Cancer Chemopreventive Effects of Cycloartane-Type and Related Triterpenoids in in Vitro and in Vivo Models

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Received September 5, 2006

Forty-eight natural and semisynthetic cycloartane-type and related triterpenoids have been evaluated for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells as a primary screening test for anti-tumor promoters. In addition, these triterpenoids have been tested for their inhibitory effects on activation of (±)-(*E*)-methtyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, as a primary screening test for anti-tumor initiators. All of the compounds tested exhibited inhibitory effects on both EBV-EA and NOR 1 activation. Six of these compounds having a C-24 hydroxylated side chain, viz., (24*R*)-cycloart-25-ene-3*β*,24-diol (**9**), (24*R*)-cycloartane-3*β*,24,25-triol (**11**), (24*S*)-cycloartane-3*β*,24,25-triol (**12**), (24*ξ*)-24-methylcycloartane-3*β*,24,24^1-triol (**14**), (24*ξ*)-24^1-methoxy-24-methylcycloartane-3*β*,24-diol (**15**), and (24*ξ*)-24,25-dihydroxycycloartan-3-one (**27**), showed higher inhibitory effects than the others tested on both EBV-EA (IC_{50} values of 6.1−7.4 nM) and NOR 1 activation. Furthermore, compounds **14** and **15** exhibited inhibitory effects on skin tumor promotion in an in vivo two-stage mouse skin carcinogenesis test using 7,12-dimethylbenz-[*a*]anthracene (DMBA) as an initiator and TPA as a promoter.

In the course of our search for potential cancer chemopreventive agents from natural sources, we have demonstrated that various types of triterpenoids exhibit inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation in Raji cells.[1,2] Inhibitory potentials against tumor promoter-induced EBV activation are well correlated with tumor promotion in some animal models, and this in vitro assay is used as a convenient primary screening test of anti-tumor promoters.[3,4] We now report the inhibitory effects on EBV-EA activation induced by TPA and on activation of (±)-(*E*)-methtyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, as a primary screening test for anti-tumor initiators,[5] for 48 natural and semisynthetic cycloartane-type and related triterpenoids. Compounds possessing inhibitory effects against activation of NOR 1 have been observed to possess inhibitory effects against tumor-initiating activity induced by NO donors using a mouse skin model.[5] In addition, we report the inhibitory effects against in vivo two-stage mouse skin carcinogenesis for two triterpenoids in mouse skin initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by TPA. Cycloartane-type triterpene alcohols (3-hydroxytriterpenoids) such as cycloartenol (**2**) exhibit more potent inhibitory effects on TPA-induced EBV-EA activation in Raji cells than the other types of tetra-[6] and pentacyclic triterpene alcohols.[1,7] Two cycloartanes, **2** and 24-methylenecycloartanol (**3**), and their 3-*O*-feruloyl esters, **17** and **18**, have been reported to exhibit potent anti-inflammatory activity on ear edema induced by TPA in mice[8,9] and, in the case of **17**, to inhibit tumor promotion in two-stage carcinogenesis.[8]

Results and Discussion

Forty-eight compounds including 31 cycloartanes (9*β*,19-cyclolanostanes; **1**−**31**), 10 29-*nor*-cycloartanes (4α,14α-dimethyl-9*β*,-19-cyclocholestanes; **32**−**41**), three 28-*nor*-cycloartanes (4*β*,14α-dimethyl-9*β*,19-cyclocholestanes; **42**−**44**), and four 28,29-*dinor*-cycloartanes (14α-methyl-9*β*,19-cyclocholestanes; **45**−**48**) were tested in this study. Thirty of these compounds are natural products isolated from various plants, and nine compounds are biotransformation products of cycloartanes by a fungus (see Experimental Section for details).

Forty-eight cycloartane-type and related triterpenoids were first evaluated for in vitro inhibition of EBV-EA activation induced by TPA, and the results are shown in Table 1, along with comparable data for *β*-carotene (a vitamin A precursor studied widely in cancer chemoprevention animal models[10]), curcumin (an acyclic diarylheptanoid known to be a potent anti-tumor promoter[11]), and retinoic acid (one of the retinoids that has been studied as a cancer chemopreventive agent for various organ site cancers[12]). All compounds tested caused high viability (70%) of Raji cells even at 32 nM (mol ratio of compound to TPA = 1000:1), indicating their low cytotoxicity at this high concentration. In addition, all compounds, except for three 3-*O*-feruloyl esters (**17**, **18**, and **37**), showed potent inhibitory effects with IC_{50} values (concentration of 50% inhibition relative to positive control) of 6.1−10.3 nM. They were more potent than the reference *β*-carotene (IC_{50} value, 12.7 nM) and retinoic acid (15.4 nM) and more potent than or almost equipotent to the reference curcumin (10.2 nM). Seven 24-hydroxylated compounds with a 3*β*-hydroxy group (**9**, **11**, **12**, **14**, **15**, and **38**) or with a 3-oxo group (**27**) exhibited higher inhibitory effects than the others tested, with IC_{50} values of 6.1−7.4 nM. Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel those against tumor promotion in vivo,[3] the highly inhibitory compounds against EBV-EA activation might turn out to be valuable anti-tumor promoters.

On the basis of the results in Table 1, the following conclusions can be drawn about the structure−activity relationship of the compounds:

(i) The 3-oxo group exerts almost no influence on the activity or reduces the activity when compared with the 3*β*-hydroxy group.

(ii) Feruloylation decreases the activity. Such reduction of the activity is also observed during benzoylation of multifloranes.[1]

(iii) Hydroxylation at C-24 enhances the activity, as has been observed for the seven compounds mentioned above.

Using an in vitro screening model for NO scavenging,[5] the inhibitory effects of 48 cycloartanes and related triterpenoids were evaluated for their scavenging activity against NO generation by NOR 1 in a cultured cell system. Table 1 shows the inhibitory ratios (InhR; InhR of NOR 1 was taken as 1.0) of these compounds and

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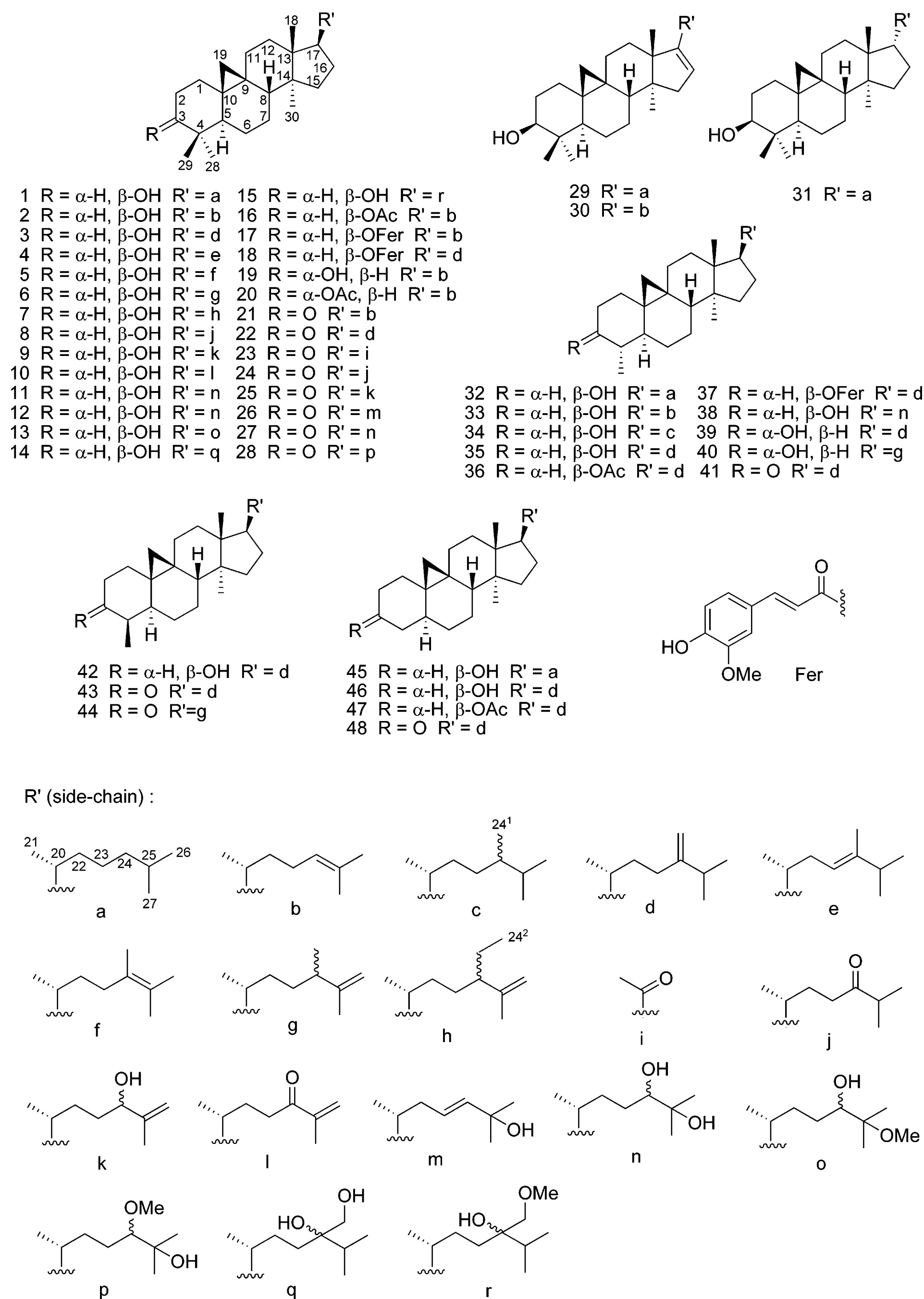
10.1021/np068044u CCC: $37.00 © 2007 American Chemical Society and American Society of Pharmacognosy
Published on Web 05/16/2007

Chart 1



two reference compounds, the natural triterpenoid glycyrrhizin and the synthetic NO scavenger carboxy-PTIO, on NOR 1 action. All compounds tested exhibited inhibitory effects with InhR of 1.4−2.2. Ten of them, all cycloartane-type compounds (**4**, **7**−**9**, **11**, **12**, **14**, **15**, **25**, and **27**), exhibited inhibitory effects (InhR = 2.0−2.2) that were almost equivalent to that of glycyrrhizin (InhR = 2.2). It is noteworthy that six (**9**, **11**, **12**, **14**, **15**, and **27**) out of these 10 compounds exhibited potent inhibitory effects also on EBV-EA induction (vide supra). The 29-*nor*-cycloartane-, 28-*nor*-cycloartane-, and 28,29-*dinor*-cycloartane-type compounds exhibited in general less inhibitory effects than those of the cycloartanes. NO is known to be involved in several potential toxic mechanisms and is a mutagen that may cause mutation in both microorganisms and mammalian cells.[13,14]

Subsequently we determined the inhibitory effects of two cycloartanes, **14** and **15** (biotransformation products of **3** by the fungus *Glomerella fusarioides*[15]), in a two-stage carcinogenesis test in mouse skin using 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 1A and 1B, respectively. The incidence of papillomas in group I (untreated) was highly significant, at 100% of mice at 10 weeks of promotion. Further, five and nine papillomas were formed per mouse at 10 and 20 weeks of promotion, respectively. The formation of papillomas in mouse skin was delayed and the mean numbers of papillomas per mouse were reduced by treatment with **14** and **15**. Thus, in groups II (treated with **14**) and III (treated with **15**), the ratios of papilloma-bearing mice were only 20% (II) and 27% (III) at 10 weeks and 80% (II) and 87% (III) at 20 weeks, and the mean papillomas per mouse were 1.2 (II and III) at 10 weeks and 4.0 (II) and 4.2 (III) at 20 weeks. The inhibitory effects of two cycloartanes, **14** and **15**, on papilloma formation in mouse skin were found to be almost equivalent to curcumin[16] and more potent than glycyrrhetic acid, an aglycone of glyzyrrhizin.[17]

Table 1. Inhibitory Effects on the Induction of Epstein−Barr Virus Early Antigen and Inhibitory Ratio (InhR) on NOR 1 Action of Cycloartane-Type and Related Triterpenoids and Reference Compounds

	oxygen-bearing functional group		percentage of EBV-EA induction[a]						
			concentration (nM)						InhR of NOR 1
compound	hydroxy	others[b]	320.00		32.00	3.20	0.32	IC_{50}[c] (nM)	activation[d]
cycloartane									
cycloartanol (**1**)	3β		0.0 ± 0.5	(70)	30.2 ± 1.2	70.2 ± 1.8	95.0 ± 0.2	9.4	1.8 ± 0.2
cycloartenol (**2**)	3β		0.0 ± 0.5	(70)	25.8 ± 1.1	69.9 ± 1.8	93.5 ± 0.3	8.8	1.6 ± 0.1
24-methylenecycloartanol (**3**)	3β		0.0 ± 0.3	(70)	23.2 ± 1.1	68.1 ± 1.8	91.4 ± 0.5	8.8	1.7 ± 0.1
cyclosadol (23*E*) (**4**)	3β		0.0 ± 0.4	(70)	31.4 ± 1.3	72.8 ± 1.9	95.2 ± 0.3	9.9	2.0 ± 0.2
cyclobranol (**5**)	3β		0.0 ± 0.4	(70)	30.5 ± 1.2	71.3 ± 1.9	94.5 ± 0.4	9.4	1.9 ± 0.2
cyclolaudenol (24*S*) (**6**)	3β		0.0 ± 0.4	(70)	30.6 ± 1.3	70.0 ± 1.9	93.6 ± 0.4	8.9	1.8 ± 0.1
polysthicol (24*S*) (**7**)	3β		0.0 ± 0.3	(70)	28.9 ± 1.3	69.1 ± 1.8	93.1 ± 0.4	8.8	2.0 ± 0.2
24-oxocycloartanol (**8**)	3β	24-oxo	0.0 ± 0.2	(70)	19.8 ± 1.1	62.7 ± 1.6	90.5 ± 0.5	8.1	2.0 ± 0.2
cycloart-25-ene-3β,24-diol (24*R*) (**9**)	3β, 24		0.0 ± 0.2	(70)	15.0 ± 1.0	57.8 ± 1.5	84.4 ± 0.6	6.4	2.0 ± 0.2
3β-hydroxycycloart-25-en-24-one (**10**)	3β	24-oxo	1.5 ± 0.2	(70)	25.4 ± 1.0	73.1 ± 1.8	97.8 ± 0.3	9.3	1.4 ± 0.1
cycloartane-3β,24,25-triol (24*R*) (**11**)	3β, 24, 25		0.0 ± 0.3	(70)	20.4 ± 1.3	68.3 ± 1.7	87.0 ± 0.5	7.3	2.1 ± 0.2
cycloartane-3β,24,25-triol (24*S*) (**12**)	3β, 24, 25		0.0 ± 0.3	(70)	16.8 ± 1.0	60.0 ± 1.7	87.4 ± 0.5	6.7	2.1 ± 0.2
25-methoxycycloartane-3β,24-diol (24*S*) (**13**)	3β, 24	25-OMe	0.0 ± 0.5	(70)	30.7 ± 1.4	74.5 ± 1.8	95.9 ± 0.3	9.5	1.8 ± 0.1
24-methylcycloartane-3β,24,24^1-triol (24ξ) (**14**)[e]	3β, 24, 24^1		0.0 ± 0.1	(70)	13.0 ± 1.0	63.2 ± 1.6	81.0 ± 0.7	6.1	2.2 ± 0.2
24^1-methoxy-24-methylcyclo-artane-3β,24-diol (24ξ) (**15**)[e]	3β, 24	24^1-OMe	0.0 ± 0.2	(70)	15.2 ± 1.2	65.4 ± 1.6	83.1 ± 0.6	6.7	2.1 ± 0.2
cycloartenol acetate (**16**)		3β-OAc	0.0 ± 0.5	(70)	32.3 ± 1.3	73.1 ± 1.7	96.9 ± 0.5	10.1	1.8 ± 0.2
cycloartenol ferulate (**17**)		3β-OFer	19.7 ± 0.7	(70)	56.0 ± 1.5	84.7 ± 2.3	100 ± 0.2	15.9	1.5 ± 0.1
24-methylenecycloartanol ferulate (**18**)		3β -OFer	17.6 ± 0.6	(70)	54.7 ± 1.6	82.0 ± 2.0	100 ± 0.3	15.8	1.8 ± 0.1
3-epicycloartenol (**19**)	3α		0.0 ± 0.3	(70)	29.7 ± 1.2	70.3 ± 1.7	93.7 ± 0.4	9.2	1.8 ± 0.1
3-epicycloartenol acetate (**20**)		3α-OAc	0.0 ± 0.3	(70)	33.2 ± 1.3	73.1 ± 1.8	96.0 ± 0.3	10.2	1.6 ± 0.1
cycloartenone (**21**)		3-oxo	2.3 ± 0.4	(70)	24.6 ± 1.2	71.3 ± 1.7	92.6 ± 0.4	8.8	1.5 ± 0.1
24-methylenecycloartan-3-one (**22**)		3-oxo	3.1 ± 0.4	(70)	26.4 ± 1.3	73.5 ± 1.8	96.1 ± 0.3	9.2	1.9 ± 0.2
4,4,14-trimethyl-9β,19-cyclopregnane-3,20-dione (**23**)		3, 20-dioxo	0.0 ± 0.3	(70)	22.1 ± 1.1	70.3 ± 1.7	94.6 ± 0.3	8.9	1.5 ± 0.1
cycloartane-3,24-dione (**24**)		3, 24-dioxo	0.0 ± 0.3	(70)	24.0 ± 1.3	72.1 ± 1.8	96.3 ± 0.3	9.0	1.9 ± 0.2
24-hydroxycycloart-25-en-3-one (24ξ) (**25**)[e]		3-oxo	0.0 ± 0.2	(70)	21.1 ± 1.2	69.7 ± 1.6	91.2 ± 0.4	8.6	2.0 ± 0.2
25-hydroxycycloart-23-en-3-one (23*E*) (**26**)	25	3-oxo	0.0 ± 0.3	(70)	20.0 ± 1.2	67.2 ± 1.7	90.1 ± 0.4	8.6	1.9 ± 0.1
24,25-dihydroxycycloartan-3-one (24ξ) (**27**)[e]	24, 25	3-oxo	0.0 ± 0.3	(70)	19.1 ± 1.0	66.8 ± 1.6	88.4 ± 0.5	7.4	2.0 ± 0.1
25-hydroxy-24-methoxycycloartan-3-one (24ξ) (**28**)[e]	25	3-oxo, 24-OMe	0.0 ± 0.3	(70)	21.0 ± 1.2	67.1 ± 1.7	94.6 ± 0.3	8.8	1.9 ± 0.2
24,25-dihydrocimicifugenol (**29**)	3β		0.0 ± 0.3	(70)	32.1 ± 1.2	72.6 ± 1.7	96.5 ± 0.3	10.1	1.6 ± 0.1
cimicifugenol (**30**)	3β		0.0 ± 0.3	(70)	26.8 ± 1.1	72.6 ± 1.8	93.8 ± 0.3	9.0	1.8 ± 0.1
17-isocycloartanol (**31**)	3β		0.0 ± 0.4	(70)	30.8 ± 1.2	71.2 ± 1.6	94.2 ± 0.4	9.4	1.6 ± 0.1
29-nor-cycloartane									
29-*nor*-cycloartanol (**32**)	3β		0.0 ± 0.3	(70)	31.5 ± 1.1	72.6 ± 1.7	95.6 ± 0.4	9.9	1.5 ± 0.1
29-*nor*-cycloartenol (**33**)	3β		0.0 ± 0.4	(70)	30.2 ± 1.1	71.4 ± 1.7	94.6 ± 0.4	9.5	1.8 ± 0.1
24-methyl-29-*nor*-cycloartanol (24ξ) (**34**)	3β		0.0 ± 0.4	(70)	31.8 ± 1.2	73.4 ± 1.6	96.8 ± 0.2	10.0	1.6 ± 0.1
cycloeucalenol (**35**)	3β		0.0 ± 0.3	(70)	26.5 ± 1.0	68.1 ± 1.6	94.8 ± 0.4	8.9	1.7 ± 0.1
cycloeucalenol acetate (**36**)		3β-OAc	0.0 ± 0.4	(70)	32.4 ± 1.3	73.5 ± 1.7	96.2 ± 0.4	10.1	1.5 ± 0.1
cycloeucalenol ferulate (**37**)		3β-OFer	14.2 ± 0.6	(70)	53.2 ± 1.6	81.5 ± 2.0	100 ± 0.4	15.5	1.5 ± 0.1
29-*nor*-cycloartane-3β,24,25-triol (24*S*) (**38**)	3β, 24, 25		0.0 ± 0.2	(70)	17.1 ± 1.1	61.3 ± 1.5	88.8 ± 0.3	6.7	1.7 ± 0.1
3-epicycloeucalenol (**39**)	3α		0.0 ± 0.4	(70)	30.5 ± 1.3	73.1 ± 1.7	95.9 ± 0.3	9.4	1.6 ± 0.1
3-epicyclomusalenol (24*S*) (**40**)	3α		0.0 ± 0.3	(70)	29.3 ± 1.2	71.5 ± 1.7	94.5 ± 0.4	9.3	1.8 ± 0.2
cycloeucalenone (**41**)		3-Oxo	0.0 ± 0.4	(70)	33.4 ± 1.3	74.0 ± 1.8	96.2 ± 0.4	10.3	1.5 ± 0.1
28-nor-cycloartane									
4-epicycloeucalenol (**42**)	3β		0.0 ± 0.2	(70)	29.9 ± 1.2	72.0 ± 1.7	95.1 ± 0.4	9.5	1.5 ± 0.1
4-epicycloeucalenone (**43**)		3-oxo	0.0 ± 0.4	(70)	32.7 ± 1.3	73.6 ± 1.8	95.1 ± 0.3	10.2	1.5 ± 0.1
4-epicyclomusalenone (24*S*) (**44**)		3-oxo	0.0 ± 0.4	(70)	32.4 ± 1.3	72.7 ± 1.7	94.6 ± 0.3	9.9	1.8 ± 0.2
28,29-dinor-cycloartane									
pollinastanol (**45**)	3β		0.0 ± 0.3	(70)	31.7 ± 1.2	73.1 ± 1.7	95.9 ± 0.4	9.9	1.5 ± 0.1
24-methylenepollinastanol (**46**)	3β		0.0 ± 0.4	(70)	30.5 ± 1.3	72.7 ± 1.8	95.8 ± 0.4	9.5	1.5 ± 0.1
24-methylenepollinastanol acetate (**47**)		3β-OAc	0.0 ± 0.4	(70)	31.4 ± 1.3	73.1 ± 1.8	96.3 ± 0.4	10.0	1.5 ± 0.1
24-methylenepollinastanone (**48**)		3-Oxo	0.0 ± 0.5	(70)	33.2 ± 1.4	73.8 ± 1.8	95.6 ± 0.4	10.2	1.5 ± 0.1
reference compound									
β-carotene			8.6 ± 0.5	(70)	34.2 ± 1.0	82.1 ± 2.0	100.0 ± 0.3	12.7	
curcumin			0.0 ± 0.5	(60)	22.8 ± 1.8	81.7 ± 1.9	100.0 ± 0.2	10.2	
retinoic acid			21.6 ± 1.0	(60)	49.3 ± 1.6	76.3 ± 1.9	100.0 ± 0.4	15.4	
glyzyrrhizin									2.2 ± 0.2
carboxy-PTIO									8.0 ± 0.3

[a] Values represent relative percentages to the positive control value. TPA (32 pM, 20 ng mL^{-1}) = 100%. Data are expressed as mean ± SD (n = 3). Values in parentheses are approximate viability percentages of Raji cells. [b] OFer = feruloyl. [c] IC_{50} represents the conentration (nM) that inhibits 50% of positive control (100%) activated with 32 pM TPA. [d] Determined at the concentration of 350 nM. Inhibitory ratio of NOR 1 (positive control; 350 nM) was taken as 1.0. Data are expressed as mean ± SD (n = 3). [e] Mixture of C-24 stereoisomers.[13]

From the results of in vitro EBV-EA induction and in vitro NOR 1 inhibition tests, and in vivo two-stage carcinogenesis, it appears that cycloartane-type and related triterpenoids, especially those with a hydroxy group at C-24 in the side chain, are valuable as chemopreventive agents in chemical carcinogenesis.

Experimental Section

General Experimental Procedures. ^{1}H NMR spectra were recorded on a JEOL LA-400 (400 MHz) spectrometer in $CDCl_3$ with TMS as internal standard. EIMS (70 eV) were recorded on a JEOL JMS-BU20 spectrometer using a direct inlet system. Reversed-phase preparative HPLC (with refractive index detector) was carried out on a C_{18} column (Hypersil ODS 5 μm column, 25 cm × 10 cm i.d.; Senshu Scientific Co., Ltd., Tokyo, Japan) at 25 °C with acetonitrile−H_2O−acetic acid (95:5:1) as mobile phase at 4.0 mL min^{-1}. Acetylation was performed in pyridine−acetic anhydride at room temperature overnight.

Test Compounds. Thirty compounds are natural products isolated from various plants: **2**, **3**, **17**, **18**, **35**, and **37** from rice bran,[7] **1** and **6** from Cucurbitaceae plants,[18] **4**, **5**, **32**, **33**, and **34** from Gramineae, Solanaceae, and other plants,[19] **7** from *Polypodium formosanum*,[20] **8** and **10** from *Bryonia dioica*,[21] **9**, **11**−**13**, and **38** from chrysanthemum flowers,[22] **30** from *Cimicifuga simplex*,[23] and **39**−**41**, **43**−**46**, and **48** from *Musa sapientum*.[23−25] Nine test compounds are biotransformation products of cycloartanes by the fungus *Glomerella fusarioides*: **14** and **15** from **3**; **21** from **2**; and **23**−**28** from **21**.[15] Seven test compounds

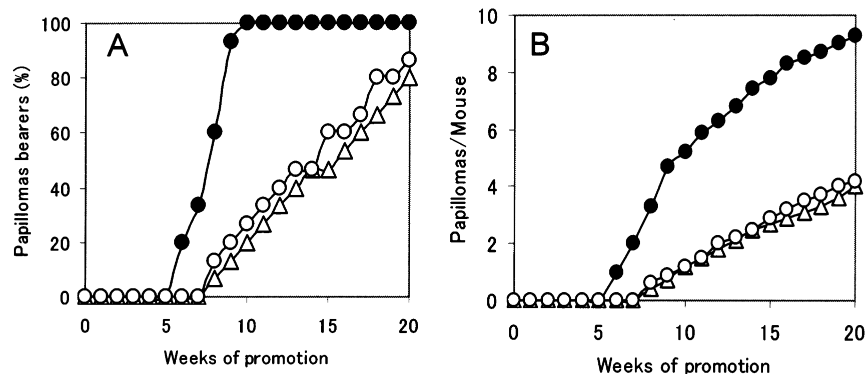


Figure 1. Inhibition of TPA-induced tumor promotion by multiple applications of (24ξ)-24-methylcycloartane-3β,24,24[1]-triol (**14**; △) and (24ξ)-24[1]-methoxy-24-methylcycloartane-3β,24-diol (**15**; ○). All mice ($n = 15$ for each of groups I, II, and III) were initiated with DMBA (390 nmol) and promoted 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 **14** (group II); ○, TPA + 85 nmol of **15** (group III). After 20 weeks of promotion, a significant difference in the number of papillomas per mouse between the groups treated with compounds **14** and **15** 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 9.3 (1.2), 4.0 (0.4), and 4.2 (0.6) for groups I, II, and III, respectively.

were semisynthesized from naturally occurring compounds: **29** and **31**,[23] **42**,[25] **47**,[26] **16** and **36**,[27] and **22**.[28]

Compound **19** was obtained from **21** by reduction with $LiAlH_4$ followed by the usual workup[24] and HPLC separation (retention time 17.6 min in HPLC), and compound **20** was prepared from **19** by acetylation.

3-Epicycloartenol (19): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.10 (1H, t, $J = 7.1$ Hz, H-24), 3.47 (1H, br s, H-3β), 1.69 (3H, s, H-26), 1.61 (3H, s, H-27), 0.96 (3H, s, H-18), 0.90 (3H, s, H-30), 0.89 (3H, d, $J = 6.4$ Hz, H-21), 0.95 and 0.89 (each 3H and s, H-28 and H-29), 0.51 (1H, d, $J = 4.2$ Hz, H-19 endo), 0.35 (1H, d, $J = 4.2$ Hz, H-19 exo); EIMS *m/z* 426 ([M]$^+$, 67), 411 (48), 408 (26), 393 (19), 365 (6), 313 (12), 297 (10), 286 (28), 271 (19), 259 (10), 69 (100).

3-Epicycloartenol acetate (20): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.10 (1H, t, $J = 6.9$ Hz, H-24), 4.69 (1H, br s, H-3β), 2.08 (3H, s, OCO*Me*), 1.68 (3H, s, H-26), 1.61 (3H, s, H-27), 0.97 (3H, s, H-18), 0.92 (3H, s, H-30), 0.88 (3H, d, $J = 6.4$ Hz, H-21), 0.93 and 0.85 (each 3H and s, H-28 and H-29), 0.51 (1H, d, $J = 4.1$ Hz, H-19 endo), 0.36 (1H, d, $J = 4.1$ Hz, H-19 exo); EIMS *m/z* 468 ([M]$^+$, 17), 453 (9), 408 (56), 393 (20), 365 (7), 339 (7), 297 (10), 286 (22), 271 (13), 203 (29), 69 (100).

Chemicals. Cell culture reagents and *n*-butanoic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan); β-carotene, glyzyrrhizin, curcumin, (*all-trans*)-retinoic acid, TPA, and DMBA from Sigma Chemical Co. (St. Louis, MO); and NOR 1 and carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide potassium salt] from Dojindo Laboratories (Kumamoto, Japan).

In Vitro EBV-EA Activation Experiment. The inhibition of EBV-EA activation was assayed using Raji cells (EBV genome-carrying human lymphoblastoid cells; nonproducer type), cultivated in 10% fetal bovine serum (FBS) RPMI-1640 medium (Nakalai Tesque, Kyoto, Japan). The indicator cells (Raji cells; 1×10^6 cells mL^{-1}) were incubated in 1 mL of the medium containing 4 mM *n*-butanoic acid as an inducer, 32 pM TPA (20 ng mL^{-1} in DMSO), and a known amount (32, 16, 3.2, 0.32 nM) of the test compound at 37 °C in a CO_2 incubator. After 48 h, the cell suspensions were centrifuged at 1000 rpm for 10 min, and the supernatant was removed. The activated cells were stained with high-titer EBV-EA-positive sera from nasopharyngeal carcinoma patients, and the conventional indirect immunofluorescence technique was employed for detection. In each assay, at least 500 cells were counted and the experiments were repeated three times. Triplicate assays were performed for each data point. The average extent of EA induction was determined and compared with that in positive control experiments in which the cells were treated with *n*-butanoic acid plus TPA, where the extent of EA induction was ordinarily more than around 40%. The viability of treated Raji cells was assessed by the trypan blue staining method. Details of the assay procedure have been reported.[28]

In Vitro NOR 1 Inhibition Experiment.[5] Chang liver cells (normal human hepatic cells; 5×10^5 mL^{-1}), derived from human liver in an MEM Eagle medium, were cultured 3 days before treatment. NOR 1 was added into the culture dish and incubated for 1 h under a CO_2 incubator as a control. For the screening assay, test samples were added to the culture dish 1 min before NOR 1 treatment. Transformed cells were observed under a light microscopy (×100). Triplicate assays were performed for each data point. All observed cells numbered more than 250. The inhibitory ratio was then calculated as follows:

$$\text{inhibitory ratio (I.R.)} = \frac{\text{transformed cell \% (NOR 1 alone)}}{\text{transformed cell \% (NOR 1 + test sample)}}$$

In Vivo Two-Stage Carcinogenesis Assay on Mouse Skin Papillomas. Each group of specific pathogen-free ICR mice obtained from Japan SLC (Shizuoka, Japan) was composed of 15 mice housed five per cage and given water ad libitum. The back of each mouse was shaved with surgical clippers, and the mouse was treated topically with DMBA (100 μg, 390 nmol) in acetone (0.1 mL) for the initiation treatment. One week after the initiation, papilloma formation was promoted by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) on the skin twice a week for 20 weeks. Group I received the TPA treatment alone, and group II received a topical application of test sample (85 nmol) in acetone (0.1 mL) 1 h before each TPA treatment. The incidence and numbers of papillomas were observed and detected weekly for 20 weeks; only typical papillomas larger than about 1 mm in diameter were counted. Details of this in vivo two-stage carcinogenesis test have been reported previously.[30]

Acknowledgment. The authors thank Dr. W. C. M. C. Kokke (Ardmore, PA) for reviewing the manuscript. Our thanks are also due to 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.

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NP068044U