Potential Antitumor-Promoting Diterpenes from the Cones of *Pinus luchuensis*

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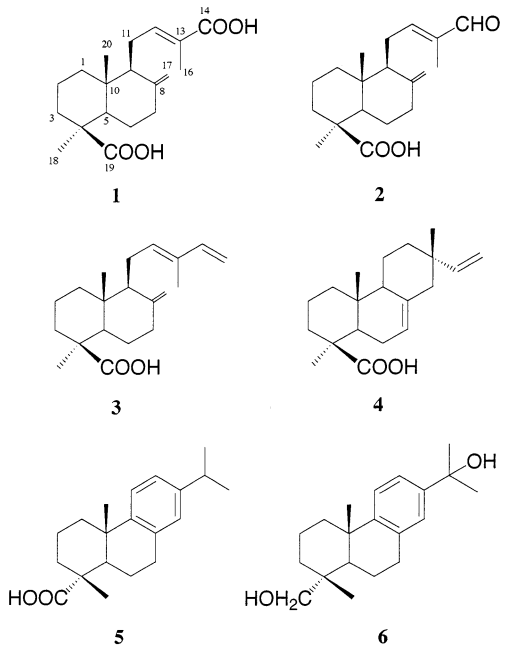
A new *nor*-labdane-type diterpene, 15-*nor*-labda-8(17),12*E*-dien-13,19-dienoic acid (**1**), along with five known diterpenes, 15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid (**2**), *trans*-communic acid (**3**), sandaracopimaric acid (**4**), dehydroabietic acid (**5**), and abieta-8,11,13-triene-15,18-diol (**6**), was isolated from the cones of *Pinus luchuensis*. The structure of **1** was established by chemical and spectroscopic methods. Among these isolates, compounds **2**, **4**, and **6** showed potent inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate.

In the course of a search for biologically active constituents from the leaves, bark, and cones of coniferous trees, which are regarded as waste materials in the forestry industry, we have found that 15,16-*bis*-*nor*-13-oxolabda-8(17),11*E*-dien-19-oic acid[1] and 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid[2] isolated from the bark of *Thuja standishii* (Cupressaceae) and 13α,14α-epoxy-3*β*-methoxyserratan-21*β*-ol[3] isolated from *Picea jezoensis* var. *jezoensis* (Pinaceae) exhibited potent antitumor-promoting activity in an in vivo mouse two-stage carcinogenesis assay. As a continuation of these studies, we have examined *Pinus luchuensis* Mayr. (Pinaceae, Japanese name: "Ryukyumatsu"), which is a common tree on Ryukyu Island in Japan. Previously, Cheng et al. have reported the isolation of some Δ^{14}-serratane triterpenes, 3*β*-methoxyserrat-14-en-21-one, serrat-14-en-3,21-dione, 3*β*-hydroxyserrat-14-en-21-one, 3*β*,21α-dimethoxyserrat-14-ene, and 3*β*-methoxyserrat-14-en-21α-ol.[4] Recently, we have reported the structures of four new *tris*-*nor*-lanostane-type triterpenes, 25,26,27-*tris*-*nor*-3α-hydroxylanost-9(11)-en-24-oic acid, 25,26,27-*tris*-*nor*-3α-methoxylanost-9(11)-en-24-oic acid, 25,26,27-*tris*-*nor*-3*β*-methoxylanost-9(11)-en-24-oic acid, and 25,26,27-*tris*-*nor*-3-oxolanost-9(11)-en-24-oic acid,[5] and two new lanostane and serratane-type triterpenes, 3-oxolanost-9(11)-ene-24*S*,25-diol and 29-acetoxy-3*β*-methoxyserrat-14-en-21α-ol, from the $CHCl_3$ extract of the stem bark of *Pinus luchuensis*.[6]

In the present investigation, we have examined the cones of *Pinus luchuensis*, which have not been studied phytochemically before. The $CHCl_3$ extract of the cones of *P. luchuensis* was carefully chromatographed over silica gel and Sephadex LH-20 and then fractionationed by medium-pressure liquid chromatography (MPLC) to afford one new (**1**) and five known (**2**−**6**) compounds. The known compounds were confirmed as 15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid (**2**), *trans*-communic acid (**3**), sandaracopimaric acid (**4**), dehydroabietic acid (**5**), and abieta-8,11,13-triene-15,18-diol (**6**), by direct comparison with authentic samples isolated from *Thuja standishii* (**2** and **3**)[2,7] and *Larix kaempferi* (**5** and **6**).[8,9] Compound **4** was identified as sandracopimaric acid by comparison of the spectral data with those reported in the literature.[10]



Compound **1** was assigned the molecular formula $C_{19}H_{28}O_4$ (M^+; *m*/*z* 320.1981) on the basis of the HREIMS. Its UV spectrum showed the presence of a HC═C−C═O chromophore. The IR spectrum exhibited absorption bands for a vinyl group and a terminal methylene and strong peaks attributable to carboxyl groups. The ^1H and ^{13}C NMR spectra (Table 1) revealed signals for two tertiary methyl groups, a vinyl methyl group, six methylene groups, two methine groups, a trisubstituted double bond [δ_H 6.82 (ddd), δ_C 146.5 (d), 126.3 (s)], an exocyclic methylene group [δ_H 4.38 (s), 4.84 (s), δ_C 107.8 (t), 147.6 (s)], and two carboxylic acids [δ_C 172.9 (s), 183.3 (s)]. Accordingly, **1** was assigned as a *nor*-labdane-type diterpene with two carboxylic acid moieties, and the ^1H and ^{13}C NMR spectra resembled those of 15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid (**2**), except for a carboxyl group in **1**, instead of an aldehyde group in **2**. Extensive 2D NMR experiments performed involving HMQC, HMBC, $^1H-^1H$ COSY, and

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Table 1. NMR Spectral Data for Compound **1** in $CDCl_3$[a]

position	δ_H[a] (*J* in Hz)	δ_C[b]	$^1H-^1H$ COSY	HMBC (H → C)	NOESY
1α	1.16 m	39.3 t	1β, 2α, 2β, 20	2, 10, 20	3α, 5α
1β	1.84 m		1α, 2α, 2β	2, 10	20
2α	1.54 m	19.9 t	1α, 1β, 2β, 3α, 3β	1, 3	
2β	1.88 m		1α, 2α, 3α	1, 3	1β, 20
3α	1.09 m	37.9 t	2α, 2β, 3β	2, 4, 19	1α, 5α
3β	2.18 m		2α, 2β	4, 5, 19	
4		44.2 s			
5α	1.37 dd (12.1, 3.0)	56.1 d	6α, 6β	4, 6, 10, 18, 19, 20	1α, 3α, 6α, 7α
6α	1.98 m	25.7 t	5α, 6β, 7α, 7β	5, 7, 10	5α, 18
6β	1.89 m		5α, 6α, 7α, 7β	5, 10	7β, 20
7α	1.95 m	38.3 t	5α, 6α, 6β, 7β	6, 8	5α, 6α, 9α
7β	2.42 m		6α, 6β, 7α	6, 8, 9	6β
8		147.6 s			
9α	1.86 m	55.8 d	7α, 7β, 11A, 11B	5, 8, 10, 11	1α, 7α, 16
10		40.3 s			
11A	2.24 dd (10.5, 7.1)	24.4 t	9α, 11B	8, 9, 12, 13	20
11B	2.39 m		9α, 11A	8, 9, 12, 13	20
12	6.82 ddd (7.1, 6.0, 1.4)	146.5 d	11A, 11B	9, 11, 13, 14, 16	11A, 11B
13		126.3 s			
14		172.9 s			
16	1.85 s	12.2 q		12, 13, 14	9α
17A	4.38 s	107.8 t	17A	7, 8, 9	12
17B	4.84 s		17B	7, 8, 9	12
18	1.26 s	29.0 q		3, 4, 5, 19	3α, 5α, 6α
19		183.3 s			
20	0.67 s	12.9 q		1, 5, 9, 10	1β, 2β, 11A, 11B

[a] Recorded at 500 MHz. [b] Recorded at 125 MHz. Signals were assighed by $^1H-^1H$ COSY, HMQC, HMBC, and NOESY specta.

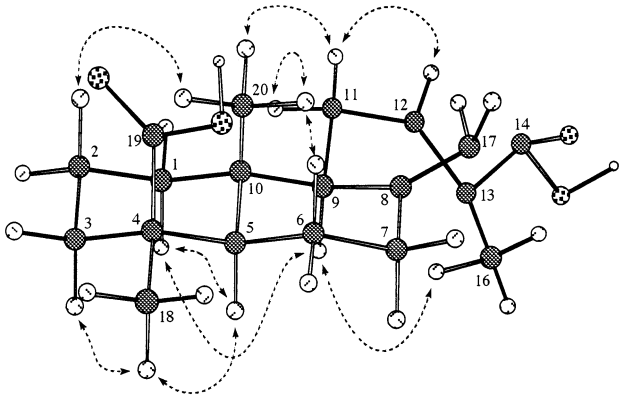

Figure 1. Important NOESY correlations observed for **1**.

NOESY spectra supported this assumption. One of carboxylic acid units was attached at C-19, on the basis of the correlation of C-19 with H-3α, H-3β, H-5α, and Me-18 in the HMBC spectrum (Table 1), and characteristic NOE enhancements were observed for Me-18 with H-3α, H-5α, and H-6α in the NOESY spectrum (Figure 1). The second carboxylic acid unit was located at C-14 on the basis of the correlation of C-14 with H-12 and Me-16 in the HMBC spectrum, and NOEs were observed between H-9α and Me-16 (Figure 1). Finally, the absolute stereochemistry of **1** was determined as 15-*nor*-labda-8(17),12*E*-dien-13,19-dienoic acid by synthesis of **1** from **2**, which has been established as 15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid[7,11,12] rather than *ent*-15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid[13] (Experimental Section). Jones oxidation of **2** in acetone gave a dicarboxyl product, with the synthetic compound and **1** identical in all respects.

The inhibitory effects of compounds **1**−**6** and the positive control substance, β-carotene, on EBV-EA activation induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were examined as a preliminary evaluation of their potential antitumor-promoting activities, and the results are provided in Table 2. As shown in Table 2, the viability percentages of Raji cells treated with the test compounds were 60−70% at the highest concentration of 1000 mol ratio/TPA, indicating that the cytotoxicities of these compounds were moderate against the in vitro cell line. Among them, compounds **2**, **4**, and **6** exhibited significant inhibitory effects on activation (100% inhibition of activation at 1000 mol ratio/TPA, more than 66% inhibition at 500 mol ratio/TPA, and more than 27% inhibition at 100 mol ratio/TPA) and were found to be more effective than that of β-carotene, a vitamin A precursor that has been intensively studied in cancer chemoprevention using animal models.[14] On the other hand, on comparison of the potential antitumor-promoting activities of **1** and **2**, **2** showed a slightly stronger effect than **1**. It is interesting to note that the presence of an aldehyde group in the side chain seems to enhance the potential antitumor-promoting activity, as found for 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid.[2] In addition, pimarane-type diterpenoids, such as sandaracopimaric acid (**4**), can be considered as appropriate lead compounds for further evaluation of their potential cancer chemopreventive activity.

Table 2. Percentage of Epstein−Barr Virus Early Antigen (EBV-EA) Induction in the Presence of Compunds **1**−**6** with Respect to a Positive Control (100%)[a]

	concentration (mol ratio/TPA)			
compound	1000	500	100	10
1	5.0 (60)	47.1	74.4	93.4
2	0 (70)	31.4	68.5	91.4
3	4.4 (60)	46.2	75.3	90.5
4	0 (70)	22.5	70.4	91.9
5	2.9 (70)	32.7	69.8	97.9
6	0 (70)	33.4	72.5	96.4
β-carotene[b]	9 (90)	34	82	100

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

Experimental Section

General Experimental Procedures. 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. UV spectra were

recorded using a Hitachi 150-20 spectrophotometer. 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 conducted with silica gel (230−400 mesh, Merck) and Cosmocil 40C_{18}-PREP (ODS, Nacarai Tesque). 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).

Plant Material. The cones of *Pinus luchuensis* were collected at Okinawa Prefecture Forestry Experiment Station, Nago City, Okinawa Prefecture, Japan, in September 1998. The extraction was carried out in October 2000. A voucher specimen (PL-98-02) is deposited at the Herbarium of the Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences.

Extraction and Isolation. The chopped cones (800 g) of *P. luchuensis* were extracted for 1 week at 50 °C with $CHCl_3$ (2 L), employing an automatic glass percolator. The $CHCl_3$ was evaporated under reduced pressure, and the resulting dark green residue (106.3 g) was subjected to silica gel column chromatography (3 kg). Elution of the column with $CHCl_3$ afforded residue A (fr. nos. 1−39, 18.33 g), and elution was continued with $CHCl_3$−EtOAc (10:1) to give residue B (fr. nos. 40−41, 20.44 g) and residue C (fr. nos. 42−61, 12.82 g). Subsequent elution with $CHCl_3$−EtOAc (7:1) afforded residue D (fr. nos. 62−72, 5.72 g), and elution with $CHCl_3$−EtOAc (5:1) gave residue E (fr. nos. 83−90, 3.50 g). Further elution with $CHCl_3$−EtOAc (2:1) and EtOAc gave residues F (fr. nos. 91−96, 3.91 g) and G (fr. nos. 92−127, 22.51 g), respectively. Repeated column chromatography of residue B on silica gel (1.2 kg) with *n*-hexane−$CHCl_3$ (1:1) furnished a colorless amorphous solid (**3**) (1.5 g) (fr. nos. 38−40) and a crystalline solid (**4**) (35.5 mg) (fr. nos. 48−51), which was identified by direct comparison with an authentic *trans*-communic acid, $[M]^+$ *m/z* 302 ($C_{20}H_{30}O_2$), $[\alpha]_D$ +28° (*c* 0.82) [lit.[15] $[\alpha]_D$ +38.1 (*c* 0.23, EtOH)] and sandarcopimaric acid, $[M]^+$ *m/z* 302 ($C_{20}H_{30}O_2$), mp 170−171.5 °C (MeOH−$CHCl_3$), $[\alpha]_D$ −20° (*c* 0.95) [lit.[10] mp 165−168 °C, $[\alpha]_D$ −19.8], respectively. Further elution with $CHCl_3$ to give a colorless gum (444.3 mg) (fr. nos. 82−86), which was subjected to MPLC using $CHCl_3$, followed by preparative TLC (plate: 20 × 20 cm, $CHCl_3$−MeOH, 100:1) gave compound **2** (22.2 mg). Compound **2** was identified by direct comparison with authentic 15-*nor*-14-oxolabda-8(17),12*E*-dien-19-oic acid, $[M]^+$ *m/z* 304 ($C_{19}H_{28}O_3$), $[\alpha]_D$ +40° (*c* 0.18) [lit.[11] $[\alpha]_D$ +39.5°]. Repeated column chromatography of residue C on silica gel (500 g) with $CHCl_3$−EtOAc (10:1) afforded a colorless gum (fr. nos. 26−30, 1.33 g). This material was subjected to MPLC with *n*-hexane−EtOAc (2:1) to give compound **6** (69.7 mg), which was identified as abieta-8,11,-13-trien-15,18-diol, $[M]^+$ *m/z* 302 ($C_{20}H_{30}O_2$), $[\alpha]_D$ −12° (*c* 0.82) [lit.[9] $[\alpha]_D$ −11]. Repeated column chromatography of residue E on silica gel (100 g) with $CHCl_3$−EtOAc (5:1) afforded a colorless gum (fr. nos. 40−43, 241.9 mg), which was rechromatographed with MPLC using *n*-hexane−EtOAc (10:1) followed by preparative TLC (plate: 20 × 20 cm, $CHCl_3$−MeOH, 100:1) to give compound **5** (33.3 mg). Compound **5** was identified by direct comparison with an authentic sample of dehydroabietic acid, $[M]^+$ *m/z* 300 ($C_{20}H_{28}O_2$), $[\alpha]_D$ +57° (*c* 0.37, EtOH) [lit.[16] $[\alpha]_D$ +62 (EtOH)]. Repeated column chromatography of residue F on silica gel (100 g) with $CHCl_3$−EtOAc (10:1) afforded a colorless gum (fr. nos. 25−30, 1.24 g), which was subjected to MPLC with *n*-hexane−EtOAc (2:1) followed by preparative TLC (plate: 20 × 20 cm, $CHCl_3$−MeOH, 100:1) to give compound **1** (9.3 mg).

15-*nor*-Labda-8(17),12*E*-dien-13,19-dienoic acid (1): colorless oil; $[\alpha]^{23}_D$ +82.8° (*c* 0.80, $CHCl_3$); UV λ_{max} 280 (log ϵ 3.8) nm; IR (firm) ν_{max} 3100−2800 and 1689 (COOH), 2930, 1646, and 890 (>C=CH_2), 1521, 1319, 1216, 1093 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* 320 [M^+, 7], 302 (34) [($M - H_2O$)$^+$, 34], 274 (31), 256 (15), 175 (18), 149 (23), 107 (28), 43 (100); HREIMS *m/z* 320.1981 [$C_{19}H_{28}O_4$, requires 320.1986].

Synthesis of 1 from 2. To a solution of compound **2** (10.1 mg) in acetone (1.5 mL) was added dropwise Jones reagent (120 μL)[17] under ice, and the resulting mixture was stirred at 50 °C for 5 h. The excess reagent was destroyed by the addition of isopropyl alcohol. Saturated sodium bicarbonate solution was added, and the resulting mixture was extracted with ether. The combined extracts were washed with brine and evaporated to give 9.2 mg of pale yellow oil, which on column chromatography over silica gel using *n*-hexane−EtOAc (2:1) afforded a dicarboxyl compound, 2.8 mg, $[\alpha]^{20}_D$ +80.5° (*c* 0.041, $CHCl_3$), EIMS *m/z* 320 $[M]^+$. The synthetic compound was identified by direct comparison with the natural compound **1**.

Assay for the Inhibition of EBV-EA Activation. The inhibition of Epstein−Barr virus early antigen (EBV-EA) activation was assayed using Raji cells (virus nonproducer), the EBV genome-carrying human lymphoblastoid cells, which were cultivated in 10% FBS RPMI 1640 medium solution (Nacalai Tesque). The indicator cells (Raji) (1 × 10^6/mL) were incubated at 37 °C for 48 h in 1 mL of the medium containing *n*-butyric acid (4 mM, inducer) and 32 pmol of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [20 ng/mL in dimethyl sulfoxide (DMSO) and a known amount of test compound in DMSO]. Smears were made from the cell suspension. The activated cells were stained by high-titer EBV-EA-positive sera from nasopharyngeal carcinoma (NPC) patients and were detected by a conventional indirect immunofluorescence technique.[18] In each assay for compounds **1**−**6**, at least 500 cells were counted and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments with *n*-butyric acid plus TPA, in which EA induction was ordinarily around 30%.

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