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Spirostanol saponins from the rhizomes of *Tacca chantrieri* and their cytotoxic activity

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Abstract

The rhizomes of *Tacca chantrieri* have been analysed for steroidal saponin constituents, resulting in the isolation of four new spirostanol saponins (**1**–**4**), along with one known saponin (**5**); their structures were elucidated on the basis of extensive spectroscopic analysis, including 2D NMR, and the results of hydrolytic cleavage. The isolated compounds were evaluated for their cytotoxic activity against HL-60 human promyelocytic leukemia cells. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Tacca chantrieri*; Taccaceae; Spirostanol saponins; Cytotoxic activity; HL-60 cells

1. Introduction

Tacca chantrieri André (Taccaceae) is a perennial plant that grows in southeastern China. Its rhizomes have been employed in traditional Chinese medicine for the treatment of gastric ulcer, enteritis, and hepatitis (Jiang Su New Medicinal College, 1977). Previously, we have reported the structural determination of two new diarylheptanoids and seven diarylheptanoid glycosides isolated from the rhizomes of *T. chantrieri*, some of which exhibited considerable cytotoxic activities against HL-60 human promyelocytic leukemia cells and HSC-2 human squamous carcinoma cells (Yokosuka et al., 2002). Further phytochemical examination of *T. chantrieri* rhizomes were carried out with particular attention to the steroidal glycosides, because a literature survey showed that some Taccaceae plants contained steroidal compounds (Zhou et al., 1983; Abdel-Aziz et al., 1990a,b). As a result, four new spirostanol saponins (**1**–**4**), along with one known saponin (**5**), were isolated. This paper deals with the structural elucidation of the new saponins on the basis of extensive spectroscopic analysis, including 2D NMR spectroscopic data, and the results of hydrolytic cleavage. The cytotoxic activity of the isolated saponins against HL-60 leukemia cells is also reported.

2. Results and discussion

A concentrated MeOH extract of *T. chantrieri* rhizomes was passed through a porous-polymer polystyrene resin (Diaion HP-20) column, and the MeOH eluate fraction, with enriched steroidal glycosides, was subjected to CC using silica gel and octadecylsilanized (ODS) silica gel to yield **1** (3.70 g), **2** (79.0 mg), **3** (9.0 mg), **4** (100 mg), and **5** (390 mg). Compound **5** was identified as (25*S*)-spirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside (Sati and Pant, 1985).

Compound **1** was isolated as an amorphous solid, $[\alpha]_D$ −86.0° ($CHCl_3$–MeOH, 1:1). The positive-ion HR–FABMS showed an accurate $[M+Na]^+$ ion at *m*/*z* 1053.5208, corresponding to the empirical molecular formula of $C_{51}H_{82}O_{21}$, which was also deduced by analysis of the ^{13}C NMR and DEPT spectral data. The glycosidic nature of **1** was suggested by strong IR absorption bands at 3400 and 1040 cm^{-1}. The ^1H NMR spectrum of **1** (C_5D_5N) exhibited two three-proton singlet signals at δ 1.04 and 0.82, indicating the presence of two angular methyl groups, as well as two three-proton doublet signals at δ 1.15 (*J* = 6.9 Hz) and 1.08

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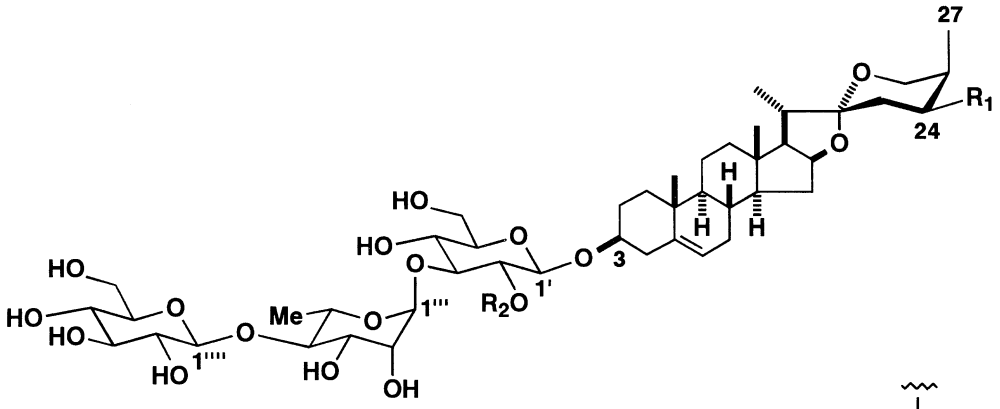
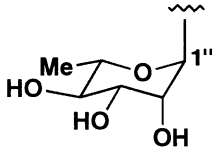
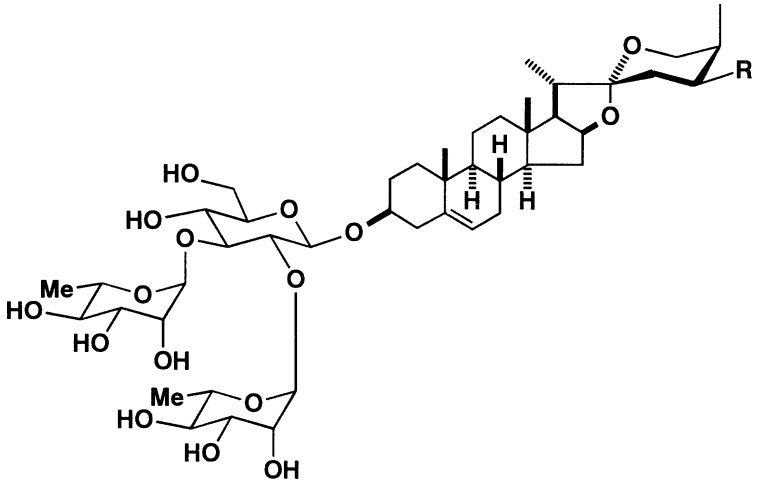
($J=7.1$ Hz) assignable to secondary methyl groups of the steroidal skeleton. In addition, signals for the anomeric protons of four monosaccharides and methyl groups of two deoxyhexopyranoses were observed at δ 5.81 (1H, *br s*), 5.76 (1H, *br s*), 5.24 (1H, *d*, $J=7.8$ Hz), and 4.90 (1H, *d*, $J=7.8$ Hz), and 1.76 (3H, *d*, $J=6.2$ Hz) and 1.69 (3H, *d*, $J=6.2$ Hz). Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) gave an aglycone identified as (25*S*)-spirost-5-en-3β-ol (yamogenin) (Agrawal et al., 1985), and D-glucose and L-rhamnose as the carbohydrate moieties. 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, with detection being carried out using a combination of refractive index (RI) and optical rotation (OR) detectors. The above data, along with four anomeric carbon signals at δ 106.5, 103.1, 102.6, and 99.9, implied that **1** was a (25*S*)-spirostanol tetraglycoside. Sequential assignments of the signals from H-1 to H_2-6 or Me-6 of each monosaccharide, including their multiplet patterns and coupling constants, in the ^1H NMR spectrum of **1**, were established by analysis of the ^1H–^1H COSY and 2D HOHAHA spectra (Table 1). Then, the HMQC spectrum was applied to associate the protons with the corresponding one-bond coupled carbon resonances. Comparison of the carbon chemical shift thus assigned with those of the reference methyl glycosides (Agrawal et al., 1985; Agrawal, 1992), taking into account the known effects of *O*-glycosylation, indicated that **1** contained a terminal β-D-glucopyranosyl, a terminal α-L-rhamnopyranosyl, a C-4 substituted α-L-rhamnopyranosyl, and a C-2 and C-3 disubstituted β-D-glucopyranosyl unit. The β-orientations of the anomeric centers of the two glucosyl moieties were supported by the relatively large J values of their anomeric protons



	R_1	R_2
1	H	Rha
2	OH	Rha
3	H	H

Rha =





	R
4	OH
5	H

Table 1
^{1}H and ^{13}C NMR chemical shift assignments of the tetraglycoside moiety of **1** in C_5D_5N

Position	^{1}H	J (Hz)	^{13}C
Glc 1′	4.90, *d*	7.8	99.9
2′	4.05, *dd*	9.3, 7.8	78.6
3′	4.18, *dd*	9.3, 9.1	86.3
4′	4.08, *dd*	9.1, 9.1	69.7
5′	3.78, *m*		78.0
6′a	4.44		
b			
Rha 1″	5.81, *br s*		102.6
2″	4.73, *br d*	3.1	72.5
3″	4.52, *dd*	3.1, 9.5	72.8
4″	4.31, *dd*	9.5, 9.5	73.8
5″	4.87, *dq*	9.5, 6.2	69.9
6″	1.76, *d*	6.2	18.7
Rha 1‴	5.76, *br s*		103.1
2‴	4.80, *br d*	3.1	72.1
3‴	4.58, *dd*	9.2, 3.1	72.4
4‴	4.44, *dd*	9.3, 9.2	84.5
5‴	4.83, *dq*	9.3, 6.2	68.7
6‴	1.69, *d*	6.2	18.3
Glc 1⁗	5.24, *d*	7.8	106.5
2⁗	4.10, *dd*	8.9, 7.8	76.4
3⁗	4.21, *dd*	8.9, 8.9	78.6
4⁗	4.28, *dd*	9.3, 8.9	71.4
5⁗	3.78, *ddd*	9.3, 6.7, 2.5	78.4
6⁗a	4.41, *dd*	12.2, 2.5	62.6
b	4.36, *dd*	12.2, 6.7	

(J = 7.8 Hz). For the rhamnosyl moieties, the large $^1J_{C,H}$ values (168.1 and 166.9 Hz) and three-bond coupled strong HMBC correlations from the anomeric proton to the C-3 and C-5 carbons (the dihedral angles between H-1 and C-3, and between H-1 and C-5 about 180°), indicated that each anomeric proton was equatorial thus possessing an α-pyranoid anomeric form (Jia et al., 1998). In the HMBC spectrum, correlation peaks were observed from δ 5.81 (H-1 of terminal rhamnosyl) to δ 78.6 (C-2 of 2,3-disubstituted glucosyl), δ 5.24 (H-1 of terminal glucosyl) to δ 84.5 (C-4 of substituted rhamnosyl), δ 5.76 (H-1 of substituted rhamnosyl) to δ 86.3 (C-3 of 2,3-disubstituted glucosyl), and δ 4.90 (H-1 of 2,3-disubstituted glucosyl) to δ 77.8 (C-3 of aglycone) (Fig. 1). Thus, the structure of **1** was determined to be (25*S*)-spirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside.

Compound **2** was shown to have the molecular formula $C_{51}H_{82}O_{22}$ on the basis of the positive-ion HR–FABMS (m/z 1069.5183 $[M+Na]^+$), ^{13}C NMR, and DEPT spectral data. The ^{1}H NMR spectrum contained signals for four steroid methyl groups at δ 1.31 (3H, *d*, J = 7.0 Hz), 1.17 (3H, *d*, J = 6.9 Hz), 1.02 (3H, *s*), and 0.80 (3H, *s*), and four anomeric protons at δ 5.80 (1H, *br s*), 5.74 (1H, *br s*), 5.23 (1H, *d*, J = 7.8 Hz), and 4.88 (1H, *d*, J = 7.9 Hz), as in **1**. Comparison of the ^{13}C NMR spectrum of **2** with that of **1** showed considerable structural similarity. However, the molecular formula of **2** was higher by one oxygen atom than that of **1** and differences were recognized in the carbon signals from the ring F portion (C-22–C-27). The ^{1}H–^{1}H COSY spectrum was carefully inspected to assign the structure of the ring F residue, with the three-proton doublet signal at δ 1.31 (J = 7.0 Hz) attributable to Me-27, being used as the starting point of analysis. As a result, the structural fragment of ring F, –$C_{(23)}H_2$–$C_{(24)}$H(–O–)–$C_{(25)}$H(–$C_{(26)}H_2$–O–)–$Me_{(27)}$, was revealed and the location of an oxygen atom at C-24 was evident. On treatment of **2** with Ac_2O in pyridine, eleven acetyl groups were introduced to the tetraglycoside residue and one acetyl to the aglycone moiety (**2a**). When the ^{1}H NMR spectrum of **2a** was compared to that of **2**, the H-24 oxymethine proton was shifted downfield by 0.88 ppm and was observed at δ 5.52, which was consistent with the presence of a C-24 hydroxyl group in **2**. The spin-coupling constants of H-24, $^3J_{H-24,H-23ax}$ = 11.6 Hz, $^3J_{H-24,H-23eq}$ = 5.1 Hz, and $^3J_{H-24,H-25}$ = 5.1 Hz, gave evidence for the 24*S* and 25*R* configurations. The structure of **2** was formulated as (24*S*,25*R*)-24-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*- [*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside.

Compound **3** was was found to be $C_{45}H_{72}O_{17}$ by combined positive-ion HR–FABMS (m/z 907.4692 $[M+Na]^+$) and ^{13}C NMR spectroscopic analyses, together with DEPT data. The ^{1}H NMR spectrum showed signals for three anomeric protons at δ 6.37 (1H, *br s*), 5.25 (1H, *d*, J = 7.8 Hz), and 4.92 (1H, *d*,

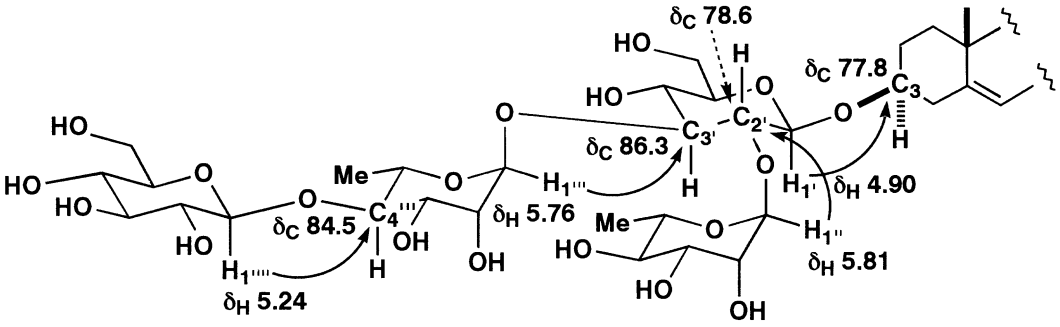


Fig. 1. HMBC correlations of the sugar moiety of **1**.

$J=7.8$ Hz), together with those for four steroid methyl groups at δ 1.15 (3H, *d*, $J=6.9$ Hz), 1.08 (3H, *d*, $J=7.1$ Hz), 0.87 (3H, *s*), and 0.82 (3H, *s*). Analysis of the ^{13}C NMR signals arising from the sugar moiety with the aid of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra revealed that **3** was a spirostanol triglycoside structurally related to **1** with the terminal rhamnosyl group attached at the inner glucosyl moiety being absent from **1**. This was confirmed by the result of acid hydrolysis of **3**, giving yamogenin, D-glucose and L-rhamnose, and by HMBC correlations from δ 5.25 (H-1 of terminal glucosyl) to δ 85.7 (C-4 of substituted rhamnosyl), δ 6.37 (H-1 of substituted rhamnosyl) to δ 82.4 (C-3 of inner glucosyl), and from δ 4.92 (H-1 of inner glucosyl) to δ 78.1 (C-3 of aglycone). The structure of **3** was determined to be (25*S*)-spirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranoside.

Compound **4** was deduced as $C_{45}H_{72}O_{17}$ from its positive-ion HR–FABMS (*m*/*z* 885.4810 $[M+H]^+$) and ^{13}C NMR spectroscopic analysis together with DEPT data. The ^{1}H NMR spectrum of **4** showed four steroid methyl signals at δ 1.32 (3H, *d*, $J=7.0$ Hz), 1.18 (3H, *d*, $J=7.0$ Hz), 1.03 (3H, *s*), and 0.81 (3H, *s*), and three anomeric proton signals at δ 5.87 (1H, *br s*), 5.76 (1H, *br s*), and 4.91 (1H, *d*, $J=7.8$ Hz). Analysis of the ^{13}C NMR spectrum of **4** and comparison with that of **2** implied that the aglycone of **4** was identical to that of **2**, but differed from **2** in terms of the glycoside structure. Two terminal α-L-rhamnopyranosyl units and a 2,3-branched β-D-glucopyranosyl unit were found to be included in the structure of **4** by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra. Accordingly, the triglycoside moiety of **4** was shown to be the same as that of **5** and the structure of **4** was assigned as (24*S*,25*R*)-24-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside.

The isolated compounds were evaluated for their cytotoxic activities against HL-60 leukemia cells. Compounds **1** and **5** showed considerable cytotoxicity with respective IC_{50} values of 1.8 and 2.1 μM, whereas etoposide used as positive control gave an IC_{50} of 0.37 μM. Compounds **2** and **4**, the corresponding C-24 hydroxy derivatives of **1** and **5**, and **3**, which is structurally related to **1** with a terminal rhamnosyl group linked to C-2 of the inner glucosyl residue absent from **1**, did not show any cell growth inhibitory activity at the sample concentration of 10 μg/ml, suggesting that the structures of both the aglycone and sugar moieties contribute to the cytotoxicity.

3. Experimental

3.1. General

FABMS: Finnigan MAT TSQ-700 (San Jose, CA, USA) (matrix: Magic Bullet, a mixture of dithiothreitol and dithioerythritol, 3:1; Tokyo-Kasei, Tokyo, Japan). NMR Bruker AM-500 (500 MHz for ^{1}H NMR, Karlsruhe, Germany). CC: Diaion HP-20 (Mitsubishi-Kasei, Tokyo, Japan); silica gel (Fuji-Silysia Chemical, Aichi, Japan); ODS silica gel (Nacalai Tesque, Kyoto, Japan). TLC: precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010) equipped with an RI detector (RI-8010, Tosoh, Tokyo, Japan) and an OR detecter (Shodex OR-2, Showa-Denko, Tokyo, Japan). The following reagents were obtained from the indicated companies: RPMI 1640 medium (Gibco, Grand Island, NY, USA); FBS (Bio-Whittaker, Walkersville, MD, USA); MTT (Sigma, St. Louis, MO, USA); penicillin and streptomycin (Meiji-Seika, Tokyo, Japan). All other chemicals used were of biochemical reagent grade.

3.2. Plant material

The rhizomes of *T. chantrieri* were collected at Yunnan Province, People's Republic of China in October 1996, and identified by one of the authors, Professor Y. Sashida. A voucher specimen has been deposited in the laboratory of Y. Sashida (voucher No. TC-96–003., Laboratory of Medicinal Plant Science).

3.3. Extraction and isolation

The plant material (7.3 kg) was extracted with hot MeOH (3 l×2). The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (115 g) was passed through a Diaion HP-20 column eluting with 30% aq MeOH, 50% aq MeOH, MeOH, EtOH, and EtOAc. Preliminary TLC analysis of these fractions suggested that the MeOH eluate portion contained several steroidal glycosides, on which further fractionation was carried out. CC of the MeOH eluate portion on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH (9:1; 4:1; 3:1; 2:1; 1:1), and finally with MeOH alone, gave five fractions (I–V). Fraction II was applied to a silica gel column which was eluted with $CHCl_3$–MeOH–H_2O (50:10:1) and ODS silica gel with MeOH–H_2O (4:1; 2:1) to give **3** (9.0 mg), **4** (100 mg), and **5** (390 mg). Fraction IV was subjected to CC on silica gel eluting with $CHCl_3$–MeOH–H_2O (40:10:1; 30:10:1; 20:10:1) and ODS silica gel with MeOH–H_2O (4:1; 2:1) to yield **1** (3.70 g) and **2** (79.0 mg).

3.4. Compound *1*

An amorphous solid. $[\alpha]_D^{25}$ −86.0° ($CHCl_3$–MeOH, 1:1; *c* 0.10). HR–FABMS (positive mode) *m*/*z*: 1053.5208 $[M+Na]^+$ ($C_{51}H_{82}O_{21}Na$ requires

1053.5246); IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2930 (CH), 1040; ^1H NMR (C_5D_5N): δ 5.81 (1H, *br s*, H-1″), 5.76 (1H, *br s*, H-1‴), 5.33 (1H, *br d*, $J=5.0$ Hz, H-6), 5.24 (1H, *d*, $J=7.8$ Hz, H-1⁗), 4.90 (1H, *d*, $J=7.8$ Hz, H-1′), 1.76 (3H, *d*, $J=6.2$ Hz, Me-6″), 1.69 (3H, *d*, $J=6.2$ Hz, Me-6‴), 1.15 (3H, *d*, $J=6.9$ Hz, Me-21), 1.08 (3H, *d*, $J=7.1$ Hz, Me-27), 1.04 (3H, *s*, Me-19), 0.82 (3H, *s*, Me-18); ^{13}C NMR (pyridine-d_5): δ 37.5 (C-1), 30.0 (C-2), 77.8 (C-3), 38.6 (C-4), 140.8 (C-5), 121.8 (C-6), 32.3 (C-7), 31.7 (C-8), 50.3 (C-9), 37.1 (C-10), 21.1 (C-11), 39.8 (C-12), 40.4 (C-13), 56.6 (C-14), 32.2 (C-15), 81.2 (C-16), 62.7 (C-17), 16.3 (C-18), 19.4 (C-19), 42.4 (C-20), 14.9 (C-21), 109.7 (C-22), 26.4 (C-23), 26.2 (C-24), 27.5 (C-25), 65.1 (C-26), 16.3 (C-27). Signals for the sugar moiety: Table 1.

3.5. Acid hydrolysis of *1*

A solution of **1** (10 mg) in 1 M HCl (dioxane–H_2O,1:1; 2 ml) was heated at 95 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and subjected to silica gel chromatography using a gradient mixture of $CHCl_3$–MeOH (19:1; 1:1) to give (25*S*)-spirost-5-en-3β-ol (3.0 mg) as the aglycone and a sugar fraction (2.7 mg). HPLC analysis of the sugar fraction under the following conditions showed the presence of D-glucose and L-rhamnose. Column: Kaseisorb LC NH_2-60–5 UG80 (4.6 mm i.d.×250 mm, 5 μm, Tokyo-Kasei, Tokyo, Japan); solvent: MeCN–H_2O (3:1); flow rate: 0.8 ml/min. R_t (min): 8.10 (L-rhamnose, negative optical rotation); 12.60 (D-glucose, positive optical rotation).

3.6. Compound *2*

An amorphous solid. $[\alpha]_D^{25}$ −108.0° ($CHCl_3$–MeOH, 1:1; *c* 0.10). HR–FABMS (positive mode) *m/z*: 1069.5138 $[M+Na]^+$ ($C_{51}H_{82}O_{22}Na$ requires 1069.5195); IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2930 (CH), 1040; ^1H NMR (C_5D_5N): δ 5.80 (1H, *br s*, H-1″), 5.74 (1H, *br s*, H-1‴), 5.32 (1H, *br d*, $J=4.8$ Hz, H-6), 5.23 (1H, *d*, $J=7.8$ Hz, H-1⁗), 4.88 (1H, *d*, $J=7.9$ Hz, H-1′), 4.64 (1H, *ddd*, $J=11.6$, 5.1, 5.1 Hz, H-24), 1.75 (3H, *d*, $J=6.2$ Hz, Me-6″), 1.69 (3H, *d*, $J=6.2$ Hz, Me-6‴), 1.31 (3H, *d*, $J=7.0$ Hz, Me-27), 1.17 (3H, *d*, $J=6.9$ Hz, Me-21), 1.02 (3H, *s*, Me-19), 0.80 (3H, *s*, Me-18); ^{13}C NMR (C_5D_5N): δ 37.4 (C-1), 30.0 (C-2), 77.8 (C-3), 38.6 (C-4), 140.7 (C-5), 121.8 (C-6), 32.2 (C-7), 31.6 (C-8), 50.2 (C-9), 37.1 (C-10), 21.0 (C-11), 39.8 (C-12), 40.4 (C-13), 56.6 (C-14), 32.1 (C-15), 81.5 (C-16), 62.3 (C-17), 16.2 (C-18), 19.3 (C-19), 42.5 (C-20), 14.8 (C-21), 111.4 (C-22), 35.9 (C-23), 66.4 (C-24), 35.8 (C-25), 64.5 (C-26), 9.7 (C-27), 99.8 (C-1′), 78.6 (C-2′), 86.2 (C-3′), 69.7 (C-4′), 78.0 (C-5′), 62.1 (C-6′), 102.6 (C-1″), 72.4 (C-2″), 72.7 (C-3″), 73.7 (C-4″), 69.9 (C-5″), 18.6 (C-6″), 103.1 (C-1‴), 72.0 (C-2‴), 72.3 (C-3‴), 84.3 (C-4‴), 68.7 (C-5‴), 18.2 (C-6‴), 106.4 (C-1⁗), 76.4 (C-2⁗), 78.5 (C-3⁗), 71.4 (C-4⁗), 78.4 (C-5⁗), 62.5 (C-6⁗).

3.7. Acetylation of *2*

Compound **2** (10 mg) was treated with Ac_2O (1 ml) and pyridine (1 ml) at room temperature for 12 h. After addition of H_2O (2 ml) into the reaction mixture followed by evaporation to dryness, it was chromatographed on silica gel eluting with hexane–Me_2CO (2:1) to give the corresponding dodecaacetate (**2a**, 10 mg).

3.8. Compound *2a*

An amorphous solid. $[\alpha]_D^{25}$ −76.0° ($CHCl_3$; *c* 0.10). IR ν_{max} (KBr) cm^{-1}: 2970 (CH), 1750 (C=O); ^1H NMR (C_5D_5N): δ 5.54 (1H, *br s*, H-1″), 5.52 (1H, *br s*, H-1‴), 5.52 (1H, *m*, H-24), 5.44 (1H, *br d*, $J=4.5$ Hz, H-6), 5.33 (1H, *d*, $J=7.9$ Hz, H-1⁗), 4.93 (1H, *d*, $J=7.8$ Hz, H-1′), 2.20, 2.18, 2.16, 2.15, 2.14, 2.07×2, 2.05, 2.04, 2.03×2, 2.01 (each 3H, *s*, Ac×12), 1.61 (3H, *d*, $J=6.1$ Hz, Me-6″), 1.47 (3H, *d*, $J=6.2$ Hz, Me-6‴), 1.16 (3H, *d*, $J=6.9$ Hz, Me-21), 1.10 (3H, *s*, Me-19), 1.10 (3H, *d*, $J=7.0$ Hz, Me-27), 0.85 (3H, *s*, Me-18).

3.9. Compound *3*

An amorphous solid. $[\alpha]_D^{25}$ −86.0° ($CHCl_3$–MeOH, 1:1; *c* 0.10). HR–FABMS (positive mode) *m/z*: 907.4692 $[M+Na]^+$ ($C_{45}H_{72}O_{17}Na$ requires 907.4667); IR ν_{max} (KBr) cm^{-1}: 3200 (OH), 2930 (CH), 1070; ^1H NMR (C_5D_5N): δ 6.37 (1H, *br s*, H-1‴), 5.30 (1H, *br d*, $J=5.1$ Hz, H-6), 5.25 (1H, *d*, $J=7.8$ Hz, H-1⁗), 4.92 (1H, *d*, $J=7.8$ Hz, H-1′), 1.77 (3H, *d*, $J=6.2$ Hz, Me-6‴), 1.15 (3H, *d*, $J=6.9$ Hz, Me-21), 1.08 (3H, *d*, $J=7.1$ Hz, Me-27), 0.87 (3H, *s*, Me-19), 0.82 (3H, *s*, Me-18); ^{13}C NMR (C_5D_5N): δ 37.4 (C-1), 30.1 (C-2), 78.1 (C-3), 39.1 (C-4), 140.8 (C-5), 121.8 (C-6), 32.2 (C-7), 31.6 (C-8), 50.2 (C-9), 37.0 (C-10), 21.1 (C-11), 39.8 (C-12), 40.4 (C-13), 56.6 (C-14), 32.1 (C-15), 81.1 (C-16), 62.7 (C-17), 16.3 (C-18), 19.3 (C-19), 42.4 (C-20), 14.8 (C-21), 109.7 (C-22), 26.4 (C-23), 26.2 (C-24), 27.5 (C-25), 65.1 (C-26), 16.3 (C-27), 102.2 (C-1′), 75.9 (C-2′), 82.4 (C-3′), 69.5 (C-4′), 78.3 (C-5′), 62.4 (C-6′), 102.1 (C-1‴), 72.6 (C-2‴), 72.0 (C-3‴), 85.7 (C-4‴), 68.0 (C-5‴), 18.6 (C-6‴), 106.8 (C-1⁗), 76.6 (C-2⁗), 78.7 (C-3⁗), 71.3 (C-4⁗), 78.5 (C-5⁗), 62.5 (C-6⁗).

3.10. Acid hydrolysis of *3*

Compound **3** (4.7 mg) was subjected to acid hydrolysis as described for **1** to give (25*S*)-spirost-5-en-3β-ol (1.3 mg) and a sugar fraction (1.6 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.

3.11. Compound **4**

An amorphous solid. $[\alpha]_D^{25}$ −112.0° ($CHCl_3$–MeOH, 1:1; *c* 0.10). HR–FABMS (positive mode) *m/z*: 885.4810 $[M+H]^+$ ($C_{45}H_{73}O_{17}$ requires 885.4848); IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 2930 (CH), 1040; ^1H NMR (C_5D_5N): δ 5.87 (1H, *br s*, H-1″), 5.76 (1H, *br s*, H-1‴), 5.32 (1H, *br d*, *J* = 5.0 Hz, H-6), 4.91 (1H, *d*, *J* = 7.8 Hz, H-1′), 4.64 (1H, *ddd*, *J* = 11.6, 5.0, 5.0 Hz, H-24), 1.76 (3H, *d*, *J* = 6.2 Hz, Me-6″), 1.65 (3H, *d*, *J* = 6.2 Hz, Me-6‴), 1.32 (3H, *d*, *J* = 7.0 Hz, Me-27), 1.18 (3H, *d*, *J* = 7.0 Hz, Me-21), 1.03 (3H, *s*, Me-19), 0.81 (3H, *s*, Me-18); ^{13}C NMR (C_5D_5N): δ 37.5 (C-1), 30.0 (C-2), 77.8 (C-3), 38.7 (C-4), 140.7 (C-5), 121.8 (C-6), 32.3 (C-7), 31.7 (C-8), 50.2 (C-9), 37.1 (C-10), 21.1 (C-11), 39.8 (C-12), 40.4 (C-13), 56.6 (C-14), 32.1 (C-15), 81.5 (C-16), 62.4 (C-17), 16.3 (C-18), 19.4 (C-19), 42.5 (C-20), 14.8 (C-21), 111.4 (C-22), 36.0 (C-23), 66.5 (C-24), 35.9 (C-25), 64.5 (C-26), 9.7 (C-27), 99.9 (C-1′), 78.3 (C-2′), 87.4 (C-3′), 69.9 (C-4′), 78.1 (C-5′), 62.3 (C-6′), 102.6 (C-1″), 72.5 (C-2″), 72.8 (C-3″), 73.8 (C-4″), 69.9 (C-5″), 18.7 (C-6″), 103.9 (C-1‴), 72.6 (C-2‴), 72.5 (C-3‴), 73.6 (C-4‴), 70.6 (C-5‴), 18.4 (C-6‴).

3.12. Cell culture assay

HL-60 cells (JCRB 0085, Human Science Research Resources Bank, Osaka, Japan) were maintained in the RPMI 1640 medium containing 10% FBS supplemented with L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The leukemia cells were washed and resuspended in the above medium to 4×10^4 cells/ml, and 196 μl of this cell suspension was placed in each well of a 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan). The cells were incubated in 5% CO_2/air for 24 h at 37 °C. After incubation, 4 μl of EtOH–H_2O (1:1) solution containing the sample was added to give the final concentrations of 0.1–10 μg/ml and 4 μl of EtOH-H_2O (1:1) was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using a modified MTT reduction assay (Sargent and Taylor, 1989). Briefly, after termination of the cell culture, 10 μl of 5 mg/ml MTT in phosphate buffered saline was added to every well and the plate was further reincubated in 5% CO_2/air for 4 h at 37 °C. The plate was then centrifuged at 1500 *g* for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μl of the supernatant was removed from every well, and 175 μl of DMSO was added to dissolve the MTT formazan crystals. The plate was mixed on a microshaker for 10 min, and then read on a microplate reader (Immuno-Mini NJ-2300, Inter Med, Tokyo, Japan) at 550 nm. A dose–response curve was plotted for **1** and **5**, which showed more than 50% of cell growth inhibition at the sample concentration of 10 μg/ml, and the concentration giving 50% inhibition (IC_{50}) was calculated.

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