Triterpene Acids from *Poria cocos* and Their Anti-Tumor-Promoting Effects

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Received January 3, 2007

The structures of six new lanostane-type triterpene acids isolated from the epidermis of the sclerotia of *Poria cocos* were established to be 15α-hydroxydehydrotumulosic acid (**5**), 16α,25-dihydroxydehydroeburicoic acid (**9**), 5α,8α-peroxydehydrotumulosic acid (**10**), 25-hydroxyporicoic acid H (**11**), 16-deoxyporicoic acid B (**12**), and poricoic acid CM (**16**) on the basis of spectroscopic methods. On evaluation of these six and 11 other known triterpene acids isolated from the sclerotium, **1**−**4**, **6**−**8**, **13**−**15**, and **17**, 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 except for **1**, **3**, **4**, and **8** exhibited inhibitory effects with IC_{50} values of 195−340 mol ratio/32 pmol TPA. Compound **12** and poricoic acid C (**13**) exhibited inhibitory effects 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.

Dried sclerotia of *Poria cocos* Wolf (Polyporaceae) are traditionally used in Chinese herbal prescriptions as diuretics and sedatives.[1,2] Whereas the inner parts of the sclerotia of *P. cocos*, called fu-ling in Chinese, were 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] Both the inner parts[2−6] and the epidermis[7,8] of the sclerotia of *P. cocos* were reported to contain lanostane-type triterpene acids,[7,8] which were suggested to be the major medicinal components of the fungus. Several of these acids have inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice[9,10] and on Epstein−Barr virus early antigen (EBV-EA) activation induced by TPA,[6] cytotoxicities against some human cancer cells,[6] and inductive effects of apoptosis in prostate cancer cells[11] and in H-ras-transformed rat2 cells.[12] In addition, some triterpene acids from the epidermis of the sclerotia of *P. cocos* have been reported to possess inhibitory effects on DNA polymerases.[13,14]

Since the lanostane-type triterpene acids from *P. cocos* were expected to be potential inhibitors of tumor promoters (cancer chemopreventive agents), we conducted a further study of these compounds and their inhibitory effects on EBV-EA induced by TPA. In this paper, we report the isolation and characterization of six new (**5**, **9**−**12**, and **16**) and 11 known (**1**−**4**, **6**−**8**, **13**−**15**, and **17**) lanostane-type triterpene acids from the epidermis of the sclerotia of *P. cocos* and their inhibitory effects on EBV-EA activation. In addition, we report the inhibitory effects of compounds **12** and **13** on *in vivo* two-stage mouse skin carcinogenesis.

Results and Discussion

Seventeen lanostane-type triterpene acids, eburicoic acid (**1**),[3] pachymic acid (**2**),[4] 3-epidehydrotrametenolic acid (**3**),[3] dehydroeburicoic acid (**4**),[3] 15α-hydroxydehydrotumulosic acid (**5**), dehydropachymic acid (**6**),[4] dehydrotrametenonic aicd (**7**),[13] dehydroeburiconic acid (**8**),[8,13] 16α,25-dihydroxydehydroeburiconic acid (**9**), 5α,8α-peroxydehydrotumulosic acid (**10**), 25-hydroxyporicoic acid H (**11**), 16-deoxyporicoic acid B (**12**), poricoic acid C (**13**),[3] poricoic acid D (**14**),[3] poricoic acid AM (**15**),[3,7] poricoic acid CM (**16**), and poricoic acid DM (**17**),[3] were isolated from an acidified $CHCl_3$-soluble fraction of a MeOH extract of the epidermis of *P. cocos* sclerotia. Among these, **5**, **9**−**12**, and **16** are new compounds. The ^{13}C and ^1H NMR data for the new compounds are shown in Table 1. Identification of all other compounds was performed by ^1H NMR and MS comparison with the corresponding compounds in the literature.

Compound **5** showed $[M]^+$ at *m*/*z* 500.3502 ($C_{31}H_{48}O_5$) in the HREIMS. The ^{13}C and ^1H NMR data and IR and UV spectra of **5** showed the presence of three secondary hydroxyls, a carboxyl, a conjugated diene,[13] a terminal methylene, five tertiary methyls, and an isopropyl group. These data, in combination with the mass fragmentations observed at *m*/*z* 345 (loss of side-chain at C-17) and 272 (loss of side-chain at C-17, ring D),[15] suggested that **5** had a 3*β*-hydroxy lanostane-type triterpene skeleton with a $\Delta^{7,9(11)}$-diene system, two additional hydroxyl groups, and a carboxyl group at C-21 in the C_9-side-chain containing a 24-methylene group.[8,13] The above evidence, coupled with comparison of ^{13}C and ^1H NMR data with those of dehydrotumulosic acid [(20ξ)-3*β*,16α-dihydroxy-24-methyllanosta-7,9(11),24(24^1)-trien-21-oic acid][8] and analysis of $^1H-^1H$ COSY, HMQC, and HMBC (Table S1, Supporting Information) spectra, indicated that **5** was (15ξ,20ξ)-3*β*,15,16α-trihydroxy-24-methyllanosta-7,9(11),24(24^1)-trien-21-oic acid. Significant NOE correlations in the NOESY experiment of **5** for H-18 (13*β*-Me) with H-15 and H-16*β* and for H-15 with H-16*β* suggested that the hydroxyl group at C-15 was oriented on the α-face. Hence, the structure of compound **5** was established as (20ξ)-3*β*,15α,16α-trihydroxy-24-methyllanosta-7,9(11),24(24^1)-trien-21-oic acid, which we named 15α-hydroxydehydrotumulosic acid.

Compound **9** gave a $[M - H]^-$ ion in the HRESIMS at *m*/*z* 497.3237, consistent with the molecular formula $C_{31}H_{46}O_5$. The ^{13}C and ^1H NMR, IR, and UV spectra of **9** showed the presence of a secondary and a tertiary hydroxyl group, a carboxylic function, a keto group, a conjugated diene,[13] a terminal methylene, and seven tertiary methyls, of which two were attached to an oxygen-bearing carbon. The EIMS of **9** exhibited diagnostic fragment ions at *m*/*z* 366 (loss of side-chain − 2H), 309 (loss of side-chain − H_2O), 269 (loss of side-chain − ring D), and 255 (269 − CH_2).[15] The above evidence, coupled with the spectroscopic comparison with **8**[8,13] and 25-hydroxy-3-epidehydrotumulosic acid [(20ξ)-3α,16α,-25-trihydroxy-24-methyllanosta-7,9(11),24(24^1)-trien-21-oic acid],[8] and analysis of $^1H-^1H$ COSY, HMQC, and HMBC (Table S1, Supporting Information) spectra, confirmed that **9** was (20ξ)-16α,-

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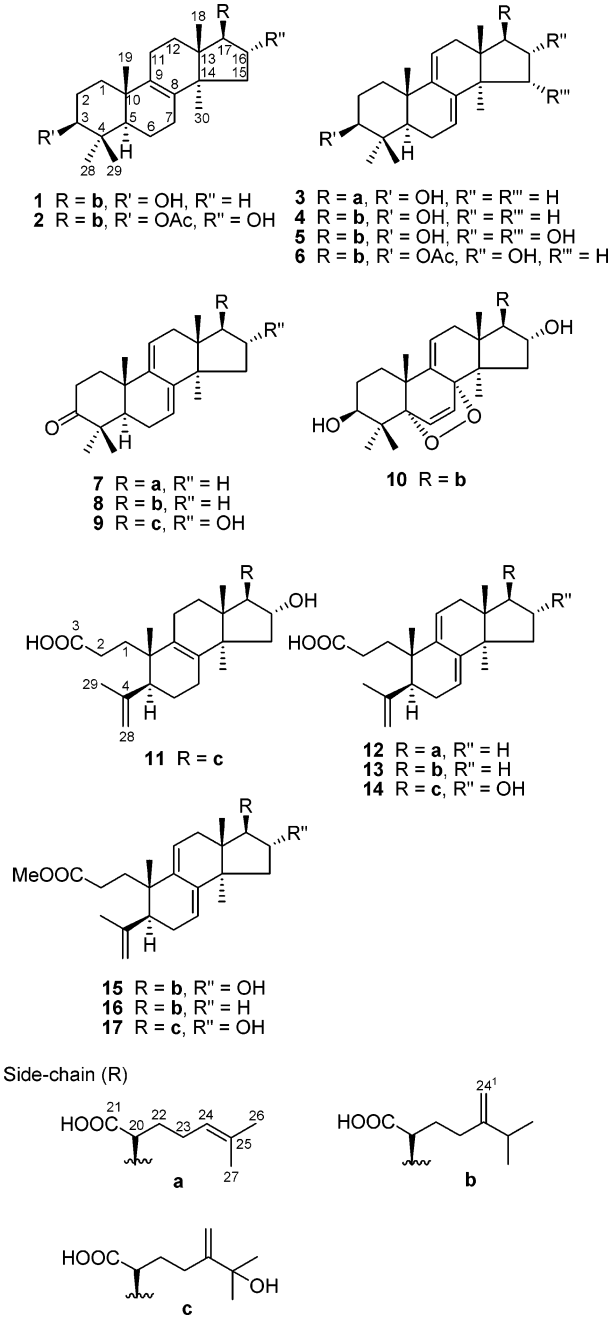
10.1021/np0780001 CCC: $37.00
Published on Web 05/09/2007



25-dihydroxy-3-oxo-24-methyllanosta-7,9(11),24(24[1])-trien-21-oic acid (16α,25-dihydroxydehydroeburicoic acid).

The molecular formula of compound **10** was determined to be $C_{31}H_{46}O_6$ from its HREIMS ([M]$^+$, *m*/*z* 514.3284). The ^{13}C and ^{1}H NMR spectra and IR spectra of **10** showed the presence of two secondary hydroxyls, a carboxyl, a *cis*-oriented disubsituted and a trisubstituted olefin, a terminal methylene, five tertiary methyls, an isopropyl group, and two oxygen-bearing tertiary carbons. The EIMS of **10** exhibited diagnostic fragment ions at *m*/*z* 341 (loss of side-chain − H_2O) and 284 (loss of side-chain − ring D − H_2O).[15] The above evidence, coupled with comparison of ^{13}C and ^{1}H NMR data with those of dehydrotumulosic acid,[8] (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol 3-*O*-β-D-glucopyranoside,[16] and 9(11)-dehydroaxinysterol [(22*E*,24*R*)-5α,8α-epidioxyergosta-6,9(11),22,25-tetraen-3β-ol],[17] suggested that **10** was (20ξ)-5α,8α-epidioxy-3β,16α-dihydroxy-24-methyllanosta-6,9(11),24(24[1])-trien-21-oic acid, which we named 5α,8α-peroxydehydrotumulosic acid. The proposed structure of **10** was supported by analyses of ^{1}H−^{1}H COSY, HMQC, and HMBC (Table S1, Supporting Information) spectra and significant NOE correlations observed in the NOESY spectrum for H-28 (4α-Me) with H-3α and for H-30 (14α-Me) with H-17α on the α-face and for H-29 (4β-Me) with H-6 and H-19 (10β-Me), for H-19 with H-6 and H-18 (13β-Me), and for H-18 with H-7 and H-16β on the β-face of the molecule (Figure 1).[18]

The molecular formula of **11** was determined to be $C_{30}H_{48}O_6$ from its HREIMS ([M]$^+$, *m*/*z* 516.3451). The ^{13}C and ^{1}H NMR spectra and IR spectra of **11** indicated the presence of secondary and tertiary hydroxyls, two carboxyls, a tetrasubstituted olefin, a terminal methylene, an isopropylidene, and five tertiary methyl groups, of which two were attached to an oxygen-bearing carbon. The mass fragmentations observed at *m*/*z* 425 [M − CH_2CH_2COOH − H_2O]$^+$ and 325 (loss of side-chain at C-17 − H_2O − 2H) suggested that **11** was 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_9-side-chain containing a 24-methylene functionality.[3,6] Comparison of ^{13}C and ^{1}H NMR data (Table 1) with those of compound **14**[3] and poricoic acid H [(20ξ)-16α-hydroxy-3,4-*seco*-24-methyllanosta-4(28),8,24(24[1])-triene-3,21-dioic acid][6] and analyses of ^{1}H−^{1}H COSY, HMQC, HMBC (Table S2, Supporting Information), and NOESY spectra indicated that **11** was (20ξ)-16α,25-dihydroxy-3,4-*seco*-24-methyllanosta-4(28),8,24(24[1])-triene-3,21-dioic acid, which we named 25-hydroxyporicoic acid H.

Compound **12** gave a [M]$^+$ ion in the HREIMS at *m*/*z* 468.3237, consistent with the molecular formula $C_{30}H_{44}O_4$. The ^{13}C and ^{1}H NMR, IR, and UV spectra of **12** showed the presence of two carboxyls, a conjugated diene,[13] an isopropenyl, an isopropylidene, and three tertiary methyl groups. The EIMS of **12** exhibited mass fragmentations at *m*/*z* 395 [M − CH_2CH_2COOH]$^+$, 325 (loss of side-chain at C-17 − 2H), and 285 (loss of side-chain at C-17 − ring D).[11] Comparison of ^{13}C and ^{1}H NMR data with those of compounds **3**[3] and **13**[3] suggested that **12** was (20ξ)-3,4-*seco*-lanosta-4(28),7,9(11),24-tetraene-3,21-dioic acid, which we named 16-deoxyporicoic acid B. The proposed structure of **12** was supported by analyses of ^{1}H−^{1}H COSY, HMQC, HMBC (Table S2, Supporting Information), and NOESY spectra.

Compound **16** gave a [M − H]$^-$ ion in the HRESIMS at *m*/*z* 495.3488 ($C_{32}H_{48}O_4$). The ^{13}C and ^{1}H NMR spectra of **16** were very similar to those of **13**,[3] except the former possessed an additional *O*-methyl group, suggesting that **16** was a methyl ester derivative of **13**. The EIMS of **16** exhibited mass fragmentations at *m*/*z* 409 [M − CH_2CH_2COOMe]$^+$ and 340 (loss of side-chain at C-17 − H). Diagnostic cross-correlations for H-2 (with C-3 and 3-O*Me*) and 3-O*Me* (with C-3) observed in the HMBC spectrum (Table S2, Supporting Information) of **16** indicated that the methyl ester group was located at C-3. Thus, the structure of **16** was methyl (20ξ)-3,4-*seco*-24-methyllanosta-4(28),7,9(11),24(24[1])-tetraene-3,21-dioic acid 3-oate, which we named poricoic acid CM. The UV, IR, ^{13}C DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra of **16** supported this structure.

The inhibitory effect on EBV-EA activation induced by TPA was examined as a preliminary evaluation of the potential anti-tumor-promoting activities for compounds **1**−**17**, and the results are shown in Table 2, along with comparable data for β-carotene (a vitamin A precursor studied widely in cancer chemoprevention animal models[19]). All compounds tested allowed high viability (60−70%) of Raji cells even at 32 nmol (mol ratio of compound to TPA = 1000:1), indicating their low cytotoxicity at this high concentration. Whereas four compounds (**1**, **3**, **4**, and **8**) exhibited only weak inhibitory effects, with IC_{50} values (concentration of 50% inhibition relative to positive control) of 405−465 mol ratio/32 pmol TPA, the other 13 compounds showed potent inhibitory effects, with IC_{50} values of 195−340 mol ratio/32 pmol TPA. They were more potent than the reference β-carotene (IC_{50} value, 397 mol ratio/32 pmol TPA). On the basis of the results in Table 2, we can draw some conclusions about the structure−activity relationship

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Data for Six Triterpenoids (C_5D_5N)[a]

	5		**9**		**10**[b]		**11**		**12**		**16**[c]	
C no.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	36.4 t	1.52 (α) 2.00 (*β*; br d, 14.1)	36.7 t	1.66 (α) 2.13 (*β*)	33.6 t	2.33 (α) 1.67 (*β*)	33.6 t	2.01 2.07	36.4 t	1.87 2.11	35.5 t	1.57 (ddd, 6.0, 13.2, 13.2) 1.82 (ddd, 4.6, 13.2, 16.0)
2	28.7 t	1.92 (2H)	34.9 t	2.34 (α) 2.75 (*β*)	28.7 t	2.00 (2H)	30.4 t	2.33 2.70	30.2 t	2.47 2.52	29.3 t	2.15 2.26
3	78.0 d	3.46 (dd, 6.5, 8.3)	215.2 s		73.0 d	4.22 (dd, 7.2, 9.1)	176.6 s		176.6 s		174.8 s	
4	39.4 s		47.5 s		41.8 s		147.9 s		149.3 s		148.9 s	
5	49.8 d	1.34	51.1 d	1.61 (dd, 3.4, 11.9)	86.8 s		47.0 d	2.31 (br dd, 2.0, 12.7)	50.8 d	2.33 (br d, 7.2)	50.6 d	2.22
6	23.5 t	2.26 (α; br dd, 6.5, 13.4) 2.19 (*β*; br dd, 12.4, 16.5)	23.9 t	2.02 (α) 2.16 (*β*)	133.8 d	6.64 (d, 8.9)	24.4 t	1.52 (α) 1.72 (*β*)	28.6 t	2.57 (α) 2.06 (*β*)	28.3 t	2.56 (α; br dd, 5.2, 18.9) 2.06 (*β*)
7	122.4 d	6.52 (d, 5.8)	120.5 d	5.58 (br d, 13.3)	132.1 d	6.97 (d, 8.9)	26.3 t	2.13 (α) 2.00 (*β*)	118.0 d	5.29	118.0 d	5.30 (br s)
8	141.8 s		142.9 s		78.7 s		139.3 s		142.0 s		141.3 s	
9	146.9 s		144.7 s		145.0 s		129.9 s		137.6 s		137.1 s	
10	37.9 s		37.5 s		41.3 s		40.7 s		38.8 s		38.4 s	
11	116.0 d	5.38 (br s)	117.8 d	5.36 (br s)	120.0 d	5.39 (br s)	21.7 t	2.14 (2H)	120.5 d	5.31	119.9 d	5.30 (br s)
12	36.9 t	2.76 (α; br d, 17.9) 2.47 (*β*)	36.2 t	2.66 (α) 2.40 (*β*)	36.0 t	2.64 (α) 2.32 (*β*)	29.9 t	2.03 (α) 2.28 (*β*)	36.8 t	2.43 2.48	36.3 t	1.98 (α) 2.21 (*β*)
13	41.7 s		45.1 s		41.8 s		46.1 s		44.7 s		44.1 s	
14	52.0 s		49.3 s		48.0 s		49.8 s		50.3 s		49.9 s	
15	73.2 d	4.58 (d, 7.9)	44.3 t	1.90 (α; br d, 12.8) 2.43 (*β*)	42.0 t	1.88 (α; d, 13.5) 2.81 (*β*; dd, 8.8, 13.5)	43.8 t	1.70 (α; br d 12.7) 2.38 (*β*; br dd, 8.6, 12.7)	31.0 t	1.39 (α) 1.74 (*β*)	30.5 d	1.37 (α) 1.67 (*β*)
16	75.3 d	4.31 (dd, 6.2, 7.9)	76.4 d	4.53 (br dd, 6.8, 7.2)	76.0 d	4.56 (br dd, 6.9, 8.8)	76.4 d	4.55 (br t, 6.9)	27.3 t	1.43 (α) 2.08 (*β*)	27.0 t	1.30 (α) 1.38 (*β*)
17	56.4 d	2.77 (br dd, 6.2, 11.3)	57.5 d	2.88	56.8 d	3.00	57.3 d	2.85 (dd like, 5.5, 11.0)	48.3 d	2.46	47.6 d	2.16
18	18.0 q	1.13 (s)	17.6 q	1.03 (s)	18.2 q	1.24 (s)	18.0 q	1.17 (s)	17.0 q	1.00 (s)	16.7 q	0.71 (s)
19	23.1 q	1.10 (s)	22.0 q	1.14 (s)	28.6 q	1.20 (s)	22.5 q	0.97 (s)	22.3 q	1.02 (s)	21.9 q	0.95 (s)
20	48.3 d	2.93 (br dd, 8.9, 11.3)	48.8 d	2.99	48.3 d	2.96	48.8 d	3.00 (br t, 10.3)	48.8 d	2.63	47.7 d	2.33
21	178.5 s		178.5 s		178.4 s		178.2 s		178.4 s		178.0 s	
22	31.7 t	2.47 2.56	32.3 t	2.57 2.80	31.5 t	2.40 2.65	32.5 t	2.58 2.80	33.2 t	1.72 1.91	31.0 t	1.33 1.69
23	33.1 t	2.35 2.55	30.0 t	2.67 2.82	33.1 t	2.36 2.55	30.1 t	2.68 2.82	26.7 t	2.23 2.35	32.1 t	1.98 2.07
24	156.2 s		158.0 s		156.0 s		158.1 s		124.8 d	5.31	155.2 s	
25	34.1 d	2.28 (dd, 6.8, 13.7)	72.6 s		34.1 d	2.30	72.6 s		131.7 s		33.8 d	2.23
26	21.9[d] q	0.99 (d, 6.8)	30.0 q	1.54 (s)	21.9 q	1.03 (d, 6.6)	30.1 q	1.55 (s)	25.8 q	1.67 (s)	21.8 q	1.02 (d, 6.8)
27	22.0[d] q	0.99 (d, 6.8)	30.0 q	1.55 (s)	22.0 q	1.01 (d, 6.6)	30.1 q	1.56 (s)	17.7 q	1.61 (s)	21.8 q	1.01 (d, 6.8)
28	28.8 q	1.19 (s)	25.6 q	1.13 (s)	24.3 q	1.44 (s)	114.2 t	4.89 (s) 4.99 (s)	112.1 t	4.75 (d, 2.4) 4.82 (s)	111.8 t	4.64 (br s) 4.66 (br s)
29	16.6 q	1.12 (s)	22.4 q	1.07 (s)	19.4 q	1.38 (s)	23.3 q	1.80 (s)	22.2 q	1.72 (s)	22.0 q	1.65 (s)
30	18.3 q	1.44 (s)	26.4 q	1.44 (s)	19.9 q	1.64 (s)	26.3 q	1.51 (s)	24.3 q	1.02 (s)	24.0 q	0.87 (s)
24[1]	107.0 t	4.85 (s) 4.97 (s)	107.0 t	5.15 (br s) 5.46 (br s)	107.0 t	4.86 (br s) 4.96 (br s)	107.0 t	5.18 (s) 5.50 (s)			106.9 t	4.69 (br s) 4.76 (br s)
OMc											51.5 q	3.62 (s)

[a] Figures in parentheses denote *J* values (hertz). [b]Determined at 125 MHz (^{13}C) and 500 MHz (^{1}H). [c]Determined in $CDCl_3$ at 125 MHz (^{13}C) and 500 MHz (^{1}H). [d]Values bearing the same superscript in each column are interchangeable.

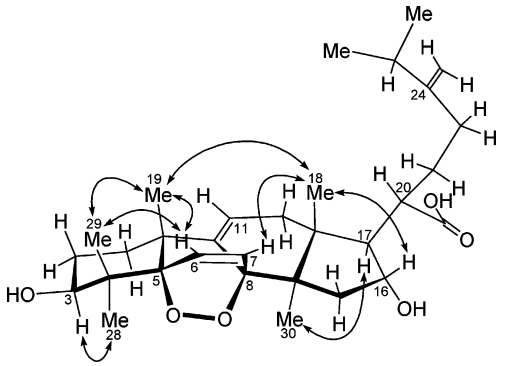


Figure 1. Major NOE correlations (↔) for compound **10**.

of the compounds: hydroxylation at C-16α (**2**, **5**, **6**, **9**−**11**, **14**, **15**, and **17**) and cleavage of ring A to form 3,4-*seco*-3-oic acid (**11**−**17**) enhance the activity. Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel those against tumor promotion *in vivo*,[20] the highly inhibitory compounds against EBV-EA activation could be valuable antitumor promoters.

Subsequently we determined the inhibitory effects of compounds **12** and **13** 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 Figures 2A and 2B, respectively. The incidence of papillomas in group I (untreated) was highly significant in 100% of mice at 11 weeks of promotion. Further, more than four and eight 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 numbers of papillomas per mouse was reduced by treatment with **12** and **13**. Thus, in groups II (treated with **12**) and III (treated with **13**), the percentage ratios of papilloma-bearing mice were only 20% (II) and 27% (III) at 11 weeks and 80% (II) and 87% (III) at 20 weeks, and the mean papillomas per mouse were 1.2 (II) and 1.5 (III) at 11 weeks and 3.2 (II) and 3.6 (III) at 20 weeks.

From the results of the *in vitro* EBV-EA induction test and *in vivo* two-stage carcinogenesis, it appears that the lanostane-type triterpene acids isolated from the sclerotium of *Poria cocos*, especially those hydroxylated at C-16α and/or those with a 3,4-*seco*-3-oic acid functionality, could be valuable as chemopreventive agents in chemical carcinogenesis.

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) or with a JEOL LA-500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer in C_5D_5N or in $CDCl_3$ with tetramethylsilane as an internal standard. EIMS (70 eV) and HREIMS were recorded on a JEOL JMS-Bu20 spectrometer using a direct inlet system. 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; fragmenter 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 with a flow rate of the eluent at 2.0 mL/min, on a TSK ODS-120A 5 μm column (Toso Co., Tokyo, Japan) [eluent: MeOH−H_2O−AcOH (90:10:1) (HPLC system I), MeOH−H_2O−AcOH (85:15:1) (system II), or MeOH−H_2O−AcOH (70:30:1) (system III)], on a Pegasil ODS II 5 μm column (Senshu Scientific Co., Ltd., Tokyo, Japan) [eluent: MeOH−H_2O−AcOH (70:30:1) (system IV)], and on a Hypersil ODS 5 μm column (Senshu Scientific Co., Ltd.) [eluent: MeOH−H_2O−AcOH (75:25:1) (system V)].

Materials. The source of the fungal material was described in a previous article.[13] Thus, dried epidermis of the sclerotia obtained from the cultivated *Poria cocos* in Yunnan was purchased from Yunnan Medicines & Health Products Imp. & Exp. Corp. (Yunnan, Kunming, China). Identification was done by Mr. Seizo Kondo (Central Research Laboratory, Kotaro Pharmaceutical Co., Ltd., Takatsuki, Japan) on the basis of the description.[1,6,21] A voucher specimen has been deposited in the College of Science and Technology, Nihon University.

Chemicals and Reagents. Compounds were purchased as follows: TPA from ChemSyn Laboratories (Lenexa, KS), β-carotene and DMBA from Sigma Chemical Co. (St. Louis, MO), and the EBV cell culture reagents and *n*-butanoic acid from Nacalai Tesque, Inc. (Kyoto, Japan).

Extraction and Isolation. 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 $NaHCO_3$ aqueous solution and then with 5% NaOH

Table 2. Inhibitory Effects of Compounds **1**−**17** on Induction of the Epstein−Barr Virus Early Angiten

compound	percentage of EBV-EA induction[a] concentration (mol ratio/32 pmol TPA) 1000	500	100	10	IC_{50} (mol ratio/32 pmol TPA)
1 eburicoic acid	9.1 (70)	45.8	75.3	100	465
2 pachymic acid	0 (70)	24.7	71.6	96.5	286
3 3-epidehydrotrametenolic acid	9.2 (70)	45.3	79.1	100	464
4 dehydroeburicoic acid	8.7 (70)	45.1	74.9	100	460
5 15α-hydroxydehydrotumulosic acid	0 (70)	22.5	70.0	91.0	268
6 dehydropachymic acid	0 (70)	28.1	72.4	95.1	284
7 dehydrotrametenonic acid	0 (70)	30.1	71.0	94.2	310
8 dehydroeburiconic acid	9.5 (70)	43.9	79.5	100	405
9 16α,25-dihydroxydehydroeburiconic acid	0 (70)	39.5	65.3	91.7	340
10 5α,8α-peroxydehydrotumulosic acid	0 (60)	17.4	57.8	83.6	202
11 25-hydroxyporicoic acid H	0 (70)	16.7	57.5	82.8	202
12 16-deoxyporicoic acid B	0 (70)	19.3	65.0	90.0	262
13 poricoic acid C	0 (70)	21.1	67.2	91.7	273
14 poricoic acid D	0 (70)	15.3	56.1	81.5	198
15 poricoic acid AM	0 (70)	9.6	57.8	81.8	195
16 poricoic acid CM	0 (70)	37.2	63.4	87.6	332
17 poricoic acid DM	0 (70)	18.1	59.0	86.3	207
reference compound					
β-carotene	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 cell.

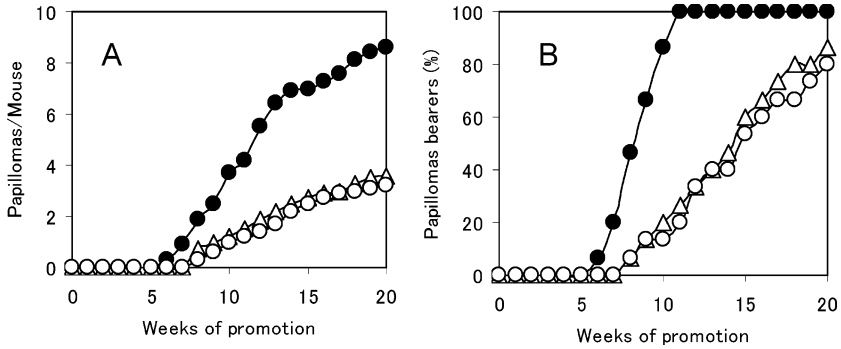


Figure 2. Inhibition of TPA-induced tumor promotion by multiple applications of 16-deoxyporicoic acid B (**12**; ○) and poricoic acid C (**13**; △). All mice ($n = 15$ for each of groups I, II, and III) were initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) given twice weekly starting 1 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 **12** (group II); △, TPA + 85 nmol of **13** (group III). After 20 weeks of promotion, a significant difference in the number of papillomas per mouse between the groups treated with compounds **12** and **13** and the control group was evident ($p < 0.05$, using Student's *t*-test). The number (standard deviations are shown in parentheses) of papillomas per mouse for each group was 8.6 (1.2), 3.2 (0.5), and 3.6 (0.6) for groups I, II, and III, respectively.

aqueous solution. The NaOH extract, after adjustment of acidity to pH 3−4 with 6 M HCl, was extracted with $CHCl_3$, which yielded 7.9 g of material. The extract was subjected to chromatography on an ODS column (300 g). Step gradient elution was conducted with H_2O−MeOH (1:1 → 0:1) to give fractions A (794 mg), B (304 mg), C (1032 mg), D (349 mg), E (336 mg), F (1624 mg), G (884 mg), H (267 mg), I (364 mg), and J (1176 mg), listed in decreasing order of polarity. Fraction C was further chromatographed on silica gel [31 g; eluent: *n*-hexane−EtOAc (9:1 → 0:1)] to afford fractions C1 (10 mg), C2 (34 mg), C3 (87 mg), C4 (146 mg), C5 (109 mg), C6 (101 mg), C7 (138 mg), and C8 (158 mg), listed in increasing order of polarity. Preparative HPLC of fractions C3 (HPLC system IV), C4 (III), and C6 (III) gave **5** (5.4 mg), **10** (2.4 mg), and **9** (2.5 mg), respectively. Preparative HPLC (HPLC system V) of fraction C8 yielded **14** (9.4 mg), **11** (4.7 mg), and **17** (1.8 mg). Fraction G, upon further chromatography on silica gel [36 g; eluent: *n*-hexane−EtOAc (4:1 → 0:1)], afforded fractions G1 (22 mg), G2 (91 mg), G3 (34 mg), G4 (62 mg), G5 (150 mg), G6 (104 mg), and G7 (73 mg). Fraction G2 was separated by HPLC system II to give **15** (6.4 mg). Preparative HPLC (HPLC system I) of fractions H and I gave **6** (6.2 mg) and **2** (64.2 mg), and **12** (50.1 mg), respectively. Fraction J was subjected to chromatography on silica gel [42 g; eluent: *n*-hexane−EtOAc (4:1 → 0:1)] to afford fractions J1 (49 mg), J2 (67 mg), J3 (110 mg), J4 (120 mg), J5 (71 mg), J6 (176 mg), and J7 (52 mg). Preparative HPLC (HPLC system I) of fractions J3, J4, and J6 yielded **7** (1.0 mg), **8** (41.9 mg), and **16** (1.7 mg); **3** (9.5 mg), **4** (6.7 mg), and **1** (2.0 mg); and **13** (108.1 mg), respectively.

15α-Hydroxydehydrotumulosic acid [(20ξ)-3β,15α,16α-trihydroxy-24-methyllanosta-7,9(11),24(24¹)-trien-21-oic acid] (5): needles, mp 208−210 °C (MeOH); $[\alpha]^{25}_D$ +29.7 (*c* 0.29, MeOH); UV λ_{max} (log ϵ) 237, 243 (3.78), 252 nm; IR (KBr) ν_{max} 3427 (OH), 1706, 1640 (>C=O) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 500 ($[M]^+$, 100), 482 ($[M]^+ - H_2O$, 31), 467 (29), 449 (29), 327 (31); HREIMS *m*/*z* 500.3502 (calcd for $C_{31}H_{48}O_5$ $[M]^+$, 500.3502).

16α,25-Dihydroxydehydroeburiconic acid [(20ξ)-16α,25-dihydroxy-3-oxo-24-methyllanosta-7,9(11),24(24¹)-trien-21-oic acid] (9): needles, mp 212−216 °C (MeOH); $[\alpha]^{25}_D$ +13.4 (*c* 0.53, MeOH); UV λ_{max} (log ϵ) 235, 242 (3.59), 250 nm; IR (KBr) ν_{max} 3434 (OH), 1704, 1646 (>C=O), 901 (>C=CH_2) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 480 ($[M]^+ - H_2O$, 48), 366 (86), 309 (77), 269 (100), 255 (32); HRESIMS (negative mode) *m*/*z* 497.3237 (calcd for $C_{31}H_{45}O_5$ $[M - H]^-$, 497.3267); ESIMS *m*/*z* 521 $[M + Na]^+$ (positive mode).

5α,8α-Peroxydehydrotumulosic acid [(20ξ)-3β,16α-dihydroxy-5α,8α-epidioxy-24-methyllanosta-6,9(11),24(24¹)-trien-21-oic acid] (10): needles, mp 196−200 °C (MeOH); $[\alpha]^{25}_D$ +99.6 (*c* 0.24, MeOH); IR (KBr) ν_{max} 3425 (OH), 1684, 1639 (>C=O), 897 (>C=CH_2) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 514 ($[M]^+$, 0.9), 284 (11), 264 (5), 256 (73), 73 (100); HREIMS *m*/*z* 514.3284 (calcd for $C_{31}H_{46}O_6$ $[M]^+$, 514.3294); HRESIMS (negative mode) *m*/*z* 513.3173 (calcd for $C_{31}H_{45}O_6$ $[M - H]^-$, 513.3216).

25-Hydroxyporicoic acid H [(20ξ)-16α,25-dihydroxy-24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24¹)-tetraene-3,21-dioic acid] (11): needles, mp 226−228 °C (MeOH); $[\alpha]^{25}_D$ +36.5 (*c* 0.17, MeOH); UV λ_{max} (log ϵ) 205 (3.49) nm; IR (KBr) ν_{max} 3422 (OH), 1706, 1640 (>C=O), 899 (>C=CH_2) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 516 ($[M]^+$, 38), 480 (26), 425 (100), 407 (65), 309 (78); HREIMS *m*/*z* 516.3451 (calcd for $C_{31}H_{48}O_6$ $[M]^+$, 516.3451).

16-Deoxyporicoic acid B [(20ξ)-3,4-*seco*-lanosta-4(28),7,9(11),-24-tetraene-3,21-dioic acid] (12): needles, mp 140−142 °C (MeOH); $[\alpha]^{25}_D$ +33.9 (*c* 0.23, MeOH); UV λ_{max} (log ϵ) 234, 241 (3.78), 250 nm; IR (KBr) ν_{max} 1708, 1640 (>C=O) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 468 ($[M]^+$, 49), 395 (100), 377 (4), 325 (4), 285 (4); HREIMS *m*/*z* 468.3237 (calcd for $C_{30}H_{44}O_4$ $[M]^+$, 468.3239).

Poricoic acid CM [(20ξ)-methyl 24-methyl-3,4-*seco*-lanosta-4(28),7,9(11),24(24¹)-tetraene-3,21-dioic acid 3-oate] (16): needles, mp 191−195 °C (MeOH); $[\alpha]^{25}_D$ +31.3 (*c* 0.27, MeOH); UV λ_{max} (log ϵ) 234, 241 (3.56), 250 nm; IR (KBr) ν_{max} 1741, 1709, 1646 (>C=O), 894 (>C=CH_2) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS (negative mode) *m*/*z* 495.3488 (calcd for $C_{32}H_{47}O_4$ $[M - H]^-$, 495.3479); HREIMS *m*/*z* 496.3550 (calcd for $C_{32}H_{48}O_4$ $[M]^+$, 496.3552); EIMS *m*/*z* 496 ($[M]^+$, 11), 465 (3), 409 (100), 340 (2), 313 (2).

In Vitro EBV-EA Activation Experiment. For the protocol for this *in vitro* assay, refer to previous articles.[22,23]

In Vivo Two-Stage Carcinogenesis Assay on Mouse Skin Papillomas. For the protocol for this *in vivo* assay, refer to a previous article.[23]

Acknowledgment. The authors thank Prof. K.-H. Lee (University of North Carolina) for his comments and advice. This work was supported, in part, by a grant "Academic Frontier" Project for Private Universities and a matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), 2002−2006.

Supporting Information Available: ^{13}C and ^1H NMR and HMBC NMR data for **5**, **9**−**12**, and **16**. This information is available free of charge via the Internet at http://pubs.acs.org.

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NP0780001