VERUCOPEPTIN†, A NEW ANTITUMOR ANTIBIOTIC ACTIVE AGAINST B16 MELANOMA

II. STRUCTURE DETERMINATION

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The structure of a new antibiotic verucopeptin has been determined by the spectroscopic analyses and chemical degradation studies. It is a 19-membered cyclodepsipeptide which is structurally related to azinothricin and A83586C.

Actinomadura verrucosospora Q886-2 produced a new antitumor antibiotic verucopeptin[1] which showed specific *in vivo* activity against B16 melanoma. The producing organism, fermentation isolation, physico-chemical and biological properties of the antibiotic were reported in the previous paper[2]. This paper describes the structure elucidation of verucopeptin.

General Characteristics

Verucopeptin (**1**) was isolated as a white amorphous powder. The molecular formula of **1** was determined to be $C_{43}H_{73}N_7O_{13}$ on the basis of elemental analysis and HRFAB-MS data (m/z 894.5217 $(M-H)^-$). The IR spectrum of **1** showed a strong absorption at 1640 cm^{-1} suggesting a peptidic structure for the antibiotic. An absorption at 1750 cm^{-1} which disappeared upon treatment of 0.1 N sodium hydroxide suggested the presence of an ester (or a lactone) in the molecule. Acid hydrolysis of **1** with 6 N hydrochloric acid gave three amino acids, glycine (1 mole), sarcosine (2 moles) and β-hydroxyleucine (1 mole) along with an unseparable mixture of lipophilic fragments.

In the ^1H NMR spectrum, all the proton and carbon signals of **1** were observed as double peaks with 4:1 ratios, which made the structural elucidation of **1** by NMR analyses difficult. In the ^{13}C NMR spectrum, **1** showed two exchangeable carbon signals at δ 98.4 (main) and δ 211.2 (minor) which suggested a hemiketal and ketone form structure, respectively.

$NaBH_4$ Reduction

1 was treated with $NaBH_4$ in methanol to afford two reduction products (**2a** and **2b**) which were separated by reversed phase column chromatography. The same molecular ion peak at m/z 896 $(M-H)^-$ was obtained in negative FAB-MS spectra of **2a** and **2b**, but they were differentiated by TLC and HPLC. Their IR and ^1H NMR are nearly identical indicating them to be stereo-isomers produced by $NaBH_4$ reduction of the ketone moiety. The ^{13}C NMR spectra of **2a** (Table 1) displayed 43 well-resolved carbons which were analyzed as $CH_3 \times 8$, $CH_2 \times 12$, $CH \times 6$, $N\text{-}CH_3 \times 2$, $O\text{-}CH_3$, $>C<$, $O\text{-}CH \times 4$, $C{=}CH$ (δ 131.7

† Verucopeptin was originally called BU-3983T (BMY-28782)[1].

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Table 1. ^{13}C NMR data of verucopeptin (**1**), reduction (**2a**) and degradation products (**3**, **4**).

Position	**1** (Main peak)	**2a**	**3**	**4**	Position	**1** (Main peak)	**2a**	**3**	**4**
1	167.1	168.5			22	176.1	174.8	176.3	175.9
2	51.7	51.7			23	77.6	76.8	76.7	76.4
3	170.9	170.8			24	98.4	76.9	78.8	78.1
4	42.4	42.3			25	27.2	26.9	25.9	25.7
5	170.2	170.7			26	24.0	26.0	25.0	25.1
6	52.4	52.7			27	80.0	82.3	83.1	83.1
7	166.9	167.0			28	75.7	76.1	75.3	75.2
8	51.3	51.4			29	130.0	131.7	131.4	131.3
9	171.8	171.5		171.6	30	137.0	134.1	133.5	133.4
9-OCH_3				51.1	31	30.2	29.4	29.4	29.4
10	48.5	48.9		52.5	32	46.1	46.0	46.0	46.0
11	23.9	23.9		26.1	33	27.7	27.8	27.9	27.9
12	21.3	21.4		21.9	34	45.0	44.8	44.8	44.8
13	46.5	46.9		47.0	35	31.7	31.6	31.7	31.7
14	171.3	171.3	170.0	170.4	36	30.4	30.4	30.3	30.4
14-OCH_3			52.3		37	11.4	11.4	11.4	11.4
15	46.9	47.2	44.8	41.8	38	20.6	20.2	21.6	21.9
16	79.9	80.1			39	56.8	57.6	57.5	57.6
17	29.6	29.6			40	11.4	13.1	13.3	13.4
18	18.3	18.5			41	21.3	21.3	21.3	21.3
19	19.2	19.3			42	19.2	19.5	19.6	19.6
20	36.7	36.6			43	19.0	19.0	19.0	19.1
21	34.7	34.7							

Recorded at 100 MHz in $CDCl_3$: ppm from solvent peak at δ 77.0.

and 134.1) and C=O×7 (δ 167.0, 168.5, 170.7, 170.8, 171.3, 171.5 and 174.8) by DEPT technique. The ^1H NMR spectrum of **2a** (Table 2) revealed the presence of two amide protons (δ 7.05 d and 7.14 br s) and two D_2O-exchangeable protons (δ 4.78 d and 9.21 s) in addition to all the protons expected from the ^{13}C NMR spectrum. These spectral data indicated that **2a** is related to azinothricin[3] and A83586C[4]. The detailed NMR analyses assisted by ^1H-^1H, ^1H-^{13}C and ^1H-^{13}C long range COSY spectra revealed the following seven structural units (a~g) in **2a**. The presence of sarcosines (a, c), glycine (b) and β-hydroxyleucine (f) was confirmed and two unusual amino acid, *N*-hydroxyglycine (d) and piperazic acid (e) were assigned as shown in Fig. 1 by comparison with the spectra of A83586C. The structure of acyl side chain (g) was elucidated by 2D NMR techniques.

Alkaline Hydrolysis of Reduction Product **2b**

2b was heated with 1.0 N sodium hydroxide at 100°C for 1 hour and the hydrolysate was treated with diazomethane. The product was purified by silica gel chromatography followed by preparative HPLC to afford degradation products **3**, **4** and **5**. In the negative FAB-MS spectra, **3**, **4** and **5** showed molecular ion peaks at *m/z* 472, 584 and 544 $(M-H)^-$, respectively. The molecular formulae of these compounds were estimated to be $C_{25}H_{47}NO_7$ for **3**, $C_{30}H_{55}N_3O_8$ for **4** and $C_{29}H_{55}NO_8$ for **5**, by FAB-MS and ^{13}C NMR spectra and their structures were determined as shown in Fig. 2 on the basis of the spectral analyses. A glycine residue is present in **3** and **4** instead of β-hydroxyleucine contained in the original compound (**2b**) and **5**. This indicated that the retro-aldol reaction occurred at the β-hydroxyleucine moiety during the alkaline hydrolysis. Compounds **3**, **4** and **5** clarified the connectivities of the structural units establishing the sequence from C-7 to C-43 in **2a**.

Table 2. ^{1}H NMR data of **2a**, **3**, **4** and **5**.

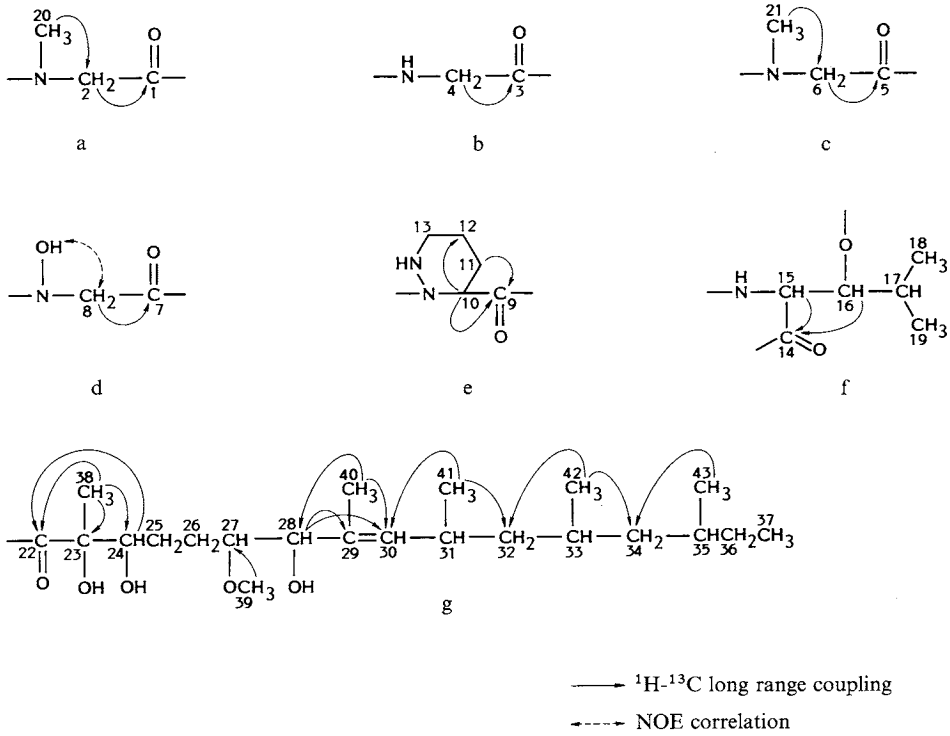
Position	2a	3	4	5
2	3.44 (d, 18.0), 4.71 (d, 18.0)			
4	3.65 (m), 4.98 (dd, 6.2, 6.9)			
4-NH	7.14 (br s)			
6	3.67 (d, 16.1), 4.16 (d, 16.1)			
8	3.88 (d, 15.3), 5.27 (d, 15.3)			
8-N-OH	9.21 (s)			
9-OCH_3			3.77 (s)	3.78 (s)
10	5.23 (m)		5.25 (m)	
11	1.86 (m), 2.23 (br d, 14.0)		1.85 (m), 2.23 (d,13.7)	
12	1.4~1.6 (m)		1.4~1.6 (m)	
13	2.67 (dt, 12.8, 12.1), 3.10 (m)		2.77 (br t, 12.4), 3.03 (br d, 12.4)	
13-NH	4.78 (br d, 12.8)			
14-OCH_3		3.75 (s)		
15	6.11 (dd, 2.9, 8.8)	4.05 (dd, 3.9, 5.5)	4.3 (m)	
15-NH	7.05 (d, 8.4)	7.33 (t, 5.5)	7.54 (br t)	7.72 (t, 7.7)
16	4.92 (dd, 2.9, 9.9)			4.69 (dd, 4.3, 7.7)
17	1.77 (m)			
18	0.88 (d, 6.6)			
19	1.11 (d, 6.6)			
20	3.11 (s)			
21	2.91 (s)			
24	3.45 (m)	3.52 (m)	3.47 (m)	3.55 (m)
25	1.5~1.8 (m)	1.40 (m), 1.85 (m)	1.5~1.8 (m)	1.5~1.8 (m)
26	1.6~1.75 (m)	1.5~1.7 (m)	1.6~1.7 (m)	1.6~1.7 (m)
27	3.28 (m)	3.18 (m)	3.18 (m)	3.18 (m)
28	4.13 (d, 4.0)	4.18 (d, 3.8)	4.17 (d, 4.3)	4.18 (d, 3.8)
30	5.23 (d, 8.5)	5.27 (d, 9.8)	5.26 (d, 9.0)	5.27 (d, 9.8)
31	2.51 (m)	2.52 (m)	2.51 (m)	2.53 (m)
32	0.95~1.30 (m)	1.0~1.3 (m)	1.0~1.3 (m)	1.0~1.3 (m)
33	1.45 (m)	1.45 (m)	1.45 (m)	1.45 (m)
34	0.9~1.1 (m)	1.0~1.1 (m)	1.0~1.1 (m)	1.0~1.1 (m)
35	1.37 (m)	1.37 (m)	1.37 (m)	1.37 (m)
36	1.0~1.3 (m)	1.2~1.4 (m)	1.0~1.3 (m)	1.1~1.3 (m)
37	0.85 (t, 7.3)	0.85 (t, 7.5)	0.85 (t, 7.2)	0.85 (t, 7.2)
38	1.40 (s)	1.34 (s)	1.36 (s)	1.33 (s)
39	3.40 (s)	3.39 (s)	3.38 (s)	3.39 (s)
40	1.63 (d, 0.7)	1.62 (s)	1.62 (s)	1.62 (s)
41	0.91 (d, 6.6)	0.91 (d, 6.8)	0.90 (d, 6.4)	0.91 (d, 6.8)
42	0.78 (d, 6.6)	0.79 (d, 6.4)	0.79 (d, 6.4)	0.79 (d, 6.4)
43	0.81 (d, 6.6)	0.80 (d, 6.8)	0.80 (d, 6.8)	0.80 (d, 6.8)

Recorded at 400 MHz in $CDCl_3$: ppm from solvent peak at δ 7.26.

Structures of **1** and **2**

The linkages of the remaining units were determined by ^{1}H-^{13}C long range COSY and NOESY spectral analyses as shown in Fig. 3. Two *N*-methyl protons and an amide proton displayed contours with following their relevant carbons, 20-CH_3 (C-2 and C-3), 21-CH_3 (C-6 and C-7) and 4-NH (C-5) to establish the linkage from C-1 to C-8. The NOE was observed between *N*-hydroxyl proton (δ 9.21) and the α-methine proton at 10-H (δ 5.23), which indicated that C-9 carbonyl group binds to the *N*-hydroxyglycine by an amide linkage. The α-methine proton of β-hydroxyleucine (16-H, δ 4.92) showed a correlation with the ester carbonyl carbon (C-1, δ 168.5) establishing a unique 19-membered cyclodepsipeptide ring (C-1 to

Fig. 1 .Partial structures of **2a**.



C-16). Thus the structure of **2a** was determined as shown in Fig. 3.

As described before, **1** exists as a mixture of two isomers in solution, and all the signals in the NMR spectra were observed as double peaks with 4:1 ratios. The main carbon signals of **1** were assigned as shown in Table 1 in comparison with **2a**. The C-24 carbon signal at δ 98.4 (80%) is interchangeable to signal at δ 211.2 (20%) suggesting that **1** formed the same hemiketal ring as A83586C between C-24 and C-28 and a part of **1** existed as a ketone form in solution. Thus, the structure of **1** is now shown in Fig. 4.

Discussion

The structure of verucopeptin has been determined based on the spectral analyses and degradation experiments. It is structurally related to azinothricin[3], A83586C[4], variapeptin[5,6], citropeptin[5,7] and L-156,602[8] which are 19-membered cyclodepsipeptides containing piperazic acids, β-hydroxyleucine and *N*-hydroxy-amino acid. Verucopeptin clearly differs from these antibiotics in having only one mole of piperazic acid and the presence of glycine and sarcosine. Furthermore, the acyl side chain conjugated to the hemiketal ring is considerably different from those of the known antibiotics.

It is also worth to note that verucopeptin showed specific *in vitro* and *in vivo* inhibition against mouse B16 melanoma and weak or no antibacterial activity, while all the other structurally related antibiotics were reported to show strong inhibitory activity against Gram-positive bacteria in addition to weak cytotoxicity.

Experimental

General

TLC was performed on precoated silica gel plates (Kieselgel $60F_{254}$, Merck). IR spectra in KBr discs

Fig. 2. $NaBH_4$ reduction and its alkaline hydrolysis.

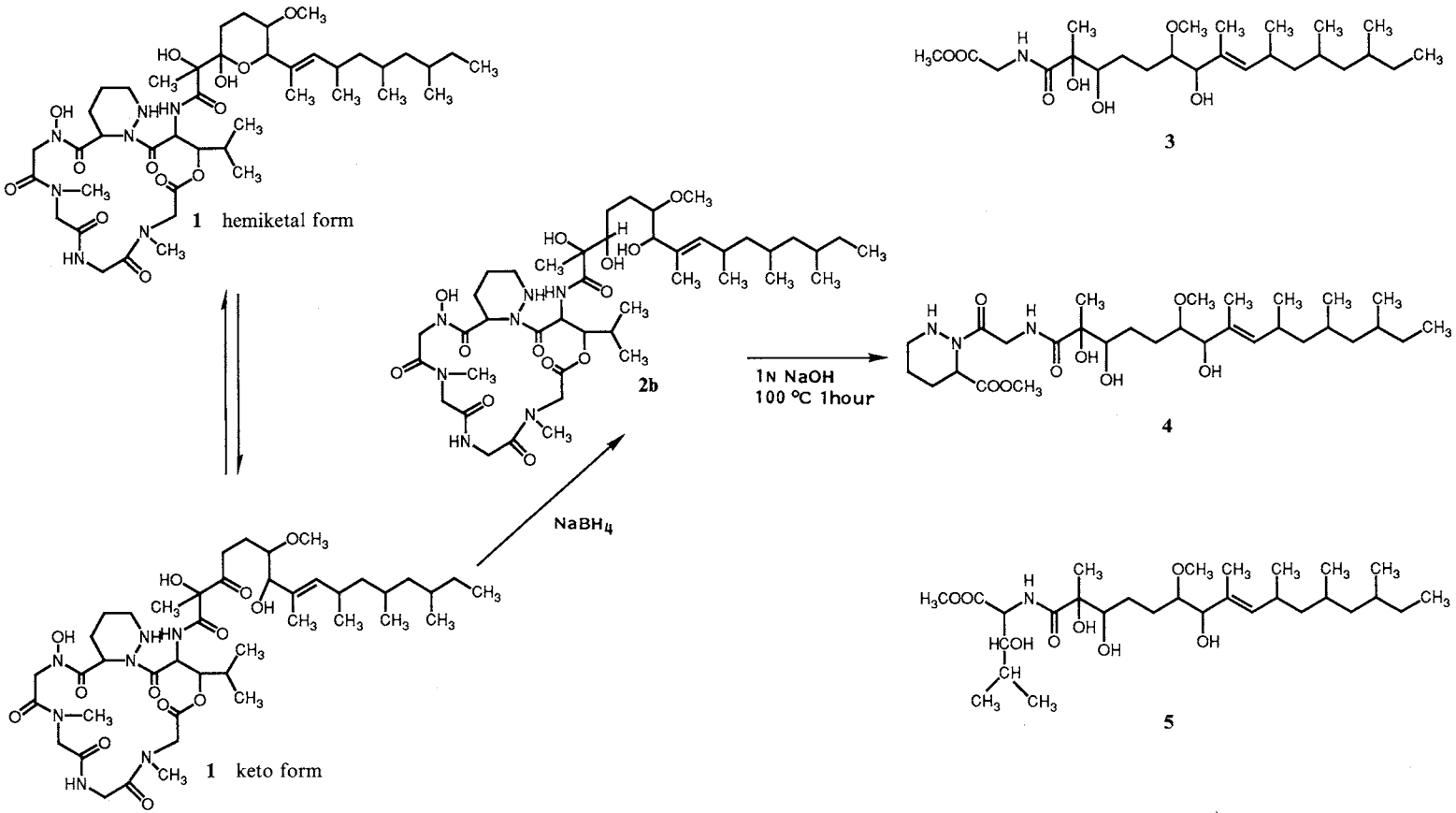


Fig. 3. Ester and amide bond assignments in **2a**.

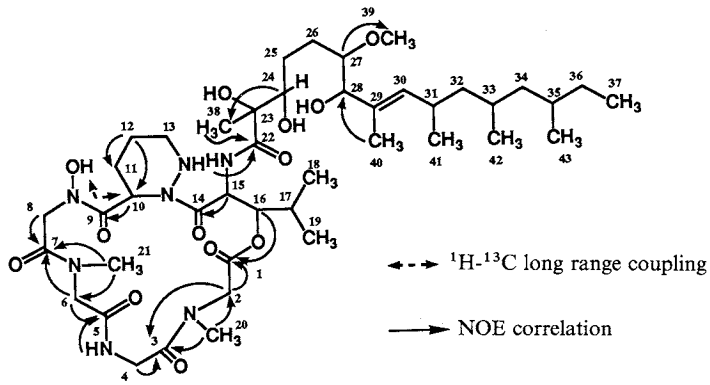
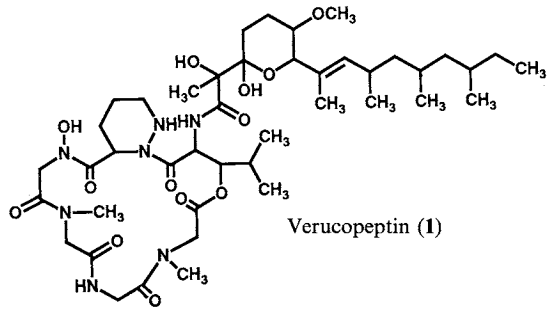
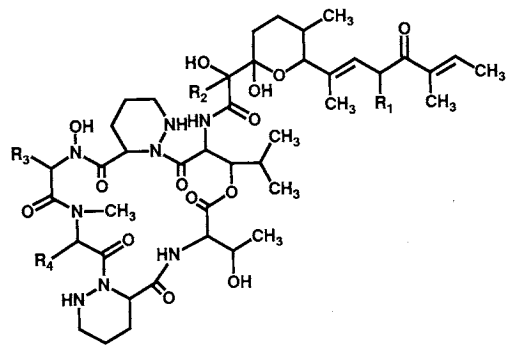
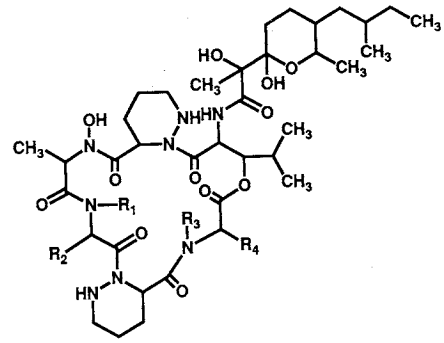


Fig. 4. Structures of verucopeptin and related antibiotics.



Verucopeptin (**1**)





	R_1	R_2	R_3	R_4
A83586C	$-CH_3$	$-CH_2CH_3$	$-CH_3$	$-CH_3$
Azinothricin	$-CH_2CH_3$	$-CH_2CH_3$	$-CH_2OCH_3$	$-CH_3$
Citropeptin	$-CH_3$	$-CH_3$	$-CH_2OCH_3$	$-CH_2CH(CH_3)_2$

	R_1	R_2	R_3	R_4
L-156,602	$-H$	$-H$	$-OH$	$-CH_3$
Variapeptin	$-CH_3$	$-CH_2C_6H_5$	$-H$	$-CH_2OH$

were recorded on a Jasco IR-810 spectrophotometer and UV spectra on a UVIDEC-610C spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-GX400 spectrometer. FAB-MS spectra were obtained on a JEOL JNM-AX 505H mass spectrometer.

Complete Acid Hydrolysis of **1**

1 (300 mg) dissolved in 6 N HCl (15 ml) was heated in a sealed tube for 16 hours at 105°C. After addition of water (90 ml), the reaction mixture was extracted with ethyl ether (50 ml × 2). Evaporation of

the ethyl ether extract afforded an oily residue (90 mg) containing unidentified lipophilic fragments. The aqueous layer was concentrated *in vacuo* to a sticky solid (240 mg) which was applied on a column of Dowex 50W × 4 (pyridine form, 1.5 i.d. × 75 cm). The column was developed with 0.1 M pyridine - formic acid (pH 3.1) and 0.2 M pyridine - formic acid (pH 3.1) successively and the eluate was monitored by TLC ($BuOH-AcOH-H_2O$, 63 : 10 : 27, ninhydrin detection). Sarcosine and glycine were eluted with 0.1 M pyridine - formic acid, β-hydroxyleucine with 0.2 M pyridine - formic acid. The corresponding fractions were pooled, evaporated and desalted with Sephadex LH-20 chromatography developed with 50% aqueous methanol to yield pure solids: sarcosine (45.9 mg); TLC Rf 0.07 ($BuOH-AcOH-H_2O$, 63 : 10 : 27) and 0.31 (phenol-H_2O, 4 : 1): glycine (15.4 mg); TLC Rf 0.06 ($BuOH-AcOH-H_2O$, 63 : 10 : 27) and 0.17 (phenol-H_2O, 4 : 1): β-hydroxyleucine (20.8 mg); TLC Rf 0.23 ($BuOH-AcOH-H_2O$, 63 : 10 : 27) and 0.31 (phenol-H_2O, 4 : 1); FAB-MS *m/z* 148 $(M+H)^+$, 129 $(M-H_2O)^+$; ^1H NMR (400 MHz, D_2O) δ 1.03 (3H, d, $J=6.6$ Hz), 1.04 (3H, d, $J=6.6$ Hz), 2.05 (1H, m), 3.59 (1H, dd, $J=2.9$ and 10.5 Hz), 4.28 (1H, d, $J=2.9$ Hz); ^{13}C NMR (100 MHz, D_2O) δ19.3 (t), 19.4 (t), 31.1 (d), 58.0 (d), 96.9 (d), 172.6 (s).

Mild Alkaline Hydrolysis of **1**

1 (5.0 mg) was dissolved in MeOH (0.45 ml) containing 1 N NaOH (0.05 ml) and stirred at room temperature for 7 hours. The mixture was acidified with 0.1 N HCl (pH 2.0) and extracted with BuOH and the extract was concentrated to yield **1**-acid (4.5 mg): IR (KBr) cm^{-1} 3370, 2950, 2920, 2860, 1640, 1520; FAB-MS (pos.) *m/z* 936 $(M+Na)^+$, 896 $(M+H-H_2O)^+$, 864; FAB-MS (neg.) *m/z* 912 $(M-H)^-$. ^1H NMR gave broad and paired signals.

$NaBH_4$ Reduction of **1**

To a stirred solution of **1** (100 mg) in 5 ml of MeOH was added $NaBH_4$ (60 mg), and the stirring was continued for 30 minutes at room temperature. The mixture was diluted with water (200 ml), acidified with 0.1 N HCl (pH 2.0) and extracted with BuOH (200 ml). The extract was concentrated *in vacuo* to give reduction product **2** (98 mg) which showed two peaks (**2a** and **2b**) in HPLC analysis (column: YMC-ODS A-301-3, 4.6 i.d. × 100 mm; solvent: CH_3CN-H_2O, 60 : 40; flow rate: 1 ml/minute; detection: UV 215 nm; Rt 8.25 minutes (**2a**, 54%) and 10.83 minutes (**2b**, 37%)). **2** (95 mg) was subjected to preparative HPLC to separate **2a** and **2b** (column: YMC D-ODS-5, 20 i.d. × 250 mm; solvent: CH_3CN-H_2O, 60 : 40; flow rate: 15 ml/minute; detection: UV 215 nm). The appropriate fractions were combined and evaporated *in vacuo* to yield **2a** (47 mg) and **2b** (32 mg). **2a**: IR (KBr) cm^{-1} 3400, 2960, 2930, 2870, 1740, 1650, 1490, 1410; FAB-MS (pos.) *m/z* 920 $(M+Na)^+$, 880 $(M+H-H_2O)^+$, 848; FAB-MS (neg.) *m/z* 896 $(M-H)^-$, 878 $(M-H-H_2O)^-$; ^1H and ^{13}C NMR spectra are shown in Tables 2 and 1, respectively. **2b**: IR (KBr) cm^{-1} 3400, 2960, 2930, 2870, 1740, 1650, 1460, 1400; FAB-MS (pos.) *m/z* 920 $(M+Na)^+$, 898 $(M+H)^+$, 880 $(M+H-H_2O)^+$, 848; FAB-MS (neg.) *m/z* 896 $(M-H)^-$, 878 $(M-H-H_2O)^-$. ^1H and ^{13}C NMR spectra are similar to **2a**.

Alkaline Hydrolysis of **2b**

A suspension of **2b** (130 mg) in aqueous 1 N NaOH (13 ml) was heated at 100°C for 1 hour in a sealed tube. The reaction mixture was diluted with water (150 ml), acidified with 1 N HCl (pH 2.0) and extracted by BuOH (150 ml × 2). The solvent extract was concentrated to a residue which was dissolved in a mixture of CH_2Cl_2-MeOH (9 : 1, 2 ml) and reacted with large excess of CH_2N_2 in ether. After concentration of the reaction mixture, the residue was dissolved in EtOAc (100 ml) and shaken with water (100 ml). The organic layer was separated and concentrated *in vacuo* to yield a hydrolysate (80 mg) which showed mainly 3 peaks in HPLC analysis (column: YMC-ODS A-301-3, 4.6 i.d. × 100 mm; solvent: CH_3CN-H_2O, 65 : 35; flow rate: 1 ml/minute; detection: UV 215 nm). They (**3**, **4** and **5**) were successfully isolated by silica gel chromatography developed with CH_2Cl_2-MeOH (98 : 2) followed by preparative HPLC (column : YMC D-ODS-5, 20 i.d. × 250 mm; solvent: CH_3CN-H_2O, 65 : 35; flow rate: 10 ml/minute; detection: UV 215 nm) to give **3** (11.3 mg), **4** (7.5 mg) and **5** (2.7 mg). **3**: TLC Rf 0.35 (CH_2Cl_2-MeOH, 95 : 5); HPLC Rt 7.98 minutes (by the above system); IR (KBr) cm^{-1} 3400, 2950, 2930, 2870, 1750, 1680, 1520, 1460, 1380, 1210, 1100; FAB-MS (pos.) *m/z* 474 $(M+H)^{-1}$, 456 $(M+H-H_2O)^+$, FAB-MS (neg.) *m/z* 472 $(M-H)^-$; ^1H and ^{13}C NMR spectra are shown in Tables 2 and 1, respectively. **4**: TLC Rf 0.27, HPLC Rt 9.19 minutes; IR (KBr) cm^{-1} 3400, 2950, 2930, 2870, 1750, 1680, 1520, 1440; FAB-MS (pos.) *m/z* 586 $(M+H)^+$, FAB-MS

(neg.) m/z 584 $(M-H)^-$; ^1H and ^{13}C NMR spectra are shown in Tables 2 and 1, respectively. **5**: TLC Rf 0.32; HPLC Rt 11.08 minutes; FAB-MS (pos.) m/z 546 $(M+H)^+$, 528 $(M+H-H_2O)^+$; FAB-MS (neg.) m/z 544 $(M-H)^-$. ^1H NMR spectrum is shown in Table 2.

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