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FR901469, a Novel Antifungal Antibiotic from an Unidentified Fungus No. 11243

III. Structure Determination

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A novel antifungal antibiotic, FR901469, was isolated from an unidentified fungus No. 11243. It is a water-soluble 40-membered macrocyclic lipopeptidolactone, consisting of D-Ala, L-Tyr, L-Val, *trans*-4OH-L-Pro, *trans*-3OH-L-Pro, *threo*-3OH-L-Gln, Gly, L-Orn, L-Thr, three residues of D-*allo*Thr and a (3*R*)-hydroxypalmitic acid. Its structure, including absolute configurations, was unequivocally determined as **1** based on chemical and spectroscopic evidence.

Until advent of FR901469, 1,3-β-glucan synthase inhibitors could be classified into two groups, the echinocandin family and the papulacandins. Spectroscopic and amino acid analysis have shown that FR901469 is a new cyclic lipopeptide, consisting of twelve amino acids and 3-hydroxypalmitic acid. The structure of FR901469 (**1**) (Fig. 1) is markedly different from those of echinocandin-like lipopeptides and therefore FR901469 adds a new structural variation to 1,3-β-glucan synthase inhibitors. Its discovery, isolation, characterization and biological properties are described in the previous publication.[1,2] In this paper we report structure elucidation of FR901469 on the basis of chemical and spectroscopic evidence.

Planar Structure Elucidation

The substance of FR901469 (**1**) was isolated from an unidentified fungus No. 11243 and obtained as a colorless powder of its mono hydrochloride salt. Compound **1** was positive to ninhydrin reagent. Low-resolution fast atom bombardment (FAB) mass spectra showed $(M+H)^+$ ion at *m/z* 1533 and $(M+Na)^+$ at 1555. High-resolution FAB mass spectral data on the $(M+Na)^+$ (calcd 1555.8235; found 1555.8240) revealed the molecular formula $C_{71}H_{116}N_{14}O_{23}$ which was consistent with the ^1H and ^{13}C NMR data (see Table 1). In the IR spectrum, a strong amide absorption at 1660 cm^{-1} with a weak shoulder at 1730 cm^{-1} suggested a peptide containing an ester function. Alkaline hydrolysis of **1** (1 N NaOH, room temperature) gave a linear peptide (**2**) whose molecular weight was 18 more than **1**, providing the first evidence that **1** contains a lactone. Amino acid analysis of the acid hydrolysate of **1** (6 N HCl, 110°C, 18 hours) revealed the presence of one residue each of Ala, Tyr, Val, Gly, Orn, NH_3 and four residues of Thr and three unknown amino acids.

The ^1H NMR spectra measured in DMSO-d_6 and D_2O displayed broadened signals, which were not suitable for a detailed analysis. Sharp and well-resolved signals in the ^1H NMR spectra were obtained in CD_3OD. Since an amide NH proton was essential in assigning the sequence of the peptide, CD_3OH was employed instead of CD_3OD. A detailed analysis of COSY, HOHAHA, HSQC and HMBC spectra revealed 13 subunits (**a**~**m**) as shown in Fig. 2.

The structural assignments of the nine common amino acid residues (**a**, **b**, **c**, **d**, **f**, **g**, **j**, **k** and **l**) were easily made. The structure elucidation of the three unusual ones (**e**, **h** and **i**) was as follows. 4-Hydroxyproline (4-OHPro) (**e**): The HMBC correlations between 5-H_2 and C-2 indicated the presence of a pyrrolidine ring and HMBC correlations 3-H_2/C-1 identified **e** as 4-OHPro. 3-Hydroxyproline (3-

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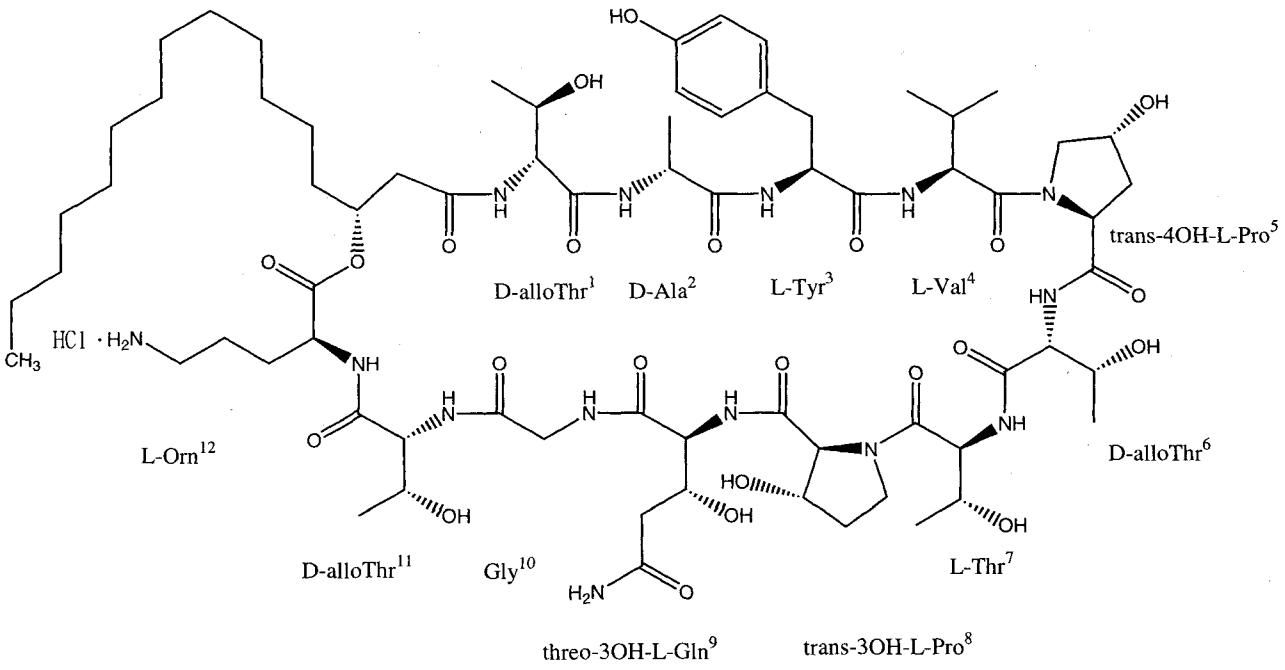
Fig. 1. Structure of FR901469 (**1**).



OHPro) (**h**): The spin systems 2-H~5-H_2 together with HMBC correlations (2-H/C-1 and 2-H/C-5) established **h** as 3-OHPro. 3-Hydroxyglutamine (3OHGln) (**i**): In the HOHAHA spectra, the doublet for the amide proton resonating at 7.97 ppm showed cross-peaks to 4-H_2 (δ 2.46 and 2.16). HMBC correlations (2-H/C-1 and 4-H_2/C-5) identified **i** as 3-OHGlx (3-OHGlu or 3-OHGln was not clear at this stage). The presence of a primary carboxamide was inferred from the peak due to NH_3 in the amino acid analysis and from the nitrogen balance required by the molecular formula. The presence of COSY cross-peaks between two exchangeable singlet protons (δ 7.61 and 7.02) showed further evidence for the existence of a primary amide. The observation of NOE between the primary amide protons and one of the 4-H_2 protons (δ 2.46) defined 3-OHGln.

Subtraction of subunits **a**~**l** from the molecular formula left $C_{16}H_{30}O_2$, corresponding to the hydroxypalmitoyl moiety. The $^1H/^{13}C$ chemical shift of the triplet methyl (δ 0.88/14.3) was typical of a terminal methyl of a straight long-chain. The chemical shift (δ 5.12/74.0) was characteristic of an acyloxymethine and the ester carbonyl was shared with Orn (**l**), *vide infra*. The multiplet proton resonating at 5.12 ppm was coupled to both 4-H_2 (δ 1.58 (2H, m)) and 2-H_2 (δ 2.54 and 2.35). The 2-H_2 methylene protons were ^1H-^{13}C long-range coupled to C-1 (δ 172.7). The 3-hydroxypalmitoyl moiety (3OHPal) (**m**) was finally confirmed by the isolation of 3-OHPal from the acid hydrolysate (*vide infra*).

The substructures **a**~**m** accounted for all atoms present in **1**. Acetylation of **1** with Ac_2O-Py gave a nonaacetate (FAB-MS *m/z* 1911 $(M+H)^+$). The number of acetyl groups introduced was compatible with the substructures. The molecular formula and the ^{13}C NMR data (see Table 1) indicated the presence of 22 exchangeable protons which was satisfied by the subunits. Twenty-one degrees of unsaturation required by the molecular formula could be accounted for with the fragments **a**~**m** and one lactone ring.

The peptide sequence and position of attachment of 3-OHPal chain was unambiguously established by a combination of ^1H-^{13}C long-range HMBC and NOESY spectra as depicted in Fig. 3. The HMBC sequence analysis revealed -Thr[1]-Ala[2]-Tyr[3]-Val[4]- and -4OHPro[5]-Thr[6]-Thr[7]-3OHPro[8]-3OHGln[9]-Gly[10]-Thr[11]-Orn[12]-. There were no HMBC cross-peaks between Val[4] and 4OHPro[5]. Linkage of Val[4] to 4OHPro[5] was obtained by NOESY cross-peaks between 2-H in Val[4] and 5-H_2 in 4OHPro[5]. With the exception of the connection of 4OHPro[5] and Thr[6], the peptide sequence was further supported by the NOESY data (Fig. 3). Linking 3OHPal to Thr[1] *via* an amide bond was evident from the HMBC correlation from NH in Thr[1]

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of FR901469.

	Position	δ_H (CD_3OH)	δ_C (CD_3OH)		Position	δ_H (CD_3OH)	δ_C (CD_3OH)
[1]Thr	1		173.8	[8]3-OH Pro	1		172.5
	2	4.40	59.9		2	4.87	68.7
	3	3.97	70.1		3	4.71	72.0
	4	1.34	21.2		4	2.31, 2.10	34.6
	NH	7.81			5	3.88, 3.84	46.3
[2]Ala	1		174.5	[9]3-OH Gln	1		173.3
	2	4.33	50.9		2	4.69	58.2
	3	1.34	17.1		3	4.70	70.0
	NH	8.87			4	2.46, 2.16	39.9
					5		176.1
[3]Tyr	1		172.6		NH	7.97	
	2	4.20	57.2				
	3	2.94, 2.77	37.5	[10]Gly	1		171.1
	4		128.4		2	4.17, 3.78	43.1
	5, 9	7.06	131.3		NH	8.43	
	6, 8	6.96	117.9				
	7		156.1	[11]Thr	1		172.0
	NH	8.05			2	4.22	60.9
					3	4.20	68.3
[4]Val	1		171.7		4	1.37	20.9
	2	4.14	57.5		NH	7.65	
	3	1.88	31.5				
	4	0.80	18.7	[12]Orn	1		171.6
	5	0.79	19.1		2	4.62	51.6
	NH	7.15			3	1.96, 1.53	28.0
					4	1.74, 1.52	25.0
[5]4-OH Pro	1		173.9		5	2.96	40.6
	2	4.28	61.3		2-NH	8.45	
	3	2.17, 1.98	38.8				
	4	4.47	70.5	3-oxyPal	1		172.7
	5	3.68, 3.56	57.8		2	2.54, 2.35	42.8
					3	5.12	74.0
[6]Thr	1		172.0		4	1.58	35.7
	2	4.44	60.4		5	1.30	25.7
	3	4.31	67.7		6 - 13	1.31-1.24	30.2-30.7
	4	1.29	18.6		14	1.25	32.9
	NH	8.17			15	1.30	23.6
					16	0.88	14.3
[7]Thr	1		172.3				
	2	5.10	56.7				
	3	4.21	69.1				
	4	1.12	20.2				
	NH	8.02					

to C-1 in 3OHPal and an NOE between NH in Thr[1] and 2-H_2 in 3OHPal. HMBC correlation between the 3-H in 3OHPal and the carbonyl of Orn formed the 40-membered macrocyclic lactone. The entire peptide sequence was confirmed by FAB MS/MS fragmentation analysis of a linear peptide (**2**) to be shown in Fig. 4. The complete ^{1}H and ^{13}C NMR signal assignments were listed in Table 1.

Fig. 2. Subunits for FR901469 (**1**).

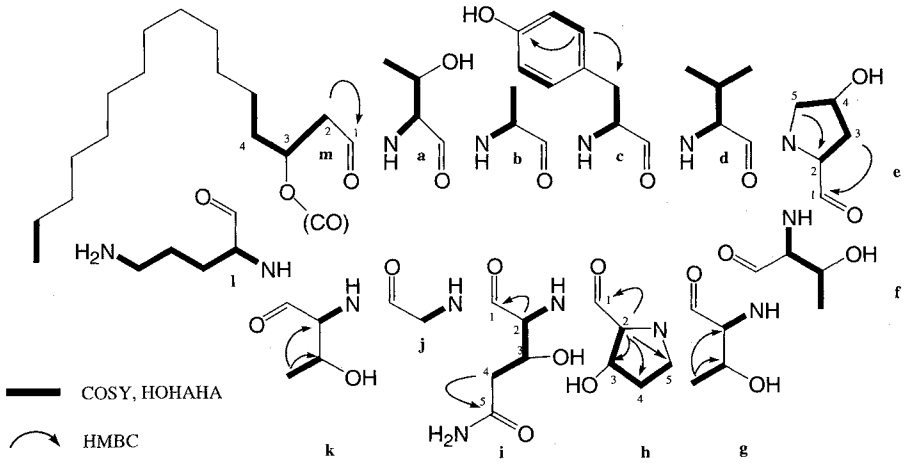


Fig. 3. Peptide sequence of **1** deduced from HMBC and NOESY.

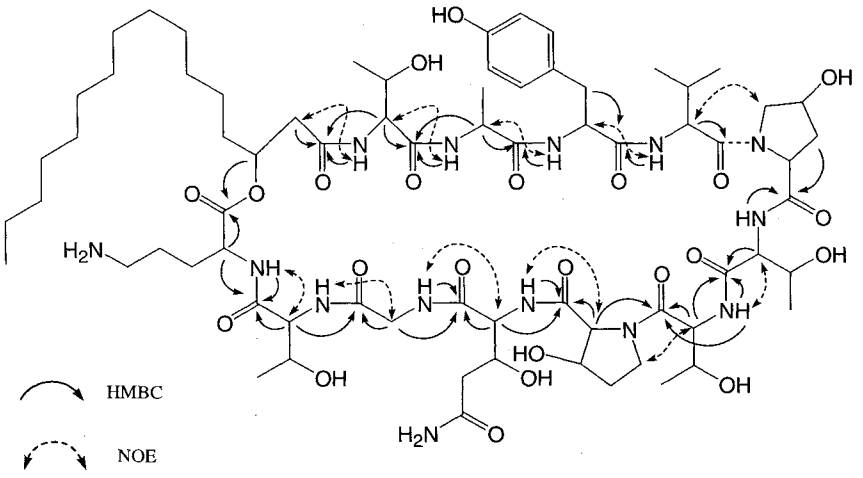
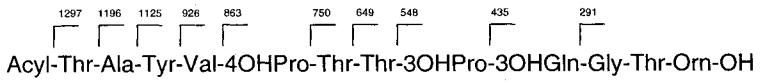


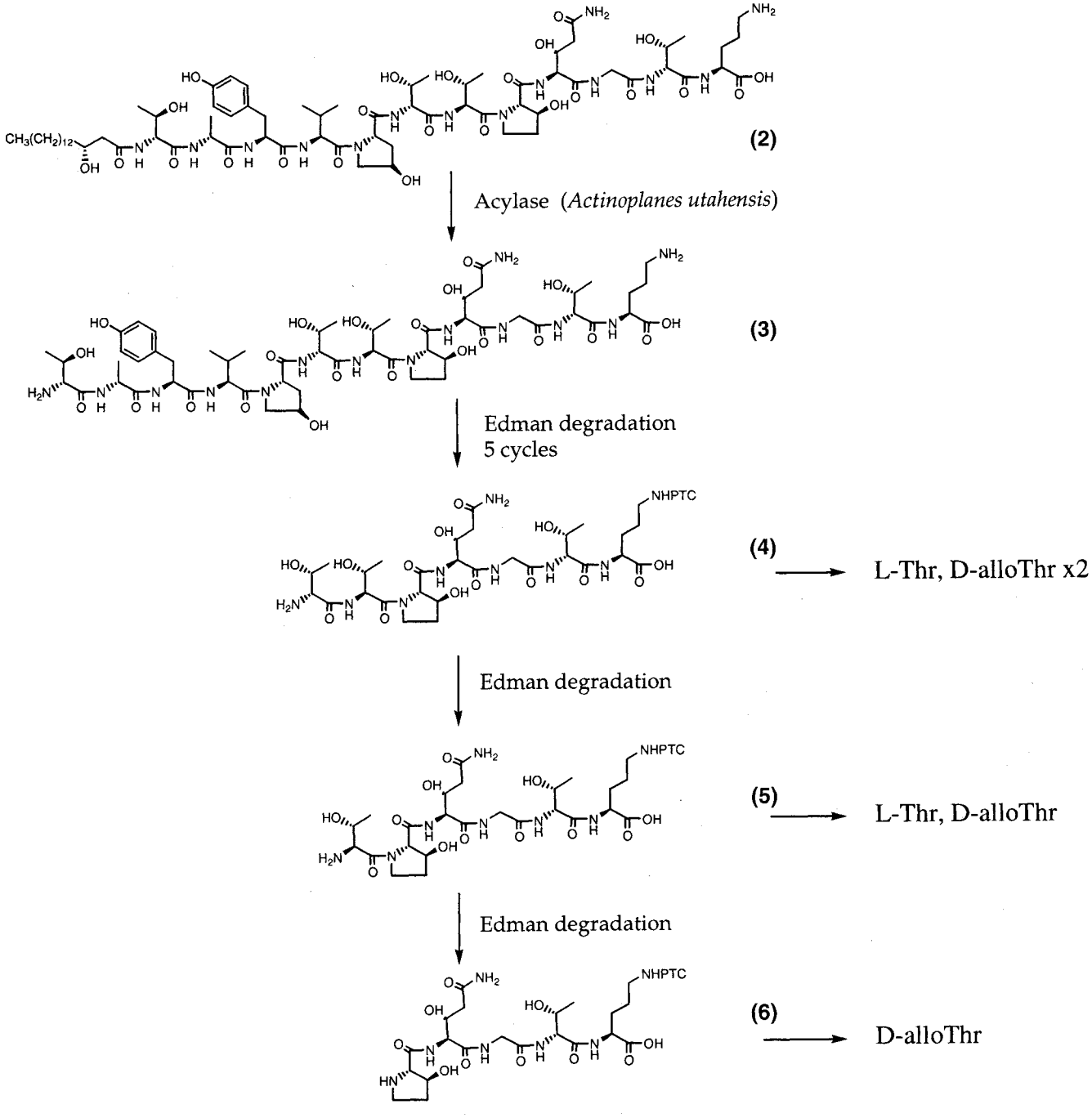
Fig. 4. FAB-MS fragmentation of **2**.



Absolute Stereochemistry

In order to determine the stereochemistry of the standard amino acids, the acid hydrolysates were derivatized with 15% HCl/*n*-BuOH followed by treatment with trifluoroacetic anhydride to give *N,O*-trifluoroacetyl *n*-butyl esters. Chiral column GC-MS analysis[3] of the derivatives allowed us to assign the L configuration for the Val, Orn, Tyr and Thr residues and D configuration for Ala and the other three *allo*Thr residues.

Fig. 5. Degradation products of **2**.



Three unusual amino acids, namely 3-hydroxyproline (3OH-Pro), 4-hydroxyproline (4OH-Pro) and 3-hydroxyglutamic acid (3OH-Glu), were isolated from the acid hydrolysate of compound **2**. Comparison of optical rotation data with literature values allowed assignment of 3OH-Glc as (2*S*,3*R*)-3-hydroxyglutamic acid[4] and 3OHPro as (2*S*,3*S*)-3-hydroxyproline,[5] respectively. The 4OH-Pro was directly identified by comparison to an authentic specimen of (2*S*,4*R*)-4-hydroxyproline. The stereochemistry of 3-hydroxypalmitic acid was found to be (*R*) by comparison of the optical rotation with the reported value.[6]

The only remaining structural problems, namely the positions of L-Thr and three residues of D-*allo*Thr, were solved by chemical methods coupled with enzyme digestion in the following manner (Fig. 5). Enzyme

digestion (*Actinoplanes utahensis*) of **2** gave deacylated dodecapeptide **3**. Edman degradation of **3** produced peptides which had removed one amino acid from *N*-terminus at each cycle. After the 5th, 6th and 7th cycles of the Edman degradation, a portion of each residue was purified by HPLC to give heptapeptide (**4**), hexapeptide (**5**) and pentapeptide (**6**). The stereochemistry of Thr contained in each peptide were determined by chiral GC-MS in the same manner. Compound **4** contained one L-Thr and two D-*allo*Thr. Compound **5** contained one L-Thr and D-*allo*Thr of each. And compound **6** contained only D-*allo*Thr. These results established that in the cyclic peptide the Thr^7 was L-Thr, and hence the other three threonines should be D-*allo*Thr.

Experimental

General Procedures

Melting points were taken using a micromelting point apparatus and are uncorrected. Infrared spectra were recorded on a JASCO 16PC FT-IR spectrophotometer. Ultraviolet spectra were measured on a 220A spectrophotometer. Optical rotations were determined with a JASCO DIP-140 polarimeter. Amino acid analyses were run on a Hitachi 835 automatic amino acid analyzer under conditions for standard amino acids in which Thr and *allo*Thr were not separated. ^1H and ^{13}C NMR spectra were measured on Bruker AM400WB and drx500 NMR spectrometers. All 2D NMR spectra were measured on a drx500 NMR spectrometer. Mass spectra were measured on a VG ZAB-SE mass spectrometer. DIAION HP20 resin was purchased from MITSUBISHI CHEMICAL CORPORATION. CD_3OH was purchased from CEA euriso-top. (2*S*,4*R*)-4-Hydroxyproline was purchased from Fluka.

Acid Hydrolysis of FR901469 (1)

For amino acid analysis, 2 mg of **1** was dissolved in 0.8 ml of 6 N HCl in an evacuated glass tube and heated at 110°C for 16 hours. After evaporation, the residue was dissolved in 0.1 N HCl and subjected to amino acid analysis. Retention times in the amino acid analysis (minute): 3OH-Pro (14.04), 3OH-Glu (15.57), 4OH-Pro (16.66), Thr (21.20), Gly (35.24), Ala (40.86), Val (44.17), Tyr (58.94), Orn (67.74) and NH_3. The molar ratio of Thr was found to be four times than that of the other amino acids.

Chiral Column GC-MS Analysis

Chiral column GC-MS was carried out by using a Chirasil-L-Val capillary column (0.25 mm×25 m) programmed from 80° to 210° at 3°C/minute and ZAB-SE mass spectrometer operating in the positive EI mode (scan range between *m/z* 30 and 600 with repetition time of 1.5 seconds). Derivatization of amino acid residues was done as follows. Acid hydrolysate of **1** was heated in 10% HCl in *n*-BuOH (0.25 ml) at 100°C for 20 minutes in a screw-capped test tube. After removal of the *n*-butanolic HCl *in vacuo*, CH_2Cl_2 (0.3 ml) and trifluoroacetic anhydride (0.2 ml) were added, and the mixture was kept at 100°C for 5 minutes. The mixture was evaporated, dissolved in EtOAc, and subjected to the analysis. Retention time (minute): D-Ala (6:49), L-Val (9:29), L-Thr (10:15), Gly (10:28), D-*allo*Thr (13:28), 3OH-Pro (14:28), 4OH-Pro (17:59), 3OH-Glu (27:55), L-Tyr (32:07), L-Orn (37:03), respectively.

Linear Peptide (2)

Compound **1** (500 mg) was dissolved in aq 1 N NaOH solution (1.0 ml) and the reaction mixture was stirred at room temperature for 2 hour. The mixture was neutralized with 1 N HCl and then applied to a column of HP-20SS (50 ml). After washed with H_2O (150 ml), the column was eluted with MeOH. The product fractions were collected and evaporated to dryness under reduced pressure. The residue was triturated with diethyl ether to give linear peptide **2** as a colorless powder (470 mg).; FABMS *m/z* 1551 $(M+H)^+$.

Separation of Each Component

450 mg of **2** was dissolved in 2 ml of 6 N HCl in an evacuated glass tube and heated at 110°C for 16 hours. After evaporation, the residue was dissolved in water and extracted with EtOAc. The organic layer was evaporated and the residue was purified by preparative TLC to give 3(*R*)-hydroxypalmitic acid (19 mg). mp 78~80°C; $[\alpha]_D^{20}$ −13.2° (*c* 0.50, $CHCl_3$) (lit.[6] $[\alpha]_D^{20}$ −13.8° (*c* 1, $CHCl_3$)); FABMS *m/z* 273 $(M+H)^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, J=7 Hz), 1.20~1.60 (24H, m), 2.48 (1H, dd, J=9, 16 Hz), 2.57 (1H, dd, J=3, 16 Hz), 4.03 (1H, m).

Each amino acid contained in the aquous layer was separated by silica gel column chromatography after protecting the *N* terminus with a Boc group and the *C* terminus with a methy ester. The *N*-Boc amino acid methyl esters separated were hydrolysed with NaOH and the Boc group was removed with TFA to give the corresponding deprotected amino acid.

(2*S*,3*R*)-3-Hydroxygultamic acid (14.7 mg): ^1H NMR

(400 MHz, D_2O) δ 2.75 (1H, dd, J=8.0, 16.5 Hz), 2.89 (1H, dd, J=4.5, 16.5 Hz), 4.18 (1H, d, J=4.5 Hz), 4.65 (1H, m), $[\alpha]_D^{21}$ +3.8° (*c* 1.0, H_2O) (lit.[4] $[\alpha]_D^{20}$ +3.3° (*c* 1.5, H_2O)).

(2*S*,3*S*)-3-Hydroxyproline (14.2 mg): ^1H NMR (400 MHz, D_2O) δ 2.05~2.23 (2H, m), 3.51~3.63 (2H, m), 4.32 (1H, br d, J=2.5 Hz), 4.77 (1H, m). $[\alpha]_D^{21}$ =−14.6° (*c* 0.35, H_2O), +16.0° (*c* 0.36, 1 N HCl) (lit.[5] $[\alpha]_D^{20}$ −15.3° (*c* 1, H_2O), +17.4° (*c* 0.5, 1 N HCl)).

(2*S*,4*R*)-4-Hydroxyproline (15.6 mg): The sample obtained was directly identified with authentic specimen. ^1H NMR (400 MHz, D_2O) δ 2.30 (1H, ddd, J=4, 10, 15 Hz), 2.51 (1H, ddd, J=2, 8, 15 Hz), 3.42 (1H, dd, J=1.5, 13 Hz), 3.54 (1H, dd, J=4, 13 Hz), 4.61 (1H, dd, J=8, 10 Hz), 4.71 (1H, m).

Enzyme Digestion of **2**

To a solution of **2** (50 mg) in 0.3 M Na_2PO_4 buffer (pH 5.8) (50 ml) was added *Actinoplanes utahensis* (5 g wet) and the solution was incubated for 5 days at 37°C. After filtration, the filtrate was charged on a column of HP20SS (30 ml) washed with water, then eluted with MeOH. The product was purified by RP-HPLC. RP-HPLC was performed with a C_{18} HPLC column and eluted with CH_3CN-0.1% aqueous TFA using a linear gradient from 10 to 90% over 30 minutes to give deacylated dodecapeptide (**3**) as a white powder (30 mg). FABMS *m/z* 1297.6 $(M+H)^+$. ^1H NMR (400 MHz, D_2O) δ 0.93 (3H, d, J=7 Hz), 0.95 (3H, d, J=7 Hz), 1.20~1.30 (15H, m), 1.68~2.17 (7H, m), 2.24~2.63 (4H, m), 2.90~3.08 (4H, m), 3.81~3.95 (4H, m), 4.00~4.74 (20H, m), 6.86 (2H, d, J=8 Hz), 7.10 (2H, d, J=8 Hz).

Edman Degradation of **3**

To a solution of **3** (30 mg) in 50% aq pyridine (1 ml) was added PITC (50 μl) and the mixture was heated for 20 minutes at 55°C . After rinsing the solution with benzene (1 ml, three times), the aq layer was dried under a N_2 stream. The residue was dissolved in TFA (200 μl) and the solution was heated for 5 minutes at 55°C. After removal of the TFA under a N_2 stream, the residue was dissolved in water (200 μl) and extracted with benzene (1 ml, twice). The aq layer was dried under a N_2 stream and used for the next step. After each 5th, 6th and 7th cycle of the Edman degradation, a portion of the products were purified by RP-HPLC to afford heptapeptide (**4**), hexapeptide (**5**) and pentapeptide (**6**).

Heptapeptide (**4**): FABMS *m/z* 885 $(M+H)^+$, ^1H NMR (400 MHz, CD_3OD) δ 1.22 (3H,d, J=6.5 Hz), 1.24 (3H, d, J=6.5 Hz), 1.25 (3H, d, J=7 Hz), 1.64~2.06 (5H, m), 2.27 (1H, m), 2.47 (2H, m), 3.59 (2H, m), 3.89 (1H, d, J=17 Hz), 4.00 (1H, d, J=17 Hz), 3.86~4.10 (4H, m), 4.20 (1H, m), 4.25 (1H, m), 4.34 (1H, d, J=3 Hz), 4.39 (1H, d, J=6 Hz), 4.36~4.61 (5H, m), 7.21 (1H, m), 7.32 (2H, m), 7.37 (2H, m). Chiral GC-MS Retention time (minute): L-Thr (10 : 22), Gly (10 : 29), D-*allo*Thr (13 : 36)×2, 3OH-Pro (14 : 26), 3OH-Glu (28 : 00), L-Orn (37 : 22), respectively.

Hexapeptide (**5**): FABMS *m/z* 784 $(M+H)^+$, ^1H NMR (400 MHz, CD_3OD) δ 1.21 (3H, d, J=6.5 Hz), 1.36 (3H, d, J=6 Hz), 1.64~1.85 (3H, m), 1.88~2.06 (2H, m), 2.28 (1H, m), 2.49 (2H, m), 3.59 (2H, m), 3.85 (2H, br d, J=7 Hz), 3.89 (1H, d, J=17 Hz), 4.01 (1H, d, J=17 Hz), 4.03~4.16 (3H, m), 4.38~4.55 (6H, m), 7.20 (1H, m), 7.31 (2H, m), 7.37 (2H, m). Chiral GC-MS Retention time (minute): L-Thr (10 : 29), Gly (10 : 38), D-*allo*Thr (13 : 37), 3OH-Pro (14 : 33), 3OH-Glu (28 : 06), L-Orn (37 : 22), respectively.

Pentapeptide (**6**): FABMS *m/z* 683 $(M+H)^+$, ^1H NMR (400 MHz, CD_3OD) δ 1.22 (3H, d, J=6 Hz), 1.65~2.15 (6H, m), 2.48 (2H, d, J=6.5 Hz), 3.45~3.63 (4H, m), 3.88 (1H, d, J=17 Hz), 4.04 (1H, d, J=17 Hz), 4.06 (1H, m), 4.28 (1H, m), 4.38~4.47 (3H, m), 4.50 (1H, br d, J=4 Hz), 4.67 (1H, m), 7.21 (1H, t, J=8 Hz), 7.31 (2H, br d, J=8 Hz), 7.37 (2H, br t, J=8 Hz). Chiral GC-MS Retention time (minute): Gly (10 : 33), D-*allo*Thr (13 : 36), 3OH-Pro (14 : 44), 3OH-Glu (28 : 04), L-Orn (37 : 15), respectively.

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