Anti-Tumor-Promoting Effects of 25-Methoxyporicoic Acid A and Other Triterpene Acids from *Poria cocos*

Toshihiro Akihisa,*[,†] Emiko Uchiyama,[†] Takashi Kikuchi,[†] Harukuni Tokuda,[‡] Takashi Suzuki,[§] and Yumiko Kimura[§]

College of Science and Technology, Nihon University, 1-8 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8308, Japan, Department of Complementary and Alternative Medicine, R&D, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan, and College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi-shi, Chiba 274-8555, Japan

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Nine new (**1**, **3**, **5**, **8**, **12**, **13**, **15**, **17**, and **18**) and nine known (**2**, **4**, **6**, **7**, **9**−**11**, **14**, and **16**) lanostane-type triterpene acids and a known diterpene acid (**19**) were isolated from the epidermis of the sclerotia of *Poria cocos*. The structures of the new compounds were established as 16α,27-dihydroxydehyrotrametenoic acid (**1**), 25-hydroxy-3-epitumulosic acid (**3**), 16α,25-dihydroxyeburiconic acid (**5**), 25-methoxyporicoic acid A (**8**), 26-hydroxyporicoic acid DM (**12**), 25-hydroxyporicoic acid C (**13**), poricoic acid GM (**15**), poricoic acid HM (**17**), and 6,7-dehydroporicoic acid H (**18**), on the basis of spectroscopic methods. On evaluation of the nine new and two of the known compounds, **4** and **19**, against the Epstein−Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, all of the compounds exhibited inhibitory effects, with IC_{50} values in the range 187−348 mol ratio/32 pmol TPA. In addition, compound **8** exhibited an inhibitory effect on skin tumor promotion in an in vivo two-stage carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. Further, 17 compounds, **1**−**14**, **16**, **18**, and **19**, were evaluated for their cytotoxic activity against two human tumor cell lines, HL60 (leukemia) and CRL1579 (melanoma).

The dried sclerotia of *Poria cocos* Wolf (Polyporaceae) are used traditionally in Chinese herbal prescriptions as a diuretic and sedative.[1,2] Whereas the inner parts of the sclerotia of *P. cocos*, called "Fu-Ling" in Chinese, are reported to have an invigorating activity in addition to diuretic and sedative activities, the epidermis ("Fu-Ling-Pi" in Chinese) of the sclerotia is reported to have only diuretic activity and no invigorating activity.[1] In a previous paper,[3] we reported the isolation and characterization of six new and 11 known lanostane-type triterpene acids from a 5% aqueous NaOH-soluble fraction extracted from a $CHCl_3$-soluble fraction of a MeOH extract of the epidermis of *P. cocos* sclerotia, with their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also evaluated. In addition, we reported the inhibitory effects of two lanostane-type triterpene acids on in vivo two-stage mouse skin carcinogenesis model.[3] In a continuation of our study on the potential anti-tumor-promoting effects of the lanostane-type triterpene acids from *P. cocos*, we report, in this paper, the isolation and characterization of nine new (**1**, **3**, **5**, **8**, **12**, **13**, **15**, **17**, and **18**) and nine known (**2**, **4**, **6**, **7**, **9**−**11**, **14**, and **16**) lanostane-type triterpene acids along with one known diterpene acid (**19**) from a saturated aqueous $NaHCO_3$-soluble fraction extracted from the $CHCl_3$-soluble fraction of a MeOH extract of the epidermis of *P. cocos* sclerotia. In addition, we report the inhibitory effects of the nine new and two known (**4** and **19**) compounds on EBV-EA activation and of compound **8** on in vivo two-stage mouse skin carcinogenesis. Evaluation of cytotoxic activities against two human tumor cell lines (HL60 and CRL1579) for 17 compounds from *P. cocos* sclerotia is also reported.

Results and Discussion

Eighteen lanostane-type triterpene acids, 16α,27-dihydroxydehydrotrametenoic acid (**1**), 25-hydroxy-3-epidehydrotumulosic acid (**2**),[4] 25-hydroxy-3-epitumulosic acid (**3**), 16α-hydroxyeburiconic acid (**4**),[5] 16α,25-dihydroxyeburiconic acid (**5**), 5α,8α-peroxydehydrotumulosic acid (**6**),[3] poricoic acid A (**7**),[6] 25-methoxyporicoic acid A (**8**), poricoic acid AM (**9**),[6] poricoic acid B (**10**),[7] poricoic acid DM (**11**),[6] 26-hydroxyporicoic acid DM (**12**), 25-hydroxyporicoic acid C (**13**), poricoic acid G (**14**),[8] poricoic cid GM (**15**), poricoic acid H (**16**),[8] poricoic acid HM (**17**), and 6,7-dehydroporicoic acid H (**18**), along with one abietane-type diterpene acid, 7-oxo-15-hydroxydehydroabietic acid (**19**),[9] were isolated from an acidified saturated aqueous $NaHCO_3$-soluble fraction extracted from a $CHCl_3$-soluble fraction of the MeOH extract of the epidermis of *P. cocos* sclerotia. Among these, nine compounds, **1**, **3**, **5**, **8**, **12**, **13**, **15**, **17**, and **18**, are new. The ^{13}C and ^1H NMR data for the new compounds are shown in Tables 1 and 2, respectively. Identification of all other compounds was performed by ^1H NMR and MS comparison with the corresponding values in the literature.

Compound **1** showed a $[M + Na]^+$ peak at *m*/*z* 509.3216 ($C_{30}H_{46}O_5Na$) in the HRESIMS. The ^{13}C (Table 1) and ^1H NMR data (Table 2) and the IR and UV spectra of **1** showed the presence of two secondary hydroxy groups, a hydroxymethylene, a carboxyl, a conjugated diene,[10] a trisubstituted double bond, five tertiary methyls, and a vinylic methyl group. Comparison of the ^{13}C and ^1H NMR data of **1** with those of 3-epidehydrotumulosic acid [(20ζ)-3α,16α-dihydroxy-24-methyllanosta-7,9(11),24(24^1)-trien-21-oic acid][4] and poricoic acid E [(20ζ)-16α,27-dihydroxy-3,4-*seco*-lanosta-4(28),7,9(11),24-tetraene-3,21-dioic acid][4] suggested that **1** has a 3α,16α-dihydroxylanostane-type triterpene skeleton with a $\Delta^{7,9(11)}$-diene system and a carboxyl group at C-21 in a 27-hydroxylated C_8-Δ^{24}-unsaturated side chain.[4] Its structure was formulated as (20ζ)-3α,16α,27-trihydroxylanosta-7,9(11),24-trien-21-oic acid, which has been named 16α,27-dihydroxydehydrotrametenoic acid. An NOE correlation in the NOESY experiment of **1** for H-24 with H-26 supported the presence of a hydroxy group at C-27, and analysis of the ^1H−^1H COSY, HMQC, HMBC, and NOESY (Table S1, Supporting Information) spectra further supported the proposed structure of **1**.

Compound **3** gave a $[M - H]^-$ ion in the HRESIMS at *m*/*z* 501.3580, consistent with a molecular formula of $C_{31}H_{50}O_5$. The ^{13}C NMR, ^1H NMR, and IR spectra of **3** showed the presence of two secondary hydroxy groups and a tertiary hydroxy group, a carboxylic function, a tetrasubstituted double bond, a terminal

* To whom correspondence should be addressed. Tel: +81-3-3259-0806. Fax: +81-3-3293-7572. E-mail: akihisa@chem.cst.nihon-u.ac.jp.
† College of Science and Technology, Nihon University.
‡ Kanazawa University.
§ College of Pharmacy, Nihon University.

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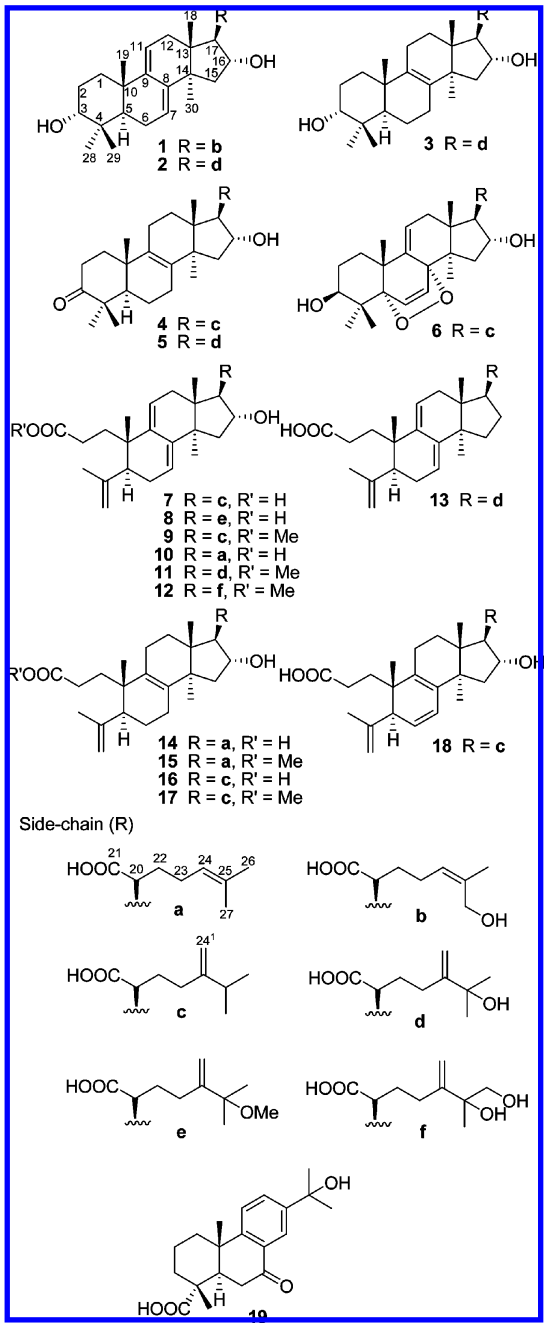

methylene, and seven tertiary methyls, of which two are attached to an oxygen-bearing carbon. The above evidence, coupled with spectroscopic comparisons with daedaleanic acid B (24-oxo-3α,16α-dihydroxylanost-8-en-21-oic acid)[11] and 25-hydroxyporicoic acid H [(20ζ)-16α,25-dihydroxy-24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid],[3] and analysis of the ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY (Table S2, Supporting Information) spectra confirmed **3** as (20ζ)-3α,16α,25-trihydroxy-24-methyllanosta-8,24(24^1)-trien-21-oic acid (25-hydroxy-3-epitumulosic acid).

The molecular formula of compound **5** was determined to be $C_{31}H_{48}O_5$, from its HRESIMS ([M − H]$^-$, *m/z* 499.3385). The ^{13}C and ^{1}H NMR spectra as well as the IR spectra of **5** showed the presence of both a secondary and a tertiary hydroxy group, a carboxyl, a ketone, a tetrasubstituted double bond, a terminal methylene, and seven tertiary methyls, of which two are attached to an oxygen-bearing carbon. Comparison of ^{13}C and ^{1}H NMR data with those of compound **3** and 16α-hydroxyeburiconic acid [(20ζ)-3-oxo-16α-hydroxy-24-methyllanosta-8,24(24^1)-dien-21-oic acid][5] suggested that **5** is the 3-oxo derivative of compound **3** (or the 25-hydroxylated derivative of 16α-hydroxyeburiconic acid), i.e., (20ζ)-3-oxo-16α,25-dihydroxy-24-methyllanosta-8,24(24^1)-dien-21-oic acid (16α,25-dihydroxyeburiconic acid). The proposed structure of **5** was supported by analysis of its ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY (Table S3, Supporting Information) spectra.

The molecular formula of **8** was determined to be $C_{32}H_{48}O_6$ from its HRESIMS ([M + Na]$^+$, *m/z* 551.3322). The ^{13}C and ^{1}H NMR data and IR and UV spectra of **8** indicated the presence of a secondary hydroxy, a tertiary methoxy, two carboxyls, a conjugated diene,[10] a terminal methylene, an isopropenyl, and five tertiary methyl groups, of which two are attached to an oxygen-bearing carbon. These data, in combination with the comparison of ^{13}C and ^{1}H NMR data of poricoic acid D [(20ζ)-16α,25-dihydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid],[6] suggested that **8** is an *O*-methylated analogue of poricoic acid D. The *O*-methyl group of **8** was located at C-25 in the side chain by a diagnostic HMBC cross-correlation for OMe-25 with C-25 (Table S4, Supporting Information). Thus, the structure of **8** was proposed as (20ζ)-25-methoxy-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid (25-*O*-methylporicoic acid D). The ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra of **8** supported this structure.

Compound **12** gave a [M + Na]$^+$ ion in the HRESIMS at *m/z* 567.3272, consistent with the molecular formula $C_{32}H_{48}O_7$. The ^{13}C and ^{1}H NMR data and IR and UV spectra of **12** showed the presence of a secondary and a tertiary hydroxy group, a hydroxymethylene, two carboxyls, a conjugated diene,[10] an isopropenyl, a terminal methylene, an *O*-methyl, and four tertiary methyl groups, of which one is attached to an oxygen-bearing carbon. The ^{13}C and ^{1}H NMR spectra of **12** were very similar to those of poricoic acid DM [methyl (20ζ)-16α,25-dihydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid 3-oate],[6] except for the absence of one of the *O*-dimethyls at C-25 and the appearance of a hydroxymethylene group. The presence of the hydroxymethylene group at C-25 in **12** was supported by diagnostic HMBC cross-correlations for H-26 (with C-25 and C-27) and H-27 (with C-25 and C-26). Thus, the structure of **12** was established as methyl (20ζ)-16α,25,26-trihydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid 3-oate (26-hydroxyporicoic acid DM) and was supported by its ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra (Table S5, Supporting Information).

Compound **13** gave a [M − H]$^-$ ion in the HRESIMS at *m/z* 497.3274, consistent with the molecular formula $C_{31}H_{46}O_5$. The ^{13}C and ^{1}H NMR data and IR and UV spectra of **13** showed the presence of a tertiary hydroxy group, two carboxyls, a conjugated diene,[10] an isopropenyl, a terminal methylene, and five tertiary methyl groups, of which two are attached to an oxygen-bearing carbon. The ^{13}C and ^{1}H NMR spectra for the overall ring system of **13** were in good agreement with those of poricoic acid C [(20ζ)-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid],[6] whereas those from the side chain are very close to those of poricoic acid D,[6] which suggested that **13** possesses the structure (20ζ)-25-hydroxy-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic acid (25-hydroxyporicoic acid C). The proposed structure for **13** was supported by its ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra (Table S6, Supporting Information).

The molecular formula of **15** was determined to be $C_{31}H_{48}O_5$ from its HRESIMS ([M − H]$^-$, *m/z* 499.3380). The ^{13}C and ^{1}H NMR spectra of **15** were very similar to those of poricoic acid G [(20ζ)-16α-hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic acid],[8] except that the former possesses an additional *O*-methyl group, suggesting that **15** is a methyl ester derivative of poricoic acid G. Diagnostic cross-correlations for H-2 (with C-3 and C-10) and OMe-3 (with C-3) observed in the HMBC spectrum (Table S7, Supporting Information) of **15** indicated that the methyl ester group is located at C-3. Thus, the structure of **15** was proposed as methyl (20ζ)-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic acid 3-oate,

Table 1. ^{13}C NMR Spectroscopic Data (δ Values; 150 MHz, C_5D_5N) for Nine Triterpene Acids from the Epidermis of *Poria cocos* Sclerotia

carbon	**1**		**3**		**5**		**8**		**12**		**13**		**15**		**17**		**18**	
1	30.6	t	30.7	t	36.1	t	36.4	t	35.9	t	36.5	t	33.1	t	33.1	t	36.2	t
2	26.8	t	26.8	t	34.7	t	30.2	t	29.5	t	29.7	t	29.9	t	29.9	t	30.5	t
3	75.2	d	75.1	d	216.3	s	176.8	s	174.5	s	176.4	s	174.4	s	174.4	s	174.5	s
4	37.9	s	38.4	s	47.3	s	149.2	s	149.7	s	149.3	s	147.7	s	147.7	s	145.7	s
5	43.7	d	44.6	d	51.3	d	50.7	d	50.7	t	50.8	d	47.0	d	47.0	d	52.4	d
6	23.4	t	18.6	t	19.6	t	28.6	t	28.5	t	28.6	t	24.2	t	24.3	t	126.2	d
7	121.1	d	26.6	t	26.6	t	118.0	d	117.9	d	118.0	d	26.2	t	26.2	t	124.8	d
8	142.8	s	134.5	s	135.5	s	141.9	s	141.8	s	142.0	s	140.0	s	139.6	s	135.5	s
9	146.6	s	135.1	s	133.3	s	137.3	s	137.2	s	137.6	s	129.5	s	129.5	s	132.3	s
10	37.8	s	37.4	s	37.0	s	38.9	s	38.8	s	38.8	s	40.6	s	40.6	s	38.2	s
11	116.8	d	21.0	t	20.9	t	120.3	d	120.5	d	120.5	d	21.6	t	21.6	t	23.4	t
12	36.2	t	29.6	t	29.6	t	37.0	t	38.0	t	36.8	t	29.8	t	29.7	t	29.3	t
13	45.2	s	46.3	s	46.2	s	45.6	s	45.6	s	44.7	s	46.1	s	46.1	s	45.5	s
14	49.4	s	38.8	s	49.7	s	49.3	s	49.2	s	50.5	s	49.7	s	49.7	s	48.0	s
15	44.3	t	43.7	t	43.6	t	43.8	t	43.7	t	31.0	t	43.8	t	43.8	t	43.4	t
16	76.4	d	76.8	d	76.4	d	76.5	d	75.9	d	27.3	t	76.3	d	76.9	d	76.8	d
17	57.6	d	57.2	d	57.3	d	57.6	d	57.5	d	48.3	d	56.5	d	57.3	d	56.8	d
18	17.7	q	17.8	q	17.8	q	18.3	q	18.3	q	17.0	q	18.0	q	18.0	q	17.7	q
19	23.0	q	19.3	q	18.6	q	22.3	q	22.2	q	22.2	q	22.4	q	22.4	q	19.4	q
20	48.8	d	48.3	d	48.8	d	48.8	d	48.4	d	49.8	d	48.5	d	48.3	d	48.8	d
21	178.6	s	178.2	s	178.6	s	178.6	s	178.6	s	178.5	s	178.6	s	178.5	s	178.6	s
22	26.6	t	32.1	t	32.4	t	31.8	t	31.8	t	32.7	t	33.2	t	31.6	t	31.4	t
23	33.2	t	29.8	t	30.1	t	28.8	t	30.3	t	28.6	t	27.1	t	33.2	t	33.2	t
24	126.8	d	157.8	s	158.0	s	153.2	s	155.0	s	157.8	s	125.3	d	156.3	s	156.1	s
25	136.9	s	72.6	s	72.6	s	77.5	s	76.3	s	72.5	s	131.7	s	34.1	d	34.1	d
26	21.9	q	30.0	q	30.0	q	25.8[a]	q	25.1	q	30.0	q	25.8	q	21.9	q	21.9[a]	q
27	60.8	t	30.0	q	30.0	q	25.9[a]	q	69.7	t	30.0	q	17.7	q	22.1	q	22.0[a]	q
28	29.2	q	29.0	q	26.4	q	112.2	t	112.2d	d	112.1	t	114.2	d	114.2	q	114.2	t
29	23.1	q	32.6	q	21.3	q	22.3	q	22.1	q	22.3	q	23.2	q	23.2	q	20.3	q
30	26.7	q	25.4	q	25.4	q	24.9	q	24.8	q	24.3	q	26.3	q	26.3	q	27.5	q
24^1			107.0	t	107.0	t	110.7	t	109.2	t	107.0	t			106.9	t	107.0	t
OCOMe-3									51.3	q			51.4	q	51.4	q		
OMe-25							50.2	q										

[a] Values bearing the same superscript in each column are interchangeable.

which has been named poricoic acid GM. The IR and $^1H-^1H$ COSY, HMQC, HMBC, and NOESY NMR spectra of **15** supported this structure.

Compound **17** gave a $[M - H]^-$ ion in the HRESIMS at *m/z* 513.3553, consistent with the molecular formula $C_{32}H_{50}O_5$. The ^{13}C and ^1H NMR spectra of **17** were in good agreement with those of poricoic acid H [(20ζ)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),8,24(24^1)-triene-3,21-dioic acid],[8] except that the former exhibited additional *O*-methyl signals, suggesting that **17** is a methyl ester derivative of poricoic acid H. The HMBC spectrum (Table S8, Supporting Information) of **17** exhibited diagnostic cross-correlations for H-2 (with C-3 and OMe-3) and OMe-3 (with C-3) and indicated that the methyl ester group is located at C-3. Hence, the structure of **17** was proposed as methyl (20ζ)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),8,24(24^1)-triene-3,21-dioic acid 3-oate (poricoic acid HM).

The molecular formula of **18** was determined to be $C_{31}H_{46}O_5$ from its HRESIMS ($[M - H]^-$, *m/z* 497.3193). The ^{13}C and ^1H NMR data and IR and UV spectra of **18** indicated the presence of a secondary hydroxy, two carboxyls, a di- and a tetrasubstitued double bond, constituting a conjugated diene system,[12] a terminal methylene, an isopropenyl, and two secondary and three tertiary methyl groups. The NMR data of **18** were very similar to those of poricoic acid H,[8] except that the former possesses an additional disubstituted double bond at C-6(7), suggesting the structure of **18** to be (20ζ)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),6,8,24(24^1)-tetraene-3,21-dioic acid (6,7-dehydroporicoic acid H). The presence of a $\Delta^{6,8}$-conjugated diene system in **18** was supported by diagnostic HMBC cross-correlations for H-5 (with C-4, C-6, and C-10), H-6 (with C-8 and C-10), and H-7 (with C-5 and C-9) (Table S9, Supporting Information). The $^1H-^1H$ COSY, HMQC, HMBC, and NOESY spectra of **18** further supported this structure.

The inhibitory effects on the induction of EBV-EA induced by TPA were examined as a preliminary evaluation of anti-tumor-promoting activity. Table 3 shows the inhibitory effects of nine new compounds, **1**, **3**, **5**, **8**, **12**, **13**, **15**, **17**, and **18**, and two known compounds, **4** and **19**, against TPA (32 pmol)-induced EBV-EA activation in Raji cells. All of the compounds caused high viability (60−70%) of Raji cells even at 32 nmol (mol ratio of compound to TPA = 1000:1), indicating low cytotoxicity at this high concentration. Each compound tested showed a potent inhibitory effect, with an IC_{50} value (concentration of 50% inhibition with respect to positive control) in the range 187−348 mol ratio/32 pmol TPA. As such, these compounds were more potent than the reference compound, β-carotene (IC_{50} = 397 mol ratio/32 pmol TPA), a vitamin A precursor studied widely in cancer chemoprevention animal models. Compounds **2**, **7**, **10**, **14**, and **16**[8] and compounds **6**, **9**, and **11**[3] have also been reported recently to possess potent inhibitory effects on EBV-EA activation induced by TPA. Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel those against tumor promotion in vivo,[13] those compounds highly inhibitory against EBV-EA activation could be valuable antitumor promoters.

Subsequently, the inhibitory effect of compound **8** was determined in a two-stage carcinogenesis test on mouse skin using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. The incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figure 1, A and B, respectively. In the positive control group (group I), there was 100% incidence of papillomas at 11 weeks of promotion. Further, 4.2 and 8.6 papillomas were formed per mouse at 11 and 20 weeks of promotion, respectively. The formation of papillomas in mouse skin was delayed and the mean number of papillomas per mouse was reduced by treatment with **8**. Thus, in group II (treated with **8**), the percentage ratios of papilloma-bearing mice were only 20% at 11 weeks and 80% at 20 weeks, and the mean papillomas per mouse were 1.1 at 11 weeks and 3.0 at 20 weeks. Two other lanostanes, poricoic acid C and 16-deoxyporicoic acid

Table 2. ^{1}H NMR Spectroscopic Data (δ Values; 600 MHz, C_5D_5N) for Nine Triterpene Acids from the Epidermis of *Poria cocos* Sclerotiaa[a]

proton(s)	**1**	**3**	**5**	**8**	**12**	**13**	**15**	**17**	**18**
1	α, 2.25 (dt, 3.1, 12.8)	α, 1.41	α, 1.44	1.87	1.70	1.84	1.80	1.79 (d, 14.6)	1.98
	β, 1.74 (br d, 10.0)	*β*, 1.78	*β*, 1.75	2.12	1.96 (ddd, 4.9, 11.4, 13.0)	2.10	1.89	1.91	2.06
2	α, 1.85 (br d, 12.9)	1.95 (2H)	α, 2.39	2.46	2.29	2.55 (2H)	2.15	2.18	2.62 (2H)
	β, 2.06 (br d, 13.3)		*β*, 2.56	2.53	2.35		2.50	2.50	
3	3.63 (br s)	3.59 (br s)							
5	2.00 (dd, 7.7, 8.0)	1.93 (dd,1.2, 11.2)	1.60 (dd, 2.3, 10.0)	2.33 (br d, 6.5)	2.27 (br d, 6.9)	2.33 (br d, 7.2)	2.21 (dd, 2.3, 12.6)	2.21 (dd, 2.0, 12.1)	2.81 (d, 5.9)
6	2.12 (2H)	α, 1.55	α, 1.50	α, 2.57	α, 2.50 (br dd, 7.5, 18.9)	α, 2.57	α, 1.52	α, 1.52	5.51 (dd, 5.4, 9.4)
		β, 1.65	*β*, 1.59	*β*, 2.07 (dd, 3.1, 18.6)	*β*, 2.05 (dd, 3.7, 18.9)	*β*, 2.06	*β*, 1.65	*β*, 1.68	
7	5.61 (t, 4.0)	α, 2.10	2.07 (2H)	5.30 (br s)	5.29 (t, 3.7)	5.29 (br s)	α, 2.08	α, 2.08	5.94 (d, 9.4)
		β, 2.15					*β*, 1.97	*β*, 1.96	
11	5.44	α, 2.09	1.94 (2H)	5.34 (br s)	5.29 (br s)	5.32 (br s)	1.87 (2H)	2.06 (2H)	α, 2.11
		β, 1.96							*β*, 2.02
12	α, 2.64 (br d, 17.2)	α, 2.27	α, 2.18	α, 2.44	α, 2.44 (dd, 5.2, 17.9)	2.42	α, 2.32	α, 2.28	α, 2.14
	β, 2.38	*β*, 2.03	*β*, 1.93	*β*, 2.64	*β*, 2.66	2.47	*β*, 2.04	*β*, 2.08	*β*, 1.97
15	α, 1.89 (br d, 12.9)	α, 1.63 (br d, 12.9)	α, 1.70 (d, 13.0)	α, 1.77 (d, 13.0)	α, 1.77 (br d, 12.8)	α, 1.38 (br dd, 9.0, 11.5)	α, 1.67 (br d, 12.9)	α, 1.64 (d, 12.6)	α, 1.82 (d, 12.6)
	β, 2.42 (dd, 8.6, 12.7)	*β*, 2.35 (dd, 8.3, 13.1)	*β*, 2.38	*β*, 2.39	*β*, 2.38 (br dd, 8.9, 13.0)	*β*, 1.72	*β*, 2.34	*β*, 2.35 (dd, 8.9, 12.9)	*β*, 2.31
16	4.48 (br t, 6.9)	4.48	4.54 (br t, 7.0)	4.48 (q, 7.9)	4.50 (br t, 7.1)	α, 1.42	4.50 (br s)	4.50 (br s)	4.54 (dd, 6.9, 7.2)
						β, 2.04			
17	2.80 (dd, 6.6, 11.2)	2.75	2.82	2.80 (dd, 6.2, 11.4)	2.85 (dd, 5.9, 11.0)	2.50	2.76	2.75	2.77 (dd, 6.0, 11.2)
18	1.03 (s)	1.10 (s)	1.12 (s)	1.05 (s)	1.04 (s)	1.00 (s)	1.13 (s)	1.12 (s)	1.14 (s)
19	1.10 (s)	1.04 (s)	1.02 (s)	1.03 (s)	0.96 (s)	1.02 (s)	0.92 (s)	0.92 (s)	0.94 (s)
20	2.92	2.93	2.98	2.92	2.97 (br t, 10.6)	2.97 (br s)	2.92	2.92	2.92 (br t, 9.2)
22	2.53 (2H)	2.58	2.57	2.43	2.56 (br t, 9.3)	2.42	1.98	2.38	2.39
		2.79	2.80	2.69	2.80	2.52	2.52	2.64	2.61
23	2.35	2.62	2.65	2.40	2.65	2.52	2.35 (2H)	2.35 (2H)	2.37
	2.51	2.85	2.81	2.63	2.86	2.62			2.52 (br t, 11.2)
24	5.47						5.33		
25								2.28	2.30 (sept, 6.9)
26	1.95 (s)	1.51[b] (s)	1.54[b] (s)	1.28[b] (s)	1.63 (s)	1.55 (s)	1.62 (s)	0.99 (d, 6.3)	0.99[b] (d, 6.9)
27	4.39 (d, 12.3)	1.52[b] (s)	1.55[b] (s)	1.29[b](s)	3.91 (d, 10.6)	1.55 (s)	1.59 (s)	1.00 (d, 6.3)	1.00[b] (d, 6.9)
	4.51 (d, 12.3)				4.01 (d, 10.6)				
28	1.19 (s)	1.19 (s)	1.14 (s)	4.74 (s)	4.77 (s)	4.75 (s)	4.82 (s)	4.81 (s)	4.76 (s)
				4.81 (s)	4.80 (s)	4.81 (s)	4.98 (s)	4.96 (s)	4.88 (s)
29	0.98 (s)	0.91 (s)	1.05 (s)	1.73 (s)	1.71 (s)	1.71 (s)	1.76 (s)	1.76 (s)	1.74 (s)
30	1.40 (s)	1.36 (s)	1.45 (s)	1.45 (s)	1.41 (s)	1.02 (s)	1.46 (s)	1.43 (s)	1.47 (s)
24[1]		5.11 (s)	5.15 (s)	5.08 (s)	5.28 (s)	5.09 (s)		4.83 (s)	4.85 (s)
		5.43 (s)	5.48 (s)	5.19 (s)	5.56 (s)	5.49 (s)		4.95 (s)	4.97 (s)
OCOMe-3					3.61 (s)		3.61 (s)	3.63 (s)	
OMe-25				3.02 (s)					

[a] *J* values (Hz) determined are shown in parentheses. [b] Values bearing the same superscript in each column are interchangeable.

Table 3. Inhibitory Effects of 11 Compounds from *Poria cocos* on Induction of the Epstein-Barr Virus Early Antigen

	percentage of EBV-EA induction[a]					
	concentration (mol ratio/ 32 pmol TPA)					
compound	1000		500	100	10	IC_{50} (mol ratio/ 32 pmol TPA)
1	0	(70)	20.8	69.2	90.1	269
3	0	(70)	20.5	69.4	93.1	238
4	2.2	(70)	28.3	70.3	96.4	348
5	0	(70)	36.1	72.2	96.1	299
8	0	(70)	17.8	58.2	84.4	268
12	0	(70)	14.1	55.3	80.2	187
13	0	(70)	16.2	57.1	83.1	201
15	0	(70)	19.9	60.1	86.4	216
17	0	(70)	20.3	61.3	87.5	219
18	0	(70)	15.2	56.0	80.9	193
19	0	(60)	19.5	69.1	93.4	238
β-carotene[b]	8.6	(70)	34.2	82.1	100	397

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

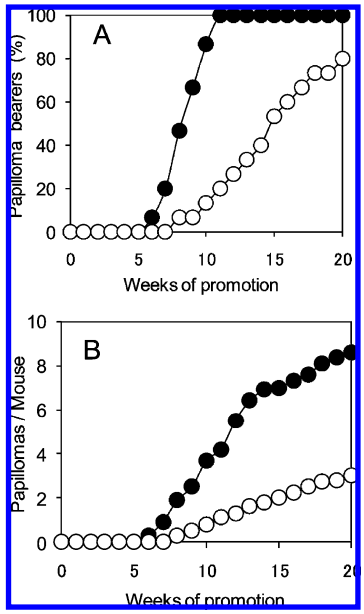

Figure 1. Inhibition of TPA-induced tumor promotion by multiple applications of 25-methoxyporioic acid A (**8**; ○). Mice (n = 15 for each of groups I and II) were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice weekly starting 1 week after initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas per mouse. ●, control TPA alone (group I); ○, TPA + 85 nmol of **8** (group II). After 20 weeks of promotion, a significant difference in the number of papillomas per mouse between the groups treated with compound **8** and the control group was evident ($p < 0.05$, using the Student's t-test). The number (standard deviations are shown in parentheses) of papillomas per mouse for each group was 8.6 (1.2) and 3.0 (0.5) for groups I and II, respectively.

B [(20ζ)-3,4-*seco*-lanosta-4(28),7,9(11),24-tetraene-3,21-dioic acid], from the epidermis of *P. cocos* sclerotia, have also been found in our laboratory to possess inhibitory effects on tumor promotion in a two-stage carcinogenesis test on mouse skin using DMBA as an initiator and TPA as a promoter.[3]

Seventeen compounds, **1**−**14**, **16**, **18**, and **19**, were evaluated for their cytotoxic activities against two human tumor cell lines, HL60 (leukemia) and CRL1579 (melanoma), in a dose-dependent manner as determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay[14] (Table S10, Supporting Information). Cisplatin was used as a positive control. Whereas seven compounds, **2**, **3**, **7**, **9**, **13**, **14**, and **16**, exhibited cytotoxicities against HL60 with IC_{50} values of 15.9−38.0 μM, the other nine compounds showed none or almost no cytotoxicity. Compound **5** (IC_{50} = 28.7 μM) showed cytotoxic potency almost comparable with that of cisplatin (IC_{50} = 21.1 μM) against CRL1579 cells, while the other 16 compounds had almost no activity against this cell line. Several lanostane-type triterpenes isolated from *P. cocos* have recently been reported to show cytotoxicity against human lung cancer (A549) and human prostate cancer (DU145) cell lines.[15]

From the results of the in vitro EBV-EA induction test and in vivo two-stage carcinogenesis in the present and in our recent studies,[3,8] it appears that the lanostane-type triterpene acids isolated from the inner parts[8] and epidermis of *Poria cocos* sclerotia may be valuable as potential chemopreventive agents in chemical carcinogenesis experiments. In contrast, these triterpene acids do not seem to be candidates as potential antitumor agents.

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. UV spectra, using a Shimadzu UV-2200 spectrometer, and IR spectra, using a JASCO FTIR-300E spectrometer, were recorded in MeOH and KBr disks, respectively. NMR spectra were recorded with a JEOL ECA-600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) spectrometer in C_5D_5N with tetramethylsilane as an internal standard. ESIMS and HRESIMS were 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) and C_{18} silica (Chromatorex-ODS, 100−200 mesh; Fuji Silysia Chemical, Ltd., Aichi, Japan) were used for open column chromatography. Reversed-phase preparative HPLC (with refractive index detector) was carried out on C_{18} silica columns (25 cm × 10 cm i.d.) at 25 °C using an eluting solvent system of MeOH−H_2O−AcOH, on a TSK ODS-120A 5 μm column (Toso Co., Tokyo, Japan) at a ratio of 70:30:1 (flow rate at 2.0 mL/min, HPLC system I; 3.0 mL/min, system II), 75:25:1 (3.0 mL/min, system III), or 80:20:1 (2.0 mL/min, system IV; 3.0 mL/min; system V) of the solvent system, and on a Pegasil ODS II 5 μm column (Senshu Scientific Co. Ltd., Tokyo, Japan) at a ratio of 65:35:1 (2.0 mL/min, system VI) or 70:30:1 (2.0 mL/min, system VII) of the solvent system.

Fungal Material. The source of the fungal material was described in a previous article.[16] Thus, the dried epidermis of the sclerotia obtained from cultivated *Poria cocos* in Yunnan was purchased from Yunnan Medicines & Health Products Import & Export Corporation (Kunming, Yunnan, People's Republic of China). The taxonomic identification was done by Mr. Seizo Kondo (Central Research Laboratory, Kotaro Pharmaceutical Co., Ltd., Takatsuki, Japan) based on the published description.[1,17] A voucher specimen (registration no. LBNR-PC-0401) has been deposited in the College of Science and Technology, Nihon University.

Chemicals and Reagents. Chemicals and reagents were purchased as follows: TPA from ChemSyn Laboratories (Lenexa, KS); MTT, β-carotene, and DMBA from Sigma Chemical Co. (St. Louis, MO); RPMI medium 1640 and 10% fetal bovine serum (FBS) from Invitrogen Co. (Auckland, New Zealand); and the EBV cell culture reagents and *n*-butanoic acid from Nacalai Tesque, Inc. (Kyoto, Japan).

Extraction and Isolation. The pulverized epidermis of the sclerotia of *P. cocos* (3.98 kg) was extracted with MeOH (12 L) under reflux (3 h) three times. The MeOH solution was evaporated in vacuo to give an extract (398 g), which was mixed with H_2O (12 L) and extracted with $CHCl_3$ (12 L × 3). The $CHCl_3$-soluble fraction was further extracted with saturated aqueous $NaHCO_3$ solution, and the $NaHCO_3$ extract, after adjustment to pH 3−4 with 6 M HCl, was extracted with $CHCl_3$ and yielded 6.60 g of material. Crystallization of the $CHCl_3$ extract from MeOH yielded crystallized (1.05 g) and filtrate portions (5.26 g). The filtrate was subjected to chromatography on an ODS column (250 g). Step gradient elution was conducted with H_2O−MeOH (3:7 → 2:8) to give fractions A (93 mg), B (114 mg), C (1412 mg), D (428 mg), E (504 mg), F (1196 mg), and G (832 mg), arranged in

decreasing order of polarity. Fraction C was further chromatographed on silica gel [45 g; eluent: *n*-hexane−EtOAc (1:1 → 0:1) and EtOAc−EtOAc (9:1)] to afford fractions C1 (11 mg), C2 (202 mg), C3 (117 mg), C4 (90 mg), C5 (104 mg), C6 (128 mg), and C7 (276 mg), listed in increasing order of polarity. Preparative HPLC of fractions C2 (HPLC system VI), C3 (I), C4 (VII), C6 (I), and C7 (VII) gave **19** [1.6 mg; retention time (t_R) 16.0 min], **4** (4.6 mg; 18.4 min) and **6** (3.6 mg; 32.0 min), **2** (10.5 mg; 41.6 min), **1** (2.2 mg; 80.0 min), and **12** (5.6 mg; 38.4 min), respectively. Fraction D, upon further chromatography on an ODS [25 g; eluent: H_2O−MeOH (4:6 → 0:1)], afforded four fractions, D1−D4. Fraction D2 (189 mg) was separated by preparative HPLC (HPLC system II) to give **5** (2.9 mg; 17.6 min) and **8** (8.7 mg; 39.2 min). Fraction E was subjected to chromatography on an ODS column [25 g; eluent: H_2O−MeOH (4:6 → 0:1)], which yielded four fractions, E1−E4. Preparative HPLC (HPLC system III) of fraction E3 (117 mg) afforded **7** (7.3 mg; 39.2 min) and **18** (2.7 mg; 36.0 min). Fraction F was subjected to chromatography on silica gel [40 g; eluent: *n*-hexane−EtOAc (6:4 → 0:1) and then EtOAc−MeOH (9:1 → 8:2)] to afford fractions F1 (83 mg), F2 (129 mg), F3 (175 mg), F4 (156 mg), F5 (102 mg), and F6 (168 mg). Preparative HPLC (HPLC system IV) of fractions F2−F5 yielded **2** (7.4 mg; 11.2 min), **3** (4.2 mg; 13.6 min), **7** (145.6 mg, 28.0 min), **10** (3.8 mg; 24.0 min), **13** (5.9 mg; 36.0 min), and **14** (19.8 mg; 31.2 min) altogether. Fraction G was subjected to chromatography on silica gel [45 g; eluent: *n*-hexane−EtOAc (7:3 → 0:1) and then EtOAc−MeOH (9:1 → 8:2)] to afford fractions G1 (78 mg), G2 (60 mg), G3 (103 mg), G4 (123 mg), G5 (80 mg), and G6 (122 mg). Preparative HPLC (HPLC system V) of fraction G1 gave **9** (3.9 mg; 21.6 min), **15** (2.0 mg; 24.8 min), and **17** (1.0 mg; 27.6 min). Preparative HPLC (HPLC system IV) of fractions G2−G6 eventually afforded **7** (82.0 mg), **10** (7.7 mg), **11** (4.5 mg; 9.6 min), **14** (10.4 mg), and **16** (18.7 mg, 38.4 min). In addition, preparative HPLC (HPLC system IV) of a moiety (100 mg) of the crystallized portion (1.05 g) of the $CHCl_3$ extract mentioned above yielded **7** (28.1 mg), **10** (1.5 mg), **14** (5.2 mg), and **16** (9.6 mg).

16α,27-Dihydroxydehydrotrametenoic Acid [(20ζ)-3α,16α,27-Trihydroxylanosta-7,9(11),24-trien-21-oic Acid] (1): amorphous solid; $[\alpha]^{25}_D$ +27.1 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 205 (3.21), 235 (3.59), 242 (3.54) nm; IR (KBr) ν_{max} 3427 (OH), 1711, 1682, 1640 (>C=O), 900 (>C=CH_2), 803 (>C=CH−) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (positive-ion mode) *m/z* 509.3216 (calcd for $C_{30}H_{46}O_5Na$ $[M + Na]^+$, 509.3242).

25-Hydroxy-3-epitumulosic Acid [(20ζ)-3α,16α,25-Trihydroxy-24-methyllanosta-8,24(24^1)-dien-21-oic Acid] (3): needles, mp 202−204 °C (MeOH); $[\alpha]^{25}_D$ +10.2 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 203 (3.54), 243 (3.34) nm; IR (KBr) ν_{max} 3421 (OH), 1714, 1640 (>C=O) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (negative-ion mode) *m/z* 501.3580 (calcd for $C_{31}H_{49}O_5$ $[M - H]^-$, 501.3580).

16α,25-Dihydroxyeburiconic Acid [(20ζ)-3-Oxo-16α,25-dihydroxy-24-methyllanosta-8,24(24^1)-dien-21-oic Acid] (5): needles, mp 209−211 °C (MeOH); $[\alpha]^{25}_D$ +22.7 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 205 (3.90), 243 (3.50) nm; IR (KBr) ν_{max} 3426 (OH), 1712, 1685 (>C=O), 903 (>C=CH_2) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (negative-ion mode) *m/z* 499.3385 (calcd for $C_{31}H_{47}O_5$ $[M - H]^-$, 499.3423).

25-Methoxyporicoic Acid A [(20ζ)-16α-Hydroxy-25-methoxy-24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic Acid] (8): needles, mp 214−216 °C (MeOH); $[\alpha]^{25}_D$ +4.0 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 205 (3.80), 242 (3.79) nm; IR (KBr) ν_{max} 3435 (OH), 1712, 1649 (>C=O), 904 (>C=CH_2), 894 (>C=CH−) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (positive-ion mode) *m/z* 551.3322 (calcd for $C_{32}H_{48}O_6Na$ $[M + Na]^+$, 551.3348).

26-Hydroxyporicoic Acid DM [Methyl (20ζ)-16α,25,27-Trihydroxy-24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic Acid 3-oate] (12): amorphous solid; $[\alpha]^{25}_D$ +18.1 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 205 (3.83), 235 (3.76), 241 (3.76) nm; IR (KBr) ν_{max} 3420 (OH), 1734, 1720, 1642 (>C=O), 900 (>C=CH_2), 820 (>C=CH−) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (positive-ion mode) *m/z* 567.3272 (calcd for $C_{32}H_{48}O_7Na$ $[M + Na]^+$, 567.3297).

25-Hydroxyporicoic Acid C [(20ζ)-25-Hydroxy-24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24^1)-tetraene-3,21-dioic Acid] (13): needles, mp 223−225 °C (MeOH); $[\alpha]^{25}_D$ −34.1 (*c* 0.22, MeOH); UV λ_{max} (log ϵ) 205 (3.80), 243 (3.98) nm; IR (KBr) ν_{max} 3447 (OH), 1718, 1641 (>C=O), 856 (>C=CH_2) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (negative-ion mode) *m/z* 497.3274 (calcd for $C_{31}H_{45}O_5$ $[M - H]^-$, 497.3267).

Poricoic Acid GM [Methyl (20ζ)-16α-Hydroxy-3,4-*seco*-lanosta-4(28),8,24-triene-3,21-dioic Acid 3-oate] (15): needles, mp 220−222 °C (MeOH); $[\alpha]^{25}_D$ −22.2 (*c* 0.18, MeOH); UV λ_{max} (log ϵ) 205 (3.81), 243 (3.86) nm; IR (KBr) ν_{max} 3421 (OH), 1742, 1709 1637 (>C=O), 902 (>C=CH−) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (negative-ion mode) *m/z* 499.3380 (calcd for $C_{31}H_{47}O_5$ $[M - H]^-$, 499.3428).

Poricoic Acid HM [Methyl (20ζ)-16α-Hydroxy-24-methyl-3,4-*seco*-lanosta-4(28),8,24(24^1)-triene-3,21-dioic Acid 3-oate] (17): needles, mp 181−183 °C (MeOH); $[\alpha]^{25}_D$ −20.7 (*c* 0.09, MeOH); UV λ_{max} (log ϵ) 205 (3.88), 243 (3.34) nm; IR (KBr) ν_{max} 3446 (OH), 1739, 1707, 1633 (>C=O), 891 (>C=CH−) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (negative-ion mode) *m/z* 513.3553 (calcd for $C_{32}H_{49}O_5$ $[M - H]^-$, 513.3585).

6,7-Dehydroporicoic Acid H [(20ζ)-16α-Hydroxy-3,4-*seco*-24-methyllanosta-4(28),6,8,24(24^1)-tetraene-3,21-dioic Acid] (18): amorphous solid; $[\alpha]^{25}_D$ −82.5 (*c* 0.4, MeOH); UV λ_{max} (log ϵ) 243 (3.52), 250 (3.50), 285 (3.39) nm; IR (KBr) ν_{max} 3427 (OH), 1704, 1647 (>C=O), 894 (>C=CH_2) cm^{-1}; ^{13}C and ^1H NMR, see Tables 1 and 2, respectively; HRESIMS (positive-ion mode) *m/z* 497.3193 (calcd for $C_{31}H_{45}O_5$ $[M - H]^-$, 497.3267).

In Vitro EBV-EA Activation Experiment. For the protocol for this in vitro assay, refer to a previous article.[18]

In Vivo Two-Stage Carcinogenesis Assay on Mouse Skin Papillomas. For the protocol for this in vivo assay, refer to a previous article.[19]

Cell Cultures. HL60 (human leukemia) and CRL1579 (human melanoma) cell lines were obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). The HL60 cells were grown in RPMI 1640 medium. The medium was supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). The cells were cultured in a 5% CO_2 humidified incubator at 37 °C.

Cytotoxicity Assay. HL60 cells (3×10^3 cells/well) and CRL 1579 cells (3×10^3 cells/well) were spread onto a 96-well culture plate with RPMI 1640 medium (with 10% FBS). Then, the compounds (final concentration 10^{-6}, 10^{-5}, 10^{-4} M) were applied for 48 h. After addition of 0.5 mg/mL MTT solution (10 μL/well), incubation was continued for 3 h. The reaction was stopped by addition of 0.04 M HCl in 2-propanol, and absorbances at 570 nm (top) and 630 nm (bottom) were measured after thorough pipetting to disperse the generated blue formazan. The 50% inhibitory concentration (IC_{50}) was the concentration that caused a 50% decrease in the absorbance of compound-treated cells compared to the vehicle control.

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Supporting Information Available: ^{13}C, ^1H, HMBC, and NOESY NMR data for compounds **1**, **3**, **5**, **8**, **12**, **13**, **15**, **17**, and **18**, and the cytotoxicity data of 17 compounds from *Poria cocos*. This information is available free of charge via the Internet at http://pubs.acs.org.

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