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FOUR NEW STEROIDAL SAPONINS FROM THE RHIZOMES OF *HELLEBORUS ORIENTALIS*

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Abstract – A phytochemical analysis has been carried out on the glycoside-enriched fraction of a MeOH extract of *Helleborus orientalis* rhizomes, resulting in the isolation of two new bisdesmosidic furostanol saponins (**1** and **2**) and two new furospirostanol saponins (**3** and **4**). The structures of **1**-**4** were determined on the basis of extensive spectroscopic analysis and acid hydrolysis followed by chromatographic and spectroscopic analysis. Compound (**3**) showed moderate cytotoxic activity against HSC-2 human squamous cell carcinoma cells with an IC_{50} value of 16 μg/mL.

INTROCUCTION

Helleborus orientalis, known by the name 'Lenten Rose' in Europe, is a perennial plant belonging to the family Ranunculaceae and is indigenous to Greece and Turkey.[1] Its rhizomes are known to contain several bufadienolide glycosides and were used as a cardiotonic agent.[2] Plants containing cardiac glycosides such as *Digitalis purpurea*[3] and *Convallaria majalis*[4] concomitantly produce steroidal glycosides. We have now made a phytochemical screening of the glycoside-enriched fraction prepared from a MeOH extract of the rhizomes of *H. orientalis* and isolated two new bisdesmosidic furostanol saponins (**1** and **2**) and two new furospirostanol saponins (**3** and **4**). This paper reports the structural elucidation of the new compounds on the basis of extensive spectroscopic analysis and acid hydrolysis followed by chromatographic and spectroscopic analysis. The cytotoxic activity of the isolated compounds against HSC-2 human squamous cell carcinoma cells is briefly described.

RESULTS AND DISCUSSION

A concentrated MeOH extract of *H. orientalis* rhizomes was passed through a porous-polymer resin (Diaion HP-20) column and the 80% MeOH eluate fraction, in which steroidal glycosides were enriched, was subjected to column chromatography over silica gel and octadecylsilanized (ODS) silica gel, as well as preparative HPLC, giving to compounds (**1**) (8.6 mg), (**2**) (12.0 mg), (**3**) (106 mg), and (**4**) (6.8 mg).

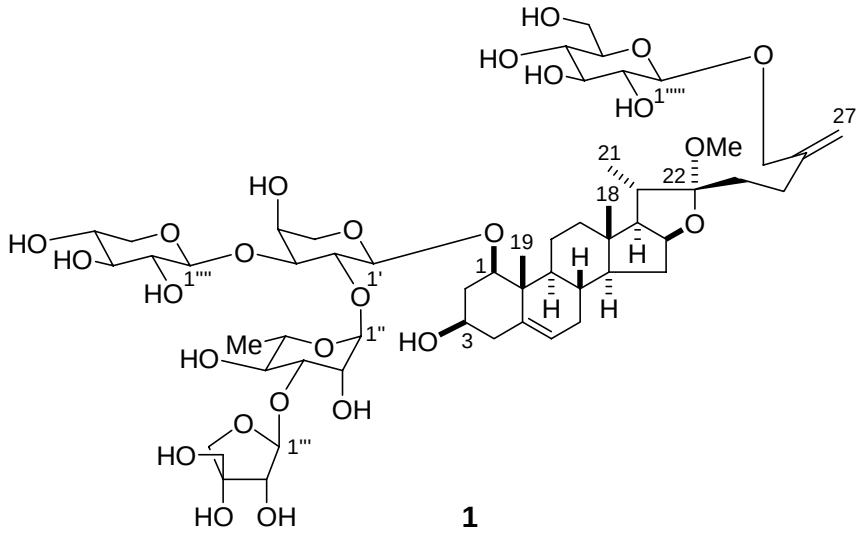
Compound (**1**) was obtained as an amorphous solid, $[\alpha]_D$ -84.0 ° (MeOH). Its molecular formula was determined to be $C_{55}H_{88}O_{26}$ by the FAB-MS (*m*/*z* 1187 $[M + Na]^+$), ^{13}C-NMR spectrum, and elemental analysis data. The glycosidic nature of **1** was suggested by strong absorption bands at 3288 and 1043 cm^{-1}. The ^{1}H-NMR spectrum of **1** contained signals for two tertiary methyl groups at δ 1.43 and 0.82 (each s), a secondary methyl group at δ 1.11 (d, *J* = 6.9 Hz), an exomethylene group at δ 5.33 and 5.04 (each br s), an olefinic proton at δ 5.58 (br d, *J* = 5.5 Hz), and five anomeric protons at δ 6.35 (d, *J* = 1.5 Hz), 6.23 (d, *J* = 2.5 Hz), 4.99 (d, *J* = 7.5 Hz), 4.91 (d, *J* = 7.8 Hz), and 4.69 (d, *J* = 7.3 Hz). Acid hydrolysis of **1** with 0.2 M HCl in dioxane-H_2O (1:1) gave a steroidal sapogenin, identified as spirosta-5,25(27)-diene-1β,3β-diol (**1a**),[5] together with D-apiose, L-arabinose, D-glucose, L-rhamnose, and D-xylose. The monosaccharides, including their absolute configurations, were identified by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column using MeCN-H_2O (17:3) as solvent system, with detection being carried out using a combination of RI and optical rotation (OR) detectors. The above spectral and chemical data, as well as a positive color reaction in Ehrlich's test, the three-proton singlet signal at δ 3.24 (3H, s), and the acetalic carbon signal at δ 112.4 (C) indicated that **1** was the corresponding bisdesmosidic 22α-methoxyfurostanol saponin[6] of **1a**, whose sugar moieties were composed of a total of five monosaccharides. The ^{1}H-^{1}H COSY and 2D TOCSY experiments with **1** allowed the sequential assignments from H-1 to CH_2-5, Me-6, and CH_2-6 of four monosaccharides. Their signal multiplet patterns and coupling constants enabled the identification of an α-L-arabinopyranosyl ($^4C_1$) unit, a β-D-glucopyranosyl ($^4C_1$) unit, an α-L-rhamnopyranosyl ($^1C_4$) unit, and a β-D-xylopyranosyl ($^4C_1$) unit (Table 1). In addition, two pairs of ABq-like signals at δ 6.23 and 4.82 (*J* = 2.5 Hz) and δ 4.64 and 4.28 (*J* = 9.2 Hz), and two-proton broad singlet signal at δ 4.15, along with the results of acid hydrolysis, were indicative of an apiofuranosyl unit. The relatively large *J* values of the anomeric protons of the arabinosyl (7.3 Hz), glucosyl (7.8 Hz), and xylosyl (7.5 Hz) moieties indicated an α anomeric orientation for the arabinosyl and β for the glucosyl and xylosyl. For the rhamnosyl moiety, the large $^1J_{C,H}$ values (170 Hz <) confirmed that the anomeric proton was equatorial thus possessing an α-pyranoid anomeric form.[7] The ^{13}C-NMR shifts of the anomeric carbon of the apiosyl at δ 111.6 indicated a β-orientation of the anomeric center.[8] All the proton signals for the sugar moiety thus assigned were associated with the one-bond coupled carbon signals using the HMQC

spectrum. The apiosyl, glucosyl, and xylosyl residues were considered to be the terminal units, as shown by the absence of any glycosylation shift for their carbon resonances, while C-2 and C-3 of the arabinosyl unit, and C-3 of the rhamnosyl unit were suggested to be substituted by comparison with those of authentic methyl glycosides.[9] In the HMBC spectrum of **1**, the anomeric proton of the apiosyl at δ 6.23 showed a $^3J_{C,H}$ correlation with C-3 of the rhamnosyl at δ 79.8, whose anomeric proton at δ 6.35, in turn, showed a long-range correlation with C-2 of the arabinosyl at δ 73.6. The anomeric proton of the xylosyl at δ 4.99 was correlated to C-3 of the arabinosyl at δ 84.7. The arabinosyl moiety was thus shown to be glycosylated at C-2 and C-3, and its anomeric proton at δ 4.69 exhibited an HMBC correlation with C-1 of the aglycone at δ 84.1. As is usual with naturally occurring furostanol bisdesmosides,[10] a glucosyl group was revealed to be linked to the C-26 hydroxyl group of the aglycone in **1** by an HMBC correlation of the anomeric proton of the glucosyl at δ 4.91 to C-26 of the aglycone at δ 72.0. Accordingly, the structure of **1** was formulated as 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurosta-5,25(27)-diene-1β-yl *O*-β-D-apiofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

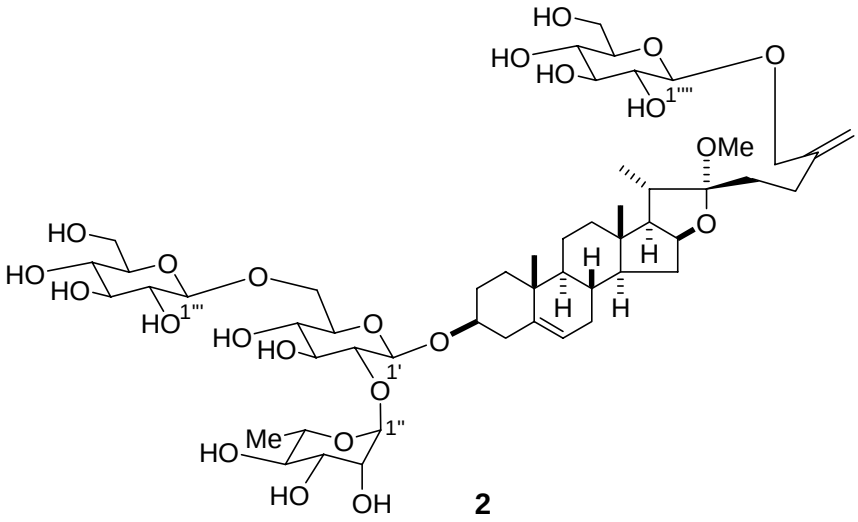
Compound (**2**) ($C_{52}H_{84}O_{23}$) was obtained as an amorphous solid, $[\alpha]_D$ -96.0 ° (MeOH). The ^{1}H-NMR spectrum of **2** showed signals for four anomeric protons at δ 6.32 (d, *J* = 0.6 Hz), 5.09 (d, *J* = 7.8 Hz), 4.96 (d, *J* = 7.7 Hz), and 4.92 (d, *J* = 7.8 Hz), as well as signals for two tertiary methyl groups at δ 1.03 and 0.79 (each s), a secondary methyl group at δ 1.17 (d, *J* = 6.9 Hz), an exomethylene group at δ 5.36 and 5.07 (each br s), an olefinic proton at δ 5.31 (br d, *J* = 4.9 Hz), and a methoxy group at δ 3.26 (s). Acid hydrolysis of **2** gave a steroidal sapogenin, identified as spirosta-5,25(27)-dien-3β-ol (**2a**),[11] and D-glucose and L-rhamnose. These ^{1}H-NMR and chemical data, together with an acetalic carbon signal at δ 112.4 and a positive color reaction with Ehrlich's reagent, implied that **2** was the corresponding bisdesmosidic 22α-methoxyfurostanol saponin of **2a**. Analysis of the ^{1}H-^{1}H COSY, 2D TOCSY, and HMQC spectra of **2** allowed the identification of the proton signals, including their multiplet patterns and coupling constant, and the carbon chemical shifts of the sugar moieties of **2** as shown in Table 1. The results indicated the presence of two terminal β-D-glucopyranosy units, an terminal α-L-rhamnopyranosyl units, and a C-2 and C-6 disubstituted β-D-glucopyranosy unit in **2**. In the HMBC spectrum of **2**, the anomeric protons at δ 6.32 (rhamnosyl) and 5.09 (terminal glucosyl) exhibited long-range correlations with C-2 (δ 77.5) and C-6 (δ 69.8) of the inner glucosyl unit, respectively. Forthermore, the anomeric protons of the inner glucosyl at δ 4.96 showed an HMBC correlation with C-3 of the aglycone at δ 78.5, whereas that of the remaining glucosyl at δ 4.92 was correlated to C-26 of the aglycone at δ 72.0. Thus, the structure of **2** was elucidated as 26-[(β-D-glucopyranosyl)oxy]-22α-methoxyfurosta-5,25(27)-diene-

3β-yl *O*-β-D-glucopyranosyl-(1→6)-*O*-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside.

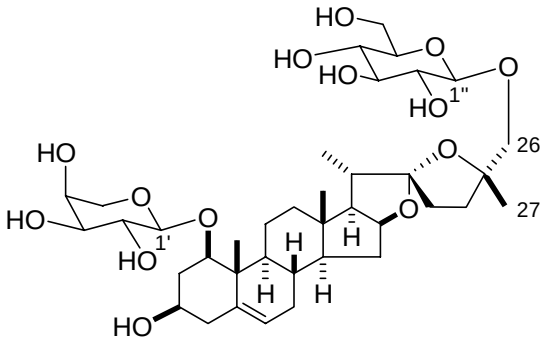
Compound (**3**) ($C_{38}H_{60}O_{14}$) was suggested to be also a steroidal saponin by its spectral properties. The ^{1}H-NMR spectrum of **3** showed signals for three tertiary methyl groups at δ 1.37, 1.20, and 0.79 (each s),



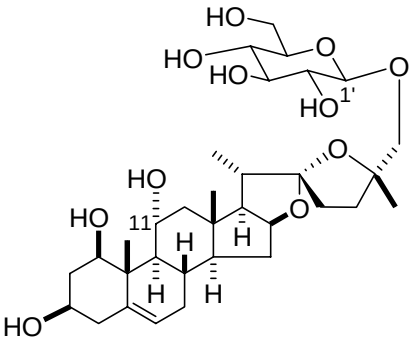
1



2



3



4

a secondary methyl group at δ 1.00 (d, *J* = 6.9 Hz), an olefinic proton at δ 5.54 (br d, *J* = 5.7 Hz), and two anomeric protons at δ 4.93 (d, *J* = 7.7 Hz) and 4.73 (d, *J* = 7.4 Hz). In the ^{13}C-NMR spectrum of **3**, the C-22 acetalic carbon signal was observed at δ 120.2, which was typical of furospirostanol type steroids.[12]

Table 1. ^{1}H- and ^{13}C-NMR spectral data of the sugar moieties of **1** and **2** in C_5D_5N

	1				**2**				
	^{1}H		*J* (Hz)	^{13}C		^{1}H		*J* (Hz)	^{13}C
1'	4.69	d	7.3	100.6	1'	4.96	d	7.7	100.6
2'	4.62	dd	8.7, 7.3	73.6	2'	4.19	dd	9.2, 7.7	77.5
3'	4.08	dd	8.7, 3.1	84.7	3'	4.23	dd	9.4, 9.2	79.4
4'	4.40	br d	3.1	69.5	4'	4.13	dd	9.4, 8.8	71.6
5' a	4.24	*		66.9	5'	4.01	ddd	8.8, 5.5, 1.7	76.8
b	3.69	br d	11.3		6' a	4.76	dd	11.6, 1.7	69.8
					b	4.33	dd	11.6, 5.5	
1"	6.35	d	1.5	101.4					
2"	4.95	dd	3.1, 1.5	71.7	1"	6.32	d	0.6	102.0
3"	4.66	dd	9.4, 3.1	79.8	2"	4.78	dd	3.3, 0.6	72.5
4"	4.38	dd	9.4, 8.5	72.5	3"	4.61	dd	9.6, 3.3	72.8
5"	4.83	dq	8.5, 6.0	69.5	4"	4.34	dd	9.6, 9.6	74.1
6"	1.68	d	6.0	19.0	5"	4.96	dq	9.6, 6.2	69.4
					6"	1.77	d	6.2	18.6
1‴	6.23	d	2.5	111.6					
2‴	4.82	d	2.5	77.7	1‴	5.09	d	7.8	105.3
3‴	-			80.2	2‴	4.02	dd	8.4, 7.8	75.1
4‴ a	4.64	d	9.2	75.1	3‴	4.19	dd	9.2, 8.4	78.4
b	4.28	d	9.2		4‴	4.21	dd	9.2, 8.3	71.6
5‴ (2H)	4.15	br s		65.5	5‴	3.91	ddd	8.3, 5.1, 2.3	78.4
					6‴ a	4.50	dd	11.8, 2.3	62.7
1⁗	4.99	d	7.5	106.4	b	4.36	dd	11.8, 5.1	
2⁗	3.90	dd	8.6, 7.5	74.6					
3⁗	4.11	dd	8.6, 8.1	78.3	1⁗	4.92	d	7.8	103.8
4⁗	4.12	*		70.9	2⁗	4.08	dd	8.5, 7.8	75.1
5⁗ a	4.26	*		67.1	3⁗	4.26	dd	8.9, 8.5	78.6
b	3.68	dd	12.5, 11.0		4⁗	4.24	dd	9.1, 8.9	71.7
					5⁗	3.96	ddd	8.9, 5.4, 2.4	78.4
1⁗'	4.91	d	7.8	103.8	6⁗ a	4.55	dd	11.9, 2.4	62.8
2⁗'	4.06	dd	7.8, 7.7	75.1	b	4.43	dd	11.9, 5.4	
3⁗'	4.24	dd	8.7, 7.7	78.5					
4⁗'	4.23	dd	8.7, 8.7	71.7					
5⁗'	3.95	ddd	8.7, 5.5, 2.5	78.6					
6⁗' a	4.55	dd	11.9, 2.5	62.8					
b	4.39	dd	11.9, 5.5						

* Multiplicities are not clear due to overlapping.

Acid hydrolysis of **3** with 2 M HCl in dioxane-H_2O (1:1) gave L-arabinose and D-glucose, as well as a

pseudo-aglycone, identified as spirost-5-ene-1β,3β,25-triol (**3a**).[13] This was assumed to be produced from the corresponding furospirostanol sapogenin of 22,25-epoxyfurost-5-ene-1β,3β-diol under acidic conditions.[14] When the ^{1}H- and ^{13}C-NMR signals arising from the F-ring part of **3a** were compared with those of (25*R*)- and (25*S*)-spirost-5-en-3β,25-diol,[15] they were in good agreement with those of the (25*S*)-isomer. Analysis of the ^{1}H- and ^{13}C-NMR spectra of **3** resulted in the identification of signals for an α-L-arabinopyranosyl unit and a β-D-glucopyranosyl unit (Table 2). In the HMBC spectrum, the anomeric proton of the arabinosyl moiety at δ 4.73 exhibited a long-range correlation with C-1 of the aglycone at δ 83.1, whereas that of the glucosyl at δ 4.93 was correlated to C-26 of the aglycone at δ 77.4. The steructure of **3** was characterized as (25*S*)-22α,25-epoxy-26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-furost-5-en-1β-yl α-L-arabinopyranoside.

Table 2. ^{1}H- and ^{13}C-NMR spectral data of the sugar moieties of **3** and **4** in C_5D_5N

	3				**4**				
	^{1}H		*J* (Hz)	^{13}C		^{1}H		*J* (Hz)	^{13}C
1'	4.73	d	7.4	102.2	1'	4.93	d	7.8	105.3
2'	4.38	dd	9.4, 7.4	72.6	2'	4.02	dd	8.9, 7.8	75.3
3'	4.12	dd	9.4, 3.4	74.7	3'	4.26	dd	8.9, 8.6	78.3
4'	4.26	br d	3.4	69.6	4'	4.27	dd	9.4, 8.6	71.5
5' a	4.29	dd	11.3, 2.1	67.5	5'	3.92	ddd	9.4, 5.0, 2.4	78.4
b	3.71	br d	11.3		6' a	4.51	dd	11.9, 2.4	62.6
					b	4.40	dd	11.9, 5.0	
1"	4.93	d	7.7	105.3					
2"	4.02	dd	8.9, 7.7	75.3					
3"	4.25	dd	9.4, 8.9	78.3					
4"	4.26	dd	9.4, 8.6	71.5					
5"	3.91	ddd	8.6, 5.2, 2.4	78.4					
6" a	4.51	dd	11.9, 2.4	62.5					
b	4.39	dd	11.9, 5.2						

Compound (**4**) was isolated as an amorphous solid with the molecular formula $C_{33}H_{52}O_{11}$. Analysis of the ^{1}H- and ^{13}C-NMR spectra of **4** and comparison with those of **3** implied that **4** was closely related to **3**. However, the signals due to the α-L-arabinopyranosyl group linked to C-1 of the aglycone could not be observed in the ^{1}H- and ^{13}C-NMR spectra of **4**, and the C-1 carbon signal of the aglycone moiety of **4** was shifted upfield by 7.3 ppm in comparison with that of **3**. Furthermore, **4** differed from **3** in the presence of one more hydroxymethine group (δ_H 4.24; δ_C 66.0), which was shown to be located at C-11 by proton spin-coupling correlations of the signal at δ 4.24 with H-9 at δ 1.47 and H_2-12 at δ 2.42 and 1.55. The large proton coupling constants between H-9 and H-11 (9.9 Hz), and between H-11 and H-12 axial (10.8

Hz) indicated that the C-11 hydroxy group was an α-equatorial-oriented configuration. The above data led to the structure of **4** as (25*S*)-22α,25-epoxy-3β,11α-dihydroxyfurost-5-en-26-yl β-D-glucopyranoside. Compounds (**1**-**4**) were evaluated for their cytotoxic activity against HSC-2 human squamous cell carcinoma cells. Only **3** showed cytotoxic activity with an IC_{50} value of 16 μg/mL, when etoposide used as a positive control, to which HSC-2 cells are relatively resistant, had an IC_{50} value of 24 μg/mL.

EXPERIMENTAL

Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. MS spectra were recorded on a Finnigan MAT TSQ-700 (San Jose, CA) or a Micromass LCT (Manchester, UK) mass spectrometer. Elemental analysis was carried out using an Elemental Vario EL (Hanau, Germany) elemental analyzer. Silica gel (Fuji-Silysia Chemical, Aichi, Japan), ODS silica gel (Nacalai Tesque, Kyoto, Japan), and Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP-18 F_{254}S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a system comprised of a Tosoh CCPM pump (Tokyo, Japan), a Tosoh CCP PX-8010 controller, a Tosoh RI-8010 detector, a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. A Capcell Pak C_{18} UG120 column (10 mm i.d. x 250 mm, 5 μm, Shiseido, Tokyo, Japan) was employed for preparative HPLC. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA); penicillin and streptomycin sulfate (Meiji-Seica, Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA). All other chemicals used were of biochemical reagent grade.

Plant material

Helleborus orientalis was purchased from a nursery in Heiwaen, Japan, in November, 1999, and was identified by an emeritus professor of Tokyo University of Pharmacy and Life Science, Dr. Y. Sashida. A voucher specimen has been deposited in our laboratory (voucher No. 99-11-7-HO, Laboratory of Medicinal Pharmacognosy).

Extraction and isolation

The plant material (fresh weight, 2.7 kg) was extracted with MeOH (20 L) at 75 °C for 3 h twice. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (300 g) was passed

through a Diaion HP-20 column (30% MeOH, 80% MeOH, MeOH, EtOH, and EtOAc). The 80% MeOH eluate portion (90 g) was chromatographed on silica gel, eluted with $CHCl_3$-MeOH gradients (9:1, 4:1, 2:1) and finally MeOH, to give subfractions I-VIII. Fraction V was subjected to silica gel column chromatography, eluted with $CHCl_3$-MeOH-H_2O (40:10:1; 30:10:1; 20:10:1), ODS silica gel with MeCN-H_2O (1:2; 1:4) and MeOH-H_2O (4:5), and finally to preparative HPLC using MeCN-H_2O (1:3) to furnish **2** (12.0 mg), **3** (106 mg), and **4** (6.8 mg). Fraction VII was subjected to silica gel column chromatography, eluted with $CHCl_3$-MeOH-H_2O (70:40:9), ODS silica gel with MeCN-H_2O (1:3; 1:5), and to preparative HPLC using MeCN-H_2O (1:3) to give **1** (8.6 mg).

Compound (1): amorphous solid; $[\alpha]_D$ -84.0 ° (*c* = 0.10, MeOH); IR (film) ν_{max} 3288 (OH), 2948 and 2888 (CH), 1043 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 5.58 (1H, br d, *J* = 5.5 Hz, H-6), 5.33 and 5.04 (each 1H, br s, H_2-27), 4.60 and 4.35 (each 1H, d, *J* = 12.7 Hz, H_2-26), 4.41 (1H, m, H-16), 3.87 (1H, m, H-3), 3.79 (1H, dd, *J* = 12.0, 3.9 Hz, H-1), 3.24 (3H, s, OMe), 1.43 (3H, s, Me-19), 1.11 (3H, d, *J* = 6.9 Hz, Me-21), 0.82 (3H, s, Me-18), signals for the sugar moieties, see Table 1; ^{13}C-NMR (C_5D_5N) δ 84.1 (C-1), 37.5 (C-2), 68.3 (C-3), 43.8 (C-4), 139.5 (C-5), 124.7 (C-6), 31.9 (C-7), 33.0 (C-8), 50.3 (C-9), 42.9 (C-10), 24.0 (C-11), 40.2 (C-12), 40.5 (C-13), 56.7 (C-14), 32.3 (C-15), 81.4 (C-16), 64.1 (C-17), 16.6 (C-18), 15.1 (C-19), 40.4 (C-20), 16.1 (C-21), 112.4 (C-22), 31.6 (C-23), 28.0 (C-24), 146.8 (C-25), 72.0 (C-26), 111.0 (C-27), 47.3 (OMe), signals for the sugar moieties, see Table 1; FAB-MS (positive-mode) *m*/*z* 1187 $[M + Na]^+$; *Anal.* Calcd for $C_{55}H_{88}O_{26}\cdot 3H_2O$: C; 54.18, H; 7.77. Found: C; 54.07, H; 8.04.

Acid Hydrolysis of 1

A solution of **1** (5.8 mg) in 0.2 M HCl (dioxane-H_2O, 1:1, 3 mL) was heated at 95 °C for 30 min under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and then passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA), eluted with H_2O followed by MeOH. The MeOH eluate portion was chromatographed on silica gel, eluted with $CHCl_3$-MeOH (9:1), to give **1a** (1.9 mg). The H_2O eluate portion was passed through a Toyopak IC-SP M cartridge (Tosoh, Tokyo, Japan), which was then analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. x 250 mm, 5 μm, Shiseido); solvent, MeCN-H_2O (17:3); flow rate, 0.9 mL/min; detection, RI and OR. Identification of D-apiose, L-arabinose, D-glucose, L-rhamnose, and D-xylose present in the sugar fraction was carried out by comparison of their retention times and optical rotations with those of authentic samples. t_R (min): 7.32 (D-apiose, positive optical rotation), 7.91 (L-rhamnose, negative optical rotation), 9.58 (L-arabinose, positive optical rotation), 9.96 (D-xylose, positive optical rotation), 15.30 (D-glucose, positive optical rotation).

Compound (2): amorphous solid; $[\alpha]_D$ -96.0 ° (*c* = 0.10, MeOH); IR (film) ν_{max} 3388 (OH), 2933 and 2851 (CH), 1041 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 5.36 and 5.07 (each 1H, br s, H_2-27), 5.31 (1H, br d, *J* = 4.9

Hz, H-6), 4.63 and 4.37 (each 1H, d, *J* = 11.0 Hz, H_2-26), 4.44 (1H, m H-16), 3.96 (1H, m, H-3), 3.26 (3H, s, OMe), 1.17 (3H, d, *J* = 6.9 Hz, Me-21), 1.03 (3H, s, Me-19), 0.79 (3H, s, Me-18), signals for the sugar moieties, see Table 1; ^{13}C-NMR (C_5D_5N) δ 37.5 (C-1), 30.3 (C-2), 78.5 (C-3), 39.1 (C-4), 140.9 (C-5), 121.6 (C-6), 32.2 (C-7), 31.6 (C-8), 50.2 (C-9), 37.1 (C-10), 21.0 (C-11), 39.7 (C-12), 40.7 (C-13), 56.5 (C-14), 32.1 (C-15), 81.4 (C-16), 64.0 (C-17), 16.2 (C-18), 19.4 (C-19), 40.3 (C-20), 16.2 (C-21), 112.4 (C-22), 29.9 (C-23), 28.1 (C-24), 146.8 (C-25), 72.0 (C-26), 111.1 (C-27), 47.3 (OMe), signals for the sugar moieties, see Table 1; HR-ESI-MS (positive-mode) *m*/*z* 1045.5214 $[M + H - MeOH]^+$ (Calcd 1045.5220 for $C_{51}H_{81}O_{22}$).

Acid hydrolysis of 2

Compound (**2**) (5.4 mg) was subjected to acid hydrolysis as described for **1** to give **2a** (1.2 mg) and a sugar fraction (1.7 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **1** showed the presence of D-glucose and L-rhamnose. t_R (min): 7.82 (L-rhamnose, negative optical rotation), 14.86 (D-glucose, positive optical rotation).

Compound (3): amorphous solid; $[\alpha]_D$ -68.0 ° (*c* = 0.10, MeOH); IR (film) ν_{max} 3363 (OH), 2890 and 2875 (CH), 1024 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 5.54 (1H, br d, *J* = 5.7 Hz, H-6), 4.67 (1H, q-like, *J* = 7.2 Hz, H-16), 4.14 and 3.87 (each 1H, d, *J* = 10.0 Hz, H_2-26), 3.89 (1H, m, H-3), 3.84 (1H, dd, *J* = 11.7, 4.0 Hz, H-1), 1.37 (3H, s, Me-27), 1.20 (3H, s, Me-19), 1.00 (3H, d, *J* = 6.9 Hz, Me-21), 0.79 (3H, s, Me-18), signals for the sugar moieties, see Table 2; ^{13}C-NMR (C_5D_5N) δ 83.1 (C-1), 37.5 (C-2), 68.0 (C-3), 43.7 (C-4), 139.4 (C-5), 124.6 (C-6), 31.8 (C-7), 32.9 (C-8), 50.1 (C-9), 42.8 (C-10), 23.7 (C-11), 40.2 (C-12), 40.2 (C-13), 56.5 (C-14), 32.3 (C-15), 80.9 (C-16), 62.5 (C-17), 16.5 (C-18), 14.7 (C-19), 38.4 (C-20), 15.1 (C-21), 120.2 (C-22), 33.0 (C-23), 33.8 (C-24), 83.8 (C-25), 77.4 (C-26), 24.3 (C-27), signals for the sugar moieties, see Table 2; FAB-MS (positive-mode) *m*/*z* 741 $[M + H]^+$; HR-ESI-TOF-MS (positive-mode) *m*/*z* 741.4066 $[M + H]^+$ (Calcd 741.4061 for $C_{38}H_{61}O_{14}$); *Anal.* Calcd for $C_{38}H_{60}O_{14}$: C; 61.60, H; 8.16. Found: C; 61.48, H; 8.20.

Acid hydrolysis of 3

Compound (**3**) (11.2 mg) was subjected to acid hydrolysis as described for **1** to give **3a** (8.6 mg) and a sugar fraction (2.9 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **1** showed the presence of L-arabinose and D-glucose. t_R (min): 9.03 (L-arabinose, positive optical rotation), 14.98 (D-glucose, positive optical rotation).

Compound (4): amorphous solid; $[\alpha]_D$ -114.0 ° (*c* = 0.10, MeOH); IR (film) ν_{max} 3347 (OH), 2921 and 2853 (CH), 1076, 1030 cm^{-1}; ^{1}H-NMR (C_5D_5N) δ 5.60 (1H, br d, *J* = 5.7 Hz, H-6), 4.70 (1H, q-like, *J* = 7.5 Hz, H-16), 4.24 (1H, ddd, *J* = 10.8, 9.9, 5.4 Hz, H-11), 4.16 and 3.87 (each 1H, d, *J* = 9.9 Hz, H_2-26), 3.93 (1H, m, H-3), 3.88 (1H, dd, *J* = 11.6, 5.4 Hz, H-1), 2.42 (1H, dd, *J* = 12.1, 5.4 Hz, H-12eq), 1.55 (1H, dd, *J* = 12.1, 10.8 Hz, H-12ax), 1.47 (1H, dd, *J* = 9.9, 9.7 Hz, H-9), 1.37 (3H, s, Me-27), 1.39 (3H, s,

Me-19), 1.02 (3H, d, *J* = 6.9 Hz, Me-21), 0.85 (3H, s, Me-18), signals for the sugar moiety, see Table 2; ^{13}C-NMR (C_5D_5N) δ 75.8 (C-1), 42.5 (C-2), 67.9 (C-3), 44.1 (C-4), 140.3 (C-5), 125.3 (C-6), 32.9 (C-7), 33.1 (C-8), 57.9 (C-9), 46.0 (C-10), 66.0 (C-11), 50.2 (C-12), 40.5 (C-13), 55.2 (C-14), 32.3 (C-15), 81.0 (C-16), 62.1 (C-17), 16.8 (C-18), 13.7 (C-19), 38.5 (C-20), 15.0 (C-21), 120.1 (C-22), 33.0 (C-23), 33.8 (C-24), 83.8 (C-25), 77.4 (C-26), 24.3 (C-27), signals for the sugar moiety, see Table 2; HR-ESI-TOF-MS (positive-mode) *m*/*z* 625.3607 $[M + H]^+$ (Calcd 625.3588 for $C_{33}H_{53}O_{11}$).

Cell culture and assay for cytotoxic activity

HSC-2 cells were maintained as monolayer cultures at 37 °C in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate in a humidified 5% CO_2 atmosphere. Cells were trypsinized and inoculated at 6×10^3 per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA, USA), and incubated for 24 h. After washing once with PBS, they were treated for 24 h without or with test compounds. The cells were washed once with PBS and incubated for 4 h with 0.2 mg/mL MTT in DMEM medium supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 mL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystems Multiskan^R (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10. The IC_{50} value, which reduces the viable cell number by 50%, was determined from the dose-response curve.

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