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Original article

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

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ABSTRACT

3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their conjugates with curcumin, kojic acid, quercetin, and baicalein (**3**–**18**), as well as new analogs (**19**–**24**) derived from **1** and **2**, 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 **16** (IC_{50} = 330 mol ratio/32 pmol/TPA), **9** (IC_{50} = 335), **10** (IC_{50} = 338), and **15** (IC_{50} = 350) were stronger than those of the other compounds and the positive control, oleanolic acid (IC_{50} = 449). Compounds **15** and **16**, which are conjugates of one molecule each of **1** or **2** and quercetin, 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

In recent years, the demand for more effective and safer agents for the chemoprevention of human cancer has been a great surge, and natural products from plants and their synthetic analogs are expected to play a vital role in creating new and better chemopreventive agents [1,2]. In particular, there is a need for chemopreventive agents that target the promotion stage of carcinogenesis in the two- or multi-stage theory [3] because it is not possible to completely avoid carcinogens in daily life and tumor promotion is a long and reversible process that can be efficiently suppressed [4].

The genus *Picea* consists of approximately 40 species worldwide. Its number is next to those of *Pinus* and *Abies* in the order Pinales. *Picea* species, however, do not have any important use besides construction materials. In our fervent search for biologically active constituents from natural sources, we previously reported several serratane-type triterpenoids that show significant anti-tumor promoting activities in an in vivo two-stage mouse skin carcinogenesis test using 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter. These include 3α-methoxyserrat-14-en-21β-ol (**1**) [5], 13α,14α-epoxy-3β-methoxyserratan-21β-ol and 21α-hydroxy-3β-methoxyserrat-14-en-29-al [6], serrat-14-en-3β,21β-diol [7], and 14β,15β-epoxy-3β-methoxyserratan-21β-ol [8], which were isolated 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). 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) are the most abundant triterpenoids in the above two *Picea* plants and *Picea glehni* (Fr. Schm.) Masters (Japanese name: Akaezomatsu) [9]. Recently, we reported that **1** significantly decreased the proliferation of adenomas and total tumors in a revised rat multi-organ carcinogenesis (DMBDD) model [10]. In addition, triterpenoids are widely distributed in vegetables [11] and fruits [12] and are attractive compounds because they have various physiological activities yet little toxicity. As active triterpenoids, Kawamori et al. reported that oleanolic acid

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can reduce AOM-induced ACF, colonic mucosal ODC activity, and AgNOR number in the colonic epithelium [13], and Senthil et al. indicated that ursolic acid has a protective effect against myocardial ischemia induced by isoproterenol in rats [14]. Chen et al. reported that the nitric oxide releasing derivatives of oleanolic acid inhibit HCC tumor growth in vivo [15]. On the other hand, betulonic acid-acetylene hybrids showed hepatoprotective and anti-inflammatory activities [16]. The conjugation of two bioactive compounds is an effective strategy that is now well accepted [17]. Natural product hybrids, including triterpenoids, play an important role in the development of drugs for the treatment of infections and cancer, as well as the development of immunosuppressive compounds [18]. Meanwhile, well-known phenolic natural products, curcumin [19], kojic acid [20], quercetin [21], and baicalein [22], possess excellent anti-tumor and anti-oxidative activities. Therefore, based on the diverse bioactivities of the terpenoids as well as the anti-oxidants described above, we initiated a study of triterpenoid-anti-oxidant conjugation. As regards well-established triterpenoid conjugations with other substances, a number of studies of hybrid connections of triterpenoids are available. Iveson and Parke reported β-glycyrrhizic acid – glucuronyl esters [23]. Baltina disclosed glycyrrhizic acid – glucopeptide conjugates having immune response [24]. Tatsuzaki et al. reported glycyrrhizic acid – dehydrozingerone conjugates as cytotoxic agents [25] and Ma et al. indicated oleanane-type triterpene – azidothymidine (AZT) or FK-3000 conjugates that inhibit the proliferation of HIV-1 and its protease [26].

In our newly published paper, we reported the synthesis of **3–18** and evaluated their anti-HIV-1 reverse transcriptase (RT) activities in infected C8166-CCR5 cells, a human $CD4^+$ T-lymphocyte cell line. Among them, **13**, which is a conjugate of two molecules of 3α-methoxyserrat-14-en-21β-ol (**1**) and one molecule of kojic acid, exerted significant anti-HIV RT activity with an EC_{50} value of 0.12 μg/mL [27]. Herein, we report the in vitro and in vivo anti-tumor promoting activity of **3–18** and new synthetic analogs (**19–24**) derived from **1** and **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 [28], and an in vivo two-stage mouse skin carcinogenesis test using DMBA as an initiator and TPA as a tumor promoter [5].

2. Results and discussion

3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) were the predominant triterpenoids in the chloroform extracts of *P. jezoensis* Carr. var. *jezoensis* and *P. jezoensis* Carr. var. *hondoensis*. Compounds **1** and **2** constituted 25% and 10% of *P. jezoensis* Carr. var. *jezoensis* extract and 18% and 30% of *P. jezoensis* Carr. var. *hondoensis* extract, respectively [29]. Although, oleanolic acid, ursolic acid, and betulinic acid were often modified into various forms, serratane-type triterpenoids, such as **1** and **2**, were not modified. Therefore, based on the diverse bioactivities of the triterpenoids as well as phenolic compounds having anti-oxidant activity, such as curcumin, kojic acid, quercetin, and baicalein, we studied the conjugation of triterpenoids and phenolic compounds on the basis of the hybrid drug strategy, and synthesized 16 compounds (**3**–**18**) (Fig. 1) as described in a recent paper [27]. In addition, six new compounds (**19**–**24**) were synthesized from **2** and subjected to the in vitro EBV-EA activation assay. Compound **19** is compound **2**-phenylcarbamate, **20** is compound **2**-2-chlorophenylcarbamate, **21** is compound **2**-carbamate, **22** is compound **2**-*N*-[(4-methylphenyl)sulfonyl]carbamate, **23** is compound **2**-phenylcarbamothioate, and **24** is compound **2**-chloroacetate (Section 3, Fig. 2).

We report herein the results of in vitro and in vivo anti-tumor promoting activities of **3**–**24**. 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 EBV-EA activation assay are shown in Table 1. Synthetic **3**–**24** had purities >99%. Their effects on the viability of Raji cells and their 50% inhibitory concentration (IC_{50}) values are shown in Table 1. All compounds exhibited dose-dependent inhibitory activities and the viability of Raji cells treated with test compounds **3**–**24** ranged from 60 to 70% except **20** at the highest concentration of 1000 mol ratio/32 pmol/TPA, suggesting that these compounds showed moderate cytotoxicities against in vitro cell lines (Table 1). The inhibitory activities of **16** (IC_{50} = 330 mol ratio/32 pmol/TPA), **9** (IC_{50} = 335), **10** (IC_{50} = 338), **15** (IC_{50} = 350), **7** (IC_{50} = 354), and **8** (IC_{50} = 358) were stronger than those of the other compounds. The relative ratios of **16** and **15** (conjugates of one molecule of **1** or **2** and quercetin using succinic acid as linker) with respect to TPA (100%) were 2.2, 30.1, 71.9, and 90.3% and 5.1, 32.3, 73.0, and 91.7% at the concentrations of 1000, 500, 100, and 10 mol ratio/32 pmol/TPA, respectively (Table 1). This means 97.8, 69.9, 28.1, and 9.7% (compound **16**) inhibition and 94.9, 67.7, 27.0, and 8.3% (compound **15**) inhibition of the TPA-induced EBV-EA activation, respectively. On the other hand, compounds **9** and **10** showed 98.4, 68.3, 29.9, and 8.4%, and 98.3, 68.9, 29.7, and 8.9% inhibition of TPA-induced EBV-EA activation at concentrations of 1000, 500, 100, and 10 mol ratio/32 pmol/TPA, respectively. Compounds **9** and **10** are conjugates of two molecules of **1** or **2** and one molecule of curcumin using malonic acid as linker and compounds **7** and **8** are conjugates of one molecule of **1** or **2** and one molecule of curcumin using malonic acid as linker. As hybrids of **1** or **2** and curcumin (Fig. 3), compound pairs **9**, **10** and **7**, **8** showed stronger inhibitory activity than compound pairs **3** (IC_{50} = 393), **4** (IC_{50} = 460), and **5** (IC_{50} = 462), **6** (IC_{50} = 395) that used succinic acid as linker. This may mean that malonic acid is a better linker than succinic acid. Compounds **11** (IC_{50} = 370) and **12** (IC_{50} = 371) are conjugates of one molecule of **1** or **2** and one molecule of kojic acid using succinic acid as linker, while **13** (IC_{50} = 463) and **14** (IC_{50} = 461) are conjugates of two molecules of **1** or **2** and one molecule of kojic acid using succinic acid as linker. Compounds **11** and **12** showed stronger inhibitory activity than **13** and **14**. Compounds **15** and **16** are conjugates of one molecule of **1** or **2** and one molecule of quercetin using succinic acid as linker, and **17** (IC_{50} = 370) and **18** (IC_{50} = 375) are conjugates of one molecule of **1** or **2** and one molecule of baicalein using succinic acid as linker. Compounds **19**–**24** are new analogs of compound **2**. Compounds **19** (IC_{50} = 459) and **20** (IC_{50} = 468) are phenylcarbamate and 2-chlorophenylcarbamate derivatives, respectively. Compounds **21** (IC_{50} = 470) and **22** (IC_{50} = 461) are carbamate and *N*-[(4-methylphenyl)sulfonyl] carbamate derivatives, respectively. Compounds **23** (IC_{50} = 460) and **24** (IC_{50} = 473) are phenylcarbamothioate and chloroacetate derivatives, respectively. The EBV-EA activation ratios of **19**–**24** were high.

In our past work, we found that the inhibitory effects on EBV-EA induction by TPA correlated well with the anti-tumor promoting activity in vivo [5–8]. Among the 22 conjugates, we selected **15** and **16** to examine their effects on the in vivo two-stage mouse skin carcinogenesis using mouse skin papillomas induced by DMBA as an initiator and TPA as a promoter. The experimental protocol is shown in Fig. 4. During the in vivo test, the body-weight gains of the mice were not influenced by the treatment with the test compounds and no toxic effects, such as lesional damage and inflammation (edema, erosion, and ulcer), were observed on the areas of mouse skin topically treated with the test compounds. As shown in Fig. 5A, papilloma-bearing mice in the positive control

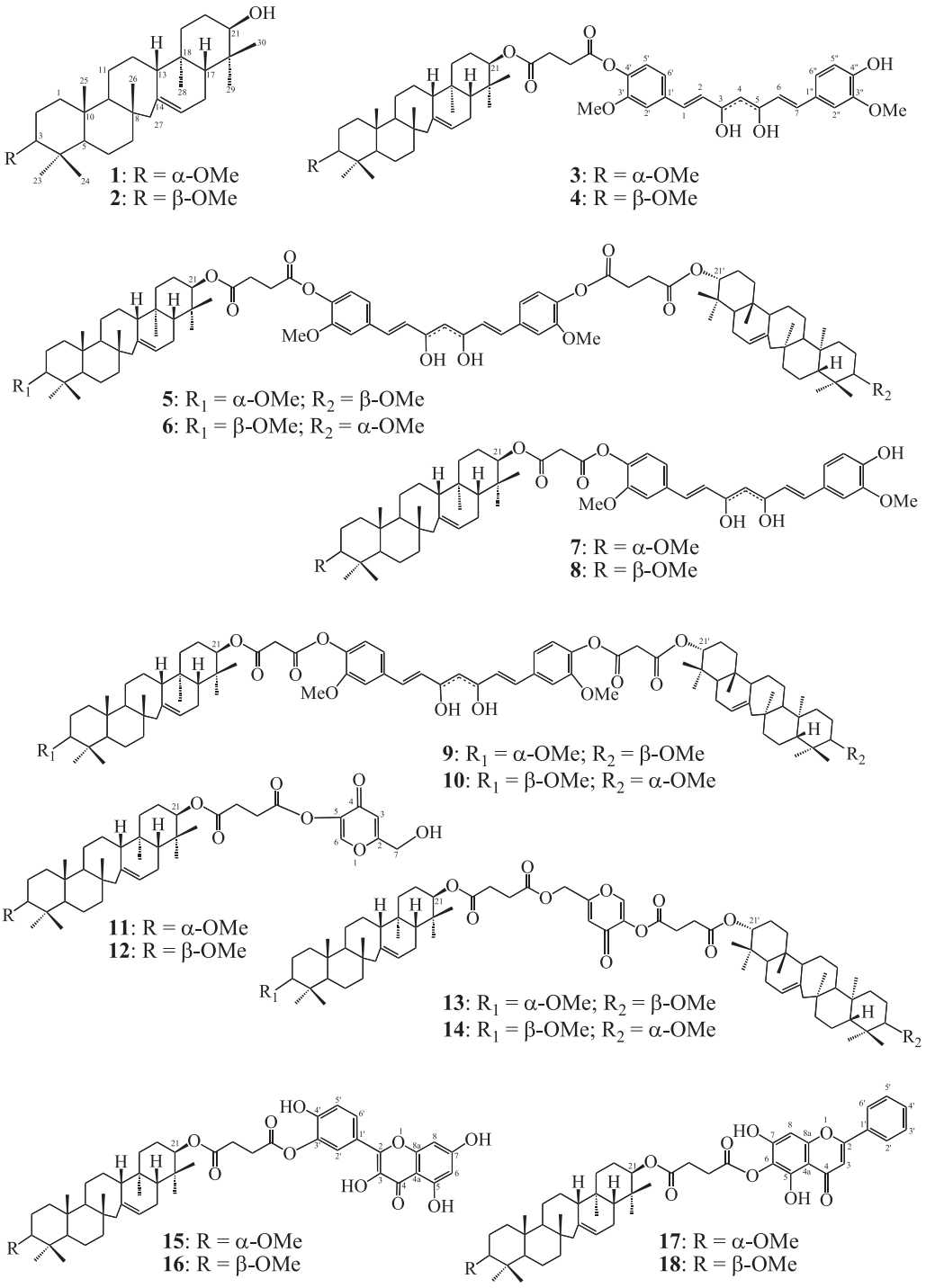

Fig. 1. 3α-Methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their curcumin, kojic acid, quercetin, and baicalein conjugates (**3**–**18**).

group treated with DMBA (390 nmol) and TPA (1.7 nmol, twice/week) appeared as early as at week 6, and the percentage of papilloma bearers increased rapidly to reach 100% after week 10. On the other hand, treatment with **15** and **16** (85 nmol) along with DMBA/TPA reduced the percentage of papilloma-bearing mice to 26.6–46.6% and 20–53.3% during weeks 10–15, respectively, and the percentage reduction was 73.3% and 86.6% at week 20. As shown in Fig. 5B, in the positive control group treated with DMBA/TPA, the number of papillomas formed per mouse increased rapidly after week 6 to reach 8.6 papillomas/mouse at week 20, whereas mice treated with **16** bore 4.7 papillomas (tumor length 26 ± 1.0 mm, tumor width 24 ± 0.7 mm) and those treated with **15** bore 5.0 papillomas (tumor length 28 ± 1.0 mm, tumor width 25 ± 0.7 mm) even at week 20. As illustrated in Fig. 5A, B, in the group treated with **1** and quercetin (each 1 mol) plus DMBA/TPA, the percentages of papilloma-bearing mice reached 93.3% and the number of papillomas/mouse was 7.0 (tumor length 28 ± 1.0 mm, tumor width 25 ± 0.7 mm) at week 20. In the in vivo two-stage mouse skin carcinogenesis test, **15** and **16** were found to delay papilloma formation. The inhibitory effects of **16** and **15** were stronger than that of the positive control, i.e., oleanolic acid and an admixture of 1 mol each of **1** and quercetin. The results of in vitro

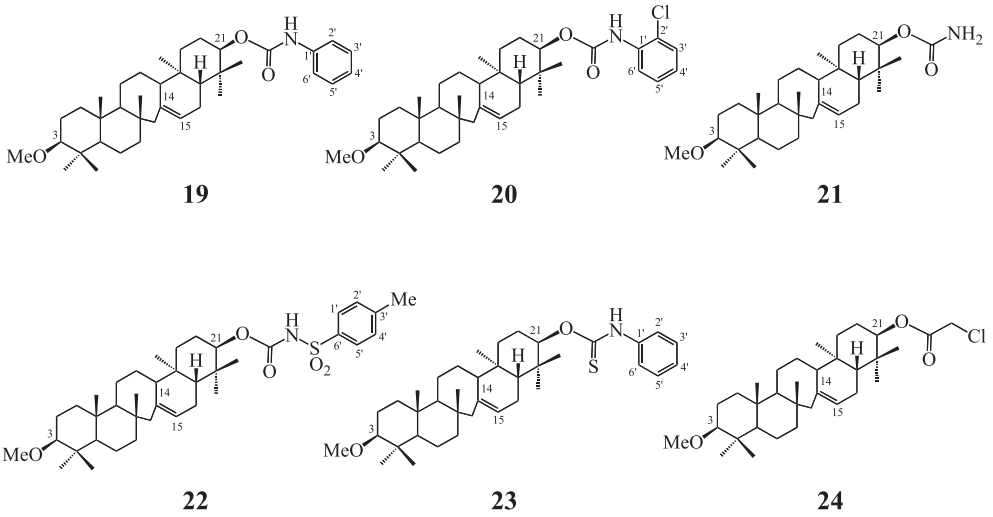

Fig. 2. New 3α-methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**) and their phenylcarbamate, 2-chlorophenylcarbamate, carbamate, *N*-[(4-methylphenyl)sulfonyl]carbamate, phenylcarbamothioate, and chloroacetate (**19**–**24**).

EBV-EA induction and in vivo two-stage mouse skin carcinogenesis test suggest that serratane-type triterpenoids **1** or **2** conjugated with quercetin, such as **15** and **16**, are useful as cancer chemopreventive agents. Further in vivo experiments of remaining effective compounds **7**–**10** are under way.

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 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).

Table 1
Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of compounds **1–24**, compound **1** plus quercetin, and oleanolic acid.

Compound	Concentration (mol ratio/32 pmol/TPA)					IC_{50}
	1000		500	100	10	
3	6.5	(60)[b]	35.6	75.4	96.2	393
4	13.9	(60)	47.9	79.7	100	460
5	14.3	(60)	48.6	81.0	100	462
6	6.2	(60)	35.2	73.9	96.8	395
7	2.9	(60)	33.1	71.7	92.3	354
8	3.1	(60)	34.5	72.8	93.7	358
9	1.6	(60)	31.7	70.1	91.6	335
10	1.7	(60)	31.1	70.3	91.1	338
11	4.9	(70)	32.0	73.1	92.1	370
12	4.8	(70)	32.1	73.8	92.6	371
13	13.1	(60)	46.8	81.1	100	463
14	12.7	(60)	47.0	80.0	100	461
15	5.1	(60)	32.3	73.0	91.7	350
16	2.2	(60)	30.1	71.9	90.3	330
17	6.5	(70)	36.0	75.3	95.7	370
18	6.3	(70)	37.6	76.1	96.9	375
19	11.7	(60)	46.2	81.3	100	459
20	13.6	(50)	47.7	83.6	100	468
21	14.2	(60)	48.2	80.7	100	470
22	12.3	(60)	46.3	82.1	100	461
23	12.0	(60)	46.1	81.1	100	460
24	14.9	(60)	48.2	84.3	100	473
1 + Quercetin (1:1)	2.9	(60)	33.1	71.7	93.4	352
Oleanolic acid[c]	12.7	(70)	30.0	80.0	100	449

[a] Values represent percentages relative to the positive control value (100%).
[b] Values in parentheses are the percentage viability of Raji cells.
[c] Positive control.

3.1.1. Chemicals

Phenyl isocyanate, chlorosulfonyl isocyanate, and *p*-toluenesulfonyl isocyanate 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 (GR) were purchased from Sigma–Aldrich Co. (USA). Cell culture reagents, *n*-butyric 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

Natural serratane-type triterpenoids, 3α-methoxyserrat-14-en-21β-ol (**1**) and 3β-methoxyserrat-14-en-21β-ol (**2**), were isolated from the stem bark of *P. jezoensis* Carr. var. *jezoensis* (Pinaceae) that was collected in Sapporo City, Hokkaido, Japan. The isolation and characterization of **1** and **2** had been reported previously [29]. A voucher specimen has been deposited at the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences. The synthesis, physical properties, and spectral data of compounds **3**–**18** were described in a recent paper [27].

3.2.1. 3β-Methoxyserrat-14-en-21β-yl phenylcarbamate (*19*)

To a solution of compound **2** (46 mg, 0.1 mmol) in pyridine (1 mL) was added phenyl isocyanate (100 μL, 0.9 mmol) and the

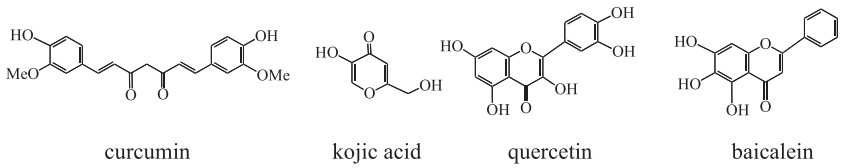

Fig. 3. Structures of curcumin, kojic acid, quercetin, and baicalein.

mixture was allowed to react at room temperature for 120 min. To the reaction mixture was added a small amount of H_2O and the whole was concentrated. To the residue was added Et_2O (10 mL) and the mixture was stirred for a while. The residue after removal of Et_2O was purified by silica gel column chromatography ($CHCl_3$) to give **19** (37 mg, 64%) as a colorless powder. mp 158–160 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ −2.0 (*c* 0.102, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, J = 11.7, 4.2 Hz, H-3α), 4.69 (1H, m, H-21α), 5.34 (1H, m, H-15), 6.61 (1H, bs, NH), 7.05 (1H, tt, J = 7.4, 1.5 Hz, H-4′), 7.30 and 7.31 (each 1H, dt, J = 7.4, 1.5 Hz, H-2′ and H-6′), 7.41 (2H, m, H-3′ and H-5′); ^{13}C NMR δ 79.3 (C-21), 88.4 (C-3), 118.5 (C-3′ and C-5′), 122.0 (C-15), 123.2 (C-4′), 129.0 (C-2′ and C-6′), 138.1 (C-1′), 138.5 (C-14), 153.4 (C═O); MS (FAB) *m/z*: 598 (M^+ + Na); HRMS calculated for $C_{38}H_{57}NO_3Na$, 598.4236; found, 598.4232.

3.2.2. 3β-Methoxyserrat-14-en-21β-yl 2-chlorophenylcarbamate (**20**)

To an ice-cooled solution of compound **2** (92 mg, 0.2 mmol) in pyridine (2 mL) was added 2-chlorophenyl isocyanate (85 μL, 0.7 mmol), and the mixture was allowed to react at room temperature for 6 hrs. To the residue obtained after concentration was added AcOEt and the mixture was washed with brine, dried, and concentrated to give a crystalline residue (200 mg). The residue was purified by silica gel column chromatography ($CHCl_3$) to give **20** (120 mg, 98%) as colorless crystals. mp 200–202 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ −22.4 (*c* 0.102, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, J = 11.7, 4.1 Hz, H-3α), 4.71 (1H, t, J = 2.3 Hz, H-21α), 5.35 (1H, m, H-15), 6.99 (1H, td, J = 7.8, 1.5 Hz, H-4′), 7.10 (1H, bs, NH), 7.26 (1H, td, J = 7.8, 1.5 Hz, H-5′), 7.35 (1H, dd, J = 7.8, 1.5 Hz, H-3′), 8.16 (1H, bd, J = 7.8 Hz, H-6′); ^{13}C NMR δ 79.9 (C-21), 88.5 (C-3), 120.3 (C-6′), 121.9 (C-15), 122.0 (C-2′), 123.6 (C-4′), 127.7 (C-5′), 129.0 (C-3′), 135.0 (C-1′), 138.5 (C-14), 153.2 (C═O); MS (FAB) *m/z*: 632 (M^+ + Na); HRMS calculated for $C_{38}H_{56}NClO_3Na$, 632.3846; found, 632.3841.

3.2.3. 3β-Methoxyserrat-14-en-21β-yl carbamate (**21**)

To an ice-cooled solution of compound **2** (92 mg, 0.2 mmol) in THF (4 mL) was added chlorosulfonyl isocyanate (30 μL, 0.3 mmol) and the mixture was allowed to react at room temperature for 40 min. To the residue obtained after concentration was added *aq*. $NaHCO_3$ and the whole was extracted with methyl ethyl ketone (MEK) several times. The extract was allowed to stand until the Rf value of the extract showed a constant value by TLC. The extract was dried and concentrated, and the residue was chromatographed on a silica gel column (AcOEt/$CHCl_3$ = 1/1). The eluted product was rechromatographed (AcOEt/$CHCl_3$ = 1/3) to afford **21** (52 mg, 52%) as colorless crystals. mp 279–281 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ −0.7 (*c* 0.107, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, J = 11.8, 4.2 Hz, H-3α), 4.56 (1H, t, J = 2.8 Hz, H-21α), 4.60 (2H, bs, NH_2), 5.33 (1H, m, H-15); ^{13}C NMR δ 79.1 (C-21), 88.5 (C-3), 122.0 (C-15), 138.4 (C-14), 156.8 (C═O); MS (FAB) *m/z*: 522 (M^+ + Na); HRMS calculated for $C_{32}H_{53}NO_3Na$, 522.3923; found, 522.3929.

3.2.4. 3β-Methoxyserrat-14-en-21β-yl N-[(4-methylphenyl)sulfonyl]carbamate (**22**)

To an ice-cooled mixture of compound **2** (92 mg, 0.2 mmol) and triethylamine (56 μL, 0.4 mmol) in THF (4 mL) was added *p*-toluenesulfonyl isocyanate (TsNCO) (40 μL, 0.26 mmol) and the mixture was allowed to react at room temperature for 60 min. To the reaction mixture was added *N*,*N*′-dimethylpropanediamine (50 μL, 0.4 mmol) and the whole was stirred until it became a clear solution. This solution was concentrated and extracted with AcOEt. The extract was successively washed with 1 M HCl and brine, dried, and concentrated. The residue was purified by silica gel column chromatography (AcOEt/$CHCl_3$ = 1/5) to give compound **22** (115 mg, 91%) as colorless crystals. mp 218–221 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ −9.4 (*c* 0.106, $CHCl_3$); ^{1}H NMR δ 2.44 (3H, s, H-4′-Me), 2.64 (1H, dd, J = 11.6, 4.1 Hz, H-3α), 4.61 (1H, t, J = 2.3 Hz, H-21α), 5.33 (1H, m, H-15), 7.33 (2H, d, J = 8.0 Hz, H-3′ and H-5′), 7.90 (2H, d, J = 8.0 Hz, H-

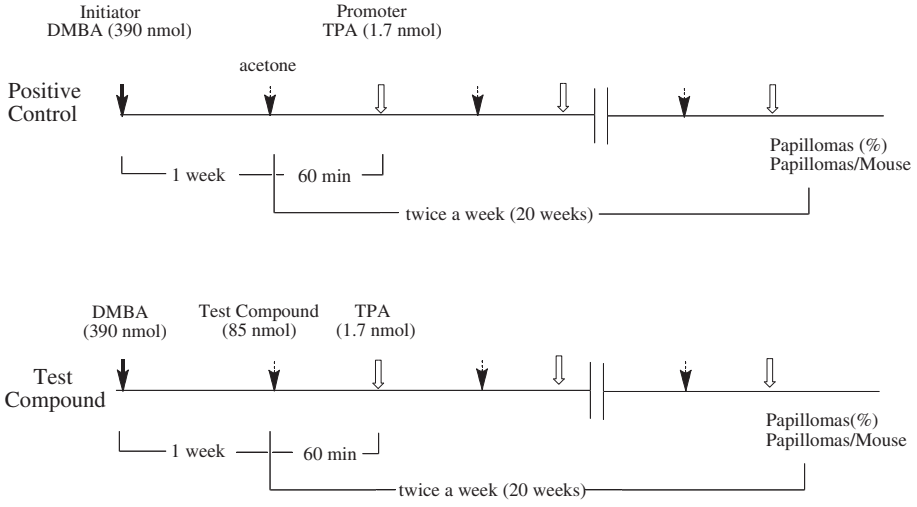

Fig. 4. Experimental design of chemopreventive activity of compounds **15** and **16** on DMBA–TPA-induced carcinogenesis.

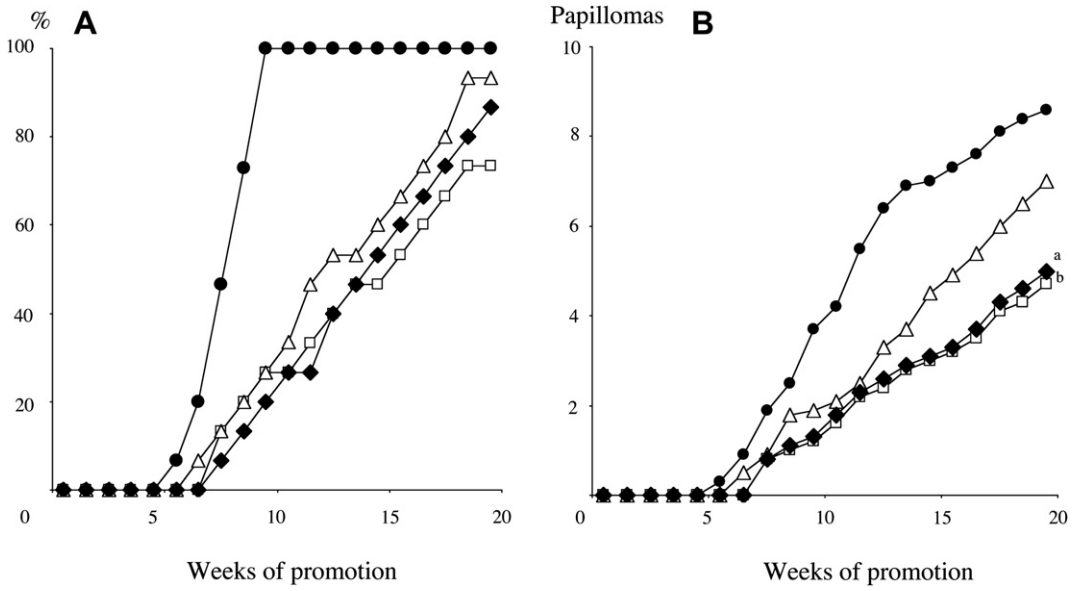

Fig. 5. Inhibition effects of compounds 13 and 14 on DMBA–TPA mouse skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (390 nmol) and promoted with TPA (1.7 nmol) twice weekly, starting 1 week after initiation. (A) Percentage of mice with papillomas. (B) Average number of papillomas/mouse. ● Control (TPA alone); △ TPA + 80 nmol of positive control (PJ-1 + quercetin (1:1)); ◆ TPA + 85 nmol of **15**; □ TPA + 85 nmol of **16**. [a,b]Statistically different from the positive control ($P < 0.05$).

2′ and H-6′); ^{13}C NMR δ 21.6 (C-4′-Me), 82.5 (C-21), 88.5 (C-3), 121.7 (C-15), 128.1 (C-2′ and C-6′), 129.6 (C-3′ and C-5′), 135.8 (C-1′), 138.5 (C-14), 144.8 (C-4′), 150.2 (C═O); MS (FAB) *m/z*: 676 ($M^+ + Na$); HRMS calculated for $C_{39}H_{59}NO_5SNa$, 676.4012; found, 676.4017.

3.2.5. 3β-Methoxyserrat-14-en-21β-yl phenylcarbamothioate (**23**)

A mixture of compound **2** (92 mg, 0.2 mmol), DMAP (13 mg), and phenyl isothiocyanate (200 μL, 1.67 mmol) in pyridine (1.5 mL) was reacted at 100 °C for 48 h. To the residue obtained after concentration was added AcOEt and the suspension was filtered. The filtrate was washed with brine, dried, and concentrated to yield a brown residue. $CHCl_3$ was added and the whole was filtered. The filtrate was concentrated and the residue was purified by silica gel column chromatography (AcOEt/$CHCl_3$ = 1/30) to give compound **23** (52 mg, 44%) as a colorless powder. mp 226–229 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ +64.3 (*c* 0.099, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, *J* = 11.7, 4.0 Hz, H-3α), 5.30 (1H, m, H-15), 5.41 (1H, m, H-21α), 7.18 (1H, t, *J* = 7.6 Hz, H-4′), 7.28 and 7.31 (each 1H, d, *J* = 7.6 Hz, H-2′ and H-6′), 7.34 (2H, t, *J* = 7.6 Hz, H-3′ and H-5′). 8.20 (1H, bs, NH); ^{13}C NMR δ 88.4 (C-3 and C-21), 121.8 (C-15), 122.9 (C-2′ or C-6′), 125.9 (C-4′), 129.0 (C-3′ and C-5′), 129.0 (C-2′ or C-6′), 136.8 (C-1′), 138.5 (C-14), 188.7 (C═S); MS (FAB) *m/z*: 614 ($M^+ + Na$); HRMS calculated for $C_{38}H_{57}NO_2SNa$, 614.4008; found: 614.4013.

3.2.6. 3β-Methoxyserrat-14-en-21β-yl chloroacetate (**24**)

To an ice-cooled mixture of compound **2** (228 mg, 0.5 mmol) and pyridine (160 μL, 2 mmol) in $CHCl_3$ (5 mL) was added chloroacetyl chloride (120 μL, 1.5 mmol) and the mixture was allowed to react at room temperature for 40 min. To the reaction mixture were added chilled H_2O and *dil.* $NaHCO_3$, and the whole was extracted with $CHCl_3$. The extract was washed with brine, dried, and concentrated to furnish a brown powder. The residue was purified by silica gel column chromatography ($CHCl_3$) to give compound **24** (206 mg, 77%) as colorless crystals. mp 231–233 °C (MeOH–$CHCl_3$); $[\alpha]^{22}_D$ −65.8 (*c* 0.102, $CHCl_3$); ^{1}H NMR δ 2.63 (1H, dd, *J* = 11.8, 4.3 Hz, H-3α), 4.77 (1H, t, *J* = 2.8 Hz, H-21α), 4.09 (2H, s, O═C−CH_2−Cl), 5.33 (1H, m, H-15); ^{13}C NMR δ 41.3 (O═C−CH_2−Cl), 80.8 (C-21), 88.5 (C-3), 121.8 (C-15), 138.5 (C-14), 166.9 (C═O); MS (FAB) *m/z*: 555 ($M^+ + Na$); HRMS calculated for $C_{33}H_{53}ClO_3Na$, 555.3581; found, 555.3586.

3.3. Assay for inhibition of EBV-EA activation

EBV-EA-positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medicinal University. Lymphoblastoid cells carrying EBV genome (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). The 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 [6]. 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 inducer, and various amounts of test compounds in 5 μL of 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 percentage relative to the control experiment (100%) that was carried out only with *n*-butyric 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. Five animals were housed in each 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 of 15 mice each. The back (2×8 cm^2) of each mouse was shaved with surgical clippers and topically treated with DMBA (100 μg, 390 nmol) in

acetone (0.1 mL) as initiation treatment. One week after the initiation, papilloma formation was promoted twice weekly by the application of 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 [6].

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