Potential Antitumor Promoting Diterpenoids from the Stem Bark of *Thuja standishii*

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Abstract

Six diterpenes, including one new natural product, were isolated from a $CHCl_3$ extract of the stem bark of *Thuja standishii*. The new compound has been characterized as 15-oxolabda-8(17),13*Z*-dien-19-oic acid. The known compounds were identified as ferruginol (**2**), sugiol (**3**), isocupressic acid (**4**), sandaracopimaric acid (**5**) and 15-oxolabda-8(17),13*E*-dien-19-oic acid (**6**). Compounds **2 – 5** and the derivatives **4a** and **4b** were tested for their inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Compounds **2**, **3**, **4** and **5** showed strong inhibitory effect on EBV-EA induction (100% inhibition at 1000 mol ratio/TPA).

In our previous study of the stem bark of *Thuja standishii* (Gord.) Carr, we reported the isolation of the novel carbon skeletal diterpenoid, standishinal [1], and five labdane-type diterpenoids [2], [3] and a *nor*-drimane-type sesquiterpene [2]. In addition, two labdane-type diterpenoids, 15,16-*bisnor*-13-oxolabda-8(17),11*E*-dien-19-oic acid [4] and 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid [3] showed significant antitumor promoting activities in an *in vivo* two-stage mouse-skin carcinogenesis assay using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a tumor promotor. Further careful examination of this extract has led to the isolation of a new labdane-type diterpene (**1**), besides five known diterpenoids, ferruginol (**2**) [5], sugiol (**3**) [6], isocupressic acid (**4**) [7], [8], sandaracopimaric acid (**5**) [9], and 15-oxolabda-8(17),13*E*-dien-19-oic acid (**6**) [10]. Known compounds were identified by comparison of their physical and spectral data with those already published.

Compound **1** was obtained as a colorless oil, and the molecular formula was assigned as $C_{20}H_{30}O_3$ by HREIMS. Its UV and IR spectra showed an α,β-unsaturated aldehyde group [λ_{max} = 238 nm (log ε 4.2); ν_{max} = 1672 cm^{-1} (>C = C-CHO)] and a carboxyl group [ν_{max} = 3200 – 2800, 1961 cm^{-1} (COOH)]. The ^{1}H- and ^{13}C-NMR spectra exhibited two tertiary methyl groups, a trisubstituted double bond [δ_H = 5.89 (d), δ_C = 129.2 (d), 164.6 (s)], an exo-

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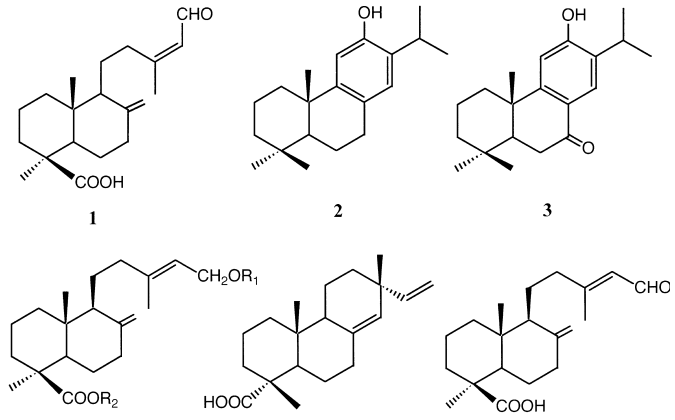

1 2 3

4 : $R_1 = R_2 = H$
4a : $R_1 = H$, $R_2 = Me$
4b : $R_1 = Ac$, $R_2 = H$

5 6

Table **1** Relative ratio[a] of EBV-EA activation with respect to positive control (100%)in the presence of compounds **2**, **3**, **4**, **4a**, **4b** and **5**

	Concentration (mol ratio/TPA)			
Compound	***1000***	***500***	***100***	***10***
2	0 (70)	35.2	73.6	91
3	0 (70)	37.4	75.5	93.4
4	3.2 (60)	45.1	76.3	97.3
4a	8.2 (60)	50.1	81.3	100
4b	7.3 (60)	49.2	79.1	100
5	0 (70)	31.4	68.5	91.4
Curcumin[b]	0 (60)	22.8	81.7	100

[a] Values represent relative percentages compared with the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells.
[b] Positive control substance [12].

cyclic methylene group [δ_H = 4.56 (s), 4.93 (s), δ_C = 106.8 (t), 147.5 (s)], an α,β-unsaturated aldehyde group [δ_H = 9.83 (d), δ_C = 191.1 (d)], and a carboxyl group [δ_C = 182.2 (s)]. The structure of **1** was established by detailed 2D NMR experiments involving HMQC, HMBC, $^1H/^1H$ COSY and NOESY spectra. In the HMBC spectrum (Fig. **1**), an aldehyde proton correlated with C-13 and C-14, Me-16 with C-12, C-13 and C-14, Me-18 with C-3, C-4, C-5 and C-19, Me-20 with C-1, C-5, C-9 and C-10, and an exomethylene proton with C-7, C-8 and C-9, respectively. In the NOESY spectrum, NOE's were observed for H-15 with H-12β, Me-16 with H-14, Me-18 with H-5α, and Me-20 with H-11β. Thus, the relative stereostructure of **1** was established as 15-oxolabda-8(17),13*Z*-dien-19-oic acid. Compound **1** was transformed into the mixture of compounds **1** and **6** (1 : 1) by exposure to air for about 2 hours. On the other hand, in $CHCl_3$ solution, the pure compound **1** was immediately changed to a mixture of compounds **1** and **6**. Compounds **1** and **6** exist as a equimolar mixture and the ratio does not change.

The inhibitory effects of **2**, **3**, **4**, **4a**, **4b** and **5** on EBV-EA activation induced by TPA were examined to screen for antitumor promoting activities, and the results are shown in Table **1**. Compound **1** was not tested in this assay because it was present in a mixture with **6**. The potencies of compounds **2**, **3**, **4** and **5** were stronger than that of the control curcumin [11] at concentrations of 100 and 10 mol ratio/TPA (Table **1**). The viability percentages of Raji cells treated with the test compounds were mostly 60% at the highest concentration of 1000 mol ratio/TPA; indicating that the cytotoxicities of these compounds seem to be quite moderate against cell lines *in vitro* (Table **1**). Compounds **4a** and **4b** were weaker than **4**, thus, the COOH group at C-19 is more effective in the enhancing antitumor promoting activity. Labdane, abietane, and pimarane-type diterpenes such as **2**, **3**, **4** and **5** may be lead compounds to more potent agents with antitumor promoting activity for clinical use.

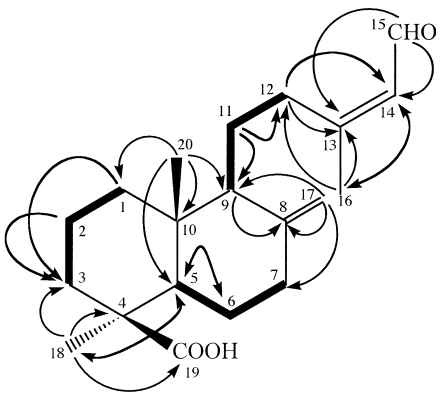

Fig. **1** ^1H-^1H COSY (▬) and HMBC correlations (→) of **1**.

Materials and Methods

The plant material (stem bark) used in this study was collected in September, 1995 at Hashimoto City, Wakayama Prefecture, Japan. A voucher specimen (TS-95-01) is deposited at the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

^1H- and ^{13}C-NMR were recorded in $CDCl_3$ on a Varian INOVA 500 spectrometer with standard pulse sequences, operating at 500 and 125 MHz, respectively. 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 MPLC were performed on silica gel (230 – 400 mesh, Merck) and Cosmocil 40 C_{18}-PREP (ODS, Nacarai Tesque). TLC and PTLC were carried out on Merck silica gel F_{254} plates. Preliminary silica gel column chromatography of the $CHCl_3$ extract (558.8 g) of the chopped stem bark (5.3 kg) of *T. standishii* has been reported previously, with separation into 13 (residues A - M) fractions [3]. Residue B (frs. No. 5 – 8, 28.7 g) was rechromatographed over silica gel (1 kg) eluting with *n*-hexane : $CHCl_3$ (1 : 1) to give residues B-a (frs. No. 22 – 54, 4.9 g) and B-b (frs. No. 55 – 70, 11.3 g). Residue B-a was rechromatographed over silica gel (200 g) eluting with *n*-hexane : EtOAc (10 : 1) to afford compounds **2** (35.3 mg) and **3** (22.1 mg) from the fractions 66 – 81 and 85 – 90, respectively. Rechromatography (2×) over silica gel (1 kg and 100 g) of residue F (frs. No. 32 – 46, 22.2 g) eluting with $CHCl_3$ gave a crystalline solid, which was recrystallized from MeOH-$CHCl_3$ to give compound **5** (1.13 g). Further elution of this column with $CHCl_3$ afforded an oil (40.4 mg) followed by HPLC (ODS) eluting with 80% MeOH to give compounds **1** (11.2 mg)

and **6** (8.2 mg), respectively. Residue I (frs. No. 60 – 71, 12.5 g) was rechromatographed twice over silica gel (500 g and 100 g) eluting with $CHCl_3$: EtOAc (10 : 1) to give compound **4** (1.77 g). Compounds **2 – 5** had the purities of over 99 %.

15-Oxolabda-8(17),13Z-dien-19-oic acid (**1**): Colorless oil; $[\alpha]_D^{25}$: + 27.3 (*c* 0.28, $CHCl_3$); HREIMS: *m/z* = 318.2206 ($C_{20}H_{30}O_3$, requires 318.2193); UV (MeOH): λ_{max} = 238 nm (log ε 4.2); IR (film): ν_{max} = 3200 – 2800 and 1961 (COOH), 1672 (> C = C-CHO), 1647 and 889 (> C = CH_2), 2937, 1448, 1395, 1263, 1165 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 0.62 (3H, s, Me-20), 1.24 (3H, s, Me-18), 1.98 (3H, d, *J* = 1.1 Hz, Me-16), 4.56 and 4.93 (each 1H, s, H-17), 5.89 (1H, d, *J* = 8.2 Hz, H-14), 9.83 (1H, d, *J* = 8.2 Hz, H-15); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 12.9 (C-20), 19.9 (C-2), 22.4 (C-11), 24.8 (C-16), 26.0 (C-6), 28.9 (C-18), 31.1 (C-12), 37.9 (C-8), 38.5 (C-9), 39.1 (C-1), 40.4 (C-10), 44.1 (C-4), 55.0 (C-9), 56.1 (C-5), 106.8 (C-17), 129.2 (C-14), 147.5 (C-17), 164.6 (C-13), 182.2 (C-19), 191.1 (C-15); EIMS: *m/z* (rel. int.): = 318 $[M]^+$ (2), 303 $[M - Me]^+$ (9), 300 (2), 285 (3), 274 (12), 235 (51), 189 (100), 121 (78).

Ferruginol (**2**): $C_{20}H_{30}O$, $[\alpha]_D^{25}$: + 39.3 (*c* 0.70, $CHCl_3$); EIMS: *m/z* = 286 $[M]^+$. Compound **2** was identical in all respects with authentic ferruginol (Lit. [5], $[\alpha]_D^{25}$: + 40.6).

Sugiol (**3**): $C_{20}H_{28}O_2$, m. p. 289 – 291 °C (*n*-hexane-EtOAc); $[\alpha]_D^{25}$: + 24.8 (*c* 0.44, EtOH); EIMS: *m/z* = 300 $[M]^+$. Compound **3** was identical in all respects with authentic sugiol (Lit. [6], m. p. 292 – 294 °C, $[\alpha]_D^{25}$: + 26).

Isocupressic acid (**4**): $C_{20}H_{32}O_3$, $[\alpha]_D^{25}$: + 51.0 (*c* 0.90, $CHCl_3$); HREIMS: *m/z* = 320.2334 $[M]^+$ ($C_{20}H_{32}O_3$, calcd. for 320.2349). Compound **4** was identical in all respects with authentic isocupressic acid (Lit. [7], $[\alpha]_D^{25}$: + 52.9).

Methyl isocupressate (**4a**): To a MeOH (2 mL) and C_6H_6 (1 mL) solution of compound **4** (15.0 mg) was added a 2.0 M trimethylsilyldiazomethane solution in *n*-hexane ($TMSCHN_2$) (0.2 mL) for 20 h at room temperature. Evaporation of the solvent under reduced pressure afforded a residue which was purified by PTLC ($CHCl_3$) to give **4a** (13.8 mg), $C_{21}H_{34}O_3$, $[\alpha]_D^{25}$: + 48.9 (*c* 0.90, $CHCl_3$); EIMS: *m/z* = 334 $[M]^+$. Compound **4a** was identical in all respects with authentic methyl isocupressate (Lit. [7], $[\alpha]_D^{25}$: + 51.2).

O-Acetylisocupressic acid (**4b**): Compound **4** (15.0 mg) was acetylated with Ac_2O-pyridine (1 : 1, 2 mL) at room temperature for 24 h. Usual work-up afforded a residue, which was purified by PTLC ($CHCl_3$) to give **4b** (15.1 mg), $C_{22}H_{34}O_4$, $[\alpha]_D^{25}$: + 47.8 (*c* 0.40, $CHCl_3$); EIMS: *m/z* = 362 $[M]^+$. Compound **4b** was identical in all respects with authentic acetyl isocupressic acid (Lit. [8], $[\alpha]_D$: + 49).

Sandaracopimaric acid (**5**): $C_{20}H_{30}O_2$, $[\alpha]_D^{25}$: – 18.1 (*c* 0.95, $CHCl_3$), m. p. 170 – 172 °C (MeOH-$CHCl_3$); EIMS: *m/z* = 302 $[M]^+$. Compound **5** was identical in all respects with authentic sandaracopimaric acid (Lit. [9], $[\alpha]_D^{25}$: -19.8, m. p. 165 – 168 °C).

*15-Oxolabda-8(17),13*E*-dien-19-oic acid* (**6**): $C_{20}H_{30}O_3$, $[\alpha]_D^{25}$: + 45.2 (*c* 0.12, $CHCl_3$); EIMS: *m/z* = 318 $[M]^+$. Compound **6** was identical in all respects with authentic 15-oxolabda-8(17),13*E*-dien-19-oic acid (Lit. [10], $[\alpha]_D$: + 47.5).

Inhibition of EBV-EA activation test: EBV-EA positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from Dr. Y. Zaizen, the Department of Biochemistry, Oita Medicinal University. The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's 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 [3]. 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 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 [11]. In each assay, at least 500 cells were included, 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*-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.

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