EURYSTATINS A AND B, NEW PROLYL ENDOPEPTIDASE INHIBITORS

II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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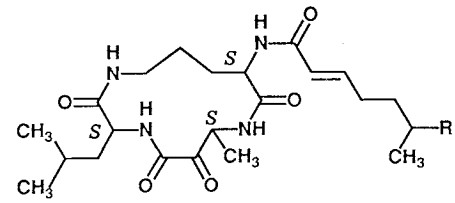
The structures of eurystatins A and B, new prolyl endopeptidase inhibitors, have been elucidated by chemical degradation and spectral studies. They have in common a unique 13-membered cyclic peptide core composed of L-leucine, L-ornithine and (*S*)-3-amino-2-oxobutyric acid, and differ from each other in the α,β-unsaturated fatty acid attached to the α-amino moiety of the ornithine.

In the preceding paper[1], we described the fermentation, isolation, and specific prolyl endopeptidase inhibitory activities of eurystatins A and B produced by *Streptomyces eurythermus*. This paper describes the physico-chemical properties and structural studies on eurystatins A and B.

Physico-chemical Properties and Spectral Analyses

Eurystatins A (**1a**) and B (**1b**) were obtained as colorless fine needles from aqueous acetonitrile. They are soluble in dimethyl sulfoxide and pyridine, slightly soluble in methanol and ethanol and practically insoluble in ethyl acetate, *n*-hexane and water. Both compounds showed positive responses to iodine and ammonium molybdate and negative to ninhydrin and Sakaguchi reagents on TLC plate. The physico-chemical properties of **1a** and **1b** are given in Table 1. Their molecular formulae were established by the EI-MS and microanalyses,

Fig. 1. Structures of eurystatins A and B.



Eurystatin A (**1a**) R=CH_3
Eurystatin B (**1b**) R=C_2H_5

Table 1. Physico-chemical properties of eurystatins A and B.

	Eurystatin A	Eurystatin B
Nature	Colorless fine needles	Colorless fine needles
MP (dec)	294~296°C	291~293°C
Optical rotation	$[\alpha]_D^{26}$ −134° (*c* 0.25, DMSO)	$[\alpha]_D^{26}$ −96° (*c* 0.25, DMSO)
EI-MS (*m/z*)	450 $(M)^+$	464 $(M)^+$
Molecular formula	$C_{23}H_{38}N_4O_5$	$C_{24}H_{40}N_4O_5$
Elemental analysis		
Calcd for:	$C_{23}H_{38}N_4O_5 \cdot \frac{3}{4}H_2O$	$C_{24}H_{40}N_4O_5 \cdot \frac{3}{4}H_2O$
	C 59.53, H 8.58, N 12.07	C 60.29, H 8.75, N 11.72
Found:	C 59.50, H 8.67, N 11.96	C 60.22, H 8.79, N 11.68
UV λ_{max} nm (ε)		
in CH_3CN-H_2O (1:1)	214 (18,900)	213 (18,400)
IR ν_{max} (KBr) cm^{-1}	3340, 2950, 1665, 1620, 1540, 1515, 1455, 1390, 1370, 1150	3340, 2950, 1665, 1640, 1620, 1540, 1515, 1455, 1390, 1375, 1150

Fig. 2. IR spectrum of eurystatin A (in KBr).

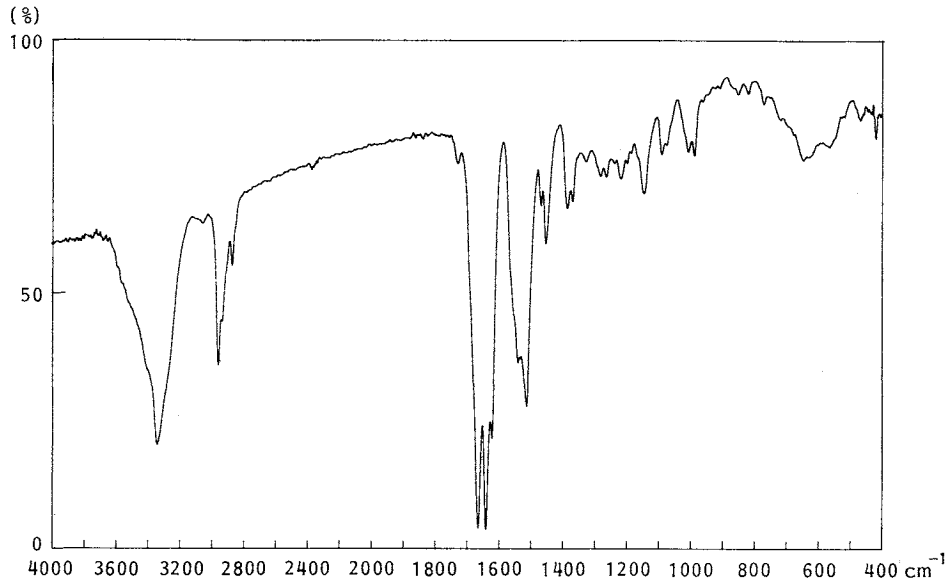
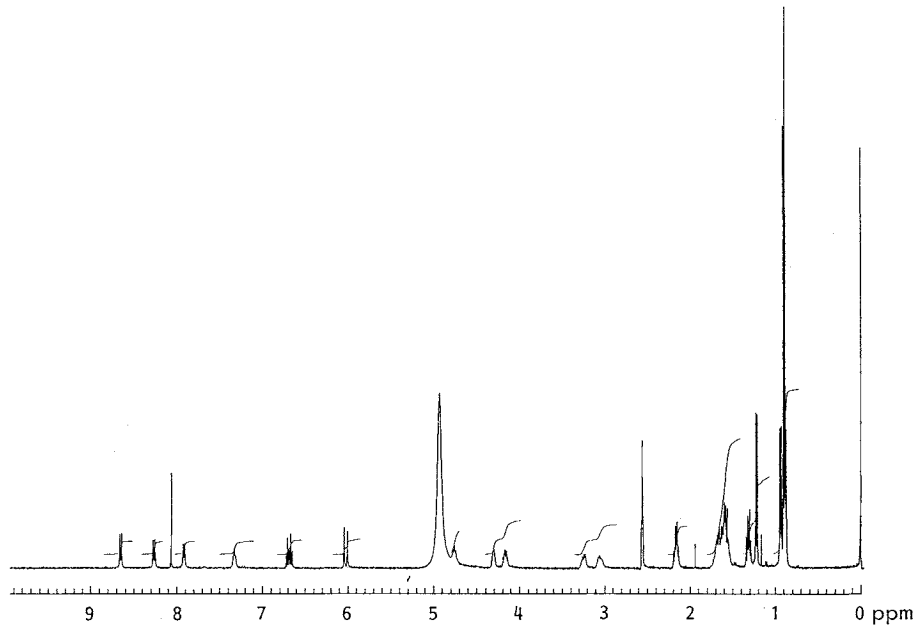


Fig. 3. ^{1}H NMR spectrum of eurystatin A (400 MHz, in DMSO-d_6-$CDCl_3$, 1:2).



indicating that **1b** was a CH_2 unit larger than **1a**. The IR spectra of both compounds (Fig. 2 (**1a**)) are similar exhibiting typical peptide absorption bands at around 1650 and 1530 cm^{-1} and their ^{1}H NMR spectra are also very similar (Fig. 3 (**1a**)). The ^{13}C NMR of **1a** was not analyzed well due to its poor solubility in the NMR solvents, but the spectrum of **1b** showed clear 24 carbon signals (Table 2) which were assignable to five methyl (δ 11.2, 14.9, 18.8, 21.5 and 22.8), seven methylene (δ 24.5, 29.0, 29.1, 29.3,

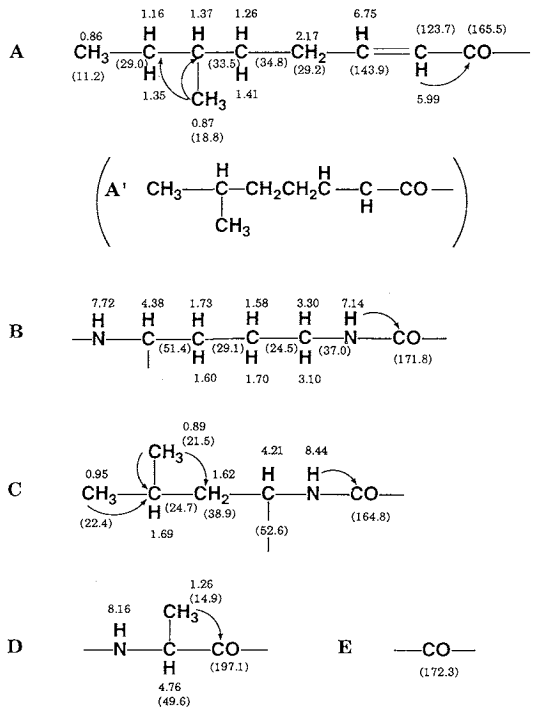
Table 2. ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR spectral data of eurystatin B in DMSO-d_6-$CDCl_3$ (1:2).

Assignment	δ_C (multiplicity)	δ_H (multiplicity)	Assignment	δ_C (multiplicity)	δ_H (multiplicity)
AOB-1	164.8 (s)		Orn-1	172.3 (s)	
-2	197.1 (s)		-2	51.4 (d)	4.38 (m)
-3	49.6 (d)	4.76 (m)	-3	29.1 (t)	1.60 (m), 1.73 (m)
-4	14.9 (q)	1.26 (d, J=6.6 Hz)	-4	24.5 (t)	1.58 (m), 1.70 (m)
-3-NH		8.16 (d, J=8.2 Hz)	-5	37.0 (t)	3.10 (m), 3.30 (m)
Leu-1	171.8 (s)		-2-NH		7.72 (d, J=7.7 Hz)
-2	52.6 (d)	4.21 (m)	-5-NH		7.14 (t, J=6.4 Hz)
-3	38.9 (t)	1.62 (m)	FA_2-1	165.5 (s)	
-4	24.7 (d)	1.69 (m)	-2	123.7 (d)	5.99 (bd, J=15.4 Hz)
-4-Me	22.8 (q)	0.95 (d, J=6.4 Hz)	-3	143.9 (d)	6.75 (dt, J=15.4, 6.8 Hz)
-4-Me	21.5 (q)	0.89 (d, J=6.6 Hz)	-4	29.3 (t)	2.17 (br q, J=6.8 Hz)
-2-NH		8.44 (d, J=7.7 Hz)	-5	34.8 (t)	1.20 (m), 1.47 (m)
			-6	33.5 (d)	1.37 (m)
			-7	29.0 (t)	1.16 (m), 1.35 (m)
			-8	11.2 (q)	0.86 (t, J=7.1 Hz)
			-6-Me	18.8 (q)	0.87 (d, J=6.4 Hz)

Abbreviations: AOB; 3-amino-2-oxobutyric acid, Leu; leucine, Orn; ornithine, FA_2; (*E*)-6-methyl-2-octenoic acid.

34.8, 37.0 and 38.9), five methine (δ 24.7, 33.5, 49.6, 51.4 and 52.6), two olefin (δ 123.7 and 143.9) and five carbonyl carbons (δ 164.8, 165.5, 171.8, 172.3 and 197.1) by the aid of DEPT experiment. The extensive ^{1}H-^{1}H, ^{13}C-^{1}H and ^{13}C-^{1}H long range COSY experiments of **1b** suggested the presence of five partial structures A, B, C, D and E in **1b** (Fig. 4). Although the 2D NMR experiments were not available for **1a**, its ^{1}H NMR indicated a 6-methyl-2-heptenoyl group (partial structure A′ in Fig. 4) instead of partial structure A of **1b**, reflecting the molecular difference between **1a** and **1b**.

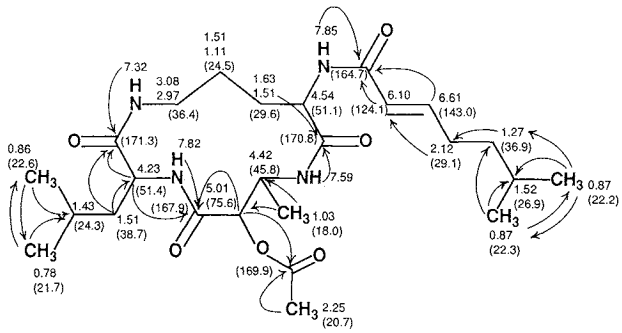
Fig. 4. Partial structures and chemical shifts δ_H (δ_C) obtained from ^{1}H-^{1}H COSY and ^{13}C-^{1}H long range (⟶) COSY experiments for eurystatin B.



Degradation Study

Upon hydrolysis with 6 N HCl at 110~120°C, **1a** yielded, after chromatography on Dowex 50 WX8, L-leucine and L-ornithine whose configuration was determined by HPLC using a chiral column. A lipophylic acidic product (FA_1) obtained from the ethereal extract of the hydrolysate exhibited the presence of one geminal dimethyl (δ 0.90, d, J=6.8 Hz, 6H), two methylene (2.24, m and 1.37, m), one methine (1.58, m) and two *trans* olefinic protons (5.83, dt, J=15.6 and 1.5 Hz, and 7.08, dt, J=15.6 and 6.8 Hz) in the ^{1}H NMR. The data combined with CI-MS (*m/z* 143 $(M+H)^+$) indicated FA_1 to be (*E*)-6-methyl-2-heptenoic acid.

Fig. 5. ^{13}C-^{1}H long range COSY analysis of **4a** (chemical shifts δ_H (δ_C)).



Acid hydrolysis of **1b** afforded a new fatty acid (FA_2) together with L-leucine and L-ornithine. The IR, ^{1}H NMR and CI-MS (*m/z* 157 $(M+H)^+$) of FA_2 indicated (*E*)-6-methyl-2-octenoic acid, substantiating the partial structures A' (of **1a**) and A (of **1b**) assigned by the NMR experiments. Thus, the difference between **1a** and **1b** was shown to be only in the fatty acid moiety.

$NaBH_4$ reduction of **1a** gave a dihydro derivative **2a** which showed two peaks in the HPLC. They were separated by preparative HPLC to $\mathbf{2a}_1$ and $\mathbf{2a}_2$ which showed the same molecular ion peak at *m/z* 452 in the EI-MS spectra. In ^{1}H NMR spectrum of $\mathbf{2a}_1$, a new doublet methine proton (δ 3.92, d, $J=1.7$ Hz) bearing a hydroxy group was observed indicating that the ketone in **1a** was reduced to a secondary alcohol giving two diastereoisomers $\mathbf{2a}_1$ and $\mathbf{2a}_2$. Acid hydrolysis of the **2a** mixture gave L-leucine, L-ornithine and a new compound **3**. The molecular formula of **3** was determined to be $C_4H_9NO_3$ by its HRFAB-MS (*m/z* 120.0657 $(M+H)^+$). Compound **3** was determined to be a diastereoisomeric mixture of 3-amino-2-hydroxybutyric acid[2] by ^{1}H NMR spectrum. Upon oxidation with Fenton reagent[3,4], **3** gave alanine, whose configuration was determined to be L by chiral HPLC. Thus the original amino acid in eurystatin (partial structures D and E) was determined to be (*S*)-3-amino-2-oxobutyric acid.

Sequence of the Structural Compounds

In order to increase solubility in NMR solvents, **2a** was acetylated to yield the monoacetate (**4a**). Upon silica gel chromatography and subsequent preparative HPLC, **4a** was separated to $\mathbf{4a}_1$ and $\mathbf{4a}_2$, both of which showed the molecular ions at *m/z* 495 $(M+H)^+$. Extensive 2D-NMR experiments were carried out for $\mathbf{4a}_1$, which exhibited higher solubility than $\mathbf{4a}_2$. It showed a new low-field methine signal at δ 5.01 (d, $J=2.1$ Hz) due to acetylation in the ^{1}H NMR, and the presence of six methyl, six methylene, six methine, two olefin, and five carbonyl carbons in the ^{13}C NMR. The ^{13}C-^{1}H long range COSY experiment combined with ^{1}H-^{1}H and ^{13}C-^{1}H 2D NMR allowed to assign all proton and carbon signals of $\mathbf{4a}_1$ and also to sequence the structural units, L-leucine, L-ornithine, (*S*)-3-amino-2-oxobutyric acid and 6-methyl-2-heptenoic acid establishing a unique 13-membered cyclic peptide structure substituted with the acyl side chain (Fig. 5). The structures of **1a** and **1b** were determined as shown in Fig. 1.

Discussion

The structures of eurystatins A and B have been determined based on the spectroscopic analysis and partial degradation experiments. They have in common a unique 13-membered cyclic peptide core and

differ from each other in the acyl side chain. As reported in the preceding paper[1], they showed specific and strong prolyl endopeptidase inhibitory activities. It is particularly interesting that reduction of the keto carbonyl of eurystatin completely lost the enzyme inhibitory activity. This indicated that the α-keto group of 3-amino-2-oxobutyric acid is very essential for the activity of the antibiotics.

Recently, a new prolyl endopeptidase inhibitor poststatin was reported by the Institute of Microbial Chemistry[5]. It is a straight chain peptide containing a (*S*)-3-amino-2-oxovaleric acid (postine) which is the homolog of (*S*)-3-amino-2-oxobutyric acid contained in eurystatin. The ketone moiety of postine has also been identified as the key functional group for the enzyme inhibitory activity. It should be noted that cyclotheonamide, a strong thrombin inhibitor recently isolated from a marine sponge, also has a β-amino-α-oxocarboxylic acid[6]. These α-keto-β-amino acids seem to be the active center in the various peptidic enzyme inhibitors.

Experimental

TLC was performed on silica gel plates (Kiesel gel $60F_{254}$, Merck). MP's were determined on a Shibayama micro melting point apparatus and were not corrected. The IR and UV spectra were determined on a Jasco IR-810 IR spectrophotometer and a Jasco UVIDEC-610C spectrometer, respectively. The ^1H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX 400 or Varian FT 80A spectrometer. The EI-MS, FAB-MS and CI-MS spectra were measured on a Jeol JMS-AX505H mass spectrometer. Amino acid analysis was carried out using the Waters PICO-TAG system.

Acid Hydrolysis of Eurystatin A (**1a**)

A suspension of **1a** (100 mg) in a mixture of 6 N HCl (10 ml) and 1,4-dioxane (4 ml) was heated at 110°C for 18 hours in a sealed tube. The reaction mixture was extracted with ether (20 ml × 4). The aqueous layer was concentrated *in vacuo* to give an oil, which was chromatographed on a column of Dowex 50WX8 (6.5 mm i.d. × 110 mm). After washing with water, the column was developed with 0.05 N HCl (200 ml), 0.1 N HCl (200 ml), 0.2 N HCl (200 ml), 0.4 N HCl (200 ml), 1.0 N HCl (200 ml) and 3.0 N HCl (200 ml), successively, and the eluate was monitored by ninhydrin test and TLC (BuOH - AcOH - H_2O, 3 : 1 : 1). Ninhydrin positive fractions containing the same amino acid were combined and evaporated *in vacuo* to yield L-leucine 25.8 mg and L-ornithine 38.5 mg. L-Leucine: TLC: Rf 0.48 (BuOH - AcOH - H_2O, 3 : 1 : 1) and 0.45 (phenol - H_2O, 4 : 1). Identified as L-leucine by amino acid analysis, ^1H NMR and HPLC using a chiral column (column: MCI GEL CRS1OW (DLAA) (Mitsubishikasei Co.), 4.6 mm i.d. × 50 mm, mobile phase: 2 mM $CuSO_4$, flow rate: 2 ml/minute, detection: UV 254 nm, temperature: 25°C, Rt: D-leucine 8.11 minutes and L-leucine 14.36 minutes). L-Ornithine: TLC: Rf 0.10 (BuOH - AcOH - H_2O, 3 : 1 : 1) and 0.02 (phenol - H_2O, 4 : 1). Identified as L-ornithine by amino acid analysis, ^1H NMR and HPLC using the same chiral column as employed for L-leucine (mobile phase; 0.1 mM $CuSO_4$, D-ornithine 4.32 minutes, L-ornithine 4.78 minutes).

The ethereal layer was re-extracted with 0.1 N NaOH and the aqueous extract was washed with ether. The aqueous layer was acidified with dil HCl to pH 2 and extracted with ether. The extract was washed with water, dried over $MgSO_4$ and evaporated to give 28 mg of an oil. It was purified by preparative TLC (Kiesel gel 60 F_{254} #5744, Merck; developed with CH_2Cl_2 - EtOAc - MeOH, 10 : 2 : 1) and then by Sephadex LH-20 chromatography eluting with CH_2Cl_2 - MeOH (1 : 1) to give pure FA_1 (8.5 mg) as a colorless oil. CI-MS *m/z* 143 $(M+H)^+$, ^1H NMR (400 MHz, $CDCl_3$), δ 7.08 (1H, dt, J=15.6 and 6.8 Hz), 5.83 (1H, dt, J=15.6 and 1.5 Hz), 2.24 (2H, m), 1.58 (1H, m), 1.37 (2H, m), 0.90 (6H, d, J=6.8 Hz).

Acid Hydrolysis of Eurystatin B (**1b**)

Acid hydrolysis of **1b** (109 mg) was carried out by the same manner as above to afford L-leucine (25 mg), L-ornithine (35 mg), and FA_2 (10.4 mg) as an oil. FA_2: CI-MS *m/z* 157 $(M+H)^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.09 (1H, dt, J=15.8 and 6.8 Hz), 5.83 (1H, dt, J=15.8 and 1.5 Hz), 2.21 (2H, m), 1.47 (1H, m), 1.36 (2H, m), 0.88 (3H, d, J=6.0 Hz), 0.87 (3H, t, J=7.3 Hz).

$NaBH_4$ Reduction of Eurystatin A (**1a**)

To a stirred solution of **1a** (70 mg) in 40 ml of 50% aqueous EtOH was added 30 mg of $NaBH_4$, and

the stirring was continued for 3 hours at room temperature. The mixture was neutralized with dil HCl and evaporated to remove organic solvent. The residue was extracted with BuOH and the extract was concentrated to give 71 mg of the reduction product (**2a**). Compound **2a** showed two peaks (**2a**$_1$ and **2a**$_2$) in HPLC analysis (column: YMC-ODS A-301-3, 4.6 mm i.d. × 100 mm; mobile phase: CH_3CN - 0.022 M phosphate buffer pH 7.0 (35 : 65); flow rate: 1 ml/minute; detection: UV 210 nm; Rt: 4.78 minutes (40%, **2a**$_2$) and 5.76 minutes (60%, **2a**$_1$)). They (10 mg) were separated by preparative HPLC (column: YMC D-ODS-5, 20 mm i.d. × 250 mm; mobile phase: CH_3CN - 0.022 M phosphate buffer pH 7.0 (37.5 : 62.5); flow rate: 10 ml/minute; detection: UV 210 nm). Appropriate fractions were desalted by solvent extraction with BuOH to give pure **2a**$_1$ (4 mg) and **2a**$_2$ (3 mg) as colorless powders. **2a**$_1$: MP >290°C, EI-MS (*m/z* 452 $(M)^+$, 437, 409, 396, 383), HPLC (Rt, 5.76 minutes by the above system), ^1H NMR (DMSO-d_6 - $CDCl_3$, 2 : 1) δ 7.78 (1H, d, J=7.7 Hz), 7.68 (1H, d, J=9.0 Hz), 7.58 (1H, br t, J=5.6 Hz), 7.40 (1H, d, J=8.5 Hz), 6.60 (1H, dt, J=15.4 and 6.8 Hz), 6.06 (1H, br d, J=15.4 Hz), 4.48 (1H, m), 4.19 (2H, m), 3.92 (1H, d, J=1.7 Hz), 3.23 (1H, m), 2.88 (1H, m), 2.12 (2H, br q, J=7.3 Hz), 1.45~1.65 (7H, m), 1.28 (2H, br q, J=7.3 Hz), 1.23~1.31 (1H, m), 1.11 (3H, d, J=6.4 Hz), 0.91 (3H, d, J=6.4 Hz), 0.88 (6H, d, J=6.8 Hz), 0.85 (3H, d, J=6.4 Hz). **2a**$_2$: MP >290°C, EI-MS (*m/z* 452 $(M)^+$, 437, 409, 396, 383), HPLC (Rt, 4.78 minutes); ^1H NMR gave paired signals.

Acid Hydrolysis of **2a**

Compound **2a** (90 mg) was hydrolyzed by the same procedure as **1a** to give L-leucine (21 mg), L-ornithine (33 mg) and **3** (14 mg) after Dowex 50WX8 chromatography. **3**: Colorless powder, TLC: Rf 0.21 (BuOH - AcOH - H_2O, 3 : 1 : 1) and 0.16 (phenol - H_2O, 4 : 1). HRFAB-MS *m/z* 120.0657, calcd for $C_4H_{10}NO_3$: 120.0661. ^1H NMR (D_2O) δ 1.25 (1H, d, J=7.0 Hz),1.37 (2H, d, J=6.6 Hz), 3.68 (0.67H, dq, J=6.6 and 4.8 Hz), 3.82 (0.33H, dq, J=7.0 and 3.7 Hz), 4.27 (0.67H, d, J=4.8 Hz), 4.46 (0.33H, d, J=3.7 Hz).

Oxidation of **3**

To a solution of **3** (3 mg) in 3% H_2O_2 (0.12 ml) was added a solution of $FeSO_4$ (0.6 mg) in 3 μl of water. The mixture was heated at 50°C for 45 minutes. After dilution with water (1 ml), the mixture was passed through a column of Dowex 50WX8 (5 mm i.d. × 30 mm). The column was washed with water and eluted with 1 N NH_4OH, and the eluate was monitored by ninhydrin. The ninhydrin positive fractions were combined and evaporated to dryness to afford L-alanine (1.1 mg), which was identified by TLC; Rf 0.25 (BuOH - AcOH - H_2O, 3 : 1 : 1) and 0.40 (pyridine - H_2O, 4 : 1), amino acid analysis and HPLC using the chiral column as used for L-leucine and L-ornithine (mobile phase: 0.1 mM $CuSO_4$, flow rate: 0.5 ml/minute, detection: UV 254 nm, temperature: 25°C, D-alanine 6.18 minutes and L-alanine 9.92 minutes).

Acetylation of **2a**

A solution of **2a** (**2a**$_1$ and **2a**$_2$ mixture, 146 mg) in a mixture of 3 ml of acetic anhydride and 10 ml of dry pyridine was stirred at room temperature for 2 days. The reaction mixture was diluted with EtOAc. After removing insoluble material by filtration, the filtrate was washed with water and dil HCl. The organic layer was evaporated *in vacuo* to give a solid which showed two peaks (**4a**$_1$ and **4a**$_2$) by HPLC (HPLC condition same as that of **2a**. Rt **4a**$_1$, 15.0 minutes and **4a**$_2$, 11.9 minutes). The solid was chromatographed on a silica gel column (Wako gel C-300, 21 mm i.d. × 220 mm) developing with CH_2Cl_2 - EtOAc - MeOH (400 : 16 : 4~400 : 20 : 5, stepwise) to afford semipure **4a**$_1$ (48 mg) and **4a**$_2$ (35 mg). The solid of **4a**$_1$ was further purified by preparative HPLC (column: YMC D-ODS-5, 20 mm i.d. × 250 mm, mobile phase: CH_3CN - 0.022 M phosphate buffer pH 7.0 (40 : 60); flow rate: 15 ml/minute; detection: UV 235 nm). The fractions showing a single peak were combined, and desalted by solvent extraction with EtOAc and subsequent Sephadex LH-20 chromatography eluting with CH_2Cl_2 - MeOH (1 : 1) to give 38 mg of pure monoacetate **4a**$_1$. MP 263~264°C, HRFAB-MS *m/z* 495.3190 $(M+H)^+$ (calcd for $C_{25}H_{43}N_4O_6$: 495.3183). ^1H and ^{13}C NMR are shown in Fig. 5. **4a**$_2$: Purified by a similar preparative HPLC. MP 277~278°C, FAB-MS *m/z* 495 $(M+H)^+$. HREI-MS *m/z* 494.3088 $(M)^+$ (calcd for $C_{25}H_{42}N_4O_6$: 494.3104).

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