Inhibition of Tumor-Promoting Effects by Poricoic Acids G and H and Other Lanostane-Type Triterpenes and Cytotoxic Activity of Poricoic Acids A and G from *Poria cocos*

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The structures of two novel 3,4-*seco*-lanostane-type triterpenes isolated from the sclerotium of *Poria cocos* were established to be 16α-hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic acid (**1**; poricoic acid G) and 16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),8,24(24[1])-triene-3,21-dioic acid (**2**; poricoic acid H) on the basis of spectroscopic methods. These two, and eight other known compounds isolated from the sclerotium, poricoic acid B (**3**), poricoic acid A (**4**), tumulosic acid (**5**), dehydrotumulosic acid (**6**), 3-epidehydrotumulosic acid (**7**), polyporenic acid C (**8**), 25-hydroxy-3-epidehydrotumulosic acid (**9**), and dehydroabietic acid methyl ester (**10**), showed potent inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Evaluation of the cytotoxicity of compounds **1** and **4** against human cancer cell lines revealed that **1** was significantly cytotoxic to leukemia HL-60 cells [GI_{50} (concentration that yields 50% growth) value 39.3 nM], although it showed only moderate cytotoxicity to the other cells. Compound **4** exhibited moderate cytotoxicity to all of the cancer cell lines tested.

The sclerotium of *Poria cocos* Wolf (Polyporaceae) is traditionally used in Chinese herbal prescriptions as a diuretic and as a sedative.[1] *P. cocos* has been reported to contain various lanostane-type triterpene acids,[1−9] several of which have been revealed to possess strong inhibitory effects against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-[6−9] and arachidonic acid[9]-induced ear inflammation in mice and against copper sulfate-induced emesis in leopard frog.[10] The methanol extract of *P. cocos*[6] and poricoic acid B (**3**), and two other lanostane-type tritepene acids isolated from *P. cocos*,[7] have been demonstrated to markedly suppress the promoting effect of TPA on skin tumor formation in mice following initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA). Since the lanostane-type triterpenes from *P. cocos* are expected to be potential inhibitors of tumor-promoters (cancer chemopreventive agents), we carried out a further isolation and a primary in vitro screening test of the isolated compounds for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) induced by TPA. In this paper, we report the isolation and characterization of two novel 3,4-*seco*-lanostane-type triteprene acids, poricoic acids G (**1**) and H (**2**), from *P. cocos* and the inhibitory effects on EBV-EA activation of these two and eight other compounds isolated from the fungal material. In addition, the cytotoxicities of **1** and poricoic acid A (**4**) against human cancer cell lines are described.

P. cocos was extracted with methanol, and the extract was partitioned between $CHCl_3$ and water. The $CHCl_3$ fraction was further extracted with saturated $NaHCO_3$ and subsequently with NaOH aqueous solutions. The aqueous extracts were acidified and then extracted with $CHCl_3$, which yielded fractions A and B from the $NaHCO_3$ and the NaOH extracts, respectively. Preparative HPLC of fraction A afforded two novel triterpenes, **1** and **2**.

The molecular formula of **1** was determined as $C_{30}H_{46}O_5$ from its HREIMS ($[M]^+$, *m/z* 486.3352) as well as from its ^{13}C NMR. The compound has a secondary hydroxyl group [ν_{max} 3436 cm^{-1}; δ_H 4.52 (1H, t, J = 7.9 Hz); δ_C 76.4 (d)], an isopropylidene group [δ_H 1.60 and 1.62 (each 3H and s), and 5.34 (1H, brt, J = 4.7 Hz); δ_C 17.7 and 25.8 (each q), and 125.2 (d)], an isopropenyl group [ν_{max} 897 cm^{-1}; δ_H 1.80 (3H, s), and 4.89 and 4.99 (each 1H and s); δ_C 23.3 (q) and 114.2 (t)], a fully substituted double bond [δ_C 129.9 and 139.3 (each s)], two carboxyls [ν_{max} 1707 cm^{-1}; δ_C 176.7 and 178.9 (each s)], and three tertiary methyls [δ_H 0.96, 1.16, and 1.50 (each s)]. These data, in combination with the mass fragmentations observed at *m/z* 413 $[M - CH_2CH_2COOH]^+$, 325 $[M - C_8H_{13}O_2$ (C-20−C-27) $- H_2O - 2H]^+$, and 69 $[CH_2CH{=}C(Me)_2]^+$ (C-23-C-27), suggested that compound **1** is a tricyclic 3,4-*seco*-lanostane-type triterpene with a fully substituted double bond and a secondary hydroxyl group in the ring system, one carboxyl group at C-3, and the other at C-21 in the C_8-side-chain containing an isopropylidene functionality.[2,5] Further fragment ions observed at *m/z* 288 $[C_{19}H_{28}O_2]^+$, corresponding to the loss of ring D by cleavage of the C-14−C-15 and C-13−C-17 bonds, and 233 $[C_{15}H_{21}O_2]^+$ formed from cleavage of C-8−C-14 and C-12−C-13 bonds with 1H loss indicated that the ring-system double bond should be located at C-8(9) and the hydroxyl group at either C-15 or C-16.[11] The above evidence, coupled with the ^{13}C and ^1H NMR data (Table 1) and analyses of $^1H-^1H$ COSY,[12] HMQC, and HMBC spectra, indicated that **1** possesses a 16α-hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic acid structure. The configuration of **1**, with the orientation of the hydroxyl group at C-16 down from the α-face of the ring system, was

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Table 1. ^{13}C, ^{1}H, and HMBC NMR Spectral Data for Triterpene Acids **1** and **2** (C_5D_5N)[a]

	1			**2**		
position	δ_C	δ_H	HMBC (H to C)	δ_C	δ_H	HMBC (H to C)
1A	33.5 t	1.99 m	10	33.8 t	1.99 m	10
1B		2.08 m	10		2.07 m	10
2A	30.5 t	2.34 m	3	30.2 t	2.30 m	1, 3
2B		2.68 m	3		2.68 m	3
3	176.7 s			176.4 s		
4	147.8 s			147.8 s		
5	46.9 d	2.31 m	1, 4, 6, 10, 19, 28	47.0 d	2.31 m	1, 4, 6, 10, 19, 28
6α	24.3 t	1.53 m		24.4 t	1.54 m	
6β		1.71 m	10		1.72 m	10
7α	26.3 t	2.12 m		26.3 t	2.13 m	
7β		1.98 m	5		2.00 m	5
8	139.3 s			139.3 s		
9	129.9 s			129.9 s		
10	40.6 s			40.7 s		
11	21.7 t	2.12 (2H)	8, 9, 12	21.7 t	2.13 (2H)	8, 9, 12
12α	29.9 t	2.23 m	18	29.9 t	2.28 m	18
12β		2.00 m	9, 14		2.02 m	9, 14
13	46.1 s			46.1 s		
14	49.7 s			49.7 s		
15α	43.8 t	1.70 m	13, 14, 16, 17, 30	43.8 t	1.70 m	13, 14, 16, 17, 30
15β		2.38 dd (8.2, 13.1)	8, 14, 16, 30		2.39 dd (7.6, 12.7)	14, 16, 30
16	76.4 d	4.52 t (7.9)	14, 17	76.5 d	4.53 t (7.6)	14, 17
17	57.4 d	2.80 dd (7.6, 10.0)	12, 13, 15, 16, 18, 20, 21, 22	57.3 d	2.81 dd (7.6, 10.0)	13, 15, 16, 18, 20, 21
18	18.0 q	1.16 s	12, 13, 14, 17	18.0 q	1.18 s	12, 13, 14, 17
19	22.5 q	0.96 s	1, 5, 9, 10	22.5 q	0.97 s	1, 5, 9, 10
20	48.5 d	2.94 br t (10.0)	17, 21	48.3 d	2.95 m	17, 21
21	178.9 s			178.4 s		
22A	33.2 t	2.34 m		31.6 t	2.38 m	23
22B		2.49 m	21		2.65 m	
23A	27.1 t	2.34 m	25	33.2 t	2.40 m	24, 24[1]
23B		2.48 m			2.53 m	
24	125.2 d	5.34 br t (4.7)	23, 26, 27	156.1 s		
25	131.5 s			34.1 d	2.29 m	26, 27
26	25.8 q	1.62 s	24, 25, 27	22.0 q	0.99 d (6.8)	24, 25, 27
27	17.7 q	1.60 s	24, 25, 26	21.9 q	1.00 d (6.6)	24, 25, 26
28A	114.2 t	4.89 s	5, 29	114.2 t	4.90 s	5, 29
28B		4.99 s	5, 29		4.99 s	5, 29
29	23.3 q	1.80 s	4, 5, 10, 28	23.3 q	1.80 s	4, 5, 10, 28
30	26.3 q	1.50 s	7, 8, 13, 14, 15	26.3 q	1.50 s	7, 8, 13, 14, 15
24[1]A				107.0 t	4.85 s	25
24[1]B					4.98 s	25

[a] Chemical shifts in ppm from internal TMS, coupling constants in Hz.

established by phase-sensitive NOESY experiment. Compound **1** showed significant NOE correlations between [H-28A–H-5α–H-7α–H-30 (14α-Me)–H-17α] on the α-face and [H-28B–H-29–H-19 (10β-Me)–H-18 (13β-Me)–H-16β and H-20] on the β-face of the molecule (Figure 1).[13] We concluded that **1** is 16α-hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic acid (20*R*) and was named poricoic acid G.

Compound **2** showed $[M]^+$ at *m*/*z* 500.3502 ($C_{31}H_{48}O_5$) in the HREIMS. The compound has a secondary hydroxyl group [ν_{max} 3442 cm^{-1}; δ_H 4.53 (1H, t, $J = 7.6$ Hz); δ_C 76.5 (d)], an isopropenyl group [ν_{max} 893 cm^{-1}; δ_H 1.80 (3H, s), and 4.90 and 4.99 (each 1H and s); δ_C 23.3 (q) and 114.2 (t)], a terminal methylene group [ν_{max} 893 cm^{-1}; δ_H 4.85 and 4.98 (each 1H and s); δ_C 107.0 (t)], a fully substituted double bond [δ_C 129.9 and 139.3 (each s)], two carboxyls [ν_{max} 1706 cm^{-1}; δ_C 176.4 and 178.4 (each s)], three tertiary methyls [δ_H 0.97, 1.18, and 1.50 (each s)], and two secondary methyls [δ_H 0.99 (d, $J = 6.8$ Hz) and 1.00 (d, $J = 6.6$ Hz)]. The EIMS of compound **2** showed diagnostic fragment ions at *m*/*z* 425 $[M - CH_2CH_2COOH - 2H]^+$, 325 $[M - C_8H_{13}O_2$ (C-20–C-27) $- H_2O - 2H]^+$, and 233 ($[C_{15}H_{21}O_2]^+$; formed from cleavage of C-8–C-14 and C-12–C-13 bonds with 1H loss). Close similarity of these spectral data with those of compound **1** described above suggested that compound **2** is a higher homologue of compound **1**. The ^{13}C and ^{1}H NMR signals for the side-chain at C-17 of compound **2** were almost identical with those of poricoic acid A (**4**),[2]

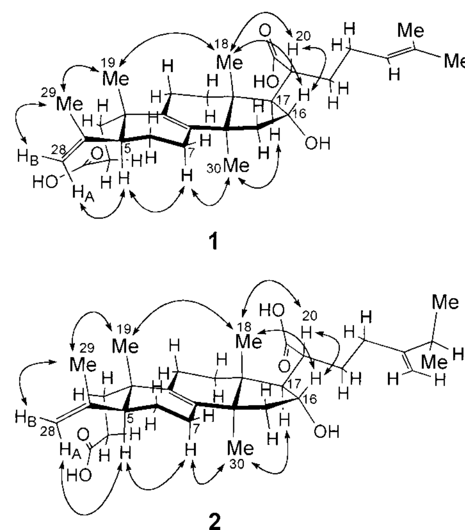
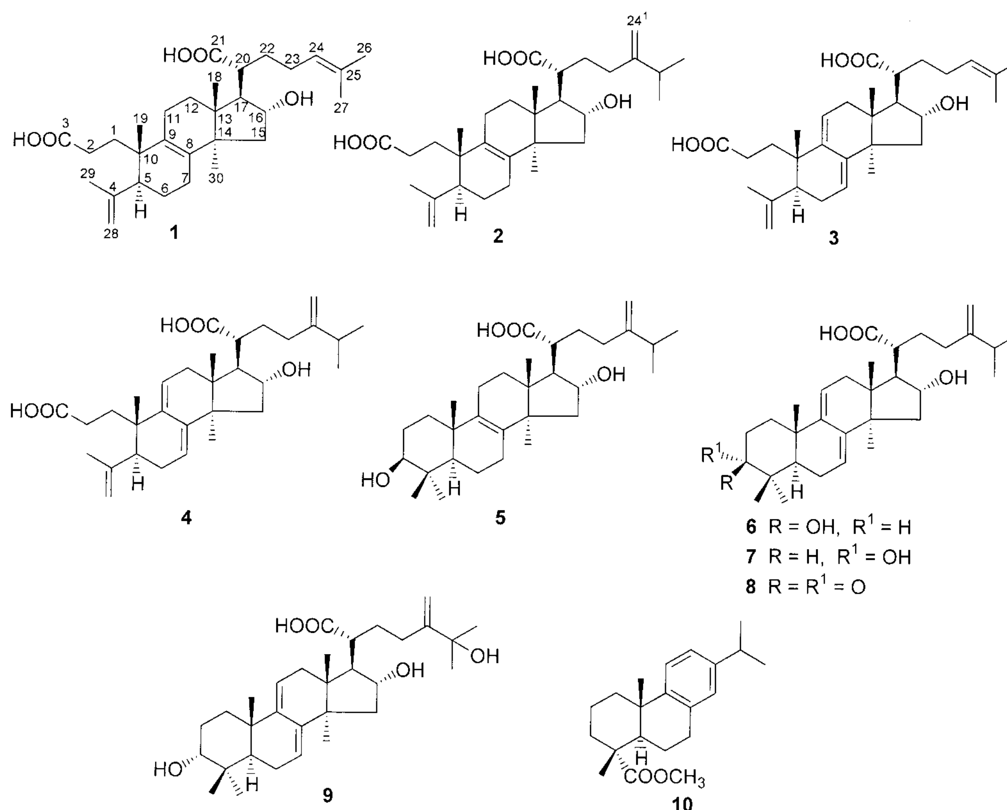


Figure 1. Major NOE correlations (←→) for **1** and **2**.

and this evidence, coupled with the analyses of ^{13}C and ^{1}H NMR data (Table 1), as well as ^{1}H–^{1}H COSY,[15] HMQC, HMBC (Table 1), and phase-sensitive NOESY (Figure 1), indicated that **2** is the 24-methylene homologue of compound **1**, 16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),8,-24(24[1])-triene-3,21-dioic acid (20*R*), which we named poricoic acid H.Five other compounds isolated from fraction A

Chart 1



were identified as the known compounds poricoic acid B (**3**),[2] poricoic acid A (**4**),[2] dehydrotumulosic acid (**6**),[5] 25-hydroxy-3-epidehydrotumulosic acid (**9**),[5] and dehydroabietic acid methyl ester (**10**).[16] From fraction B, five additional known compounds were isolated: tumulosic acid (**5**),[17] **6**, 3-epidehydrotumulosic acid (**7**),[5] polyporenic acid C (**8**),[18] and **10**. Identification of the known compounds was carried out by spectral comparison with the data reported in the literature for each compound.

Although several 3,4-*seco*-lanostane-type triterpenes with the $\Delta^{7,9(11)}$-conjugated diene system have been reported from *Poria cocos*,[2,5] the two new triterpenes isolated in this study, poricoic acids G (**1**) and H (**2**), are the first examples of the occurrence of Δ^{8}-unsaturated 3,4-*seco*-lanostane-type triterpenes in *P. cocos*. Thus far, only two 3,4-*seco*-lanost-8-enes have been reported as natural products: manwuweizic acid from *Schisandra propinqua*[19] and *Kadsura heteroclita*,[20] and schisanlactone F from *K. longipeduaculata*.[21]

The inhibitory effects of nine lanostane-type triterpenes, **1**−**9**, and one abietane-type diterpene, **10**, on EBV-EA activation induced by TPA were examined as a preliminary evaluation of their potential to inhibit tumor-promoting activities, and the results are shown in Table 2. All compounds showed potent dose-related inhibitory effects on EBV-EA induction by TPA, while not affecting the viability of Raji cells even at 1000 mol ratio/TPA (0.032 μM). The inhibitory effects of all compounds tested were found to be stronger than those of β-carotene (Table 2), a vitamin A precursor that has been intensively studied in cancer prevention using animal models.[22] Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel inhibition of tumor promotion in vivo,[23,24] the nine triterpenes and one diterpene from *Poria cocos* may be viable inhibitors of tumor-promotion (potential cancer chemopreventive agents). Accordingly, it is noteworthy that A-ring cleavage to give 3,4-*seco*-3-oic acids (compounds **1**−**4**) or C-25 hydroxylation (compound **9**) appears to enhance the inhibitory effects of the lanostane-type tritepene acids.

Table 2. Percentage of Epstein−Barr Virus Early Antigen (EBV-EA) Induction in the Presence of Compounds **1**−**10**[a]

	concentration (mol ratio/TPA)				
compound	1000		500	100	10
1	0	(70)	16.0	55.9	82.8
2	0	(70)	15.7	53.8	80.8
3	0	(70)	14.3	54.0	80.7
4	0	(70)	18.5	58.9	83.8
5	0	(70)	22.7	64.9	90.5
6	0	(70)	22.7	64.9	90.5
7	0	(70)	25.5	67.0	93.8
8	0	(70)	26.3	68.7	92.6
9	0	(70)	13.2	51.6	79.4
10	0	(60)	23.2	68.3	91.6
β-carotene[b]	8.6	(70)	34.2	82.1	100

[a] Values represent relative percentages to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells. [b] Reference compound.

Evaluation of the cytotoxicity of poricoic acids G (**1**) and A (**4**) against the NCI panel of 60 human cancer cell lines[25] revealed that compound **1** was significantly cytotoxic to leukemia HL-60 cells [GI_{50} (concentration that yields 50% growth), 39.3 nM], although it showed only moderate cytotoxicity to the other cell lines [GI_{50}, >22 μM]. Compound **4** exhibited moderate cytotoxicity [GI_{50}, 15−30 μM] to all of the 60 human cancer cell lines tested. These results suggest that compound **1** may be a potentially useful anticancer agent for human leukemia (HL-60 cells) in addition its potential use as a chemopreventive agent.

Experimental Section

General Experimental Procedures. Crystallizations were performed in methanol (MeOH), and melting points measured are uncorrected. UV spectra on a Shimadzu UV-300 spectrom-

eter and IR spectra on a JASCO IR-300 IR spectrometer were recorded in EtOH and KBr disks, respectively. Optical rotations were measured on a JASCO DIP-370 polarimeter in MeOH at 20 °C. Reversed-phase HPLC was carried out on an octadecyl silica column (Superiorex ODS S-5 μm column, 25 cm × 10 mm i.d.; Shiseido Co., Ltd., Tokyo) at 25 °C with MeOH−H_2O−AcOH (80:20:0.1, v/v/v; 1.8 mL/min) as a mobile phase. Dehydrotumulosic acid (**6**; retention time 33.0 min) was the standard for the determination of Rt_R in HPLC of the compounds described in this paper. EIMS and HREIMS were recorded on a Hitachi M-80B double-focusing gas chromatography−mass spectroscopy (GC−MS) instrument (70 eV) using a direct inlet system. NMR spectra were recorded on a JEOL LA-500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in C_5D_5N with tetramethylsilane (TMS) as internal standard. Chemical shifts are δ values.

Materials. Dried sclerotium of *Poria cocos* was purchased from Kinokuniya Kan-Yaku Kyoku Co. (Tokyo, Japan). Identification was done by description, chemical analysis, and purity test (total ash: less than 1.0%).[1,26] A voucher specimen has been deposited in the College of Science and Technology, Nihon University.

Extraction and Isolation. Pulverized sclerotium of *P. cocos* (1 kg) was extracted with water (2 L) under reflux (3 h) three times. The residual material was extracted twice with MeOH (2 L) under reflux (3 h). The MeOH solutions were combined and evaporated in vacuo to give an extract (5.9 g) that was mixed with water (3 L) and extracted with $CHCl_3$ (3 L × 3). The $CHCl_3$-soluble fraction was further extracted with saturated $NaHCO_3$ aqueous solution and then with 5% NaOH aqueous solution. The aqueous extracts, after adjustment to pH 3−4 with 6 M HCl solution, were extracted with $CHCl_3$, which yielded fraction A (85 mg) from the $NaHCO_3$ extract and fraction B (108 mg) from the NaOH extract. Reversed-phase HPLC of the fractions enabled the isolation of compounds **1** (3.3 mg; Rt_R 1.54), **2** (2.8 mg; Rt_R 2.07), **3** (6.6 mg; Rt_R 1.10), **4** (5.0 mg; Rt_R 1.41), **6** (2.1 mg; Rt_R 1.00), **9** (1.1 mg; Rt_R 0.61), and **10** (4.1 mg; Rt_R 0.35) from fraction A and compounds **5** (2.5 mg; Rt_R 1.14), **6** (6.3 mg), **7** (2.9 mg; Rt_R 2.04), **8** (1.3 mg; Rt_R 1.20), and **10** (8.5 mg) from fraction B. Some physical characteristics and the spectral data of two novel compounds, **1** and **2**, are shown below.

Poricoic acid G (**1**): colorless needles, mp 260 °C (dec); $[\alpha]^{20}_D$ +38° (*c* 0.36, MeOH); UV (EtOH) λ_{max} (log ϵ) 205 (2.9); IR ν_{max} 3436, 1707, 1639, 897 cm^{-1}; ^{13}C and ^{1}H NMR, see Table 1; EIMS *m*/*z* 486 [M]$^+$ (46), 453 (8), 413 (47), 395 (24), 325 (3), 311 (4), 288 (31), 273 (3), 253 (10), 233 (16), 220 (15), 201 (7), 187 (12), 69 (79), 41 (100); HREIMS *m*/*z* 486.3352 (calcd for $C_{30}H_{46}O_5$, 486.3343), 413.2954 (assignment $C_{27}H_{41}O_3$), 325.2114 ($C_{22}H_{29}O_3$), 288.2059 ($C_{19}H_{28}O_2$), 233.1585 ($C_{15}H_{21}O_2$), 69.0705 (C_5H_9).

Poricoic acid H (**2**): colorless needles, mp 270 °C (dec); $[\alpha]^{20}_D$ +43° (*c* 0.34, MeOH); UV (EtOH) λ_{max} (log ϵ) 205 (2.9); IR ν_{max} 3442, 1706, 1639, 893 cm^{-1}; ^{13}C and ^{1}H NMR, see Table 1; EIMS *m*/*z* 500 [M]$^+$ (20), 467 (3), 425 [M − CH_2CH_2COOH − 2H]$^+$ (100), 409 (9), 325 (3), 311 (4), 295 (3), 269 (3), 253 (7), 237 (4), 233 (5), 225 (5), 211 (9), 159 (26), 95 (18), 81 (24), 69 (35); HREIMS *m*/*z* 500.3502 (calcd for $C_{31}H_{48}O_5$, 500.3500).

EBV-EA Induction Tests. The inhibition of EBV-EA activation was assayed using Raji cells (virus nonproducer type), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% fetal bovine serum−Roswell Park Memorial Institute (FBS RPMI) 1640 medium solution (Nacalai Tesque, Inc., Kyoto, Japan). The indicator cells (Raji) (1 × 10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, inducer) and 32 pmol of TPA (20 ng/mL) in dimethyl sulfoxide (DMSO) and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients and were detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments with *n*-butyric acid plus TPA, in which EA induction was ordinarily around 30%.[27]

In Vitro Cytotoxicity Assay. Compounds **1** and **4** were tested at a minimum of five concentrations at 10-fold dilutions starting from a high of 10^{-4} M. Cytotoxicity was evaluated against 60 human tumor cell lines derived from seven cancer types (lung, colon, melanoma, renal, ovarian, brain, and leukemia).[24] A 48 h continuous drug exposure protocol was used, and a sulforhodamine B protein assay was used to estimate cell viability or growth. Details of the assay procedure have been previously reported.[25]

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(12) Compound **1** showed ^{1}H−^{1}H COSY correlations between [H-1−H-2], [H-5−H-6−H-7], [H-11−H-12], and [H-15−H-16−H-17−H-20−H-22−H-23−H-24].
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