Structural Determination of Stevastelins, Novel Depsipeptides from *Penicillium* sp.

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Structures of novel immunosuppressants, stevastelin A, B and B3[1] were determined by their spectroscopic and chemical studies. Three stevastelins were shown to be cyclic depsipeptides composed of a fatty acid and three amino acid moieties. The sequence of these moieties was determined to be as 3,5-dihydroxy-2,4-dimethylstearylvalylthreonyl (or *O*-sulfonylthreonyl in stevastelin A)-*O*-acetylserine. Cyclic structures were shown to be formed by ester linkages between the carboxylic group of the *O*-acetylserine moiety and the 5-hydroxy group of the fatty acid moiety in stevastelin A and B, and the 3-hydroxy group of the fatty acid moiety in stevastelin B3.

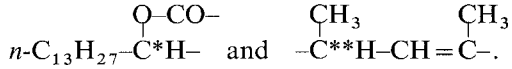
The stevastelins are novel immunosuppressants produced by a *Penicillium* sp. NK374186[1]. The isolation, and preliminary physico-chemical and biological properties of the stevastelins were previously reported[1]. In this paper we describe the determination of the novel depsipeptide structures of stevastelin A (**1**), B (**2**) and B3 (**3**) in detail.

Physico-chemical Properties of **1**, **2** and **3**

The NMR and MS spectroscopic data are summarized in Table 1. These data of the three stevastelins are very similar, implying that they are related compounds. Therefore, the studies for the basic structure of stevastelin was performed on one compound, **2**. The molecular formula of **2** was confirmed as $C_{34}H_{61}N_3O_9$ (655.85) from HRFAB-MS data and ^{13}C NMR spectrum data (Table 1). Since **2** gave a positive color in Rydon-Smith's reaction, and showed signals due to five carbonyl, seven methyl, thirteen aliphatic methylene and nine aliphatic methine carbons in its ^{13}C NMR spectrum, **2** was deduced to be a peptide related compound.

Acid Hydrolysis of **2**

Amino acid analysis of the acid hydrolysate of **2** revealed the presence of one mole each of serine, threonine and valine (Fig. 1). From the ethereal extract of the acid hydrolysate **4** was furnished (Fig. 1). The molecular formula of **4** was determined from HRFAB-MS data to be $C_{20}H_{36}O_2$. ^1H and ^{13}C NMR analysis including 2D analysis showed that **4** had two partial structures:



The presence of an ester bond was supported by the chemical shift of carbon with asterisk (δ_C* 80.28) and IR absorption of **4** (1735 cm^{-1}). The connectivity of these two partial structures were elucidated by proton coupling between C*H (δ_H 4.29) and C**H(δ_H 2.24) detected in an ^1H-^1H COSY analysis. From these results, the structure of **4** was deduced to be as shown in Fig. 1.

Thus, the four degradation products of serine, threonine, valine and **4** were obtained from the acid hydrolysate of **2**.

Alkaline Hydrolysis and Lithium Borohydride Reduction of **2**

Since **2** gave no coloration to ninhydrin and was not susceptible to carboxylpeptidase Y (Oriental Yeast) digestion, we speculated that **2** had a cyclic structure. In order to confirm this cyclic structure, degradations were carried out by alkaline hydrolysis and lithium borohydride ($LiBH_4$) reduction.

Alkaline hydrolysis gave ether extractable **5** as a minor product following acidification (Fig. 1). The molecular formula of **5** was determined to be $C_{20}H_{40}O_4$ by HRFAB-MS data detected at *m/z* 345.5246 $(M+H)^+$. Treatment of **5** with diazomethane furnished a compound which gave a protonated molecular ion at *m/z* 359 $(M+H)^+$, indicating the presence of a carboxylic group in **5**. ^1H NMR and ^1H-^1H COSY NMR analysis showed a partial structure of **5**:

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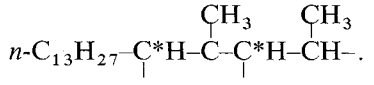


The two carbons with asterisks (C*) were considered to be substituted with hydroxyl groups, since the chemical shifts of these carbons were detected at δ_C 73.01 and 78.13. From these results, the structure of **5** was deduced to be 3,5-dihydroxy-2,4-dimethylstearic acid as shown Fig. 1.

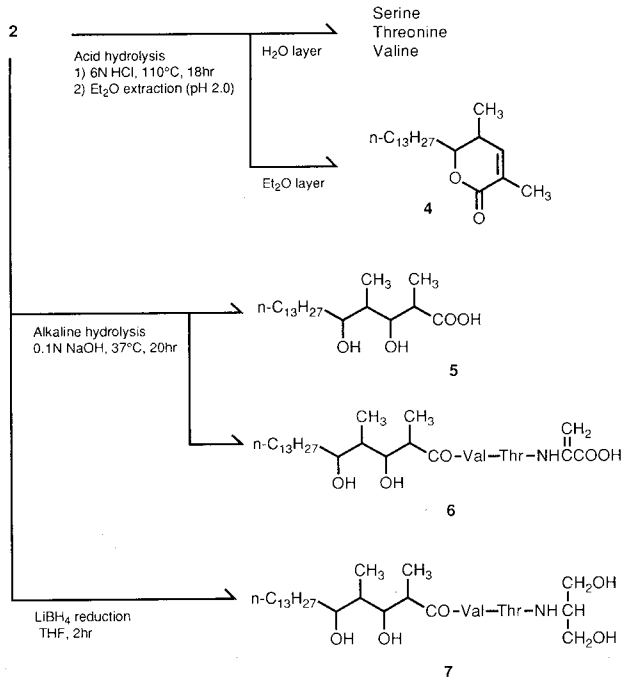
Alkaline hydrolysis of **2** gave **6** as a major product, and $LiBH_4$ reduction gave **7** (Fig. 1). The molecular formulas and weights of **6** and **7** were determined by ^{13}C NMR and HRFAB-MS as $C_{32}H_{59}N_3O_8$ (613.81) and $C_{32}H_{63}N_3O_8$ (617.85), respectively. Assignments of the NMR spectroscopic data of **6** and **7** were carried out with reference to the degradation products obtained. Data showed that **6** and **7** contain 3,5-dihydroxy-2,4-dimethylstearic acid moiety. And also, in the ^{13}C NMR spectra of **6** and **7**, the signals due to valine moiety were detected at δ_C 19.23 (q), 19.40 (q), 30.18 (d), 58.70 (d),

Table 1. Physico-chemical properties of stevastelins.

NMR data[a] Carbon No.[b]	A (**1**)		B (**2**)		B3 (**3**)	
	C ppm	H ppm m H (J=(Hz))	C ppm	H ppm m H (J=(Hz))	C ppm	H ppm m H (J=(Hz))
[Fatty acid]						
F1	174.89 s	—	174.77 s	—	170.43 s	—
F2	46.28 d	2.17 m	46.31 d	2.19 m	41.01 d	2.90 m
F3	74.24 d	3.58 m	75.29 d	3.62 br	80.13 d	4.91 m
		–OH, 5.09 d (5.5)		–OH, 5.53 d (5.5)		
F4	38.74 d	1.76 m	40.04 d	1.73 m	38.78 d	1.73 m
F5	78.43 d	4.87 m	78.73 d	4.92 m	68.85 d	3.84 br
						–OH, 4.37 d (5.8)
F6	31.21 t	1.42 m	31.59 t	1.43 m	34.79 t	1.20 m
		1.57 m		1.55 m		1.34 m
F7	25.41 t	1.23 2H	25.32 t	1.23 2H	25.62 t	1.24 2H
F8	28.6	1.23 16H	28.7	1.23 16H	28.6	1.24 16H
~15	~29.0 t ×8		~29.0 t ×8		~29.0 t ×8	
F16	31.21 t	1.23 2H	31.22 t	1.23 2H	31.21 t	1.24 2H
F17	22.01 t	1.23 2H	22.01 t	1.23 2H	22.01 t	1.24 2H
F18	13.86 q	0.85 t 3H (6.9)	13.86 q	0.85 t 3H (6.6)	13.86 q	0.86 t 3H (6.8)
F19	15.70 q	1.13 d 3H (6.6)	16.42 q	1.14 d 3H (7.3)	13.72 q	1.12 d 3H (7.3)
F20	6.85 q	0.72 d 3H (7.0)	6.50 q	0.74 d 3H (7.0)	9.11 q	0.54 d 3H (6.6)
[Valine]						
V1	170.89 s	—	171.28 s	—	170.76 s	—
V2	61.14 d	3.96 m	61.11 d	4.00 dd (9.9, 9.9)	61.51 d	4.03 m
		–NH, 7.89 d (10.0)		–NH, 7.93 d (9.9)		–NH, 7.67 br
V3	29.61 d	2.07 m	29.74 d	2.10 m	28.63 d	2.05 m
V4a	18.97 q	0.83 d 3H (6.6)	18.94 q	0.83 d 3H (7.0)	18.87 q	0.90 d 3H (6.6)
V4b	19.40 q	0.89 d 3H (6.6)	19.32 q	0.89 d 3H (6.6)	19.11 q	0.94 d 3H (6.6)
[Threonine]						
T1	169.31 s	—	170.31 s	—	170.58 s	—
T2	56.98 d	4.36 m	57.64 d	4.28 dd (2.6, 9.5)	59.30 d	4.16 m
		–NH, 8.20 d (9.5)		–NH, 8.33 d (9.5)		–NH, 7.72 d (9.9)
T3	72.21 d	4.56 m	66.73 d	4.19 m	65.13 d	4.03 m
				–OH, 4.90 d (4.8)		–OH, 5.24 d (4.4)
T4	17.97 q	1.11 d 3H (7.3)	20.42 q	1.00 d 3H (6.2)	20.84 q	1.08 d 3H (6.2)
[*O*-acetylserine]						
S1	169.17 s	—	169.31 s	—	168.72 s	—
S2	49.89 d	4.67 m	49.83 d	4.73 m	50.06 d	4.91 m
		–NH, 7.94 d (8.4)		–NH, 7.81 d (8.1)		–NH, 7.49 d (8.8)
S3	61.99 t	3.96 m	62.31 t	3.94 m	63.15 t	4.20 m
		4.38 m		4.40 m		4.35 m
S4	169.99 s	—	170.05 s	—	170.04 s	—
S5	20.51 q	2.00 s 3H	20.45 q	1.98 s 3H	20.55 q	2.01 s 3H
HRFAB-MS						
Found	780.3708 $(M+2Na-H)^+$		678.4290 $(M+Na)^+$		678.4319 $(M+Na)^+$	
Calcd	780.3693		678.4306		678.4306	
mol formula	$C_{34}H_{61}N_3O_{12}S$		$C_{34}H_{61}N_3O_9$		$C_{34}H_{61}N_3O_9$	
mol weight	735.91		655.85		655.85	

[a] Recorded in DMSO-d_6. [b] Refered to Fig. 2.

Fig. 1. Degradation studies of stevastelin B (**2**).



Degradations were carried out as described in the Experimental section. Abbreviations: Val and Thr of **6** and **7** mean valine and threonine moieties, respectively. The sequence of these moieties was determined by ^{1}H-^{13}C long range coupling analysis in the same manner as described for **2** (Fig. 2).

171.49 (s) and at δ_C 18.11 (q), 19.11 (q), 30.05 (d), 57.88 (d), 179.08 (s), respectively. Similarly, the signals of **6** and **7** due to threonine moiety were detected at δ_C 17.81 (q), 57.34 (d), 65.34 (d), 168.35 (s) and at δ_C 17.62 (q), 57.34 (d), 72.13 (d), 168.69 (s), respectively. Furthermore, ^{1}H-^{13}C long range coupling analysis showed the sequence of these moieties as shown in Fig. 1. Therefore, another signals of **6** detected at δ_C 101.50 (t), 136.66 (s), 165.15 (s) and those of **7** detected at δ_C 53.10 (d), 59.88 (t, $\times 2$), were considered to be due to a terminal moiety and were analysed.

In the ^{1}H NMR spectrum of **6**, two multiplet signals (δ_H 5.50 (1H), 6.05 (1H)), attributed to olefinic protons, were detected. This was supported by the ^{13}C NMR signals detected at δ_C 136.66 (s) and 101.50 (t). And also, a singlet amide proton (δ_H 9.27 (s)) and the carbonyl carbon (δ_C 165.15 (s)) were detected. From these results, a terminal moiety of **6** was deduced to be the dehydroalanine, $-NH-\overset{\overset{CH_2}{\|}}{C}-COOH$ (Fig. 1).

Compound **7** also gave signals due to a terminal moiety. The methine carbon (δ_C 53.10) coupled to a proton (δ_H 3.67) in its ^{1}H-^{13}C COSY analysis. This methine proton coupled to four protons of two methylenes (δ_H 3.42 (4H)) and an amide proton (δ_H 7.78 (d, $J=9.5$ Hz)) in ^{1}H-^{1}H COSY analysis. These couplings showed a partial structure: $-NH-CH(CH_2-)_2$. Chemical shifts of these two methylene carbons at δ_C 59.88 (t, $\times 2$) indicated the presence of two hydroxymethyl groups. Thus, a terminal moiety of **7** was deduced to be 2-amino-1,3-propanediol, $-NH-CH(CH_2OH)_2$ (Fig. 1).

The degradation studies of **2** are summarized in Fig. 1. These results showed that **2** contains a 3,5-dihydroxy-2,4-dimethylstearic acid moiety. Compound **4** obtained by the acid hydrolysis of **2** was considered to be generated by β-elimination and lactonization of this fatty acid. The structures of the degradation products, **6** and **7**, showed the presence of valine and threonine moieties, which were transformed to amino acids by acid hydrolysis. Furthermore, it was suggested that **2** had another moiety which were transformed to serine by acid hydrolysis, to a dehydroalanine moiety by alkaline hydrolysis and to 2-amino-1,3-propanediol moiety by $LiBH_4$ reduction, respectively.

NMR Analysis of 2

NMR studies (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, ^{1}H-^{13}C long range coupling and HMBC analysis) were carried out on **2**. Assignments shown in Table 1 revealed the presence of 3,5-dihydroxy-2,4-dimethylstearic acid, valine and threonine moieties. Data also showed presence of serine and an acetyl group (Table 1, S1, 2, 3 and S4, 5). In HMBC analysis, signals between S3 protons and S4 carbon was detected (Fig. 2). These data clearly showed the presence of an *O*-acetylserine moiety. It is reasonable that the *O*-acetylserine moiety was transformed to serine, the dehydroalanine moiety and the 2-amino-1,3-propanediol moiety by the corresponding degradation reactions (Fig. 1).

The sequence of the four moieties was elucidated by ^{1}H-^{13}C long range coupling analysis. Each amide proton of valine, threonine and *O*-acetylserine coupled to each carbonyl carbon of the fatty acid, valine and threonine, respectively (Fig. 2). Therefore, the sequence was concluded to be as 3,5-dihydroxy-2,4-dimethylstearylvalyl-threonyl *O*-acetylserine (Fig. 2).

A cyclic structure for **2** was supported by the ^{1}H-^{13}C correlation map obtained by the HMBC experiment. In the map, F5 proton of **2** coupled to S1 carbon as shown in Fig. 2. Furthermore in ^{1}H NMR analysis of **2**, there was no signal of F5 hydroxy group (Table 1), which was detected in **5**, **6** and **7** (Fig. 1). This result suggested that

Fig. 2. NMR studies of stevastelin B (2).

Typical correlations were shown by arrows. Data were recorded in DMSO-d_6.

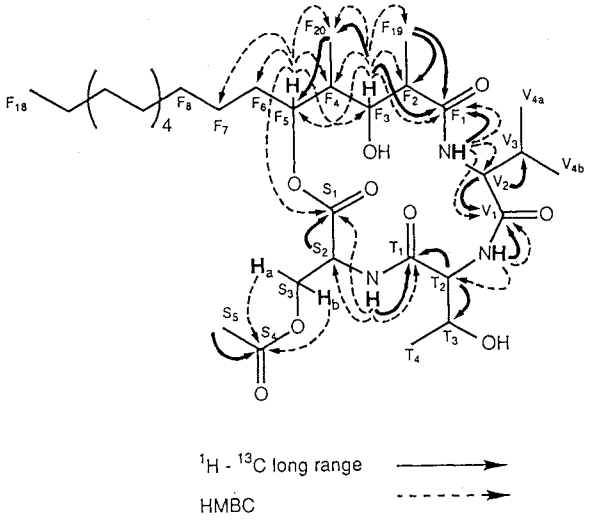
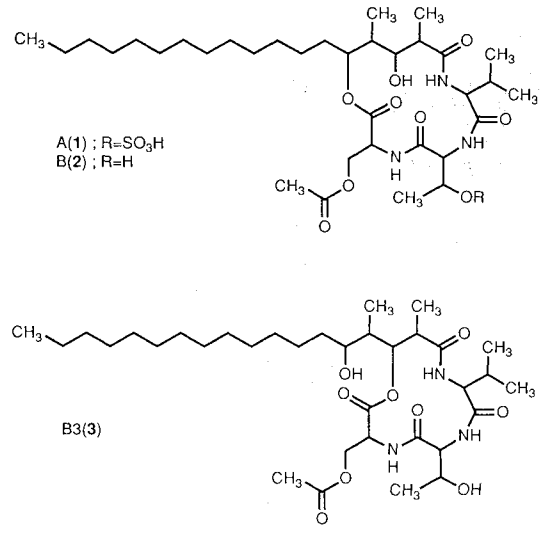


Fig. 3. Structures of stevastelin A (1), B (2) and B3 (3).



the F5 hydroxy group was used for ester linkage formation. Based on these results, an ester linkage was deduced to be formed between a carboxylic group of the *O*-acetylserine moiety and the F5 hydroxy group of 3,5-dihydroxy-2,4-dimethylstearic acid moiety (Fig. 2). Compounds **6** and **7** are considered to be generated by breakage of this ester linkage in alkaline hydrolysis and $LiBH_4$ reduction, respectively (Fig. 1). Thus, a cyclic depsipeptide structure of **2** was determined as shown in Fig. 3.

Spectroscopic Analysis of 1 and 3

The NMR data and molecular formulas of **1** and **3** are shown in Table 1. NMR data showed that **1** also contains the fatty acid, valine and *O*-acetylserine moieties as does **2** (Table 1). However, in the ^{1}H NMR spectrum of **1**, the T3 carbon (δ_C 72.21, Table 1) was shifted downfield when compared with that of **2** (δ_C 66.73, Table 1), and a signal for the T3 hydroxy group was not detected (Table 1). These results suggested substitution of T3 hydroxy group of **1**. The molecular formula of **1** ($C_{34}H_{61}N_3O_{12}S$, Table 1) showed that **1** contained one more sulfur and three more oxygen atoms than **2**. Therefore, **1** was concluded to have an *O*-sulfonyl-threonine moiety instead of the threonine moiety of **2** (Fig. 3).

Compound **3** showed quite identical molecular weight and formula with **2** (Table 1). NMR data of **3** was also similar to that of **2** (Table 1). However, differences were observed at the F3 and F5 positions. In ^{13}C NMR analysis, the F3 carbon of **3** (δ_C 80.13, Table 1) was shifted downfield when compared with that of **2** (δ_C 75.29, Table 1). In ^{1}H NMR analysis of **3**, the F3 hydroxyl group was not detected (Table 1), whereas the F5 hydroxyl group was detected (δ_H 4.37) and coupled to the F5 proton (δ_H 3.84) in ^{1}H-^{1}H COSY analysis. These results suggested that the F3 hydroxy group is used for an ester linkage formation. This was finally confirmed by an HMBC experiment in which a signal between the F3 proton (δ_H 4.91) and the S1 carbon (δ_C 168.72) was detected. Based on these results, the cyclic structure of **3** was concluded to be formed by an ester linkage between the F3 hydroxy group of the fatty acid moiety and the carboxylic group of the *O*-acetylserine moiety (Fig. 3).

Thus, the structures of **1** (stevastelin A), **2** (stevastelin B) and **3** (stevastelin B3) were determined to be as shown in Fig. 3. The absolute structural elucidation of **2** by synthetic studies will be reported in a following report[2].

Experimental

MS and NMR

Low-resolution and high resolution FAB-MS spectra were carried out with VG MM-ZAB HF and VG Autospec-Q mass spectrometers. Generally NMR spectra were observed with a JEOL JNM-GX400 spectrometer. The HMBC experiment was carried out with a JEOL JNM-A600.

Acid Hydrolysis

16 mg of **2** was suspended in 2 ml of 6 N HCl solution and treated at 110°C for 18 hours in a sealed tube. The hydrolysate was diluted with 4 ml of water and extracted

twice with 5 ml of Et_2O. The aqueous layer was evaporated to dryness under reduced pressure. The residue was dissolved in 0.1 N HCl solution and examined with an ATTO amino acid analyser, MCL-203. The Et_2O layer was evaporated to dryness and the residue was chromatographed on silica gel (cyclohexan - Me_2CO = 20 : 1) and Sephadex LH-20 (MeOH) to give **4** (4 mg).

Compound **4**: FAB-MS *m/z* 309 $(M+H)^+$, 331 $(M+Na)^+$. HRFAB-MS *m/z* Found 309.2792 $(M+H)^+$, Calcd 309.2794 for $C_{20}H_{37}O_2$. ^1H NMR (400 MHz, $CDCl_3$) δ 0.81 (3H, t, J=6.8 Hz), 0.92 (3H, d, J=7.1 Hz), 1.19 (22H), 1.45 (1H, m), 1.75 (1H, m), 1.84 (3H, s), 2.24 (1H, m), 4.29 (1H, m), 6.58 (1H, dd, J=1.46, 6.31 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.40 (q), 14.14 (q), 17.01 (q), 22.72 (t), 25.27 (t), 29.4~29.7 (t, ×8), 31.46 (t), 31.95 (t), 32.40 (d), 80.28 (d), 127.05 (s), 145.81 (d), 166.40 (s).

Alkaline Hydrolysis

50 mg of **2** was suspended in 6 ml of 0.1 N NaOH solution and treated at 37°C for 20 hours. The hydrolysate was adjusted to pH=2.0 by HCl solution and extracted twice with 6 ml of Et_2O. After evaporation of the Et_2O layer, the residue was chromatographed on a silica gel ($CHCl_3$ - MeOH = 100 : 0, 50 : 1, 20 : 1 and 10 : 1, stepwise), to yield **5** (2 mg) and crude **6** (30 mg). Crude **6** was further chromatographed on Sephadex LH-20 ($CHCl_3$) to afford pure **6** (21 mg) as a colorless powder.

Compound **5**: FAB-MS *m/z* 345 $(M+H)^+$. HRFAB-MS *m/z* Found 345.5246 $(M+H)^+$, Calcd 345.5240 for $C_{20}H_{41}O_4$. ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, J=6.8 Hz), 0.98 (3H, d, J=7.3 Hz), 1.25 (22H), 1.33 (3H, d, J=7.3 Hz), 1.48 (1H, m), 1.72 (1H, m), 2.04 (1H, m), 2.65 (1H, m), 3.92 (1H, s), 4.74 (1H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.08 (q), 12.14 (q), 14.09 (q), 22.66 (t), 25.40 (t), 29.3~29.7 (t, ×8), 31.89 (t), 31.95 (t), 36.48 (d), 36.86 (d), 73.01 (d), 78.13 (d), 173.71 (s).

Compound **6**: FAB-MS *m/z* negative 612 $(M-H)^-$, positive 636 $(M+Na)^+$. HRFAB-MS *m/z* Found 636.4162 $(M+Na)^+$, Calcd 636.41998 for $C_{32}H_{59}N_3O_8$-Na. ^1H NMR (400 MHz, DMSO-d_6) δ 0.83 (3H,d, J=7.0 Hz), 0.83~0.89 (6H, m), 0.90 (3H, d, J=7.0 Hz), 0.98 (3H, d, J=6.3 Hz), 1.03 (3H, d, J=7.0 Hz), 1.24 (22H), 1.34 (2H, br), 1.42 (1H, m), 2.09 (1H, m), 2.59 (1H, m), 3.45 (2H, br), 3.50 (1H, m), 3.58 (1H, m), 4.05 (1H, m), 4.28 (1H, m), 4.32 (1H, m), 4.58 (1H, br), 5.50 (1H, s), 6.05 (1H, s), 7.93 (1H, d, J=9.5 Hz), 7.96 (1H, d, J=9.2 Hz), 9.27 (1H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 7.39 (q), 13.86 (q), 15.13 (q), 17.81 (q), 19.23 (q), 19.40 (q), 21.99 (t), 25.44 (t), 28.6~29.1 (t, ×8), 30.18 (d), 31.19 (t), 34.68 (t), 39.6 (d), 42.70 (d), 57.34 (d), 58.70 (d), 65.34 (d), 72.34 (d), 74.59 (d), 101.50 (t), 136.66 (s), 165.15 (s), 168.35 (s), 171.49 (s), 175.47 (s).

Lithium Borohydoride Reduction

12.6 mg of **2** was dissolved in 1 ml of tetrahydrofuran and 1.0 mg of lithium borohydride was added. The reaction mixture was stirred for 2 hours at room temperature. After removal of solvent, the residue was chromatographed on silica gel ($CHCl_3$ - MeOH = 20 : 1) to yield **7** (5 mg) as a colorless powder.

Compound **7**: FAB-MS *m/z* negative 616 $(M-H)^-$, positive 640 $(M+Na)^+$. HRFAB-MS *m/z* Found 640.4445 $(M+Na)^+$, Calcd 640.4496 for $C_{32}H_{63}N_3O_8$-Na. ^1H NMR (400 MHz, DMSO-d_6) δ 0.82 (3H, d, J=7.0 Hz), 0.83~0.87 (9H, m), 0.95 (3H,d, J=7.2 Hz), 1.12 (3H, d, J=6.4 Hz), 1.24 (22H), 1.34 (2H, br), 1.40 (1H, br), 2.00 (1H, m), 2.56 (1H, m), 3.42 (4H, m), 3.50 (1H, m), 3.58 (1H, m), 3.67 (1H, m), 4.12 (1H, m), 4.21 (1H, m), 4.34 (1H, d, J=6.0 Hz), 4.44 (2H, m), 4.52 (3H, m), 7.78 (1H, d, J=9.5 Hz), 7.87 (1H, d, J=9.3 Hz), 8.32 (1H, d, J=9.2 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ 7.35 (q), 13.89 (q), 14.91 (q), 17.62 (q), 18.11 (q), 19.11 (q), 22.01 (t), 25.43 (t), 28.6~29.2 (t, ×8), 30.04 (d), 31.21 (t), 34.65 (t), 39.66 (d), 42.76 (d), 53.10 (d), 57.35 (d), 57.88 (d), 59.88 (t, ×2), 72.13 (d), 72.59 (d), 74.53 (d), 168.69 (s), 170.08 (s), 175.77 (s).

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