OF4949, NEW INHIBITORS OF AMINOPEPTIDASE B

II. ELUCIDATION OF STRUCTURE

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The structures of OF4949-**I**, **II**, **III** and **IV** were identified by analysis of the products of their chemical degradation and by ^{1}H NMR, ^{13}C NMR, and mass spectrometry. These compounds were new cyclic peptides containing diphenyl ether as a chromophore. OF4949-**I** had two amino acids, β-hydroxy-L-asparagine and 4-methylisodityrosine. The structural differences between **I** and **II** and between **III** and **IV** lay solely in the diphenyl ether moiety; the phenolic hydroxyl group in **II** and **IV** was methylated in **I** and **III**. OF4949-**III** and **IV** contained L-asparagine instead of the β-hydroxy-L-asparagine moiety of **I** and **II**.

OF4949-**I**, **II**, **III** and **IV** are inhibitors of aminopeptidase B from Ehrlich ascites carcinoma cells, and are produced by a fungus, *Penicillium rugulosum* OF4949. We reported on the taxonomy of the organism, and on the fermentation, isolation, and physico-chemical and biological properties of these compounds.[1] Physico-chemical data on the four components showed that they were water-soluble amphoteric peptides with similar structures. This paper deals with the structural elucidation of these four compounds.

Results

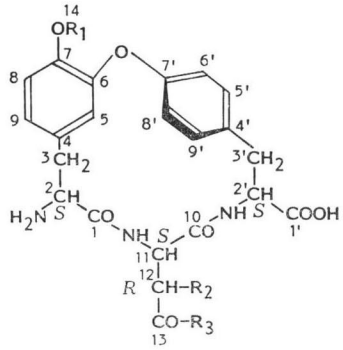
The molecular formula of **I** was identified as $C_{23}H_{26}N_4O_8$ and that of **II**, $C_{22}H_{24}N_4O_8$, judging from elemental analysis and secondary ion mass spectrometry (SI-MS).[1]

In the ^{1}H NMR data for **II** in ND_4OD (Table 1), the signals at δ 6.74, 6.67 and 5.78 indicated the presence of a tri-substituted benzene ring, and the signals at δ 7.41, 7.22, 7.05 and 6.85 indicated the presence of a di-substituted benzene ring. The signals at δ 4.46, 3.36 and 2.65 showed an AMX system, and those at δ 3.67, 2.88 and 2.76 an ABX system, which suggested the presence of two similar carbon chains, both with $-\overset{|}{\underset{|}{C}}CH_2\overset{|}{\underset{|}{C}}H\overset{|}{\underset{|}{C}}-$. The ^{1}H NMR spectrum of **I** corresponded to that of **II**, the only difference being the signal at δ 3.93, which we attributed to a methoxy group in **I** (Table 1).

The ^{13}C NMR spectrum of **II** in ND_4OD showed 22 well-defined carbon signals including two methylene carbons, four methine carbons (one of these four an aliphatic carbon bound to an oxygen function), four carbonyl carbons, and twelve carbons in two benzene rings, including five singlets and seven doublets (Table 2). These data are consistant with those from ^{1}H NMR studies of **II**. The ^{13}C NMR spectrum of **I** corresponded well to the signals of **II** except for one quartet signal at δ 56.7 (Table 2).

Amino acid analysis of the acid hydrolysate of **I** disclosed three unusual amino acids, **A**, $\mathbf{B}_1$ and

Fig. 1. Structures of OF4949-I, II, III, IV, D and F.

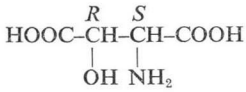


OF4949-I	$R_1=CH_3$	$R_2=OH$	$R_3=NH_2$
OF4949-II	$R_1=H$	$R_2=OH$	$R_3=NH_2$
OF4949-III	$R_1=CH_3$	$R_2=H$	$R_3=NH_2$
OF4949-IV	$R_1=H$	$R_2=H$	$R_3=NH_2$
OF4949-D	$R_1=CH_3$	$R_2=OH$	$R_3=OH$
OF4949-F	$R_1=H$	$R_2=OH$	$R_3=OH$

$\mathbf{B}_2$. Two of the same amino acids, **A** and $\mathbf{B}_1$, appeared in the hydrolysate of **II**. Both $\mathbf{B}_1$ and $\mathbf{B}_2$ had UV absorption, but **A** was not chromophoric.

Structure of A

The molecular formula of **A** was $C_4H_7NO_5$. Its ^1H NMR and ^{13}C NMR spectra indicated the presence of one alcoholic hydroxyl group, one free amino group, and two free carboxylic groups. The gas chromatography mass spectrometry (GC-MS) spectrum of the trimethylsilyl (TMS) derivative of **A** was in agreement with that of authentic β-hydroxyaspartic acid: m/z 422 (M^+-CH_3), 320 ($M^+-COOTMS$), 218 ($^+CH\langle^{COOTMS}_{NHTMS}$). The L-configuration was assigned to **A** with data on the specific rotation. We concluded that the absolute configuration of **A** was the L-*erythro* (1*S*, 2*R*) form by direct comparison with authentic racemic *erythro*-β-hydroxyaspartic acid synthesized from glycine and glyoxalate[2] using HPLC andTLC.



Structure of $\mathbf{B}_1$ and $\mathbf{B}_2$

The chromophoric amino acid, $\mathbf{B}_1$ ($C_{17}H_{20}N_2O_6$) had UV absorption similar to that of the 4-hydroxydiphenyl ether. The presence of a phenolic hydroxyl group in $\mathbf{B}_1$ was shown by the reaction with $FeCl_3$ reagent. The ^1H NMR spectrum of $\mathbf{B}_1$ in ND_4OD had seven ring proton signals at δ 7.00,

Table 1. ^1H NMR of OF4949-I and II in ND_4OD.

δ, intensity, multiplicity, J (Hz)		Assignment
I	II	
2.66, 1H, t, J=13.0	2.65, 1H, t, J=13.0	H-3′
2.82, 1H, dd, J=14.0, 6.0	2.76, dd, J=13.0, 5.5	H-3
2.94, 1H, dd, J=14.0, 2.5	2.88, 1H, dd, J=13.0, 3.0	H-3
3.37, 1H, dd, J=13.0, 4.0	3.36, 1H, dd, J=13.0, 4.0	H-3′
3.70, 1H, dd, J=6.0, 2.5	3.67, 1H, dd, J=5.5, 3.0	H-2
3.93, 3H, s		H-14
4.42, 1H, d, J=5.0	4.40, 1H, d, J=4.0	H-11
4.48, 1H, dd, J=13.0, 4.0	4.46, 1H, dd, J=13.0, 4.0	H-2′
5.83, 1H, d, J=2.0	5.78, 1H, d, J=2.0	H-5
6.87, 2H, m	6.67, 1H, dd, J=8.0, 2.0	H-9
	6.74, 1H, d, J=8.0	H-8
7.07, 2H, m	6.85, 1H, dd, J=8.0, 2.5	H-6′ or H-8′
	7.05, 1H, dd, J=8.0, 2.5	H-6′ or H-8′
7.26, 1H, dd, J=8.0, 2.5	7.22, 1H, dd, J=8.0, 2.5	H-5′ or H-9′
7.46 1H, dd, J=8.0, 2.5	7.41, 1H, dd, J=8.0, 2.5	H-5′ or H-9′

Assignment of H-2 and H-2′ was done with the ^1H NMR spectra of I and II in DMSO-d_6+D_2O.

Table 2. ^{13}C NMR of OF4949-**I**, **II**, $\mathbf{B}_1$ and **A** in ND_4OD.

δ, multiplicity				Assignment
I	**II**	$\mathbf{B}_1$	**A**	
178.7, s	178.8, s	181.6, s		C-1 or 1′
176.0, s	176.0, s	181.4, s		C-1 or 1′
174.9, s	175.8, s		176.0, s	C-10 or 13
168.3, s	168.3, s		171.4, s	C-10 or 13
153.4, s	154.1, s	158.1, s		C-7′
149.4, s	149.0, s	155.7, s		C-7
148.0, s	145.6, s	145.8, s		C-6
136.5, s	136.1, s	131.8, s		C-4 or 4′
132.9, d	132.8, d	131.4, d×2		C-5′ or 9′
131.5, d	131.4, d			C-5′ or 9′
128.2, s	127.4, s	127.5, s		C-4 or 4′
125.0, d	125.2, d	125.3, d		C-9
123.1, d	123.1, d	123.3, d		C-6′ or 8′
122.1, d	122.0, d	120.8, d		C-6′ or 8′
116.2, d	116.8, d	117.2, d×2		C-5′
113.0, d	117.0, d			C-8
72.9, d	73.0, d		71.3, d	C-12
57.9, d	57.9, d		58.3, d	C-2 or 2′ or 11
56.7, q				C-14
55.1, d	55.1, d	58.0, d×2		C-2 or 2′ or 11
53.8, d	54.0, d			C-2 or 2′ or 11
39.7, t	39.7, t	39.8, t		C-3 or 3′
38.9, t	39.1, t	39.4, t		C-3 or 3′

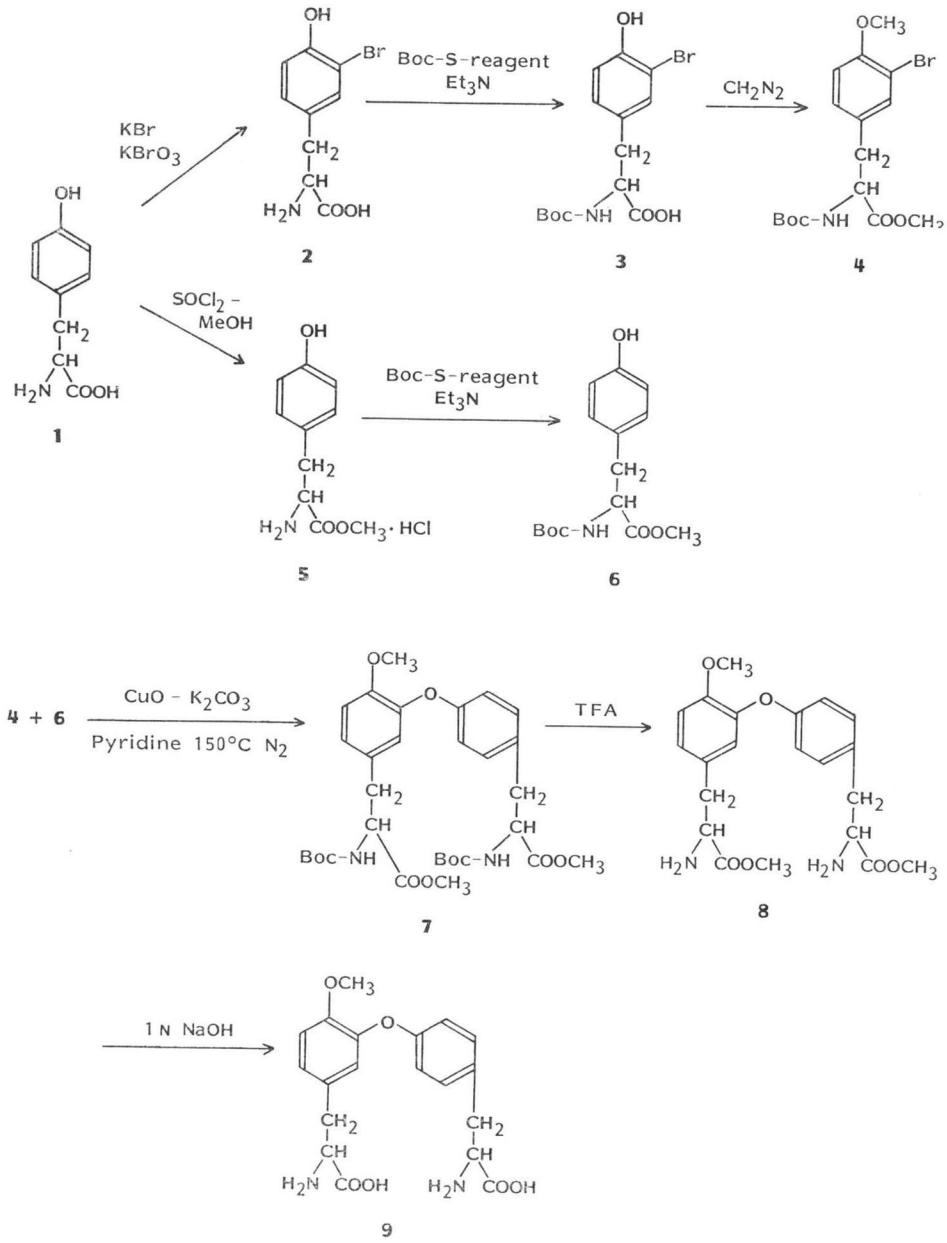
6.70, 6.62 and 6.58. In the ^{13}C NMR spectrum, three (δ 158.1, 155.7 and 145.8) of the five singlet signals were attributed to carbons bound to oxygen atoms. From these findings, we deduced that $\mathbf{B}_1$ has a diphenyl ether formed by one tri-substituted benzene ring and one di-substituted benzene ring. The GC-MS spectral data of a $\mathbf{B}_1$-trimethylsilyl derivative showed ion peaks at m/z 705 (M^+-CH_3), 603 ($M^+-COOTMS$), and 503 ($MH^+-CH\langle^{COOTMS}_{NHTMS}$).

Another chromophoric compound, $\mathbf{B}_2$ ($C_{18}H_{22}N_2O_6$), had properties similar to those of $\mathbf{B}_1$, except for the presence of methoxy protons in ^1H NMR. In the GC-MS spectrum of TMS-$\mathbf{B}_2$, one methoxy group on the diphenyl ether moiety was apparent from the ion peaks at m/z 647 (M^+-CH_3), 545 ($M^+-COOTMS$) and 445 ($MH^+-CH\langle^{COOTMS}_{NHTMS}$). The methylated product of the $\mathbf{B}_1$-methyl ester was identified to be $\mathbf{B}_2$-methyl ester by HPLC and TLC. So, $\mathbf{B}_2$ was an *O*-methyl derivative of $\mathbf{B}_1$.

To confirm the structure of $\mathbf{B}_2$, the diphenyl ether was cleaved with sodium in liquid ammonia. Two chromophoric fragments resulting from the reductive cleavage of $\mathbf{B}_2$ were identified to be L-tyrosine and *O*-methyl-L-tyrosine by HPLC and TLC. The configurations of these two amino acids were found to be the L-form by the method of KINOSHITA *et al.*[3]

Next, a synthetic study was done to locate the ether bond and methoxy group in the diphenyl ether moiety of $\mathbf{B}_2$. The synthesis of $\mathbf{B}_2$ was based on the coupling of *tert*-butyloxycarbonyl(Boc)-3-bromo-4-methoxyphenyalanine methyl ester (**4**) and Boc-L-tyrosine methyl ester (**6**) by the Ullmann reaction (Chart 1). The synthesized componuds **7**, **8** and **9** were identical to Boc-$\mathbf{B}_2$-methyl ester, $\mathbf{B}_2$-methyl ester, and natural $\mathbf{B}_2$, respectively, in HPLC and TLC. Consequently, $\mathbf{B}_2$ was identified as 4-methylisodityrosine, and $\mathbf{B}_1$ as isodityrosine, as shown below.

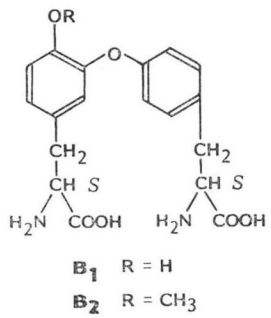
Chart 1. Synthesis scheme of B_2.



Structures of OF4949-I and II

Controlled hydrolysis of **I** (or **II**) afforded the new peptides **C** and **D** (or **E** and **F**, Chart 2).

The following series of reactions permitted us to identify the numbers and locations of peptide bonds and of free carboxyl and amino groups in **I** and **II**. Methylation of **D** gave a **D**-dimethyl ester, which gave **I**-amide upon treatment with NH_4OH. Methylation of **I** gave **I**-methyl ester, which produced *N,O*-diacetyl-**I**-methyl ester when treated with acetic anhydride in pyridine. OF4949-**I** was treated with acetic anhydride in MeOH to give a *N*-acetyl-**I**-methyl ester. These data, together



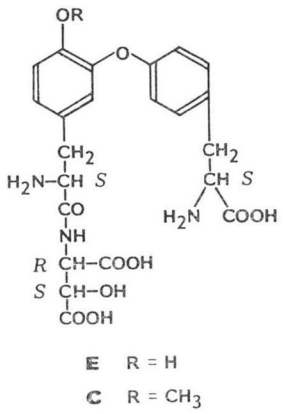
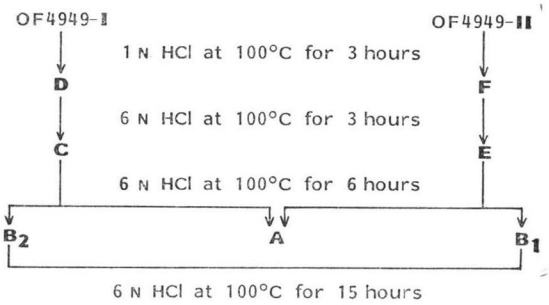


Chart 2. Controlled hydrolysis of OF4949-I and II.



with the spectral data from the similar derivatives of **II**, confirmed the presence of a free carboxyl, a free amino, a carboxamide, and a secondary alcoholic hydroxyl group, and of two peptide bonds in the structure of **I** and **II**. This evidence enabled us to conclude that **I** (**II**) was a cyclic peptide incorporating **A** and $\mathbf{B}_2$ (**A** and $\mathbf{B}_1$) fragments that had two peptide bonds.

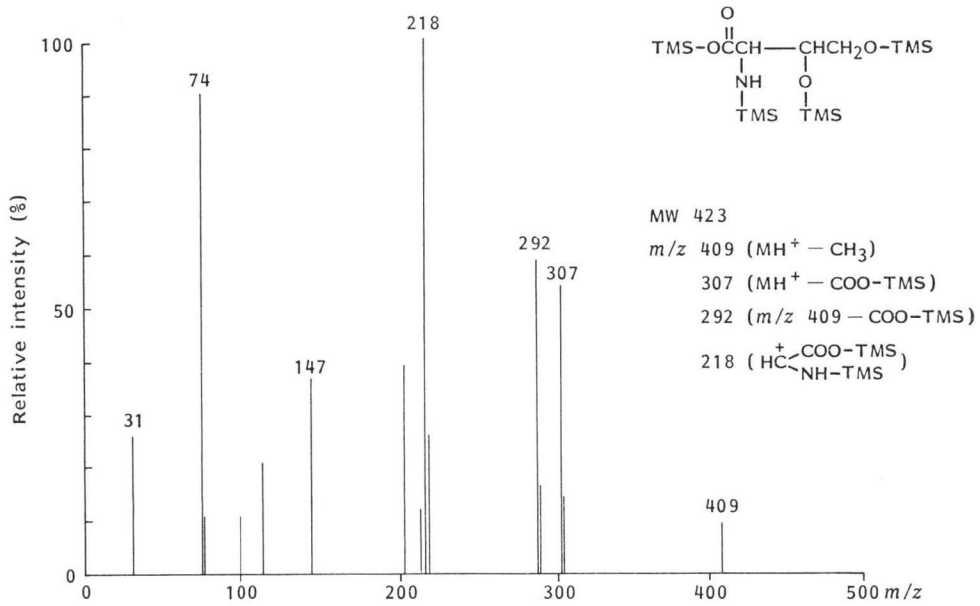
Compound **D** is, we think, a hydroxylated derivative of the carboxamide group of **I**. This is supported by the potentiometric titration data for **D**, with a *pKa'* of 2.55, 4.60 (both carboxyl groups), and 7.64 (amino group), and by those for **I**, with a *pKa'* of 3.12 (carboxyl group) and 7.56 (amino group). Similarly, **F** had properties similar to those of **D** except for having a methoxy group instead of a phenolic hydroxyl group. Thus we decided that **F** was a demethylated derivative of **D**.

To locate the free amino group in **I** and **II**, they were reacted with 2,4-dinitrofluorobenzene to give their dinitrophenyl (DNP) derivatives. Upon hydrolysing DNP-**I** (or DNP-**II**), we found that only $\mathbf{B}_2$ (or $\mathbf{B}_1$) was modified with DNP, and that **A** was unchanged. The acid hydrolysis of DNP-**I** followed by cleavage with sodium in liquid ammonia gave L-tyrosine. Thus, the free amino group of **I** was derived from the amino group of the *O*-methyl-L-tyrosine moiety of $\mathbf{B}_2$.

To decide on the location of the free carboxyl group, **I**-methyl ester (or **II**-methyl ester) was reduced with lithium borohydride ($LiBH_4$) and then hydrolyzed with HCl to give reduced $\mathbf{B}_2$ (or reduced $\mathbf{B}_1$) and unchanged **A**. This result suggested that the free carboxyl group was also located on the $\mathbf{B}_2$ (or $\mathbf{B}_1$) fragment and that one of the two carboxyl groups on β-hydroxyaspartic acid was substituted by a carboxamide group in **I** and **II**.

Compound **C** was treated with MeOH - HCl to afford **C**-trimethyl ester, which indicated that **C** is a linear peptide resulting from the cleavage of one of the two peptide bonds in the cyclic peptide **D**. DNP-**C** was hydrolyzed with HCl to afford DNP-$\mathbf{B}_2$ and unchanged **A**. The cleavage of **C** with sodium in liquid ammonia gave L-tyrosine but no *O*-methyl-L-tyrosine. The results of these reactions were sufficient evidence to allow us to conclude that the peptide bond of **C** was between the α-amino group of β-hydroxy-L-asparagine and the carboxyl group of *O*-methyl-L-tyrosine moiety, and that the terminal aromatic amino acid of **C** was L-tyrosine. Another chromophoric linear peptide **E** was determined

Fig. 2. GC-MS spectrum of TMS-reduced **A**.



to be a demethylated derivative of **C** by spectrometric analysis with the aid of the reactions above. Consequently, **C** and **E** have the structures shown below.

The only remaining uncertainty about the structures of **I** and **II** was the linkage between β-hydroxyasparagine and $\mathbf{B}_1$ (or $\mathbf{B}_2$).

Both structures shown here were theoretically possible. We decided between them as follows. **F**-Dimethyl ester was reduced with $LiBH_4$ to give **F**-diol and then hydrolyzed with HCl to afford reduced β-hydroxyaspartic acid. The GC-MS spectrum of the TMS derivative of the β-hydroxyaspartic acid (see Fig. 2) indicated that structure (a) is correct. To confirm this, the **F**-diol was treated with sodium periodate ($NaIO_4$) and then with sodium borohydride ($NaBH_4$) followed by hydrolysis, which gave serine. Thus we confirmed that one peptide bond of **I** or **II** formed with the α-amino group of β-hydroxyasparagine and the α-carboxyl group of *O*-methyl-L-tyrosine moiety, and that the other formed with the α-carboxyl group of β-hydroxyasparagine and the α-amino group of L-tyrosine moiety.

From these results, the structures of **I**, **II**, **D** and **F** were deduced to be as shown in Fig. 1.

Structures of OF4949-III and IV

OF4949-**III** (or **IV**) was hydrolyzed with HCl to yield L-aspartic acid, $\mathbf{B}_1$, and $\mathbf{B}_2$ (or L-aspartic acid and $\mathbf{B}_1$). By comparison of ^{1}H NMR analysis, **III** and **IV** were found to be the deoxy derivatives of the alcoholic hydroxyl group of **I** and **II**, respectively. The UV, IR and ^{1}H NMR spectra of **III** and **IV** showed that **III** was the 14-methyl derivative of **IV**. Consequently, **III** and **IV** have the structures shown in Fig. 1.

Discussion

OF4949-**I** and **II** each contained two unusual amino acids, β-hydroxyasparagine and either 4-methylisodityrosine ($\mathbf{B}_2$) or isodityrosine ($\mathbf{B}_1$). β-Hydroxyasparagine is one of the normal constituents of mammalian urine.[4] OKAI found the spacial configuration of the natural product from human urine to be that of *erythro*-β-hydroxy-L-asparagine.[5] The β-hydroxyasparagine moiety of **I** and **II** was the same optical isomer as that of urine. Isodityrosine coupled with two tyrosine units linked by diphenyl ether has been found in potato callus and in carrot cell walls.[6,7] A few natural products, including piperazinomycin,[8,9] cyclic hexapeptides RA,[10,11] and bouvardin,[12] have an analog of isodityrosine in their large cyclic structures.

When the aromatic proton lies over the other benzene ring at very close range as in the C5 proton δ 5.76 (1H, d, J=2.0 Hz) of piperazinomycin,[9] the C6 proton δ 5.84 (1H, d, J=2.0 Hz) of acerogenin A,[13,14] and the Tyr-6δ_b proton δ 4.40 (1H, d, J=1.8 Hz) of bouvardin,[12] it gives a signal much more upfield in the ^{1}H NMR spectrum than ordinary aromatic protons. Therefore, the doublet proton signals (1H, d, J=2.0 Hz) at δ 5.83 of **I** and at the 5.78 of **II** are C5 protons.

The complete structure and the absolute configuration of **I** was confirmed by X-ray crystallographic analysis of its monobromo derivative.*

Experimental

General

UV spectra were recorded on a Shimadzu double-beam UV-200 spectrophotometer, and IR spectra on a Hitachi 270-30 spectrophotometer. Optical rotation was measured with a Jasco DPI181, and potentiometric titration was obtained with a Toa autotitrator HTS-10A. ^{13}C (50.3 MHz) and ^{1}H (200 MHz) NMR spectra were recorded on a Varian XL-200. Secondary ion mass spectra were recorded on a Hitachi M-80A, and GC-MS measurements were done on a Hitachi M-70. Preparative HPLC was done on a Nucleosil 30C_{18} column (20×250 mm, Macherey Nagel Co.) or a Prep-PAK-500/C_{18} column (Waters Co.). For analysis, a Nucleosil 5C_{18} column (4×150 mm) or a ISC-09/S2504 column (4×250 mm, Toyo Soda Mfg. Co.) was used and monitored by a Soma UV detector S-310 or flurometric reaction with *o*-phthalaldehyde (OPA).

III: ^{1}H NMR (DMSO-d_6) δ 2.37 (2H, m), 2.68 (2H, br s), 3.79 (3H, s), 4.06 (1H, m), 4.55 (1H, m), 5.85 (1H, d, J=2 Hz), 6.56~6.74 (2H, m), 6.80~6.94 (2H, m), 7.04~7.30 (2H, m), 6.76, 7.43 (each 1H, br s, $CONH_2$), 7.52 (1H, d, J=9 Hz, CONH), 7.63 (1H, d, J=9 Hz, CONH).

IV: ^{1}H NMR (DMSO-d_6) δ 2.32 (2H, m), 2.62 (2H, br s), 3.95 (1H, m), 4.50 (1H, m), 5.82 (1H, d, J=2 Hz), 6.53 (1H, m), 6.62 (1H, m), 6.69 (1H, m), 6.85 (1H, m), 7.07 (1H, m), 7.18 (1H, m), 6.73, 7.45 (each 1H, br s, $CONH_2$), 7.39 (1H, d, J=8 Hz, CONH), 7.49 (1H, d, J=8 Hz, CONH).

Total Hydrolysis of I and II: Isolation of A, $\mathbf{B}_1$ and $\mathbf{B}_2$

A solution of **I** (200 mg) in 5 ml of 6 N HCl was heated at 100°C for 24 hours in a sealed tube. For separation of each amino acid, the residue was put on a Nucleosil 30C_{18} column and eluted with a 97.5:2.5 mixture of 0.1 M citrate buffer, pH 5.7, and acetonitrile. The fractions containing compound **A** were further purified on a Dowex 50W (H^+) column by gradient elution with 0~1.0 N HCl, and on a Dowex 1 ($formate^-$) column with 0.05 N formic acid, giving 63 mg of **A** as a colorless powder. The chromophoric amino acids $\mathbf{B}_1$ and $\mathbf{B}_2$ were separately chromatographed on a Dowex 50W (H^+) column with 1 N ammonia followed by desalting on a Nucleosil 30C_{18} column with H_2O, giving 28 mg of $\mathbf{B}_1$ and 51 mg of $\mathbf{B}_2$.

II (200 mg) was hydrolyzed and purified in the same way, which gave 69 mg of **A** and 73 mg of $\mathbf{B}_1$.

A: $[\alpha]_D^{25}$ +42.7° (*c* 0.77, 1 N HCl); IR ν_{max} (KBr) cm^{-1} 3450, 3000, 1700, 1610, 1480, 1310, 1270, 1160, 1090, 1040; ^{1}H NMR (1 N ND_4OD) δ 3.49 (1H, d, J=4 Hz), 4.09 (1H, d, J=4 Hz); ^{13}C NMR see Table 2.

$\mathbf{B}_1$: $[\alpha]_D^{25}$ −6.0° (*c* 0.83, 1 N HCl); UV λ_{max} nm (ε), H_2O and 0.05 N HCl 274 (2,960), 0.05 N

* HIROTSU, K.: Personal communication.

NaOH 297 (3,900); IR ν_{max} (KBr) cm^{-1} 3400, 3000, 1580, 1500, 1405, 1320, 1290, 1260, 1220, 1170, 1120, 1060; ^1H NMR (1.22 N ND_4OD) δ 2.40~3.00 (4H, m), 3.20~3.60 (2H, m), 6.58 (1H, d, J=9 Hz), 6.62 (1H, d, J=3 Hz), 6.70 (3H, d, J=9 Hz), 7.00 (2H, d, J=9 Hz); ^{13}C NMR see Table 2.

B_2: $[\alpha]_D^{25}$ −6.6° (*c* 1.0, 1 N HCl); UV λ_{max} nm (ε), H_2O and 0.05 N HCl 273 (3,290), 0.05 N NaOH 273 (3,440); IR ν_{max} (KBr) cm^{-1} 3400, 3000, 1610, 1580, 1510, 1400, 1280, 1230, 1130, 1030; ^1H NMR (1 N ND_4OD) δ 2.60~3.10 (4H, m), 3.30~3.50 (2H, m), 3.81 (3H, s), 6.70 (2H, d, J=8 Hz), 6.93 (1H, d, J=2 Hz), 7.08 (1H, dd, J=2, 8 Hz), 7.16 (1H, d, J=8 Hz), 7.27 (2H, d, J=8 Hz).

Mild Acid Hydrolysis of **I** and **II**: Isolation of **C**, **D**, **E** and **F**

Compound **I** (500 mg) was hydrolyzed in 20 ml of 1 N HCl in a sealed tube for 7 hours at 100°C. The hydrolysate was chromatographed on a Prep-PAK-500/C_{18} column with 0.1 M citrate buffer - acetonitrile (95 : 5). The fractions containing **C** or **D** were separately desalted on a Diaion HP-20 column using 50% MeOH, giving 61 mg of **C** as a colorless powder and 90.6 mg of **D** as a columnar crystal.

Hydrolyzation of **II** (500 mg) in the same way gave 53.2 mg of **E** and 78.8 mg of **F**. The purification of **E** and **F** was done as for **B_1** and **B_2**.

C: $[\alpha]_D^{25}$ −3.4° (*c* 1.0, H_2O); UV λ_{max} nm (ε), H_2O and 0.05 N HCl 273 (3,430), 0.05 N NaOH 273 (3,540); IR ν_{max} (KBr) cm^{-1} 3400, 1720, 1620, 1500, 1390, 1260, 1160, 1120, 1020; ^1H NMR (D_2O) δ 3.0~3.4 (4H, m), 3.84 (3H, s), 3.98 (1H, dd, J=6, 8 Hz), 4.30 (1H, t, J=7 Hz), 4.35 (1H, d, J=4 Hz), 4.63 (1H, d, J=4 Hz), 6.87 (1H, d, J=2 Hz), 6.92 (2H, d, J=8 Hz), 7.08 (1H, dd, J=2, 8 Hz), 7.14 (1H, d, J=8 Hz), 7.27 (2H, d, J=8 Hz).

D: UV λ_{max} nm (ε), H_2O and 0.05 N HCl 273 (2,480), 0.05 N NaOH 273 (2,570); IR ν_{max} (KBr) cm^{-1} 3400, 1720, 1660, 1500, 1265, 1235, 1130, 1020; *Anal* calcd for $C_{23}H_{25}N_3O_9 \cdot 2H_2O$; C 52.77, H 5.58, N 8.03, found; C 53.01, H 5.83, N 8.09; ^1H NMR (DMSO-d_6) δ 3.83 (3H, s), 4.56 (1H, m), 4.66 (1H, m), 5.81 (1H, br s), 6.67 (1H, dd, J=2, 8 Hz), 6.76 (1H, d, J=8 Hz), 6.80~7.10 (3H, m), 7.17 (1H, d, J=8 Hz), 7.32 (1H, d, J=8 Hz), 7.97 (1H, d, J=10 Hz), 8.11 (1H, d, J=10 Hz).

E: UV λ_{max} nm (ε), H_2O and 0.05 N HCl 273 (3,190), 0.05 N NaOH 296 (3,390); IR ν_{max} (KBr) cm^{-1} 3400, 1720, 1600, 1550, 1405, 1320, 1300, 1260, 1220, 1170, 1120, 1060; ^1H NMR (1 N ND_4OD) δ 2.50~3.20 (4H, m), 3.40~3.70 (2H, m), 4.18 (1H, d, J=4 Hz), 4.52 (1H, d, J=4 Hz), 6.72 (1H, d, J=8 Hz), 6.77 (1H, d, J=2 Hz), 6.86 (3H, d, J=8 Hz), 7.16 (2H, d, J=8 Hz).

F: UV λ_{max} nm (ε), H_2O and 0.05 N HCl 273 (2,840), 0.05 N NaOH 296 (3,450); IR ν_{max} (KBr) cm^{-1} 3350, 1630, 1590, 1500, 1395, 1230, 1115, 960; ^1H NMR (1 N ND_4OD) δ 2.5~3.0 (3H, m), 3.34 (1H, dd, J=4, 13 Hz), 3.36 (1H, br s), 4.16 (1H, d, J=5 Hz), 4.48 (1H, dd, J=4, 13 Hz), 5.75 (1H br s), 6.64 (2H, br s), 6.83 (1H, dd, J=3, 8 Hz), 7.03 (1H, dd, J=3, 8 Hz), 7.21 (1H, dd, J=2, 8 Hz), 7.40 (1H, dd, J=2, 8 Hz).

TMS-A, TMS-**B_1** and TMS-**B_2**

A solution of **A**, **B_1**, or **B_2** (0.5 mg) in a mixture of 0.1 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 0.1 ml of acetonitrile was heated at 150°C for 15 minutes in a sealed tube. The reaction mixture was tested by GLC or GC-MS analysis.

B_1-Methyl Ester and **B_2**-Methyl Ester

First, 1 ml of $SOCl_2$ was added dropwise into 10 ml of MeOH cooled over a bath of ice and MeOH. After stirring it in the cold for 10 minutes, we added 74.6 mg of **B_1**; then stirring was continued at room temperature overnight. The reaction mixture was chromatographed on a Nucleosil 30C_{18} column with 0.1 M citrate buffer - acetonitrile (82.8 : 17.5) and desalted on a Diaion HP-20 column by gradient elution with H_2O - MeOH, which gave 44.2 mg of **B_1**-methyl ester as a colorless powder.

Then, **B_2** was esterified in the same way to give **B_2**-methyl ester. **B_2**-Methyl ester was also obtained from **B_1**-methyl ester by reaction with CH_2N_2 in a mixture of MeOH and ether (1 : 1).

B_1-Methyl Ester: IR ν_{max} (KBr) cm^{-1} 3400, 2900, 1735, 1600, 1500, 1430, 1280, 1110, 820.

B_2-Methyl Ester: ^1H NMR (CD_3OD) δ 3.60 (3H, s, $COOCH_3$), 3.66 (3H, s, $COOCH_3$), 3.74 (3H, s, OCH_3).

Reductive Cleavage of **B_2**

To a solution of 3.0 mg of **B_2** and 2 ml of liquid ammonia being stirred over a bath of dry ice and

acetone, we added 200 mg of metal sodium. Stirring was continued in the cold for 60 minutes; then the reaction mixture was purified on a Diaion HP-20 column with a gradient of H_2O - 50% MeOH, and monitored by HPLC on a Nucleosil $5C_{18}$ column (mobile phase, 2.5% acetonitrile in 0.1 M citrate buffer, pH 5.7; flow rate, 0.5 ml/minutes; detection, at 275 nm). Fractions showing the same retention time (Rt) as L-tyrosine (Rt, 5.1 minutes) and *O*-methyl-L-tyrosine (Rt, 18.2 minutes) were concentrated separately to give 1.0 mg and 0.8 mg of these amino acids, respectively.

The optical configuration of both amino acids were found to be L by their 2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate (GITC) derivatives by the method of KINOSHITA *et al.*[3]

Synthesis of $\mathbf{B_2}$

3-Bromo-L-tyrosine (**2**):[15] L-Tyrosine (**1**, 9.05 g) was dissolved in 1.15 liters of 0.5 N HCl. A solution containing 2.8 g of $KBrO_3$ and 11.9 g of KBr in 1 liter of H_2O was added dropwise. The solution was concentrated and purified on a Diaion HP-20 column with 20% MeOH, giving 11.5 g of **2** as a colorless powder.

L-Tyrosine Methyl Ester (**5**): Compound **1** (19.0 g) was esterified with 3.9 ml of $SOCl_2$ in MeOH in the same way as for $\mathbf{B_1}$-methyl ester to give 10.2 g of **5** as colorless needles.

Boc-3-bromo-L-tyrosine (**3**), Boc-L-tyrosine Methyl Ester (**6**) and Boc-$\mathbf{B_2}$: The preparation of **3** (10.4 g), **6** (8.5 g) and Boc-$\mathbf{B_2}$ (3.5 mg) was done by the method of NAGASAWA *et al.*,[16] with *tert*-butyl-(4,6-dimethylpyrimidyl-2-thiol)carbonate and triethylamine from 10.4 g of **2**, 9.26 g of **5**, and 3.8 mg of $\mathbf{B_2}$, respectively.

Boc-3-bromo-4-methoxy-L-phenylalanine Methyl Ester (**4**) and Boc-$\mathbf{B_2}$-methy Ester: Through a solution of 10.4 g of **3** or 3.5 mg of Boc-$\mathbf{B_2}$ in a mixture of MeOH and ether (1 : 1), we bubbled CH_2N_2 until the solution turned yellow. After being washed with 10% citrate, 4% $NaHCO_3$, and water, in that order, the reaction mixture was chromatographed on a silica gel column with a mixture of benzene and $CHCl_3$ (3 : 7), which gave 8.5 g of **4** or 3.2 mg of Boc-$\mathbf{B_2}$-methyl ester.

Ullmann reaction[17] between **4** and **6**: A solution of 310 mg of **4** and 472 mg of **6** in pyridine (2.5 ml) was heated on an oil bath (145~150°C) with stirring under a nitrogen atmosphere in the presence of CuO (57.2 mg) and anhydride K_2CO_3 (1.5 g). After 4 hours, the reaction mixture was poured into $CHCl_3$ (15 ml), and the resultant solution was washed with 2% NaOH, 1% HCl, and then water. The residue was chromatographed on a Nucleosil $5C_{18}$ column with 70% MeOH to give 7.2 mg of **7**.

Compounds **7**, **8** and **9** were analyzed by HPLC (Nucleosil $5C_{18}$ column; flow rate, 0.5 ml/minute; detection, at 275 nm) and TLC (silica gel; detection at 254 nm). They had the same Rt and Rf as Boc-$\mathbf{B_2}$-methyl ester, $\mathbf{B_2}$-methyl ester and $\mathbf{B_2}$, respectively.

	HPLC (Rt, minutes)	TLC (Rf)
7, Boc-$\mathbf{B_2}$-methyl ester	13.5[a]	0.44[d]
8, $\mathbf{B_2}$-methyl ester	10.46[b]	—
9, $\mathbf{B_2}$	7.72[c]	0.48[e]

Mobile phase: [a] MeOH - H_2O (7 : 3), [b] 0.1 M citrate buffer (pH 5.7) - acetonitrile (7 : 3), [c] 0.1 M citrate buffer (pH 5.7) - acetonitrile (11 : 1), [d] $CHCl_3$ - EtOAc (9 : 1), [e] PrOH - 28% NH_4OH (4 : 1).

2: ^{1}H NMR (1 N ND_4OD) δ 3.00 (2H, m), 3.80 (1H, m), 6.71 (1H, d, J=8 Hz), 6.99 (1H, dd, J=2, 8 Hz), 7.29 (1H, d, J=2 Hz).

3: ^{1}H NMR (CD_3OD) δ 1.46 (9H, s), 2.95 (2H, br d), 4.32 (1H, m), 6.75 (1H, d, J=8 Hz), 6.99 (1H, dd, J=2, 8 Hz), 7.32 (1H, d, J=2 Hz).

4: ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 2.97 (2H, m), 3.69 (3H, s), 3.72 (3H, s), 4.50 (1H, m), 5.29 (1H, br d), 6.73 (1H, d, J=8 Hz), 7.04 (1H, d, J=2 Hz), 7.30 (1H, d, J=2 Hz).

5: ^{1}H NMR (CD_3OD) δ 2.92 (2H, br d), 3.64 (3H, s), 6.55 (2H, d, J=8 Hz), 6.85 (2H, d, J=8 Hz).

6: ^{1}H NMR ($CDCl_3$) δ 1.14 (9H, s), 2.90 (2H, br d), 3.69 (3H, s), 4.32 (1H, m), 5.35 (1H, br d), 6.60 (2H, d, J=8 Hz), 6.88 (2H, d, J=8 Hz).

N-Formyl-**II**

To a solution of 60 mg of **II** and 5 ml of 98% formic acid being stirred over an ice bath, we added 5 ml of acetic anhydride. Stirring was continued in the cold for 30 minutes and then at room temperature for 60 minutes. The reaction mixture was purified on Nucleosil $30C_{18}$ with 0.1 M citrate buffer - acetonitrile (92.5 : 7.5) and desalted on HP-20 column, giving 24 mg of *N*-formyl-**II** as colorless needles: ^{1}H NMR (CD_3OD) δ 7.87 (1H, s, O*H*C-NH).

N-Formyl-**I**-methyl Ester and *N*-Formyl-**II**-methyl Ester

A solution of 267 mg of *N*-formyl-**II** in a mixture of 20 ml of DMF, 200 mg of K_2CO_3, and 0.2 ml of methyl iodide was stirred at room temperature for 24 hours. The reaction mixture was neutralized with 2 N HCl, chromatographed on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (75 : 25), and desalted on an XAD-2 column to give 52.3 mg of *N*-formyl-**II**-methyl ester and 137.2 mg of *N*-formyl-**I**-methyl ester.

A solution of 42.2 mg of *N*-formyl-**I**-methyl ester in 4 N NaOH was stirred for 30 minutes at room temperature. After neutralization, the reaction mixture was desalted on a Diaion HP-20 column to give 3.9 mg of **I**.

N-Formyl-**I**-methyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.72 (3H, s, $COOCH_3$), 3.80 (3H, s, OCH_3), 7.98 (1H, s, O*H*C-NH).

N-Formyl-**II**-methyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.72 (3H, s, $COOCH_3$), 7.94 (1H, s, O*H*C-NH).

I-Methyl Ester, **II**-Methyl Ester, **D**-Dimethyl Ester, **F**-Dimethyl Ester, and **C**-Trimethyl Ester

A solution of 68.7 mg of **I** in 10 ml of 0.2 N methanolic HCl was stirred at room temperature for 15 hours. The reaction mixture was chromatographed on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (85 : 15) and desalted on a XAD-2 column, giving 30.6 mg of **I**-methyl ester as a colorless powder. When 91 mg of **II**, 33.8 mg of **D**, 90.4 mg of **F**, and 12.7 mg of **C** were separately esterified and purified in the same way, 74.3 mg of **II**-methyl ester, 22.3 mg of **D**-dimethyl ester, 55.5 mg of **F**-dimethyl ester, and 2.0 mg of **C**-trimethyl ester were obtained.

I-Methyl ester (3.4 mg) was also obtained from **I** (11.0 mg) by reaction with CH_2N_2 in a mixture of MeOH and ether (1 : 1) followed by chromatography on Nucleosil $30C_{18}$ and XAD-2 columns. In the same way, 1.9 mg of **D**-dimethyl ester was obtained from 10.5 mg of **D**.

I-Methyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.70 (3H, s, $COOCH_3$), 3.79 (3H, s, OCH_3).

II-Methyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.71 (3H, s, $COOCH_3$).

D-Dimethyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.55 (3H, s, $COOCH_3$), 3.70 (3H, s, $COOCH_3$), 3.79 (3H, s, OCH_3).

F-Dimethyl Ester: ^{1}H NMR (DMSO-d_6) δ 3.55 (3H, s, $COOCH_3$), 3.70 (3H, s, $COOCH_3$).

C-Trimethyl Ester: ^{1}H NMR (CD_3OD) δ 3.61 (3H, s, $COOCH_3$), 3.76 (3H, s, $COOCH_3$), 3.80 (3H, s, $COOCH_3$), 3.81 (3H, s, OCH_3).

I-Amide

A solution of 59.6 mg of **D**-dimethyl ester in 28% ammonia was stirred at room temperature for 15 hours. After neutralization with 6 N HCl, the reaction mixture was chromatographed on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (87.5 : 12.5) and desalted on a Diaion HP-20 column to give 30.8 mg of **I**-amide as a colorless powder; ^{1}H NMR (DMSO-d_6) δ 3.78 (OCH_3), 6.5~7.5 (4H, m, $CONH_2\times2$), 7.55 (1H, d, J=10 Hz, CONH), 7.93 (1H, d, J=10 Hz, CONH).

N-Acetyl-**I**-methyl and *N*-Acetyl-**II**-methyl Esters

Compound **I** (100 mg) in a mixture of 10 ml of MeOH and 5 ml of acetic anhydride was stirred at room temperature for 15 hours. The reaction mixture was purified on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (75 : 25) and desalted on a XAD-2 column to give *N*-acetyl-**I**-methyl ester (50.7 mg) as a colorless powder. *N*-Acetyl-**II**-methyl ester (23.3 mg) was obtained from **II** (50.0 mg) in the same way.

N-Acetyl-**I**-methyl Ester: ^{1}H NMR (CD_3OD) δ 1.98 (3H, s, CH_3CONH), 3.76 (3H, s, $COOCH_3$), 3.84 (3H, s, OCH_3).

N-Acetyl-**II**-methyl Ester: ^{1}H NMR (CD_3OD) δ 1.95 (3H, s, CH_3CONH), 3.80 (3H, s, $COOCH_3$).

N,O-Diacetyl-**I**-methyl Ester

I-Methyl ester (30.6 mg) was dissolved in a mixture of 1 ml of pyridine and 0.1 ml of acetic anhydride and stirred at room temperature for 4 hours. The reaction mixture was purified on Nucleosil $30C_{18}$ with 0.1 M citrate buffer - acetonitrile (78.5 : 22.5) and desalted on an XAD-2 column, giving 12.9 mg of *N,O*-diacetyl-**I**-methyl ester as a colorless crystal. Electron impact mass spectrometry (EI-MS) *m/z* 584 (M^+); ^{1}H NMR (DMSO-d_6) δ 1.82 (3H, s, CH_3CONH), 2.07 (3H, s, $COOCH_3$), 3.72 (3H, s, $COOCH_3$), 3.80 (3H, s, OCH_3).

Hydrolysis of DNP-**I**, DNP-**II** and DNP-**C**

To a solution of **I** (1 mg) in 0.5 ml of 1% aqueous triethylamine, we added 1 ml of 5% 2,4-dinitrofluorobenzene solution in EtOH. The mixture was stirred for 2 hours at room temperature. After being extracted three times with ether, the aqueous layer was hydrolyzed with 6 N HCl at 100°C for 18 hours in a sealed tube. In the same way, 1.0 mg of **II** and 1.5 mg of **C** were treated with 2,4-dinitrofluorobenzene and hydrolyzed. These hydrolysate was extracted with ether, and then the aqueous layer was analyzed by HPLC and TLC. DNP-$\mathbf{B}_2$ prepared from $\mathbf{B}_2$ in the same way gave a yellow spot with an Rf of 0.24 on a silica gel plate with a mixture of $CHCl_3$, benzyl alcohol, and acetate (70 : 30 : 3), and had a retention time of 14.5 minutes on a Nucleosil $5C_{18}$ column of HPLC (mobile phase, 30% acetonitrile in 0.1 M citrate buffer, pH 5.7; flow rate, 0.5 ml/minute; detection at 360 nm; HPLC-1 system). The acid hydrolysate of DNP-**C** had the same Rf value in TLC and retention time in HPLC as did that of DNP-$\mathbf{B}_2$. All of these hydrolysates were also analyzed by HPLC on a ISC-09/S2504 (mobile phase, 0.2 M citrate buffer, pH 3.25; flow rate, 0.5 ml/minute; detection, *o*-phthalaldehyde; HPLC-2 system) and had the same retention time as **A** (Rt, 4.83 minutes).

Hydrolysis of Reduced **I**-Methyl Ester (**II**-Methyl Ester)

To 2 mg of **I**-methyl ester dissolved in 2 ml of THF, we added 10 mg of $LiBH_4$; the mixture was refluxed for 6 hours. It was then concentrated to dryness and the residue was hydrolyzed in 1 ml of 6 N HCl at 100°C for 15 hours in a sealed tube. The hydrolysate was analyzed by the HPLC-1 and -2 systems and gave reduced $\mathbf{B}_2$ (HPLC-1; Rt, 25.92 minutes) and **A** (HPLC-2), but no $\mathbf{B}_2$. Similarly, the hydrolysate of reduced **II**-methyl ester gave reduced $\mathbf{B}_1$ (HPLC-1; Rt, 6.78 minutes) and **A**, but no $\mathbf{B}_1$.

D-Diol and **F**-Diol

First, 462 mg of **D**-dimethyl ester was refluxed in THF (40 ml) with 500 mg of $LiBH_4$ for 6 hours. The reaction mixture was treated with 0.2 N HCl, and chromatographed on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (82.5 : 17.5), and desalted on a Diaion HP-20 column, giving 234 mg of **D**-diol as a colorless powder. When 40 mg of **F**-dimethyl ester was reduced and chromatographed in the same way, 12.0 mg of **F**-diol resulted.

D-Diol: ^{1}H NMR (D_2O) δ 3.40~3.80 (4H, m, $CH_2OH \times 2$).

F-Diol: ^{1}H NMR (CD_3OD) δ 3.55 (2H, m, CH_2OH), 3.68 (2H, m, CH_2OH).

Serine from **F**-Diol

A solution of 1 mg of **F**-diol in a mixture of 0.5 ml of H_2O and 0.5 ml of MeOH, also containing 3.12 mg of $NaIO_4$, was stirred at 5°C overnight. To the solution was added 5 mg of $NaBH_4$ in 0.5 ml of H_2O. After the mixture was stirred at room temperature for 3.5 hours, a drop of acetate was added to the mixture, which was then concentrated to dryness. The residue was hydrolyzed in 1 ml of 6 N HCl at 100°C for 15 hours. The hydrolysate was analyzed by the HPLC-2 system. It had the same retention time as that of L-serine (Rt, 10.7 minutes).

Bromo-**I**

To a solution of **I** (200 mg) dissolved in 20 ml of H_2O was added 250 mg of *N*-bromosuccinylimide. This solution was stirred at room temperature for 60 minutes, and a few drops of 4 N ammonia were added to the reaction mixture, which was then chromatographed on a Nucleosil $30C_{18}$ column with 0.1 M citrate buffer - acetonitrile (85 : 15) and desalted on an XAD-2 column to give 75.8 mg of bromo-

I as a colorless columnar crystal: *Anal* calcd for $C_{23}H_{25}N_4O_8Br \cdot H_2O$; C 47.35, H 4.67, N 9.60, found; C 46.91, H 4.93, N 9.57; ^{1}H NMR (1 N ND_4OD) δ 2.50~2.90 (2H, m), 3.20~3.40 (2H, m), 3.76 (1H, dd, J=2, 8 Hz), 3.90 (3H, s), 4.19 (1H, d, J=5 Hz), 4.48 (1H, dd, J=5, 12 Hz), 5.94 (1H, s), 6.85 (1H, dd, J=3, 8 Hz), 7.03 (1H, dd, J=3, 8 Hz), 7.21 (1H, dd, J=2, 8 Hz), 7.32 (1H, s), 7.42 (1H, dd, J=2, 8 Hz).

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