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Two New Anti-Tumor Promoting Serratane-Type Triterpenoids from the Stem Bark of *Picea jezoensis* var. *jezoensis*

Abstract

Two new serratane-type triterpenoids, **1** and **2**, were isolated from the stem bark of *Picea jezoensis* Carr. var. *jezoensis* (Pinaceae). Their structures were determined to be 3β-methoxyserrat-13-en-21β-ol (**1**) and 13β, l4β-epoxy-3β-methoxyserratan-21β-ol (**2**) on the basis of spectroscopic methods and partial syntheses. Compounds **1** and **2** and their acetates were screened as potential anti-tumor promoters by using the *in vitro* short-term 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) activation assay. IC_{50} value evaluation showed that compound **1** was more effective than others. In addition, compounds **1** and **2** were examined for anti-tumor promoting activities in a two-stage carcinogenesis assay of mouse skin tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. Compounds **1** and **2** exhibited significant anti-tumor promoting effects on mouse skin carcinogenesis.

Key words
3β-Methoxyserrat-13-en-21β-ol · 13β,14β-epoxy-3β-methoxyserratan-21β-ol · *Picea jezoensis* var. *jezoensis* · Pinaceae · 2D NMR · *in vitro* EBV-EA activation assay · *in vivo* two-stage mouse skin carcinogenesis test

Introduction

Chemoprevention is regarded as one of the efficient strategies for cancer prevention [1]. Inhibition of the tumor promotion stage in multistage chemical carcinogenesis has been regarded as the most promising method for cancer chemoprevention [2]. In the search for cancer chemopreventive agents, the inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) induction by the tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), have been studied as a primary screening test [3]. In our search for naturally occurring cancer chemopreventive agents, the stem bark of *Picea jezoensis* Carr. var. *jezoensis* (Japanese name: Ezomatsu) (Pinaceae) was selected for detailed investigation. In previous papers, we had reported a considerable number of serratane-type triterpenenoids from this stem bark [4], [5], [6], [7]. Recently we reported two novel compounds, jezananals A and B from this bark [8]. Among them, 13α,14α-epoxy-3β-methoxyserratan-21β-ol (**3**) showed significant anti-tumor promoting activity in the *in vivo* two-stage mouse-skin carcinogenesis assay using 7,12-dimethylbenz[α]anthracene (DMBA) as an initiator and TPA as a tumor promoter [9].

Therefore, serratane-type triterpenoids were considered to represent appropriate lead compounds to develop more potent agents with anti-tumor promoting activity for clinical use. In our further search for naturally occurring cancer chemopreventive agents, a detailed investigation of this stem bark was carried out.

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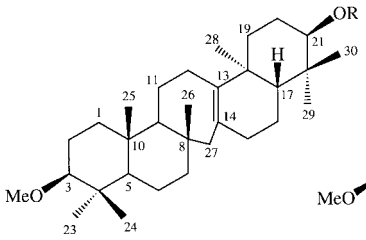

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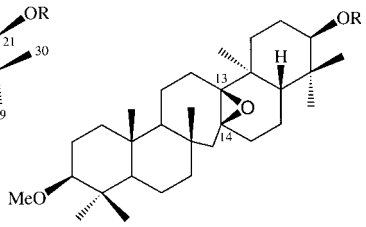
1a : R = Ac



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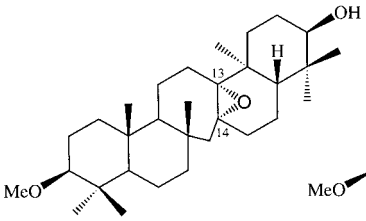

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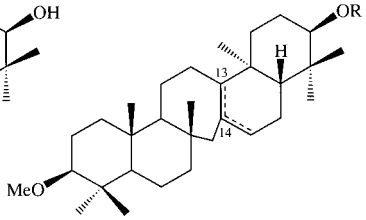

4 : R = H, 14-ene
4a : R = Ac, 14-ene
4b : R = Ac, 13-ene
4c : R = H, 13-ene = **1**
4d : R = H, 13α,14α-epoxy
4e : R = H, 13β,14β-epoxy

Material and Methods

General experimental procedures

Melting points were measured with a Yanagimoto micro-melting point apparatus without correction. Optical rotations were determined with a JASCO DIP-IOOO 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 MHz and 125 MHz, respectively. $CDCl_3$ was used as the solvent and Me_4Si (TMS) as the internal standard. EI-MS 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). TLC and preparative TLC were carried out on Merck silica gel F_{254} plates.

Plant material

Cuticles of *P. jezoensis* (Sieb. et Zucc.) Carr. var. *jezoensis* were collected at ca. 1000 m in the mountains of Hidaka town, Saryu district, Hokkaido, Japan, in June 2001. A voucher specimen (PJJ-01-01) is deposited at the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

Extraction and isolation

The freshly chopped cuticles (12 kg) of *P. jezoensis* var. *jezoensis* were extracted with $CHCl_3$ (20 L) employing an automatic percolator for 7 days at 50 °C. The $CHCl_3$ solution was evaporated under reduced pressure and the resulting dark green residue (685.0 g) was subjected to silica gel (13 kg) column chromatography. Elution of the column with $CHCl_3$ afforded residues A (39.3 g), B (67.2 g), C (28.9 g), D (71.0 g), E (51.6 g), and F (23.8 g), from fractions 1 – 13, 14 – 26, 27 – 30, 31 – 40, 41 – 60 and 61 – 74 (each 2 L). Elution was continued with $CHCl_3$-EtOAc (5 : 1) to give residues G (60.1 g), H (52.9 g) and I (12.6 g) from fractions 75 – 83, 84 – 90 and 91 – 105 (each 2 L). Further elution with EtOAc gave a residue J (83.1 g) from fractions 106 – 111 (each 2 L). Recrystallization of residue E gave 3*β*-methoxyserrat-14-en-21*β*-ol (**4**) (33.8 g), and the filtrate (17.8 g) of compound **4** was subjected to column chromatography on silica gel (600 g) using *n*-hexane-$CHCl_3$ (1 : 1) and afforded a crystalline solid (fractions 48 – 52, 2.032 g) (each 300 mL), which was subjected to MPLC with *n*-hexane-EtOAc (10 : 1) followed by recrystallization from MeOH-$CHCl_3$ to give compound **1** (1.518 g). Repeated column chromatography of residue G on silica gel (2 kg) eluting with $CHCl_3$-EtOAc (10 : 1) afforded a crystalline solid (fractions 38 – 55, 1.220 g) (each 500 mL), which was subjected to MPLC using *n*-hexane-EtOAc (10 : 1) to give crude compound **2** (fractions 77 – 83, 312 mg) and recrystallized from MeOH-$CHCl_3$ to give compound **2** (246 mg).

3β-Methoxyserrat-13-en-21β-o1 (**1**): Colorless prisms; m.p. 257 – 259 °C (from MeOH-$CHCl_3$); $[\alpha]_D^{23.5}$: +59.1 (*c* 0.28, $CHCl_3$); HR-El-MS: *m/z* = 456.3965 $[M]^+$ (calcd. for $C_{31}H_{52}O_2$: 456.3965); lR (KBr) ν_{max} = 3497 (OH), 2960, 2850, 1457, 1383, 1224, 1201, 1181, 1098, 991, 963 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table **1**. El-MS: *m/z* (rel. int.) = 456 (100) $[M^+]$, 441 (66), 438 (42), 423 (41), 409 (7), 391 (14), 285 (8), 255 (17), 221 (73), 203 (57), 189 (69), 135 (42), 121 (31).

3*β-Methoxyserrat-13-en-21β-yl acetate* (**la**): Compound **1** (25 mg) was acetylated as usual (Ac_2O-pyridine, 1 : 1, 2 mL) to yield a crystalline solid. Purification by PTLC ($CHCl_3$-MeOH, 30 : 1) afforded a corresponding monoacetate (**1a**), m.p. 206 – 209 °C (from MeOH-$CHCl_3$); $[\alpha]_D^{23.5}$: –9.7 (*c* 0.11, $CHCl_3$); HR-El-MS: *m/z* = 498.4072 $[M^+]$ (calcd. for $C_{33}H_{54}O_3$: 498.4070); lR (KBr): ν_{max} = 1727, 1246 cm^{-1} (OAc); ^{1}H- and ^{13}C-NMR: see Table **1**. El-MS: *m/z* (rel. int.): = 498 (42) $[M]^+$, 438 (36),423 (75), 221 (52), 203 (100), 189 (90), 121 (35).

Conversion of 3β-methoxyserrat-13-en-21β-ol (**1**) *to 3β-methoxyserrat-14-en-21β-ol* (**4**): Compound **4** (140.1 mg) was acetylated as usual to give a corresponding mono-acetate (**4a**) (147.0 mg). A solution of compound **4a** (100 mg) in glacial HOAc (30 mL) was added H_2SO_4 (3.0 mL) under ice cooling, and the mixture was kept at room temperature for 16 h. Then, the mixture was poured into ice/water, and the resulting precipitate was extracted with $CHCl_3$ (100 mL × 3). The $CHCl_3$ extract was neutralized with 5 % NaOH solution, washed with H_2O, and dried over Na_2SO_4. Evaporation of $CHCl_3$ yielded a crystalline solid (94.8 mg) which was subjected to MPLC (230 – 400 mesh, silica gel, Merck) using *n*-hexane:EtOAc (20 : 1) to give 3*β*-methoxyserrat-13-en-21*β*-yl acetate (**4b**) (fractions 13 – 25, 85.5 mg). Compound **4b** (70 mg) was hydrolyzed with KOH (200 mg) in MeOH (30 mL) at 80 °C in 8 h. Work-up at usual gave a residue (64.2 mg) which was purified by MPLC using *n*-hexane : EtOAc (10 : 1) to afford 3*β*-methoxyserrat-13-en-21*β*-ol (**4c**) (fractions 37 – 47, 62.5 mg), and **4c** was identified by direct comparison with an authentic sample of **1**.

13β,14β-Epoxy-3β-methoxyserratan-21β-ol (**2**): Colorless prisms; m.p. 264 – 267 °C (from MeOH-$CHCl_3$); $[\alpha]_D^{23.5}$: +4.7 (*c* 0.11, $CHCl_3$); HR-El-MS: *m/z* = 472.3913 $[M^+]$ (calcd. for $C_{31}H_{52}O_3$: 472.3914); lR (KBr): ν_{max} = 3500 (OH), 2963, 2937, 2874, 1460, 1385 and 1365 *(gem.* dimethyl), 1221, 1182, 1103,

Table **1** NMR Data for compound 1 (125 and 500 MHz, $CDCl_3$)[a,b]

		1		*1c*	
Position	δ_C	δ_H	***NOESY***	δ_C	δ_H
Lα	38,2 t	0.85 m		38.2 t	0.86m
1β		1.81 m	11β		1.80 m
2α	22.3 t	1.79 m		22.3 t	1.80 m
2β		1.41 m			1.40 m
3α	88.6 d	2.63 dd (11.9, 4.1)	5α, 23	88.5 d	2.62 dd (11.9, 3,9)
4	38.9 s			37.0 s	
5α	56.2 d	0.78 m	9α	56.2 d	0.78 m
6α	18.8 t	1.49 m	23	18.8 t	1.48 m
6β		1.44 m			1.43 m
7α	44.9 t	1.21 ddd (13,3, 13.3, 4.3)	5α	44.9 t	1.23 ddd (13.3, 13.3, 4.1)
7β		1.41 m			1.42 m
8	35.8 s			35.7 s	
9α	65.0 d	0.91 d (11.9)	lα, 5α	64.8 d	0.92 dd (11.9, 1.8)
10	38.3 s			38.2 s	
11α	21.6 t	1.64 m	1β, 9α	21.5 t	1.66 m
11β		1.03 m			1.01 m
12α	28.2 t	1.73 m	9α, 27α, 28	28.1 t	1.76 m
12β		2.26 dd (14.2, 7.8)	19α, 19β		2.25 dd (14.4, 8.0)
13	143.1 s			142.9 s	
14	129.6 s			129.6 s	
15α	36.1 t	1.90 dd (17.8, 5.5)		36.2 t	1.90 m
15β		2.07 m	26		2.07 m
16α	19.2 t	1.36 m	28, 29	19.0 t	1.52 m
16β		1.52 m	30		1.34 m
17β	45.3 d	1.52 m		46.4 d	1.52 m
18	38.4 s			38.3 s	
19α	29.5 t	1.61 m		30.1 t	1.64 m
19β		1.61 m			1.46 m
20α	25.8 t	1.98 dddd (12.6, 12.6, 5.5, 2.3)	28, 29	23.4 t	1.91 m
20β		1.64 m			1.68 m
21α	75.8 d	3.44 t (2.8)	29, 30	77.8 d	4,68 t (2.7)
22	37.7 s			36.8 s	
23	28.0 q	0.94 s	6α	28.0 q	0.96 s
24	16.1 q	0.74 s	23	16.1 q	0.74 s
25	16.3 q	0.76 s	2β, 11β, 26	16.3 q	0.79 s
26	19.4 q	0.84 s	7β, 11β, 15β, 17β	19.2 q	0.85 s
27α	52.8 t	2.16 d (14.2)	7α	52.8 t	2.16 d (14.2)
27β		1.35 d (14.2)			1.37 d (14.2)
28	19.2 q	0.89 s	12α, 16α, 19α, 20α	19.2 q	0.90 s
29	22.2 q	0.85 s	16α, 16β	27.6 q	0.88 s
30	28.0 q	0.97 s	16β, 29	21.8 q	0.70 s
OMe	57.5 q	3.35 s		57.5 q	3.35 s
OAc				21.4 q	2.06s
				171.0 s	

[a] Assignments confirmed by H/H COSY, NOESY, HMQC and HMBC spectra.

[b] *J* values are given in Hz.

1078, 1070, 995, 976 cm^{-1}; ^1H- and ^{13}C-NMR: see Table **2**. El-MS: *m/z* (rel. int.) = 472 (1) [M^+], 454 (1), 421 (3), 367 (1), 319 (1), 287 (4), 269 (8), 221 (6), 189 (11) 154 (19), 136 (100), 121 (45).

13β,14β-Epoxy-3β-methoxyserratan-21β-yl acetate (**2a**): Compound **2** (15 mg) was acetylated as usual (Ac_2O-pyridine, 1 : 1, 2 mL) to yield a crystalline solid, which was recrystallized from MeOH-$CHCl_3$ to give a corresponding monoacetate (**2a**), m. p. 205 – 207 °C (from MeOH-$CHCl_3$); $[\alpha]_D^{23.5}$: –18.9 (*c* 0.11, $CHCl_3$); HR-El-MS: *m/z* = 514.4015 [M^+] (calcd for $C_{33}H_{54}O_4$: 514.4020); lR (KBr): ν_{max} = 1728, 1244 cm^{-1} (OAc); ^1H- and ^{13}C-NMR: see Table **2**; El-MS: *m/z* (rel. int.) = 514 (6) [M^+], 496 (10), 454 (22), 436 (9), 421 (68), 221 (6), 136 (100), 121 (47).

Preparation of 13β,14β-epoxy-3β-methoxyserratan-21β-ol (**2**) *from 3β-methoxyserrat-13-en-21β-ol* (**4c**): A solution of *m-CPBA*

Table **2** NMR data for compound **2** (125 and 500 MHz, $CDCl_3$)[a,b]

		2			2a	
Position	δ_C	δ_H	*HMBC (C → H)*	*COSY*	δ_C	δ_H
1α	38.3 t	0.84m	5α, 9α, 25		38.3 t	0.84 m
1β		1.86 dt (16.2, 3.2)		1α,2β		1.87 m
2α	22.3 t	1.78 m		1α, 2α, 2β	22.3 t	1.80 m
2β		1.42 m		1α, 1β, 2α, 3α		1.42 m
3α	88.5 d	2.61 dd (11.9, 4.3)	23, 24, OMe		88.5 d	2.62 dd (11.7, 4.3)
4	38.9 s		3α, 5α, 23, 24		38.9 s	
5α	56.1 d	0.67 dd (10.1, 3.9)	23, 24, 25	6α, 6β	56.1 d	0.67 dd (10.5, 3.0)
6α	18.1 t	1.44 m	5α	5α, 7α	18.1 t	1.45 m
6β		1.44 m		5α, 7α		1.45 m
7α	45.0 t	1.43 m	27β		44.9 t	1.44 m
7β		1.14 td (13.3, 5.3)		6α, 6β, 7α		1.15 td (13.0, 3.0)
8	37.0 s		7β, 9α, 27α		36.9 s	
9α	61.7 d	0.55 d (10.5)	12β, 25, 26	11β	61.4 d	0.57 d (10.3)
10	38.3 s		5α, 9α, 25		38.3 s	
11α	19.4 t	1.50 m	9α	11β	19.4 t	1.51 m
11β		1.32 m		9α, 11α, 12α		1.32 m
12α	27.9 t	1.56 m	9α	11α, 11β, 12β	27.9 t	1.55 m
12β		2.14 ddd (14.6, 7.6, 1.8)		11α, 12α		2.12 ddd (15.1, 8.0, 2.5)
13	70.0s		12β, 15α, 27α, 27β, 28		70.0 s	
14	63.9 s		12β, 15α, 27α, 27β		63.7 s	
15α	31.2 t	1.91 m	17β	15β, 16α, 16β	31.1 t	
15β		1.74 m		15α, 16α, 16β		
16α	17.3 t	1.28 m	15β, 17α	15α, 15β, 17β	17.1 t	1.26 m
16β		1.28 m		15α, 15β, 17β		1.26 m
17β	36.5 d	1.81 dd (9.6, 5.0)	28,29,30	16α, 16β	37.6 d	1.88 m
18	37.8 s		19β, 28		37.3 s	
19α	27.4 t	1.54 m	28	19β, 20α	28.0 t	1.55 m
19β		1.68 m		19α, 20β		1.55 m
20α	25.3 t	1.65 m		19α, 19β, 20β	22.9 t	1.85 m
20β		1.89 m				1.70 ddd (14.9, 6.9, 3.4)
21α	75.5 d	3.36 t (2.7)	29, 30		77.4 d	4.62 t (2.7)
22	37.4 s		29, 30		36.5 s	
23	28.1 q	0.95 s	3α, 24		28.1 q	0.95 s
24	16.1 q	0.73 s	1α, 3α, 5α, 23		16.1 q	0.77 s
25	15.7 q	0.77 s	1α, 9α		15.7 q	0.74 s
26	21.9 q	1.02 s	7α, 7β, 9α, 27α		22.0 q	1.01 s
27α	53.8 t	1.62 d (15.1)	9α, 26	27β	53.7 t	1.63 d (15.1)
27β		1.42 d (15.1)		27α		1.42 d (15.1)
28	16.7 q	1.00 s	19β		16.7 q	0.81 s
29	22.0 q	0.83 s	30		21.7 q	0.95 s
30	28.5 q	0.91 s	29		28.0 q	1.03 s
OMe	57.5 q	3.35 s	3α		57.5 q	3.35 s
OAc					21.4 q	2.06 s
					171.0s	

[a] Assignments confirmed by decoupling, H/H COSY, NOESY, HMQC, and HMBC spectra.
[b] *J* values are given in Hz.

(100 mg) in dry $CHCl_3$ (5 mL) was gradually added over a solution of **4c** (100 mg) in dry $CHCl_3$ (10 mL) under stirring at room temperature and allowed to stand for 24 h. Work-up as usual yielded a residue (97 mg), which was subjected to MPLC with *n*-hexane-EtOAc (10 : 1) to give compounds **4e** (4 mg) (fractions 7 – 8) and **4d** (89 mg) (fractions 25 – 40). The synthetic compounds **4d** and **4e** were identified as natural compounds **3** and **2**, respectively, by direct comparison.

Inhibition of EBV -EA activation test

EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from Dr. Y. Zaizen, 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 [9]. 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 dimethyl sulfoxide (DMSO)], as inducer and various amounts of test compound 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 [2]. In each assay, at least 500 cells were counted, the number of stained cells were counted, and the number of stained cells (positive cells) present 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*-butyric acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was ordinarily around 35%. The viability of treated Raji cells was assayed by Trypan blue staining methods.

Two-stage mouse-skin carcinogenesis test

Specific pathogen-free female ICR mice (6 weeks old, body weight approx. 30 g) were obtained from Japan SLC Inc., Shizuok, Japan, and the animals were housed, 5 per polycarbonate cage, in a temperature-controlled room at 24 ± 2 °C and given food and water ad libitum throughout the experiment. 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 DMBA (100 μg, 390 nmol) in acetone (0.1 mL) as an initiating 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. Group 1 received the TPA treatment alone, and groups II and III received a topical application of compounds **1** and **2** (85 nmol), in acetone (0.1 mL), respectively, 1 h before the TP A treatment. The incidence and numbers of papillomas were monitored weekly for 20 weeks.

Results and Discussion

Structure determination of 1 and 2

The molecular formula of compound **1** was assigned as $C_{31}H_{52}O_2$ (M^+; m/z = 456.3965) by HR-EI-MS. The IR spectrum showed a hydroxy (ν_{max} = 3497 cm^{-1}) absorption. The ^{1}H- and ^{13}C-NMR spectra of **1** (Table **1**) exhibited seven tertiary methyls, eleven methylenes, three methines, five quaternary carbons, a secondary hydroxy group [δ_H = 3.44 (1H, t); δ_C = 75.8 (d)], a secondary methoxy group [δ_H = 2.63 (dd), 3.35 (3H, s); δ_C = 57.5 (q), 88.6 (d)] and a tetrasubstituted double bond [δ_C = 129.6 (s), 143.1 (s)]. Acetylation of **1** gave a monoacetate (**la**), $C_{33}H_{54}O_3$ (M^+; *m/z* = 498.4072), in which the hydroxymethine proton signal was shifted to δ = 4.68 (t). In the ^{1}H-^{1}H COSY spectra of **1**, H-12α (δ_H = 1.73) was related only to H-11α, H-11β and H-12β, and H-12β (δ_H = 2.26) was related only to H-11α, H-11β and H-12α. In the NOESY spectrum of **1**, significant NOEs were observed between H-3α with H-5α and Me-23, and between H-21α with Me-29 and Me-30, which suggested that the methoxy group had C-3β and the hydroxy group had C-21β configurations. All these data suggested that the structure of **1** should be 3β-methoxyserrat-13-en-21β-ol. In order to confirm this structure, we tried the synthesis of **1** from 3β-methoxyserrat-14-en-21β-ol (**4**), which is the most abundant triterpenoid of this stem bark. Isomerization of 3β-methoxyserrat-14-en-21β-yl acetate (**4a**) with conc. H_2SO_4/HOAc furnished the corresponding 3β-methoxyserrat-13-en-21β-yl acetate (**4b**) in almost quantitative yield, and then hydrolysis gave the corresponding alcohol **4c**. The above synthetic **4b** and **4c** were identical with **la** and **1** in all respects. To the best of our knowledge, other serrat-13-ene derivatives isolated so far are 3α-methoxyserrat-13-en-21β-ol from the bark of *Picea sitchensis* (Sitka sparuce) [10] and 21α-methoxyserrat-13-en-3-one from the stem bark of *Picea jezoensis* Carr. var. *hondoensis* [11] and *P. jezoensis* Carr. var. *jezoensis* [7].

Compound **2** was assigned as $C_{31}H_{52}O_3$ (M^+; *m/z* = 472.3913) by HR-EI-MS. The IR spectrum showed a hydroxy (**1**: ν_{max} = 3500 cm^{-1}) absorption. The ^{1}H- and ^{13}C-NMR spectra of **1** (Table **2**) exhibited seven tertiary methyls, eleven methylenes, three methines, five quaternary carbons, a secondary hydroxy group [δ_H = 3.36 (1H, t); δ_C = 75.5 (d)], a secondary methoxy group [δ_H = 2.61 (dd), 3.35 (3H, s); δ_C = 57.5 (q), 88.5 (d)] and two sp^3 quaternary carbons combined with one oxygen atom [δ_C = 63.9 (s), 70.0 (s)]. Acetylation of **2** furnished a monoacetate (**2a**), $C_{33}H_{54}O_4$ (M^+; *m/z* = 514.4015), in which the hydroxymethine proton signal was shifted to δ_H = 4.69 (t). The DEPT and HMQC spectra of **2** showed the same carbon composition as 13α,14α-epoxy-3β-methoxyserratan-21β-ol (**3**) [7]. The difference between **2** and **3** is assumed to come from the bonding of the epoxy ring at C-13 and C-14, considering that these carbons in **2** appeared at δ = 70.0 (s, C-13) and 63.9 (s, C-14) whereas they appeared at δ = 72.9 (s, C-13) and 65.7 (s, C-14) in **3**, and H-12α and H-12β appeared at δ = 2.52 (dd) and 0.98 (m) in **2** whereas they appeared at δ = 1.56 (m) and 2.14 (ddd) in **3** [7]. The HMBC spectrum of **2** (Table **2**) supported this assumption, accordingly, the structure of **2** was suggested as the epoxy epimer of **3**. In the NOESY spectrum of **2** (Fig. **1**), significant NOEs were observed between H-12α (δ_H = 1.56) with H-9α and Me-28, between H-27α (δ_H = 1.62) with H-7α and H-9α, between H-3α (δ_H = 2.61) with H-5α and Me-23, and between H-21α (δ_H = 3.36) with Me-29 and Me-30. However, the cross peaks of H-12α, H-15α and H-17β which decide the conformation of the C and D rings of **2** were not observed clearly. The configuration of an epoxy ring and the conformation of the C, D rings were determined by employing the NOE difference experiment. Upon selective irradia-

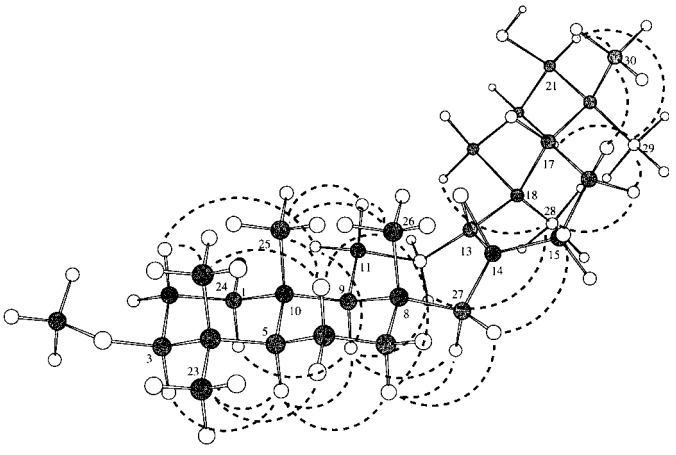

Fig. **1** NOESY correlations of **2**.

tion of the signal of H-12β (δ_H = 2.14), 1.71%, 4.53%, 3.45% NOEs were observed for the signals of H-16α, H-16β and Me-28. Irradiation of the signal for H-15α (δ_H = 1.91) showed 1.91%, 3.85%, 1.97%, 1.93% and 0.87% NOEs for H-12α, H-27α, H-27β, Me-28 and Me-29. Irradiation of the signal for H-17β (δ_H = 1.81) showed 1.40%, 0.96%, 2.56% and 3.95% NOEs for H-12α, H-19β, H-27β and Me-30. Thus, the stereostructure of **2** was established as shown in Fig. **1**.

The El-MS of **2** showed the fragment ion peaks due to cleavage of the D and C rings at *m/z* = 287.2366 $[C_{20}H_{31}O]^+$, 221.1902 $[C_{15}H_{25}O]^+$, and 189.1654 $[C_{14}H_{21}]^+$, 154.1358 $[C_{10}H_{18}O]^+$, 136.1245 $[C_{10}H_{16}]^+$, 121.1021 $[C_9H_{13}]^+$, and the fragment pattern was close to that of **3** [7]. All these data indicated that the structure of **2** as 13β,14β-epoxy-3β-methoxyserratan-21β-ol, which was confirmed by synthesis. Oxidation of synthetic 3β-methoxyserrat-13-en-21β-ol (**4c**) (100 mg) with *m*-CPBA gave 13α,14α-epoxy-3β-methoxyserratan-21β-ol (**4d**) (89 mg) as a major product and the 13β,14β-epoxy epimer (**4e**) (6 mg) of **4d** as a minor product, which was identical in all respects with **2**. It is interesting to note that *Picea jezoensis* Carr. var. *jezoensis* produces both 13α,14α-epoxy-3β-methoxyserratan-21β-ol and its 13β,14β-epoxy epimer in the plant organ.

In vitro EBV-EA activation

The primary screening test was carried out utilizing a short-term *in vitro* assay on EBV-EA activation. Table **3** lists inhibitory effects of compounds **1**, **la**, **2** and **2a** on the EBV-EA activation induced by TPA and the associated viability of Raji cells. The inhibitory effects of all compounds (**1**, **1a**, **2**, **2a)** were stronger at every concentration than that of oleanolic acid [12] known as a representative anti-tumor promoting agent. All compounds exhibited dose-dependent inhibitory activities, and the viability percentages of Raji cells treated with the test compounds (**1**, **la**, **2**, **2a**) were 70% at the highest concentration of 1000 mol ratio/TPA, suggesting that the cytotoxicities of all compounds were rather moderate against *in vitro* cell lines (Table **3**). Among them, compound **1** exhibited a stronger inhibitory activity than others (**la**, **2**, **2a**). The relative ratios of compound **1** with respect to TPA (100%) were 0, 20.3, 74.1 and 89.7% at the concentrations of 1000, 500, 100 and 10 mol ratio/TPA, respectively (Table **3**); meaning 100, 79.7, 25.9 and 10.3% inhibition of the EBV-EA activation by TPA, respectively.

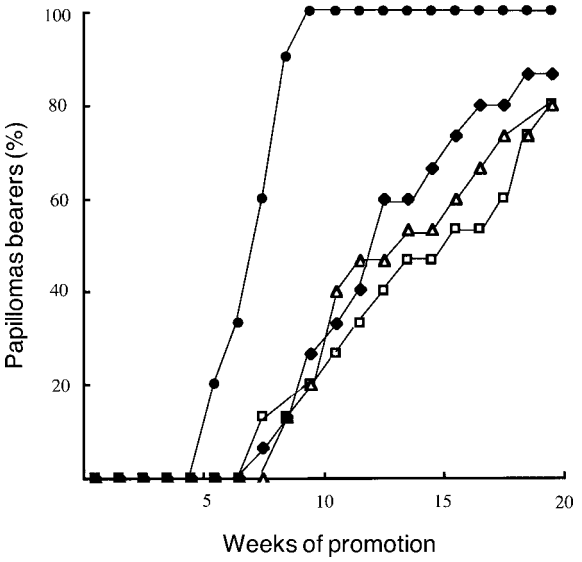

Fig. **2** Inhibition of TP A-induced tumor promotion by multiple application of 3β-methoxyserrat-13-en-21β-ol (**1**) and 13β,14β- epoxy-3β-methoxyserratan-21β-ol (**2**). All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TP A, given twice weekly starting 1 week after initiation. Percentage of mice bearing papillomas. ● control (TPA alone); ■ TPA + 85 nmol of oleanolic acid; △ TPA + 85 nmol of **1**; □ TPA + 85 nmol of **2**.

In vivo two-stage carcinogenesis test on mouse skin papillomas initiated by DMBA

The results of the *in vitro* experiments and structure-activity relationship of serratane-type triterpenoids prompted us to examine the effects of compounds **1** and **2** on the *in vivo* two-stage carcinogenesis bioassay on mouse skin using DMBA as an initiator and TPA as a promotor. The incidence (%) of papilloma-bearing mice and the average numbers of papillomas per mouse are presented in Figs. **2** and **3**, respectively. No significant toxic effects, such as inflammation and lesional damages, on the areas of mouse skin topically treated with the test compounds were observed at the end of treatment except for the formation of papillomas, and also the body weight gains were not influenced during the treatment. As demonstrated in Fig. **2**, the percentage of papilloma bearers in the control group (DMBA and TPA only) increased rapidly from week 6 and reached 100% after week 9, whereas the treatment with compound **1** (85 nmol) along with

Table **3** Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of compounds **1, 1a, 2** and **2a**

Compounds	*EBV-EA positive cells (% viability)* *Compound concentration (mol ratio/32 pmol TPA)*				IC_{50}
	1000	*500*	*100*	*10*	*(mol ratio/32 pmol TPA)*
1	0 (70)[b]	20.3	74.1	89.7	271
1a	0 (70)	27.5	78.0	91.5	290
2	0 (70)	25.7	74.9	92.6	288
2a	0 (70)	27.0	78.4	94.7	291
Oleanolic acid	12.7 (70)	30.0	80.0	100	360

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

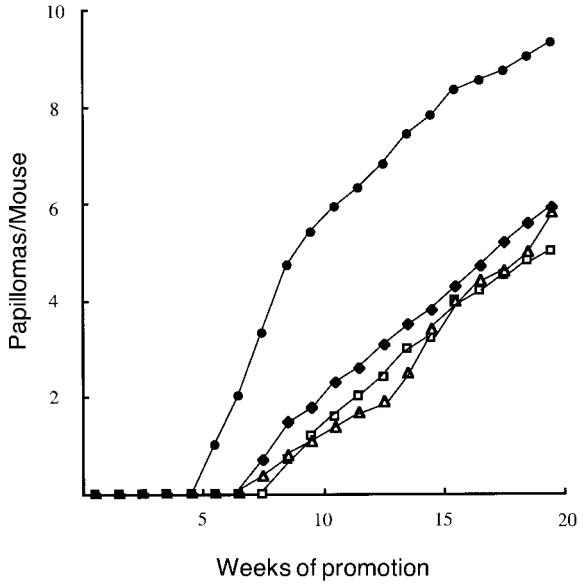

Fig. **3** Inhibition of TP A-induced tumor promotion by multiple application of 3β-methoxyserrat-13-en-21β-ol (**1**) and 13β, 14β-epoxy-3β-methoxyserratan-21β-ol (**2**). All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TP A, given twice weekly starting 1 week after initiation. Average number of papillomas per mouse. ● control (TPA alone); ■ TPA + 85 nmol of oleanolic acid; △ TPA + 85 nmol of **1**; □ TPA + 85 nmol of **2**.

DMBA/TPA inhibited the formation of papillomas until week 8 and reduced the percentage of papilloma-bearing mice to approximately 47% during weeks 12 and 13 and thereafter 80% over the period of week 20. On the other hand, the treatment with compound **2** (85 nmo1) along with DMBA/TPA inhibited the formation of papillomas until week 7 and reduced the percentage of papilloma-bearing mice to approximately 47% during weeks 14 and 15 and thereafter 80% over the period of week 20. As shown in Fig. **3**, in the control group, the number of papillomas formed per mouse increased rapidly after week 6 and reached 10.0 papillomas/mouse at week 20. On the other hand, the mice treated with compound **1** or compound **2** bore 5.0 or 5.8 papillomas over the period of week 20, although the antitumor-promoting activities of compounds **1** and **2** seem to be weaker than that of **3**. It is interesting to note that the 13α,14α-epoxyserratane framework is important to enhance the activity expression.

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