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Cytotoxic and Apoptosis-Inducing Activities of Triterpene Acids from *Poria cocos*

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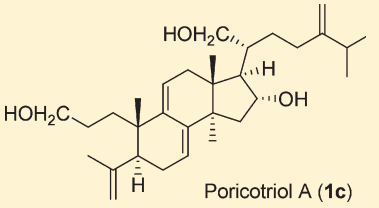
ABSTRACT: Six lanostane-type triterpene acids (**1a**−**6a**), isolated from *Poria cocos*, and their methyl ester (**1b**−**6b**) and hydroxy derivatives (**1c**−**6c**) were prepared. Upon evaluation of the cytotoxic activity of these compounds against leukemia (HL60), lung (A549), melanoma (CRL1579), ovary (NIH:OVCAR-3), breast (SK-BR-3), prostate (DU145), stomach (AZ521), and pancreas (PANC-1) cancer cell lines, 11 compounds (**5a**, **6a**, **2b**−**5b**, **1c**, and **3c**−**6c**) exhibited activity with single-digit micromolar IC_{50} values against one or more cell lines. Poricotriol A (**1c**), a hydroxy derivative of poricoic acid A (**1a**), exhibited potent cytotoxicities against six cell lines with IC_{50} values of 1.2−5.5 μM. Poricotriol A induced typical apoptotic cell death in HL60 and A549 cells on evaluation of the apoptosis-inducing activity by flow cytometric analysis. Western blot analysis in HL60 cells showed that poricotriol A activated caspases-3, -8, and -9, while increasing the ratio of Bax/Bcl-2. This suggested that poricotriol A induced apoptosis via both mitochondrial and death receptor pathways in HL60. On the other hand, poricotriol A did not activate caspases-3, -8, and -9, but induced translocation of apoptosis-inducing factor (AIF) from mitochondria and increased the ratio of Bax/Bcl-2 in A549. This suggested that poricotriol A induced apoptosis via the mitochondrial pathway mostly by translocation of AIF, independent from the caspase pathway in A549. Furthermore, poricotriol A was shown to possess high selective toxicity in lung cancer cells since it exhibited only weak cytotoxicity against a normal lung cell line (WI-38).



Poricotriol A (**1c**)

The dried sclerotia of *Poria cocos* Wolf (Polyporaceae) are used traditionally in Chinese herbal prescriptions as a diuretic, sedative, and analgesic.[1,2] Investigations by us and by other workers have shown that both the inner parts[2−6] and the epidermis[7−10] of the sclerotia of *P. cocos* contain lanostane-type triterpene acids, which were suggested to be one of the major medicinal components of the fungus.[2] Several of these acids have potent inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice,[11,12] and on Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA.[6,9,10] In addition, in a skin tumor promotion assay using an *in vivo* two-stage carcinogenesis test employing 7,12-dimethylbenz[*a*]-anthracene (DMBA) as an initiator and TPA as a promoter, 16-deoxyporicoic acid B, poricoic acid C, and 25-methoxyporicoic acid A showed activity.[9,10] Poricoic acid A showed moderate cytotoxicities against some human cancer cells,[6] and pachymic acid and dehydrotrametenolic acid exhibited inductive effects of apoptosis in prostate cancer cells[13] and in H-ras-tranformed rat2 cells.[14] In addition, we have reported that some triterpene acids from *P. cocos* possess inhibitory effects on DNA polymerases.[15,16] In this paper, we report the conversion of six lanostane-type triterpene acids (**1a**−**6a**), isolated from *P. cocos*,[6,9,10,15] into their methyl ester (**1b**−**6b**) and hydroxy derivatives (**1c**−**6c**) and evaluation of the cytotoxic activities of these compounds (**1a**−**6a**, **1b**−**6b**, and **1c**−**6c**) against eight human cancer cell lines. In addition, we report the induction of apoptosis in HL60 and A549 cells by poricotriol A (**1c**), a hydroxy derivative of poricoic acid A (**1a**), and the mechanisms of the apoptotic cell death.

RESULTS AND DISCUSSION

Six lanostane-type triterpene acids, poricoic acid A (**1a**), poricoic acid C (**2a**), poricoic acid G (**3a**), poricoic acid H (**4a**), 25-hydroxy-3-epidehydrotumulosic acid (**5a**), and dehydroeburiconic acid (**6a**), were isolated from the sclerotia of *P. cocos*.[6,9,10,15] Dimethyl poricoate A (**1b**) was prepared from poricoic acid A by methylation with trimethylsilyl (TMS)-diazomethane, and poricotriol A (**1c**) from **1b** by reduction with $LiAlH_4$.

Compound **1b** gave a $[M + Na]^+$ ion in the HRESIMS at m/z 549.3629, consistent with a molecular formula of $C_{33}H_{50}O_5$. The ^{13}C and ^1H NMR data (Table 1) of **1b** were very similar to those of poricoic acid A,[7] except for the presence of additional signals of two methoxy groups [δ_C 51.2 (q) and 51.4 (q); δ_H 3.61 (3H, s) and 3.78 (3H, s)]. The above evidence, coupled with analyses of $^1H-^1H$ COSY, HMQC, and HMBC (Table 1) spectra, indicated that **1b** was dimethyl (20*R*)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioate. Significant NOE correlations (Figure 1)[17] were observed in the NOESY experiment of **1b** for H-18 (13β-Me) with H-16β, H-19 (10β-Me), and H-20 and for H-30 (14α-Me) with H-17α. These NOE correlations suggested that **1b** possesses the 20*R*-stereochemistry at C-20, and the structure was established as dimethyl (20*R*)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24-(24^1)-tetraene-3,21-dioate (dimethyl poricoate A).

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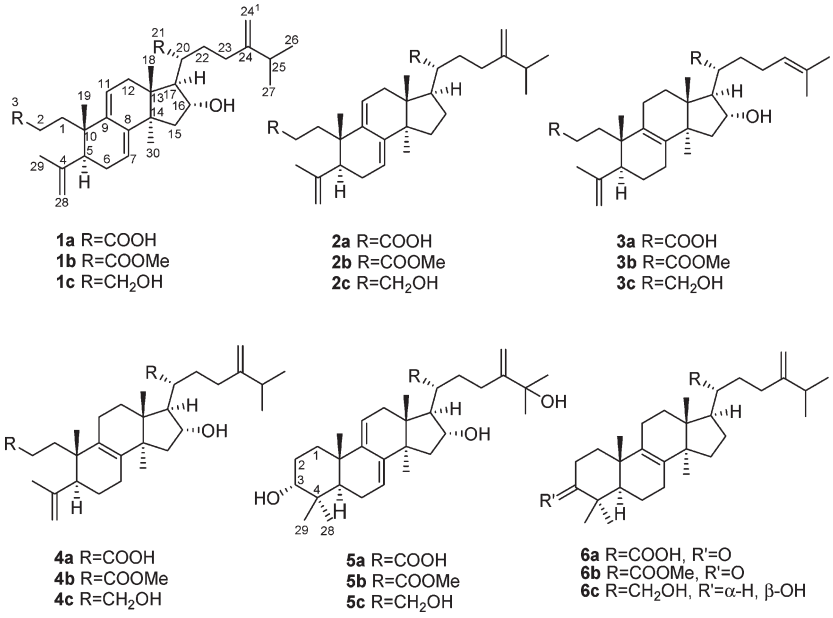
Chart 1



The molecular formula of compound **1c** was determined to be $C_{31}H_{50}O_3$ from its HRESIMS ($[M + Na]^+$, m/z 493.3674). The appearance of the signals for two primary hydroxy groups [δ_C 63.0 (t) and 63.6 (t); δ_H 3.82 (2H, m), 3.90 (1H, dd, J = 4.0, 10.6 Hz), and 4.13 (1H, br d, J = 8.3 Hz)] in the ^{13}C and ^1H NMR spectra and the disappearance of two carboxy signals in the ^{13}C NMR spectrum of **1c** when compared with those of poricoic acid A[7] and **1b** suggested that **1c** is the 3,21-dihydroxy derivative. This evidence, coupled with the analysis of $^1H-^1H$ COSY, HMQC, HMBC (Table 1), and NOESY (Figure 1)[17] spectra, surpports the conclusion that **1c** is (20*R*)-3,4-*seco*-24-methyllanosta-4-(28),7,9(11),24(24^1)-tetraene-3,16α,21-triol (poricotriol A).

The other five methyl ester (**2b**−**6b**) and five hydroxy (**2c**−**6c**) derivatives were prepared from the corresponding triterpene acids (**2a**−**6a**), respectively, in the same way as described above.

The cytotoxic activities of compounds **1a**−**6c** along with two anticancer drugs, cisplatin and 5-fluorouracil, were evaluated against the following human cancer cell lines: HL60 (leukemia), A549 (lung), CRL1579 (melanoma), NIH:OVCAR-3 (ovary), SK-BR-3 (breast), DU145 (prostate), AZ521 (stomach), and PANC-1 (pancreas), by means of a thiazolyl blue tetrazolium bromide (MTT) assay, and the results are summarized in Table 2. Compounds **5a**, **6a**, **2b**−**5b**, **1c**, and **3c**−**6c** exhibited single-digit micromolar cytotoxicities with IC_{50} values of 1.2−9.5 μM against one or more cancer cell lines. Thus, compound **5a** was active against A549 and NIH:OVCAR-3 cells, **6a** against PANC-1 cells, **2b**, **4b**, **5b**, **3c**, and **5c** against HL60 cells, **3b** against SK-BR-3 cells, **1c** against HL60, A549, CRL1579, NIH:OVCAR-3, SK-BR-3, and AZ521 cells, **4c** against HL60 and CRL1579 cells, and **6c** was against HL60, A549, CRL1579, and NIH:OVCAR-3 cells. In particular, the cytotoxicities of compound **5a** against A549 and NIH:OVCAR-3 cells, **6a** against PANC-1 cells, **1c** against A549, CRL1579, NIH:OVCAR-3, SK-BR-3, and AZ521 cells, **4c** against CRL1579 cells, and **6c** against A549, CRL1579, and NIH:OVCAR-3 cells were superior to those of the reference compounds, cisplatin and/or 5-fluorourasil, tested in the same assay. The prostate cancer cells (DU145) were less sensitive to the compounds tested in this study, and **2a**, **4b**, **5b**, **1c**, and **2c** showed only moderate cytotoxicities (IC_{50} 14.5−28.9 μM), being less active than cisplatin (IC_{50} 9.2 μM).

Poricotriol A (**1c**) exhibited potent cytotoxicities against most of the cell lines tested (Table 2) and was evaluated for its apoptosis-inducing activity against HL60 and A549 cells. HL60 and A549 cells were incubated with **1c** for 24 and 48 h, and then the cells were analyzed in terms of cell cycle distribution by means of flow cytometry.[18] The sub-G_1 peak, i.e., fragmented DNA, was quantified as the apoptosis index. At 48 h after treatment, the sub-G_1 peak increased [HL60 (Figure 2A): from 3.0% to 38.7%; A549 (Figure 2B): from 3.1% to 17.4%]. The cell cycle analysis of HL60 and A549 cells also demonstrated that **1c** induced a depletion of cells in the S phase [HL60 (Figure 2A): from 42.6% to 28.9% after 24 h; A549 (Figure 2B): from 27.7% to 7.8% after 24 h and 6.0% after 48 h] and accumulation of cells in the G_0/G_1 phase (HL60: from 51.4% to 62.3% after 24 h; A549: from 64.3% to 77.2% after 24 h). These results suggest that the cells had undergone cell cycle arrest in the G_0/G_1 phase and apoptosis after treatment with **1c**.

Exposure of the membrane phospholipid, phosphatidylserine, to the external cellular environment is one of the earliest markers of apoptotic cell death.[19] Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface. Propidium iodide (PI) does not enter whole cells with intact membranes and was used to differentiate between early apoptotic (annexin V positive, PI negative), late apoptotic (annexin V, PI double positive), and necrotic cell death (annexin V negative, PI positive). The ratio of early apoptotic cells (Figure 3A, lower right) was increased after treatment with **1c** in HL60 for 24 h (from 7.6% to 48.1%) and 48 h (52.5%), and that of late apoptotic cells (upper right) was increased after 48 h (from 3.3% to 30.4%) (Figure 3A). On the other hand, the proportion of early apoptotic cells did not increase after treatment with **1c** for 24 h in A549 cells (Figure 3B). This was, however, increased after 48 h (from 4.3% for 0 h to 24.1%) along with the increase of late apoptotic cells (from 1.1% for 0 h

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Table 1. ^{13}C (125 MHz), ^{1}H (500 MHz), and HMBC Spectroscopic Data (δ values; C_5D_5N) for Dimethyl Poricoate A (1b) and Poricotriol A (1c)[a]

	1b				1c			
carbon	δ_C		δ_H	HMBC (H to C)	δ_C		δ_H	HMBC (H to C)
1	35.9	t	1.71 m	5	37.9	t	1.55 dt (5.1, 12.3)	2, 5, 9, 10
			1.94 m	2, 5, 10, 19			1.66 dt (4.0, 12.3)	2, 19
2	29.6	t	2.29 m	1, 3	28.5	t	1.67 m	1, 3
			2.37 m	1, 3			1.75 m	1, 3, 10
3	174.5	s			63.0	t	3.82 m (2H)	
4	149.6	s			149.7	s		
5	50.7	d	2.27 br d (5.7)	1, 4, 6, 7, 9, 10, 28	50.6	d	2.36 dd (8.6, 14.6)	4, 6, 7, 9, 10, 28
6	28.5	t	α, 2.51 m	4, 5, 7, 8, 10	28.7	t	α, 2.55 m	4, 7, 8
			β, 2.05 dd (3.6, 19.0)	4, 5, 7, 8			*β*, 2.08 dd (4.0, 19.0)	5, 7, 8, 10
7	118.2	d	5.25 t (3.0)	5, 9	117.6	d	5.25 br s	5, 6, 9, 14
8	141.5	s			142.2	s		
9	137.3	s			138.5	s		
10	38.8	s			39.0	s		
11	120.1	d	5.30 br d (3.0)	8, 10, 12	119.5	d	5.43 br d (2.8)	8, 10, 13
12	36.7	t	α, 2.37 m	9, 11, 13, 14, 18	37.7	t	α, 2.51 m	9, 11, 13, 18
			β, 1.91 dd (5.1, 17.5)	9, 11, 13, 18			*β*, 2.27 dd (5.1, 17.4)	9, 11, 13, 14, 18
13	45.3	s			46.0	s		
14	49.3	s			49.4	s		
15	43.7	t	α, 1.72 br d (10.9)	13, 14, 30	43.3	t	α, 1.77 br d (12.6)	13, 16, 17, 30
			β, 2.32 m	14, 16, 30			*β*, 2.34 m	14, 16, 30
16	76.1	d	4.37 t-like (7.3)	14, 20	74.6	d	4.54 t-like (7.5)	14, 20
17	57.5	d	2.60 dd (6.1, 11.2)	12, 13, 18, 20, 22	56.7	d	2.52 dd (6.3, 13.2)	12, 13, 16, 20, 21
18	18.2	q	0.87 s	12, 13, 14, 17	18.7	q	0.84 s	12, 13, 14, 17
19	22.2	q	1.00 s	1, 5, 9, 10	22.8	q	1.10 s	1, 5, 9, 10
20	47.6	d	2.77 dt (3.1, 10.8)		41.3	d	1.87 m	
21	176.4	s			63.6	t	3.90 dd (4.0, 10.6)	22
							4.13 br d (8.3)	17, 22
22	31.1	t	2.16 m	20, 23	27.8	t	2.18 m (2H)	21, 23
			2.49 m	20, 23				
23	32.9	t	2.15 m (2H)	22, 24^1	32.9	t	2.20 m	20, 22, 24, 25, 24^1
							2.53 m	20, 22, 24
24	155.5	s			157.0	s		
25	34.0	d	2.24 m	26, 27, 24^1	34.1	d	2.33 m	24, 26, 27, 24^1
26	22.0	q	0.99 d (7.0)	24, 25, 27	22.2	q	1.04 d (6.9)	24, 25, 27
27	21.9	q	0.98 d (7.0)	24, 25, 26	22.0	q	1.03 d (6.9)	24, 25, 26
28	112.3	t	4.75 s	4, 5, 29	112.0	t	4.78 br s	4, 5, 29
			4.79 d (2.2)	4, 29			4.84 s	4, 5, 29
29	22.2	q	1.70 s	4, 5, 28	22.4	q	1.77 s	4, 5, 28
30	24.7	q	1.32 s	8, 13, 14, 15	25.3	q	1.38 s	13, 14, 15
24^1	107.3	t	4.82 br s	23, 24, 25	106.7	t	4.83 s	23, 24, 25
			4.87 s	23, 24, 25			4.92 s	23, 24, 25
3-COOMe	51.4	q	3.61 s	3				
21-COOMe	51.2	q	3.78 s	21				

[a] *J* values (Hz) determined are shown in parentheses.

to 20.2%). A small number of necrotic cells (upper left) also were detected in A549 cells at 48 h (from 3.4% for 0 h to 6.3%). These results revealed that most of the cytotoxicty of **1c** to HL60 and A549 cells is due to inducing apoptotic cell death.

Caspases are known to mediate the apoptotic pathway.[20,21] In order to clarify the mechanism by which compound **1c** induces apoptotic cell death, activation of caspases-3, -8, and -9 was evaluated by western blot analysis. After treatment of HL60 cells with **1c** (30 μM) for 8 h, the level of procaspases-3, -8, and -9 diminished and cleaved caspases-3, -8, and -9 were detected (Figure 4A). In contrast, diminution of procaspases-3, -8, and -9 and expression of cleaved caspases-3, -8, and -9 were not deteced after treatment with **1c** in A549 (Figure 4B). These results suggest that compound **1c**-induced cell death occurs through

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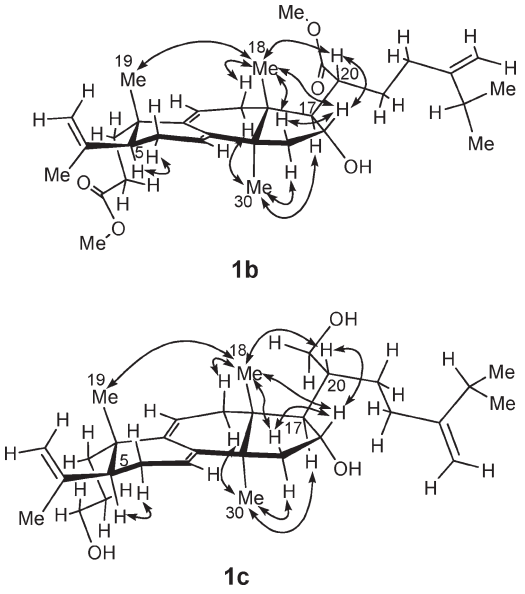

Figure 1. Major NOE correlations (↔) for dimethyl poricoate A (**1b**) and poricotriol A (**1c**).[17]

activation of caspases-3, -8, and -9 in HL60, although not in the case of A549.

Next, we investigated the effect of compound **1c** on Bax and Bcl-2. The proapoptotic proteins Bax and Bid and the antiapoptotic mitochondrial protein Bcl-2 are important regulators of cytochrome *c* release from mitochondria.[22,23] Expression of these proteins was examined by western blot analysis. Treatment of HL60 cells by compound **1c** (30 μM) decreased the level of Bcl-2 and increased the level of Bax in a time-dependent manner (Figure 4A). In A549 cells, compound **1c** had no effect on the expression of Bax, but diminished Bcl-2, though to a small extent, after 24 h of treatment (Figure 4B).[24] The Bax/Bcl-2 ratio is one of the indices of the intrinsic mechanism of apoptosis in mitochondria.[25] Since compound **1c** increased this ratio in HL60 and A549 cells, it seems that compound **1c**-induced apoptosis involved Bax/Bcl-2 signal transduction. Compound **1c** is, therefore, suggested to induce apoptosis in HL60 and A549 cells by involving the mitochondrial signal transduction pathway.

Translocation of AIF (apoptosis-inducing factor) was investigated in A549 cells treated with compound **1c**. AIF and endonuclease G (EndoG) are capable of inducing apoptotic DNA fragmentation in a caspase-independent pathway.[26] AIF, released

Table 2. Cytotoxic Activity on Eight Human Cancer Cell Lines of Triterpene Acids from *Poria cocos* and Their Methyl Ester and Hydroxy Derivatives[a]

	IC_{50} (μM)[b]							
compound	HL60 (leukemia)	A549 (lung)	CRL1579 (melanoma)	NIH:OVCAR-3 (ovary)	SK-BR-3 (breast)	DU145 (prostate)	AZ521 (stomach)	PANC-1 (pancreas)
triterpene acid								
1a	38.0	>100	>100	>100	>100	>100	>100	>100
2a	25.9	27.0	>100	53.1	23.9	14.5	45.9	68.9
3a	32.6	>100	>100	>100	>100	>100	>100	>100
4a	28.4	>100	>100	>100	>100	>100	>100	>100
5a	29.4	4.0	84.0	9.5	62.3	>100	51.5	67.4
6a	29.2	10.0	89.7	37.7	12.2	>100	>100	5.5
methyl ester derivative								
1b	18.2	79.6	>100	91.8	32.2	>100	32.1	>100
2b	2.4	83.8	27.8	23.6	23.9	>100	19.8	>100
3b	8.8	66.0	42.4	26.3	4.3	>100	34.5	>100
4b	3.6	13.5	97.6	49.5	15.9	28.9	14.6	44.9
5b	6.9	41.4	76.5	67.5	27.5	28.9	14.4	71.3
6b	23.5	96.5	20.9	>100	27.5	>100	53.4	>100
hydroxy derivative								
1c	1.9	4.2	5.5	2.2	1.2	14.5	1.4	26.3
2c	12.3	80.0	76.4	70.3	11.0	20.3	13.3	91.3
3c	7.5	11.0	11.6	43.5	13.0	>100	13.4	17.9
4c	2.9	41.2	9.9	>100	51.3	>100	>100	>100
5c	2.5	>100	14.0	>100	>100	>100	>100	>100
6c	6.7	4.0	2.8	6.4	11.7	>100	12.5	22.6
reference compound								
cisplatin	1.9	24.9	21.1	17.9	9.8	9.2	2.7	79.0
5-fluorouracil	9.5	11.3	>100	>100	>100	>100	>100	54.1

[a] Cells were treated with compounds (1×10^{-4} to 1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. [b] IC_{50} value is the concentration of compound required to inhibit the growth of the cells by 50%. This was obtained on the basis of triplicate assay results.

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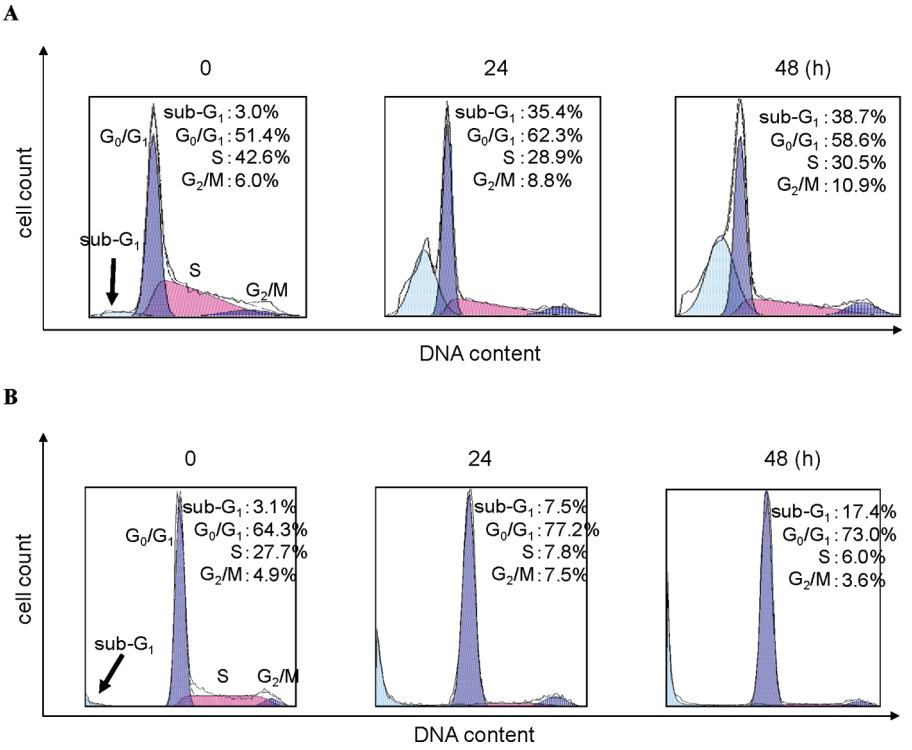

Figure 2. Cell cycle analysis of HL60 and A549 cells treated with poricoriol A (**1c**). (A) HL60 cells were treated with **1c** (30 μM) for 0, 24, and 48 h. (B) A549 cells were treated with **1c** (30 μM) for 0, 24, and 48 h. Each value is the mean of three experiments.

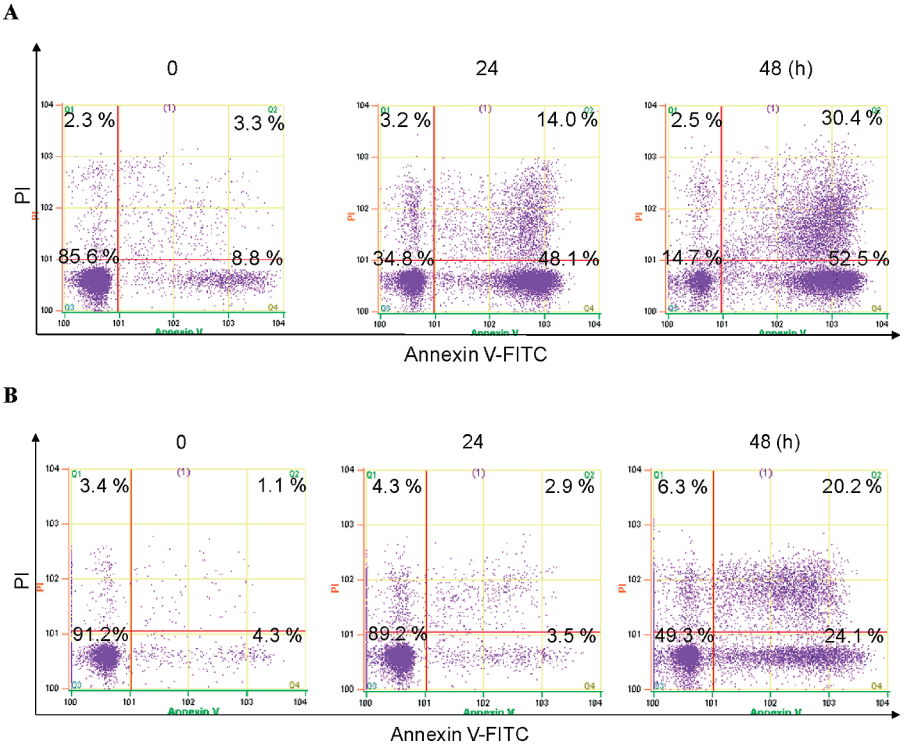

Figure 3. Poricotriol A (**1c**) induced apoptosis against HL60 and A549 cells. (A) HL60 cells were cultured with **1c** (30 μM) for 24 and 48 h. (B) A549 cells were cultured with **1c** (30 μM) for 24 and 48 h. Each value is the mean of three experiments.

from mitochondria, is translocated into cytosol and then into the nucleus. This induces DNA fragmentation and chromatin condensation.[27] Mitochondrial AIF diminished in a time-dependent manner, and cytosolic AIF increased after treatment for 24 h, although it decreased after 48 h (Figure 4C). It seems that AIF was released into the cytosol from mitochondria and translocated

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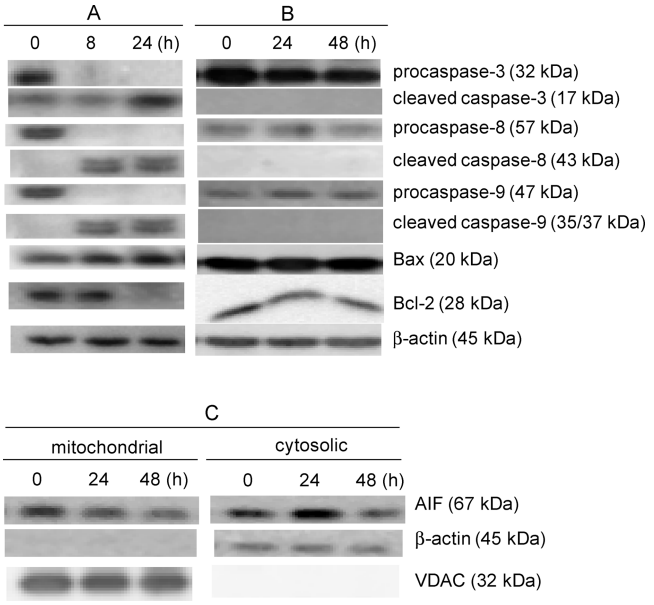

Figure 4. Western blot analysis of HL60 and A549 cells treated with poricotriol A (**1c**). (A) Western blot analysis of caspases-3, -8, and -9, Bax, and Bcl-2 in HL60 cells with **1c** (30 μM) for 8 and 24 h. (B) Western blot analysis of caspases-3, -8, and -9, Bax, and Bcl-2 in A549 cells with **1c** (30 μM) for 24 and 48 h. (C) Translocation of AIF from mitochondria to cytosol in A549 treated with **1c**. The results are from one representative experiment among three runs, which showed similar patterns to one another.

to the nucleus after treatment with compound **1c** in A549 cells. Compound **1c** is, therefore, suggested to induce apoptosis in A549 via the mitochondrial pathway mostly by translocation of AIF, and possibly also by EndoG,[26] independent from the caspase pathway.

Compound **1c** and cisplatin were tested for their cytotoxicity against a normal lung fibroblast cell line, WI-38, and the selectivity index (SI) value,[28] which was obtained by dividing the IC_{50} value for the normal cell line (WI-38) by the IC_{50} value for the cancer cell line (A549), was determined. As shown in Table 3, compound **1c** exhibited an SI value of 13.2, greater than that of cisplatin (SI 4.5).

Plant secondary metabolites and their semisynthetic derivatives continue to play an important role in anticancer drug therapy.[29] From the results of the cytotoxicity test of the lanostane-type triterpene acids from *P. cocos*, and their methyl ester and hydroxy derivatives against human cancer cell lines, it appears that some of these may be valuable anticancer agents. Furthermore, poricotriol A (**1c**), which induced apoptotic cell death in leukemia and lung cancer cells and displayed high selective toxicity against lung cancer cells, may be a promising lead compound for developing an effective drug for leukemia and lung cancer. Induction of apoptosis and the mechanisms of apoptotic cell death by poricotriol A in other cancer cell lines are under investigation.

EXPERIMENTAL SECTION

General Experimental Procedures. Crystallizations were performed in MeOH, and melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 polarimeter in MeOH at 25 °C. IR

Table 3. Cytotoxicity of Poricotriol A (1c) and Cisplatin against Lung Cancer (A549) and Lung Nomal Cell Lines (WI-38)[a]

	IC_{50} (μM)[b]		
compound	A549	WI-38	SI[c]
1c	4.2	55.7	13.2
cisplatin[d]	24.9	113.7	4.5

[a] Cells were treated with compounds (1×10^{-4} to 1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. [b] IC_{50} based on triplicate assay results. [c] SI refers to the selectivity index, which was obtained by dividing the IC_{50} value for the normal cells by the IC_{50} value for the cancer cells. [d] Reference compound.

spectra, using a JASCO FTIR-300E spectrometer, were recorded in KBr disks. NMR spectra were recorded with a JEOL LA-500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) or with a JEOL ECX-400 (^{1}H, 400 MHz) spectrometer in C_5D_5N with tetramethylsilane as an internal standard. HRESIMS was recorded on an Agilent 1100 LC/MSD TOF (time-of-flight) system [ionization mode: positive; nebulizing gas (N_2) pressure: 35 psig; drying gas (N_2): flow, 12 L/min; temp: 325 °C; capillary voltage: 3000 V; fragmentor voltage: 225 V]. Silica gel (silica gel 60, 220−400 mesh, Merck) was used for open column chromatography.

Materials, Chemicals, and Reagents. Six lanostane-type triterpene acids, poricoic acid A (**1a**),[6,10] poricoic acid C (**2a**),[9] poricoic acid G (**3a**),[6,10] poricoic acid H (**4a**),[6,10] 25-hydroxy-3-epidehydrotumulosic acid (**5a**),[6] and dehydroeburiconic acid (**6a**),[9,15] were isolated from the sclerotia of *Poria cocos* (the fungal material was purchased from Kinokuniya Kan-Yaku Kyoku Co., Tokyo, Japan).[6] Chemicals and reagents were purchased as follows: fetal bovine serum (FBS) from Roswell Park Memorial Institute (RPMI); 1640 medium, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and non essential amino acid (NEAA) from Invitrogen Co. (Auckland, New Zealand); Dulbecco's modified Eagle's medium (D-MEM), Eagle's minimal essential medium (MEM), and thiazoyl blue tetrazolium bromide (MTT) from Sigma-Aldrich Japan Co. (Tokyo, Japan); rh annexin V/FITC kit (Bender MedSystems) from Cosmo Bio Co. Ltd. (Tokyo, Japan); and digitonin and TMS-diazomethane from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Preparation of Methyl Ester Derivatives. To a solution of poricoic acid A (**1a**) (51 mg) in EtOAc−MeOH (1:2; 3.0 mL) was added TMS-diazomethane (10% *n*-hexane solution; 0.5 mL). The reaction mixture was stirred for 1 h at room temperature and evaporated under reduced pressure to give a crude methyl ester (56 mg). Chromatography over silica gel [eluting solvent: *n*-hexane−EtOAc (6:4)] of the crude methyl ester gave purified dimethyl poricoate A (**1b**) (18.5 mg). Five other methyl ester derivatives, dimethyl poricoate C (**2b**), dimethyl poricoate G (**3b**), dimethyl poricoate H (**4b**), methyl 25-hydroxy-3-epidehydrotumulosate (**5b**), and methyl dehydroeburiconate (**6b**), were prepared from **2a**, **3a**, **4a**, **5a**, and **6a**, respectively, in the same way as described above for the preparation of **1b**.

Preparation of Hydroxy Derivatives. To a solution of dimethyl poricoate A (**1b**; 40 mg) in dried THF (1 mL) was added $LiAlH_4$ (30 mg), and the mixture was refluxed for 6 h under N_2. HCl (1 M, 2−3 mL) was added to the reaction mixture and stirred for a few minutes, then extracted twice with Et_2O. The Et_2O extract was washed with aqueous $NaHCO_3$ and water, dried over Na_2SO_4, and concentrated under reduced pressure. The crude reaction mixture (32 mg) was subjected to chromatography on silica gel [eluting solvent: *n*-hexane−EtOAc (6:4)] to afford purified poricotriol A (**1c**; 8.1 mg). Five other hydroxy derivatives, poricotriol C (**2c**), poricotriol G (**3c**), poricotriol H (**4c**), 25-hydroxy-3-epidehydrotumulosol (**5c**), and dehydroburicol

(**6c**), were prepared from **2b**, **3b**, **4b**, **5b**, and **6b**, respectively, in the same way as described above for the preparation of **1c**.

Characterization of the methyl ester (**1b**−**6b**) and hydroxy (**1c**−**6c**) derivatives was performed by spectroscopic methods, and their physical characteristics and spectral data are shown below. Assignments of the ^{1}H NMR signals for compounds **2b**−**6b** and **2c**−**6c** were performed by comparison of their ^{1}H NMR data with those of compounds **1b** and **1c** (Table 1) and relevant compounds in the literature.[3,6−10,15]

Dimethyl Poricoate A [dimethyl (20*R*)-16α-hydroxy-3,4-*seco*-24- methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioate] (1b): amorphous solid; $[\alpha]^{25}_D$ +20.6 (*c* 0.41, MeOH); IR (KBr) ν_{max} 3436 (OH), 1645 (>C═O), 899 (>C═CH$_2$) cm^{-1}; ^{13}C and ^{1}H NMR, see Table 1; HRESIMS *m*/*z* 549.3629 (calcd for $C_{33}H_{50}O_5Na$ [M + Na]$^+$, 549.3555).

Dimethyl Poricoate C [dimethyl (20*R*)-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioate] (2b): amorphous solid; $[\alpha]^{25}_D$ +42.7 (*c* 0.90, MeOH); IR (KBr) ν_{max} 1738, 1732, 1645 (>C═O), 892 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.37 (1H, br s, H-11), 5.25 (1H, br s, H-7), 4.88 and 4.85 (each 1H and br s, H-24^1), 4.80 and 4.76 (each 1H and br s, H-28), 3.80 (3H, s, OMe-21), 3.66 (3H, s, OMe-3), 1.71 (3H, s, H-29), 1.02 and 1.01 (each 3H and d, *J* = 6.8 Hz; H-26, H-27), 1.00 (3H, s, H-30), 0.91 (3H, s, H-19), 0.83 (3H, s, H-18); HRESIMS *m*/*z* 533.3586 (calcd for $C_{33}H_{50}O_4Na$ [M + Na]$^+$, 533.3606).

Dimethyl Poricoate G [dimethyl (20*R*)-16α-hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioate] (3b): amorphous solid; $[\alpha]^{25}_D$ +8.4 (*c* 0.38, MeOH); IR (KBr) ν_{max} 3436 (OH), 1732, 1668 (>C═O) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.24 (1H, br t, *J* = 7.6 Hz, H-24), 4.91 and 4.84 (each 1H and br s, H-28), 4.43 (1H, m, H-16), 3.82 (1H, s, OMe-21), 3.67 (1H, s, OMe-3), 1.77 (3H, s, H-29), 1.63 (3H, s, H-26), 1.58 (3H, s, H-27), 1.42 (3H, s, H-30), 0.98 (3H, s, H-18), 0.94 (3H, s, H-19); HRESIMS *m*/*z* 537.3538 (calcd for $C_{32}H_{55}O_5Na$ [M + Na]$^+$, 537.3555).

Dimethyl Poricoate H [dimethyl (20*R*)-16α-hydroxy-3,4-*seco*-24- methyllanosta-4(28),8,24(24^1)-triene-3,21-dioate] (4b): amorphous solid; $[\alpha]^{25}_D$ +12.6 (*c* 0.26, MeOH); IR (KBr) ν_{max} 3436 (OH), 1730, 1668 (>C═O), 896 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 4.91 and 4.84 (each 1H and s, H-24^1), 4.98 and 4.84 (each 1H and s, H-28), 4.43 (1H, m, H-16), 3.81 (3H, s, OMe-21), 3.67 (3H, s, OMe-3), 1.78 (3H, s, H-29), 1.42 (3H, s, H-30), 1.00 (3H, s, H-18), 1.00 and 0.99 (each 3H and d, *J* = 6.6 Hz; H-26, H-27), 0.94 (3H, s, H-19); HRESIMS *m*/*z* 551.3645 (calcd for $C_{33}H_{52}O_5Na$ [M + Na]$^+$, 551.3712).

Methyl 25-Hydroxy-3-epidehydrotumulosate [methyl (20*R*)-3α,16α-dihydroxy-24-methyllanosta-7,9(11),24(24^1)-triene-21-oate] (5b): amorphous solid; IR (KBr) ν_{max} 3368 (OH), 1679 (>C═O) cm^{-1}; $[\alpha]^{25}_D$ +16.8 (*c* 0.19, MeOH)]; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.61 (1H, br t, *J* = 3.8 Hz, H-7), 5.49 (1H, br d, *J* = 5.6 Hz, H-11), 5.44 and 5.08 (each 1H and s, H-24^1), 4.44 (1H, m, H-16), 3.74 (3H, s, OMe-21), 3.66 (1H, m, H-3), 1.55 and 1.54 (each 3H and s, H-26, H-27), 1.37 (3H, s, H-30), 1.21 (3H, s, H-28), 1.12 (3H, s, H-19), 1.00 (3H, s, H-18), 0.91 (3H, s, H-29); HRESIMS *m*/*z* 537.3570 (calcd for $C_{32}H_{50}O_5Na$ [M + Na]$^+$, 537.3555).

Methyl Dehydroeburiconate [methyl (20*R*)-3-oxo-24-methyllanosta-8,24(24^1)- diene-21-oate] (6b): fine needles; mp 102−104 °C; IR (KBr) ν_{max} 1723, 1640 (>C═O), 901 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.56 (1H, br d, *J* = 6.3 Hz, H-7), 5.37 (1H, br d, *J* = 6.1 Hz, H-11), 4.89 and 4.87 (each 1H and br s, H-24^1), 3.79 (3H, s, OMe-21), 1.16 (3H, s, H-28), 1.15 (3H, s, H-19), 1.08 (3H, s, H-29), 1.04 and 1.03 (each 3H and d, *J* = 6.8 Hz; H-26, H-27), 0.97 (3H, s, H-30), 0.83 (3H, s, H-18); HRESIMS *m*/*z* 503.3456 (calcd for $C_{32}H_{48}O_3Na$ [M + Na]$^+$, 503.3501).

Poricotriol A [(20*R*)-3,4-*seco*-24-methyllanosta-4(28),7,9-(11),24(24^1)-tetraene- 3,16α,21-triol] (1c): fine needles; mp 197−199 °C; $[\alpha]^{25}_D$ +20.8 (*c* 0.24, MeOH); IR (KBr) ν_{max} 3339 (OH), 886 (>C═CH$_2$) cm^{-1}; ^{13}C and ^{1}H NMR, see Table 1; HRESIMS *m*/*z* 493.3674 (calcd for $C_{31}H_{50}O_3Na$ [M + Na]$^+$, 493.3657).

Poricotriol C [(20*R*)-3,4-*seco*-24-Methyllanosta-4(28),7,9(11),-24(24^1)-tetraene- 3,21-diol] (2c): amorphous solid; $[\alpha]^{25}_D$ +27.0 (*c* 0.38, MeOH); IR (KBr) ν_{max} 3368 (OH), 886 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.40 (1H, m, H-11), 5.29 (1H, m, H-7), 4.92 and 4.88 (each 1H and br s, H-24^1), 4.86 and 4.79 (each 1H and br s, H-28), 4.08 (1H, br d, *J* = 10.5 Hz) and 3.93 (1H, m) (H-20), 3.87 (2H, m, H-20), 1.78 (3H, s, H-29), 1.10 (3H, s, H-30), 1.08 and 1.07 (each 3H and d, *J* = 6.8 Hz; H-26, H-27), 1.00 (3H, s, H-19), 0.84 (3H, s, H-18); HRESIMS *m*/*z* 477.3618 (calcd for $C_{31}H_{50}O_2Na$ [M + Na]$^+$, 477.3708).

Poricotriol G [(20*R*)-3,4-*seco*-lanosta-4(28),8,24-triene-3,16α,21-triol] (3c): fine needles; mp 125−127 °C; $[\alpha]^{25}_D$ +49.8 (*c* 0.19, MeOH); IR (KBr) ν_{max} 3368 (OH), 891 (>C═CH−) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.32 (1H, t, *J* = 7.6 Hz, H-24), 4.99 and 4.85 (each 1H and s, H-28), 4.56 (1H, br t, *J* = 7.3 Hz, H-16), 4.14 and 3.87 (eacch 1H and m, H-20), 1.81 (3H, s, H-29), 1.68 (3H, s, H-26), 1.63 (3H, s, H-27), 1.41 (3H, s, H-30), 1.01 (3H, s, H-18), 0.90 (3H, s, H-19); HRESIMS *m*/*z* 481.3586 (calcd for $C_{30}H_{55}O_3Na$ [M + Na]$^+$, 481.3657).

Poricotriol H [(20*R*)-3,4-*seco*-24-methyllanosta-4(28),8,24-(24^1)-triene-3,16α,21-triol] (4c): fine needles; mp 167−169 °C; $[\alpha]^{25}_D$ +54.0 (*c* 0.20, MeOH); IR (KBr) ν_{max} 3394 (OH), 889 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 4.99 and 4.87 (each 1H and br s, H-28), 4.95 and 4.87 (each 1H and s, H-24^1), 4.60 (1H, br t, *J* = 8.3 Hz, H-16), 4.19 and 3.92 (each 1H and m, H-20), 3.92 (2H, m, H-3), 1.82 (1H, s, H-29), 1,44 (1H, s, H-30), 1.06 and 1.05 (each 3H and d, *J* = 6.8 Hz; H-26, H-27), 1.02 (3H, s, H-18), 0.92 (3H, s, H-19); HRESIMS *m*/*z* 495.3857 (calcd for $C_{31}H_{52}O_3Na$ [M + Na]$^+$, 495.3814).

25-Hydroxy-3-epidehydrotumulosol [(20*R*)-3,4-*seco*-24-methyllanosta-4(28),8, 24(24^1)-triene-3,16α,21-triol] (5c): amorphous solid; $[\alpha]^{25}_D$ +19.6 (*c* 0.09, MeOH); IR (KBr) ν_{max} 3435 (OH), 901 (>C═CH$_2$) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.61 (1H, br t, *J* = 3.8 Hz, H-7), 5.54 (1H, br d, *J* = 5.8 Hz, H-11), 5.45 and 5.13 (each 1H and br s, H-24^1), 4.58 (1H, br t, *J* = 9.3 Hz, H-16), 4.21 and 3.95 (each 1H and m, H-21), 3.67 (1H, m, H-3), 1.60 and 1.59 (each 3H and s, H-26, H-27), 1.40 (3H, s, H-30), 1.21 (3H, s, H-28), 1.15 (3H, s, H-19), 1.00 (3H, s, H-18), 0.84 (3H, s, H-29); HRESIMS *m*/*z* 509.3653 (calcd for $C_{31}H_{50}O_4Na$ [M + Na]$^+$, 509.3606).

Dehydroeburicol [(20*R*)-24-methyllanosta-7,9(11),24-(24^1)-triene-3*β*,21-diol] (6c): fine needles; mp 164−166 °C; IR (KBr) ν_{max} 3421 (OH), 888 (>C═CH−) cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 5.61 (1H, br s, H-7), 5.,46 (1H, br d, *J* = 5.6 Hz, H-11), 4.93 and 4.89 (each 1H and br s, H-24^1), 4,87 (1H, br d, *J* = 10.7 Hz) and 3.91 (1H, m) (H-20), 3.48 (1H, t, *J* = 7.5 Hz, H-3), 1.24 (3H, s, H-28), 1.15 (3H, s, H-29), 1.12 and 1.09 (each 3H and s, H-19, H-30), 1.04 and 1.03 (each 3H and d, *J* = 6.8 Hz; H-26, H-27), 0.82 (3H, s, H-18); HRESIMS *m*/*z* 475.3628 (calcd for $C_{31}H_{48}O_2Na$ [M + Na]$^+$, 475.3552).

Cell Cultures. Cell lines HL60 (leukemia), A549 (lung), CRL1579 (melanoma), NIH:OVCAR-3 (ovary), SK-BR-3 (breast), DU145 (prostate), AZ521 (stomach), and PANC-1 (pancreas) and a normal cell line, WI-38 (lung), were obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). Cell lines HL60, CRL1579, NIH:OVCAR-3, SK-BR-3, DU145, and PANC-1 were grown in RPMI 1640 medium, while A549, AZ521, and WI-38 cell lines were grown in D-MEM, 90% D-MEM + 90% MEM, and MEM, respectively. The medium was supplemented with 10% FBS and antibiotics. The cells were incubated at 37 °C in a 5% CO_2 humidified incubator.

Cytotoxicity Assay. Cytotoxicity assay was performed according to the method previously reported.[30,31] Briefly, the cell lines cited above [each 3 × 10^3 cells/well except for NIH:OVCAR-3 (1 × 10^4 cells/well)] were treated with test compounds for 48 h, and then MTT

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solution was added to the well. After incubation for 3 h, the generated blue formazan was solubilized with 0.04 M HCl in 2-propanol. The absorbances at 570 nm (top) and 630 nm (bottom) were measured with a microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan).

Annexin V−Propidium Iodide Double Staining. Apoptosis was detected using an rh annexin V/FITC kit. HL60 (1×10^5 cells) and A549 (6×10^4 cells) were exposed to test compound (final concentration: 30 μM). To prepare the cell sample for flow cytometry, cells were washed with annexin-binding buffer and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 10 min. The cell samples were analyzed by flow cytometer (Cell Lab Quanta SC; Beckman Coulter K.K., Tokyo, Japan) using the FL1 and FL2 ranges for annexin V-FITC and PI, respectively.

Cell Cycle Analysis. Aliquots of HL60 (1×10^5 cells) and A549 (1.5×10^5 cells) were incubated with medium containing test compounds. The cells were then washed with PBS with centrifugation. DNA was stained with Nim-DAPI (NPE Systems, FL) staining solution for 10 min at room temperature. Fluorescence intensity was measured using a flow cytometer (Cell Lab Quanta SC). Cell cycle distribution was analyzed using DNA Cell Analysis software (Multi Cycle AV; Phoenix Flow System, San Diego, CA).

Whole Cell Extraction and Subcellular Fractionation for Western Blot Analysis. Whole cell extraction and subcellular fractionation were performed according to the method previously reported.[32] For isolation of whole cell lysates, HL60 (1×10^6 cells) and A549 (1.5×10^5 cells) were incubated for 24 h. Then, test compound (final concentration: 30 μM) was added and incubated. Cells were collected and lysed with extraction buffer. For isolation of cytosolic and mitochondria-rich fractions, A549 cells (4.5×10^5) were collected and suspended in lysis buffer containing 0.05% digitonin. After incubation for 20 min on ice, the cell suspentions were centrifuged. The supernatants were saved as a cytosolic fraction, and the pellets were further resuspsnded in lysis buffer containing 1% NP-40. After 20 min on ice, the supernatants were obtained as a mitochondria-rich fraction by centrifugation.

Western Blotting. Western blot analysis was performed according to the method previously reported.[30,31] Briefly, lysates of total protein were separated by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated with anticaspase-3, anticaspase-8, and anti-AIF (Sigma-Aldrich Japan Co., Tokyo, Japan) and anticleaved caspase-3, anticaspase-9, anti-Bax, anti-Bcl-2, anti-β-actin, and anti-VDAC (Cell Signaling Technology, Beverly, MA) primary antibodies at 4 °C overnight. The blots were then detected with enhanced chemiluminescence (ECL) plus a western blotting detection system (GE Healthcare, Buckinghamshire, U.K.).

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REFERENCES

(1) Shan, Z.; Yuan, Y. X., Eds. *Zhong Shan Medical College, Clinical Application of Chinese Medicine*; Guang Dong People's Publisher: Guang Dong, 1975; p 136.

(2) Namba, T. *The Encyclopedia of Wakan-Yaku (Traditional Sino-Japanese Medicines) with Color Pictures*, revised ed.; Hoikusya: Osaka, 1994; Vol. II, pp 241−243.

(3) Tai, T.; Akahori, A.; Shingu, T. *Phytochemistry* **1993**, *32*, 1239–1244.

(4) Tai, T.; Shingu, T.; Kikuchi, T.; Tezuka, Y.; Akahori, A. *Phytochemistry* **1995**, *40*, 225–231.

(5) Yasukawa, K.; Kaminaga, T.; Kitanaka, S.; Tai, T.; Nunoura, Y.; Natori, S.; Takido, M. *Phytochemistry* **1998**, *48*, 1357–1360.

(6) Ukiya, M.; Akihisa, T.; Tokuda, H.; Hirano, M.; Oshikubo, M.; Nobukuni, Y.; Kimura, Y.; Tai, T.; Kondo, S.; Nishino, H. *J. Nat. Prod.* **2002**, *65*, 462–465.

(7) Tai, T.; Akahori, A.; Shingu, T. *Phytochemistry* **1991**, *30*, 2796–2797.

(8) Tai, T.; Shingu, T.; Kikuchi, T.; Tezuka, Y.; Akahori, A. *Phytochemistry* **1995**, *39*, 1165–1169.

(9) Akihisa, T.; Nakamura, Y.; Tokuda, H.; Uchiyama, E.; Suzuki, T.; Kimura, Y.; Uchikura, K.; Nishino, H. *J. Nat. Prod.* **2007**, *70*, 948–953.

(10) Akihisa, T.; Uchiyama, E.; Kikuchi, T.; Tokuda, H.; Suzuki, T.; Kimura, Y. *J. Nat. Prod.* **2009**, *72*, 1786–1792.

(11) Kaminaga, T.; Yasukawa, K.; Takido, M.; Tai, T.; Nunoura, Y. *Phytother. Res.* **1996**, *10*, 581–584.

(12) Kaminaga, T.; Yasukawa, K.; Kanno, H.; Tai, T.; Nunoura, Y.; Takido, M. *Oncology* **1996**, *53*, 382–385.

(13) Gapter, L.; Wang, Z.; Glinski, J.; Ng., K.-y. *Biochem. Biophys. Res. Commun.* **2005**, *332*, 1153–1161.

(14) Kang, H.-M.; Lee, S.-K.; Shin, D.-S.; Lee, M.-Y.; Han, D. C.; Baek, N.-I.; Son, K.-H.; Kwon, B.-M. *Life Sci.* **2006**, *78*, 607–613.

(15) Akihisa, T.; Mizushina, Y.; Ukiya, M.; Oshikubo, M.; Kondo, S.; Kimura, Y.; Suzuki, T.; Tai, T. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 448–450.

(16) Mizushina, Y.; Akihisa, T.; Ukiya, M.; Murakami, C.; Kuriyama, I.; Xu, X.; Yoshida, H.; Sakaguchi, K. *Cancer Sci.* **2004**, *95*, 354–360.

(17) Drawings correspond to energy-minimized conformation of the (20*R*)-epimers of **1b** and **1c**. Calculation was performed using CAChe CONFLEX with the MM2 force field (*CAChe* version 5.5; Fujitsu Co., Tokyo, Japan).

(18) Darzynkiewicz, Z.; Bruno, S.; Del Bino, G.; Gorczyca, W.; Hotz, M. A.; Lassota, P.; Traganose, F. *Cytometry* **1992**, *13*, 795–808.

(19) Martin, S. J.; Reutelingsperger, C. P.; McGahon, A. J.; Rader, J. A.; van Schie, R. C. A. A.; LaFace, D. M.; Green, D. R. *J. Exp. Med.* **1995**, *182*, 1545–15556.

(20) Salvesen, G. S.; Dixit, V. M. *Cell* **1997**, *91*, 443–446.

(21) Thornberry, N. A.; Lazebnik, Y. *Science* **1998**, *281*, 1312–1316.

(22) Kluck, R. M.; Bossy-Wetzel, E.; Green, D. R.; Newmeyer, D. D. *Science* **1997**, *275*, 1132–1137.

(23) Kluck, R. M.; Esposti, M. D.; Perkins, G.; Renken, C.; Kuwana, T.; Bossy-Wetzel, E.; Goldberg, M.; Allen, T.; Barber, M. J.; Green, D. R.; Newmeyer, D. D. *J. Cell Biol.* **1999**, *147*, 809–822.

(24) Bcl-2 band was quantified with CS Analyzer Ver. 3.00 (ATTO&Rise Corporation, Tokyo, Japan). The level of bcl-2 decreased from 1.00 to 0.81 ± 0.11 after 24 h and 0.69 ± 0.07 after 48 h (means ± standard error) (n = 3).

(25) Oltvai, Z. N.; Milliman, C. L.; Korameyer, S. J. *Cell* **1993**, *74*, 609–619.

(26) Cregan, S. P.; Dawson, V. L.; Slack, R. S. *Oncogene* **2004**, *23*, 2785–2796.

(27) Norberg, E.; Orrenius, S.; Zhivotovsky, B. *Biochem. Biophys. Res. Commun.* **2010**, *396*, 95–100.

(28) Pati, H. N.; Das, U.; Kawase, M.; Sakagami, H.; Balzarini, J.; De Clercq, E.; Dimmock, J. R. *Bioorg. Med. Chem.* **2008**, *16*, 5747–5753.

(29) Pan, L.; Chai, H.; Kinghorn, A. D. *Phytochem. Lett.* **2009**, *2*, 15–18.

(30) Tabata, K.; Motani, K.; Takayanagi, N.; Nishimura, R.; Asami, S.; Kimura, Y.; Ukiya, M.; Hasegawa, D.; Akihisa, T.; Suzuki, T. *Biol. Pharm. Bull.* **2009**, *28*, 1404–1407.

(31) Kikuchi, T.; Nihei, M.; Nagai, H.; Fukushi, H.; Tabata, K.; Suzuki, T.; Akihisa, T. *Chem. Pharm. Bull.* **2010**, *58*, 568–571.

(32) Motani, K.; Tabata, K.; Kimura, Y.; Okano, S.; Shibata, Y.; Nagai, H.; Akihisa, T.; Suzuki, T. *Biol. Pharm. Bull.* **2008**, *31*, 618–626.