Cardenolide Glycosides from *Pergularia tomentosa* and Their Proapoptotic Activity in Kaposi's Sarcoma Cells

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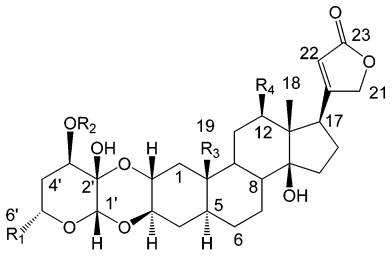
Received May 24, 2006

Continuing our investigations on plants belonging to the Asclepiadaceae family, three new cardenolide glycosides, 3′-*O*-*β*-D-glucopyranosylcalactin (**1**), 12-dehydroxyghalakinoside (**2**), and 6′-dehydroxyghalakinoside (**3**), along with the known ghalakinoside (**4**) and calactin (**5**), were isolated from the roots of *Pergularia tomentosa*. The structures of these compounds were elucidated by extensive spectroscopic methods including 1D- and 2D-NMR experiments as well as ESIMS analysis. The isolated cardenolides caused apoptotic cell death of Kaposi's sarcoma cells.

Plants belonging to the family Asclepiadaceae are rich in steroidal glycosides.[1,2] *Pergularia tomentosa* L. (Asclepiadaceae) is an Egyptian wild perennial shrub that is traditionally used as a laxative, as an abortive, and in the treatment of some skin diseases.[3] Previous studies reported the presence of the cardenolide glycosides ghalakinoside, calactin, uzarigenin, and pergularoside in the roots and desglucouzarin, coroglaucigenin, and uzarigenin along with a *β*-sitosterol glucoside in the leaves.[4,5]

In our ongoing research for new compounds from medicinal plants from Egyptian flora,[6−8] we have investigated the EtOH extract of the roots of *P. tomentosa*. Here we report the occurrence of three new cardenolide glycosides (**1**−**3**), along with the known cardenolide glycosides ghalakinoside (**4**) and calactin (**5**). Since cardenolides are active against a large range of cancer cell types,[9−12] the antiproliferative effects of compounds **1**−**5** were examined against the Kaposis'sarcoma (KS) cell line.



1	R_1 = Me	R_2 = β-D-Glc	R_3 = CHO	R_4 = H
2	R_1 = CH_2OH	R_2 = H	R_3 = CH_2OH	R_4 = H
3	R_1 = Me	R_2 = H	R_3 = CH_2OH	R_4 = OH
4	R_1 = CH_2OH	R_2 = H	R_3 = CH_2OH	R_4 = OH
5	R_1 = Me	R_2 = H	R_3 = CHO	R_4 = H

Results and Discussion

The roots of *P. tomentosa* were extracted with EtOH−H_2O (4:1), and the extract was partitioned between hexane−H_2O, $CHCl_3$−H_2O, and *n*-BuOH−H_2O (1:1). The $CHCl_3$ extract was chromatographed on silica gel to yield three new cardenolide glycosides (**1**−**3**), along with the known cardenolide glycosides ghalakinoside (**4**) and calactin (**5**)[4] (see Experimental Section).

Compound **1** was obtained as an amorphous white solid, and its molecular formula was unequivocally established as $C_{35}H_{50}O_{14}$ by HRMALDIMS. Its IR spectrum exhibited absorption bands for hydroxyl (3446 cm^{-1}), carbonyl (1742 cm^{-1}), and olefinic (1623 cm^{-1}) groups. The UV spectrum indicated the presence of an α,*β*-unsaturated carbonyl group (λ_{max} 218 nm). The ^1H NMR spectrum of the aglycone portion of **1** showed characteristic signals of a butenolactone ring at *δ* 5.93 (1H, t, J = 1.5 Hz), 5.00 (1H, dd, J = 18.4, 1.5 Hz), and 4.90 (1H, dd, J = 18.4, 1.5 Hz), as well as a one-proton singlet at *δ* 10.07 and a methyl signal at 0.83 (3H, s), indicating a cardenolide skeleton with an aldehyde function. The ^{13}C NMR spectrum of **1** (Table 1) showed 35 carbon signals, of which 23 were assigned to the aglycone moiety and 12 to a sugar portion. The ^{13}C NMR chemical shifts of all the hydrogenated carbons could be unambiguously assigned by the HSQC spectrum. In particular, the analysis of the ^{13}C NMR spectrum on the basis of the HSQC correlations clearly confirmed the occurrence of an aldehydic carbon (*δ* 209.3) along with resonances typical of a butenolactone ring: a carbonyl group (*δ* 177.2), an olefinic quaternary function (*δ* 178.1), an olefinic methine (*δ* 117.9), and an oxymethylene group (*δ* 75.3). The ^1H NMR spectrum of **1** was similar to that of calactin (**5**)[4] with the exception of additional signals at *δ* 4.33 (1H, d, J = 7.9 Hz), 3.92 (1H, dd, J = 12.0, 2.5 Hz), 3.66 (1H, dd, J = 12.0, 4.5 Hz), 3.36 (1H, dd, J = 9.0, 9.0 Hz), 3.31 (1H, m), 3.28 (1H, dd, J = 9.0, 9.0 Hz), and 3.25 (1H, dd, J = 9.0, 7.9 Hz). Detailed analysis of HSQC, 1D-TOCSY, and DQF-COSY experiments led to the identification of a *β*-glucopyranosyl unit. The linkage position of this sugar unit was established from the downfield shift of C-3′ (*δ* 78.6), when compared to calactin (**5**) (*δ* 71.6), and the HMBC correlation of $H\text{-}1_{glc}$ at *δ* 4.33 and the C-3′ resonance at *δ* 78.6. The relative configurations of C-3′ and C-17 were established by analysis of ^1H NMR coupling constants. The coupling constant of H-3′ (*δ* 3.83, t, J = 2.0 Hz) indicated an equatorial position of H-3′ and, thus, a *β*-orientation of the *O*-glucosyl moiety.[13] The α-orientation of H-17 was assigned on the basis of the coupling constants of the signal at *δ* 2.85 (1H, dd, J = 5.7, 3.1 Hz), in agreement with published data for 17*β*-cardenolides.[14] The configuration of the glucopyranosyl unit was assigned after hydrolysis with 1 N HCl. The hydrolyzate was trimethylsilylated, and the GC retention time of the sugar unit was

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10.1021/np060228l CCC: $33.50
Published on Web 08/24/2006

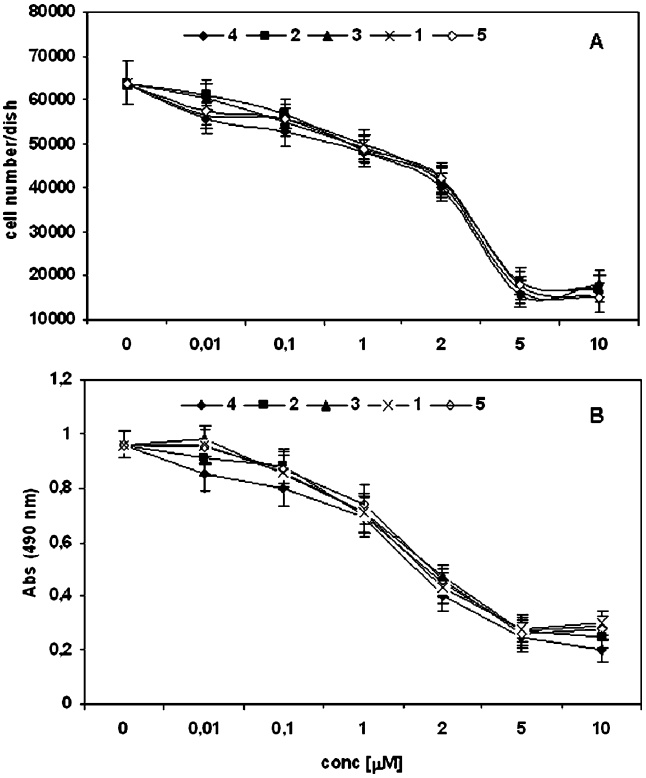

Figure 1. Effect of compounds **1**−**5** on KS cell survival. Cell were exposed to the indicated concentration of each compound, as indicated in the Experimental Section. The cell number was determined by both XTT colorimetric assay and Coulter counter. Data represents the means ± SEM from at least three independent experiments in duplicate with $P < 0.01$ for cells treated with compounds at a concentration higher than 1 μM vs cells treated with DMSO.

Table 1. ^{13}C NMR Data of Compounds **1**−**3** in CD_3OD[a]

position	**1** δ_C	**2** δ_C	**3** δ_C
1	36.9, t	37.2, t	36.7, t
2	70.2, d	69.7, d	69.7, d
3	73.2, d	73.3, d	73.4, d
4	34.5, t	33.6, t	33.5, t
5	44.4, d	47.1, d	47.3, d
6	28.8, t	28.3, t	28.3, t
7	28.6, t	28.1, t	28.1, t
8	43.4, d	41.3, d	41.3, d
9	49.6, d	46.0, d	46.0, d
10	53.9, s	41.9, s	41.9, s
11	22.8, t	22.5, t	31.7, t
12	40.1, t	40.9, t	75.6, d
13	50.6, s	50.7, s	57.0, s
14	85.7, s	86.0, s	86.4, s
15	32.7, t	32.3, t	33.1, t
16	27.8, t	27.5, t	27.5, t
17	51.6, d	51.8, d	46.3, d
18	15.8, q	15.9, q	9.6, q
19	209.3, d	60.0, t	60.1, t
20	178.1, s	178.1, s	178.3, s
21	75.3, t	75.3, t	74.9, t
22	117.9, d	117.9, d	117.8, d
23	177.2, s	177.2, s	177.9, s
1′	96.1, d	97.1, d	95.4, d
2′	90.8, s	92.8, s	91.6, s
3′	78.6, d	73.7, d	71.3, d
4′	35.7, t	33.6, t	39.1, t
5′	67.4, d	70.7, d	69.6, d
6′	21.1, q	65.3, t	20.9, q
		β-D-Glc	
1	102.5, d		
2	74.6, d		
3	78.1, d		
4	71.6, d		
5	78.1, d		
6	62.6, t		

[a] Assignments were confirmed by HSQC and HMBC experiments.

compared with that of an authentic sample prepared in the same manner. In this way the sugar unit of **1** was determined to be D-glucose. On the basis of this evidence, the structure of compound **1** was established as 3′-*O*-β-D-glucopyranosylcalactin.

Compound **2**, obtained as a colorless, amorphous solid, displayed the molecular formula $C_{29}H_{42}O_{10}$ from HRMALDIMS. The ^{1}H and ^{13}C NMR data (Table 1) of **2** closely resembled those of ghalakinoside (**4**)[4] and showed that compound **2** differed from **4** only by the absence of the C-12 hydroxyl group. Thus, compound **2** was identified as the new cardenolide glycoside 12-dehydroxyghalakinoside.

Compound **3** was obtained as an amorphous, white solid. Its molecular formula was deduced as $C_{29}H_{42}O_{10}$ from HRMALDIMS. The ^{1}H NMR spectrum of **3** in comparison to that of ghalakinoside (**4**) showed just a few differences. In particular, it displayed a doublet at δ 1.26 (3H, d, $J = 6.2$ Hz), indicating the presence of

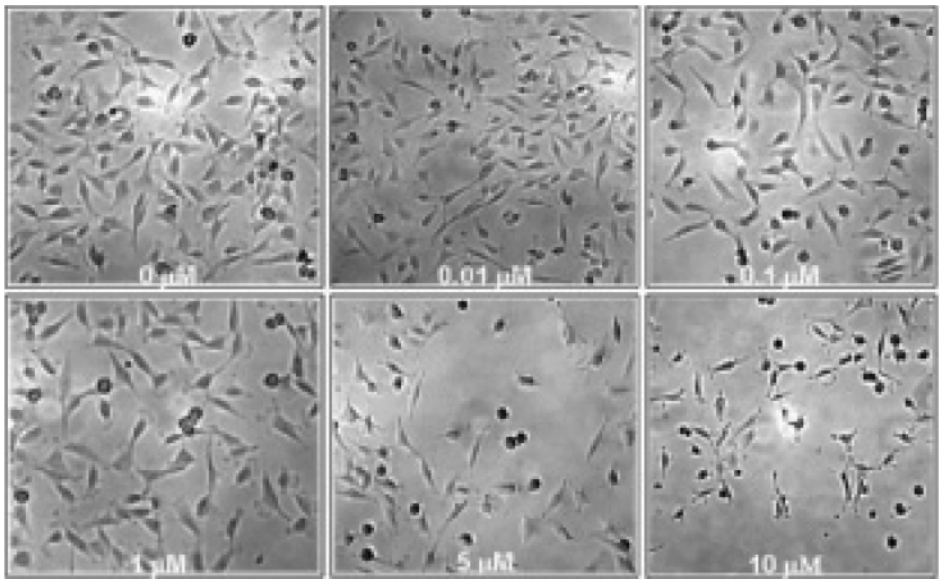

Figure 2. Dose-dependent effect of compound **1** on KS cell survival. Micrographs show control cells and KS cells treated with various concentrations of **1**. Results are representative of four independent experiments.

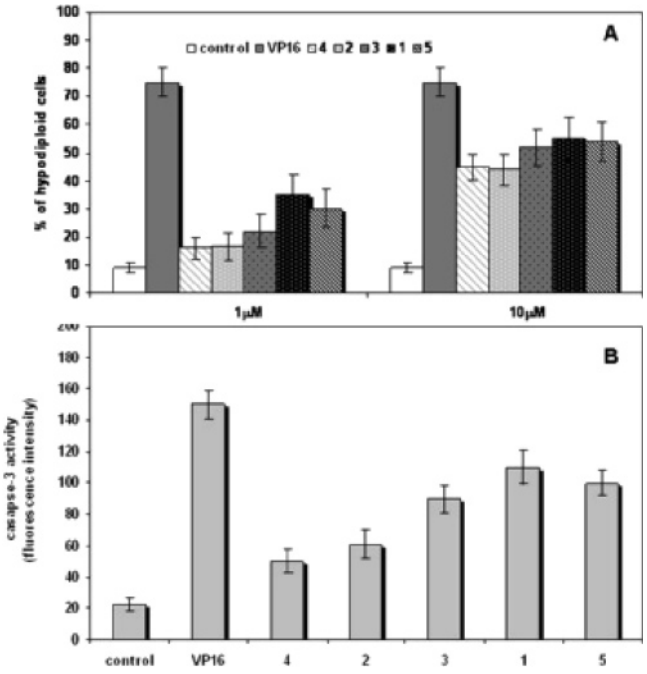

Figure 3. Effect of compounds **1**−**5** on KS cell apoptosis. (A) KS cells were treated with two different concentrations of each compound (1 and 10 μM) or with VP-16 (60 μg/mL), as described in the Experimental Section. Hypodiploid cells were quantified by propidium iodide staining followed by FACS analysis. (B) Cell lysates from KS cells treated with each compound (10 μM) were used to determine caspase-3 activity by a fluorimetric protease assay procedure with the synthetic substrate DEVD-AFC, as described in the Experimental Section. Results shown are the means ± SEM from three independent experiments in duplicate ($n = 6$). *P* value was <0.01 for cells treated with **1**, **3**, and **5** vs cells treated with DMSO, and <0.05 for cells treated with **2** or **4** vs cells treated with DMSO.

a methyl group instead of the hydroxymethyl group in **4**. It was also evident that the resonances at δ 3.63 and 3.60 ascribable to the C-6′ hydroxymethyl group in **4** were absent in **3**. 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments showed the occurrence of a 4,6-dideoxyhexosulose. Thus, compound **3** was identified as 6-dehydroxyghalakinoside.

On the basis of the cytotoxic activity reported for cardenolides,[9−12] the isolated compounds were examined for their effects on cell survival toward the Kaposi's sarcoma (KS) cell line. KS is the most common neoplasm arising in the HIV-1-infected host. Its pathogenesis has been attributed to HIV-1 Tat protein, to cytokines, particularly vascular endothelial growth factor (VEGF), platelet-derived growth factor, oncostatin M (OSM), and interleukin 6 (IL-6), and more recently to KS herpes virus (KSHV).[15] Results shown in Figure 1 indicated that compounds **1**−**5** reduced KS cell growth in a dose-dependent manner, as determined by both Coulter counter (panel A) and XTT colorimetric assay (panel B). When the in vitro cytotoxic activity was determined by XTT and trypan blue dye assays, compounds **1**−**5** did not show any significant cytotoxic activity up to a concentration of 1 μM. However, at a concentration higher than 1 μM the compounds induced a noticeable reduction of KS cell survival. As shown in Figure 2, visual examination of KS cells treated with compound **1** revealed that this compound at a concentration higher than 1 μM caused them to bleb and detach from tissue culture plates. In order to determine whether the cytotoxicity of the compounds was due to an induction of apoptotic cell death, KS cells were treated with each compound at a concentration of 1 or 10 μM. The antineoplastic agent etoposide (VP-16) was used as positive control. Apoptosis was quantified by propidium iodide staining followed by FACS analysis, which measures cells with fragmented DNA (hypodiploid cells) within the gate before the G1 phase of the cell cycle. As shown in Figure 3 (panel A) all compounds induced a concentration-dependent DNA fragmentation. At a concentration of 1 μM the apoptotic cell death was scarcely induced, and the highest effect was observed for compound **1** (35% of hypodiploid cells compared to control cells). The potency order of the compounds at 1 μM was **1** > **5** > **3** > **2** = **4**. When compounds were tested at a concentration of 10 μM, all compounds induced a consistent apoptotic cell death (about 45% to 55% of hypodiploid cells compared to control cells) with the same potency order shown at 1 μM. To investigate the involvement of caspase activation in compound-induced apoptosis, we treated KS cells with each compound (10 μM) and determined the caspase-3 activity by DEVD-AFC fluorimetric assay. As shown in Figure 3 (panel B), compounds **1**−**5** at a concentration of 10 μM stimulated protease activation. Compounds **1**, **3**, and **5** were more active than compounds **2** and **4**, as shown by the increase in fluorescence intensity (about 4.5-fold and about 2.5-fold, respectively). These data are consistent with the FACS results (panel A) and indicate that the cytotoxic activity of **1**−**5** was mediated by an apoptotic effect.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 1000 polarimeter. UV spectra were obtained on a Beckman DU 670 spectrometer. IR measurements were obtained on a Bruker IFS-48 spectrometer. Accurate mass estimations were measured by a Voyager DE mass spectrometer. Samples were analyzed by MALDIMS. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18−39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard. ESIMS analyses were performed using a ThermoFinnigan LCQ Deca XP Max ion trap mass spectrometer equipped with Xcalibur software. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D-NMR spectra were acquired in CD_3OD. The standard pulse sequence and phase cycling were used for DQF-COSY, HSQC, HMBC, and ROESY spectra. 1D-TOCSY selective excitation spectra were acquired using waveform generator-based GAUSS-shaped pulses, with mixing times ranging from 100 to 120 ms and with a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. TLC was performed on silica gel F254 (Merck) plates, and reagent grade chemicals (Carlo Erba) were used throughout. GC analysis was performed on a Thermo Finnigan Trace GC apparatus using a 1-Chirasil-Val column (0.32 mm × 25 m). For biological assays, XTT Cell Proliferation Kit II from Roche was used. All culture reagents were from Invitrogen, Inc. Propidium iodide and etoposide were from Sigma. Caspase-3 apoptosis detection kit was from Santa Cruz.

Plant Material. Fresh roots of *P. tomentosa* L. were collected in Wadi Um Hebal, Egypt, in January 2001 and identified by one of the authors (I.V.S.). A voucher specimen (No. 10298) was deposited at the Faculty of Science Herbarium of the South Valley University.

Extraction and Isolation. The dried and powdered roots of *P. tomentosa* (1580 g) were extracted at room temperature with EtOH−H_2O (4:1) by maceration until exhaustion. The alcoholic extract was concentrated under reduced pressure to a syrupy consistency (195 g). The crude extract was partitioned between hexane−H_2O, $CHCl_3$−H_2O, and *n*-BuOH−H_2O (1:1), to afford hexane (38 g), $CHCl_3$ (90 g), and *n*-BuOH (67 g) extracts. Part of the $CHCl_3$ extract (50 g) was slurried with 20 g of silica gel (E Merck) and transferred to the top of a column (120 × 4.5 cm) of activated silica gel (600 g) previously packed by the wet method in $CHCl_3$. Elution was started by $CHCl_3$, and the polarity was gradually increased by using MeOH. The effluents (100 mL) were collected, and each fraction was concentrated under reduced pressure to about 10 mL and screened for its content by TLC using a suitable solvent system. Fractions were then grouped according to similar contents. Fractions eluted with $CHCl_3$−MeOH (8:2) gave calactin (**5**) (20 mg) and ghalakinoside (**4**) (15 mg). Fractions eluted with $CHCl_3$−MeOH (7:3) were combined (fraction A, 10 g). Part of fraction A (1

g) was loaded on a C-18 column and eluted with CH_3CN (20−25%) to give compounds **2** (10 mg), **3** (10 mg), and **1** (100 mg).

Compound 1: white, amorphous powder; $[\alpha]_D^{25}$ +33.7 (*c* 1.0, MeOH); UV max (MeOH) 218 nm; IR (KBr) ν_{max} 3446 (O-H), 1742 (>C═O), 1623 (>C═C) cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 10.07 (1H, s, H-19), 5.93 (1H, t, J = 1.5 Hz, H-22), 5.00 (1H, dd, J = 18.4, 1.5 Hz, H-21a), 4.90 (1H, dd, J = 18.4, 1.5 Hz, H-21b), 4.71 (1H, s, H-1′), 4.16 (1H, m, H-5′), 4.33 (1H, d, J = 7.9 Hz, H-1_{glc}), 3.92 (1H, dd, J = 12.0, 2.5 Hz, H-$6b_{glc}$), 3.83 (1H, t, J = 2.0 Hz, H-3′), 3.66 (1H, dd, J = 12.0, 4.5 Hz, H-$6a_{glc}$), 3.36 (1H, dd, J = 9.0, 9.0 Hz, H-3_{glc}), 3.31 (1H, m, H-5_{glc}), 3.28 (1H, dd, J = 9.0, 9.0 Hz, H-4_{glc}), 3.25 (1H, dd, J = 9.0, 7.9 Hz, H-2_{glc}), 2.85 (1H, dd, J = 5.7, 3.1 Hz, H-17), 1.86 (1H, m; H-4′a), 1.69 (1H, m, H-4′b), 1.23 (3H, d, J = 6.2 Hz, Me-6′), 0.83 (3H, s, Me-18); ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m*/*z* 695 $[M + H]^+$; HRMALDIMS *m*/*z* $[M + H]^+$ calcd for $C_{35}H_{51}O_{14}$ 695.3279, found 695.3294.

Compound 2: white, amorphous powder; $[\alpha]_D^{25}$ +35.5 (*c* 0.7, MeOH); UV max (MeOH) 215 nm; IR (KBr) ν_{max} 3440 (O-H), 1745 (>C═O), 1633 (>C═C) cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 5.93 (1H, d, J = 1.5 Hz, H-22), 5.00 (1H, dd, J = 18.4, 1.5 Hz, H-21a), 4.90 (1H, dd, J = 18.4, 1.5 Hz, H-21b), 4.51 (1H, s, H-1′), 3.90 (1H, d, J = 11.4 Hz, H-19a), 3.65 (1H, m, H-5′), 3.64 (1H, d, J = 11.4 Hz, H-19b), 3.63 (1H, m, H-6′a), 3.61 (1H, t, J = 2.0 Hz, H-3′), 3.60 (1H, m, H-6′b), 2.86 (1H, dd, J = 5.7, 3.1 Hz, H-17), 1.76 (1H, m; H-4′a), 1.68 (1H, m, H-4′b), 0.96 (3H, s, Me-18); ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m*/*z* 551 $[M + H]^+$, HRMALDIMS *m*/*z* $[M + H]^+$ calcd for $C_{29}H_{43}O_{10}$ 551.2856, found 551.2873.

Compound 3: white, amorphous powder; $[\alpha]_D^{25}$ +39.2 (*c* 0.8, MeOH); UV max (MeOH) 217 nm; IR (KBr) ν_{max} 3448 (O-H), 1748 (>C═O), 1620 (>C═C) cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 5.93 (1H, d, J = 1.5 Hz, H-22), 5.00 (1H, dd, J = 18.4, 1.5 Hz, H-21a), 4.90 (1H, dd, J = 18.4, 1.5 Hz, H-21b), 4.71 (1H, s, H-1′), 4.14 (1H, m, H-5′), 3.89 (1H, d, J = 11.4 Hz, H-19a), 3.69 (1H, t, J = 2.0 Hz, H-3′), 3.64 (1H, d, J = 11.4 Hz, H-19b), 3.35 (1H, dd, J = 5.7, 3.1 Hz, H-17), 3.30 (1H, dd, J = 10.0, 4.0 Hz, H-12), 1.76 (1H, m; H-4a), 1.61 (1H, m, H-4′b), 1.26 (3H, d, J = 6.2 Hz, Me-6′), 0.87 (3H, s, Me-18); ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m*/*z* 551 $[M + H]^+$, HRMALDIMS *m*/*z* $[M + H]^+$ calcd for $C_{29}H_{43}O_{10}$ 551.2856, found 551.2863.

GC Analysis to Determine Absolute Configuration of Glucose. A solution (2 mg) of compound **1** in 1 N HCl (0.5 mL) was stirred at 80 °C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue was separated by H_2O and CH_2Cl_2 (1 mL, 1:1 v/v). The CH_2Cl_2 layer was analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. The peak of the hydrolyzate of **1** was detected at 14.72 min (D-glucose).

Cell Culture and Treatment. Kaposi's sarcoma (KS) cells, derived from HIV-1 patients and spontaneously immortalized, were a kind gift of Dr. Giovanni Camussi (University of Torino, Italy). Cells were cultured in 75 cm^2 flasks in 20 mL of RPMI 1640 with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 in air. Subconfluent monolayers were prepared by seeding KS in 24-well plates at the density of 5×10^3 cells/dish.

The day before the experiments, subconfluent monolayers of KS cells were starved for 12 h in serum-free media. Rested cells were then washed twice with 5 mL of Hank's balanced salt solution (HBSS) and 10 mM HEPES and preincubated at 37 °C for 1 h with compounds **1**−**5** dissolved in DMSO. At the end of the incubation, the cells were washed twice with 5 mL of HBSS−10 mM Hepes and cultured for an additional 24 h in media containing 1% FBS. The final DMSO concentration in the media was less than 0.1%, and the cell viability was >95%, as assessed by trypan blue dye exclusion.

Cytotoxic Activity. KS cells were seeded in 24-well plates (5×10^3 cells/well) and allowed to attach for 24 h. Cells were then washed twice with 2 mL of HBSS and 10 mM Hepes, rested for 12 h in serum-free media, and then incubated at 37 °C for 24 h in fresh serum-free media with or without different concentrations of compounds **1**−**5**. At the end of the incubation, the media were removed and the cells were washed twice with 2 mL of HBSS−10 mM Hepes before determining cell number by both XTT colorimetric assay and Coulter counter. The optical density (OD) of each well was measured with a microplate spectrophotometer (Biorad) equipped with a 490 nm filter.

DNA Fragmentation Analysis. DNA fragmentation was quantified by propidium iodide staining and FACS analysis. Cells treated with compounds **1**−**5**, as described above, were resuspended in PBS containing 50 μg/mL propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4 °C for 12 h and vortexed prior to FACS analysis (Becton Dickinson FACSCalibur).

Caspase-3 Activity. A fluorimetric protease assay procedure with a synthetic substrate DEVD-AFC (Santa Cruz) was followed. Briefly, cell lysates prepared from cells treated with or without compounds **1**−**5** were incubated in a reaction mixture containing DEVD-AFC. This substrate is composed of a synthetic tetrapeptide, DEVD, which corresponds to the upstream amino acid sequence of the caspase-3 cleavage site in poly(ADP-ribose) polymerase (PARP) and a fluorophore, AFC (7-amino-4-trifluoromethylcoumarin). Cleavage of the substrate between D and AFC by caspase-3 releases AFC. Measure of free AFC was performed using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength range of 480−520 nm.

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NP060228L