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Anti-tumor Promoting Diterpenes from the Stem Bark of *Thuja standishii* (Cupressaceae)

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Abstract—Three new labdane-type diterpenoids, labda-8(17),13-dien-15,12*R*-olid-19-oic acid (**1**), 12*S*-hydroxylabda-8(17),13(16),14-trien-19-oic acid (**2**) and 13-ethoxylabda-8(17),11,14-trien-19-oic acid (**3**), along with known diterpenoids, *trans*-communic acid (**4**), totarol (**5**), 12-methoxyabieta-8,11,13-trien-11-ol (**6**), and 7α,8α-epoxy-6α-hydroxyabieta-9(11),13-dien-12-one (**7**) were isolated from the stem bark of *Thuja standishii*. The structures of **1**–**3** were established by spectroscopic methods and chemical conversion. These compounds together with standishinal (**8**), 12-hydroxy-6,7-*seco*-abieta-8,11,13-trien-6,7-dial (**9**) and 6α-hydroxysugiol (**10**) were tested for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as a test for potential cancer chemopreventive agents. Compound **10** showed strong inhibitory effect on EBV-EA induction (100% inhibition at 1000 mol ratio/TPA), and compounds **2** and **6** showed moderate inhibitory effects on EBV-EA induction. In addition, 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid (**11**) was found to exhibit the anti-tumor promoting activity in two-stage mouse skin carcinogenesis test using 7,12-dimethylbenz[*a*]anthracene and TPA.

Introduction

Cancer chemoprevention is regarded as one of the efficient strategies for cancer prevention.[1] Inhibition of the tumor promotion stage in the multistage of 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 promotor, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), have been studied as a primary screening test.[3] Recently, we reported that the novel carbon skeletal diterpenoid, standishinal [6α,12-dihydroxy-6(7→11)*abeo*-abieta-8,11,13-trien-7-al].[4] and three new compounds, 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid, 15-oxolabda-8(17),11*Z*,13*Z*-trien-19-oic acid, and 12-oxo-11-*nor*-drim-8-en-14-oic acid.[5] from the stem bark of *Thuja standishii* (Gord.) Carr (Cupressaceae), and 15,16-*bisnor*-13-oxolabda-8(17),11*E*-dien-19-oic acid from the stem bark of this tree showed significant anti-tumor promoting activity of in vivo two-stage mouse-skin carcinogenesis assay using 7,12-dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a tumor promotor.[6]

Further careful examination of this extract has led to the isolation of three new labdane-type diterpenoids, **1**–**3**, besides four known diterpenoids. The structures of the new compounds (**1**–**3**) were determined on the basis of spectroscopic data and chemical conversion. The known compounds were identified as *trans*-communic acid (**4**),[7,8] totarol (**5**),[9] 12-methoxyabieta-8,11,13-trien-11-ol (**6**),[10] and 7α,8α-epoxy-6α-hydroxyabieta-9(11),13-dien-12-one (**7**)[11] by comparison of their spectral data with those reported in the literature.

This paper deals with the structure determination of **1**–**3** and results of in vitro and in vivo anti-tumor promoting activities of compounds **1**–**7**, along with standishinal (**8**), 12-hydroxy-6,7-*seco*-abieta-8,11,13-trien-6,7-dial (**9**), 6α-hydroxysugiol (**10**)[4] and 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid (**11**)[5,6] using 7,12-dimethylbenz[*a*]anthracene and TPA.1

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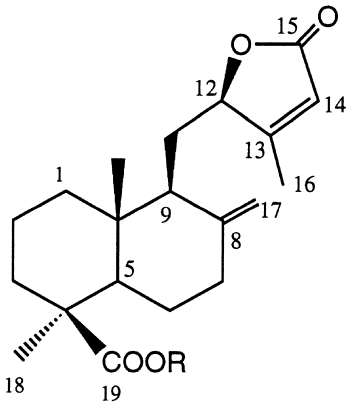

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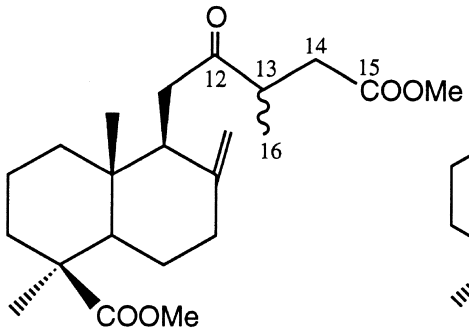
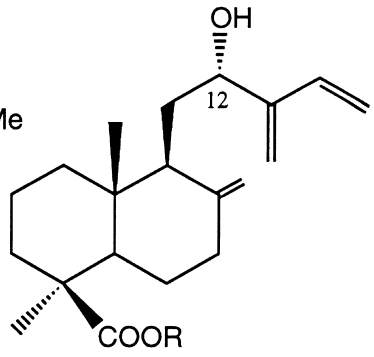
1a : R = Me



1b



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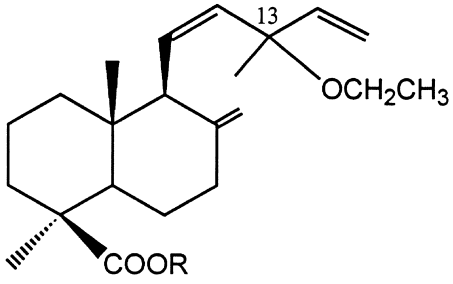

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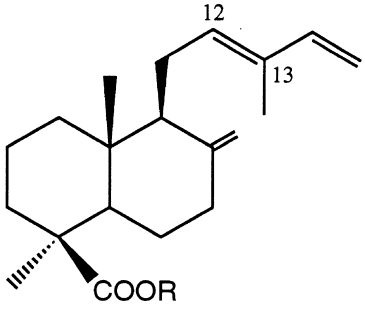

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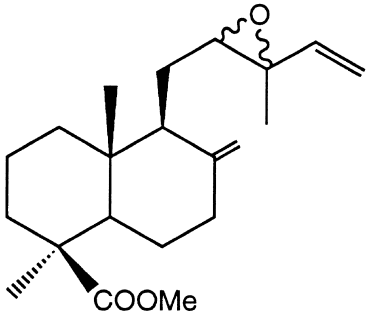

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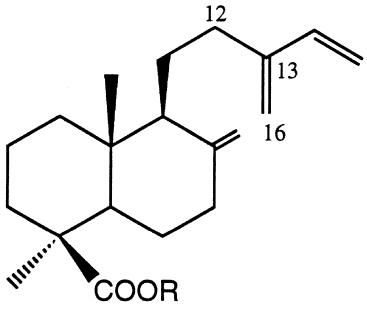
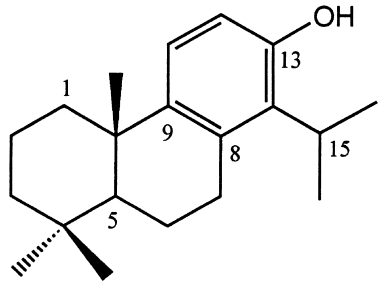


4c : R = Me, 12*S*-OH
4d : R = Me, 12*R*-OH
4e : R = H, 12*S*-OH
4f : R = CH_2·N, 12*S*-OH
4g : R = Me, 12*S*-MTPA ester
4h : R = Me, 12*R*-MTPA ester



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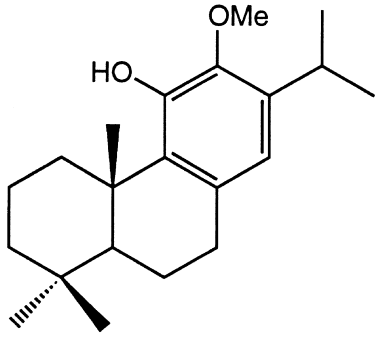

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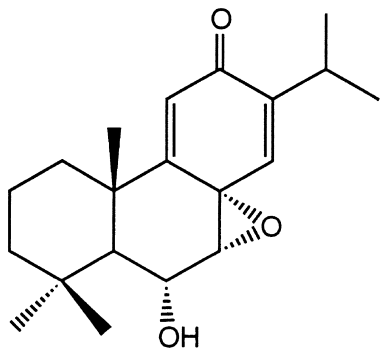

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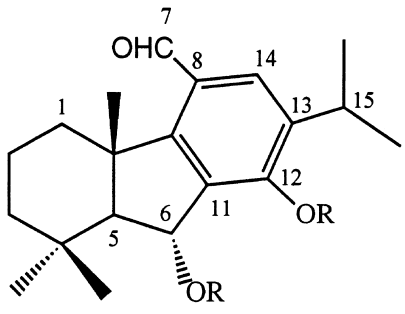
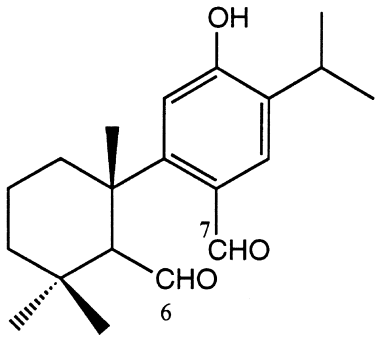


8 : R = H
8a : R = Ac



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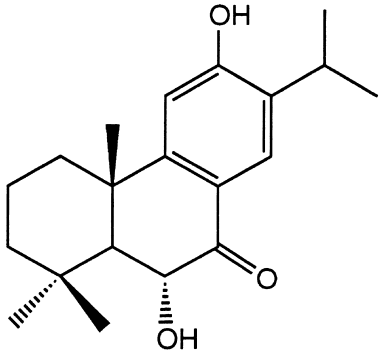

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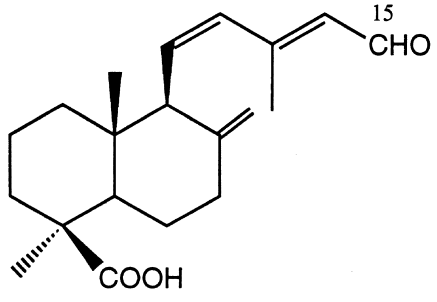


11

Results and Discussion

The $CHCl_3$ extract of the stem bark of *T. standishii* was carefully chromatographed over Si gel, Sephadex LH-20, and then fractionated by medium-pressure liquid chromatography to give three new (**1**–**3**) and four known diterpenoids (**4**–**7**). The known compounds were confirmed as *trans*-communic acid (**4**),[7,8] totarol (**5**),[9] 12-methoxyabieta-8,11,13-trien-11-ol (**6**),[10] and 7α,8α-epoxy-6α-hydroxyabieta-9(11),13-dien-12-one (**7**).[11] Compound **4** was reported in ref 6, but no chemical description was written, in this study, we synthesized compound **2** from compound **4**.

The molecular formula of compound **1** was assigned as $C_{20}H_{28}O_4$ based on HREI-MS. The IR spectrum of **1** exhibited bands indicative of terminal methylene group, αβ-unsaturated γ-lactone ring and carboxyl group. The ^1H and ^{13}C NMR spectra (Table 1) exhibited two tertiary methyl groups, six methylenes, two methine groups, an oxymethine [δ_H 4.87 (d); δ_C 83.4 (d)], a β-methyl-αβ-unsaturared γ-lactone [δ_H 2.10 (3H, brs), 5.78 (quintet); δ_C 13.9 (q), 116.5 (d), 169.3 (s), 173.1 (s)], a terminal methylene group [δ_H 4.44 (s), 4.94 (d); δ_C 106.3 (t), 147.9 (s)] and a carboxyl group [δ_C 183.3 (s)]. Its EI-MS (Fig. 1) showed a prominent ion peak at *m/z* 98 (β-methyl-αβ-unsaturated-γ-lactone) and a base ion peak at *m/z* 235 corresponding to a M^+-β-methyl-αβ-unsaturared-γ-lactone moiety. The gross structure of **1** was established by extensive 2D NMR experiments involving HMQC, HMBC, $^1H/^1H$ COSY and NOESY spectra. In the HMBC spectrum (Fig. 1), H-12 was correlated with C-9, C-11, C-13, C-14 and C-16. In the $^1H-^1H$ COSY spectrum (Fig. 1), H-12 oxymethine signal (δ_H 4.87) was correlated with H-11α and H-11β. On the other hand, methylation of **1** with trimethylsilyldiazomethane gave a methyl ester (**1a**) and subsequent alkaline hydrolysis furnished 12-oxo-13-methyl-15-oic acid (**1b**) (Table 1) whose C-13 methyl group was epimeric mixture. The relative stereostructure was determined by NOESY spectrum (Fig. 2). Hence, compound **1** was proved to be labda-8(17),13-dien-15,12-olid-19-oic acid, and the configuration of C-12 was determined as *R* because the negative cotton effect curve was observed at 214 nm ($\Delta\varepsilon-32.4$).[12] To the best of our knowledge, evillosin is a labdane-type diterpenoid possessing a 15,12-lactone ring was isolated from *Eupatrium villosum* (Compositae),[13] thus, compound **1** was the second instance of labdane-type diterpene having a 15,12-lactone.

Compound **2** was isolated as the *N*-(chloromethyl)phthalimide (NMP) ester[14] of **2** (**2a**), and its molecular formula was assigned as $C_{20}H_{29}O_3$ [*m/z* 317.2111, M^+-*N*-(methyl) phthalimide)] by HREI-MS. The IR spectrum showed absorption bands for exocyclic methylene group and hydroxyl group. The ^1H and ^{13}C NMR spectra (Table 2) showed two tertiary methyl groups, six methylene groups, two methine groups, two sp^3 quaternary carbons, a hydroxymethine [δ_H 4.41

Table 1. ^1H and ^{13}C NMR data for compounds **1** and **1b** ($CDCl_3$)[a]

	1		**1b**	
Position	δ_C	δ_H	δ_C	δ_H
1α	38.6t	1.20 ddd (4.0, 13.0, 13.3)	39.15 t, 39.19 t	1.15 ddd (4.0, 13.0, 13.3)
1β		1.64 m		1.63 m
2α	19.7 t	1.51 m	19.9 t	1.52 m
2β		1.81 m		1.84 m
3α	37.7 t	1.07 ddd (4.0, 13.5, 13.5)	38.1 t	1.06 ddd (4.0, 13.5, 13.5)
3β		2.15 m		2.18 m
4	44.3 s		44.3 t	
5	55.9 d	1.42 m	55.96 d, 55.98 d	1.41 t (3.0), 1.43 t (3.0)
6α	25.9 t	2.10 m	25.76 t, 25.84 t	2.01 m
6β		1.92 m		1.78 m
7α	38.4 t	2.44 m	38.01 t, 38.04 t	2.41 m
7β		1.98 m		2.06 m
8	147.9s		148.4s, 148.8s	
9	51.7 d	2.22 brd (11.5)	49.7 d, 50.1 d	2.45 brd (11.0)
10	40.1 s		39.43 s, 39.46 s	
11A	28.0 t	1.44 m	36.8 t, 37.0 t	2.27 m
11B		1.93 m		2.76 m
12	83.4 d	4.87 d (11.0)	177.7 s	
13	169.3 s		41.9 d, 42.4 d	3.06 m
14	116.5 d	5.78 quintet (1.0)	37.5 t, 37.8 t	2.66 m
				2.74 m
15	173.1 s		211.8 s, 211.9 s	
16	13.9 q	2.10 brs	17.1 q, 17.4 q	1.14 d (7.0), 1.15 d (7.0)
17A	106.3 q	4.94 d (1.0)	105.9 t, 106.5 t	4.75 d (1.0), 4.77 brs
17B		4.44 s (11.0)		4.32 brs, 4.39 brs
18	29.0 q	1.26 s	28.8	1.20 s
19	183.3 s		172.71 s, 172.75 s	
20	12.8 q	0.61 s	12.95 q, 12.96 q	0.54 s
OMe		3.62 s	51.2 q	
OMe		3.639 s, 3.642 s	51.7 q	

[a]Operated at 500 and 125 MHz for ^1H and ^{13}C NMR experiments, respectively; δ in ppm, *J* (in parentheses) Hz; assignments made from $^1H-^1H$ COSY, HMQC, HMBC, and NOESY data.

(dd), δ_C 72.4 (d)], three exocyclic methylene groups [δ_H 4.70 (brs), 4.88 (d), δ_C 106.9 (t), 148.7 (s); δ_H 5.11 (d), 5.15 (brs), δ_C 115.0 (t), 149.2 (s); δ_H 5.10 (d), 5.41 (dd), δ_C 115.1 (t), 135.7 (d)], and a NMP ester group [δ_H 4.69 (d), 5.64 (d), 7.79 (m), 7.93 (m); δ_C 60.6 (t), 123.9 (d), 131.7 (s), 134.6 (d), 166.7 (s)]. The above data showed **2** should be a myrcerocommunic acid[15] derivative having a secondary hydroxyl group. The secondary hydroxyl group was attached at C-12 based on the cross peaks between C-12 with H-9α, H-11α, H-11β, H-16A and H-16B in the HMBC spectrum, and cross peaks between H-12 with H-11α and H-11β in the ^{1}H–^{1}H COSY spectrum of **2a** (Fig. 3). In the NOESY spectrum of **2a** (Table 2) NOEs were observed for Me-18 with H-3α, H-5α, and for Me-20 with H-1β, H-2β, H-11β, since the carboxylic acid should be placed at C-19. Accordingly, the structure of **2** was assumed to be 12-hydroxy-labda-8(17),13(16),14-trien-19-oic acid.

The absolute stereo structure was determined by the synthesis of **2a** from *trans*-communic acid methyl ester (**4a**) and the use of the modified Mosher method. *M*-CPBA oxidation of **4a** gave a 12,13-epoxy derivative (**4b**), and subsequent treatment with aluminium isoproxide[16] afforded compounds **4c** and 12-epi hydroxyl derivative (**4d**). Compound **4c** was hydrolysed with potassium *t*-butoxide to give compound **4e**, and then esterified with *N*-(chloromethyl)phthalimide to give NMP ester (**4f**). The above synthetic **4f** was identical with **2a** in all respects. On the other hand, the absolute configuration of C-12 hydroxyl group was determined by the modified Mosher method. The (*S*)- and (*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl)-acetic acid (MTPA) esters (**4g** and **4h**) were prepared from **4a** by a standard method.[17] The ^{1}H chemical-shift differences between (*S*)- and (*R*)-MTPA esters **4g** and **4h**) are shown in Figure 4.

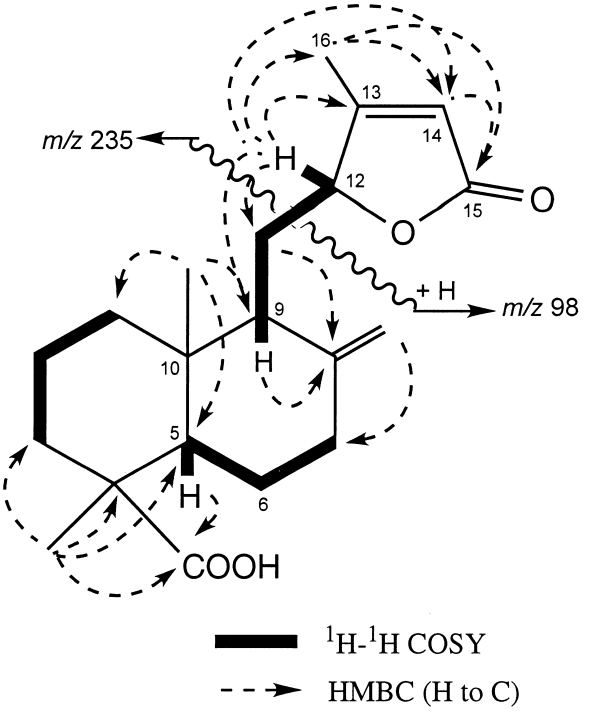


Figure 1. EI-MS, ^{1}H–^{1}H COSY and HMBC correlations for **1**.

The result determined the *S* configuration for the asymmetric center at C-12, and consequently, led to the absolute stereo structure of **2**.

Compound **3** was also isolated as the NMP ester of **3** (**3a**), and its molecular formula was assigned as $C_{22}H_{34}O_3$ (*m*/*z* 345.2431, $M^+ - N$-(methyl)-phthalimide) by HREI-MS. The IR spectrum showed absorption bands for *cis*-ditsubstituted olefin, exocyclic methylene groups, ester group and ether oxygen. The ^{1}H and ^{13}C NMR spectra (Table 3) showed three tertiary methyl groups, five methylene groups, two sp^3 methine groups, three sp^3 quaternary carbons, $\Delta^{11,12}$-disubstituted olefin [δ_H 5.607 (dd), 5.45 (d) δ_C 128.4 (d), 136.63 (d); δ_H 5.614 (dd), 5.45 (d), δ_C 128.5 (d), 136.64 (d)], two exocyclic methylene groups [δ_H 4.72 (d), 4.41 (d), δ_C 149.4 (s), 108.0 (t); δ_H 4.72 (d), 4.45 (d), δ_C 108.1 (t), 149.5 (s)], a tertiary ethoxy group [δ_H 1.15 (2H, t), 3.367 (3H, quartet), δ_C 16.0 (q), 58.2 (t); δ_H 1.16 (2H, t), 3.370 (3H, quartet), δ_C 16.0 (q), 58.3 (t)] and a NMP ester group [δ_H 5.65 (d), 5.71 (d), 7.80 (m), 7.94 (m), δ_C 60.9 (t), 123.9 (d), 131.7 (d), 134.6 (d), 166.7 (s)]. An ethoxy group was attached at C-13, since C-13 correlated with H-1′, H-11, H-12, H-14, H-15 and Me-16 in the HMBC spectrum (Table 3). Compound **3** gave a mixture of two stereoisomers on ethoxy groups at C-13*S* and C-13*R* as is shown in Table 3.

Antitumor-promoting activity

Compounds **1**–**10** were tested for their inhibitory effects on Epstein–Barr virus early antigen (EBV-EA), induced by the tumor promotor, TPA, in Raji cells. Their inhibitory effects on the activation of the early antigen and the viability of Raji cells are shown in Table 4.

The potency of compound **10** was stronger than that of a representative control β-carotene[18] at every concentration. Compounds **2** and **11** showed strong inhibitory effect on EBV-EA induction, even at 10 mol ratio/TPA (100% inhibition activity at 1000 mol ratio/TPA, and more than 25% even at 100 mol ratio/TPA, respectively), and compounds **5** and **6** showed moderate inhibitory effects (more than 28% even at 100 mol ratio/TPA, respectively) on EBV-EA induction. The viability percentages of Raji cells treated with the test compounds (**1**–**11**) were mostly 60% at the highest concentration of 1000 mol ratio/TPA; indicating that the cytotoxicities of these compounds seem to be considerably moderate against in vitro cell lines (Table 4).

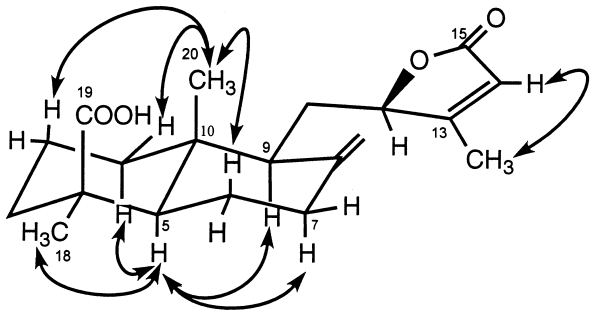


Figure 2. NOESY correlations for **1**.

Table 2. ^{1}H, ^{13}C and NOESY data for compound **2** ($CDCl_3$)[a]

Position	δ_C	δ_H	NOESY
1α	38.8 t	0.93 m	9α
1β		1.72 m	20
2α	19.9 t	1.46 m	1α, 2α
2β		1.78 m	
3α	37.9 t	2.13 m	18
3β		1.00 m	
4	44.5 s		
5	56.4 d	1.26 m	6α, 9α, 18
6α	26 t	1.93 m	5α, 18
6β		1.75 m	7β
7α	38.6 t	1.80 m	5a
7β		2.36 m	6α, 6β, 17A
8	148.7 s		
9	52.5 d	1.54 m	7α, 11β, 12*R*
10	40.2 s		
11A	30.7 t	1.73 m	1α, 17A
11B		1.81 m	18
12	72.4 d	4.41 dd (6.0, 8.5)	9α, 15A, 15B
13	149.2 s		
14	135.7 d	6.32 ddd (1.0, 11.0, 17.5)	15A, 15B, 16B
15A	115.1 t	5.10 d (11.0)	14
15B		5.41 dd (1.0, 18.0)	12, 14, 16B
16A	115.0 t	5.11 d (1.5)	9α, 15
16B		5.15 brs	14, 15
17A	106.9 t	4.70 brs	11A, 12*R*
17B		4.88 d (1.5)	7β
18	28.8 q	1.18 s	3α, 5α, 6α, 7β
19	176.0 s		
20	12.7 q	0.51 s	1β, 2β, 11β, 17A, 17B
N-(Chloromethyl)phthalimide	60.6 t, 123.9 d, 131.7 s, 134.6 d, 166.7 s	5.64 d (10.5), 4.69 d (10.5), 7.79 m, 7.93 m	1β, 2β, 11β, 17A, 17B

[a]Operated at 500 and 125 MHZ for ^{1}H and ^{13}C NMR experiments, respectively; δ in ppm, *J* (in parentheses) Hz; assignments made from ^{1}H–^{1}H COST, HMQC, HMBC, and NOESY data.

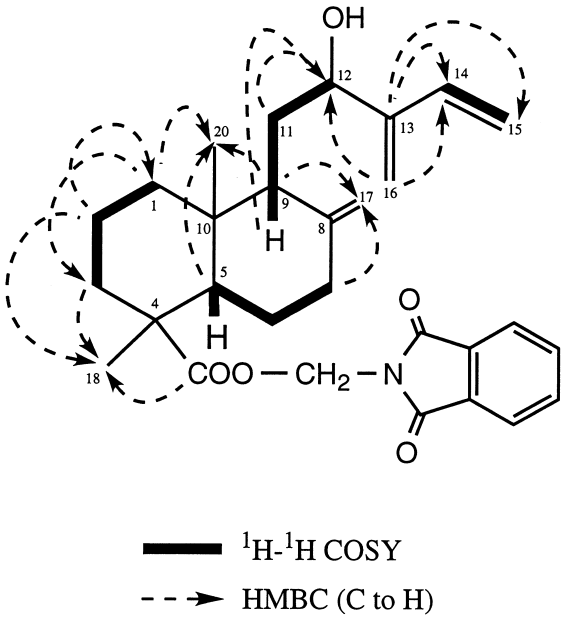
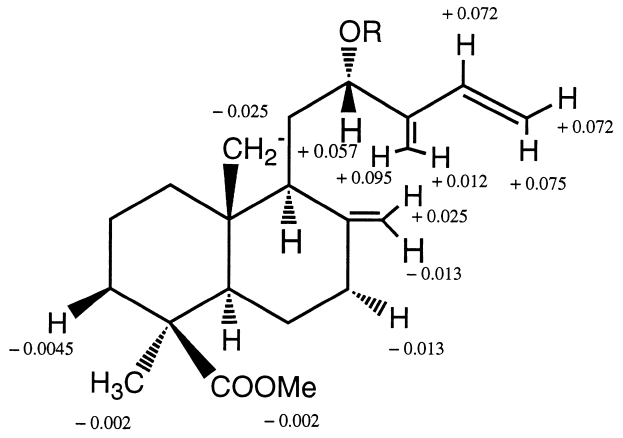


Figure 3. ^{1}H–^{1}H COSY and HMBC correlations of **2a**.

The results of the in vitro experiments prompted us to examine the effect of compound **11** on the in vivo two-stage carcinogenesis bioassay on mouse skin using DMBA as an initiator and TPA as a promotor. Compound **2** was not tested in this in vivo assay because it was obtained as a minor product. No significant toxic effects, such as inflammation and lesional damages, on the areas of mouse skin topically treated with the test compound was observed, except for the formation of papillomas, at the end of the treatment, and also the body weight gains was not influenced during the treatment. As demonstrated in Figure 5, the percentage of papilloma bearers in the control group (DMBA and TPA only) increased rapidly from week 6 and reached 100% after week 8, whereas the treatment with compound **11** along with the initiator and the promotor reduced the percentage of papilloma-bearing mice to approximately 25% during weeks 7–11 and thereafter 73.3% over the period of week 20. As shown in Figure 6, in the control group, the number of papillomas per mouse formed increased rapidly after 6 and reached 10.0 papillomas/mouse at week 20. On the other hand, the mice treated with compound **11** bore 2.4 papillomas over the period of week 20.

Konishi et al. reported the inhibitory effect of a beyerane-type diterpene, *ent*-3β-hydroxy-15-beyeren-2-one, isolated from *Excoecaria agallocha* in the in vivo test under similar experimental conditions described above; when treated with *ent*-3β-hydroxy-15-beyeren-2-one, the percentage of papilloma-bearing mice was approximately 70% and the number of papilloma/mouse was about 4.0 at week 18.[18] Thus, as demonstrated above, the in vivo anti-tumor promoting activity of compound **11** seemed to be stronger than that of the beyerene diterpenoid.

The labdane or abietane-type diterpenoids including compound **11** and 15,16-*bisnor*-13-oxolabda-8(17),11*E*-dien-19-oic acid[6] can be considered to become appropriate lead compounds for developing more potent agents with anti-tumor promoting activity for clinical employment.



4g R = (*S*)-MTPA
4h R = (*R*)-MTPA

Figure 4. ^{1}H Chemical-shift differences ($\Delta\delta = \delta_S - \delta_R$) between the (*S*)- and (*R*)-MTPA esters **4g** and **4h** of **4a**. $\Delta\delta$ values are expressed in Hz (500 MHz).

Experimental

General

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 Si gel (70–230 mesh, Merck) and medium-pressure liquid chromatography (MPLC) was conducted with Si gel (230–400 mesh, Merck) and Cosmocil 40C_{18}-PREP (ODS, Nacalai 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 stem bark of *T. standishii* (Gord.) Carr. was collected at Hashimoto City, Wakayama Prefecture, Japan, in September 1995. A voucher specimen (TS-95-01) is deposited at the Herbarium of the Laboratory of

Table 3. ^{1}H, ^{13}C and HMBC NMR data for compounds **3a** ($CDCl_3$)[a]

Position	δ_C	δ_H	HMBC (C→H)
1α	19.7 t	1.43 m	2α, 3β
1β		1.75 m	
2α	40.6 t	1.03 m	20
2β		1.51 m	
3α	38.1 t	2.16 m	18
3β		1.04 m	
4	44.4 s		2α, 3β, 5, 18
5	55.9 d	1.30 dd (2.5, 7.5)	1β, 6β, 18, 20
6α	24.9 t	1.93 m	18
6β		1.77 m	
7α	37.2 t	1.98 m	17A, 17B
7β		2.40 m	
8	149.4 s, 149.5 s		7α, 7β, 9
9	60.2 d	2.30 brd (10.0)	12, 17A, 17B, 20
10	39.2 s, 39.3 s		1β, 5, 9, 20
11	128.4 d, 128.5 d	5.607 dd (10.0, 16.0), 5.1614 dd (10.0, 16.0)	9, 12
12	136.63 d, 136.64 d	5.45 d (16.0)	11, 16
13	77.6 s, 77.7 s		1′, 11, 12, 14, 15A, 15B, 16
14	142.5 d, 142.6 d	5.871 dd (11.0, 17.5), 5.875 dd (11.0, 17.5)	1′, 12, 15B
15A	113.7 t, 113.8 t	5.107 dd (1.0, 10.5), 5.108 (1.0, 10.5)	16
15B		5.16 dd (1.0, 17.5), 5.17 dd (1.0, 17.5)	
16	24.6 q, 24.7 q	1.36 s	12
17A	108 t, 108.1 t	4.72 d (1.5)	6α, 7β, 9
17B		4.41 d (1.5), 4.45 d (1.5)	
18	28.7 q	1.21 s	2α, 3β
19	176.1 s		2α, 3β, 5, 18
20	13.41 q, 13.43 q	0.64 s	2α, 5, 9
1′	58.2 t, 58.3 t	3.367 quartet (7.0), 3.370 quartet (7.0)	2′
2′	16.0 (2H) q	1.15 t (7.0), 1.16 t (7.0)	1′
N-(Chloromethyl)phthalimide	60.9 t, 123.9 d, 131.7 d, 134.6 d, 166.7 s	5.65 d (10), 5.71 d (10), 7.80 m, 7.94 m	

[a]Operated at 500 and 125 MHz for ^{1}H and ^{13}C NMR experiments, respectively; δ in ppm, *J* (in parentheses) Hz; assignments made from ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY data.

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Bioassays

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 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.[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), 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 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 Tripan Blue staining methods.

Table 4. Relative ratio[a] of EBV-EA activation with respect to positive control (100%) in the presence of compounds **1**–**11** and related compounds (**1a**, **3a**, **8a**)

Compounds	Concentration (mol ratio/TPA)[b]			
	1000	500	100	10
	% to control (% viability)			
1	19.4(60)[c]	64.8	84.1	100.0
1a	26.6(60)	67.9	88.5	100.0
2	0(60)	50.6	72.7	92.6
3a	13.4(60)	55.9	87.4	100.0
4	4.4(60)	46.2	75.3	90.5
5	3.5(70)	40.4	71.5	89.9
6	2.3(70)	30.7	68.4	97.0
7	3.1(70)	50.9	88.9	100
8	14.1(60)	68.0	83.3	100
8a	15.5(60)	69.5	88.9	100
9	11.7(70)	54.0	82.7	100
10	0(70)	22.7	63.9	92.6
11	0(60)	45.1	72.0	88.3
β-Carotene	9(90)	34.0	82.0	100.0

[a]Values represent percentages relative to the positive control value (100%).
[b]TPA concentration was 20 ng/ml (32 pmol/mL).
[c]Values in parentheses are the viability percentages of Raji cells.

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., Shizuoka, 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 ($2\times8\,cm^2$) of each mouse was

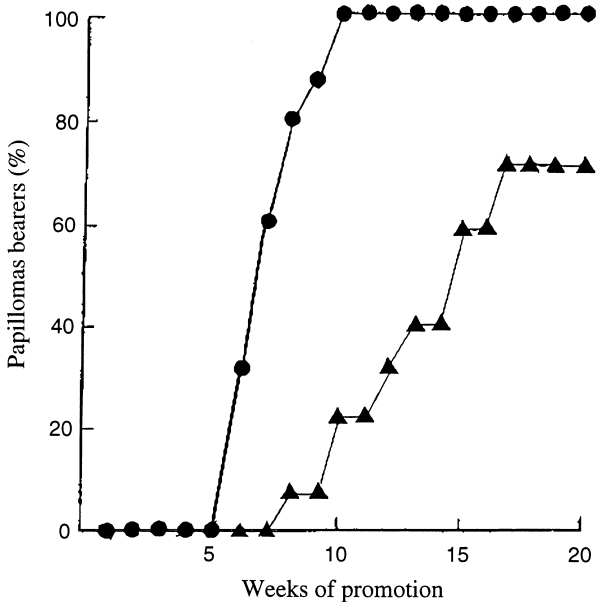

Figure 5. Inhibition of TPA-induced tumor promotion by multiple application of 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid (**11**). All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA, given twice weekly starting 1 week after initiation. Percentage of mice bearing papillomas. ●, Control (TPA alone); ▲, TPA + 85 nmol of **11**.

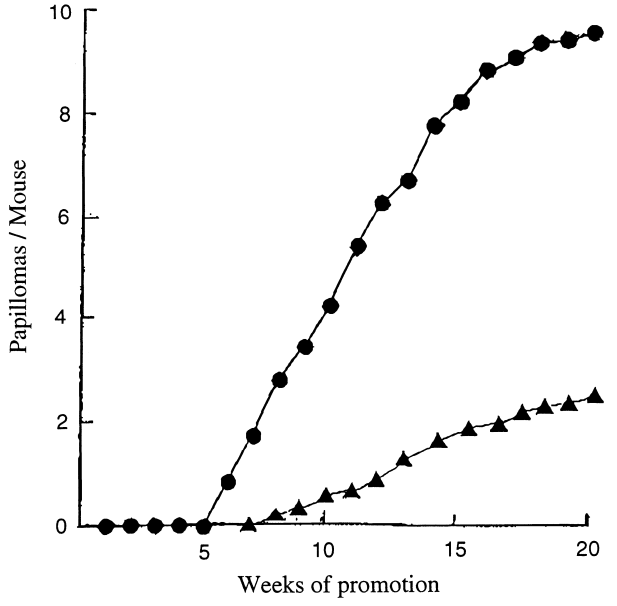

Figure 6. Inhibition of TPA-induced tumor promotion by multiple of 15-oxolabda-8(17),11*Z*,13*E*-trien-19-oic acid (**11**). All mice initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA, given twice weekly starting 1 week after initiation. Average number of papillomas per mouse. ●, Control (TPA alone); ▲, TPA + 85 nmol of **11**. Papillomas per mouse of **11** treatment were significantly different from the positive control at 20 weeks after promotion, $p < 0.005$.

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. One hour before each treatment, 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.

Extraction and isolation. Preliminary Si 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.[5] Residue C (fraction nos 9–11, 28.7 g) was rechromatographed over Si gel (1 kg) eluting with $CHCl_3$ to give compound **4** (12.78 g). Twice rechromatography over Si gel (500 and 100 g) of residue E (fraction nos 21–31, 15.4 g) eluting with $CHCl_3$ gave a crude oil (17.6 mg) followed by esterification with *N*-(chloromethyl)phthalimide (NCMP) followed by preparative TLC with $CHCl_3$/MeOH (50:1) to give compound **2** NMP ester (**2a**) (4.1 mg). Repeated CC of residue G (fraction nos 47–53, 53.2 g) over Si gel, Sephadex LH-20, and ODS column chromatography has already reported,[5] and afforded 15-oxolabda-8(17),11(*Z*),13(*E*)-trien-19-oic acid, 15-oxolabda-8(17),11(*Z*),13(*Z*)-trien-19-oic acid, and 15-*nor*-14-oxolabda-8(17),12(*E*)-dien-19-oic acid. At this stage, we had collected diterpene mixture (residue c, fraction nos 31–33, 155.0 mg), which was subjected to preparative TLC with $CHCl_3$/MeOH (50:1) to afford compound **1** (50.4 mg). Residue H (fraction nos 54–59, 15.5 g) was rechromatographed over silica gel eluting with *n*-hexane/EtOAc (50:1) to yield a crude oil (10.6 mg), which was esterification with *N*-(chloromethyl)phthalimide, and this was purified by HPLC with ODS, eluting with 85% MeOH to give compound **3**-NMP ester (**3a**) (4.3 mg).

Labda-8(17),13-dien-15,12*R*-olid-19-oic acid (1). Colourless oil; $[\alpha]_D^{25}$ −150 (*c* 0.72, $CHCl_3$), HR-EI–MS *m*/*z* 332.1981 $[M]^+$ ($C_{20}H_{28}O_4$ requires 332.1986), IR ν_{max} cm^{-1} 2942 br and 1693 (COOH), 1760 and 1693 (αβ-unsaturated γ-lactone), 1643 and 960 (>C=CH_2); ^1H and ^{13}C NMR, see Table 1; EI-MS *m*/*z* (rel. int) 332 $[M]^+$ (4), 286 (6), 235 (100), 217 (17), 189 (72), 121 (47), 98 (53), 81 (53), 41 (42). CD nm (*c* 4.156×10^{-5} M, EtOH) (Δε) 238 (0), 214 (−32.4).

Methyl labda-8(17),13-dien-15,12*R*-olid-19-oate (1a). A MeOH (2 mL) and C_6H_6 (1 mL) solution of compound **1** (14.7 mg) was added a trimethylsilyldiazomethane 2.0 M 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 (*n*-hexane–$CHCl_3$, 1:2) to afford compound **1a** (5.1 mg). Colorless powder; $[\alpha]_D^{25}$ −69 (*c* 0.10, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 2342, 1762 (αβ-unsaturated γ-lactone), 1723 (COOMe), 1645 and 959 (>C=CH_2), 1309, 1134, 1094, 1076, 1043, 959, 915, 875; ^1H NMR δ 0.51 (3H, s, H-20), 1.20 (3H, s, H-18), 2.10 (3H, m, H-16), 3.62 (3H, s, COOMe), 4.43 (1H, s, H-17A), 4.94 (1H, d, *J* = 1.5 Hz, H-17B); ^{13}C NMR δ 12.6 (q, C-20), 13.9 (q, C-16), 19.8 (t, C-2), 26.1 (t, C-6), 28.0 (t and s C-11 and C-18), 28.8 (q, C-18), 38.0 (t, C-3), 38.5 (t, C-7), 38.7 (t, C-1), 40.0 (s, C-10), 44.3 (s, C-4), 51.2 (q, OMe), 55.9 (d, C-5), 83.4 (d, C-12), 106.2 (t, C-17), 116.5 (d, C-14), 148.1 (s, C-8), 169.3 (s, C-13), 173.1 (s, C-15), 177.6 (s, C-19); EI-MS *m*/*z* (rel. int.) 346 (18) $[M]^+$, 286 (25), 249 (75), 217 (26), 189 (100), 161 (16), 147 (14), 133 (22), 121 (93), 107 (28), 41 (17).

Alkaline hydrolysis of 1a. Compound **1** (5.1 mg) was refluxed with a solution of 1 N KOH/MeOH on a steam bath for 24 h. Evaporation of the solvent under reduced pressure afforded a residue which was purified by PTLC (*n*-hexane–$CHCl_3$, 1:2) to afford compound **1b** (5.0 mg). Colorless oil; $[\alpha]_D^{25}$ −34 (*c* 0.18, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 2932, 2874, 2850, 2360, 2243, 1723 (COOMe), 1645 and 989 (>C=CH_2), 1462, 1379, 847; ^1H and ^{13}C NMR, see Table 1; EI-MS *m*/*z* (rel. int.) 378 (34) $[M]^+$, 346 (15), 287 (15), 234 (49), 175 (24), 129 (100), 121 (48), 59 (31).

Esterification of 12*S*-hydroxylabda-8(17),13(16),14-trien-19-oic acid (2) with *N*-(chloromethyl)phthalimide. A mixture of crude compound **2** (17.6 mg) in MeCN (0.5 mL) solution and Et_3N (24 μg) and *N*-(chloromethyl)phthalimide (35.6 mg) was stirred at 60 °C for 1 h. Evaporation of the solvent under reduced pressure afforded a residue which was purified by PTLC (*n*-hexane–$CHCl_3$–EtOH, 10:1:1) followed by HPLC (ODS) using 70% CH_3CN to give compound **2a**. Colorless oil; $[\alpha]_D^{25}$ +13 (*c* 0.15, $CHCl_3$); HR-EI-MS *m*/*z* 317.2111 ($C_{20}H_{29}O_3$, M^+−*N*-(methyl) phthalimide, calcd for *m*/*z* 317.2115); UV λ_{max} nm 218, 292 (log ε 4.4, 3.1); IR ($CHCl_3$) ν_{max} cm^{-1} 3650 (OH), 1733 (–COO), 1637 and 902 (>C=CH_2); ^1H and ^{13}C NMR, see Table 2; EI-MS *m*/*z* (rel. int.): 446 (1) $[M]^+$, 400 (1), 317 (2) [M−*N*-(methyl)phthalimide]$^+$, 299 (5) [317–H_2O], 271 (3), 253 (3), 160 (100).

Esterification of 13-ethoxylabda-8(17),11,14-trien-19-oic acid (3) with *N*-(chloromethyl)phthalimide. A mixture of crude compound **3** (12.6 mg) in MeCN (0.4 mL) solution and Et_3N (18 μg) and *N*-(chloromethyl)phthalimide (22.4 mg) was stirred at 60 °C for 1 h. Evaporation of the solvent under reduced pressure afforded a residue which was purified by HPLC (ODS) using 85% MeOH to give compound **3a**. Colorless oil; $[\alpha]_D^{25}$ −5 (*c* 0.15, $CHCl_3$); HR-EI-MS *m*/*z* 505.2836 ($C_{31}H_{39}NO_5$, calcd for 505.2826), *m*/*z* 345.2431 ($C_{22}H_{33}O_3$, M^+−*N*-(methyl)-phthalimide, calcd for *m*/*z* 345.2428); UV λ_{max} nm 217, 283 (log ε 4.5, 3.3); IR ($CHCl_3$) ν_{max} cm^{-1} 1733 (–COO), 1632 and 891 (>C=CH_2), 1222 (–C–O); ^1H and ^{13}C NMR, see Table 3; EI–MS *m*/*z* (rel. int.) 505 (3) $[M]^+$, 490 (1) $[M-Me]^+$, 459 (1), 345 (4)[M−*N*-(methyl)phthalimide]$^+$, 300 (7), 160 (100), 99 (40).

Methylation of *trans*-communic acid (4). A solution of diazomethane in ether was added to a solution of crude compound **4** (2.55 g) in ether (100 mL), and the mixture was allowed to stand over night. The removal of the solvent gave a residue (2.66 g) which was purified by Si gel CC (*n*-hexane–EtOAc, 5:1), and recrystallized from MeOH–ether to give **4a** (1.67 g). $[\alpha]_D^{25}$ +48 (*c* 1.26,

$CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 1644 and 889 (>C=CH_2), 1446, 1385, 1318, 1229, 1153; ^1H NMR: δ 1.19 (3H, s, H-18), 3.62 (3H, s, OMe).

***M*-CPBA oxidation of compound 4a.** A CH_2Cl_2 solution of compound **4a** (402.5 mg) was gradually added to a 80% *m*-CPBA (411.3 mg) and $NaHCO_3$ (160.2 mg) with stirring at −10 °C for 1.5 h. The reaction mixture was washed with saturated $NaHCO_3$ and H_2O. Evaporation of the solvent under reduced pressure afforded a residue (457.1 mg) that was purified by silica gel CC (*n*-hexane–EtOAc, 5:1) to yield 12,13-epoxylabda-8(17),14-dien-19-oic acid (**4b**) (281.5 mg), $C_{20}H_{30}O_3$, $[\alpha]_D^{25}$ +35 (*c* 0.19, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 3583, 2945, 2874, 2849, 1727 (COOMe), 1644 and 891 (>C=CH_2), 1230, 1154, 1135; ^1H NMR: δ 3.74 (m), 4.28 (m); EI-MS *m*/*z* (rel. int.) 332 (2) $[M]^+$, 317 (3), 249 (9), 203 (19), 189 (35), 161 (21), 121 (100), 107 (31), 93 (34).

Synthesis of methyl-12(*R*), (*S*)-hydroxylabda-8(17), 13(16),14-trien-19 oate (4c), (4d) from 4b. A mixture of dry toluene solution (10 mL) of compound **4b** (65.5 mg) and $Al(O\text{-}i\text{-}Pr)_3$ (56.2 mg) was refluxed for 4.5 h. Evaporation of the solvent under reduced pressure afforded a residue which was resolved in EtOAc (25 mL) and the organic layer was washed with 1 M HCl (15 mL), H_2O and $NaHCO_3$. The crude residue (65.4 mg) was purified by PTLC (*n*-hexane–ether, 4:1), followed by HPLC (ODS) eluting with 85% MeOH to give methyl-12*S*-hydroxylabda-8(17),11*Z*,13(16)-trien-19-oate (**4c**) (13.1 mg) and methyl-12*R*-hydroxylabda-8(17),11*Z*,13(16)-trien-19-oate (**4d**) (6.6 mg). (**4c**): $C_{21}H_{32}O_3$; $[\alpha]_D^{25}$ +41 (*c* 0.70, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1} 1725 (COOMe), 1645 and 901 (>C=CH_2), 1466, 1449, 1229, 1154, 1134. ^1H NMR :δ 0.51 (3H, s, H-20), 1.17 (3H, s, H-18), 4.44 (1H, dd, *J* = 5.4, 8.1 Hz, H-12*R*), 4.74 and 4.91 (each 1H, s, H-17), 5.12 (1H, d, *J* = 11.4 Hz, H-15), 5.14 and 5.17 (each 1H, s, H-16), 5.43 (1H, d, *J* = 18 Hz, H-15), 6.34 (1H, dd, *J* = 11.1, 18.0 Hz, H-14); EI-MS *m*/*z* (rel. int.) 332 (7) $[M]^+$, 328 (10), 314 (7), 299 (7), 255 (14), 189 (21), 121 (100), 81 (37), 52 (39). (**4d**): $C_{21}H_{32}O_3$; $[\alpha]_D^{25}$ +41 (*c* 0.27, $CHCl_3$); IR ($CHCl_3$) ν_{max} cm^{-1}: 1724 (COOMe), 1645 and 891 (>C=CH_2), 1448, 1229, 1154, 1036. ^1H NMR: δ 0.50 (3H, s, H-20), 1.12 (3H, s, H-18), 4.42 (1H, dd, *J* = 5.7, 11.4 Hz, H-12*S*), 4.53 (1H, s, H-17), 4.89 (1H, d, *J* = 1.5 Hz, H-17), 5.12 (1H, d, *J* = 11.0 Hz, H-15), 5.14 and 5.21 (each 1H, s, H-16), 5.42 (1H, d, *J* = 18 Hz, H-15), 6.34 (1H, dd, *J* = 11.0, 18.0 Hz, H-14); EI-MS *m*/*z* (rel. int.) 332 (7) $[M]^+$, 314 (21), 262 (21), 189 (21), 132 (32), 121 (100), 81 (37), 52 (39).

Alkaline hydrolysis of 4c. Compound **4c** (1.5 mg) was hydrolyzed with potassium *t*-butoxide (4.5 mg) in ether (0.5 mL) and H_2O (0.2 μL) at 0 °C, and then stirred at room temperature for 26 h. Work up as described above gave a residue which was purified by HPLC (ODS, 80% CH_3CN) to afford compound **4e** (1.1 mg). Colorless oil; EI-MS *m*/*z* 318 [M^+, $C_{20}H_{30}O_3$].

Esterification of 4e with *N*-(chloromethyl)phthalimide. A mixture of compound **4e** (1.0 mg) in MeCN (0.4 mL) solution and Et_3N (1.5 μg) and *N*-(chloromethyl)phthalimide (2.2 mg) was stirred at 60 °C for 1 h. Evaporation of the solvent under reduced pressure afforded a residue which was purified by PTLC (*n*-hexane–$CHCl_3$–EtOH, 10:1:1) to give compound **4f**. Colorless oil; $[\alpha]_D^{25}$ +13 (*c* 0.08, $CHCl_3$). The synthetic **4f** was identified by direct comparison with an authentic sample of **2a**.

Formation of the (*S*)- and (*R*)-MTPA ester 4e and 4f from 4a. (*S*)-MTPA (4.0 mg), dicyclohexylcarbodiimide (DCC) (3.5 mg) and 4-(dimethylamino)pyridine (DMAP) (1.5 mg) were added to a dry CH_2Cl_2 solution (200 μL) of compound **4a** (1.4 mg), and the reaction mixture was left at room temperature for over night. The solvent was evaporated off under reduced pressure, and the residue was purified by HPLC (ODS) using 95% MeOH to afford (*S*)-MTPA ester of **4a** (**4g**) (1.0 mg). The same reaction with **4a** (1.8 mg) using (*R*)-MTPA ester (3.8 mg) gave (*R*)-MTPA ester of **4a** (**4h**) (0.5 mg).

(*S*)-MTPA ester (4g). Amorphous powder; ^1H NMR: δ 1.16 (3H, s, H-18), 5.13 and 5.43 (each 1H, d, *J* = 11.5, 17.5 Hz, H-15), 5.75 (1H, dd, *J* = 4.0, 11.0 Hz, H-12), 6.29 (1H, ddd, *J* = 1.0, 11.5, 18.0 Hz, H-14).

(*R*)-MTPA ester (4h). Amorphous powder; ^1H NMR: δ 1.16 (3H, s, H-18), 5.06 and 5.36 (each 1H, d, *J* = 11.5, 17.5 Hz, H-15), 5.69 (1H, dd, *J* = 4.5, 11.0 Hz, H-12), 6.22 (1H, dd, *J* = 11.5, 18.0 Hz, H-14).

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