagenic in TA1537. Although the amino substituent at position 9 dominated the activity of the acridines, amino groups at positions 2 and 3 were also effective, particularly when a methyl group was also present at the ring nitrogen.

Finally, the processes which were induced by mammalian microsomal enzymes affected the acridines with amino groups at positions 2 and 3 (6), as seen by a depression in TA1537 frameshift activity and an induction of TA1538 frameshift activity, especially for 2-aminoacridine, 2-amino-10-methylacridinium chloride, 3-aminoacridine and 3,6-diaminoacridine. The 10-methylated forms of 3-aminoacridine and 3,6-diaminoacridine were slightly less active in TA1537 and TA1538 than their non-methylated counterparts. The metabolically activated 2-aminoacridine, and to a lesser extent, 3-aminoacridine also reverted the base-pair substitution strain TA1535. Thus, deactivation of mutagenic activity for TA1537 resulted when the acridines were assayed in the presence of S9 mammalian enzyme fraction, yet a broader spectrum of activity was seen by the reversion of both frameshift strains, TA1537 and TA1538, and the base-pair substitution strain, TA1535.

Very little is known about the mechanisms responsible for acridine mutagenesis. Much information has accumulated recently concerning the mutagenic activity of 9-aminoacridines,[14] and structure–function relationships of acridines and mutagenic activity found by

the spot-test method.[4,15] The spot-test method involved overlaying bacteria in top agar with 10 μl of an acridine solution of 2 mg/ml DMSO placed in the center of the plate. Using our procedure, all of the compounds which were active in the spot-test method showed enhanced levels of mutagenesis. In addition, some acridines demonstrated mutagenicity which was not observed by the spot-test method. More importantly, the mutagenicity of covalently bound acridines formed by either metabolic activation of proflavin[16] or light activation of azido analogs of acridines[14] has been studied. The results presented in this communication emphasize the importance of amino substituents in the mutagenic process involving: 1) non-covalent, and presumably simple intercalative, interactions between acridines and DNA; and 2) covalent and non-covalent adduct formation between acridines and DNA following S9 metabolic activation.

In conclusion, the results presented here suggest that mammalian metabolizing enzymes cause both activation and deactivation of aminoacridines. Further, an amino substituents, particularly at position 9, but also at positions 2 and 3 (6), and a methyl substituent at position 10 play a major role in regulating the activities of aminoacridines undergoing mammalian metabolism.

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Interaction of Cationic Surfactant with Arabate and Chondroitin Sulfate[1)]

CHIKAKO YOMOTA,*[,a] YOSHIO ITO,[a] and MASAYUKI NAKAGAKI[b]

National Institute of Hygienic Sciences, Osaka,[a] 1-1-43 Hoenzaka, Higashi-ku, Osaka 540, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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Interactions of cationic surfactants with a branched polymer, arabate (Ar), and a linear polymer, chondroitin sulfate (Chs), were investigated by the potentiometric technique using surfactant ion-selective electrodes. The results indicated a highly cooperative nature in the binding process of *n*-dodecyltrimethylammonium ion ($DOTMA^+$) with Chs, but not Ar, in spite of the similarity of the charge density parameter, ξ, between the two polymers. The cooperativity parameter, u, was estimated from the Zimm–Brag theory in the present experimental system to be 3.5 for Ar and 200 for Chs, each of which was independent of the concentration of added salt. The binding constant, K, in the case of the Chs–$DOTMA^+$ system was considerably larger than that of the Ar–$DOTMA^+$ system. This difference of binding behavior of $DOTMA^+$ to Ar and to Chs was considered to be mainly due to the structural difference between the two polymers.

Keywords——arabic acid; chondroitin sulfate; cationic surfactant; binding isotherm

Gum arabic is an important material in drug processing as an emulsifying agent, suspending agent, binding agent and raw materials for preparing microcapsules.[2)] The main component of gum arabic is arabic acid, which is a branched heteropolysaccharide containing one glucuronic acid for every seven glycosides.[3)]

In the previous papers,[3,4)] in order to clarify the fundamental properties of arabic acid as a polyelectrolyte, the osmotic coefficient, ϕ,[3)] counterion activity coefficient, γ_+,[3)] and transport parameter, f,[4)] of arabate solution were measured. The results suggested that the interaction of arabic acid with counterions is due to electrostatic force alone, and further that Manning's theory for cylindrical polyelectrolytes could be applicable to the branched polysaccharide, if the intercharge distance, b, can be assumed to be a parameter reflecting a spatial intercharge distance.

In the present work, the interaction of arabate with cationic surfactants was investigated as a model system to examine the effect of the structural properties of arabate, having low charge density and branching, on the binding behavior with large-molecular ionic substances. For this purpose, binding isotherms of cationic surfactants to arabate were measured by using a surfactant ion-selective electrode as reported previously.[5,6)]

Materials and Methods

Materials——Sodium arabate (Ar) was purified from acacia senegal gums as reported previously.[3)] Sodium chondroitin sulfate (Chs) of the c type was purchased from Seikagaku Kogyo Co., Ltd. and used without further purification. Cationic surfactants such as *n*-octyltrimethylammonium bromide (OTMABr), *n*-decyltrimethylammonium bromide (DETMABr) and *n*-dodecyltrimethylammonium bromide (DOTMABr) were purchased from Tokyo Kasei Kogyo Co., Ltd. and purified by recrystallization from acetone. All the other chemicals used in this work were of reagent grade and were used without further purification.

Measurements of Free Surfactant Concentration——Surfactant-selective electrodes developed recently were

applied to determine the free surfactant concentration. Alkyltrimethylammonium cation-selective membrane, or a solid state-membrane made of poly(vinyl chloride) (PVC), was prepared by the following method.[5,6] A mixture of 0.2 g of PVC, 0.79 g of bis(2-ethylhexyl) phthalate, and 10 mg of the carrier described below was dissolved in an aliquot of 6 ml of tetrahydrofuran by heating. The obtained clear viscous solution was poured into a Petri dish of 10 cm in diameter. The solvent, tetrahydrofuran, was allowed to evaporate off gradually overnight at room temperature, and a thin membrane was obtained. The carrier used for preparing the membrane was propionyl-α-cyclodextrin, which was prepared by the esterification of α-cyclodextrin.[5] The electrode membrane was annealed at 40—50 °C under reduced pressure for 4 h, and cut to a suitable size. A piece of the PVC membrane was glued onto the bottom of a PVC tube by using a tetrahydrofuran solution of PVC as an adhesive.

The membrane potential was measured with the following cell: | calomel electrode | agar, 1 M NH_4Cl | test solution | PVC membrane | reference solution | agar, 1 M NH_4Cl | calomel electrode |. The electromotive force (EMF) of the cell was measured with a Metrohm 654 potentiometer. The EMF value reached a constant within a few minutes under the present experimental conditions and the values at various concentrations of surfactants were reproducible with accuracy of within ±0.1 mV over 2 months. With regard to the PVC membrane, its selectivity was confirmed not to be interfered with by any of the wide range of inorganic ions used in the experiments. All the measurements in the present experiments were carried out at 25 °C.

Measurements of Sodium Ion Activity Coefficients——Activity coefficients of sodium ion, γ_{Na+}, were determined at 25 °C by using an Orion combination electrode, model 97—12, in the usual way.[3]

Results

Precipitation of Polyanions by Surfactants

Interactions between polyanions and cationic surfactants were investigated in terms of the turbidity change measured as the optical density at 400 nm. In these experiments, we used sodium Ar and sodium Chs as polyanions and OTMABr, DETMABr and DOTMABr as cationic surfactants.

In the case of OTMABr, no precipitation and no turbidity change in the polyelectrolyte solutions occurred at any concentration of the surfactant added. On the other hand, in the case of the other two surfactants, both polyanions showed a turbidity change following precipitation in a certain concentration range as shown in Fig. 1.

In the Ar–$DETMA^+$ system, the turbidity increases in the concentration range above the critical micelle concentration (cmc) of DETMABr (0.065 M), while in the Ar–$DOTMA^+$ system, the turbidity increases at concentrations below the cmc of DOTMABr (0.016 M). Figure 1 also showed that the concentration of surfactants needed to cause the turbidity change or precipitation was dependent on the concentration of Ar.

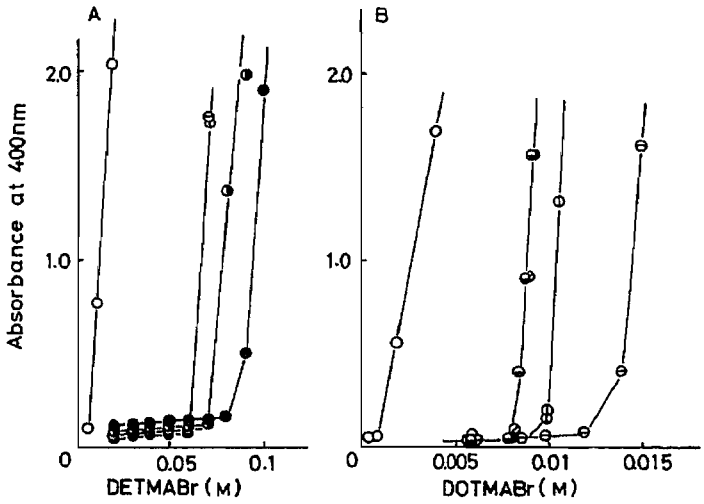

Fig. 1. Changes in the Absorbance at 400 nm of 0.01 eq/dm³ Chs and Ar with Various Concentrations of DETMABr (A) or DOTMABr (B)

Chs: ○. Ar (eq/dm³): ◒, 0.00125; ⊘, 0.0025; ⊕, 0.05; ⊖, 0.01; ◑, 0.02; ●, 0.03.

In the case of Chs, the Chs–DETMA$^+$ and Chs–DOTMA$^+$ systems both showed similar behavior to that found in Fig. 1, but in each system, the turbidity change of the Chs solution started at a very low concentration of the added surfactant compared to the case of Ar solution. These results indicated that the interaction of polyanions with cationic surfactants depends on the characteristics of the polyions and on the carbon number of the side chain in the surfactant molecule, which is similar to the results reported on anionic surfactants.[7]

Binding Isotherms of Cationic Surfactants to Polysaccharides

The amounts of three cationic surfactants bound to Ar and Chs were determined by using the cationic surfactant-selective electrode, and the results obtained are shown in Figs. 2—4. In Fig. 2A, EMF, the response of the PVC membrane electrode to OTMA$^+$, is plotted against the concentration of surfactant, $\log m_D$, where the solid line indicates the calibration curve for OTMABr. It is clear from Fig. 2A that the electrode response curves in the presence of Ar and Chs were identical with each other and that the observed values of EMF deviated from the calibration curve. The deviation is considered to reflect the binding degree of surfactants to the polyanion at the corresponding equilibrium molar concentration of surfactant, m_D^f, as illustrated by the dotted line in Fig. 2A. From the data, the binding degree, β, was calculated as a function of free surfactant concentration, m_D^f, where β equals the fraction of ionic sites on the polyanion occupied by the bound surfactants: $\beta=(m_D-m_D^f)/c_p$, where c_p indicates the polyanion concentration, which was kept constant at 1×10^{-2} eq/dm^3 in this experiment. The calculated results are presented in Fig. 2B. In the case of OTMA$^+$, binding isotherms for Ar and Chs were identical with each other and the values of β increased gradually with increasing m_D^f. From the standpoint of the counterion, or Na, a slow dissociation of Na from the polyions was observed with increase of m_D^f, as is also clear from Fig. 2B.

In the Ar–DETMA$^+$ and Ar–DOTMA$^+$ systems shown in Figs. 3 and 4, the electrode response curves were similar to that of the Ar–OTMA$^+$ system, except that the deviations from the corresponding calibration curves were larger than that of the Ar–OTMA$^+$ system in the order of Ar–OTMA$^+$ < Ar–DETMA$^+$ < Ar–DOTMA$^+$, as is reflected in the behavior

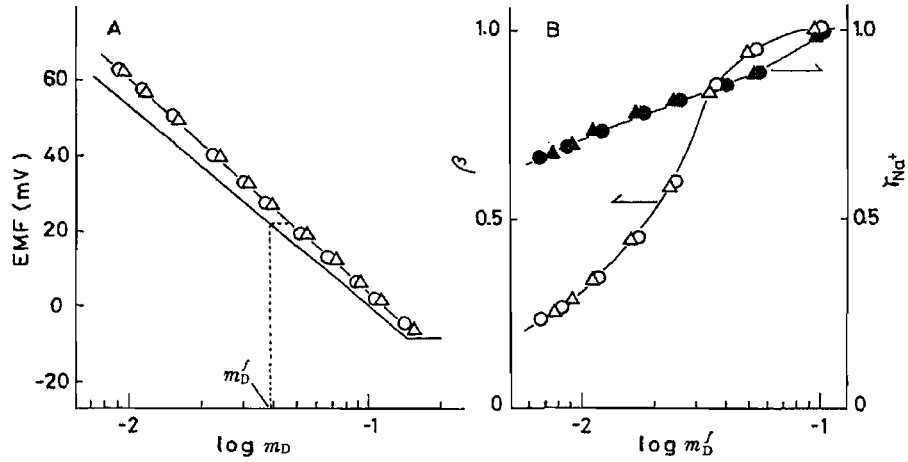

Fig. 2. (A) Response Curves of the Surfactant-Selective Electrode to OTMABr in the Presence of 0.01 eq/dm^3 Ar and/or 0.01 eq/dm^3 Chs

The solid line indicates the calibration curve.

(B) Binding Isotherms of OTMA$^+$ for Ar and Chs, and the Corresponding Sodium Ion Activity Coefficients

Ar: (△, ▲). Chs: (○, ●).

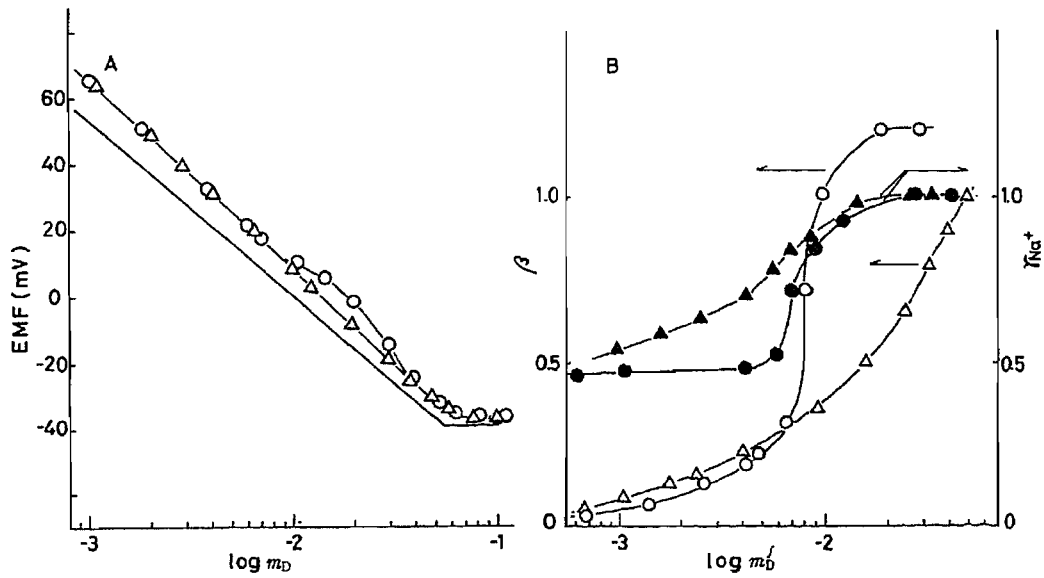

Fig. 3. (A) Response Curves of the Surfactant-Selective Electrode to DETMABr in the Presence of 0.01 eq/dm^3 Ar and/or 0.01 eq/dm^3 Chs

The solid line indicates the calibration curve.

(B) Binding Isotherms of $DETMA^+$ for Ar and Chs, and the Corresponding Sodium Ion Activity Coefficients

Ar: (△, ▲). Chs: (○, ●).

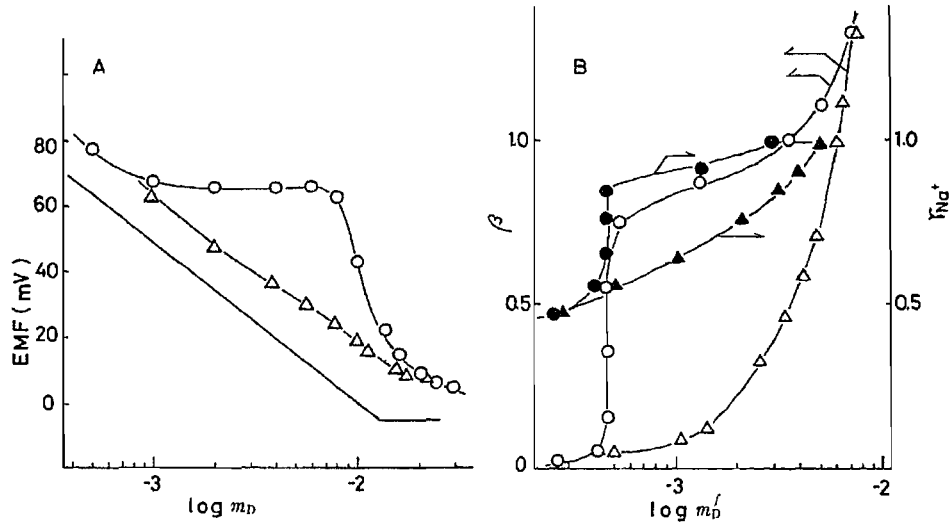

Fig. 4. (A) Response Curves of the Surfactant-Selective Electrode to DOTMABr in the Presence of 0.01 eq/dm^3 Ar and/or 0.01 eq/dm^3 Chs

The solid line indicates the calibration curve.

(B) Binding Isotherms of $DOTMA^+$ for Ar and Chs, and the Corresponding Sodium Ion Activity Coefficients

Ar: (△, ▲). Chs: (○, ●).

of the β–m_D^f curve. In other words, the slope of the β–m_D^f curve becomes steeper with the increase of the carbon number of surfactants, as shown in Figs. 2B—4B.

On the other hand, the Chs–$DETMA^+$ and Chs–$DOTMA^+$ systems showed different behavior. As is clear from the electrode response curves in Figs. 3A and 4A, the deviation of

the observed values from the calibration curve is larger at a certain concentration of surfactant, that is, 0.01 M in the case of $DETMA^+$ and 0.001 M in the case of $DOTMA^+$. These phenomena were represented as a steeper rise of β in the binding isotherms, (Figs. 3B and 4B), which is thought to be due to the cooperative binding of surfactants to the polymer, as discussed elsewhere.[5-12] As for the γ_{Na+}–m_D^f curve, the increase of the value of γ_{Na+} was observed to start parallel with that of the β, as shown in Figs. 3B and 4B.

Effect of Added Salt on the Binding Isotherms

In order to elucidate how the electrostatic or hydrophobic forces contribute to the binding of surfactant ions to polyanions, the interactions between cationic surfactants, $DETMA^+$ or $DOTMA^+$, and the polyanions, Ar or Chs, were investigated in the presence of NaCl. In these experiments, polyanion concentration was kept constant at 1×10^{-3} eq/dm^3, since this concentration was low enough not to produce precipitation even at $\beta = 1$. The results obtained were represented as binding isotherms, plotted as the degree of binding, β, against the free surfactant concentration, m_D^f, in Figs. 5—8.

In the Ar–$DETMA^+$ (Fig. 5), the binding curves increased monotonously to reach a plateau region near $\beta = 1$ under the experimental conditions tested, and the initial slope increases with increasing concentration of NaCl. In the Chs–$DETMA^+$ system (Fgi. 6), the binding behavior was similar to that of the Ar–$DETMA^+$ system except that the plateau region appears near $\beta = 0.7$. The behavior in the Chs–$DETMA^+$ system (Fig. 6) is fairly different from the result at higher concentrations of Chs (Fig. 3B), which suggested that the interaction of Chs with $DETMA^+$ was distinctly affected by the concentration of Chs.

With $DOTMA^+$ as a surfactant ion, the binding isotherms for Ar were apparently different from those for Chs, as is clear from comparing the results in Figs. 7 and 8. The binding isotherms for Ar gave gentle slopes, and adding NaCl resulted in a shift of the binding isotherms to higher concentrations of free surfactant. The slopes increased with increasing concentration of NaCl.

In contrast, the binding isotherms for the Chs–$DOTMA^+$ system began to level off near $\beta \simeq 0.6$ in the absence of NaCl and the presence of NaCl caused a shift in the binding

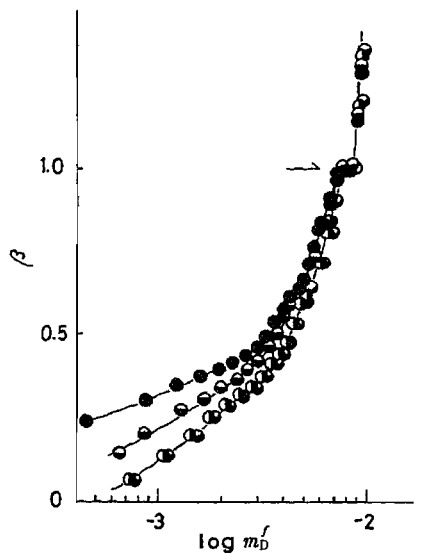

Fig. 5. Binding Isotherms of $DETMA^+$ to Ar (0.001 eq/dm^3) in the Absence and Presence of NaCl

NaCl (M): ●, 0; ◒, 0.0025; ◑, 0.01; ◕, 0.1.

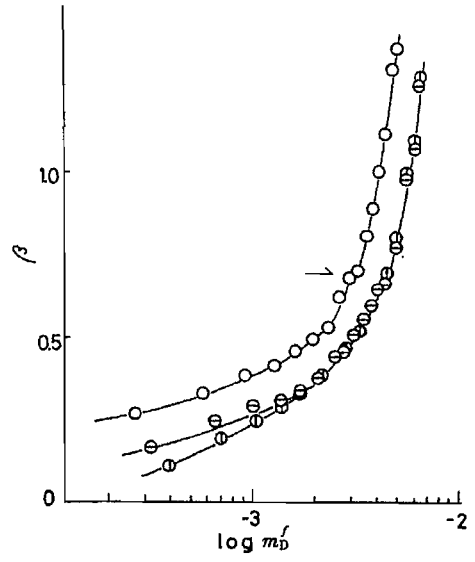

Fig. 6. Binding Isotherms of $DETMA^+$ to Chs (0.001 eq/dm^3) in the Absence and Presence of NaCl

NaCl (M): ○, 0; ⊖, 0.01; ⊙, 0.1.

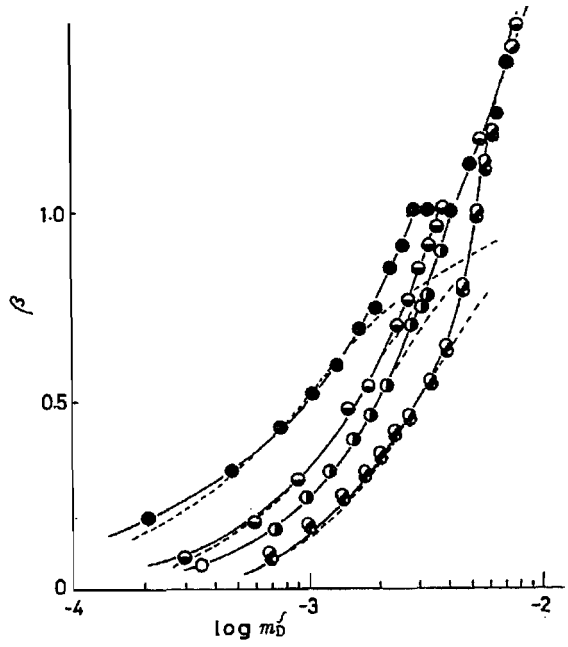

Fig. 7. Binding Isotherms of $DOTMA^+$ to Ar (0.001 eq/dm^3) in the Absence and Presence of NaCl

NaCl (M): ●, 0; ◒, 0.001; ⓓ, 0.0025; ◪, 0.01; ◔, 0.1.

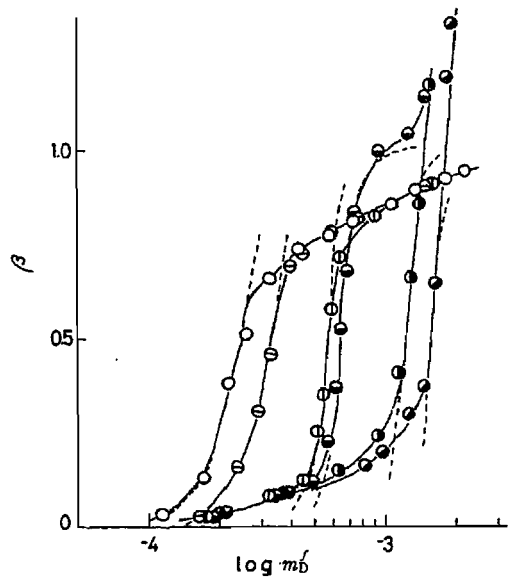

Fig. 8. Binding Isotherms of $DOTMA^+$ to Chs (0.001 eq/dm^3) in the Absence and Presence of NaCl

NaCl (M): ○, 0; ⊖, 0.001; ⓓ, 0.005; ◒, 0.01; ◑, 0.05; ◪, 0.1.

isotherms for the Chs–$DOTMA^+$ system to higher free surfactant concentrations, following the changes in the slopes of the isotherms. Above 0.01 M added NaCl, the degree of binding, β, went up to and over unity.

As can be seen in Figs. 5—8, every binding isotherm increased over unity, where the surface charges of the polymer should be saturated with cationic surfactants. This is presumably because additional surfactant could be bound to the surfactant–polymer complex by hydrophobic interaction.

Discussion

In the previous reports,[3,4] we investigated the various colligative properties of Ar and determined the values of b, a parameter reflecting a spatial intercharge distance, and the charge density parameter, ξ, according to Manning's theory to be 6.64 Å and 1.19, respectively for Ar. These values are similar to those of Chs ($b=6.3$ Å, $\xi=1.13$). Hayakawa and Kwak[8] suggested that the charge density parameters ξ is the dominant factor in the binding isotherms for polyion–surfactant systems. However, the binding isotherms for the Ar–$DOTMA^+$ and Chs–$DOTMA^+$ systems were unexpectedly not similar in the presence and absence of added salts, as shown in Figs. 7 and 8. In order to clarify the reasons for this, the binding isotherms for the polyion–surfactant ion systems were investigated semi-quantitatively according to the theories reported by Hayakawa and Kwak,[6,8] and Satake and Yang,[13] as described below.

As for the cooperative binding of small ions to polyions, a convenient form based on the nearest-neighbor interaction model provided by Zimm and Bragg[14] can be expressed as follows: $(OO)+A \overset{K}{\rightleftharpoons} (OA)$, $(OA)+A \overset{Ku}{\rightleftharpoons} (AA)$, where O represents a binding site on the polyion, A the surfactant ion, K the binding constant between the surfactant and an isolated

TABLE I. Binding Constants and Cooperativity Parameters

Sodium Ar			Sodium Chs		
NaCl (M)	log Ku	u	NaCl (M)	log Ku	u
0	2.99	1.5	0	3.60	45
0.001	2.78	2.9	0.001	3.48	60
0.0025	2.70	3.6	0.005	3.24	200
0.01	2.52	3.5	0.01	3.19	200
0.1	2.52	3.5	0.05	2.92	200
			0.1	2.80	200

binding site on the polyion, and u the cooperativity parameter. As Ku can be seen as the binding constant between a surfactant and a site adjacent to the site already occupied by a surfactant ion, $kT\ln Ku$ corresponds to the free energy which can transfer the surfactant ions from the bulk solution to the polymer–surfactant complex. When u is larger than unity, the proposed model means that the first surfactant bound will help the second surfactant to bind to a site adjacent to the site already occupied by the first surfactant ion. With reference to the cooperative binding process described above, the following relations for the degree of binding, β, were derived by Satake and Yang[13]

$$\beta=\frac{1}{2}\{1+(s-1)/((1-s)^2+4s/u)^{1/2}\} \tag{1}$$

$$(m_D^f)_{0.5}=(Ku)^{-1} \tag{2}$$

$$(d\beta/d\ln m_D^f)_{0.5}=u^{1/2}/4 \tag{3}$$

where s equals Kum_D^f, and $(m_D^f)_{0.5}$ is the concentration of free surfactant at the half-bound point ($\beta=0.5$). In these equations applied to the simple model, the electrostatic potential of the polymer is considered to be kept constant during the binding process of surfactant ions to the polymer, and therefore complicated phenomena such as conformational change of the polymer and the formation of hydrophobic regions within the polymer are not taken into account. Best fitting values of Ku and u for the binding isotherms in the Ar–DOTMA$^+$ or Chs–DOTMA$^+$ systems were estimated from the β–log m_D^f curves and are given in Table I. The predicted binding isotherms, calculated from Eq. 1 by using the observed values of Ku and u listed in Table I, are represented by the broken lines in Figs. 7 and 8.

In the case of the Ar–DOTMA$^+$ system (Fig. 7), Eq. 1 gave an excellent fit to the data for the degree of binding, β, below $\beta\simeq0.6$ in the presence of NaCl as well as in the absence of NaCl, and above $\beta\simeq0.6$ the observed values increased sharply above the broken line. On the other hand, in the case of the Chs–DOTMA$^+$ system, there was some difference from the results on Ar. Although below $\beta\simeq0.6$ the observed binding isotherms agree well with the expected curves in the regions below 0.001 M NaCl, above $\beta\simeq0.6$ the observed curve deviates from the expected values. At NaCl concentrations above 0.05 M, there was poor agreement between the two curves. This discrepancy between the Ar and Chs systems can be explained as follows. The binding constant K is expected to be a function of the electrostatic potential on the polyion surface. According to Manning's condensation theory for polyelectrolyte solution,[15] a constant fraction of counterion will be trapped in the close vicinity of the polymer, reducing the polymer's effective charge density parameter ξ to unity for monovalent counterions. Thus, in the case of Chs the bound surfactant counterions would not affect the effective linear charge density parameter until β reaches 0.6, because of its charge density parameter $\xi=1.13$. On the basis of this theory, K should be constant up to $\beta=0.6$ and

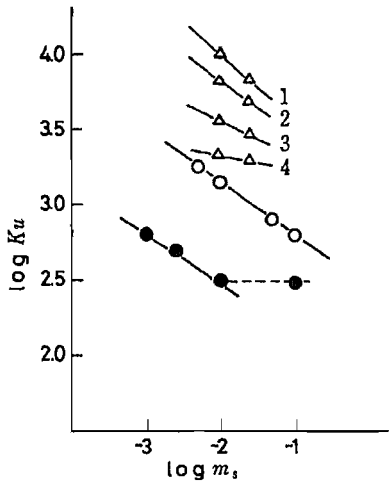

Fig. 9. NaCl-Concentration Dependence of the Binding Constant *Ku* for $DOTMA^+$

○: Chs. ●: Ar. △: date from ref. 10 for pectinate–tetradecylpyridium$^+$ systems, 1, $\xi = 1.61$; 2, $\xi = 1.28$; 3, $\xi = 0.87$; 4, $\xi = 0.48$.

decrease at higher degrees of binding. In fact, the observed isotherms for Chs, at NaCl concentrations below 0.005 M were in very reasonable agreement with this predicted behavior, while those of Ar did not agree in spite of the similar value of ξ to Chs. This discrepancy between Ar and Chs may be due to the difference of structure (linear or branching). This point is further discussed below.

The phenomena described above can be considered in terms of the parameters, *u* and *Ku*. As presented in Table I, the relationship between the concentration of added salt and the values of *Ku* and *u* in both the Ar–$DOTMA^+$ and Chs–$DOTMA^+$ systems was similar to the reported results[6] for dextran sulfate and polystyrene sulfate. That is, the values of log *Ku* for Ar systems are much smaller than those of the Chs systems, and log *Ku* for each system decreased with increasing concentration of added NaCl, which may be due to the diminished electrostatic potential on the polymer surface resulting from compression of the electric double layer. With respect to *u*, in both cases (Ar and Chs), the values of *u* are independent of the concentration of added salt in the concentration range of NaCl above 0.005 M and the value of *u* was constant at about 3.5 for the Ar system and about 200 for Chs. This may indicate that the cooperative effect in the binding of $DOTMA^+$ to Ar or Chs is due to the hydrophobic mutual interaction among surfactant ions bound to the polyion backbone and not due to the electrostatic interaction between them, although the cooperative effect in the binding of $DOTMA^+$ by Ar seemed considerably smaller than that of Chs.

We now turn our attention to the ionic strength dependence of the binding constant *Ku*, since the values of charge density parameter ξ for both polyions are very similar to each other. The values of log *Ku* were plotted against log m_s and the results obtained are shown in Fig. 9, where m_s indicates the molar concentration of added salt. Figure 9 also shows literature values on the pectinate-tetradecylpyridium$^+$ system[10] for comparison. In this system, four kinds of pectinate with differently esterified carboxyl groups were used in order to investigate the effect of ξ, and the results demonstrated that the added salt concentration dependence of *Ku* became smaller with decreasing charge density of the polyion. Considering that the slope for Ar–$DOTMA^+$ was comparable with that for Chs–$DOTMA^+$, the spatial charge density, or intercharge distance, *b*, of Ar seems to be similar to the value of *b* for Chs even in the binding process of $DOTMA^+$ to the polyions. In the present work, we showed that the binding behaviors of Ar and Chs to cationic surfactant ions are obviously different although both polyions show very similar binding behavior with inorganic cations. In other words, this suggests that the charge density parameter ξ may not always be dominant in the process of binding described here.

Several rod-like molecules, carboxy methyl cellulose (CMC), alginate and pectinate,[8]

have similar values of ξ in the 1.32—1.61 range. The values of *Ku* for the binding isotherms of $DOTMA^+$ were similar, but the cooperativity parameter, *u*, of CMC was only about one-eighteenth of that of the other two polyions. In the cases of dextran-sulfate (Dxs) and polystyrene sulfonate (PS),[6] both polyions have the same value of $\xi=2.80$ but the binding constant, *Ku*, for the Dxs–$DOTMA^+$ system was smaller than that of the PS–$DOTMA^+$ system in the presence of 0.01 M NaCl and the value of the cooperativity parameter, *u*, for the Dxs–$DOTMA^+$ was 3 times larger than that of the PS–$DOTMA^+$ system. This may be because both polymers, CMC and PS, have a hydrophobic polymer backbone based on the carboxymethyl group and styrene group, respectively. In addition, these reported results strongly suggest that the detailed structural properties as well as the charge density of the polymer play an important role in the polymer–surfactant binding process. We may now consider the observed binding difference with surfactants between Ar and Chs as follows. Both polymers have a similar hydrophilic backbone, but Chs is a linear polysaccharide and Ar is a branched heteropolysaccharide. Considering that the surfactant has a long hydrophobic chain and a fairly large molecular size, it is suggested that the interaction between Ar and surfactant will be affected by steric hindrance arising from the branching, resulting in lower values of *Ku* and *u* for Ar compared to those for Chs.

In the above discussion on the binding behavior of cationic surfactants with polysaccharides, the difference of binding behavior between Ar and Chs was suggested to be caused by the structural difference. This structural difference may also account for the difference of visually detected phenomena, precipitation and gelation, after mixing of the polyions and cationic surfactants, $DETMA^+$ and $DOTMA^+$. As shown in Fig. 1, surfactant was added to each polysaccharide solution up to $\beta=1$ in the test tube and the mixture was left overnight at room temperature. Ar formed a gel on the bottom of the test tube, but in the case of Chs the gel was observed to lie flat on the wall of the tube. In addition, the Ar–surfactant gel could be easily dispersed simply by light shaking by hand and the suspension returned again to the gel phase on standing. Further the gel was water-soluble. On the other hand, the gel of Chs–surfactant could not be dispersed by shaking and did not dissolve even in a large amount of water. Such characteristics may be due to the structural difference of the polyanions as discussed above. In the case of the linear polymer Chs, surfactant molecules bound to the polymer can easily interact hydrophobically with other surfactant molecules bound to another polymer molecule because of the linear backbone. In the case of the branched polymer Ar, however, hydrophobic interaction among surfactant molecules bound to different polymer molecules does not seem to occur easily because of the steric hindrance due to the side chains or branches of Ar, which may prevent close mutual contact of the polymers molecules. Therefore the gel formed by the interaction between Chs and surfactant may be more stable than that in the case of Ar.

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Macromolecular Complexes of Drugs. I. Doxorubicin–Heparin Complex

TOSHINOBU AOYAMA,*[,a] MASAYOSHI HORIOKA[a] and SHINGO NAGAMITSU[b]

Hospital Pharmacy, Kyushu University Hospital[a] and Department of Surgery I, Faculty of Medicine, Kyushu University,[b] Maidashi 3–1–1, Higashi-ku, Fukuoka 812, Japan

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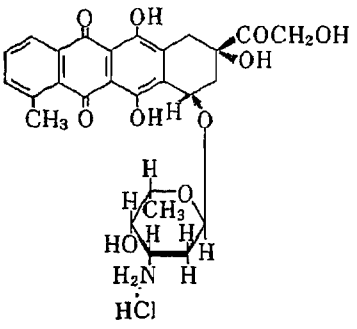
As a part of our studies to develop anticancer agents with prolonged action and reduced side effects, we examined the macromolecular complex of doxorubicin and heparin. Formation of a complex of doxorubicin and heparin was confirmed by HIAC, infrared, ultraviolet, differential scanning calorimetry, energy micro analysis X-ray and high performance liquid chromatography analysis. If the molecular weight of heparin is assumed to be 9800, one molecule of heparin binds 16 molecules of doxorubicin.

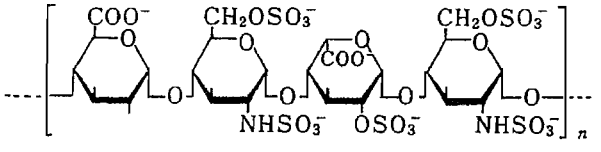
Keywords——complex; heparin; doxorubicin; anticancer drug; macromolecular complex; particle count; energy dispersing X-ray

Generally, anticancer drugs do not exhibit prolonged action at the tumor site because of their short retention time in the blood and tissues due to their low-molecular charactes. Side effects, however, can be severe if high drug concentrations are sustained in the blood for a long time. In an effort to develop safer and more effective drugs, the use of anticancer agents bound with macromolecular compounds has been suggested, such as methotrexate–poly(l-lysine) complex,[1] polystyrene maleic acid–neocarzinostatin complex,[2] polyglycolic acid–5-fluorouracil complex,[3] and mitomycin C–dextran conjugate.[4]

While studying macromolecular complexes of anticancer compounds and biological components in order to find anticancer substances which can be administered locally, we have noted reports that heparin form complexes with various drugs having an NH_2 moiety, such as streptomycin,[5,6] polymixin M,[5] monomycin, kanamycin and neomycin.[6] We have investigated the ability of heparin to form complexes with some anticancer drugs and report here the results on the complex of doxorubicin and heparin (Chart 1).



doxorubicin hydrochloride



heparin

Chart 1

Experimental

Materials——Doxorubicin hydrochloride (Dox–HCl) was a gift from Kyowa Hakko Co., Ltd. (Tokyo, Japan). Heparin sodium (Hep–Na), a reagent, was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Novo-heparin injection was obtained from Kodama Co., Ltd. (Tokyo, Japan). An 8.4% sodium bicarbonate ($NaHCO_3$) injection was obtained from Otsuka Pharmaceutical Co., Ltd. (Meylon 84, Tokyo, Japan).

Doxorubicin–Heparin (Dox–Hep) Complex——Hep–Na solution (50 mg/5 ml) was added to Dox–HCl solution (50 mg/5 ml). After cooling, the precipitate was collected by centrifugation, washed several times with a small volume of cold distilled water, and dried *in vacuo*. To show that the precipitate is not the base of DOX, $NaHCO_3$-treated Dox–HCl was processed in the same manner without Hep–Na.

Particle Counts of Dox–Hep Complex and $NaHCO_3$-Treated Dox–HCl——Dox–HCl (10 mg) was dissolved in saline solution and filtered through a 0.22 μm Millipore filter. The filtrate was made up to 150 ml with saline solution. Novo-heparin (1 ml) was added to the solution with stirring. Five minutes later, the number of particles formed was measured on a HIAC PC-305 (HIAC/Royco Instrument Division, Pacific Scientific Co., Ltd., California, U.S.A.). Another 1 ml of Novo-heparin was added and the number of particles was again measured. Separately, Dox–HCl solution was treated with $NaHCO_3$ (8.4% solution) and the number of particles was similarly measured. Measurement was done four times with 10 ml of sample solution in each case, and the last three measured values were used to calculate the mean value; the first measurement was discarded.

Energy Micro Analysis X-Ray (EMAX) (Na, Cl and S)——Na, Cl and S in Dox–HCl, Dox–Hep and $NaHCO_3$-treated Dox–HCl were determined by using a Hitachi S-50 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at 20 kV with an EMAX-1770 energy dispersing X-ray analyzer (Horiba Seisakusho Co., Ltd., Tokyo, Japan) at 20 eV for 600 s. The sample was fixed on an aluminum stub (1 × 1 cm) with double-side adhesive tape and coated with carbon at 150 Å thickness in a Hitachi 5GB high vacuum evaporator (30 A and 1×10^{-6} Torr).

Infrared (IR) Spectra——IR spectra were obtained on an IR-810 spectrometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

Visible Spectra——The visible spectra of solutions of test materials (Dox–HCl, Dox–Hep, $NaHCO_3$-treated Dox–HCl) and a mixture of dimethyl sulfoxide (DMSO) and water (1 : 1) were determined (Shimadzu UV-240, Shimadzu Co., Ltd., Kyoto, Japan). Dox–HCl solution was added to Hep–Na solution and the mixture was filtered through a Mol-cut II SJGC (molecular weight cut-off, 10000; Nihon Millipore Co., Ltd., Tokyo, Japan). Hep–Na of low molecular weight in the filtrate was identified by methylene blue metachromasis.

Differential Scanning Calorimetry (DSC)——Measurements were made under 40 ml/min of N_2 (SSC/580-II, DSC-20, DS-50 Data Processor, Seiko Instruments & Electronics Co., Ltd., Tokyo, Japan).

High Performance Liquid Chromatography (HPLC)——A Nucleosil 5C18 (5 μm) column, 4 mm i.d. × 25 cm (Nagel, W. Germany), was used. The column was eluted with MeOH–H_2O–acetic acid (40 : 35 : 5, v/v%) at the flow rate of 1 ml/min (λ = 495 nm, × 0.04 a.u.f.s.). A 10 μl aliquot of a test material (5 mg in 100 ml of DMSO–H_2O, 1 : 1) was injected. A Hitachi HPLC-633, a Hitachi wavelength-tunable effluent monitor (Hitachi Ltd., Tokyo, Japan) and a Shimadzu Chromatopac-CRIB (Shimadzu Co., Kyoto, Japan) were used.

Results and Discussion

Particle Counts by HIAC

When Novo-heparin solution was added to Dox–HCl solution, an increase in the number of 2 to 5 μm particles was evident. Addition of 2 ml of Novo-heparin solution increased the

TABLE I. Particle Counts in Doxorubicin Solution Mixed with Novo-heparin or Sodium Bicarbonate Solution

Admixture		Particle diameter (μm)				
		>2	>5	>10	>20	>30
Novo-heparin	Blank (Dox)	184	37	16	5	0
	1 ml added	79935	36125	2063	9	3
	Another 1 ml added	179835	34676	75	18	2
$NaHCO_3$ (8.4%)	Blank (Dox)	211	45	7	0	0
	1 ml added	608	81	26	5	1
	Another 1 ml added	2251	172	30	5	3

Particle counts were measured per 10 ml in a HIAC PC-305 apparatus. Values are averages of 3 samples.

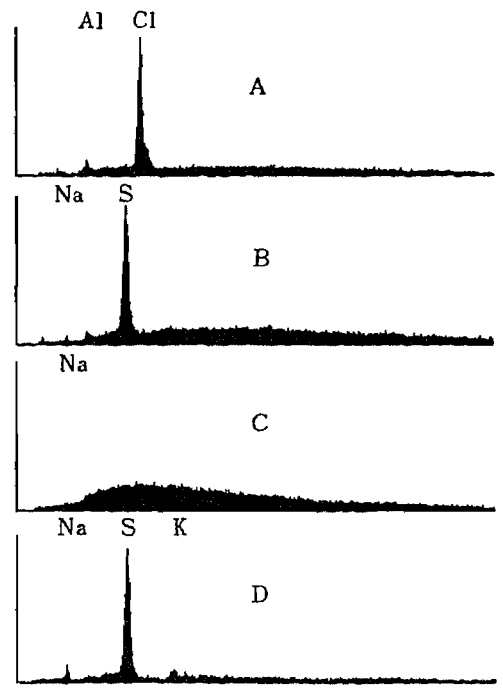


Fig. 1. EMAX of Dox–HCl (A), Dox–Hep Complex (B), $NaHCO_3$-Treated Dox–HCl (C) and Hep–Na (D)

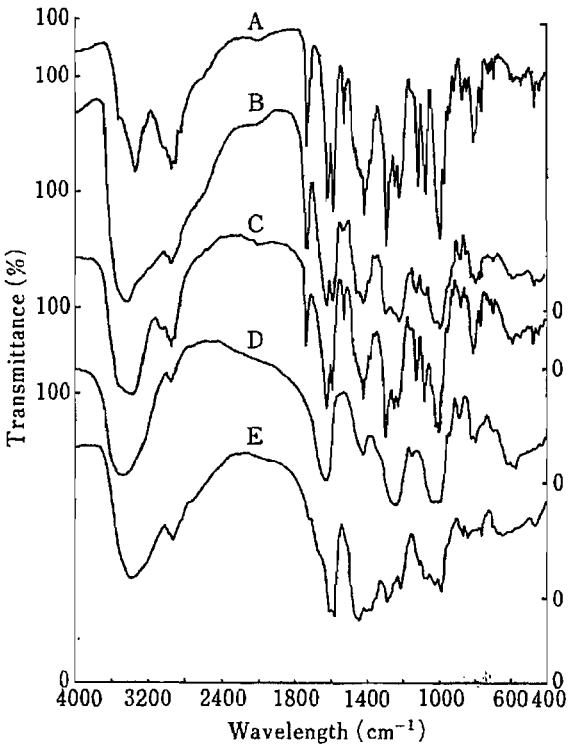


Fig. 2. IR Spectra of Dox–HCl, Hep–Na and Related Compounds

A, Dox–HCl; B, Dox–Hep complex; C, doxorubicin and heparin mixture; D, Hep–Na; E, $NaHCO_3$-treated Dox–HCl.

number to 1.5 to 2 times that when 1 ml of the solution was added, while addition of $NaHCO_3$ gave a particle number of about 1/90 of that determined with the Novo-heparin solution. Thus, the precipitation with heparin appears to be different from that with $NaHCO_3$ (Table I).

EMAX

X-Ray signals specific for Cl (as hydrochloride) and Na (as sodium salt) and for S (as SO_3^{2-}) were observed for Dox–HCl and Hep–Na, respectively. No Cl was detected in Dox–Hep, but S and a trace amount of Na resulting from heparin were noted. A trace amount of sodium, probably from $NaHCO_3$, was found in the $NaHCO_3$-treated Dox–HCl, but Cl was not identified (Fig. 1). These results suggest the possibility of complex formation of Dox–HCl and Hep–Na.

IR Spectra

The IR spectrum of Dox–Hep differed from those of Dox–HCl, Hep–Na, the mixture of Dox–HCl and Hep–Na, and $NaHCO_3$-treated Dox–HCl (Fig. 2).

Since IR ν_{max}^{KBr} cm^{-1}: 1732 (C=O of hydroxymethylketone) and 1618 (C=O of quinone) still remain in Dox–Hep, the reaction may be considered to be a salt formation reaction. We designated Dox–Hep as a complex, as Govorovich and Bogomolova[5] and Bychkov[6] did in the case of binding of antibiotics to heparin.

Visible Spectrum

Dox–Hep in DMSO–H_2O (1 : 1) showed a similar spectrum to Dox–HCl (Fig. 3). When a Hep–Na solution was filtered through Mol-cut II and subjected to the methylene blue

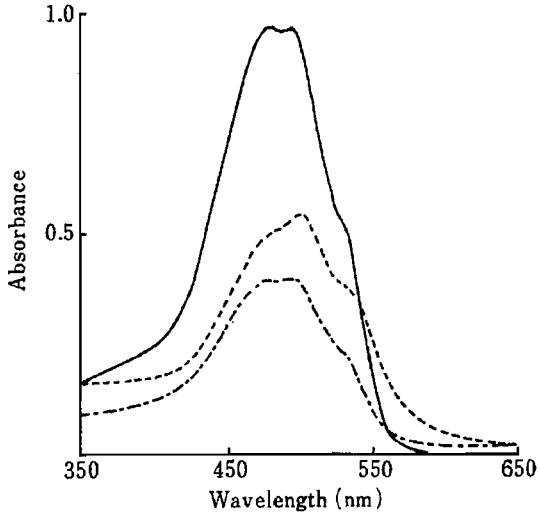


Fig. 3. Absorption Spectra of Dox–HCl, Dox–Hep Complex, and $NaHCO_3$-Treated Dox–HCl

——, Dox–HCl (0.498 mg/10 ml); ------, $NaHCO_3$-treated Dox–HCl (0.465 mg/10 ml); – – – –, Dox–Hep complex (0.422 mg/10 ml).

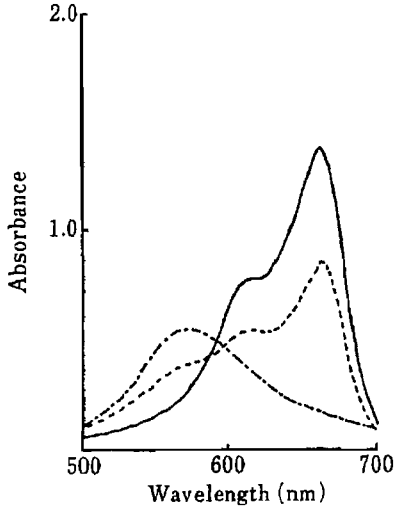


Fig. 4. Absorption Spectra of Dox–HCl and Hep–Na

After filtration through a Millipore Mol-cut II, methylene blue solution was added and the mixture was diluted with water.

——, Dox–HCl (2.8 mg) and Hep–Na (2.0 mg); ------, Dox–HCl (1.0 mg) and Hep–Na (2.0 mg); – – – –, Hep–Na (2.0 mg).

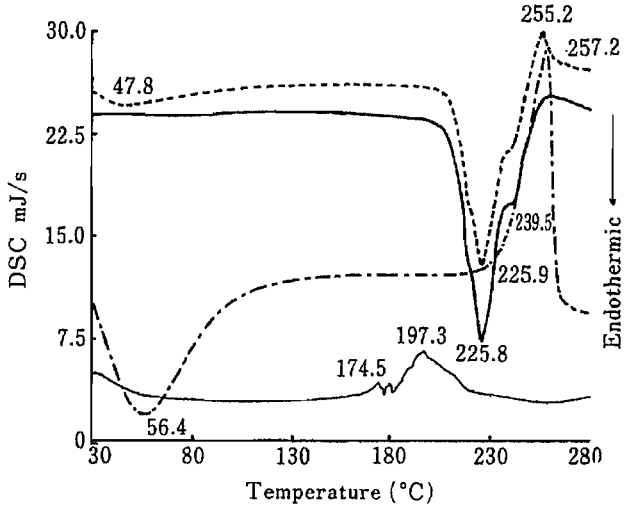


Fig. 5. DSC of Dox–HCl, Dox–Hep Complex and Related Compounds

——, Dox–HCl; ------, Dox–HCl (85%) and Dox–Na (15%) mixture; – – – –, Hep–Na; ——, Dox–Hep complex.

reaction, metachromasis developed (purple coloration). This is probably because Hep–Na of low molecular weight passed through the filter. When Dox–HCl was added to Hep–Na in an amount slightly larger than the complex-forming amount, metachromasis was not observed (Fig. 4). Presumably, Hep–Na remained as Dox–Hep complex on the filter and the filtrate was free from Hep–Na.

DSC

The mixture of Dox–HCl and Hep–Na should give a thermogram pattern corresponding to the sum of those of Dox–HCl and Hep–Na if there is no interaction. However, Dox–Hep showed a thermogram different from those of Dox–HCl and Hep–Na, indicating that this product is a distinct complex (Fig. 5).

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Relationship between the Structures and the Antitumor Activities of Tannins

KENICHI MIYAMOTO,*[,a] NOBUHARU KISHI,[a] RYOZO KOSHIURA,[a] TAKASHI YOSHIDA,[b] TSUTOMU HATANO[b] and TAKUO OKUDA[b]

Department of Pharmacology, Hokuriku University School of Pharmacy,[a] Kanagawa-machi, Kanazawa 920–11, Japan and Faculty of Pharmaceutical Sciences, Okayama University,[b] Tsushima, Okayama 700, Japan

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Sixty-three tannins and related polyphenols were intraperitoneally injected into mice at 4d before intraperitoneal sarcoma-180 cell inoculation, and their antitumor activities were evaluated. The condensed tannins and related compounds all showed negligible activity. As regards the hydrolyzable tannins, active compounds were found among ellagitannins. In particular, dimeric ellagitannins such as oenothein B, rugosin E, rugosin D, gemin A and coriariin A showed significantly higher antitumor activity than agrimoniin, which we previously reported as an antitumor tannin. Coriariin A, which has four galloyl groups instead of two hexahydroxydiphenoyl groups of agrimoniin, showed the strongest activity. It seems to be essential for marked antitumor activity that ellagitannins should possess a dimeric structure with several galloyl groups on the glucose core.

Keywords——tannin; antitumor activity; sarcoma-180; mice; structure–activity relationship; hydrolyzable tannin; ellagitannin; dimeric ellagitannin; coriariin A

A number of medicinal plants have been traditionally used for treatment of various ailments, and it has been suggested that tannins may be the active principles of many of them. Since it has recently become possible to isolate pure tannins from plants, we can now examine the characters and activities of each tannin. As regards biological activities of tannins, inhibition of lipid peroxidation,[1] decrease of mutagenicity of several mutagens,[2] antiviral activity,[3] decrease of blood urea-nitrogen content,[4] inhibition of plasmin activity,[5] various effects on lipolysis in fat cells,[6] and several others have been presented. However, there are very few reports on the antitumor activity of tannins, and on the correlation of their structures with the antitumor activities. Recently, we have reported that the methanol extract from roots of *Agrimonia pilosa* LEDEB. showed some host-mediated antitumor activities,[7] and we showed that the main antitumor constituent in the plant is agrimoniin,[8] which is also contained in *Agrimonia pilosa japonica* and *Potentilla kleiniana*.[9]

The present paper deals with the antitumor activities of various types of tannins, and discusses the relationship between structure and antitumor activity.

Experimental

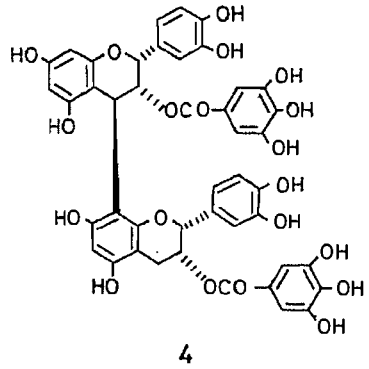
Materials——Sixty-three tannins and related polyphenols were isolated from plants according to the cited methods (indicated in the right-hand column of Table I). OK-432, a streptococcal preparation, was obtained from Chugai Pharmaceutical Co. and used as a positive control.[12] Materials were dissolved in sterilized physiological saline before use.

Antitumor Experiments——Sarcoma-180 was maintained by intraperitoneal passage at weekly intervals in female ddY mice (Shizuoka Laboratory Animal Center). Six female mice (6-weeks old) in a group were intraperitoneally administered with 5 or 10 mg/kg of a test compound once at 4 d before intraperitoneal inoculation of the tumor cells (1×10^5), because agrimoniin, a dimeric ellagitannin, was more effective when given by intraperitoneal injection once at 4 d before the tumor cell inoculation than three times on 1, 4 and 7 d after the inoculation.[8] Sixty days after the tumor cell inoculation, survivors were killed and autopsied. The antitumor activity of each compound was evaluated in terms of the number of regressors and the percent increase in the life span (%ILS) calculated according to the following formula:

$$\%\text{ILS} = 100 \times [(\text{mean survival days of the treated group}) - (\text{mean survival days of the vehicle control group})] / (\text{mean survival days of the vehicle control group})$$

Results and Discussion

The results on the *in vivo* antitumor activity of sixty-three tannins and related compounds are shown in Table I. The data for OK-432 as a positive control[12] were obtained under similar experimental conditions. We have reported that agrimoniin (**55**) is an antitumor tannin.[8] In this study, **55** gave sarcoma-180-bearing mice 75.3% and 108.8% ILS at 5 and 10 mg/kg, respectively. Thus, a value of %ILS > 70 was chosen as the minimum requirement for a compound to be considered as active. All the condensed tannins and the related polyphenols (**1**—**7**) were practically inactive. As regards the hydrolyzable tannins, caffeic



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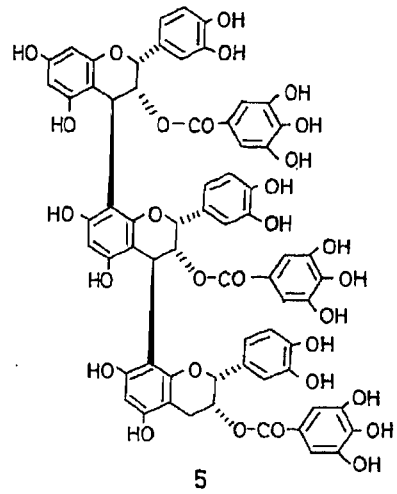

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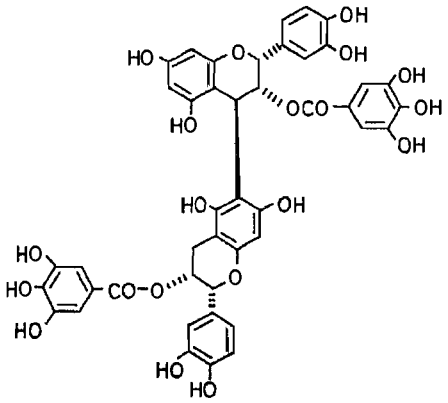

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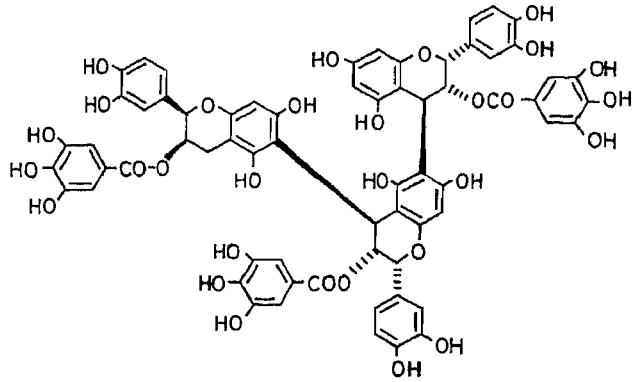

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Chart 1

TABLE I. Antitumor Activities of Tannins and Related Compounds against Sarcoma-180 in Mice

Tannin or related compound	Dose (mg/kg)	%ILS	Regressors[a]	Reference of isolation
[A] Condensed tannins and related polyphenols				
(−)-Epicatechin (**1**)	5	35.5	0	10*a*
(−)-Epigallocatechin (**2**)	5	−19.4	0	10*a*
(−)-Epigallocatechin gallate (**3**)	5	18.1	0	10*a*
ECG-(4β→8)-ECG (**4**)	5	48.7	0	10*a*
ECG-(4β→8)-ECG-(4β→8)-ECG (**5**)	5	16.8	0	10*b*
ECG-(4β→6)-ECG (**6**)	5	57.4	0	10*b*
ECG-(4β→6)-ECG-(4β→6)-ECG (**7**)	5	13.3	0	10*b*
[B] Hydrolyzable tannins and related polyphenols				
Caffeic acid derivatives				
Rosmarinic acid (**8**)	5	32.8	0	10*c*
Chlorogenic acid (**9**)	5	−3.2	0	1*c*
3,5-Di-*O*-caffeoylquinic acid (**10**)	5	23.0	0	1*c*
Bergenin and its derivatives				
Bergenin (**11**)	5	−5.2	0	10*d*
Dimethylbergenin (**12**)	5	19.4	0	10*d*
11-*O*-Galloylbergenin (**13**)	5	31.0	0	10*d*
Gallotannins and related polyphenols				
3-*O*-Digalloylquinic acid (**14**)	5	6.8	0	10*e*
3-*O*-Trigalloylquinic acid (**15**)	5	52.6	0	10*e*
3-*O*-Tetragalloylquinic acid (**16**)	5	134.2	1	10*e*
3-*O*-Pentagalloylquinic acid (**17**)	5	15.0	0	10*e*
3-*O*-Hexagalloylquinic acid (**18**)	5	38.9	0	10*e*
3-*O*-Heptagalloylquinic acid (**19**)	5	65.5	0	10*e*
1,2,6-Tri-*O*-galloyl-β-D-glucose (**20**)	5	15.7	0	10*f*
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (**21**)	10	27.2	0	10*f*
Penta-*O*-galloyl-β-D-glucose (**22**)	10	81.9	0	10*g*
Ellagitannins				
Casuarinin (**23**)	5	8.2	0	10*h*
Isovalolaginic acid (**24**)	5	−3.3	0	10*i*
Castalagin (**25**)	5	41.8	0	10*i*
Vescalagin (**26**)	5	58.2	0	10*i*
Strictinin (**27**)	5	44.2	0	10*h*
Gemin D (**28**)	5	1.8	0	10*j*
Tellimagrandin I (**29**)	10	35.2	0	10*h*
Tellimagrandin II (**30**)	5	73.1	0	10*k*
Rugosin A (**31**)	5	110.3	1	10*l*
Pedunculagin (**32**)	10	27.3	0	10*h*
Casuarictin (**33**)	10	75.8	0	10*h*
Praecoxin A (**34**)	5	70.8	0	10*l*
Rugosin C (**35**)	5	91.7	0	10*l*
Alnusiin (**36**)	5	74.3	0	10*m*
Punicalagin (**37**)	5	41.6	0	10*n*
Teroblongin (**38**)	5	16.8	0	10*o*
Corilagin (**39**)	10	36.1	0	10*p*
1-*O*-Galloyl-2,2:3,6-bis-*O*-HHDP-β-D-glucose (**40**)	10	25.0	0	10*q*
Geraniin (**41**)	10	23.8	0	10*r*
Mallotusinic acid (**42**)	5	77.0	0	10*s*
Granatin B (**43**)	5	66.4	0	10*t*
Furosinin (**44**)	5	83.2	0	10*u*
Dehydrogeraniin (**45**)	5	35.4	0	10*u*
Isoterchebin (**46**)	5	57.4	0	10*k*

TABLE I. (continued)

Tannin or related compound	Dose (mg/kg)	%ILS	Regressors[a]	Reference of isolation
Oligomeric ellagitannins				
Campthotin A (47)	5	48.7	0	11*a*
Cornusiin A (48)	10	181.1	2	10*h*
Oenothein B (49)	5	235.6	1	11*a*
Coriariin E (50)	10	53.9	0	11*b*
Rugosin E (51)	10	234.7	2	11*c*
Rugosin D (52)	10	171.5	1	11*c*
Isorugosin D (53)	5	146.5	2	11*d*
Rugosin F (54)	10	35.2	0	11*c*
Agrimoniin (55)	10	108.8	0	9
	5	75.3	0	
Gemin A (56)	10	176.1	1	11*e*
Coriariin A (57)	5	238.0	3	11*f*
Nonacosa-*O*-methylcoriariin A (58)	5	21.3	0	11*f*
Coriariin C (59)	10	156.2	2	11*g*
Medinillin B (60)	5	108.8	1	10*o*
Nobotanin A (61)	10	126.6	0	11*h*
Nobotanin F (62)	5	76.4	0	11*h*
Cornusiin C (63)	10	108.2	0	11*i*
OK-432	100 (KE/kg)	208.4	2	

The vehicle control mice died 12.9 ± 0.8 d. *a*) Number of mice negative for tumor at 60 d after the tumor cell inoculation, out of six mice. ECG: (−)-epicatechin gallate. HHDP: hexahydroxydiphenoyl.

acids (**8**—**10**) and bergenin and its derivatives (**11**—**13**) showed low activities. 3-*O*-Tetragalloylquinic acid (**16**) markedly prolonged the life span of mice and cured one out of six mice, but further galloylation of **16** did not lead to an increase of the antitumor activity (**17**—**19**). Penta-*O*-galloyl-*β*-D-glucose (**22**), in which the glucose core is saturated with galloyl groups, was active compared with other galloylglucoses. Several monomeric ellagitannins showed relatively high antitumor activities. Tellimagrandin II (**30**) has more galloyl groups and showed higher activity than strictinin (**27**), gemin D (**28**) and tellimagrandin I (**29**). Rugosin A (**31**), which has a valloneic acid at the same location on the glucose core as a hexahydroxydiphenoyl (HHDP) group of **30**, cured one mouse. A similar correlation was observed among a group of tannins; pedunculagin (**32**), casuarictin (**33**), praecoxin A (**34**) and rugosin C (**35**). Only negligible activity was shown by **32**, but the other tannins prolonged the life span of mice by over 70% as compared with the control.

On the other hand, alnusiin (**36**) showed relatively high activity, but punicalagin (**37**) and teroblongin (**38**) were almost inactive. Among dehydroellagitannins, mallotusinic acid (**42**) and furosinin (**44**) showed higher activities than geraniin (**41**), granatin B (**43**), dehydrogeraniin (**45**) and isoterchebin (**46**). In these cases, the enhancement of the antitumor activity is attributable to factors other than the presence of gallic acid. Compounds with the open chain form of glucose moiety (**23**—**26**) hardly showed any antitumor activity.

Most of the oligomeric ellagitannins showed strong activities compared with the monomeric tannins. The addition of galloyl groups on the glucose core increased the antitumor activity: cornusiin A (**48**) and oenothein B (**49**) showed higher activities than campthothin A (**47**); rugosin E (**51**) and rugosin D (**52**) showed higher activities than coriariin E (**50**). Isorugosin D (**53**) cured two out of six mice. It seems therefore that the antitumor activity of tannins is not much influenced by the isomerism between **52** and **53**. While

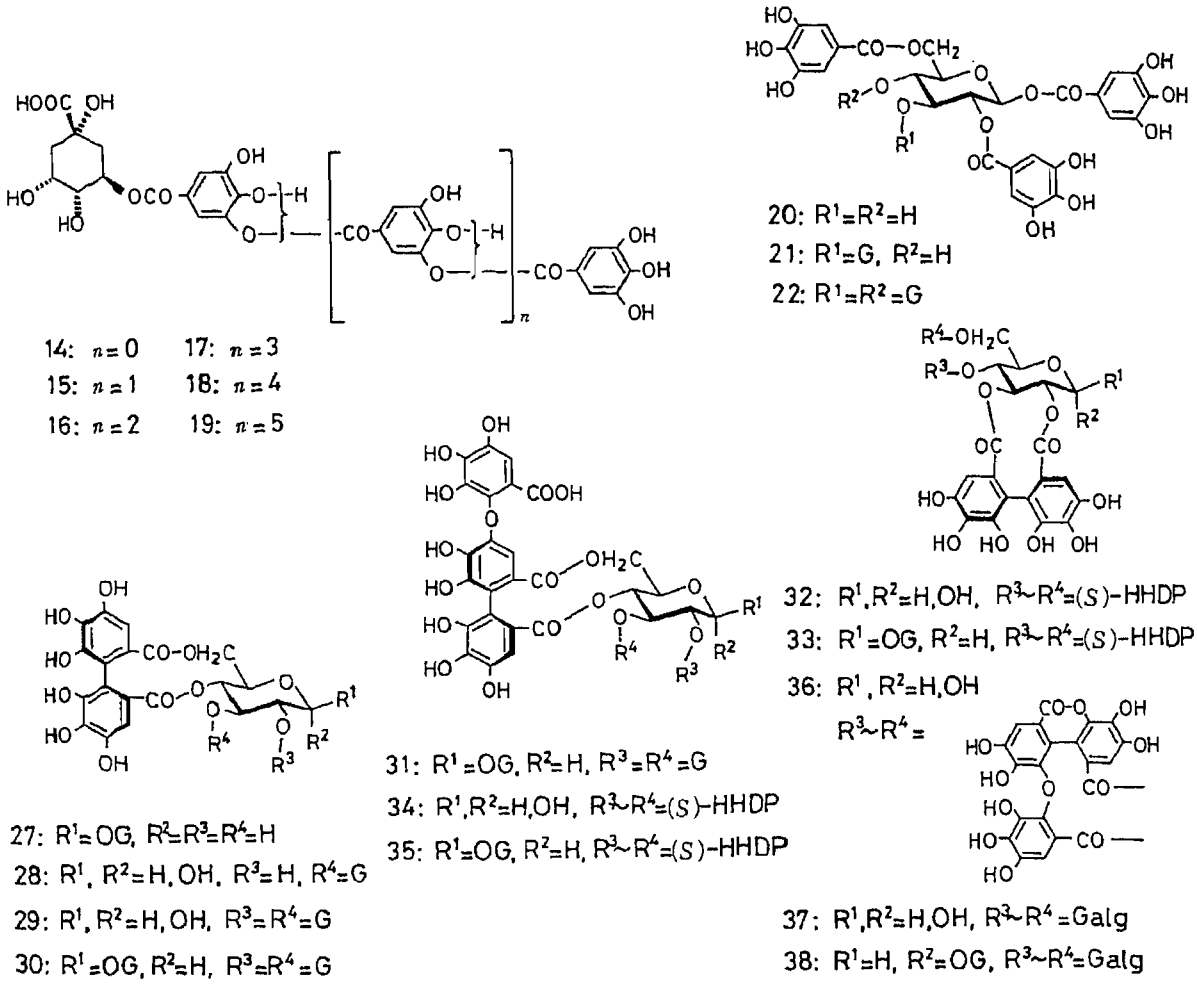

Chart 2

medinillin B (**60**), nobotanin A (**61**) and nobotanin F (**62**) possess two, three and four galloyl groups in their molecule, respectively, all of them showed similar degrees of activity, and in this case, the correlation of the antitumor activity with the galloylation was not clear.

On the other hand, it was observed that the antitumor activity of several tannins was potentiated by the conversion of one HHDP group into two galloyl groups (see the chart for a comparison of structures): rugosin F (**54**) < rugosin D (**52**); agrimoniin (**55**) < gemin A (**56**) < coriariin A (**57**). Coriariin C (**59**) which has a valloneic acid instead of an HHDP in **57** cured two mice, but its activity was lower than that of **57**. The activity of **48**, a dimeric tannin, was significantly higher than that of **29**, a monomeric tannin, but the activity of cornusiin C (**63**), a trimeric tannin, was not higher than that of **48**. These results suggest that the antitumor activity of tannins does not simply increase with the molecular weight. Nonacosa-*O*-methylcoriariin A (**58**), in which the phenolic hydroxyl groups of **57** are completely methylated, was inactive, while **57** showed the strongest antitumor activity among tannins and the related polyphenols examined in this study. It is clear that the presence of free phenolic hydroxyl groups is essential for the antitumor activity.

It has been thought that tannins show a number of pharmacological activities due to their astringent activity. Our previous report indicated that the binding activities of tannins with hemoglobin and methylene blue increased with increase of the molecular weight of tannins, particularly upon galloylation.[13] We have also reported that the methanol extract from

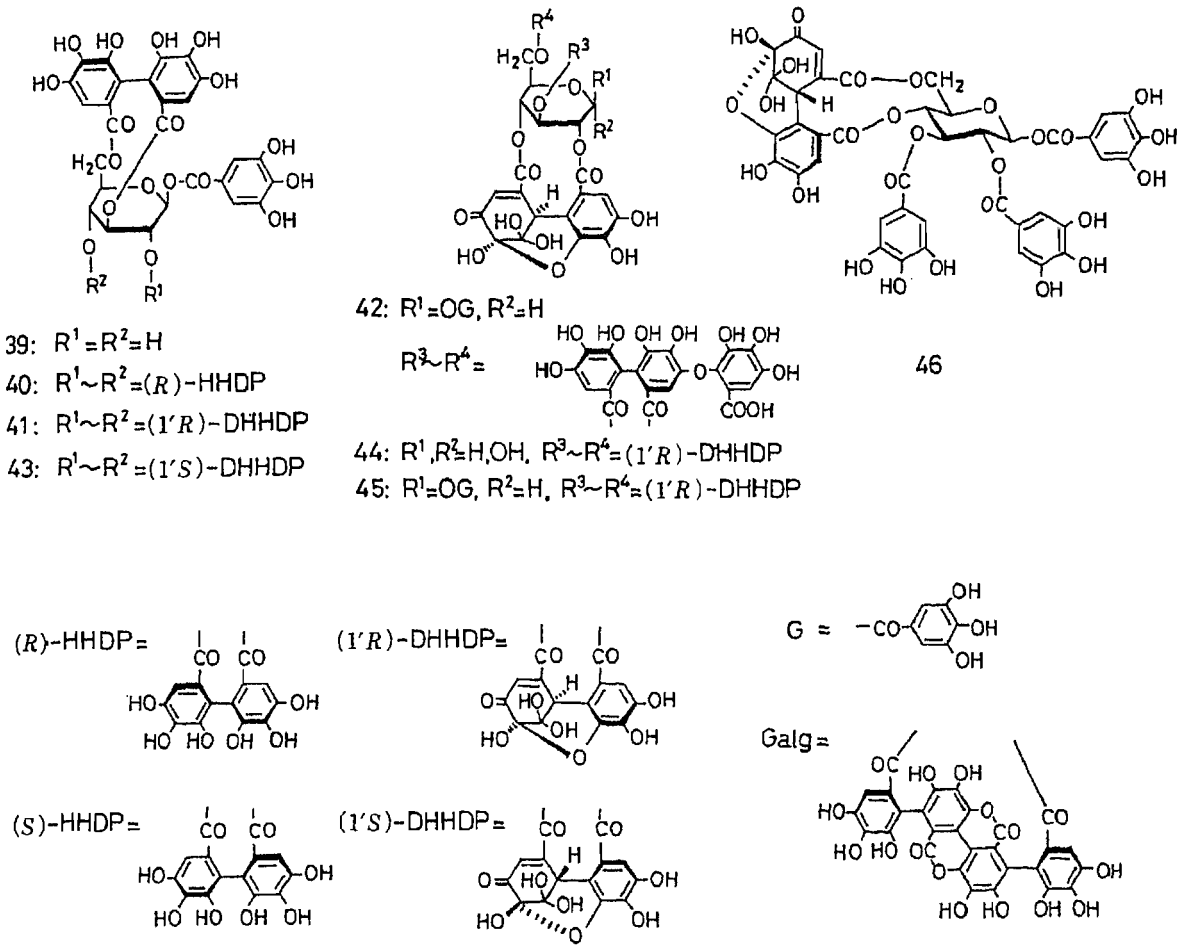

Chart 3

Agrimonia pilosa LEDEB. and agrimoniin showed cytocidal activity *in vitro*, but the activity was significantly decreased by the addition of calf serum to the culture; further, they increased macrophage-like cells with cytostatic activity and lymphocytes with growing ability *in vivo*.[7,8] Tannins in this study were injected into the mice at 4 d before the tumor cell inoculation. Consequently, it is difficult to consider that the tannins acted directly on the tumor cells. It appears that a suitable molecular mass with a high level of galloylation may be essential for the antitumor activity of ellagitannins, and the stereochemical composition of free phenolic hydroxyl groups may also be an important factor. It is likely that these tannins show antitumor activity through potentiation of the immunity of host animals.

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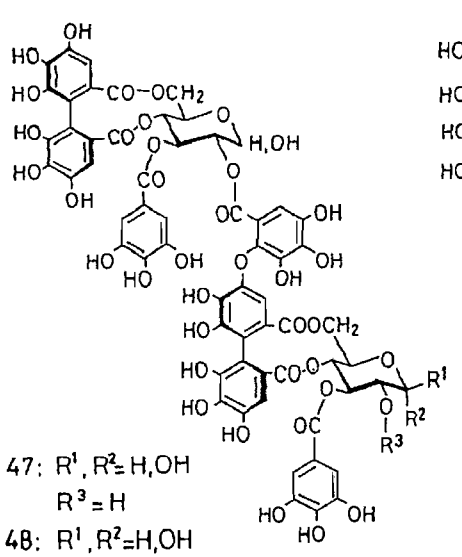
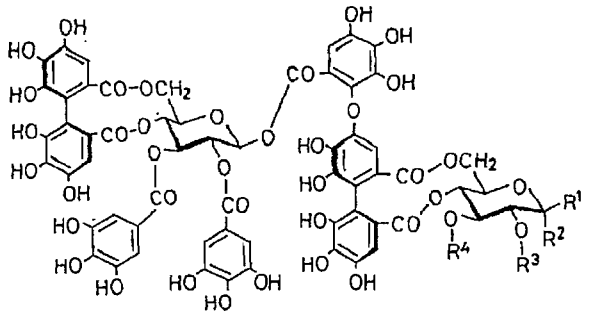
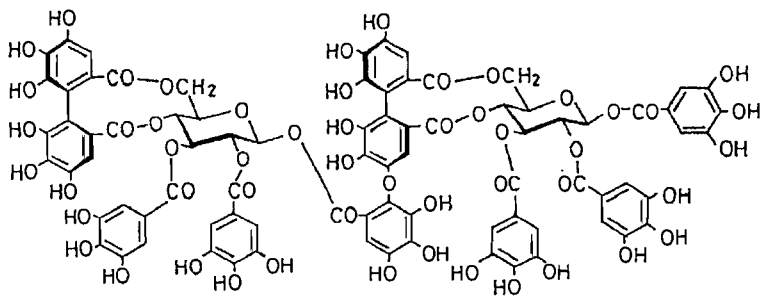


47: R^1, R^2 = H,OH
R^3 = H
48: R^1, R^2 = H,OH
R^3 = G
49: R^1 = R^3 = H, R^2 = OG



50: R^1, R^2 = H,OH, R^3 = H, R^4 = G
51: R^1, R^2 = H,OH R^3 = R^4 = G
52: R^1 = OG, R^2 = H, R^3 = R^4 = G
54: R^1 = OG, R^2 = H
R^3 ~ R^4 = (S)-HHDP



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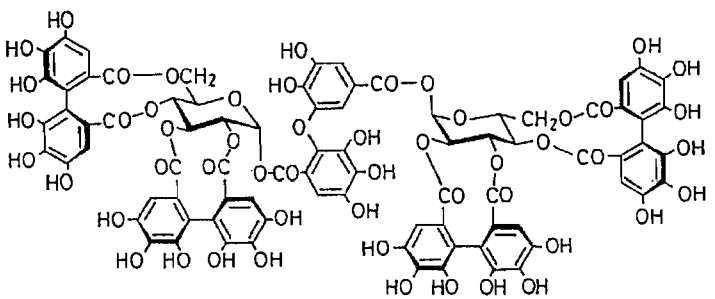

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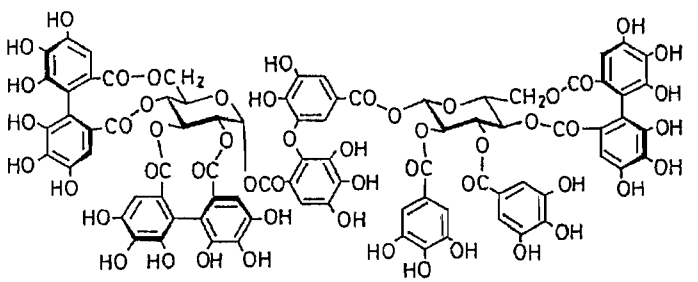

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Chart 4

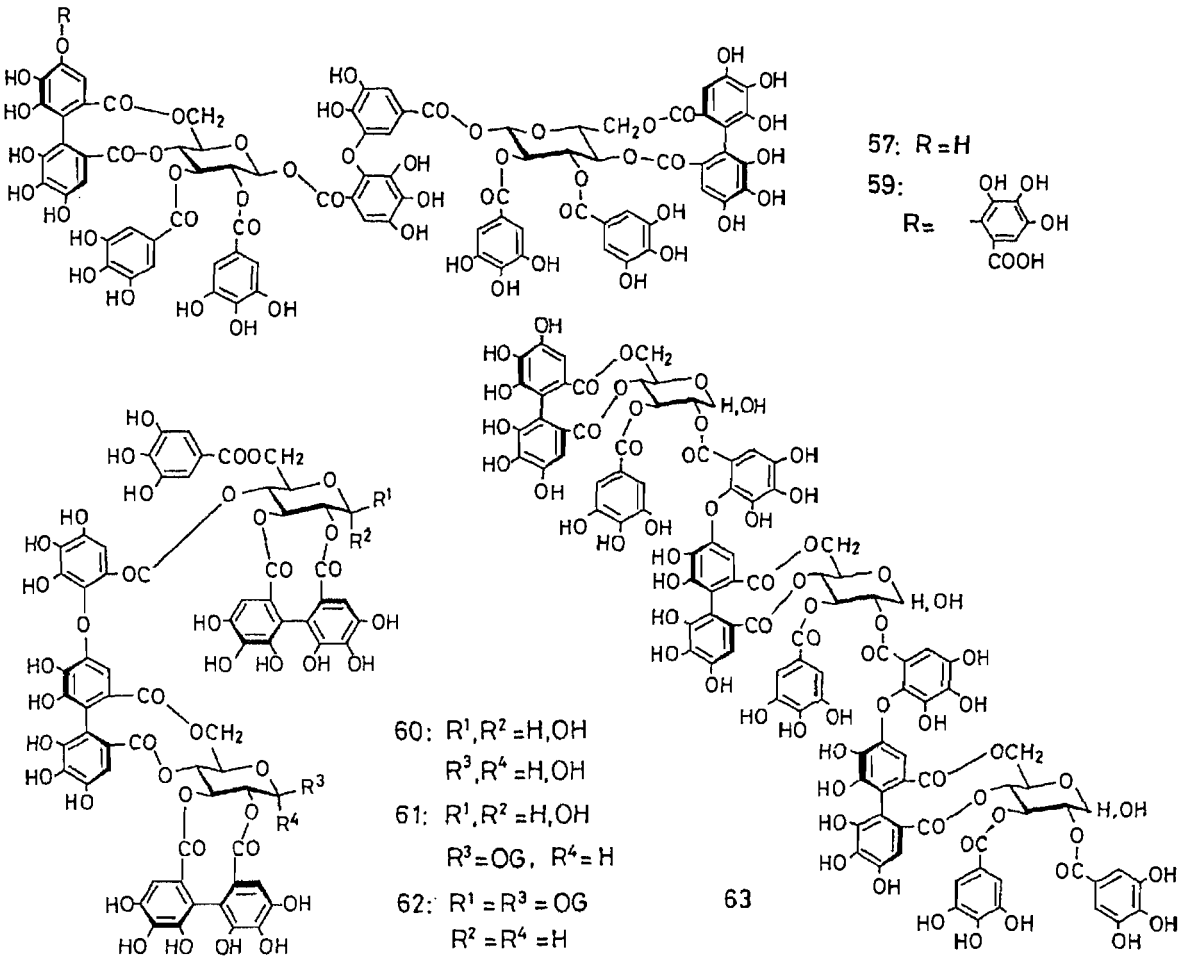

Chart 5

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Notes

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Palladium-Catalyzed Reactions of Terminal Acetylenes and Olefins with Halo-1,3-azoles

TAKAO SAKAMOTO, HIDEO NAGATA, YOSHINORI KONDO, MASAFUMI SHIRAIWA, and HIROSHI YAMANAKA*

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

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The palladium-catalyzed reactions of 4-bromo- and 5-bromothiazoles, as well as 4-bromo- and 5-bromooxazoles with terminal acetylenes gave ethynyl derivatives in 43—89% yields, whereas the reactions of 2-bromothiazoles and iodo-*N*-methylimidazoles afforded the products in poor yields. The reaction of the halo-1,3-azoles with terminal olefins was also examined.

Keywords——palladium-catalyzed reaction; bromothiazole; bromooxazole; iodo-*N*-methylimidazole; ethynylthiazole; ethynyloxazole; ethynyl-*N*-methylimidazole; ethenylthiazole; ethenyloxazole; ethenyl-*N*-methylimidazole

The palladium-catalyzed reaction of aryl halides with terminal acetylenes is a powerful method to introduce ethynyl groups into aromatic nuclei. This reaction was reported independently by Casser[1] and Dieck and Heck,[2] and later was improved by Sonogashira *et al.*[3] On the other hand, Heck *et al.*[4] developed a similar palladium-catalyzed reaction of aryl halides with olefins. These reactions were applied to six-membered *N*-heteroaryl halides by several groups, using acetylenes[5] and olefins.[6]

This article is concerned with an approach to the introduction of an unsaturated side-chain into 1,3-azole nuclei by the use of the palladium-catalyzed reaction of halo-1,3-azoles with terminal acetylenes and olefins.

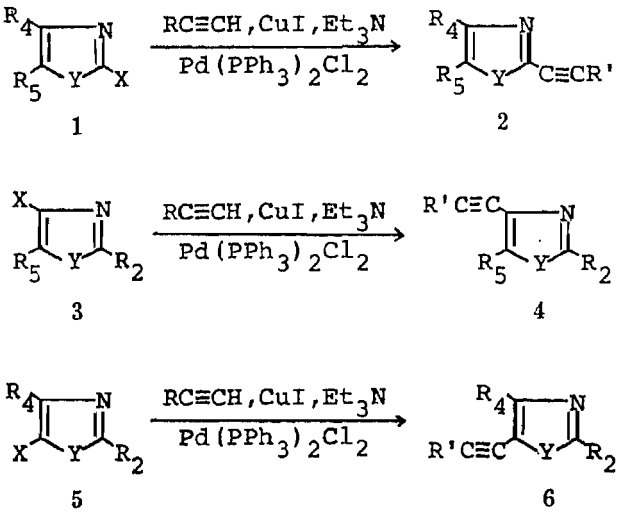

Chart 1

TABLE I. Palladium-Catalyzed Reaction of 2-Halo-1,3-azoles with Acetylenes

2-Halo-1,3-azole					Reaction temp. (°C)	Reaction time (h)	Product		
No.	Y	X	R_4	R_5			No.	R′	Yield (%)
1a	S	Br	H	H	90	3	**2aa**	Ph	24
1b	S	Br	Ph	H	70	3	**2ba**	Ph	5
1a	S	Br	H	H	80	3	**2ab**	H	17
1b	S	Br	Ph	H	70	3	**2bb**	H	36
1d	NMe	I	H	H	90	7	**2da**	Ph	16

TABLE II. Palladium-Catalyzed Reaction of 4-Halo-1,3-azoles with Acetylenes

4-Halo-1,3-azole					Reaction temp. (°C)	Reaction time (h)	Product		
No.	Y	X	R_2	R_5			No.	R′	Yield (%)
3a	S	Br	H	H	80	3	**4aa**	Ph	71
3a	S	Br	H	H	80	3	**4ab**	H	43
3b	O	Br	Me	Ph	90	15	**4ba**	Ph	83
3c	NMe	I	H	H	90	8	**4ca**	Ph	25
3c	NMe	I	H	H	100	17	**4cb**	H	13

TABLE III. Palladium-Catalyzed Reaction of 5-Halo-1,3-azoles with Acetylenes

5-Halo-1,3-azole					Reaction temp. (°C)	Reaction time (h)	Product		
No.	Y	X	R_2	R_4			No.	R′	Yield (%)
5a	S	Br	H	Me	80	14	**6aa**	Ph	65
5a	S	Br	H	Me	70	3	**6ab**	H	70
5b	S	Br	Ph	Me	90	12	**6bb**	H	51
5c	O	Br	Me	Ph	90	4	**6ca**	Ph	89
5d	NMe	I	H	H	90	7	**6da**	Ph	26
5d	NMe	I	H	H	100	3	**6db**	H	25

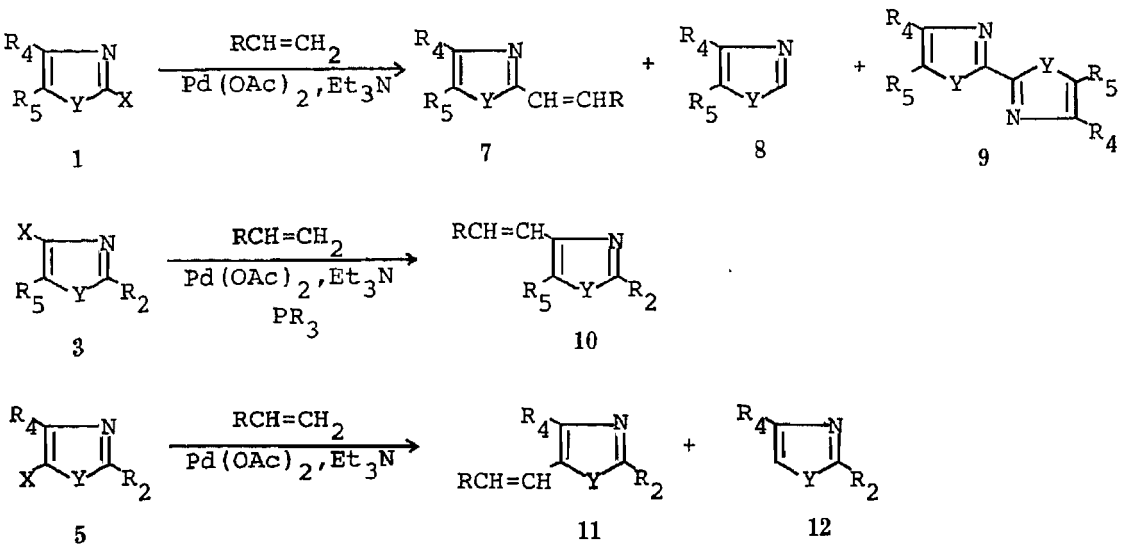

Chart 2

Firstly, the palladium-catalyzed reaction of phenylacetylene with halo-1,3-azoles such as bromothiazoles, bromooxazoles, and iodo-*N*-methylimidazoles was examined as a preliminary experiment.

As shown in Tables I, II, and III, the reaction of the 1,3-azoles under conventional conditions [dichlorobis(triphenylphosphine)palladium–cuprous iodide–triethylamine] afforded the desired products in moderate yields at the 4- and 5-positions, but in the case of the 2-

TABLE IV. Palladium-Catalyzed Reaction of 2-Halo-1,3-azoles with Olefins

2-Halo-1,3-azole					Solvent	Phosphine	Reaction temp. (°C)	Reaction time (h)	Product (yield %)			
No.	Y	X	R_4	R_5					7	(R)	8	9
1b	S	Br	Ph	H	DMF	—	130	24	**7ba** (*E*)	(COOEt)	19 22	6
1b	S	Br	Ph	H	DMF	—	100	48	**7bb** (*E*/*Z*)	(CN)[a]	15 12	15
1b	S	Br	Ph	H	DMF	—	100	48	**7bc** (*E*)	(Ph)	5 7	Trace
1d	NMe	I	H	H	MeCN	—	110	55	**7dc**	(Ph)	0 0	15

a) Ratio of *E*-isomer to *Z*-isomer was 3 to 1 as evaluated from the ^{1}H-NMR (CCl_4) spectrum.

TABLE V. Palladium-Catalyzed Reaction of 4-Halo-1,3-azoles with Olefins

4-Halo-1,3-azole					Solvent	Phosphine[a] (2 eq)	Reaction temp. (°C)	Reaction time (h)	Product		
No.	Y	X	R_2	R_5					No.	(R)	Yield (%)
3a	S	Br	H	H	DMF	TOP	130	24	**10aa** (*E*)	(COOEt)	19
3a	S	Br	H	H	DMF	TOP	100	48	**10ab** (*E*/*Z*)	(CN)[b]	15
3a	S	Br	H	H	DMF	TOP	100	48	**10ac** (*E*)	(Ph)	8
3b	O	Br	Me	Ph	DMF	TPP	120	52	**10ba** (*E*)	(COOEt)	65
3b	O	Br	Me	Ph	DMF	TPP	120	115	**10bb** (*E*)	(CN)	43
3b	O	Br	Me	Ph	DMF	TPP	120	47	**10bc** (*E*)	(Ph)	64
3c	NMe	I	H	H	MeCN	—	110	50	**10cc** (*E*)	(Ph)	23

a) TPP, triphenylphosphine; TOP, tri(*o*-tolyl)phosphine. *b*) Ratio of *E*-isomer to *Z*-isomer was 8 to 1 as evaluated from the ^{1}H-NMR (CCl_4) spectrum.

TABLE VI. Palladium-Catalyzed Reaction of 5-Halo-1,3-azoles with Olefins

5-Halo-1,3-azole					Solvent	Phosphine[a]	Reaction temp. (°C)	Reaction time (h)	Product (yield %)		
No.	Y	X	R_2	R_4					11	(R)	12
5b	S	Br	Ph	Me	DMF	—	115	24	**11ba** (*E*)	(COOEt)	61 0
5b	S	Br	Ph	Me	DMF	—	130	24	**11bb**	(CN)	0 70
5b	S	Br	Ph	Me	DMF	TPP (4 eq)	100	48	**11bb** (*E*/*Z*)	(CN)[b]	47 19
5b	S	Br	Ph	Me	DMF	—	100	48	**11bc** (*E*)	(Ph)	1 81
5b	S	Br	Ph	Me	DMF	TPP (4 eq)	100	48	**11bc** (*E*)	(Ph)	37 32
5b	S	Br	Ph	Me	DMF	TOP (4 eq)	100	48	**11bc** (*E*)	(Ph)	53 31
5c	O	Br	Me	Ph	—	TPP (2 eq)	120	67	**11ca** (*E*)	(COOEt)	83 0
5c	O	Br	Me	Ph	—	TPP (2 eq)	120	44	**11cb** (*E*)	(CN)	50 0
5c	O	Br	Me	Ph	—	TPP (2 eq)	120	28	**11cc** (*E*)	(Ph)	87 0
5d	NMe	I	H	H	MeCN	—	120	24	**11da** (*E*)	(COOEt)	9 0
5d	NMe	I	H	H	MeCN	—	130	24	**11db** (*E*)	(CN)	2 0
5d	NMe	I	H	H	MeCN	—	110	51	**11dc** (*E*)	(Ph)	25 0

a) TPP, triphenylphosphine; TOP, tri(*o*-tolyl)phosphine. *b*) Ratio of *E*-isomer to *Z*-isomer was 2 to 1 as evaluated from the ^{1}H-NMR (C_6D_6) spectrum.

TABLE VII. Physical Constants and Spectral Data for Ethynyl- and Ethenyl-1,3-azoles

No.	bp/mmHg [mp] (°C)	IR (cm^{-1}) ($CHCl_3$)	^{1}H-NMR δ (ppm) (CCl_4 or $CDCl_3$, J=Hz)
2aa	147/4 [43—45]	2210	7.35 (1H, d, J=3), 7.78 (1H, d, J=3), 7.2—7.8 (5H, m)[a]
2ba	[97—102]	2210	7.3—8.3 (11H, m)[b]
2ab	118/55	2120	3.45 (1H, s), 7.33 (1H, d, J=3), 7.78 (1H, d, J=3)[a]
2bb	[59—61]	2120	3.38 (1H, s), 7.2—7.6 (4H, m), 7.7—8.1 (2H, m)[a]
2da	170/5	2220	3.80 (3H, s), 6.97 (1H, s), 7.12 (1H, s), 7.3—7.8 (5H, m)[b]
4aa	150/5	2220	7.1—7.7 (6H, m), 8.67 (1H, d, J=2)[a]
4ab	108/50	2120	3.04 (1H, s), 7.47 (1H, d, J=2), 8.67 (1H, d, J=2)[a]
4ba	175—180/1	2200	2.47 (3H, s), 7.0—7.8 (8H, m), 7.8—8.3 (2H, m)[b]
4ca	[129—130]	2220	3.67 (3H, s), 7.1—7.7 (7H, m)[b]
4cb	130/3	2110	3.04 (1H, s), 3.68 (3H, s), 7.09 (1H, s), 7.31 (1H, s)[b]
6aa	130—132/4	2220	2.53 (3H, s), 7.1—7.7 (5H, m), 8.50 (1H, s)[a]
6ab	91—92/48	2120	2.53 (3H, s), 3.43 (1H, s), 8.47 (1H, s)[a]
6bb	[66—67]	2100	2.53 (3H, s), 3.45 (1H, s), 7.3—7.6 (3H, m), 7.8—8.1 (2H, m)[a]
6ca	[58—59]	2200	2.50 (3H, s), 7.7—7.9 (8H, m), 7.9—8.3 (2H, m)[b]
6da	160/5	2220	3.73 (3H, s), 7.9—8.3 (2H, m)[b]
6db	116/25	2110	3.48 (1H, s), 3.66 (3H, s), 7.30 (1H, s), 7.43 (1H, s)[b]
7ba (*E*)	170/4	1720 980	1.33 (3H, t, J=7), 4.22 (2H, q, J=7), 6.70 (1H, d, J=16) 7.2—8.1 (7H, m)[a]
7bb (*E*)	160/3	2220 960	5.01 (1H, d, J=12), 6.33 (1H, d, J=16), 7.3—7.7 (5H, m) 7.8—8.1 (2H, m)[a]
7bc (*E*)	[128]	960	7.3—7.8 (11H, m), 7.8—8.1 (2H, m)[b]
10aa (*E*)	[71—72]	1710 980	1.35 (3H, t, J=7), 4.24 (2H, q, J=7), 6.76 (1H, d, J=16) 7.42 (1H, d, J=2), 7.55 (1H, d, J=16), 8.77 (1H, d, J=2)[a]
10ab (*E*/*Z*)	[85—86]		5.48 (1H, d, J=12), 6.35 (1H, d, J=16), 7.37 (1H, d, J=16) 7.49 (1H, d, J=12), 8.79 (1H, d, J=2)[a]
10ac (*E*)	158/15	970	6.9—7.7 (8H, m), 8.70 (1H, d, J=2)[a]
10ba (*E*)	[81—82]	1700 980	1.31 (3H, t, J=7), 2.51 (3H, s), 4.23 (2H, q, J=7), 6.33 (1H, d, J=16), 7.2—7.9 (5H, m), 7.61 (1H, d, J=16)[b]
10bb (*E*)	[115—116]	2220	2.50 (3H, s), 6.18 (1H, d, J=16), 7.1—7.8 (6H, m)[b]
10bc (*E*)	170—175/1 [71—72]	960	2.50 (3H, s), 6.7—7.8 (12H, m)[b]
10cc (*E*)	185/3	960	3.67 (3H, s), 6.8—7.7 (9H, m)[a]
11ba (*E*)	180/4 [59—60]	1710 970	1.31 (3H, t, J=7), 2.53 (3H, s), 4.20 (2H, q, J=7), 6.00 (1H, d, J=16), 7.2—8.1 (6H, m)[a]
11bb (*E*)	[146—147]	2215 955	2.54 (3H, s), 5.52 (1H, d, J=16), 7.3—7.7 (4H, m), 8.8—9.1 (2H, m)[b]
11bb (*E*)	[112—114]	2210	2.54 (3H, s), 5.30 (1H, d, J=12), 7.3—7.7 (4H, m), 7.8—8.2 (2H, m)[b]
11bc (*E*)	[102—104]	950	2.47 (3H, s), 6.63 (1H, d, J=16), 6.9—7.6 (9H, m), 7.6—8.0 (2H, m)[a]
11ca (*E*)	[80—81]	1710 970	1.30 (3H, t, J=7), 2.52 (2H, q, J=7), 2.51 (3H, s), 6.40 (1H, d, J=16), 7.1—8.0 (6H, m)[b]
11cb (*E*)	[62.5—64]	2215 960	2.54 (3H, s), 5.85 (1H, d, J=16), 6.9—7.9 (6H, m)[b]
11cc (*E*)	155—160/1	960	2.50 (3H, s), 6.6—8.2 (10H, m), 7.10 (2H, s)[b]
11da (*E*)	154/4	1710 975	1.32 (3H, t, J=7), 3.72 (3H, s), 4.25 (2H, q, J=7), 6.25 (1H, d, J=16), 7.4—7.7 (3H, m)[b]
11db (*E*)	140/3	2210 960	3.70 (3H, s), 5.70 (1H, d, J=16), 7.20 (1H, d, J=16) 7.46 (1H, s), 7.55 (1H, s)[b]
11dc (*E*)	170/3	950	3.67 (3H, s), 6.92 (2H, s), 7.2—7.7 (7H, m)[b]

a) In CCl_4. *b*) In $CDCl_3$.

TABLE VIII. Analytical Data for Ethynyl- and Ethenyl-1,3-azoles

No.	Formula	Analysis (%) Calcd C	H	N	Found C	H	N
2aa	$C_{17}H_{10}N_4O_7S$ (picrate)[a]	49.28	2.43	13.52	49.36	2.39	13.66
2ba	$C_{17}H_{11}NS$	78.13	4.24	5.36	78.06	4.28	5.27
2ab	C_5H_3NS	55.03	2.77	12.84	54.63	2.96	12.64
2bb	$C_{11}H_7NS$	71.32	3.81	7.56	71.20	3.76	7.02
2da	$C_{12}H_{10}N_2$	79.10	5.53	15.37	79.05	5.38	15.63
4aa	$C_{11}H_7NS$	71.32	3.81	7.56	71.69	3.83	7.51
4ab	C_5H_3NS	55.03	2.77	12.84	55.05	2.72	12.99
4ba	$C_{18}H_{13}NO$	83.37	5.05	5.40	83.71	5.15	5.23
4ca	$C_{12}H_{10}N_2$	79.10	5.53	15.37	78.83	5.44	15.46
4cb	$C_6H_6N_2$	67.91	5.70	26.39	67.87	5.52	26.64
6aa	$C_{12}H_9NS$	72.33	4.55	7.03	72.16	4.68	6.84
6ab	C_6H_5NS	58.51	4.09	11.37	58.53	4.10	11.31
6bb	$C_{12}H_9NS$	72.33	4.55	7.03	72.21	4.30	6.70
6ca	$C_{18}H_{13}NO$	83.37	5.05	5.40	83.70	4.97	5.72
6da	$C_{18}H_{13}N_5O_7$ (picrate)[b]	52.56	3.19	17.03	52.74	3.12	16.92
6db	$C_{12}H_9N_5O_7$ (picrate)[c]	42.99	2.71	20.89	43.18	2.61	20.92
7ba (*E*)	$C_{14}H_{13}O_2S$	64.84	5.05	5.40	64.98	5.09	5.45
7bb (*E*)	$C_{12}H_8N_2S$	67.90	3.80	13.20	68.10	4.01	13.35
7bc (*E*)	$C_{17}H_{13}NS$	77.53	4.97	5.32	77.36	5.08	5.35
10aa (*E*)	$C_8H_9NO_2S$	52.44	4.96	7.65	52.48	5.09	7.39
10ab (*E*/*Z*)	$C_6H_4N_2S$	52.93	2.96	20.57	53.10	2.92	20.58
10ac (*E*)	$C_{11}H_9NS$	70.54	4.84	7.48	70.64	5.11	7.40
10ba (*E*)	$C_{15}H_{15}NO_3$	70.02	5.88	5.44	70.15	5.85	5.29
10bb (*E*)	$C_{13}H_{10}N_2O$	74.27	4.79	13.33	74.42	5.06	13.21
10bc (*E*)	$C_{18}H_{15}NO$	82.73	5.79	5.36	82.36	6.05	5.11
10cc (*E*)	$C_{12}H_{12}N_2$	78.23	6.57	15.20	78.16	6.75	15.07
11ba (*E*)	$C_{15}H_{15}NO_2S$	65.91	5.53	5.13	65.85	5.47	4.78
11bb (*E*)	$C_{13}H_{10}N_2S$	69.00	4.45	12.38	68.76	4.41	12.10
11bb (*Z*)	$C_{13}H_{10}N_2S$	69.00	4.45	12.38	69.11	4.88	12.01
11bc (*E*)	$C_{18}H_{15}NS$	77.94	5.45	5.05	77.80	5.43	4.80
11ca (*E*)	$C_{15}H_{15}NO_3$	70.02	5.88	5.44	70.19	5.62	5.68
11cb (*E*)	$C_{13}H_{10}N_2O$	74.27	4.79	13.33	74.64	4.67	13.30
11cc (*E*)	$C_{18}H_{15}NO$	82.73	5.79	5.36	82.57	5.79	5.30
11da (*E*)	$C_9H_{12}N_2O_2$	59.99	6.71	15.54	59.61	6.56	15.40
11db (*E*)	$C_7H_7N_3$	63.14	5.30	31.56	62.84	5.43	31.75
11dc (*E*)	$C_{12}H_{12}N_2$	78.23	6.57	15.20	78.21	6.57	15.12

a) mp 135—136 °C (dec.). *b*) mp 196—197 °C (dec.). *c*) mp 173—174 °C (dec.).

position, the reaction afforded the products in low yields. In general, aryl iodides are the best substrates among the corresponding chlorides, bromides, and iodides in acetylene cross-coupling reaction,[5a] but in the case of iodo-*N*-methylimidazoles, the reaction gave an unsatisfactory result at any position.

On the basis of these findings, the reaction with trimethylsilylacetylene[3b,5f] was carried out in order to synthesize the 1,3-azole derivatives containing an unsubstituted ethynyl group. The 4- and 5-bromothiazoles could be converted into the corresponding trimethylsilylethynyl compounds in moderate yields, whereas the products obtained by the reaction of 4- and 5-bromooxazoles with trimethylsilylacetylene resinified during the purification process.

Next, the reaction of halo-1,3-azoles with terminal olefins such as ethyl acrylate, acrylonitrile, and styrene under conventional conditions [palladium acetate–triphenylphosphine (TPP)–triethylamine] was investigated. As compared with the acetylene cross-coupling reaction described above, the reaction with the olefins appears to be less valuable as a synthetic method except at the 4- and 5-positions of oxazole. It is difficult to generalize in some cases, the synthesis of the olefinic compounds was disturbed by the formation of biazoles. Since standard conditions for the olefin cross-coupling reaction of 1,3-azoles have not yet been established, some modification of the catalyst ligands and solvents may be necessary to obtain good results as indicated in Tables IV, V, and VI. The reaction of iodo-*N*-methylimidazoles with the olefins, like the reaction with the acetylenes, afforded poor results.

Heck *et al.*[6a] reported that the reaction of 2-bromothiazole with methyl acrylate gave no significant product using tri-(*o*-tolyl)phosphine (TOP) as the ligand of the palladium catalyst. Similarly, in our present investigation, the reactions of 2-halo-1,3-azoles with the acetylenes and the olefins were found to give large amounts of resinous substances, probably due to ring-cleavage caused by palladation at the 2-position of the substrates.

Experimental

All melting points are uncorrected. Infrared (IR) spectra were measured with a JASCO IRA-1 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken at 60 MHz with a JEOL JMN-PMX 60 spectrometer. Chemical shifts are expressed in δ (ppm) values. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, and br = broad.

Palladium-Catalyzed Reaction of Halo-1,3-azoles with Phenylacetylene (General Procedure A)——A mixture of a halo-1,3-azole (10 mmol), phenylacetylene (12 mmol), $PdCl_2(PPh_3)_2$ (280 mg), CuI (150 mg), and Et_3N (3 ml) was heated in a sealed tube or an open-vessel at an appropriate temperature for an appropriate time as shown in Tables I, II, and III. The reaction mixture was diluted with H_2O and extracted with C_6H_6, ether, or CH_2Cl_2. The residue obtained from the extract was purified by SiO_2 column chromatography, and the product was distilled or recrystallized.

Palladium-Catalyzed Reaction of Halo-1,3-azoles with Trimethylsilylacetylene (General Procedure B)——A crude trimethylsilylethynyl-1,3-azole obtained according to general procedure A, was dissolved in a mixture of 1 N KOH (6 ml) and MeOH (20 ml) and the solution was stirred at room temperature with monitoring by SiO_2 thin-layer chromatography. After evaporation of the solvent, the aqueous residue was extracted with ether. The residue obtained from the ethereal extract was purified according to general procedure A.

Palladium-Catalyzed Reaction of Halo-1,3-azoles with Olefins (General Procedure C)——A mixture of a halo-1,3-azole (10 mmol), an olefin (12 mmol), $Pd(OAc)_2$ (110 mg), Et_3N (1.2 ml), and dimethylformamide (DMF, 4 ml) or MeCN (6 ml) was heated in a sealed tube at an appropriate temperature for an appropriate time as shown in Tables IV, V, and VI. After evaporation of the solvent, the residue was purified by SiO_2 column chromatography, and the product was distilled or recrystallized.

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Synthesis of Homodolichosterone and Related 2-Deoxysteroids

SUGURU TAKATSUTO*,[a] and NOBUO IKEKAWA[b]

Department of Chemistry, Joetsu University of Education,[a] Joetsu, Niigata 943, Japan and Department of Chemistry, Tokyo Institute of Technology,[b] Ookayama, Meguro-ku, Tokyo 152, Japan

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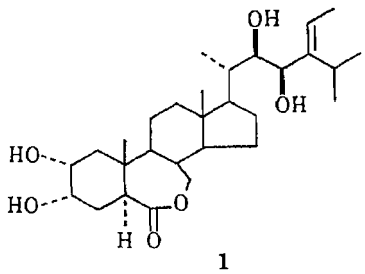
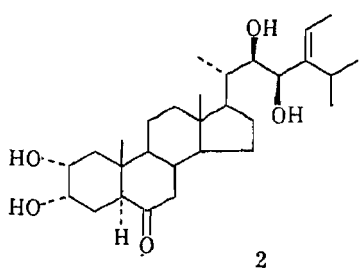
Homodolichosterone (**2**) and the related 2-deoxysteroids **9** and **13** were synthesized from (22*R*,23*R*,24*S*)-3β-acetoxy-22,23-epoxy-5α-stigmastan-6-one (**3**). Reaction of the (22*R*,23*R*)-epoxide **4** with phenylselenyl anion followed by heating with 30% H_2O_2 afforded a mixture of the allylic alcohols **5** and **6**, which were then epoxidized with *m*-chloroperbenzoic acid. The isolated hydroxyepoxide **7** was heated with aluminum isopropoxide to yield the 3β,22,23-triol **9**. Acetonide formation of **9** and mesylation gave the sulfonate **10**, which was refluxed with lithium carbonate and dimethylformamide and then saponified to give the 3α-ol **11** and the 2,24(28)-diene **12**. Acid hydrolysis of **11** provided the 3α,22,23-triol **13**. Selective α-face hydroxylation of **12** with osmium tetroxide and deprotection gave homodolichosterone (**2**).

Keywords——brassinolide; brassinosteroid; homodolicholide; homodolichosterone; plant growth promoter; 2-deoxysteroid

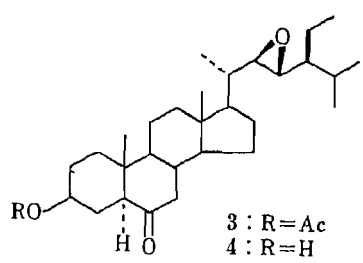
Since the discovery of brassinolide and castasterone as plant growth promoters,[1] the related 2-deoxysteroids, typhasterol (2-deoxycastasterone) and teasterone, the 3β-isomer of typhasterol, have been isolated and identified in several higher plants.[2] Similarly, in connection with homodolicholide (**1**)[3] and homodolichosterone (**2**),[4] it is possible that the related 2-deoxysteroids **9** and **13**, which correspond to teasterone and typhasterol, respectively, may exist in nature. As a part of our program for the identification and characterization of brassinosteroids from plant sources, we required standard samples of **2**, **9**, and **13**. In this paper we describe the synthesis of these heretofore unknown 2-deoxysteroids **9** and **13** and of homodolichosterone (**2**).



1



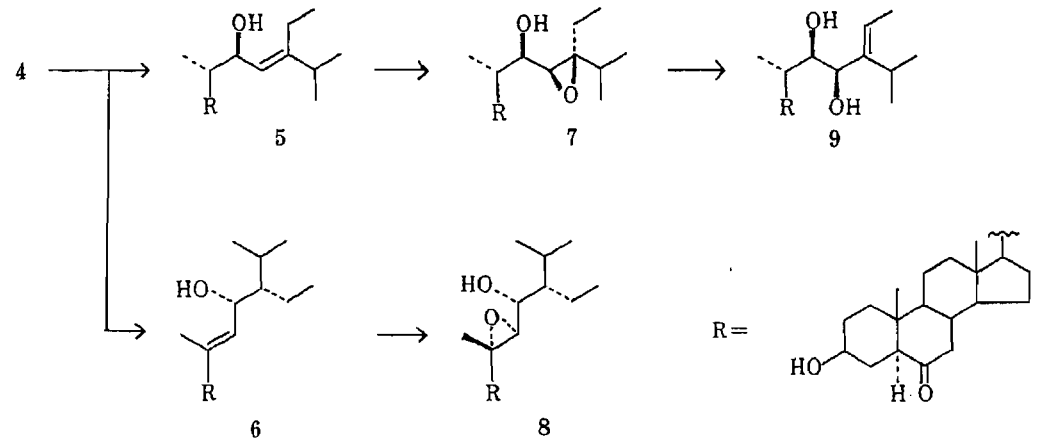
2



3 : R=Ac
4 : R=H

For the construction of the side chain part of **2**, **9**, and **13**, the method[5] developed by Sakakibara and Mori for the synthesis of homodolichosterone (**2**) seems to be convenient. Thus, we applied their method to the isomerically pure (22*R*,23*R*)-epoxide (**4**). In their synthesis of the side chain of **2**,[5] an inseparable mixture of the (22*R*,23*R*)- and (22*S*,23*S*)-epoxy compounds was used as an intermediate so that the subsequent reactions and purification were complicated. In our case, the isomerically pure compound was employed in order to avoid these problems. Reaction of **4**, derived from the known acetate **3**,[6] with an

excess of phenylselenyl anion[7] was carried out in refluxing 1-butanol for 5 d. The resulting α-hydroxyselenides were then heated with 30% H_2O_2 to effect the *syn*-elimination reaction. The regioisomeric alcohols **5** and **6** thus obtained were epoxidized with *m*-chloroperbenzoic acid and the products were easily separated by column chromatography to give the less polar hydroxyepoxide **8** [28%, δ_H 3.05 (1H, d, $J=8$ Hz, 22-H)] and the more polar isomer **7** [21%, δ_H 2.88 (1H, d, $J=7$ Hz, 23-H)], along with recovery of **4** (40%). The stereochemistry of **7** and **8** was confirmed by comparison of the proton nuclear magnetic resonance (^{1}H-NMR) data with those of reference compounds.[5] Treatment of **7** with aluminum isopropoxide in refluxing toluene[8] provided (22*R*,23*R*,24(28)*E*)-3β,22,23-trihydroxy-5α-stigmast-24(28)-en-6-one (**9**), mp 228—230 °C, in 22% yield and the starting material **7** was recovered (25%). The rearrangement of the epoxide into the allylic alcohol proceeded in low yield. The reason for this might be ascribed to the presence of the free 3β-hydroxyl group, judging from the reported results, in which the 2α,3α-diol of the substrate was protected as the acetonide.[5] Improvement of the yield was not attempted since we simply wanted to obtain standard sample of **2**, **9**, and **13**.

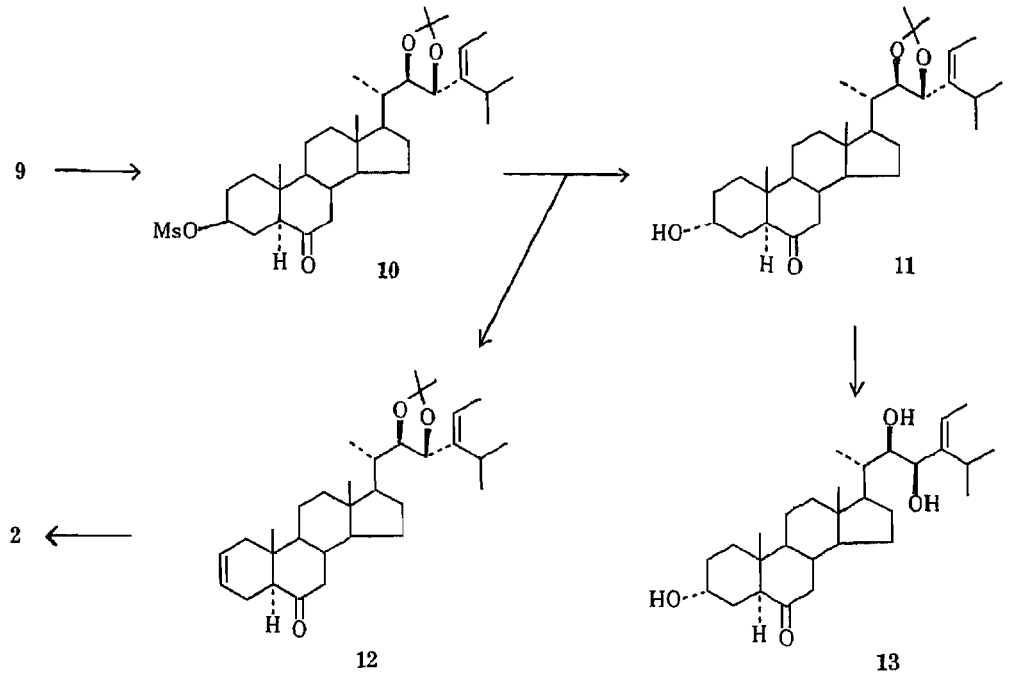


Transformation of the 3β,22,23-triol **9** into its 3α-isomer **13** and homodolichosterone (**2**) was achieved according to our procedure[6] as follows. The triol **9** was submitted to acetonide formation and then mesylation to give the mesylate **10**, which was further treated with lithium carbonate and dimethylformamide under reflux. The resulting products were saponified and then purified by chromatography on silica gel to afford the 2,24(28)-diene **12** and the 3α-ol **11** in 44 and 35% yields, respectively. Heating of **11** with 80% aqueous AcOH provided (22*R*,23*R*,24(28)*E*)-3α,22,23-trihydroxy-5α-stigmast-24(28)-en-6-one (**13**), mp 209—210 °C, in 91% yield. Regio- and stereoselective *cis*-hydroxylation of the diene **12** was carried out with a catalytic amount of OsO_4 and *N*-methylmorpholine *N*-oxide in *tert*-BuOH–tetrahydrofuran(THF)–H_2O (10:3:1). Removal of the protecting group of the resulting 2α,3α-diol gave homodolichosterone (**2**), mp 217—219 °C, sinter at 208 °C (lit.,[5] mp 218—219°C, sinter at 208 °C), in 70% yield. Its spectral data were in good agreement with the reported data.[4,5]

In conclusion, we were able to synthesize homodolichosterone (**2**) and the related 2-deoxysteroids **9** and **13**, which were required as standard samples for studies to identify them in plant sources.

Experimental

Melting points were determined on a Yazawa hot stage microscope and are uncorrected. ^{1}H-NMR spectra were



run on a Hitachi R-24B (60 MHz) spectrometer unless otherwise noted. All NMR spectra were taken in $CDCl_3$ solution with tetramethylsilane as an internal standard. Mass spectra (MS) were obtained with a Shimadzu LKB-9000S mass spectrometer. Thin-layer chromatography (TLC) was carried out on precoated plates of silica gel (Merck, Kieselgel $60F_{254}$, 0.25 mm thickness) and column chromatography on Kieselgel $60F_{254}$ (70—230 mesh, Merck). Work-up refers to dilution with water, extraction with an organic solvent, washing of the extract to neutrality, drying over $MgSO_4$, filtration, and removal of the solvent under reduced pressure.

(20*R*,22*S*,23*R*,24*S*)-20,22-Epoxy-3β,23-dihydroxy-5α-stigmastan-6-one (7) and (22*R*,23*S*,24*R*)-23,24-Epoxy-3β,22-dihydroxy-5α-stigmastan-6-one (8)——Sodium borohydride (2.4 g, 63.2 mmol) was added to a suspension of diphenyl diselenide (9.9 g, 31.7 mmol) in 1-butanol (150 ml) and the mixture was stirred at room temperature for 20 min. A solution of **4** (2.3 g, 5.14 mmol), derived from the known acetate **3**,[6] in THF (10 ml) was added to the reagent solution. The mixture was refluxed for 5 d. THF (100 ml) and 30% H_2O_2 (30 ml) were added to the cooled reaction mixture and it was further stirred at 80 °C for 2 h. Work-up (ether) gave crude products, which showed four spots of *Rf* 0.42, 0.33, 0.27, and 0.19 on TLC (benzene–EtOAc, 1 : 1, developed once). The spot of *Rf* 0.42 was identical with **4**. The mixture in CH_2Cl_2 (100 ml) was treated with *m*-chloroperbenzoic acid (300 mg) at room temperature for 1 h. TLC analysis of the reaction mixture showed no spot of *Rf* 0.33. $Ca(OH)_2$ (1.0 g, powder) was added to the mixture and it was stirred for 1 h. Filtration and evaporation of the solvent gave crude products (2.1 g), which were applied to a column of silica gel (2.5 cm i,d, × 28 cm). Elution with benzene–EtOAc (3 : 1) gave **4** (917 mg, 40% recovery), which was identified by ^{1}H-NMR. Further elution with benzene–EtOAc (2.5 : 1) gave **8** (671 mg, 28%), mp 185—187 °C (EtOAc), *Rf* 0.27. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.72 (3H, s, 19-H_3), 1.32 (3H, s, 21-H_3), 3.05 (1H, d, J = 8 Hz, 22-H), 3.20—3.80 (2H, m, 3-H and 23-H). *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.60; H, 10.50. Found: C, 75.35; H, 10.34. Further elution with benzene–EtOAc (2 : 1) provided **7** (498 mg, 21%), mp 209—211 °C (EtOAc), *Rf* 0.19. ^{1}H-NMR δ: 0.68 (3H, s, 18-H_3), 0.73 (3H, s, 19-H_3), 2.90 (1H, d, J = 7 Hz, 23-H), 3.20—3.80 (2H, m, 3-H and 22-H). *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.60; H, 10.50. Found: C, 75.42; H, 10.34.

(22*R*,23*R*,24(28)*E*)-3β,22,23-Trihydroxy-5α-stigmast-24(28)-en-6-one (9)——A mixture of **8** (410 mg, 0.891 mmol) and aluminum isopropoxide (410 mg, 2.01 mmol) in toluene (30 ml) was refluxed for 1 h. Work-up (CH_2Cl_2) gave crude products, which were applied to a column of silica gel (1.5 cm i.d. × 20 cm). Elution with $CHCl_3$–MeOH (15 : 1) recovered **8** (103 mg, 25%), which was identified by ^{1}H-NMR and TLC. Further elution with the same solvent gave **9** (92 mg, 22%), mp 228—230 °C (EtOAc). ^{1}H-NMR δ: 0.62 (3H, s, 18-H_3), 0.74 (3H, s, 19-H_3), 1.69 (3H, d, J = 7 Hz, 29-H_3), 2.74 (1H, m, 25-H), 3.55 (1H, m, 3-H), 3.63 (1H, d, J = 8 Hz, 22-H), 3.92 (1H, d, J = 8 Hz, 23-H), 5.46 (1H, q, J = 7Hz, 28-H). EI-MS (as the methaneboronate–TMS derivative)[9] *m/z* (20 eV): 556 (M^+, 14%), 541 (18), 527 (8), 513 (80), 466 (6), 167 (100). *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.60; H, 10.50. Found: C, 75.32; H, 10.44.

(22*R*,23*R*,24(28)*E*)-22,23-Isopropylidenedioxy-5α-stigmasta-2,24(28)-dien-6-one (11) and (22*R*,23*R*,24(28*E*)-3α-Hydroxy-22,23-isopropylidenedioxy-5α-stigmast-24(28)-en-6-one (12)——A solution of **9** (117 mg, 0.255 mmol) in acetone (10 ml) was treated with *p*-TsOH (5 mg) at room temperature for 1 h. Work-up (ether) gave a crude product, which was taken up in pyridine (2 ml) and reacted with MsCl (0.1 ml) at room temperature for 0.5 h.

Work-up (EtOAc) gave the mesylate **10** (147 mg). ^{1}H-NMR δ: 0.63 (3H, s, 18-H_3), 0.76 (3H, s, 19-H_3), 1.37 and 1.40 (6H, s×2, acetonide), 1.72 (3H, d, J=7 Hz, 29-H_3), 2.98 (3H, s, mesyl), 3.72 (1H, d, J=9 Hz, 22-H), 4.08 (1H, d, J=9 Hz, 23-H), 4.60 (1H, m, 3-H), 5.50 (1H, q, J=7 Hz, 28-H). A mixture of **10** (147 mg), dimethylformamide (3 ml), and lithium carbonate (100 mg) was refluxed for 1 h. Work-up (EtOAc) gave crude products, which were treated with 5% KOH/MeOH (5 ml) at room temperature for 0.5 h. Work-up (ether) and chromatography on silica gel (1.5 cm i.d. × 14 cm) with benzene–EtOAc (40 : 1) gave **12** (55 mg, 44%), mp 205—206 °C (MeOH). ^{1}H-NMR δ: 0.66 (3H, s, 18-H_3), 0.73 (3H, s, 19-H_3), 1.41 and 1.43 (6H, s×2, acetonide), 1.75 (3H, d, J=7 Hz, 29-H_3), 2.70 (1H, m, 25-H), 3.80 (1H, d, J=9 Hz, 22-H), 4.35 (1H, d, J=9 Hz, 23-H), 5.30—5.93 (3H, 2-H, 3-H, and 28-H). *Anal.* Calcd for $C_{32}H_{50}O_3$: C, 79.62; H, 10.44. Found: C, 79.55; H, 10.39. Further elution with benzene–EtOAc (20 : 1) gave **11** (46 mg, 35%), mp 189—190 °C (MeOH). ^{1}H-NMR δ: 0.65 (3H, s, 18-H_3), 0.73 (3H, s, 19-H_3), 1.41 and 1.43 (6H, s×2, acetonide), 1.75 (3H, d, J=7 Hz, 29-H_3), 2.50—2.95 (2H, m, 5α-H and 25-H), 3.80 (1H, d, J=9 Hz, 22-H), 4.35 (1H, d, J=9 Hz, 23-H), 4.37 (1H, m, $W_{1/2}$=8 Hz, 3β-H), 5.57 (1H, q, J=7 Hz, 28-H). *Anal.* Calcd for $C_{32}H_{52}O_4$: C, 76.75; H, 10.47. Found: C, 76.68; H, 10.53.

(22*R*,23*R*,24(28)*E*)-3α,22,23-Trihydroxy-5α-stigmast-24(28)-en-6-one (13)——A mixture of **12** (38 mg, 0.075 mmol) and 80% aqueous AcOH (4 ml) was heated at 60 °C for 1.5 h. Removal of the solvent under reduced pressure and chromatography on silica gel (1.5 cm i.d. × 15 cm) with EtOAc gave **13** (32 mg, 91%), mp 209—210 °C (EtOAc). ^{1}H-NMR δ: 0.63 (3H, s, 18-H_3), 0.73 (3H, s, 19-H_3), 1.72 (3H, d, J=7 Hz, 29-H_3), 2.40—3.00 (2H, m, 5α-H and 25-H), 3.67 (1H, d, J=8 Hz, 22-H), 3.96 (1H, d, J=8 Hz, 23-H), 4.15 (1H, m, $W_{1/2}$=8 Hz, 3β-H), 5.52 (1H, q, J=7 Hz, 28-H). EI-MS (as the methaneboronate–TMS derivative)[9] m/z(20 eV): 556 (M^+, 23%), 541 (19), 527 (2), 513 (100), 466 (4), 167 (96).

(22*R*,23*R*,24(28*E*)-2α,3α,22,23-Tetrahydroxy-5α-stigmast-24(28)-en-6-one (2)——A solution of **11** (47 mg, 0.097 mmol) in *tert*-BuOH–THF–H_2O (10 : 3 : 1, 10 ml) was treated with a catalytic amount of OsO_4 and *N*-methylmorpholine *N*-oxide (33 mg, 0.24 mmol) at room temperature for 1 h, then sat. $NaHSO_3$ solution (5 ml) was added and the mixture was kept at room temperature for 1 h. Work-up (CH_2Cl_2) gave a crude product, which was further treated with 80% aqueous AcOH (10 ml) at 60 °C for 2 h. Removal of the solvent and chromatography on silica gel (1 cm i.d. × 24 cm) with EtOAc–MeOH (20 : 1) provided homodolichosterone (**2**) (32 mg, 70%), mp 217—219 °C, sinter at 208 °C (MeOH) (lit.,[5] mp 218—219 °C, sinter at 208 °C). ^{1}H-NMR (200 MHz) and EI-MS (as the bismethaneboronate)[9] of the synthetic **2** were in good agreement with the reported data.[4,5]

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Blockwise Mechanical Synthesis of Oligonucleotides by the Phosphoramidite Method[1)]

KAZUNOBU MIURA, KAZUYUKI SAWADAISHI, HIDEO INOUE and EIKO OHTSUKA*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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For use in automated synthesis of oligodeoxyribonucleotides, a dimer containing the triesterified internucleotide linkage and the 3′-phosphoramidite has been prepared. 5′-*O*-Dimethoxytritylthymidylyl-(3′-5′)-(*o*-chlorophenyl)-thymidine was converted to the 3′-(methyl)*N*,*N*-diisopropylphosphoramidite, which has been used as a condensing unit for the synthesis of pentadecathymidylate on controlled pore glass by a deoxyribonucleic acid (DNA) synthesizer.

Keywords——solid-phase synthesis; *o*-chlorophenyl phosphotriester; 3′-terminal phosphite; mixed dinucleotide unit; DNA synthesizer

Solid-phase synthesis of polydeoxyribonucleotides has made it feasible to obtain a large number of gene fragments, as described in review articles.[2a,b)] The solid-phase phosphotriester synthesis using dinucleotide units has been applied to oligomers with 10—20 nucleotides,[2c,d)] and the use of the phosphoramidite synthesis on a machine can yield oligodeoxyribonucleotides with at least 20 nucleotides.[3)] Products obtained by the blockwise phosphotriester synthesis usually contain less by-products than those obtained by machine synthesis. Although high pressure liquid chromatography (HPLC) can be used to purify oligonucleotides, it is desirable to isolate oligonucleotides in quantity without extensive chromatography. In this paper, we describe a machine synthesis of pentadecathymidylate by condensation of dimers containing an internucleotidic triester using the phosphoramidite method to test the feasibility of automatic blockwise oligonucleotide syntheses. The purity of products is considered to be increased as a result of using fewer condensation steps, and this should be especially advantageous in large scale syntheses of oligonucleotides.

Preparation of a Dimer Unit

As a dimer unit, 5′-*O*-dimethoxytritylthymidylyl-(3′-5′)-(*o*-chlorophenyl)thymidine 3′-(methyl)*N*,*N*-diisopropylphosphoramidite (**5**) was prepared. For this preparation a 3′-unprotected dimer containing a triesterified internucleotide phosphate (**3**) was required. Compound **3** was synthesized by condensation of 5′-dimethoxytritylthymidine 3′-(*o*-chlorophenyl)phosphate (**1**)[4)] with an excess of thymidine (**2**) using 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT)[5)] as the condensing reagent (Chart 1). Although some papers have reported the absence of symmetrical 3′-3′-dinucleoside phosphates in the reaction of 3′,5′-unprotected nucleosides,[6)] formation of a few percent of 3′-3′-dinucleoside phosphates has been described.[7)] In the present experiment the desired product (**3**) was separated from the 3′-3′ by-product by chromatography on a column of silica gel in a yield of 71%.

The 3′-unprotected dimer (**3**) was converted to the phosphoramidite (**5**) by treatment with (methyl)*N*,*N*-isopropylchlorophosphoramidite (**4**) at room temperature for 30 min, and the phosphoramidite (**5**) was purified by silica gel chromatography in a yield of 85%.

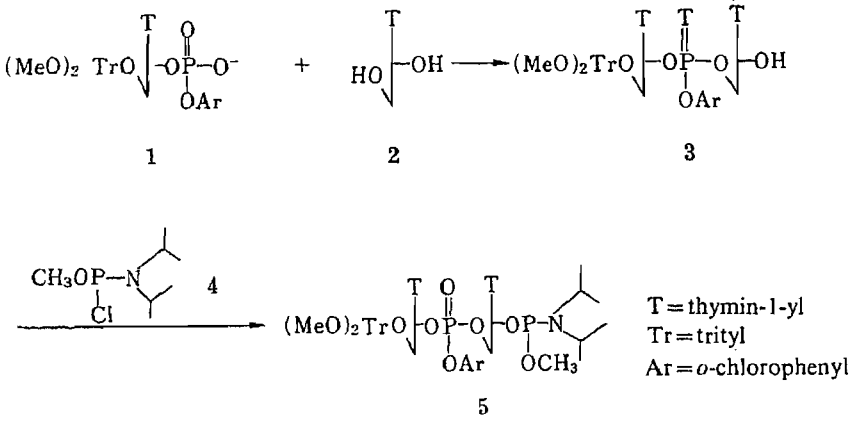

Chart 1

TABLE I. DNA Synthetic Cycle and Cleavage

Step manipulation	Solvent or reagent	Time[a]
(1) Synthetic cycle		
1 Wash resin (×1)	Acetonitrile	39 s
2 Detritylation	Trichloroacetic acid	50 s
3 Wash resin (×2)	Acetonitrile	232 s
4 Condensation	Amidite+tetrazole	60 s
5 Filtration		20 s
6 Capping	Acetic anhydride+dimethyl-aminopyridine	140 s
7 Filtration		26 s
8 Oxidation	Iodine–water	53 s
9 Wash resin (×3)	Acetonitrile	165 s (13 min/cycle)
(2) Cleavage		
1 Demethylation	Thiophenol	600 s×3+43 s
2 Wash resin (×3)	Methanol, acetonitrile	157 s
3 Cleavage and collection	Conc. aqueous ammonia	900 s×8+74 s
4 Wash resin (×1)	Acetonitrile	50 s

a) At room temperature (*ca.* 25 °C).

Condensation of the Dimer on a Support by a Deoxyribonucleic Acid (DNA) Synthesizer

The phosphoramidite dimer (**5**) was used to synthesize pentadecathymidylic acid. The condensation to the 3′-succinylthymidine on controlled-pore glass was performed at room temperature for 50 s using a DNA synthesizer and repeated 6 times. Manipulations for a cycle of chain elongation and the cleavage procedure of the synthesized oligonucleotide from the polymer support are summarized in Table I. The overall yield was estimated as 95.7% by means of the dimethoxytrityl color reaction.[8] The product cleaved from the polymer support was treated with ammonia to remove *o*-chlorophenyl protecting groups on phosphate. Then, the dimethoxytritylated pentadecamer was isolated by reversed phase chromatography and the completely deblocked product was analyzed by reversed phase HPLC (Fig. 1a). Impurities were removed by fractionation by the same chromatography, and the product was analyzed by reversed phase and anion-exchange HPLC (Fig. 1b). Purity and the chain length were confirmed by mobility shift analysis.[9]

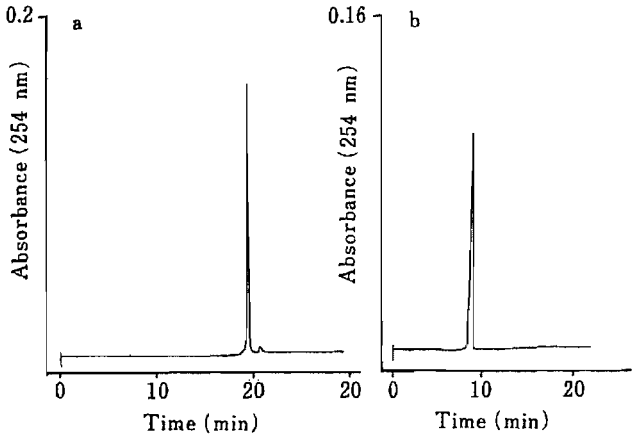

Fig. 1. a) Reversed-Phase HPLC of Pentadecathymidylate

Column: 10×300 mm. Solvent: A, 5% CH_3CN–0.1 M triethylammonium acetate; B, 25% CH_3CN–0.1 M triethylammonium acetate. Gradient: (B/A), 30—60%, 1.5%/min. Flow rate: 2 ml/min.

b) Anion-Exchange HPLC of Pentadecathymidylate

Column: 4.6×250 mm. Solvent: A, 20% CH_3CN; B, 1 M $HCOONH_4$–20% CH_3CN. Gradient: (B/A), 30—50%, 1%/min. Flow rate: 1 ml/min.

Conclusion

The dimer unit for a machine synthesis of pentadecathymidylate has been prepared by using unprotected thymidine. *o*-Chlorophenyl and methyl groups were incorporated alternatively during the synthesis as protecting group for internucleotide phosphates. The condensation yield of this relatively bulky dimer was comparable to those reported in condensations involving 3′-phosphitylated dimers,[10] despite the presence of the bulky phenyl group. The yield of the isolated product after reversed phase HPLC was about 30%. In the present particular case, anion-exchange chromatography was not required. It seems likely that a combination of a conventional dimer synthesis using 3′,5′-unprotected nucleosides and the phosphoramidite condensation of preformed protected oligonucleotides has a practical value.

Experimental

General Method——Preparation of protected nucleosides and the 3′-phosphodiester were described previously.[11] Characterization of nucleotides by thin layer chromatography (TLC) and HPLC was performed as described. Preparative silica gel chromatography was performed by using Wakogel C-300 (Wako Pure Chemical Industries) in chloroform–methanol. The phosphitylating reagent was obtained from American BioNuclear Inc. A mechanical synthesis of oligonucleotides was performed by using an Applied BioSystems synthesizer, model 380A. Mobility shift analysis was performed after partial digestion of the 5′-phosphorylated oligonucleotide with venom phosphodiesterase.[9b] HPLC was performed by using columns of C-18 silica gel (M & S Pack, M & S Co.) and diethylaminoethyl (DEAE)-silica gel (TSK Gel DEAE-2SW, Toyo Soda Co.).

Preparation of the Dimer Unit (5)——5′-*O*-Dimethoxytritylthymidine 3′-(*o*-chlorophenyl)phosphate (1, 2 mmol) and thymidine (2, 10 mmol) were dried by evaporation of pyridine twice and dissolved in pyridine (4.8 ml). The solution was cooled in an ice bath, and MSNT (4 mmol) was added. The mixture was kept at room temperature for 1 h and treated with aqueous pyridine (30%). The product was extracted with chloroform, washed with water and applied to a column of silica gel. The 3′-5′ linked dimer (3) was eluted with 1.5% methanol in chloroform and analyzed by TLC (*Rf* 0.7 in 10 : 1 chloroform–methanol). The 3′-3′ linked by-product, which had a higher *Rf* value in TLC, was separated. The product (3) was precipitated with ether–hexane (1 : 1). The yield was 71% (1.36 g).

The dimer (3) (0.48 g, 0.5 mmol) was dried with benzene azeotropically and dissolved in dichloromethane (5 ml). *N*,*N*-diisopropylethylamine (0.35 ml, 2.0 mmol) was added to the mixture and the phosphitylating reagent (4) (0.196 ml, 1 mmol) was added through a septum by using a syringe. The reaction mixture was kept at room temperature for 30 min. The solvent was evaporated off, and the residue was dissolved in ethyl acetate. The solution was washed twice with sat. sodium bicarbonate and then with water. The product was isolated by silica gel column chromatography using a mixture of dichloromethane–ethyl acetate–triethylamine (1 : 1 : 0.05) and analyzed by TLC in ethyl acetate–triethylamine (1 : 0.05). The yield was 85% (0.475 g).

Synthesis of Pentadecathymidylic Acid——Dimethoxytritylthymidine (1 μmol) linked to controlled pore glass through a succinyl group (Applied BioSystems) in the machine was used as the support. The dimer (5) (0.25 g) was dissolved in acetonitrile (2 ml) and the solution was passed to the synthesizer. The condensation was repeated seven times and subsequently, the oligonucleotide was partially deblocked with thiophenol and cleaved from the support as

shown in Table I. Then, the partially deprotected pentadecathymidylate was treated with aq. ammonia (2 ml) at 50°C for 5 h. The dimethoxytritylated oligonucleotide was separated by reversed phase chromatography on a column of C-18 Silica gel using a gradient of acetonitrile (5—35%) in 0.01 M triethylammonium bicarbonate. The product was completely deblocked by treatment with 80% acetic acid and analyzed by reversed phase HPLC. The product was fractionated by similar chromatography (39.3 A_{260} units). The pentadecathymidylate thus obtained was homogeneous in reversed phase and ion-exchange chromatography.

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Preparation of a Tricyclic A-Ring Analog of Quassin

YOHEI KURATA, HIROSHI HIROTA,* TADASHI HONDA, and TAKEYOSHI TAKAHASHI*

Department of Chemistry, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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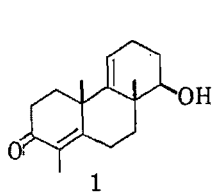
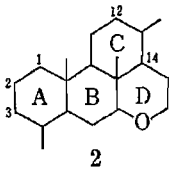
(±)-3-Methoxy-1α,4aβ,8aβ-trimethyl-1,4,4a,6,7,8,8a,9,10,10aα-decahydrophenanthrene-4,8-dione, a tricyclic A-ring analog of quassin, was synthesized from a known tricyclic ketone through seven reaction steps, including phenylselenation in a neutral medium and the Wharton reaction.

Keywords——synthesis; quassinoid; Wharton reaction; 1,3-carbonyl transposition; phenylselenation

In the course of our synthetic studies on quassinoids, an easily available tricyclic hydroxy ketone (**1**) has been used as a key intermediate.[1,2] The hydroxyl group of **1** was utilized to construct the D-ring of the picrasane skeleton (**2**) and to functionalize the C- and D-rings. On the other hand, the carbonyl group at the C-3[3] position is considered to provide a foothold for A-ring functionalization. We have already obtained 12β-hydroxypicrasan-3-one (**3**) on the synthetic route to quassin (**4**) from **1**.[2] The next step of this synthetic study would be a transposition of the carbonyl group from the C-3 to the C-1 position. A number of procedures for 1,3-carbonyl transposition have already been developed.[4] 17β-Hydroxy-5α-androst-1-en-3-one was converted into 2-methoxy-4α-methyl-5α-androst-2-ene-1,17-dione[5] by successive reactions including the Wharton reaction.[6] This paper deals with a method for preparation of a tricyclic A-ring analog (**5**) of quassin from the hydroxy ketone (**1**).



1



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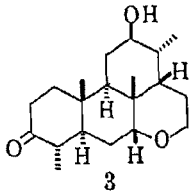

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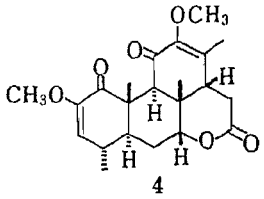

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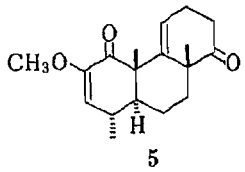
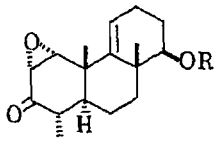


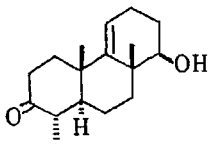
5

A transformation of **1** into the 1α,2α-epoxy ketone (**6**)[3] was initially attempted by the use of successive reactions: namely, i) protection of the hydroxyl group at C-14 with methoxymethyl ether, ii) the Birch reduction, iii) phenylselenation of the C-2 position with lithium diisopropylamide/benzeneselenenyl chloride, and then iv) treatment with alkaline hydrogen peroxide.[7] However, the yield of the phenylselenation reaction in the third step was found to be poor (the best yield: 43%) and more than half of the starting material was recovered even when hexamethylphosphoric triamide was added to the reaction solution. Sharpless *et al.* reported that cholestan-3-one was transformed into cholest-1-en-3-one by utilizing a substitution reaction of the phenylselenenyl group followed by oxidation–elimination reaction in a neutral medium.[8] This substitution reaction of the phenylselenenyl group under neutral conditions was now examined. The hydroxy ketone (**7**), obtained from **1** by Birch reduction (88% yield), was stirred with 1.2 mol equivalent of benzeneselenenyl chloride in ethyl acetate,

followed by addition of pyridine and *m*-chloroperbenzoic acid to afford the α,β-unsaturated ketone (**8**) in 80% yield. When the 14-*O*-methoxymethyl analog of **7**[3] was subjected to the same reaction, the corresponding α,β-unsaturated ketone was obtained in *ca.* 27% yield. Epoxidation of the α,β-unsaturated ketone (**8**) was carried out with alkaline hydrogen peroxide to give the epoxide (**9**) in 83% yield. Treatment of **9** with hydazine hydrate afforded the allylic alcohol (**10**) by the Wharton reaction. The Collins oxidation of **10** gave a diketone (**11**)



6 : R=CH_2OCH_3
9 : R=H



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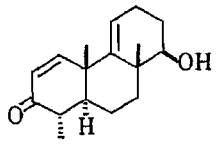

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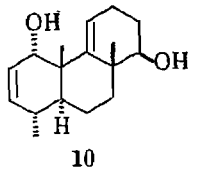

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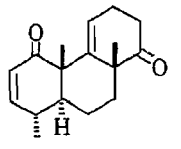
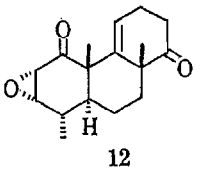


11



12

in 38% yield from **9**. Epoxidation of **11** was carried out under the same conditions as used for **8** to give the epoxide (**12**) in 73% yield. A mixture of **12** and sodium methoxide in methanol was refluxed under a nitrogen atmosphere to afford the title compound (**5**) in 50% yield. Overall yield of **5** from **1** was 8.1%.

Experimental

General Procedures——All melting points were determined on a Mel-temp capillary melting point apparatus (Laboratory Devices) and are uncorrected. Ultraviolet absorption (UV) and infrared (IR) spectra were measured on Hitachi 340 and Hitachi 260-30 spectrometers, respectively. Mass spectra (MS) were run on a JEOL JMS-D300 mass spectrometer operating at 70 eV. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken using a Varian EM390 (90 MHz) spectrometer. Chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane as an internal standard and coupling constants in Hz. Thin-layer chromatography (TLC; including preparative) was carried out on Kieselgel 60 GF_{254} (0.25 mm thickness). Wakogel C-200 (Wako) and Florisil (100—200 mesh) were used for column chromatography. High-performance liquid chromatography (HPLC) was carried out with an NP-DX-8 pump (Nihon Seimitsu Kagaku Co.) and an ERC-7520 type RI detector (Erma Optical Works) using a YMC-Pack A-012 SIL column. All the samples taken for high resolution MS were pure on TLC and/or HPLC examination.

(±)-8β-Hydroxy-1α,4aβ,8aβ-trimethyl-1,2,3,4,4a,6,7,8,8a,9,10,10aα-dodecahydrophenanthren-2-one (7)——At −78 °C, dry liquid ammonia (100 ml) was added to lithium (400 mg) under a nitrogen atmosphere, and the whole was stirred for 30 min to form a solution. A solution of α,β-unsaturated ketone (**1**; 3.96 g) in tetrahydrofuran (50 ml) was added and the mixture was refluxed for about 1 h. After addition of ammonium chloride, ammonia was evaporated off, and the reaction mixture was extracted with chloroform as usual to afford a ketone (**7**; 3.52 g; 88% yield): white crystals, mp 152.5—154.5 °C (recrystallized from chloroform–hexane). IR (KBr) 3520, 1700 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.00 (3H, d, J=7 Hz), 1.18 (3H, s), 1.31 (3H, s), 3.41 (1H, dd, J=9, 8 Hz), 5.38 (1H, t, J=4 Hz). MS *m/z* (rel. intensity) 262 (M^+, 8), 247 (7), 244 (70), 218 (100), 203 (67). High-resolution MS. Found: *m/z* 262.1945. Calcd for $C_{17}H_{26}O_2$: M, 262.1933. *Anal.* Found: C, 77.47; H, 10.04. Calcd for $C_{17}H_{26}O_2$: C, 77.82; H, 9.99.

(±)-8β-Hydroxy-1α,4aβ,8aβ-trimethyl-1,2,4a,6,7,8,8a,9,10,10aα-decahydrophenanthren-2-one (8)——Benzeneselenenyl chloride (2.94 g, 1.2 eq) was added to a solution of the keto alcohol (**7**; 3.36 g) in dry ethyl acetate (200 ml) at 0 °C, and the mixture was stirred at room temperature for 2 h. When the color of the solution changed from brown into yellow, a saturated aqueous solution of sodium hydrogen carbonate was added to bring the solution to pH>7, and most of the aqueous layer was removed. Pyridine (2.6 ml) and *m*-chloroperbenzoic acid (6.9 g; 2.5 eq) were added to the organic layer. After being stirred for 30 min at 40—50 °C, the reaction mixture was washed with a saturated aqueous solution of sodium hydrogen carbonate and saturated brine successively, and dried ($MgSO_4$). After removal of the solvent, the resulting yellow oily product was crystallized from hexane–ether to give a white

crystalline compound (**8**; 2.67 g; 80% yield). **8**: white needles, mp 151—153 °C (recrystallized from chloroform–hexane). UV (EtOH) 235 nm (ε 11000). IR (KBr) 3540, 1675 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.14 (3H, d, J=7 Hz), 1.20 (3H, s), 1.32 (3H, s), 3.42 (1H, dd, J=8, 7 Hz), 5.57 (1H, t, J=4 Hz), 5.81 (1H, d, J=10 Hz), 7.29 (1H, d, J=10 Hz). MS m/z (rel. intensity) 260 (M^+, 66), 242 (75), 216 (40), 201 (100). High resolution MS. Found: m/z 260.1769. Calcd for $C_{17}H_{24}O_2$: M, 260.1774. *Anal.* Found: C, 75.54; H, 9.16. Calcd for $C_{17}H_{24}O_2 \cdot 1/2H_2O$: C, 75.80; H, 9.35.

(±)-3α,4α-Epoxy-8β-hydroxy-1α,4aβ,8aβ-trimethyl-1,2,3,4,4a,6,7,8,8a,9,10,10aα-dodecahydrophenanthren-2-one (9)——A 30% aqueous solution (23 ml) of hydrogen peroxide in methanol (50 ml) was added dropwise at 0 °C to a mixture of the α,β-unsaturated ketone (**8**, 2.67 g) in tetrahydrofuran (200 ml) and 2 M aqueous sodium hydroxide (30 ml), and the mixture was stirred at room temperature for 3 h. After addition of a saturated aqueous solution of sodium hydrogen sulfite at 0 °C and then a 5% aqueous solution of sodium hydroxide, the organic solvents were evaporated off and the residue was extracted with ether. The ethereal extract was treated as usual to afford the epoxy ketone (**9**; 2.33 g; 83% yield): white crystals, mp 132—134 °C (recrystallized from chloroform). IR (KBr) 3450, 1705, 1100, 1075, 1010, 990, 920, 900 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.15 (3H, d, J=6 Hz), 1.17 (6H, s), 3.32 (1H, d, J=4 Hz), 3.48 (1H, t, J=7.5 Hz), 3.77 (1H, d, J=4 Hz), 5.78 (1H, t, J=3 Hz). MS m/z (rel. intensity) 276 (M^+, 10), 258 (45), 232 (100). High resolution MS. Found: m/z 276.1768. Calcd for $C_{17}H_{24}O_3$: M, 276.1726. *Anal.* Found: C, 71.74; H, 8.59. Calcd for $C_{17}H_{24}O_3 \cdot 1/2H_2O$: C, 71.55; H, 8.83.

(±)-1α,4aβ,8aβ-Trimethyl-1,4,4a,6,7,8,8a,9,10,10aα-decahydrophenanthrene-4α,8β-diol (10)——Hydrazine hydrate (6 ml) was added to the epoxy ketone (**9**; 493 mg), and the mixture was heated for 1 h at 150—160 °C (bath temperature) under a nitrogen atmosphere. After the addition of saturated brine at 0 °C, chloroform extraction was carried out as usual to give the allylic alcohol as a crude oil (**10**; 474 mg). The crude oily compound (**10**) was used for the next oxidation reaction without purification. On the other hand, to obtain pure **10**, a part of this crude oil (36 mg) was charged on top of a silica gel (2 g) column. Elution with hexane–ethyl acetate (1 : 1) gave 12 mg of the pure allylic alcohol (**10**): pale yellow crystals, mp 150—151.5 °C (recrystallized from chloroform–hexane). IR (KBr) 3380, 1640 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.05 (3H, d, J=7 Hz), 1.06 (3H, s), 1.14 (3H, s), 3.50 (1H, t, J=8 Hz), 4.03 (1H, d, J=4.5 Hz), 5.47 (1H, t, J=4 Hz), 5.73 (2H, m). MS m/z (rel. intensity) 262 (M^+, 19), 244 (49), 229 (13), 226 (14), 211 (21), 134 (100). High resolution MS. Found: m/z 262.1961. Calcd for $C_{17}H_{26}O_2$: M, 262.1933. *Anal.* Found: C, 77.58; H, 9.98. Calcd for $C_{17}H_{26}O_2$: C, 77.82; H, 9.99.

(±)-1α,4aβ,8aβ-Trimethyl-1,4,4a,6,7,8,8a,9,10,10aα-decahydrophenanthrene-4,8-dione (11)——A mixture of dry dichloromethane (130 ml), dry pyridine (15 ml), and anhydrous chromium (VI) oxide (8.7 g) was stirred at room temperature for 30 min. A solution of the crude allylic alcohol (**10**; 1.70 g) in dichloromethane (40 ml) was added, and the whole was stirred at room temperature for 4 h. After addition of sodium hydrogen sulfate (32 g), the mixture was filtered and then passed through a Florisil column (ether elution) to give 763 mg of a yellow oily substance, which was subjected to silica gel (15 g) column chromatography. Elution with hexane–ethyl acetate (3 : 1) gave 624 mg (38% yield from **9**) of the conjugated ketone (**11**): a pale yellow oil. IR (neat) 1710, 1670, 1635 cm^{-1}. ^{1}H-NMR (CCl_4) δ=1.15 (3H, d, J=7 Hz), 1.31 (3H, s), 1.33 (3H, s), 5.72 (1H, dd, J=11, 3 Hz), 6.45 (1H, dd, J=11, 2 Hz), 6.63 (1H, t, J=3 Hz). MS m/z (rel. intensity) 258 (M^+, 20), 230 (95), 215 (100), 134 (90). Found: m/z 258.1633. Calcd for $C_{17}H_{22}O_2$: M, 258.1620.

(±)-2α,3α,-Epoxy-1α,4aβ,8aβ-trimethyl-1,2,3,4,4a,6,7,8,8a,9,10,10aα-dodecahydrophenanthrene-4,8-dione (12)——A 2 M aqueous solution (5.4 ml) of sodium hydroxide and a mixture of 30% aqueous hydrogen peroxide (4 ml) and methanol (8.8 ml) were added to a solution of the conjugated ketone (**11**; 482 mg) in tetrahydrofuran (34 ml) at 0 °C. The mixture was stirred at room temperature for 3.5 h, then an aqueous solution of sodium hydrogen sulfite (4 g) and a saturated aqueous solution of sodium hydrogen carbonate were added. After removal of the organic solvents *in vacuo*, the residue was extracted with ether. The ethereal extract was treated as usual to give a crystalline compound (**12**; 375 mg; 73% yield): white crystals, mp 98—99 °C (recrystallized from chloroform–methanol). IR (KBr) 3000, 1700, 1000, 845 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.25 (3H, d, J=6 Hz), 1.27 (3H, s), 1.35 (3H, s), 3.34 (2H, br s), 6.01 (1H, t, J=3 Hz). MS m/z (rel. intensity) 274 (M^+, 95), 259 (18), 246 (48), 232 (100). High resolution MS. Found: m/z 274.1570. Calcd for $C_{17}H_{22}O_3$: M, 274.1573. *Anal.* Found: C, 74.41; H, 7.97. Calcd for $C_{17}H_{22}O_3$: C, 74.42; H, 8.08.

(±)-3-Methoxy-1α,4aβ,8aβ-trimethyl-1,4,4a,6,7,8,8a,9,10,10aα-decahydrophenanthrene-4,8-dione (5)——Sodium (30 mg) was dissolved in 0.5 ml of methanol under a nitrogen atmosphere. A solution of the epoxy ketone (**12**; 22 mg) in methanol (2.5 ml) was added, and the mixture was refluxed for 18 h. After addition of 2 M hydrochloric acid for neutralization, methanol was removed *in vacuo* and the residue was extracted with ether. The ethereal extract was treated as usual to afford, after purification by preparative TLC [hexane–ethyl acetate (4 : 1)], 11.5 mg (50% yield) of **5**: a colorless oil. UV (EtOH) 263 nm (ε 5100). IR (neat) 1700, 1680, 1630 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ=1.15 (3H, d, J=7 Hz), 1.36 (3H, s), 1.42 (3H, s), 3.60 (3H, s), 5.40 (1H, d, J=2 Hz), 6.55 (1H, dd, J=7, 3 Hz). MS m/z (rel. intensity) 288 (M^+, 18), 260 (100), 245 (70). Found: m/z 288.1739. Calcd for $C_{18}H_{24}O_3$: M, 288.1726.

References and Notes

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Two New Triterpenoid Glycosides from Leaves of *Ilex chinensis* SIMS[1)]

AKIRA INADA, MARI KOBAYASHI, HIROKO MURATA, and TSUTOMU NAKANISHI*

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-01, Japan

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Two new triterpenoid glycosides were isolated (one of them as the methyl ester) as major components from leaves of *Ilex chinensis* SIMS (Aquifoliaceae). The structures of these glycosides, termed ilexoside A (**1**) and ilexoside B methyl ester (**2a**), have been established to be 3β-*O*-(β-D-xylopyranosyl)siaresinolic acid and 3β-*O*-(β-D-xylopyranosyl)pomolic acid methyl ester, respectively, based on chemical and spectral evidence. High-resolution ^{13}C-nuclear magnetic resonance data for the methyl ester of **1** (**1a**) and **2a** are also reported.

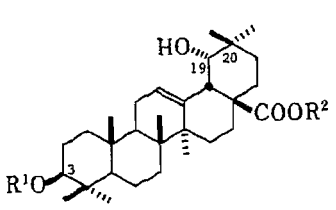
Keywords——*Ilex chinensis*; Aquifoliaceae; leaf; triterpenoid glycoside; siaresinolic acid xyloside; pomolic acid xyloside

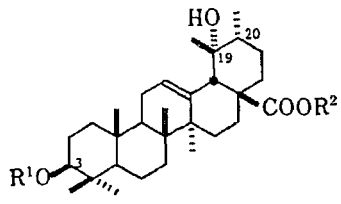
A Chinese crude drug (Dong-Qing-Ye), leaves of *Ilex chinensis* SIMS (Nanaminoki in Japanese)[2)] (Aquifoliaceae) has been used in China as a remedy (when given internally) for bronchitis, pneumonia, and ulceration, and as an external treatment for scald, chilblain, *etc.*[3)] As a part of our continuing phytochemical research on plants in the genus Ilex,[4)] we examined leaves of *I. chinensis* and isolated two new and major glycosides (one of them as the methyl ester). We have named them ilexoside A and ilexoside B methyl ester, and established their structures as **1** and **2a**.

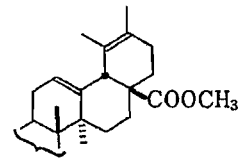
The glycoside mixture (fraction No. 7; see Experimental) isolated from the methanol extract was recrystallized from methanol to afford ilexoside A (**1**), $C_{35}H_{56}O_8$, mp 264—267 °C, $[\alpha]_D$ +7.8° (MeOH) as colorless crystals. The mother liquor of the recrystallization was methylated with diazomethane in a usual manner and the resulting methyl ester mixture was subjected to high-performance liquid chromatography (HPLC) to afford ilexoside A methyl ester (**1a**),[5)] $C_{36}H_{58}O_8$, mp 229—232 °C, $[\alpha]_D$ +16.7° (MeOH) and ilexoside B methyl ester (**2a**), $C_{36}H_{58}O_8$, mp 155—158 °C, $[\alpha]_D$ +15.9° (MeOH), respectively.

Field desorption mass spectroscopic (FD-MS) experiments suggested that both methyl esters (**1a** and **2a**) possess the same molecular weight [(M^+ +H), *m/z* 619]. Furthermore, on electron impact mass spectroscopy (EI-MS), both methyl esters gave two common significant and intense fragment ions at *m/z* 278 and 201 (278 − COOMe − H_2O), both due to the typical retro-Diels–Alder type cleavage[6)] of the olean- or urs-12-en-28-oic acid methyl ester framework with a hydroxy group on ring D or E. In addition, both methyl esters also afforded another common significant fragment peak at *m/z* 486, corresponding to M^+ − pentose unit (132). These lines of evidence provided useful information concerning the aglycone and the glycone structures of both **1a** and **2a**.

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1a** showed signals due to seven tertiary methyls, two methines bearing an oxygen function (δ 3.36 and 3.57), and an anomeric proton (δ 4.87, d, J=7.5 Hz) (Table I). That of **2a** showed the presence of six tertiary methyls, a secondary methyl (δ 1.10, J=6.7 Hz), a methine bearing an oxygen function (δ 3.37), and an anomeric proton (δ 4.86, d, J=7.6 Hz) (Table I). These ^{1}H-NMR data suggest







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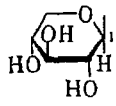
1: R^1 =  , R^2=H

(β-D-xylopyranosyl)

1a: R^1=β-D-xylopyranosyl, R^2=CH_3

3: R^1=H, R^2=CH_3

2: R^1=β-D-xylopyranosyl, R^2=H

2a: R^1=β-D-xylopyranosyl, R^2=CH_3

4: R^1=H, R^2=CH_3

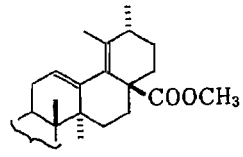


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Chart 1

TABLE I. ^{1}H-NMR Spectral Data for **1a** and **2a** (400 MHz, in pyridine-d_5, δ)[a]

	1a	**2a**
Aglycone region protons		
3α-H	3.36 (dd, 11.5, 4.5)	3.37 (dd, 11.3, 4.3)
12-H	5.50 (m)	5.50 (m)
18β-H	3.45 (br s)	2.86 (s)
19α-OH	6.11 (d, 6.3)	5.25 (s)
19β-H	3.57 (t-like)[b]	
$COOCH_3$	3.74 (s)	3.74 (s)
20α-CH_3		1.10 (d, 6.7)
CH_3	0.88, 0.92, 1.02, 1.05, 1.18, 1.33, 1.63 (s×7)	0.88, 0.93, 1.03, 1.34, 1.40, 1.71 (s×6)
Xylose protons		
1′-H	4.87 (d, 7.5)	4.86 (d, 7.6)
2′-H	4.05 (dd, 8.7, 7.5)	4.05 (dd, 8.5, 7.6)
3′-H	4.19 (t, 8.7)	4.20 (t, 8.5)
4′-H	4.27 (ddd, 10.2, 8.7, 5.1)	4.26 (ddd, 10.4, 8.5, 4.9)
5′α-H	3.82 (dd, 11.4, 10.2)	3.81 (dd, 11.0, 10.4)
5′β-H	4.42 (dd, 11.4, 5.1)	4.41 (dd, 11.0, 4.9)

a) Multiplicity and coupling constant (Hz) in parentheses. b) This signal changed to a doublet (J= 4.0 Hz) on deuteration.

that **1a** and **2a** are triterpene mono-glycosides. Furthermore, olean- and urs-12-en-28-oic acid methyl ester frameworks can be assigned, as the aglycone structures of **1a** and **2a**, respectively.

Acidic hydrolysis of **1a** with 4N H_2SO_4–MeOH (1:5) gave 1 mol each of methyl siaresinolate (**3**)[7] and xylose. Analogous acidic hydrolysis of **2a** afforded an artifact aglycone, *i.e.*, a dienic mixture of methyl tomentosolate (**5**) and vanguerolate (**6**).[8,9] However, on enzymic hydrolysis with protease [type XIII from *Aspergillus saitoi* (Molsin)],[10] **2a** gave methyl pomolate (**4**)[4,11] as the genuine aglycone.

On methanolysis, both **1a** and **2a** afforded methyl xyloside. This suggests that the common glycone unit of **1a** and **2a** is xylopyranose. Furthermore, the difference in molecular rotation between **1a** and **3** ($\Delta[M]_D$ −58.63°) and that between **2a** and **4** ($\Delta[M]_D$ −93.22°) indicated that both xylose moieties in **1a** and **2a** are of D-configurations.[12]

The final structures for **1a** and **2a** were established as follows. In a carbon-13 nuclear magnetic resonance (^{13}C-NMR) study (Table II), the C-3 signals (δ 88.70 and 88.75 ppm,

TABLE II. ^{13}C-NMR Spectral Data for **1a**, **2a**, **3**, and **4** (100.5 MHz, in pyridine-d_5, δ_c)

Carbon No.	1a	3	2a	4
Aglycone				
C-1	38.64	38.81	38.83	39.43
C-2	26.72	28.09	26.72	28.16
C-3	88.70	78.16	88.75	78.24
C-4	39.61	39.41	39.61	39.06
C-5	56.00	55.96	55.93	55.89
C-6	18.65	18.98	18.64	18.97
C-7	33.19[a]	33.27	33.38	33.49
C-8	39.94	39.98	40.27	40.31
C-9	48.21	48.30	47.62	47.71
C-10	37.18	37.55	37.02	37.38
C-11	24.10	24.12	23.96	24.01
C-12	123.76	123.81	128.31	128.36
C-13	144.26	144.29	139.45	139.46
C-14	41.97	41.97	41.93	41.97
C-15	28.95	28.95	29.04	29.06
C-16	28.09	28.09	26.04	26.08
C-17	46.40	46.42	48.61	48.63
C-18	44.66	44.66	54.44	54.48
C-19[b]	80.98 (d)	81.03 (d)	72.59 (s)	72.60 (s)
C-20[b]	35.61 (s)	35.61 (s)	42.21 (d)	42.23 (d)
C-21	28.95	28.95	26.79	26.74
C-22	33.27[a]	33.27	38.15	38.15
C-23	28.20	28.75[c]	28.24	28.82
C-24	16.88	16.48	16.97[d]	16.55[d]
C-25	15.45	15.51	15.56	15.64
C-26	17.28	17.34	17.03[d]	17.06[d]
C-27	24.67	24.69	24.67	24.67
C-28	178.71	178.73	178.47	178.47
C-29	28.77	28.79[c]	26.99	26.98
C-30	24.84	24.87	16.68[d]	16.68[d]
$COO\underline{C}H_3$	51.68	51.68	51.54	51.55
Xylose				
C-1′	107.70		107.72	
C-2′	75.53		75.57	
C-3′	78.58		78.64	
C-4′	71.23		71.27	
C-5′	67.12		67.14	

a, c, d) Assignments may be interchanged in each column. *b*) Signal multiplicities of C-19 and C-20 in compounds **1a**, **2a**, **3**, and **4** were determined in the off-resonance mode and are listed in parentheses.

respectively) of the glycosides **1a** and **2a** appeared at lower field than those (δ 78.16 and 78.24 ppm, respectively) of the corresponding aglycones **3** and **4**, indicating that in both **1a** and **2a**, xylopyranose was linked to the 3-OH group of the respective aglycones (**3** and **4**) by an ether bond *via* the anomeric hydroxyl group of xylose. The chemical shift values of the anomeric carbons (Table II) of **1a** and **2a** together with the large coupling constants of the anomeric protons of **1a** and **2a** (*vide supra* and Table I) showed that the anomeric configurations in **1a** and **2a** are both β.

Based on the combined evidence, the structures of **1a** and ilexoside B methyl ester (**2a**) are now defined as 3β-*O*-(β-D-xylopyranosyl)siaresinolic acid methyl ester and 3β-*O*-(β-D-xylopyranosyl)pomolic acid methyl ester, respectively. The ^{13}C-NMR assignments for **1a** and **2a** (Table II) are in agreement with these established structures (**1a** and **2a**). Needless to say,

ilexoside A (1) corresponds to the natural carboxylic acid form of **1a** . Ilexoside A (1) is the first example of the isolation of a siaresinolic acid glycoside from plants in the genus Ilex.

A triterpene glycoside, pedunculoside, has been isolated as a bitter principle from leaves of *I. chinensis*, and characterized as 28-*O*-β-D-glucopyranosyl rotundic acid.[13] In the present study, we isolated free rotundic acid from the methanol extract as a major component and identified it by comparison with data reported for an authentic sample.[14] However, we could not confirm the existence of pedunculoside in the methanol extract.

Experimental

All melting points were determined on a Yanagimoto micro-apparatus and are uncorrected. Infrared (IR) spectra were run with a JASCO A-302 instrument. Unless otherwise mentioned, ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100.5 MHz) spectra were measured with a JEOL GX-400 spectrometer with pyridine-d_5 as a solvent and tetramethylsilane (TMS) as an internal standard. Unless otherwise stated, EI-MS spectra were obtained from a JEOL DX-300 spectrometer equipped with a direct inlet system at 20 eV, and FD-MS from the same mass spectrometer using carbon emitters under the following conditions: accelerating voltage, 3 kV; emitter current, 15—29 mA; chamber temperature, room temperature. Optical rotations were determined on a JASCO DIP-140 digital polarimeter. Gas liquid chromatography (GLC) was carried out on a Shimadzu GC-7AG gas chromatograph under the following operating conditions: column, 1.5% SE-52 on Chromosorb WAW DMCS (2 m × 3 mm i.d.); detector, FID; column temperature, 182 °C; carrier N_2 gas, 32 ml/min. For column and thin layer chromatography (TLC), Merck Kieselgel 60 (230—400 mesh) and precoated silica gel plates (Merck HF-254) were used, respectively. Preparative HPLC was performed on a Kusano instrument with a KPW-10 micro-pump and a Shodex SE-31 differential refractometer. A pre-packed Kusano ODS column (22 mm i.d. × 10 cm) was used, with an eluant flow of 3 ml/min of MeOH–H_2O (12 : 1). Protease [type XIII from *Aspergillus saitoi* (Molsin)] was a commercial product (Sigma Chem. Co.; Lot. No. 104F-0124).

Plant Material——Leaves of *Ilex chinensis* were collected in 1984 from a male tree growing in the Medicinal Plant Garden of Setsunan University (Faculty of Pharmaceutical Sciences, Hirakata, Osaka, Japan). The plant used in this study was identified by one of us (H. Murata), and specimens are deposited in the herbaria of the Faculty of Science, the University of Tokyo, and the Faculty of Pharmaceutical Sciences, Setsunan University.

Isolation of 1,1a, and 2a——The fresh leaves (2 kg) were extracted three times with MeOH (50 l) at room temperature for a week, and the solvent was removed under reduced pressure. The combined extract (466 g) was suspended in H_2O, and the aqueous suspension was extracted with *n*-BuOH. The *n*-BuOH layer was evaporated to dryness to give a residue (338 g), a portion (63.5 g) of which was chromatographed on silica gel (1.5 kg) with $CHCl_3$–MeOH (10 : 1). Of the 11 separated fractions (from No. 1 to No. 11), fraction No. 7 afforded a glycoside mixture (2.05 g) comprised of **1** and **2** (assumed to be the natural form of **2a**). Recrystallization of the mixture from MeOH gave pure **1** (830 mg) as colorless crystals, mp 264—267 °C, $[\alpha]_D^{19}$ +7.8° (MeOH, c=0.20). The mother liquor of the recrystallization was treated with diazomethane–ether and the reaction mixture was allowed to stand overnight. Evaporation of the solvents afforded a methylated product (1.2 g), a part (0.35 g) of which was subjected to preparative HPLC to give **1a** (230 mg), **2a** (67 mg), and a mixture (50 mg) of them. The physical and spectral data are as follows. **1**: IR ν_{max}^{KBr} cm^{-1}: 1675 (COOH), 1190, 1155, 1040. FD-MS *m/z* (%): 627 (M^+ +Na, 56), 605 (M^++H, 100), 559 (39), 473 (M^++H−132, 5). ^{1}H-NMR δ: 0.89, 1.00, 1.06, 1.14, 1.22, 1.32, 1.69 (3H each, all s, 7 × *tert*-Me), 3.36 (1H, dd, J=11.8, 4.3 Hz, 3α-H), 3.65 (1H, m, 19β-H), 3.82 (1H, dd, J=11.1, 10.1 Hz, 5′α-H), 4.04 (1H, dd, J=8.7, 7.5 Hz, 2′-H), 4.19 (1H, t, J=8.7 Hz, 3′-H), 4.25 (1H, ddd, J=10.1, 8.7, 4.9 Hz, 4′-H), 4.42 (1H, dd, J=11.1, 4.9 Hz, 5′β-H), 4.86 (1H, d, J=7.5 Hz, 1′-H), 5.57 (1H, m, 12-H). **1a**: Colorless crystals of mp 229—232 °C (MeOH), $[\alpha]_D^{19}$ +16.7° (MeOH, c=0.20). IR ν_{max}^{KBr} cm^{-1}: 1710 (COOMe), 1190, 1160, 1040. EI-MS *m/z* (%): 618 (M^+, 29), 600 (M^+-H_2O, 42), 559 (28), 486 (M^+−132, 4), 468 (M^+−132−H_2O, 12), 278 (99), 201 (100), 200 (45). FD-MS *m/z* (%): 619 (M^++H, 100), 618 (M^+, 46). ^{1}H- and ^{13}C-NMR: given in Tables I and II, respectively. *Anal.* Calcd for $C_{36}H_{58}O_8 \cdot H_2O$: C, 67.89; H, 9.50. Found: C, 67.92; H, 9.17. **2a**: Colorless crystals of mp 155—158 °C (MeOH), $[\alpha]_D^{19}$ +15.9° (MeOH, c=0.15). IR ν_{max}^{KBr} cm^{-1}: 1720 (COOMe), 1200, 1145, 1030. EI-MS *m/z* (%): 618 (M^+, 2), 600 (M^+-H_2O, 2), 559 (3), 486 (M^+−132, 12), 468 (M^+−132−H_2O, 27), 278 (25), 201 (26), 179 (100). FD-MS *m/z* (%): 619 (M^++H, 100), 618 (M^+, 26). ^{1}H- and ^{13}C-NMR: given in Tables I and II, respectively.

Acidic Hydrolysis of 1a——A solution of **1a** (120 mg) in 4 N H_2SO_4–MeOH (1 : 5, 30 ml) was refluxed for 8 h, poured into ice-water, and extracted with Et_2O. The Et_2O layer was washed with 5% aqueous $NaHCO_3$ and then H_2O, dried over $MgSO_4$, and concentrated to give a crude aglycone (90 mg). Recrystallization from MeOH furnished pure **3** (47 mg), colorless fine crystals, mp 184—186 °C (ref. 7, mp 184—186 °C), $[\alpha]_D^{19}$ +47.7° ($CHCl_3$, c=0.15) (ref. 7, +45.0° ($CHCl_3$)) and $[\alpha]_D^{19}$ +33.3° (MeOH, c=0.20). IR $\nu_{max}^{CCl_4}$ cm^{-1}: 3625 (OH), 1725 (COOMe), 1190, 1165. EI-MS (30 eV) *m/z* (%): 486 (M^+, 3), 468 (M^+-H_2O, 12), 278 (83), 260 (99), 201 (100), 200 (65). ^{1}H-NMR ($CDCl_3$) δ: 0.67, 0.78, 0.90, 0.96, 0.97, 0.99, 1.25 (3H each, all s, 7 × *tert*-Me), 3.21 (1H, dd, J=10.6, 5.1 Hz, 3α-H), 3.33 (1H, t-like,[15]

19β-H), 3.63 (3H, s, COOMe), 5.45 (1H, m, 12-H). ^{13}C-NMR: given in Table II. The melting point, optical rotation, IR, ^{1}H-NMR, and EI-MS spectra of **3** were consistent with the published data for authentic methyl siaresinolate.[7] The aqueous layer of the hydrolysate was neutralized with Amberlite IRA-45 (OH^- form) and subjected to paper chromatography [a) *n*-BuOH : AcOH : H_2O = 4 : 1 : 5 (upper layer) and b) iso-PrOH : *n*-BuOH : H_2O = 7 : 1 : 2 as developing solvent systems; aniline hydrogen phthalate for detection] to demonstrate the presence of xylose.

Acidic Hydrolysis of 2a——A solution of **2a** (10 mg) in 4 N H_2SO_4–MeOH (1 : 5, 3 ml) was refluxed for 8 h. The reaction mixture was worked up in the same manner as in the case of acidic hydrolysis of **1a**. Based on ^{1}H-NMR study and TLC comparison, the aglycone obtained was identified as a dienic mixture of methyl tomentosolate (**5**) and vanguerolate (**6**).[4,8,9]

Enzymic Hydrolysis of 2a——Protease [type XIII from *Aspergillus saitoi* (Molsin)][10] (1 g) in 0.2 M citric acid–0.2 M Na_2HPO_4 buffer (pH 4.0, 30 ml) was added to a solution of **2a** (120 mg) in EtOH (5 ml). The reaction mixture was stirred at 37 °C for 6 d, then poured into H_2O, and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried over $MgSO_4$, and evaporated to dryness. The residue (99.8 mg) was purified by silica gel column chromatography to give pure **4** (60 mg) and the recovered starting material (25 mg). The genuine aglycone (**4**) obtained, $[\alpha]_D^{19}$ +44.2° ($CHCl_3$, c = 0.20) and +39.4° (MeOH, c = 0.15), was shown to be identical with authentic methyl pomolate[4] by comparisons of melting point (recrystallized from MeOH), optical rotation ($CHCl_3$), IR (KBr) and ^{1}H-NMR (pyridine-d_5) spectra, and TLC behavior (3 different solvent systems). The ^{13}C-NMR data are also given in Table II.

Methanolysis of 1a——A solution of **1a** (5 mg) in 5% anhydrous HCl–MeOH (1.3 ml) was refluxed for 4 h. The reaction mixture was neutralized with Ag_2CO_3. The inorganic precipitate was filtered off and the filtrate was evaporated to dryness. The residue was trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide–pyridine and subjected to a GLC analysis to demonstrate the presence of Me xyloside.

Methanolysis of 2a——A solution of **2a** (5 mg) in 5% anhydrous HCl–MeOH (1.3 ml) was refluxed for 4 h. As in the methanolysis of **1a**, the reaction mixture was worked up and analyzed by GLC, and Me xyloside was identified.

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Studies on Active Substances in Herbs Used for Oketsu ("Stagnant Blood") in Chinese Medicine. V. On the Anticoagulative Principle in Moutan Cortex[1)]

HITOSHI ISHIDA,* MIKI TAKAMATSU, KUNIRO TSUJI and TAKUO KOSUGE

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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The anticoagulative principle in Moutan Cortex, which is one of the most important herbs and is used particularly for the treatment of Oketsu "stagnant blood" in traditional Chinese medicine, was investigated. Plasma recalcification time in mice was used for the bioassay. By following the anticoagulative activities, paeonol (2′-hydroxy-4′-methoxyacetophenone) was isolated as a major active principle in the herb.

Keywords——Moutan Cortex; stagnant blood; anticoagulative principle; plasma recalcification time; paeonol

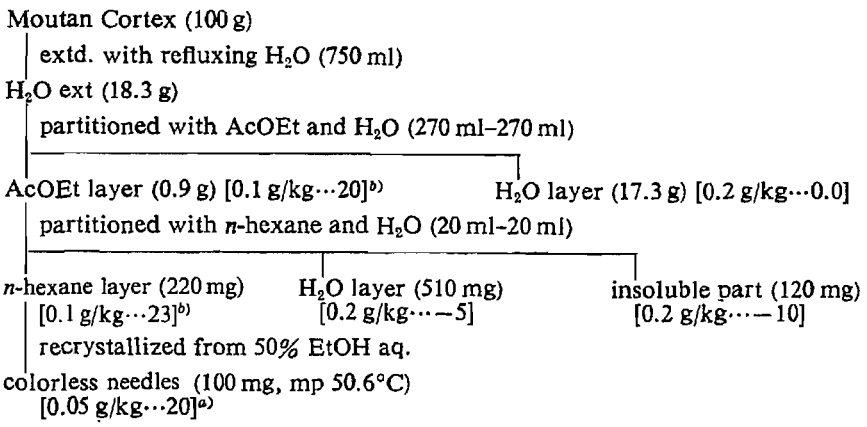
As reported previously,[2)] we have found that the extracts of herbs which are commonly used for Oketsu ("stagnant blood")[3)] in traditional Chinese medicine have an anti-aggregation activity *in vivo*, and that the extract of Moutan Cortex, one of them, has a significant anticoagulative activity. The present paper presents the isolation and identification of the anticoagulative principle in the herb.

Moutan Cortex, which is a component of prescriptions used for treatment of Oketsu, such as Keishi-bukuryô-gan (桂枝茯苓丸), Daiô-shachû-gan (大黄䗪虫丸) and Kepputsuio-tô (血府逐瘀湯), is frequently used as an analgesic, sedative, antiinflammatory agent, hemostatic and remedy for female diseases in traditional oriental medicine.[4)] Previous studies on this herb have been focused mostly on elucidation of the chemical constituents, *e.g.*, paeonol, paeonoside, paeoniflorin and benzoylpaeoniflorin.[5-8)] Pharmaceutical studies on the herb showed that the extracts of this herb and paeonol, a constituent, have antiinflammatory activity,[9a)] hemostatic activity[10)] and inhibit blood platelet coagulation induced by endotoxin, collagen and adenosine diphosphate (ADP) *in vitro*.[9a,b)] It has been suggested that paeonol might play an important role in the antiaggregation effect of the herb.[11)] However no detailed study on the anticoagulative principles in the herb has appeared.

In this paper, we wish to describe the isolation process and identification of the anticoagulative principle in the herb. During the isolation process, plasma recalcification time in mice was used for following the anticoagulative activity of the material, as reported previously.[2)] Isolation of the active principle was achieved by a combination of partitions and recrystallization, as summarized in Chart 1.

The hot-water extract of Moutan Cortex was partitioned between water and ethyl acetate. The active fraction I (ethyl acetate part) was extracted with *n*-hexane and then the soluble part was partitioned between *n*-hexane and water. As significant activity emerged only in the *n*-hexane part (active fraction II), this fraction was recrystallized from 50% ethanol to afford colorless needles.

From the spectral data of the active compound, it was concluded to be paeonol (2′-



( ) indicates yields. [/] indicates dose and blood coagulation-inhibitory activity (%). Significant difference from the control group: *a*) $p<0.05$, *b*) $p<0.02$. The coagulation time of control group was 2.40 ± 10 min (mean ± S.E. from 5 mice).

Chart 1. Isolation of the Active Principle

hydroxy-4′-methoxyacetophenone). This identification was confirmed by direct comparison with an authentic sample of paeonol (obtained from Wako Co.). The anticoagulative activity of the authentic sample was equal to that of the natural product.

Paeonol has been isolated from various plants, *e.g.*, *Bathysameridionalis*,[12] *Morus alba*,[13] *Betula platyphylla* var. *Japonica*,[14] *Primula viscosa* VILL.,[15] and *Cynanchum paniculatum*.[16] Pharmaceutical studies of the compound have examined the central effects[17] and stress-preventing action *in vivo*,[18] and the antiaggregation activity, hemostatic activity and so on *in vitro*.[9-11] Earlier reports, indicating that paeonol inhibits induced platelet aggregation *in vitro*,[9] suport our finding that this compound is responsible for the blood anticoagulative activity *in vivo*. This inhibitory effect of paeonol *in vivo* may result partially from its action on the formation of thromboxane A2 from arachidonic acid, as reported previously.[11] However, further studies are necessary to examine in detail the mechanism of the antiaggregation effect of paeonol *in vivo*, because there is a report that paeonol does not affect blood coagulation *in vitro*.[9b]

In summary, our present results clearly indicate that paeonol is a major constituent with *in vivo* blood anticoagulative activity in Moutan Cortex, and confirms the assumption by Hirai *et al.*, concerning the role of paeonol in the herb.[11]

Experimental

The melting point is uncorrected. The infrared (IR) spectrum was recorded on a JASCO A-2 spectrophotometer. The nuclear magnetic resonance (NMR) spectra were run on a JEOL FX-90 Fourier transform spectrometer (90 MHz for ^{1}H-NMR and 22.5 MHz for ^{13}C-NMR). The mass spectrum (MS) was recorded on a JEOL JMS D-100 spectrometer. Elemental analysis was done with a Perkin Elmer 240 analyzer. Ultraviolet (UV) spectra were taken with a Shimadzu UV-360 recording spectrophotometer. The anticoagulative activity was determined by the method reported in Part I.[2]

Material——Moutan Cortex (*Paeonia moutan* SIMS) used in this study was a commercial product.

Extraction——Ground Moutan Cortex (100 g) was extracted with water (500 ml) under reflux for half an hour. The mixture was centrifuged at 2500 rpm for 20 min, and the supernatant was lyophilized to give the crude extract (18.3 g).

Partition of the Water Extract between Water and Ethyl Acetate——The water extract (18.3 g) was dissolved in water (270 ml) and extracted with ethyl acetate (270 ml) four times. Evaporation of the ethyl acetate layer gave the active fraction I (0.9 g) as a brown gum.

Partition of Active Fraction I between Water and *n*-Hexane——Active fraction I (900 mg) was extracted with *n*-

hexane (50 ml) three times, and the soluble part (780 mg) was partitioned between *n*-hexane (20 ml) and water (20 ml). Concentration of the upper layer under reduced pressure afforded active fraction II (220 mg). This fraction was recrystallized from 50% ethanol to yield colorless needles (100 mg).

Identification of the Active Principle——The active principle (mp 50.6 °C (49.5—50.0 °C)[8]) was identified as paeonol by direct comparison of the physical properties (IR, NMR, UN and MS) with those of an authentic sample.

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Studies on Active Substances in Herbs Used for Oketsu ("Stagnant Blood") in Chinese Medicine. VI. On the Anticoagulative Principle in Paeoniae Radix[1)]

HITOSHI ISHIDA,* MIKI TAKAMATSU, KUNIRO TSUJI and TAKUO KOSUGE

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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The anticoagulative principles in Paeoniae Radix, which is one of the most important herbs and is used commonly for the treatment of female diseases in traditional oriental medicine, were investigated. In this study the measurement of plasma recalcification time in mice was found to be useful for following the anticoagulative activity of the material, and the active principles were isolated from the water extract of the herb by a combination of a partition and repeated silica gel column chromatographies.

Keywords——Paeoniae Radix; stagnant blood; anticoagulative principle; plasma recalcification time; paeoniflorin; benzoylpaeoniflorin

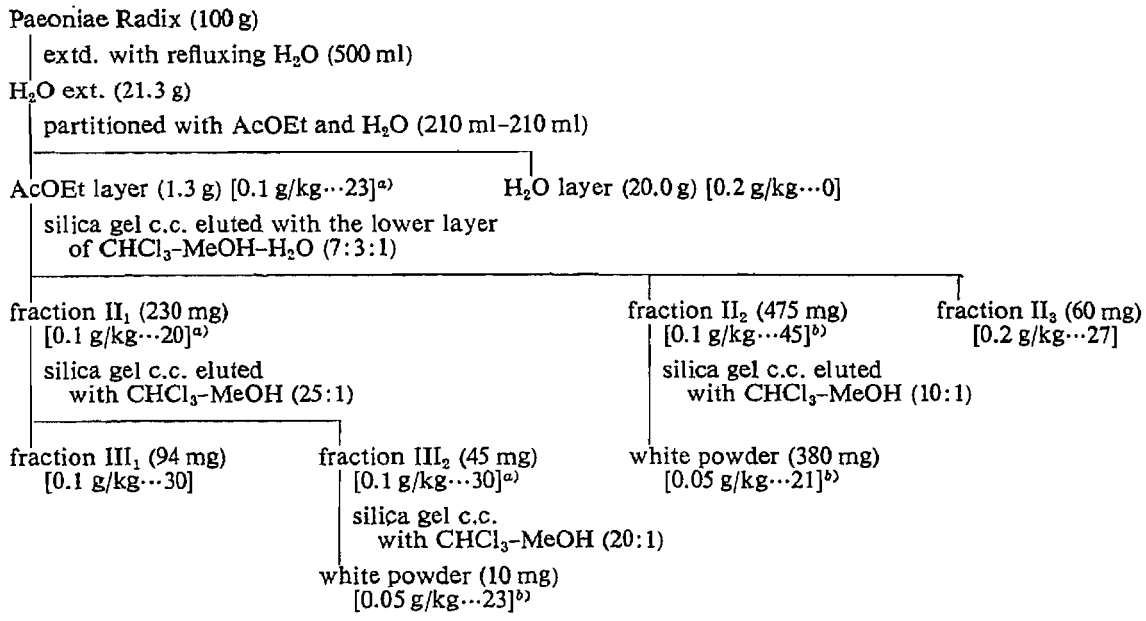
In the course of our study on the isolation of anticoagulative constituents in herbs used for treatment of Oketsu ("stagnant blood") in traditional Chinese medicine,[2)] we have so far reported the isolation and identification of several active principles, triolein in Persicae Semen, some curcuminoids in Curcumae Rhizoma, *d*-catechin in Rhei Rhizoma and paeonol in Moutan Cortex.[3)] In this paper, we will deal with the isolation and identification of anticoagulative principles in Paeoniae Radix (*Paeoniae lactiflora* PALL).

Paeoniae Radix is well known as one of the important herbs showing anticoagulant, anodyne and sedative action, and is used frequently treatment of female diseases in traditional oriental medicine.[4)] Previous studies on the herb have been mostly focused on the chemical components, such as terpenoids (*e.g.*, paeoniflorin, benzoylpaeoniflorin, and albiflorin),[5)] tannin[6)] and paenol.[7)] Pharmacological studies on the antiinflammatory effect of the water-soluble part of the 70% MeOH extract of the herb, and on the antiallergic and anti-platelet aggregation effects of paeoniflorin and benzoylpaeoniflorin *in vitro*[8)] and so on[9)] have been reported. However, no detailed study on the anticoagulative principles in the herb has appeared.

We describe here the isolation and identification of anticoagulative principles in Paeoniae Radix. During the isolation process, the anticoagulative activity of the material was determined by means of the plasma recalcification time method reported previously.[3)] Isolation of the active principles was achieved by a combination of partition and repeated column chromatographies on silica gel. The procedures are summarized in Chart 1.

The active fraction I (ethyl acetate-soluble part of the water extract) was subjected to column chromatography on silica gel with the lower layer of $CHCl_3$–MeOH–H_2O (7 : 3 : 1) to afford three fractions, namely fractions II_1, II_2 and II_3.

The activity emerged in all fractions. In this work, we dealt with the significantly active fractions, II_1 and II_2, which contained the bulk of the activity of fraction I. Column chromatography of active fraction II_1 on silica gel using $CHCl_3$–MeOH as an eluent gave two



( ) indicates yields. [/] indicates dose and blood coagulation-inhibitory activity (%). Significant difference from the control group: *a*) $p<0.05$, *b*) $p<0.02$. The coagulation time of the control group was 2.40 ± 0.10 min (the mean ± S.E. from 5 mice). Silica gel c.c., silica gel column chromatography.

Chart 1. Isolation of the Active Principles

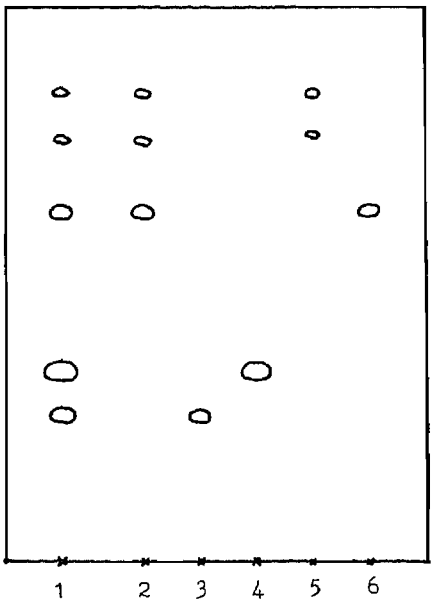


Fig. 1. Thin Layer Chromatograms of Fraction II_1—II_3, Fraction III_1 and Fraction III_2

Plate: Kieselgel H60 (Merck Art 5721). Solvent: Lower layer of $CHCl_3$–MeOH–H_2O (7:3:1). Color reagent: Vaughan's reagent.
1: AcOEt-layer. 2: fraction II_1. 3: fraction II_2. 4: fraction II_3. 5: fraction III_1. 6: fraction III_2.

fractions, III_1 and III_2. Significant activity was found only in fraction III_2. Final purifications of active fractions II_2 and III_2 were achieved by silica gel column chromatographies with $CHCl_3$–MeOH to afford two active compounds, **1** from fraction II_2 and **2** from fraction III_2. As shown in the thin layer chromatograms of each fraction in Fig. 1, silica gel column chromatography is very effective for purifying the active compounds. Anticoagulative activities of the fractions described above are shown in Chart 1. Work on other active fractions is still in progress.

Compound **1** is one of the major constituents and **2** is a minor principle in Paeoniae

Radix. Their spectral data indicated **1** to be paeoniflorin[5b,10] and **2** to be 6'-*O*-benzoylpaeoniflorin (benzoylpaeoniflorin).[11] The identifications were confirmed by direct comparisons of the spectral data (IR, NMR, UV and MS) with those of the authentic samples of paeoniflorin, obtained from Wako Co., and benzoylpaeoniflorin, prepared from **1** by the known method.[12] The anticoagulative activities of authentic samples of paeoniflorin and benzoylpaeoniflorin are equal to those of **1** and **2**, respectively.

Paeoniflorin and benzoylpaeoniflorin have been isolated from *Paeonia lactiflora* PALL[11] and *Paeonia moutan* SIMS.[5a] Pharmacological studies on the antiinflammatory and anti-aggregatory effects of these compounds *in vitro* have been reported.[8,13] They were found to show an inhibitory effect on collagen-, endotoxin- and adenosine diphosphate (ADP)-induced blood platelet coagulation but not on blood aggregation *in vitro*.[13] Further work is necessary to elucidate in detail the mechanism of the anticoagulative effects *in vivo*, in view of the reported lack of effect on blood coagulation *in vitro*.

In summary, it is demonstrated that paeoniflorin and benzoylpaeoniflorin have anti-coagulative activity *in vivo* and might play an important role in the antiaggregatory effect of Paeoniae Radix.

Experimental

Ultraviolet (UV) spectra were taken with a Shimadzu UV-360 recording spectrophotometer. Mass spectra (MS) were recorded on a JEOL JMS D-100 spectrometer. The infrared (IR) spectra were recorded on a JASCO A-2 spectrophotometer. The nuclear magnetic resonance (NMR) spectra were measured on a JEOL FX-90 Fourier-transform spectrometer (90 MHz for ^{1}H-NMR and 22.5 MHz for ^{13}C-NMR). Elemental analyses were done with a Perkin Elmer 240 analyzer. The anticoagulative activity of the material was determined by the method reported previously.[3a]

Material——A commercial product of Paeoniae Radix (*Paeonia lactiflora* PALL) was used in this study.

Extraction——Ground Paeoniae Radix (100 g) was extracted with water (500 ml) under reflux for half an hour. The mixture was centrifuged at 2500 rpm for 20 min, and the supernatant was lyophilized to give the crude extract (21.3 g).

Partition of the H_2O Extract between Water and Ethyl Acetate——The H_2O extract (21.3 g) was dissolved in water (210 ml) and extracted with ethyl acetate (210 ml) four times. Concentration of the ethyl acetate layer under reduced pressure afforded the active fraction I (1.3 g) as a brown gum.

Silica Gel Column Chromatography of Active Fraction I——Active fraction I (1.3 g) was subjected to column chromatography on silica gel (2.4 × 28 cm) using the lower layer of $CHCl_3$–MeOH–H_2O (7 : 3 : 1) as an eluent to afford three active fractions, namely fractions II_1 (230 mg), II_2 (475 mg) and II_3 (60 mg).

Column Chromatography of Active Fraction II_1 on Silica Gel——Active fraction II_1 (230 mg) was chromatographed on silica gel (0.9 × 25 cm) with $CHCl_3$–MeOH (20 : 1) as an eluent to give the active fractions, fractions III_1 (94 mg) and III_2 (45 mg).

Silica Gel Column Chromatography of Active Fraction II_2——Column chromatography of active fraction II_2 (475 mg) over silica gel (1.1 × 27 cm) using $CHCl_3$–MeOH (10 : 1) as an eluent gave the active compound **1** (380 mg) as a white powder.

Column Chromatography of Active Fraction III_2 over Silica Gel——Active fraction III_2 (45 mg) was chromatographed on silica gel (0.7 × 20 cm) with $CHCl_3$–MeOH (20 : 1) as an eluent to afford the active compound **2** (10 mg) as a white powder.

Identification of the Active Principles, 1 and 2——The active compounds, **1** and **2**, were identified as paeoniflorin and benzoylpaeoniflorin by direct comparisons of their physical data (IR, UV, MS and NMR) with those of their authentic samples.

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Studies on 2(1*H*)-Quinolinone Derivatives as Gastric Antiulcer Active Agents. Synthesis and Antiulcer Activities of Optically Active α-Amino Acid Derivatives of 2(1*H*)-Quinolinone and Oxindole

MINORU UCHIDA, FUJIO TABUSA,* MAKOTO KOMATSU, SEIJI MORITA, TOSHIMI KANBE and KAZUYUKI NAKAGAWA

Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Kagasuno 463-10, Kawauchi-cho, Tokushima 771-01, Japan

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In order to study the relationship of structure to antiulcer activity, optical active α-amino acid derivatives of 2(1*H*)-quinolinone and oxindole were synthesized and tested for antiulcer activity against acetic acid-induced gastric ulcer in rats. The enantiomers of 2(1*H*)-quinolinone derivatives were obtained by optical resolution with (−)-brucine. The oxindole derivatives having different absolute configurations at the α-amino acid moiety were synthesized by oxidation of *N*-(4-chlorobenzoyl)-L- or -D-tryptophan. The antiulcer activity did not seem to be influenced by the α-amino acid chirality.

Keywords——2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid; α-(4-chlorobenzoylamino)-2,3-dihydro-2-oxo-1*H*-indole-3-propionic acid; antiulcer activity; optical resolution

Introduction

In biologically active compounds having chirality, it is often found that each enantiomer exhibits different activity because of the participation of a specific enzyme, receptor and so on in the action. However, in the field of antiulcer agents, little information is available[1] on the difference of activity between enantiomers. We have already described the synthesis and antiulcer activity of racemic α-amino acid derivatives of 2(1*H*)-quinolinone[2] and oxindole.[3] Among them, 2-(4-chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic acid (OPC-12759) (**1**) and α-(4-chlorobenzoylamino)-2,3-dihydro-2-oxo-1*H*-indole-3-propionic acid (**2**) exhibited potent activity. OPC-12759 (**1**) is now under clinical trial.

Then we turned our attention to the α-amino acid moiety of **1** and **2**, and investigated the preparation of the compounds having different absolute configurations of the α-amino acid moiety in order to study the influence of chirality on the antiulcer activity.

Synthesis

First, the optically active 2(1*H*)-quinolinone derivatives (**1a** and **1b**) were obtained by resolution of **1** using (−)-brucine. The optical purities of **1a** and **1b** were determined by high-performance liquid chromatographic (HPLC) analysis as follows. The enantiomers (**1a** and **1b**) were condensed with (+)-α-methylbenzylamine (98% purity) in the presence of diethyl phosphorocyanidate (DEPC). For the separation of the diastereomers thus obtained, various analytical conditions were examined with prepacked octadecylsilica gel columns (YMC A-303, YMC A-312, Cosmosil 5C-18, Nucleosil 5C-18) using aq. MeOH or aq. MeCN as an eluent. Among the conditions investigated, the best result was obtained with the use of a Yamamura YMC type A-303 (ODS, 6.0 i.d. × 100 mm) column eluted with aq. MeCN

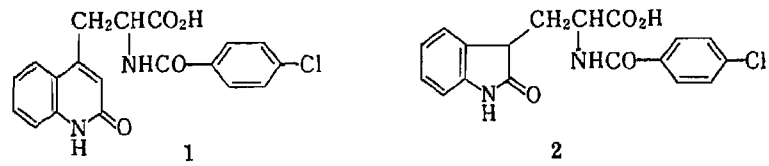

Fig. 1

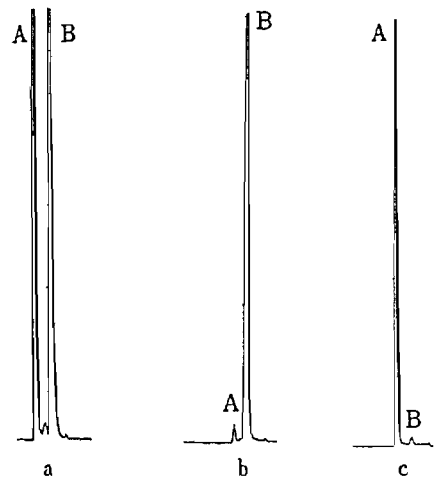

Fig. 2. HPLC Chromatograms of **1a**, **1b** and Racemic **1**

a) HPLC chromatogram of racemic **1**. b) HPLC chromatogram of **1a**: optical purity, 98% e.e. c) HPLC chromatogram of **1b**: optical purity, 98% e.e.

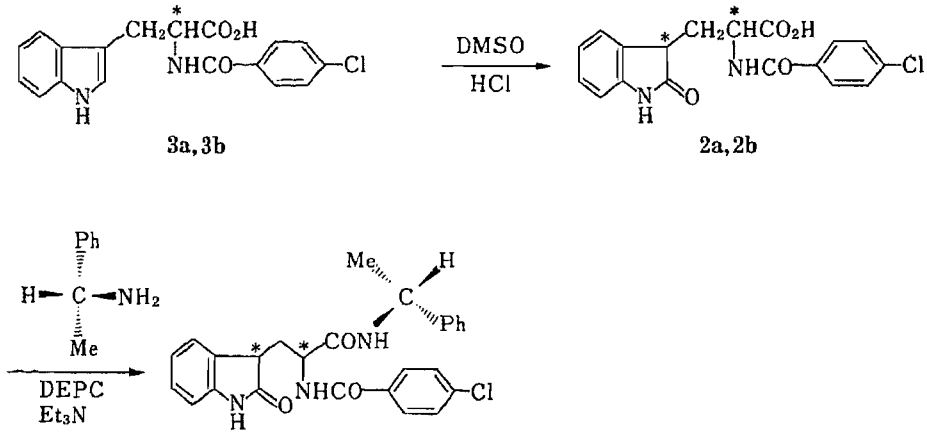

Chart 1

(MeCN : 0.03 M Na_2SO_4 = 32 : 68). The results are shown in Fig. 2.

Next, the oxindole derivatives having different absolute configuration at the α-amino acid moiety were synthesized. Compound **3a**, which is known as Benzotript,[4] was obtained by the acylation of L-tryptophan in the presence of $NaHCO_3$, and showed negative optical rotation ($[\alpha]_D^{20} = -30.7°$ ($c = 1$, MeOH)). Next, **3a** was oxidized by using a known method[5] to the oxindole derivative (**2a**), which was a mixture of two diastereomers arising from the new asymetric center at the C-3 position of the oxindole ring (*vide infra*). Similarly, **2b** was prepared from D-tryptophan as a mixture of two diastereomers and was found to have positive optical rotation ($[\alpha]_D^{25} = +92.4°$ ($c = 1$, MeOH)). The configurational purities at the α-amino acid moiety of **2a** and **2b** were determined by a method similar to that described for **1a** and **1b** using aq. MeOH–THF (MeOH : THF : 0.03 M Na_2SO_4 = 45 : 15 : 40) as an eluent. The results are shown in Fig. 3. Since **2a** and **2b** showed two major peakes in Fig. 3, it was concluded that **2a** and **2b** were diastereomixtures in the ratio of *ca.* 1 : 1.

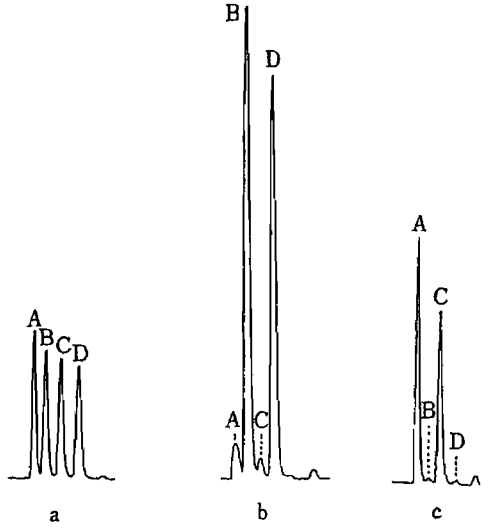

Fig. 3. HPLC Chromatograms of **2a**, **2b** and Racemic **2**

a) HPLC chromatogram of racemic **2**. b) HPLC chromatogram of **2a**: configurational purity at α-amino acid moiety, 85%. c) HPLC chromatogram of **2b**: configurational purity at α-amino acid moiety, 95%.

TABLE I. Antiulcer Activity of Racemic and Optically Active **1** and **2**

Compd. No.	Antiulcer activity[a]
Racemic **1**	++
1a	++
1b	++
Racemic **2**	++
2a	++
2b	++

a) For details of the evaluation, see the previous report.[2]

Biological Results

Next, the antiulcer activities of **1a**, **b** and **2a**, **b** against acetic acid-induced gastric ulcer,[6] as a model of chronic ulcer, were examined. The results are shown in Table I; the pairs of enantiomers or diastereomers showed no significant difference in activity.

Experimental

Melting points were determined with a Yamato MP-21 apparatus and are uncorrected. Infrared (IR) spectra were recorded on a JASCO IR-810 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390, JEOL JMN FX-200 or Brucker WH-400 spectrometer in $CDCl_3$ with tetramethylsilane or in d_6-dimethyl sulfoxide (d_6-DMSO) with 3-(trimethylsilyl)propionic acid-d_6 as an internal standard. Mass spectra (MS) were obtained on a Shimadzu GCMS-QP 1000 instrument. High-performance liquid chromatograms were obtained with a Toyo Soda HLC-803D. Optical rotations were measured with a JASCO DIP-140 digital polarimeter.

Preparation of (−)-2-(4-Chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic Acid (1a)——(−)-Brucine (23 g) was added to a suspension of **1** (21.6 g) in MeOH (1100 ml) and the resulting clear solution was stirred for 4 h at room temperature. The solution was stored overnight under cooling, and the precipitated crystals were separated by filtration, then washed with chilled MeOH. Four further recrystallizations gave the pure salt (7.6 g), $[\alpha]_D^{20} = +60.4°$ ($c=1.0$, $CHCl_3$). Then 10% HCl was added to the MeOH suspension of the salt, and the precipitates were filtered off and washed with water. Three recrystallizations from dimethylformamide (DMF)–H_2O gave colorless needles (1.6 g, 14.8%), mp 305—306 °C (dec.). $[\alpha]_D^{20} = -116.7°$ ($c=1.0$, DMF). NMR (d_6-DMSO) δ: 3.05—3.73 (2H, m), 4.62—4.96 (1H, m), 6.48 (1H, s), 7.10—7.60 (4H, m), 7.53 (2H, d, $J=8$ Hz), 7.86 (2H, d, $J=8$ Hz), 8.92 (1H, d, $J=9$ Hz), 12.00 (1H, br). IR ν(KBr): 1710, 1670, 1640 cm^{-1}. *Anal.* Calcd for $C_{19}H_{15}ClN_2O_4$: C, 61.55; H, 4.08; N, 7.55. Found: C, 61.66; H, 3.89; N, 7.85.

Preparation of (+)-2-(4-Chlorobenzoylamino)-3-[2(1*H*)-quinolinon-4-yl]propionic Acid (1b)——The recovered salt from the first mother liquor of **1a** was dissolved in MeOH, and 10% HCl was added. The precipitates were filtered off, washed with water and suspended in MeOH. (−)-Brucine was added to the suspension, and the mixture was stirred for 1 h at room temperature. The insoluble materials were removed by filtration. Then conc. HCl was added to the filtrate and the resulting precipitates were separated and washed with water. Four recrystallizations from DMF–H_2O gave colorless needles (3 g, 27.8%), mp 305—306 °C (dec.). $[\alpha]_D^{20} = +116.9°$ ($c=1.0$, DMF). NMR (d_6-DMSO) δ: 3.06—3.73 (2H, m), 4.62—4.96 (1H, m), 6.47 (1H, s), 7.12—7.60 (4H, m), 7.53 (2H, d, $J=8$ Hz), 7.86 (2H, d, $J=8$ Hz), 8.95 (1H, d, $J=9$ Hz), 12.00 (1H, br). IR ν(KBr): 1710, 1670, 1640 cm^{-1}. *Anal.* Calcd for $C_{19}H_{15}ClN_2O_4$: C, 61.55; H, 4.08; N, 7.55. Found: C, 61.34; H, 3.90; N, 7.62.

Determination of the Optical Purity of 1a or 1b——Under stirring, 0.4 g of (+)-α-methylbenzylamine (98% purity), 0.6 g of DEPC, 0.6 g of triethylamine were added successively to a DMF (20 ml) solution of **1a** or **1b** (1 g) on an ice-water bath. The mixture was stirred for 3 h at room temperature, then the solvent was removed under reduced pressure. Water was added to the residue and the resulting precipitates were filtered off and washed with water. The crude material was dissolved in MeOH and analyzed by HPLC. The result is shown in Fig. 2.

Preparation of a Mixture of (2*S*,3'*R*)- and (2*S*,3'*S*)-2-(4-Chlorobenzoylamino)-2',3'-dihydro-2'-oxo-1*H*-indole-3'-propionic Acid (2a)——Concentrated HCl (6.5 ml) was added to a solution of **3a**[4] (20 g) in DMSO (25 ml) in one portion under stirring at room temperature. Stirring was continued for 2 h, the reaction mixture was poured into water, and the resulting precipitates were collected by filtration and washed with water. The precipitates were dissolved in dil.NaOH, and conc. HCl was again added. The resulting precipitates were filtered off and washed with water. The crude material was purified by silica gel column chromatography (elute; $CHCl_3$: MeOH = 50 : 1). Recrystallization from MeOH–H_2O gave a pale yellow powder (2.4 g, 11%), mp 133—135 °C. $[\alpha]_D^{25} = -88.9°$ ($c = 1$, MeOH). NMR (d_6-DMSO) δ: 1.90—2.63 (2H, m), 3.37—3.63 (1H, m), 4.63—5.00 (1H, m), 6.70—7.40 (4H, m), 7.47 (2H, dd, $J = 2$, 9 Hz), 7.85 (2H, dd, $J = 2$, 9 Hz), 8.83 (1H, dd, $J = 8$, 9 Hz), 10.35 (1H, d, $J = 2$ Hz) IR ν (KBr): 1730, 1720, 1710, 1700, 1660, 1650 cm^{-1}. *Anal.* Calcd for $C_{18}H_{15}ClN_2O_4 \cdot 1/4\ H_2O$: C, 59.51; H, 4.30; N, 7.71. Found: C, 59.43; H, 4.14; N, 7.62.

Preparation of a Mixture of (2*R*,3'*R*)- and (2*R*,3'*S*)-2-(4-Chlorobenzoylamino)-2',3'-dihydro-2'-oxo-1*H*-indole-3'-propionic Acid (2b)——Compound **2b** (21%) was prepared by the same procedure as used for **2a**, starting from D-α-(4-chlorobenzoylamino)-1*H*-indole-3-propionic acid, as a white powder after recrystallization from MeOH–H_2O, mp 136 °C. NMR (d_6-DMSO) δ: 1.93—2.60 (2H, m), 3.33–3.60 (1H, m), 4.60—4.97 (1H, m), 6.73—7.42 (4H, m), 7.53 (2H, dd, $J = 2$, 9 Hz), 7.91 (2H, dd, $J = 2$, 9 Hz), 8.94 (1H, dd, $J = 8$, 9 Hz), 10.42 (1H, d, $J = 2$ Hz). IR ν (KBr): 1720, 1710, 1700, 1660, 1650 cm^{-1}. *Anal.* Calcd for $C_{18}H_{15}ClN_2O_4 \cdot 3/4\ H_2O$: C, 58.07; H, 4.20; N, 7.52. Found: C, 58.06; H, 4.21; N, 7.65.

Determination of the Optical Purity of 2a or 2b——Determination of the optical purity of **2a** or **2b** was carried out in the same manner as in the case of **1a** or **1b**. The result is shown in Fig. 3.

Biological Method——Antiulcer activity was measured by the reported method[2] using acetic acid-induced gastric ulcer in rats.

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Studies on Antihemorrhagic Substances in Herbs Classified as Hemostatics in Chinese Medicine. VI. On the Antihemorrhagic Principle in *Sophora japonica* L.[1a)]

HITOSHI ISHIDA,* TAKAYUKI UMINO, KUNIRO TSUJI and TAKUO KOSUGE

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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The antihemorrhagic principle of *Sophora japaonica* L. was isolated from the aqueous extract of dried buds by partition and Sephadex LH-20 column chromatography, and identified as quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one].

Keywords——hemostatic; *Sophora japonica* L.; antihemorrhagic principle; quercetin

In the course of our work on the chemical elucidation of antihemorrhagic principles in herbs which are classified as hemostatics in Chinese medicine, we have so far reported the isolation of five antihemorrhagic principles, β-*N*-oxallyl-α,β-diaminopropionic acid (neurotoxin) from Sanchi Ginseng Radix,[1b)] 3,3′,4-tri-*O*-methylellagic acid from *Sanguisorba officinallis* L.,[1c)] wedelolactone and desmethylwedelolactone from *Hypericum erectum* THUMB[1d)] and quercitrin from *Biota orientallis* (L.) ENDL. In this paper, we describe the isolation process and identification of the antihemorrhagic principle in *Sophora japonica* L.

Sophora japaonica L. is well known as an antihemorrhagic and antihypertensive agent, and is commonly used for the treatment of bleeding due to hemorrhoid and ulcerative colitis, and hypertension.[2)] There have been previous studies on the chemical elucidation of the components of the herb, such as flavonoids[3)] and terpenoids,[4)] and pharmacological studies to examine the effect of flavonoids in the herb *e.g.*, rutin, quercetin, *etc.*, on capillary fragility.[5)] However, no pharmacological study on the antihemorrhagic principle has been reported. We wish to describe here the isolation and identification of the antihemorrhagic principle in the herb.

During the isolation process, Tajima *et al.*'s method[6)] using mice was employed for following the antihemorrhagic activity of the material, as reported previously.[1b)] Isolation of the active principle was achieved by partition and Sephadex LH-20 column chromatography. The procedures are summarized in Chart 1.

As shown in Chart 1, the ground buds of *Sophora japonica* L. were extracted with water. The extract was partitioned between *n*-butanol and water. As the activity emerged predominantly in the *n*-butanol layer (active fraction I), this fraction was subjected to Sephadex LH-20 column chromatography with methanol. The elution pattern and activities of the fractions are shown in Fig. 1. Figure 1 shows that in the present case, column chromatography over Sephadex LH-20 with methanol effectively removes inactive material from fraction I. The active fraction II(K_d value = 3.15—7.10) was recrystallized from methanol to afford the active principle as yellow needles.

The spectral data (nuclear magnetic resonance (NMR), infrared (IR), mass and ultraviolet (UV)) of the active principle suggested that this principle is quercetin. This was

Sophora japonica L. (100 g)
↓ extd. with refluxing H_2O (1000 ml)
H_2O extract (32.7 g) [0.5 g/kg···2.9 min]
↓ partition with *n*-BuOH and H_2O (60 ml–60 ml)
active fraction I (*n*-BuOH layer) (9.588 g) [0.29 g/kg···5.2 min]
↓ gel filtration of Sephadex LH-20 with MeOH
active fraction II (531 mg) [1.34 mg/kg···3.3 min]
↓ recrystallized from MeOH
yellow needles (437 mg) [1.25 mg/kg···4.1 min]

( ) indicates yield. [ ] indicates dose and activity (shortening of bleeding time).

Chart 1. Isolation of the Active Principle

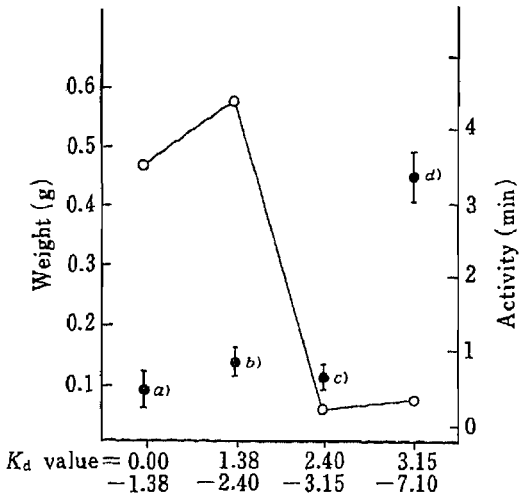

Fig. 1. Elution Pattern of the *n*-BuOH Layer (1.30 g on Sephadex LH-20 Gel Filtration (4.1 × 40 cm) and Activities of the Fractions

O—O, chromatogram; ●, activity (shortening of bleeding time after i.p. administration at each dose [*a*) 0.110, *b*) 0.130, *c*) 0.012, *d*) 0.013 g/kg weight]). Each point represents the mean of the antihemorrhagic activity in five different experiments with the S.E. indicated.

confirmed by direct comparison of physical properties (mp, IR, NMR, UV and IR) with those of an authentic sample (obtained from Wako Co.). The antihemorrhagic activity of the authentic sample was equal to that of the natural product.

Quercetin has been isolated from many plants, *e.g.*, *Polygonum polystachyum*,[7] tea leaves,[8] *Vitis vinifera*,[9] *Mallotus japonicus*,[10] *Euphora longana*,[11] *etc.* Pharmacological studies of quercetin, describing its capillary-stabilizing effect, effect on capillary fragility,[6] effect on adrenaline metabolism,[12] effect on bacteriostatic action,[13] and so on have been done, but there has been no previous report that it has an antihemorrhagic effect on bleeding *in vivo*.

Dose response curves of the water extracts of heated buds and untreated buds of *Sophora japonica* L. and quercetin are shown in Fig. 2. The water extracts of unheated buds and heated buds of *Sophora japonica* L. contain about 0.44% and 0.58% quercetin, as described in Chart 1.[14] However, the antihemorrhagic activity of the extract of heated herb is about two and a half times of that of the unheated herb. Since quercetin is responsible for the antihemorrhagic activity of the herb, it is concluded that the water extract of uhneated buds contains a material, which depresses the antihemorrhagic action of quercetin and is unstable to heating. Our finding supports the description that the parched buds of *Sophora japonica* L. show higher antihemmorhagic activity than the untreated buds.[2] A study to identify the component(s) which depresses the antihemorrhagic action of quercetin is in progress.

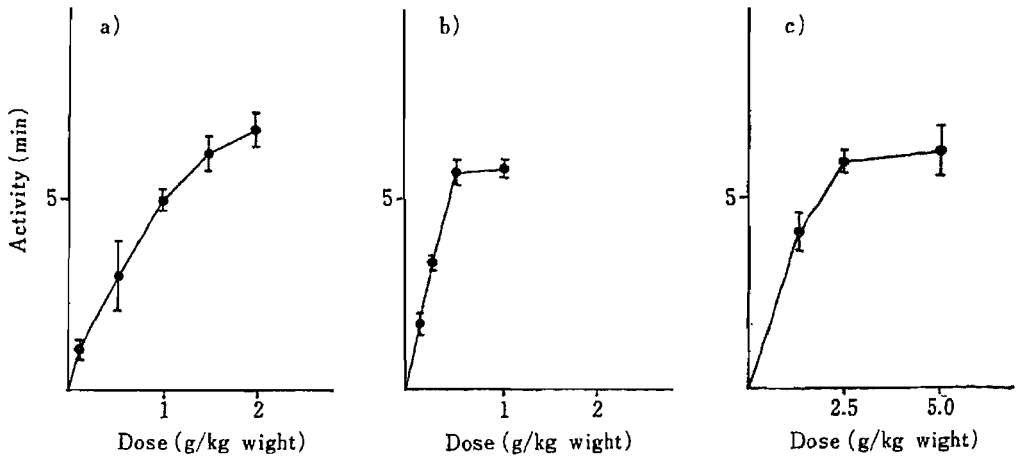

Fig. 2. Dose–Response Relationships for Antihemorrhagic Activity of Water Extracts of Parched Herb and Untreated Herb, and Quercetin

Each point represents the mean of the antihemorrhagic activity in five different experiments, with the S.E. indicated. a) Water extract of unheated herb. b) Water extract of perched herb. c) Quercetin.

In summary, it is demonstrated that quercetin is an antihemorrhagic principle in *Sophora japonica* L., and firing of the herb before use is recommended when the herb is to be used for treatment of bleeding, as noted in the literature.[2)]

Experimental

Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded with a JEOL FX-90 Fourier-transform nuclear magnetic spectrometer. The IR spectrum was recorded on a JASCO IRA-2 grating IR spectrophotometer. Mass spectra (MS) were recorded on a Hitachi M-80A instrument. UV spectrum were recorded on a Shimadzu UV-360 recording spectrophotometer. The melting point is uncorrected.

Assay of Hemostatic Activity——Hemostatic tests were carried out according to Tajima *et al.*[6)] with male mice weighing 18—20 g. Test material homogenized in 1% methylcellulose–0.9% sodium chloride aq. was given interperitoneally by injection. The bleeding time was determined according to Tajima *et al.*[6)]

Material——The herb used in this study was a commercial product and was identified as *Sophora japonica* L. by an expert. Parched buds were prepared by heating on a hot plate at 200 °C for 15 min.

Extraction——Ground buds of *Sophora japonica* L. (100 g) were extracted with 1 l of water under reflux for 30 min. The mixture was centrifuged at 2500 rpm for 20 min and the supernatant was lyophilized to afford a yellow-brown powder (32.7 g). From 100 g of parched herb, 41.0 g of water extract was obtained by means of the same extraction procedure as noted above.

Partition between *n*-Butanol and Water——The crude extract (32.7 g) was dissolved in 700 ml of water and extracted with 700 ml of *n*-butanol three times. Upon concentration of the active fraction (*n*-butanol layer) in a rotary evaporator, 9.588 g of fraction I was obtained as a light brown gum.

Gel Filtration on Sephadex LH 20——A portion (1.30 g) of the active fraction was dissolved in 20 ml of methanol and subjected to gel filtration through Sephadex LH-20 (4.1 × 40 cm) with methanol. In total, 531 mg of active fraction II (K_d value = 3.15—7.10) was obtained by repeated chromatographies of active fraction I. It was recrystallized from dil. ethanol to afford 437 mg of the active principle as yellow needles. The physical properties of the active principle are described below.

Identification of the Active Principle——The active compound (mp 313 °C (dec.) (314 °C (dec.)[15)]) was identified as quercetin by direct comparison with an authentic sample.

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Studies on Antihemorrhagic Substances in Herbs Classified as Hemostatics in Chinese Medicine. VII. On the Antihemorrhagic Principle in *Cirsium japonicum* DC.[1)]

HITOSHI ISHIDA,* TAKAYUKI UMINO, KUNIRO TSUJI and TAKUO KOSUGE

Shizuoka College of Pharmacy, 2–2–1 Oshika, Shizuoka 422, Japan

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The antihemorrhagic principle in *Cirsium japonicum* DC. was isolated by partition, gel filtration on Sephadex LH-20 and silica gel column chromatography, and identified as pectolinarin [7-[6-*O*-(6-deoxy-β-mannopyranosyl)-β-D-glucopyranosyl]-5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one].

Keywords——hemostatic; *Cirsium japonicum*; antihemorrhagic principle; pectolinarin

In the previous paper, we reported that the antihemorrhagic principle had been isolated from *Sophora japonica* L., which is one of the herbs used as hemostatics in Chinese medicine, and identified as quercetin.[1)] The present paper describes the isolation of the antihemorrhagic principle in *Cirsium japonicum* DC.

Cirsium japonicum DC. is an important herb, used as an antihemorrhagic agent and uretic in Chinese medicine.[2)] Previous work on the herb focused only on the chemical components, such as pectolinarin and acetin rhamnoglucoside,[3)] and no pharmacological study on the antihemorrhagic principle in the herb has been reported. We would like to describe here the isolation and identification of the hemostatically active principle in the herb.

In the isolation process, Tajima *et al.*'s method[4)] with mice was used to follow the antihemorrhagic activity of the material.[5)] Isolation of the active principle was achieved by partition, gel filtration through Sephadex LH-20 and column chromatography over silica gel. The procedures are summarized in Chart 1.

As shown in Chart 1, ground herb of *Cirsium japonicum* DC. was extracted with water. The extract was partitioned between *n*-butanol and water. The activity emerged only in the *n*-butanol layer (active fraction I). By using a small quantity of methanol, this fraction was fractionated into soluble and insoluble parts, namely fraction II_1 and fraction II_2. The activity emerged in both fractions. Firstly, we dealt with fraction II_1, because it showed higher antihemorrhagic activity than fraction II_2. The active fraction II_1 was subjected to Sephadex LH-20 column chromatography with methanol. The elution pattern and activities of the fractions are shown in Fig. 1. As the activity was present only in fraction III (K_d value = 1.14—1.76), this was chromatographed over silica gel to afford highly active fraction IV. After gel filtration of this fraction through Sephadex LH-20, the product was recrystallized from methanol to afford the active principle as light yellow needles. This active principle was also obtained by silica gel column chromatography of the hot methanol extract of fraction II_2, as shown in Chart 1. Thin layer chromatograms of fractions II_1, II_2, III and IV, and the active principle on silica gel are shown in Fig. 2. As shown in Figs. 1 and 2, the combination of column chromatographies is very effective for the purification of the active principle.

Cirsium japonicum DC. (200 g)

extracted with refluxing H_2O (2000 ml)

H_2O ext. (19.8 g) [0.5 g/kg···4.0 min]

partition with *n*-BuOH and H_2O (350 ml–350 ml)

active fraction I (*n*-BuOH layer (1.87 g) [0.05 g/kg···4.0 min]

fractionated with MeOH (20 ml)

active fraction II_1 (soluble part) (1.075 g) [25 mg/kg···3.9 min]

gel filtration on Sephadex LH-20 with MeOH

active fraction III (136 mg) [7 mg/kg···3.0 min]

silica gel c.c. with lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10)

active fraction IV (51.2 mg) [4 mg/kg···3.6 min]

1) gel filtration on Sephadex LH-20 with MeOH
2) recrystallized from MeOH

light yellow needles (17.4 mg) [1 mg/kg···3.4 min]

active fraction II_2 (precipitates) (755.3 mg) [25 mg/kg···2.1 min]

1) silica gel c.c. with lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10)
2) gel filtration on Sephadex LH-20 with MeOH
3) recrystallized from MeOH

light yellow needles (25.6 mg) [1 mg/kg···3.6 min]

( ) indicates yield. [ ] indicates dose and activity (shortening of bleeding time).
Silica gel c.c., silica gel column chromatography.

Chart 1. Isolation of the Active Principle

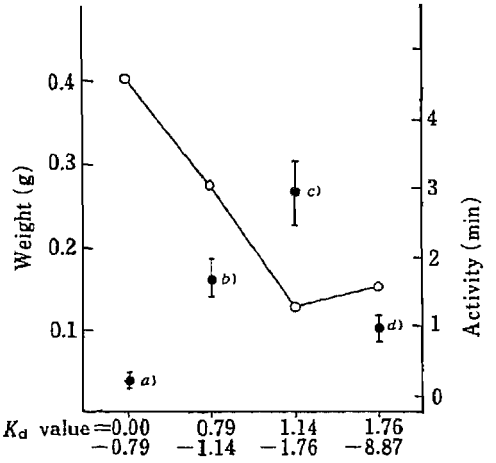

Fig. 1. Elution Pattern of the *n*-BuOH Layer (1.07 g) on a Sephadex LH-20 Column (4.4 × 80 cm) and Activities of the Fractions

O—O, chromatogram; ●, activity (shortening of bleeding time after i.p. administration at each dose [*a*) 0.042, *b*) 0.030, *c*) 0.007, *d*) 0.017 g/kg weight]). Each point represents the mean of the antihemorrhagic activity in five different experiments with the S.E. indicated.

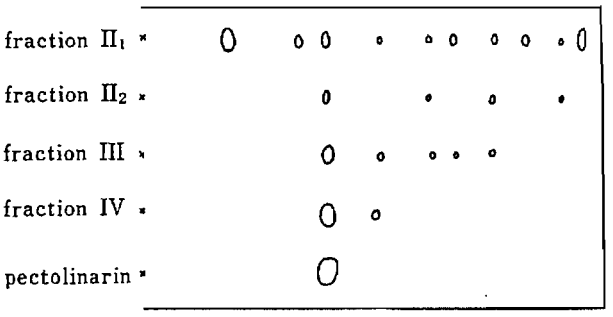

Fig. 2. Thin Layer Chromatograms of Fractions II_1, II_2, III and IV, and Pectolinarin

Plate: Kieselgel H60 (Merck Art. 5721). Solvent: lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10).

From the spectral data (nuclear magnetic resonance (NMR), infrared (IR), mass (MS) and ultraviolet (UV)), the active principle was apparently identical with pectolinarin [7-[6-*O*-(6-deoxy-β-mannopyranosyl)-β-D-glucopyranosyl]-5,7-dihydroxy-6-methoxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one]. This was confirmed by a direct comparison (mp,

NMR, MS, IR and UV) with an authentic sample. The antihemorrhagic activity of the authentic sample is equal to that of the natural product.

Pectolinarin has been isolated from leaves of *Sophora angustifolia* SIEB. *et* ZUCC,[6] *Cirsium microspicatum, C. otayae, C. Yoshizawae, C. olderaceum,* and *Lindra japonica*[3] and dried plant of *Lindra vulgaris* L.[7] There has been no pharmacological study of pectolinarin, so this is the first report that pectolinarin has a biological (hemostatic) activity *in vivo*.

In summary, we have identified pectolinarin as a hemostatically active principle of *Cirsium japonicum* DC.

Experimental

The melting point is uncorrected. The IR spectrum was recorded on a JASCO IRA-2 grating IR spectrophotometer. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded with a JEOL FX-90 Fourier-transform nuclear magnetic spectrometer and are calibrated in parts per million (δ) downfield from tetramethylsilane as an internal standard. The MS were recorded on a Hitachi M-80A instrument. The UV spectrum was recorded on a Shimadzu UV-360 recording spectrophotometer.

Assay of Hemostatic Activity——Hemostatic tests were carried out by Tajima *et al.*'s method[4] on male mice weighing 18—20 g. Test material homogenized in 1% methylcellulose–0.9% sodium chloride aq. was given interperitoneally by injection. The bleeding time was determined according to Tajima *et al.*'s method.[4]

Material——The herb used in this study was a commercial product available in China and was identified as *Cirsium japonicum* DC. by an expert.

Extraction——Ground herb of *Cirsium japonicum* DC. (200 g) was extracted with 2 l of water under reflux for 30 min. The mixture was centrifuged at 2500 rpm for 20 min and the supernatant was lyophilized to afford brown needles (19.8 g).

Partition between *n*-Butanol and Water——The crude extract (19.8 g) was dissolved in 350 ml of water and extracted with 350 ml of *n*-butanol three times. Concentration of the upper layer under reduced pressure afforded active fraction I (1.87 g).

Gel Filtration on Sephadex LH-20——Active fraction I (1.87 g) was fractionated into the soluble part (fraction II_1) (1.075 g) and precipitates (fraction II_2) (0.755 g) with 20 ml of methanol. Fraction II_1 was subjected to gel filtration through Sephadex LH-20 (4.4 × 80 cm) with methanol to afford active fraction III (K_d value = 1.14—1.76) (136.4 mg).

Silica Gel Column Chromatography of Active Fraction III——Active fraction III (136.4 mg) was chromatographed over silica gel (2.3 × 14 cm) with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10) as an eluent to afford active fraction IV (51.2 mg).

Recrystallization of Active Fraction——Active fraction V (fractions with K_d value = 1.00—1.20) (24.1 mg) was obtained by Sephadex LH-20 (2.1 × 30 cm) column chromatography of active fraction IV (51.2 mg) with methanol. This product was recrystallized from methanol to afford the active principle as light yellow needles (17.1 mg). The physical properties are described below.

Column Chromatography of Active Fraction II_2 on Silica Gel——Active fraction II_2 (755 mg) was extracted with methanol (50 ml) under reflux twice to give a light brown gum (240.6 mg). Column chromatography of this extract over silica gel (2.3 × 17 cm), using the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10) as an eluent, gave a product (51.4 mg) which contained a component with the same *Rf* value as the active principle. This fraction was gel-filtered through Sephadex LH-20 with methanol to afford a fraction with K_d 1.00—1.25. Recrystallization from methanol gave yellow needles (25.6 mg). This product was identified as pectolinarin by direct comparison with an authentic sample.

Identification of the Active Principle——The active compound has the following properties. mp 241—244 °C (248—250 °C).[6] *Anal* Calcd for $C_{29}H_{34}O_{15}$: C, 55.94; H, 5.50. Found: C, 55.67; H, 5.57. MS *m/z*: 314, 299, 271, 133, 69. High resolution MS: 314.0799 (error −1.0 mMU) for $C_{17}H_{14}O_6$. ^{1}H-NMR (in DMSO-d_6) δ (ppm): 12.95—12.50 (1H, br), 8.04 (2H, d, J = 8.8 Hz), 7.16 (2H, d, J = 8.8 Hz), 6.92 (2H, s), 5.6—4.2 (8H, m), 3.86 (3H, s), 3.77 (3H, s), 4.0—3.5 (2H, m), 1.05 (3H, d, J = 7.7 Hz). Other signals overlapped with those of the solvent. IR ν_{max}^{KBr} cm^{-1}: 3400, 2930, 1660, 1640, 1460, 1355, 1295, 1180, 835. UV λ_{max}^{MeOH} nm (log ε): 272 (4.18), 327 (4.33).

The active compound was identified as pectolinarin by direct comparison of the physical properties with those of an authentic sample.

Acetylation of Pectolinarin——The natural product (10 mg) was acetylated with pyridine (1.5 ml) and acetic anhydride (3 ml) at 90 °C for 1 h. Recrystallization of the crude product from dilute methanol gave the hepta-acetylated derivative of pectolinarin as white needles (16 mg). This derivative has the following properties. mp 131—133 °C (130—135 °C).[6] *Anal.* Calcd for $C_{43}H_{48}O_{22}$: C, 56.33; H, 5.27. Found: C, 55.80; H, 5.17. MS *m/z*: 356, 314, 273, 213, 171, 153, 127. ^{1}H-NMR (in $CDCl_3$) δ(ppm): 7.80 (2H, d, J = 8.8 Hz), 7.06 (1H, s), 7.00 (2H, d, J = 8.8 Hz),

6.51 (1H, s), 4.6—5.5 (10H, m), 3.88 (3H, s), 3.6—4.1 (2H, m), 3.82 (3H, s), 2.47 (3H, s), 2.05 (15H, s), 1.85 (3H, s), 1.18 (3H, d, J=6.6 Hz). ^{13}C-NMR (in $CDCl_3$) δ(ppm): 176.4 (s), 170.1 (s), 169.9 (s), 169.7 (s), 169.3 (s), 169.1 (s), 162.4 (s), 154.2 (s), 153.3 (s), 142.7 (s), 140.7 (s), 128.0 (d), 123.7 (s), 114.5 (d), 113.7 (d), 106.8 (d), 103.3 (d), 99.3 (d), 98.0 (s), 73.5 (d), 72.4 (d), 70.9 (d), 69.5 (d), 69.0 (d), 66.4 (d), 61.8 (d), 55.5 (d), 21.0 (q), 20.6 (q), 17.4 (q). IR ν_{max}^{KBr} cm^{-1}: 2940, 1755, 1640, 1220, 835. UV λ_{max}^{MeOH} nm (log ε): 257 (4.13), 318 (4.44).

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Application of *o*-Hydroxyhydroquinonephthalein–Iron(III) Complex to Determination of Pyridone Carboxylic Acids[1)]

YOSHIKAZU FUJITA, ITSUO MORI,* KINUKO FUJITA and TAKESHI TANAKA

Osaka University of Pharmaceutical Sciences, 2–10–65, Kawai, Matsubara, Osaka 580, Japan

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A simple spectrophotometric method for the determination of pyridone carboxylic acids (PCA) using *o*-hydroxyhydroquinonephthalein(Qnph)–iron(III) complex was developed. The present method is based on the ternary complex formation among Qnph, iron(III) and PCA in the presence of sodium dodecylbenzenesulfonate in weakly basic media. In the determination of nalidixic acid(NA), Beer's law held up to *ca.* 20 μg of NA in the final volume of 10 ml, with an apparent molar absorptivity of 1.3×10^5 l mol^{-1} cm^{-1} for NA {1.8×10^5 l mol^{-1} cm^{-1} for piromidic acid (PA), 6.6×10^4 l mol^{-1} cm^{-1} for pipemidic acid}. The proposed method was applied to the assay of NA or PA in pharmaceutical preparations and calf serum. The use of a membrane filter preconcentration technique was also investigated in an attempt to improve the proposed method.

Keywords——spectrophotometry; pyridone carboxylic acid; nalidixic acid; piromidic acid; naphthyridine; *o*-hydroxyhydroquinonephthalein; iron(III); ternary complex; anionic surfactant; membrane filter

Pyridone carboxylic acids (PCA)[2)] have recently attracted much interest as new antibacterial agents, and various compounds have been prepared. Some work has been on the determination of PCA by gas-liquid chromatography[3)] and high-performance liquid chromatography,[4)] but little work has been done on simple chemical methods, such as spectrophotometry[5)] and fluorometry.[6)] In addition, most of the reported methods[3–6)] are procedures for the determination of only nalidixic acid (NA).

On the other hand, we have shown that the absorbances of xanthene dye–iron(III) complexes are remarkably enhanced by addition of substances having the donor atoms O,O, and we reported[7)] some simple and highly sensitive methods for the determination of phosphate ion, organophosphorus compounds, citric acid and 1,2-diphenols by using the *o*-hydroxyhydroquinonephthalein(Qnph)–iron(III) or phenylfluorone(Phfl)–iron(III) complexes. In further investigations, similar phenomena were observed when PCA, especially naphthyridines such as NA and piromidic acid (PA), were used instead of phosphate ion, citric acid and so on.

In this work, a simple spectrophotometric method for the determination of PCA using the Qnph–iron(III) complex was developed. The proposed method was applied to the assay of NA or PA in pharmaceutical preparations and calf serum. The use of a membrane filter preconcentration technique[8)] was also investigated in an attempt to improve the proposed method.

Experimental

Reagents, Materials and Apparatus——Stock solutions (1.0×10^{-2} M, M = mol l^{-1}) of NA and PA were prepared by dissolving appropriate quantities of NA (Maruishi Pharmaceutical Co., Ltd.) and PA (Dainippon Pharmaceutical

Co., Ltd.) in small amounts of 1 N sodium hydroxide solution and were diluted to 10 ml with water. The working solutions were made by diluting these stock solutions as required. These solutions were stored in a cool, dark place. A Qnph solution and an iron(III) solution were prepared as 1.0×10^{-3} M methanol solution and 5.0×10^{-4} M aqueous solution, respectively, as described in the previous reports.[7a,b] A 1.0% aqueous solution of sodium dodecylbenzenesulfonate (SDBS, Tokyo Kasei Kogyo Co., Ltd.) was also prepared. A 0.2 M ammonia–0.2 M ammonium chloride buffer solution was used for pH adjustment. All other reagents and materials were of analytical reagent grade, and deionized water was used. All apparatus used was as described in the previous reports.[7a,b]

Standard Procedure——The following components were added to a 10-ml volumetric flask; a sample solution containing up to *ca.* 20 μg NA or PA, 1.0 ml of 1.0% SDBS solution, 0.5 ml of 5.0×10^{-4} M iron(III) solution, 3.0 ml of ammonium buffer solution (pH 8.2) and 0.5 ml of 1.0×10^{-3} M Qnph solution. The mixture was made up to the total volume of 10 ml with water, kept at 60 °C for 25 min, and cooled for 5 min in water. The absorbance of the Qnph–iron(III) –NA (or –PA) solution (Solution A) was measured at 615 nm against a similarly prepared Qnph–iron(III) solution (Solution B).

Results and Discussion

Figure 1 shows the absorption spectra of Solutions A and B, and their absoption difference (curve C) under the standard conditions. The absorption maximum of the curve C was at 615 nm, which was selected for the determination of PCA. A constant absorbance was obtained between pH 7.8—8.6 with 0.2 M ammonia–0.2 M ammonium chloride buffer solution.[7b]

The effect of various surfactants was examined. Surfactants used were nonionic [Tween 20, polyvinyl alcohol and gum arabic], cationic[hexadecyltrimethylammonium chloride and hexadecylpyridinium chloride] and anionic surfactants[SDBS, sodium dodecyl sulfate (SDS), sodium bis-(2-ethylhexyl)sulfosuccinate, sodium choleate and sodium oleate]. Compared with other surfactants, SDBS and SDS gave about 5—10 times better sensitivity. SBDS was somewhat superior to SDS as regards the stability of the ternary complex and rapidity of color development. A constant absorbance was observed upon addition of more than 0.5 ml of 1.0% SDBS solution in the final volume of 10 ml.

The effect of xanthene dyes in this reaction system was studied. Among the xanthene dyes[9] tested—Qnph, 4,5-dibromohydroxyhydroquinonephthalein, 3′,4′,5′,6′-tetrachlorohydroxyhydroquinonephthalein, Phfl, salicylfluorone, pyrogallol red and gallein—, Qnph was the most effective dye in terms of sensitivity and reproducibility. In this reaction system, only iron(III) (or iron(II)) was effective among various metal ions tested—iron(III,II), copper(II), zinc(II), manganese(II), palladium(II), zirconium(IV) and aluminum(III). Maximum absorbance was obtained when the molar ratio of iron(III) to Qnph was 1 : 2.

The color formation in this reaction system did not occur instantaneously at room

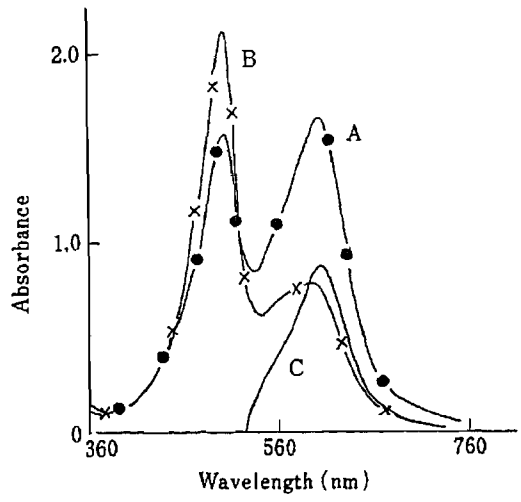

Fig. 1. Absorption Spectra

Curve A, Solution A [Qnph–Fe(III)–NA]; curve B, Solution B [Qnph–Fe(III)]; curve C, Solution A minus Solution B; NA, 7.5×10^{-6} M; Fe(III), 2.5×10^{-5} M; Qnph, 5.0×10^{-5} M; SDBS, 1.0 ml of 1.0% SDBS solution; pH 8.2; reference, water.

TABLE I. Apparent Molar Absorptivities for Some PCA

Compound	PCA classification	$\varepsilon^{a)}$
NA	Naphthyridine	1.3×10^5
PA	Naphthyridine	1.8×10^5
Pipemidic acid[b)]	Naphthyridine	6.6×10^4
Norfloxacin[c)]	Quinoline	4.7×10^3
Ofloxacin[d)]	Quinoline	7.5×10^3

a) Apparent molar absorptivity ($l\,mol^{-1}\,cm^{-1}$) at 615 nm. *b*) Dainippon Pharmaceutical Co., Ltd. *c*) Kyorin Pharmaceutical Co., Ltd. *d*) Daiichi Pharmaceutical Co., Ltd.

TABLE II. Determination of NA and PA in Commercial Pharmaceutical Preparations

Sample	Amount (mg)		Recovery test[a)]	
	Nominal	Found[a)]	Added (μg/10 ml)	%
A	250	250.0	5.8	98.6
B	50	51.9	5.8	102.8
C	250	248.7	7.2	101.1
D	200	200.5	7.2	102.1

Sample A, NA tablet (in 1 Tab. ≒ 380 mg); sample B, NA dry syrup (in 1 g); sample C, PA tablet (in 1 Tab. ≒ 300 mg); sample D, PA dry syrup (in 1 g). *a*) Mean of 5 determinations.

temperature. Experiments on the effect of time and temperature showed that maximum and constant absorbance was obtained on heating at 60 °C for 20—30 min. The absorbance remained unchanged for at least 2 h after Solutions A and B had been cooled to room temperature.

Calibration curves were constructed for PCA at 615 nm under the standard conditions. In the determination of NA, a good linear relationship was obtained over the range up to *ca.* 20 μg of NA in the final volume of 10 ml. The apparent molar absorptivity for NA was 1.3×10^5 l mol^{-1} cm^{-1}, which is about 5 times higher than that obtained by the method [5b)] using copper(II) and sodium diethyldithiocarbamate. The relative standard deviation ($n=5$) was 1.4% at 11.6 μg of NA. The other PCA were also determined under the same conditions. The sensitivities for naphthyridines were far larger than those for quinolines, as shown in Table I.

The iron(III)-to-Qnph ratio and the NA-to-iron(III) ratio as determined by the molar ratio method and the continuous variation method were 1 : 2 and 1 : 1, respectively. Thus, the ternary complex formed in this reaction system may be expressed as (NA) (Fe^{III}) $(Qnph)_2$.

Under the standard procedure, the effects of various interfering substances on the determination of 11.6 μg of NA were examined. The coexistence of metal ions such as zinc(II), copper(II) and cobalt(II) interfered with the determination when present in an equimolar amount with respect to NA. These interfering metal ions could be masked by addition of iminodiacetic acid solution. Among the anions tested, phosphate ion and hydroxypolycarboxylate ions such as citrate and tartrate gave positive errors in small amounts, but other anions did not interfere at 100- to 200-fold molar excess with respect to NA. Caffeine, taurine, cephalexin, lactose and glucose did not interfere in 100-fold molar excess. The coexistence of up to 200 μg of human albumin had no effect.

The proposed method was applied to the assays of NA and PA in commercial pharmaceutical preparations, and the experimental results were in good agreement with those

obtained by the ultraviolet (UV) method.[5c] Recoveries of NA and PA added to the samples were satisfactory (98—103%). The results are given in Table II. On the other hand, recovery of 5.8 μg of NA added to calf serum was examined, but was low (*ca.* 50—70%).

Next, the use of a membrane filter preconcentration technique[8] was investigated in an attempt to improve the proposed method with respect to sensitivity, *etc.*

This procedure did not much improve the sensitivity as compared with that in aqueous media, but had the advantage that the absorbance of the reagent blank was remarkably low. The recommended procedure is as follows: Solutions A and B obtained according to the standard procedure were each filtered through a membrane filter (nitrocellulose of 0.20 μm pore size), and the filtered substances were washed with about 20 ml of water. The membrane filters were dissolved in 5 ml of dimethyl sulfoxide, and then the absorbance at 650 nm was measured against the reagent blank.

In conclusion, though further investigations are necessary for the application of this method to biological samples, including development of the membrane filter preconcentration technique, the method should be useful and convenient for the simple determination and detection of PCA, especially naphthyridines such as NA and PA, in pharmaceutical preparations.

Acknowledgement We are grateful to Maruishi Pharmaceutical Co., Ltd., Dainippon Pharmaceutical Co., Ltd., Kyorin Pharmaceutical Co., Ltd. and Daiichi Pharmaceutical Co., Ltd. for supplying PCA.

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Peroxidase-like Catalytic Activity of Anion-Exchange Resins Modified with Metalloporphyrins in the Dye Formation Reaction of *N,N*-Diethylaniline with 4-Aminoantipyrine by Hydrogen Peroxide

YUTAKA SAITO,*[,a] MASAKI MIFUNE,[a] SUZUYO NAKASHIMA,[a] HIROMI NAKAYAMA,[a] JUNICHI ODO,[a] YOSHIMASA TANAKA,[a] MASAHIKO CHIKUMA,[b] and HISASHI TANAKA[c]

Faculty of Pharmaceutical Sciences, Okayama University,[a] 1-1-1 Tsushima-Naka Okayama 700, Japan, Osaka College of Pharmacy,[b] 2-10-65 Kawai, Matsubara-City 580, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[c] Sakyo-ku, Kyoto 606, Japan

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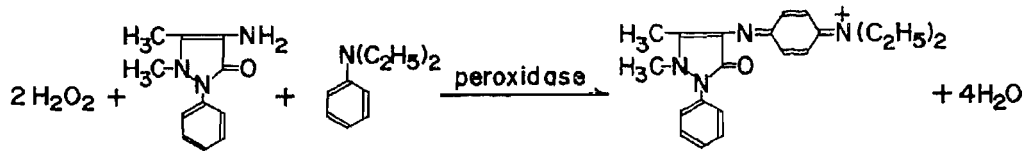
In an attempt to develop a practically useful mimesis of peroxidase, the peroxidase-like catalytic activity of anion exchange resins (Amberlite IRA 900) modified with manganese, cobalt, iron, copper and zinc complexes of some porphyrins was examined in terms of the accelerating effect on dye formation in the following reaction. The evaluation of the activity was carried out by comparison of the effect obtained by using the resins with that obtained by using peroxidase.

$$\text{4-aminoantipyrine} + \textit{N,N}\text{-diethylaniline} + 2H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinoid dye} + 4H_2O$$

Amberlite IRA 900 modified with manganese-tetrakis(sulfophenyl)porphine was found to be the best among the resins tested.

Keywords——peroxidase mimesis; resin modified metalloporphyrin; peroxidase-like activity; quinoid dye formation; functionalization ion-exchange resin

In the previous paper,[1] the peroxidase-like catalytic activity of anion-exchange resins modified with metalloporphyrins in the dye formation of phenol with 4-aminoantipyrine (AAP) by hydrogen peroxide was reported. Amberlite IRA 900 modified with manganese-tetrakis(sulfophenyl)porphine ($MnTPPS_r$) was found to be useful in the determination of hydrogen peroxide by the use of these chromogens.[2] However, in this color reaction, a large excess of phenol was necessary to obtain a good result, because phenol and the dye formed were adsorbed by the resin. Accordingly, we consider that it is more advantageous to use *N,N*-diethylaniline (DEA) which is a kind of base, as a chromogen in place of phenol (see the reaction in Fig. 1).[3]



4-aminoantipyrine (AAP) | *N,N*-diethylaniline (DEA) | quinoid dye ($\lambda_{max} = 553$ nm)

Fig. 1. Dye Formation Reaction

Experimental

Materials——Cu^{2+}- and Fe^{3+}-chlorophyllins (Cu^{2+}- and Fe^{3+}-CP) were obtained from Wako Junyaku Co., Ltd. Metal-tetrakis(sulfophenyl)porphines (M-TPPS, M=Mn^{3+}, Co^{3+}, Fe^{3+}, Zn^{2+} and Cu^{2+}), metal-tetra(*p*-carboxyphenyl)porphines (M-TCPP, M=Mn^{3+}, Co^{3+}, Fe^{3+}, Zn^{2+} and Cu^{2+}), metal-protoporphyrins (M-PP, M=Mn^{3+}, Co^{3+}, Fe^{3+}, Zn^{2+} and Cu^{2+}) and metal-tetrasulfophthalocyanines (M-PCS, M=Co^{3+} and Cu^{2+}) were prepared by methods similar to those given in the literature.[4]

Anion-exchange resins (Amberlite IRA 900) modified with metalloporphyrins (25 μmol/g) were prepared as described in the previous papers.[1,2] Peroxidase (from horseradish, type IV) was purchased from Sigma Co., Ltd. Other reagents were of analytical or reagent grade.

The chromogenic reagent solution was a 1:1:3 mixture of DEA (1.0 mg/ml), AAP (1.0 mg/ml) and 0.05 M borate buffer (pH 7.0).

Apparatus——The absorption spectra and the absorbances were measured on a Shimadzu UV-180 double-beam spectrophotometer with 10 mm quartz cells.

Evaluation of the Peroxidase-like Activity——The chromogenic reagent solution (5.0 ml) and a resin (100 mg) modified with a metalloporphyrin were added to hydrogen peroxide solution (20 μg/ml, 1.0 ml) and the mixture was incubated at 35 °C for 60 min. The absorbance of the supernatant was measured at 555 nm against the reagent blank. The control value was obtained by the use of the chromogenic solution containing 2.5 U of peroxidase (5.0 ml) and a solution of hydrogen peroxide (20 μg/ml, 1.0 ml) according to the literature.[3]

The peroxidase-like activity of each resin was evaluated in terms of the absorbance of the quinoid dye formed by the use of the resin and peroxidase.

Results and Discussion

Selection of the Conditions for Evaluation

Effect of pH——The absorbance of the resulting dye depended upon pH as shown in Fig. 2. The evaluation of the activity was carried out at pH 7.0, because the highest value was obtained at this pH (Fig. 2).

Effect of Incubation Time——With most of the resins, incubation for 30 min was sufficient, but we adopted 60 min, because in the case of iron(III)-porphyrin, a longer time was necessary as shown in Fig. 3. In the phenol–AAP system, the absorbance decrease at incubation times longer than 30 min because of the adsorption of the dye onto the modified resin. However, in the DEA–AAP system, no decrease of the absorbance was observed even at long time incubation times. This result indicates that the quinoid dye formed in this system is not adsorbed by the resins and the DEA–AAP system is more advantageous than the phenol–AAP system.

Effect of Incubation Temperature——We carried out the experiments of 35 °C, because the absorbance of the dye exhibited a maximum and constant value at the incubation

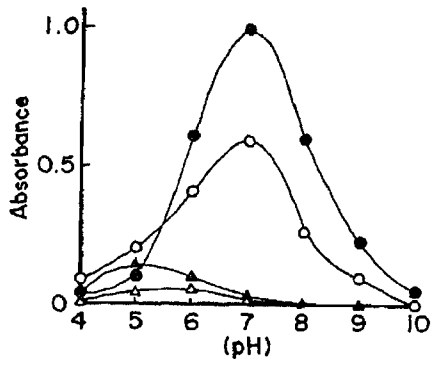


Fig. 2. Effect of pH (Incubation for 20 min at 35 °C)

—●—, Mn-TPPS$_r$; —○—, Co-TCPP$_r$; —△—, Cu-PP$_r$; —▲—, Zn-PP$_r$, Cu-PCS$_r$.

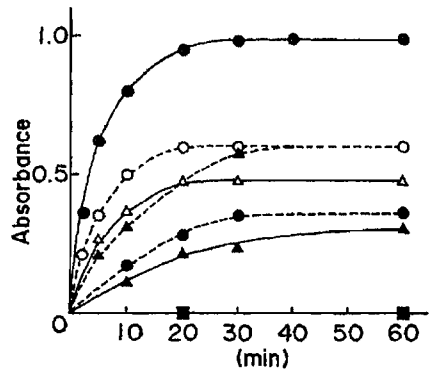


Fig. 3. Effect of Incubation Time at 35 °C in Borate Buffer (pH 7.0)

—●—, Mn-TPPS$_r$; ---○---, Mn-TCPP$_r$; ---●---, Mn-PP$_r$; ---▲---, Co-TCPP$_r$; —△—, Co-TPPS$_r$; —▲—, Fe-CP$_r$, Fe-TCPP$_r$; —■—, H_2-TPPS$_r$.

TABLE I. Absorbance of Quinoid Dye Formed[a]

Resin[b]	Absorbance[c]	Resin[b]	Absorbance[c]
Mn^{3+}-$TPPS_r$	0.978 (84.3)	Cu^{2+}-$TPPS_r$	0.010 (0.9)
Mn^{3+}-$TCPP_r$	0.611 (52.6)	Cu^{2+}-$TCPP_r$	0.050 (4.3)
Mn^{3+}-PP_r	0.370 (31.8)	Cu^{2+}-PP_r	0.008 (0.7)
Co^{3+}-$TPPS_r$	0.480 (41.3)	Cu^{2+}-CP_r	0.014 (1.2)
Co^{3+}-$TCPP_r$	0.600 (51.7)	Cu^{2+}-PCS_r	0.000 (0.0)
Co^{3+}-PP_r	0.270 (23.2)	Zn^{2+}-$TPPS_r$	0.010 (0.9)
Co^{3+}-PCS_r	0.272 (23.4)	Zn^{2+}-$TCPP_r$	0.011 (0.9)
Fe^{3+}-$TPPS_r$	0.230 (19.8)	Zn^{2+}-PP_r	0.004 (0.4)
Fe^{3+}-$TCPP_r$	0.370 (31.8)	H_2-$TPPS_r$	0.013 (1.1)
Fe^{3+}-PP_r	0.184 (15.8)	H_2-$TCPP_r$	0.062 (5.3)
Fe^{3+}-CP_r	0.314 (27.0)	H_2-PP_r	0.003 (0.3)
Control (Peroxidase)	1.160 (100)	IRA 900	0.001 (0.1)

a) Quinoid dye formed by the use of 1.0 ml of 20 μg/ml H_2O_2 solution; incubation at 35 °C for 60 min in borate buffer (pH 7.0). *b*) Subscript "r" indicates the resin modified with the corresponding metalloporphyrin. *c*) Parentheses indicate relative activity.

temperature between 30 and 40 °C.

Effect of the Amount of DEA——The same amount of DEA as used in the control peroxidase method was enough, because DEA is not adsorbed on the resin.

Peroxidase-like Activity of the Modified Resin

Table I summarizes the absorbances of the quinoid dye formed by the use of the resins, together with the value obtained by using peroxidase. The resin modified with Mn^{3+}-porphyrins exhibited strong peroxidase-like activity in most cases. The activity of Mn-$TPPS_r$ was the highest and reached more than 85% of that obtained by peroxidase, as in the case of the phenol–AAP system. Manganese as a central metal is also essential for strong peroxidase-like activity to form the quinoid dye in this system. Previously, we have found in the phenol–AAP system that the resins modified with Fe^{3+}-porphines exhibited stronger activity than the resins modified with Co^{3+}-porphines. In the DEA–AAP system, the activities of the resins modified with Fe^{3+}-porphines were found to be 49—69% of the corresponding values of the resins with Co^{3+}-porphines. This order of activity is the same as that in the case of the catalase-like activity reported previously.[5]

Repeated Use of the Resins

In order to confirm that the resins behave as catalysts, the effect of repeated use on the absorbance generated was examined. During ten cycles of use, essentially no difference in the absorbance of the dye formed was observed. On the other hand, lower absorbance caused by the adsorption of the dye was observed in the phenol–AAP system. The above result indicates clearly that the resins behave as the catalysts, and the dye formed is not adsorbed on the resin in the DEA–AAP system.

In conclusion, the DEA–AAP system presented here is better as a color reaction than the phenol–AAP system for the practical use of resins modified with metalloporphyrins in the determination of hydrogen peroxide.

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Antitumor Activity and Lethal Toxicity of Chemically Synthesized Tetraacetyl-2-keto-3-deoxyoctonic Acid-(α2→6)-D-glucosamine Analogues of Lipid A

TADAYORI SHIMIZU,* SHIN-ICHIRO AKIYAMA, TOSHIYUKI MASUZAWA, YASUTAKE YANAGIHARA, SHIN-ICHI NAKAMOTO, and KAZUO ACHIWA

Shizuoka College of Pharmacy, 2-2-1 Oshika, Shizuoka 422, Japan

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The antitumor activity and lethal toxicity of three acylated derivatives of chemically synthesized 4-*O*-monophosphorylglucosamine-linked tetraacetyl 2-keto-3-deoxyoctonic acid (KDO) were determined. Although the antitumor activity of the compounds against ascites form of Ehrlich carcinoma in ddY mice was weaker than that of natural lipopolysaccharide, compound A-203, with di-3-tetradecanoyloxytetradecanoyl at the C-2 and C-3 positions, was effective. Compounds A-204 (di-3-dodecanoyloxytetradecanoyl) and A-205 (3-dodecanoyloxytetradecanoyl and 3-tetradecanoyloxytetradecanoyl) produced longer survival than the control group, but the differences were not significant. On the other hand, the lethal toxicity of A-205 in C57BL/6 mice loaded with galactosamine was most potent among the three compounds, whereas A-203 did not show lethality even at a high dose (50 μg/mouse). However, the antitumor activity and lethal toxicity of the derivatives of 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO did not differ from those of KDO-free 4-*O*-monophosphorylglucosamine derivatives.

Keywords——lipopolysaccharide; lipid A monosaccharide; 2-keto-3-deoxyoctonic acid; antitumor activity; lethal toxicity

Introduction

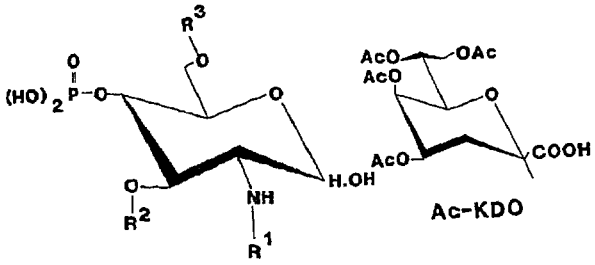
Lipid A is known to be responsible for the expression of many biological activities of lipopolysaccharide (LPS) of gram-negative bacteria.[1] Recently, it has been shown that the biological activities of chemically synthesized lipid A analogues[2] are almost the same as those of natural lipid A.[3] Furthermore, there are several reports on the biological activities of natural[4] and chemically synthesized[5] monosaccharide analogues of lipid A.

Recent chemical analyses revealed that the C-6 position of the nonreducing glucosamine moiety in lipid A of the Re mutants of *Salmonella typhimurium*[6] and *Escherichia coli*[7] is the attachment site to the C-2 position of 2-keto-3-deoxyoctonic acid (KDO), which is well known as the acidic sugar of the core region of LPS. However, the role(s) of KDO in the various biological activities of LPS is still unclear.

In the previous paper,[8] we reported that the acylated derivatives of the 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO are capable of increasing the incorporation of ^{3}H-thymidine into splenocytes *in vitro*. In this paper, we describe the antitumor activity against ascites form of Ehrlich carcinoma and the lethal toxicity of three synthetic derivatives of 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO in comparison with those of bacterial LPS.

Materials and Methods

Test Materials——All compounds tested in this study were synthesized as previously described[9] and their



Compounds	Substituents		
	R^1 (N–)	R^2 (3-O–)	R^3 (6-O–)
A-203	C_{14}–O–C_{14}	C_{14}–O–C_{14}	Ac-KDO
A-204	C_{14}–O–C_{12}	C_{14}–O–C_{12}	Ac-KDO
A-205	C_{14}–O–C_{12}	C_{14}–O–C_{14}	Ac-KDO
A-105	C_{14}–O–C_{12}	C_{14}–O–C_{14}	H

Ac-KDO, tetraacetyl-2-keto-3-deoxyoctonic acid; C_{14}–O–C_{12}, (*R*)-3-dodecanoyloxytetradecanoyl; C_{14}–O–C_{14}, (*R*)-3-tetradecanoyloxytetradecanoyl.

Fig. 1. Structures of Synthetic Monosaccharide Analogues, Tetraacetyl-KDO-(α2→6)-glucosamine-4-phosphate, of Lipid A

chemical structures are shown in Fig. 1. Before experiments, all compounds were suspended in pyrogen-free saline (Ohtsuka Seiyaku Co., Ltd., Osaka) and sonicated for 20—30 s. Reference LPSs of *S. typhimurium* LT-2 and *Vibrio anguillarum* PT-514 were isolated from dried cells by the hot phenol–water technique of Westphal *et al.*[10] and purified by ultracentrifugation (105000 × *g*, 1 h).

Animals and Tumor Cells——Male C57BL/6 mice and ddY mice weighing 20—25 g, purchased from the Shizuoka Agricultural Cooperative for Laboratory Animals, Hamamatsu, were used. Ehrlich carcinoma cells in ascites form were maintained by weekly passage in ddY mice.

Test for Antitumor Activity——To examine the antitumor activity of synthetic compounds, each group of mice was inoculated intraperitoneally (i.p.) with 1×10^4 Ehrlich carcinoma cells. The test compounds and reference materials,[11] whole cells of *Vibrio anguillarum* and its LPS, were injected i.p. into mice twice prior to tumor inoculation and 3 times after the inoculation. Control mice inoculated with tumor cells were given i.p. physiological saline alone at the same times. Survivors were counted every day. Twenty-five days after the tumor inoculation, the number of survivors was determined.

Lethal Toxicity——Lethality tests were carried out according to the method of Galanos *et al.*[12] In brief, groups of C57BL/6 mice were sensitized by intraperitoneal injection of 640 mg/kg of D-galactosamine hydrochloride (Wako Pure Chemical, Osaka) in 0.5 ml of saline, then intravenous (i.v.) injection of a test compound in 0.2 ml of saline was immediately performed. The death of mice was observed over a 24 h period.

Results and Discussion

To determine the antitumor activity of the three derivatives of 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO, the compounds were administered i.p. on days −5, −2, +1, +3, and +5 (Table I). The dose of 100 μg/d/mouse of the compounds was used in the experiment on antitumor effect, because *V. anguillarum* LPS, used as the reference material, showed marked activity at the dose of 100 μg or more/d/mouse.[11] Although the antitumor activity of the synthetic compounds was weaker than that of whole cells of *V. anguillarum* and its LPS, A-203 with two acyloxyacyl groups (C_{14}-O-C_{14} and C_{14}-O-C_{14}) at the C-2 and C-3 positions, respectively, was effective ($p < 0.05$). Compounds A-204 (C_{14}-O-C_{12} and C_{14}-O-C_{12}) and A-205 (C_{14}-O-C_{12} and C_{14}-O-C_{14}) produced longer survival than the control group, but the differences were not significant ($p > 0.05$).

Few studies have dealt with *in vivo* antitumor activity of synthetic lipid A analogues.[13] In the previous paper,[14] we reported that acylated derivatives of KDO-free 4-*O*-monophosphorylglucosamine are effective against ascites form of Ehrlich carcinoma cells. However, it was found here that the antitumor activity of 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO is not enhanced by the attachment of the tetraacetyl-KDO.

Next, we determined the lethal toxicity of the synthetic compounds in C57BL/6 mice loaded with galactosamine–HCl (640 mg/kg) according to the method of Galanos *et al.*[12] Injection of LPS of *S. typhimurium* LT-2 caused the death of all mice within 6—8 h after the

TABLE I. Antitumor Activity of Synthetic Monosaccharide Analogues, Tetraacetyl-KDO-(α2→6)-glucosamine-4-phosphate, of Lipid A against Ascites Form of Ehrlich Carcinoma

Preparation[a)]	Dose (μg/d)	Mean survival days (mean±S.E.)	25-d survivors/ No. of mice tested
A-203	100	23.2±1.0 ($p<0.05$)	1/6
A-204	100	19.8±0.7	0/6
A-205	100	21.3±1.3	1/6
V. anguillarum PT-514 LPS	100	25.0	8/8
V. anguillarum PT-514 whole cells	500	25.0	8/8
Saline (control)		18.1±0.9	0/8

Ehrlich carcinoma cells (1×10^4) were inoculated i.p. into ddY mice on day 0. *a*) Each preparation was given i.p. to mice on days −5, −2, +1, +3, and +5.

TABLE II. Lethal Toxicity of Synthetic Monosaccharide Analogues, Tetraacetyl-KDO-(α2→6)-glucosamine-4-phosphate, of Lipid A in Galactosamine-Sensitized Mice

Preparations	No. of deaths/No. of mice tested with indicated dose (μg)				
	0.1	5	10	25	50
A-203	n.t.	0/3	0/3	0/3	0/4
A-204	n.t.	0/3	0/3	0/3	2/4
A-205	n.t.	0/3	0/3	3/3	3/3
A-105	n.t.	n.t.	2/3	4/4	n.t.
S. typhimurium LT-2 LPS	5/5	n.t.	n.t.	n.t.	n.t.

Mice were treated i.p. with D-galactosamine HCl (640 mg/kg) and, within 30 min, the test preparations were injected intravenously. n.t.; not tested.

treatment at a dose of 0.1 μg (Table II).

Compound A-205 showed potent lethal toxicity in the high dose range of 25 to 50 μg/mouse within 10 h after the treatment, while 2 out of 4 mice died at the dose of 50 μg of A-204. However, A-203 did not show lethality at 50 μg/mouse. The findings indicate that the lethal toxicity of 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO is no greater than that of the KDO-free compound (A-105).

Amano *et al.*[15] have reported that the presence of KDO may be necessary for the expression of antitumor activity and mitogenic activity of bacterial LPS. In a previous report,[8] we showed that the presence of tetraacetyl-KDO enhances the mitogenic activity of acylated derivatives of 4-*O*-monophosphorylglucosamine. However, we have found here that the presence of tetraacetyl-KDO did not affect the expression of antitumor activity and lethal toxicity.

Of the compounds used in the experiments, A-203 (C_{14}-O-C_{14} and C_{14}-O-C_{14}) and A-205 (C_{14}-O-C_{12} and C_{14}-O-C_{14}) have the same chemical structures as the nonreducing glucosamine moiety of *Proteus*[16] and that of *E. coli* or *Salmonella*,[16] respectively, but A-204 (C_{14}-O-C_{12} and C_{14}-O-C_{12}) has not been found in LPS of any bacteria. As shown in Table II, the lethal toxicity of A-205 (*E. coli* and *Salmonella* type) is higher than that of A-203 (*Proteus* type) or A-204. In contrast, the antitumor activity of A-203 was most effective among the

compounds (Table I). The reason why the biological activities of the three compounds were different is not clear, but it may be related to the different acyloxyacyl groups in each compound.

The 4-*O*-monophosphorylglucosamine-linked tetraacetyl-KDO showed higher solubility than the tetraacetyl-KDO-free compound (A-105). However, the enhancement of the solubility by the attachment of acetyl-KDO did not affect the *in vivo* antitumor activity or lethal toxicity. To determine the role(s) of KDO in the biological activity of LPS, the synthesis of acylated derivatives of 4-*O*-monophosphorylglucosamine-linked acetyl-free KDO is in progress in our laboratory.

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A Rapid and Simple Procedure for Purification of S-II, a Transcription Factor of Ribonucleic Acid Polymerase II

SHOJI HIRASHIMA,[a] MASAMI HORIKOSHI,[a] YOSHINOBU NAKANISHI,[a] HIROSHI HIRAI,[a] KAZUHISA SEKIMIZU,[a] DEN'ICHI MIZUNO,[b] and SHUNJI NATORI*,[a]

Faculty of Pharmaceutical Sciences, University of Tokyo,[a] Bunkyo-ku, Tokyo 113, Japan and Faculty of Pharmaceutical Sciences, Teikyo University,[b] Sagamiko, Tsukui-gun, Kanagawa 199-01, Japan

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We previously reported the purification of eukaryotic transcription factor S-II and its phosphorylated form S-II′ from Ehrlich ascites tumor cells. In this work, we modified the previous purification procedure to make it quicker and simpler. By this improved procedure, involving a different solubilization method, and improved buffer solutions and centrifugation processes, S-II could be purified to homogeneity from 1 kg of frozen cells in about 10 d instead of 1 month. The purity, specific activity and total yield of S-II purified by the new method were comparable to those obtained by using the previous method.

Keywords——transcription; S-II; stimulatory factor; RNA polymerase II; Ehrlich ascites tumor cell

Introduction

Multiple factors have been found to participate in transcription mediated by ribonucleic acid (RNA) polymerase II.[1,2] For elucidation of the mechanism of transcription, it is therefore indispensable to isolate and characterize these factors. Although chromatographic fractionations of these factors have been reported,[1-7] none of the factors has yet been purified to homogeneity.

We have purified a stimulatory protein of RNA polymerase II, named S-II and its phosphorylated form S-II′, and another related protein S-I(b) to homogeneity from Ehrlich ascites tumor cells.[8-10] Experiments using antibody against S-II showed that S-II is one of the essential transcription factors for RNA polymerase II.[11,12] Furthermore, S-II was demonstrated to form a stoichiometric complex with RNA polymerase II in the initiation step, and this complex was shown to be stable during the elongation step of transcription.[13] Moreover, results suggested that the function of S-II is regulated by phosphorylation and dephosphorylation of the protein molecule.[14-16] Therefore, for studies on the mechanism of eukaryotic transcription, S-II must be obtained in quantity. However, our previous method for purification of S-II was laborious and about 30 d were required to purify 1—2 mg of S-II from 1 kg of Ehrlich ascites tumor cells.

This paper describes a rapid, simple method for the purification of S-II. By modification of the solubilization procedure and several other conditions, almost the same amount and the same quality of S-II could be obtained from 1 kg of Ehrlich ascites tumor cells in about 10 d.

Materials and Methods

Cells, Buffer Solutions, and Chemicals——Ehrlich ascites tumor cells were grown and harvested as described

before.[17] Buffer I was composed of 50 mM Tris–HCl, pH 7.9, 5 mM 2-mercaptoethanol, and 0.1 mM ethylenediaminetetracetic acid (EDTA). Buffer II consisted of 10 mM Tris–HCl, pH 7.9, containing 5 mM 2-mercaptoethanol. Buffer III had the same composition as buffer I, but was supplemented with 5 mM $MgCl_2$. Buffer IV consisted of 50 mM Tris–HCl, pH 7.9, 5 mM 2-mercaptoethanol, and 0.1% (v/v) Triton X-100. Buffer V was the same as buffer IV except that the Tris–HCl concentration was reduced to 10 mM. Buffer VI was composed of 10 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl, and 50% (v/v) glycerol.

Dimethylaminoethyl (DEAE)-cellulose (DE 52), phosphocellulose (P 11), and carboxymethyl (CM)-cellulose (CM 52) were purchased from Whatman. ^{3}H-uridine triphosphate (UTP) was from Amersham Japan Corp.

Assay of Stimulatory Factor of RNA Polymerase II——This assay was done essentially as described before, using partially purified RNA polymerase II from Ehrlich ascites tumor cells[18] and CsCl-purified Ehrlich deoxyribonucleic acid (DNA).[8] One unit of stimulatory activity was defined as the amount that increased the activity of 10 units of RNA polymerase II to 11 units under the standard assay conditions.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis and Protein Determination

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli in 12.5% acrylamide gel.[19] After electrophoresis, proteins were located by staining with Coomassie Brilliant Blue by the method of Fairbanks *et al.*[20] Protein was determined by a modification of the method of Lowry *et al.*,[21] as described before.[8]

Results and Discussion

Purification of S-II

All subsequent procedures were performed at 0—4 °C unless otherwise stated.

Solubilization of S-II——About 1 kg of Ehrlich ascites tumor cells was homogenized in 1 l of buffer I containing 0.2% (w/v) sodium deoxycholate in a Polytron homogenizer (Kinematica, Switzerland) at a power setting of 10 for 10 min. Then, 128 ml of saturated $(NH_4)_2SO_4$ solution was added, and the mixture was homogenized again under the same conditions for 15 min. Then 3.4 l of buffer I was added and the mixture was centrifuged for 30 min at $10000 \times g$. The supernatant was saved for the next step. The efficiency of solubilization of stimulatory factors was monitored by radioimmunoassay with antibody against S-II,[22] since stimulatory activity could not be quantitated under the standard assay conditions due to contamination with other proteins in the preparation. Results showed that the efficiency of solubilization was comparable to that in the previous method (data not shown). Thus, the procedures of homogenization in a motor-driven homogenizer and sonication used previously could be replaced by disruption in a Polytron. We used a regular refrigerated centrifuge instead of an ultracentrifuge to obtain solubilized samples and did not add glycerol to buffers. These improvements of the procedure saved much time.

First $(NH_4)_2SO_4$ Precipitation——About 5 l of supernatant was mixed with a half volume of saturated $(NH_4)_2SO_4$ solution and the mixture was stirred for 30 min. The resulting precipitate was removed by centrifugation for 50 min at $10000 \times g$, and the supernatant was collected. Solid $(NH_4)_2SO_4$ was added to the resulting clear supernatant at 540 g per liter, and the mixture was stirred for 1 h. Precipitated proteins were collected by centrifugation for 50 min at $10000 \times g$, dissolved in 100 ml of buffer II, and dialyzed against 5 l of the same buffer for 18 h with two changes of the outer solution. Insoluble materials appearing during dialysis were removed by centrifugation for 30 min at $10000 \times g$, and the buffer was changed to buffer III by adding concentrated solutions of constituents.

DEAE-Cellulose Column Chromatography and Second $(NH_4)_2SO_4$ Precipitation——The dialyzed material was applied to a column of DEAE-cellulose (7.5×25 cm) equilibrated with buffer III at a flow rate of 180 ml/h, and the column was then washed with 2 l of buffer III. Unadsorbed fractions with optical densities at 280 nm of more than 0.5 were collected and combined. Then 1.5 volumes of saturated $(NH_4)_2SO_4$ solution was added to this unadsorbed fraction with thorough mixing. Then solid $(NH_4)_2SO_4$ was added at 300 g per liter of the mixture with stirring for 1 h. The resulting precipitate was collected by centrifugation for 50 min at $10000 \times g$, dissolved in 50 ml of buffer II, and dialyzed against 5 l of buffer II for 10 h with two changes of the outer solution. Insoluble materials that appeared

during dialysis were removed by centrifugation for 30 min at $10000 \times g$, and the clear supernatant was collected.

Ethanol Precipitation——The supernatant was mixed with a half volume of ethanol that had been kept at -20 °C, and stirred for 1 min. The mixture was kept at -20 °C for 1 h to complete protein precipitation, and then centrifuged for 10 min at $2000 \times g$. The resulting pellet was suspended in 100 ml of buffer IV, and homogenized in a glass homogenizer by 10 strokes of a Teflon pestle. The homogenate was centrifuged for 30 min at $10000 \times g$, and the clear supernatant was collected.

Phosphocellulose Column Chromatography——The soluble fraction thus obtained was loaded on a column of phosphocellulose (2.5 × 10 cm) equilibrated with buffer IV. Then the column was washed successively with one column volume of buffer IV, three column volumes of buffer IV containing 0.1 M NaCl, and five column volumes of buffer IV containing 0.3 M NaCl. S-I(b) activity was detected in the eluate with 0.3 M NaCl, indicating that S-I(b) was also solubilized by this procedure. S-II and S-II′ were then eluted with 800 ml of a linear gradient of 0.3—0.8 M NaCl in buffer IV. Fractions of 5 ml were collected and 5 μl of each fraction was assayed for activity to stimulate RNA polymerase II. S-II and S-II′ were eluted as a single peak with 0.45 M NaCl. About 50 ml of active fraction was dialyzed against 5 l of buffer V for 10 h with two changes of the outer solution.

CM-Cellulose Column Chromatography——The resulting dialyzate was loaded on a column of CM-cellulose (1.5 × 8 cm) equilibrated with buffer V. The column was washed with three column volumes of buffer V, and adsorbed materials were eluted with 600 ml of a linear gradient of 0—0.1 M NaCl in buffer V. Fractions of 3 ml were collected and the stimulatory activity of 5 μl of each fraction was assayed. As shown in Fig. 1, S-II′ and S-II were eluted with 35 and 45 mM NaCl, respectively, as found previously. However, the amount of S-II′ relative to S-II was less than with the previous method and it varied from preparation to preparation. Since the recovery of S-II was about the same, the new method was probably less efficient for solubilization of S-II′. Active fractions of S-II and S-II′ were pooled separately, dialyzed

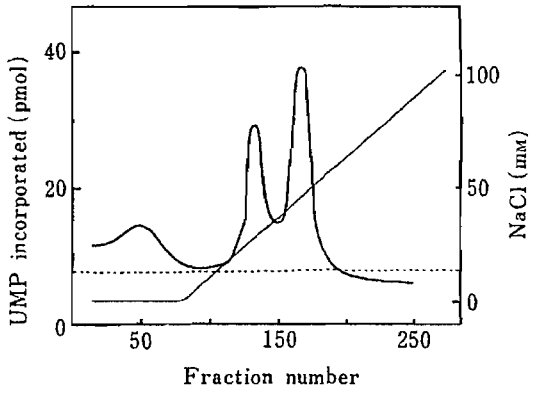

Fig. 1. CM-Cellulose Column Chromatography of S-II and S-II′

Active fractions from the phosphocellulose column were combined and loaded on a column of CM-cellulose as described in the text. The RNA polymerase II stimulatory activity of each fraction (thick line) and the NaCl concentration (thin line) are indicated. The broken line indicates the amount of uridine monophosphate (UMP) incorporated with RNA polymerase II alone.

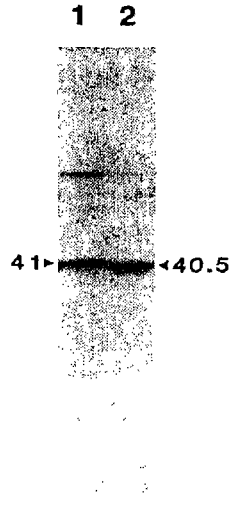

Fig. 2. SDS-Polyacrylamide Gel Electrophoresis of the Final Preparations of S-II′ and S-II

Active fractions of S-II′ (lane 1) and S-II (lane 2) from the CM-cellulose column were pooled and analyzed by SDS-polyacrylamide gel electrophoresis. The molecular weights determined with reference to various standard marker proteins are shown in kilodaltons.

TABLE I. Summary of Purification of S-II

Purification step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg protein)	Recovery (%)
DEAE-cellulose	890	4700	1800000	380	100
Second $(NH_4)_2SO_4$ precipitation	270	1900	1500000	790	83
Ethanol precipitation	990	420	910000	2200	51
Phosphocellulose	106	21	220000	10000	12
CM-cellulose	7.8	1.2	36000	30000	2

against buffer VI, and stored at −20 °C.

Evaluation of the New Purification Method

As shown in Fig. 2, at the CM-cellulose step, S-II was almost homogeneous, but S-II′ was not still pure, when pooled fractions of S-II and S-II′ from CM-cellulose step were analyzed by SDS-polyacrylamide gel electrophoresis. Therefore, with this new method after the CM-cellulose step we could omit the second DEAE-cellulose column chromatography, a step that was essential for obtaining homogeneous S-II in the previous method.[8] The purity of S-II′ at the CM-cellulose step was always lower than that obtained by the previous method. Therefore, we concluded that this new procedure is not suitable for purification of S-II′. A summary of a typical purification of S-II is shown in Table I. The specific activity and yield of purified S-II were comparable with those obtained by the previous method. Thus, we conclude that the new method for purification of S-II is better than the previous one. Starting from the same amount of frozen cells, it was possible to save more than two weeks by improving the procedure for solubilizing stimulatory proteins and omitting the second DEAE-cellulose column chromatography. Use of a regular refrigerated centrifuge instead of an ultracentrifuge and omission of glycerol from buffer solutions also made the purification procedure much easier.

We believe that this new method for purifying S-II should be useful in studies on the regulation of eukaryotic transcription and transcription factors.

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Study of the Interactions between Sulfamethizole and Alclofenac, Diclofenac or Tiaprofenic Acid in Rats

KAZUMOTO CHIBA, SATOSHI KIKUCHI, SEIKUN NISHIMURA, NAOYO HIGUCHI, SHOZO MIYAZAKI and MASAHIKO TAKADA*

Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Kanazawa, Ishikari-Tobetsu, Hokkaido 061-02, Japan

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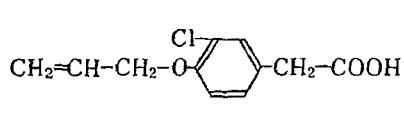
In this study, the effects of three non-steroidal anti-inflammatory drugs, alclofenac, diclofenac and tiaprofenic acid, on the plasma level of sulfamethizole were investigated in rats. The persistence of sulfamethizole in plasma was prolonged by coadministration of each anti-inflammatory drug. In experiments to investigate the renal excretion of sulfamethizole, the clearance ratio of sulfamethizole was markedly decreased after infusion of each anti-inflammatory drug. From these results, it is speculated that prolongation of the persistence of sulfamethizole in plasma by alclofenac, diclofenac or tiaprofenic acid is mainly caused by competitive interactions between sulfamethizole and alclofenac, diclofenac or tiaprofenic acid at the renal secretory level.

Keywords——drug interaction; alclofenac; diclofenac; tiaprofenic acid; sulfamethizole; pharmacokinetic parameter; proximal tubular secretion; renal excretion

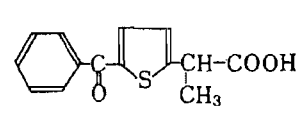
Pharmacokinetic drug interaction is the modification of the pharmacokinetic behavior of one drug by another drug. Therefore, elucidation of pharmacokinetic drug interactions is very important for ensuring the effectiveness and safety of clinical drug therapy.[1]

Alclofenac (ACF) and diclofenac (DCF), which are phenylacetic acid derivatives, and tiaprofenic acid (TPA), which is a propionic acid derivative, are relatively new and effective non-steroidal anti-inflammatory drugs.[2] These drugs have been frequently applied clinically in combination with other drugs. We previously reported studies on the interactions between sulfonamides and several non-steroidal anti-inflammatory drugs in rats.[3,4]

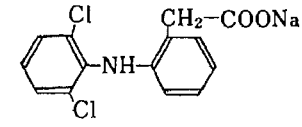
In this study, we investigated the interactions between sulfamethizole (SMZ) and ACF, DCF or TPA by means of the determination of plasma drug concentration and renal clearance in rats.



alclofenac (ACF)



tiaprofenic acid (TPA)



diclofenac sodium (DCF)

Chart 1

Experimental

Materials——ACF: Commercially available ACF was recrystallized from EtOH, mp 91—94 °C. DCF: Commercially available DCF was recrystallized from EtOH as the sodium salt, dichlofenac sodium, mp 280—282 °C. TPA: Commercially available TPA was recrystallized from EtOH, mp 94—97 °C. SMZ was recrystallized from EtOH, mp 207—208 °C. All other chemicals were of reagent grade and were used without further purification.

Plasma Level of SMZ in Rats——Male Wister rats weighing 250—350 g were used in this study. They were anesthetized with sodium pentobarbital (30 mg/kg body weight) and were intubated to allow free respiration. After

the intubation, the left femoral vein and right femoral artery were catheterized with polyethylene tubing (PE-50). SMZ at a dose of 30 mg/kg was administered to the rats through the left femoral vein. In coadministration experiments, an anti-inflammatory agent at a dose of 10 mg/kg was also administered through the left femoral vein immediately after administration of SMZ. SMZ was used as an aqueous solution of sodium sulfamethizole. Each anti-inflammatory drug was used as a suspension in 0.5% Tween 80. At each sampling time, about 0.2 ml of blood was withdrawn through the right femoral artery, and the plasma was obtained by centrifugation.

Pharmacokinetic Analysis——The elimination half-life ($t_{1/2}$) was estimated by least-squares fitting of the plasma levels using a microcomputer (NEC PC-8801).

Renal Clearance Experiment——Standard methods for renal clearance[5] were employed. Male Wistar rats weighing 240—350 g were used in these experiments. Sodium pentobarbital at the dose of 30 mg/kg was used for anesthetization. After intubation and catheterization of the left femoral vein and right femoral artery, the rats were primed with SMZ (20 mg/body) and inulin (40 mg/body) through the left femoral vein, and sustained infusion of SMZ (0.35 mg/min) and inulin (0.2 mg/min) in saline was continued throughout the experiment. The left ureter was catheterized with polyethylene tubing (PE-10) by the retroperitoneal approach procedure.[6] Constant urine flow was usually obtained within 1 h after the start of infusion. After attainment of constant urine flow, three control clearance periods were carried out. Immediately thereafter, an anti-inflammatory drug (2 mg/body) was primed through the femoral vein for blockade of proximal tubular secretion of SMZ, and sustained infusion of the anti-inflammatory drug (0.35 mg/min) was continued. About 30 min after primed infusion of the anti-inflammatory drug, three experimental clearance periods were carried out. Drug clearance (C, ml/min) was calculated as $C = UV/P$, where U, P and V are the urine and plasma concentrations of the drug in mg/ml, and the urine flow rate in ml/min, respectively. To estimate the renal handling of the drug, clearance ratio (CR) has been conventionally used and was expressed as $CR = C/GFR$, where GFR is the glomerular filtration rate in ml/min.

Analytical Method——For the determination of SMZ, 60 μl of MeOH and 100 μl of H_2O were added to 50 μl of plasma. The mixture was shaken and centrifuged at 3000 rpm for 10 min. Then 10 μl of the supernatant or 10 μl of urine sample was taken, 420 μl of H_2O and 10 μl of internal standard solution (sulfamethoxazole) were added, and the mixture was filtered through a Millipore Filter HV (0.45 μm diameter). A 20 μl aliquot of the filtrate was subjected to high-performance liquid chromatography (HPLC). A high-performance liquid chromatograph (Shimadzu LC-5A) equipped with a ultraviolet (UV) detector (245 nm, Shimadzu SPD-2A) was used with a stationary phase of Zorbax C_8 (5—6 μm particle diameter) packed in 25 cm × 4.6 mm i.d. stainless-steel tubing. The mobile phase was 0.2 M sodium phosphate (monobasic pH 5.6) mixed with acetonitrile at a volume ratio of 3/2, and the flow rate was maintained at 0.5 ml/min.

Inulin was determined by a modification of the method described by Dische and Borenfreund.[7] The temperature at which the reaction mixture was left standing, was modified at 40 °C (room temperature is recommended in the original procedure[7]).

Results

Elimination Profile of SMZ with or without ACF, DCF or TPA from Plasma

The plasma level of SMZ after coadministration with or without ACF, DCF or TPA was studied in rats. In this study, when SMZ was coadministered with ACF, DCF or TPA, we could not calculate pharmacokinetic parameters of the distribution phase of plasma SMZ precisely, and calculation of the elimination half-life ($t_{1/2}$) of plasma SMZ was carried out from the slope of the elimination phase of palsma SMZ. Partial time courses of plasma concentration of SMZ after intravenous administration of SMZ with or without ACF, DCF or TPA coadministration are shown in Fig. 1. The elimination half-life ($t_{1/2}$) of SMZ is shown in Table I. When SMZ was coadministered with ACF, DCF or TPA, its elimination half-life ($t_{1/2}$) was prolonged as compared with the control value, as shown in Table I. In particular, when SMZ was coadministered with DCF, its elimination half-life ($t_{1/2}$) was 17.0 ± 1.15 h and this value was approximately 5 times the control value (3.38 ± 0.206 h). On the other hand, when SMZ was coadministered with ACF or TPA, its elimination half-life ($t_{1/2}$) was 10.3 ± 0.273 h or 8.52 ± 0.602 h, respectively, and the prolongation of elimination half-life ($t_{1/2}$) of SMZ was less marked.

Interactions between SMZ and ACF, DCF or TPA at the Renal Level

For renal clearance experiments, nine rats were used to determine whether the renal excretion of SMZ could be altered by ACF, DCF or TPA infusion. The results are shown in

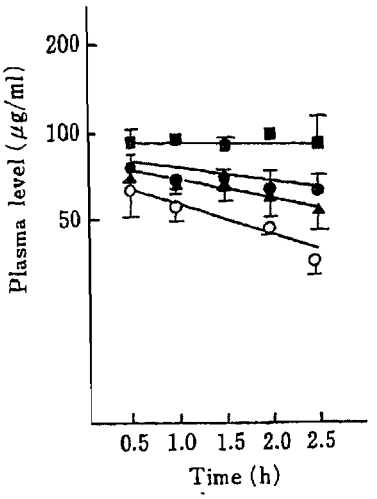


Fig. 1. Effect of ACF, DCF or TPA on the Plasma Levels of SMZ in Rats

○, SMZ alone ($n=3$); ●, SMZ with ACF ($n=3$); ■, SMZ with DCF ($n=3$); ▲, SMZ with TPA ($n=4$). The S.D. is indicated by a bar.

Table I. Values of Elimination Half-life for SMZ with or without Coadministration of ACF, DCF or TPA in Rats[a]

Drug	$t_{1/2}$ (h^{-1})
SMZ ($n=3$)	3.38 ± 0.206
SMZ+ACF ($n=3$)	10.3 ± 0.273[b]
SMZ+DCF ($n=3$)	17.0 ± 1.15[b]
SMZ+TPA ($n=4$)	8.52 ± 0.602[b]

a) Each value is the mean ± S.D. b) Significantly different from the control rats, $p<0.001$.

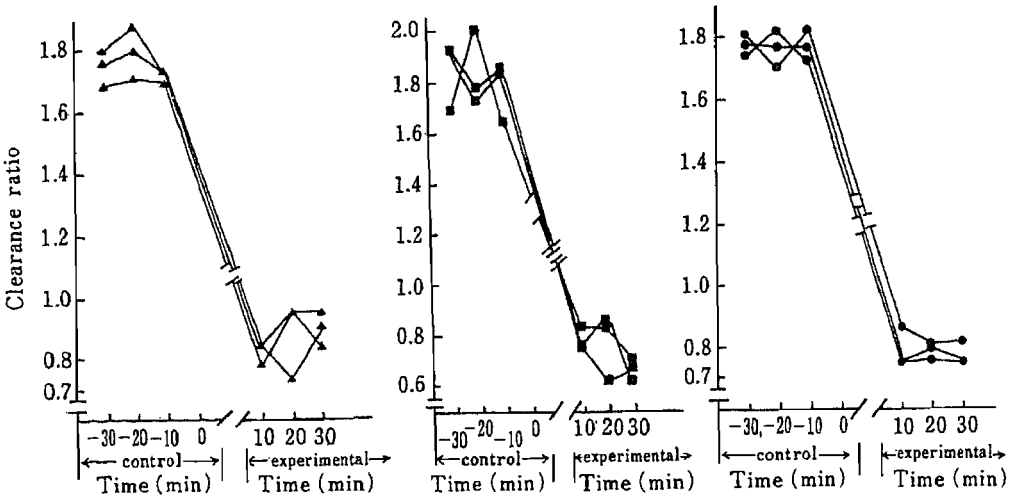


Fig. 2. Clearance Ratio of SMZ before and after Blockade of Proximal Tubular Secretion by ACF, DCF, or TPA in Rats

▲, SMZ with ACF; ■, SMZ with DCF; ●, SMZ with TPA.

Table II. Effect of ACF on Renal Clearance of SMZ in a Rat[a]

	Time (min)	GFR[c] (ml/min)	C[d] (ml/min)	CR[e]
Control	30—20	10.8	18.0	1.67
	20—10	9.47	16.2	1.71
	10— 0	10.5	17.8	1.70
Exptl[b]	30—40	13.1	10.1	0.771
	40—50	15.1	14.5	0.960
	50—60	15.7	13.1	0.834

a) Weight: 250 g. b) Experimental. c) Glomerular filtration rate. d) Drug clearance. e) Clearance ratio.

TABLE III. Effect of DCF on Renal Clearance of SMZ in a Rat[a]

	Time (min)	GFR[c] (ml/min)	C[d] (ml/min)	CR[e]
Control	30—20	11.4	19.4	1.70
	20—10	13.7	28.6	2.09
	10— 0	15.5	25.9	1.65
Exptl[b]	30—40	17.3	14.3	0.827
	40—50	14.4	11.8	0.819
	50—60	20.2	14.1	0.698

a) Weight: 250 g. *b*) Experimental. *c*) Glomerular filtration rate. *d*) Drug clearance. *e*) Clearance ratio.

TABLE IV. Effect of TPA on Renal Clearance of SMZ in a Rat[a]

	Time (min)	GFR[c] (ml/min)	C[d] (ml/min)	CR[e]
Control	30—20	14.1	24.6	1.74
	20—10	14.5	26.5	1.83
	10— 0	14.8	25.7	1.74
Exptl[b]	30—40	16.8	12.6	0.750
	40—50	15.0	11.4	0.760
	50—60	14.3	10.8	0.775

a) Weight: 250 g. *b*) Experimental. *c*) Glomerular filtration rate. *d*) Drug clearance. *e*) Clearance ratio.

Fig. 2. As shown in Fig. 2, a marked decline in the clearance ratio of SMZ after ACF, DCF or TPA infusion was observed. The data are summarized in Tables II—IV.

Discussion

Propionic acid derivatives and phenylacetic acid derivatives are new groups of non-steroidal anti-inflammatory drugs. They may offer significant advantages over indomethacin and pyrazolone derivatives for many patients, since they are less toxic and better tolerated.[2] They are usually coadministered with other drugs to patients suffering from various diseases, and the possibility of drug interactions must be taken into consideration in all patients receiving non-steroidal anti-inflammatory drugs with other drugs. The aim of the present study was to investigate the effect of the three non-steroidal anti-inflammatory drugs on the persistence of plasma SMZ and the renal excretion of SMZ in rats, and to compare the results with those for other non-steroidal anti-inflammatory drugs.[3,4]

In order to obtain preliminary information on the presence or absence of interaction between SMZ and each anti-inflammatory drug, variation of the plasma elimination profile of SMZ caused by coadministration of each anti-inflammatory drug was examined. Marked prolongation of the persistence of SMZ in plasma as a result of coadministration of ACF, DCF or TPA is shown in Fig. 1. Judging from this result and the fact that SMZ is mostly excreted unchanged, it seems likely that each anti-inflammatory drug exerts its effect on SMZ plasma level by altering the renal excretion of SMZ. As shown in Fig. 2, a marked difference was observed in the clearance ratio of SMZ before and after ACF, DCF or TPA infusion in

rats. It is well known that tubular secretion of one drug may be decreased by competition with another drug for the same transport system.[8] SMZ is known to be excreted through the renal proximal tubules in the rat.[3,4] It seems likely that ACF, DCF or TPA competitively interferes with the proximal tubular secretion of SMZ. These three non-steroidal anti-inflammatory drugs are weak organic acids and might be actively excreted by the same tubular transport mechanism as that proposed for the secretion of *p*-aminohippuric acid and some other organic acids, such as SMZ. This consideration is consistent with previous data obtained on propionic acid derivatives such as ibuprofen,[3] ketoprofen and fenbufen,[4] and a phenylacetic acid derivative, 4-biphenylacetic acid.[9] The prolonging effect of ACF or TPA on the persistence of plasma SMZ was less than that of DCF, in spite of the remarkable inhibitory effect of ACF or TPA on renal handling of SMZ. It was reported that DCF is eliminated very slowly in rats.[10] On the other hand, rapid elimination of both ACF[11] and TPA[12] in rats was reported. This difference might be partly responsible for the difference in the prolonging effects of the respective anti-inflammatory drugs on the persistence of plasma SMZ. More detailed investigation will be necessary, to confirm this.

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Communications to the Editor

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THE ICHTHYOTOXICITY AND CORONARY VASODILATOR ACTION OF 3,3'-DIHYDROXY-α,β-DIETHYLSTILBENE[1)]

Yoshihiko Inamori,*,[a] Mayuri Kubo,[a] Hiroshi Tsujibo,[a] Masafumi Ogawa,[a] Yoshinobu Saito,[b] Yasuyoshi Miki,[b] and Shoji Takemura[b]

Osaka University of Pharmaceutical Sciences,[a] Kawai, Matsubara-shi, Osaka 580, Japan and Faculty of Phrmaceutical Sciences, Kinki University,[b] 3-4-1, Kowakae, Higashiosaka-shi, Osaka 577, Japan

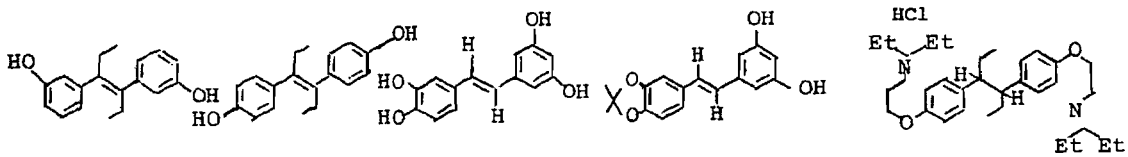
Like diethylstilbestrol (II), 3,3'-dihydroxy-α,β-diethylstilbene (I), an isomer of II, had strong ichthyotoxicity and coronary vasodilator action on the isolated guinea-pig heart. The median tolerance limit (TLm) at 48 h was 3.00 ppm in *Oryzias latipes* and 3.20 ppm in *Carassius auratus*. The ichthyotoxic activity of I was stronger than that of II. The vasodilator activity of I in the isolated guinea-pig heart (ED_{50}: 4.30 μg/heart) was weaker than that of II (ED_{50}: 0.26 μg/heart). However, the action of I was stronger than that of papaverine (ED_{50}: 7.0 μg/heart), used as a standard. On the other hand, I, unlike II and other oxystilbene derivatives so far examined, had a cardiotonic effect. In this respect, the effect of I on the isolated guinea-pig heart is noteworthy. Owing to its negligible hormonal side effect, the above-mentioned activities of I are of considerable interest.

KEYWORDS ——— 3,3'-dihydroxy-α,β-diethylstilbene; diethylstilbestrol; coralgil (diethylaminoethoxy hexestrol dihydrochloride); phospholipidosis; hormonal side effect; ichthyotoxicity; vasodilator action; 3,3',4,5'-tetrahydroxystilbene; 3,4-*O*-isopropylidene-3,3',4,5'-tetrahydroxystilbene

As reported previously, 3,3',4,5'-tetrahydroxystilbene (III, Chart 1)[2,3)] isolated from the heartwood of *Cassia garrettiana* and its derivatives, 3,4-*O*-isopropylidene-3,3',4,5'-tetrahydroxystilbene (IV, Chart 1)[4,5)] have strong ichthyotoxic and coronary vasodilator effect on the isolated guinea-pig heart.

Recently, we reported regarding these activities that diethylstilbestrol (II, Chart 1),[6,7)] a nonsteroidal estrogen, is much stronger than III and IV. However, since 1970 coralgil (diethylaminoethoxy hexestrol dihydrochloride, V, Chart 1), which has the same basic skeleton as I, has been discontinued in spite of its strong coronary vasodilator action. This is because of the severe side effects of phospholipidosis on the liver[8)] and lung[9)] cells, and the foam cell syndrome.[10)] It is well known that II also has the hormonal side effect.

Subsequently, various oxystilbene compounds were synthesized and their biological activities were examined to obtain more strongly active substances. As a result, 3,3'-dihydroxy-α,β-diethylstilbene (I, Chart 1),[11)] an isomer of II, was found to have rather strong ichthyotoxicity and coronary vasodilator action on the isolated guinea-pig heart. In this paper we report the ichthyotoxicity of I and its coronary vaso-



I II III IV V HCl

Chart 1

dilator action on the isolated guinea-pig heart from the view-points of comparative toxicology and the common biological activity. It was already reported that by shifting the phenolic hydroxyl groups to *m*-position (from II to I), the hormonal character of I was largely lost, while the antimicrobial properties were fully retained.[12] However, the ichthyotoxicity and coronary vasodilator action of I have not been reported.

The ichthyotoxicity of I to *Oryzias latipes* and *Carassius auratus* was examined by the method of Sugawara and Koyama.[13] The median tolerance limit (TLm) was calculated according to the Doudoroff method.[14] As shown in Table I, the TLm at 48 h was 3.00 ppm in *O. latipes* and 3.20 ppm in *C. auratus*. Compound I was toxic to both fishes. In addition to I, compounds II,[6,7] III,[2] IV,[4,5] resveratrol,[15] piceid[15] and rhapontin[15] were already confirmed to have a strong effect on both fishes. Among these, I had the strongest action. These findings suggest that ichthyotoxicity may be a common biological activity of oxystilbene compounds. The mechanisms of the ichthyotoxicity of I and other oxystilbene compounds have been investigated.

Table I. The Ichthyotoxicity of 3,3'-Dihydroxy-α,β-diethylstilbene (I)

Fish	TLm (48 h, ppm)				
	I	II[6,7]	III[2]	IV[4,5]	Rotenone
Oryzias latipes	3.00	3.30	26.5	14.0	0.030
Carassius auratus	3.20	4.50	31.5	18.4	0.033

Calculation of TLm: Doudoroff method.
Temperature: 25°C.
Experimental size: 10 fishes/group, 2 groups.

The effect of I on the isolated guinea-pig heart was examined by the Langendorff method. The results are summarized in Table II. The action of I was weaker than that of II. However, the activity of I was stronger than that of papaverine used as a standard. On the other hand, I, unlike the oxystilbene compounds so far tested, had a cardiotonic effect (Fig. 1). Although the positive inotropic effect of I was transient, its effect (170%) was stronger than that (150%) of papaverine (as 100% before treatment). This effect of I on the isolated guinea-pig heart should be emphasized. The coronary vasodilator action of I may be intrinsic to oxystilbene compounds for the following reasons: 1) compounds II,[6,7] III[3] and IV[4,5] had a coronary vasodilator effect on the isolated guinea-pig heart, and 2) the oxystilbene compounds, piceid[15] and rhapontin[15] also had the same action. These results indicate that the hydroxyl groups attached to the benzene ring and the *trans*-olefin structure in the molecule are necessary for stilbene derivatives to have coronary vasodilator action. Compound I meets these requirements. These considerations are supported by the following findings: 1) phloroglucinol,[16] which has a polyphenol structure in common with I, II, III and IV, relaxes the smooth muscle of rats, and 2) curcumine,[17] which has the same polyphenol and *trans*-olefin structure in the molecule as I, II, III and IV, also relaxes the smooth muscle.

As mentioned above, the coronary vasodilator, coralgil (V) was reported to have the severe side effects of phospholipidosis of the liver[8] and lung[9] cells, and foam cell syndrome.[10] It was also reported by W.A. Phillips et al.[18] that V lowers the

cholesterol level in blood serum, while increasing the desmosterol content in liver. The findings were consistent with the report that V blocks the route from desmosterol to cholesterol in the biosynthesis of cholesterol.[19] The high levels of the peculiar acidic glycerophospholipids lysobisphosphatidic acid and phosphatidyl inositol in liver and other tissues also occurs in rats fed V.[20] The increase in lysobisphosphatidic acid and phosphatidyl inositol seems to arise secondarily from the increase of desmosterol.[21] However, unlike hexestrol compounds such as V, the side effects of the oxystilbene compounds (I-IV) in causing phospholipidosis of the liver and lung cells and the foam cell syndrome have not been investigated as yet. Work on these side effects in rats fed I and other oxystilbene compounds (II-IV) are in progress.

Table II. Cardiac Effect of 3,3'-Dihydroxy-α,β-diethylstilbene (I) on Isolated Guinea-Pig Heart

Compound	Coronary vasodilation (ED_{50}: μg/heart)	Cardiotonic effect
I	4.30	p.i.
II[6,7]	0.26	n.e.
III[3]	13.00	n.e.
IV[4,5]	4.50	n.e.
Papaverine	7.00	p.i.

Each value represents the mean of 3 guinea-pigs. Animals: Male Hartley strain guinea-pig (body weight: 400-500 g). The guinea-pig hearts were rapidly isolated and perfused with Krebs-Henseleit solution according to the Langendorff method. Drugs (0.1 ml in 10% DMSO) were administered directly into the perfused solution through a connecting rubber tube. DMSO (10%) has no effect on coronary vasodilation. Transducer: Force-Diplacement Transducer 45196 (SAN-EI Instrument Co., Ltd.) and MPU-0.5-290-0-3 (Nihon Kohden Kogyo Co., Ltd.). The relative potency of the test compounds was determined at that perfusion pressure which induced vasodilation by 50% of the maximum response produced by papaverine at 33 μg/heart (ED_{50}). n.e.: no effect, p.i.: positive inotropic effect.

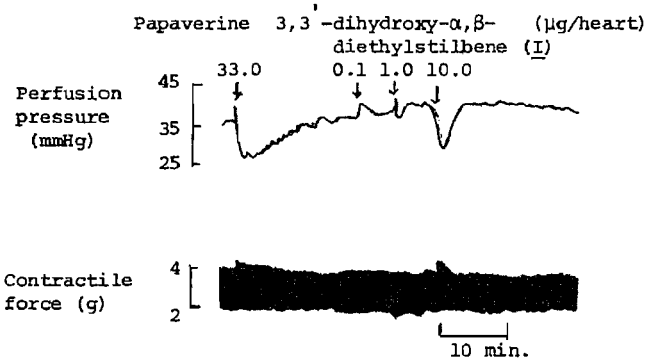


Fig. 1. Effect of 3,3'-Dihydroxy-α,β-diethylstilbene (I) on Perfusion Pressure and Contractile Force in Isolated Guinea-Pig Heart

In conclusion, it is apparent that I has strong ichthyotoxic and coronary vasodilator action on isolated guinea-pig hearts. Shifting the phenolic hydroxyl groups to the *m*-position (from II to I) eliminates the hormonal character of I was largely lost,[12] but the above-mentioned activities of I are fully retained. In this respect, the biological activities of I are of considerable interest.

ACKNOWLEDGEMENT

The authors express their gratitude to Fumio Sato Ph.D., Suntory Institute for Biomedical Research and Professor Mitsugi Kozawa Ph.D., Osaka University of Pharmaceutical Sciences for valuable advice during this project.

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Communications to the Editor

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THE SYNTHESIS OF LUPIN ALKALOIDS. I. TOTAL SYNTHESIS OF (±)-LEONTIFORMINE AND (±)-LEONTIFORMIDINE

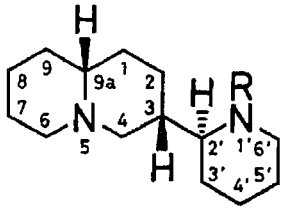
Noriyuki Takatsu, Shigeru Ohmiya, and Hirotaka Otomasu*
Faculty of Pharmaceutical Sciences, Hoshi University,
2-4-41 Ebara, Shinagawa-ku, Tokyo, 142, Japan

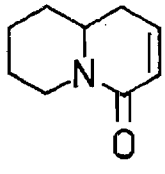
The novel total synthesis of the lupin alkaloids (±)-leontiformine (**1**) and (±)-leontiformidine (**2**) has been achieved by using the 1,3-dipolar cycloaddition reaction of 1-piperine 1-oxide (**4**) twice in the synthesis process.

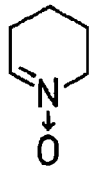
KEYWORDS ——— lupin alkaloid; 3-(2'-piperidyl)quinolizidine; (±)-leontiformine; (±)-leontiformidine; 1,3-dipolar cycloaddition; 1-piperine 1-oxide; 2,3-dehydroquinolizidin-4-one

Lupin alkaloids are usually found in plants of the Leguminosae family. Most of them have a trans-quinolizidine ring system and some have a cis-ring system. Many methods for synthesizing various types of lupin alkaloids have been reported, but there are only a few reports on 3-(2'-piperidyl)quinolizidine derivatives, such as leontiformine (**1**)[1a] and leontiformidine (**2**).[1b]

Recently, we have reported[2] on the alternative total synthesis of indolizidine alkaloids, elaeokanine homologues, using the 1,3-dipolar cycloaddition reaction of 1-pyrroline 1-oxide as a key reaction. As an extension of this work, we have synthesize some lupin alkaloids. Kakisawa et al.[3] reported the syntheses of the lupin alkaloids (±)-lupinine and (±)-α-isosparteine using the cycloaddition reaction of cyclic nitrone. In our first trial, a total synthesis of (±)-**1** and (±)-**2** was attained by using the cycloaddition reaction of the conjugated enone (**3**) with 1-piperine 1-oxide (nitrone **4**) as a key step. The key intermediate (**3**) was prepared by three steps from the 1,3-dipolar cycloaddition product (**6**) formed from the same nitrone (**4**) with ethyl 3-butenoate (**5**).





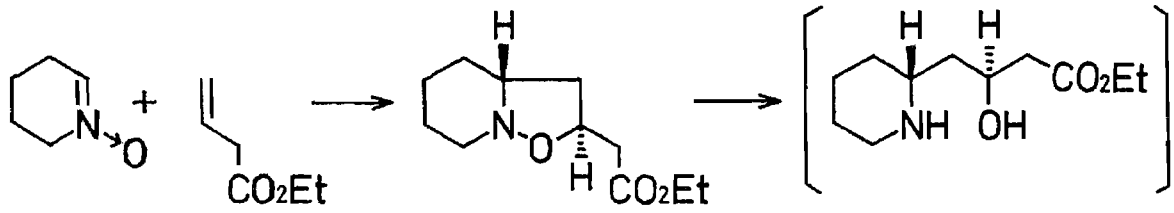


leontiformine (**1**) R = CHO
leontiformidine (**2**) R = H

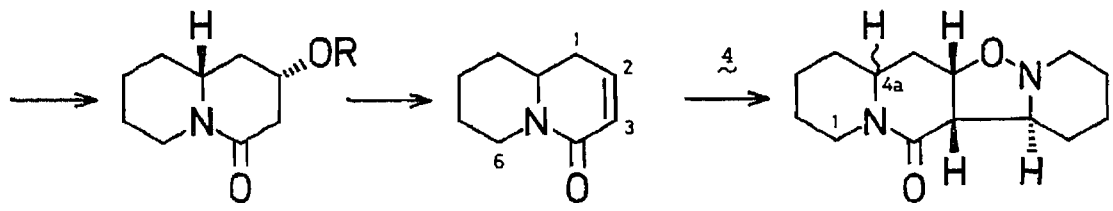
3

4

Procedures for preparing the intermediate (3) are as follows. The cycloaddition reaction of **4** with **5** was carried out according to the method of Tufariello et al.[4] [reflux in toluene, 15 h, N_2], and the exo-adduct (**6**) was obtained[5] in 87% yield. The stereochemistry of **6** is supported by the following evidence. The next recyclized product **7** formed from **6** has a rigid structure, and appeared in the ^{1}H-NMR spectrum at δ3.93 [1H, m, $W_{1/2}$=24 Hz, which was assigned to axial C2-βH]. And the spectral data of both **6** and **7** indicated the presence of a single isomer. Catalytic hydrogenolysis of **6** [10% Pd-C, H_2, 100 Kg/cm^2, in EtOH, r.t., 3 day] caused a reductive N-O bond cleavage resulting in a recyclized product (**7**) in 90% yield. The dehydration of **7** applying acidic conditions [for example, 75% H_2SO_4, 130°C, 3 h, 76% yield] gave the unconjugated enone (**8**) [1,2-dehydroquinolizidin-4-one (**8**); MS m/e 151 (M^+, base peak), 136, 122, 108; ^{1}H-NMR($CDCl_3$) δ2.96 (2H, m, C3-H), 4.86 (1H, m, C6-eqH), 5.63 (2H, m, C1-H and C2-H); IRν_{max}^{film} 1620 cm^{-1}].



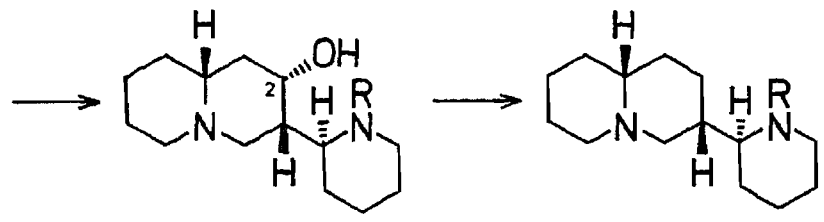
4 5 6



7 R = H
7' R = OMs

3

9a C4a - βH
9b C4a - αH



10 R = H
11 R = $PhCH_2$-

12 R = $PhCH_2$-
(±)-**2** R = H
(±)-**1** R = CHO

The desired **3** was finally prepared under basic conditions *via* the methanesulfonate (**7'**) [1) methanesulfonyl chloride 1.25 eq, excess Et_3N, in CH_2Cl_2, r.t., 2 h; 2) DBU 1.1 eq, in fresh dry THF, reflux, 2 h, N_2] in 89% yield [2,3-dehydroquinolizidin-4-one (**3**); MS *m/e* 151 (M^+), 136, 122, 84 (base peak); ^{1}H-NMR($CDCl_3$) δ4.50 (1H, dm, J=13.8 Hz, C6-eqH), 5.87 (1H, ddd, J=1.5, 2.3, and 9.8 Hz, C3-H), 6.87 (1H, ddd, J=3.4, 4.9, and 9.8 Hz, C2-H); IRν_{max}^{film} 1668, 1615 cm^{-1}].

Next, the 1,3-dipolar cycloaddition reaction of the key intermediate (**3**) with **4** [reflux in $CHCl_3$, over night, N_2] afforded an inseparable stereoisomeric mixture of the adducts (**9**) in 99% yield. The formation ratio of **9a** and **9b** was shown to be 5.7:1 by its ^{1}H-NMR (400 MHz) spectrum[6], comparing the signals due to C1-H. The structures **9a** and **9b** were identified as C4a-βH and C4a-αH forms, respectively, by comparison of the spectral data of the final synthetic products and authentic samples prepared by the method of Bohlmann.[7a] Formation of the stereoisomers (**9a** and **9b**) is attributable to the exo-addition of nitrone (**4**) from both sides of the enone system of **3**. The major product **9a** is probably derived from the less steric effect of the C9a-position of **3**. The adduct (**9**) was treated with $LiAlH_4$ [3 eq, reflux in dry Et_2O, 12 h, N_2], and the resulting product was converted to (±)-2-hydroxyleontiformidine (**10**) by catalytic hydrogenolysis [10% Pd-C, H_2, 6.5 Kg/cm^2, in MeOH, r.t., 3 day] in 80% yield. The aminoalcohol (**10**) was converted into the *N*-benzyl derivative (**11**) by successive benzoylation and reduction [1) 3 eq PhCOCl, Et_3N, r.t., over night, 79% yield; 2) 8 eq $LiAlH_4$, reflux in dry Et_2O, over night, N_2, 96% yield]. Then the hydroxyl group of **11** was replaced with bromine followed by hydrogenation to give (±)-*N*-benzylleontiformidine (**12**) [1) 10 eq PBr_3, reflux in CCl_4, 10 h; 2) 3 eq Super-H, r.t., over night, N_2] in 90% yield from **11**.

The conversion of (±)-**12** to (±)-leontiformidine (**2**) was carried out by catalytic hydrogenolysis in AcOH [1) preparation of (±)-**12**-HCl salt, 2) Pd black, H_2, 6-7 Kg/cm^2, in AcOH, r.t., 3 day]. The product thus obtained was subjected to column chromatography [SiO_2, CH_2Cl_2-MeOH-28% NH_4OH(90:9:1)] to separate (±)-**2** and its C-9a epimer along with a fair amount of unchanged (±)-**12**. The sum of the yields of (±)-**2** and its C-9a epimer was 32% in the ratio of 5:1 obtained by GC [(±)-**2** ; MS *m/e* 222 (M^+), 164, 150, 138, 110, 98, 84 (base peak); ^{13}C-NMR($CDCl_3$) δ24.70 (t), 25.12 (t), 25.97 (t), 26.81 (t), 27.37 (t), 30.02 (t), 33.29 (t), 33.32 (t), 42.02 (d), 47.62 (dd), 56.80 (dd), 59.47 (t), 60.52 (d), 62.96 (d); IR$\nu_{max}^{CCl_4}$ 2928, 2853, 2802, 2767, 1445, 1115 cm^{-1} : C-9a epimer ; MS *m/e* 222 (M^+), 164, 150, 138, 110, 98, 84 (base peak); ^{13}C-NMR($CDCl_3$) δ24.79 (t), 24.95 (t), 25.06 (t), 25.71 (t), 26.82 (t), 29.60 (t), 31.15 (t), 32.85 (t), 40.06 (d), 47.47 (dd), 56.27 (d), 56.89 (dd), 58.26 (dd), 63.22 (d); IR$\nu_{max}^{CCl_4}$ 3315, 2933, 2857, 2800, 2763, 1443, 1114 cm^{-1}]. These products were identified with the authentic samples[1a,7a] by comparison of all available spectroscopic data (MS, ^{13}C-NMR, IR, TLC, and GC).

On the other hand, catalytic hydrogenolysis of (±)-**12** in formic acid [10% Pd-C, H_2, 6-7 Kg/cm^2, in 98% HCO_2H, r.t., 3 day] was performed in a similar manner and (±)-leontiformine (**1**) was obtained in 24% yield as a result of formylation of released (±)-**2** with the solvent [(±)-**1** ; IR$\nu_{max}^{CCl_4}$ 2935, 2855, 2800, 2770, 1673 cm^{-1}]. In this case, much (±)-**12** was recovered but there was no C-9a epimer. The product was identified with the formylation product of (±)-**2** by comparison of IR,[1b,7b] TLC, and GC. The debenzylation of (±)-**12** is being improved.

Acknowledgment We wish to thank Miss Satomi Ishizawa for preparing the starting materials.

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Communications to the Editor

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NMR EVIDENCE FOR THE UNEXPECTED INTERCONVERSION OF 2-DEOXY-2-FLUORO-D-GLUCOSE AND 2-DEOXY-2-FLUORO-D-MANNOSE IN MICE

Yoko Kanazawa,[a] Yuko Momozono,[a] Hideki Yamane,[b]
Terushi Haradahira,[a] Minoru Maeda,[a] and Masaharu Kojima[a]

Faculty of Pharmaceutical Sciences,[a] and Faculty of Medicine[b],
Kyushu University 62, Higashi-ku, Fukuoka 812, Japan

2-Deoxy-2-fluoro-D-glucose (FDG) or its 6-phosphate (FDG-6-P) was detected by ^{19}F NMR in the organs and urine of mice previously injected with 2-deoxy-2-fluoro-D-mannose(FDM). This conversion of FDG(-6-P) to FDM(-6-P) in vivo was reversible.

KEYWORDS ——— 2-deoxy-2-fluoro-D-glucose; 2-deoxy-2-fluoro-D-mannose; epimerization; in vivo NMR; ^{19}F NMR

Regional glucose utilization has been determined in man with the aid of 2-deoxy-2-[F-18]-fluoro-D-glucose(^{18}FDG) and positron emission tomography (1). This procedure is based on the principle that FDG is trapped in tissues of high glucose-metabolism activity in the form of FDG-6-phosphate(FDG-6-P) and that further metabolism is inhibited by the absence of an OH group at the C(2) position. However, we have shown by ^{19}F NMR the formation of α- and β-FDM and/or FDM-6-P in significant amounts in tissues of FDG-injected mice (2). This may require modification of the compartment model used in the rate analysis of glucose metabolism in vivo. Moreover, in the present study we have demonstrated the reverse conversion, from FDM(or FDM-6-P) to FDG(or FDG-6-P). Thus, in mice these fluorinated hexoses undergo significant interconversion.

FDG and FDM were prepared by the method reported previously (3) and FDG-6-phosphate (FDG-6-P) was prepared according to a reported procedure (4). Male mice (6-7 week-old, ddY strain) were used after 16 hours of fasting. Following the intravenous injection of fluorinated hexoses (0.2 g/kg), the animals were sacrificed at various time intervals, the organs were removed, washed immediately, and kept frozen pending NMR measurements. ^{19}F NMR spectra of organs and of urine were taken with a JEOL FX-100 standard high resolution spectrometer operated at 93.7 MHz at ambient temperature (25 ± 1°C) in a 10-mm OD sample tube at the spectral width of 50 kHz. Hexafluorobenzene (HFB) was used as an external standard to measure chemical shifts.

α-, β-FDG, α-, β-FDM, α-, and β-FDG-6-P dissolved in water gave spectra centered at -32.3, -32.2, -37.6, -56.0, -32.4, and -32.2 ppm, respectively. The separation between FDG and α- or β-FDM was sufficient for their identification

in vivo, whereas the positions for the signals of α-, β-FDG, α-, and β-FDG-6-P severely overlapped. Extra small signals were observed in the sample of FDG-6-P in the vicinity of α- and β-FDM were attributed to the 6-phosphate of FDM(FDM-6-P) produced by the phosphorylation of FDM present in the FDG starting meterial (ca. 4%). Again, those 6-phosphates were not distinguishable from FDM in tissue.

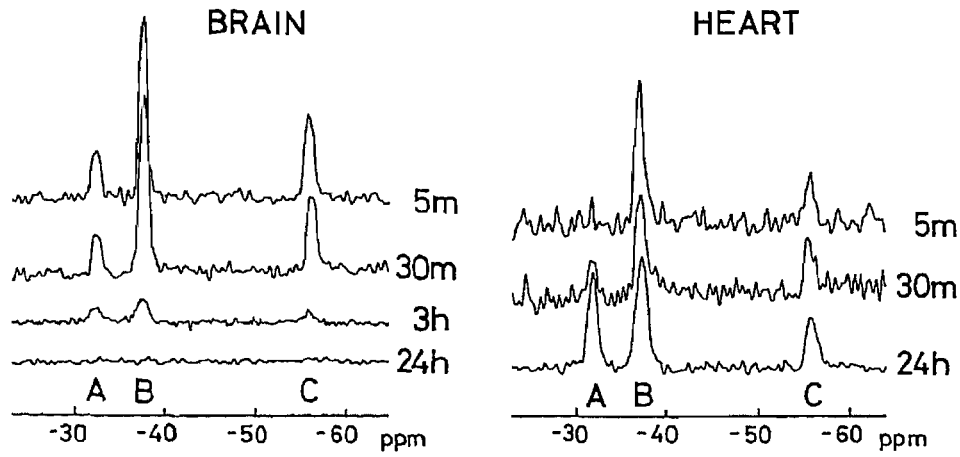

Fig. 1. ^{19}F NMR Spectra (93.7 MHz) of the Organs of FDM Injected Mice. The Chemical Shifts were Measured from External HFB. A, FDG(-6-P); B, α-FDM(-6-P); C, β-FDM(-6-P).

The ^{19}F spectra of organs and urine excised or collected at various time after FDG or FDM injection are shown in Figs. 1 - 3. The spectra of specific organs of FDM-injected mice showed clearly the presence of FDG (Fig. 1). The concentration of FDG relative to FDM increased with time prior to the excretion of entire fluorinated compounds from these organs. This was similar to the earlier finding that FDG-injected mice formed FDM (Fig. 2)(2). The spectra for the urine samples

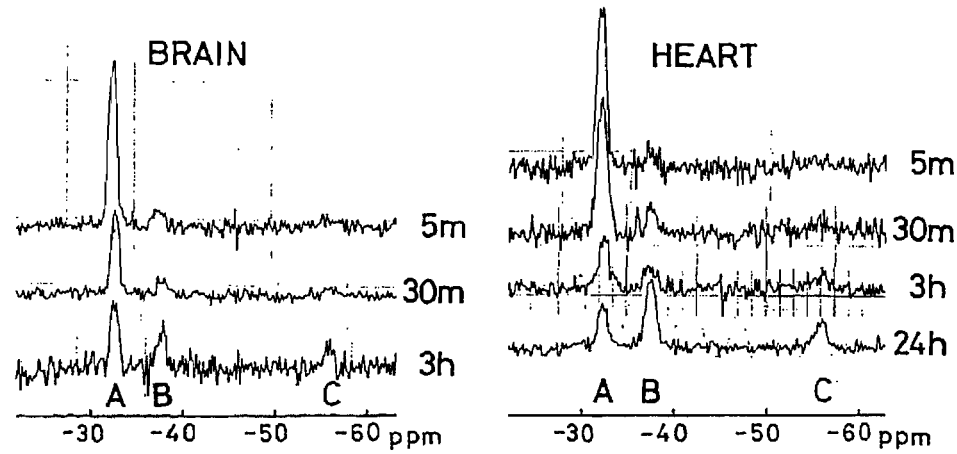

Fig. 2. ^{19}F NMR Spectra of the Organs of FDG Injected Mice The Notation of the Signals are as in Fig. 1.

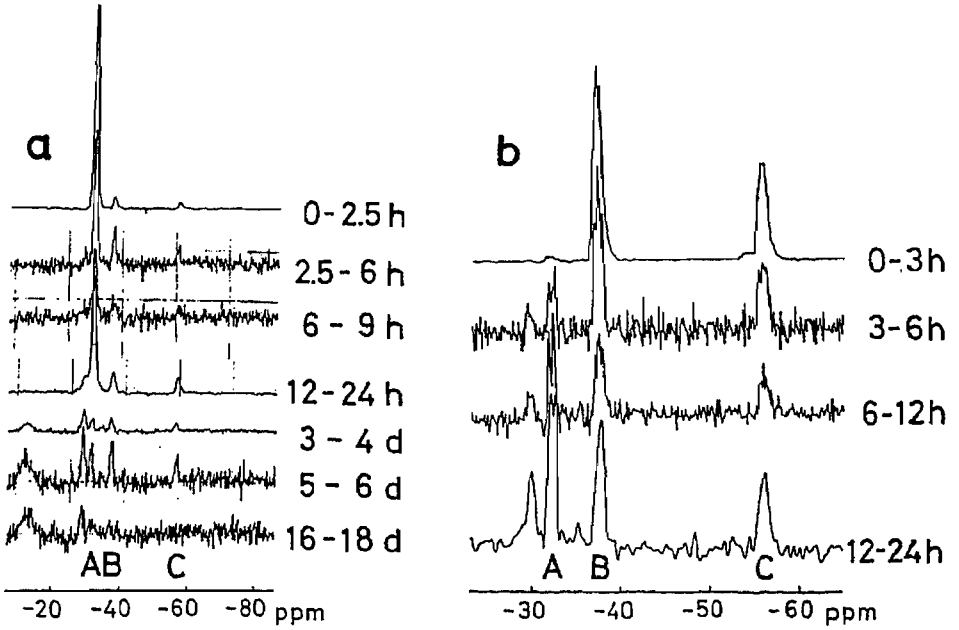

Fig. 3. ^{19}F NMR Spectra of Urine Excreted from FDG- or FDM-Injected Mice (a) Urine of FDG-Injected Mice. (b) Urine of FDM-Injected Mice.

are shown in Fig. 3. The excretion of the main metabolites α- and β-FDM in the case of FDG injection and of FDG in the case of FDM injection is clearly evident. The other metabolite at -31 ppm is presumably the oxidation product of FDG, 2-deoxy-2-fluoro-δ-phosphogluconolactone and/or 2-deoxy-2-fluoro-6-phosphogluconate, as suggested by Nakada et al.(5). The origin of the other signals were not identified, but may include oxidation products of FDM.

A preliminary experiment showed that FDG-6-P was converted to FDM-6-P in the buffer solution containing phosphoglucose isomerase while no change was observed in the FDG-6-P solution without the enzyme. This indicates the site of interconversion.

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Communications to the Editor

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DIHYDROSHIKONOFURAN, AN UNUSUAL METABOLITE OF QUINONE BIOSYNTHESIS IN LITHOSPERMUM CELL CULTURES

Kazufumi Yazaki,[1] Hiroshi Fukui, and Mamoru Tabata
Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, 606, Japan

A novel compound named dihydroshikonofuran (1), which is biogenetically related to the naphthoquinone pigment shikonin, was isolated from shikonin-free cell suspension cultures of Lithospermum erythrorhizon grown in LS medium. When the cells were transferred to the production medium M9 to induce shikonin synthesis, the content of the compound **1** decreased with time. The significance of this unusual metabolite in relation to shikonin biosynthesis is discussed.

KEYWORDS—Lithospermum erythrorhizon; Boraginaceae; plant cell culture; biosynthesis; intermediate; dihydroshikonofuran; m-geranyl-p-hydroxybenzoic acid; geranylhydroquinone

In Linsmaier-Skoog (LS) medium, cell suspension cultures of Lithospermum erythrorhizon Sieb. et Zucc.[2] stop producing red naphthoquinone pigments, the derivatives of shikonin.[3] However, they begin producing the pigments when transferred to M9 medium [4,5] containing no NH_4^+, only NO_3^- as the sole nitrogen source. From these shikonin-producing cells, we have isolated not only two intermediates of shikonin biosynthesis, m-geranyl-p-hydroxybenzoic acid (2) and geranylhydroquinone (3), but also two unusual metabolites, deoxyshikonofuran and shikonofuran E.[6] We now report the isolation and structure of a new compound dihydroshikonofuran (1) from the cells cultured in LS medium.

Shikonin-free cells of strain M18 (300 g dry wt), cultured in LS liquid medium for 3 weeks at 25° in the dark, were extracted with MeOH. After concentration, the extract was separated with Et_2O and H_2O. The Et_2O layer was evaporated to dryness to yield a syrupy extract, which was subjected to silica gel column chromatography using benzene - acetone (5 : 1) as the solvent. An oily substance eluted from the column was further purified by preparative TLC (silica gel, benzene - EtOAc 8 : 2) to give dihydroshikonofuran (1, 152 mg).

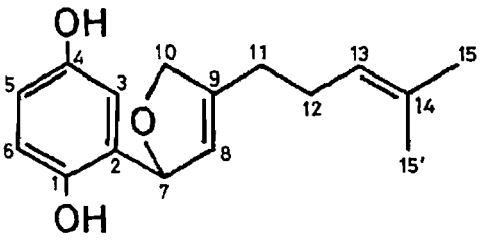
Dihydroshikonofuran (1): $C_{16}H_{20}O_3$ (m/z 260, M^+), $[\alpha]_D$ -90.2° (c=0.4, $CHCl_3$), UV λ_{max}^{MeOH}nm (log ε): 204 (4.15), 294 (3.44), colorless oil. The Rf values of 1 on TLC (silica gel) were 0.36 (benzene - EtOAc 8 : 2) and 0.37 ($CHCl_3$ - MeOH 20 : 1), and the spot of 1 developed a pale orange color on standing overnight. This indicates that 1 has a hydroquinone moiety in the molecule, which is easily oxidized to yield a benzoquinone derivative.[6]

^{1}H-NMR of 1 (200 MHz, $CDCl_3$) showed three protons assignable to 1,2,4-tri-

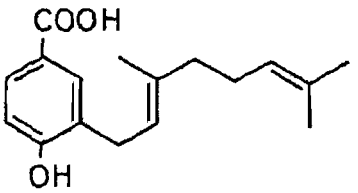
substituted benzene ring [δ 6.67 (1H, d, J=8 Hz, H-6), 6.60 (1H, dd, J=3 and 8 Hz, H-5), 6.53 (1H, d, J=3 Hz, H-3)], one benzyl methine proton having an O-functional group [δ 5.99 (1H, broad t, J=4 Hz, H-7)], two vinyl protons [δ 5.60 (1H, d, J=1 Hz, H-8), 5,09 (1H, m, H-13)], two methylene protons having an O-functional group [δ 4.66 (2H, m, H-10)], four methylene protons [δ 2.00 - 2.30 (4H, m, H-11, 12)], and two methyl groups on a double bond [δ 1.68 (3H, s,), 1.60 (3H, s), H-15, 15']. This spectrum was very similar to that of geranylhydroquinone (3) except that the former had methylene protons and a benzyl proton which were attached to the O-functional group, instead of the methyl protons and benzyl methylene protons in 3. This indicates that 1 is probably either a dihydroxyl derivative of 3 or a geranylhydroquinone analogue having an ether bond on the side chain of the molecule. Furthermore, the ^{1}H-NMR spectrum showed that one of the three methyl residues and the carbon located at the benzyl position are bound to the O-function. The assignment of 1 was confirmed by a homo-decoupling experiment and by the H-C selective decoupling spectrum at low power in the gated decoupling mode.

The ^{13}C-NMR spectrum of 1[7] also indicated that 1 is one of the derivatives of 3 having an O-functional group. One of the two carbons bound to the O-function was observed at 84.58 ppm (C-7), and the other at 77.79 ppm (C-10); they must have one and two protons, respectively, according to off-resonance and INEPT spectrum measurements. The occurrence of the two carbons in a field in the ^{13}C-NMR spectrum much lower than an ordinary carbon with a hydroxyl group suggests that they are connected by an ether bond to form a five-membered ring. The validity of the estimated partial structure is supported by the large homoallyl coupling constant (4 Hz) between H-7 and H-10,[8] which is characteristic of a dihydrofuran ring. In addition, the parent peak of m/z 260 in the EI and CI (NH_3) mass spectra shows that 1 has a dihydrofuran moiety in the molecule. Furthermore, this structure was confirmed by the acetylation of 1 to give its diacetyl derivative: $C_{20}H_{24}O_5$ (m/z 344, M^+), $[\alpha]_D$ -90.4 (c=1.2, $CHCl_3$), UV λ_{max}^{MeOH}nm (log ε): 204 (4.29), 269 (3.03), ^{1}H-NMR (200 MHz, $CDCl_3$): δ 2.31 (3H, s, OAc), 2.27 (3H, s, OAc). Therefore, the structure of dihydroshikonofuran was determined as 1, although the stereochemistry at C-7 remains undetermined.

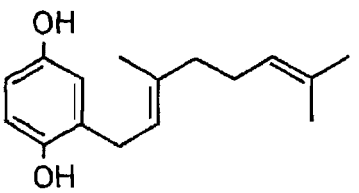
Compound 1 and the two key intermediates of shikonin biosynthesis, m-geranyl-p-hydroxybenzoic acid (2) and geranylhydroquinone (3), from shikonin-producing and non-producing cell cultures have been quantitatively analyzed by HPLC. In addition to strain M18, the cell suspension cultures of two other strains (M130 and LY) of L. erythrorhizon were also investigated. Strain M130 was cultured in LS medium under the same conditions as applied to M18, while strain LY was cultured in LS medium containing 10^{-6}M 2,4-D, instead of IAA and kinetin, as the sole growth regulator. Both cultures were incapable of producing shikonin derivatives even in M9 medium. The shikonin-producing cells cultured in M9 medium at 25° in the dark, were harvested 2 weeks after inoculation. Fresh cells of both cultures were lyophilized followed by extraction with MeOH. The MeOH extracts were subjected to HPLC analysis: stainless column (6 mm i. d. x 150 mm) packed with YMC A312 (ODS), solvent system MeOH - CH_3CN - H_2O - AcOH (34 : 28 : 37 : 1), flow rate 1.5 ml/min, pressure 100 kg/cm^2, and UV detection at 257 nm for 2 and at 294 nm for 1 and 3.



1



2



3

Table I. Contents of intermediates and the related compound 1 of shikonin biosynthesis in Lithospermum cell suspension cultures of strains M18, M130, and LY cultured in LS or M9 medium

Compound	Strain M18		Strain M130		Strain LY	
	LS[a]	M9[a]	LS	M9	LS	M9
	(mg / g dry wt)					
1	0.87	0.08	trace	trace	trace	trace
2	0.02	0.47	0.02	0.02	0.06	0.04
3	-[b]	0.04	-	-	-	-

a) culture medium used
b) none detected

As shown in Table 1, the content of dihydroshikonofuran (1) was much higher in the shikonin-free cultures of strain M18 in LS medium than in the shikonin-producing cultures of that strain in M9 medium. In contrast to 1, more 2 and 3 were found in the M9 than in the LS medium. Compound 3 was detected only in the pigmented cultures of M18. Strains M130 and LY accumulated hardly any compound 1 irrespective of the medium, and 2 was also scarce in these variant strains incapable of forming shikonin. Compound 3 was not detected in these two strains.

Inouye et al.[9] suggested, on the basis of tracer experiments on Lithospermum callus cultures, that the synthetic auxin 2,4-D blocks the biosynthesis of shikonin at the step of decarboxylation and hydroxylation of 2, since 2 was detected in a minute amount but no 3 was detected in the white cells. However,

the isolation of **1**, which is presumably an unusual metabolite of **3**, from the shikonin-free cells of M18 suggests that the cells cultured in LS liquid medium containing IAA can convert **2** into **1** via **3**. The reason **3** was not detected in the cells grown in LS medium may be a quick turnover of **3** or its strong binding to a protein such as an enzyme.

Compound **1** is apparently not a precursor of shikonin, but it is considered to be the precursor of the shikonofuran derivatives previously isolated from Lithospermum cultures.[6,10] This indicates that the reaction step which is suppressed in the biosynthetic route of shikonin in Lithospermum cells cultured in LS medium may be the ring closure of **3** leading to the formation of naphthoquinone.

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THREE NEW 10-PHENYL-[11]CYTOCHALASANS, CYTOCHALASINS N, O, AND P FROM PHOMOPSIS SP.

Takako Tomioka,[1] Yoshihiko Izawa, Kiyotaka Koyama, and Shinsaku Natori*
Meiji College of Pharmacy, Yato-cho, Tanashi-shi, Tokyo 188, Japan

From Phomopsis sp.(68-GO-164) three new cytochalasans named cytochalasins N, O, and P were isolated, besides the four known cytochalasans, cytochalasins H and J and epoxycytochalasins H and J. The structures of the new compounds were determined by spectral analysis, especially by NMR, and by correlation reactions with known compounds.

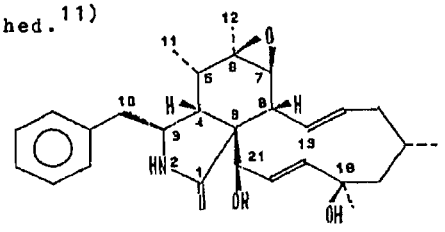
KEYWORDS——cytochalasan; cytochalasin; Phomopsis sp.; ^{1}H-NMR; ^{13}C-NMR; cytotoxicity

In the course of our studies on mycotoxin-production by food-borne fungi collected in Japan, Chaetomium spp. were found to produce eight novel cytochalasans with an indol-3-yl group.[2] They were designated chaetoglobosins A-G and J. Twelve strains of the molds exhibiting cytotoxicity to HeLa cells with polynuclear cell formation[3] were investigated with regard to the production of metabolites that affect microfilaments (actin) and microtubules (tubulin). Nine of the strains showed no such effects, probably due to the loss of metabolic activity during storage. But Phomopsis sp., Diaporthe phaseolorum, and Pithomyces sacchari exhibited the same type of the cytotoxicity.[4]

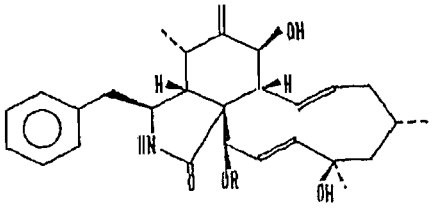
The metabolites of Phomopsis sp. (68-GO-164) were investigated in detail. The culture conditions were examined by monitoring cytotoxicity. Culturing on wheat for 20 days at 26°C was found to give a good yield of the metabolites. The moldy wheat thus obtained was extracted with dichloromethane and the extracts were chromatographed on silica gel (hexane-acetone). The toxic fractions were further separated by HPLC using Nucleosil 50-5 (hexane-acetone). After spraying with 50% methanolic sulfuric acid and heating, the fractions containing cytochalasans were detected on TLC as bright yellow fluorescent spots under UV light. Nine compounds were thus separated and characterized. First two compounds, which were not cytotoxic after purification, were identified as (3S,4S)cis- and trans-4-hydroxymellein.[5] The other seven compounds (1 - 7) were proved to belong to the 10-phenyl-[11]cytochalasans, a group of cytotoxic mycotoxins that bind specifically with actin.

According to elemental analyses and mass spectrometry, three of these compounds(1 - 3) had the same molecular formula $C_{30}H_{39}NO_5$, three others (4 - 6) had the formula $C_{28}H_{37}NO_4$, and the seventh (7) had $C_{30}H_{41}NO_6$. Nuclear magnetic resonance (NMR) data revealed that the three (1 - 3) are the acetyl derivatives of the other three (4 - 6) respectively. Detailed ^1H- and ^{13}C-NMR studies of the metabolites indicated that the compounds (1, 2, 4, and 5) were epoxycytochalasin H, cytochalasin H (kodocytochalasin-1, paspalin P1), epoxycytochalasin J (epoxydeacetylcytochalasin H), and cytochalasin J (kodocytochalasin-2, paspalin P2, deacetylcytochalasin H). These had been previously isolated from Phomopsis spp.,[6-9] and their identities were established by direct comparison with authentic samples.

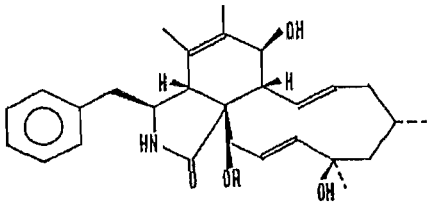
The other three compounds were new: cytochalasin N (3), colorless powder, mp 253-254°C (acetone), $[\alpha]_D$ + 85.4° (MeOH), λ_{max}^{MeOH} 208 nm (ε 19800), $\nu_{max}^{KBr}cm^{-1}$: 3400, 2725, 1690, 1470, 1235, 1150, 960, 700; cytochalasin O (6), colorless needles, mp 187-188 °C (hexane-acetone), $[\alpha]_D$ +59.6° (MeOH), λ_{max}^{MeOH}: 206 nm (ε 20800), $\nu_{max}^{KBr}cm^{-1}$: 3425, 1670, 1250, 1140, 960, 700; and cytochalasin P (7), mp 117-118°C ($CHCl_3$), $[\alpha]_D$ -116° (MeOH), λ_{max}^{MeOH} 208 nm (ε 15200), $\nu_{max}^{KBr}cm^{-1}$: 3400, 2920, 1680, 1370, 1230, 960, 700. The NMR spectra of 3 and 6 (Table I) indicated that, excepting the cyclohexane part of the molecule, they have the same structures as 1 and 2 and 4 and 5 respectively, and they are 5(6)-en-7β-ol derivatives like cytochalasin C[10] and chaetoglobosin B.[2] To confirm the structures, the correlation reactions shown in Chart 1 were performed and the structures of cytochalasins N (3) and O (6) were established.[11]



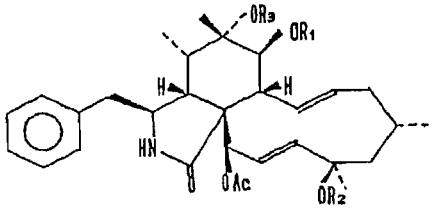
(1) R=Ac epoxycytochalasin H
(4) R=H epoxycytochalasin J



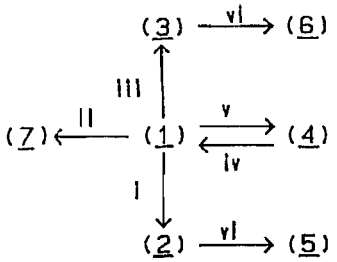
(2) R=Ac cytochalasin H
(5) R=H cytochalasin J



(3) R=Ac cytochalasin N
(6) R=H cytochalasin O



(7) R_1=R_2=R_3=H cytochalasin P
(8) R_1=benzoyl R_2=R_3=H
(9) R_1=R_2=benzoyl R_3=H
(10) R_1=R_2=R_3=benzoyl



Reagents : I) Al[OCH(CH$_3$)$_2$]$_3$ / toluene
II) CF_3CO_2H / CH_3CN
III) H_2SO_4 / DMSO
iv) Ac_2O / pyridine
v) NaOH / t-BuOH
vi) 5% KOH

Chart I

Comparison of the physical data and the molecular formulae indicated that the seventh congener (7) corresponds to the hydrate of 1, 2, and 3. The ^{1}H- and ^{13}C-NMR data of 7 were precisely analyzed using COSY, C-H COSY, and DEPT. The results (Table I) clearly indicated the presence of a tert-hydroxyl and a methyl group at C_6 besides secondary hydroxyl group at C_7. Treatment of epoxycytochalasin H (1) with trifluoroacetic acid in acetonitrile[12] gave 7 in a 30% yield, besides 2 and 3. Thus the compound (7) was proved to be a 6,7-diol-type compound, which had not been found previously among the forty-three cytochalasans so far characterized.[13]

Table I ^{1}H- and ^{13}C-NMR Data of Cytochalasins N (3), O (6), and P (7) (in DMSO-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C)

	Cytochalasin N (3)	Cytochalasin O (6)	Cytochalasin P (7)
		^{1}H	
2	8.15 (s)	7.83 (s)	7.92 (s)
3	3.17 (ddd, 4.3, 10.5, --)	3.12 (ddd, 5.0, 9.8, --)	4.02 (ddd, 4.6, 5.4, 5.0)
4	2.27 (m)	2.85 (m)	1.75 (dd, 5.0, 5.1)
5	-----	-----	1.63 (dq, 5.1, 7.0)
7	3.60 (m)	3.57 (m)	3.27 (dd, 5.9, 11.4)
8	2.36 (dd, 10.0, 10.0)	2.36 (dd, 10.1, 10.1)	2.46 (dd, 11.4, 10.2)
10	2.96 (dd, 12.8, 4.3) 2.72 (dd, 12.8, 10.5)	2.92 (dd, 12.9, 5.0) 2.70 (dd, 12.9, 9.8)	2.57 (dd, 13.8, 5.4) 2.78 (dd, 13.8, 4.6)
11	1.51 (s)	1.51 (s)	0.81 (d, 7.0)
12	0.87 (s)	0.95 (s)	0.98 (s)
21	5.66 (dd, 1.8, 2.0)	5.27 (s)	4.77 (dd, 2.4, 2.1)
21-Ac	2.25 (s)	-----	2.01 (s)
		^{13}C	
1	174.5 (s)	176.6 (s)	174.3 (s)
3	49.1 (d)	48.5 (d)	52.6 (d)
4	60.0 (d)	59.8 (d)	48.8 (d)
5	125.5 (s)	126.4 (s)	37.8 (d)
6	133.2 (s)	132.4 (s)	75.0 (d)
7	68.3 (d)	68.6 (d)	75.5 (d)
8	48.5 (d)	48.0 (d)	45.4 (d)
9	51.4 (s)	53.1 (s)	52.9 (s)
10	43.3 (t)	43.4 (t)	42.9 (t)
11	14.4 (q)	14.3 (q)	12.8 (q)
12	16.5 (q)	16.6 (q)	22.4 (q)
21	75.1 (d)	73.4 (d)	77.6 (d)
21-Ac	20.4 (q) 170.4 (s)	----- -----	20.5 (q) 169.2 (s)

The stereochemistry of the glycol part was established as follows: The coupling constant of the C_7 and C_8 protons (11.4 Hz) showed the β-configuration of the C_7-hydroxyl group. Although the formation of 7 from 1 by the cleavage of the epoxide indicated the trans configuration of the glycol,[14] further experiments to confirm the configuration were carried out by two methods. First, the relative stereochemistry of the C_6-methyl group and protons at C_5 and C_8 were confirmed by NOE difference spectra. Second, the dibenzoate chirality rule was applied as follows: Treatment of cytochalasin P (7) with benzoyl chloride in pyridine gave 7-monobenzoate (8), $[\theta]$ -1.93×10^3(280 nm), 7,18-dibenzoate (9), $[\theta]$ -3.69×10^3(277 nm), and 6,7,18-tribenzoate (10), $[\theta]$ $+3.65\times10^4$(270 nm), according to the reaction conditions. Comparing the CD spectra of these compounds showed the positive effect of the 6,7-dibenzoate, indicating the α-configuration of the C_6-hydroxyl. Thus the stereochemistry of the glycol part was established.

The ED_{50} values of the cytochalasins (1 - 6) to HeLa cells were in the range of 0.32-3.2 μg/ml. However, cytohalasin P (7), with an additional hydroxyl group at the 6-position, was much less cytotoxic. The structure-activity relationship of these compounds to cell phenomena and actin in vitro[15] is under investigation.

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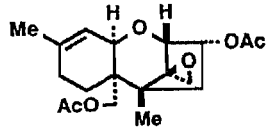
SYNTHETIC STUDIES TOWARD TRICHOTHECENE SESQUITERPENES. SYNTHESIS OF AN OPTICALLY PURE KEY INTERMEDIATE FOR CALONECTRIN USING HIGHLY STEREOSELECTIVE CYCLIZATION

Kiyoshi Tomioka, Masamichi Sugimori, and Kenji Koga*
Faculty of Pharmaceutical Sciences, University of Tokyo,
Hongo, Bunkyo-ku, Tokyo 113, Japan

Using highly stereoselective cis-cyclization, the optically pure key intermediate for calonectrin was synthesized.

KEYWORDS —— stereoselectivity; Michael reaction; tetrahydrochromane; cis-cyclization; trichothecene

A family of trichothecene sesquiterpenes is known to be endowed with remarkable biological properties including anticancer activity[1] and represents challenging targets in synthetic organic chemistry.[2] We are studying the total synthesis of antileukemic natural products by the novel use of optically pure γ-hydroxymethyl-γ-butyrolactone (2 and its enantiomer) as a chiral synthon.[3] Thus far this has lead to the successful asymmetric total synthesis of lignans,[4a] neolignan,[4b] burbonene sesquiterpenes,[4c] marine spatane diterpenes,[4d] and verrucarinolactone, a constituent of a macrocyclic trichothecene verrucarin A.[4e] We describe here the synthesis of an optically pure key intermediate (15) for calonectrin (1), one of the representative trichothecene sesquiterpenes.



(+)-1

Previously we have reported the preparation of 4 as a major product of two diastereomers starting from 3.[5] Diastereomerically pure 5 (mp 126.5-127 °C, $[\alpha]_D^{20}$ -104.9 °(acetone)) was obtained in 33% overall yield from 3 by the recrystallization ($CHCl_3$) of a detritylated mixture of 4.[6] Since an attempted direct cyclization of 5 was fruitless, the lactone part in 5 was reduced.

Reduction of 5 with DIBAH in THF at -78 °C, followed by protection, afforded 6. Without purification 6 was converted to 7 (mp 104.5-105 °C, $[\alpha]_D^{22}$ -18.5 ° ($CHCl_3$)) in 30% overall yield in five steps by successive treatment with $LiAlH_4$ in THF, $PhCH_2Br$-NaH in DMF, PPTs in MeOH , Ph_3CCl in pyridine, and PDC in CH_2Cl_2.

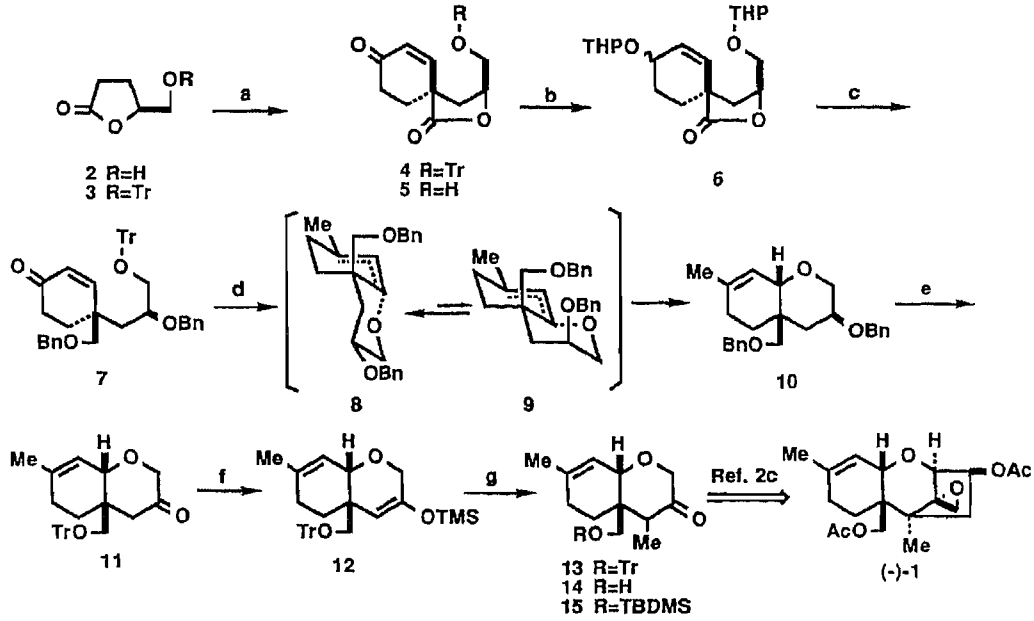
The stage was thus set for the construction of the cis-fused tetrahydrochromane skeleton of calonectrin (1). Successive treatment of 7 with MeLi in THF, and then with c. HCl in MeOH at -20 °C for 12 h and at 50 °C for 2 h afforded the cis-cyclization product (10: ^1H NMR ($CDCl_3$) δ=5.44 (vinyl H, d, J=5 Hz)) as the sole product in 97% yield. The coupling constant of the olefinic proton of 10 is in good agreement with that of 1. Virtually complete cis-cyclization may be

attributable to the developing intermediate structure (8) where the secondary OBn substituent takes an equatorial-like orientation, not axial as in trans-cyclization (9).

The dibenzyl ether (10) was converted to 11 ($[\alpha]_D^{18}$ +19.6 °($CHCl_3$)) in 72% yield through reductive debenzylation, selective tritylation of the primary alcohol, and then oxidation. Regioselective introduction of the methyl group into 11 was achieved via the silyl enol ether (12).

The silyl enol ether (12) was highly selectively prepared in quantitative yield by treating 11 with TMSCl-Et_3N in DMF at 90 °C for 4 h. Other methods were found to afford a mixture of regioisomers with poor selectivity.[7] Methylation was carried out by the successive treatment of 12 with MeLi and then with MeI-HMPA in THF to afford 13 ($[\alpha]_D^{22}$ +66.2 °($CHCl_3$)) in 14% yield along with the corresponding methyl enol ether (33%). The structure of 13 was established by the conversion to the reported key intermediate (15) for the calonectrin (1) synthesis.

Detritylation of 13 and silylation afforded 15 ($[\alpha]_D^{22}$ +56.7 °($CHCl_3$)) in 50% yield. The spectral data (NMR, IR) of 15 were indistinguishable from those of dl-15 reported by Kraus.[2c] Since Kraus reported the total synthesis of dl-



Tr=CPh_3, Bn=CH_2Ph, TMS=$SiMe_3$, TBDMS=$SiMe_2Bu$-t

a) i. NaH-HCOOEt/toluene, ii. MVK-NEt_3/toluene, -78 °C, iii. K_2CO_3-Et_4NBr/toluene (60%), iv. 10% H_2SO_4-acetone (55%); b) i. DIBAH-THF, ii. DHP-p-TsOH/CH_2Cl_2-THF; c) i. LAH/THF, ii. BnBr-NaH/DMF, iii. PPTS/MeOH, iv. TrCl-DMAP/pyridine, v. PDC/CH_2Cl_2 (30%); d) i. MeLi/THF, ii) c. HCl/MeOH, -20 °C (12 h) then 50°C (2 h) (97%); e) i. Na/NH_3-THF, ii. TrCl-DMAP/pyridine, iii. PDC/CH_2Cl_2 (66%); f) TMSCl-NEt_3/DMF, 90 °C (quant); g) i. MeLi/THF, then MeI-HMPA (14%), ii. c. HCl/MeOH-CH_2Cl_2, iii. TBDMSCl-imidazole/DMF (50%).

calonectrin (1) from dl-15, the present synthesis of optically active 15 constitutes the formal total synthesis of optically active calonectrin ((-)-1). Since we have already established the synthesis of the optical antipode of 2 starting from 2, synthesis of both enantiomers of 15 is possible.

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5. K. Tomioka, Y.-s. Cho, F. Sato, and K. Koga, Chemistry Lett., **1981**, 1621. The spiroenone (4) was obtained in an improved ratio of 7.8:1 when the Michael reaction was conducted at -78°C.
6. All new compounds reported here have been fully characterized by IR, ^{1}H NMR, high resolution MS and/or combustion analyses.
7. Treatment of 11 with LDA and TMSCl in THF afforded a mixture of 12 and regioisomer in a ratio of 2:3. Thermodynamically controled silylation also afforded a mixture in a ratio of 5:1. M. E. Krafft and R. A. Holton, Tetrahedron Lett., **24**, 1345 (1983).
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THE DIELS-ALDER REACTION OF 1-PHENYLSULFONYL-3,3,3-TRIFLUOROPROPENE

Takeo Taguchi, Akihiko Hosoda, Ginjiro Tomizawa, Akihiro Kawara, Toshio Masuo, Yoshimitsu Suda, Masaharu Nakajima, and Yoshiro Kobayashi*
Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

The Diels-Alder reaction of 1-phenylsulfonyl-3,3,3-trifluoropropene (1) with 1,3-dienes was carried out to give the [4+2]cycloadducts in good yields. The carbon chain extension of the adducts by the reaction of the sulfonyl-stabilized carbanion with alkyl halides and the reductive desulfonylation proceeded without affecting the trifluoromethyl group. Trifluoromenadione (6h) was prepared in 71% yield through the reaction of 1 with 1-methoxyisobenzofuran (2h).

KEYWORDS 1-phenylsulfonyl-3,3,3-trifluoropropene; trifluoropropene; Diels-Alder reaction; trifluoromethyl; trifluoromenadione; vitamin K

Trifluoromethylated compounds have been attracting attention due to their characteristic properties, particularly related to biologically active compounds.[1] Trifluoromethylation,[2] fluorination,[3] and the halogen exchange reaction[4] are possible methods for introducing the trifluoromethyl group into a molecule. However, such methods are sometimes accompanied by low reactivity and low selectivity. Another promising approach is to use a proper building block which has the trifluoromethyl group in it. Recently, we reported the Michael-type addition reaction of 1-phenylsulfonyl-3,3,3-trifluoropropene (1) as a useful building block for preparing trifluoromethylated compounds related to the amino acids[5] and the vitamin D_2 analog.[6] Further development of the synthetic utility of 1 has been focused on the Diels-Alder reaction to prepare the functionalized six-membered carbocyclic systems. A part of this study is related to the fluorine-modified retinoids[7] and vitamin K analogs. In this paper, we report the Diels-Alder reaction of 1 and some reactions of the cycloadducts involving an efficient preparation of trifluoromenadione (6h).

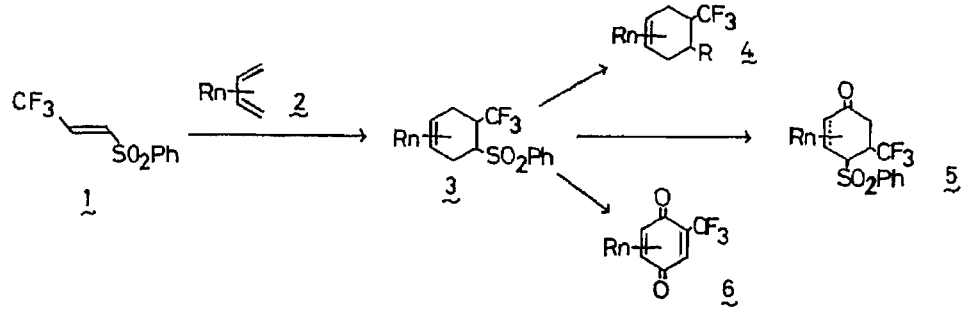


Chart 1

The phenylsulfonyl group in 1 would be expected to play the following roles: Enhancing the reactivity with dienes and affecting the regiochemistry with unsymmetrical dienes. Furthermore, the introduction of alkyl substituents using sulfonyl stabilized carbanion and the β- or reductive elimination of the phenylsulfonyl group in the cycloadducts may possibly be carried out to give a variety of trifluoromethylated six-membered compounds (Chart 1).

Reaction of 1 with cyclopentadiene (2a) proceeded at 0°C to give the cycloadduct (3a) in 80% yield. Similar reactions of 1 with 1,3-butadiene (2b), o-quinodimethane (2c), 2,5-bistrimethylsilyloxyfuran (2d) and 2-methoxyfuran (2e) gave the cycloadducts in good yields (Table 1). The Diels-Alder reaction of 3,3,3-trifluoropropene itself with dienes requires high temperature and a long reaction period to give the cycloadducts in moderate yields.[8] Thus, the presence of the phenylsulfonyl group greatly enhances the reactivity.[9] Since the phenylsulfonyl group in the cycloadducts can be replaced by hydrogen, as described below, 1 serves as a reactive synthetic equivalent of trifluoropropene with symmetrical dienes.

Reaction of 1 with 2-trimethylsilyloxy-1,3-butadiene (2f) gave the regioisomeric mixture of the cycloadducts. These were isolated as the ethylene ketals (7 and 8) in 66% and 23% yield, respectively (Chart 2). Desulfonylation of 7 and 8 with Na(Hg) afforded 3-trifluoromethyl- and 4-trifluoromethylcyclohexanone ethylene ketals. Their structures were confirmed by their ^{13}C-nmr spectra on the basis of the symmetrical structure of the 4-trifluoromethyl derivative. On the other hand, when the mixture of the cycloadducts was treated with 1 N HCl to convert to the ketones, only the 4-phenylsulfonyl derivative (5f) was isolated as a sulfone compound in 68% yieid. But the 3-phenylsulfonyl derivative was further converted to 4-trifluoromethylcyclohexenone by the elimination of benzenesulfinic acid. Similar reaction of 1 with 2,4-bistrimethylsilyloxy-1,3-pentadiene (2g) at 140°C for 55 h, followed by the acidic work-up, gave the cyclohexenone (5g) in 78% yield (Table 1).

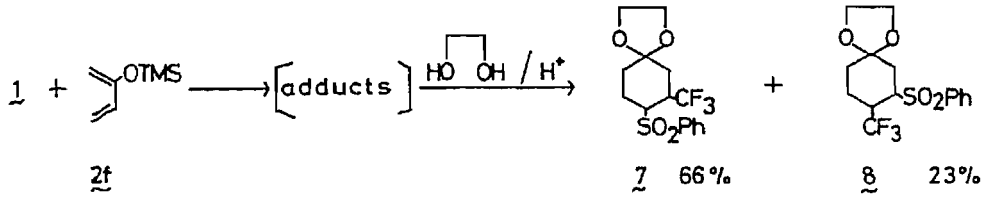


Chart 2

Introduction of the alkyl substituent into the cycloadduct (3) was achieved by the standard method.[9] Treatment of 3c with LDA followed by the reaction with prenyl bromide gave the coupling product in 83% yield, which was desulfonylated by the reaction with Na(Hg) to afford 4c in 89% yield. A similar reaction sequence was carried out using 3b (Chart 3).

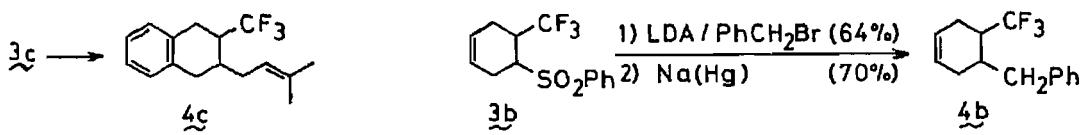
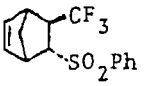
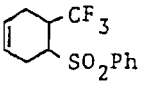
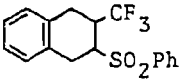
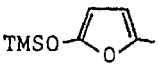
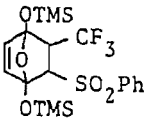
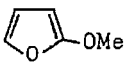
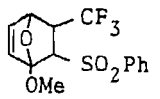
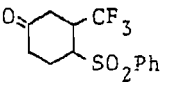
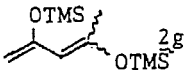
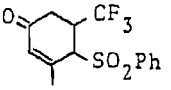


Chart 3

Table 1. The Diels-Alder Reaction of 1 with 1,3-Dienes(2)

2		Temp(°C)	Time(h)	Product		Yield(%)[a]
	2a	0	1		3a[b]	80
	2b	180	72		3b[e]	94
	2c[c]	70	0.5		3c[e]	86
	2d[d]	50	24		3d[e]	50
	2e	60	3		3e[e]	91
	2f	110	4		5f[f]	68
	2g	140	55		5g[f]	78

a) isolated yield b) CF_3-exo/CF_3-endo=1/4 c) To a mixture of 1 and Zn powder in DMF at 70°C was added o-xylylene dibromide. K. Alder and D. Fremercy, Tetrahedron, 14, 190(1961). d) P. Brownbridge and T-H. Chan, Tetrahedron Lett., 21, 3423(1980). e) On the basis of the ^{1}H-nmr (400 MHz) and ^{19}F-nmr (56.4 MHz) spectrum, a single isomer was isolated. f) After the Diels-Alder reaction, the reaction mixture was treated with 1 N HCl at room temperature.

Next, we turned our attention to the preparation of the trifluoromethylated quinones (6). Several attempts to convert the tetraline derivative (4c') to trifluoromenadione (6h) failed. For example, reaction of 4c' with CrO_3 in acetic acid gave the tetralone derivative (5c) in 61% yield.[10] Further oxidation of 5c under enforced conditions resulted in the formation of a messy mass. The existence of the trifluoromethyl group caused severe resistance against the oxidation at the benzylic position. Alternatively, the Diels-Alder reaction of 1 with 1-methoxyisobenzofuran (2h) was carried out. Reaction of 1 with 2h generated by the reported method (acetic acid catalized reaction)[11] required a long reaction period for the completion of the reaction to give a complex mixture. Adding AcOK generates 2h in proper concentration to activate the Diels-Alder reaction. Thus, a mixture of 1 (1 equiv.), 1,3-dihydro-1,1-dimethoxyisobenzofuran (2 equiv.), AcOK (4 equiv.) and AcOH in toluene was refluxed for 3 h to give the cycloadducts.[12] This was, in turn, treated with 1 N HCl and then oxidized with MnO_2 to give trifluoromenadione (6h)[13] in 71% yield (Chart 4).

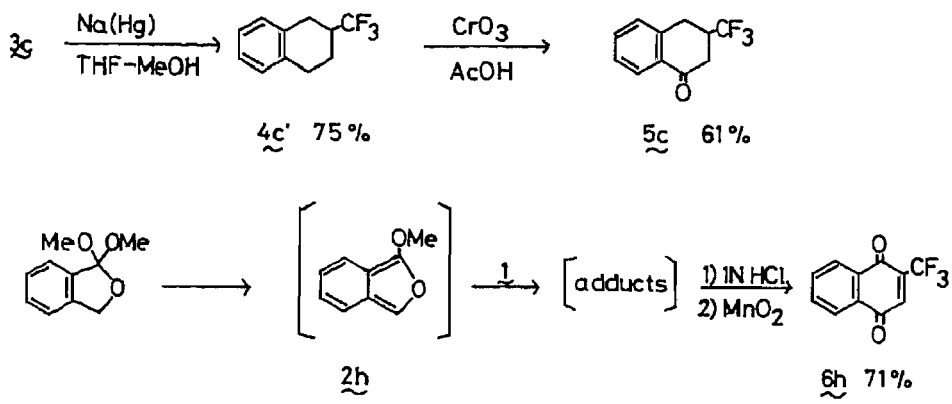


Chart 4

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Communications to the Editor

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A NEW AND FACILE SYNTHESIS OF 2-ALKENENITRILES USING 3-BUTEN-2-ONE CYANOHYDRIN DIETHYL PHOSPHATE; APPLICATION TO (±)-(E)-NUCIFEROL SYNTHESIS

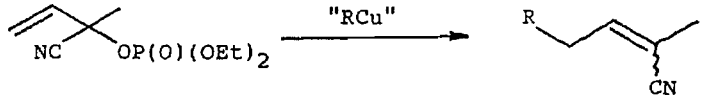
Ryuji Yoneda, Shinya Harusawa, and Takushi Kurihara*
Osaka University of Pharmaceutical Sciences, 2-10-65, Kawai, Matsubara, Osaka 580, Japan

3-Buten-2-one cyanohydrin diethyl phosphate reacted regio- and stereo-selectively with a variety of dialkyl cuprates and ethoxycarbonylmethylcopper giving γ-coupling products, 2-alkenenitriles, one of which was used to synthesize (±)-(E)-Nuciferol.

KEYWORDS ——— 3-buten-2-one cyanohydrin diethyl phosphate; organocopper reagent; 2-alkenenitrile; stereoselectivity; regioselectivity; (±)-(E)-Nuciferol

Recently we reported a novel regio- and stereo-specific arylation of α-cyanoallylic phosphate such as 3-buten-2-one cyanohydrin diethyl phosphate (**1**), via S_N2' reaction, to give 4-arylangelonitriles.[1] Although the regio- and stereospecific coupling of primary and secondary allylic phosphates with Grignard reagents under catalytic[2] and non-catalytic[3] conditions have been investigated, little is known about the formation of the carbon-carbon bond of α,β-unsaturated cyanohydrin diethyl phosphate (cyanophosphate) with organometallic nucleophile. In a continuation of our work on the synthetic utility of cyanophosphates, we describe here a new highly regioselective γ-alkylation of **1** with a variety of organocopper reagents,[4] which have been widely used for carbon-carbon bond formation in organic synthesis.[5] The process was found to be a new method for regioselective homologation of C_5-unit. Its application to (E)-Nuciferol synthesisis also described.

First, **1**, prepared from methyl vinyl ketone and diethyl phosphorocyanidate in the presence of lithium cyanide,[1] with a slight excess (1.2 equiv.) of lithium dibutylcuprate ($Bu^n{}_2CuLi$) was coupled in ether at -78 °C. After quenching, the crude reaction mixture was analyzed by ^{1}H-NMR spectrum, which showed the presence of the γ-coupling products (**2**) with a 27 : 73 mixture of E- and Z-stereoisomers.



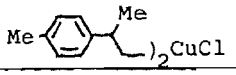
1 **2**

Chart 1

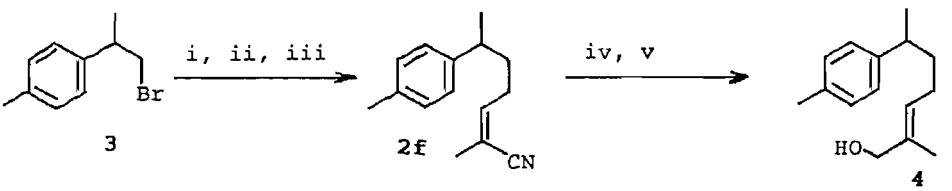
The structures of the products (E- and Z-**2a**) were confirmed by ^{1}H-NMR spectral evidence of the pure E- and Z-2-methyloctenenitriles (**2a**) separated by flash column chromatography on silica gel in 57% combined yield. When tetrahydrofuran

(THF) was used as the solvent, the stereoselectivity as well as the combined yield of E- and Z-**2a** was improved to 18 : 82 in 71%. No α-coupling product could be found, even in the ^{1}H-NMR spectra of the crude products of the above reactions. Other cuprate species that might have a useful effect on the E/Z ratios were investigated, such as Lewis acid-mediated organocoppers[6] (Bu^nCu. BF_3 and $Bu^n{}_2CuLi$. BF_3) and particulary the so-called higher-order cuprates[7] ($Bu^n{}_2CuLi_2CN$). Unfortunately, however, prominent enhancement has scarcely been achieved in either stereoselectivity or yield. Thus, reactions of **1** with other R_2CuLi were carried out in THF, the results of which are summarized in the Table. It should be noted that only a γ-coupling product was obtained regioselectively in all cases. The γ-coupling of **1** with ethoxycarbonylmethylcuprate (entry 6) was also observed in satisfactory stereoselectivity and yield. As is shown in entry 7, the present regioselective alkylation of **1** with lithium dialkylcuprate, prepared from the bromide (**3**) and giving **2f** in good yield, would be applicable to the synthesis of (Z)-Nuciferol[8] via a sequence of reduction and hydroxylation.[9] However, attempts to reduce the nitrile group in **2f** to primary amine were

Table I. Reaction of 3-Buten-2-one Cyanophosphate (**1**) with Organocopper Reagents[a]

Run	Reagent ("RCu")	Solvent	Yield (%)	Product	Product ratio[b] E : Z
1	$Bu^n{}_2CuLi$	Et_2O	57	**2a**	27 : 73
2	$Bu^n{}_2CuLi$	THF	83	**2a**	18 : 82
3	$Bu^s{}_2CuLi$	THF	88	**2b**	14 : 86
4	$Hex^n{}_2CuLi$[c]	THF	72	**2c**	24 : 76
5	$Phen_2CuLi$[c]	THF	96	**2d**	15 : 85
6	EtO_2CCH_2Cu	THF	87	**2e**	12 : 88
7		THF	88	**2f**	11 : 89

a) All new compounds (except for **2a**[10]) exhibited satisfactory spectroscopic data and were analyzed by high-resolution mass spectra. The following procedure is representative: A solution of **1** (1 mmol) in THF (3 ml) was added dropwise to a solution of $Bu^n{}_2CuLi$ (1.2 mmol) in THF (5 ml) under N_2 at -78°C. The mixture was stirred at -78 °C for 30 min. After the usual work-up, the E- and Z-isomers were separated by column chromatography. b) The ratio was determined by ^{1}H-NMR measurements. c) Hex=hexyl, Phen=phenethyl



3 **2f** **4**

Reagents and conditions: i, Li; ii, CuI; iii, **1**, -78 °C;
iv, KOH/ethyleneglycol, reflux, 5 h; v, $LiAlH_4$, ether, r.t., 1 h

Chart 2

unsuccessful. Thus, hydrolysis[11] of **2f** with KOH in refluxing ethyleneglycol and subsequent $LiAlH_4$ reduction gave Nuciferol (**4**) [^{1}H-NMR (CCl_4) δ 1.51 (3H, s, CH_3) and 5.26 (1H, s, =CH); M^+, m/z 218.1670 (calc. 218.1671)] in 77% overall yield. Its ^{1}H-NMR spectrum was identical with that of (E)-Nuciferol[8]:[^{1}H-NMR (CCl_4) δ 1.50 (3H, s, CH_3) and 5.28 (1H, s, =CH)].

The above results demonstrate that 3-buten-2-one cyanophosphate (**1**) would be a useful synthon for the regioselective homologation of the C_5-unit, including α,β-unsaturated nitrile moiety, by reaction with an organocopper reagent. Further work is now in progress to extend the reaction to α,β-unsaturated aldehyde cyanohydrin phosphate.

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OXYGENATION OF BIPHENYL BY THE REAGENT SYSTEM $Fe(MeCN)_6^{2+}$-H_2O_2-Ac_2O: AN IMPLICATION TO THE MECHANISM IN MAMMALIAN METABOLISM

Eiichi Kotani,[a] Atsushi Midorikawa,[a] Akira Tanaka,[b] and Seisho Tobinaga[a]*

[a]Showa College of Pharmaceutical Sciences, Tsurumaki, Setagaya-ku, Tokyo 154, Japan and [b]National Institute of Hygienic Sciences, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

Oxygenation of biphenyl with $Fe(MeCN)_6^{2+}$-H_2O_2-Ac_2O, a model enzyme system for mono-oxygenase, was explored in connection with mammalian metabolism. The reaction yielded 2- and 4-acetoxy derivatives in a ratio of 2.5 : 1. The mechanism of this reaction may be a laboratory model explaining the biological mechanism whereby biphenyl is oxygenated by heme-containing mono-oxygenase.

KEYWORDS —— oxygenation; biphenyl; $Fe(MeCN)_6^{2+}$-H_2O_2-Ac_2O; model enzyme; mono-oxygenase

Considerable interest has been focused in organic chemistry on the oxygenation of biphenyls from the viewpoints of mechanics and synthesis,[1] and of mammalian metabolism.[2] Chemical acetoxylation of biphenyl usually affords the 2- and 4-acetoxy derivatives, preferentially the 4- compound,[1a-e] except for acetoxylation with $Pd(OAc)_2$ which gives the 3-acetoxybiphenyl.[1f] Biphenyl is normally metabolized by hydroxylation to either the 2-, 4-, or both, hydroxylated derivatives. It was reported, however, that young rats hydroxylate biphenyl to both the 2- and 4-hydroxy compounds whereas adult rats preferentially form the 4-hydroxy derivative.[2a] Also, the *in vivo* hydroxylation of biphenyl is usually stimulated when animals are pretreated with inducing agents. For example, it has been reported that the formation of 2-hydroxybiphenyl is stimulated when rats and mice are pretreated with benzo[a]pyrene and that 4-hydroxylation is stimulated when they are pretreated with phenobarbital.[2b] In addition, a recent paper indicated that pretreatment with 3-methylcholanthrene caused simultaneous increases in both biphenyl 2- and 4-hydroxylation in the *in vivo* assay system with rat liver microsomes.[2c] It has also been reported that *in vitro* hydroxylation of biphenyl is genetically associated with the induction of aryl hydrocarbon hydroxylase (which may be cytochrome P-450) in responsive strains of mice.[2d] Concerning these metabolic observations, we investigated oxygenation of biphenyl by the reagent system $Fe(AN)_6^{2+}$-H_2O_2-Ac_2O; AN= MeCN, a model reagent for mono-oxygenase. It afforded 2- and 4-acetoxybiphenyls in a ratio of 2.5 : 1.

Oxygenation of biphenyl by the reagent system was carried out as follows. To a solution of $Fe(ClO_4)_2$ and biphenyl in AN and Ac_2O, a solution of H_2O_2 in AN was added dropwise, keeping the temperature at 25~30 °C, in appropriate molar ratios (Fe^{2+} : biphenyl : H_2O_2) (Table I). The resulting solutions were worked up in the way reported previously,[3] then purified by silica gel column chromatography ($CHCl_3$: hexane = 1 : 1 as an eluent) to divide the monoacetate and diacetate fractions. The separated fractions were hydrolyzed to give free hydroxylated forms and then chromatographed with silica gel to separate the 2- from the 4-hydroxybiphenyls, and the 2,2'-dihydroxy- from a mixture of 2,4'- and 2,5-dihydroxybiphenyls. Further, the 2,4'- was separated from the mixture of 2,4'- and 2,5-dihydroxybiphenyls by oxidation with Ag_2O to give the quinone (from the 2,5-dihydroxy compound) and the unchanged dihydroxy compound (i. e. the 2,4'-dihydroxy compound).

Table I. Oxygenation of Biphenyl with $Fe(AN)_6^{2+}$-H_2O_2-Ac_2O in AN

Run	Molar ratio biphenyl: Fe^{2+}: H_2O_2	Product[a] (Yield, %[b]) monoacetate 2-	monoacetate 4-	diacetate 2,2'-	diacetate 2,4'-	diacetate 2,5-	Recovery (%)
1	1 : 0.5 : 1.5	24.9	11.9	1.2	2.8	0.7	36.0
2	1 : 0.1 : 1.5	15.3	9.0	0.7	2.4	0.6	38.6
3	1 : 0.5 : 3.0	19.0	3.8	4.7	8.6	2.2	5.0
4	1 : 0.1 : 3.0	11.2	7.1	2.0	2.7	0.7	6.8
5	1 : 1 : 1.5	34.6	13.7	-	-	-	41.1

a) Identified by direct comparison with authentic samples.
b) Isolated yields.

In like manner, oxygenation of 2-acetoxybiphenyl with the reagent system acetoxybiphenyl : Fe^{2+} : H_2O_2 in AN in a molar ratio of 1 : 0.5 : 1.5 gave 2,2'-, 2,4'-, 2,5-diacetoxybiphenyls, 2-hydroxybiphenyl (a hydrolyzed compound), and unchanged acetoxybiphenyl in yields of 4.1, 1.8, 0.5, 7.6, and 52.3%, respectively.

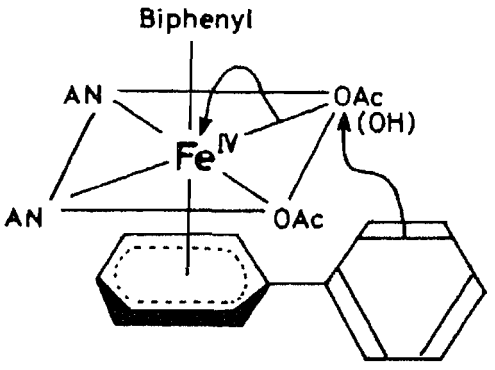

Chart 1

The mechanism of the preferential formations of 2-acetoxybiphenyl and 2,2'-diacetoxybiphenyl in the oxygenations of biphenyl and 2-acetoxybiphenyl was assumed from the following observation. Absorption spectral changes were observed when biphenyl was added to a 1×10^{-4} M solution of the iron(III) solvate[4] $Fe(AN)_6(ClO_4)_3$ in AN at 20 °C. In these

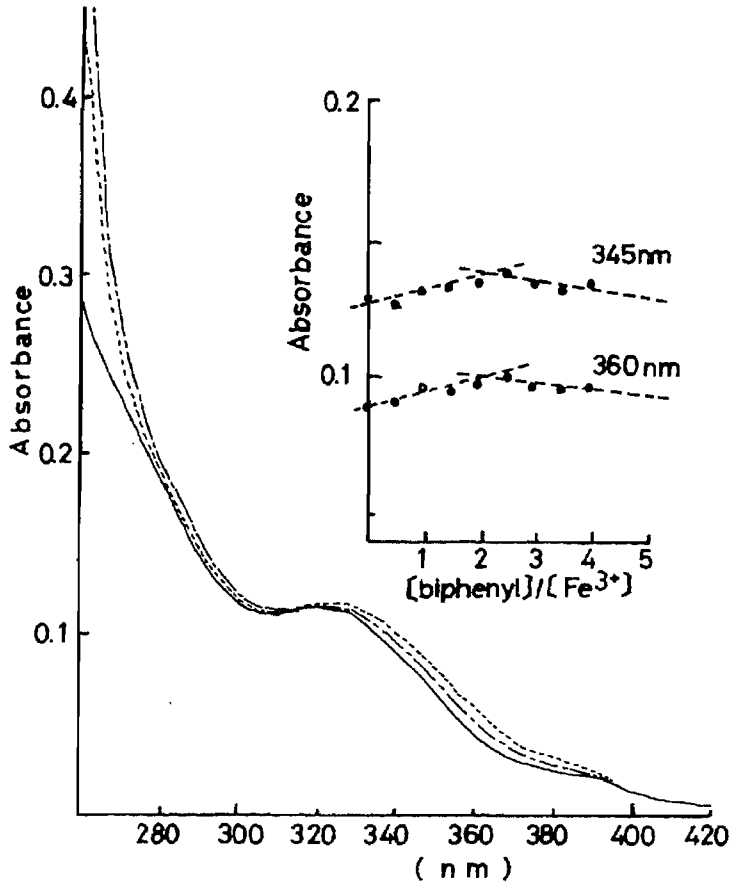

Figure 1. Absorption spectrum change of Fe^{3+} - biphenyl in AN solution; The concentrations of biphenyl are 1 x 10^{-4} (·····), 2 x 10^{-4} M (——). The concentration of $Fe(AN)_6(ClO_4)_3$ in AN is 1 x 10^{-4}M (——). Mole ratio plots for the solution of $Fe(AN)_6(ClO_4)_3$ -biphenyl in AN. The concentration of $Fe(AN)_6(ClO_4)_3$ is 1 x 10^{-4}.

spectra isosbestic points existed at 310 and 400 nm (Fig. 1). This indicates the formation of a complex, weak but significant, between the iron(III) solvate and biphenyl in a mole ratio of 1 : 2 in equilibrium (assumed by mole ratio method). As already established, the reactive species of this oxygenation reagent system may be $Fe^{IV}(OH)(OAc)^{2+}$ or $Fe^{IV}(OAc)_2^{2+}$, and the reaction may proceed in either an ionic or a radical mode, depending on the organic substrate.[3] Thus, the preferential formation of the 2-acetoxy derivative in the oxygenation of biphenyl may be due to the complex formation followed by acetoxylation with $Fe^{IV}(OH)(OAc)^{2+}$ or $Fe^{IV}(OAc)_2^{2+}$, as shown in Chart 1.[5,6] These results may explain the in vivo mechanism whereby biphenyl is oxygenated by heme-containing mono-oxygenase.

On the other hand, the preferential formation of 2,2'-diacetoxybiphenyl by the reaction with 2-acetoxybiphenyl may indicate that the complex is formed either between iron(III) and the acetoxy group rather than the phenyl group, or between iron(III) and the phenyl group having electron-donating acetoxy moiety rather than the phenyl group with no substituent. Then the 2' position is acetoxylated with $Fe^{IV}(OAc)_2^{2+}$.

Further, the formation of 2- and 4-acetoxybiphenyls was confirmed also in

an experiment using U-^{14}C-biphenyl. Since the 2-hydroxy compound is a carcinogen in rats,[7] an easy supply of ^{14}C-2-hydroxybiphenyl by this synthesis method would be useful to study the biochemical mechanisms by which rat bladder tumor is induced.

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Communications to the Editor

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TWO NOVEL FLAVANONES, METHYLLINDERATONE AND ISOLINDERATONE, FROM LINDERA UMBELLATA THUNB. VAR. MEMBRANACEA (MAXIM.) MOMIYAMA

Kazuhiko Ichino, Hitoshi Tanaka, and Kazuo Ito*
Faculty of Pharmacy, Meijo University, Tenpaku-ku, Nagoya 468, Japan

From the fresh leaves of Lindera umbellata Thunb. var. membranacea (Maxim.) Momiyama two new flavanones, methyllinderatone (**1**) and isolinderatone (**2**), were isolated. Their structures were established by chemical and spectroscopic means.

KEYWORDS —— Lauraceae; Lindera umbellata var. membranacea; linderatin; linderatone; methyllinderatone; isolinderatone; flavanone; p-menthene

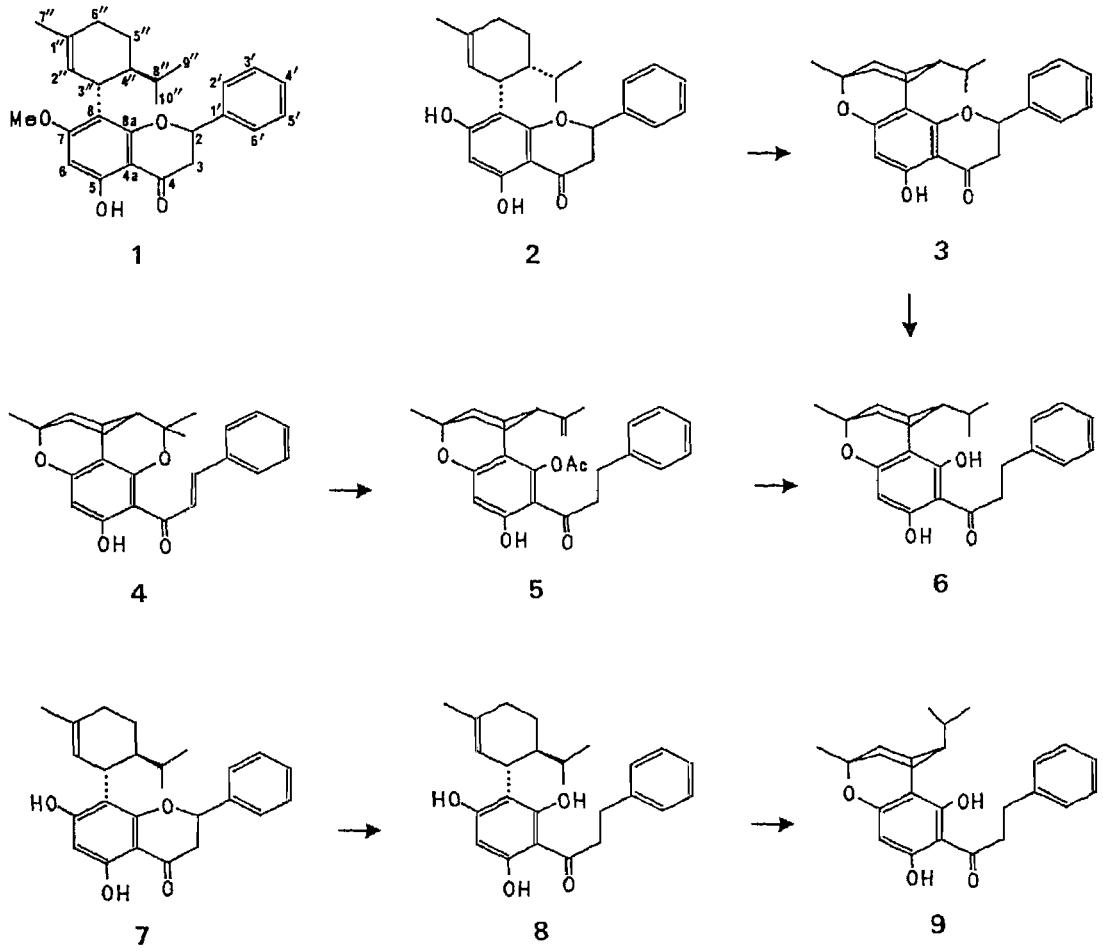
In a previous paper,[1] we reported the isolation and the structural determination of a dihydrochalcone, linderatin (**8**), from Lindera umbellata Thunb. var. lancea Momiyama and a flavanone, linderatone (**7**), from Lindera umbellata Thunb. Both compounds are novel flavonoids having a p-menthene substituent. In the course of further investigation of the genus Lindera, we isolated two new flavanone derivatives, named methyllinderatone (**1**) and isolinderatone (**2**) from the fresh leaves of Lindera umbellata Thunb. var. membranacea (Maxim.) Momiyama. Here we describe the structure elucidation of these compounds.

Methyllinderatone (**1**), a viscous oil, $[\alpha]_D$ +68.6° (c=0.35, $CHCl_3$), gave a bluish color with ethanolic ferric chloride and was positive to the magnesium-hydrochloric acid test. The molecular formula was determined to be $C_{26}H_{30}O_4$ by the high-resolution mass spectrum (m/z 406.2158). The ^{13}C-NMR spectrum indicated the presence of twenty-six carbons (Table I). Other spectra of this compound are: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600, 1630, 1570, 1495; UV λ_{max}^{MeOH} nm: 232 (sh), 292, 341 ($\lambda_{max}^{MeOH+AlCl_3}$ nm: 315, 364); ^{1}H-NMR ($CDCl_3$) δ: 0.79, 0.84 (6H, d x 2, J=7 Hz, two 8"-Me), 1.66 (3H, br s, 1"-Me), 2.76 (1H, dd, J=4, 17 Hz, 3-H_{eq}), 3.10 (1H, dd, J=13, 17 Hz, 3-H_{ax}), 3.77 (3H, s, 7-OMe), 5.16 (1H, br s, 2"-H), 5.43 (1H, dd, J=4, 13 Hz, 2-H), 6.08 (1H, s, 6-H), 7.44 (5H, s, Ar-H), 12.34 (1H, s, 5-OH); CD (c=0.012, MeOH): $[\theta]_{310}$ +2.00 x 10^3, $[\theta]_{289}$ -6.32 x 10^3, $[\theta]_{255}$ +6.99 x 10^3.

The mass spectrum of **1** showed a molecular ion at m/z 406 indicating an increase of fourteen mass units in comparison with **7**. This spectrum also had the characteristic peak at m/z 336 which was formed by the retro Diels-Alder reaction[2] of a p-menthene unit as in **7**. The ^{1}H-NMR and ^{13}C-NMR spectra of this compound were very similar to those of **7** except for the signal due to a methoxyl function. These results suggest that **1** may be a 7-O-methyl ether of **7** (^{1}H-NMR

δ: 3.77 (7-OMe), 12.34 (5-OH)). And treatment of 7 with CH_2N_2 in ether afforded a monomethyl derivative which was identical with 1 in all respects. Therefore, the structure of methyllinderatone is represented by the formula 1.

Next, isolinderatone 2, a viscous oil, $[\alpha]_D$ -67.1° ($CHCl_3$, c=1.25), gave a bluish color with ethanolic ferric chloride and was positive to the magnesium-hydrochloric acid test. The molecular formula was determined to be $C_{25}H_{28}O_4$ by the high-resolution mass spectrum (m/z 392.1975). The ^{13}C-NMR spectrum indicated the presence of twenty-five carbons (Table I). Other spectra of this compound are: IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3370, 1640, 1600, 1445; UV λ_{max}^{MeOH} nm: 222, 294, 325 ($\lambda_{max}^{MeOH+AlCl_3}$ nm: 218 (sh), 315, 384); ^1H-NMR ($CDCl_3$) δ: 0.55, 0.79 (6H, d x 2, J=7 Hz, two 8"-Me), 1.77 (3H, br s, 1"-Me), 2.75 (1H, dd, J=4, 17 Hz, 3-H_{eq}), 3.07 (1H, dd, J=13, 17 Hz, 3-H_{ax}), 3.66-3.92 (1H, m, 3"-H), 5.30 (1H, dd, J=4, 13 Hz, 2-H), 5.50 (1H, br s, 2"-H), 6.04 (1H, s, 6-H), 7.39 (5H, s, Ar-H), 12.34 (1H, s, 5-OH); CD (c=0.333, MeOH): $[\theta]_{308}$ +2.12 x 10^3, $[\theta]_{288}$ -9.42 x 10^3, $[\theta]_{248}$ +4.71 x 10^2.



1 2 3 4 5 6 7 8 9

Chart 1

Table I. ^{13}C-NMR Chemical Shifts of 1, 2 and 7 in Acetone-d_6

Carbon	1	2	7
C-2	80.1	79.9	79.7
C-3	43.8	43.8	43.7
C-4	197.4	197.5	196.7
C-4a	103.6	103.5	103.0
C-5	162.6*	165.6*	165.6*
C-6	92.1	96.8	95.8
C-7	167.4*	163.1*	163.3*
C-8	113.3	110.7	111.8
C-8a	162.2*	162.1*	161.7*
C-1'	140.1	140.2	139.9
C-2'	129.5	129.2	129.2
C-3'	127.3	129.1	127.1
C-4'	126.5	127.0	126.2
C-5'	127.3	129.1	127.1
C-6'	129.5	129.2	129.2
C-1"	132.5	133.5	134.0
C-2"	126.5	126.4	126.2
C-3"	35.9	36.3	35.7
C-4"	42.4	43.2	42.4
C-5"	23.6	23.5	23.6
C-6"	31.5	30.3	31.4
C-7"	23.9	23.5	23.9
C-8"	29.3	29.3	29.1
C-9"	16.7	16.7	16.7
C-10"	21.8	21.9	21.9
OMe	56.4	-	-

* Assignments may be interchanged.

Like 7, the mass spectrum of 2 showed a molecular ion at m/z 406. This spectrum also had a characteristic peak at m/z 322 which was formed by the retro Diels-Alder reaction of a p-menth-1-ene unit as in 1 and 7. The ^{13}C-NMR spectrum was similar to that of 7 except for a few signals assigned to the carbons of monoterpene unit (Table I). The ^{1}H-NMR spectrum also showed close similarity to that of 7 except for the chemical shifts of the geminal methyl groups. Comparison of the ^{13}C- and ^{1}H-NMR spectra of 2 with those of 5,7-dihydroxyflavanone also showed close similarity. This compound also seemed to be a 5,7-dihydroxyflavanone containing a monoterpene substituent on the A ring. The stereochemistry of its flavanone skeleton was the 2S configuration as in 7 according to the CD spectrum.[3] And the negative result of the Gibbs test and the bathochromic shift in the UV spectrum support the idea that 2 has a p-menth-1-ene group, not on the C-6 but on the C-8 position in the A ring, as mentioned[1b] about 7.

Next, from careful investigation by ^{1}H-NMR spectroscopy, we found that the signal of $C_{3''}$-H was observed as multiplet[4] in **2**, while it was found to be doublet in **7**[1b] ($J_{3''H,4''H}$ = 10 Hz) and **8**[1a] ($J_{3''H,4''H}$ = 12 Hz). This suggests that **2** might have been a C-4" epimer (cis-isomer) of **7** concerning on the substitution pattern of the monoterpenoid unit. We already reported[1] that hydrogenolysis of **7** followed by acid treatment of the resulting product **8** gave cyclolinderatin (**9**). Isolinderatone (**2**), the C-4" epimer (cis-isomer) of **7**, also can be converted into the corresponding dihydrochalcone **6** (C-4" epimer of **9**), which can be obtained from the readily available rubranin (**4**).[5]

Thus, catalytic reduction of **4** with Pd-C in AcOEt followed by selective cleavage of the pyran ring with Ac_2O afforded an isopropenyl derivative **5**. And compound **5** was transformed into the cis-isomer **6**[6] by catalytic reduction and subsequent hydrolysis. Next, acid treatment of **2**, followed by hydrogenolysis of the resulting cyclization product **3**, gave a compound which was identical in all respects, not with **9** but with product **6** derived from **4**.

Therefore, the structure of isolinderatone must be represented by the formula **2**. It is particularly interesting that we could obtain the two epimers **2** and **7** from the same natural source.

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4) In the ^{1}H-NMR spectrum of **7** or **8**, the signal of $C_{3''}$-H was observed as doublet ($J_{3''H,4''H}$ = 10~12 Hz) and no coupling was found between $C_{2''}$-H and $C_{3''}$-H. Further investigation with molecular models (Dreiding models) revealed the fact that the dihedral angle between the two protons was approximately a right angle. On the other hand, the signal of $C_{3''}$-H in **2** was observed as complicated pattern, in which the dihedral angle between $C_{2''}$-H and $C_{3''}$-H was not a right angle.
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6) The spectral data of **10** were as follows: high-resolution mass spectrum m/z: 394.2145 ($C_{25}H_{30}O_4$); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3580, 1620; UV λ_{max}^{MeOH} nm: 228 (sh), 294; ^{1}H-NMR ($CDCl_3$) δ: 0.76, 1.14 (6H, d x 2), 1.33 (3H, s), 2.86-3.48 (4H, m), 5.66 (1H, s), 7.15 (5H, s), 13.76 (1H, s).

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A REINVESTIGATION OF THE STRUCTURE OF ZEDERONE, A FURANOGERMACRANE-TYPE SESQUITERPENE FROM ZEDOARY

Hirotaka Shibuya,[a] Yoshihiro Hamamoto,[b] Yang Cai,[a] and Isao Kitagawa*,[a]

Faculty of Pharmaceutical Sciences, Osaka University,[a] 1-6, Yamada-oka, Suita, Osaka 565, Japan and Suntory Institute for Biomedical Research,[b] Shimamoto-cho, Mishima-gun, Osaka 618, Japan

The stereochemical structure of zederone, a furanogermacrane-type sesquiterpene isolated from various zedoary (Zedoariae Rhizoma), has been revised from a previously proposed structure (8a) to 8 on the basis of the NOE experiments in the ^{1}H NMR study at 500 MHz, and the absolute configuration has been determined by X-ray analysis of 12-bromozederone (9).

KEYWORDS — Zedoariae Rhizoma; zederone; epoxide configuration; furanogermacrane sesquiterpene ^{1}H NMR; furanogermacrane sesquiterpene X-ray analysis

During the course of our chemical study of the bioactive constituents of zedoary (Zedoariae Rhizoma), we isolated furanogermenone (7) as an experimental anti-hepatitis principle from Zedoariae Rhizoma imported from China.[1] Afterwards, we isolated (4S,5S)-(+)-germacrone 4,5-epoxide (2) from zedoary[2] cultivated in Yakushima, Japan, and elucidated the absolute stereostructure.[3] Since the epoxide (2) was considered to be a plausible biogenetic intermediate for concomitant sesquiterpenoids in the zedoary, the epoxide (2) was subjected to a biogenetic-type conversion reaction providing three guaiane-type compounds [e.g. procurcumenol (4) and GU-2 (5)] and an eudesmane-type compound.[4] Furthermore, the essential oil constituents in various zedoary from China, Taiwan, and Yakushima (Japan) were analytically compared by gas-liquid chromatography-mass spectrometry.[5]

Among more than a dozen of sesquiterpenoids hitherto elucidated from various zedoary, zederone was initially isolated from Zedoariae Rhizoma imported from Taiwan and was assigned a furanogermacrane-epoxide structure 8a mainly on the basis of ^{1}H NMR studies at 100 MHz.[6] Since the 4,5-epoxide configuration in 8a appeared exceptional, considering zedoary sesquiterpenoids biogenetically, we have re-investigated this matter.

Thus, a group of sesquiterpenoids, which were isolated from zedoary cultivated in Yakushima[2] and lack a furan-ring, comprise germacrone (1),[7] (4S,5S)-(+)-germacrone 4,5-epoxide (2),[3] dehydrocurdione (3),[8] procurcumenol (GU-1, 4),[4] and GU-2 (5).[4] Their biogenetic correlation may be arrayed as shown in Chart 1.

Epoxidation at C-4,5 in germacrone (1) may give rise to the epoxide (2), which, via the epoxide-ring opening at C-4 followed by a hydride-shift from C-5, may be converted to 3. On the other hand, the epoxide-ring opening at C-5, followed by a transannular reaction from $C_{1(10)}$ and deprotonation, may yield 4 and 5.

As for the furanogermacrane-type sesquiterpenoids, furanodiene (6),[9] furanogermenone (7),[1] and zederone have been characterized from zedoary cultivated in Yakushima.[1,5] These furanosesquiterpenoids may be biogenetically related in a sequence as shown in Chart 2. A furanosesquiterpene 4,5-epoxide (i), although not isolated so far, is anticipated as a biogenetic intermediate 2. Similar biogenetic conversion from i, as from 2 to 3, may give rise to 7 while oxidation at C-6 may yield zederone which is anticipated to have a (4S,5R)-4,5-epoxide moiety (as shown in 8) but is inconsistent with the previously proposed structure 8a.[6]

The ^{1}H NMR spectra of zederone (8) were taken at both 90 MHz and 500 MHz[10] and the assignments are given in Table I. As is apparent from these data, the signals due to 2α,β-H_2 and 3α,β-H_2 (appearing approximately between δ 1.3 and 2.5) are not well resolved and overlap the signal of 4-CH_3 at 90 MHz. Consequently, the results of the selective NOE experiments between 4-CH_3 and 5-H at 90 MHz appear to be improbable. On the other hand, all proton signals of zederone obtained at 500 MHz are well resolved and assigned as given in Table I. It has become clear that the signal of 3β-H is observed at δ 1.29 which is close to the chemical shift (δ 1.34) of 4-CH_3.

In the NOE experiments at 500 MHz, irradiation of 1-H or 3β-H caused a 6% or 7% increase in the signal of 5-H, whereas irradiation of 4-CH_3 resulted in a 7% increase in the 3α-H signal. However, no NOE was observed between the 4-CH_3 signal and the 5-H signal, although the NOE was reported in the previous experiments as occurring at 100 MHz.[6] Consequently, it has become evident that the previously proposed structure (8a) of zederone should be revised to 8.

In order to determine the absolute configuration of zederone (8), it was treated with N-bromosuccinimide (1 equiv.) in $CHCl_3$ to furnish 12-bromozederone

Table I. ^{1}H NMR Data for Zederone (8) [δ in $CDCl_3$, J values in Hz]

	at 90 MHz	at 500 MHz
1-H	5.48 (dd, J=11.5, 4.0)	5.48 (dd, $J_{1,2\alpha}$=11.5, $J_{1,2\beta}$=4.0)
2α-H	a)	2.52 (dddd, $J_{2\alpha,1}$=11.5, $J_{2\alpha,2\beta}$=12.0, $J_{2\alpha,3\alpha}$=3.5, $J_{2\alpha,3\beta}$=13.5)
2β-H	a)	2.23 (br d)
3α-H	a)	2.29 (ddd, $J_{3\alpha,2\alpha}$=3.5, $J_{3\alpha,2\beta}$=3.5, $J_{3\alpha,3\beta}$=13.0)
3β-H	a)	1.29 (ddd, $J_{3\beta,2\alpha}$=13.5, $J_{3\beta,2\beta}$=4.0, $J_{3\beta,3\alpha}$=13.0)
5-H	3.81 (s)	3.81 (s)
9-H_2	3.72 (br s)	3.69, 3.75 (ABq, J=16.5)
12-H	7.09 (q, J=1.3)	7.08 (q, J=1.2)
4-CH_3	1.34 (br s)	1.34 (br s)
10-CH_3	1.60 (br s)	1.60 (br s)
11-CH_3	2.11 (d, J=1.3)	2.11 (d, J=1.2)

a) These signals could not be assigned due to their overlapping.

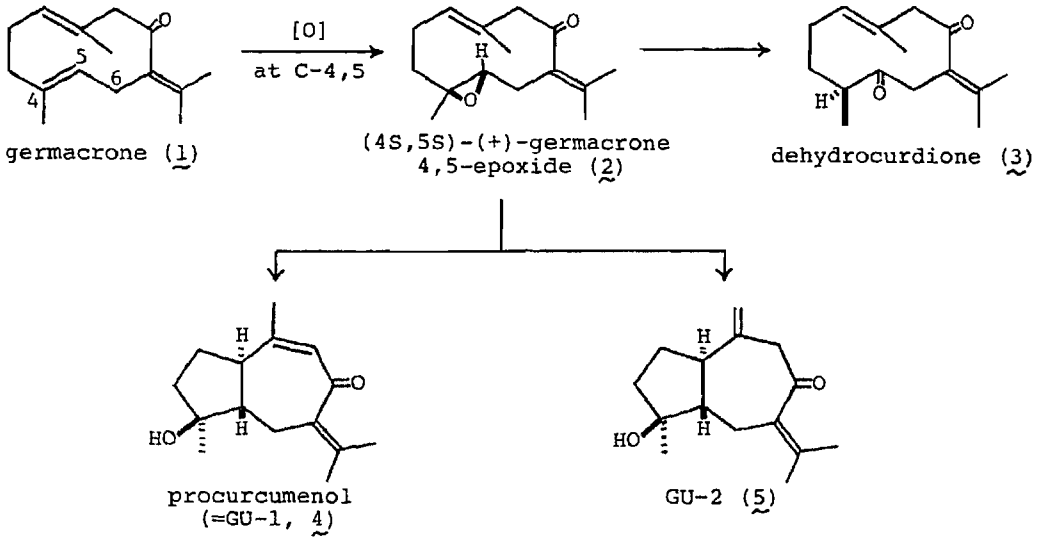

Chart 1

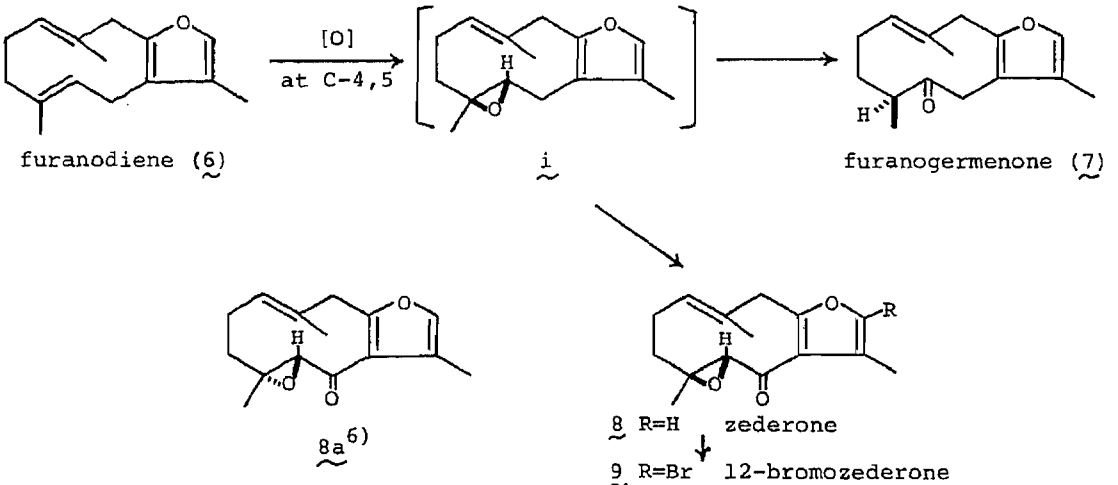

Chart 2

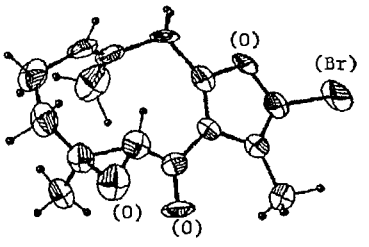

Fig. 1: 9

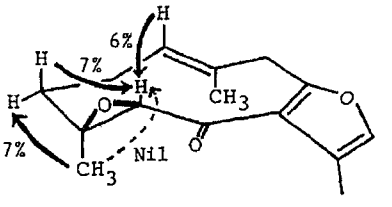

Fig. 2: 8 (➡ NOE)

(9)[11] in 50% yield. The bromo derivative (9) was subjected to X-ray analysis.[12] As shown in a perspective view (Fig. 1), the absolute stereostructure of 12-bromozederone has been clarified as 9, so that the structure of zederone is now depicted as 8 having a (4S,5R)-4,5-epoxide moiety.

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10) Measured with a JEOL JNM-FX-90Q NMR spectrometer and a JEOL JNM-GX 500 NMR spectrometer.

11) Colorless prisms of mp 118-119°C (from hexane-AcOEt), $[\alpha]_D^{23}$ +171° (c=1.0, $CHCl_3$). $C_{15}H_{17}BrO_3$, MS m/z (%): 326 (53), 324 (56). High resolution MS: Calcd. for $C_{15}H_{17}{}^{81}BrO_3$ and $C_{15}H_{17}{}^{79}BrO_3$: 326.034 and 324.036. Found: 326.036 and 324.037. UV λ_{max}^{MeOH} nm (ε): 292 (2500), 211 (18500). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1665. ^1H NMR (500 MHz, $CDCl_3$, δ): 1.35 (3H, br s, 4-CH_3), 1.62 (3H, br s, 10-CH_3), 2.08 (3H, s, 11-CH_3), 3.69, 3.75 (2H, ABq, J=16.5 Hz, 9-H_2), 3.79 (1H, s, 5-H), 5.50 (1H, dd, J=11.5, 4.0 Hz, 1-H).

12) The crystals were recrystallized from hexane-AcOEt. $C_{15}H_{17}Br_1O_3$, orthorhombic, $P2_12_12_1$, a=10.132(1), b=19.080(2), c=7.559(2) Å, V=1461.2(4) $Å^3$, z=4, Dc=1.48, F(000)=166, μ=38.71 cm^{-1}.

The X-ray diffraction intensity data from a crystal (0.2x0.2x0.3 mm) of 9 was obtained on a Rigaku AFC diffractometer equipped with a rotating anode X-ray generater (50 kV - 200 mA), using graphite-monochromated Cu-Kα radiation (λ= 1.5418 Å). A total of 1385 independent reflections with $2\theta \leq 126°$ were collected by the ω scanning mode ($2\theta < 45°$) and the ω/2θ scanning mode ($2\theta \geq 45°$).

The crystal structure was solved by the Patterson method. Hydrogen atoms were determined by difference Fourier synthesis. The refinement was carried out by the block-diagonal least-squares method with anisotropic thermal parameters for non-hydrogen atoms and with isotropic thermal parameters for hydrogen atoms. The R factor was reduced to 0.049 using 1317 reflections with $|Fo| > 3\sigma(Fo)$. Computations were performed on PANAFACOM U-1200 II of Rigaku RASA-5RP system.

The determination of the absolute structure was carried out on the microcomputer, NEC PC-9801 VM-2, using the unpublished program CHIRAL, developed by Prof. Y. Katsube et al.

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FOUR NEW BISABOLENE-TYPE AMINOSESQUITERPENES FROM AN OKINAWAN MARINE SPONGE, *THEONELLA* SP. (THEONELLIDAE)

Isao Kitagawa,*[,a] Norihito Yoshioka,[a] Chikayo Kamba,[a] Masayuki Yoshikawa,[a] and Yoshihiro Hamamoto[b]

Faculty of Pharmaceutical Sciences, Osaka University,[a] 1-6, Yamada-oka, Suita, Osaka 565, Japan and Suntory Institute for Biomedical Research,[b] Shimamoto-cho, Mishima-gun, Osaka 618, Japan

Four new aminosesquiterpenes, aminobisabolene (1), aminobisabolenol (2), isoaminobisabolenol-a (3), and isoaminobisabolenol-b (4), were isolated from an Okinawan marine sponge, *Theonella* sp. (Theonellidae) and their absolute configurations were determined.

KEYWORDS —— aminobisabolene; aminobisabolenol; isoaminobisabolenol-a; isoaminobisabolenol-b; aminosesquiterpene; *Theonella* sp.; marine sponge; allyl benzoate chirality method; sesquiterpene X-ray analysis

In a continuing study in search of bioactive constituents from marine organisms,[1] we recently isolated five peptide-lactones, theonellapeptolides Ia, Ib, Ic, Id, and Ie, from an Okinawan marine sponge of *Theonella* sp. (Theonellidae) collected on the coral reef of Zamami-jima, Okinawa and determined the chemical structure of the major constituent theonellapeptolide Id.[2]

For comparison, we have investigated another sponge specimen of *Theonella* sp. collected in July on the coral reef of Hatoma-jima, Okinawa.[3] This specimen does not contain peptide-lactone but instead contains four new aminosesquiterpenes, aminobisabolene (1), aminobisabolenol (2), isoaminobisabolenol-a (3), and isoaminobisabolenol-b (4). This paper deals with their structure.[4]

The cold acetone extract of the fresh sponge was partitioned into an AcOEt-water mixture. The AcOEt soluble portion was purified by column chromatography (SiO_2, $CHCl_3$-MeOH) to afford aminobisabolene (1, 9.6% from the AcOEt ext.). The water-soluble portion was extracted with 1-BuOH and the 1-BuOH extract was purified by column chromatography (SiO_2: $CHCl_3$-MeOH, $CHCl_3$-MeOH-aq. NH_4OH; SiO_2-$AgNO_3$: $CHCl_3$-MeOH-H_2O) to furnish aminobisabolenol (2) and a mixture of isoaminobisabolenol -a and -b (both 2.0% from the 1-BuOH ext.). The latter mixture was treated with 2,4-dinitrofluorobenzene in aq. Na_2CO_3-acetone (r.t.) and the DNP derivatives were separated by HPLC (Zorbax ODS, MeOH-H_2O=7:1). Removal of the DNP residue with Dowex 1x2 (OH^- form) in aq. acetone furnished isoaminobisabolenol-a (3, 0.8%) and -b (4, 0.8% from the 1-BuOH ext.).

Aminobisabolene (1), colorless oil, $C_{15}H_{27}N$,[5] $[\alpha]_D$ +39° (MeOH), IR ($CHCl_3$): 3480-3100, 1579 cm^{-1}, colored yellowish orange with the Ninhydrin reagent. The ^1H

NMR spectrum[6] of 1 showed signals due to one *tert*. methyl (δ 1.05, s, 8-CH_3), three olefinic methyls [δ 1.62, 1.63, 1.67, all 3H s, 1-CH_3, 13-$(CH_3)_2$], and two olefinic protons (δ 5.11, t, J=7.0 Hz, 12-H; δ 5.38, br s, 2-H). Acetylation of 1 afforded an N-acetate (1a), oil, $C_{17}H_{29}NO$, IR ($CHCl_3$): 3450, 1667, 1500 cm^{-1}, and SeO_2 oxidation of 1a in dioxane at 32°C gave a conjugated aldehyde (5), oil, $C_{17}H_{27}NO_2$, IR ($CHCl_3$): 3436, 1670. Detailed comparisons of ^1H and ^{13}C NMR data (Table I) for 1, 1a, and 5 indicate that 1 has a bisabolene structure with an amino group at C-8. To determine the structure, the bromobenzoyl derivative (1b), mp 114-116°C, $C_{17}H_{28}NOBr$, was subjected to X-ray analysis.[7] As depicted in Fig. 1, the absolute configuration of 1b has been confirmed. Thus, the absolute stereostructure of aminobisabolene (1) with 4*R*, 8*S* configuration has been established.

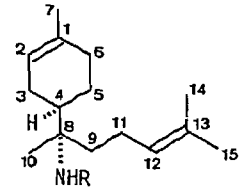
Aminobisabolenol (2), colorless oil, $C_{15}H_{27}NO$, $[\alpha]_D$ +29° (MeOH), IR ($CHCl_3$): 3580, 3320 (br) cm^{-1}, colored yellowish orange with the Ninhydrin reagent. The ^1H NMR spectrum of 2 showed signals due to three *tert*. methyls [δ 1.08, 3H s, 8-CH_3; δ 1.28, 6H s, 13-$(CH_3)_2$], one olefinic methyl (δ 1.63, s, 1-CH_3), and three olefinic protons (δ 5.38, br s, 2-H; δ 5.66, dt, J=15.5, 6.7 Hz, 11-H; δ 5.70, d, J=15.5 Hz, 12-H). Ordinary acetylation of 2 provided an N-acetate (2a), oil, $C_{17}H_{29}NO_2$, IR ($CHCl_3$): 3591, 3443, 1663, 1498 cm^{-1} and an N,O-diacetate (2b), oil, $C_{19}H_{31}NO_3$, IR ($CHCl_3$): 3442, 1718, 1666, 1495, 1238 cm^{-1}, in a 2:1 ratio. Treatment of 2 with 9% HCl-dry MeOH (r.t.) gave isomeric methoxyl derivatives: 2c (50%), oil, $C_{16}H_{29}NO$ and 6 (15%)(a mixture of C-11 isomers). Acetylation of 2c yielded an N-acetate (2d), oil, $C_{18}H_{31}NO_2$, IR ($CHCl_3$): 3450, 1665, 1498 cm^{-1}. Comparisons of the physical data for 2, 2a - 2d, and 6 revealed the plane structure of 2.

Catalytic hydrogenation (5% Pd-C) of 2 (r.t.) and subsequent treatment of the product with $SOCl_2$-pyridine (0°C, 5 min, N_2) provided 7. Acetylation of 7 followed by catalytic hydrogenation (5% Pd-C, r.t.) afforded 8 (63% from 2, a mixture of C-1 isomers in a 5:2 ratio).[8] On the other hand, catalytic hydrogenation (5% Pd-C) of N-acetylaminobisabolene (1a) yielded 8 (a diastereomeric mixture in a 7:2 ratio). Finally, constant-current electrolysis [Pt electrode, $(PhSe)_2$, Et_4NBr, 1 mA/cm^2, 1 h, 0-5°C] of 1a in MeOH furnished 2d (conversion 50%, yield 80%). Thus, the absolute configuration of aminobisabolenol (2) has been determined.

Isoaminobisabolenol-a (3), colorless oil, $C_{15}H_{27}NO$, $[\alpha]_D$ +34° (MeOH), IR ($CHCl_3$): 3665, 3360 (br), 1598 cm^{-1} and isoaminobisabolenol-b (4), colorless oil, $C_{15}H_{27}NO$, $[\alpha]_D$ +40° (MeOH), IR ($CHCl_3$): 3665, 3365 (br), 1608 cm^{-1}, are diastereomers. The ^1H NMR spectra (in $CDCl_3$) of both compounds showed signals due to one *tert*. methyl (3: δ 1.21; 4: δ 1.26; each s), exomethylene protons (3: δ 4.82, 4.98; 4: δ 4.83, 4.98; all 1H, br s), two olefinic methyls (3: δ 1.64, 1.73; 4: δ 1.64, 1.71; all 3H s), and one olefinic proton (3, 4: δ 5.36; both 1H, br s).

Acetylation followed by NaOMe-MeOH treatment (r.t.) of 3 or 4 provided 3a, oil, $C_{17}H_{29}NO_2$, IR ($CHCl_3$): 3660, 3600, 3450, 1664, 1493 cm^{-1} or 4a, oil, $C_{17}H_{29}NO_2$, IR ($CHCl_3$): 3660, 3600, 3450, 1661, 1494 cm^{-1}. CrO_3 oxidation of 3a or 4a in pyridine yielded the same conjugated ketone (9), oil, $C_{17}H_{27}NO_2$, IR ($CHCl_3$): 3450, 1666, 1497 cm^{-1}. Thus, isoaminobisabolenol-a (3) and -b (4) have been shown to be C-12 configurational isomers.

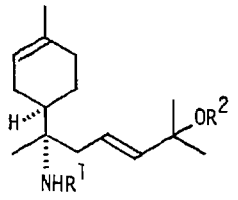
Treatment of N-acetylaminobisabolene (1a) with *m*-chloroperbenzoic acid in



1 : R = H (aminobisabolene)

1a : R = Ac

1b : R = $\overset{O}{\overset{\|}{C}}CH_2Br$



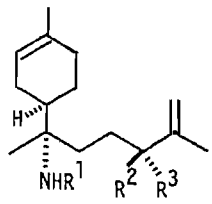
2 : $R^1 = R^2 = H$ (aminobisabolenol)

2a : R^1 = Ac, R^2 = H

2b : $R^1 = R^2$ = Ac

2c : R^1 = H, R^2 = Me

2d : R^1 = Ac, R^2 = Me



3 : $R^1 = R^3$ = H, R^2 = OH (isoaminobisabolenol-a)

3a : R^1 = Ac, R^2 = OH, R^3 = H

3b : R^1 = Ac, R^2 = OBz, R^3 = H

4 : $R^1 = R^2$ = H, R^3 = OH (isoaminobisabolenol-b)

4a : R^1 = Ac, R^2 = H, R^3 = OH

4b : R^1 = Ac, R^2 = H, R^3 = OBz

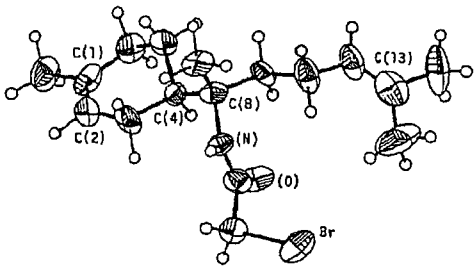
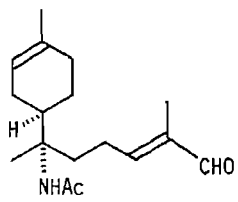


Fig 1 : 1b



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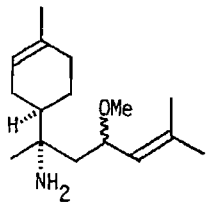
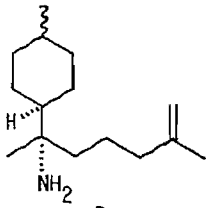
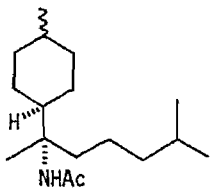


6



7



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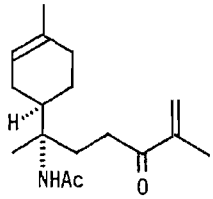

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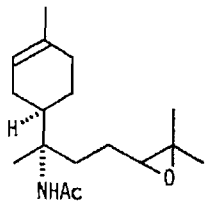

10

Table I. ^{13}C NMR Data[a)]

	1	1a	2	2a	3	3a	4	4a	5	9
1	133.4	133.9	133.9	134.0	134.0	134.1	133.8	134.1	134.1	134.1
2	120.1	120.7	119.7[c)]	120.5	120.0	120.5	120.0	120.5	120.3	120.4
3	25.8[b)]	26.9[b)]	25.9[b)]	26.4[b)]	26.2[b)]	26.6[b)]	26.0[b)]	26.6[b)]	26.4[b)]	26.6[b)]
4	41.6	40.9	41.1	40.2	42.4	40.9	40.5	40.8	40.5	40.8
5	23.5[b)]	24.3[b)]	23.6[b)]	24.4[b)]	23.7[b)]	24.4[b)]	23.7[b)]	24.4[b)]	24.3[b)]	24.4[b)]
6	30.8	31.4	30.7	31.2	30.9	31.3[c)]	30.8	31.3[c)]	31.2	31.2
7	22.9	23.0	23.2	23.2	23.2	23.3	23.2	23.3	23.2	23.2
8	55.6	59.0	59.0	58.9	57.3	58.8	58.2	58.8	58.7	58.6
9	38.4	36.2	39.4	38.6	29.2	29.4	28.7	29.1	34.5	29.7
10	21.9	20.9	21.5	20.8	22.2	21.1	22.1	21.0	20.7	20.8
11	23.3[b)]	22.7[b)]	119.4[c)]	122.2	33.7	31.9[c)]	33.4	31.5[c)]	23.9[b)]	32.7
12	123.8	124.7	144.7	141.9	75.6	76.1	75.0	75.9	139.3	202.7
13	131.3	131.3	70.7	70.7	147.3	147.6	146.9	147.5	154.8	144.4
14	17.3	17.5	29.3	30.1	110.9	111.1	111.1	111.1	9.2	124.9
15	25.3	25.5	29.3	30.1	18.2	17.6	18.3	17.8	195.1	17.7

a) Measured at 22.5 MHz in $CDCl_3$ with a JEOL FX-90Q NMR spectrometer.

b,c) The assignments for these signals within the same column may be interchanged.

CH_2Cl_2 (r.t.) furnished a diastereomeric mixture of 12,13-epoxides (10) (conversion 45%, yield 67%), which, on treatment with *p*-TsOH·H_2O in CH_2Cl_2, provided 3a and 4a in a 4:3 ratio (conversion 49%, yieid 45%). Thus, the structures of 3a and 4a have been elucidated except for their C-12 configurations which have been determined by application of the allylic benzoate chirality method.[9]

Benzoylation (benzoyl chloride-pyridine, 0°C, 1 h) of 3a or 4a afforded 3b, oil, $C_{24}H_{33}NO_3$ or 4b, oil, $C_{24}H_{33}NO_3$. The CD spectra (in MeOH) of 3b and 4b respectively showed a first positive maximum: $[\theta]_{226}$ +2100 and a first negative maximum: $[\theta]_{226}$ -3500. Consequently, the 12*S* and 12*R* configurations of 3 and 4 respectively have been determined.

Two bisabolene sesquiterpenes having an isothiocyanate group or a formamide group at C-1 were isolated from a marine sponge of *Theonella* sp. (collected in Kerama Rettō, Okinawa).[10] The present bisabolene sesquiterpenes characteristically have an amino group at C-8. This may be of interest from the chemotaxonomical viewpoint.

REFERENCES AND NOTES

1) A recent paper on these subjects: I. Kitagawa, Z. Cui, B. W. Son, M. Kobayashi, and Y. Kyogoku, Chem. Pharm. Bull., 35, 124 (1987), and the preceding papers of this series.
2) I. Kitagawa, M. Kobayashi, N. K. Lee, H. Shibuya, Y. Kawata, and F. Sakiyama, Chem. Pharm. Bull., 34, 2664 (1986). This sponge species was abbreviated Theo-83-ZM-1 in our laboratory.
3) This marine sponge (abbreviated Theo-84-HM-1) looks very similar to, but differs from, the one collected in Zamami-jima (Theo-83-ZM-1). It was identified as *Theonella* sp. (Theonellidae) by Dr. T. Hoshino, Mukaijima Biological Station, Hiroshima University, to whom the authors are grateful.
4) I. Kitagawa, M. Kobayashi, N. K. Lee, H. Shibuya, C. Kamba, M. Yoshikawa, Y. Kawata, and F. Sakiyama, presented at the 28th Symposium on the Chemistry of Natural Products, held at Sendai, Oct. 1986. Symposium Papers, p. 144.
5) The molecular compositions of the new compounds, with their chemical formulae, were determined by high resolution mass spectrometry.
6) The ^1H NMR spectra were measured at 500 MHz in CD_3OD (unless specified otherwise) with a JEOL GX-500 NMR spectrometer.
7) The crystals were recrystallized from n-hexane: prisms, orthorhombic, space group $P2_12_12_1$, a=9.7655(9), b=19.987(2), c=9.0470(7) Å, v=1765.8(3) Å^3, z=4, D_c= 1.29 g/cm^3, F(000)=152, μ(Cu Kα)=31.476. The X-ray diffraction intensity data from the crystal (0.1x0.2x0.3 mm) were obtained on a Rigaku AFC diffractometer equipped with a rotating anode X-ray generator (50 kV-200 mA), using graphite-monochromated Cu Kα radiation (λ=1.5418 Å). A total of 1663 independent reflections with $2\theta \leq 126°$ were collected by the ω scanning mode ($2\theta < 45°$) and the ω/2θ scanning mode ($2\theta \geq 45°$). The structure was solved by the direct method MULTAN80 (Main, *et al.*, 1980). The refinement of the structure using 1584 reflections with $|Fo| \geq 3\sigma(Fo)$ was carried out by the block-diagonal least-squares method with anisotropic thermal parameters and the R factor was reduced to 0.065. Computations were performed on the PANAFACOM U-1200 II of RIGAKU RASA-5RP system. The determination of the absolute structure was carried out on a microcomputer NEC PC-9801 VM2 using the unpublished program "CHIRAL" developed by Prof. Y. Katsube, *et al.* The authors are grateful to Profs. Y. Katsube and N. Tanaka for providing them with the program.
8) Two diastereomers: 8a, oil, $C_{17}H_{34}NO$, IR ($CHCl_3$): 3670, 3450, 1664, 1497 and 8b, oil, $C_{17}H_{34}NO$, IR ($CHCl_3$): 3670, 3440, 1661, 1493 cm^{-1}, were separated by HPLC (Zorbax SIL, n-hexane-AcOEt). However, the C-1 configuration of each compound has not yet been clarified.
9) a) N. Harada and K. Nakanishi, "Circular Dichroic Spectroscopy-Exciton Coupling in Organic Stereochemistry," Tokyo Kagaku Dojin, Tokyo, 1982; b) N. Harada, Kagaku to Seibutsu, 22, 329 (1984).
10) H. Nakamura, J. Kobayashi, Y. Ohizumi, and Y. Hirata, Tetrahedron Lett., 25, 5401 (1984).

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