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Steroidal Saponins from the Rhizomes of *Ruscus hypophyllum*

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Phytochemical screening of the rhizomes of *Ruscus hypophyllum* (Liliaceae) has resulted in the isolation of five spirostanol saponins (**1**-**5**) including a new saponin (**4**) and nine furostanol saponins (**6**-**14**) including three new saponins (**9**-**11**), together with a known cholestane glycoside (**15**) and a new cholestane glycoside (**16**). The structures of the new compounds were determined on the basis of extensive spectroscopic analysis, including 2D NMR data, and chemical evidence. The spirostanol saponins **1**, **2**, and **5** showed moderate cytotoxic activity against HL-60 human promyelocytic leukemia cells.

Keywords: *Ruscus hypophyllum*, Liliaceae, spirostanol saponins, furostanol saponins, cholestane glycosides, HL-60 cells.

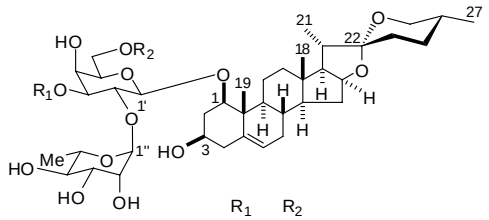
Plants of the genus *Ruscus* in the family Liliaceae are composed of about six species and are known to be rich sources of steroidal saponins. The representative species of the genus *Ruscus*, *R. aculeatus*, has been shown to contain a variety of steroidal saponins based upon spirost-5-ene-1β,3β-diol (ruscogenin) [1-6]. Previously, we have made a phytochemical screening of the rhizomes of *Ruscus hypophyllum* L., which is distributed from the Madeira Island to the Caucasian mountains, and isolated a series of cholestane glycosides [7]. Our further chemical examination of *R. hypophyllum* rhizomes has resulted in the isolation of five spirostanol saponins (**1**-**5**) including a new saponin (**4**) and nine furostanol saponins (**6**-**14**) including three new saponins (**9**-**11**), together with a known cholestane glycoside (**15**) and a new cholestane glycoside (**16**). This paper reports the structural determination of the new compounds on the basis of extensive spectroscopic analysis, including 2D NMR data, and chemical evidence. The cytotoxic activity of the isolated compounds is also described.

The rhizomes of *R. hypophyllum* (1.2 kg) were extracted with hot MeOH. The concentrated MeOH extract (221 g) was passed through a porous-polymer polystyrene resin (Diaion HP-20) column, eluted with 30% MeOH in H_2O, EtOH, and EtOAc. The EtOH eluate fraction (25 g), in which steroidal saponins were enriched, was subjected to a series of chromatographic separations, resulting in the isolation of compounds **1** (33.2 mg), **2** (20.3 mg), **3** (44.1 mg), **4** (95.7 mg), **5** (100 mg), **6** (54.3 mg), **7** (26.2 mg), **8** (47.5 mg), **9** (110 mg), **10** (178 mg), **11** (23.2 mg), **12** (90.6 mg), **13** (22.0 mg), **14** (20.1 mg), **15** (59.7 mg), and **16** (10.0 mg).

Compounds **1**-**3**, **5**-**8**, and **12**-**15** were identified as (25*R*)-3β-hydroxyspirost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside (**1**) [3], (25*R*)-3β-hydroxyspirost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-6-*O*-acetyl-β-D-galactopyranoside (**2**) [3], (25*R*)-3β-hydroxyspirost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl (1→3)]-β-D-galactopyranoside (**3**) [8], 3β-hydroxyspirosta-5,25(27)-dien-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**5**) [2], (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→4)-β-D-gluco-

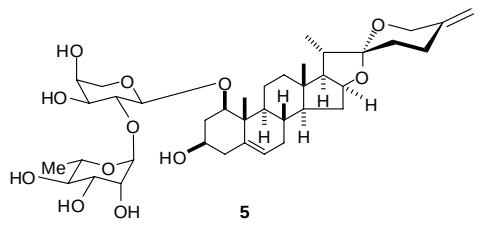
pyranoside (**6**) [9], (25*R*)-26-[(β-D-glucopyranosyl)-oxy]-3β-hydroxy-22α-methoxyfurost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranoside (**7**) [3], (25*R*)-26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-6-*O*-acetyl-β-D-galacto-pyranoside (**8**) [3], 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurosta-5,25(27)-dien-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**12**) [2], 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurosta-5,25(27)-dien-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside (**13**) [2], 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxy-furosta-5,25(27)-dien-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-4-*O*-[(2*S*,3*S*)-2-hydroxy-3-methylpentanoyl]-α-L-arabinopyranoside (**14**) [2], and (22*S*)-16β-[(β-D-glucopyranosyl)oxy]-3β,22-dihydroxycholest-5-en-1β-yl α-L-rhamnopyranoside (**15**) [10], respectively. Although **14** was obtained by mild alkaline hydrolysis of 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurosta-5,25(27)-dien-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-3-*O*-acetyl-4-*O*-[(2*S*,3*S*)-2-hydroxy-3-methylpentanoyl]-α-L-arabino-pyranoside [2], there has been no report on the isolation of **14** from a natural source.

Compound **4** was isolated as an amorphous solid and its molecular formula was determined to be $C_{46}H_{72}O_{18}$ on the basis of the HRMS-ESITOF data (m/z 913.4802 $[M + H]^+$). The IR spectrum of **1** was indicative of a glycoside (3376 and 1046 cm^{-1}) and showed an absorption band due to an ester carbonyl group (1749 cm^{-1}). The ^1H NMR spectrum of **1** showed signals for four typical steroid methyl groups; two appeared as singlets at δ 1.39 and 0.85, and the other two as doublets at δ 1.04 (J = 6.2 Hz) and 0.65 (J = 5.1 Hz). An olefinic proton and three anomeric proton signals were also noted at δ 5.59 (br d, J = 5.5 Hz), and δ 6.33 (br s), 4.97 (d, J = 7.6 Hz) and 4.71 (d, J = 7.8 Hz). In the ^{13}C NMR spectrum of **4**, a quaternary carbon signal assignable to C-22 of the spirostanol skeleton, together with three anomeric carbon signals were observed at δ 109.2, and δ 106.6, 101.6 and 100.9, respectively. These spectral features of **4** were similar to those of **3**. The presence of an acetyl group in the molecule of **4** was shown by the IR (1749 cm^{-1}), ^1H NMR [δ 1.98 (3H, s)], and ^{13}C NMR [δ 170.5 (C=O) and 20.8 (Me)] spectral data.

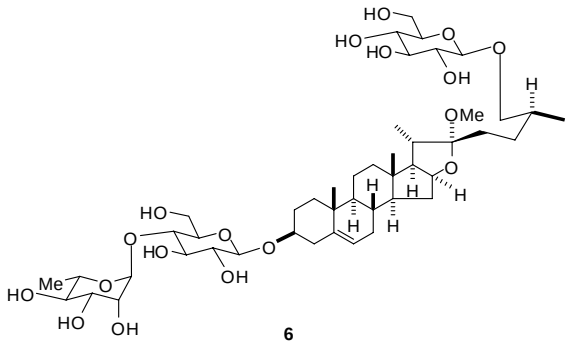


	R_1	R_2
1	H	H
2	H	Ac
3	Xyl	H
4	Xyl	Ac

Xyl: β-D-xylopyranosyl
Ac: acetyl



5



6

Treatment of **4** with 3% NaOMe in MeOH gave **3**. Therefore, **4** was found to be a monoacetate of **3**. In the ^{13}C NMR spectrum of **4**, the signal due to C-6 of the galactosyl moiety was shifted to lower field by 3.1 ppm, whereas the signal due to C-5 occurred at higher shift by 3.1 ppm, as compared with those of **3**. Furthermore, long-range correlations were observed between the carbonyl carbon at δ 170.5 and the unequivalent H_2-6 methylene protons of the galactosyl residue at δ 4.93 (dd, J = 11.2, 7.7 Hz) and 4.49 (dd, J = 11.2, 4.2 Hz) in the HMBC spectrum of **4**. Thus, the acetyl moiety was revealed to be linked to the galactose C-6 hydroxy position, and the structure of **4** was determined to be (25*R*)-3β-hydroxyspirost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-6-*O*-acetyl-β-D-galactopyranoside.

Compound **9** was analyzed for $C_{51}H_{84}O_{23}$ by the MS-FAB, HRMS-ESITOF, and elemental analysis data.

Table 1. ^{13}C NMR spectral data of **4, 9-11**, and **16** in C_5D_5N.

carbon	**4**	**9**	**10**	**11**	**16**
1	85.5	84.7	85.4	84.6	83.1
2	38.1	37.9	38.0	37.8	37.5
3	68.1	68.1	68.2	68.2	68.0
4	43.6	43.7	43.7	43.5	43.8
5	139.4	139.4	139.4	139.2	139.4
6	124.7	124.7	124.7	124.6	124.9
7	32.0	32.0	32.0	31.8	31.8
8	33.1	32.9	33.0	33.0	33.1
9	50.5	50.4	50.5	50.3	50.3
10	42.7	42.7	42.7	42.5	42.7
11	24.0	23.9	24.0	24.2	23.8
12	40.1	40.2	40.0	40.5	40.5
13	40.2	40.4	40.5	40.6	42.1
14	57.1	56.9	57.0	57.0	55.2
15	32.4	32.2	32.3	32.3	37.2
16	81.1	81.2	81.3	81.3	82.7
17	62.9	64.1	64.3	64.4	58.0
18	16.8	16.7	16.7	16.8	13.7
19	15.0	15.0	14.9	14.9	14.7
20	41.9	40.4	40.5	40.6	36.0
21	14.9	16.0	16.1	16.2	12.6
22	109.2	112.6	112.6	112.6	73.2
23	31.7	30.7	30.8	30.7	33.7
24	29.2	28.0	28.0	28.0	36.7
25	30.5	34.1	34.1	34.1	28.9
26	66.7	75.1	75.1	75.1	23.1
27	17.2	17.0	17.0	17.0	23.0
OMe		47.1	47.1	47.1	
	Gal	*Gal*	*Gal*	*Gal*	*Xyl*
1	100.9	100.7	100.9	100.1	102.2
2	73.2	73.5	73.2	73.8	75.2
3	84.9	85.5	84.9	81.7	78.8
4	70.1	69.6	70.1	71.6	71.1
5	72.8	75.9	72.8	71.5	67.5
6	64.7	61.6	64.7	63.2	
Ac	170.5		170.5	170.3	
	20.8		20.8	20.7	
				170.2	
				20.5	
	Rha	*Rha*	*Rha*	*Rha*	*Glc*
1	101.6	101.5	101.6	101.7	107.0
2	72.3	72.3	72.3	72.2	75.6
3	72.3	72.3	72.3	72.3	78.7
4	74.2	74.2	74.2	74.1	71.7
5	69.3	69.2	69.2	69.5	78.2
6	19.0	19.0	19.0	19.1	62.9
	Xyl	*Xyl*	*Xyl*	*Xyl*	
1	106.6	106.4	106.6	106.3	
2	74.6	74.6	74.6	74.7	
3	78.2	78.2	78.2	78.1	
4	70.9	70.9	70.9	70.8	
5	66.9	66.8	66.9	67.0	
		Glc	*Glc*	*Glc*	
1		104.8	104.8	104.8	
2		75.1	75.1	75.1	
3		78.4	78.5	78.5	
4		71.6	71.6	71.6	
5		78.3	78.4	78.3	
6		62.7	62.7	62.7	

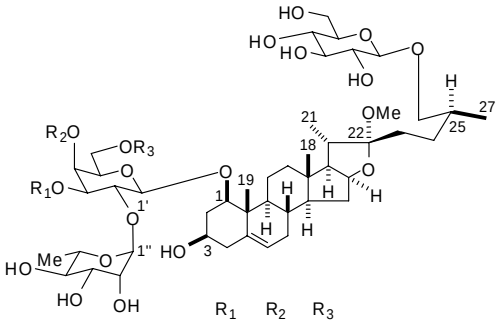
The fundamental structure of **9** was shown to be a 22-methoxyfurostanol saponin by a positive color reaction with Ehrlich's reagent, and by the ^{1}H NMR [δ 3.22 (3H, s)] and ^{13}C NMR [δ 112.6 (C-22) and 47.1 (OMe)] spectra [11]. The ^{1}H NMR spectrum of **9** showed signals for four steroid methyl groups at δ 1.39 (s), 1.06 (d, *J* = 6.9 Hz), 0.95 (d, *J* = 6.7 Hz), and 0.82 (s), as well as those for four anomeric protons at δ 6.33 (br s), 4.97 (d, *J* = 7.5 Hz), 4.80 (d, *J* = 7.8 Hz), and 4.72 (d, *J* = 7.7 Hz). Enzymatic hydrolysis of β-D-glucosidase resulted in the production of **3** and D-glucose. In addition, a long-range correlation was observed between the anomeric proton of the terminal glucosyl unit at δ 4.80 and the C-26 methylene carbon at δ 75.1. Thus, **9** was revealed to be the corresponding 22-methoxy-furostanol saponin of **3** with a glucosyl unit at C-26. An NOE correlation between the methoxy protons at δ 3.22 and the H-16 proton at δ 4.86 was consistent with the 22α configuration. Accordingly, the structure of **9** was elucidated as 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxy-furost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-β-D-galactopyranoside.

Compound **10** was deduced to be $C_{53}H_{86}O_{24}$ on the basis of the MS-FAB, HRMS-ESITOF, and elemental analysis data. Analysis of the ^{1}H and ^{13}C NMR spectra of **10** showed that the structure of **10** is closely related to that of **9** except for the presence of an acetyl group [IR: 1742 cm^{-1} (C=O); ^{1}H NMR: δ 1.99 (3H, s); ^{13}C NMR: δ 170.5 (C=O) and 20.8 (Me)]. Alkaline methanolysis of **10** with 3% NaOMe in MeOH furnished **9**. When the ^{13}C NMR spectrum of **10** was compared with that of **9**, the signal due to C-6 of the galactosyl moiety was shifted to lower field by 3.1 ppm, whereas the signal due to C-5 occurred at higher shift by 3.1 ppm, suggesting an ester linkage of an acetyl group at C-6 of the galactosyl unit. This was confirmed by HMBC correlations between the carbonyl carbon at δ 170.5 and the H_2-6 methylene protons of the galactosyl residue at δ 4.93 (dd, *J* = 11.2, 7.8 Hz) and 4.50 (m). Thus, the structure of **10** was determined to be 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-6-*O*-acetyl-β-D-galactopyranoside.

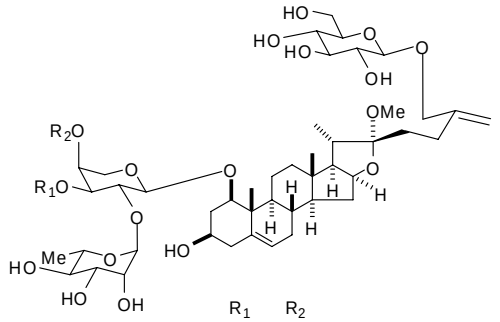
Compound **11** exhibited a molecular formula of $C_{55}H_{88}O_{25}$ based on the MS-FAB, HRMS-ESITOF, and elemental analysis data. The ^{1}H and ^{13}C NMR spectral data of **11** suggested that its structure is essentially analogous to that of **9**. The presence of two acetyl groups in **11** was shown by two three-proton singlet signals at δ 2.00 and 1.93, which, respectively, correlated to the δ 20.7 and 20.5 carbon resonances, and to the carbonyl carbons at δ 170.3 and 170.2 in the HMBC spectrum. Alkaline methanolysis of **11** with NaOMe in MeOH gave **9**.

On comparison of the ^{13}C NMR spectrum of **11** with that **9**, the resonances assignable to C-4 and C-6 of the galactosyl moiety were moved downfield by 2.0 ppm and 1.6 ppm, respectively, whereas those due to C-3 and C-5 were displaced upfield by 3.8 ppm and 4.4 ppm, suggesting that the two acetyl groups are involved in ester linkages at C-4 and C-6 of the galactosyl group. Furthermore, in the HMBC spectrum of **11**, long-range correlations were observed between the carbonyl carbon at δ 170.2 and the H-4 proton of the galactosyl group at δ 5.86 (br d *J* = 2.8 Hz), and between the carbonyl carbon at δ 170.3 and the H_2-6 methylene protons of the galactosyl group at δ 4.50 (dd, *J* = 12.2, 2.0 Hz) and 4.30 (m). These data allowed the structure of **11** to be assigned as 26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy-22α-methoxyfurost-5-en-1β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-4,6-di-*O*-acetyl-β-D-galactopyranoside.

Compound **16** was shown to have the molecular formula $C_{38}H_{64}O_{13}$ on the basis of the HRMS-ESITOF data. The ^{1}H NMR spectrum showed signals for five typical methyl groups of the cholestane skeleton at δ 1.24 (s), 1.15 (d, *J* = 7.0 Hz), 1.03 (s), 0.93 (d, *J* = 6.3 Hz), and 0.92 (d, *J* = 6.2 Hz), as well as those for an olefinic proton at δ 5.52 (br d, *J* = 5.5 Hz) and four methine protons geminally bearing an oxygen atom at δ 4.56 (m), 4.29 (m), 3.89 (br m), and 3.86 (dd, *J* = 11.6, 3.7 Hz). Signals for two anomeric protons were also noted at δ 4.85 (d, *J* = 7.6 Hz) and 4.73 (d, *J* = 7.8 Hz). These ^{1}H NMR spectral properties and ^{13}C NMR information implied that the aglycone moiety of **16** is the same as that of **15** and that the sugar moieties of **16** are composed of a β-D-glucopyranosyl unit and a β-D-xylopyranosyl unit. Acid hydrolysis of **16** with 1.0 M HCl in dioxane-H_2O (1:1) yielded (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol (**16a**) [12] as the aglycone, and D-glucose and D-xylose as the sugar moieties. In the HMBC spectrum of **16**, long-range correlations were observed between the anomeric proton of the xylosyl unit at δ 4.85 and the C-1 carbon of the aglycone at δ 83.1, and between the anomeric proton of the glucosyl unit at δ 4.73 and the C-16 carbon of the aglycone at δ 82.7. The structure of **16** was formulated as (22*S*)-16β-[(β-D-glucopyranosyl)oxy]-3β,22-dihydroxycholest-5-en-1β-yl β-D-xylopyranoside.

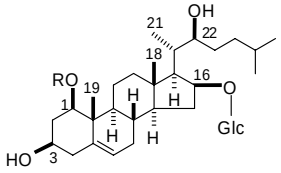


	R_1	R_2	R_3
7	H	H	H
8	H	H	Ac
9	Xyl	H	H
10	Xyl	H	Ac
11	Xyl	Ac	Ac



	R_1	R_2
12	H	H
13	Xyl	H
14	H	HMP

HMP: (2*S*,3*S*)-2-hydroxy-3-methylpentanoyl



	R
15	Rha
16	Xyl

Glc: β-D-glucopyranosyl
Rha: α-L-rhamnopyranosyl

The isolated compounds were evaluated for their cytotoxic activity against HL-60 leukemia cells using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction method [13]. Compounds **1**, **2**, and **5**, which are the spirostanol derivatives with the sugar unit of rhamnosyl-(1→2)-galactosyl or rhamnosyl-(1→2)-arabinosyl at C-1 of the aglycone moiety, showed moderate cytotoxic activity with IC_{50} values of 9.6 μM, 9.2 μM, and 6.5 μM, respectively, when etoposide and cisplatin used as positive controls gave IC_{50} values of 0.46 and 1.6 μM.

Experimental

General experimental procedures: Optical rotations were measured using a JASCO DIP-1030 (Tokyo, Japan) automatic digital polarimeter. Elemental analysis was carried out using an Elemental Vario EL (Hanau, Germany) elemental analyzer. 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 the δ-value with reference to tetramethylsilane (TMS) as an internal standard. MS-ESITOF data were obtained on a Waters-Micromass LCT mass spectrometer (Manchester, U.K.). Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and octadecylsilanized (ODS) silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm or 0.5 mm thick, Merck, Darmstadt, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. HPLC was performed using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh) and/or 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 materials and reagents were used for cell culture and assay of cytotoxic activity: microplate reader, Spectra Classic, Tecan (Salzburg, Austria); 96-well flat-bottom plate, Iwaki Glass (Chiba, Japan); HL-60 cells, Human Science Research Resources Bank (JCRB 0085, Osaka, Japan); fetal bovine serum (FBS), Bio-Whittaker (Walkersville, MD, U.S.A.); RPMI 1640 medium, etoposide, cisplatin, and MTT, Sigma (St. Louis, MO, U.S.A.); penicillin G and streptomycin sulfate, Meiji-Seika (Tokyo, Japan). All other chemicals used were of biochemical reagent grade.

Plant material: The rhizomes of *R. hypophyllum* were collected in the field of Giresun, Tirebolu, and Inkoy wards, Turkey, in March 2002. The plant was identified by one of the authors (M.C.). A voucher specimen has been deposited in Ankara Universitesi Eczacilik Fakultesi Herbaryumu (No. 22844).

Extraction and isolation: The powdered rhizomes (dry weigh, 1.2 kg) were extracted with MeOH (4.5 L) at room temperature for 1 week. The extract was concentrated under reduced pressure, and the viscous concentrate (221 g) was passed through a Diaion HP-20 column (70 mm i.d. x 380 mm), successively eluted with 30% MeOH in H_2O, EtOH, and EtOAc (each 3 L). The EtOH eluate portion (25 g) was chromatographed on silica gel (70 mm i.d. x 270 mm) and eluted with $CHCl_3$-MeOH gradients (5:1; 3:1; 1:1), and finally with MeOH alone, to give 8 fractions (I-VIII). Fraction IV was separated by a silica gel column (40 mm i.d. x 200 mm) eluted with $CHCl_3$-MeOH-H_2O (30:10:1) to give four subfractions (IV-1–IV-4). Fraction IV-1 was subjected to ODS silica gel column chromatography (20 mm i.d. x 230 mm) eluted with MeOH-H_2O (3:1) and MeCN-H_2O (1:2) to yield **2** (20.3 mg) and **5** (100 mg). Fraction IV-2 was chromatographed on ODS silica gel (20 mm i.d. x 230 mm) eluted with MeOH-H_2O (8:5; 4:3) and MeCN-H_2O (5:11) to afford **15** (59.7 mg) and **16** (10.0 mg). Fraction IV-4 was subjected to an ODS silica gel column (20 mm i.d. x 230 mm) eluted with MeOH-H_2O (8:5) to give **6** (54.3 mg) and **13** (22.0 mg). Fraction V was chromatographed on silica gel (25 mm i.d. x 220 mm) eluted with $CHCl_3$-MeOH-H_2O (20:10:1) and ODS silica gel (25 mm i.d. x 220 mm) with MeCN-H_2O (2:5) to furnish **1** (33.2 mg), **4** (95.7 mg), and **8** (47.5 mg). Fraction VI was subjected to column chromatography on silica gel (20 mm i.d. x 240 mm) eluted with $CHCl_3$-MeOH-H_2O (25:10:1) and ODS silica gel (20 mm i.d. x 230 mm) with MeCN-H_2O (2:5) to give **3** (44.1 mg). Fraction VII was subjected to ODS silica gel column chromatography (50 mm i.d. x 250 mm) eluted with MeCN-H_2O gradients (1:4; 1:2; 1:1) followed by silica gel column chromatography (40 mm i.d. x 230 mm) eluted with $CHCl_3$-MeOH-H_2O (30:10:1) to give three subfractions (VII-1–VII-3). Fraction VII-1 was purified by preparative HPLC using MeCN-H_2O (10:27) to yield **11** (23.2 mg) and **12** (90.6 mg). Fraction VII-2 was purified by preparative HPLC using MeCN-H_2O (5:14) to yield **7** (26.2 mg) and **9** (110 mg). Fraction VII-3 was chromatographed on silica gel (25 mm i.d. x 250 mm) eluted with $CHCl_3$-MeOH-H_2O (20:10:1) and ODS silica gel (25 mm i.d. x 250 mm) eluted with MeCN-H_2O (1:4) to afford **10** (178 mg) and **14** (20.1 mg).

All the furostanol saponins were obtained as a mixture of 22-hydroxy- and 22-methoxyfurostanol saponins. The 22-hydroxy form present in the

mixture was completely converted to the 22-methoxy form by treatment with hot MeOH, and the structural determination was carried out with the 22-methoxy form.

Compound 4

Amorphous solid.
$[\alpha]_D^{25}$: -56.0 (*c* 0.10, MeOH).
IR (film): 3376 (OH), 2925 and 2854 (CH), 1749 (C=O), 1046 cm^{-1}.
^1H NMR (500 MHz, C_5D_5N): δ 6.33 (1H, br s, H-1 of Rha), 5.59 (1H, br d, *J* = 5.5 Hz, H-6), 4.97 (1H, d, *J* = 7.6 Hz, H-1 of Xyl), 4.93 (1H, dd, *J* = 11.2, 7.7 Hz, H-6a of Gal), 4.71 (1H, d, *J* = 7.8 Hz, H-1 of Gal), 4.55 (1H, m, H-16), 4.49 (1H, dd, *J* = 11.2, 4.2 Hz, H-6b of Gal), 3.78 (1H, m, H-3), 3.71 (1H, dd, *J* = 12.0, 3.9 Hz, H-1), 1.98 (3H, s, Ac), 1.70 (3H, d, *J* = 6.1 Hz, Me-6 of Rha), 1.39 (3H, s, Me-19), 1.04 (3H, d, *J* = 6.2 Hz, Me-21), 0.85 (3H, s, Me-18), 0.65 (3H, d, *J* = 5.1 Hz, Me-27).
^{13}C NMR: Table 1.
HRMS-ESITOF: m/z $[M + H]^+$ calcd for $C_{46}H_{73}O_{18}$:913.4797; found: 913.4802.

Alkaline methanolysis of 4: Compound **4** (5.0 mg) was treated with 3% NaOMe in MeOH (3 mL) at room temperature for 2 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B (Organo, Tokyo, Japan) column and then chromatographed on silica gel eluted with $CHCl_3$-MeOH-H_2O (20:10:1) to yield **3** (3.9 mg).

Compound 9

Amorphous solid.
$[\alpha]_D^{25}$: -37.5 (*c* 0.10, MeOH).
IR (film): 3388 (OH), 2927 (CH), 1046 cm^{-1}.
^1H NMR (500 MHz, C_5D_5N): δ 6.33 (1H, br s, H-1 of Rha), 5.54 (1H, br d, *J* = 5.5 Hz, H-6), 4.97 (1H, d, *J* = 7.5 Hz, H-1 of Xyl), 4.86 (1H, m, H-16), 4.80 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 4.72 (1H, d, *J* = 7.7 Hz, H-1 of Gal), 3.81 (1H, m, H-3), 3.76 (1H, dd, *J* = 11.9, 3.6 Hz, H-1), 3.22 (3H, s, OMe), 1.68 (3H, d, *J* = 6.1 Hz, Me-6 of Rha), 1.39 (3H, s, Me-19), 1.06 (3H, d, *J* = 6.9 Hz, Me-21), 0.95 (3H, d, *J* = 6.7 Hz, Me-27), 0.82 (3H, s, Me-18).
^{13}C NMR: Table 1.
MS-FAB (positive): m/z 1087.6 $[M + Na]^+$.
HRMS-ESITOF: m/z $[M - OMe]^+$ calcd for $C_{50}H_{81}O_{22}$: 1033.5220; found: 1033.5199.
Analysis: $C_{51}H_{84}O_{23}\cdot 2H_2O$, Calc: C, 55.61; 8.06. Found: C, 55.16; H, 8.25.

Enzymatic hydrolysis of 9: A mixture of **9** (7.7 mg) and β-D-glucosidase (EC 232-589-7, Sigma) (16.7 mg) in AcOH-AcONa buffer (pH 5.0, 3 mL) was incubated at room temperature for 20 h. The crude mixture was chromatographed on silica gel eluted with $CHCl_3$-MeOH-H_2O (20:10:1) to yield **8** (5.8 mg) and D-glucose. TLC: D-glucose, R_f 0.40 (*n*-BuOH-Me_2CO-H_2O (4:5:1).

Compound 10

Amorphous solid.
$[\alpha]_D^{25}$: -42.0 (*c* 0.10, MeOH).
IR (film): 3387 (OH), 2933 (CH), 1742 (C=O), 1070, 1045 cm^{-1}.
^1H NMR (500 MHz, C_5D_5N): δ 6.32 (1H, br s, H-1 of Rha), 5.59 (1H, br d, *J* = 5.5 Hz, H-6), 4.97 (1H, d, *J* = 7.6 Hz, H-1 of Xyl), 4.93 (1H, dd, *J* = 11.2, 7.8 Hz, H-6a of Gal), 4.81 (1H, d, *J* = 7.2 Hz, H-1 of Glc), 4.71 (1H, d, *J* = 7.8 Hz, H-1 of Gal), 4.50 (1H, m, H-6b of Gal), 4.47 (1H, m, H-16), 3.78 (1H, m, H-3), 3.71 (1H, dd, *J* = 12.0, 3.7 Hz, H-1), 3.21 (3H, s, OMe), 1.99, (3H, s, Ac), 1.68 (3H, d, *J* = 6.1 Hz, Me-6 of Rha), 1.39 (3H, s, Me-19), 1.10 (3H, d, *J* = 6.8 Hz, Me-21), 0.95 (3H, d, *J* = 6.6 Hz, Me-27), 0.85 (3H, s, Me-18).
^{13}C NMR: Table 1.
MS-FAB (positive): m/z 1129.9 $[M + Na]^+$.
HRMS-ESITOF: m/z $[M - OMe]^+$ calcd for $C_{52}H_{83}O_{23}$: 1075.5325; found: 1075.5364.
Analysis: $C_{53}H_{86}O_{24}\cdot 2H_2O$, Calc: C, 55.67; 7.94. Found: C, 55.65; H, 8.30.

Alkaline methanolysis of 10: Compound **10** (5.5 mg) was treated with 3% NaOMe in MeOH (3 mL) at room temperature for 2 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B column and then chromatographed on silica gel eluted with $CHCl_3$-MeOH-H_2O (20:10:1) to yield **9** (4.3 mg).

Compound 11

Amorphous solid.
$[\alpha]_D^{25}$: -30.1 (*c* 0.10, MeOH).
IR (film): 3382 (OH), 2935 and 2907 (CH), 1744 (C=O), 1070, 1047 cm^{-1}.
^1H NMR (500 MHz, C_5D_5N): δ 6.31 (1H, br s, H-1 of Rha), 5.86 (1H, br d, *J* = 2.8 Hz, H-4 of Gal), 5.59 (1H, br d, *J* = 5.9 Hz, H-6), 4.95 (1H, m, H-16), 4.94 (1H, d, *J* = 7.4 Hz, H-1 of Xyl), 4.82 (1H, d, *J* = 7.7 Hz, H-1 of Glc), 4.76 (1H, d, *J* = 7.6 Hz, H-1 of Gal), 4.50 (1H, dd, *J* = 12.2, 2.0 Hz, H-6a of Gal), 4.30 (1H, m, H-6b of Gal), 3.79 (1H, m, H-3), 3.72 (1H,

dd, *J* = 12.0, 3.8 Hz, H-1), 3.22 (3H, s, OMe), 2.00 and 1.93 (each 3H, s, Ac), 1.72 (3H, d, *J* = 5.6 Hz, Me-6 of Rha), 1.38 (3H, s, Me-19), 1.14 (3H, d, *J* = 6.8 Hz, Me-21), 0.97 (3H, d, *J* = 6.2 Hz, Me-27), 0.92 (3H, s, Me-18).
^{13}C NMR: Table 1.
MS-FAB (positive): m/z 1171.6 $[M + Na]^+$.
HRMS-ESITOF: m/z $[M - OMe]^+$ calcd for $C_{54}H_{85}O_{24}$: 1117.5431; found: 1117.5499.
Analysis: $C_{55}H_{88}O_{25}\cdot 1/2H_2O$, Calc: C, 57.02; 7.75. Found: C, 56.98; H, 8.43.

Alkaline methanolysis of 11: Compound **11** (5.3 mg) was treated with 3% NaOMe in MeOH (3 mL) at room temperature for 2 h. The reaction mixture was neutralized by passage through an Amberlite IR-120B column and then chromatographed on silica gel eluted with $CHCl_3$-MeOH-H_2O (20:10:1) to yield **9** (3.9 mg).

Compound 16

Amorphous solid.
$[\alpha]_D^{28}$: -13.7 (*c* 0.10, MeOH).
IR (film): 3362 (OH), 2925 (CH), 1038 cm^{-1}.
^{1}H NMR (500 MHz, C_5D_5N): δ 5.52 (1H, br d, *J* = 5.5 Hz, H-6), 4.85 (1H, d, *J* = 7.6 Hz, H-1 of Xyl), 4.73 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 4.56 (1H, m, H-16), 4.29 (1H, m, H-22), 3.89 (1H, m, H-3), 3.86 (1H, 1H, dd, *J* = 11.6, 3.7 Hz, H-1), 1.24 (3H, s, Me-19), 1.15 (3H, d, *J* = 7.0 Hz, Me-21), 1.03 (3H, s, Me-18), 0.93 (3H, d, *J* = 6.3 Hz, Me-26), 0.92 (3H, d, *J* = 6.2 Hz, Me-27).
^{13}C NMR: Table 1.
HRMS-ESITOF: m/z $[M + Na]^+$ calcd for $C_{38}H_{64}O_{13}Na$: 751.4245; found: 751.4218.

Acid hydrolysis of 16: A solution of **16** (5.5 mg) in 1.0 M HCl (dioxane-H_2O, 1:1, 2 mL) was heated at 95°C for 1 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo) column and then was subjected to a Diaion HP-20 column eluted with H_2O-MeOH (3:2) followed by EtOH-Me_2CO (1:1) to give **16a** (2.0 mg) and a sugar mixture. The sugar mixture was analyzed by HPLC under the following conditions: column, a 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-glucose and D-xylose present in the mixture was carried out by comparison of their retention times and polarities with those of authentic samples. t_R (min): 10.09 (D-xylose, positive optical rotation) and 16.75 (D-glucose, positive optical rotation).

HL-60 Cell culture assay: HL-60 cells were maintained in the RPMI 1640 medium containing 10% FBS supplemented with L-glutamine, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate. The leukemia cells were washed and resuspended in the above medium to 4 x 10^4 cells/mL, and 196 μL of this cell suspension was placed in each well of a 96-well flat-bottom plate. 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 0.1-20 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. Briefly, after termination of the cell culture, 10 μL of 5 mg/mL MTT in phosphate-buffered saline (PBS) 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 rpm 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 dimethyl sulfoxide (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 at 550 nm.

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