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STEROIDAL GLYCOSIDES FROM THE UNDERGROUND PARTS OF *HOSTA PLANTAGINEA* VAR. *JAPONICA* AND THEIR CYTOSTATIC ACTIVITY ON LEUKAEMIA HL-60 CELLS

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Key Word Index—*Hosta plantaginea* var. *japonica*; Liliaceae; steroidal glycosides; steroidal saponins; C_{22}-steroid glycoside; cytostatic activity; HL-60 cells.

Abstract—A new C_{22}-steroid glycoside was isolated from the underground parts of *Hosta plantaginea* var. *japonica*, together with a known furostanol saponin and three known spirostanol saponins. The structure of the new steroid glycoside was characterized by spectroscopic analysis and acid-catalysed hydrolysis as 2α,3β,16β-trihydroxy-5α-pregn-20(21)-ene-carboxylic acid γ-lactone 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}. The isolated compounds were assayed for their cytostatic activity on leukaemia HL-60 cells. The spirostanol saponins showed cytostatic activity in a dose-dependent manner with the IC_{50} values ranging between 1 and 3 μg ml^{-1}.

INTRODUCTION

The genus *Hosta* contains *ca* 20 species and has a distribution in east Asia. We have already examined the underground parts of *H. longipes* and isolated a total of 12 steroidal saponins [1, 2], which appeared to be the first examples of the steroidal saponins from the *Hosta* species. As a part of our contribution to the study of this genus, we have now established phytochemical screening of the underground parts of *H. plantaginea* var. *japonica*, resulting in the isolation of a new C_{22}-steroid triglycoside, together with a known furostanol saponin and three known spirostanol saponins. This paper reports the identification of the known steroidal saponins and the structural determination of the new steroid glycoside based on spectroscopic data including various two-dimensional NMR techniques and the result of acid-catalysed hydrolysis. Cytostatic activity of the isolated compounds on leukaemia HL-60 cells is also described.

RESULTS AND DISCUSSION

A methanol extract of the fresh underground parts of *H. plantaginea* var. *japonica* was partitioned between 1-butanol and water. The 1-butanol-soluble phase was fractionated by a combination of Diaion HP-20, silica gel and octadecylsilanized (ODS) silica gel column chromatographies to give compounds **1–5**.

The structures of **1–4** were identified as (25*R*)-22-*O*-methyl-5α-furostane-2α,3β,22ξ,26-tetrol 26-*O*-β-D-glucopyranoside [3], (25*R*)-5α-spirostane-2α,3β-diol (gitogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} [4], gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} [5–7] and gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} [7], respectively. The ^{13}C NMR data indicated that small amounts of the corresponding 25*S*-isomer coexisted in **1** and **2** (Table 1). Copies of the original spectra are obtainable from the authors.

Compound **5** was obtained as an amorphous solid, $[\alpha]_D$ −32.5° (methanol). The molecular formula ($C_{40}H_{62}O_{19}$) was determined by the FAB mass spectra (positive-FAB *m/z* 869 $[M + Na]^+$; negative-FAB *m/z* 846 $[M]^-$) and elemental analysis. (Found: C, 55.21; H, 7.40. Calc.: C, 55.55; H, 7.26.) The ^1H NMR spectrum of **5** in pyridine-d_5 featured signals for an exo-methylene group at δ 6.35 and 5.51 (each *br s*), two angular methyl groups at δ 0.67 and 0.58 (each *s*) and three anomeric protons at δ 5.28 (*d*, *J* = 7.4 Hz), 5.16 (*d*, *J* = 7.8 Hz) and 4.94 (*d*, *J* = 7.9 Hz). Acid hydrolysis of **5** with 1 M hydrochloric acid in dioxane–water gave D-glucose and D-galactose, in a ratio of 2:1,

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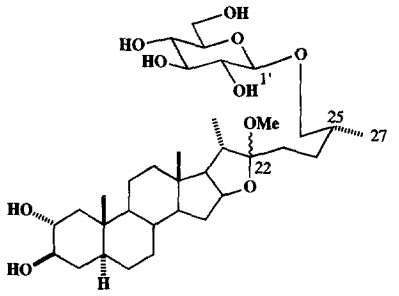

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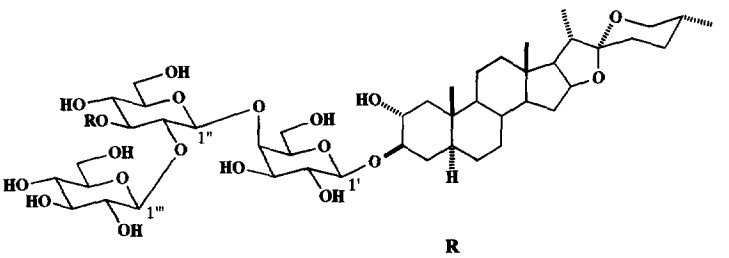
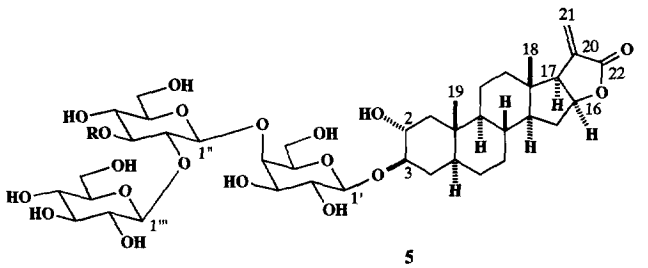


	R
2	H
3	β-D-Xyl*p*
4	α-L-Rha*p*-(1→4)-β-D-Xyl*p*



5

as the carbohydrate compounds. The ^{13}C NMR spectrum showed a total of 40 resonance lines, 18 of which could be assigned to two glucoses and one galactose, and three anomeric carbons were observed at δ 106.9, 105.1 and 103.4. This implied a $C_{22}H_{32}O_4$ molecular formula for the aglycone moiety, which allowed us to confirm **5** to be a C_{22}-steroid.

Attempted analysis of the ^1H–^1H COSY in conjunction with the HOHAHA data for **5**, which were recorded in a mixed solvent of pyridine-d_5 and methanol-d_4 (11:1) to remove exchangeable protons and minimize signal overlap, led to the sequential assignment of the proton resonances stemming from 1-H_2 to 17-H of the steroid skeleton as shown in Table 2. The ^{13}C shifts could be assigned by detecting $^1J_{C,H}$ correlations in the HMQC spectrum. The NMR data thus assigned indicated the location of oxygen atoms at the C-2, C-3 and C-16 positions on the steroid skeleton. Further information was obtained from the HMBC spectrum. The quaternary carbon signal observed at δ 44.3 showed $^2J_{C,H}$ and $^3J_{C,H}$ correlation peaks with the proton signals at δ 4.84 (*ddd*, J = 7.9, 7.9 and 5.4 Hz, 16-H), 2.76 (*br d*, J = 7.9 Hz, 17-H), 2.13 (15α-H), 1.45 (11 eq-H) and 0.57 (*s*, 18-Me) and was assigned to C-13. Another quaternary carbon signal at δ 36.9 was assignable to C-10, which was correlated to the proton signals at δ 2.12 (*dd*, J = 12.9 and 4.8 Hz, 1 eq-H), 1.10 (*dd*, J = 12.9 and 11.4 Hz, 1 ax-H), 1.83 (*ddd*, J = 12.8, 4.9 and 2.6 Hz, 4 eq-H) and 0.68 (*s*, 19-Me). The correlation peaks from each of the exomethylene proton signals at δ 6.33 and 5.55 (each *br s*) to C-17 at δ 55.4, and from 17-H at δ 2.76 to the exomethylene carbon signals at δ 137.8 (quaternary) and 122.0 (methylene) allowed the location of the exomethylene group to be assigned to C-20(21). The $^3J_{C,H}$ correlations from the exomethylene protons at δ 6.33 and 5.55 to δ 171.5 assignable to an ester carbonyl carbon indicated that the ester carbonyl group was conjugated with the exomethylene group. Unfortunately, no correlation was detectable between the ester carbonyl carbon (C-22) and 16-H in the HMBC spectra measured in the two $^nJ_{C,H}$ parameters optimized for 8 and 5 Hz. However, the formation of a γ-lactone ring between C-16 and C-22 was confirmed by the characteristic IR absorption at 1755 cm^{-1}, the prominent deshielded shift of the 16-H proton appearing at δ 4.84, and by seven degrees of unsaturation calculated from the molecular formula $C_{22}H_{32}O_4$; the conjugated carbonyl group consumed two degrees and consequently the aglycone was concluded to have a five-ring system. Thus, the plane structure of the aglycone moiety of **5** was assigned as shown in Fig. 1.

The A/B *trans* ring junction was shown by the ^{13}C shift of the C-19 methyl at δ 13.3 [8, 9]. The NOE correlations, 9-H/5-H and 14-H, 12ax-H/17-H, 14-H/16-H and 17-H, in the phase-sensitive NOESY spectrum, made the relative stereochemistry, B/C *trans*, C/D *trans* and D/E *cis* (16α-H/17α-H) ring junctions. The 2α and 3β orientations of the oxygen atoms were deduced from the multiplicities of the 2-H proton, $^3J_{2\text{-H,1ax-H}} = 11.4$ Hz, $^3J_{2\text{-H,1eq-H}} = 4.8$ Hz and $^3J_{2\text{-H,3-H}} = 9.0$ Hz. The 3β orientation was further supported by the NOE correlations from 3-H to 1α(ax)-H and 5α(ax)-H (Fig. 2). Accordingly, the aglycone structure was formulated as 2α,3β,16β-trihydroxy-5α-pregn-20(21)-ene-carboxylic acid γ-lactone.

Next, our attention was focused on the triglycoside structure and its linkage position to the aglycone in **5**. Inspection of the the ^1H–^1H COSY spectrum combined with the HOHAHA data allowed the sequential assign-

Table 1. ^{13}C NMR spectral data for compounds **1–5***

C	**1**	(25*S*)	**2**	(25*S*)	**3**	**4**
1	46.5		45.6		45.6	45.6
2	73.0		70.4		70.4	70.4
3	76.7		84.7		84.3	84.3
4	37.2		34.1		34.1	34.1
5	45.2		44.6		44.6	44.6
6	28.3		28.1		28.1	28.1
7	32.1[a]		32.2[a]		32.2[a]	32.2[a]
8	34.6		34.6		34.6	34.6
9	54.6		54.4		54.4	54.3
10	37.5		36.9		36.9	36.9
11	21.4		21.4		21.4	21.4
12	40.0		40.0		40.1	40.0
13	41.1		40.8		40.8	40.8
14	56.3		56.3		56.3	56.3
15	32.3[a]		32.1[a]		32.1[a]	32.1[a]
16	81.3		81.2		81.3	81.3
17	64.3		63.0	(62.8)	63.0	63.0
18	16.3		16.6		16.6	16.6
19	13.7		13.4		13.4	13.4
20	40.5		42.0	(42.4)	42.0	42.0
21	16.5		15.0	(14.8)	15.0	15.0
22	112.6		109.2	(109.7)	109.2	109.2
23	30.8	(31.0)	31.8	(26.4)	31.8	31.8
24	28.2		29.2	(26.2)	29.3	29.3
25	34.2	(34.5)	30.6	(27.5)	30.6	30.6
26	75.2	(74.9)	66.8	(65.1)	66.9	66.9
27	17.1	(17.5)	17.3	(16.3)	17.3	17.3
OMe	47.2	(47.3)				
1′	105.0	(105.1)	103.4		103.3	103.3
2′	75.2		72.8		72.5	72.5
3′	78.5[b]		75.5		75.7	75.7
4′	71.7		80.9		79.4	79.4
5′	78.6[b]		75.5		76.1	76.1
6′	62.9		60.5		60.6	60.6
1″			105.1		104.8[b]	104.7
2″			86.0		81.1	81.1
3″			78.5		87.0	86.7
4″			71.8		70.4	70.4
5″			78.2		77.6	77.5
6″			63.2		62.7	62.7
1‴			106.9		104.7[b]	104.7
2‴			76.6		75.1	75.2
3‴			77.8		78.5	78.5
4‴			70.5		71.4	71.4
5‴			79.0		78.1	78.1
6‴			61.8		63.0	62.9
1⁗					105.0	104.7
2⁗					75.5	75.5
3⁗					78.7	74.8
4⁗					70.8	76.1
5⁗					67.3	64.1
1⁗′						99.8
2⁗′						72.5
3⁗′						72.5
4⁗′						73.9
5⁗′						70.0
6⁗′						18.6

*Spectra were measured in pyridine-d_5.
[a,b]Assignments may be interchanged.

Table 2. ^1H and ^{13}C NMR chemical shift assignment of **5***

Position	^1H	*J* (Hz)	^{13}C
1 ax	1.10 *dd*	12.9, 11.4	45.6
1 eq	2.12 *dd*	12.9, 4.8	
2	3.91 *ddd*	11.4, 9.0, 4.8	70.5
3	3.78		84.5
4 ax	1.46		34.0
4 eq	1.83 *ddd*	12.8, 4.9, 2.6	
5	1.00		44.7
6 ax	1.07		28.0
6 eq	1.16		
7 ax	0.78 *dddd*	12.3, 12.3, 12.3, 4.1	32.2
7 eq	1.47		
8	1.28		34.6
9	0.58 *ddd*	11.8, 11.8, 4.0	54.3
10			36.9
11 ax	1.13		21.0
11 eq	1.45		
12 ax	1.11		38.2
12 eq	1.67 *br d*	11.5	
13			44.3
14	0.97		54.5
15a	2.13		33.3
15b	1.43		
16	4.84 *ddd*	7.9, 7.9, 5.4	82.0
17	2.76 *br d*	7.9	55.4
18	0.57 *s*		14.5
19	0.68 *s*		13.3
20			137.8
21a	6.33 *br s*		122.0
21b	5.55 *br s*		
22			171.5
1′	4.87 *d*	7.9	103.2
2′	4.44 *dd*	9.7, 7.9	72.6
3′	4.06 *dd*	9.7, 3.7	75.3
4′	4.50 *br d*	3.7	80.8
5′	4.03		75.5
6′	4.61 *dd*	10.6, 8.8	60.5
	4.18		
1″	5.06 *d*	7.8	105.0
2″	4.02		85.5
3″	4.17 *dd*	8.9, 8.9	78.3
4″	3.88		71.6
5″	3.88		78.1
6″	4.51 *br d*	12.2	63.1
	4.04		
1‴	5.18 *d*	7.7	106.8
2‴	3.96 *dd*	8.9, 7.7	76.4
3‴	4.05 *dd*		77.6
4‴	4.08 *dd*	8.9, 8.9	70.4
5‴	3.80		78.9
6‴	4.51 *br d*	12.2	61.7
	4.31 *dd*	12.2, 4.1	

*Spectra were measured in pyridine-d_5–methanol-d_4 (11:1).

ment of the proton resonances for each monosaccharide to be made, starting from the easily distinguished anomeric protons. The HMQC spectrum correlated all the ^1H resonances with those of the corresponding one-bond coupled carbons. Comparison of the ^{13}C NMR shifts thus assigned with those of the reference methyl glycosides [8, 10], taking into account the

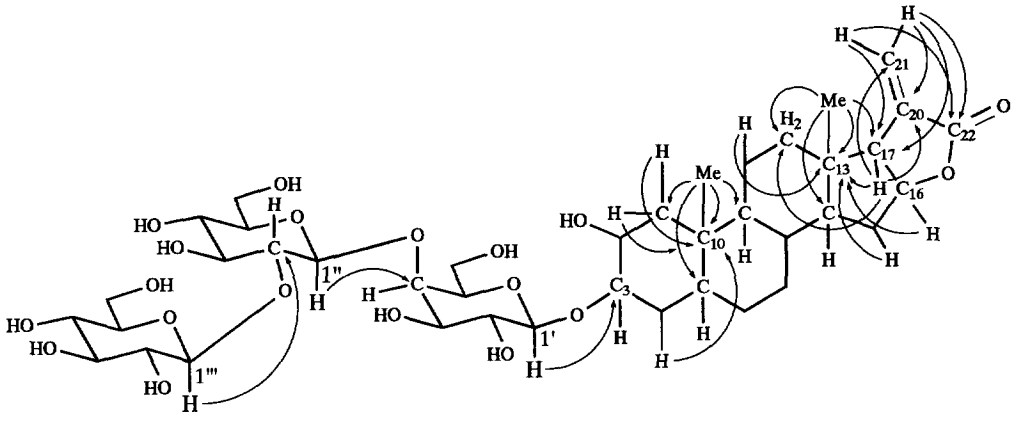
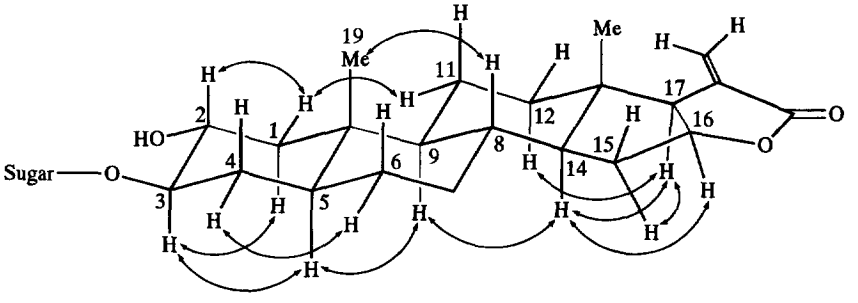


Fig. 1. HMBC correlations of **5** in pyridine-d_5–methanol-d_4 (11:1).



$^3J_{2\text{-H,1ax-H}} = 11.4$ Hz, $^3J_{2\text{-H,1eq-H}} = 4.8$ Hz, $^3J_{2\text{-H,3-H}} = 9.0$ Hz

Fig. 2. NOE correlations of **5** in pyridine-d_5–methanol-d_4 (11:1).

known effects of *O*-glycosylation and the result of acid hydrolysis, indicated that **5** contained a terminal β-D-glucopyranosyl unit (δ 106.8, 76.4, 77.6, 70.4, 78.9 and 61.7), a 2-substituted β-D-glucopyranosyl unit (δ 105.0, 85.8, 78.3, 71.6, 78.1 and 63.1) and a 4-substituted β-D-galactopyranosyl unit (δ 103.2, 72.6, 75.3, 80.8, 75.5 and 60.5). In the HMBC spectrum, the anomeric proton signals at δ 5.18 (*d*, $J = 7.7$ Hz, terminal glucose), 5.06 (*d*, $J = 7.8$ Hz, 2-substituted glucose) and 4.87 (*d*, $J = 7.9$ Hz, 4-substituted galactose) exhibited correlations with the carbon signals at δ 85.8 (C-2 of 2-substituted glucose), 80.8 (C-4 of 4-substituted galactose) and 84.5 (C-3 of aglycone), respectively, leading to the triglycoside structure as *O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside and its linkage to the C-3 hydroxyl group of the aglycone. Finally, the full structure of **5** was characterized as 2α,3β,16β-trihydroxy-5α-pregn-20(21)-ene-carboxylic acid γ-lactone 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

A survey of the literature resulted in finding two examples of a C_{22}-steroid, vespertilin [11] and 22*S*-hydroxyvespertilin [12] from *Solanum vespertilio*. However, no publication could be traced concerning the glycoside of a C_{22}-steroid. Thus, to the best of our knowledge, **5** is the first example of a C_{22}-steroid glycoside.

The isolated compounds were examined for their cytostatic activity on human promyelocytic leukaemia HL-60 cells (Fig. 3). The results are shown in Table 3.

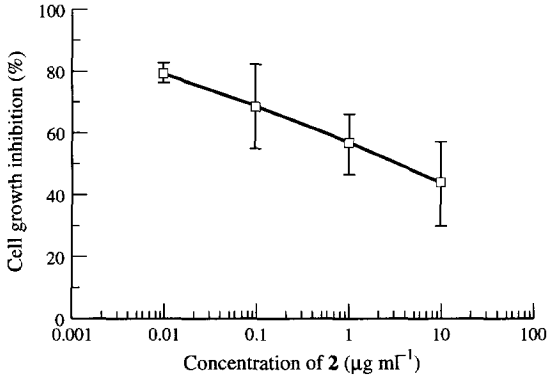


Fig. 3. Cytostatic activity of **2** on leukaemia HL-60 cells. HL-60 cells were incubated in the presence of various concentrations (0.01–10 μg ml^{-1}) of **2** for 72 hr. The percentages of cell growth (ordinate) vs **2** concentrations (abscissa) are plotted. The results are shown as ±S.D. of three or four experiments.

Table 3. Cytostatic activity of the isolated compounds on leukaemia HL-60 cells

Compounds	IC_{50} ($\mu g\ ml^{-1}$)
1	>10.0
2	2.9
3	1.0
4	1.7
5	>10.0

The spirostanol saponins (**2**–**4**) showed cytostatic activity in a dose-dependent manner; IC_{50} values ranging between *ca* 1 and 3 $\mu g\ ml^{-1}$, while the furostanol saponin (**1**) and C_{22}-steroid (**5**) showed no activity ($IC_{50} > 10\ \mu g\ ml^{-1}$).

EXPERIMENTAL

General. NMR (ppm, *J* Hz): 1D (Bruker AM-400, 400 MHz for ^1H NMR) and 2D (Bruker AM-500 or JEOL A-500, 500 MHz for ^1H NMR). CC: silica gel (Fuji-Silysia Chemical), ODS silica gel (Nacalai Tesque) and Diaion HP-20 (Mitsubishi-Kasei). TLC: precoated Kieselgel 60 F_{254} (0.25 mm, Merck) and RP-18 $F_{254}S$ (0.25 mm, Merck). HPLC: Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000) equipped with a TSK-gel ODS-Prep column (Tosoh, 250 × 4.6 mm i.d., ODS, 5 μm). Microplate reader: Immuno-Mini NJ-2300 (Inter Med, Japan). HL-60 cells: ICN Biomedicals, Japan. RPMI 1640 medium: Gibco, U.S.A. All other chemicals used were of biochemical-reagent grade.

Plant material. Hosta plantaginea var. *japonica* was purchased from Nichi-En, Japan, and the plant specimen is deposited in our laboratory.

Extraction and isolation. Plant material (fresh wt 4.8 kg) was extracted with hot MeOH. The MeOH extract was concd under red. pres., and the viscous concentrate was partitioned between H_2O and *n*-BuOH. CC of the *n*-BuOH-soluble phase on silica gel and elution with a gradient of $CHCl_3$–MeOH, and finally with MeOH, gave 5 frs (I–V). Fr. III was further divided by subjecting it to silica gel CC, eluting with $CHCl_3$–MeOH (4:1) into frs IIIa and IIIb. Fr. IIIa was subjected to CC on ODS silica gel eluting with MeOH–H_2O (8:3), and on silica gel with $CHCl_3$–MeOH (6:1) to give **1** (230 mg). Fr. IIIb was subjected to CC on ODS silica gel CC, eluting with MeOH–H_2O (4:1), and on silica gel with $CHCl_3$–MeOH–H_2O (40:10:1) to give **2** (307 mg). Fr. IV was passed through a Diaion HP-20 column, eluting with H_2O with increasing concns of MeOH in H_2O and finally with MeOH only. The 80% MeOH eluate fr. was subjected to CC on silica gel, eluting with $CHCl_3$–MeOH–H_2O (30:10:1; 20:10:1), and on ODS silica gel with MeOH–H_2O (2:1) and MeCN–H_2O (1:3) to yield **5** (14.8 mg). The MeOH eluate fr. was subjected CC on to ODS silica gel with MeOH–H_2O (4:1) and MeCN–H_2O (5:8), and on silica gel with $CHCl_3$–MeOH–H_2O (40:10:1) to yield **3** (42.6 mg) and **4** (12.3 mg). The spectral data for **1**–**4** were consistent with indicated lit. values in the text.

Compound **5**. Amorphous solid, $[\alpha]_D^{26}$ −32.5° (MeOH; *c* 0.10). Anal. calc. for $C_{40}H_{62}O_{19} \cdot H_2O$: C, 55.55; H, 7.46. Found: C, 55.21; H, 7.40. Positive-FABMS *m/z* 869 $[M+Na]^+$; Negative-FABMS *m/z* 846 $[M]^-$; IR ν_{max}^{KBr} cm^{-1}: 3405 (OH), 2930 and 2860 (CH), 1755 (C=O), 1445, 1410, 1380, 1305, 1285, 1260, 1070, 1035, 920, 885, 815, 695; ^1H NMR (pyridine-d_5): δ 6.35 and 5.51 (each 1H, *br s*, 21-H_2), 5.28 (1H, *d*, *J* = 7.4 Hz, 1‴-H), 5.16 (1H, *d*, *J* = 7.8 Hz, 1″-H), 4.94 (1H, *d*, *J* = 7.9 Hz, 1′-H), 4.82 (1H, *ddd*, *J* = 7.8, 7.8, 5.5 Hz, 16-H), 2.75 (1H, *br d*, *J* = 7.8 Hz, 17-H), 0.67 (3H, *s*, 19-Me), 0.58 (3H, *s*, 18-Me).

Acid hydrolysis of **5**. A soln of **5** (3.0 mg) in 1 M HCl [dioxane–H_2O (1:1), 1 ml] was heated at 100° for 2 hr under Ar. After cooling, the reaction mixt. was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column and subjected to CC on silica gel eluting with a gradient of $CHCl_3$–MeOH (19:1; 1:1) to give several unidentified compounds and a mixt. of monosaccharides (1.1 mg). The monosaccharide mixt. (1.1 mg) was diluted with H_2O (1 ml) and treated with (−)-α-methylbenzylamine (5 mg) and $Na[BH_3CN]$ (8 mg) in EtOH (1 ml) at 40° for 4 hr, followed by acetylation with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixt. was passed through a Sep-Pak C_{18} cartridge (Waters) with H_2O–MeCN (4:1; 1:1; 1:9, each 10 ml). The H_2O–MeOH (1:9) eluate fr. was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixt. of 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides [13, 14], which was then analysed by HPLC under the following conditions: solvent, MeCN–H_2O (2:3); flow rate, 0.8 ml min^{-1}; detection, UV 230 nm. The derivatives of D-galactose and D-glucose were detected. R_t (min): D-galactose, 19.66; D-glucose, 23.18.

Cell culture and assay for cytostatic activity. HL-60 cells were maintained in RPMI 1640 medium containing 10% foetal bovine serum supplemented with L-glutamine, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin. Leukaemia cells were washed and resuspended in the above medium to 3×10^4 cells ml^{-1}, and 196 μl cell suspension was placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 hr at 37° in 5% CO_2/air. After incubation, 4 μl EtOH soln containing the sample were added to give final concns of 0.01–10 $\mu g\ ml^{-1}$; 4 μl of EtOH was added to control wells. The cells were incubated for a further 72 hr in the presence of each agent, and then cell growth was evaluated with an MTT assay procedure. The MTT assay was carried out according to a modified method of ref. [15] as follows. After termination of cell culture, 10 μl MTT (5 $\mu g\ ml^{-1}$) in phosphate buffered saline was added to each well and the plate reincubated at 37° in 5% CO_2/air for a further 4 hr. The plate was then centrifuged at 1500 *g*

for 5 min to ppt. cells and formazan. Supernatant (150 μl) was removed from every well, and 175 μl DMSO was added to dissolve formazan crystals. The plate was mixed on a microshaker for 10 min and then read on a microplate reader at 550 nm. A dose–response curve was plotted for each sample, and a concn which gave IC_{50} was calcd.

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