Steroidal Glycosides from the Bulbs of *Allium jesdianum*

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Phytochemical analysis of the fresh bulbs of *Allium jesdianum* yielded four steroidal glycosides, (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1,16-di-*O*-β-D-glucopyranoside (**1**), (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1-*O*-α-L-rhamnopyranosyl 16-*O*-β-D-glucopyranoside (**2**), (25*R*)-5α-spirostane-2α,3β-diol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (F-gitonin) (**3**), and (25*R*)-5α-spirostane-2α,3β,6α-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (**4**). Compound **2** is a new natural product, and **4** is a new spirostanol saponin. Compound **3** was found to exhibit cytostatic and cytotoxic activities against several malignant tumor cells.

The steroidal saponins are naturally occurring glycosides that possess properties such as producing a form, hemolytic activity, toxicity to fish, and complex formation with cholesterol. Plants of the genus *Allium* (Liliaceae) are well-known for their production of steroidal saponins.[1,2] We have previously performed a systematic phytochemical screening study of nine *Allium* species, *A. giganteum*,[3−5] *A. aflatunense*,[4] *A. schubertii*,[6,7] *A. albopilosum*,[8] *A. ostrowskianum*,[8] *A. chinense*,[9] *A. macleanii*,[10] *A. senescens*,[10] and *A. sphaerosephalon*,[11] and have isolated a variety of novel steroidal saponins and cholestane glycosides. In a continuation of our studies on steroidal glycosides with medicinal potential from the genus *Allium*, we have now examined the fresh bulbs of *Allium jesdianum* Boissier & Buhse, which is native to Iran and Iraq and cultivated in Japan as a garden plant with purple-lilac flowers. This study has resulted in the isolation of two cholestane glycosides (**1** and **2**) and two spirostanol saponins (**3** and **4**) (Chart 1), among which **2** has been isolated from a plant source for the first time and **4** is a new spirostanol saponin. In this paper, we report the identification and structural determination of **1**−**4** on the basis of spectroscopic analysis, including 2D NMR techniques, and the results of acid hydrolysis. The activity of **1**−**4** against HL-60 human promyelocytic leukemia cells was also investigated.

The concentrated *n*-BuOH-soluble extract of the methanolic extract of *A. jesdianum* bulbs (fresh weight of 22.2 kg) was repeatedly subjected to column chromatography on porous-polymer resin (Diaion HP-20), silica gel, and on octadecylsilanized (ODS) silica gel to yield **1** (54.5 mg), **2** (71.3 mg), **3** (4.12 g), and **4** (110 mg).

Compounds **1** and **3** are known steroidal glycosides, and the structures were identified as (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1,16-di-*O*-β-D-glucopyranoside[12] and (25*R*)-5α-spirostane-2α,3β-diol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} (F-gitonin),[13] respectively, and the physical and spectral data obtained were consistent with the literature values.

Compound **2** ($C_{39}H_{66}O_{13}$, negative-ion FABMS *m*/*z* 741 $[M - H]^-$) was obtained as an amorphous solid. The ^{1}H NMR spectrum of **2** in pyridine-d_5 showed signals for five steroid methyls; three appeared as doublets at δ 1.16 (J = 6.9 Hz), 0.94 (J = 6.0 Hz), and 0.93 (J = 6.1 Hz) and the other two as singlets at δ 1.22 and 1.05. Two anomeric proton signals were also noted at δ 5.63 (br s) and 4.75 (d, J = 7.7 Hz). Analysis of the ^{13}C NMR spectrum of **2** indicated that the structure of the aglycon of **2** was identical to that of **1** bearing a monosaccharide at each of the C-1 and C-16 hydroxyl groups and that **2** contained a terminal α-L-rhamnopyranosyl moiety (δ 97.7, 72.9 × 2, 73.7, 70.7, and 18.7) and a terminal β-D-glucopyranosyl moiety (δ 107.0, 75.6, 78.7, 71.7, 78.2, and 62.9). The above data were consistent with the structural assignment of **2** as (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1-*O*-α-L-rhamnopyranosyl 16-*O*-β-D-glucopyranoside.[14] Although it has been already obtained as a partial hydrolysate of the bisdesmosidic cholestane triglycoside isolated from *A. albopilosum*,[8] this is the first example of the isolation of **2** from a plant source.

Compound **4** was isolated as an amorphous solid, $[\alpha]_D$ −42.0° (methanol). The molecular formula was determined to be $C_{50}H_{82}O_{24}$ by the ^{13}C NMR spectrum with 50 signals, from the high-resolution positive-ion FABMS that showed a quasimolecular ion peak at *m*/*z* 1085.5111 $[M + Na]^+$ (+Δ 1.7 mmu), and by the results of elemental analysis. The ^{1}H NMR spectrum in pyridine-d_5 of **4** showed signals for two tertiary methyl groups at δ 0.82 and 0.77 (each 3H, s); two secondary methyl groups at δ 1.13 (3H, d, J = 6.9 Hz) and 0.69 (3H, d, J = 5.8 Hz); and four anomeric protons at δ 5.57 (1H, d, J = 7.7 Hz), 5.25 (1H, d, J = 7.8 Hz), 5.22 (1H, d, J = 7.9 Hz), and 4.89 (1H, d, J = 7.8 Hz). These ^{1}H NMR data and a quaternary ^{13}C NMR signal at δ 109.3 suggested **4** to be a spirostanol tetraglycoside. Acid hydrolysis of **4** with 1 M hydrochloric acid in dioxane−H_2O (1:1) yielded an aglycon (**4a**) and D-glucose, D-galactose, and D-xylose as carbohydrate moieties. The monosaccharides were identified by HPLC analysis following their conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives.[15] Compound **4a** gave an accurate molecular ion peak at *m*/*z* 448.3190 in the high-resolution EIMS, appropriate for a molecular formula of $C_{27}H_{44}O_5$. From a preliminary inspection of the ^{13}C NMR spectrum of **4a**, seven signals at δ 109.2 (C), 81.1 (CH), 77.1 (CH), 73.0 (CH), 68.4 (CH), 66.9 (CH_2), and 63.0 (CH) could be identified between 60 and 100 ppm, and four of them at δ 109.2 (C), 81.1 (CH), 66.9 (CH_2), and 63.0 (CH) were assigned to the C-22, C-16, C-26, and C-17 positions, respectively, in a spirostanol skeleton. Consequently, the remaining three signals at δ 77.1 (CH), 73.0 (CH), 68.4 (CH) were due to hydroxy methine groups. The complete

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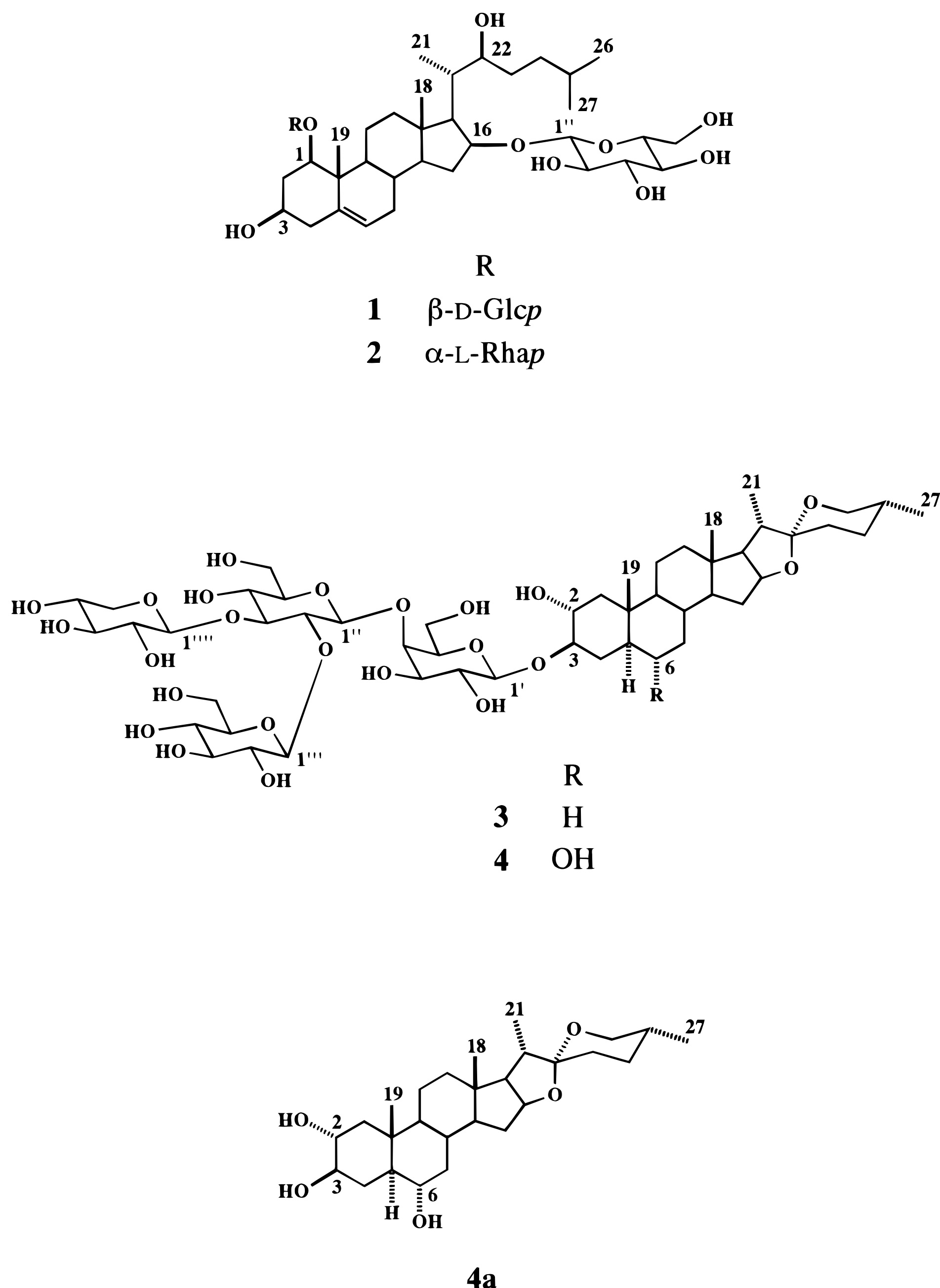
Chart 1



assignment of the ^{13}C NMR spectrum of **4a** was made, which was carried out by the combined use of ^{1}H-^{1}H COSY, HOHAHA, and HMQC spectra. A close similarity to signals of the aglycon region of **3** (25*R*)-5α-spirostane-2α,3β-diol (gitogenin) was observed,[16] with the exception of the signals due to C-6 and its neighboring carbons. The methylene ^{13}C NMR signal at δ 28.4 assignable to C-6 in gitogenin was replaced by the oxymethylene signal at δ 68.4 in **4a**, accompanied by downfield or upfield shifts of the signals attributable to C-4, C-5, and C-7 by −5.2, +7.6, and +10.4 ppm, respectively. This led to the assignment of the location of a hydroxyl group at C-6 in addition to the C-2α and C-3β hydroxyl groups. The C-6α equatorial orientation of the hydroxyl group was confirmed by the coupling constant between the H-6 proton and its adjacent protons of H-5 and H$_2$-7 ($^{3}J_{H\text{-}6,H\text{-}5}$ = 10.6 Hz, $^{3}J_{H\text{-}6,H\text{-}7\alpha(ax)}$ = 10.6 Hz, and $^{3}J_{H\text{-}6,H\text{-}7\beta(eq)}$ = 4.4 Hz). The NOE correlations, H-6/H-4β(ax), H-8, and Me-19, which were observed in the phase-sensitive NOESY spectrum of **4a**, were consistent with a C-6α configuration. The ^{1}H NMR shifts, including the proton multiplicities, and the NOE correlations between Me-19/H-2 and H-4β(ax) and H-5/H-7α(ax) and H-9 also supported the C-2α and C-3β hydroxy configurations and the A/B trans (H-5α) ring junction pattern. Thus, the structure of **4a** was shown to be (25*R*)-5α-spirostane-2α,3β,6α-triol. The tetraglycoside sequence composed of two β-D-glucopyranose, one β-D-galactopyranose, and one β-D-xylopyranose was presumed to be identical to that of **3** by the precise agreement of the ^{13}C NMR shifts between the two compounds.[17] This was confirmed by the following three-bond coupled ^{1}H/^{13}C correlations, δ 5.57 (anomer proton of terminal glucose)/δ 81.3 (C-2 of 2,3-disubstituted glucose), δ 5.25 (anomer proton of xylose)/δ 87.0 (C-3 of 2,3-disubstituted glucose), and δ 5.22 (anomer proton of 2,3-disubstituted glucose)/δ 79.3 (C-4 of galactose), in the HMBC spectrum of **4**. Furthermore, a correlation between the resonances at δ 4.89 (anomer proton of galactose) and δ 85.1 (C-3 of aglycon) gave additional evidence for the

tetraglycoside linkage position at C-3 of **4a**. Accordingly, the structure of **4** was formulated as (25*R*)-5α-spirostane-2α,3β,6α-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}. This is a new spirostanol saponin.

The cytostatic activity of **1**–**4** was evaluated against HL-60 human promyelocytic leukemia cells. Compound **3** exhibited considerable cytotoxic activity with an IC_{50} value of 1.5 μg/mL compared with etoposide used as a positive control (IC_{50} 0.3 μg/mL), while **4** was inactive ($IC_{50} > 10$ μg/mL), indicating that introduction of a hydroxyl group at C-6 of the aglycon of **3** caused the activity to decrease. The cholestane glycosides (**1** and **2**) showed no activity. Subsequent evaluation of **3** in the National Cancer Institute 60 cell line assay[18] showed that the mean concentrations required to achieve GI_{50}, TGI, and LC_{50} levels[19] against the panel of cells tested were 4.5, 18, and 54 μM, respectively. The differential cellular sensitivity of **3** was moderate for the cell lines, and it did not exhibit a specific activity toward any particular subpanel of cells. However, some cell lines were relatively sensitive to it, namely, the leukemia CCRF-CEM (GI_{50}, 0.25 μM; TGI, 0.55 μM; LC_{50}, 2.0 μM), nonsmall cell lung cancer HOP-62 (GI_{50}, 1.2 μM; TGI, 2.8 μM; LC_{50}, 6.2 μM), and breast cancer MCF-7 (GI_{50}, 0.31 μM; TGI, 0.62 μM; LC_{50}, 1.9 μM) cell lines.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a JASCO A-100 spectrophotometer. 1D NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^{1}H NMR) and 2D NMR on a Bruker DRX-500 (500 MHz for ^{1}H NMR). Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. MS were recorded on a VG AutoSpec E mass spectrometer. Elemental analysis was carried out using an Elementar Vario EL elemental analyzer. Diaion HP-20 (Mitsubishi-Kasei, Tokyo, Japan), silica gel (Fuji-Silysia Chemical, Aichi, Japan), and 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 thick, Merck, Darmstadt, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a Tosoh HPLC system comprised of a CCPM pump, a CCP controller PX-8010, a UV-8000, and Rheodyne injection port with a 20 μL sample loop. A Capcell Pak C_{18} column (Shiseido, Tokyo, Japan, 4.6 mm i.d. × 250 mm, ODS, 5 μm) was employed for HPLC analysis. The following materials and reagents were used for cell culture and assay of cytostatic activity: microplate reader, Inter Med Immuno-Mini NJ-2300 (Tokyo, Japan); 96-well flat-bottom plate, Iwaki Glass (Chiba, Japan); HL-60 cells, ICN Biomedicals (Costa Mesa, CA); RPMI 1640 medium, GIBCO BRL (Rockville, MD); MTT, Sigma (St. Louis, MO). All other chemicals used were of biochemical reagent grade.

Plant Material. The bulbs of *A. jesdianum* were purchased from a nursery in Heiwaen, Nara, Japan, in September 1995. The bulbs were cultivated, and a voucher of the plant is on file in our laboratory.

Extraction and Isolation. The plant material (fresh weight, 22.2 kg) was extracted with hot MeOH. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate was partitioned between H_2O and *n*-BuOH. The *n*-BuOH-soluble phase was passed through a Diaion HP-20 column eluting with increased amounts of MeOH in H_2O. Column chromatography of the MeOH eluate portion on silica gel and elution with a stepwise gradient mixture of $CHCl_3$–MeOH (19:1; 9:1; 6:1; 4:1; 2:1), and finally with MeOH alone, gave five fractions (I–V). Fraction III was chromatographed on silica gel eluting with $CHCl_3$–MeOH–H_2O (20:10:1) and ODS silica gel with MeOH–H_2O (8:5) and MeCN–H_2O (5:13) to yield **2** (71.3 mg). Fraction IV was subjected to column chromatography on silica gel eluting with $CHCl_3$–MeOH–H_2O (20:10:1; 7:4:1) and ODS silica gel with MeOH–H_2O (4:1) to give **3** (4.12 g) and **4** (110 mg), and **1** with a few impurities. Final purification of **1** (54.5 mg) was carried out on an ODS silica gel column eluting with MeOH–H_2O (4:3) and MeCN–H_2O (1:2; 1:3). Compounds **1** and **3** were identified by comparison with literature data.[8,12,17]

Table 1. ^{13}C NMR Spectral Data for Compounds **2**, **4**, and **4a**[a]

carbon	2	4	4a
1	81.3	46.2	47.0
2	36.0	70.4	73.0
3	68.1	85.1	77.1
4	43.7	28.9	32.0
5	139.1	52.3	52.9
6	125.1	68.3	68.4
7	31.5	42.6	42.8
8	33.5	34.2	34.2
9	50.8	54.2	54.3
10	42.9	38.0	38.6
11	24.8	21.5	21.5
12	40.6	40.0	40.1
13	42.3	40.9	40.8
14	55.3	56.3	56.3
15	37.2	32.2	32.2
16	82.6	81.1	81.1
17	58.1	63.0	63.0
18	13.9	16.7	16.6
19	14.6	14.7	14.9
20	36.0	42.1	42.0
21	12.6	15.1	15.0
22	73.1	109.3	109.2
23	33.8	31.9	31.8
24	36.8	29.3	29.3
25	28.9	30.7	30.6
26	23.0	66.9	66.9
27	23.1	17.4	17.3
1′	97.7	103.3	
2′	72.9	72.8	
3′	72.9	75.6	
4′	73.7	79.3	
5′	70.7	75.6	
6′	18.7	60.6	
1″	107.0	104.7	
2″	75.6	81.3	
3″	78.7	87.0	
4″	71.7	70.5	
5″	78.2	77.6	
6″	62.9	63.0	
1‴		104.9	
2‴		76.1	
3‴		78.2	
4‴		71.5	
5‴		78.5	
6‴		62.8	
1⁗		105.0	
2⁗		75.2	
3⁗		78.8	
4⁗		70.8	
5⁗		67.4	

[a] Spectra were measured in pyridine-d_5.

Compound 2: amorphous solid; $[\alpha]^{27}_D$ −36.0° (*c* 0.10, MeOH); IR (KBr) ν_{max} 3360 (OH), 2925 (CH), 1060, 1025 cm^{-1}; ^{1}H NMR (C_5D_5N) δ 5.63 (1H, br s, H-1′), 5.54 (1H, br d, *J* = 5.5 Hz, H-6), 4.75 (1H, d, *J* = 7.7 Hz, H-1″), 1.65 (3H, d, *J* = 5.8 Hz, Me-6′), 1.22 (3H, s, Me-19), 1.16 (3H, d, *J* = 6.9 Hz, Me-21), 1.05 (3H, s, Me-18), 0.94 (3H, d, *J* = 6.0 Hz, Me-26 or Me-27), 0.93 (3H, d, *J* = 6.1 Hz, Me-26 or Me-27); ^{13}C NMR, see Table 1; FABMS (negative mode) *m/z* 741 [M − H]$^-$.

Compound 4: amorphous solid; $[\alpha]^{27}_D$ −42.0° (*c* 0.10, MeOH); IR (KBr) ν_{max} 3400 (OH), 2930 (CH), 1445, 1365, 1235, 1145, 1045, 970, 910, 890, 855 (intensity 910 < 890, 25*R*-spiroacetal) cm^{-1}; ^{1}H NMR (C_5D_5N) δ 5.57 (1H, d, *J* = 7.7 Hz,

H-1‴), 5.25 (1H, d, $J = 7.8$ Hz, H-1⁗), 5.22 (1H, d, $J = 7.9$ Hz, H-1″), 4.89 (1H, d, $J = 7.8$ Hz, H-1′), 1.13 (3H, d, $J = 6.9$ Hz, Me-21), 0.82 (3H, s, Me-18), 0.77 (3H, s, Me-19), 0.69 (3H, d, $J = 5.8$ Hz, Me-27); ^{13}C NMR, see Table 1; HRFABMS (positive mode) m/z 1089.5111 $[M + Na]^+$ (calcd for $C_{50}H_{82}O_{24}Na$, 1089.5094); *anal.* C 54.79%, H 8.09%, calcd for $C_{50}H_{82}O_{24}\cdot 3/2H_2O$, C 54.88%, H 7.83%.

Acid Hydrolysis of 4. A solution of **4** (63.1 mg) in 1 M HCl (dioxane−H_2O 1:1, 8 mL) was heated at 80 °C for 4 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and chromatographed over silica gel using a discontinuous gradient of $CHCl_3$−MeOH (9:1−1:1) to give a sugar fraction (31.2 mg) and an aglycon (**4a**) (8.2 mg). The sugar fraction (2.1 mg) was dissolved in H_2O (1 mL), to which (−)-α-methylbenzylamine (5 mg) and $Na[BH_3CN]$ (8 mg) in EtOH (1 mL) were added. After being set aside at 40 °C for 4 h followed by addition of AcOH (0.2 mL) and evaporation to dryness, the reaction mixture was acetylated with Ac_2O (0.3 mL) in pyridine (0.3 mL) at room temperature for 12 h. The crude mixture was passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA) with H_2O−MeCN (4:1; 1:1, each 5 mL) mixtures as solvents. The H_2O−MeCN (1:1) eluate was further passed through a Toyopak IC-SP M cartridge (Tosoh, Tokyo, Japan) with EtOH (10 mL) to give a mixture of the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides,[15] which was then analyzed by HPLC under the following conditions: solvent, MeCN−H_2O (2:3); flow rate, 0.8 mL/min; detection, UV 230 nm. The derivatives of D-xylose, d-galactose, and D-glucose were detected as follows: t_R (min) 13.66 (derivative of D-xylose); 14.73 (derivative of D-galactose); 17.14 (derivative of D-glucose).

Compound 4a: amorphous solid; $[\alpha]^{22}_D$ −46.4° (*c* 0.25, MeOH); IR (KBr) ν_{max} 3380 (OH), 2925 (CH), 1445, 1375, 1240, 1170, 1145, 1055, 1020, 975, 955, 915, 895, 855 (intensity 915 < 895, 25*R*-spiroacetal) cm^{-1}; ^{1}H NMR (C_5D_5N) δ 4.55 (1H, q-like, $J = 7.6$ Hz, H-16), 4.13 (1H, ddd, $J = 11.5, 8.9, 4.7$ Hz, H-2), 3.94 (1H, ddd, $J = 11.2, 8.9, 5.0$ Hz, H-3), 3.69 (1H, ddd, $J = 10.6, 10.6, 4.4$ Hz, H-6), 3.58 (1H, dd, $J = 10.6, 3.7$ Hz, H-26eq), 3.50 (1H, dd, $J = 10.6, 10.6$ Hz, H-26ax), 1.14 (1H, d, $J = 7.0$ Hz, Me-21), 0.96 (3H, s, Me-19), 0.85 (3H, s, Me-18), 0.70 (3H, d, $J = 5.7$ Hz, Me-27); ^{13}C NMR, see Table 1; EIMS m/z 448 $[M]^+$ (6), 376 (13), 334 (10), 305 (14), 139 (100), 115 (20); HREIMS m/z 448.3190 $[M]^+$ (calcd for $C_{27}H_{44}O_5$, 448.3189).

Cell Culture Assay. HL-60 cells were maintained in the RPMI 1640 medium containing 10% fetal bovine serum 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 3×10^4 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 0.01−10 μg/mL; 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 by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay procedure.[20] The MTT assay was carried out according to a modified method of Sargent and Tayler as follows. 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 formazan. An aliquot of 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. Compound **3** showed more than 50% of cell growth inhibition at the sample concentration of 10 μg/mL. A dose−response curve was plotted for **3**, and the concentration giving 50% inhibition (IC_{50}) was calculated.

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Supporting Information Available: Table of the GI_{50}, TGI, and LC_{50} values of **3** in the NCI 60 cell line panel (1 page). Ordering information is given on any current masthead page.

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