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Original article

Conjugates of 3α-methoxyserrat-14-en-21β-ol (PJ-1) and 3β-methoxyserrat-14-en-21β-ol (PJ-2) as cancer chemopreventive agents

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In vivo two-stage mouse-skin carcinogenesis test

ABSTRACT

3α-Methoxyserrat-14-en-21β-ol (**PJ-1**) and 3β-methoxyserrat-14-en-21β-ol (**PJ-2**) were conjugated with well-known phenolic compounds, narigenin, hesperetin, genistein, and daidzein (**1**–**8**). Other conjugates of **PJ-2**–3,5-dihydroxy-4-methoxybenzoic acid (**9**), **PJ-2**–pyrogallol (**10**), and derivatives of **PJ-1**, **PJ-2** –3,3-dimethyl-succinates (**11**, **12**), **PJ-1**, **PJ-2**–succinates (**13**, **14**), **PJ-2**–glycine (**15**), **PJ-2**–piperidine acetic acid (**16**), and **PJ-1 epoxy**–3,3-dimethyl-succinate (**17**) were tested for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The inhibitory effects of **11** ($IC_{50} = 251$), **12** ($IC_{50} = 248$), and **17** ($IC_{50} = 230$ mol ratio/32 pmol/TPA), were 2-fold stronger than those of the other compounds such as oleanolic acid ($IC_{50} = 449$). Compounds **10**, **11**, and **17** inhibited mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis model. The *in vivo* two-stage mouse-skin carcinogenesis test employed 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter.

1. Introduction

It is said that the conjugation of two bioactive compounds is an effective strategy that is now well accepted [1]. Hybrids including natural triterpenoids play an important role in the development of drugs for treatment against infections and cancer, particularly immunosuppressive compounds [2]. As well-known triterpenoid hybrids, Iveson et al. reported β-glycyrrhizic acid–glucuronyl esters [3], Baltina et al. found glycyrrhizic acid–glucopeptide conjugates with an immune response [4], Tatsuzaki et al. reported glycyrrhizic acid–dehydrozingerone conjugates as cytotoxic agents [5] and Ma et al. found triterpene–azidothymidine conjugates on the proliferation of HIV-1 and its protease [6]. We previously reported 17 hybrids of 3α-methoxyserrat-14-en-21β-ol (**PJ-1**) or 3β-methoxyserrat-14-en-21β-ol (**PJ-2**), and kojic acid, quercetin, and baicalein were used to evaluate their anti-HIV-1 reverse transcriptase (RT) activities in infected C8166-CCR5 cells, a human $CD4^+$ T-lymphocyte cell line. Among them, a conjugate of two molecules of **PJ-1** and one molecule of kojic acid using succinic acid as a linker had significant anti-HIV activity with an EC_{50} value of 0.12 μg/mL [7]. Additionally, we recently reported the effect of *in vitro* and *in vivo* anti-tumor promoting activity of the 17 hybrids, along with phenylcarbamate, 2-chlorophenylcarbamate, carbamate, *N*-[(4-methylphenyl)sulfonyl]carbamate, phenylcarbamothioate and chloroacetate derivatives. Among the hybrids, one molecule of **PJ-1** or **PJ-2**, and one molecule of quercetin using succinic acid as a linker showed strong anti-tumor promoting activity *in vivo* [8]. In our follow-on paper, herein we report the *in vitro* and *in vivo* anti-tumor promoting activity of new synthetic analogs (**1**–**17**) derived from **PJ-1** and **PJ-2**. The screening methods employed were a convenient primary *in vitro* assay to estimate the inhibitory effect on Epstein–Barr virus early antigen (EBV-EA) activation induced by a well-known tumor promoter, TPA [9], and an *in vivo* two-stage mouse-skin carcinogenesis test using DMBA as an initiator and TPA as a tumor promoter [10].

2. Results and discussion

The genus *Picea* consists of approximately 40 species, similar to those of genera *Pinus* and *Abies* in the order *Pinales*. *Picea* species do not have any use except in construction materials. In recent years, more effective and safer agents have been intensively required for

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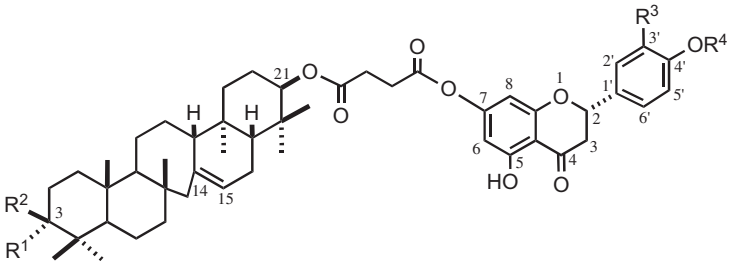
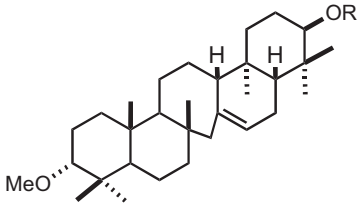
the chemoprevention of human cancer, while natural products from plants and their synthetic derivatives are expected to play an important role in developing innovative agents to inhibit the onset of cancer [11,12]. In particular, there is a need for agents targeting for the promoting stage of carcinogenesis in the two- or multi-stage theory [13] since it is difficult to avoid detrimental contact with tumor-initiating agents in human life, but tumor promotion is a long and reversible stage that can be efficiently suppressed [10,14].

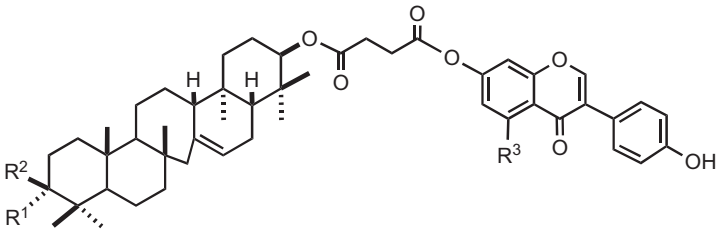
In our quest for biologically active constituents from natural sources, we found compounds showing significant anti-tumor promoting activities in an *in vivo* two-stage mouse-skin carcinogenesis assay using 7,12-dimethylbenz[a]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA): 13α,14α-epoxy-3β-methoxy 21β-ol and 21α-hydroxy-3β-methoxyserrat-14-en-29-al [10], serrat-14-en-3β,21β-diol [15], 3α-methoxyserrat-14-en-21β-ol (**PJ-1**) and 3β-methoxyserrat-14-en-21β-ol (**PJ-2**) [16], and 14β,15β-epoxy-3β-methoxyserratan-21β-ol [17] from the bark of *Picea jezoensis* (Sieb. et Zucc) Carr. var. *jezoensis* (Pinaceae, Japanese name: Ezomatsu), and *P. jezoensis* (Sieb. et Zucc) Carr. var. *hondoensis* (Mayr) Rehder (Pinaceae, Japanese name: Touhi). **PJ-1** and **PJ-2** are the most abundant triterpenoid constituents from two of the above two *Picea* plants and the total yields of **PJ-1** and **PJ-2** accounted for more than 1/3 of the chloroform extract of the above two plants. It was reported that **PJ-1** significantly decreased the size of adenomas and total tumors in a revised rat multi-organ carcinogenesis (DMBDD) model [18]. Recently, we reported that 13α,14α-epoxy-3β-methoxyserratan-21β-



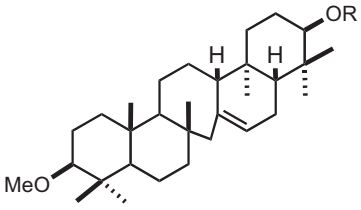
1 : R^1 = OMe, R^2 = R^3 = R^4 = H
2 : R^1 = H, R^2 = OMe, R^3 = R^4 = H
3 : R^1 = OMe, R^2 = H, R^3 = OH, R^4 = Me
4 : R^1 = H, R^2 = OMe, R^3 = OH, R^4 = Me



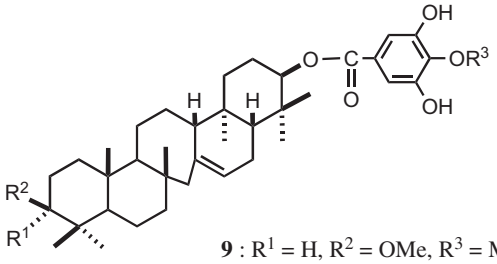
PJ-1: R = H
11 : R = $COCH_2C(CH_3)_2CO_2H$
13 : R = $COCH_2CH_2CO_2H$



5 : R^1 = OMe, R^2 =H, R^3 = OH
6 : R^1 = H, R^2 = OMe, R^3 = OH
7 : R^1 = OMe, R^2 = R^3 =H
8 : R^1 = R^3 = H, R^2 = OMe



PJ-2: R = H
12 : R = $COCH_2C(CH_3)_2CO_2H$
14 : R = $COCH_2CCH_2CO_2H$
15 : R = $COCH_2NH_2$
16 : R = $COCH_2$-N



9 : R^1 = H, R^2 = OMe, R^3 = Me
10 : R^1 = R^3 = H, R^2 = OMe

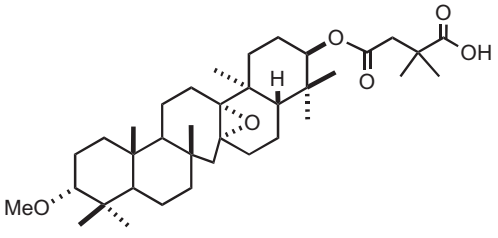


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Fig. 1. 3α-methoxyserrat-14-en-21β-ol (**PJ-1**) and 3β-methoxyserrat-14-en-21β-ol (**PJ-2**) and their conjugates or derivatives (**1**-**17**).

Table 1
Relative ratio[a] of EBV-EA activation levels (%) in the presence of compounds **1**-**17** and oleanolic acid.

Compound	Concentration (mol ratio/TPA)				IC_{50} (nM)
	1000	500	100	10	
1	14.3 (60)[b]	48.7	81.0	100	473
2	14.0 (60)	47.3	80.0	100	470
3	13.6 (60)	47.3	80.3	100	473
4	13.2 (60)	47.0	80.1	100	460
5	12.2 (60)	45.9	81.0	100	458
6	12.0 (60)	45.2	79.6	100	455
7	15.8 (60)	50.3	83.4	100	498
8	15.0 (60)	49.4	82.6	100	495
9	4.7 (60)	37.8	72.5	93.4	375
10	2.2 (60)	36.5	71.3	91.5	290
11	0 (60)	29.7	67.5	91.5	251
12	0 (60)	28.5	67.0	90.3	248
13	0 (60)	32.4	68.3	92.4	281
14	0 (60)	33.6	69.5	93.8	285
15	12.0 (60)	42.5	80.3	100	449
16	13.5 (60)	46.3	82.0	100	460
17	0 (60)	26.6	68.4	89.6	230
Oleanolic acid[c]	12.7 (70)	30.0	80.0	100	449

[a] The value obtained in the assay where the EBV-EA activation was performed by treatment with TPA (32 pmol) alone (without adding any triterpenoids) was evaluated as 100%.
[b] Values in parentheses are the percentage viability of Raji cells.
[c] A standard sample to compare the inhibitory activities of **1**–**17** against EBV-EA activation.

ol (PJJ-34) is chemopreventive against lung and colon carcinogenesis without exerting apparent toxicity [19]. Although oleanolic acid, ulsoric acid and betulinic acid were often modified into various forms, *e.g.* 3-*O*-(3,3′-dimethyl-succinyl)-betulinic acid (**PA-457**) as an anti-HIV agent [20,21], serratane-type triterpenoids, such as **PJ-1** and **PJ-2**, were not modified. Now, we continued the study of **PJ-1** and **PJ-2** conjugates on the basis of the hybrid drug strategy, targeting for anti-tumor promoting agents. In the present study, we synthesized 17 new compounds (**1**–**17**) on the basis of the diverse bioactivities of the triterpenoids as well as phenolic or benzoic acid compounds having anti-oxidant activity, such as naringenin, hesperetin, genistein, daidzein, 3,5-dihydroxy-4-methoxybenzoic acid, glycine, pyrogallol, and piperidineacetic acid (Fig. 1). Being encouraged by the fact that **PA-457** (*vide info*) is now assessed in a Phase II clinical trial, **PJ-1** and **PJ-2**, **PJ-1 epoxy**–3,3-dimethyl-succinyl analogs (**11**,**12**,**17**) were also prepared along with **PJ-1** and **PJ-2**–succinyl derivatives (**13**,**14**) for anti-tumor promoting assay.

First, these compounds (**1**–**17**) were subjected to the *in vitro* inhibition assay against EBV-EA activation. Compounds **1** and **2** are **PJ-1**, **PJ-2**–naringenin [22] conjugates, compounds **3** and **4** are **PJ-1**, **PJ-2**–hesperetin [23] conjugates, compounds **5** and **6** are **PJ-1**, **PJ-2**–genistein [24] conjugates, compounds **7** and **8** are **PJ-1**, **PJ-2**–daidzein [25] conjugates, compound **9** is a **PJ-2**–3,5-dihydroxy-4-methoxybenzoic acid [26] conjugate, compound **10** is a **PJ-2**–pyrogallol [27] conjugate, compounds **11** and **12** are **PJ-1**, **PJ-2**–3,3-dimethyl-succinyl derivatives, compounds **13** and **14** are **PJ-1**, **PJ-2**–succinyl derivatives, compound **15** is a **PJ-2**–glycine [28] conjugate, compound **16** is a **PJ-2**–piperidine acetic acid [29] derivative, and compound **17** is a synthetic **PJ-1-13α,14α-epoxy** (**PJ-1 epoxy**)–3,3-dimethyl-succinyl derivative (Experimental Section, Fig. 1).

We report the results of *in vitro* and *in vivo* anti-tumor promoting activities of new synthetic analogs (**1**–**17**) (Fig. 1). EBV-EA is activated by tumor promoters, producing viral early antigen (EA), and the evaluation of its inhibitors is used as a primary screen for *in vivo* anti-tumor promoting activities [30]. The results of the *in vitro* inhibitory activities of synthetic **1**–**17** against EBV-EA activation are shown in Table 1. Herein, the value obtained when the EBV-EA activation was performed by treatment with TPA (32 pmol) alone was evaluated as 100%. Oleanolic acid was used as a comparison standard on screening the test compounds **1**–**17**. Moreover, their effects on the viability of Raji cells and their 50% inhibitory concentration (IC_{50}) values are also shown in Table 1. All compounds exhibited dose-dependent inhibitory activities, and the viability of Raji cells treated with the test compounds **1**–**17** was 60% at the highest concentration of 1000 mol ratio/TPA, of which the unit, mol ratio/TPA, presented mol volume of each test compound per that of TPA (32 pmol). The results suggested that these compounds showed moderate cytotoxicity against cell lines *in vitro* (Table 1). The inhibitory activities were as follows: **10** (IC_{50} = 290 mol ratio/32 pmol/TPA), **11** (IC_{50} = 251), **12** (IC_{50} = 248), **13** (IC_{50} = 281), **14** (IC_{50} = 285), and **17** (IC_{50} = 230). Compounds **11**–**14** and **17** are 3,3-dimethyl-succinyl derivatives, or succinyl derivatives, and their EBV-EA activation was stronger than those of the other compounds. The relative rates of **11**, **12** and **17** (**PJ-1**, **PJ-2** and **PJ-1-epoxy**–3,3-dimethyl-succinyl derivatives) with respect to TPA (100%) were 0, 29.7, 67.5, 91.5%, and 0, 28.5, 67.0, 90.3%, and 0, 26.6, 68.4 and 89.6% at concentrations of 1000, 500, 100, and 10 mol ratio/TPA, respectively (Table 1), showing 100, 70.3, 32.5, and 8.5% (compound **11**), and 100, 71.5, 33.0, and 9.7% (compound **12**), and 100, 73.4, 31.6, and 10.4% (compound **17**) inhibition of TPA-induced EBV-EA activation, respectively. On the other hand, compound **10** (**PJ-2**–pyrogallol hybrid) showed 97.8, 63.5, 28.7, 8.5% inhibition of TPA-induced EBV-EA activation at concentrations of 1000, 500, 100, and 10 mol ratio/TPA, respectively. Although hybrids of **PJ-1** or **PJ-2** and naringenin, hesperetin, genistein, daidzein (**1**–**8**) did not show preferable inhibitory activities, the comparable compact size of modification or hybrids, *e.g.*, compounds **10**–**14**, exhibited potent inhibitory activities.

In our previous works, we found that the inhibitory effects on EBV-EA induction by TPA correlated well with the anti-tumor promoting activity *in vivo* [15–17]. Thus, we selected compounds **10**, **11** and **17** among the 17 compounds to examine their effects on *in vivo* two-stage carcinogenesis using mouse-skin papillomas induced by DMBA as an initiator and TPA as a promoter. The experimental protocol is shown in Fig. 2. During the *in vivo* test, the body-weight gains of the mice were not influenced by treatment with the test compounds and no toxic effects, such as lesional damage and inflammation (edema, erosion and ulcer), were observed in areas of mouse skin topically treated with the test compounds. As shown in Fig. 3A, papilloma-bearing mice in the positive control group treated with DMBA (390 nmol) and TPA (1.7 nmol, twice/week) appeared as early as at week 6, and the percentage of papillomas bearers increased rapidly to reach 100% after week 11. On the other hand, treatment with compound **11** (**PJ-1**–3,3-dimethyl-succinyl derivative) (85 nmol) along with DMBA/TPA reduced the percentage of papilloma-bearing mice to 13.3–46.6% during weeks 10–15, and thereafter 80.0% during week 20. As shown in Fig. 3B, in the positive control group treated with DMBA/TPA, the number of papillomas formed per mouse increased rapidly after week 6 to reach 8.0 papillomas/mouse at week 20, whereas mice treated with **11** bore only 3.8 papillomas (tumor length 16 ± 1.0 mm, tumor width 14 ± 0.6 mm). As shown in Fig. 3A, treatment with compounds **10** (**PJ-2**–pyrogallol hybrid), **17** (**PJ-1 13α,14α-epoxy**–3,3,-dimethyl-succinyl derivative) (85 nmol), along with DMBA/TPA reduced the percentage of papilloma-bearing mice to 20.0–53.3% during weeks 10–15 in **10**, and 26.6–66.6% during weeks 10–15 in **17**, and thereafter 86.6 and 93.3% in **10** and **17** during week 20. As shown in Fig. 3B, after treatment with compounds **10** or **17**, the number of papillomas formed per mouse increased rapidly after week 8 to reach 4.1 papillomas/mouse at week 20 in **10** (tumor length 18 ± 1.1 mm,

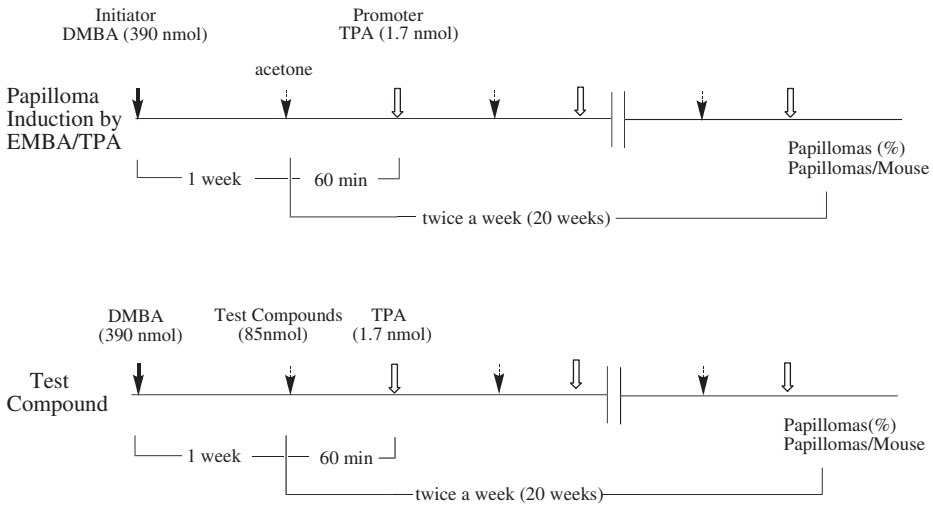

Fig. 2. Experimental design of chemopreventive activity of compounds **10**, **11** and **17** on DMBA-TPA-induced carcinogenesis.

tumor width 15 ± 0.7 mm), and 4.8 papillomas/mouse at week 20 in **17** (tumor length 18 ± 1.0 mm, tumor width 17 ± 0.7 mm). In the *in vivo* two-stage mouse-skin carcinogenesis test, **10**, **11** and **17** were found to delay papilloma formation. The results of *in vitro* EBV-EA induction and the *in vivo* two-stage mouse-skin carcinogenesis test suggest that serratane-type triterpenoids, **PJ-1**–3,3-dimethylsuccinyl derivative, and **PJ-2**–pyrogallol hybrid, such as **10** and **11**, are useful cancer chemopreventive agents. Further *in vivo* tests of the remaining effective compounds **12**–**14** are in progress.

3. Experimental

3.1. Chemistry

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter. IR spectra were recorded using a Perkin–Elmer 1720X FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. $CDCl_3$ was used as the solvent and TMS as the internal standard. EIMS were recorded on a Hitachi 4000H double-focusing mass spectrometer (70 eV). Column chromatography was carried out over silica gel (70–230 mesh; Merck) and medium-pressure liquid chromatography (MPLC) was carried out with silica gel (230–400 mesh; Merck). HPLC was run on a JASCO PU-1586 instrument equipped with a differential refractometer (RI 1531). Fractions obtained from column chromatography were monitored by TLC (silica gel 60 F_{254}; Merck). Preparative TLC was carried out on Merck silica gel F_{254} plates (20 × 20 cm, 0.5 mm thick).

3.1.1. Chemicals

Phenyl isocyanate, chlorosulfonyl isocyanate, *p*-toluenesulfonyl isocyanate and chloroacetyl chloride (GR) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2-Chlorophenyl isocyanate and phenyl isothiocyanate (GR) were obtained from Tokyo Chemical Industry (TCI) Ltd. (Tokyo, Japan). *N,N*-dimethylaminopyridine (DMAP) and chloroacetyl chloride were purchased from Sigma–Aldrich Co. (USA). The cell culture reagents, *n*-butyric

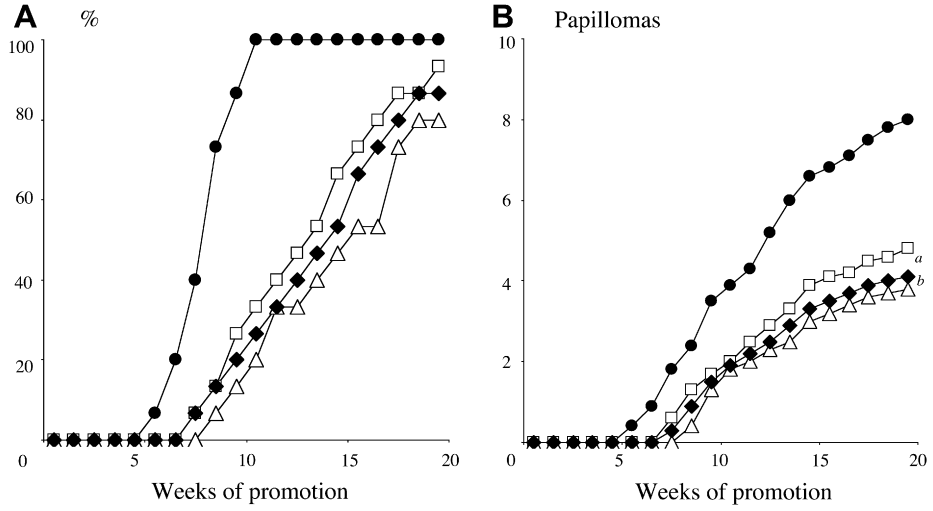

Fig. 3. Inhibition effects of compounds **10**, **11** and **17** on DMBA-TPA-induced carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly, 1 week after initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas/mouse. ● control (TPA alone); ◆ TPA+85 nmol of **10**; △ TPA+85 nmol of **11**; □ TPA+85 nmol of **17**. [a,b]Statistically different from the positive control ($P < 0.05$).

acid and other reagents for the bioassay were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). TPA and DMBA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.2. Test compounds

The natural serratane-type triterpenoids, 3α-methoxyserrat-14-en-21β-ol (**PJ-1**), 3β-methoxyserrat-14-en-21β-ol (**PJ-2**) were isolated from the stem bark of *P. jezoensis* Carr. var. *jezoensis* (Pinaceae) collected in Sapporo City, Japan. The isolation and characterization of **PJ-1** and **PJ-2** were reported previously [10]. A voucher specimen was deposited in the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

3.2.1. *PJ-1*–*naringenin conjugate* (*1*)

Naringenin (174 mg, 0.62 mmol), WSC·HCl (79 mg, 0.42 mmol), and a small amount of *N,N*-dimethylaminopyridine (DMAP) were added to a solution of **PJ-1** succinate ester (180 mg, 0.32 mmol) in tetrahydrofuran (2.0 mL), and stirred at 60 °C for 2.5 h under a nitrogen atmosphere. A small amount of distilled water was added to the reaction mixture, which was extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, and condensed *in vacuo* to give an amorphous powder, which was purified by silica gel column chromatography (*n*-hexane : AcOEt = 5/1–4/1), followed by HPLC (*n*-hexane : AcOEt = 2/1) to afford compound **1** (133.6 mg, 51%) as a colorless powder. Mp 145–147 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −57.4 (*c* 0.05, $CHCl_3$). ^{1}H NMR δ 2.75–2.85 (6H, m, H-3β, naringenin H-3α, and −O−CO−C$\underline{H}_2$−C$\underline{H}_2$−CO−O−), 3.03 (1H, dd, *J* = 13.0, 16.5 Hz, naringenin H-3β), 3.31 (3H, s, OMe), 4.73 (1H, br t, *J* = 2.6, H-21α), 5.31 (1H, m, H-15), 5.39 (1H, dt, *J* = 13.0, 3.2 Hz, naringenin H-2), 5.98 (1H, br s, naringenin H-8), 6.00 (1H, br s, naringenin H-6), 6.85 (1H, br s, naringenin O$\underline{H}$), 7.15 (2H, d, *J* = 8.7 Hz, naringenin H-3′ and H-5′), 7.44 (2H, d, *J* = 8.7 Hz, naringenin H-2′ and H-6′), 12.02 (1H, s, naringenin O$\underline{H}$); ^{13}C NMR δ 29.4 and 29.5 (−O−CO−C$\underline{H}_2$−C$\underline{H}_2$−CO−O−), 43.3 (naringenin C-3), 57.1 (OMe), 78.6 (naringenin C-2), 79.3 (C-21), 86.0 (C-3), 95.5 (naringenin C-8), 96.9 (naringenin C-6), 121.7 (C-15), 121.9 (naringenin C-3′ and C-5′), 127.3 (naringenin C-2′ and C-6′), 138.7 (C-14), 171.0 and 171.8 (−O−C$\underline{O}$−CH_2−CH_2−C$\underline{O}$−O−), 195.4 (naringenin C-4); MS (FAB) *m/z*: 779 [M − OMe]$^+$; HRFABMS ($C_{49}H_{63}O_8$, 779.4515; calculated for 779.4523) [M − OMe]$^+$.

3.2.2. *PJ-2*–*naringenin conjugate* (*2*)

PJ-2–naringenin conjugate (**2**) (151 mg, 51%) was synthesized from naringenin and **PJ-2** succinic acid (187 mg, 0.34 mmol), similarly to compound **1**. Mp 155–157 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −12.4 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.64 (1H, dd, *J* = 11.7, 4.1 Hz, H-3α), 4.74 (1H, dif t, H-21α), 5.33 (1H, m, H-15), 5.40 (1H, dd, *J* = 13.0, 3.0 Hz, naringenin H-2), 5.97–6.10 (2H, m, naringenin H-6 and H-8), 7.16 (2H, dd, *J* = 8.8 Hz, naringenin H-3′, 5′), 7.45 (2H, d, *J* = 8.8 Hz, naringenin H-2′, 6′), 12.0 (1H, d, *J* = 1.4 Hz, naringenin OH); ^{13}C NMR δ 79.2 (C-21), 88.7 (C-3), 121.9 (C-3′, C-5′, C-15, and C-4′), 127.2, 127.3 (C-2′ and C-6′), 136.0 (C-1′), 138.5 (C-14), 195.4 (naringenin C-4); MS (FAB) *m/z*: 779 [M − OMe]$^+$; HRFABMS ($C_{49}H_{63}O_8$, 779.4515; calculated for 779.4523) [M − OMe]$^+$.

3.2.3. *PJ-1*–*hesperetin conjugate* (*3*)

PJ-1–hesperidine conjugate (**2**) (151 mg, 51%) was synthesized from hesperetin (108 mg, 0.43 mmol) and **PJ-1** succinate (200 mg, 0.35 mmol), similarly to compound **1**. Mp 138–140 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −36.1 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.77 (1H, m, H-3β), 2.77 (1H, dd, *J* = 17.0, 3.2 Hz, hesperetin H-3α), 2.78–2.96 (4H, m, −O−CO−C$\underline{H}_2$C$\underline{H}_2$−CO_2), 3.02 (1H, m, hesperetin H-3β), 4.73 (1H, t, *J* = 2.7 Hz, H-21α), 5.32 (2H, m, H-15 and hesperetin H-2), 5.94 (1H, d, *J* = 2.0 Hz, hesperetin H-8), 5.97 (1H, d, *J* = 2.0 Hz, hesperetin H-6), 6.98 (1H, d, *J* = 8.4 Hz, hesperetin H-5′), 7.16 (1H, d, *J* = 2.3 Hz, hesperetin H-2′), 7.25 (1H, dd, *J* = 8.4, 2.3 Hz, hesperetin H-6′), 12.01 (1H, d, *J* = 1.4 Hz, OH); ^{13}C NMR δ 29.1 and 29.5 -O−CO−C$\underline{H}_2$−C$\underline{H}_2$−CO−O−), 43.1 (hesperetin C-3), 77.2 (hesperetin C-2), 78.3 (C-21), 85.9 (C-3), 95.5 (hesperitin C-8), 96.8 (hesperetin C-6), 103.0 (hesperetin C-10), 112.5 (hesperetin C-5′), 121.0 (hesperetin C-2′), 121.7 (C-15), 124.8 (hesperetin C-6′), 130.9 (hesperetin C-3′), 138.7 (C-14), 151.4 (hesperetin C-1′), 163.0 (hesperetin C-9), 164.3 (hesperetin C-5), 164.9 (hesperetin C-7), 170.6, 171.6 (−O−C$\underline{O}$−CH_2−CH_2−C$\underline{O}$−O−) 195.6 (hesperetin C-4); MS (FAB) *m/z*: 841 [M + H]$^+$; HRFABMS ($C_{51}H_{69}O_{10}$, 841.4893; calculated for 841.4891) [M + H]$^+$.

3.2.4. *PJ-2*–*hesperetin conjugate* (*4*)

PJ-2–hesperetin conjugate (**4**) (137 mg, 68%) was synthesized from hesperidine (108 mg, 0.43 mmol) and **PJ-2** succinate (200 mg, 0.35 mmol), similarly to compound **1**. Mp 136–138 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −3.2 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.64 (1H, dd, *J* = 11.9, 4.3 Hz, H-3α), 2.76 (1H, dd, *J* = 3.2, 17.0 Hz, hesperetin H-3α), 2.80–2.97 (4H, m, -O−CO−C$\underline{H}_2$−C$\underline{H}_2$−CO−O−), 3.02 (1H, dd, *J* = 12.8, 17.0 Hz, hesperetin H-3β), 3.84 (3H, s hesperetin OMe), 4.73 (1H, t, *J* = 2.5 Hz, H-21α), 5.30 (1H, dd, *J* = 3.2, 12.8 Hz, hesperetin H-2), 5.31 (1H, m, H-15), 5.94 (1H, br s, hesperetin H-8), 5.97 (1H, br s, hesperetin H-6), 6.88 (1H, br s, hesperetin O$\underline{H}$), 6.98 (1H, d, *J* = 8.5 Hz, hesperetin H-5′), 7.16 (1H, d, *J* = 2.0 Hz, hesperetin H-2′), 7.25 (1H, dd, *J* = 2.0, 8.5 Hz, hesperetin H-6′), 12.0 (1H, s, hesperetin H-5-O$\underline{H}$); ^{13}C NMR δ 29.1 and 29.5 −O−CO−C$\underline{H}_2$−C$\underline{H}_2$−CO−O−), 43.1 (hesperetin C-3), 57.5 (OMe), 78.3 (hesperetin C-2), 79.1 (C-21), 88.7 (C-3), 95.5 (hesperetin C-8), 96.8 (hesperetin C-6), 102.9 (hesperetin C-10), 112.5 (hesperetin C-5′), 121.0 (hesperetin C-2′), 121.9 (C-15), 124.8 (hesperetin C-6′), 130.9 (hesperetin C-3′), 138.5 (C-14), 151.3 (hesperetin C-1′), 162.9 (hesperetin C-9), 164.2 (hesperetin C-5), 165.1 (hesperetin C-7), 195.5 (hesperetin C-4); MS (FAB) *m/z*: 841 [M + H]$^+$; HRFABMS ($C_{51}H_{69}O_{10}$, 841.4899; calculated for 841.4891) [M + H]$^+$.

3.2.5. *PJ-1*–*genistein conjugate* (*5*)

PJ-1–genistein conjugate (**5**) (221.3 mg, 76%) was synthesized from genistein (126 mg, 0.47 mmol) and **PJ-1** succinate (205 mg, 0.36 mmol), similarly to compound **1**. Mp 143–145 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −32.7 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.77 (1H, t, *J* = 2.5 Hz, H-3β), 4.74 (1H, t, *J* = 2.6 Hz, H-21α), 5.31 (1H, m, H-15), 6.33 (1H, d, *J* = 2.3 Hz, genistein H-6), 6.38 (1H, d, *J* = 2.3 Hz, genistein H-8), 7.16 (2H, dt, *J* = 8.9, 2.4 Hz, genistein H-3′ and H-5′), 7.53 (2H, dt, *J* = 8.9, 2.4 Hz, genistein H-2′ and H-6′), 7.84 (1H, s, genistein H-2), 12.79 (1H, s, genistein OH); ^{13}C NMR δ 79.2 (C-21), 85.8 (C-3), 94.2 (genistein C-8), 99.8 (genistein C-6), 105.9 (genistein C-10), 121.7 (C-15, genistein C-3′ and C-5′), 123.0 (genistein C-3), 128.5 (genistein C-1′), 130.0 (genistein C-2′ and C-6′), 138.7 (C-14), 150.7 (genistein C-4′), 153.1 (genistein C-2), 157.0 (genistein C-5), 158.0 (genistein C-9), 162.9 (genistein C-7), 171.0, 171.7 (−O−C$\underline{O}$−CH_2CH_2−C$\underline{O}$−O−), 180.3 (C-4); MS (FAB) *m/z*: 809 [M]$^+$; HRFABMS ($C_{49}H_{61}O_8$, 777.4363; calculated for 777.4366) [M − OMe]$^+$.

3.2.6. *PJ-2*–*genistein conjugate* (*6*)

PJ-2–genistein conjugate (**6**) (122 mg, 41%) was synthesized from **PJ-2** succinate (200 mg, 0.35 mmol) and genistein (126 mg, 0.47 mmol), similarly to compound **1**. Mp 261–263 °C (MeOH–$CHCl_3$); $[\alpha]_D^{22}$ −136.9 (*c* 0.10, $CHCl_3$). ^{1}H NMR δ 2.63 (1H, dd, *J* = 11.8, 4.0 Hz, H-3β), 4.74 (1H, t, *J* = 2.6 Hz, H-21α), 5.33 (1H, m, H-15), 6.35 (1H, br s, genistein H-6), 6.40 (1H, br s, H-8), 7.16 (2H, d, *J* = 8.7 Hz, genistein H-3′ and H-5′), 7.53 (2H, d, *J* = 8.7 Hz, genistein H-2′ and H-6′), 7.82 (1H, br s, genistein H-2), 12.77 (1H, s, genistein OH); ^{13}C NMR δ 79.1 (C-21), 88.4 (C-3), 94.2 (genistein C-8), 99.9 (genistein C-6), 105.7 (genistein C-10), 121.9 (C-15), 122.9 (C-3′ and

C-5′), 122.9 (genistein C-3), 128.6 (genistein C-1′), 130.0 (genistein C-2′ and C-6′), 138.5 (C-14), 150.6 (genistein C-4′), 153.0 (genistein C-2), 157.1 (genistein C-5), 158.0 (genistein C-9), 162.8 (genistein C-7), 171.0, 171.7 (−O−CO−CH_2CH_2−CO−O−), 180.3 (genistein C-4); MS (FAB) *m/z*: 809 $[M]^+$; HRFABMS ($C_{49}H_{61}O_8$, 777.4359; calculated for 777.4366) $[M - OMe]^+$.

3.2.7. *PJ-1–daidzein conjugate (**7**)*

PJ-1–daidzein conjugate (**7**) (120 mg, 42%) was synthesized from daidzein (110 mg, 0.43 mmol) and **PJ-1** succinate (201 mg, 0.36 mmol), similarly to compound **1**. Mp 246–248 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −24.2° (*c* 0.10, $CHCl_3$). ^{1}H NMR δ 2.77 (1H, t, *J* = 2.5 Hz, H-3β), 4.74 (1H, t, *J* = 2.8 Hz, H-21α), 5.31 (1H, m, H-15), 6.82 (1H, d, *J* = 2.3 Hz, daidzein H-8), 6.91 (1H, dd, *J* = 8.7, 2.3 Hz, daidzein H-6), 7.14 (2H, dt, *J* = 8.7, 2.3 Hz, daidzein H-3′, 5′), 7.53 (2H, dt, *J* = 8.7, 2.3 Hz, daidzein H-2′, 6′), 7.90 (1H, s, daidzein H-2), 8.13 (1H, d, *J* = 8.7 Hz, daidzein H-5); ^{13}C NMR δ 79.2 (C-21), 85.9 (C-3), 102.9 (daidzein C-8), 115.3 (daidzein C-6), 118.0 (daidzein C-10), 121.5 (daidzein C-3′ and C-5′), 121.7 (C-15), 124.2 (daidzein C-3), 128.1 (daidzein C-5), 129.5 (daidzein C-1′), 130.0 (daidzein C-2′ and C-6′), 138.7 (C-14), 150.5 (daidzein C-4′), 152.9 (daidzein C-2), 157.9 (daidzein C-9), 161.4 (daidzein C-7), 171.1, 171.8 (−O−CO−CH_2CH_2−CO−O−), 175.9 (daidzein C-4); MS (FAB) *m/z*: 793 $[M + H]^+$; HRFABMS ($C_{50}H_{65}O_8$, 793.4674; calculated for 793.4680) $[M + H]^+$.

3.2.8. *PJ-2–daidzein conjugate (**8**)*

PJ-2–daidzein conjugate (**8**) (97.0 mg, 34%) was synthesized from **PJ-2** succinic acid (203 mg, 0.36 mmol) and daidzein (110 mg, 0.43 mmol), similarly to compound **1**. Mp 243–245 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −1.1 (*c* 0.11, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, *J* = 11.9, 4.1 Hz, H-3α), 4.74 (1H, t, *J* = 2.5 Hz, H-21α), 5.33 (1H, m, H-15), 6.82 (1H, d, *J* = 2.3 Hz, daidzein H-8), 6.91 (1H, dd, *J* = 8.7, 2.2 Hz, daidzein H-6), 7.14 (2H, d, *J* = 8.7 Hz, daidzein H-3′ and H-5′), 7.54 (2H, d, *J* = 8.7 Hz, H-2′ and H-6′), 7.90 (1H, s, daidzein H-2), 8.12 (1H, d, *J* = 8.7 Hz, daidzein H-5); ^{13}C NMR δ 79.2 (C-21), 88.7 (C-3), 102.8 (daidzein C-8), 115.4 (daidzein C-6), 117.8 (daidzein C-10), 121.5 (daidzein C-3′ and C-5′), 121.9 (C-15), 124.1 (daidzein C-3), 128.0 (daidzein C-5), 129.6 (daidzein C-1′), 130.1 (daidzein C-2′ and C-6′), 138.5 (C-14), 150.5 (daidzein C-4′), 153.0 (daidzein C-2), 158.0 (daidzein C-9), 161.8 (daidzein C-7), 171.1, 171.8 (−O−CO−CH_2CH_2−CO−O−), 176.0 (C-4); MS (FAB) *m/z*: 793 $[M + H]^+$; HRFABMS ($C_{50}H_{65}O_8$, 793.4684; calculated for 793.4680) $[M + H]^+$.

3.2.9. *PJ-2–3,5-dihydroxy-4-methoxy-benzoic acid conjugate (**9**)*

3,5-Dimethoxymethyl-4-methoxybenzoic acid (238 mg, 0.88 mmol), DCC (200 mg, 0.96 mmol), and DMAP (cat. amount) were added to a solution of **PJ-2** (200 mg, 0.44 mmol) in chloroform (2 mL), and stirred for 7.5 h at room temperature and for another 12 h at 60 °C. The reaction mixture was condensed *in vacuo* and the residue was purified by silica gel column chromatography (*n*-hexane:ethyl acetate = 8/1) to afford **PJ-2 3,5-dimethoxymethyl-4-methoxybenzoate** (170 mg, 55%). Methanesulfonic acid (64 μL, 1 mmol) was added to a solution of **PJ-2 3,5-dimethoxymethyl-4-methoxybenzoate** (70.6 mg, 0.1 mmol) in methanol (0.3 mL) and chloroform (3.0 mL) and the mixture was stirred for 22 h at room temperature. After the reaction, a saturated aqueous solution of sodium bicarbonate was added to the reaction mixture, which was extracted with ethyl acetate after removing methanol *in vacuo*. The organic layer was washed with brine, dried over magnesium sulfate, and evaporated. The residue was purified by column chromatography over silica gel (chloroform:methanol = 5/1) to afford compound **9** (36.0 mg, 59%). Mp 276–278 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −6.7 (*c* 0.10, $CHCl_3$). ^{1}H NMR δ 2.78 (1H, t, *J* = 2.7 Hz, H-3β), 3.31 (3H, s, 3-OMe), 3.98 (3H, s, 4′-OMe), 4.85 (1H, t, *J* = 2.5 Hz, H-21α), 5.35 (1H, m, H-15), 5.97 (2H, br d, 3′, 5′-OH), 7.28 (2H, s, H-2′, 6′); ^{13}C NMR δ; 79.7 (C-21), 85.9 (C-3), 109.7 (C-2′, 6′), 121.8 (C-15), 126.8 (C-1′), 138.4 (C-4′), 138.8 (C-14), 148.7 (C-3′, 5′), 165.8 (C=O); MS (FAB) *m/z*: 622 $[M]^+$; HRFABMS ($C_{39}H_{58}O_6$, 622.4239; calculated for 622.4233) $[M]^+$.

3.2.10. *PJ-2–3,4,5-trihydroxybenzoic acid conjugate (**10**)*

PJ-2 (69.0 mg) was condensed with 3,5,4-trimethoxymethy lbenzoic acid to afford **3,4,5-trimethoxymethylbenzoyl–PJ-2** (71.0 mg, 64%), which was treated with methanesulfonic acid (46 μL, 0.7 m mol) to afford compound **10** (43.0 mg, 79%), similarly to the synthesis of compound **9**. Mp 208–210 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ +18.2 (*c* 0.03, $CHCl_3$). ^{1}H NMR δ 2.64 (1H, dd, *J* = 11.8, 4.2 Hz, H-3α), 3.37 (3H, s, OMe), 4.83 (1H, t, *J* = 2.5 Hz, H-21α), 5.37 (1H, m, H-15), 7.31 (2H, s, H-2′, 6′); ^{13}C NMR δ 79.6 (C-21), 88.7 (C-3), 109.8 (C-2′, 6′), 122.0 (C-15), 122.4 (C-1′), 136.4 (C-4′), 138.6 (C-14), 143.5 (C-3′, 5′), 166.4 (C=O); MS (FAB) *m/z*: 608 $[M]^+$; HRFABMS ($C_{38}H_{56}O_6$, 608.4076; calculated for 608.4077) $[M]^+$.

3.2.11. *PJ-1 3,3-dimethyl-succinyl derivative (**11**)*

2,2-Dimethyl succinic anhydride (1.28 g, 10 mmol) and DMAP (cat. amount) were added to a solution of **1** (456 mg, 1 mmol) in pyridine (5.0 mL), and stirred for 22.5 h under refluxed conditions. The reaction was quenched and worked up as usual. The residue was purified by silica gel column chromatography (*n*-hexane: AcOEt = 7/1) to afford compound **11** (328 mg, 56%). Mp. 186–188 °C; $[\alpha]_D^{22}$ −35.0° (*c* 0.009, $CHCl_3$). ^{1}H NMR δ 1.32 and 1.33 (each 3H, s, −OCO−CH_2−C(CH_3)$_2$−COOH), 2.62 and 2.70 (each 1H, d, *J* = 16.2 Hz, H_2-2′), 2.78 (1H, t, *J* = 2.5 Hz, H-3β), 4.71 (1H, t, *J* = 2.7 Hz, H-21α), 5.31 (1H, m, H-15); ^{13}C NMR δ 25.1 and 25.5 (−OCO−CH_2−C(CH_3)$_2$−COOH, C-3′), 44.2 (C-2′), 78.7 (C-21), 88.7 (C-3), 121.7 (C-15), 138.7 (C-14), 170.9 (C-1′), 182.0 (C-4′); MS (FAB) *m/z*: 607 $[M + Na]^+$; HRFABMS ($C_{37}H_{60}O_5Na$, 607.4343; calculated for 607.4338) $[M + Na]^+$.

3.2.12. *PJ-2 3,3-dimethyl-succinyl derivative (**12**)*

PJ-2 3,3-dimethyl-succinyl derivative (**12**) (230 mg, 45%) was synthesized from **PJ-2** succinic acid (400 mg, 0.87 mmol), similarly to compound **11**. Mp 218–220 °C (MeOH–$CHCl_3$); $[\alpha]_D^{22}$ −40.9 (*c* 0.098, $CHCl_3$); ^{1}H NMR δ 1.32 and 1.33 (each 3H, s, −OCO−CH_2−C(CH_3)$_2$−COOH), 2.62 and 2.70 (each 1H, d, *J* = 16.2 Hz, H_2-2′), 2.63 (1H, dd, *J* = 11.9, 4.1 Hz, H-3α), 4.71 (1H, t, *J* = 2.5 Hz, H-21α), 5.31 (1H, m, H-15); ^{13}C NMR δ 25.1 and 25.2 (−OCO−CH_2−C(CH_3)$_2$−COOH), 40.4 (C-3′), 44.5 (C-2′), 78.7 (C-21), 88.7 (C-3), 121.7 (C-15), 138.7 (C-14), 170.9 (C-1′), 182.2 (C-4′); MS (FAB) *m/z*: 607 $[M + Na]^+$; HRFABMS ($C_{37}H_{60}O_5Na$, 607.4344; calculated for 607.4338) $[M + Na]^+$.

3.2.13. *PJ-1 succinyl derivative (**13**)*

PJ-1 succinyl derivative (**13**) (345 mg, 94%) was synthesized from succinic anhydride (666 mg, 6.66 mmol) and **PJ-1** (360 mg, 0.657 mmol), similarly as compound **11**. Mp. 175–177 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −41.9 (*c* 0.10, $CHCl_3$). ^{1}H NMR δ 2.65–2.72 (4H, m, H-2′ and H-3′), 2.77 (1H, t, *J* = 2.5 Hz, H-3β), 4.71 (1H, t, *J* = 2.7 Hz, H-21α), 5.32 (1H, m, H-15); ^{13}C NMR δ 28.9 and 29.3 (C-2′ and 3′), 79.0 (C-21), 85.8 (C-3), 121.7 (C-15), 138.7 (C-14), 171.6 (C-1′), 176.6 (C-4′); MS (FAB) *m/z*: 556 $[M]^+$; HRFABMS ($C_{35}H_{56}O_5$, 556.4127; calculated for 556.4128) $[M]^+$.

3.2.14. *PJ-2 succinyl derivative (**14**)*

PJ-2 succinyl derivative (**14**) (1.57 g, 65%) was synthesized from succinic anhydride (4.4 g, 4.4 mmol) and **PJ-2** (2.0 g, 4.4 mmol), similarly as compound **11**. Mp. 172–174 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −5.5 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.64–2.72 (4H, m, H-2′ and

H-3′), 2.64 (1H, dd, J = 11.8, 4.2 Hz, H-3α), 4.71 (1H, t, J = 3.0 Hz, H-21α), 5.33 (1H, m, H-15); ^{13}C NMR δ 29.0 and 29.3 (C-2′ and 3′), 78.9 (C-21), 88.5 (C-3), 121.9 (C-15), 138.5 (C-14), 171.7 (C-1′), 176.9 (C-4′); MS (FAB) *m/z*: 556 $[M]^+$; HRFABMS ($C_{35}H_{56}O_5$, 556.4133; calculated for 556.4128) $[M]^+$.

3.2.15. ***PJ-1*–*glycine conjugate* (*15*)**

N-Boc-glycine (35.0 mg, 0.1 mmol), WSC·HCl (35.0 mg, 0.2 mmol), and DMAP (24.0 mg, 0.2 mmol) were added to a solution of **PJ-2** (46.0 mg, 0.1 mmol) in tetrahydrofuran (2 mL), which was stirred for 5 days at room temperature. The reaction was quenched and worked up as usual. The residue was purified by column chromatography over silica gel (*n*-hexane:ethyl acetate = 3:1) to afford **Boc-Glycine**–**PJ-2** (55.0 mg, 90%). Subsequent treatment of **Boc-Glycine**–**PJ-2** with methanesulfonic acid (37 μL, 0.55 mmol) in methanol (0.5 mL) and chloroform (2 mL) afforded compound **15** (33.0 mg, 64%). Mp 158–160 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −10.3 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 2.63 (1H, dd, J = 11.8, 4.1 Hz, H-3α), 3.46 (2H, s, O=C–C$\underline{H}_2$–NH_2) 4.74 (1H, t, J = 2.5 Hz, H-21α), 5.33 (1H, m, H-15); ^{13}C NMR δ 44.5 (O=C–$\underline{C}H_2$–NH_2), 79.0 (C-21), 88.5 (C-3), 121.9 (C-15), 138.5 (C-14), 173.9 (C=O); MS (FAB) *m/z*: 514 $[M + H]^+$; HRFABMS ($C_{33}H_{56}NO_3$, 514.4262; calculated for 514.4260) $[M + H]^+$.

3.2.16. ***PJ-1* piperidineacetic acid derivative (*16*)**

Chloroacetyl chloride (12 mL) was added to a solution of **PJ-2** (46.0 mg, 0.1 mmol) in pyridine (30 mL) and chloroform (1 mL) at 0 °C, which was stirred for 1 h at the same temperature. The reaction was quenched and worked up as usual. The residue was purified by column chromatography over silica gel (chloroform) to afford **chloroacetyl**–**PJ-2** (46.0 mg, 87%). Subsequent reaction of **chloroacetyl**–**PJ-2** (54.0 mg, 0.1 mmol) and piperidine (30 mL, 0.3 mmol) in DMF (1.0 mL), and purification of the residue by column chromatography over silica gel (chloroform:AcOEt = 5/1) afforded compound **16** (47.0 mg, 81%). Mp 222–224 °C (MeOH–$CHCl_3$); $[\alpha]_D^{23}$ −3.8 (*c* 0.11, $CHCl_3$). ^{1}H NMR δ 1.44 (2H, m, piperidine-4), 1.63 (4H, m, piperidine-3 and -5), 2.55 (4H, t, J = 5.5 Hz, piperidine-2 and -6), 2.63 (1H, dd, J = 11.8, 4.2 Hz, H-3), 3.21, 3.25 (each 1H, d, J = 16.5 Hz, CO–CH_2–N), 3.36 (3H, s, OMe), 4.75 (1H, t, J = 2.6 Hz, H-21), 5.33 (1H, m, H-15); ^{13}C NMR δ ppm ($CDCl_3$): 24.0 (piperidine-4), 25.9 (piperidine-3 and -5), 54.2 (piperidine-2 and -6), 60.3 (CO–CH_2–N), 78.4 (C-21), 88.5 (C-3), 121.9 (C-15), 138.5 (C-14), 170.4 (O–$\underline{C}$O). MS (FAB) *m/z*: 582 $[M + 2H]^+$; HRFABMS ($C_{38}H_{62}NO_3$, 582.4880; calculated for 582.4886) $[M + 2H]^+$.

3.2.17. ***PJ-1*-13α,14α-epoxy- 3,3-dimethyl-succinyl derivative (*17*)**

PJ-1 (1.0 g) was acetylated (Ac_2O/pyridine 1:1, 10 mL) to give 3β-methoxyserrat-14-en-21β-yl acetate (**PJ-1** acetate: 1.04 g). A mixture of glacial AcOH (100 mL) and *c*-H_2SO_4 (10 mL) was gradually added to **PJ-1** acetate and was kept at room temperature for 24 h. Then, the mixture was poured into ice water and the resulting precipitate was extracted with $CHCl_3$. The extract was neutralized with 5% NaOH, washed with H_2O and dried over Na_2SO_4. Evaporation of $CHCl_3$ yielded a crystalline mass (752.9 mg), which was subjected to SiO_2 column chromatography to afford 3α-methoxyserrat-13-en-21β-yl acetate (**PJ-1**–**13-en Ac**: 715.1 mg). Treatment of PJ-1–13-en Ac in boiling 0.3 N KOH/MeOH (100 ml) for 8 h and the subsequent usual workup furnished 3β-methoxyserrat-13-en-21β-ol (655.7 mg), followed by adding a solution of *m*-chloroperbenzoic acid (*m*-CPBA) to dry $CHCl_3$. After 24 h, the reaction was quenched, worked up as usual, and then the residue was purified by MPLC (240–400 mesh SiO_2), eluting with *n*-hexane:AcOEt (10/1) to afford 13α,14α-epoxy-3α-methoxyserratan-21β-ol (**PJ-1 epoxy**) (525.0 mg) and 13β,14β-epoxy-3α-methoxyserratan-21β-ol (29.1 mg). 2,2-dimethyl succinic anhydride (567.4 mg, 4.43 mmol) and DMAP (cat. amount) were added to a solution of **PJ-1 epoxy** (224.1 mg, 0.519 mmol) in pyridine (4.0 mL), and stirred for 24 h under refluxed conditions. The reaction mixture was condensed *in vacuo* and 1 M hydrochloric acid was added to the residue, which was extracted with ethyl acetate. The organic layer was treated as usual and the residue was purified by silica gel column chromatography (chloroform) and then HPLC (ODS, MeOH:H_2O = 95/5) to afford compound **17** (79.5 mg, 26%). Mp 126–128 °C (MeOH–$CHCl_3$); $[\alpha]_D^{22}$ −11.2° (*c* 0.097, $CHCl_3$); ^{1}H NMR δ 1.35 (6H, s, −OCO–CH_2–C($\underline{C}H_3$)$_2$–COOH), 2.61 and 2.66 (2H, d, J = 16.1 Hz, H-2′), 2.78 (1H, t, J = 2.5 Hz, H-3β), 4.69 (1H, t, J = 2.5 Hz, H-21α), 5.31 (1H, m, H-15); ^{13}C NMR δ 25.5 and 25.7 (−OCO–CH_2–C($\underline{C}H_3$)$_2$–COOH), 40.4 (C-3′), 44.5 (C-2′), 78.3 (C-21), 88.7 (C-3), 121.7 (C-15), 138.7 (C-14), 170.5 (C-1′), 182.7 (C-4′); MS (EI) *m/z*: 600 $[M]^+$; HREIMS ($C_{37}H_{60}O_6$, 600.4385; calculated for 600.4390) $[M]^+$.

3.3. Inhibition of EBV-EA activation assay

EBV-EA-positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medicinal University. The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitts lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). Spontaneous activation of EBV-EA in our sub-line Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus non-producer type) as described previously [10]. The indicator cells (Raji cells, 1 × 10^6/mL) were incubated at 37 °C for 48 h in 1 ml of a medium containing *n*-butyric acid (4 mmol), TPA (32 pmol = 20 ng in dimethylsulfoxide (DMSO), 2 μL) as an inducer and various amounts of test compounds in 5 μL DMSO. Smears were made from the cell suspension, and the activated cells that were stained by EBV-EA-positive serum from NPC patients were detected by an indirect immunofluorescence technique [31]. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%) which was carried out only with *n*-butylic acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by the Trypan Blue staining method.

3.4. Animals

Specific pathogen-free female ICR mice (6 weeks old, body weight approx. 30 g) were obtained from Japan SLC Inc. (Shizuoka, Japan), and the animals were housed, five animals per polycarbonate cage, in a temperature-controlled room at 24 ± 2 °C and given food and water ad libitum throughout the experiment.

3.5. Two-stage mouse-skin carcinogenesis test

Animals were divided into three experimental groups containing 15 mice each. The back (2 × 8 cm^2) of each mouse was shaved with surgical clippers, and the mice were topically treated with 7,12-dimethylbenz[a]anthracene (DMBA) (100 μg, 390 nmol) in acetone (0.1 mL) as the initial treatment. One week after initiation, papilloma formation was promoted twice weekly by the application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (1 μg, 1.7 nmol) in acetone (0.1 mL) to the skin. One hour before each treatment with TPA, the mice were treated with the samples (85 nmol) in acetone (0.1 mL). The incidence of papillomas was examined weekly over a period of 20 weeks [10].

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