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NEW LABDANE-TYPE DITERPENOIDS FROM *CROTON OBLONGIFOLIUS* AND THEIR CYTOTOXIC ACTIVITY

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Abstract – Eight new labdane-type diterpenoids (**1**-**3**) and (**5**-**9**) and hardwickiic acid (**4**), a clerodane, were isolated from the stem bark of *Croton oblongifolius*. Their structures were established to be 3-oxygenated *ent*-manoyl oxide derivatives with a 8,13-epoxytricyclic ring system and hydroxylabdandienes on the basis of spectroscopic and X-Ray crystallographic analysis. The absolute stereochemistry of the core labdane skeleton was deduced to be (5*S*,8*S*,9*S*,10*R*,13*S*) from the X-Ray crystallographic analysis of the *p*-bromobenzoate of *ent*-3α-hydroxymanoyl oxide (**3**) and mutual chemical correlation. Cytotoxicity of these compounds was tested against a panel of human tumor cell lines.

INTRODUCTION

Croton oblongifolius (Euphorbiaceae) has been used as a traditional medicine in Thailand for many purposes such as dysmenorrheal, a purgative, dyspepsia, and dysenteria. Our continuing investigation on chemical constituents of *C. oblongifolius* available in various area of Thailand has yielded several new and different types of diterpenoids with cytotoxic activity, for example, labdanes,[1] cembranes,[2] clerodanes,[3] kauranes,[4] and halimanes.[5] In this paper we describe the isolation of eight new labdane-type diterpenoids and hardwickiic acid from *C. oblongifolius* collected at Loei Province in the north-eastern part of Thailand. The absolute configurations of the isolates and their cytotoxic activities against human tumor cell lines are also discussed.

RESULTS AND DISCUSSION

Isolation and structural determination of chemical constituents. The hexane extract of the resinous stem bark of *C. oblongifolius* was subjected to the separation of chemical constituents by repeated column chromatography on silica gel using a gradient solvent system of hexane-ethyl acetate followed by recrystallization, to result in the isolation of nine pure crystalline compounds (**1-9**) (Figure 1).

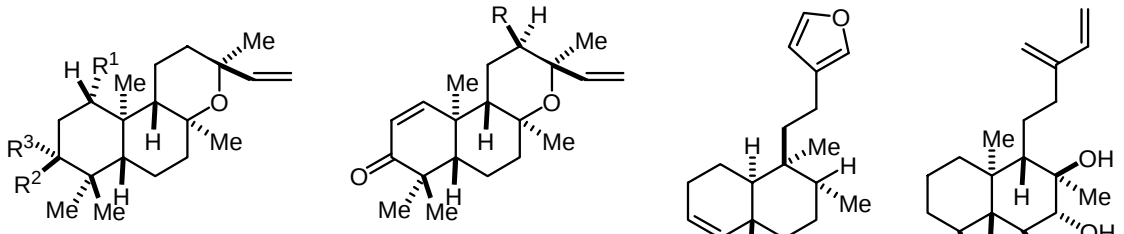


Figure 1. Chemical structures of the isolates from *C. oblongifolius*

The structures were determined by spectroscopic means (see, Table 1 for ^{1}H NMR spectrum, Table 2 for ^{13}C NMR spectrum) including X-Ray crystallography in some cases. The absolute stereochemistry is assigned by X-Ray crystallographic analysis of the *p*-bromobenzoate of compound (**3**) and chemical correlation among the isolates in addition to specific rotation $[\alpha]_D$. Compound (**4**) was identified with hardwickiic acid obtained previously.[6]

Compound (**1**) was obtained as colorless prisms, mp 98-99 ºC, with a molecular formula of $C_{20}H_{32}O_2$ based on ^{1}H and ^{13}C NMR spectra and FABMS spectrum [*m/z* 304 (M^+)]. The IR spectrum shows a characteristic absorption due to carbonyl group (1696 cm^{-1}). The ^{13}C NMR spectrum and DEPT experiments reveal the presence of non-equivalent twenty carbons, among which the seventeen are sp^3

Table 1. ^{1}H NMR spectral data of the isolates (**1**-**3**) and (**5**-**9**) (400 MHz, in $CDCl_3$)[a]

C#	**1**	**2**	**3**	**5**	**6**	**7**	**8**	**9**
1	1.45 (m)	7.10 (d, *J*=10)	1.32 (m)	3.89 (dif. t, *J*=*ca*. 6)	7.13 (d, *J*=10)	3.84 (dd, *J*=11, 5)	0.96 (m)	1.03 (m)
	1.86 (m)	-	1.35 (m)	-	-	-	1.66 (m)	1.65 (m)
2	2.43 (m)	5.86 (d, *J*=10)	1.58 (m)	2.36 (dd, *J*=15, 5)	5.88 (d, *J*=10)	1.80 (m)	1.42 (m)	1.41 (m)
	2.53 (m)	-	1.94 (m)	2.92 (dd, *J*=15, 8)	-	1.84 (m)	1.59 (m)	1.60 (m)
3	-	-	3.41 (d, *J*=2)	-	-	3.50 (t, *J*=3)	1.15 (m)	1.23 (m)
	-	-	-	-	-	-	1.40 (m)	1.38 (m)
4	-	-	-	-	-	-	-	-
5	1.50 (m)	1.78 (m)	1.42 (m)	1.49 (m)	1.85 (dd, *J*=12, 3)	1.42 (m)	1.08 (m)	1.16 (m)
6	1.05 (m)	1.52 (m)	1.30 (m)	1.50 (m)	1.52 (m)	1.44 (m)	1.30 (m)	3.63 (t, *J*=10)
	1.08 (m)	1.70 (m)	1.57 (m)	1.54 (m)	1.72 (m)	1.58 (m)	1.88 (m)	-
7	1.48 (m)	1.53 (m)	1.49 (m)	1.35 (m)	1.58 (m)	1.76 (m)	3.53 (dd, *J*=12, 4)	3.38 (d, *J*=9)
	1.86 (m)	1.92 (m)	1.83 (dt, *J*=12, 3)	1,50 (m)	1.92 (m)	1.88 (m)	-	-
8	-	-	-	-	-	-	-	-
9	1.40 (m)	1.62 (m)	1.44 (m)	1.52 (m)	2.09 (dd, *J*=9, 6)	1.68 (m)	1.09 (m)	1.21 (m)
10	-	-	-	-	-	-	-	-
11	0.91 (m)	1.70 (m)	1.42 (m)	1.55 (m)	1.94 (m)	1.70 (m)	1.45 (m)	1.42 (m)
	1.84 (m)	1.84 (m)	1.62 (m)	2.18 (m)	1.98 (m)	2.16 (m)	1.65 (m)	1.63 (m)
12	1.66 (m)	1.72 (m)	1.61 (m)	1.54 (m)	3.80 (dd, *J*=7, 4)	1.70 (m)	2.30 (m)	2.30 (m)
	1.80 (m)	1.90 (m)	1.77 (m)	1.56 (m)				
13	-	-	-	-	-	-	-	-
14	5.87 (dd, *J*=14, 9)	5.89 (dd, *J*=18, 11)	5.87 (dd, *J*=18, 11)	5.87 (dd, *J*=18, 11)	5.81 (dd, *J*=17, 11)	5.87 (dd, *J*=18, 11)	6.35 (dd, *J*=18, 11)	6.35 (dd, *J*=18, 11)
15	4.93 (dd, *J*=11, 2)	4.94 (dd, *J*=11, 1)	4.90 (dd, *J*=11, 2)	4.93 (dd, *J*=11, 2)	5.26 (dd, *J*=11, 2)	4.92 (dd, *J*=11, 2)	5.08 (d, *J*=11)	5.08 (d, *J*=11)
	5.15 (dd, *J*=18, 2)	5.16 (dd, *J*=17, 2)	5.11 (dd, *J*=17, 2)	5.15 (dd, *J*=18, 2)	5.44 (dd, *J*=18, 2)	5.13 (dd, *J*=18, 2)	5.30 (d, *J*=18)	5.30 (d, *J*=18)
16	1.29 (s)	1.32 (s)	1.28 (s)	1.28 (s)	1.39 (s)	1.25 (s)	5.02 (s)	5.04 (s)
17	1.34 (s)	1.38 (s)	1.30 (s)	1.33 (s)	1.38 (s)	1.30 (s)	1.11(s)	1.16 (s)
18	1.09 (s)	1.16 (s)	0.95 (s)	1.08 (s)	1.16 (s)	0.92 (s)	0.80 (s)	0.86 (s)
19	1.03 (s)	1.08 (s)	0.83 (s)	1.04 (s)	1.09 (s)	0.83 (s)	0.89 (s)	1.17 (s)
20	0.91 (s)	1.05 (s)	0.80 (s)	0.86 (s)	1.04 (s)	0.86 (s)	2.17 (s)	0.99 (s)

[a]Coupling constant is denoted by Hz. Assignment is based on 2D NMR spectral experiments.

Table 2. ^{13}C NMR spectral data of the isolates (**1-3**) and (**5-9**) (125 MHz, in $CDCl_3$)[a]

C#	1	2	3	5	6	7	8	9
1	37.8	157.6	32.0	77.8	157.3	74.6	39.3	39.4
2	33.8	125.8	25.2	44.9	125.9	42.7	18.3	18.1
3	217.2	205.1	76.1	214.9	205.0	76.7	41.7	43.5
4	47.3	42.3	37.5	47.1	44.6	37.3	33.4	33.8
5	54.7	53.2	55.3	51.0	53.2	47.6	53.7	57.6
6	20.7	20.2	19.6	20.5	20.1	19.6	28.0	72.0
7	42.3	44.6	43.1	41.9	41.5	35.9	80.7	85.4
8	74.5	75.0	74.9	74.5	75.3	74.9	78.1	76.9
9	55.0	49.9	48.9	55.1	43.5	54.8	59.5	58.8
10	36.5	39.4	36.8	42.5	38.7	43.1	39.4	39.2
11	15.6	15.5	15.2	17.6	22.9	18.4	23.8	24.0
12	35.8	35.4	35.7	35.4	70.0	34.9	34.8	34.8
13	73.5	73.7	73.2	73.4	76.7	73.3	147.2	147.2
14	147.6	147.4	147.8	147.6	142.5	147.7	138.7	138.7
15	110.4	110.7	110.4	110.5	115.8	110.5	113.6	113.6
16	28.3	28.6	28.4	28.6	27.4	29.0	115.8	115.8
17	24.9	25.6	25.5	24.9	25.3	25.8	17.8	19.5
18	26.5	27.6	28.2	27.2	27.6	27.8	15.6	16.9
19	20.9	21.3	21.8	20.2	21.2	21.7	33.2	36.2
20	15.0	18.6	15.2	10.7	18.9	10.9	21.5	22.1

[a]Assignment is based on 2D NMR spectral experiments.

carbons (five methyl, six methylene, two methane, and four quaternary carbons). The remaining three carbons are attributable to sp^2 carbons composed of one methylene, one methine, and one carbonyl carbons. The relative stereochemistry was established by X-Ray crystallography (Figure 2a), indicating that **1** was 3-oxomanoyl oxide, firstly isolated as (+)-form, $[\alpha]_D$ +54º, from *Xylia dolabriformis.*[7] In this study **1** was isolated as (-)-form ($[\alpha]_D$ -53º) and, as a result, established to be *ent*-3-oxomanoyl oxide. The structurally related ribenone (*ent*-3-oxo-13-epimanoyl oxide), a 13-epimer of **1**, had been chemically derived in the course of the structural determination of ribenol[8] and was also isolated as a natural product.[9]

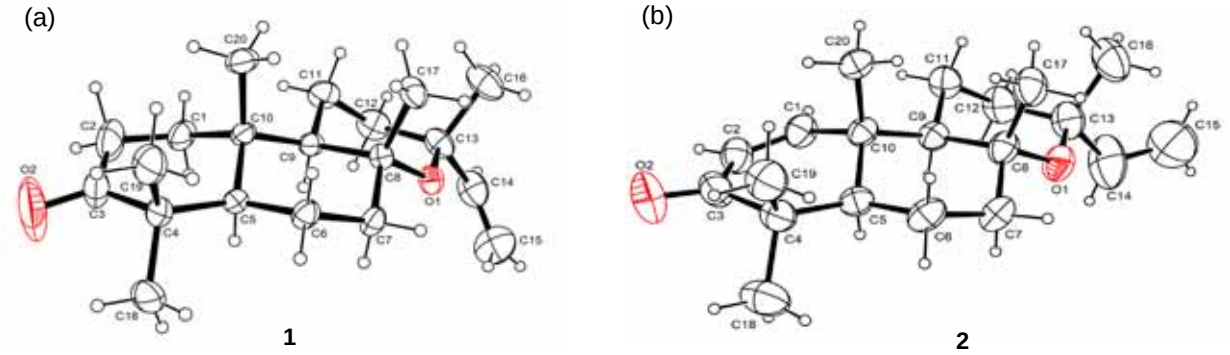


Figure 2. ORTEP drawings of compounds (**1**) and (**2**) with enantiomeric stereochemistry

The molecular formula of compound (**2**) was similarly assigned to be $C_{20}H_{30}O_2$ [*m/z* 303.2324 $(M+H)^+$ in HRFABMS]. The IR spectrum showed a conjugated carbonyl absorption (1655, 1615 cm^{-1}), which was supported by NMR spectra [δ_H 5.86, 7.10 (each 1H, d, *J*=10 Hz); δ_C 125.8 (CH), 157.6 (CH), 205.1 (CO)]. Precise examination of 2D NMR spectra and X-Ray crystallographic analysis (Figure 2b) allowed us to depict **2** as *ent*-1,2-dehydro-3-oxomanoyl oxide. The 13-epimer of **2** had been obtained as a metabolite in the microbial transformation of ribenone.[10]

Compound (**3**) {$C_{20}H_{34}O_2$ [*m/z* 307.2637 $(M+H)^+$ in HRFABMS]} was assigned to be 3-hydroxylmanoyl oxide by characteristic absorption of hydroxyl function (3432 cm^{-1}), in place of carbonyl one of **1**, in the IR spectrum and a methine carbinol signal [δ_H 3.41 (d, *J*=2 Hz); δ_C 76.1 (CH)] in the NMR spectra. The axial orientation of the hydroxyl group was assigned by the small coupling constant of the methine proton, suggesting that **3** was 3-hydroxymanoyl oxide. 3-Hydroxymanoyl oxide had been prepared from 2α,3α;14,15-bisepoxymanoyl oxide with lithium aluminum hydride.[11] The structure of **3**, including absolute stereochemistry, was confirmed by the X-Ray crystallographic analysis of the *p*-bromobenzoate derived from **3** mentioned later (see, Figure 4). Thus, **3** was established to be *ent*-3α-hydroxymanoyl oxide. The structurally related *ent*-3β-hydroxymanoyl oxide, a 3-epimer of compound (**3**), had been isolated from *Sideritis varoi* (Labiatae).[12] Ribenol, a 13-epimer of **3**, is a wide-spread component in nature and was firstly isolated from *S. canariensis*.[8]

Compound (**5**) {$C_{20}H_{32}O_3$ [*m/z* 321.2430 $(M+H)^+$ in HRFABMS]} was assigned to be a hydroxylated 3-oxomanoyl oxide by the presence of methine carbinol [ν_{max} 3400 cm^{-1}; δ_H 3.89 (dif. q, *J*=*ca.* 6 Hz); δ_C 77.8 (CH)] and carbonyl [ν_{max} 1719 cm^{-1}; δ_C 214.9 (CO)] functions. The presence of a pair of double doublets at δ 2.36 (*J*=15, 5 Hz) and 2.92 (*J*=15, 8 Hz), coupled with the methine carbinol proton, allowed us to deduce **5** as 1-hydroxy-3-oxomanoyl oxide. A diastereomeric pair of 1-hydroxy-3-oxo-13-epimanoyl oxides, *ent*-1α-hydroxy-3-oxo-13-epimanoyl oxide[9] and *ent*-1β-hydroxy-3-oxo-13-epimanoyl oxide,[10,13] had been obtained as metabolites in the microbial transformation of ribenone. The ABX signal pattern of this sequence [-CH_2CH(OH)-] is similar to that of the latter 1β-epimer carrying equatorial hydroxyl group between the metabolites. Thus, compound (**5**) was determined to be *ent*-1β-hydroxy-3-oxomanoyl oxide.

Compound (**6**) {$C_{20}H_{30}O_3$ [*m/z* 319.2273 $(M+H)^+$ in HRFABMS]} showed the presence of a secondary hydroxyl group [ν_{max} 3521 cm^{-1}; δ_H 3.80 (dd, *J*=7, 4 Hz); δ_C 70.0 (CH)] and a conjugated carbonyl system [ν_{max} 1660, 1463 cm^{-1}; δ_H 5.88, 7.13 (each 1H, d, *J*=10 Hz); δ_C 125.9 (CH), 157.3 (CH), 205.0 (CO)] in its molecule. Location of the hydroxyl group at the C12 position was suggested by precise 2D NMR spectral experiments. Finally, compound (**6**) was determined to be *ent*-1,2-dehydro-12α-hydroxy-3-oxomanoyl oxide by X-Ray crystallographic analysis (Figure 3). The structurally related *ent*-1,2-dehydro-12β-hydroxy-3-oxo-13-epimanoyl oxide had also been obtained as a

metabolite in the microbial transformation of ribenone.[10,14]

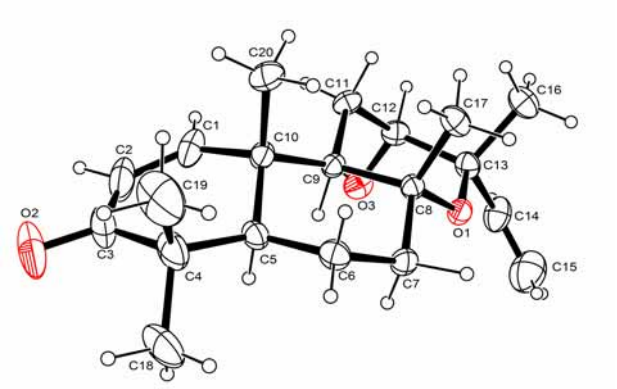


Figure 3. ORTEP drawing of compound (**6**) with enantiomeric stereochemistry

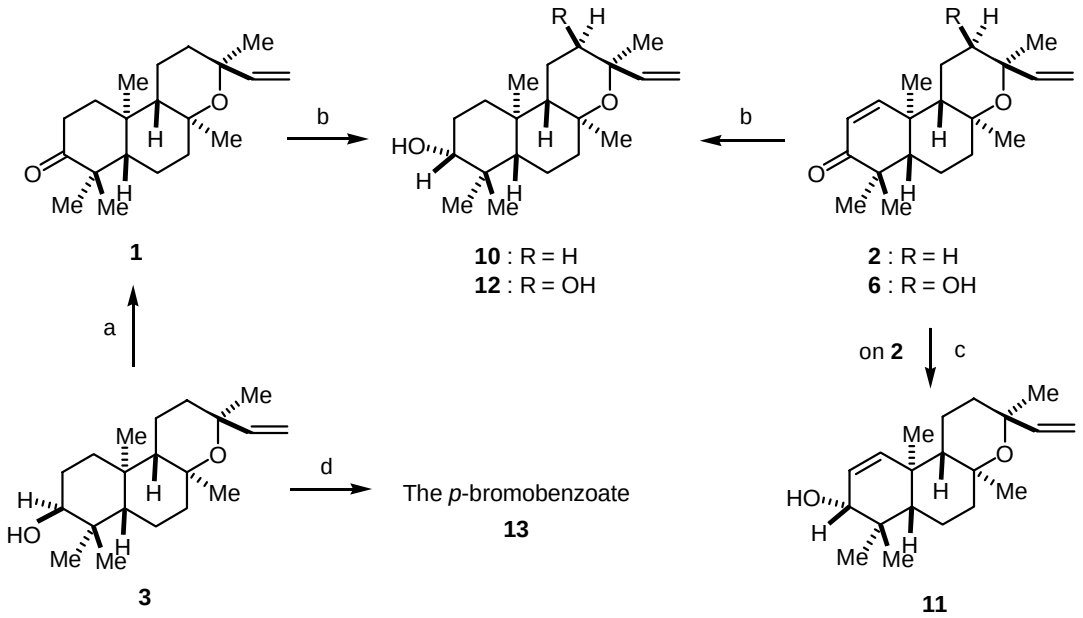
Compound (**7**) {$C_{20}H_{34}O_3$ [*m/z* 323.2564 $(M+H)^+$ in HRFABMS]} was reasonably assignable to be dihydroxylated manoyl oxide by the presence of two characteristic methine carbinol functions [ν_{max} 3410 cm^{-1}; δ_H 3.84 (dd, *J*=11, 5 Hz); δ_C 74.6 for C1-H; δ_H 3.50 (t, *J*=3 Hz); δ_C 76.7 for C3-H]. Precise examination of 2D NMR spectra (HMBC, HMQC, COSY, and NOESY) revealed that the two hydroxyl groups were located at the C1 and the C3 positions with equatorial and axial configurations, respectively. Thus, compound (**7**) was determined to be *ent*-1β,3α-dihydroxymanoyl oxide. To our knowledge the structurally related 1,3-dihydroxymanoyl oxides have never been known until now.

Compound (**8**) {$C_{20}H_{34}O_2$ [*m/z* 307.2637 $(M+H)^+$ in HRFABMS]} showed four degree of unsaturations, among which the two were attributable to 2-substituted 1,3-diene function [δ_H 5.02 (s), 5.08 (d, *J*=11 Hz), 5.30 (d, *J*=18 Hz), 6.35 (dd, *J*=18, 11 Hz); δ_C 113.6 (CH), 115.8 (CH), 138.7 (CH), 147.2 (C)] by the NMR spectra. In addition, the presence of vicinal glycol system [ν_{max} 3419 cm^{-1}; δ_H 3.53 (dd, *J*=12, 4 Hz); δ_C 80.7 (CH) for C7-H, δ_C 78.1 (C) for C8] at C7 and C8 positions was indicated by 2D NMR spectral experiments (HMBC and HMQC etc) and the equatorial configuration of the C7-secondary alcohol was assigned by the splitting pattern in the ^1H NMR spectrum. These data are similar to those of nidorellol, 7β,8α-dihydroxylabda-13(16),14-diene, isolated from *Nidorella auriculata.*[15] Although the C8 stereochemistry of nidorellol had not been determined, the C8 stereochemistry of compound **8** could be reasonably assumed to be the same configuration that those of the isolates discussed above.

Compound (**9**) {$C_{20}H_{34}O_3$ [*m/z* 345.2406 $(M+Na)^+$ in HRFABMS]} contains sequential triol system with equatorial orientations [δ_H 3.63 (t, *J*=10 Hz); δ_C 72.0 for C6-H, δ_H 3.38 (d, *J*=9 Hz); δ_C 85.4 for C7-H, δ_C 76.9 for C8] in addition to the same 2-substituted 1,3-diene function that in **8**, like 6α-hydroxynidorellol.[15] Thus, **9** is assigned to be *ent*-6α,7β,8α-trihydroxylabda-13(16),14-diene.

Chemical correlation among the isolates. The isolates were chemically correlated to each other as

shown in Scheme 1. Thus, the pyridinium chlorochromate (PCC) oxidation of **3** afforded **1** in 90% yield. Treatment of not only a ketone (**1**) but also an unsaturated ketone (**2**) with sodium borohydride ($NaBH_4$) gave the same saturated alcohol (**10**) as a single product in 80 and 75% yield, respectively. Reduction of **6** under the same conditions gave the corresponding saturated alcohol (**12**), too. It should be noted here that the stereochemistry at the C3 in the semisynthetic product (**10**) was opposite to that of natural **3** by comparison of their NMR spectral data [δ_H 3.41 (d, *J*=2 Hz) in **3** and 3.20 (dd, *J*=11, 5 Hz) in **10**]. On the other hand, reduction of **2** with $NaBH_4$ in the presence of cerium chloride ($CeCl_3$) gave α,β-unsaturated alcohol (**11**) with an equatorial hydroxyl group [δ_H 3.85 (d, *J*=8 Hz, C3-H)] in 83% yield. In addition, the *p*-bromobenzoate (**13**) was prepared from compound (**3**) for the determination of the absolute configuration of the isolates. The X-Ray crystallographic analysis (Figure 4) shows that they have (5*S*,8*S*,9*S*,10*R*,13*S*) configuration as a common stereochemistry.



Scheme 1. Chemical correlation among some isolates: a) PCC/CH_2Cl_2 rt, 1 h (90%); b) $NaBH_4$/MeOH, rt, 1 h (80% from **1**; 75% from **2**; 40% from **6**); c) $NaBH_4$, $CeCl_3 \cdot 7H_2O$/MeOH, rt, 3 h (83%); d) i) $SOCl_2$, *p*-$BrPhCO_2H$, reflux, 0.5 h; ii) 3, rt, 2 h (86%)

Cytotoxic activity tests. The isolates (**1-9**) as well as semisynthetic products (**10-12**) obtained in this work were tested on cytotoxic activity using human tumor cell lines, among which compounds (**5-7**) and (**12**) were inactive (>10 μg/mL). The data of the remaining samples are shown in Table 3. Compounds (**1-3** and **8-9**) exhibited strong activity (<1 μg/mL) against gastric carcinoma (KATO-3) and moderate activity against breast ductal carcinoma (BT474), undifferentiated lung carcinoma (CHAGO), liver hepatoblastoma (HEP-G2), and colon adenocarcinoma (SW620). Although compound (**4**) showed strong activity (<1 μg/mL) against BT474, compounds (**10**) and (**11**) moderate activity against all cell lines.

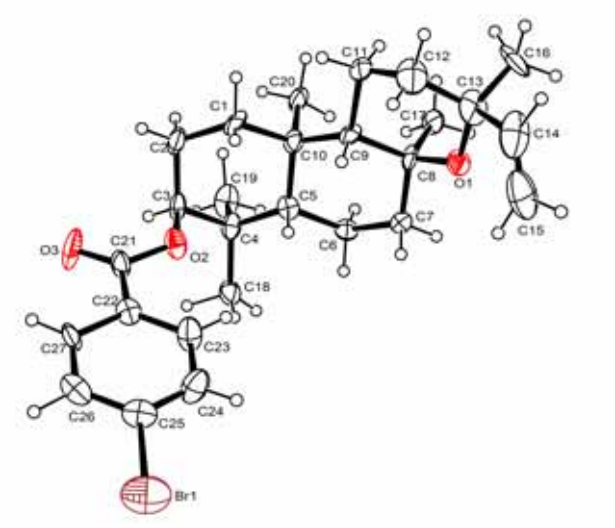


Figure 4. ORTEP drawing of compound (**13**) with correct stereochemistry

Structure activity relationship of the tested compounds could not be reasonably drawn in this stage; however, the presence of either equatorial hydroxyl group at the C1 position or axial hydroxyl group at the C12 position obviously renders these molecules inactive. It should be noted here that a number of labdane-type diterpenoids shows cytoxicity.[16-18]

Table 3. Cytotoxic activity of selected compounds (**1-4**) and (**8-11**)[a]

Compounds	BT474[b]	CHAGO[c]	HEP-G2[d]	KATO-3[e]	SW620[f]
1	8.0	5.8	4.6	0.8	6.9
2	6.3	6.0	5.0	0.6	5.6
3	4.2	6.0	4.1	0.8	4.8
4	0.7	5.3	2.1	3.6	4.2
8	6.3	6.4	4.7	0.1	4.6
9	4.7	5.8	4.4	0.7	4.9
10	2.5	5.5	3.3	2.5	4.3
11	7.8	5.7	4.1	1.5	5.1
doxorubicin HCl	1.0	0.6	0.1	0.7	0.5

[a]Results are expressed as IC_{50} values (μg/mL). [b]BT-474: human breast ductal carcinoma ATCC No. HTB 20. [c]CHAGO: human undifferentiated lung carcinoma. [d]HEP-G2: human liver hepatoblastoma ATCC No. HB 8065. [e]KATO-3: human gastric carcinoma ATCC No. HTB 103. [f]SW620: human colon adenocarcinoma ATCC No. CCL 227.

CONCLUSION

Eight labdane-type diterpenoids together with hardwickiic acid were newly isolated from *C. oblongifolius* and their structures were established by chemical correlation in addition to spectroscopic means including X-Ray crystallographic analysis. Three ring-fused products (**1-3**) and (**5-7**) were deduced to have a

(5*S*,8*S*,9*S*,10*R*,13*S*)-8,13-epoxylabda-14-ene system as a common absolute stereochemistry. Examination of cytotoxic activity of these products using human tumor cell lines showed that the five (**1-3**) and (**8-9**) exhibited strong activity (<1 μg/mL) against KATO-3 (stomach) and moderate activity against BT474 (breast), CHAGO (lung), HEP-G2 (liver), SW620 (colon).

EXPERIMENTAL

IR spectra were recorded on JASCO FT/IR-230 infrared spectrophotometer. Optical rotations were determined on a JASCO J1010 polarimeter (cell length 10 mm). FABMS were measured with a JEOL HX-110 using m-nitrobenzyl alcohol as a matrix unless otherwise indicated. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM GSX-400A and -500A spectrometer. Column chromatography was carried out SiO_2 (Fuji Silycia, FD-100).

Isolation of products. The dried and powdered resinous stem bark of *C. oblongifolius* (2 kg) collected at latitude N 17 08 32.7 and longitude E 101 10 18.6, Loei Province in the north-eastern part of Thailand in October, 2000 (voucher specimen: BKF No 084729) and macerated with hexane (2 L x 2) at rt for 3 days for each to give the extract (213.7 g). A part (10 g) of the hexane extract was subjected to silica gel column chromatography using hexane as eluting solvent with increasing polarities by adding ethyl acetate to give 6 fractions [F1 (0.92 g), F2 (3.85 g), F3 (0.86 g), F4 (1.53 g), F5 (0.81 g), and F6 (1.94 g)]. Fractional crystallization of F2 from ethyl acetate gave *ent*-1,2-dehydro-3-oxomanoyl oxide (**2**) (0.95 g, 1.0% w/w) and *ent*-3-oxomanoyl oxide (**1)** (0.38 g, 0.4% w/w), respectively.

F3 was separated by column chromatography with 5% ethyl acetate-hexane to give 4 fractions [F3-1 (0.04 g), F3-2 (0.52 g), F3-3 (0.25 g), and F3-4 (0.01 g)]. Trituration of F3-2 with ethyl acetate afforded *ent*-3α-hydroxymanoyl oxide (**3**) (0.32 g, 0.34% w/w). Column chromatography of F3-3 with 5% ethyl acetate in hexane gave hardwickiic acid (**4**) (0.11 g, 0.12% w/w).

Separation of F4 by column chromatography with 5% ethyl acetate in hexane gave 5 fractions [F4-1 (0.22 g), F4-2 (0.26 g), F4-3 (0.14 g), F4-4 (0.54 g), and F4-5 (0.20 g)]. Recrystallization of F4-2 and F4-4 from ethyl acetate yielded *ent*-1β-hydroxy-3-oxomanoyl oxide (**5**) (0.14 g, 0.15% w/w) and *ent*-1,2-dehydro-12α-hydroxy-3-oxomanoyl oxide (**6**) (0.38 g, 0.4% w/w), respectively.

Column chromatography of F5 with 2% methanol in chloroform gave 3 fractions [F5-1 (0.30 g), F5-2 (0.32 g), and F5-3 (0.18 g)], among which F5-2 was recrystallized from ethyl acetate to give *ent*-1β,3α-dihydroxymanoyl oxide (**7**) (0.10 g, 0.11% w/w).

Column chromatography of F6 with 5% methanol in chloroform gave 5 fractions [F6-1 (0.22 g), F6-2 (0.26 g), F6-3 (0.14 g), F6-4 (0.35 g), and F6-5 (0.49 g)]. Recrystallization of F6-2 and F6-4 from ethyl acetate yielded *ent*-7β,8α-dihydroxylabda-13(16),14-diene (**8**) (0.08 g, 0.08% w/w) and *ent*-6α,7β,8α-trihydroxylabda-13(16),14-diene (**9**) (0.12 g, 0.13% w/w).

***ent*-3-Oxomanoyl oxide** (**1**). Colorless prisms, mp 98-99 ºC; IR ν_{max} (KBr) cm^{-1}: 1696; $[\alpha]_D$ -53.1º (c 0.6, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); FABMS *m/z*: 304 (M^+).

***ent*-1,2-Dehydro-3-oxomanoyl oxide** (**2**). Colorless prisms, mp 147-148 ºC; IR ν_{max} (KBr) cm^{-1}: 1655, 1615; $[\alpha]_D$ -53.6º (c 1.0, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 303.2324 $(M+H)^+$ ($C_{20}H_{31}O_2$ requires 303.2316).

***ent*-3α-Hydroxymanoyl oxide (3).** Colorless needles, mp 135-138 ºC; IR ν_{max} (KBr) cm^{-1}: 3432; $[\alpha]_D$ -3.53º (c 1.0, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 307.2637 $(M+H)^+$ ($C_{20}H_{35}O_2$ requires 307.2628).

***ent*-1β-Hydroxy-3-oxomanoyl oxide (5).** Colorless needles, mp 124-125 ºC; IR ν_{max} (KBr) cm^{-1}: 3521, 1713; $[\alpha]_D$ -66.8º (c 0.6, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 321.2430 $(M+H)^+$ ($C_{20}H_{33}O_3$ requires 321.2421).

***ent*-1,2-Dehydro-12α-hydroxy-3-oxomanoyl oxide (6).** Colorless prisms, mp 141-142 ºC; IR ν_{max} (KBr) cm^{-1}: 3521, 1660; $[\alpha]_D$ -54.1º (c 1.0, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 319.2273 $(M+H)^+$ ($C_{20}H_{31}O_3$ requires 319.2265).

***ent*-1β,3α-Dihydroxymanoyl oxide (7).** White solid, mp 59-60 ºC; IR ν_{max} (KBr) cm^{-1}: 3410; $[\alpha]_D$ -12.4º (c 0.6, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z* : 323.2564 $(M+H)^+$ ($C_{20}H_{35}O_3$ requires 323.2577).

***ent*-7β,8α-Dihydroxylabda-13(16),14-diene (8).** White solid, mp 82-83 ºC; IR ν_{max} (KBr) cm^{-1}: 3419; $[\alpha]_D$ -27.5º (c 0.6, $CHCl_3$); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 307.2637 $(M+H)^+$ ($C_{20}H_{35}O_2$ requires 307.2628).

***ent*-6α,7β,8α-Trihydroxylabda-13(16),14-diene (9).** White solid, mp 99-100 ºC; IR ν_{max} (KBr) cm^{-1}: 3390; $[\alpha]_D$ -8.8º (c 0.6, DMF); ^1H NMR (Table 1) and ^{13}C NMR (Table 2); HRFABMS *m/z*: 345.2406 $(M+Na)^+$ ($C_{20}H_{34}O_3Na$ requires 345.2499).

Oxidation of *ent*-3α-hydroxymanoyl oxide (3). A mixture of **3** (50 mg) and PCC (70 mg) in methylene chloride (2 mL) was stirred at rt for 1 h, then diluted with anhydrous ether, and decanted through Florisil (washed twice with anhydrous ether). Evaporation of the solvent gave **1** (45 mg, 90%) as white solid.

Reduction of *ent*-3-oxomanoyl oxide (1) [or *ent*-1,2-dehydro-3-oxomanoyl oxide (2)] with $NaBH_4$. $NaBH_4$ (10 mg) was added to a stirred solution of **1** (or **2**) (10 mg) in methanol (2 mL) and the mixture was stirred at rt for 1 h, poured into water, and extracted with ether. The extract was worked up in the normal manner to give *ent*-3β-hydroxymanoyl oxide (**10**) (7.5 mg, 75% from **2**). White solid, mp 62-63 ºC; IR ν_{max} (KBr) cm^{-1}: 3499; $[\alpha]_D$ -15.4º (c 1.0, $CHCl_3$); ^1H NMR δ: 0.77, 0.80, 0.98, 1.30, 1.32 (each, s), 1.38, 1.42, 1.55, 1.58, 1.61, 1.62, 1.63, 1.66, 1.76, 1.79, 1.83, 1.86 (each m), 3.20 (dd, *J*=11, 5 Hz), 4.90 (dd, *J*=11, 2 Hz), 5.14 (dd, *J*=17, 2 Hz), 5.87 (dd, *J*=17, 11 Hz); ^{13}C NMR δ: 15.2 (CH_3), 15.3 (CH_2), 19.6

(CH_2), 21.8 (CH_3), 25.2 (CH_2), 25.5 (CH_3), 28.2 (CH_3), 28.4 (CH_3), 32.0 (CH_2), 35.7 (CH_2), 36.8 (C), 37.5 (C), 43.1 (CH_2), 48.9 (CH), 55.3 (CH), 73.2 (C), 74.9 (C), 76.1 (CH), 110.4 (CH_2), 147.8 (CH); FABMS *m/z*: 307.2637 $(M+H)^+$ ($C_{20}H_{35}O_2$ requires 307.2628).

Reduction of *ent*-1,2-dehydro-3-oxomanoyl oxide (2) with $NaBH_4$ in the presence of $CeCl_3$. A mixture of **2** (10 mg) and $CeCl_3 \cdot 7H_2O$ (80 mg) in methanol (2 mL) at rt for 1 h. To the mixture $NaBH_4$ (5 mg) was added and then the whole was stirred at rt for 2 h, poured into water, and extracted with ether. The extract was worked up in the normal manner to give *ent*-3β-hydroxy-1,2-dehydromanoyl oxide (**11**) (8.3 mg, 83%). White solid, mp 89-90 ºC; IR ν_{max} (KBr) cm^{-1}: 3316; $[\alpha]_D$ -59.6º (c 1.0, $CHCl_3$); ^1H NMR δ: 0.80, 0.94, 1.00, 1.27, 1.32 (each, s), 1.59, 1.64, 1.71, 1.72, 1.84, 1.88, 1.90, 1.92 (each m), 3.85 (d, *J*=8 Hz), 4.92 (dd, *J*=11, 1 Hz), 5.14 (dd, *J*=17, 1 Hz), 5.35 (dd, *J*=10, 2 Hz), 5.81 (d, *J*=10 Hz), 5.86 (dd, *J*=17, 11 Hz); ^{13}C NMR δ: 15.7 (CH_2), 17.2 (CH_3),18.1 (CH_3),19.1 (CH_2), 26.4 (CH_3), 27.4 (CH_3), 28.8 (CH_3), 35.2 (CH_2), 38.8 (C), 43.4 (CH_2), 52.2 (CH), 73.5 (C), 75.3 (C), 77.1 (CH), 110.6 (CH_2), 126.5 (CH), 136.8 (CH), 147.6 (CH); HRFABMS *m/z*: 343.2039 $(M+K)^+$ ($C_{20}H_{32}O_2K$ requires 343.2094).

Reduction of *ent*-1,2-dehydro-12α-hydroxy-3-oxomanoyl oxide (6) with $NaBH_4$. According to the procedure mentioned above the reduction of **6** (10 mg) with $NaBH_4$ (10 mg) for 1 h afforded *ent*-3β,12α-dihydroxymanoyl oxide (**12**) (4.0 mg, 40%). White solid, mp 63-64 ºC; IR ν_{max} (KBr) cm^{-1}: 3423; $[\alpha]_D$ -35.6º (c 0.5, $CHCl_3$); ^1H NMR δ: 0.78, 0.80, 0.97, 1.30, 1.35 (each, s), 1.34, 1.58, 1.59, 1.69, 1.70, 1.76, 1.77, 1.83, 1.86, 1.87 (each m), 3.25 (dd, *J*=11, 5 Hz), 3.65 (d, *J*=5 Hz), 5.24 (dd, *J*=11, 2 Hz), 5.43 (dd, *J*=17, 2 Hz), 5.79 (dd, *J*=17, 5 Hz); ^{13}C NMR δ: 15.2 (CH_2), 15.8 (CH_3), 19.5 (CH_2), 22.8 (CH_3), 25.2 (CH_3), 27.1 (CH_2), 27.4 (CH_3), 28.0 (CH_3), 36.5 (C), 37.0 (CH_2), 38.8 (C), 42.5 (CH_2), 48.8 (CH), 55.2 (CH), 70.2 (CH), 75.0 (C), 75.8 (C), 78.8 (CH), 115.5 (CH_2), 143.0 (CH); HRFABMS *m/z* : 361.2144 $(M+K)^+$ ($C_{20}H_{34}O_3K$ requires 361.2199).

Preparation of the *p*-bromobenzoate of 3 (Compound 13). A mixture of thionyl chloride (2 mL) and *p*-bromobenzoic acid (100 mg) was refluxed for 30 min under stirring. The reaction mixture was cooled down to rt and **3** (100 mg) was added into the mixture. The whole was stirred for 2 h and worked up as usual to give the *p*-bromobenzoate (**13**) as colorless crystal (86 mg), mp 125-126 ºC, which was recrystallized from ethyl acetate. IR ν_{max} (KBr) cm^{-1}: 1786, 1716; ^1H NMR δ: 0.86, 0.91, 0.95, 1.34, 1.42 (each 3H, s), 4.88 (1H, t, *J*= 3 Hz), 4.96 (1H, dd, *J*=11, 2 Hz), 5.19 (1H, dd, *J*=18, 2 Hz), 5.92 (1H, dd, *J*=18, 11 Hz), 7.62 (2H, d, *J*=8 Hz), 7.91 (2H, d, *J*=8 Hz); FABMS *m/z*: 489 $(M+H)^+$.

Crystallographic data collections of 1, 2, 6, and 13. Crystal data are shown in Table 4. Reflection data were collected at 293 K, on a 1K Bruker SMART CCD area diffractometer using graphite-monochromated MoKα radiation (λ = 0.71073 Å). The crystal structures were solved by direct methods and refined by full-matrix least-square method on $(F_{obs})^2$ with anisotropic thermal parameters for all non-hydrogen atoms. The hydrogen atoms were located by difference synthesis and refined

isotropically using SHELXS-97. Crystallographic data for structure (**1**, **2**, **6**, and **13**) reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication. Copies of the data can be obtained free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (Fax: +44-(0)1223-336033, e-mail: deposit@ccdc.cam.ac.uk).

Table 4 Crystal data of compounds (**1**, **2**, **6**, and **13**).

	1	**2**	**6**	**13**
Identification code	CCDC 253556	CCDC 253557	CCDC 253555	CCDC 253558
Empirical formula	$C_{20}H_{32}O_2$	$C_{20}H_{30}O_2$	$C_{20}H_{30}O_3$	$C_{27}H_{37}O_2Br$
Formula weight	304.46	302.44	318.44	489.49
Wave length (Mo Ka), (Å)	0.71073	0.71073	0.71073	0.71069
Crystal system	orthorhombic	orthorhombic	orthorhombic	monoclinic
Space group	P(21)(21)(21)	P(21)(21)(21)	P(21)(21)(21)	C2
Unit cell dimensions				
a (Å)	6.30450(10)	6.3271(4)	6.1692(3)	22.836(5)
b (Å)	13.7686(2)	11.4575(7)	14.2262(2)	7.762(2)
c (Å)	20.7350(2)	24.6115(15)	20.4812(3)	18.289(4)
b (°)				127.588(3)
Volume (Å3)	1799.88(4)	1784.16(19)	1797.52(9)	2518(1)
Z	4	4	4	4
D(cal), g/cm^3	1.124	1.126	1.177	1.291
Absorption coefficient (cm^{-1})	0.070	0.070	0.077	16.61
F(000)	672	664	696	1032
Crystal size (mm)	0.25x0.28x0.48	0.20x0.28x0.50	0.15x0.40x0.43	0.45x0.30x0.28
Reflections collected / unique	13527 / 5222 [R(int)=0.0292]	8749 / 2920 [R(int)=0.0348]	13405 / 5107 [R(int)=0.0221]	15,142 / 3154 [R(int)=0.036]
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data / restraints / parameters	5222 / 0 / 327	2920 / 7 / 257	5107 / 0 / 268	2331/0/282
Goodness-of-fit on F^2	1.091	1.042	1.100	1.84
Final R indices [I>2sigma(I)]	R1=0.0564, wR2=0.1162	R1=0.0616, wR2=0.1454	R1=0.0699, wR2=0.1998	R1=0.052, wR2=0.083
R indices (all data)	R1=0.0920, wR2=0.1358	R1=0.0795, wR2=0.1571	R1=0.0823, wR2=0.2147	R1=0.057
Absolute structure parameter	-0.2(17)	0(3)	-0.1(16)	-
Largest diff. peak and hole (e.Å3)	0.150 and -0.132	0.168 and -0.133	0.948 and -0.383	0.52 and -0.44

Bioassay of cytotoxic activity. Bioassay of cytotoxic activity against human cell cultures *in vitro* was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric

method.[19] Doxorubicin hydrochloride was used as a positive control substance. The human tumor cell line was harvested from exponential-phase maintenance culture (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100 μL volumes using a repeating pipette. Following a 24 hours incubation at 37 ºC, with 5% CO_2, 100% relative humidity and 100 μL of culture medium. Culture medium containing sample was dispensed within appropriate wells (control group, N=6; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=2) and medium/tetrazolium reagent blank (N=6) "background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/mL PBS was steriled and filtered with 0.45 μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1 : 5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μL) was added to each culture well resulting in 50 μg MTT/250 μL total medium volume) and cultures were incubated at 37 ºC for 4 to 24 h depending upon individual cell line requirements. Following incubation, cell monolayers and formazan were inspected microscopically: culture paltes containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μL of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μL of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor=1.00). Cell line growth and growth inhibition were expressed in terms of mean (± 1 SD) absorbance units and/or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance.

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