Candicanoside A, a Novel Cytotoxic Rearranged Cholestane Glycoside from *Galtonia candicans*

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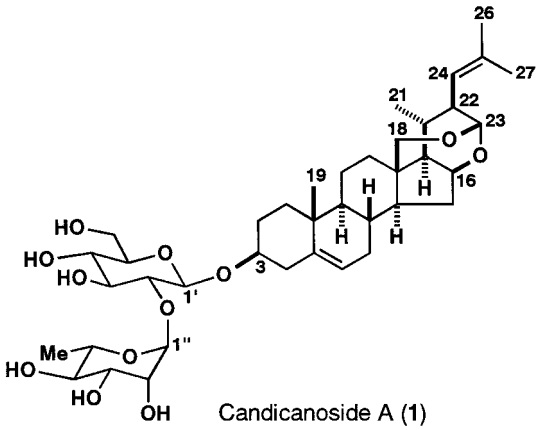
A novel cholestane diglycoside, named candicanoside A (**1**), was isolated from the bulbs of *Galtonia candicans* by monitoring the cytotoxic activity on HL-60 leukemia cells. Candicanoside A (**1**) is very unique in structure, having two epoxy functionalities in its rearranged cholestane skeleton, and showed differential cytotoxicity in the Japanese Foundation for Cancer Research 38 cell-line assay.

1. Introduction. – The acylated cholestane glycoside, tentatively designated OSW-1, isolated by us from the bulbs of *Ornithogalum saundersiae* (Liliaceae), has been revealed to show remarkable cytotoxicity against a variety of tumor-cell lines and experimental-animal tumors [1]. OSW-1 promises to be a candidate for a new anticancer agent from a plant source. During our ongoing project focused on higher-plant antineoplastic constituents [2], we found that a MeOH extract of the bulbs of *Galtonia candicans*, which is indigenous to South Africa and taxonomically related to *O. saundersiae*, exhibited potent cell-growth-inhibitory activity on HL-60 human promyelocytic leukemia cells. A cytotoxicity-guided fractionation procedure performed on the MeOH extract led to the isolation of a novel hexacyclic rearranged cholestane diglycoside, named candicanoside A (**1**), as an active constituent responsible for HL-60 cells cytostasis. This paper deals with the structural elucidation of candicanoside A (**1**) and with its cytostatic and cytotoxic activities against the cultured 38 malignant tumor cells.

2. Results and Discussion. – The bulbs of *G. candicans* (fresh weight of 5.5 kg) were extracted with hot MeOH. The MeOH extract showed cytotoxic activity against HL-60 cells with an IC_{50} value of 0.017 μg ml^{-1}. On fractionation of the MeOH extract into BuOH-soluble phase and H_2O phase, the cytocidal activity appeared only in the BuOH phase (0.0056 μg ml^{-1}). A series of chromatographic separations of the BuOH-soluble phase led to the isolation of candicanoside A (**1**; 0.00076%, fresh weight).

Candicanoside A (**1**) was obtained as an optically active amorphous powder. Its molecular formula was determined to be $C_{39}H_{60}O_{12}$ on the basis of the positive-ion HR-FAB-MS, showing an accurate $[M + Na]^+$ ion, and ^{13}C-NMR analysis. The IR spectrum of **1** was consistent with the presence of OH groups (3395 cm^{-1}). The ^{1}H-NMR



Candicanoside A (**1**)

spectrum ((D$_5$)pyridine; see *Table 1*) displayed signals arising from a tertiary Me group (δ 1.03), two secondary Me groups (δ 1.78 and 1.22), two Me groups at a C=C bond (δ 1.77 and 1.61), two olefinic protons (δ 5.43 and 5.32), and two anomeric protons (δ 6.40 and 5.05). The signal at δ 1.78 was assignable to the Me group of a 6-deoxyhexopyranose. The presence of two trisubstituted C=C bonds in **1** was established by ^{13}C-NMR data (δ 140.9, 134.4, 125.7, and 121.7; see *Table 1*). Acid hydrolysis of **1** with 1M HCl in dioxane/H$_2$O 1:1 resulted in the production of D-glucose and L-rhamnose in a ratio of 1:1 as the carbohydrate components, while the genuine aglycone was decomposed under acidic conditions. The monosaccharides, including their absolute configurations, were identified by HPLC analysis following their conversion to the 1-{acetyl[(1*S*)-1-phenylethyl]amino}-1-deoxyalditol acetate derivatives [3]. The ^{13}C-NMR spectrum of **1** showed a total of 39 resonance lines, 27 of which were assigned to the aglycone moiety and 12 to the two monosaccharides. This implied a $C_{27}H_{40}O_3$ composition for the aglycone moiety, possessing eight degrees of unsaturation. The presence of two C=C bonds accounted for two degrees. Consequently, the aglycone of **1** was predicted to have a C_{27} steroid skeleton with a six-ring system. Treatment of **1** with Ac_2O in pyridine introduced six acetyl groups only into the sugar moiety, which implied that no OH group was present in the aglycone moiety. The C(1) to C(17) portion (A to D rings) of **1** was readily shown to be identical to that of usual cholest-5-en-3-ol by analysis of the COSY, TOCSY, HMQC, and HMBC data. Analysis of the phase-sensitive NOESY spectrum made the configuration assignable. A ^{1}H,^{1}H-COSY experiment allowed the sequential assignment of the resonances for each monosaccharide, starting from the easily distinguished anomeric protons. Multiplet patterns and measurements of coupling constants confirmed the presence of a β-D-glucopyranosyl unit ($^4C_1$) and an α-L-rhamnopyranosyl unit ($^1C_4$). Thus, the structure of **1** was established as (16β,22*S*,23*S*)-16,23:18,23-diepoxy-22-(2-methylprop-1-enyl)-24-norchol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 $\rightarrow$ 2)-β-D-glucopyranoside. Compound **1** is very unique in structure, having two epoxy functionalities in the rearranged cholestane skeleton.

Table 1. *^{1}H- and ^{13}C-NMR Data for Compound* **1**[a])

	δ(H)[b])		δ(C)		δ(H)		δ(C)
CH_2(1)	1.70	(H_{eq})	37.6	Me(19)	1.03	(*s*)	19.3
	0.97	(H_{ax})		H−C(20)	1.91		30.5
CH_2(2)	2.14	(br. *d*, *J* = 11.4, H_{eq})	30.2	Me(21)	1.22	(*d*, *J* = 7.1)	24.1
	1.88	(H_{ax})		H−C(22)	2.57		45.6
H−C(3)	3.96	(*m*, $w_{1/2}$ = 20.1)	77.9	H−C(23)	5.37	(*d*, *J* = 4.0)	98.2
CH_2(4)	2.28	(br. *dd*, *J* = 12.0, 3.1, H_{eq})	39.0	H−C(24)	5.43	(*d*, *J* = 9.2)	125.7
	2.74	(br. *dd*, *J* = 12.0, 12.0, H_{ax})		C(25)	–		134.4
C(5)	–		140.9	Me(26)	1.77	(*s*)	26.1
H−C(6)	5.32	(br. *d*, *J* = 5.1)	121.7	Me(27)	1.61	(*s*)	18.2
CH_2(7)	1.55	(H_α)	32.1				
	1.93	(H_β)		H−C(1′)	5.05	(*d*, *J* = 7.3)	100.4
H−C(8)	1.72		31.9	H−C(2′)	4.29	(*dd*, *J* = 9.3, 7.3)	77.7
H−C(9)	0.99		51.0	H−C(3′)	4.31	(*dd*, *J* = 9.3, 9.3)	79.7
C(10)	–		37.2	H−C(4′)	4.18	(*dd*, *J* = 9.3, 9.3)	71.8
CH_2(11)	1.48	(H_{eq})	21.2	H−C(5′)	3.91	(*ddd*, *J* = 9.3, 4.6, 2.3)	78.3
	1.17	(br. *d*, *J* = 11.6, H_{ax})		CH_2(6′)	4.52	(*dd*, *J* = 11.9, 2.3, H_a)	62.7
CH_2(12)	1.69	(H_α)	35.4		4.36	(*dd*, *J* = 11.9, 4.6, H_b)	
	1.00	(H_β)					
C(13)	–		47.4	H−C(1″)	6.40	(*d*, *J* = 1.1)	102.0
H−C(14)	1.02		52.6	H−C(2″)	4.81	(*dd*, *J* = 3.3, 1.1)	72.6
CH_2(15)	2.25	(*ddd*, *J* = 13.5, 8.6, 8.6, H_α)	39.1	H−C(3″)	4.64	(*dd*, *J* = 9.4, 3.3)	72.8
	1.90	(H_β)		H−C(4″)	4.36	(*dd*, *J* = 9.4, 9.4)	74.2
H−C(16)	4.66		73.2	H−C(5″)	5.00	(*dq*, *J* = 9.4, 6.3)	69.5
H−C(17)	1.34	(br. *d*, *J* = 7.0)	53.5	Me(6″)	1.78	(*d*, *J* = 6.3)	18.7
CH_2(18)	4.04	(*d*, *J* = 13.0, H_a)	66.8				
	3.55	(*d*, *J* = 13.0, H_b)					

[a]) Spectra were measured in (D_5)pyridine. [b]) δ in ppm; *J* in Hz.

In the HMBC spectrum of **1**, the *s* at δ 1.03 (3*H*) showed long-range correlations with the signals of C(1) (δ 37.6), C(5) (δ 140.9), C(9) (δ 51.0), and C(10) (δ 37.2), and was assigned to CH_3(19). The NMR data were indicative of the lack of the angular Me(18) group typical of the steroid skeleton in **1**. Instead of it, the presence of an oxymethylene moiety was shown by *AB*(*q*) signals at δ 4.04 and 3.55 (*J* = 13.0 Hz) in the ^{1}H-NMR spectrum, which was correlated to the one-bond-coupled C-atom at δ 66.8 in the HMQC spectrum. The quaternary C-atom at δ 47.4 was assignable to C(13), at which the OCH_2(18) group was shown to be located by the observation of *J*(C,H) correlations from its signal at δ 4.04 to those of C(13) (δ 47.4), C(14) (δ 52.6), and C(17) (δ 53.5). The downfield-shifted signal at δ 4.66 was assigned to *H*−C(16), which was correlated to the C-atom at δ 73.2 in the HMQC spectrum. This indicated that C(16) was bonded to an O-atom. Tracing out the proton spin-coupling system from the distinctive *H*−C(16) signal through the COSY and TOCSY plots allowed us to formulate the structure of the rearranged cholestane portion. The signal due to *H*−C(23) at δ 5.37 (*d*, *J* = 4.0 Hz) showed an HMQC correlation with δ 98.2. The HMBC correlations from *H*−C(23) to both C(16) and C(18) gave confirmative evidence for the formation of a six-membered acetal ring between C(16) and C(23), and a seven-membered acetal ring between C(18) and C(23) (*Fig.*). The H−C(22) at δ 2.57 was coupled not only to *H*−C(20) and *H*−C(23), but also to an olefinic proton assignable to *H*−C(24) at δ 5.43 with a *J* value of 9.2 Hz. *H*−C(24), in turn, showed long-range correlations with the two Me groups at δ 26.1 (C(26)) and 18.2 (C(27)). A long-range correlation from *H*−C(22) to the olefinic C(25) (δ 134.4) was also observed. These data indicated that a 2-methylprop-1-enyl group was attached at C(22). Thus, the plane structure of the aglycone of **1** was shown to be 16,23:18,23-diepoxy-22-(2-methylprop-1-enyl)-24-norchol-5-en-3-ol.

NOE Correlations from *H*−C(8) to H_a−C(18) and CH_3(19), and of H−C(9) to *H*−C(14) indicated that **1** had the usual B/C *trans* and C/D *trans* steroidal ring junctions (*Fig.*). Other NOE networks from *H*−C(14) to *H*−C(17), H−C(16) to *H*−C(17) and CH_3(21), *H*−C(22) to CH_3(21), *H*−C(23) and CH_3(27), and *H*−C(24) to

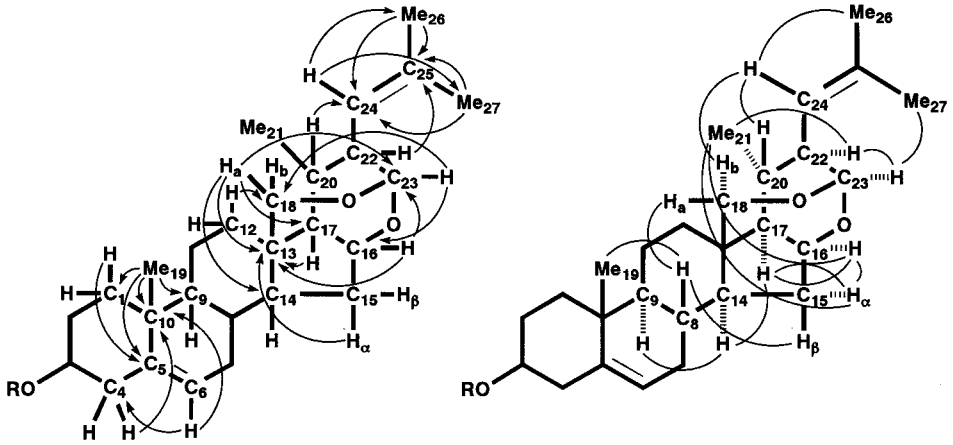


Figure. *HMBC* (arrows) *and NOE* (curved lines) *correlations in* **1**

H_b–C(18) and H–C(20) provided evidence for the (16*S*), (17*R*), (20*S*), (22*S*), and (23*S*) configurations. The orientation of O–C(3) was ascertained to be β from the multiplicity of the H–C(3) signal ($W_{1/2} = 20.1$ Hz).

In the HMBC spectrum, the anomeric-proton signals at δ 6.40 (rhamnose) and 5.05 (glucose) were correlated to δ 77.7 (C(2) of glucose) and 77.9 (C(3) of aglycone), respectively, indicating the structure of the glycosyl moiety of **1** as α-L-rhamnosyl-(1 → 2)-β-D-glucosyl and its linkage to C(3) of the aglycone.

The cytotoxic activity of **1** on HL-60 human promyelocytic leukemia cells was evaluated. The cells were continuously treated with **1** for 72 h, and the cell growth was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay procedure [4]. The IC_{50} value was calculated from a dose-dependent curve as 0.032 μM, which was as potent as those of the clinically applied anticancer agents etoposide (IC_{50} 0.025 μM) and methotrexate (IC_{50} 0.012 μM). Subsequent evaluation of **1** in the Japanese Foundation for Cancer Research 38 cell-line assay [5] showed that the mean concentrations required to achieve GI_{50}, *TGI*, and LC_{50} levels against the panel cells tested were 1.01, 8.97, and 39.1 μM, respectively. Compound **1** displayed differential cytotoxicities, with breast cancer, CNS cancer, and lung cancer subpanel cell lines showing particular sensitivity, but with colon cancer, ovarian cancer, and stomach cancer subpanel cell lines being relatively resistant to it (*Table 2*). The pattern of differential cytotoxicity of **1** was evaluated by the Compare Program and was revealed not to be correlated with that shown by any of the other compounds including currently used anticancer drugs (correlation coefficient value is *ca.* 0.5). This indicates that **1** may have a unique mode of action and the potentiality as the lead of a new anticancer agent. The yield of **1**, however, was not high enough to explain the potent cytotoxic activity of the BuOH phase. It suggests the presence of other, more potent principles in the fraction. Further investigation is now under way.

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Table 2. *The* GI_{50}, *TGI, and* LC_{50} *Values of Compound* **1** *against the 38 Cell Lines*[a])

Panel/cell Line	GI_{50} [μM]	*TGI* [μM]	LC_{50} [μM]	Panel/cell Line	GI_{50} [μM]	*TGI* [μM]	LC_{50} [μM]
Breast cancer				Melanoma			
HBC-4	0.39	–[b])	–	LOX IMVI	0.19	0.82	–
BSY-1	0.040	0.13	9.61				
HBC-5	0.10	0.84	–	Ovarian Cancer			
MCF-7	0.34	4.71	–	OVCAR-3	–	–	–
MCF-7	0.34	4.71	–	OVCAR-4	2.30	–	–
MDA-MB-231	1.05	–	–	OVCAR-5	2.20	–	–
				OVCAR-8	–	–	–
CNS Cancer				SK-OV-3	2.65	–	–
U251	0.12	–	–				
SF-268	4.60	–	–	Renal cancer			
SF-295	0.039	0.12	4.19	RXF-631L	0.55	4.29	–
SF 539	0.051	0.43	6.50	ACHN	–	–	–
SNB-75	0.047	0.88	–				
SNB-78	0.37	3.74	–	Stomach cancer			
				St-4	4.60	–	–
Colon cancer				MKN1	–	–	–
HCC-2998	–	–	–	MKN7	3.11	–	–
KM12	0.63	–	–	MKN28	–	–	–
HT29	2.53	–	–	MKN45	4.19	–	–
HCT-15	3.11	–	–	MKN74	0.19	–	–
HCT-116	1.92	–	–				
				Prostate cancer			
Lung cancer				DU-145	–	–	–
NCI-H23	–	–	–	PC-3	0.92	–	–
NCI-H226	2.10	–	–				
NCI-H522	0.36	3.18	–	Mean conc.	1.01	8.97	39.1
NCI-H460	0.081	–	–				
A549	0.71	–	–				
DMS273	0.027	0.071	0.33				
DMS114	0.20	–	–				

[a]) LC_{50} = concentration at which only 50% of the cells are viable, GI_{50} = concentration that yields 50% growth, and *TGI* (total growth inhibition) = concentration at which no growth is observed. [b]) The value is more than 10 μM.

Experimental Part

General. The following materials and reagents were used for HL-60 cell culture and assay of cytotoxic activity: microplate reader, *Inter Med Immuno-Mini NJ-2300* (Japan); 96-well flat-bottom plate, *Iwaki Glass* (Japan); HL-60 cells, *ICN Biomedicals* (USA); *RPMI 1640* medium, *GIBCO BRL* (USA); MTT, *Sigma* (USA). All other chemicals used were of biochemical reagent grade. Column chromatography: silica gel (*Fuji-Silysia Chemical*, Japan) and octadecylsilanized (ODS) silica gel (*Nacalai Tesque*, Japan). TLC: Precoated silica gel *60* F_{254} (0.25-mm thick, *Merck*, Germany) and *RP-18-*F_{254}*-S* (0.25 mm thick, *Merck*) plates; visualization by spraying with 10% H_2SO_4 soln., followed by heating. HPLC: *CCPM* pump (*Tosoh*, Japan), *CCP PX-8010* controller (*Tosoh*), *UV-8000* detector (*Tosoh*), and *Rheodyne* injection port with a 20-μl sample loop; anal. HPLC, *Capcell-Pak-*C_{18} column (4.6 mm × 250 mm, 5 μm, *Shiseido*, Japan); t_R in min. Optical rotation: *Jasco-DIP-360* automatic digital polarimeter. IR Spectra: *Jasco-A-100* spectrophotometer. NMR Spectra: *Bruker-DRX-500* (500 MHz for ^{1}H-NMR) spectrometer using standard *Bruker* pulse programs; chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard. MS: *VG-AutoSpec-E* mass spectrometer.

Plant Material. The bulbs of *G. candicans* were purchased from a nursery in *Heiwaen*, Japan. The bulbs were cultivated, and the flowered plant was identified by one of the authors, Prof. *Yutaka Sashida*. A voucher of the plant is on the file in our laboratory (97-7-GC-F).

Extraction and Isolation. The plant material (fresh weight 5.5 kg) was extracted with hot MeOH twice. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate was partitioned between BuOH and H_2O. The BuOH phase was submitted to CC (silica gel, stepwise gradient $CHCl_3$/MeOH 9:1, 4:1 and 2:1, and finally MeOH): four fractions (*I–IV*). *Fr. II* was subjected to CC (silica gel, $CHCl_3$/MeOH/H_2O 30:10:1; then ODS silica gel, MeOH/H_2O 3:1): 41.7 mg of *candicanoside A* (=*(16β,22S,23S)-16,23:18,23-Diepoxy-22-(2-methylprop-1-enyl)-24-norchol-5-en-3β-yl* O-α-L-*Rhamnopyranosyl-(1→2)-β-D-glucopyranoside*; **1**). Amorphous solid. $[\alpha]_D^{25} = -32.0$ ($c = 0.10$, MeOH). IR (KBr): 3395 (OH), 2940 and 2880 (CH), 1435, 1365, 1340, 1150, 1120, 1060, 1025, 985, 955, 835, 800. HR-FAB-MS (pos.): 743.3975 ($[M+Na]^+$, $C_{39}H_{60}O_{12}Na^+$; calc. 743.3983).

Acid Hydrolysis of Candicanoside A (**1**). A soln. of **1** (2.5 mg) in 1M HCl (dioxane/H_2O 1:1 2 ml) was heated at 100° for 1 h under Ar. After cooling, the mixture was neutralized by passage through an *Amberlite-IRA-93ZU* (*Organo*) column and chromatographed (silica gel, $CHCl_3$/MeOH 19:1 followed by MeOH only) to give an aglycone fraction (1.2 mg) and a sugar fraction (0.8 mg). TLC Analysis of the aglycone fraction showed that it contained several unidentified artifactual sapogenols. After the sugar fraction was passed through a *Sep-Pak-C_{18}* cartridge (*Waters*; with H_2O/MeOH 4:1 (5 ml)), the residue was dissolved in H_2O (1 ml), and (−)-α-methylbenzylamine (5 mg) and Na[BH_3CN] (8 mg) in EtOH (1 ml) were added. After being set aside at 40° for 4 h followed by addition of AcOH (0.2 ml) and evaporation, the mixture was acetylated with Ac_2O (0.3 ml) in pyridine (0.3 ml) at r.t. for 12 h. The crude mixture was passed through a *Sep-Pak-C_{18}* cartridge (with H_2O/MeCN 4:1 and 1:1 (each 5 ml)). The H_2O/MeCN 1:1 eluate was further passed through a *Toyopak-IC-SP-M* cartridge (*Tosoh*; with EtOH (5 ml)) to give a mixture of the 1-{acetyl[(1*S*)-1-phenylethyl]amino}-1-deoxyalditol acetate derivatives of the monosaccharides [3], which was then analyzed by HPLC (MeCN/H_2O 2:3, flow rate 0.8 ml min^{-1}, detection at 230 nm): t_R 22.87 (derivative of D-glucose), 26.02 (derivative of L-rhamnose).

Acetylation of Candicanoside A (**1**). Compound **1** was treated with Ac_2O (2 ml) in pyridine (2 ml) for 12 h. The mixture was diluted with H_2O and extracted with Et_2O, the Et_2O phase evaporated, and the residue chromatographed (silica gel, hexane/Me_2CO 3:2): 6.2 mg of *candicanoside A hexaacetate* (=*(16β,22S,23S)-16,23:18,23-Diepoxy-22-(2-methylprop-1-enyl)-24-norchol-5-en-3β-yl* O-α-L-*Rhamnopyranosyl-(1→2)-β-D-glucopyranoside Hexaacetate*). Amorphous solid. IR (KBr): 2940 and 2820 (CH), 1755 (C=O), 1475, 1460, 1435, 1370, 1200, 1125, 1010, 830, 790. ^{1}H-NMR ($CDCl_3$): 5.40 (br. *d*, $J = 5.2$, H−C(6)); 5.24 (*dd*, $J = 9.5$, 9.5, H−C(3′)); 5.23 (*dd*, $J = 10.1$, 3.5, H−C(3″)); 5.15 (*d*, $J = 9.2$, H−C(24)); 5.07 (*dd*, $J = 10.1$, 10.1, H−C(4″)); 5.00 (*dd*, $J = 3.5$, 1.8, H−C(2″)); 4.99 (*d*, $J = 4.3$, H−C(23)); 4.96 (*d*, $J = 1.8$, H−C(1″)); 4.95 (*dd*, $J = 9.5$, 9.5, H−C(4′)); 4.58 (*d*, $J = 7.8$, H−C(1′)); 4.56 (*ddd*, $J = 8.5$, 7.8, 2.7, H−C(16)); 4.38 (*dq*, $J = 10.1$, 6.2, H−C(5″)); 4.26 (*dd*, $J = 12.2$, 4.9, H_a−C(6′)); 4.08 (*dd*, $J = 12.2$, 2.4, H_b−C(6′)); 3.96 (*d*, $J = 13.0$, H_a−C(18)); 3.71 (*dd*, $J = 9.5$, 7.8, H−C(2′)); 3.68 (*ddd*, $J = 9.5$, 4.9, 2.4, H−C(5′)); 3.61 (*m*, $w_{1/2} = 22.8$, H−C(3)); 3.46 (*d*, $J = 13.0$, H_b−C(18)); 2.12, 2.06, 2.05, 2.01, 2.00, 1.99 (6*s*, 6 MeCO); 1.73 (*s*, Me(27)); 1.65 (*s*, Me(26)); 1.19 (*d*, $J = 6.2$, Me(6″)); 1.18 (*d*, $J = 7.0$, Me(21)); 1.01 (*s*, Me(19)).

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^{-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 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°. After incubation, 4 μl of an EtOH/H_2O 1:1 soln. containing the sample was added to give the final concentrations of 0.0001–10 μg ml^{-1}, 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 the modified MTT reduction assay [4]. Briefly after termination of the cell culture, 10 μl of 5 mg ml^{-1} 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°. The plate was then centrifuged at 1500 *g* for 5 min to precipitate cells and MTT formazan. An aliquot of 150 μl of the supernatant was removed from every well, and DMSO (175 μl) 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. A dose/response curve was plotted for the fractions and **1**, and the concentration giving 50% inhibition (IC_{50}) was calculated.

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