ภาคผนวก

Chemistry of Natural Compounds, Vol. 45, No. 5, 2009

NEW CLEISTANTHANE DITERPENOIDS AND 3,4-*seco*-CLEISTANTHANE DITERPENOIDS FROM *Croton oblongifolius*

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UDC 547.596

*Two new cleistanthane-type diterpenoids, 3-hydroxycleistantha-13(17),15-diene (1) and 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid (2), were isolated from the stem bark of* Croton oblongifolius *Roxb. Epoxidation of 2 gave two epoxides 3 and 4. Their structures were established on the basis of spectroscopic data. Compound 1 showed nonspecific strong cytotoxicity against human tumor cell lines, compound 2 showed weak activity, and compounds 3 and 4 were inactive.*

Key words: *Croton oblongifolius*, cleistanthane, diterpenoids, cytotoxicity.

In our previous work, the chemical constituents of *Croton oblongifolius* from various parts of Thailand were investigated and several diterpenoid compounds were reported, for example, cembranes [1, 2], labdane [3], halimane [4], clerodane [5], and kaurane [6]. Most of the compounds are obtained in good yield and possess biological activities such as cytotoxicity and inhibition of Na^+, K^+-ATPase activity. Our preliminary study of *C. oblongifolius* from various locations in Thailand suggested that this plant is a very good source of diterpenoid compounds. As part of our continuing search for novel diterpenoids in this plant, we found two new cleistanthane diterpenoids for the first time from *C. oblongifolius*. In this paper, we report the structural determination and cytotoxicity of the new compounds.

Compound **1** was isolated as colorless needle crystals. The molecular formula of $C_{20}H_{32}O$ was established by elemental analysis, ^1H and ^{13}C NMR (Table 1 and 2), and MS $[M+H]^+$ (*m/z* 289) data. The IR spectrum of **1** showed strong absorption bands at 3413 cm^{-1} (OH stretching) and 1633 cm^{-1} (C=C stretching). ^{13}C NMR spectrum indicated the presence of 20 carbons.

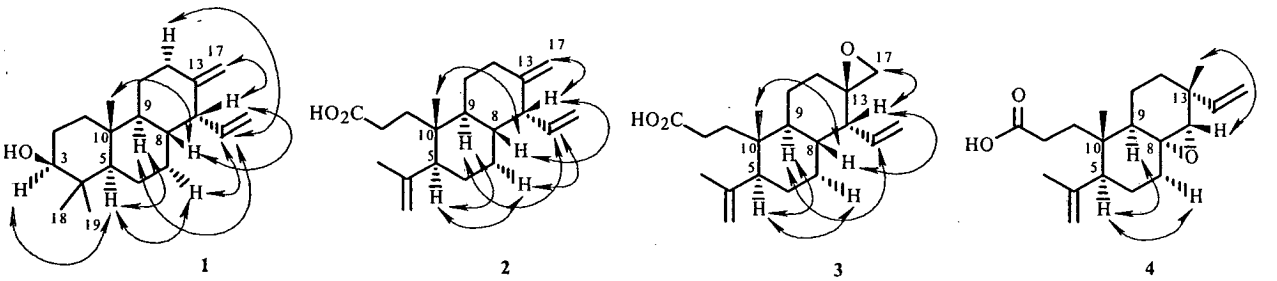

Fig. 1. Key NOESY correlations of **1**–**4**.

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0009-3130/09/4505-0641

TABLE 1. ^{1}H NMR ($CDCl_3$, 400 MHz) Data of Compounds **1**–**4**[a,b]

Position	1	2	3	4
1α	1.06 (m)	1.65 (m)[c]	1.65 (m)[c]	1.66 (m)[c]
1β	1.73 (m)			
2α	1.52 (m)	2.42 (m)	2.28 (m)	2.22 (m)
2β	1.63 (ddd, J = 3.4, 7.3, 14.8)	2.30 (m)	2.43 (m)	2.44 (m)
3	3.22 (dd, J = 11.6, 4.5)			
5	0.78 (m)	1.96 (dd, J = 12.8, 2.4)	1.96 (dd, J = 12.4, 2.8)	2.15 (dd, J = 12.4, 2.4)
6α	1.34 (m)	1.44 (m)	1.42 (m)	1.49 (m)
6β	1.62 (m)	1.67 (m)	1.71 (m)	2.02 (m)
7α	1.24 (m)	1.24 (m)	1.19 (m)	1.27 (m)
7β	1.50 (m)	1.46 (m)	1.39 (m)	1.89 (m)
8	1.49 (m)	1.57 (m)	1.86 (m)	
9	1.04 (m)	1.26 (m)	1.25 (m)	1.02 (m)
11α	1.75 (m)	1.17 (m)	1.16 (m)	1.22 (m)[c]
11β	1.77 (m)	1.72 (m)	2.02 (m)	
12α	2.17 (m)	2.20 (m)[c]	1.46 (m)	1.38 (m)[c]
12β	2.17 (m)		1.64 (m)	
14	2.81 (dd, J = 9.2, 4.2)	2.83 (dd, J = 8.8, 4.4)	1.67 (m)	2.55 (m)
15	6.01 (ddd, J = 9.2, 9.6, 17.0)	6.02 (ddd, J = 17.6, 10.4, 8.8)	5.90 (ddd, J = 17.2, 10.4, 10.0)	5.87 (ddd, J = 17.6, 10.4, 10.0)
16α	5.02 (dd, J = 9.6, 1.6)	5.03 (dd, J = 10.4, 1.6)	5.03 (dd, J = 10.4, 2.0)	5.01 (dd, J = 10.4, 2.0)
16β	5.03 (dd, J = 17.0, 1.6)	5.04 (dd, J = 17.6,1.6)	5.11 (dd, J = 17.2, 2.0)	5.04 (dd, J = 17.6, 2.0)
17α	4.57 (s)	4.59 (d, J = 0.9)	2.60 (d, J = 4.4)	1.10 (s)[d]
17β	4.65 (s)	4.67 (d, J = 0.9)	2.63 (d, J = 4.8)	
18α	0.80 (s)[d]	4.67 (s)	4.68 (s)	4.74 (s)
18β		4.88 (s)	4.86 (s)	4.90 (s)
19	0.97 (s)[d]	1.74 (s)[d]	1.74 (s)[d]	1.78 (s)[d]
20	0.79 (s)[d]	0.84 (s)[d]	0.90 (s)[d]	0.98 (s)[d]

[a]Chemical shifts in ppm, J values in Hz are in parentheses; [b]the assignments were based on ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

[c]Intensity of two protons; [d]intensity of three protons.

The ^{13}C NMR spectrum showed a signal of oxygenated methine carbon (δ 79.1 ppm), the signals of exocyclic methylene (106.4, CH_2; 152.4, C), and a vinyl group (137.7, CH; 115.9, CH_2). ^{1}H NMR spectrum indicated two olefinic protons of terminal methylene at δ 4.57 (s) and 4.65 (s), vinyl protons at δ 6.01 (1H, ddd, J = 17.0, 9.6 and 9.2 Hz), 5.02 (1H, dd, J = 9.6 and 1.6 Hz), and 5.03 (1H, dd, J = 17.0 and 1.6 Hz), and three signals of methyl groups at δ 0.80 (3H, s), δ 0.97 (3H, s), and δ 0.79 (3H, s). The DEPT NMR spectra indicated the presence of three methyl carbons, six methylene carbons, and four methine carbons. The molecular formula of $C_{20}H_{32}O$ indicated five double-bond equivalents. The ^{1}H and ^{13}C NMR spectra indicated an exocyclic double-bond and a vinyl double-bond; therefore, **1** must consist of three rings. The 1D and 2D NMR, including COSY, NOESY, HMQC, and HMBC spectral data, showed that compound **1** has a cleistanthane-type diterpenoid structure. The stereochemistry of **1** was established by the coupling constants in the ^{1}H NMR spectrum and NOESY spectra. The coupling constant of H-3 (dd, J = 11.6 and 4.5 Hz) indicated that the hydroxyl group was equatorially oriented. The observed NOEs between H-3 and H-5 and between H-5 and H-9 and the absence of NOEs between H-5 and the methyl of C-20 in the NOESY experiment suggested that H-5, H-9, and C-20 was axially oriented. The coupling constant of H-14 with H-15 ($J_{14,15}$ = 9.2 Hz) and with H-8 ($J_{14,8}$ = 4.2 Hz) and the observed NOEs between H-14 and H-17, between H-14 and H-8, between H-15 and H_{ax}-7, and between H-15 and H_{ax}-12 in the NOESY experiment suggested that the vinyl group and H-8 were axially oriented. Thus, compound **1** was assigned the (5α,8β,9α,10β,14α)-cleistantha-13(17),15-dien-3β-ol structure.

TABLE 2. ^{13}C NMR ($CDCl_3$, 100 MHz) Data of Compounds **1–4**[a]

Position	1	2	3	4
1	37.6 (t)	32.1 (t)	32.1 (t)	32.1 (t)
2	27.5 (t)	28.1 (t)	28.0 (t)	28.0 (t)
3	79.1 (d)	181.0 (s)	180.1 (s)	178.7 (s)
4	38.9 (s)	148.4 (s)	147.4 (s)	146.9 (s)
5	54.3 (d)	50.7 (d)	50.7 (d)	50.6 (d)
6	21.3 (t)	27.6 (t)	27.5 (t)	25.6 (t)
7	32.1 (t)	31.5 (t)	31.1 (t)	34.7 (t)
8	40.5 (d)	40.4 (d)	36.6 (d)	61.4 (d)
9	49.2 (d)	41.0 (d)	40.2 (d)	43.3 (d)
10	36.9 (s)	39.0 (s)	39.0 (s)	40.3 (s)
11	27.0 (t)	27.4 (t)	29.3 (t)	16.4 (s)
12	31.3 (t)	31.3 (t)	23.0 (t)	34.9 (t)
13	152.4 (s)	151.9 (s)	61.5 (s)	35.6 (s)
14	54.6 (d)	54.7 (d)	53.0 (d)	63.7 (d)
15	137.7 (d)	137.3 (d)	135.5 (d)	146.8 (d)
16	115.9 (t)	116.2 (t)	118.3 (t)	112.0 (t)
17	106.4 (t)	106.8 (t)	53.9 (t)	22.0 (q)
18	15.8 (q)	113.8 (t)	113.7 (t)	114.0 (t)
19	28.4 (q)	23.9 (q)	23.9 (q)	23.6 (q)
20	14.0 (q)	16.8 (q)	16.8 (q)	18.3 (q)

[a]The assignments were based on HSQC and HMBC experiments.

TABLE 3. Cytotoxicity Data of Compounds **1–4**[a]

Compounds	Cell lines[b]				
	KATO-3	SW620	BT474	HEP-G2	CHAGO
1	6.0	>10	6.1	0.5	5.5
2	9.6	>10	10	8.6	>10
3	>10	>10	>10	>10	>10
4	>10	>10	>10	>10	>10
Doxorubicin hydrochloride	0.5	1.2	2.2	1.7	0.1

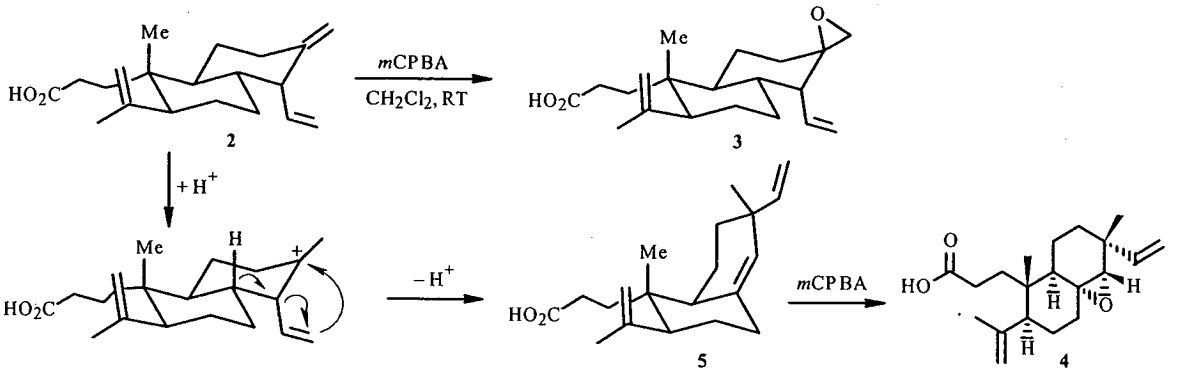
[a]Results are expressed as IC_{50} values (μg/mL); [b]KATO-3, human gastric carcinoma ATCC No. HTB 103; SW620, human colon adenocarcinoma ATCC No. CCL 227; BT474, human breast ductal carcinoma ATCC No. HTB20; HEP-G2, human liver hepatoblastoma ATCC No. HB 8056; CHAGO, human undifferentiated lung carcinoma.

Compound **2** was isolated as a colorless oil. The molecular formula of compound **2** was assigned as $C_{20}H_{30}O_2$ based on HRESIMS, ^1H and ^{13}C NMR (Table 1 and 2) and MS $[M+H]^+$ (*m/z* 303). The IR spectrum showed the presence of a carboxylic acid group (3400–2400 and 1704 cm^{-1}). The ^{13}C NMR spectrum and DEPT experiments revealed the presence of 20 carbons consisting of two methyl groups, six methylene sp^3-carbons, four methine sp^3-carbons, a quaternary sp^3-carbon, three methylene sp^2-carbons, a methine sp^2-carbon, two quaternary sp^2-carbons, and a carbonyl carbon of carboxylic group. Since the six degrees of unsaturation were accounted for, it was implied that **2** should contain two rings. The ^1H NMR spectrum revealed the presence of two terminal methylene groups [two olefinic protons at δ 4.59 (1H, d, J = 0.9 Hz) and δ 4.67 (1H, d, J = 0.9 Hz) and two olefinic protons at δ 4.67 (1H, s) and δ 4.88 (1H, s)]. The ^1H NMR also showed the presence of a vinyl group at δ 6.02 (1H, ddd, J = 17.6, 10.4, and 8.8 Hz), δ 5.03 (1H, dd, J = 10.4 and 1.6 Hz) and δ 5.04 (1H, dd, J = 17.6 and 1.6 Hz) and two methyl groups at δ 1.74 (3H, s) and δ 0.84 (3H, s). By comparison of spectral data of **2** with those of **1**, including a detailed analysis of 2D NMR spectra, it seems like the structure of **2** was quite similar to of **1**, except that the ring A of **2** was broken at C-3 and C-4 and C-3 became a carboxylic group. The HMBC experiment with the assistance of COSY, TOCSY, and NOESY led to the structure of **2** which was assigned as 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid.

The relative stereochemistry of **2** at positions 5, 8, 9, 10, and 14 was determined on the basis of the coupling constant and NOESY experiments (Fig. 1).

Epoxidation of **2** with *m*CPBA in CH_2Cl_2 gave epoxide **3** as a major product and epoxide **4** as a minor product without the presence of epoxidation at the isopropylene moiety. The molecular formula of **3** was proposed to be $C_{20}H_{30}O_3$ based on microanalysis and ^1H and ^{13}C NMR data (Table 1 and 2). The spectral data of **3** were quite similar to those of compound **2**, except that the exocyclic double bond (C-13 and C-17) was replaced by the epoxide moiety. By comparison of the spectral data of **3** with those of **2** and a detailed analysis of 2D NMR spectra, it can be concluded that the structure of **3** was an epoxide of compound **2**. NOESY analysis with the assistance of the coupling constants led to the relative structure of **3** (Fig. 1) as 13,17-epoxy-3,4-*seco*-cleistantha-4(18),15-dien-3-oic acid.

Compound **4** showed a molecular ion with *m/z* 318 ($C_{20}H_{30}O_3$). The ^{13}C NMR spectrum and HSQC experiments revealed the presence of 20 carbons consisting of three methyl groups, six methylene sp^3-carbons, two methine sp^3-carbons, an oxygenated methine sp^3-carbon, a quaternary sp^3-carbon, an oxygenated quaternary sp^3-carbon, two methylene sp^2-carbons, a methine sp^2-carbon, a quaternary sp^2-carbon, and a carbonyl carbon of carboxylic group. Since the six degrees of unsaturation were accounted for, it was implied that **4** should contain one epoxide ring and two additional rings. The HMBC experiment with the assistance of the coupling constants, COSY, TOCSY, and NOESY led to the structure of **4** (Fig. 1), and it can be concluded that compound **4** was 8,14-epoxypimara-4(18),15-dien-3-oic acid (Fig. 1). Compound **4** was derived from the intermediate **5**, which was thought to arise through an acid-catalyzed process during the epoxidation, as shown in Sheme 1.



Scheme 1.

The cytotoxicity of compounds **1–4** was tested against human tumor cell lines (Table 3). Compound **1** showed high activity for HEP-G2 and moderate activity for KATO-3, BT474, and CHAGO. Compound **2** had moderate activity for KATO-3, BT474, and HEP-G2. Compounds **3** and **4** did not show any strong cytotoxicity. Doxorubicin hydrochloride was used as positive control in all experiments and showed strong cytotoxicity for all cell lines.

EXPERIMENTAL

General Experimental Procedures. All commercial grade solvents were distilled prior to use. Melting points were determined on a Fisher-Johns melting point apparatus. The optical rotation was determined on a Perkin–Elmer 341 polarimeter. Measurements of UV spectra were carried out on a Hewlett Packard 8452A diode array spectrophotometer. IR spectra were recorded on a Nicolet Impact 410 spectrophotometer. Spectra of solid samples were recorded as KBr pellets. ^1H and ^{13}C NMR spectra were recorded at 400.00 and 100.00 MHz, respectively, on a Varian Model Mercury 400 MHz instrument. Low-resolution MS were obtained with a MALDI/TOF mass spectrometer (Biflex Bruker, Germany). Microanalyses were determined on a Perkin–Elmer PE 2400 Series II.

Plant Material. The *C. oblongifolius* sample used in this study was collected from Amphur Viengsa, Nan Province, Thailand, in November 2003. Botanical identification was done by comparison with voucher specimen No. 9607 in the Herbarium of the Royal Forest Department of Thailand, Bangkok, Thailand.

Extraction and Isolation. The powdered sun-dried stem bark (0.5 kg) of *C. oblongifolius* was repeatedly extracted with hexane (2×5 L) at room temperature. The hexane extract was filtered and evaporated under reduced pressure to obtain a yellowish green oil (15 g). The plant residue was further re-extracted with EtOAc (2 × 5 L) and MeOH (2 × 5 L) to give the EtOAc (25 g) and MeOH (20 g) crude extracts, respectively. The hexane crude extract (15 g) was fractionated by silica gel column chromatography using Merck Si gel 60 (0.5 kg). The column was eluted with hexane–EtOAc gradient in a stepwise fashion, and 90 × 25 mL fractions were collected. After the solvent was separated by rotary evaporation under reduced pressure, the similar fractions were combined into three fraction: I (2.1 g, 0–20% EtOAc in hexane, F25–F42), II (5.4 g, 25–30% EtOAc in hexane, F43–F57), and III (1.3 g, 50% EtOAc in hexane, F58–F74). Compound **1** (200 mg, 0.04% based on dried plant) was obtained by recrystallization from methanol. Compound **2** (2.2 g, 0.4% based on dried plant) was obtained as a colorless oil by re-column chromatography of 5.4 g of fraction II on SiO_2 (100 g) and eluted with 30% EtOAc in hexane.

3-Hydroxycleistantha-13(17),15-diene (1), white needle crystal (200 mg, 0.04% based on the dried plant material), mp 102–104°C; $[\alpha]_D^{25}$ +11.06° (*c* 0.32, EtOAc); UV (MeOH, λ_{max}): 213 (log ε 2.89) nm; IR (KBr, ν_{max}, cm^{-1}): 3400–3200 (OH), 1627 (C=C); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Table 1 and 2; MS *m/z*: 289 $[M+H]^+$; anal. C 83.24%, H 11.20%, calcd for $C_{20}H_{32}O$, C 83.27%, H 11.18%.

3,4-*seco*-Cleistantha-4(18),13(17),15-trien-3-oic acid (2), colorless oil (2 g, 0.4% based on the dried plant material; $[\alpha]_D^{25}$ +9.36° (*c* 0.26, EtOAc); UV (MeOH, λ_{max}): 217, (log ε 3.40) nm; IR (ν_{max}, neat, cm^{-1}): 3400–2400 (OH), 1699 (C=O), 1637 (C=C); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Table 1 and 2; HRESIMS *m/z:* 303.2333 $[M+H]^+$; calcd for $C_{20}H_{30}O_2$+H 303.2324.

Epoxidation of Compound 2. *m*CPBA (70%) (230 mg, 0.93 mmol) was added to a solution of **2** (235 mg, 0.78 mmol) in CH_2Cl_2 (5 mL). After stirring for 5 h at room temperature the reaction mixture was washed with saturated sodium carbonate solution and water, respectively. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a colorless residue (145 mg). The residue was separated by silica gel column chromatography eluted with a 1:1 ratio of hexane–EtOAc to yield compound **3** (120 mg, 50%) and then compound **4** (30 mg, 21%).

13,17-Epoxy-3,4-*seco*-cleistantha-4(18),15-dien-3-oic acid (3), colorless oil; $[\alpha]_D^{25}$ +97.17° (*c* 1.83, EtOAc); UV (MeOH, λ_{max}): 211 (log ε 6.65) nm; IR (ν_{max}, neat, cm^{-1}): 3130–3000 (OH), 1699 (C=O), 1632 (C=C); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Table 1 and 2; MS *m/z:* 341 $[M+Na]^+$; anal. C 75.41%, H 9.56%, calcd for $C_{20}H_{30}O_3$, C 75.43%, H 9.50%.

Compound (4), colorless oil; $[\alpha]_D^{25}$ –1.56° (*c* 2.60, EtOAc); UV (MeOH, λ_{max}): 222 (log ε 3.54); IR (ν_{max}, neat, cm^{-1}): 3000 (OH), 2926, 2865, 1699 (C=O), 1632 (C=C), 1283 (C-O); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Table 1 and 2; MS *m/z*: 319 $[M+H]^+$.

Cytotoxicity Testing. Bioassay of cytotoxic activity against human tumor cell cultures *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetic method [7, 8]. Doxorubicin hydrochloride was used as a positive control substance.

ACKNOWLEDGMENT

We thank the Thailand Research Fund (DBG4880013), the Thai Government (Nan), and the Graduate School and Department of Chemistry, Faculty of Science, Chulalongkorn University for financial support.

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