[CANCER RESEARCH 48, 5210-5215, September 15, 1988]

Inhibition of the Tumor-promoting Action of 12-*O*-Tetradecanoylphorbol-13-acetate by Some Oleanane-type Triterpenoid Compounds

Hoyoku Nishino, Atsuko Nishino, Junko Takayasu, Teiko Hasegawa, Akio Iwashima, Kazuhiro Hirabayashi, Susumu Iwata, and Shoji Shibata

Department of Biochemistry, Kyoto Prefectural University of Medicine, Kawaramachi-dori, Kamigyo-ku, Kyoto 602 [H. N., A. N., J. T., T. H., A. I.]; Research Laboratory, Minophagen Pharmaceutical Co., Komatsubara 2-5233, Zama, Kanagawa-ken 228 [K. H., S. I.]; Meiji College of Pharmacy, Nozawa 1-35-23, Setagaya-ku, Tokyo 154 [S. S.], Japan

ABSTRACT

Since glycyrrhetinic acid was proved to suppress tumor promoter effects, several oleanane-type triterpenes which were chemically derived from oleanolic acid and hederagenin were tested *in vitro* and *in vivo* against the action of tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate.

By *in vitro* experiment monitoring with 12 - *O* - tetradecanoylphorbol-13-acetate-induced stimulation of $^{32}P_i$ incorporation into phospholipids and an *in vivo* test on skin tumor formation in mice initiated with 7,12-dimethylbenz[*a*]anthracene and promoted with 12-*O*-tetradecanoylphorbol-13-acetate, 18β-olean-12-ene-3β,28-diol (=erythrodiol), 18β-olean-12-ene-3β,23,28-triol, 18α-olean-12-ene-3β,28-diol, and 18α-olean-12-ene-3β,23,28-triol showed remarkable suppressive effects. Especially 18α-oleanane derivatives having a CH_2OH grouping converted from the COOH group initially allocated at C-17 were 100 times more effective than glycyrrhetinic acid both *in vitro* and *in vivo*.

INTRODUCTION

Several antiinflammatory substances have been known to inhibit the action of tumor promoters in the two-stage mouse skin carcinogenesis. Recently, some triterpenoid compounds were reported to have antitumor promoter action (1). Independently, glycyrrhetinic acid, which is known to have antiinflammatory activity, has been tested *in vitro* and *in vivo* against the tumor-promoting action induced by TPA[1] and teleocidin on the carcinogenic system initiated by DMBA (2). The time course of skin tumor formation in mice initiated by DMBA and promoted by either teleocidin or TPA was observed for 18–20 weeks with or without glycyrrhetinic acid. The experimental results showed that glycyrrhetinic acid remarkably suppressed the skin tumor formation. For example, at week 18 of tumor promotion, the group treated with DMBA (100 μg, single application) plus TPA (0.5 μg/painting, twice a week) produced 10.9 tumors/mouse, whereas the group treated with DMBA plus TPA and glycyrrhetinic acid (10 μmol, applied topically 40 min before each promoter application) had 1.5 tumors/mouse.

It has been known that the tumor promoter induces primarily the change of phospholipid metabolism *in vitro* and *in vivo* (3, 4), and the enhancement of phospholipid metabolism is suggested to play an important role in tumor promotion *in vivo*. Glycyrrhetinic acid was tested on TPA-induced $^{32}P_i$ incorporation into phospholipids of cultured cells. The experimental results showed 30.9% inhibition by the 25 μg/ml of glycyrrhetinic acid (2). This would suggest that the same mechanism would be involved in the antitumor promoter activity of glycyrrhetinic acid *in vivo*. Furthermore, besides glycyrrhetinic acid, various kinds of chemicals, which modulate cellular phospholipid metabolism, were proved to have antitumor-promoting activity *in vivo*, and the evaluation of the inhibitory potency for the TPA-enhanced phospholipid metabolism has been shown to be valuable for the screening of new antitumor promoters.

In reducing the pseudoaldosteronism of glycyrrhetinic acid while enhancing its therapeutic effects, several triterpenoid compounds were prepared by partial chemical modification of glycyrrhetinic acid (5, 6) and some related triterpenoids. These compounds were tested for the antitumor promoter effects on *in vitro* phospholipid synthesis enhanced by TPA. Some compounds which strongly antagonized the phospholipid synthesis promoted by TPA were subjected to the *in vivo* experiments on mouse skin carcinogenesis.

MATERIALS AND METHODS

Preparation of Triterpenoid Compounds

Erythrodiol (18β-Olean-12-ene-3β,28-diol) (III). Oleanolic acid (18β-olean-12-en-3β-ol-28-oic acid) (I) (1 g) dissolved in hexamethylphosphoric acid triamide (10 ml) was added with 25% NaOH (1 ml) to keep stirring for 1 h at room temperature. Ethyl bromide (1 ml) was added to the reaction mixture to allow the reaction to proceed for 20 h under stirring at room temperature. By acidification of the reaction mixture with 5% HCl (20 ml) followed by dilution with water, ethyl oleanolate was obtained quantitatively.

The dried ethyl oleanolate (1 g) dissolved in dried THF (20 ml) was dropped into a refluxed mixture of sodium aluminum bis(ethoxymethoxy) hydride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] dissolved in 70% toluene (3 ml) and THF (10 ml) under a stream of N_2. The reaction mixture was refluxed further for 1 h on an oil bath. After cooling to room temperature, 10% HCl was added gradually to the reaction mixture to adjust the acidity to pH 3–4. The precipitates formed by the decomposition of the aluminum complex were filtered off, and the filtrate was extracted with chloroform 3 times (50 ml each). The solvent was removed in a vacuum below 40°C to obtain erythrodiol (III) almost quantitatively (Fig. 1). Erythrodiol (III), m.p. 236–237°C, $[\alpha]_D$ +77°C (in $CHCl_3$), M^+.

$C_{30}H_{50}O_2$

Calculated: 442.3811
Found: 442.3850

18β-Olean-12-ene-3β,23,28-triol (IV). IV was prepared starting from hederagenin (18β-olean-12-ene-3β,23-diol-28-oic acid) (II) by the same procedure used for obtaining III from I (Fig. 1).

18β-Olean-12-ene-3β,23,28-triol (IV), m.p. 216-217°C, $[\alpha]_D$ +78.4°C ($CHCl_3$), M^+.

$C_{30}H_{50}O_3$

Calculated: 458.3743
Found: 458.3763

18α-Olean-12-ene-3β,28-diol (V). Oleanolic acid monoacetate (I') (4

Received 3/2/88; revised 6/17/88; accepted 6/21/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[*a*]anthracene; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet.

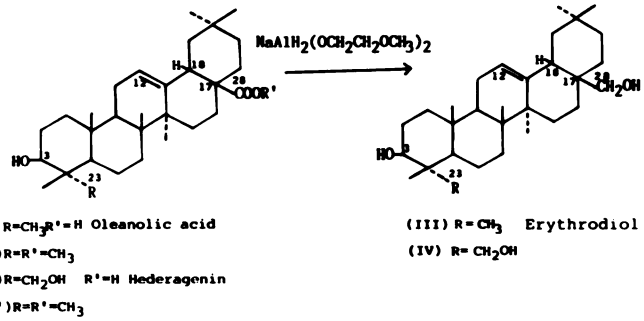


Fig. 1. Preparation of 18β-olean-12-ene-3β,28-diol (erythrodiol) (III) and 18β-olean-12-ene-3β,23-28-triol (IV).

g) dissolved in acetic acid (200 ml) was reacted with *tert*-butyl chromate (20 ml) at 60°C for 4 h on a water bath under shading. The reaction mixture was extracted with chloroform to obtain 11-oxooleanolic acid monoacetate (VIII) which was recrystallized from 95% ethanol (yield, 3 g).

Compound VIII (3 g) was added into a mixture of ethyleneglycol (180 ml) and NaOH (3 g/60 ml H_2O) and was allowed to react for 12 h at 105°C in an oil bath. After cooling, the reaction mixture was acidified with concentrated HCl and extracted 3 times with $CHCl_3$ (80 ml each). The solvent was distilled off after drying to obtain 18α-olean-12-ene-3β-ol-11-oxo-28-oic acid (X) which was recrystallized from chloroform or methanol (yield, 1 g).

Compound X (1 g) was methylated in chloroform solution with CH_2N_2 in ether. The methyl ester (X') (1 g) dissolved in dried THF was added dropwise into the refluxing mixture of $NaAlH_2(OCH_2CH_2OCH_3)_2$ in 70% toluene (3 ml) and dried THF (10 ml) under stirring in N_2. The reaction mixture was refluxed further for 1 h. After the reaction mixture had cooled to room temperature, its acidity was adjusted to pH 3–4 to decompose the aluminum complex. The precipitates formed were filtered and the filtrate was extracted with chloroform 3 times (50 ml each). The chloroform solution was evaporated below 40°C to obtain 18α-olean-12-ene-3β,11α or 11β-28-triol (XII).

Compound XII dissolved in ethanol (100 ml) was stirred with palladium-carbon (10%, 3 g) for 8 h under a stream of H_2. The reaction mixture was filtered and evaporated in a vacuum to obtain 18α-olean-12-ene-3β-28-diol (V), which was recrystallized from methanol (yield, 0.6 g) (Fig. 2), m.p. 264–266°C, $[\alpha]_D$ +41°C ($CHCl_3$), M^+.

$C_{30}H_{50}O_2$

Calculated: 442.3811
Found: 442.3781

^{13}C NMR δ ppm ($CDCl_3$): 142.6 (s, C-13), 117.4 (d, C-12), 78.1 (d, C-3), 56.8 (t, C-28), 55.9 (d, C-5), 47.7 (d, C-9).

18α-Olean-12-ene-3β,23,28-triol (VI). Hederagenin diacetate (18β-olean-12-ene-3β,23-diol-28-oic acid diacetate) (II') prepared from hederagenin (II) (1.5 g) by acetylation with acetic anhydride and pyridine was dissolved in acetic acid (30 ml) and reacted with *tert*-butyl chromate (2 ml) at 60°C for 4 h. The reaction mixture was extracted with chloroform 3 times (50 ml each) to separate 18β-olean-12-en-11-oxo-3β,23-diol-28-oic acid diacetate (IX) (yield, 1.58 g), m.p. 154–161°C.

Compound IX (1.58 g) was dissolved in acetic acid (30 ml) and added with concentrated HCl (30 ml) to react at 120°C for 4 h. The reaction mixture was extracted with chloroform 3 times (50 ml each). The dried chloroform extract was purified by means of silica gel chromatography using hexane-acetone mixture as the solvent to obtain 18α-olean-12-en-11-oxo-3β,23-diol-28-oic acid diacetate (XI) (yield, 0.35 g).

Methyl ester of XI (XI') dissolved in dried THF was dropped into the refluxed solution of $NaAlH_2(OCH_2CH_2-OCH_3)_2$ (2 ml) in THF (3 ml) under stirring in N_2. Stirring was continued for 1 h, and the reaction mixture was acidified to pH 3–4 by the gradual addition of 10% HCl at room temperature. The aluminum complex was decomposed in liberating gas to form precipitates. The filtrate was extracted with chloroform 3 times (50 ml each), and from the dried extract 18α-olean-12-ene-3β,11,23,28-tetraol (XIII) was separated, which was reduced catalytically in ethanol using 10% palladium-carbon (5 g) as the catalyst under stirring for 8 h. The reaction product separated from the catalyst was chromatographed over silica gel to yield 18α-olean-12-ene-3β,23,28-triol (VI) (yield, 0.57 g) (Fig. 2). 18α-Olean-12-ene-3β,23,28-triol (VI), m.p. 258–263°C, $[\alpha]_D$ +56°C M^+.

$C_{30}H_{50}O_3$

Calculated: 458.3743
Found: 458.3770

^{13}C NMR δ ppm ($CDCl_3$): 141.38 (s, C-13), 116.26 (D, C-12), 72.14 (d, C-3), 66.64 (t, C-23), 55.60 (d, C-28), 47.42 (d, C-5), 46.48 (d, C-9), 39.08 (d, C-18).

18α-Olean-12-ene-3β,30-diol (VII). 18β-Glycyrrhetinic acid (18β-olean-12-en-11-oxo-3β-ol-30-oic acid) (XIV) (2 g) was added into a mixture of ethylene glycol (60 ml) and NaOH (10 g in 20 ml H_2O), and reacted (60 ml) for 12 h at 110–120°C. After cooling, the reaction mixture was acidified with concentrated HCl and extracted 3 times with $CHCl_3$ (80 ml each). The residue obtained on evaporation of the solvent was recrystallized from $CHCl_3$ to obtain 18α-glycyrrhetinic acid (XV) (yield, 1 g).

18α-Glycyrrhetinic acid acetate or methyl 18α-glycyrrhetinate was subjected to reduction using $NaAlH_2(OCH_2CH_2OCH_3)_2$ in dried THF

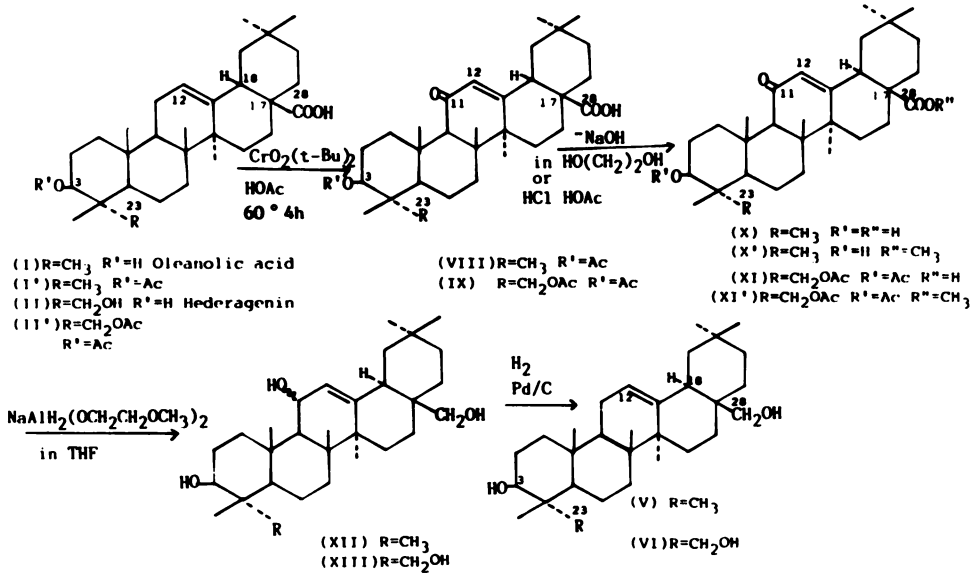


Fig. 2. Preparation of 18α-olean-12-ene-3β,28-diol (V) and 18α-olean-12-ene-3β,23,28-triol (VI).

by the procedure as mentioned above to produce 18α-olean-12-ene-3β,11,30-triol (XVI), which was catalytically reduced using 10% palladium-carbon in ethanol to yield 18α-olean-12-ene-3β,30-diol (18α-deoxoglycyrrhetol) (VII). The product was purified by silica gel chromatography followed by recrystallization from methanol (yield, 0.1 g) (Fig. 3).

18α-Olean-12-ene-3β,30-diol (VII), m.p. 254–256°C, $[\alpha]_D$ +63.2°C ($CHCl_3$) M^+.

$C_{30}H_{50}O_2$

Calculated: 442.3811
Found: 442.3814

From the crude product, the diacetate, m.p. 231–232°C, was prepared, which was recrystallized from methanol. ^{13}C-NMR ppm ($CDCl_3$): 171.3 (s, $CH_3\underline{C}O$), 176.9 (s, $CH_3\underline{C}O$), 142.6 (s, C-13), 117.3 (d, C-13), 80.9 (d, C-3), 75.3 (t, C-30), 55.4 (d, C-5), 47.2 (d, C-9), 43.7 (s, C-14), 39.6 (s, d, C-8 and C-18).

Compound VII was recovered quantitatively from the acetate (yield, 0.4 g).

$^{32}P_i$ Incorporation into Phospholipids of Cultured Cells

Incorporation of $^{32}P_i$ into phospholipids of HeLa cells, C3H10T½ cells or Swiss 3T3 cells was assayed as described previously (2).

Analysis of $^{32}P_i$-labeled phospholipids was carried out as follows. Aliquots of phospholipid fraction extracted from $^{32}P_i$-labeled cells were chromatographed on Silica Gel 60 thin-layer plates (E. Merck, Darmstadt, Federal Republic of Germany) in chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, by volume), and the autoradiograms of the thin-layer plates were prepared and then scanned by a densitometer.

In Vivo Two-Stage Carcinogenesis Experiment

The back of ICR mice (purchased from Shizuoka Laboratory Animal Center) at 7 weeks of age was shaved with an electric clipper. After 2 days, initiation was accomplished by a single application of 100 μg of DMBA on a shaved area. TPA, at the dose of 0.5 μg (0.81 nmol)/painting, was applied twice a week starting 1 week after the initiation. 18α- or 18β-olean-12-ene-3β,23,28-triol (VI or IV) (81 nmol; molar ratio to TPA, 100:1) dissolved in 100 μl of a vehicle consisting of acetone:dimethyl sulfoxide (99:1) was applied topically 40 min before each promoter application. Mice in control group were treated with the vehicle alone. The experiments was continued for 18 weeks. Each experimental group consisted of 15 mice. The number and size of tumors at the back of mice were determined once a week, and the body weight of mice was measured once a month.

RESULTS

Based on an idea for drug design that the conversion of COOH into CH_2OH may enhance the biological activity of triterpenoids (5, 6), a COOH group attached at C-17 of oleanolic acid and hederagenin was converted into CH_2OH by the action of $NaAlH_2(OCH_2CH_2OCH_3)_2$. By the *in vitro* system, the inhibiting activities of the original compounds and their modified derivatives against tumor promoter were compared. The idea was approved by the experimental results as shown in Table 1.

Contrary to the expectation, 18β-olean-12-ene-3β,30-diol (=18β-deoxoglycyrrhetol) which is a pharmacologically active key compound of the chemical modification of glycyrrhetinic acid revealed no inhibiting activity at 25 μg/ml against $^{32}P_i$ incorporation into phospholipids of HeLa cells enhanced by TPA (data not shown). However, by the stereochemical conversion at position 18 of the oleanane skeleton, 18α-olean-12-ene-3β,30-diol (=18α-deoxoglycyrrhetol) (VII) showed a strong inhibition of $^{32}P_i$ incorporation by 81.3% at the concentration of 25 μg/ml (Table 2).

The potentiation of biological activity by the conversion of 18β-H into 18α-H of oleanane-triterpenoids was demonstrated to be a general phenomenon (Table 2; Fig. 4).

Thus 18α-olean-12-ene-3β,23,28-triol (VI) showed the highest inhibition rate on the TPA action thus far tested. The 50% inhibitory dose was calculated as around 6 μM (3 μg/ml) (Fig. 4).

The inhibitory effect of VI on phospholipid synthesis was confirmed in other cell lines, such as C3H10T½ cells and Swiss 3T3 cells (Table 3).

Analysis of the 18α-olean-12-ene-3β,23,28-triol-induced change in phospholipid fractions revealed that $^{32}P_i$ incorporation into phosphatidylethanolamine and phosphatidylcholine was remarkably suppressed (Fig. 5). Inhibition of $^{32}P_i$ incorporation into phosphatidylinositol by treatment with VI was also distinct.

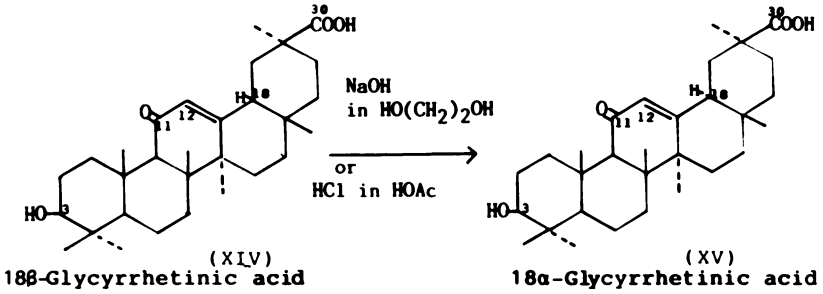


Fig. 3. Preparation of 18α-olean-12-ene-3β,30-diol (VII).

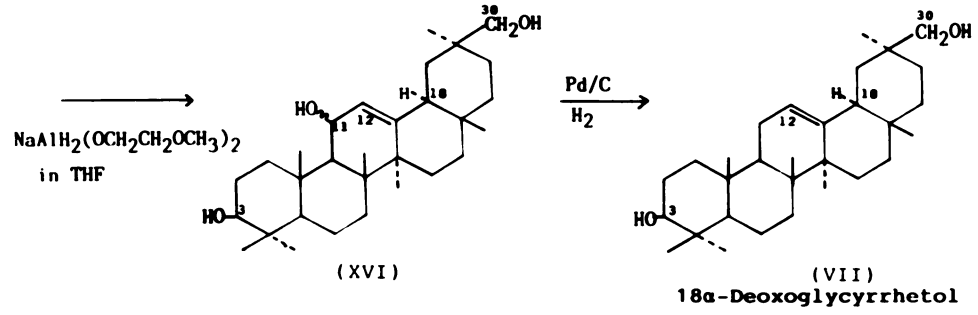


Table 1 *Effects of oleanane-type triterpenoids on the enhanced $^{32}P_i$ incorporation into phospholipids of HeLa cells induced by TPA*

HeLa cells were incubated with one of test compounds (25 μg/ml), and after 1 h, $^{32}P_i$ (20 μCi/culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, and then the radioactivity incorporated into phospholipid fraction was measured. Data, expressed as percentage of inhibition on TPA-enhanced $^{32}P_i$ incorporation, are mean values of duplicate experiments.

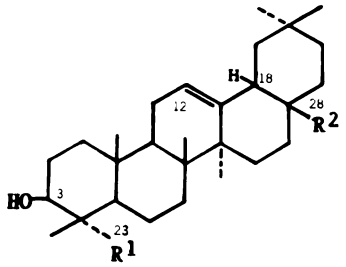
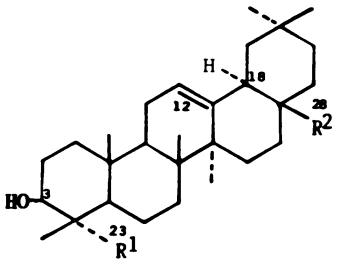
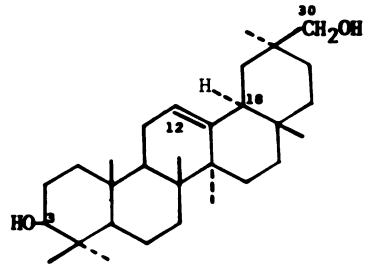
Compound	Inhibition %
	
Oleanolic acid (R^1=CH_3,R^2=COOH) (I)	48.0
Hederagenin (R^1=CH_2OH,R^2=COOH) (II)	72.6
Erythrodiol (R^1=CH_3,R^2=CH_2OH) (III)	81.1
18β-Olean-12-ene-3β,23,28-triol (R^1=R^2=CH_2OH) (IV)	100.0

Table 2 *Effects of oleanane-type triterpenoids on the enhanced $^{32}P_i$ incorporation into phospholipids of HeLa cells induced by TPA*

Experimental conditions were the same as those in Table 1.

Compound	Inhibition %
	
18α-Olean-12-ene-3β,28-diol (R^1=CH_3, R^2=CH_2OH) (V)	100.0
18α-Olean-12-ene-3β,23,28-triol (R^1=R^2=CH_2OH) (VI)	100.0
	
18α-Olean-12-ene-3β,30-diol (VII)	81.3

Under the experimental conditions in this study, glycyrrhetinic acid and the related triterpenoids did not show any cytotoxic effect on cultured cells; the number of cells was not decreased, and viability of these cells was not affected by treatment with these test compounds, either.

These results of *in vitro* experiments prompted us to examine the effect of VI and IV on *in vivo* carcinogenesis. Fig. 6 shows the time course of skin tumor formation of the groups treated with DMBA plus TPA, with or without VI or IV. The first tumor appeared at week 6 in the group treated with DMBA plus TPA. In the groups treated with DMBA plus TPA and VI or IV, the first tumor appeared at week 11. The comparison of control and triterpenoid-treated groups at week 18 is summarized as follows.

Since the tumor response had not reached to the plateau at week 18, the comparison was tentative. The percentage of

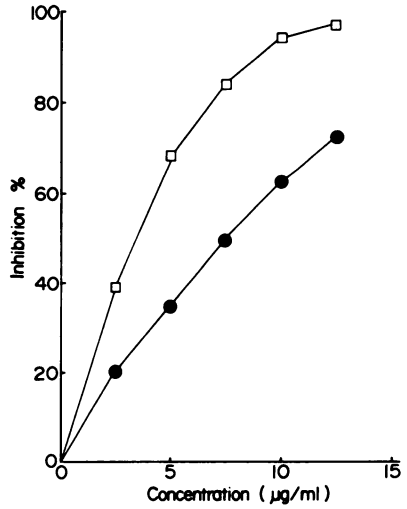


Fig. 4. Effect of 18α-olean-12-ene-3β,23,28-triol (□) and 18β-olean-12-ene-3β,23,28-triol (●) on the enhanced $^{32}P_i$ incorporation into phospholipids of HeLa cells induced by TPA.

HeLa cells cultured as a monolayer in Petri dishes (35 mm diameter) were incubated with various concentrations of 18α- and 18β-olean-12-ene-3β,23,28-triol. After 1 h, $^{32}P_i$ (20 μCi/culture) was added with or without TPA (50 nM). After 4 h, the radioactivity incorporated into phospholipids of cells was measured. Data, expressed as percentage of inhibition on TPA-stimulated $^{32}P_i$ incorporation, are mean values of duplicate experiments.

Table 3 *Effects of 18α- and 18β-olean-12-ene-3β,23,28-triol on enhanced $^{32}P_i$ incorporation into phospholipids of cultured cells induced by TPA*

Experimental conditions were the same as those in Table 1.

Condition	$^{32}P_i$ incorporation (cpm/mg protein × 10^{-4})	Inhibition %
C3H10T½ cells		
Control	4.51	
+ TPA	32.10	
TPA + 18β-olean-12-ene-3β,23,28-triol (IV)	18.27	50.1
+ TPA + 18α-Olean-12-ene-3β,23,28-triol (VI)	10.03	80.0
Swiss 3T3 cells		
Control	6.11	
+ TPA	15.54	
+ TPA + 18β-olean-12-ene-3β,23,28-triol (IV)	10.01	58.6
+ TPA + 18α-Olean-12-ene-3β,23,28-triol (VI)	7.12	89.3

tumor-bearing mice treated with DMBA plus TPA was 100%, whereas that of the group treated with DMBA and TPA plus VI or IV were 20 and 40%, respectively (Fig. 6*A*). Compounds VI and IV also decreased the average number of tumors per mouse (Fig. 6*B*). The group treated with DMBA plus TPA without triterpenoid compounds produced 10.6 tumors/mouse at week 18, whereas the group treated with DMBA plus TPA and VI or IV had 0.6 ($P < 0.001$, Student's t test) or 1.8 ($P < 0.001$) tumors/mouse, respectively. The size of tumors was smaller in the group treated with triterpenoid compounds than that in the control group (Table 4). Body weight measurement during the experiment showed that both 18α- and 18β-olean-12-ene-3β,23,28-triol did not cause any growth retardation (data not shown). Furthermore, we observed that the triterpenoid-treated mice had smooth skin, while those treated with tumor promoter alone had multiple foci of inflammation, necrosis, and scarring. Therefore, the terpenoids seemed not to have general toxicity but rather to prevent the toxic and inflammatory reaction induced by tumor promoter. Thus, VI and IV were proved to have remarkable antitumor-promoting activity *in vivo* without any toxicity. Compared with the previous results of glycyrrhetinic acid (2), these compounds were much more

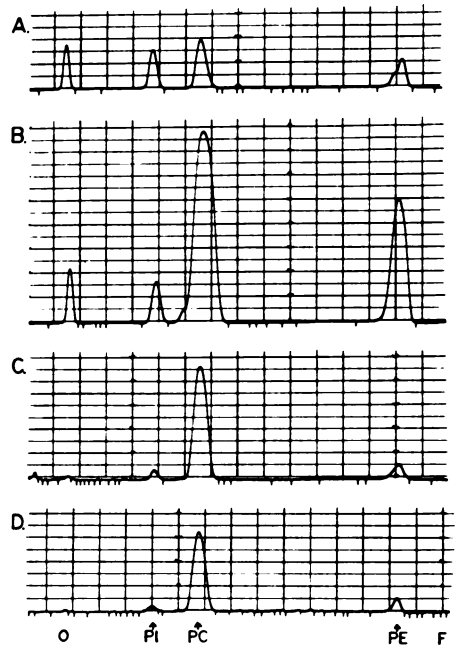


Fig. 5. Analysis of phospholipids containing $^{32}P_i$ in HeLa cells incubated with TPA in the presence or absence of triterpenoid compounds.

HeLa cells were incubated with $^{32}P_i$ with or without TPA (50 nM) for 4 h after pretreatment with or without triterpenoid compounds (12.5 μg/ml) for 1 h. Phospholipids were extracted and analyzed by thin-layer chromatography, followed by autoradiography and densitometry. *A*, control; *B*, +TPA; *C*, +18β-olean-12-ene-3β,23,28-triol + TPA; *D*, +18α-olean-12-ene-3β,23,28-triol + TPA. *PI*, phosphatidylinositol; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine.

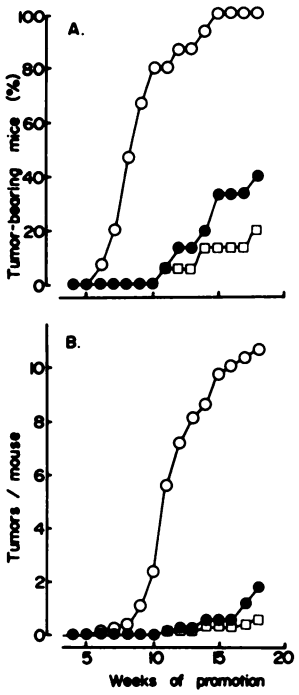


Fig. 6. Effect of triterpenoid compounds on the promotion of skin tumor formation by TPA in DMBA-initiated mice.

From 1 week after initiation by a single application of 100 μg of DMBA, 0.5 μg (0.81 nmol) of TPA was applied twice a week. 18α- and 18β-olean-12-ene-3β,23,28-triol (81 nmol; molar ratio to TPA, 100:1) was applied topically 40 min before each TPA application. *A*, percentage of tumor-bearing mice; *B*, tumor incidence. ○, group treated with DMBA plus TPA; □ and ●, groups treated with DMBA plus TPA and 18α- or 18β-olean-12-ene-3β,23,28-triol, respectively.

active in suppressing tumor promotion *in vivo*; the activity of VI was potentiated more than 100 times that observed in glycyrrhetinic acid (XIV).

Table 4 *Effect of 18α- and 18β-olean-12-ene-3β,23,28-triol on the promotion of skin tumor formation by TPA in DMBA-initiated mice.*

Experimental conditions were as described in Fig. 6. At week 18 of promotion, the diameter and number of tumors were measured.

Condition	Av. no. of tumors/mouse				
	Size of tumors (mm)				
	1–3	3–5	5–7	7–9	Total
Control	6.20	3.20	1.07	0.13	10.6
+ 18β-Olean-12-ene-3β,23,28-triol (IV)	1.73	0.07			1.8
+ 18α-Olean-12-ene-3β,23,28-triol (VI)	0.53	0.07			0.6

The inhibitory potency of VI against tumor promoter *in vivo* was higher than that of IV, which correlated well with the activity *in vitro*.

DISCUSSION

Cancer prevention is now the most urgently required for public health. It is especially worthy to develop the method for prevention at the promoting stage of carcinogenesis, since such method might be applicable even after the hit of tumor-initiating agents, which seems, in many cases, unavoidable in human life. In this context, the application of antitumor-promoting triterpenoids is highly promising for protection against tumor formation; the terpenoids developed in this study seem to be especially valuable, since they did not show any particular toxicity *in vivo*.

Although the mechanism of the antitumor-promoting effect of 18α- and 18β-olean-12-ene-3β,23,28-triol and other related triterpenes used in this study is not known, the modulation of phospholipid metabolism appeared to be one of the important aspects; it was proved that the inhibitory potency of the triterpenoids for the TPA-enhanced phospholipid synthesis correlated well to their antitumor-promoting activity.

The triterpenoid compounds were found to inhibit the inflammatory reaction induced by tumor promoters. This antiinflammatory activity may play an important role in the mechanism of antitumor promotion as demonstrated in many cases (7).

Recently, we found that glycyrrhetinic acid and its related compounds interact with calmodulin, an intracellular Ca^{2+}-binding protein (published elsewhere). Since calmodulin is an essential protein for cell proliferation, the calmodulin-interacting property of the triterpenoids seems to be important for antitumor-promoting action.

Glycyrrhetinic acid was proved to inhibit the binding of TPA to its receptor (8), which has been suggested to be Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C) (9). Therefore, glycyrrhetinic acid and the related triterpenes may also affect the TPA binding in modulating the activity of protein kinase C. Since protein kinase C has been suggested to be one of the key enzymes in the signal-transducting system to control cellular functions including the expression of oncogenes, cell proliferation, and transformation (10), the analysis of the interaction of triterpenoids with protein kinase C is worthy to know their mechanism of action.

ACKNOWLEDGMENTS

The authors are grateful to Dr. T. Sugimura, President of National Cancer Center, Tokyo, and to T. Mori, Head of the Research Laboratory, Minophagen Pharmaceutical Co., for their advice and encouragement.

REFERENCES

1. Ohigashi, H., Takamura, H., Koshimizu, H., Tokuda, H., and Ito, Y. Search for possible antitumor promoters by inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation. Ursolic acid and oleanolic acid from an anti-inflammatory Chinese medicinal plant, *Glechoma hederaceae* L. Cancer Lett., *30:* 143–151, 1986.
2. Nishino, H., Yoshioka, K., Iwashima, A., Takizawa, H., Konishi, S., Okamoto, H., Okabe, H., Shibata, S., Fujiki, H., and Sugimura, T. Glycyrrhetic acid inhibits tumor-promoting activity of teleocidin and 12-*O*-tetradecanoylphorbol-13-acetate in two-stage mouse skin carcinogenesis. Jpn. J. Cancer Res., *77:* 33–38, 1986.
3. Rohrschneider, L. R., and Boutwell, R. K. The early stimulation of phospholipid metabolism by 12-*O*-tetradecanoylphorbol-13-acetate and its specificity for tumor promotion. Cancer Res., *33:* 1945–1953, 1973.
4. Rohrschneider, L. R., O'Brien, D. H., and Boutwell, R. K. The stimulation of phospholipid metabolism in mouse skin following phorbol ester treatment. Biochim. Biophys. Acta, *280:* 57–70, 1972.
5. Shibata, S., Takahashi, K., Yano, S., Harada, M., Saito, H., Tamura, Y., Kumagai, A., Hirabayashi, K., Yamamoto, M., and Nagata, N. Chemical modification of glycyrrhetinic acid in relation to the biological activities. Chem. Pharm. Bull. (Tokyo), *35:* 1910–1918, 1987.
6. Takahashi, K., Shibata, S., Yano, S., Saito, H., Tamura, Y., and Kumagai, A. Chemical modification of glycyrrhetinic acid in relation to the biological activities (preliminary report). Chem. Pharm. Bull. (Tokyo), *28:* 3449–3452, 1980.
7. Nishino, H., Kitagawa, K., Fujiki, H., and Iwashima, A. Berberine sulfate inhibits tumor-promoting activity of teleocidin in two-stage carcinogenesis on mouse skin. Oncology, *43:* 131–134, 1986.
8. Kitagawa, K., Nishino, H., and Iwashima, A. Inhibition of the specific binding of 12-*O*-tetradecanoylphorbol-13-acetate to mouse epidermal membrane fractions by glycyrrhetinic acid. Oncology, *43:* 127–130, 1986.
9. Blumberg, P. M. Protein kinase C as the receptor for the phorbol ester tumor promoters. Cancer Res., *48:* 1–8, 1988.
10. Persons, D. A., Wilkinson, W. O., Bell, R. M., and Finn, O. J. Altered growth regulation and enhanced tumorigenicity of NIH3T3 fibroblasts transfected with protein kinase C-1 cDNA. Cell, *52:* 447–458, 1988.