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PF1163A and B, New Antifungal Antibiotics Produced by *Penicilium* sp.

II. Physico-chemical Properties and Structure Elucidation

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The structures of new antifungal antibiotics, PF1163A and B, were elucidated by spectroscopic analyses of the degradation products and by X-ray crystallography of the de-2-hydroxyethyl derivative of PF1163B. Both antibiotics consist of a 13-membered macrocyclic structure containing a derivative of *N*-methyl tyrosine and a hydroxy fatty acid. PF1163A differs from PF1163B by having an additional hydroxyl group on the side chain.

During the course of our screening for inhibitors of fungal ergosterol biosynthesis from microbial metabolites, PF1163A (**1**) and B (**2**) were isolated as new antifungal antibiotics from the mycelia of cultured *Penicillium* sp. PF1163. These compounds inhibit ergosterol biosynthesis in *Candida albicans*. In the preceding paper[1], we described the taxonomy and fermentation of the producing strain and isolation and the biological activities of the antibiotics. The physico-chemical properties and elucidation of structures of **1** and **2** are presented in this paper.

Results

Physico-chemical Properties

The physico-chemical properties of PF1163A (**1**) and B (**2**) are summarized in Table 1. The HR-FAB-MS established the molecular formulae of **1** and **2** as $C_{27}H_{43}NO_6$ and $C_{27}H_{43}NO_5$, respectively. The IR spectra of **1** and **2** showed the absorption peaks characteristic for hydroxyl group (3400 cm^{-1}), lactone or ester carbonyl (1740 cm^{-1}) and amide carbonyl (1635 cm^{-1}). The UV spectra of **1** and **2** indicated the existence of same chromophore in both compounds. The ^1H NMR spectra of **1** and **2** resembled each other and all the signals were very broad as shown in Fig. 2. Attempts to improve the resolution of the NMR spectra of **1** and **2** were unsuccessful. Low resolution of the NMR spectra of **1** and **2** prevented us from elucidating the structures completely. Therefore, we tried to obtain the chemical degradation products of **1** and **2**.

The Structure of PF1163A (**1**)

The chemical conversions of **1** are summarized in Fig. 3. Acid hydrolysis of **1** with hydrochloric acid followed by the extraction with diethyl ether gave a mixture of hydroxy fatty acids. Mild degradation gave a single product (as mentioned below), so, we deduced that elimination of hydroxyl groups from the fatty acid of **1** had occurred randomly in acid hydrolysis. The aqueous layer was applied to a cation exchange resin and the column was eluted with 0.5 N NH_4OH. The eluate was concentrated to give an amino acid (**3**). The structure of **3** was determined as *N*-methyl-tyrosine 2-hydroxyethyl ether by spectroscopic data.

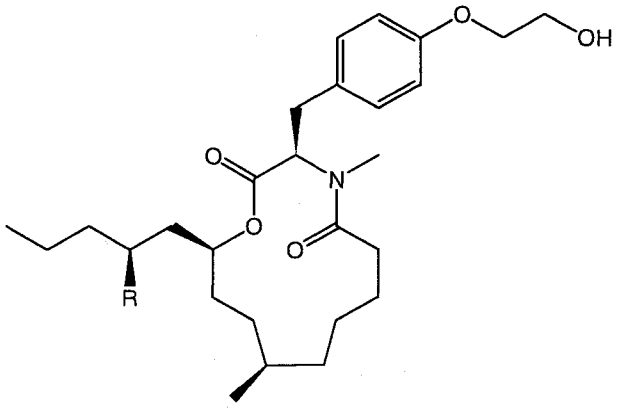
The strong absorption at 1740 cm^{-1} in the IR spectrum of **1** suggested the existence of ester or lactone function in **1**. Treatment of **1** with 1 N NaOH gave an alkaline

Table 1. Physico-chemical properties of PF1163A (**1**) and B (**2**).

	1	**2**
Appearance	colorless oil	colorless oil
Molecular formula	$C_{27}H_{45}NO_6$	$C_{27}H_{43}NO_5$
FAB-MS (*m/z*)	478 $[M+H]^+$	462 $[M+H]^+$
HRFAB-MS (*m/z*)		
found	478.3173	462.3218
Calcd.	478.3169	462.3219
$[\alpha]_D^{25}$	−91.84 (*c* 1.0, MeOH)	−111.59 (*c* 1.2, MeOH)
UV λ_{max}^{MeOH} nm (ε)	224 (11500), 276 (1700), 282 (1400)	224 (11000), 276 (1500), 282 (1300)
IR ν_{max} cm^{-1} (KBr)	3400, 1735, 1635, 1510,	3400, 1735, 1635, 1510,
TLC Rf*	0.43	0.61

* Silica gel TLC Merck art. No.5715: hexane-ethyl acetate, 1:1

Fig. 1. Structures of PF1163A (**1**) and B (**2**).



PF1163A (**1**) R = OH

PF1163B (**2**) R = H

hydrolysis product (**4**). Molecular ions 18 mass units higher than the starting compound were observed in the SI-MS of **4**. The resolution of the NMR spectra of **4** are much better than that of **1**. The structure of **4** was determined using NMR (^1H, ^{13}C, COSY, HMQC and HMBC) and MS data of **4**. The HMBC correlations of **4** are summarized in Fig. 4. The NMR spectrum of **4** indicated the existence of two conformers in the CD_3OD solution (see Table 2). The *N*-methylamide group in **4** can cause the existence of *cis* and *trans* conformers.

There are two secondary hydroxyl groups in **4**, so, there remained two possibilities for the structure of **1**. Namely, the oxygen atom at C-9 hyroxyl group connected to the carbonyl carbon of amino acid **3** *via* ester bondage to form a 13-membered ring structure or the oxygen atom at C-11 connected to form a 15-membered macrocyclic ring structure in **1**. To determine the position of free hydroxyl group, the following chemical conversions of **1** were carried out as shown in Fig. 3. Methylation of **1** with methyl iodide and Ag_2O gave monomethyl ether derivative. Oxidation of monomethyl ether of **1** with pyridinium chlorochromate followed by alkaline hydrolysis gave an α,β-unsaturated ketone derivative (**5**).

The structure of **5** was elucidated by analyzing NMR data including 2D NMR (COSY, HSQC and HMBC). The α,β-unsaturated ketone structure in **5** indicated that the oxidation had occurred at C-11 hydroxyl group followed by β-elimination of C-9 hydroxyl group during alkaline hydrolysis. These results indicated the oxygen atom at C-9 was connected to the carbonyl carbon in **1**. Thus, the gross structure of **1** was determined as a 13-membered macrocyclic structure as shown in Fig. 1. The stereochemistry of **1** was not determined at this stage.

Fig. 2. ^{1}H NMR spectra of PF1163A (**1**) and B (**2**) in d_6-DMSO.

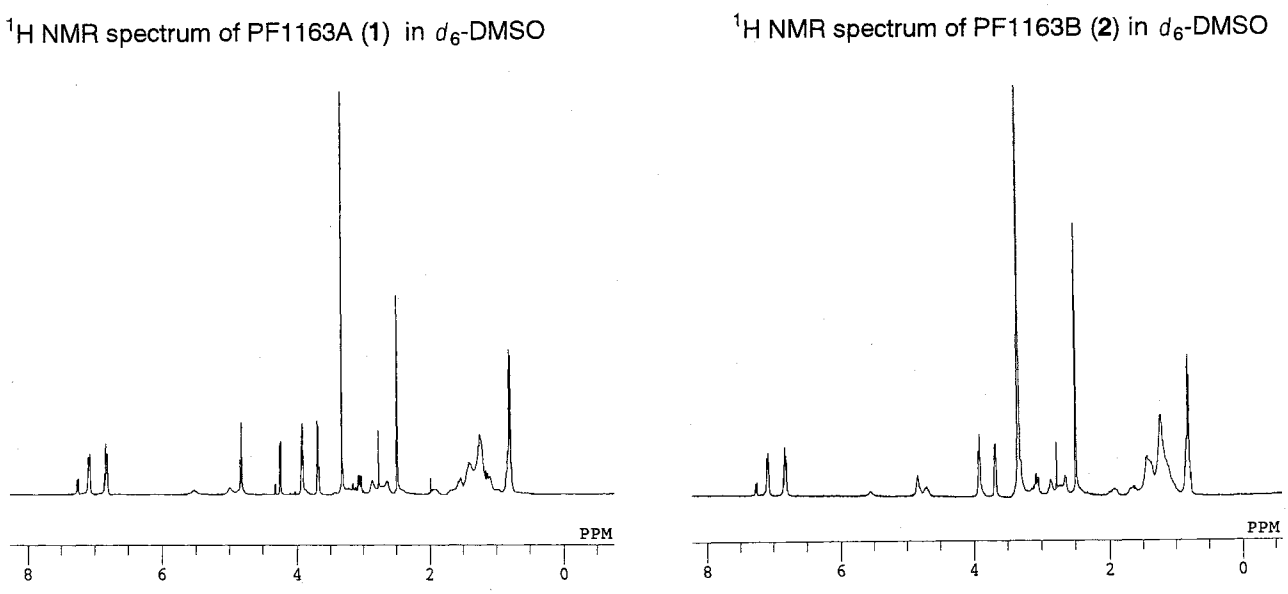
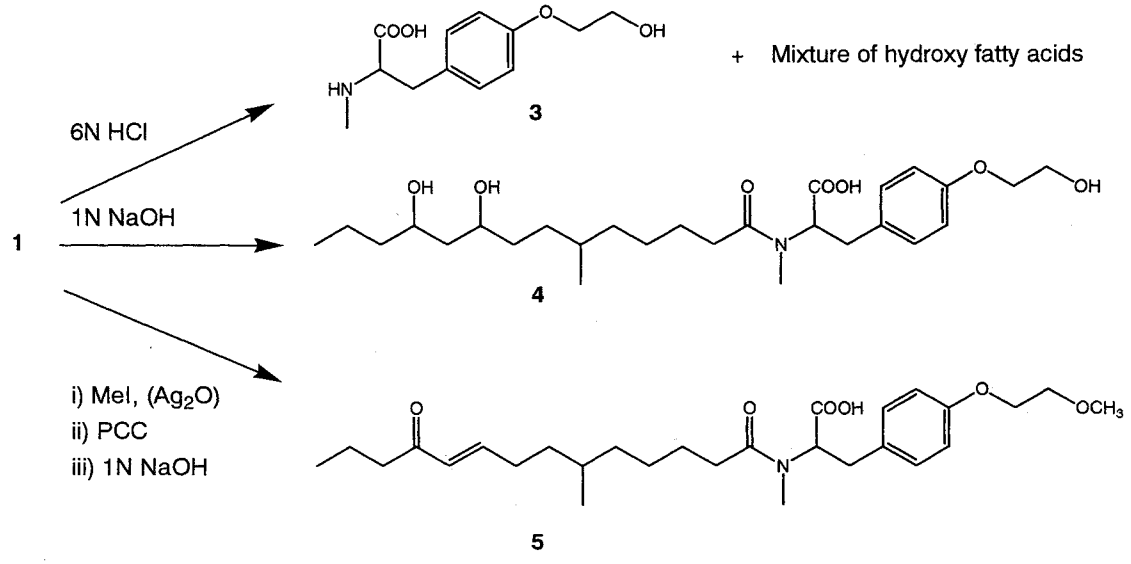


Fig. 3. The chemical conversion of PF1163A (**1**).

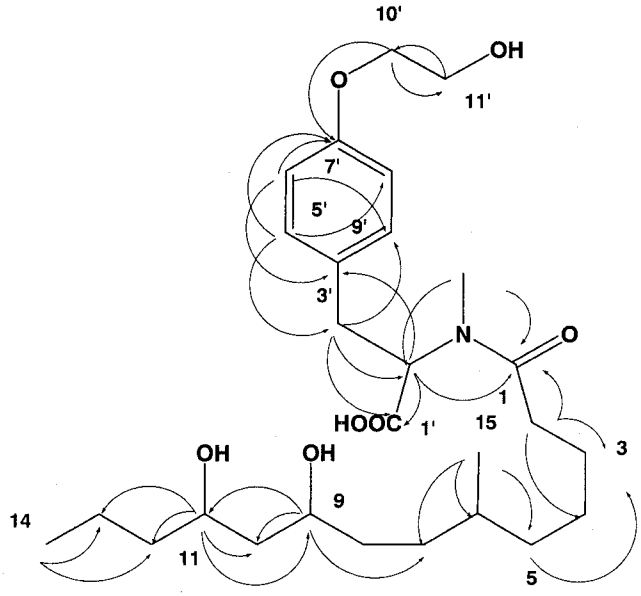


Structure of PF1163B (**2**)

^{1}H NMR spectrum of **1** in DMSO showed two exchangeable protons which disappeared upon addition of D_2O, indicating the existence of a primary hydroxyl group (δ_H 4.82, 1H, t) and a secondary hydroxyl group (δ_H 4.22, 1H, d) in **1**, while only one exchangeable proton (δ_H 4.80, 1H, t) was observed in ^{1}H NMR spectrum of **2**. In addition, molecular ions 16 mass units less than **1** were observed in the FAB-MS of **2**. Based on these results, we deduced that the hydroxyl group at C-11 in **1** was replaced by hydrogen in **2**.

This was confirmed by the following degradation studies. Acid hydrolysis of **2** with hydrochloric acid gave an amino acid and a hydroxy fatty acid methyl ester (**6**). The amino acid was identical with **3** which was obtained by the acid

Fig. 4. HMBC correlations for compound **4**.



degradation of **1**. The structure of **6** was deduced by the fragmentation pattern of EI-MS of **6** as shown in Fig. 5.

In order to determine the stereochemistry of three chiral centers on the macrocyclic ring, we tried to obtain crystals suitable for X-ray diffraction analysis by chemical conversion of **2**. This was achieved by converting **2** into the de-2-hydroxyethyl derivative (**7**). Tosylation of **2** followed by treatment with NaI and zinc[2,3] gave crystalline solid of **7** (Fig. 6). Recrystallization of **7** from a mixture of acetone and water gave colorless crystals which were suitable for X-ray diffraction analysis. The molecular structure of **7** calculated from the crystallographic measurements is shown in Fig. 7. Furthermore, upon acid hydrolysis of **7**, an optically active *N*-methyltyrosine (**8**) was obtained. The specific rotation of **8** ($[\alpha]_D^{22}$ +10.3° (*c* 0.4, 1 N HCl)) indicated the *S*-configuration for the amino acid. Thus, the structure of **2**, including absolute stereochemistry, was determined as shown in Fig. 1.

Table 2. NMR data for alkaline hydrolysis product (**4**) of PF1163A.

	major	minor	major	minor
Position	^{13}C (δ)	^{13}C (δ)	^{1}H (δ)	^{1}H (δ)
1	176.3	176.5		
2	34.3	33.6	2.32	1.87, 2.19
3	26.4	26.4	1.48	1.48
4	27.7	27.7	1.3	1.3
5	37.9	37.9	1.17, 1.37	1.17, 1.37
6	33.8	33.8	1.44	1.44
7	33.9	33.9	1.28, 1.44	1.28, 1.44
8	36.5	36.5	1.46, 1.56	1.46, 1.56
9	69.6	69.6	3.84	3.84
10	45.6	45.6	1.53	1.53
11	69.0	69.0	3.87	3.87
12	41.4	41.4	1.53	1.53
13	19.9	19.9	1.44, 1.58	1.44, 1.58
14	14.5	14.5	1.09	1.09
15	20.0	20.0	0.94	0.92
1'	173.4	174.0		
2'	60.4	63.8	5.22	4.84
3'	34.7	35.1	3.10, 3.35	3.04, 3.34
4'	131.0	130.9		
5' and 9'	130.9	131.3	7.19	7.21
6' and 8'	115.6	115.8	6.93	6.96
7'	159.2	159.5		
10'	70.5	70.5	4.07	4.07
11'	61.7	61.7	3.92	3.92
N-CH3	34.1	30.3	2.93	2.93

400 MHz for ^{1}H and 100 MHz for ^{13}C in CD_3OD.

Fig. 5. Acid hydrolysis of PF1163B (**2**) and EI-MS fragmentation of compound **6**.

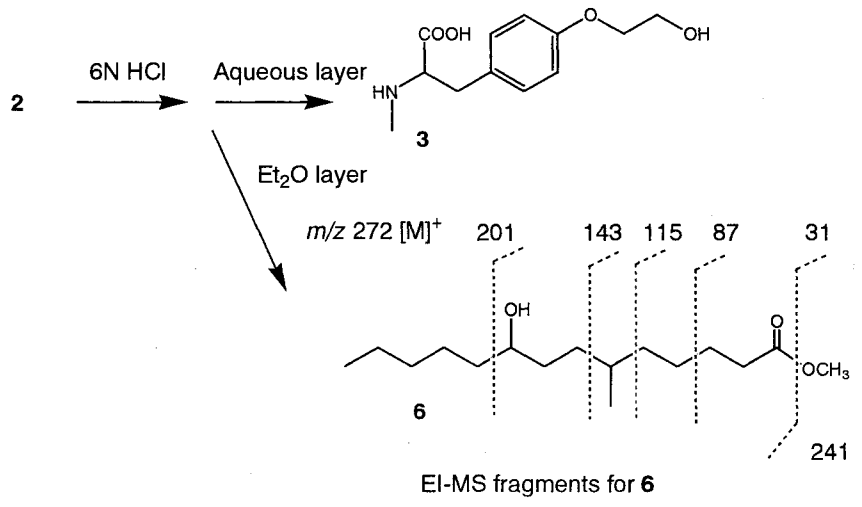


Fig. 6. The chemical conversion of PF1163B (**2**) to the de-2-hydroxyethyl derivative (**7**).

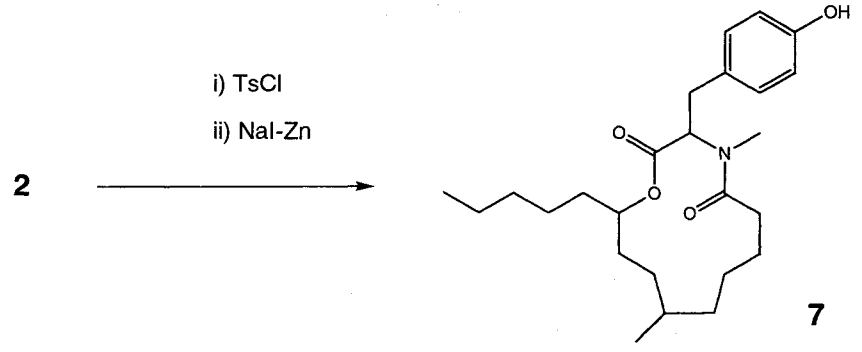
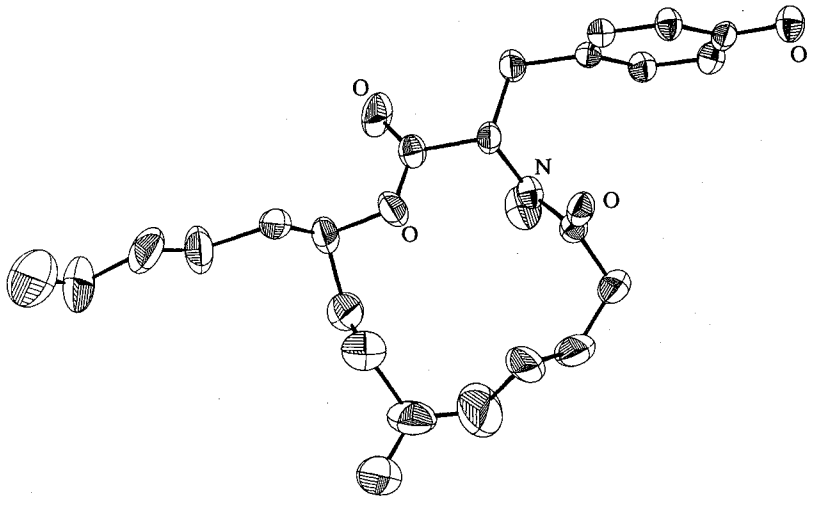


Fig. 7. Molecular structure of PF1163B de-2-hydroxyethyl derivative (**7**).



Discussion

The structure of new antifungal antibiotics PF1163A (**1**) and B (**2**) were determined by chemical degradation studies and X-ray diffraction analysis. The resolution of the NMR spectra of **1** and **2** were too low to analyze their structures. The *N*-methylamide function can cause the existence of *cis* and *trans* conformers in solution[4]. The interconversion of the conformers may be restricted by the 13-membered rigid macrocyclic structure in **1** and **2**. We attributed the low resolution of NMR spectra of **1** and **2** to the restricted interconversion of conformers, because the resolution of the NMR spectrum of **4**, the alkaline hydrolysis product of **1**, was good enough for structural analysis.

The stereochemistry of **2** was determined by single crystal X-ray diffraction analysis of the de-2-hydroxyethyl derivative of **2**. Attempts to crystallize the same derivative of **1** gave only amorphous powders. The absolute structure of **1** was determined by the total synthesis. The details on the total synthesis of **1** and **2** will be reported in a separate paper[5].

Experimental

General

UV and IR spectra were recorded on Shimadzu UV-260 and Shimadzu FTIR-8100 spectrophotometers, respectively. ^1H and ^{13}C NMR spectra were recorded on JEOL JNM-GSX 400 spectrometer. SI and FD mass spectra were recorded with a Hitachi M-80B mass spectrometer and HR-FAB-MS were measured on a JEOL JMS-700 mass spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter using a 10 cm cell.

Acid Hydrolysis of **1**

To a solution of 48.8 mg of **1** in 0.5 ml of methanol was added 0.5 ml of conc. hydrochloric acid. The mixture was kept at 110°C for 18 hours. The reaction mixture was diluted with 10 ml of water then extracted with 10 ml of diethyl ether twice. The solvent layer was concentrated to dryness to give 28.5 mg of a mixture of oily materials. The aqueous layer was applied to a cation exchange resin column (PK208; Mitsubishi Kagaku Ltd.). The column was washed with water and eluted with 0.5 N NH_4OH. The eluate was concentrated to give 17.9 mg of colorless powder of **3**. CI-MS *m/z* 240 $[M+H]^+$, $[\alpha]_D^{23}$ +16.7° (*c* 0.1, 1 N HCl), ^1H NMR (D_2O) δ_H 7.24 (2H, d, 7.9 Hz), 7.00 (2H, d, 7.9 Hz), 4.17 (2H, t, 4.8 Hz), 3.92 (2H, t, 4.8 Hz), 3.82 (1H, t, 5.8 Hz), 3.18 (2H, t, 5.8 Hz).

Alkaline Hydrolysis of **1**

To a solution of 19 mg of **1** in 4.8 ml of ethanol was added 0.6 ml of 1 N NaOH. The mixture was stirred at 50°C for 20 minutes. The reaction mixture was diluted with 10 ml of H_2O, and after the pH was adjusted to 2.6 with 1 N HCl the product was extracted with ethyl acetate. The solvent layer was dried to give 13 mg of **4**. SI-MS *m/z* 496 $[M+H]^+$, ^1H NMR (CD_3OD) observed as a mixture of conformers; see Table 2.

The Chemical Conversion of **1**, α,β-Unsaturated Ketone Derivative (**5**)

To a solution of 36.2 mg of **1** in CH_3CN were added 22 mg of Ag_2O and 50 μl of CH_3I and the mixture was refluxed for 96 hours. The reaction mixture was filtered and concentrated. The residue was purified by preparative silica gel TLC to give 27.5 mg of monomethyl ether derivative of **1**. To a solution of 27.5 mg of monomethyl ether in CH_2Cl_2 were added 22.8 mg of PCC and MS4A and stirred at 24°C for 19 hours. The reaction mixture was filtered and concentrated to give 27.8 mg of ketone derivative. To a solution of 27.8 mg of ketone derivative in 0.99 ml of dioxane was added 0.05 ml of 1 N NaOH and the mixture was stirred at 40°C for 1 hour. After acidification with 1 N HCl, the reaction mixture was extracted with ethyl acetate. The solvent layer was concentrated and the residual solid was purified by silica gel TLC to give 8.4 mg of **5**. SI-MS *m/z* 490 $[M+H]^+$, ^1H NMR (CD_3OD): observed as a mixture of conformers: 7.05 (4H, d), 7.00 (2H, dt), 6.90 (4H, d), 6.15 (2H, d), 5.42 (1H, dd), 4.45 (1H, dd), 4.12 (4H, t), 3.78 (4H, t), 3.46 (6H, s), 3.40 (2H, dd), 2.98 (N-CH_3, s), 2.96 (N-CH_3, s), 2.95 (2H, dd), 2.63 (4H, t), 2.1~2.4 (6H, m), 1.10~1.80 (22H, m), 1.01 (3H, t), 1.00 (3H, t), 0.94 (3H, d), 0.93 (3H, d).

Acid Hydrolysis of **2**

To a solution of 56.0 mg of **2** in 0.5 ml of MeOH was added 0.5 ml of conc. hydrochloric acid. The mixture was kept at 110°C for 14.5 hours. The reaction mixture was diluted with 10 ml of water, then extracted twice with 10 ml of diethyl ether. The solvent layer was concentrated to dryness to give 35.0 mg of colorless oil (**6**): FAB-MS *m/z* 273 $[M+H]^+$, ^1H NMR ($CDCl_3$) δ 3.70 (3H, s), 3.58 (1H, m), 2.30 (2H, t), 1.60 (2H, m), 1.10~1.40 (18H, m), 1.90 (3H, t), 1.87 (3H, d). The aqueous layer was applied to a cation exchange resin column (PK208; Mitsubishi Kagaku Ltd.). The column was washed with water and eluted with 0.5 N NH_4OH. The eluate was concentrated to give 9.4 mg

of colorless powder. The physico-chemical properties of the powder are identical with those of **3**.

De-2-hydroxyethyl Derivative (7) of PF1163B

To a solution of 44 mg of **2** in 0.5 ml of pyridine was added 58 mg of *p*-toluenesulfonyl chloride in 0.5 ml of dichloromethane at 0°C, and stirred for 16 hours at room temperature. After the reaction was quenched with 0.05 ml of H_2O for 2 hours, ethyl acetate (20 ml) was added to the solution, and the ethyl acetate layer was washed with 10% $KHSO_4$, saturated aqueous $NaHCO_3$, and 10% NaCl successively. The organic layer was dried over Na_2SO_4 and concentrated to give 51 mg of a tosylate as colorless oil.

To a solution of 50 mg of the above tosylate in 1 ml of *N*,*N*-dimethylformamide was added 130 mg of NaI and 75 mg of zinc dust. The mixture was sonicated for 5 minutes and stirred for 1 hour at 90°C. The reaction mixture was filtered with the aid of ethyl acetate (10 ml), and the filtrate was washed with water, 5% $Na_2S_2O_3$, saturated $NaHCO_3$ and 10% NaCl. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give 30 mg of **7**. ES-MS *m/z* 418 $[M+H]^+$, 440 $[M+Na]^+$ $[\alpha]_D^{23}$ −119° (*c* 0.46, $CHCl_3$) ^1H NMR ($CDCl_3$, 400 MHz) δ_H; 7.1 (d), 6.75 (d), 5.9 (m), 4.85 (m), 3.01, 2.90 (–NCH_3), 2.2~3.2 (m), 1.1~1.8 (m), 0.8~0.95 (m).

Acid Hydrolysis of 7

To a solution of 32 mg of **7** in 0.4 ml of dioxane was added 1.5 ml of 6 N HCl. The mixture was stirred vigorously at 110°C for 3 hours. The reaction mixture was washed with ethyl acetate. The aqueous layer was concentrated under reduced pressure to give 13.5 mg of solids. The residue was applied on an ODS column (AQ120-S5, YMC, 3 ml) and the column was eluted with 5% aqueous MeCN. The eluate was concentrated to give 8.6 mg of colorless powder. The powder was crystallized from H_2O at 5°C to give 3.9 mg of *N*-methyltyrosine (**8**): $[\alpha]_D^{22}$ +10.3° (*c* 0.4, 1 N HCl) Lit. $[\alpha]_D^{23}$ +16.0° (*c* 0.42, 1 N HCl)[6], ^1H NMR (D_2O) δ_H: 7.15 (1H, d), 6.82 (1H, d), 4.20 (1H, t), 3.22 (2H, dd), 2.70 (3H, s).

Single-crystal X-Ray Diffraction Analysis of 7

Crystals of **7** were obtained from an acetone-water solution. A colorless rod-like crystal having the approximate dimensions of 0.5×0.1×0.1 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated CuKα radiation and rotating anode generator. The crystal data are as follows: Empirical formula; $C_{25}H_{39}NO_4$. Formula weight; 417.59. Crystal system; monoclinic. Lattice parameters; a=13.076(1) Å, b=5.911(1) Å, c=16.4957(9)Å, β=91.519(6) Å, V=1274.5(3) Å. Space group; $P2_1$. Z value; 2. D_{calc}; 1.088 g/cm^3. The intensity data were collected by using the ω–2θ scan technique to a maximum 2θ value of 120.2°.

A total of 2225 reflections were collected, of which 2123 were unique. The structure was determined by direct methods (SHELXS)[7] and expanded using Fourier technics (DIRDIF)[8]. The non hydrogen atoms were refined by the methods of full-matrix least-squares with anisotropic thermal parameters. The final R value was 0.093.

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