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STEROIDAL SAPONINS FROM THE UNDERGROUND PARTS OF *RUSCUS ACULEATUS* AND THEIR CYTOSTATIC ACTIVITY ON HL-60 CELLS

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Key Word Index—*Ruscus aculeatus*; Liliaceae; underground parts; steroidal saponins; spirostanol saponins; furostanol saponins; cytostatic activity; HL-60 cells.

Abstract—Phytochemical examination of the underground parts of *Ruscus aculeatus* has been undertaken as part of a systematic study of plants of the Liliaceae. Six new spirostanol saponins and five new furostanol saponins were isolated, and their structures were assigned on the basis of spectroscopic analysis, including two-dimensional NMR techniques, and hydrolysis. Ruscogenin diglycoside with three acetyl groups attached to the inner galactosyl moiety and its corresponding 26-glucosyloxyfurostanol saponin showed cytostatic activity on leukemia HL-60 cells.

INTRODUCTION

Ruscus aculeatus belongs to the subfamily Asparagoideae in the Liliaceae and is a widely distributed European plant. An alcoholic extract of its rhizome has been used for the treatment of some veinous ailments for decades. Previous chemical analysis made on the plant disclosed the occurrence of a series of steroidal sapogenins and saponins based upon spirost-5-ene-1β,3β-diol [1–8], and sterols and triterpenes [9] in the rhizomes, among which aculeoside A, a bisdesmosidic spirostanol saponin isolated by us is unique in structure having 6-deoxy-D-glycero-L-threo-4-hexosulose as a carbohydrate component [7]. Our continuing investigation of the underground parts of *R. aculeatus* has led to the isolation of six new spirostanol saponins and five new furostanol saponins. In this report, we deal with the structural assignment of the new saponins on the basis of spectroscopic analysis, including two-dimensional NMR techniques, and hydrolysis. The cytostatic activity of the isolated saponins on leukemia HL-60 cells is also described.

RESULTS AND DISCUSSION

Fresh underground parts of the plant material (3.1 kg) were extracted with hot methanol. The crude extract was partitioned between water and 1-butanol. The 1-butanol-soluble phase was fractionated through the combined use of repeated column chromatography on silica gel, octadecylsilanized (ODS) silica gel, as well as preparative HPLC to furnish 11 saponins (**1**–**11**).

Compound **1** ($C_{39}H_{62}O_{13}$; negative-ion FABMS *m/z* 737 $[M-H]^-$), $[\alpha]_D$ −74.0° (MeOH), was obtained as an amorphous solid. The glycosidic nature of **1** was suggested by the strong absorption band at 3410 and 1050 cm^{-1} in the IR spectrum. The ^1H NMR spectrum showed signals for four typical steroid methyl groups; two appeared as singlets at δ 1.48 and 0.90, and the other two as doublets at δ 1.08 (J = 6.9 Hz) and 0.72 (J = 5.2 Hz). An olefinic proton and two anomeric proton signals were also noted at δ 5.60 (*br d*, J = 5.5 Hz), and 6.45 (*br s*) and 4.82 (*d*, J = 7.8 Hz), respectively. Acetylation of **1** with acetic anhydride in pyridine introduced seven acetyl groups (**1a**). The fundamental steroid structure of **1**, based upon (25*R*)-spirostanol, was suggested by a quaternary carbon signal at δ 109.2 assignable to C-22 of the spirostanol skeleton in the ^{13}C NMR spectrum [10], and the oxymethylene proton signals at δ 3.61 (*dd*, J = 10.4, 2.4 Hz) and 3.52 (*dd*, J = 10.4, 10.4 Hz) attributable to 26-H_2, as well as the above ^1H NMR data. When **1** was submitted to acid hydrolysis with 1M hydrochloric acid in dioxane–H_2O (1:1), it was hydrolysed to yield D-galactose and L-rhamnose as the carbohydrate compounds, and an aglycone (**1b**). The ^1H NMR spectrum of **1b** showed two exchangeable proton signals at δ 6.24 and 6.06, and the ^{13}C NMR spectrum was consistent with the structure of **1b** as

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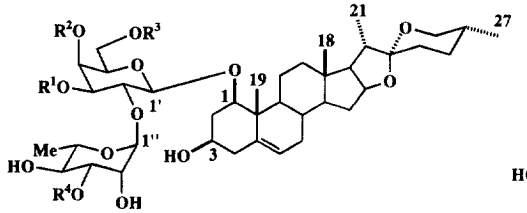
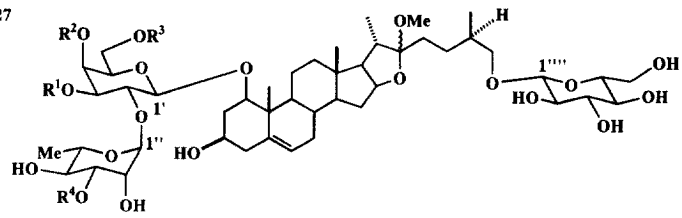
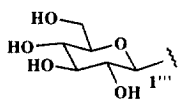


	R^1	R^2	R^3	R^4
1	H	H	H	H
2	H	H	Ac	H
3	H	Ac	Ac	H
4	Ac	Ac	Ac	H
5	H	H	H	β-D-Glc*p*
6	H	Ac	Ac	β-D-Glc*p*
11a	Ac	Ac	Ac	β-D-Glc*p*



	R^1	R^2	R^3	R^4
7	H	H	H	H
8	H	H	Ac	H
9	Ac	Ac	Ac	H
10	H	H	H	β-D-Glc*p*
11	Ac	Ac	Ac	β-D-Glc*p*

β-D-Glc*p*:



(25*R*)-spirost-5-ene-1β,3β-diol, that is, ruscogenin [10]. Thus, **1** was ruscogenin diglycoside. Analysis of the ^{1}H-^{1}H COSY spectrum allowed the sequential assignment of the resonances for the two monosaccharides, starting from the anomeric proton signals at δ 6.45 and 4.82. Multiplet patterns and measurements of coupling constants gave evidence for the presence of an α-L-rhamnopyranosyl unit (^{1}C$_4$) and a β-D-galactopyranosyl unit (^{4}C$_1$) (Table 2). The HMQC spectrum correlated all the proton resonances with those of the corresponding one-bond coupled carbons. In the HMBC spectrum optimized for an $^nJ_{C,H}$ parameter of 8 Hz, the anomeric proton of the rhamnose showed a $^3J_{C,H}$ correlation with C-2 (δ 74.9) of the galactose and that of the galactose with C-1 (δ 84.2) of the aglycone, indicating the rhamnosyl-(1 → 2)-galactosyl structure attached to C-1 of the aglycone. The presence of a terminal rhamnosyl unit was supported by the fragment ion peak at *m/z* 591 in the negative-ion FAB mass spectrum. Accordingly, the structure of **1** was determined to be ruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside}.

The spectral features of **2** ($C_{41}H_{64}O_{14}$) were similar to those of **1**. The presence of an acetyl group in the molecule was shown by the IR (1730 cm^{-1}), ^{1}H NMR [δ 2.02 (3H, *s*)] and ^{13}C NMR [δ 170.5 (C=O) and 20.9 (Me)] spectral data. Treatment of **2** with 3% sodium methoxide in methanol gave **1**. Therefore **2** was found to be a monoacetate of **1**. In the ^{13}C NMR spectrum of **2**, the signal due to C-6 of the galactose moiety was shifted to lower field by 2.7 ppm, whereas the signal due to C-5 occurred at higher shift by 3.1 ppm, as compared with those of **1**. Furthermore, the unequivalent methylene protons of the galactose 6-H_2, which were observed at δ 4.55 (*dd*, *J* = 10.3, 6.9 Hz) and 4.40 (*dd*, *J* = 10.3, 5.7 Hz) in the ^{1}H NMR spectrum of **1**, were moved downfield by 0.40 and 0.13 ppm, respectively, to appear at δ 4.95 (*dd*, *J* = 11.2, 7.7 Hz) and 4.53 (*dd*, *J* = 11.2, 4.8 Hz) in that of **2**. Thus, the acetyl moiety was revealed to be linked at the galactose C-6 hydroxy position, and the structure of **2** was assigned as ruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-galactopyranoside}.

Compound **3** ($C_{43}H_{66}O_{15}$) was shown to have two acetyl groups by the ^{1}H NMR [δ 2.03 and 1.99 (each 3H, *s*)] and ^{13}C NMR [δ 170.9 and 170.2 (C=O), and 20.8 and 20.6 (Me)] spectra. Mild alkaline treatment of **3** with 10% ammonia solution cleaved one acetyl group to give **2**. On comparison of the ^{1}H NMR spectrum of **3** with that of **2**, 4-H of the galactose was shifted downfield by 1.51 ppm to be observed at δ 5.68 (*br d*, *J* = 3.4 Hz), indicating the addition acetyl group was located at 4-H of the galactose. Thus, the structure of **3** was formulated as ruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-4,6-di-*O*-acetyl-β-D-galactopyranoside}.

The ^{1}H NMR [δ 2.08, 1.97 and 1.95 (each 3H, *s*)] and ^{13}C NMR [δ 170.6 and 170.1 × 2 (C=O), and 20.6, 20.5 and 20.3 (Me)] spectra of **4** ($C_{45}H_{68}O_{16}$) showed the presence of three acetyl groups in the molecule. Alkaline hydrolysis of **4** with 3% sodium methoxide gave **1**. Analysis of the whole ^{13}C NMR spectrum of **4** allowed the assignment of the signals due to a terminal α-L-rhamnopyranosyl moiety and a 1-*O*-glycosylated ruscogenin, suggesting that the three acetyl groups were located at the galactosyl residue. The downfield-shifted proton signals at δ 5.49 (*dd*, *J* = 9.8, 3.4 Hz) and 5.76 (*br d*, *J* = 3.4 Hz) were assigned to the galactose 3-H and 4-H protons, which were displaced downfield by more than 1 ppm in comparison with those of **1** and **2** through *O*-acetylation. Thus, the structure of **4** was shown to be ruscogenin 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranoside}.

Table 1. ^{13}C NMR spectral data for compounds **1**, **1b**, **2–11** and **11a** in pyridine-d_5

C	1	1b	2	3	4	5	6	7	8	9	10	11	11a
1	84.2	78.1	84.8	84.2	84.4	85.3	85.3	84.1	84.8	84.5	85.3	85.4	85.4
2	38.0	44.0	38.1	37.9	37.9	38.7	38.4	37.9	38.0	37.9	38.6	38.3	38.3
3	68.1	68.1	68.3	68.3	68.1	68.2	68.3	68.2	68.2	68.1	68.2	68.1	68.1
4	43.8	43.6	43.8	43.7	43.6	43.9	43.7	43.9	43.8	43.6	43.8	43.6	43.5
5	139.5	140.3	139.6	139.4	139.2	139.6	139.3	139.6	139.6	139.2	139.5	139.1	139.1
6	124.7	124.4	124.8	124.7	124.9	124.8	124.8	124.7	124.8	124.9	124.8	124.9	125.0
7	31.9	32.3	32.2	32.0	31.9	32.0	32.0	32.0	32.1	31.9	32.0	31.9	31.9
8	33.0	33.0	33.2	33.2	33.1	33.1	33.2	33.0	33.2	33.1	33.0	33.1	33.1
9	50.5	51.4	50.6	50.4	50.4	50.8	50.6	50.6	50.6	50.4	50.7	50.6	50.6
10	42.8	43.6	42.9	42.7	42.7	42.9	42.7	42.8	42.8	42.6	42.8	42.7	42.7
11	24.0	24.2	24.1	24.4	24.4	24.0	24.3	24.0	24.1	24.4	23.9	24.2	24.3
12	40.4	40.6	40.3	40.6	40.5	40.5	40.4	40.4	40.2	40.4	40.4	40.4	40.4
13	40.1	40.2	40.3	40.5	40.4	40.2	40.4	40.5	40.6	40.7	40.5	40.7	40.4
14	57.1	57.0	57.2	57.2	57.2	57.2	57.3	57.0	57.1	57.1	57.0	57.2	57.3
15	32.4	32.5	32.5	32.4	32.4	32.4	32.4	32.4	32.5	32.4	32.3	32.4	32.4
16	81.1	81.1	81.2	81.2	81.2	81.1	81.2	81.3	81.4	81.4	81.3	81.4	81.2
17	62.9	63.2	63.1	63.3	63.3	63.1	63.3	64.3	64.4	64.5	64.3	64.5	63.2
18	16.8	16.6	16.9	17.0	17.0	16.9	17.0	16.8	16.8	16.9	16.8	16.9	17.0
19	15.0	13.9	15.0	15.0	15.0	15.1	15.0	15.0	15.0	14.9	15.1	15.0	15.0
20	41.9	42.0	42.0	42.0	42.0	42.0	42.0	40.5	40.6	40.6	40.5	40.6	42.0
21	14.9	15.0	15.0	15.0	14.9	14.9	15.0	16.1	16.2	16.3	16.1	16.3	15.0
22	109.2	109.3	109.3	109.3	109.3	109.3	109.3	112.7	112.7	112.7	112.7	112.7	109.3
23	31.7	31.8	31.9	31.9	31.9	31.8	31.9	30.7	30.9	30.8	30.8	30.8	31.8
24	29.2	29.3	29.3	29.3	29.3	29.3	29.3	28.1	28.2	28.2	28.2	28.2	29.2
25	30.6	30.6	30.6	30.6	30.6	30.6	30.6	34.2	34.2	34.2	34.2	34.2	30.6
26	66.8	66.9	66.9	66.9	66.9	66.8	66.9	75.2	75.2	75.2	75.2	75.2	66.8
27	17.2	17.3	17.3	17.3	17.3	17.3	17.3	17.1	17.1	17.1	17.1	17.1	17.3
OMe								47.2	47.2	47.2	47.2	47.2	
1′	100.6		100.7	100.1	99.8	101.2	100.7	100.6	100.7	99.8	101.2	100.1	100.2
2′	74.9		74.6	75.0	72.9	74.1	74.1	75.0	74.5	72.8	74.0	71.8	71.8
3′	76.8		76.4	74.1	75.2	76.8	74.1	76.8	76.4	75.2	76.8	75.2	75.3
4′	70.4		70.6	72.0	68.5	70.5	72.0	70.4	70.6	68.5	70.4	68.4	68.4
5′	76.3		73.2	71.3	70.8	76.4	71.4	76.3	73.2	70.7	76.4	70.8	70.8
6′	61.9		64.6	63.0	62.2	62.6	63.1	61.9	64.6	62.2	62.6	62.2	62.2
1″	101.7		101.7	102.0	102.2	101.3	101.7	101.7	101.7	102.1	101.3	101.7	101.7
2″	72.6		72.5	72.4	72.3	72.1	72.0	72.6	72.5	72.3	72.1	71.6	71.6
3″	72.6		72.7	72.6	72.4	82.7	82.7	72.6	72.7	72.4	82.7	82.3	82.3
4″	74.2		74.3	74.2	73.8	73.3	73.2	74.3	74.2	73.8	73.3	72.9	72.9
5″	69.3		69.3	69.6	70.1	69.2	69.5	69.3	69.3	70.1	69.2	69.8	69.8
6″	19.0		19.0	19.1	19.0	18.7	18.8	19.0	19.0	19.0	18.7	18.7	18.7
1‴						106.6	106.6				106.5	106.5	106.4
2‴						76.2	76.1				76.2	76.1	76.0
3‴						78.4	78.4				78.3	78.4	78.3
4‴						71.8	71.7				71.7	71.7	71.7
5‴						78.3	78.3				78.2	78.4	78.3
6‴						62.0	62.6				61.9	62.5	62.5
1⁗								105.0	105.0	105.0	105.0	105.0	
2⁗								75.2	75.2	75.2	75.2	75.2	
3⁗								78.6	78.6	78.6	78.6	78.6	
4⁗								71.8	71.8	71.8	71.8	71.8	
5⁗								78.6	78.5	78.5	78.5	78.5	
6⁗								62.9	62.9	62.9	62.9	62.9	
Ac			170.5	170.9	170.6		170.9		170.5	170.6		170.6	170.6
				170.2	170.1		170.3			170.1		170.2	170.2
					170.1					170.1		170.1	170.2
			20.9	20.8	20.6		20.8		20.9	20.6		20.6	20.6
				20.6	20.5		20.6			20.5		20.5	20.5
					20.3					20.3		20.3	20.3

Table 2. ^{1}H NMR chemical shift assignment of the saccharide moiety of **1**, **1a** and **2–5** in pyridine-d_5

Position	**1**		**1a**		**2**		**3**		**4**		**5**	
1′	4.82 *d*	(7.8)	4.81 *d*	(7.7)	4.73 *d*	(7.5)	4.78 *d*	(7.6)	4.86 *d*	(7.7)	4.75 *d*	(7.8)
2′	4.70 *dd*	(9.3, 7.8)	4.32 *dd*	(10.3, 7.7)	4.58 *dd*	(8.8, 7.5)	4.45 *dd*	(9.5, 7.6)	4.43 *dd*	(9.8, 7.7)	4.68 *dd*	(9.1, 7.8)
3′	4.24 *dd*	(9.3, 9.3)	5.52 *dd*	(10.3, 3.3)	4.17		4.33 *dd*	(9.5, 3.4)	5.49 *dd*	(9.5, 3.4)	4.20 *dd*	(9.1, 3.3)
4′	4.54 *br d*	(3.3)	5.84 *br d*	(3.3)	4.17		5.68 *br d*	(3.4)	5.76 *br d*	(3.4)	4.55 *br d*	(3.3)
5′	3.99 *br dd*	(6.9, 5.7)	4.31 *br dd*	(6.7, 6.0)	3.96 *br dd*	(7.7, 4.8)	4.12 *br dd*	(7.6, 5.2)	4.22 *br dd*	(6.9, 6.1)	3.99 *br dd*	(7.1, 5.7)
6′a	4.55 *dd*	(10.3, 6.9)	4.58 *dd*	(10.9, 6.7)	4.95 *dd*	(11.2, 7.7)	4.57 *dd*	(11.4, 7.6)	4.56 *dd*	(11.3, 6.9)	4.55 *dd*	(10.4, 7.1)
b	4.40 *dd*	(10.3, 5.7)	4.37 *dd*	(19.9, 6.0)	4.53 *dd*	(11.2, 4.8)	4.34 *dd*	(11.4, 5.2)	4.32 *dd*	(11.3, 6.1)	4.41 *dd*	(10.4, 5.7)
1″	6.45 *br s*		5.62 *br s*		6.34 *br s*		6.27 *d*	(0.9)	5.69 *br s*		6.47 *br s*	
2″	4.81 *br d*	(3.5)	5.50 *br d*	(3.4)	4.72 *br d*	(3.4)	4.69 *dd*	(3.3, 0.9)	4.51 *br d*	(3.4)	5.03 *br d*	(2.9)
3″	4.71 *dd*	(9.4, 3.5)	5.76 *dd*	(10.1, 3.4)	4.63 *dd*	(9.4, 3.4)	4.58 *dd*	(9.5, 3.3)	4.48 *dd*	(9.5, 3.4)	4.96 *dd*	(9.5, 2.9)
4″	4.38 *dd*	(9.4, 9.4)	5.62 *dd*	(10.1, 10.1)	4.30 *dd*	(9.4, 9.4)	4.31 *dd*	(9.5, 9.5)	4.30 *dd*	(9.5, 9.5)	4.59 *dd*	(9.5, 9.5)
5″	4.97 *dq*	(9.4, 6.1)	4.75 *dq*	(10.1, 6.2)	4.90 *dq*	(9.4, 6.2)	4.89 *dq*	(9.5, 6.2)	4.75 *dq*	(9.5, 6.1)	5.01 *dq*	(9.5, 6.1)
6″	1.80 *d*	(6.1)	1.49 *d*	(6.2)	1.74 *d*	(6.2)	1.78 *d*	(6.2)	1.78 *d*	(6.1)	1.72 *d*	(6.1)
1‴											5.76 *d*	(7.8)
2‴											4.17 *dd*	(8.4, 7.8)
3‴											4.32 *dd*	(8.4, 8.4)
4‴											4.30 *dd*	(8.4, 8.4)
5‴											4.22 *ddd*	(8.4, 5.0, 1.8)
6‴a											4.54 *dd*	(11.8, 1.8)
b											4.38 *dd*	(11.8, 5.0)
Ac			2.21 *s*		2.02 *s*		2.03 *s*		2.08 *s*			
			2.18 *s*				1.99 *s*		1.97 *s*			
			2.15 *s*						1.95 *s*			
			2.08 *s*									
			2.03 *s*									
			2.03 *s*									
			2.01 *s*									

J values in parentheses are expressed in Hz.

Compound **5** ($C_{45}H_{72}O_{18}$), $[\alpha]_D$ −46.0° (methanol), was a more polar constituent than **1**. The negative-ion FAB mass spectrum showed an $[M-H]^-$ ion at *m/z* 899, shifted 162 mass units with respect to **1**. The ^1H NMR spectrum showed three anomeric proton signals at δ 6.47 (*br s*), 5.76 (*d*, J = 7.8 Hz) and 4.75 (*d*, J = 7.8 Hz), as well as the signals for four steroid methyls at δ 1.49 (*s*), 1.07 (*d*, J = 6.9 Hz), 0.90 (*s*) and 0.72 (*d*, J = 5.1 Hz). In comparison of the ^{13}C NMR spectrum of **5** with that of **1**, a set of additional signals, corresponding to a terminal β-D-glucopyranosyl unit, appeared at δ 106.6, 76.2, 78.4, 71.8, 78.3 and 62.0, and the signals due to the rhamnosyl moiety varied, while all other signals remained almost unaffected. Total acid hydrolysis of **5** gave ruscogenin (**1b**), D-glucose, D-galactose and L-rhamnose, and partial acid hydrolysis with 0.2 M hydrochloric acid gave **1** and D-glucose. In the HMBC spectrum of **5**, a correlation peak between the signals of the anomeric proton of the glucose at δ 5.76 and the C-3 carbon of the rhamnose at δ 82.7 indicated that the C-3 hydroxy position of the rhamnose was the glycosylated position to which the additional D-glucose was linked. This led to the monosaccharide sequence as glucosyl-(1 → 3)-rhamnosyl-(1 → 2)-galactosyl, which was consistent with the fragment ion peaks observed in the negative-ion FAB mass spectrum at *m/z* 737 and 591. The structure of **5** was characterized as ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyransoyl-(1 → 2)-β-D-galactopyranoside}.

Compound **6** ($C_{49}H_{76}O_{20}$) was suggested to be a spirostanol saponin related to **3** from its spectral data. On comparison between the ^{13}C NMR data of **6** with those of **3**, the presence of an additional β-D-glucopyranosyl unit was readily recognized by the characteristic six signals at δ 106.6, 76.1, 78.4, 71.7, 78.3 and 62.6. The glycosidic linkage in the rhamnose C-3 hydroxy position was formed from β-D-glucopyranose as was evident in the good agreement of the ^{13}C NMR shifts of the rhamnosyl moiety between **6** and **5**, in which the occurrence of a significant downfield shift due to glycosylation was recognized at C-3 of the rhamnose. The structure of **6** was shown to be ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyransol-(1 → 2)-4,6-di-*O*-acetyl-β-D-galactopyranoside}.

Compound **7** ($C_{46}H_{76}O_{19}$) was shown to be a 22-methoxyfurostanol saponin by Ehrlich's test [11, 12], and the ^1H NMR [δ 3.25 (3H, *s*)] and ^{13}C NMR [δ 112.7 (C-22) and 47.2 (Me)] spectra [10]. The ^1H NMR spectrum showed three anomeric proton signals at δ 6.39 (*br s*), 4.84 (*d*, J = 7.7 Hz) and 4.79 (*d*, J = 7.7 Hz), as well as four steroid methyls at δ 1.44 (*s*), 1.11 (*d*, J = 6.8 Hz), 0.99 (*d*, J = 6.6 Hz) and 0.87 (*s*). Enzymatic hydrolysis of **7** with β-glucosidase gave **1** and D-glucose. Thus, the structure of **7** was assigned as 26-*O*-β-D-glucopyranosyl-22-*O*-methyl-(25*R*)-furost-5-ene-1β,3β, 22ξ,26-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside}.

Compound **8** ($C_{48}H_{78}O_{20}$), **9** ($C_{52}H_{82}O_{22}$), **10** ($C_{52}H_{86}O_{24}$) and **11** ($C_{58}H_{92}O_{27}$) were also 22-methoxyfurostanol saponins. Enzymatic hydrolysis of **8**–**10** with β-glucosidase furnished the corresponding spirostanol saponins, **2**, **4** and **5**, respectively, along with D-glucose. The structures of **8**–**10** were 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-galactopyranoside}, 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranoside} and 1-*O*-{*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside} of 26-*O*-β-D-glucopyranosyl-22-*O*-methyl-(25*R*)-furost-5-ene-1β,3β,22ξ,26-tetrol, respectively.

Treatment of **11** with β-glucosidase gave the corresponding spirostanol saponin (**11a**). Compound **11a** was shown to be an analogous spirostanol saponin to **4**. It differed from **4** in the presence of an additional terminal β-D-glucopyranosyl unit. In the ^{13}C NMR spectrum of **11a**, the signals arising from the rhamnosyl moiety were in good accordance with those of **5** bearing a glucosyl moiety at C-3 of its rhamnose residue. The structure of **11a** was assigned as ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranoside}. Consequently, the structure of **11** was characterized as 26-*O*-β-D-glucopyranosyl-22-*O*-methyl-(25*R*)-furost-5-ene-1β,3β,22ξ,26-tetrol 1-*O*-{*O*-β-D-glucopyranosyl-(1 → 3)-*O*-α-L-rhamnopyranosyl-(1 → 2)-3,4,6-tri-*O*-acetyl-β-D-galactopyranoside}.

Table 3. Cytostatic activity of the isolated saponins on leukemia HL-60 cells

Compounds	Inhibition (%)*	IC_{50} (μg ml^{-1})
1	18.4	—†
2	24.1	—
3	43.7	—
4	98.2	3.1
5	22.3	—
6	10.3	—
7	0.1	—
8	0	—
9	82.5	3.7
10	9.2	—
11	0.6	—

* Data expressed as percentage of cell growth inhibition at the sample concentration of 10 μg ml^{-1}.

† Not measured.

Cytostatic activity of the isolated saponins on human promyelocytic leukemia HL-60 cells was evaluated. The cells were continuously treated with the each sample for 72 h and the cell growth was measured with an MTT assay procedure. Percentage inhibition at the sample concentration of 10 μg ml^{-1} is listed in Table 3. Ruscogenin diglycoside with three acetyl groups attached to the inner galactosyl moiety (**4**) and its corresponding 26-glucosyloxyfurostanol saponin (**9**) exhibited 98.2% and 82.5% inhibition at

10 μg ml^{-1}, respectively, while the other saponins were far less potent. The IC_{50} values of **4** and **9** were calculated from the dose-response curve as about 3.1 μg ml^{-1} and 3.7 μg ml^{-1}, respectively. The saponins isolated from other Liliaceae plants are now being assayed and detailed structure-activity relationships will be reported in the near future.

EXPERIMENTAL

General

NMR (ppm, *J* Hz): 1D (Bruker AM-400, 400 MHz for ^{1}H NMR) and 2D (Bruker DPX-400 using XWIN-NMR 1.3 pulse programs, 400 MHz for ^{1}H 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 thick or 0.5 mm thick, Merck) and RP-18 $F_{254}S$ (0.25 mm thick, Merck). HPLC: a Tosoh HPLC system (pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000 or RI-8010) equipped with a CAPCELL PAK C_{18} column (Shiseido, 10 mm i.d. × 250 mm, ODS, 5 μm) for prep. HPLC and a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) for analytical HPLC. Microplate reader: Immuno-Mini NJ-2300 (Inter Med, Japan). HL-60 cells: ICN Biomedicals, USA. RPMI 1640 medium: Gibco, USA. All other chemicals used were of biochemical reagent grade.

Plant material

The underground parts of *R. aculeatus* used for this experiment were collected at Chiba prefecture, Japan, in June 1992, and the plant specimen is on file in our laboratory.

Extraction and isolation

The plant material (fr wt, 3.1 kg) was extracted with hot MeOH. The MeOH extract was concentrated 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 stepwise gradient mixture of $CHCl_3$–MeOH system (9:1; 6:1; 4:1; 2:1), and finally with MeOH, gave six fractions (I–VI). Fr. IV was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (40:10:1) and ODS silica gel with MeOH–H_2O (4:1) to give **4** (31.7 mg). Fr. V was also subjected to silica gel CC eluting with $CHCl_3$–Et_2O–MeOH–H_2O (10:10:6:1) and ODS silica gel CC with MeOH–H_2O (4:1; 3:1) to result in the isolation of **2** (35.5 mg) and **3** (45.7 mg). Fr. VI contained considerable amounts of saccharides, the removal of which from it was performed by passage through a Diaion HP-20 column using gradients of MeOH in H_2O. The 80% MeOH and MeOH eluate frs were combined and further fractionated by a silica gel column eluting with $CHCl_3$–MeOH (4:1) into three frs (VIa–VIc). Fr. VIa was subjected to silica gel CC eluting with $CHCl_3$–MeOH–H_2O (40:10:1) to give **5** (43.3 mg), and mixture of **1** and **6**. Separation of **1** and **6** was carried out by means of prep. HPLC using MeOH–H_2O (4:1) to yield **1** (118 mg) and **6** (32.8 mg). Fr. VIb was purified by CC on silica gel eluting with $CHCl_3$–MeOH–H_2O (40:10:1) and $CHCl_3$–Et_2O–MeOH–H_2O (10:10:7:1), and ODS silica gel with MeOH–H_2O (7:3) and MeCN–H_2O (3:7) to yield **9** (474 mg) and **11** (851 mg), and a mixture of **7** and **8**. The mixture was separated by prep. HPLC using MeCN–H_2O (3:7) to furnish **7** (310 mg) and **8** (35.8 mg). Fr. VIc was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (30:10:1) and ODS silica gel with MeOH–H_2O (13:7) and MeCN–H_2O (1:3) to give **10** with a few impurities. Final purification of **10** was established by prep. HPLC using MeCN–H_2O (1:3) to provide **10** (520 mg) as a pure compound.

Compound **1**. Amorphous solid. $[\alpha]_D^{25}$ −74.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 737 $[M-H]^-$, 591 $[M-$rhamnosyl$]^-$. IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 2935 (CH), 1445, 1375, 1235, 1130, 1050, 980, 960, 915, 895, 860, 830, 805. ^{1}H NMR (pyridine-d_5): δ 5.60 (1H, *br d*, *J* = 5.5 Hz, 6-H), 4.50 (1H, *q*-like, *J* = 7.5 Hz, 16-H), 3.88 (1H, *dd*, *J* = 11.6, 4.0 Hz, 1-H), 3.86 (1H, *m*, 3-H), 3.61 (1H, *dd*, *J* = 10.4, 2.4 Hz, 26a-H), 3.52 (1H, *dd*, *J* = 10.4, 10.4 Hz, 26b-H), 1.48 (3H, *s*, 19-Me), 1.08 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.90 (3H, *s*, 18-Me), 0.72 (3H, *d*, *J* = 5.2 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Acetylation of **1**. Compound **1** (26 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (0.5 ml) and the crude acetate was chromatographed on silica gel eluting with hexane–Me_2CO (5:2) to yield the corresponding heptaacetate (**1a**) (24.3 mg). Compound **1a**: amorphous solid. IR ν_{max}^{KBr} cm^{-1}: 2905 (CH), 1745 (C=O), 1435, 1365, 1225, 1135, 1065, 1040, 970, 895, 855, 825, 795. ^{1}H NMR (pyridine-d_5): δ 5.66 (1H, *br d*, *J* = 5.4 Hz, 6-H), 4.84 (1H, *m*, 3-H), 4.60 (1H, *q*-like, *J* = 7.3 Hz, 16-H), 3.70 (1H, *dd*, *J* = 11.9, 3.9 Hz, 1-H), 3.60 (1H, *dd*, *J* = 10.2, 2.7 Hz, 26a-H), 3.52 (1H, *dd*, *J* = 10.2, 10.2 Hz, 26b-H), 1.31 (3H, *s*, 19-Me), 1.14 (3H, *d*, *J* = 6.7 Hz, 21-Me), 0.98 (3H, *s*, 18-Me), 0.71 (3H, *d*, *J* = 5.1 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Acid hydrolysis of **1**. A soln of **1** (10 mg) in 1 M HCl (dioxane–H_2O, 1:1, 5 ml) was heated at 100° for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column and chromatographed on silica gel eluting with a gradient of $CHCl_3$–MeOH (19:1; 1:1) to give ruscogenin (**1b**) (4.2 mg) and a mixture of monosaccharides (4.1 mg). Ruscogenon (**1b**): amorphous solid. Negative-ion FABMS *m/z* 429 $[M-H]^-$. ^{1}H NMR (pyridine-d_5): δ 6.24 and 6.06 (each 1H, *br s*, OH), 5.62 (1H, *br d*, *J* = 5.6 Hz, 6-H), 4.55 (1H, *q*-like, *J* = 7.4 Hz, 16-H), 3.97 (1H, *m*, 3-H), 3.83 (1H, *br d*, *J* = 10.9 Hz, 1-H), 3.59 (1H, *dd*, *J* = 10.4, 3.5 Hz, 26a-H), 3.51 (1H, *dd*,

J = 10.4, 10.4 Hz, 26b-H), 1.36 (3H, *s*, 19-Me), 1.11 (3H, *d*, J = 7.0 Hz, 21-Me), 0.93 (3H, *s*, 18-Me), 0.70 (3H, *d*, J = 5.4 Hz, 27-Me). The monosaccharide mixture (2 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 h, followed by acetylation with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixture was passed through a Sep-Pak C_{18} cartridge (Waters) with H_2O–MeCN (4:1; 1:9, each 10 ml). The H_2O–MeCN (1:9) eluate fr. was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixture of 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides [13, 14], which were then analyzed 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 L-rhamnose were detected. R_t (min): 19.32 (D-galactose derivative); 26.02 (L-rhamnose derivative).

Compound **2**. Amorphous solid. $[\alpha]_D^{25}$ −76.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 779 $[M-H]^-$, 737 $[M-acetyl]^-$, 616 $[M-rhamnose]^-$, 591 $[M-rhamnosyl-acetyl]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2940 (CH), 1730 (C=O), 1445, 1370, 1235, 1135, 1050, 980, 960, 915, 895, 860, 830, 805. ^1H NMR (pyridine-d_5): δ 5.61 (1H, *br d*, J = 5.3 Hz, 6-H), 4.59 (1H, 16-H), 3.82 (1H, *m*, 3-H), 3.77 (1H, *dd*, J = 11.9, 3.9 Hz, 1-H), 3.56 (1H, *dd*, J = 10.3, 2.6 Hz, 26a-H), 3.49 (1H, *dd*, J = 10.3, 10.3 Hz, 26b-H), 1.42 (3H, *s*, 19-Me), 1.07 (3H, *d*, J = 6.4 Hz, 21-Me), 0.89 (3H, *s*, 18-Me), 0.68 (3H, *d*, J = 5.1 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Alkaline hydrolysis of **2**. Compound **2** (5 mg) was treated with 3% NaOMe in MeOH (5 ml) at room temp. for 30 min. The reaction mixture was neutralized by passage through an Amberlite IR-120B (Organo) column and chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (30:10:1) to yield **1** (3.8 mg).

Compound **3**. Amorphous solid. $[\alpha]_D^{25}$ −62.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 821 $[M-H]^-$, 779 $[M-acetyl]^-$, 737 $[M-acetyl\times2]^-$, 675 $[M-rhamnosyl]^-$, 616 $[M-rhamnose-acetyl]^-$, 591 $[M-rhamnosyl-acetyl\times2]^-$. IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 2945 (CH), 1745 (C=O), 1450, 1375, 1240, 1180, 1130, 1065, 1055, 980, 965, 915, 900, 865, 835, 810. ^1H NMR (pyridine-d_5): δ 5.62 (1H, *br d*, J = 5.5 Hz, 6-H), 4.59 (1H, 16-H), 3.81 (1H, *m*, 3-H), 3.78 (1H, *dd*, J = 12.0, 4.1 Hz, 1-H), 3.58 (1H, *dd*, J = 10.4, 2.6 Hz, 26a-H), 3.50 (1H, *dd*, J = 10.4, 10.4 Hz, 26b-H), 1.44 (3H, *s*, 19-Me), 1.13 (3H, *d*, J = 6.6 Hz, 21-Me), 0.98 (3H, *s*, 18-Me), 0.69 (3H, *d*, J = 5.3 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Mild alkaline hydrolysis of **3**. Compound **3** (4 mg) was treated with 10% NH_3 soln (MeOH–H_2O, 1:1) at room temp. for 8 min. The reaction mixture was evaporated under red. pres. and chromatographed on ODS silica gel eluting with MeCN–H_2O (1:1) to yield **2** (2.5 mg).

Compound **4**. Amorphous solid. $[\alpha]_D^{25}$ −78.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 863 $[M-H]^-$, 821 $[M-acetyl]^-$, 779 $[M-acetyl\times2]^-$, 737 $[M-acetyl\times3]^-$, 633 $[M-rhamnosyl-acetyl\times2]^-$, 591 $[M-rhamnosyl-acetyl\times3]^-$. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2945 (CH), 1755 (C=O), 1455, 1375, 1235, 1140, 1075, 1055, 980, 965, 920, 900, 865, 835, 810. ^1H NMR (pyridine-d_5): δ 5.62 (1H, *br d*, J = 5.4 Hz, 6-H), 4.58 (1H, *q*-like, J = 7.0 Hz, 16-H), 3.81 (1H, *m*, 3-H), 3.77 (1H, *dd*, J = 12.0, 3.7 Hz, 1-H), 3.58 (1H, *dd*, J = 10.3, 2.6 Hz, 26a-H), 3.50 (1H, *dd*, J = 10.3, 10.3 Hz, 26b-H), 1.40 (3H, *s*, 19-Me), 1.14 (3H, *d*, J = 6.7 Hz, 21-Me), 0.97 (3H, *s*, 18-Me), 0.69 (3H, *d*, J = 4.8 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Alkaline hydrolysis of **4**. Compound **4** (5 mg) was subjected to alkaline hydrolysis as described for **2** to give **1** (3.8 mg).

Compound **5**. Amorphous solid. $[\alpha]_D^{25}$ −46.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 899 $[M-H]^-$, 737 $[M-glucosyl]^-$, 591 $[M-glucosyl-rhamnosyl]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2930 (CH), 1445, 1375, 1235, 1150, 1055, 975, 960, 915, 895, 860, 830. ^1H NMR (pyridine-d_5): δ 5.59 (1H, *br d*, J = 5.6 Hz, 6-H), 4.51 (1H, *q*-like, J = 7.5 Hz, 16-H), 3.86 (1H, *m*, 3-H), 3.78 (1H, *dd*, J = 11.8, 3.9 Hz, 1-H), 3.61 (1H, *dd*, J = 10.5, 2.4 Hz, 26a-H), 3.52 (1H, *dd*, J = 10.5, 10.5 Hz, 26b-H), 1.49 (3H, *s*, 19-Me), 1.07 (3H, *d*, J = 6.9 Hz, 21-Me), 0.90 (3H, *s*, 18-Me), 0.72 (3H, *d*, J = 5.1 Hz, 27-Me). Signals for the saccharide moiety: Table 2.

Acid hydrolysis of **5**. Compound **5** (5.2 mg) was subjected to acid hydrolysis as described for **1** to give **1b** (2.1 mg) and a mixture of monosaccharides (1.1 mg). The monosaccharides were identified as D-glucose, D-galactose and L-rhamnose by HPLC analysis of their corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives. R_t (min): 17.57 (D-galactose derivative); 20.90 (D-glucose derivative); 23.59 (L-rhamnose derivative).

Partial acid hydrolysis of **5**. Compound **5** (5 mg) was treated with 0.2 M HCl in dioxane–H_2O (1:1, 5 ml) at 100° for 30 min. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU column and chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (30:10:1) to give **1** (0.8 mg).

Compound **6**. Amorphous solid. $[\alpha]_D^{25}$ −50.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 983 $[M-H]^-$, 941 $[M-acetyl]^-$, 738 $[M-glucosyl-acetyl\times2]^-$. IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 2930 (CH), 1735 (C=O), 1445, 1370, 1240, 1065, 1050, 975, 960, 915, 895, 860, 830, 800. ^1H NMR (pyridine-d_5): δ 6.29 (1H, *br s*, 1″-H), 5.69 (1H, *br d*, J = 3.5 Hz, 4′-H), 5.64 (1H, *d*, J = 7.9 Hz, 1‴-H), 5.61 (1H, *br d*, J = 5.4 Hz, 6-H), 4.74 (1H, *d*, J = 7.7 Hz, 1′-H), 4.59 (1H, *q*-like, J = 7.3 Hz, 16-H), 3.82 (1H, *m*, 3-H), 3.72 (1H, *dd*, J = 11.9, 4.0 Hz, 1-H), 3.58 (1H, *dd*, J = 10.5, 2.4 Hz, 26a-H), 3.50 (1H, *dd*, J = 10.5, 10.5 Hz, 26b-H), 2.04 and 1.94 (each 3H, *s*, Ac), 1.73 (3H, *d*, J = 6.2

Hz, 6″-Me), 1.45 (3H, *s*, 19-Me), 1.12 (3H, *d*, *J* = 6.5 Hz, 21-Me), 0.96 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 5.1 Hz, 27-Me).

Compound **7**. Amorphous solid. $[\alpha]_D^{25}$ −36.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 931 $[M-H]^-$. IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 2930 (CH), 1455, 1375, 1260, 1225, 1060, 980, 905, 835, 810. ^1H NMR (pyridine-d_5): δ 6.39 (1H, *br s*, 1″-H), 5.58 (1H, *br d*, *J* = 5.4 Hz, 6-H), 4.84 (1H, *d*, *J* = 7.7 Hz, 1‴, 4.79 (1H, *d*, *J* = 7.7 Hz, 1′-H), 3.85 (1H, *dd*, *J* = 12.0, 3.9 Hz, 1-H), 3.82 (1H, *m*, 3-H), 3.25 (3H, *s*, OMe), 1.75 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.44 (3H, *s*, 19-Me), 1.11 (3H, *d*, *J* = 6.8 Hz, 21-Me), 0.99 (3H, *d*, *J* = 6.6 Hz, 27-Me), 0.87 (3H, *s*, 18-Me).

Enzymatic hydrolysis of **7**. Compound **7** (5 mg) was treated with β-glucosidase (5 mg) in HOAc–NaOAc buffer (pH 5, 5 ml) at room temp. for 24 h. The reaction mixture was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (30:10:1) to yield **1** (2.5 mg) and D-glucose. D-Glucose: TLC, R_f 0.40 (*n*-BuOH–Me_2CO–H_2O, 4:5:1).

Compound **8**. Amorphous solid. $[\alpha]_D^{25}$ −46.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 973 $[M-H]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2930 (CH), 1735 (C=O), 1445, 1370, 1245, 1130, 1065, 980, 905, 835, 810. ^1H NMR (pyridine-d_5): δ 6.35 (1H, *br s*, 1″-H), 5.63 (1H, *br d*, *J* = 5.5 Hz, 6-H), 4.84 (1H, *d*, *J* = 7.7 Hz, 1⁗-H), 4.74 (1H, *d*, *J* = 7.6 Hz, 1′-H), 3.83 (1H, *m*, 3-H), 3.78 (1H, *dd*, *J* = 11.9, 4.0 Hz, 1-H), 3.24 (3H, *s*, OMe), 2.03 (3H, *s*, Ac), 1.73 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.43 (3H, *s*, 19-Me), 1.14 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.99 (3H, *d*, *J* = 6.6 Hz, 27-Me), 0.89 (3H, *s*, 18-Me).

Compound **9**. Amorphous solid. $[\alpha]_D^{25}$ −160° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1057 $[M-H]^-$, 623 [M − rhamnosyl − glucosylacetyl × 3]$^-$. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2930 (CH), 1745 (C=O), 1445, 1370, 1235, 1130, 1045, 980, 910, 835, 810. ^1H NMR (pyridine-d_5): δ 5.76 (1H, *br d*, *J* = 3.2 Hz, 4′-H), 5.69 (1H, *br s*, 1″-H), 5.64 (1H, *br d*, *J* = 5.3 Hz, 6-H), 5.50 (1H, *dd*, *J* = 9.9, 3.2 Hz, 3′-H), 4.87 (1H, *d*, *J* = 7.5 Hz, 1⁗-H), 4.85 (1H, *d*, *J* = 7.6 Hz, 1′-H), 3.83 (1H, *m*, 3-H), 3.77 (1H, *dd*, *J* = 12.0, 3.8 Hz, 1-H), 3.26 (3H, *s*, OMe), 2.08, 1.98 and 1.95 (3H, *s*, Ac), 1.78 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.40 (3H, *s*, 19-Me), 1.20 (3H, *d*, *J* = 6.8 Hz, 21-Me), 1.00 (3H, *d*, *J* = 6.5 Hz, 27-Me), 0.97 (3H, *s*, 18-Me).

Compound **10**. Amorphous solid. $[\alpha]_D^{25}$ −98.0° (MeOH; *c* 0.10). Negative-iron FABMS *m/z* 1093 $[M-H]^-$, 931 [M − glucosyl]$^-$. IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 2925 (CH), 1455, 1375, 1265, 1225, 1160, 1065, 980, 910, 835. ^1H NMR (pyridine-d_5): δ 6.40 (1H, *br s*, 1″-H), 5.69 (1H, *d*, *J* = 7.8 Hz, 1‴-H), 5.57 (1H, *br d*, *J* = 5.4 Hz, 6-H), 4.84 (1H, *d*, *J* = 7.7 Hz, 1⁗-H), 4.71 (1H, *d*, *J* = 7.8 Hz, 1′-H), 3.82 (1H, *m*, 3-H), 3.75 (1H, *dd*, *J* = 11.9, 3.9 Hz, 1-H), 3.25 (3H, *s*, OMe), 1.67 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.45 (3H, *s*, 19-Me), 1.10 (3H, *d*, *J* = 6.9 Hz, 21-Me), 0.99 (3H, *d*, *J* = 6.6 Hz, 27-Me), 0.86 (3H, *s*, 18-Me).

Compound **11**. Amorphous solid. $[\alpha]_D^{25}$ −40.0° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1219 $[M-H]^-$. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 2930 (CH), 1745 (C=O), 1450, 1370, 1245, 1070, 1045, 980, 910, 835, 805. ^1H NMR (pyridine-d_5): δ 5.78 (1H, *br d*, *J* = 3.4 Hz, 4′-H), 5.67 (1H, *br s*, 1″-H), 5.62 (1H, *d*, *J* = 7.6 Hz, 1‴-H), 5.61 (1H, *br d*, *J* = 5.3 Hz, 6-H), 5.43 (1H, *dd*, *J* = 9.9, 3.4 Hz, 3′-H), 4.85 (1H, *d*, *J* = 7.7 Hz, 1⁗-H), 4.77 (1H, *d*, *J* = 7.7 Hz, 1′-H), 3.84 (1H, *m*, 3-H), 3.72 (1H, *dd*, *J* = 12.1, 3.8 Hz, 1-H), 3.26 (3H, *s*, OMe), 2.04, 1.98 and 1.94 (3H, *s*, Ac), 1.72 (3H, *d*, *J* = 6.1 Hz, 6″-Me), 1.43 (3H, *s*, 19-Me), 1.19 (3H, *d*, *J* = 6.9 Hz, 21-Me), 1.00 (3H, *d*, *J* = 6.6 Hz, 27-Me), 0.96 (3H, *s*, 18-Me).

Enzymatic hydrolysis of **8**–**11**. Compounds **8** (5 mg), **9** (20 mg), **10** (10 mg) and **11** (30 mg) were subjected to enzymatic hydrolysis with β-glucosidase (5–30 mg) in HOAc–NaOAc buffer (pH 5, 5–15 ml) at room temp. for 32–72 h, followed by purification with CC on silica gel using $CHCl_3$–MeOH–H_2O system as the solvent to yield the corresponding spirostanol saponins, **2** (2.5 mg), **4** (1.5 mg), **5** (2.2 mg) and **11a** (4.5 mg), respectively, along with D-glucose.

Compound **11a**. Amorphous solid. $[\alpha]_D^{25}$ −105° (MeOH; *c* 0.10). Negative-ion FABMS *m/z* 1025 $[M-H]^-$, 983 [M − acetyl]$^-$, 941 [M − acetyl × 2]$^-$, 779 [M − glucosyl − acetyl × 2]$^-$, 591 [M − glucosyl − rhamnosyl − acetyl × 3]$^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2925 (CH), 1750 (C=O), 1450, 1370, 1255, 1235, 1070, 1050, 980, 960, 910, 900, 835, 800. ^1H NMR (pyridine-d_5): δ 5.78 (1H, *br d*, *J* = 3.4 Hz, 4′-H), 5.68 (1H, *br s*, 1″-H), 5.62 (1H, *d*, *J* = 7.6 Hz, 1‴-H), 5.61 (1H, *br d*, *J* = 5.3 Hz, 6-H), 5.43 (1H, *dd*, *J* = 9.9, 3.4 Hz, 3′-H), 4.77 (1H, *d*, *J* = 7.7 Hz, 1′-H), 3.82 (1H, *m*, 3-H), 3.72 (1H, *dd*, *J* = 11.8, 3.7 Hz, 1-H), 3.58 (1H, *dd*, *J* = 10.3, 2.5 Hz, 26a-H), 3.50 (1H, *dd*, *J* = 10.3, 10.3 Hz, 26b-H), 2.04, 1.98 and 1.95 (3H, *s*, Ac), 1.73 (3H, *d*, *J* = 6.2 Hz, 6″-Me), 1.43 (3H, *s*, 19-Me), 1.14 (3H, *d*, *J* = 6.7 Hz, 21-Me), 0.97 (3H, *s*, 18-Me), 0.69 (3H, *d*, *J* = 5.1 Hz, 27-Me).

Cell culture and assay for cytostatic activity

HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The leukemia cells were washed and resuspended in the above medium to 3×10^4 cells ml^{-1}, and 196 μl of this cell suspension were placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 h at 37° in 5% CO_2/air. After incubation, 4 μl of EtOH–H_2O (1:1) soln containing the sample was added to give final concentrations of 0.01–10 μg ml^{-1}; 4 μl of EtOH–H_2O (1:1) was added into control wells. The cells were incubated for a further 72 h in the presence of each agent, and then cell growth was evaluated with an MTT assay procedure [15]. The MTT assay was carried out according to a modified method of Sargent and Tayler as follows. After termination of cell culture, 10 μl of 5 mg ml^{-1} MTT in phosphate buffered saline was added to every

well and the plate reincubated at 37° in 5% CO_2/air for a further 4 h. The plate was then centrifuged at 1500 g for 5 min to precipitate cells and formazan. 150 μl of the supernatant was removed from every well, and 175 μl of DMSO was added to dissolve the 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 the samples of **4** and **9**, and a concentration which gave 50% inhibition of cell growth (IC_{50}) was calculated.

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